



A comparison between adjuvant and delivering functions of calcium phosphate, aluminum hydroxide and chitosan nanoparticles, using a model protein of *Brucella melitensis* Omp31

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ABSTRACT

Vaccination is the most efficient and economic approach used to hinder infection and intense consequences caused by viruses, bacteria, or other pathogenic organisms. Since the intrinsic immunogenicity of recombinant antigens is usually low, safe and potent vaccine adjuvants are needed to ensure the success of those recombinant vaccines. Nanoparticles (NPs) have attracted much interest as adjuvants and delivery systems. Previous studies have shown that calcium phosphate (CP), aluminum hydroxide (AH) and chitosan (CS) NPs are promising delivery systems for immunization. In addition, it has been determined that Omp31 is a good candidate for inducing protection against *Brucella (B) melitensis* and *B. ovis*. Our aim in the present study was to compare the functions of CP, AH and CS NPs for stimulation of the immune response and protection against *B. melitensis* by using omp31 as a model protein. Based on the cytokine profile and subclasses of the antibody, vaccination with Omp31 load CP (CP/Omp31) and Omp31 load AH (AH/Omp31) NPs induced T helper type 1 (Th1)-T helper type 2 (Th2) immune response, whereas immunization by Omp31 load CS (CS/Omp31) NPs induced Th1 immune response. CP/Omp31 NPs elicited protection toward *B. melitensis* challenge equivalent to the vaccine strain *B. melitensis* Rev.1. Compared to CS/Omp31 NPs, CP/Omp31 NPs elicited a low increase in protection level against *B. melitensis* 16 M. In conclusion, the obtained results indicated that CP NPs were potent antigen delivery systems to immunize brucellosis.

1. Introduction

Vaccination is the most efficient and economic approach used to hinder infections caused by viruses, bacteria, or other pathogenic organisms. A variety of vaccines cause a decrease in the global mortality along with the cost for infection treatments. Development of vaccines is accompanied with a fast progression in the fields of immunology, biotechnology and biomaterials [1].

Zoonotic infections are common diseases between humans and animals, one of those most important of which is brucellosis. Due to possible airborne transfer of brucellosis, there has been growing concern about its usage as a potential factor in bioterrorism; along with lack of approved vaccines, there is an ever growing need for an effective brucellosis vaccine for human [2,3]. Many efforts have consistently indicated that live-attenuated vaccines provide the best protection against brucellosis; however, the available animal brucellosis vaccines (*B. melitensis* Rev.1 for small ruminants and *B. abortus* S19 and *B.*

abortus RB51 for cattle) have still remained imperfect. These live-attenuated vaccine strains have the drawbacks of residual virulence, antibiotic resistance, pathogenicity for human beings, limited efficacy and interference with supervision and diagnosis [3,4].

Recombinant vaccines are an interesting approach without the drawbacks of conventional vaccines, and a number of recombinant vaccines have been released on the market. Intensive researches are in progress for development of efficient vaccines toward a large number of illnesses based on recombinant DNA strategy. Recombinant vaccines are expanded by rationally designed recombinant antigens through epitopes focusing, genomic screening or structure-based design. Beside the improved understanding of the genes and facilitations in the identification of determinants (such as protective immune responses), these approaches have provided novel routes to develop new vaccines against infectious, metabolic or parasitic diseases [5,6].

Inherent immunogenicity of the recombinant antigens is low, compared to the more live-attenuated or killed vaccines. Therefore, safe

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and robust vaccine adjuvants are needed for ensuring the success of the recombinant vaccines [7]. The low immunogenicity observed in recombinant antigens is related to the lack of activators of exogenous antigens. Recombinant antigens are loaded on different adjuvants, and their immunomodulatory effects are related to the particular adjuvant applied in conjunction with the specific antigens [8].

Loading antigens into adjuvants has the advantages of dose sparing, enhanced efficiency in the elderly, and expanding cellular and humoral immune responses. Different adjuvants have been examined to be used in veterinary vaccines, such as aluminum, Montanide, biodegradable polymeric microparticles (MPs), and nanoparticles (NPs) [9].

NPs have attracted interests in various fields of nanomedicine [10–15] including adjuvants and delivery systems [13–16]. The use of NPs in vaccine formulations is based on the fact that most pathogenic microorganisms have a dimension in a nanometer scale, and are effectively processed by the immune system; the latter leads to potent immune responses [17,18]. Calcium phosphate (CP) NPs are among the most commonly applied inorganic nano-adjuvants. Their advantages include low cost, resistance to degradation by lipase and bile salts, excellent biodegradability, and non-toxicity. Furthermore, CP has a proper adjuvant potential in inducing immune responses against different infectious illnesses [19,20]. Due to larger specific surface area, stronger adsorption capacity, higher surface reactivity, and smaller particle size, aluminium hydroxide (AH) NPs can adsorb more antigens than the traditional AH-based adjuvants [21,22]. In addition, it has been shown that AH NPs represent a stronger vaccine adjuvant activity than traditional AH MPs [23]. Due to chitosan (CS) mucoadhesive properties, biodegradability, immune-stimulatory potential and safety profile, its NPs have been used as adjuvant and vaccine delivery systems. The usage of CS NPs to increase the efficiency and response to vaccination with bacterial derived toxins and antigens, model protein antigens, viral antigens and DNA plasmids are well documented [24,25]. Meanwhile, it has been determined that Omp31 is a good antigen candidate to elicit protection against *B. melitensis* and *B. ovis* [26].

