CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Clin Exp Vaccine Res 2024;13:232-241
https://doi.org/10.7774/cevr.2024.13.3.232
pISSN 2287-3651 • eISSN 2287-366X

Intranasal and intraperitoneal immunization against *Brucella* infection using niosome and mannosylated niosomes containing *Brucella* recombinant trigger factor/Bp26/Omp31 chimeric protein in a mouse model

Fahimeh Sharif[1], Razieh Nazari[1], Mahdi Fasihi-Ramandi[2], Ramezan Ali Taheri[3], Mohsen Zargar[1]

[1]Department of Microbiology, Islamic Azad University Qom Branch, Qom; [2]Molecular Biology Research Center and [3]Nanobiotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

Received: February 19, 2024
Revised: May 22, 2024
Accepted: June 29, 2024

Corresponding author: Mahdi Fasihi-Ramandi, PhD
Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, No. 14359 15371, Mollasadra St., Vanak Square, Sheykh Bahayi st, Tehran, Iran
Tel: +98-9029029868, Fax: +98-03155505551
E-mail: fasihi.m@bmsu.ac.ir

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

Thanks to guidance and advice from the "Clinical Research Development Unit of Baqiyatallah Hospital" and the officials of the laboratories of Qom Islamic Azad University.

Purpose: Brucellosis, a zoonotic infectious disease, is a worldwide health issue affecting animals and humans. No effective human vaccine and the complications caused by the use of animal vaccines are among the factors that have prevented the eradication of the disease worldwide. However, bio-engineering technologies have paved the way for designing new targeted and highly efficacious vaccines. In this regard, the study aimed to evaluate immunity induced by mannosylated niosome containing *Brucella* recombinant trigger factor/Bp26/Omp31 (rTBO) chimeric protein in a mouse model.

Materials and Methods: rTBO as chimeric antigen (Ag) was expressed in *Escherichia coli* BL21 (DE3) and, after purification, loaded on niosome and mannosylated niosome. The characteristics of the nanoparticles were assessed. The mice were immunized using rTBO, niosome, and mannosylated niosome-rTBO in intranasal and intraperitoneal routes. Serum antibodies (immunoglobulin [Ig]A, IgG, IgG1, and IgG2a) and splenocyte cytokines (interferon-gamma, interleukin [IL]-4, and IL-12) were evaluated in immunized mice. Finally, immunized mice were challenged by *B. melitensis* and *B. abortus*. A high antibody level was produced by niosomal antigen (Nio-Ag) and mannosylated noisomal antigen (Nio-Man-Ag) compared to the control after 10, 24, and 38 days of immunization. The IgG2a/IgG1 titer ratio for Nio-Man-Ag was 1.2 and 1.1 in intraperitoneal and intranasal methods and lower than one in free Ag and Nio-Ag. Cytokine production was significantly higher in the immunized animal with Ag-loaded nanoparticles than in the negative control group ($p<0.05$). Moreover, cytokine and antibody levels were significantly higher in the injection than in the inhalation method ($p<0.05$).

Results: The combination of mannosylated noisome and rTBO chimeric proteins stimulate the cellular and humoral immune response and produce cytokines, playing a role in developing the protective acquired immune response in the *Brucella* infectious model. Also, the intraperitoneal route resulted in a successful enhancement of cytokines production more than intranasal administration.

Conclusion: Designing an effective vaccine candidate against *Brucella* that selectively induces cellular and humoral immune response can be done by selecting a suitable nanoniosome formulation as an immunoadjuvant and recombinant protein as an immune response-stimulating Ag.

Keywords: Brucellosis, Mannose, Niosome, Vaccine, Recombinant proteins

Introduction

Despite many studies worldwide, as one of the most prevalent bacterial zoonotic infectious diseases, brucellosis is a serious problem in endemic areas, including Latin America, the Middle East, Africa, Asia, and the Mediterranean [1]. The disease causes offspring mortality, reduced milk production, and infertility in domestic animals and wild mammals [2]. Human brucellosis, caused by direct contact with various species of infected animals or consumption of infected dairy products, is a systemic illness with undulant fever. Although brucellosis is managed using antibiotics, recovery needs long treatment duration and different combinations of antibiotics in case of relapses [3,4]. The disease is caused by *Brucella*, a small intracellular Gram-negative, nonmotile, non-spore-forming aerobic coccobacillus. DNA sequence among the *Brucella* genus has 94% homology; therefore, immunity by any effective brucellosis vaccine can induce cross-protection against other species [5,6]. However, *Brucella* can successfully escape immune responses and resists antimicrobial agents by entering the mononuclear phagocyte cells [7]. These features are challenging to develop effective vaccines due to the need for improved drug development strategies.

As an important goal for many research groups, new molecular techniques have been introduced to create safe, effective, engineered therapeutic agents like vaccines to prevent and control microbial infections [8-10]. In this regard, recombinant subunit proteins based on *Brucella* antigens have been designed and expressed in eukaryotic or prokaryotic systems indicating a safer profile than live organism-based vaccines and acceptable immunoreactivity in laboratory conditions; however, these may confer less efficacy [11,12]. Therefore, adjuvant systems have been suggested to enhance their efficacy by improving immunogenicity [13,14]. Niosomes have been presented as strong immunogenic-adjuvant agents. These nonionic amphiphilic vesicles are into closed bilayer structures which are applied as well targeted delivery systems by their stability, easy modification, and delayed clearance [15,16]. On the other hand, research-based evidence demonstrated that the mannosylated niosomes serve as a useful strategy for improving the efficacy of vaccines due to the possibility of antigen delivery by mucosal or topical routes and better properties for the controlled release of the loaded molecule. Moreover, mannose receptors are highly expressed in macrophages and dendritic cells as the major antigen-presenting cells (APCs); therefore, mannosylated vaccine delivery systems increase the attraction of immune cells and, as a result, improve immunogenicity [17,18].

