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Immunogenicity of glycine nanoparticles containing a chimeric antigen as *Brucella* vaccine candidate

Purpose: Brucellosis as a worldwide zoonotic illness affect domestic animals and humans doesn't have any vaccine for the prevention of infection in humans yet. The aim of this study was to evaluate the specific immune response following the administration of glycine nanoparticles as adjuvant and delivery system of a chimeric antigen contained trigger factor, Omp31, and Bp26 in murine model.

Materials and Methods: The chimeric antigen of *Brucella* was cloned and expressed in *Escherichia coli* (*E. coli*) BL21 (DE3). Purification and characterization of recombinant protein was conducted through Ni-NTA (nickel-nitrilotriacetic acid) agarose, SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and Western blot. Nanoparticle characteristics including morphology, particle size distribution, zeta potential, protein retention rate, and release rate were measured *in vitro*. Subsequently, nanoparticle contained antigen was administered to mice and blood sample was taken to measured the antibody level.

Results: The protein retention in the nanoparticles was successfully done and the nanoparticle characteristics were appropriate. The average size of glycine particles containing antigen was about 174 nm, and the absorption of protein was approximately 61.27% of the initial value, with a release rate of approximately 70% after 8 hours. Enzyme-linked immunosorbent assay result proved that the immunized sera of mice which were administered with nano-formula contains high levels of antibodies (immunoglobulin G) against recombinant chimeric antigen and also a high level of mucosal antibody (immunoglobulin A) in the oral group, which showed a desirable immunity against *Brucella*.

Conclusion: The results showed that chimeric antigen-loaded glycine nanoparticles can act as a vaccine candidate for inducing the cellular and humoral immune response against brucellosis.

Keywords: Nanoparticles, Vaccine, Brucellosis, Glycine, Chimeric antigens

Introduction

Brucellosis is known as a worldwide zoonotic illness contributes to abortion and infertility in domestic animals and Malta fever in humans [1]. Human brucellosis is often caused by *Brucella suis*, *B. melitensis*, and *B. abortus*. Among them, *B. melitensis* has the most intense pathogenicity for humans; while other species cause human infection occasionally [2]. Generally, some factors such as skin breakage, mucosal contact, and inhalation of contaminated ingredient contribute to human's infection through contacting with infected animals or consumption of contaminated dairy products [3].

Nowadays, brucellosis is a crucial health issue in many countries and the problem is that there is no vaccine available for prevention in humans yet. Besides, due to *Brucella*'s intracellular residence, there are a few efficient antibiotic accessible [4]. Thanks to live-attenuated or killed vaccines (for instance, *B. melitensis* Rev.1, *B. abortus* RB51, and *B. abortus* S19), brucellosis has been approximately controlled in domestic animals [5]. However, some disadvantages considered for these vaccines which can limit their usage, such as causing sickness in humans through transferring to the pathogenic form as well as abortion in domestic animals [6]. In this regard, there is a significant demand to produce effective vaccines against human brucellosis or even the animal ones. Recent researches have proven that recombinant subunit vaccines candidates are so beneficial, due to their nonpathogenic essence and their possibility of manipulating to reach the most desirable properties parallel with decreasing the unpleasant ones. On the other hand, recombinant subunit vaccines have been known as well-defined vaccines with a high level of yield and purity [7]. There are only few antigenic components which have desirable immunogenic function against brucellosis such as trigger factor (TF, a cytoplasmic protein), Bp26 (a periplasmic immunogenic protein), and Omp31 (an outer membrane protein), known as protective immunogenic antigens and their efficacy have been proven by many researches [8].

In contrast to whole bacterial vaccine, recombinant vaccines are approximately less immunogenic. In order to enhance efficacy, they demand immune-stimulating or adjuvant-stimulating compounds which can specifically increase the immune responses of these weakened antigens. Additionally, the importance of the delivery system and the route of administration in immunization of the vaccines are taken for granted [9]. It should be mentioned that, incorporation of antigens into nanoparticles as an adjuvant and efficient delivery system leads to increase the antigen uptake by antigen presenting cells. Furthermore, nano-adjuvants can protect these antigens inside them against undesirable conditions, such as low pH and enzymes activity. For these reasons, they can be so beneficial for oral and nasal vaccines which their entrance to the body is through the mucosal surfaces [10-12]. Spray freeze dryer (SFD) is a drying technology with ultra-fast freezing rates, formed through low-liquid nitrogen temperature. This can result in homogeneous embedding of the antigens and minimizing the probability of phase separation between protein and the phase of excipients surrounded on a molecular scale [13-15]. Moreover, stability, dissolution rates, and aro-