Since CP, AH and CS NPs have shown promising activity as adjuvant and vaccine delivery system in different diseases, and to the best of our knowledge there has not been any report on the delivering functions of these NPs using the *Brucella melitensis* Omp31 protein, in the present study and for the first time, their function to stimulate the immune response and protection against *B. melitensis* using Omp31 as a model protein was compared.

2. Experimental section

2.1. Plasmids, bacteria and animals

Four- to six-week female BALB/c mice were received from the center of comparative and experimental medicine (Shiraz University of Medical Sciences). They were acclimated and randomly divided into experimental groups. The animals were kept in standard care and condition in accordance with the protocol of the local Ethics of Animal Experiments of Shiraz University of Medical Sciences. The animals were housed at a controlled temperature ($24 \pm 2^\circ\text{C}$) and humidity (40–70%) with weekly floor exchange. They had free access to water and food. A 12:12 light:dark cycle was observed.

B. melitensis 16 M and *B. melitensis* Rev.1 were received from Razi Vaccine and Serum Research Institute (Iran). *Escherichia coli* (*E. coli*) BL21 (DE3) and pET28a vector from Novagen (USA) were used to express recombinant Omp31 (rOmp31).

2.2. Production of antigen

Omp31 was expressed in *E. coli* BL21 (DE3). In brief, the respective gene *omp31* was cloned into a pET28a expression vector and the resultant plasmid was brought in *E. coli* BL21 (DE3) from Novagen (USA).

The positive clones were then selected, and the recombinant proteins were expressed in transformed bacteria by isopropyl- β -D-thiogalactopyranoside (IPTG) induction in LB medium. It was then purified by affinity chromatography using Ni-agarose beads from Qiagen (UK). The recombinant protein expression was validated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was stored at -70°C until use for enzyme-linked immunosorbent assay (ELISA) or *in vitro* splenocytes induction.

2.3. Synthesis and characterization of rOmp31-loading NPs

CP NPs were synthesized according to a previously described method [27]. Briefly, a mixture containing 12.5 mmol L^{-1} calcium chloride from Merck (Germany), 12.5 mmol L^{-1} disodium hydrogen phosphate from Sigma (USA) and 15.6 mmol L^{-1} sodium citrate from Sigma (USA) were prepared, slowly mixed and stirred for 48 h to obtain a NP suspension. Then, the mixture was sonicated for 30 min in an ultrasonic bath. In order to prepare Omp31 load CP (CP/Omp31) NPs, 1.0 mg Omp31 dissolved in $300 \mu\text{L}$ phosphate buffer (PBS), pH 7.5 was mixed with $1000 \mu\text{L}$ NP suspension (5 mg mL^{-1}) and shaken at 4°C for 16 h. The mixture was then centrifuged at $12,000 \text{ g}$ and washed with distilled water.

AH NPs were synthesized based on a previously reported method [21]. Equal volumes of 3.6 mg mL^{-1} $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.04 mol L^{-1} NaOH solutions were mixed. After adjusting pH to 7.0, the mixture was stirred at room temperature for 30 min. In the next step, the mixture was sonicated for 10 min in an ultrasonic bath. The byproduct NaCl was removed by a AmiconVR Ultra-4 centrifugal filter from Merck Millipore (Germany). To prepare Omp31 load AH (AH/Omp31) NPs, we added a certain volume of AH dispersion to a 3.33 mg mL^{-1} Omp31 solution prepared in PBS so that the ratio of the protein to AH NPs was 1.5 to 1.1. The mixture was stirred for 20 min and then stored at 4°C until use.

CS and Omp31 load CS (CS/Omp31) NPs were prepared by ionic complexation with pentasodium tripolyphosphate (TPP) from Merck (Germany). To prepare CS/Omp31 NPs, we dissolved CS and Omp31 in a 0.1 mol L^{-1} acetate buffer, pH 5 to a final concentration of 1.0 and 0.1 mg mL^{-1} , respectively. A TPP solution (5 mg mL^{-1}) was added under continuous stirring to attain a weight ratio of CS:TPP:Omp31 of 10:4:1. NPs were collected by centrifugation at $12,000 \text{ g}$ for 30 min on a glycerol bed [28]. CS NPs were prepared similarly; otherwise, Omp 31 was not employed.

Morphology and size of NPs were characterized by field emission scanning electron microscopy (FESEM), using a TESCAN Mira 3-XMU microscope (Czech Republic).

Omp31 content of CP/Omp31, AH/Omp31 and CS/Omp31 was determined with a Bradford protein assay, according to the manufacturer's instructions. The loading efficiency (LE) was obtained by the equation of $\text{LE}(\%) = (\text{total amount of Omp31-free Omp31}) / \text{total amount of Omp31}$.

2.4. Immunization

Female BALB/c mice were randomly divided into different groups ($n = 10$). The animals were immunized by subcutaneous injection with CP/Omp31, AH/Omp31 or CS/Omp31 NPs. The negative control groups were immunized with CP, AH and CS NPs in subcutaneous immunization route. Vaccination was repeated twice by $30 \mu\text{g}$ antigen injection on days 0 and 15. Also, the positive control group animals ($n = 5$) were immunized subcutaneously on the 15th day with 10^5 CFU of *B. melitensis* Rev.1.