There is currently no effective and approved vaccine for human brucellosis. Therefore, researchers intend to develop a safe and effectual vaccine using new nanostructured drug delivery systems and engineered antigens to protect at-risk workers and general populations in endemic areas. As carbohydrates enhance the immunogenicity of a vaccine by binding to specific glycan-binding receptors on the surface of APCs [19], this study aimed to develop a niosome-based nanoparticle vaccine candidate containing recombinant trigger factor/Bp26/Omp31 (rTBO) chimeric protein and assess its potency in the induction of immune response in BALB/c mice.

Materials and Methods

Materials

Anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate (Sigma Corp., Kawasaki, Japan), cholesterol (Sigma Corp.), bovine serum albumin (Merck, Darmstadt, Germany) Span 80, Tween 60 (Merck), 3,3',5,5'-Tetramethylbenzidine (TMB; Sigma Corp.), and enzyme-linked immunosorbent assay (ELISA) plates (NUNC; Thermo Fisher Scientific, Waltham, MA, USA) were used in this study. Live attenuated *Brucella abortus* RB51 and *B. melitensis* Rev.1 vaccines were obtained from Razi Vaccine & Serum Research Institute (Karaj, Iran) and *B. abortus* strain 544 and *B. melitensis* 16M bacterial strains were obtained from the microbial collection of Pasteur Institute of Iran (Tehran, Iran). Female BALB/c laboratory mice with a lifespan of 6–8 weeks obtained from the Pasteur Institute of Iran were used.

Preparation of mannosylated rTBO-niosomes

Niosomes encapsulated with rTBO chimeric protein was used to create mannosylated niosomes nanostructures. Briefly, the rTBO chimeric gene was expressed in the expression vector pET28a (+) on *Escherichia coli* BL21 (DE3) strain. The recombinant protein produced with a 6×His-tag was purified by nickel-nitrilotriacetic acid affinity chromatography. The Bradford protein assay was performed to measure proteins concentration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting were used for confirmation and analysis of purified protein.

Niosomes containing rTBO were prepared by thin film hydration as previously described with some modification [20]. Briefly, Tween 60, Span 80, and cholesterol in 1:1:0.85 molar

ratios were dissolved in 10 mL chloroform into a long-necked quick-fit round-bottom flask. The solvent was slowly evaporated at 56°C, using a rotary evaporator (Heidolph Instruments, Schwabach, Germany) at 140 revolutions per minute (rpm) such that a thin, dry film of the components was formed on the inner wall of the flask. The dried thin layer was then hydrated with 10 mL of phosphate-buffered saline (PBS, pH 7.4) containing 10 mg rTBO by rotating the flask in a water bath under normal pressure to ensure complete hydration of the film. The niosomal suspension was left to mature overnight at 4°C.

To synthesize mannosylated niosome, all the above process was done, and mannosylated cholesterol was used (generous gift of Dr. Mehdi Rahimi-Nasrabadi) instead of cholesterol.

Determining the encapsulation efficacy of antigen in niosomes

Antigen-containing nanoparticles were washed using PBS to remove the free and unencapsulated antigen and mixed with isopropyl alcohol at a volume ratio of 1:9 to break the lipid wall around the antigen and release the protein. In the next step, the amount of absorption of the encapsulated antigen was calculated using a Bradford assay. At the end, by using the standard curve of antigen in isopropyl alcohol and the formula EE=B/A×100, the percentage of antigen inclusion in nanoparticles was calculated. Where EE is encapsulation efficacy, A is the amount of protein used initially, and B is the amount of protein in isopropyl alcohol dissolved nanoparticles.

Determination of protein release rate

To check the release pattern of the recombinant protein, 30 mg of protein-containing nanoparticles were poured into a 2 mL microtube in a volume of 1 mL of PBS solution and dispersed by stirring. The microtube was placed in a beaker at 37°C and 100 rpm, and the solution was sampled at regular intervals. Then, the solution containing nanoparticles was centrifuged for 30 seconds at a speed of 10,000, and 700 μL of the supernatant solution were removed and stored at -20°C. To the amount of the collected solution, PBS buffer was added to the microtube, and after vortex, it was placed in a shaker Bain-Marie. At first, sampling was done for 1 and 2 hours, and then every 4, 6, 12, and 24 hours. After 96 hours, the concentration of the samples was determined using a Bradford assay. The percentage curve of protein released from nanoparticles was drawn in the specified periods.

Characterization of nanoparticles

The nanoparticles were centrifuged at 18,000 rpm for 20 minutes and the supernatant was discarded. To wash and disperse the precipitated nanoparticles by adding deionized water, the centrifugation process was repeated 3 times. After centrifugation, the remaining suspension was turned into a powder by a freeze-drying machine and resuspended in 5 mL of deionized distilled water to determine the particle size with a DLS device according to the number, intensity, and volume of the particles at a viscosity of 0.8872.

After sample preparation on a microscope slide, a 30 kW XL scanning electron microscope photographed the nanoparticles to determine the size and shape of them.

Immunization experiments

Female 6–8 weeks old BALB/c mice were used for *in-vivo* studies. The study was carried out under the protocols approved by the Institutional Animal Ethics Committee of the Islamic Azad University of Qom under ethical approval number IR.IAU.QOM.REC.1398.026.

In this study, two intraperitoneal and intranasal routes were used for immunization. To determine the effect of nanostructures to induce the immune response, four groups of ten BALB/c mice were immunized with the intraperitoneal injection or intranasal inoculation of 20 μg of vaccine compounds on days 0, 14, and 28. The first group received antigen, the second and third groups received rTBO loaded niosome and mannosylated niosome, respectively, and the fourth group received PBS as a control. Positive control groups were immunized with attenuated *B. abortus* S19 and *B. melitensis* Rev vaccines with a dose of 1×10^5 colony-forming units (CFU).

Blood sampling and evaluating the IgG1, IgG2a, and IgG antibodies

Blood was collected from immunized mice on days 10, 24, and 38 and their serum was separated and stored at -70°C. These samples were used to evaluate immunoglobulin A (IgA), total IgG, IgG1, and IgG2a subclasses.

