Gelatin Micro/Nanoparticles-Based Delivery of Urease and Omp31 in Mice Has a Protective Role Against *Brucella melitensis* 16 M Infection

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Abstract

Brucellosis is one of the most endemic diseases in many regions of the world. Due to its serious medical and economic consequences, many attempts have been made to prevent infection in domestic animals through vaccines. In this study, vaccine formulations of urease-loaded gelatin (urease/gelatin) micro/nanoparticles (MNPs) and Omp31-loaded gelatin (Omp31/gelatin) MNPs against brucellosis were developed. Urease/gelatin MNPs and Omp31/gelatin MNPs, separately or in combination (MNPs cocktail), were orally administered. The formulations comprised 165-nm to 1.3-μm particles. Antibody detection, cytokine measurement, and lymphocyte proliferation assay were performed. Finally, the immunized mice were challenged with the virulent *B. melitensis* 16 M. All the immunized mice elicited titers of specific IgG and IgA. According to cytokine assay and antibody isotypes, oral administration with all vaccine formulations stimulated a Th1-Th17 immune response. In lymphocyte proliferation assay, splenocytes from all-immunized mice indicated a robust recall proliferative response. The MNPs cocktail conferred protection against *B. melitensis* challenge equivalent to that of vaccine strain *B. melitensis* Rev.1. In comparison to gelatin/Omp31 MNPs alone, MNPs cocktail induced only a low increase in protection level. Altogether, the results showed that MNPs cocktail might be a promising candidate for the subunit vaccines development against brucellosis. Furthermore, gelatin MNPs are a suitable delivery system for orally-administered *Brucella* antigens.

1 Introduction

Brucellosis is one of the most prevalent endemic diseases in many regions of the world, especially Africa, Southern Europe, Latin America, and Asia including the Middle East. The World Health Organization (WHO) estimates that this illness is responsible for more than 500,000 new human cases worldwide. The disease is caused by gram-negative and facultative intracellular pathogen bacilli of the genus *Brucella* (*B.*). It is transmitted to human beings via contact with infected animals, infection from a contaminated environment, and consumption of contaminated and unpasteurized dairy products. Four species have been identified to cause human brucellosis of *B. melitensis* (being the most common cause), *B. canis*, *B. suis*, and *B. abortus* [1–3].

Brucellosis's clinical symptoms vary from an asymptomatic symptom to a multi-organ involvement, and it might mimic other illnesses, making the diagnosis much harder. The most common symptoms are anorexia, fever (in 99% of the cases), headache, night sweats, weakness, chills, malaise, and joint pain [4].

At the turn of the twentieth century, vaccination as a preventive strategy became the major approach to eradicate illness. At the same time, scientific researches focusing on the design and development of a *B.* vaccine to prevent its transmission from animals to humans was initiated. Due to its re-emergence throughout the world, it is essential to discover novel therapies, such as prophylactic tools to counter the illness occurrence [5].

Although studies have continuously showed that live-attenuated vaccines induces the best protective responses against brucellosis infection, the recent live animal

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vaccines for the control of brucellosis, *B. melitensis* Rev.1 for small ruminants, and *B. abortus* S19 and *B. abortus* RB51 for cattle have been proved to be less than optimal. These *B.* strains have a number of disadvantages, including the possibility of regional spread, pathogenicity for human beings, abortion in pregnant animals, antibiotic resistance, and interference with diagnosis [6]. Hence, a vaccine that is nonpathogenic to human and domestic animals, but is effective in inducing a wide protective immunity, is essential to control brucellosis.

Recombinant protein-based subunit vaccines are regarded as valuable alternatives for effective and safer intervention products against human brucellosis. While different intracellular and cell surface components of *B.* spp. were surveyed as potential protective antigens against brucellosis challenge, only a few proteins have provided proper protection [7, 8]. Additionally, it was shown that Omp31 can induce protection against *B. ovis* and *B. melitensis* challenges [8, 9].

The main drawbacks of recombinant protein-based subunit vaccines are short-term immune responses and low immunogenicity [10]. Therefore, there is an urgent demand for formulations and adjuvants those can elicit long-term cellular and humoral immune responses. In this regard, nanomaterials-based platforms are being developed as vaccine and drug carriers [8, 11–18]. Nanoparticles (NPs) and microparticles (MPs) have gained attention for the subunit vaccines delivery due to their ability in forming a depot at the immunization site, preventing antigens from degradation and targeting antigen to dendritic cells [13, 14]. Numerous studies have been performed using gelatin as an effective carrier system for proteins and drugs. Due to high uptake of gelatin particles by dendritic cells and antigen targeting to antigen-presenting cells (APCs), these particles are considered as proper immune adjuvants and specific antigen delivery systems [15–17]. In the present study, we aimed to examine the immunogenicity and protective efficacy of Omp31 and urease, either alone or in combination, loaded on gelatin micro/nanoparticles (MNPs) in a mouse model.