sol function of dry powder inhalers can improve through utilization of SFD [16-19]. Adding different amino acids like glycine, to the spray drying formulation leads to the prevention of powder aggregation as well as improvement of spray properties dramatically [20,21]. This can be an impressive overview which used to combine low dosage antigens in a suitable matrix like glycine [22]. The aim of present research was to conduct assessment of specific immune responses against a recombinant chimeric antigen TF/Bp26/Omp31 (TBO) with glycine nanoparticles as an effective adjuvant and delivery system. Furthermore, other purpose was to investigate the most influential administration routes and their efficacy on immune responses. For this purpose, immunization conducted in BALB/c mice, and different isotypes of antibodies production were measured by indirect enzyme-linked immunosorbent assay (ELISA), based on the previous research.

Materials and Methods

Plasmid and bacterial strains

B. abortus 544 and *B. melitensis* 16 M which obtained from Razi Vaccine and Serum Research Institute, Karaj, Iran. *E. coli* BL21 (DE3) were applied in the protection assay. pET28a vector (Novagen, Madison, WI, USA) were also used for the expression of recombinant protein.

Mice and ethics statement

The 6-8-week-old female BALB/c mice (Pasteur Institute of Iran, Tehran, Iran) housed in standard polypropylene cages at 20°C to 22°C and 12-hour light/dark cycles. All experimental procedures on animals were officially agreed by the Iran National Committee for Ethics in Biomedical Research (IR. IAU.PS.REC.1397.114).

Expression and purification of recombinant protein

The TBO recombinant protein was characterized and prepared as previously described [6]. Briefly, *E. coli* BL21 (DE3) containing pET28a-TBO was inoculated into 1,000 mL of LB medium containing kanamycin (50 µg/mL). The incubation was continued with agitation (180 rpm) to 0.5 optical density value at 600 nm and then gene expression was induced by IPTG (isopropyl β-d-1-thiogalactopyranoside) in 37°C for 4 hours. The IPTG concentration was optimized by adding different quantity of IPTG to define the best concentration. The bacteria were separated by centrifugation at 6,000 rpm, 10 minutes, 4°C, then resuspended in lysis buffer (8 M urea, 0.1

M NaH₂PO₄, 0.01 M Tris hydrochloride, and 0.02 M imidazole). The recombinant subunit protein was purified using Ni-NTA (nickel-nitrilotriacetic acid) resin (Qiagen, Manchester, UK). For this purpose, proteins were eluted by elution buffer (1 mL buffer containing 250 mM imidazole; Sigma-Aldrich, St. Louis, MO, USA) after several washing steps using different concentrations of imidazole. To remove imidazole and urea, the protein elution was dialyzed in the de-ionized water for 72 hours in cold room, and then stored at -70°C. After all, Bradford protein assay conducted to estimate recombinant protein concentration.

Protein (antigen) characterization

The purified protein *E. coli* BL21 (DE3) containing pET28a-TBO were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot using horseradish peroxidase (HRP) conjugated anti His-tag antibody (Sigma-Aldrich).

Glycine nanoparticle preparation

Glycine at 40 mg concentration was dissolved on stirring in 7 mL of deionized water containing 4 mg of purified TBO protein. The solution was sprayed (with the capillary diameter of 0.1 μm) into a container with liquid nitrogen. The solid mixture phases formed through liquid nitrogen, and then freeze-drying was done with a laboratory scale freeze dryer (NIIC SB RAS, Novosibirsk, Russia) at -5°C and continued for 5 hours. Finally, the sample was weighted and stored at room temperature after transferring into a pre-weighted vial [23].

Nanoparticle characterization

Dynamic light scattering (DLS) (Malvern 3000; Malvern Instruments, Malvern, UK) was used to determine zeta potential and size of the glycine nanoparticles. Furthermore, scanning electron microscope (SEM) illustrated morphology and the size range of nanoparticles.