The indirect ELISA method investigated the amount of total IgG, IgA, IgG1, and IgG2a antibodies in immunized mice to determine immune responses. In this regard, 5 μg of recombinant protein (antigen) were dissolved in 100 μL of coating buffer (sodium carbonate-bicarbonate buffer, 0.05 M; pH=9.6) and poured into each well of the microplate, except the control well, incubated overnight at 4°C. After washing, the wells were blocked with 100 μL of blocking buffer (containing PBST

 https://doi.org/10.7774/cevr.2024.13.3.232

containing 3% gelatin) and 1 hour of incubation at 37°C. A volume of 100 μL of serially diluted serum samples were added to each well and placed in a shaker incubator at 37°C for 45 minutes. Then, 100 μL of the conjugated antibodies were added to each well and incubated at 37°C for 1 hour and washed with PBST buffer. A volume of 100 μL of TMB substrate was added to each well, and the microplate was transferred to a dark place to conduct the reaction. At the end, after the color of the solution changed to blue, the reaction was stopped with 2.5 M sulfuric acid, and an ELISA reader read the optical density of the wells at a wavelength of 450 nm.

Spleen cells culturing and cytokine evaluation

Three mice from each group were sacrificed 2 weeks after the last immunization, and their spleens were obtained and aseptically homogenized. Then, the red blood cells were removed from splenocytes suspension using ammonium chloride buffer. Splenocytes were counted, and a suspension containing 4×10^6 cells/mL was prepared for each sample, and 100 μL containing 4×10^5 cells was added to each 96-well cell culture plate and incubated with 5% carbon dioxide for 1 hour in RPMI-1640 medium (GIBCO; Thermo Fisher Scientific Inc., Loughborough, UK; containing 10% fetal bovine serum and penicillin/streptomycin). Then, 10 μg of the specific antigen related to each cell group was added to the and kept in an incubator at 37° and 5% CO_2 for 72 hours. Spleen cells stimulated with phytohemagglutinin-A were considered positive control, and spleen cells incubated with RPMI 1640 were negative control. After 72-hour incubation, supernatants of cultured cells were used to determine interferon-gamma (IFN-γ), interleukin 4 (IL-4), and interleukin 12 (IL-12) cytokines levels. The kit was used to measure cytokines, and the method was according to the kit's instructions [21].

Bacterial challenges

Four weeks after the last exposure, mice were infected intraperitoneally with a suspension containing 10^5 CFU/mL of the pathogenic strain of *Brucella* (*B. abortus* 544 or *B. melitensis* 16M), following the quarantine precautions. After 4 weeks, mice were sacrificed and the homogenized spleens were cultured in different dilutions on *Brucella* agar containing 10% fetal bovine serum at 37°C for 3–4 days. The $\log_{10}$ number of CFUs per sample was determined. The following formula obtained units of protection: mean $\log_{10}$ CFU of PBS control group–mean $\log_{10}$ CFU of the experimental group.

Statistical analysis

The mean and dispersion indices were calculated, and a comparison between groups was made through one-way analysis of variance, Games-Howell, and least significant difference tests. Two-by-two data comparisons were made using the non-parametric method and comparing two independent variables.

Results

The characteristics of the nanoparticle

The western blot indicated a single band of the rTBO protein with an approximate weight of 70 kD, consistent with the predicted weight and antigenic similarities with the natural form of this protein. Moreover, the recombinant protein was soluble.

The nanoparticles produced were often spherical and had a relatively smooth surface, and the accumulation of nanoparticles and the formation of clots did not observe (Fig. 1). The zeta potential of nanoparticles containing recombinant protein was about -20 mV, and its average size was 100 nm.

The encapsulation efficiency was 81.96%±1.4%. Examining the release rate showed that the protein was released explosively at first, and on the second day, about 23% of the total loaded protein was released. In total, during the 96-hour study period, about 97% of the protein trapped in the nanoparticles was released.

Measurement of IgA, IgG, and serum IgG isotypes

The ELISA analysis of the serum was performed to evaluate the concentration of the immunoglobulins. Based on the serum serial dilutions against antigens and nanostructures, an antibody titer of 1/128,000 niosome was detected, indicating high immune system stimulation. Ten days after the intraperitoneal injection, a high antibody level produced by niosomal antigen (Nio-Ag) and mannosylated noisomal antigen (Nio-Man-Ag) was produced compared to the control. Similarly, in the 24th and 38th days after the injection, nanostructures had higher immunogenicity than free antigens and control. Similar results were observed for the intranasal method. The results are shown in Fig. 2.

In the examination of IgG isotypes (IgG1 and IgG2a) to determine the type of immune stimulation, the results showed a high antibody titer on the 38th day, which indicated a no significant difference between intraperitoneal and nasal route of antigen administration ($p>0.05$). Results are shown in Fig. 3.

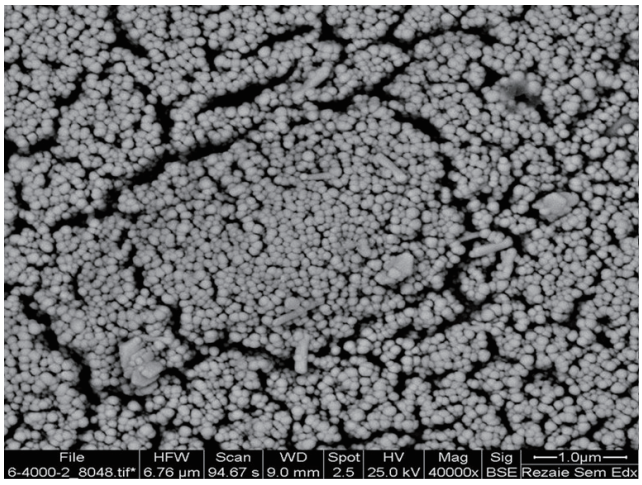

Fig. 1. Electron microscope image of nanoparticles containing protein.