2 Materials and Methods

2.1 Animals

Four to six weeks old female BALB/c mice were obtained from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences. Animals were kept under conventional animal facilities with free access to food and water during the experiment and cared according to institutional policies for animal health and welfare.

2.2 Source of Bacteria

Escherichia coli (E. coli) strain DH5α (Invitrogen, Carlsbad, CA, USA) was used as host during the cloning experiments and for plasmids propagation. *E. coli* BL21 (DE3) and pET28a expression vector (Novagen, Madison, WI, USA) were applied for the recombinant proteins expression. Bacterial strains were routinely grown at 37 °C in LB broth or agar, supplemented when required with 50 μg mL^{-1} of kanamycin. *B. melitensis* 16 M and *B. melitensis* Rev.1 were obtained from Razi Vaccine and Serum Research Institute, Iran.

2.3 Recombinant Proteins

Recombinant Omp31 and urease were expressed in *E. coli* and purified. Briefly, 482 bp and 708 bp long open reading frames of urease alpha middle part subunit gene (Ala$_{201}$ to Leu$_{350}$) and Omp31 were amplified by specific primers and cloned into the pET28a (Novagen, Madison, WI, USA). To express the recombinant proteins, DE3 cells were transformed using purified recombinant plasmids. Three colonies from each one were incubated in LB broth containing kanamycin (50 μg mL^{-1}) and grown overnight at 37 °C by shaking at 170 rpm. Five hundred microliter of overnight cultures were then diluted inside 4.5 mL of LB broth containing kanamycin (50 μg mL^{-1}). Protein expression induction was performed with 1.0 mmol mL^{-1} of isopropyl b-D-thiogalactoside (IPTG) in a culture of bacteria with a 600-nm absorbance value of unity. Bacteria were incubated at 37 °C for 15 h by shaking at 170 rpm. The recombinant protein expression was evaluated using 12% SDS-PAGE gel. The proteins were purified and refolded by affinity chromatography on Ni-agarose beads (Qiagen, Dorking, UK).

2.4 Preparation of Gelatin MNPs

Gelatin (200 mg) was dissolved in deionized water (10 mL) under constant heating at 40 ± 1 °C, pH = 3 (by adding 0.3 mol mL^{-1} HCl). The recombinant protein was added, followed by the drop-wise addition of acetone (30 mL) to form gelatin MNPs. At the end of the process, glutaraldehyde solution (25% V/V in 3 mL distilled water) was added as a cross-linking agent. Then, the solution was stirred for 1 h. The obtained dispersion was centrifuged at 16,000 g for 30 min to sediment particles. The particles were purified by threefold centrifugation and redispersion in water. After the final redispersion, the particles were freeze-dried to obtain white freely flowing powder of antigen-loaded gelatin MNPs.

2.5 Characterization of Antigen-Loaded Particles

The size and morphology of gelatin MNPs were examined using field emission scanning electron microscopy (FESEM, TESCAN Mira 3-XMU microscope, Czech Republic).

Loading efficiency (LE) of antigen on gelatin MNPs using the formula:

$$LE = (total\ protein - free\ protein)/total\ protein \qquad (1)$$

and release evaluation of antigen from gelatin MNPs were performed through protein determination by the Bradford protein assay, according to the manufacturer's instructions. For antigen release inspection, antigen-loaded gelatin MNPs were dispersed in 10 mL phosphate buffer saline (PBS) of 100 mmol L^{-1} (pH 7.4) by stirring at 37 °C. MNPs were centrifuged at 16,000 g and 4 °C for 25 min, and the supernatant was removed. The resultant particles were resuspended into 10 mL PBS of 100 mmol L^{-1} (pH 7.4) by stirring and kept in the same way at 37 °C. At intervals of one day and until 5 days, 0.5 mL of the stirring mixture was separated followed by centrifugation at 16,000 g for 20 min, and the antigen level in the supernatant was measured by the Bradford assay. The same volume of PBS was replaced in the release medium to keep the volume of the release medium constant. As a negative control, antigen-free gelatin MNPs were assayed.

2.6 Immunization of Mice

The mice were orally immunized with gelatin MNPs loaded by 75 µg of Omp31 (Omp31/gelatin MNPs) and urease (urease/gelatin/ MNPs), either alone or in combination (MNPs cocktail) on days 0, 7, and 14. The negative control group was immunized with a solution containing only gelatin MNPs on those days. The positive control group was administered orally on the 15th day with 5×10^8 CFU of *B. melitensis* Rev.1.

The sera samples were collected from the retro-orbital plexus, and fecal exact pellets were obtained from each mouse on days 15, 30, and 45 after the first vaccination.