2.5. Antibody responses

To analyze the antibody production, we took serum samples from the vaccinated animals 15, 30 and 45 days after the first vaccination. The total IgG, IgG1 and IgG2a isotype levels were examined against

Omp31 by an indirect ELISA method. Wells of microplates were coated with 100 μL of Omp31 in carbonate-bicarbonate buffer, pH 9.6, at 37 °C for 1 h. The microplates were washed three times with PBS containing 0.1% Tween 20. After 1 h of blocking at 37 °C with 3% (w/v) skim milk in PBS containing 0.1% Tween 20 to prevent nonspecific binding, the microplates were incubated with serially diluted sera (1:250 to 1:6000) at 37 °C for 2 h. Anti-mouse IgG, IgG1 and IgG2a HRP conjugated antibodies from Santa Cruz Biotechnology Inc. (USA) were added to the wells and incubated at 37 °C for 1 h. After addition of 100 μL of o-phenylenediamine dichloride from Sigma (USA) dissolved in phosphate-citrate buffer, pH 5.5 and H_2O_2 as a substrate, the microplates were incubated at 37 °C for 15 min. The reaction was terminated by addition of 50 μL of 1 mol L^{-1} H_2SO_4 , and the absorbance values were measured at 450 nm by a microplate reader of BioTek (USA).

2.6. Cytokine quantitation

One month after the final immunization, the subjects were sacrificed and their spleens were removed under aseptic conditions. Suspensions of single-cell were made from the spleens followed by lysis of the red blood cells using a ACK solution (1 mmol L^{-1} KHCO_3 , 0.1 mmol L^{-1} Na_2EDTA , 150 mmol L^{-1} NH_4Cl , pH 7.3). Splenocytes were cultured in a 5% CO_2 atmosphere at 37 °C in a 96-well microplate with a concentration of $4 \times 10^6 \text{ mL}^{-1}$ in duplicate wells in RPMI 1640 medium supplemented with 2 mmol L^{-1} L-glutamine and 10% heat-inactivated FBS from Eurobio (France) with Omp31 (10 $\mu\text{g mL}^{-1}$). In murine splenocyte culture supernatants, the levels of IFN- γ , IL-12, IL-4 and IL-10 were determined after 48 h [29]. The cytokines were assayed according to the manufacturer's instructions using specific ELISA kits from R&D Systems Inc. (USA).

2.7. Protection experiments

Thirty days after the final booster injection, the subjects were subcutaneously stimulated with of *B. melitensis* 16 M (4×10^4 CFU). 30 days after stimulation, the subjects were sacrificed by cervical dislocation, and their spleens were aseptically removed. The spleens were homogenized in a stomacher bag, diluted in a serial manner, plated on Brucella agar, and incubated at 37 °C for 4 days. CFU numbers per spleen were counted, and the results are presented as the mean of log CFU \pm standard deviation in each group.

2.8. Assay of lymphocyte proliferation

All the subjects were sacrificed one month after the last immunization. The spleen cells from subcutaneous vaccinated mice (2×10^5 cells/well) were cultured in quadruplicate wells. The spleen cells were stimulated with Omp31 (0.1 μg /well) and incubated in a 5% CO_2 atmosphere at 37 °C for 72 h. For lymphocyte proliferation determination, the MTT assay was employed. 1 mL MTT of 5.0 $\mu\text{g mL}^{-1}$ MTT was introduced to the incomplete media; then, 5 μL of this solution was added to the wells and incubated in dark in a 5% CO_2 atmosphere with 95% humidity at 37 °C for 2 h. The media were removed from the wells and formazane crystals were dissolved using 90% acidified isopropanol (0.5% W/V sodium dodecyl sulfate and 25 mmol L^{-1} HCl in 90% isopropanol). The absorbance intensity (OD) was finally measured at 540 nm.

2.9. Statistics analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS software. Differences were considered to be statistically significant at p value ≤ 0.01 .

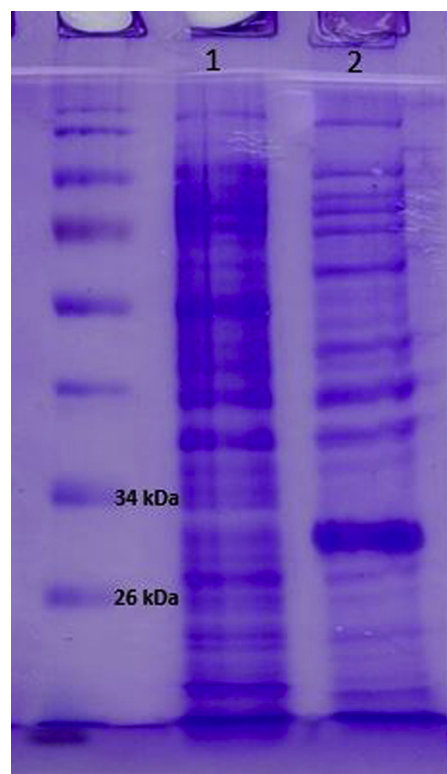


Fig. 1. SDS-PAGE analysis of Omp31 protein expression. Lane 1 shows uninduced and Lane 2 shows induced cell lysates of Omp31 expressing *E. coli* cells.