Although all niosomal combinations induced IgG2a and IgG1 production, the IgG2a/IgG1 titer ratio was 1.2 and 1.1 in Nio-Man-Ag after the intraperitoneal and intranasal injection methods, respectively. However, other groups revealed a ratio of less than one.

Examination of serum IgA on day 24 showed a significant increase in the Nio-Man-Ag group compared to other groups and control in both injection methods ($p<0.05$). The results are shown in Fig. 4.

Cytokine assay

Based on Fig. 5, IL-4, IL-12, and IFN-γ production in the intraperitoneal injection were significantly higher in the immunized mouse models with nanoparticles containing rTBO

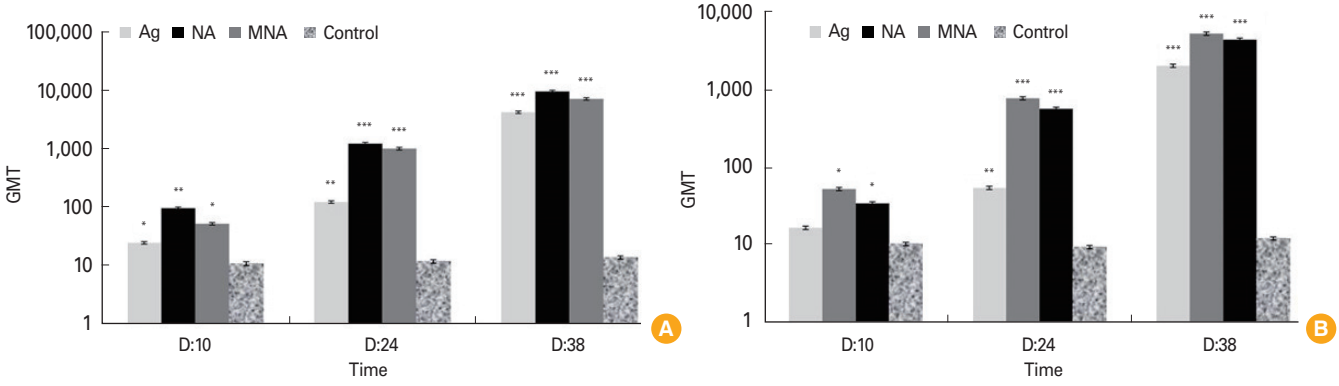

Fig. 2. Serum immunoglobulin G-antibody titers against recombinant trigger factor/Bp26/Omp31 (rTBO) antigen were measured in mice immunized intraperitoneal **(A)** or intranasal **(B)**. Formulations: phosphate-buffered saline (control), free antigen (Ag), niosomal antigen (NA), and mannosylated noisomal antigen (MNA). The immune responses were determined for days 10, 24, and 38. The geometric mean titer (GMT) for each group of mice was calculated and is shown on the y-axis. 95% confidence intervals are indicated by error bars. Significant differences between groups were expressed as *$p<0.05$, **$p<0.001$, and ***$p<0.0001$, respectively.

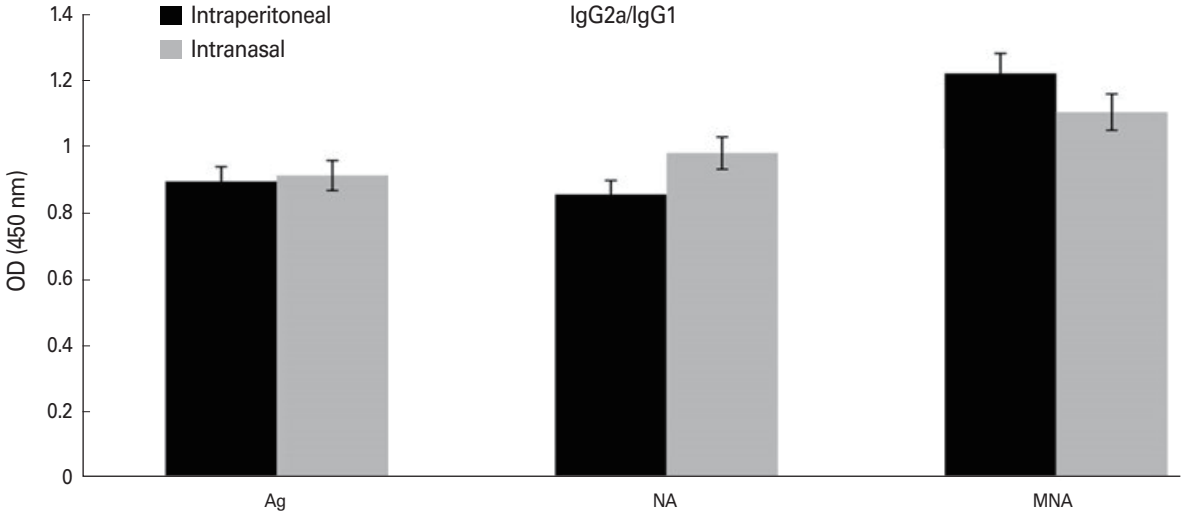

Fig. 3. Evaluation of immunoglobulin G (IgG)2a/IgG1 antibody ratio to specify the type of immune response. Formulations: phosphate-buffered saline (control), free antigen (Ag), niosomal antigen (NA), and mannosylated noisomal antigen (MNA). OD, optical density.

 https://doi.org/10.7774/cevr.2024.13.3.232

protein than in the negative control group ($p<0.05$). However, the cytokines production was similar between the study groups and the positive control group receiving *B. abortus* RB51 and *B. melitensis* Rev.1 vaccine ($p>0.05$). Intranasal ad-