2.7 End-Point Antibody Titer and Isotyping

Specific antibody IgG, IgG1, and IgG2a titers in serum and specific antibody secretory IgA (sIgA) titers in fecal samples were determined by an enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates were coated at 4 °C overnight with purified recombinant proteins in a carbonate buffer, pH = 9.6. The plates were washed three times with PBS containing 0.05% tween 20 and then saturated with PBS containing 5% skim milk at 37 °C for 1 h. The serially diluted serum

and fecal samples were added in duplicate and incubated for 1 h at 37 °C. After the plates were washed three times with PBS containing 0.05% tween 20, horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, and IgA antibodies (Sigma, USA) were added to each well and incubated for 2 h at 37 °C. Then, the plates were washed three times and incubated for 15 min with 100 µL of 3,3',5,5'-tetramethyl-benzidine (TMB) substrate in dark. The reaction was terminated using 4 mol L^{-1} H_2SO_4. Finally, color intensity was measured at 450 nm with an ELISA plate reader.

2.8 Cytokine Responses

One month after the last immunization, five mice in each group were sacrificed; then, their spleens were aseptically removed, crushed and splenocytes were isolated in RPMI medium. Briefly, the spleens cell suspensions from immunized or control mice were plated at 4×10^6 cell/well. The cells were stimulated in vitro at 37 °C in 5% CO_2 with recombinant antigen (10 µg mL^{-1}). Supernatants were harvested from the cultures after 48 h of incubation. IFN-γ, IL-12, IL-4, and IL-17 in culture supernatants were measured by sandwich ELISA, using paired cytokine-specific mAbs according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN, USA).

2.9 Protection Experiments

One month after the last vaccination, mice were challenged orally with 3×10^8 CFU live *B. melitensis* 16 M. Four weeks of post-challenge, their spleens were aseptically removed, homogenized in sterile PBS, diluted, plated, and incubated; then, the number of *B. melitensis* 16 M CFU was counted. The obtained results were represented as mean $\log_{10}$CFU ± SD in each group. The protection levels were calculated as the difference between the mean of $\log_{10}$CFU from the negative control group and the mean of $\log_{10}$CFU from the experimental groups.

2.10 Lymphocyte Proliferation

As described in above, the spleens were dissected from the animals and suspended in sterile and cold PBS containing 2% FBS. Red blood cells were lysed with a lysis buffer, and splenocytes were cultured in vitro (2×10^5 cells/well) in 96-well microtiter plates in RPMI medium. Then, the cell suspension was incubated with 10 µg mL^{-1} of the recombinant proteins to antigen recall for 72 h. Before incubation ending, 100 µL of 0.5 mg mL^{-1} 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide was added per well and incubated in dark in humidified chamber at 37 °C supplied with 5% CO_2 for 30 min. After removing the media from each well, formazane crystals were solubilized using

90% acidified isopropanol (0.5% W/V SDS and 25 mmol L^{-1} HCl in 90% isopropanol). Absorbance was measured by a spectrophotometric plate reader at 590 nm. The stimulation index (S.I.) was calculated according to the average OD of stimulated wells/average OD of unstimulated wells.

2.11 Statistical Analysis of the Data

Data obtained from antibody detection, cytokine assay, cell proliferation assay, and protection experiments were analyzed using ANOVA and the statistical significance level was set at $p \leq 0.01$. The CFU data were normalized by log transformation and evaluated by analysis of variance, followed by Dunnett's post hoc test.

3 Results

Omp31 and urease were successfully expressed in bacterial cells after induction with 1.0 mmol L^{-1} IPTG. Figure 1 shows SDS-PAGE analysis of the proteins indicating the successful recombination. The recombinant proteins were then purified by the Ni-agarose beads.

The gelatin MNPs were characterized by FESEM, and the related image is shown in Fig. 2. The particles contained both 165 ± 36 nm-NPs and 1.3 ± 0.3 μm-MPs.

LEs of urease and Omp31 on the gelatin MNPs were obtained as 68 ± 3 and $65 \pm 4\%$, respectively. Moreover, Omp31/gelatin and urease/gelatin MNPs represented respectively 38 and 42% releasing amount after one first day without release on the further 4 next days.

After oral immunization of the mice, the levels of antibodies IgG, IgG1, and IgG2a in serum, and sIgA in fecal samples were determined by ELISA and compared to the negative control group. Figure 3 shows the levels of IgG (A), IgG1 (B), IgG2a (C), and sIgA (D) measured at 15, 30, and 45 days after the first vaccination. The specific IgG titer against recombinant proteins in the serum of vaccinated mice was higher than the negative control group. The IgG antibody isotypes showed higher IgG2a titers over IgG1 in all vaccinated groups. Furthermore, all immunized groups showed increased IgA titers in fecal samples.