3. Results

After induction with IPTG, Omp31 protein expressed by recombinant cells was analyzed by SDS-PAGE, and the results are shown in Fig. 1. Lanes 1 and 2 show the uninduced and induced cell lysates of Omp31 expressing *E. coli* cells, respectively. The results indicated that Omp31 was produced using the expression host *E. coli* BL21 (DE3) and pET-28a (+) vector.

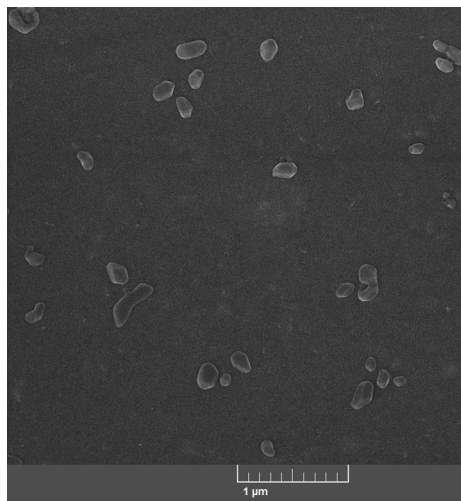
Fig. 2 shows FESEM images of CP/Omp31 (A), AH/Omp31 (B) and CS/Omp31 (C) NPs. The images show the average sizes for CP/Omp31 and CS/Omp31 NPs as 176.5 ± 11 and 99.1 ± 15 nm, respectively. AH/Omp31 NPs had a rod-like structure with an average diameter of 81.3 ± 17 nm.

LEs for Omp31 on CP, AH and CS were obtained as 56.2 ± 5.1 , 73.8 ± 4.9 and $61.2 \pm 6.7\%$, respectively.

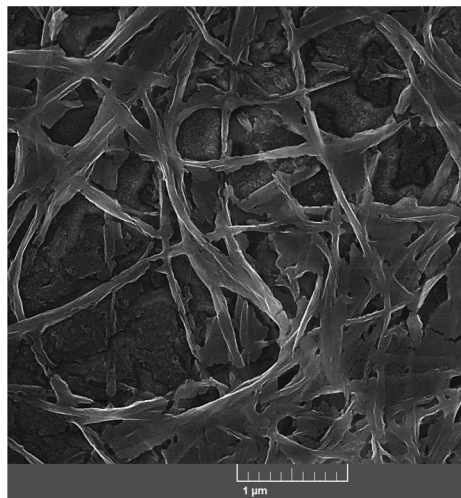
The levels of anti-Omp31 antibody total IgG (A), IgG1 (B) and IgG2a (C) are presented in Fig. 3. Sera obtained from mice belonging to different experimental groups were collected at regular intervals up to day 45 post-primary immunization, dilution 1:250. The sera were analyzed in triplicates for Omp31 specific IgG antibodies by ELISA. Although immunization with all vaccine formulations induced the total IgG level, the highest IgG titer belonged to AH/Omp31 NPs. The highest titer of IgG1 and IgG2a was observed in the mice immunized with CP/Omp31 and AH/Omp31 NPs, respectively ($p \leq 0.01$).

Fig. 4 depicts the levels of IFN- γ (A), IL-12 (B), IL-10 (C) and IL-4 (D) determined by ELISA in the supernatants of the spleen cell of the immunized mice of different groups stimulated with Omp31 for 48 h. The results indicated that the supernatants of the splenocyte cultures from immunized mice with CP/Omp31 and AH/Omp31 NPs contained significant levels of IL-4, IL-10, IFN- γ and IL-12, compared to the adjuvants alone, whereas vaccination with CS/Omp31 NPs only induced secretion of IFN- γ and IL-12, compared to the adjuvants ($p \leq 0.01$).

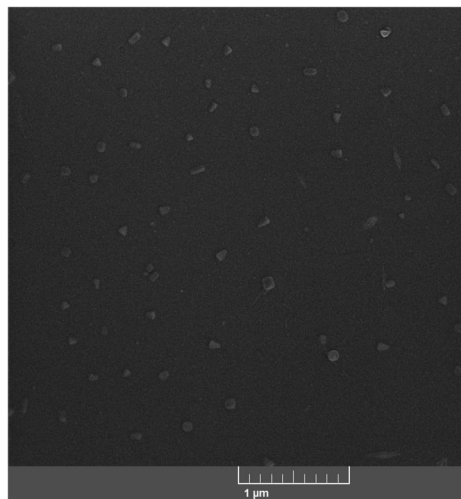
The BALB/c mice immunized with different vaccine formulations were subcutaneously stimulated with *B. melitensis* 16 M 30 days after



(A)