In order to analyze protein loading in the nanoparticles, triplicate samples were examined after encapsulation and the amount of protein entrapped in the nanoparticles was calculated by the difference between the total protein added to the solution and the amount of non-entrapped protein remaining in the supernatant. A non-loaded nanoparticle suspension (without recombinant protein) was used as a blank to correct any unwanted interference by glycine nanoparticle. The loading efficacy (LE) and the loading capacity (LC) of the antigen loaded nanoparticles were calculated from following

equations, respectively.

$$LE (\%) = \frac{Protein_{total} - Protein_{free}}{Protein_{total}} \times 100$$

$$LC (\%) = \frac{Protein_{total} - Protein_{free}}{Nano\ particles\ dry\ weight} \times 100$$

To determine *in vitro* release study, synthesized nanoparticles containing 1 mg of subunit recombinant antigens were re-suspended in 5 mL of 0.1 M phosphate-buffered saline (PBS) buffer (pH 7.4) and maintained at 37°C under stirring (100 rpm). Then, at various and specified intervals, 1 mL of the suspension was separated and centrifuged (16,000×g, 15 minutes) to measure the concentration of released protein in the supernatant with the Bradford protein assay. The same volume of fresh PBS buffer was added to the release medium to reach the original volume. A sample consisting of only non-loaded glycine nanoparticles re-suspended in PBS was used as a blank.

Mice vaccination by subunit recombinant antigens

BALB/c mice aged 6 to 8 weeks old (Pasteur Institute, Tehran, Iran) were immunized 3 times at days 0, 14, and 28. The mice were classified into five groups: first group was immunized directly by nasal administration of 20 μg protein along with glycine nanoparticles, the second group received 20 μg protein along with glycine orally, and the third and fourth group were injected to intraperitoneal same amount of protein along with glycine and complete Freund's adjuvant (Sigma-Aldrich; considered as standard group), respectively (protein was mixed with an equal volume of complete Freund's adjuvant). Last group was administered intraperitoneally only with PBS served as negative control. Blood samples were collected at days 11, 24, and 38, centrifuged at 1,000 g for 5 minutes; sera were separated and stored at -20°C.

Enzyme-linked immunosorbent assay

Specific isotypes (total immunoglobulin G [IgG], IgG1, IgG2a, IgG2b, and immunoglobulin A [IgA]) were determined by isotyping ELISA kit (Sigma-Aldrich). ELISA 96-well microplates were coated with 10 μg/mL of purified protein and kept at 37°C for 1 hour. Microplate washing was repeated 3 times after each step. In order to prevent nonspecific binding, blocking buffer (5% skim milk in PBS) was added to plates and maintained for 1 hour at 37°C. Microplates incubation was conducted with serially diluted sera (1:500 to 1:64,000) at

37°C for 1 hour. Wells were incubated by 100 µL/well rabbit anti-mouse HRP conjugated antibody at 37°C for 1 hour. Finally, 100 µL tetramethylbenzidine substrate (TMB; Sigma-Aldrich) was added to each well and incubated at room temperature for 15 minutes, the reaction was stopped after color development and the absorbance was read at 450 nm using microplate reader (Bio-Rad, Hercules, CA, USA).

Protection assay

To determine the potency of TBO-loaded glycine nanoparticles in protection against virulent *B. melitensis* 16 M and *B. abortus* 544, challenge was measured in vaccinated BALB/c mice. For this purpose, 1 month after the final immunization, three mice from each group were challenged with 10⁴ colony-forming unit (CFU) of *B. abortus* 544 and the other three mice were challenged to the same extent of *B. melitensis* 16 M through intraperitoneal injection route of the administration. Four weeks after the challenge, mice were sacrificed by cervical dislocation, then their spleens were extracted, homogenized, diluted, and plated out on *Brucella* agar at 37°C for 2–3 days to specify the number of *Brucella* colonies. The results were illustrated as the mean log₁₀ CFU ± standard deviation of protection per group and protection units were measured by subtracting the mean log₁₀ CFU for the immunized groups from the mean log₁₀ CFU of the negative control group [24] (Table 1).

Statistical analysis

Data obtained from protection assay and antibody determination were analyzed using the independent-sample T-test analysis. All p-values ≤0.05 were considered as statistically significant. The CFU data were normalized by log transformation and evaluated by analysis of variance, followed by Dunnett’s post hoc test.