Fig. 1 SDS-PAGE expression analysis of the recombinant Omp31 and urease. Lane 1 is for the uninduced and lane 2 is for the induced cell lysates of Omp31 (**A**) and urease (**B**) expressing *E. coli* cells
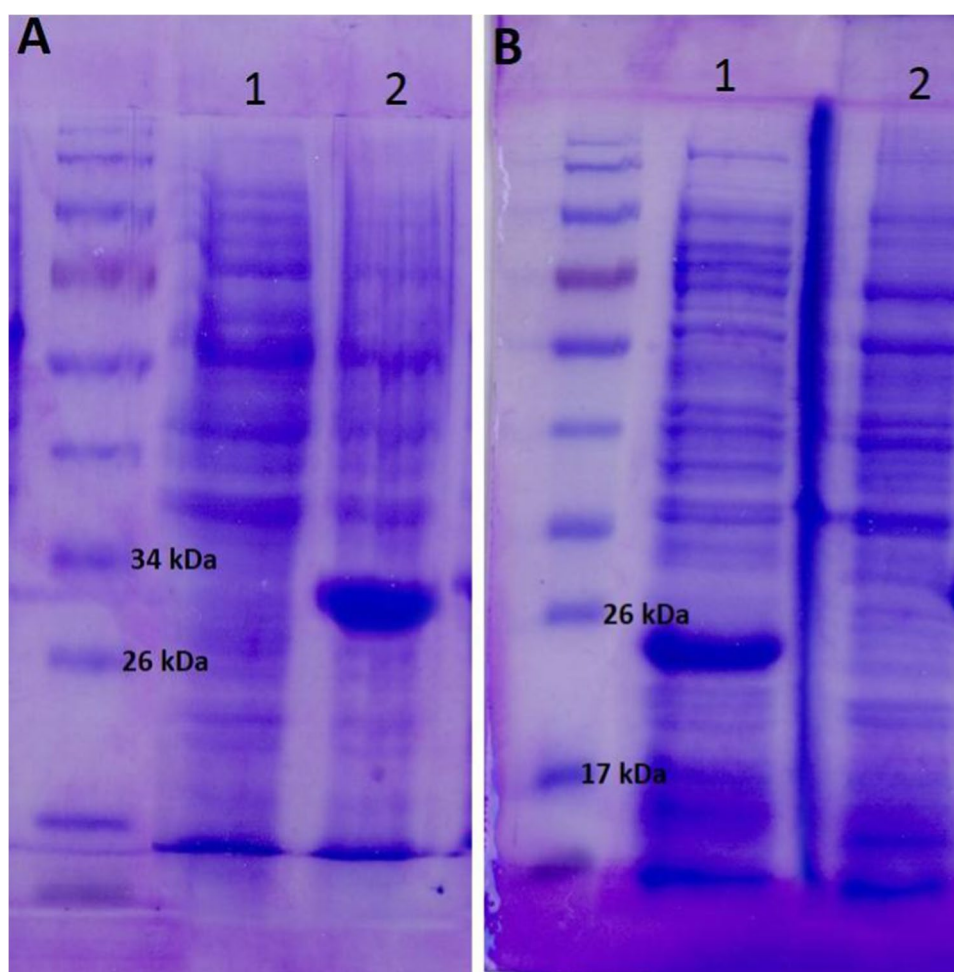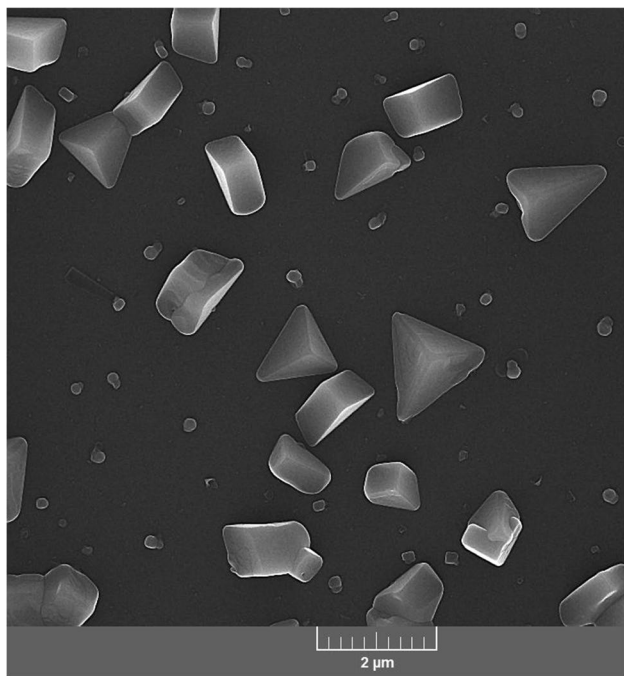
Fig. 2 A FESEM image of the gelatin MNPs

To assay the cellular immune response against *B.* antigens, we measured cytokine levels of IFN-γ, IL-17, IL-12, and IL-4 in splenocytes of different groups of mice were measured after stimulation with recombinant antigens, as shown in Fig. 4. The IL-12, IL-17, and IFN-γ secretions showed a remarkable increase in all vaccinated groups. All vaccinated groups did not induce any detectable specific IL-4 immune response. Overall, there was no significant difference between immunized groups with MNPs cocktail and Omp31/gelatin MNPs.