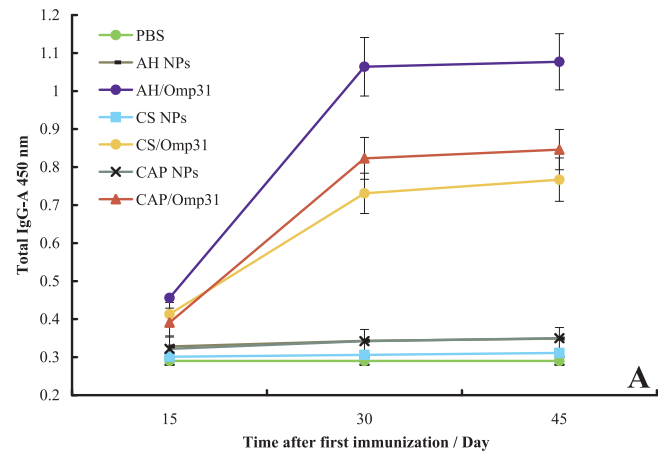
(B)



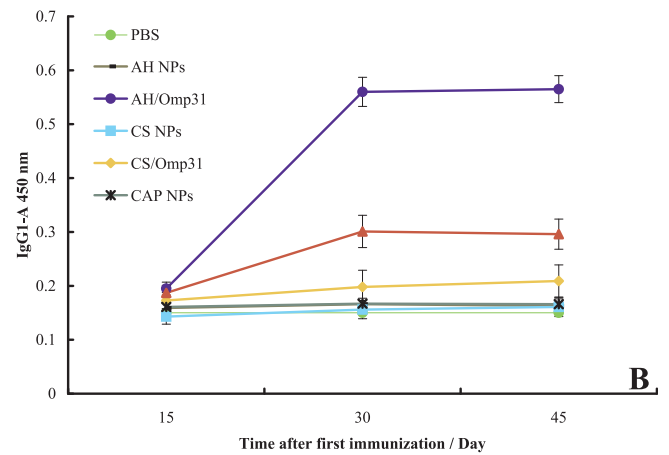
(C)

Fig. 2. FESEM images of CP/Omp31, AH/Omp31 and CS/Omp31.

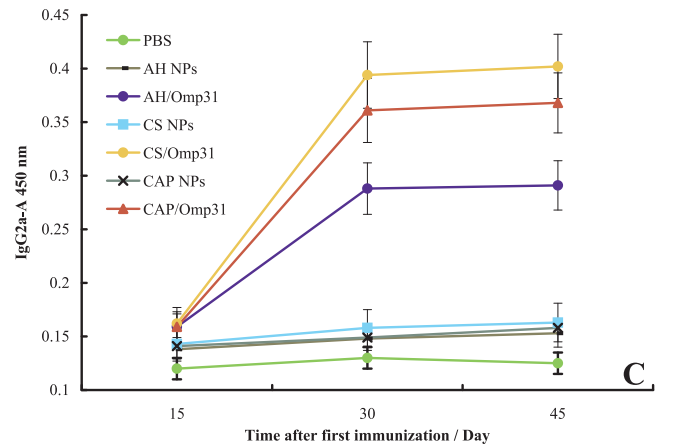
the final booster injection. Protection against *B. melitensis* 16 M in the subjects was compared with those who received vaccine strain Rev.1, as presented in Table 1. CP/Omp31 and CS/Omp31 NPs conferred equivalent protection one month after the challenge; however, the protection obtained from AH/Omp31 NPs was significantly lower than



A



B



C

Fig. 3. The levels of anti-Omp31 antibody total IgG (A), IgG1 (B) and IgG2a (C) for the sera obtained from the mice belonging to different experimental groups collected at regular intervals of 15, 30, 45 days of post-primary immunization. The antibody levels in the sera from the mice which received PBS, CP/Omp31, AH/Omp31 and CS/Omp31 were nearly the same.

that of the *B. melitensis* Rev.1 vaccine strain ($p \leq 0.01$). Animals immunized with CP/Omp31 NPs showed a somewhat higher degree of protection than those immunized with CS/Omp31 NPs, albeit without reaching statistical significance.

In order to check the ability of various vaccine formulations to elicit antigen specific cellular immunity, we performed an *in vitro* cell proliferation assay 30 days after the last immunization. Splenocytes of the