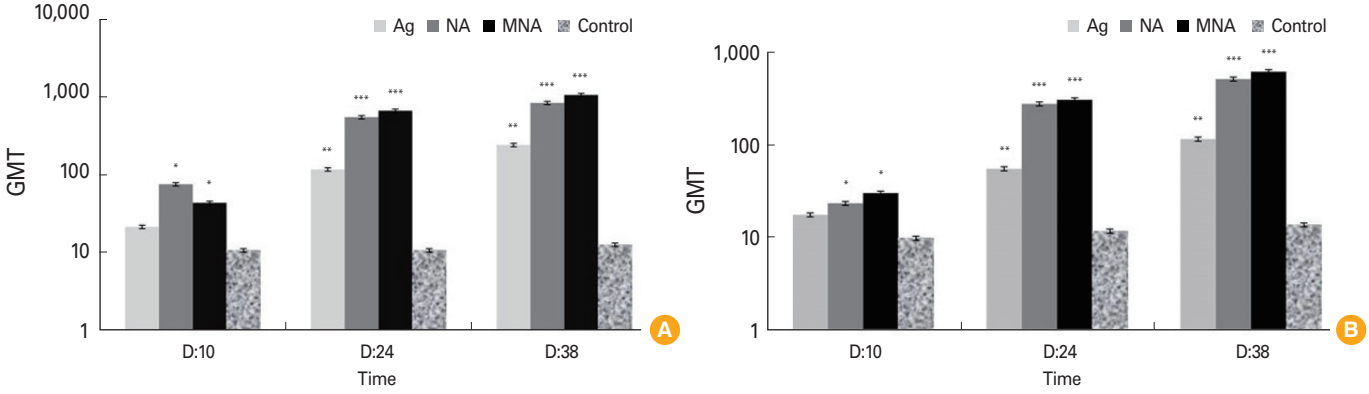

Fig. 4. Serum immunoglobulin A antibody titers against recombinant trigger factor/Bp26/Omp31 antigen were measured in mice immunized intraperitoneal **(A)** or intranasal **(B)**. Formulations: phosphate-buffered saline (control), free antigen (Ag), niosomal antigen (NA), and mannosylated noisomal antigen (MNA). The immune responses were determined for days 10, 24, and 38. The geometric mean titer (GMT) for each group of mice was calculated and is shown on the y-axis. 95% confidence intervals are indicated by error bars. Significant differences between groups were expressed as *p<0.05, **p<0.001, and ***p<0.0001, respectively.

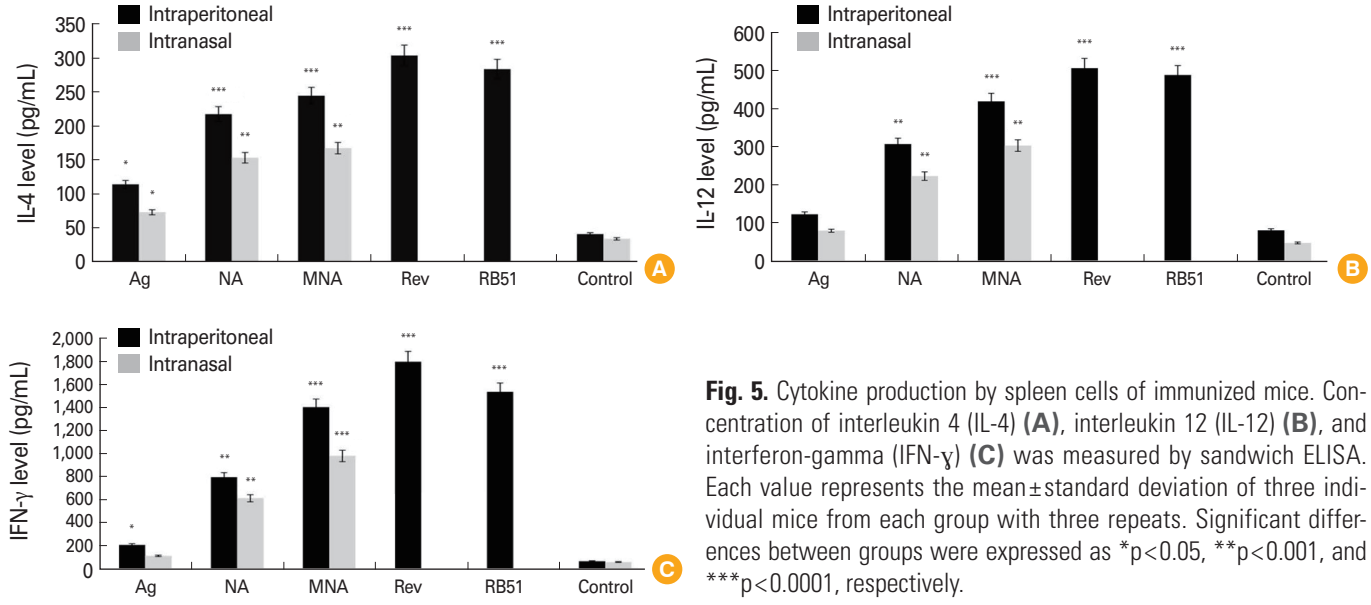

Fig. 5. Cytokine production by spleen cells of immunized mice. Concentration of interleukin 4 (IL-4) **(A)**, interleukin 12 (IL-12) **(B)**, and interferon-gamma (IFN-ɣ) **(C)** was measured by sandwich ELISA. Each value represents the mean±standard deviation of three individual mice from each group with three repeats. Significant differences between groups were expressed as *p<0.05, **p<0.001, and ***p<0.0001, respectively.