One month after the last vaccination, the mice of different groups were orally challenged with live *B. melitensis* 16 M. Table 1 represents the level of protection against *B. melitensis* 16 M that expressed with the *B. melitensis* 16 M CFU counts in spleens of mice. Compared to the negative control group, mice vaccinated with Omp31/gelatin or urease/gelatin MNPs represented a high degree of protection against *B. melitensis* (1.14 and 1.51 units of protection, respectively). Animals vaccinated with urease/gelatin MNPs were protected less than *B. melitensis* Rev.1-immunized mice. The mice immunized with MNPs cocktail (1.69 units of protection) displayed a somewhat higher degree of protection than animals vaccinated with Omp31/gelatin MNPs alone, albeit without reaching a statistical significance (Table 1).

To survey the ability of vaccine formulations for stimulating antigen-specific cellular immune responses, we
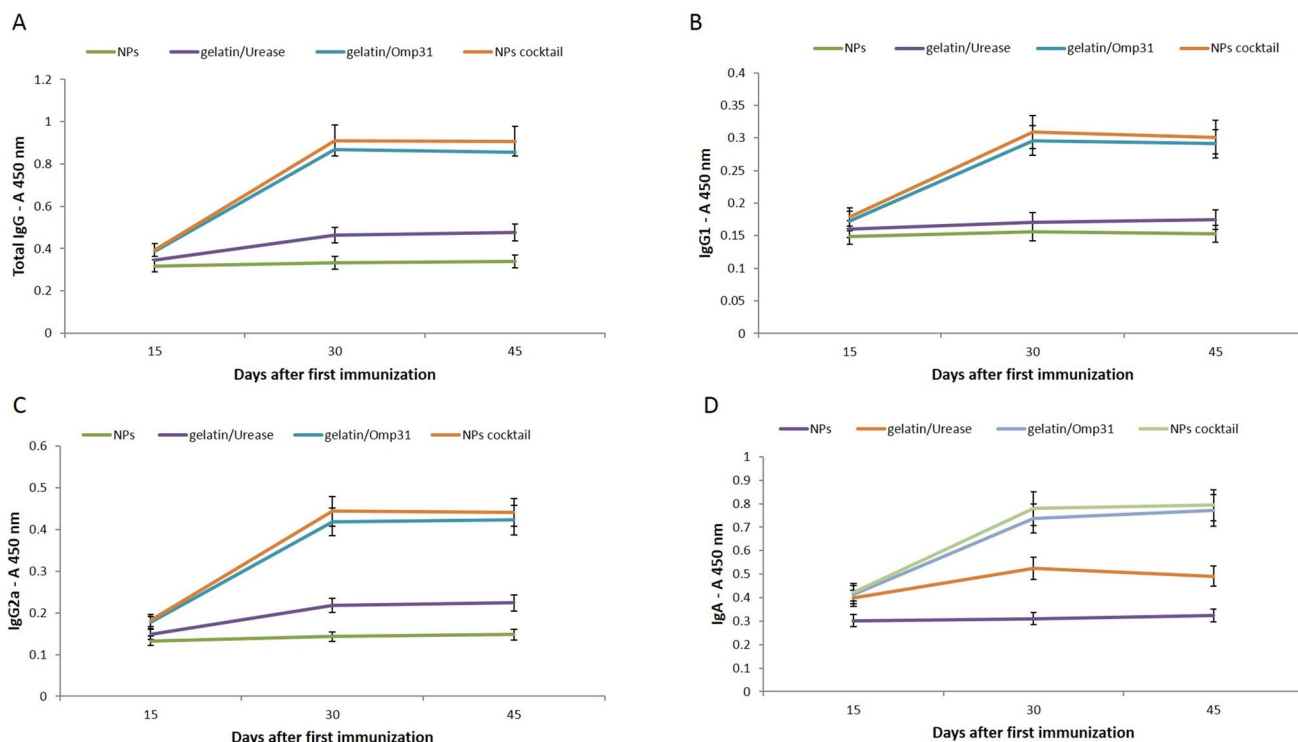