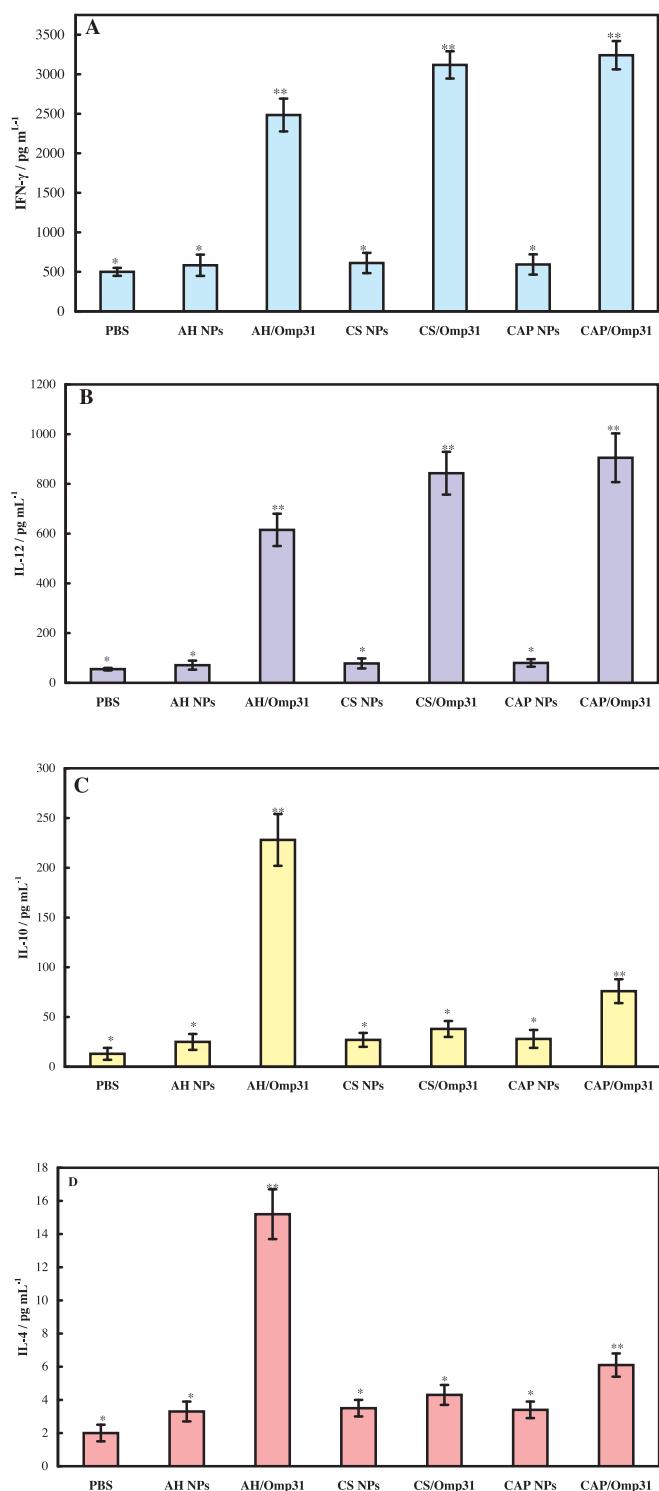


Fig. 4. The levels of IFN- γ (A), IL-12 (B), IL-10 (C) and IL-4 (D) determined by ELISA in the supernatants of the spleen cell of the immunized mice of different groups stimulated with Omp31 for 48 h. Values with significant differences are indicated with different number of star(s) ($p \leq 0.01$).

vaccinated mice were stimulated with Omp31 (0.1 $\mu\text{g}/\text{well}$) for 72 h and the proliferative response was determined by in vitro-MTT assay, as shown in Fig. 5. The data were presented as stimulation indices (S.I.) \pm SD ($p \leq 0.01$). The stimulation index (S.I.) corresponds to the count per minute of stimulated spleen cells divided by the count per minute of unstimulated spleen cells. According to these results, a significantly higher cell proliferation rate in all vaccinated mice was

observed, compared to the obtained results of the mice immunized with NPs alone. The high proliferation index indicates the cell stimulatory ability of various vaccine formulations due to the robust immune response.

4. Discussion

NPs can induce immune responses for both prophylactic and therapeutic effects. They can be applied as delivery systems to increase the antigen processing and protect the antigen from degradation, and as an immune-stimulant to trigger immune responses. Nanotechnology has allowed the customization of the NPs properties of the shape, size and surface charges, resulting in a great variety of NPs applications [13–16]. Different synthetic and biological NPs have been approved for human application, and some others are in pre-clinical or clinical investigations [18,30]. In the present study, we evaluated the CP, AH and CS NPs' ability as antigen delivery systems and adjuvant via subcutaneous administration route.

The results of antibody assay showed that the IgG titer after immunization with AH/Omp31 NPs was higher than that of CS/Omp31 and CP/Omp31 NPs. Previous studies showed that aluminum-containing adjuvants stimulate a robust humoral response, which is mainly recognized by the antigen-specific antibodies secretion [22]. Therefore, our results are consistent with previous studies indicating that AH significantly stimulates humoral immune response.

Since IgG isotypes are determined by the cytokines pattern generated by CD4+ helper T cells, both Omp31-specific IgG1 and IgG2a antibodies titers increased after vaccination were examined. IgG1 titer significantly increased after vaccination with AH/Omp31 and CP/Omp31 NPs, while IgG2a titer was raised in all the vaccinated mice sera. It has been determined that due to facilitating phagocytosis, IgG2a isotype has a key role in anti-*Brucella* immunity. Thus, the high IgG2 titer after vaccination with CS/Omp31 and CP/Omp31 NPs can be one of the effective factors in creating better immunity against *Brucella*.

Due to the intracellular residence of *Brucella*, it is difficult to entirely eradicate a pathogen by the host cellular immune responses or eliminated it by antimicrobial drugs. Indeed, IFN- γ protects the host against *Brucella* infection by upregulating both macrophage effector function and the iron molecules and TNF- α secretion. Furthermore, T cell subsets have a key role in immunity against *Brucella* infection. CD4+ T cells produce a robust cytokine such as IFN- γ , which is recognized to promote protection against *Brucella* infection [31]. Our results indicate that immunization with CP/Omp31 and AH/Omp31 NPs can induce secretion of IL-4, IL-12, IL-10 and IFN- γ , whereas immunization with CS/Omp31 NPs stimulated only IL-12 and IFN- γ secretion. Hence, depending on the antibody subclass and cytokine profile, CS/Omp31 NPs immunization induces T helper type 1 (Th1) immune response, whereas vaccination with CP/Omp31 and AH/Omp31 NPs induces Th1-T helper type 2 (Th2) immune response. Thus, the results obtained from antibody and cytokine assays are in accordance with the data obtained from previous studies, indicating that immunization with CP NPs can induce Th1-Th2 immune responses [32,33].