Results

Recombinant protein expression

Different concentrations of IPTG were evaluated and 1 mM was selected as the optimal concentration, induced *E. coli* BL21 which was transformed with pET28a-TBO with the N-terminal 6X-His tag. After that, recombinant TBO was successfully expressed in *E. coli* cells and the SDS-PAGE analysis illustrated the attendance of recombinant protein as a significant band (Fig. 1A). The average yield of recombinant protein was about 0.6 mg/mL of culture. Also, western blotting results

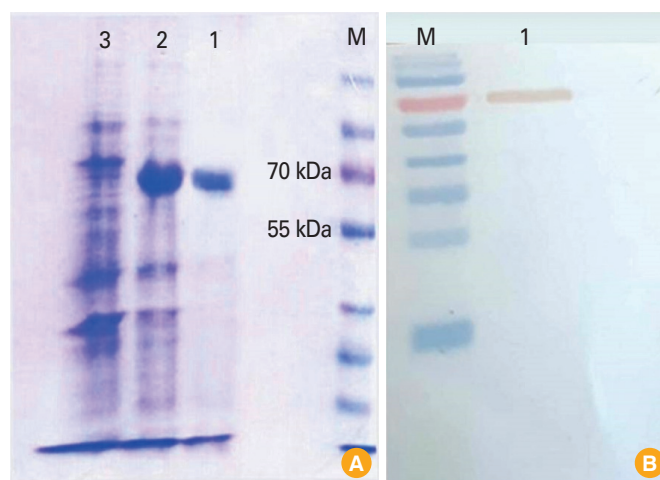


Fig. 1. (A) An expected rTBO protein was specified by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis); lane 1 illustrates the elution of purified rTBO at 250 mM imidazole concentration. Lanes 2 and 3 define the induced and uninduced cell lysate of rTBO transformed *E. coli*, respectively. As can be seen in the figures, a 67 kDa band indicates the correct expression of the desired protein. (B) Western blot using an anti-His antibody showed a single band, with expected size of recombinant protein; lane 1 illustrates the IPTG (isopropyl β-d-1-thiogalactopyranoside) induced *E. coli* contained pET28a-TBO. Lane 2 defines uninduced *E. coli*. M, protein size marker; rTBO, recombinant trigger factor/Bp26/Omp31.

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

Studied groups	Log ₁₀ CFU of <i>B. abortus</i> 544 in spleen	Protection unit in spleen (log units)	Log ₁₀ CFU of <i>B. melitensis</i> 16 M in spleen	Protection unit in spleen (log units)
Negative control-PBS	5.98 ± 0.55	-	5.33 ± 0.51	-
NP+Ag (injection)	4.25 ± 0.47	1.73	3.74 ± 0.33	1.59
NP+Ag (nasal)	4.43 ± 0.33	1.55	3.86 ± 0.41	1.47
NP+Ag (oral)	4.55 ± 0.25	1.43	3.92 ± 0.31	1.37
Freund+Ag (injection)	4.85 ± 0.25	1.13	4.22 ± 0.19	1.11

Units of protection were specified by detracting the mean log CFU of the immunized groups from the mean log CFU of negative control groups. The difference between groups was evaluated by independent-sample T-test and comparisons were considered significant at p < 0.05.

CFU, colony-forming unit; *B. abortus*, *Brucella abortus*; *B. melitensis*, *Brucella melitensis*; PBS, phosphate-buffered saline; NP, nanoparticle; Ag, antigen.

showed that anti-His-tag antibody detected recombinant chimeric antigens (Fig. 1B).

Nanoparticle characterization

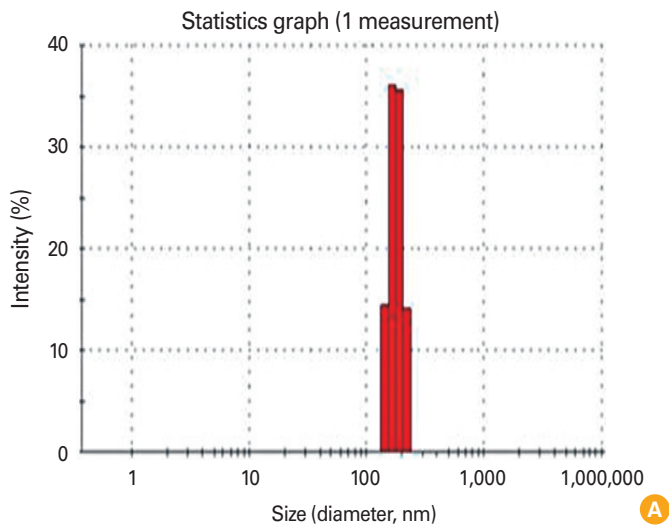
DLS showed that most of glycine/TBO nanoparticles had a mean size distribution of 150–200 nm with the zeta potential of -14.1 (Fig. 2A, B). Furthermore, SEM images displayed the size of particles smaller than measured with DLS (between 90 and 140 nm). Also, smooth surface and spherical morphology of nanoparticles were illustrated by SEM images (Fig. 2C).