Fig. 3 The levels of IgG (**A**), IgG1 (**B**), IgG2a (**C**), and sIgA (**D**) measured on days 15, 30, and 45 after the first vaccination of different experimental groups
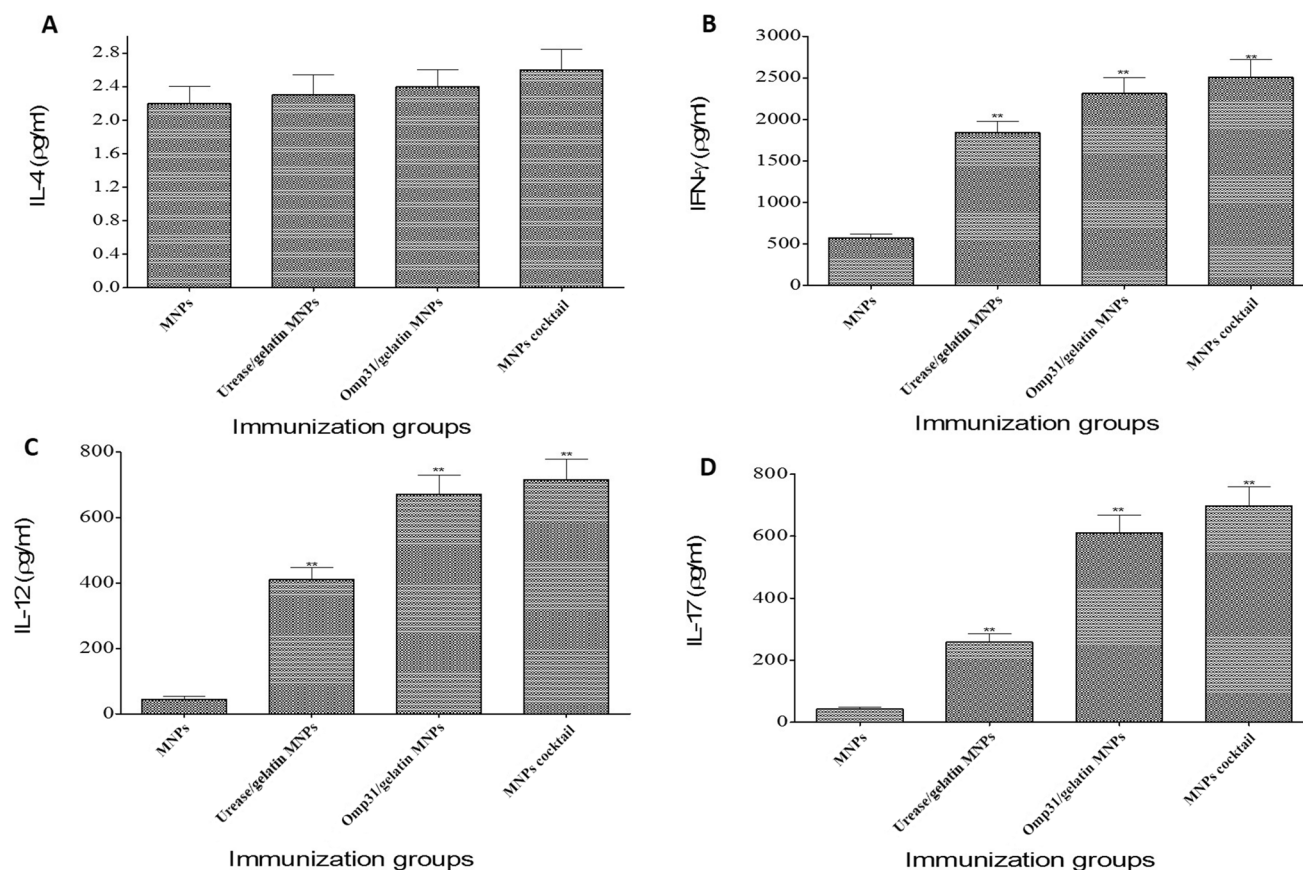
Fig. 4 The levels of IL-4 (**A**), IFN-γ (**B**), IL-12 (**C**), and IL-17 (**D**) in cell supernatants determined by ELISA

Table 1 Protection against *B. melitensis* 16 M in orally vaccinated BALB/c mice ($n = 5$)

Vaccine	Log_{10}CFU of *B. melitensis* 16 M in spleen	Protection unit*
Gelatin MNPs	$5.61 \pm 0.17^{a**}$	0
Urease/gelatin MNPs	4.47 ± 0.11^{b}	1.14
Omp31/gelatin MNPs	4.1 ± 0.16^{c}	1.51
MNPs cocktail	3.92 ± 0.13^{c}	1.69
B. melitensis Rev.1	3.99 ± 0.15^{c}	1.62

*Units of protection were determined by deducting the mean log_{10}CFU of the vaccinated groups from the mean log_{10}CFU of negative control groups