Due to biodegradability, biocompatibility, non-toxicity and high charge density, chitosan and its derivatives have gained particular attention for drug and vaccine delivery [34]. A recent study showed that intraperitoneal vaccination with tri-methyl chitosan (TMC), a derivative of chitosan, NPs with Omp31 (TMC/Omp31) induced Th1-Th2 immune responses, whereas oral administration of TMC/Omp31 stimulated Th1-Th17 immune responses [35]. However, in the present study, it was shown that subcutaneous vaccination with CS/Omp31 NPs induced the Th1 response. The reason for the difference in the immune response type can be due to the use of NPs in different vaccination routes, as well as different physicochemical properties of NPs.

Aluminum salts are an example of a family of prevalent adjuvants, mainly comprising AH, potassium aluminum sulfate, or aluminum phosphate particles. These salts, because of their high safety and low-

Table 1
Protection against *B. melitensis* 16 M in BALB/c mice immunized with different vaccine formulations compared with the vaccine strain Rev.1.

Vaccine (n = 5)	Adjuvant	log CFU of <i>B. melitensis</i> 16 M in spleen [†]	Protection units [‡]	p value
AH NPs	AH NPs	5.86 ± 0.13 ^a	0	≤ 0.01
AH/Omp31	AH NPs	4.32 ± 0.12 ^b	1.54	≤ 0.01
CS NPs	CS NPs	5.69 ± 0.15 ^a	0	≤ 0.01
CS/Omp31	CS NPs	3.7 ± 0.14 ^c	1.99	≤ 0.01
CAP NPs	CP NPs	5.66 ± 0.17 ^a	0	≤ 0.01
CAP/Omp31	CP NPs	3.58 ± 0.15 ^c	2.08	≤ 0.01
PBS	–	5.95 ± 0.18 ^a	0	≤ 0.01
<i>B. melitensis</i> Rev.1	–	3.9 ± 0.16 ^c	2.05	≤ 0.01

The difference between groups was assessed by the ANOVA and comparisons were considered significant at $p \leq 0.01$. Different letters (a, b and c) represent significant difference between groups.

[†] The content of bacteria in spleens is represented as the mean log CFU ± SD per group.

[‡] Units of protection were determined by deducting the mean log CFU of the vaccinated groups from the mean log CFU of negative control groups.

cost, are applied as adjuvants for many types of vaccines. Aluminum salts are still the most commonly used adjuvants in licensed human vaccines, including commonly known vaccines against hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis E virus (HEV) and human papilloma virus (HPV) [22]. However, aluminum salts have had many drawbacks including IgE mediated allergic responses, occasional induction of local reactions such as erythema or granulomatous inflammation, variability in production of aluminum salts precipitated toxoids/vaccines, lack of stimulating cytotoxic T lymphocyte (CTL) responses, and stability issues during freeze drying [36,37]. Moreover, aluminum salts mainly elicit humoral immune responses (Th2 type, IgG1 response) and very low cellular immune response (Th1 type, IgG2a response). In agreement with previous studies, our results also showed that AH stimulated the Th1-Th2 (Th1 < Th2) immune responses [38]. Therefore, it has limited value in vaccination against viral infections, intracellular bacteria or cancer that requires cellular immunity. However, according to high capability in inducing humoral immunity, it seems that AH NPs are suitable as an adjuvant and vaccine delivery system for control and prevention of extracellular bacteria.

CP has been applied as an antigen carrier adjuvant in tetanus toxoid for long-term vaccination, like many investigations with promising result in the induction of systemic immunity. Significant cellular and cytotoxic (CD8) T-cell immune responses were obtained upon addition of CP adjuvant to antigen formulations [39,40]. As an alternative adjuvant, CP adjuvant might be effective in vaccines against intracellular pathogens wherein an immune response alone is not sufficient for

protective immunity. Biocompatible CP NPs with tunable characteristics have potentials to function as adjuvants for inducing Th1-Th2 immune responses [20]. In agreement with previous reports, our data also showed that CP NPs stimulated the Th1-Th2 (Th1 > Th2) immune responses. CP is a compound naturally present in organisms; it is safe and has already been used in human vaccination [41]. In comparison with other adjuvants, CP can be a good candidate in replacing for alum salts as a vaccine adjuvant. In line with previous studies, our results showed CP NPs had a better function in stimulation of cellular immunity and protection than AH NPs.

Our data showed that CP and CS NPs provide protection against *B. melitensis* infection equal to that of traditional vaccine strain *B. melitensis* Rev.1. The protection degree obtained after immunization with AH/Omp31 NPs was lower than that of the other two vaccinated mice groups and the positive control group. In comparison to CS/Omp31 NPs, CP/Omp31 NPs provided a bit increment in the protection level against *B. melitensis* infection. Although such increase in the protection level was not found to be statistically meaningful, CP NPs provided in the present study might confer better efficiency due to cumulative effects on the IgG2a titer, IFN- γ induction and stimulation index.