Generally, both DLS and SEM define the size range under 200 nm. The loading efficiency and loading capacity of TBO was measured as 61.26% and 12%, respectively. The protein release study that was conducted at simulated body temperature and pH, followed a time dependent manner. According

to the graph, the release of protein loaded in the nanoparticles after about 8 hours was approximately 70%, which was gradually released and being accessible to the immune system due to the biodegradability of the nanoparticles (Fig. 2D).

Serum antibodies detection

The ELISA results after each sampling at days 11, 24, and 38 indicated that total serum antibodies against chimeric antigen in the groups of mice subjected to injection, nasal and oral administration of TBO-loaded glycine nanoparticles increased significantly compared to the group subjected to intraperitoneal injections of PBS alone (as negative control) (p-value <0.05), besides, in all three groups that received nanoformulated antigens, the immune response was not statically significant in comparison with those received intraperitoneal



Results	Mean (mV)	Area (%)	Width (mV)
Zeta potential (mV): -14.1	Peak 1: -14.1	100.0	4.20
Zeta deviation (mV): 4.20	Peak 2: 0.00	0.0	0.00
Conductivity (ms/cm): 0.975	Peak 2: 0.00	0.0	0.00
Result quality: good			

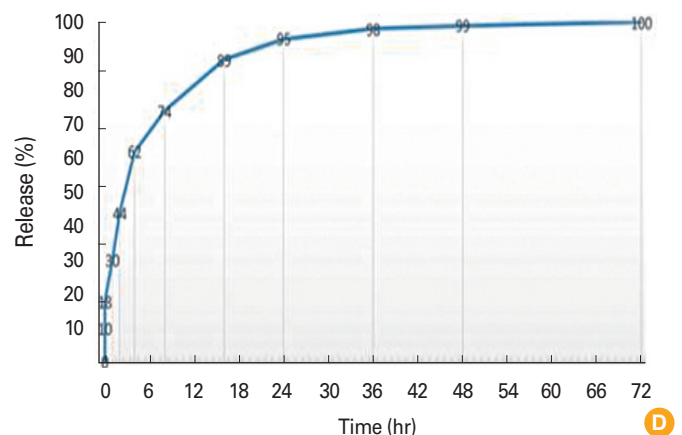
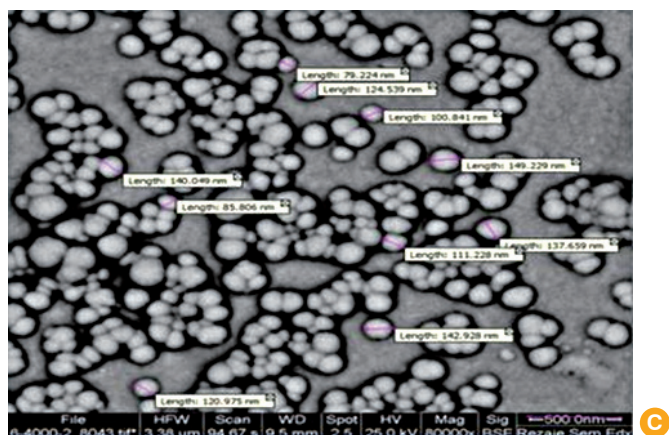
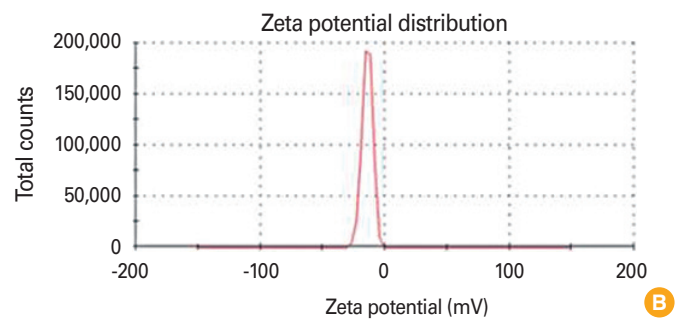


Fig. 2. (A) Particle size distribution chart for glycine nanoparticle. According to the chart, 80% of particles size is between 150 to 200 nm, (B) glycine nanoparticles zeta potential graph, (C) scanning electron microscope view of glycine nanoparticles containing antigens, and (D) release profiles of TBO from TBO–glycine NPs at pH 7.4 at 37°C for 72 hours, calculated as a cumulative percent. TBO, trigger factor/Bp26/Omp31; NP, nanoparticle.