**Different letters of a, b, and c represent a significant difference between the groups ($p \leq 0.01$)

measured in vitro lymphocyte proliferation. For this purpose, 4 weeks after the last immunization, splenocytes were stimulated with recombinant antigens for 72 h, and the proliferative response was determined by the MTT assay. Figure 5 shows S.I. level of different groups of mice that corresponds to the count per minute of stimulated spleen
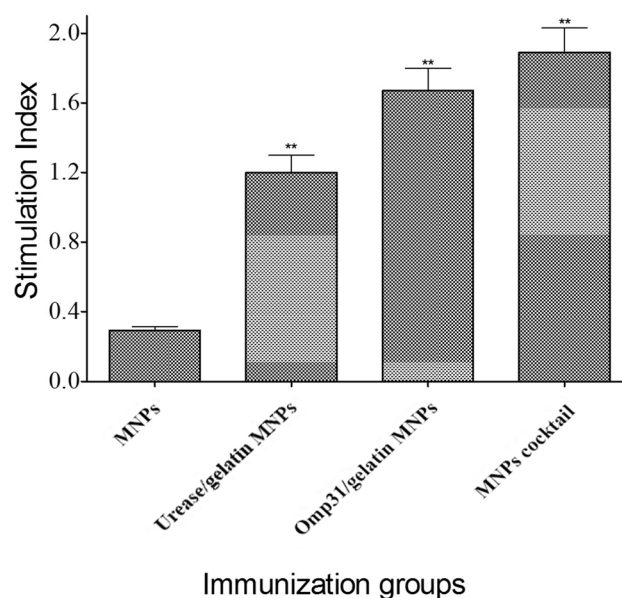

Fig. 5 Lymphocyte proliferation assay of splenocytes from mice immunized with recombinant antigens that represented as S.I

cells divided by the count per minute of unstimulated ones. Based on the results, a significantly higher cell proliferation rate in all vaccinated groups was observed, compared to the results obtained when the animals were immunized with gelatin MNPs alone. The high S.I. obviously points out the cell stimulatory activity of urease/gelatin, Omp31/gelatin MNPs and MNPs cocktail, as a reason behind the robust immune responses.

4 Discussion

Although *B*. spp. does not reside in the intestine, oral infection in animals and human is the major route of the related illness. This pathogen needs mucosal vaccine approaches that enable multiple arms of the adaptive and innate immune responses [19, 20]. In this regard, oral administration of brucellosis antigens is an attractive approach since vaccination induces the mucosal immunity for eradicating *B*. infection before they become systemic and induce the systemic immunity to inhibit release into host cells. Oral vaccination is very easy without going on working with contaminated syringes and needles. Additionally, the easy logistics of oral vaccines are significantly compatible with mass vaccination campaigns, and in most communities, children and adults are more easily compatible with oral administrations than with parenteral immunizations. However, oral immunization due to the vaccine components experiences a very degradative situation in the stomach, and encounter a hard transport obstacle extended by the tightly juxtaposed epithelial cells that are situated in the gut mucosa [21]. To resolve these problems, live attenuated microorganisms like viruses and bacteria, and particulate vaccine delivery formulations like polymeric MPs and NPs, virosomes, immune-stimulating complexes, and liposomes have been surveyed [8, 22, 23]. In the present study, we surveyed the immunogenicity and protective immune responses of Omp31 and urease, either alone or in combination, along with gelatin MNPs via oral administration route.

One of the objectives of the present study was to induce anti-*B*. IgA in mucosal sites. There is no determined role for IgA in generating protective responses against *B*. infection. However, IgA is a result of the stimulation of mucosal immunity. We examined the induction of mucosal immune responses in immunized mice by the detection of Omp31- and urease-specific IgA titers in the fecal extract. In comparison to the negative control group, animals vaccinated with urease/gelatin/, Omp31/gelatin MNPs, and MNPs cocktail showed higher IgA titers. The results were in contrast with a recent study data, indicating that oral immunization with urease/N-trimethyl chitosan (urease/NMC) NPs does not stimulate a measurable IgA immune response [24]. This shows that gelatin MNPs have the potential to stimulate IgA

immune response in the oral administration route. All vaccinated mice groups showed high IgG titer in sera. The IgG isotyping showed IgG2a was the main subtype produced in all immunized groups after oral vaccination, whereas IgG1 showed a slight increase.

Acquired cellular immunity has a key role in *B*. eradication from the infected host cells. It has been indicated that IFN-γ has a critical importance in *B*. growth inhibition. Furthermore, it has been shown that the *B*. growth inhibition is stable when IFN-γ is available even after bacterial infection. IFN-γ elicits the macrophages for effective killing; thus, making itself a necessary effector cytokine that assists in intracellular *B*. clearance in susceptible mouse model [25]. Furthermore, it was indicated that IL-17 generation plays a main role in producing immunity against *B*. infection [26]. Our results indicated that after in vitro induction, the splenocytes from all vaccinated animals generated high levels of IL-17, IFN-γ, and IL-12, whereas IL-4 level was not increased in any vaccinated group. Hence, our data indicated that oral vaccination with all formulations in gelatin MNPs stimulates a mixed Th1-Th17 immune response; this is consistent with the data by other researchers, which had indicated that oral immunization with Omp19/NMC, urease/NMC NPs and plant-expressed Omp19 elicits a remarkable cellular mixed Th1–Th17 immune response [24, 26]. However, the data was in contrast with those of a study conducted by Sudheesh et al. suggesting that subcutaneous administration of tetanus toxoid-loaded gelatin NPs induces Th1-Th2 immune responses [27]. The reason for this difference is that the type of antigen and administration route can affect the immune response type.