Table 1. Investigating protection against *Brucella melitensis* 16M and *B. abortus* 544 in immunized mice

Study groups	Log_{10} CFU of *B. abortus* 544 in spleen	Protection unit in spleen (log units)	Log_{10} CFU of *B. melitensis* 16M in spleen	Protection unit in spleen (log units)
Negative control-PBS	5.98±0.61	-	6.03±0.31	-
Niosomeal antigen	4.41±0.51	1.57*	4.82±0.31	1.21*
Mannosilated noisomal antigen	4.30±0.27	1.68*	4.23±0.52	1.80*
Rev.1	-	-	3.92±0.28	2.11*
RB51	4.02±0.21	1.96*	-	-

Values are presented as mean±standard deviation unless otherwise stated.
CFU, colony-forming units; PBS, phosphate-buffered saline.
*p<0.05.

ministration indicated a higher cytokine production by nanostructure than in the negative control group (PBS). However, IL-4, IL-12, and IFN-γ production levels were significantly higher in the intraperitoneal injection than in the intranasal route ($p<0.05$).

Spleen bacteria count

The bacterial challenge test was performed to evaluate the protective effect of different formulations compared with live attenuated *B. melitensis* Rev.1 vaccine and *B. abortus* RB51. After *B. melitensis* 16M exposure, Log_{10} CFU was 4.82±0.31 and 4.23±0.52 in immunized mice receiving Nio-Ag and Nio-Man-Ag, respectively. Exposure to *B. abortus* 544 also indicated a Log_{10} CFU equal to 4.41±0.51 and 4.3±0.27 for Nio-Ag and Nio-Man-Ag, respectively. These values were similar to the positive control group receiving live attenuated *B. melitensis* Rev.1 vaccine and *B. abortus* RB51 (Table 1).

Discussion

Brucellosis is a global problem, and the lack of access to an effective human vaccine and the complications caused by animal vaccines are among the factors that have prevented the eradication of this disease worldwide. Inventing new genetic engineering methods, using recombinant chimeric proteins with antigenic and adjuvant properties, nanostructures as drug delivery carriers, and designing new vaccines based on the natural pathogenesis pattern have caused reduced risks of available vaccines, increasing immunogenic properties, and targeting antigen transfer to immune cells. In this regard, the design of an effective vaccine against *Brucella* that selectively induces cellular and humoral immune response can be done by selecting a suitable nanoniosome formulation as an immunoadjuvant and recombinant protein as an immune response-stimulating antigen. Therefore, the present study investigated the intranasal and intraperitoneal immunization of the *Brucella* vaccine candidate designed based on mannosylated niosome nanostructures containing rTBO chimeric protein in the mouse model. The results presented the cellular and humoral immune response and cytokine production in the *Brucella* infectious model, whit a higher immune response in the intraperitoneal than intranasal administration.

The present study applied mannose in the noisome nanostructures to target the delivery of recombinant proteins to immune cells. Receptors can recognized carbohydrates on the surface of APCs and, when associated with an antigen, can enhance uptake via endocytosis/phagocytosis, as mannose can be recognized by mannose receptors [22]. Furthermore, coating the surface of nanoparticles and nanocapsules with mannose has enhanced antigen uptake by APCs [23]. *Brucella* mannose also activates the complement system through the lectin pathway. Mannose-binding lectin is one of the important components in innate immunity, which is produced by hepatocytes in the liver and triggers the complement activation cascade, resulting in various antibodies [24]. Therefore, targeting by mannose is used to prevent non-specific interactions and, on the other hand, to increase absorption by immune cells, which by binding to their receptors on the surface of immune system cells, especially APCs, activates these cells and initiates inflammatory responses.

Serum IgG indicated a high antibody titer in immunized animals using nanostructures compared with free antigens and negative control. Low stimulation using free antigen may arise from removing it by digestive enzymes and the blood refining system before effective contact with immune cells. These findings confirm the efficacy of nanoparticles as a strong delivery and adjuvant system in stimulating the immune system, in addition to rising protein stability. Therefore, the formulation of immunological proteins with nanoparticles increases the immunogenic power with small antigen concentrations. Furthermore, comparing the immunogenicity between formulations indicated higher immune system stimulation by Nio-Ag compared with the Nio-M-Ag; however, the endpoint detection was similar. As previously reported, mannose can interact with mannose receptors displayed on APCs and, as a result, activate the cellular immune response, which in return activates a more robust and prolonged immunization. In line with these findings, a previous study on mannosylated niosomes as a topical vaccine delivery carrier and adjuvant for the induction of both humoral and cellular immunity in albino rats reported a niosomal formulations elicited a significantly higher serum IgG titer upon topical application as compared with controls [25]. However, inconsistent with our results, this study presented the potential higher efficacy of mannosylated niosomes than plain uncoated niosomes [25]. Some other reports have also emphasized the effectiveness of nanovaccines modified by mannose moiety [23,26]. Although the present study rejected the superiority of mannosylated modification to free nanoparticles, this observation may result from the methodology and physicochemical structure of created Nio-Man.

 https://doi.org/10.7774/cevr.2024.13.3.232

Based on immunological studies, IgG2a/IgG1 ratio represents cellular and humoral immune response proportion. In this regard, T helper type 1 (Th1)-dependent cytokines, such as IL-12 and IFN-γ, stimulate IgG2a, whereas T helper type 2 (Th2) cytokines inhibit it by IL-4, IL-5, and IL-10. In the present study, the IgG2a/IgG1 ratio was more than one in intranasal and intraperitoneal immunization by Nio-Man-Ag, indicating an immune shift towards cellular immunity in immunized mice and a tendency towards stimulating the T cell response. Conversely, free antigen, Nio-Ag, and standard vaccines Rev.1 and RB51, with a ratio lower than 1, stimulated humoral immunity under the antigen-stimulated spleen cell producing a higher proportion of IgG1 than IgG2a. In line with our results, oral immunization with mannosylated nanoparticles resulted in higher IgG2a levels (Th1 response) related to the mannosylation strategy of the nanoparticles [27]. A previous study has also reported serum IgG2a/IgG1 response by niosomal formulations; however, inconsistent with our data, IgG1 response was predominant, indicating superiority of humoral response. These findings support the stimulation of Th1 and Th2 lymphocytes and activate cellular and humoral responses by particulate antigens, whereas soluble antigens stimulate Th2 and humoral response [28,29]. Consequently, combining antigens in mannosylated niosomes may provoke both humoral and cellular immunity. Although, further investigations should consider T lymphocyte proliferation and cytokine production to completely characterize the immune response stimulated by the niosomal system.