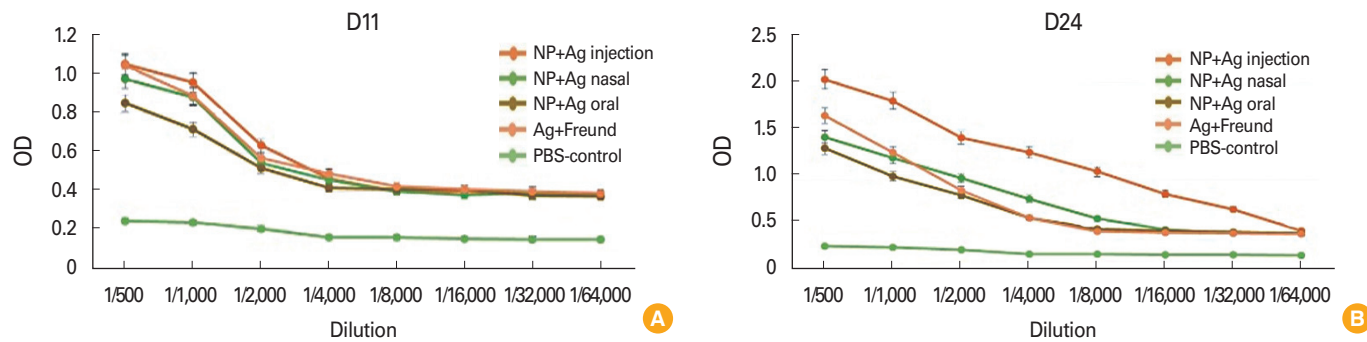


Fig. 3. Anti-TBO antibody levels: enzyme-linked immunosorbent assay was conducted to analyze the sera in triplicates for glycine loaded TBO specific IgG antibodies with comparison to the control and antigens-Freund groups. (A–C) the IgG titration results in the sera of all five groups after each blood-sampling on days 11, 24, and 38, respectively. TBO, trigger factor/Bp26/Omp31; IgG, immunoglobulin G; OD, optical density; NP, nanoparticle; Ag, antigen; PBS, phosphate-buffered saline.