The obtained results indicated that the MNPs cocktail induced a degree of protection against *B. melitensis* 16 M infection equivalent to that of vaccine strain *B. melitensis* Rev.1. The protection level generated after vaccination with urease/gelatin MNPs alone was lower than the other two immunized groups and the positive control group. Compared to Omp31/gelatin MNPs alone, MNPs cocktail produced only a slight increase in the degree of protection. Although this level of increase was not found to be statistically significant, MNPs cocktail presented in this study might confer better efficiency due to cumulative effects on the antibody titers, IL-17 and IFN-γ elicitation, and S.I.

A significant S.I. was observed that represents an increase in the lymphocytic inflammatory cell milieu following immunization. It is very favorable; hence, it can be one of the efficient agents for the *B*. infection clearance.

In a study, Ghasemi et al. examined the immunogenicity of trigger factor (Tf) and Omp31 proteins with Freund's adjuvant, alone and in combination, after intraperitoneal (i.p.) immunization in mice. The data showed that mice vaccinated with Tf protein and a combination of Tf and Omp31 proteins exhibited a protective level equivalent to the amount of protection

offered by vaccine strain *B. melitensis* Rev.1. However, the protection level caused by vaccination with Omp31 alone was lower than the protection level obtained by commercial vaccine *B. melitensis* Rev.1 [28].

Cassataro et al. showed that intraperitoneal (IP) vaccination of BALB/c mice with the chimeric protein containing *B. lumazine synthase (BLS)* and Omp31 (BLSOmp31) in Freund's adjuvant-induced the highest protective responses level against *B. ovis* challenge, which was more than the co-delivery of both antigens (Omp31 + BLS) and equivalent to the positive control vaccine strain *B. melitensis Rev.1*. Additionally, chimeric protein BLSOmp31 elicited protective responses against *B. melitensis* infection, but to a lesser degree than *B. melitensis Rev.1* strain. To sum up, their results showed that the chimeric protein exhibited a better protection than the combination of its components against *B. ovis* and *B. melitensis* [29]. According to Cassataro's results, it seems that urease and Omp31 fusion can generate better protection unit than their cocktail form. Additionally, their results showed that the protection created by the antigens cocktail against *B. melitensis* was equivalent to the protection provided by Omp31 alone. Therefore, our results are consistent with those in the course of protection against *B. melitensis*, so that the protection created during the use of urease and Omp31 cocktail was equivalent to the protection created by Omp31 alone.

To date, the use of several NPs and MPs has been reported in the field of vaccination against brucellosis. For example, Singh et al. used poly(lactic-co-glycolic acid) (PLGA) NPs to deliver the L7/L12 recombinant antigen in the IP route. The results showed Th1 immune response was induced. In addition, a high level of protection by this nanoformulation was obtained against the pathogenic strain of *B. abortus* 544 in mice [30]. In 2013, Goel et al. used liposomes and PLGA MPs to deliver Omp25 antigen in the intradermal administration route. In both cases, Th1-Th2 immune response was stimulated, but the amount of protection induced by the liposome MPs was higher than PLGA MPs [31]. In another study, it was shown that oral vaccination with NMC/Omp31 NPs induces Th1-Th17 immune responses, wherein oral immunization with NMC/Omp31 NPs confer equivalent protection to the control vaccine *B. melitensis Rev.1* in mice against *B. melitensis* 16 M infection [18]. Hence, gelatin MNPs, like NMC NPs, can stimulate the Th1-Th17 immune response via the oral route. In comparison to NMC NPs, gelatin MNPs seem to have a higher ability to stimulate Th17 and IgA immune responses in the oral immunization route.

5 Conclusion

The obtained results in this study showed that the MNPs cocktail induces robust immune responses against its components. Furthermore, the vaccination with MNPs cocktail generated a significant decrease in bacterial load in mouse spleens. Hence, the antigenic cocktail might be a suitable vaccine candidate for the design and development of a chimeric subunit vaccine that induces a high protection level against *B. melitensis*. Furthermore, the results showed the potential of gelatin MNPs as a potent and appropriate adjuvant and delivery system for orally administered *B.* antigens.

Data Availability All data generated or analysed during this study are included in this published article.

Declarations

Informed Consent None.

Conflict of Interest The authors declare no competing interests.

Research Involving Humans and Animals Statement All procedures performed in this study involving animals were in accordance with the ethical standards of the medical research ethics committee at Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1396. S209).

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