The current study showed a significantly increased serum IgA by intranasal and intraperitoneal administration of the Nio-Man-Ag. Similarly, a study on oral genetic immunization against hepatitis B also reported the effectiveness of nanoparticles, especially mannosylated niosomes, in the mucosal immune response, as the highest salivary IgA level was observed by administration of *o*-palmitoyl mannan-coated niosomes containing DNA vaccine [30]. Another study investigating nanoparticles for oral antigen delivery revealed that oral immunization with mannosylated niosomes induced a higher mucosal IgA response by lymphocytes [27].

Evaluation of cytokine production revealed significantly higher levels of IL-4, IL-12, and IFN-γ in the intranasal and intraperitoneal immunized mouse models with nanoparticles containing rTBO than in the negative control. Moreover, cytokine levels were similar between intraperitoneal injection and positive controls receiving *B. abortus* RB51 and *B. melitensis* Rev.1 vaccines. These findings showed the immune response stimulation of the immunized mice through cellular and humoral pathways by the stimulation of Th1 and Th2, which is the most favorable result regarding the production of a vaccine against *Brucella*. As well to the injection approach, there was a significant difference in the production of cytokines between the immunized mice and the negative controls in the intranasal method. In the serum of immunized mice, there was a high production of IL-4, IL-12, and IFN-γ compared to the negative control group. These observations also demonstrated the activation of both humoral and cellular immunity by immunization via the intranasal method using Nio-Man-Ag. In line with these findings, previous investigations revealed increased levels of Th1 cytokines, including IL-12 and IFN-γ production after parenteral administration of mannosylated cationic liposomes [27] and mannan-coated liposome-protamine-DNA nanoparticles [28]. This observation can be illustrated by the efficacious endocytosis of mannosylated peptides to dendritic cells more than non-mannosylated ones by mannose receptors [31].

Higher IL-4, IL-12, and IFN-γ levels in the intraperitoneal injection than in the intranasal method presented a better stimulation of the immune response in mice immunized with nanoparticles containing rTBO protein using the injection method. Also, the combination of Nio-Man-Ag had the highest stimulating potency of the immune system. Intranasal vaccination has introduced one of the most favorable methods presenting advantages, including prevention of enzymatic and proteolytic degradation and a large surface for deposition and absorption [32]. A study on intestinal parasites of lambs has shown a strong immune response and significant protection [33]. Another study in cattle using intranasal and subcutaneous vaccination revealed the effect of the delivery mechanism in stimulating the systemic and mucosal immune responses [34]. In this regard, some evidence indicated the efficacious potential function of intranasal administration using subunit and live vaccines for *Brucella* vaccination [35-37].

Considering bacterial count revealed protection in immunized mice against *B. melitensis* 16M and *B. abortus* 544, confirming the potential role of niosomes containing rTBO recombinant protein to induce immunity as similar live attenuated *B. melitensis* Rev.1 vaccine and *B. abortus* RB51 compared with negative control.

In conclusion, the present study indicated that the combination of mannosylated noisome and *Brucella* rTBO recombinant chimeric proteins induce IgG and IgA production during infection; as a result, stimulating the cellular and humoral

immune response. Moreover, it can create significant lymphocyte responses during the natural course of pathogenic bacteria infection by producing cytokines, playing a role in developing the protective acquired immune response in the infectious model. These findings present the rTBO chimeric protein as a suitable candidate for the design of immunogenic compounds against *Brucella*. Although the parenteral method resulted in a successful enhancement of cytokines production than intranasal administration, further studies suggested developing strategies to increase the antibodies levels and reduce their removal by digestive enzymes using a proper delivery system or changing the vaccination method to more effectively reduce bacterial colonization in the host.

ORCID

Fahimeh Sharif *https://orcid.org/0000-0002-8059-996X*
Razieh Nazari *https://orcid.org/0000-0002-3487-1416*
Mahdi Fasihi-Ramandi *https://orcid.org/0000-0002-8242-4880*
Ramezan Ali Taheri *https://orcid.org/0000-0003-1394-6412*
Mohsen Zargar *https://orcid.org/0000-0002-3108-5655*