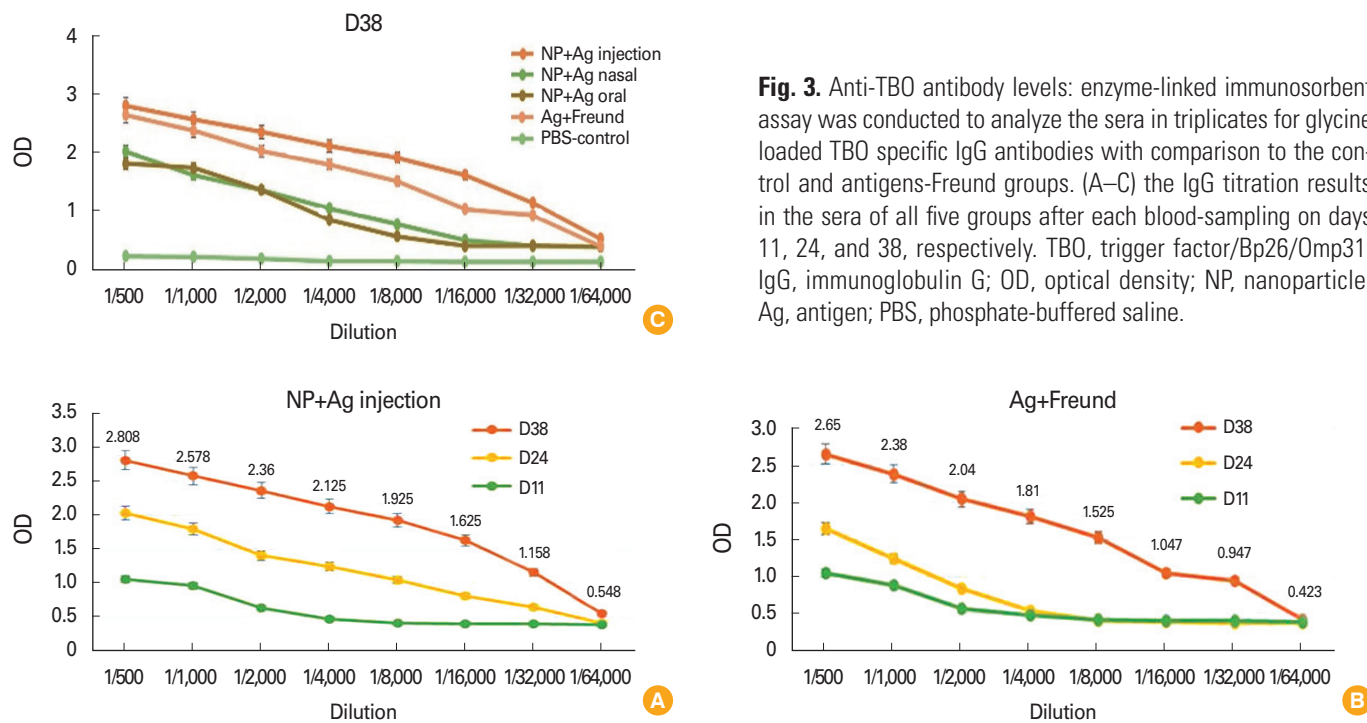


Fig. 4. Comparison of immunoglobulin G titration antibodies after blood-sampling in different days in (A) intraperitoneal injections of nanoformulated antigen and (B) antigen-Freund. OD, optical density; NP, nanoparticle; Ag, antigen.

injections of antigen-Freund (standard group) (p -value >0.05) (Fig. 3A–C). Total serum antibodies titer in per group per administration was statically significant compared to the previous steps, in those subjected intraperitoneal injections of nanoformulated antigen and antigen-Freund (p -value <0.05) (Fig. 4A, B). In order to determine the type of immune response, the ratio of IgG2a to IgG1 was calculated. All groups determine this ratio less than 1, which indicates the induction of humoral immunity against the recombinant antigens (Fig. 5A). IgA/IgG1 ratio was calculated in each group and it was shown that intraperitoneal injection of nanoformulated antigen and nasal administration did not elicit any detectable IgA levels in the serum while oral immunization with glycine loaded TBO showed increased levels of IgA (Fig. 5B).

Protection test

Counting the bacterial colonies indicates a significant increase in protective response in the spleen of all immunized mice subjected to nanoformulated antigen and antigens-Freund compared to the control group (p -value <0.05). Compared with the negative control group, the mice that were administered TBO-loaded glycine intraperitoneally showed a higher degree of protection units when challenged with *B. melitensis* 16 M and *B. abortus* 544, with the protection units of 3.74 and 4.25, respectively. Moreover, log protection units in spleen in those groups immunized through nasal and oral administration was more than antigens-Freund group.

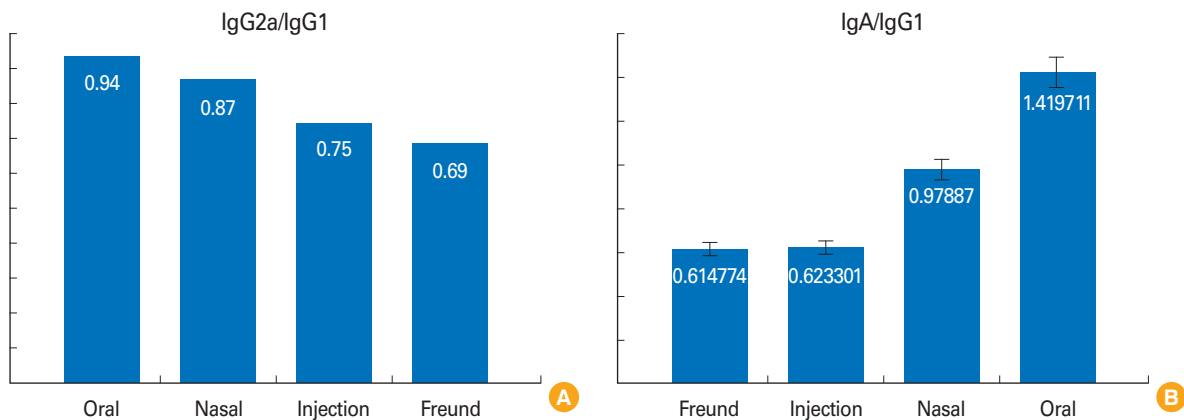


Fig. 5. (A) Evaluation of IgG2a/IgG1 antibody ratio to specify the type of immune response. (B) IgA/IgG1 antibody ratio to determine mucosal immunization. IgG, immunoglobulin G; IgA, IgG, immunoglobulin A.