In a study conducted by Volkova et al., CP NPs and CS MPs adjuvant activities were examined after intranasal vaccination to commercial chickens with inactivated Newcastle disease virus (NDV) vaccine. Compared with the immunization with NDV antigen only, both CP NPs and CS MPs enhanced the antibody titers in mucosal and blood samples. Due to better function of CS MPs than CP NPs in stimulation of humoral and mucosal immunity and protective responses, they suggested CS usage as a potential adjuvant for poultry vaccines production [42]. Our data showed that CP NPs were slightly better than CS NPs in stimulating cellular protection and immune response toward *B. melitensis*. The reason for the difference between our results and those of Volkova et al.'s study can be the usage of different host, antigen and immunization route. For example, recent studies showed that TMC NPs with Omp31 protein (TMC/Omp31 NPs) in the oral immunization route had a better function than the intraperitoneal immunization route, whereas TMC/Urease NPs in this immunization route has a better function than the oral vaccination one [35,43]. Hence, in line with previous studies, each antigen or particle based delivery system can exhibit a different function in each immunization route. In addition, due to mucoadhesive properties, CS is expected to have a better function than CP NPs in intranasal vaccination route, while the immunization via subcutaneous route might produce different results.

Particle shape, charge and size are the main physicochemical factors which play a key role in immune response induction via interaction between antigen-presenting cells and particles. Previous studies showed that spherical particles were better endocytosed by the immune cells

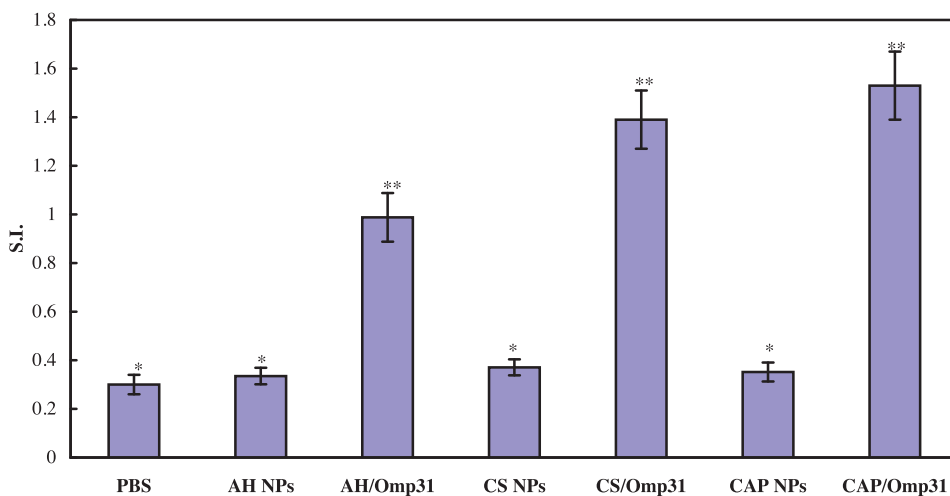


Fig. 5. The stimulation index of lymphocyte proliferation of the splenocytes from vaccinated mice 30 days after the last immunization. Splenocytes from the vaccinated mice were stimulated with Omp31 for 72 h and the proliferative response was determined by in vitro-MTT assay. The stimulation index (S.I.) corresponds to the count per minute of stimulated spleen cells divided by the count per minute of unstimulated spleen cells. The data are the mean S.I. ± SD of five individual mice from each group with three repeats. Values with significant differences are indicated with different number of star(s) ($p \leq 0.01$).

than rod-shaped particles [44,45]. Kumar et al. examined the role of NPs' shape and size in antigen presentation and next processing by the immune cells. They used Ovalbumin as a model protein. Spherical polystyrene NPs of 521 nm and 193 nm diameters were stretched to produce rod-like particles of 1530 nm and 376 nm in length, respectively. The results of vaccination study indicated that small spherical NPs (193 nm in diameter) induced Th1 immune responses, whereas rod-shaped particles (1530 nm in length) induced Th2 immune responses [48]. Previous studies attempting to associate the particles size and their adjuvant property have been debatable. Some authors indicated that larger particles were better than smaller ones, whereas others indicated the opposite. Interestingly, it has been determined that the size of particles affects the immune responses type, however, it still remains debatable as to whether large or small particles favor Th1 versus Th2 or cellular immunity vs. antibody responses [46–49]. Our results showed that NPs with larger diameter had a better performance than smaller NPs.

5. Conclusion

CP, AH and CS NPs act as Omp31 delivery systems and adjuvant for vaccination and protection against *B. melitensis* infection in mice. A significant increase was found in the level of antibodies and cytokines during vaccination with CP/Omp31, AH/Omp31, and CS/Omp31. CP/Omp31 and CS/Omp31 induce protection against *B. melitensis* infection mice, similar to traditional vaccine strain *B. melitensis* Rev.1. Immunization of mice with CP/Omp31, AH/Omp31, and CS/Omp31 stimulates supreme humoral immune response and protection against *B. melitensis* infection, while CP NPs is the best option due to cumulative effects on the IgG2a titer, IFN- γ induction and stimulation index.

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