References

1. Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The new global map of human brucellosis. Lancet Infect Dis 2006;6:91-9.
2. Corbel MJ, Elberg SS, Cosivi O. Brucellosis in humans and animals. Geneva: World Health Organization; 2006.
3. Al-Tawfiq JA. Therapeutic options for human brucellosis. Expert Rev Anti Infect Ther 2008;6:109-20.
4. Alavi SM, Alavi L. Treatment of brucellosis: a systematic review of studies in recent twenty years. Caspian J Intern Med 2013;4:636-41.
5. Gee JE, De BK, Levett PN, Whitney AM, Novak RT, Popovic T. Use of 16S rRNA gene sequencing for rapid confirmatory identification of Brucella isolates. J Clin Microbiol 2004;42:3649-54.
6. Korle SL. The design, construction, and testing of a recombinant DNA vaccine for Brucella abortus and Brucella melitensis [dissertation]. Baton Rouge (LA): Louisiana State University and Agricultural & Mechanical College; 2022.
7. Hoover DL, Borschel RH. Medical protection against brucellosis. In: Lindler LE, Lebeda FJ, Korch GW, editors. Biological weapons defense: infectious disease. Totowa (NJ): Humana Press; 2005. p. 155-84.
8. Hou H, Liu X, Peng Q. The advances in brucellosis vaccines. Vaccine 2019;37:3981-8.
9. Eshtiaghi S, Nazari R, Fasihi-Ramandi M. In-silico and in-vitro evaluation of antibacterial, cytotoxic, and apoptotic activity and structure of modified CM11 peptide. Int J Pept Res Ther 2021;27:1069-78.
10. Heidary M, Dashtbin S, Ghanavati R, et al. Evaluation of brucellosis vaccines: a comprehensive review. Front Vet Sci 2022;9:925773.
11. Hop HT, Arayan LT, Huy TX, et al. Immunization of BALB/c mice with a combination of four recombinant Brucella abortus proteins, AspC, Dps, InpB and Ndk, confers a marked protection against a virulent strain of Brucella abortus. Vaccine 2018;36:3027-33.
12. Zhu L, Wang Q, Wang Y, et al. Comparison of immune effects between Brucella recombinant Omp10-Omp28-L7/L12 proteins expressed in eukaryotic and prokaryotic systems. Front Vet Sci 2020;7:576.
13. Conacher M, Alexander J, Brewer JM. Niosomes as immunological adjuvants. In: Uchegbu LF, editor. Synthetic surfactant vesicles. London: CRC Press; 2000. p. 219-42.
14. Shilpa S, Srinivasan BP, Chauhan M. Niosomes as vesicular carriers for delivery of proteins and biologicals. Int J Drug Deliv 2011;3:14-24.
15. Sankhyan A, Pawar P. Recent trends in niosome as vesicular drug delivery system. J Appl Pharm Sci 2012;2:20-32.
16. Pardakhty A, Moazeni E. Nano-niosomes in drug, vaccine and gene delivery: a rapid overview. Nanomed J 2013;1:1-12.
17. Irache JM, Salman HH, Gamazo C, Espuelas S. Mannose-targeted systems for the delivery of therapeutics. Expert Opin Drug Deliv 2008;5:703-24.
18. Hatami E, Mu Y, Shields DN, et al. Mannose-decorated hybrid nanoparticles for enhanced macrophage targeting. Biochem Biophys Rep 2019;17:197-207.
19. Mosaiab T, Farr DC, Kiefel MJ, Houston TA. Carbohydrate-based nanocarriers and their application to target macrophages and deliver antimicrobial agents. Adv Drug Deliv Rev 2019;151-152:94-129.
20. Baillie AJ, Florence AT, Hume LR, Muirhead GT, Rogerson A. The preparation and properties of niosomes: non-ionic surfactant vesicles. J Pharm Pharmacol 1985;37:863-8.
21. Fasihi-Ramandi M, Ghobadi-Ghadikolaee H, Ahmadi-Renani S, Taheri RA, Ahmadi K. Vibrio cholerae lipopolysaccharide loaded chitosan nanoparticle could save life by induction of specific immunoglobulin isotype. Artif

Cells Nanomed Biotechnol 2018;46:56-61.
22. Gao H, Goncalves C, Gallego T, et al. Comparative binding and uptake of liposomes decorated with mannose oligosaccharides by cells expressing the mannose receptor or DC-SIGN. Carbohydr Res 2020;487:107877.
23. Xing L, Fan YT, Zhou TJ, et al. Chemical modification of chitosan for efficient vaccine delivery. Molecules 2018;23:229.
24. Lee SG, Yum JS, Moon HM, et al. Analysis of mannose-binding lectin 2 (MBL2) genotype and the serum protein levels in the Korean population. Mol Immunol 2005;42:969-77.
25. Jain S, Vyas SP. Mannosylated niosomes as carrier adjuvant system for topical immunization. J Pharm Pharmacol 2005;57:1177-84.
26. Yang R, Xu J, Xu L, et al. Cancer cell membrane-coated adjuvant nanoparticles with mannose modification for effective anticancer vaccination. ACS Nano 2018;12:5121-9.
27. Hattori Y, Kawakami S, Suzuki S, Yamashita F, Hashida M. Enhancement of immune responses by DNA vaccination through targeted gene delivery using mannosylated cationic liposome formulations following intravenous administration in mice. Biochem Biophys Res Commun 2004;317:992-9.
28. Morokata T, Ishikawa J, Yamada T. Antigen dose defines T helper 1 and T helper 2 responses in the lungs of C57BL/6 and BALB/c mice independently of splenic responses. Immunol Lett 2000;72:119-26.
29. Singh P, Prabakaran D, Jain S, Mishra V, Jaganathan KS, Vyas SP. Cholera toxin B subunit conjugated bile salt stabilized vesicles (bilosomes) for oral immunization. Int J Pharm 2004;278:379-90.
30. Jain S, Singh P, Mishra V, Vyas SP. Mannosylated niosomes as adjuvant-carrier system for oral genetic immunization against hepatitis B. Immunol Lett 2005;101:41-9.
31. Lam JS, Mansour MK, Specht CA, Levitz SM. A model vaccine exploiting fungal mannosylation to increase antigen immunogenicity. J Immunol 2005;175:7496-503.
32. Nian X, Zhang J, Huang S, Duan K, Li X, Yang X. Development of nasal vaccines and the associated challenges. Pharmaceutics 2022;14:1983.
33. Mohamed Fawzi E, Cruz Bustos T, Gomez Samblas M, et al. Intranasal immunization of lambs with serine/threonine phosphatase 2A against gastrointestinal nematodes. Clin Vaccine Immunol 2013;20:1352-9.
34. Rebelatto MC, Siger L, Hogenesch H. Kinetics and type of immune response following intranasal and subcutaneous immunisation of calves. Res Vet Sci 2001;71:9-15.
35. Yang X, Walters N, Robison A, Trunkle T, Pascual DW. Nasal immunization with recombinant Brucella melitensis bp26 and trigger factor with cholera toxin reduces B. melitensis colonization. Vaccine 2007;25:2261-8.
36. Tabynov K, Sansyzbay A, Kydyrbayev Z, et al. Influenza viral vectors expressing the Brucella OMP16 or L7/L12 proteins as vaccines against B. abortus infection. Virol J 2014;11:69.
37. Clapp B, Yang X, Thornburg T, Walters N, Pascual DW. Nasal vaccination stimulates CD8(+) T cells for potent protection against mucosal Brucella melitensis challenge. Immunol Cell Biol 2016;94:496-508.