Discussion

Various researches in brucellosis control have indicated that an efficient application of vaccines is required to prevent the spread of this disease in animals and humans. Many cell surface and intracellular components have been designed and evaluated as protective subunit vaccines against brucellosis in BALB/c mice [25-27]. Recent studies have shown that proteins like Bp26, TF, and Omp31 have been known to induce a protective and impressive immune response, and are used as *Brucella* vaccine candidates. In the present study, the immune response stimulation of TBO-loaded glycine nanoparticles with the size range of 100–200 nm in BALB/c mice was investigated. In this study, for the first time glycine nanoparticle is used as an adjuvant and antigen carrier. The recombinant subunit antigen was produced in *E. coli* BL21 (DE3) and confirmed by SDS-PAGE and western blotting assay (Fig. 1). Glycine nanoparticles had appropriate characteristics and size (Fig. 2). Previous studies also have proven that particles smaller than one micron are easily absorbed by phagocytic cells [28]. These particles will be ideal for swallowing through immune cells. Due to biodegradability of nanoparticles, the results obtained from release study in 72 hours, indicated that the process of antigen releasing from nanoparticles was gradual. The above antigen does not spontaneously dissipate from nanoparticles and only released through biological degradation of above particles. Due to the body entrance of *Brucella* via consumption of contaminated water and food, mucosal immunity can monitor the infection as first and the most crucial line of defense before bacteria reaches the bloodstream [29]. As IgA titration can demonstrate the stimulation of common mucosal immune system, one of the most

significant goals of this experiment was the induction of anti-*Brucella* IgA. Our results showed that orally administration of TBO-loaded glycine nanoparticles was, significantly enhanced the specific anti-TBO IgA in comparison with the other groups (Fig. 5B). This outcome indicates that the nanoparticles have been able to protect antigens against digestive enzymes and increased the production of mucosal antibodies. On the other hand, intraperitoneal oral and nasal administration of TBO-glycine is able to stimulate a strong IgG response comparing to negative control groups. It should be noted that the Freund adjuvant, in spite of some disadvantages such as severe pain in the injection site and the potential for serious complications (abscess, chronic granuloma, and wound necrosis) that limits its usage not only in humans but also in animals, is one of the most popular adjuvants thanks to its potential to stimulate the humoral immune response with high production titer of antibodies. Therefore, the absence of significant difference in titration of antigens-Freund with the other groups showed the high efficacy of nanoformulated antigen. Our results are in agreement with observations by Chen et al. [30], showing that intraperitoneally injection of antigens-loading nanoparticles into mice generated high levels of IgG titers but low IgA titers. By contrast, oral administration of antigens-loading nanoparticles elicited high titers of IgA [30]. Moreover, the isotype antibody responses (IgG1 and IgG2a) offer that oral immunization with glycine nanoparticles may direct the antigen-specific immune response towards cellular immunity (Fig. 5A). Similar results were observed after a nasal immunization [31]. Vaccination route selection contributes to effectiveness of antigens. Generally, protective effect of nanoformulated TBO is proven by significant increase in protective response

against pathogenic bacteria compared with the control group. According to protection obtained in this experiment, intraperitoneal administration of TBO-loaded glycine nanoparticles can generate a better immune response in comparison with oral and nasal administration and the administration of antigens-Freund adjuvant. In this study we show that the protection units of all groups which were administered with nanoparticles were higher than the Freund group. In accordance with the previous research, the advantageous efficacy of nanoparticles as a carrier and delivery system was clearly observed in this study, through providing an effective protection to the body by gradual release of antigen and conserving this antigen against digestive enzymes which contributes to high titer of mucosal antibodies production [32]. The vaccination studies, indicated that although oral administration of TBO-loaded glycine nanoparticles was able to induce high level of IgA immune responses and a shift in immune response to cellular immunity, it failed to induce the highest level of protection against *B. melitensis* 16 M and *B. abortus* 544 in comparison with intraperitoneal injection of nano vaccine. Similar to our report, low degree of protection and local immune responses through nasal administration of Bp26 plus trigger factor with cholera toxin is reported in a study [33]. This could happen through the entrance of bacteria to the body that has been systemically administered (intraperitoneal injection), and naturally systemic immunity (IgG) is the protective antibody. Since the entrance of bacteria is through the mucosal membrane (oral or inhalable), the oral or nasal rout administration indicate more effective protection regard to IgA secretion as protective antibody in mucosal immune system.

In conclusion, all groups administrated with TBO-loaded glycine nanoparticles increased specific immune responses and protection. The present study also supplies a hint that glycine nanoparticles can be served as components of future vaccines to prevent infection via oral administration to induce high level of local immune responses. This is an ongoing project and brucellosis demand more investigations focusing on the context of increasing the effectiveness of nano vaccine with using various nano-adjuvants or specific delivery systems against *Brucella*.

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