

Immunogenicity evaluation of rBoNT/E nanovaccine after mucosal administration

Mohamad Javad Bagheripour¹, Firouz Ebrahimi^{1*}, Abbas Hajizade¹, Shahram Nazarian¹

¹ Biology Research Centre, Basic Science Faculty, Imam Hossein University, Tehran, Iran

ARTICLE INFO

Article type:

Original article

Article history:

Received: Jul 30, 2017

Accepted: Nov 12, 2018

Keywords:

Botulinum toxin type E

Chitosan

Mucosal administration

Nanoparticles

Recombinant protein

ABSTRACT

Objective(s): The Botulism syndrome is caused by types A to G of botulinum neurotoxins. The binding domains of these neurotoxins are immunogenic and considered as appropriate candidate vaccines. Due to the low immunogenicity of recombinant vaccines, there have been many studies on the use of biocompatible carriers such as chitosan nanoparticles for the delivery of these vaccines. The aim of this study was evaluating the efficiency of chitosan nanoparticles as carriers for a candidate vaccine, binding domain of BoNT/E, through oral and intranasal routes.

Materials and Methods: Chitosan nanoparticles containing rBoNT/E binding domain, were synthesized via ionic gelation. After administration of the nanoparticles to mice through oral and intranasal routes, antibody titers were assessed by ELISA and, finally, all groups were challenged by active botulinum neurotoxin type E.

Results: The groups that received nanoparticles containing the antigen, through oral and intranasal routes, and the group that received the bare antigen orally, were able to tolerate 5×10^2 folds of MLD. The intranasally immunized mice by the bare antigen were able to tolerate 2×10^3 folds of the toxin's MLD.

Conclusion: It seems that the use of chitosan nanoparticles has no significant effect on the protective immunization of the mice against botulinum BoNT/E in either route ($P > 0.05$), even intranasal administration of the bare antigen gives better mice immunization against the toxin.

► Please cite this article as:

Bagheripour MJ, Ebrahimi F, Hajizade A, Nazarian SH. Immunogenicity evaluation of rBoNT/E nanovaccine after mucosal administration. Iran J Basic Med Sci 2019; 22:353-359. doi: 10.22038/ijbms.2019.25333.6258

Introduction

Human botulism is caused by A, B, E, and F types of botulinum neurotoxins (BoNTs). These toxins act principally on the peripheral nervous system and cause flaccid paralysis and ultimately, the botulism syndrome (1, 2).

The problems of toxoid vaccines, such as high production costs, the need to use several serotypes, the risks of working with dangerous strains, and the entrance of unwanted proteins to the body during the immunization, have led the researchers to focus on recombinant vaccines (3).

Studies have shown that the binding domain of the botulinum neurotoxins has high immunogenicity so that it can be used as an efficient recombinant vaccine. Several recombinant serotype-specific subunit vaccines against BoNTs have been developed, including a subunit vaccine comprising the receptor binding domain of the BoNTs (4, 5). Due to the low immunogenicity of recombinant proteins in comparison to the toxoid vaccines, the use of an appropriate vehicle for the delivery of these vaccines is inevitable. Therefore, the use of delivery systems based on nano and microparticles is widely exploited by researchers (6, 7).

Mucosal vaccination would offer several advantages over the parenteral route. In this case, the pathogens will be blocked at the site of entry and as a result, the general efficacy of the vaccines will be increased.

Furthermore, by avoiding the traumatic procedure of injection, it would increase the compliance and safety of the administration (8). However, mucosal vaccines have to overcome several formidable barriers in the form of significant dilution and dispersion, competition with a myriad of various live replicating bacteria, viruses, inert food, and dust particles, enzymatic degradation, and low pH before reaching the target cells. Therefore, efforts were made to generate a mucosal vaccine that provides protection against these conditions (5). Indeed, it has long been known that vaccination through mucosal membranes requires potent adjuvants to enhance the immune system potentiation. For this, mucosal vaccines have been administered using a broad spectrum of nanocarriers, such as multiple emulsions, liposomes, polymeric nanoparticles, dendrimers, ISCOMs (immune stimulating complexes), etc (9). Among these carriers, biodegradable nanoparticles have been the most extensively investigated delivery systems, and it has been shown that they can be used as adjuvants in vaccine formulations (10-12). There have been many studies on the use of chitosan and its derivatives for the delivery of antigens through the mucosal routes. Due to their better stability, low toxicity, simple and mild preparation method, and providing versatile routes of administration, ie, oral, nasal, and ocular mucosa, which are non-invasive routes, these nanoparticles are the most used nanocarriers for vaccine delivery. Indeed,

*Corresponding author: Firouz Ebrahimi. Biology Research Centre, Basic Science Faculty, Imam Hossein University, Tehran, Iran. Tel/Fax: +98-21-77104934; Email: febrhimi@ihu.ac.ir

chitosan-based polymers are mucoadhesive and, therefore, are capable of opening the tight junctions of epithelial cells (13-16).

The aim of this study was evaluating the efficiency of chitosan nanoparticles as carriers for mucosal administration of recombinant BoNT/E binding domain as a candidate vaccine.

Materials and Methods

In this study, medium molecular weight chitosan was purchased from Sigma-Aldrich (USA), Sodium tripolyphosphate (TPP) was obtained from Scharlau (Spain), glacial acetic acid and other chemical materials were bought from Merck (Germany), anti-Mouse HRP secondary antibodies were purchased from Abcam (USA), and *BoNT/E binding domain gene* was synthesized by ShineGene Molecular Biotech, Inc. (Shanghai, China). Female NMRI & NIH mice aged 6 to 8 weeks, were obtained from Pasteur Institute of Iran.

Expression and purification of rBoNT/E-Hc

The synthetic gene was subcloned into pET28a (+) (Novagen) and recombinant plasmids were introduced into *E. coli* BL21(DE3) competent cells (Novagen) by the heat shock method. The transformed host cells were selected on Luria-Bertani (LB) agar media, containing 80 µg kanamycin/ml. Several of the selected colonies were cultured in LB broth, supplemented with 80 µg/ml of kanamycin. Expression of the recombinant protein was induced by addition of 0.5 mM IPTG and analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The expressed protein was purified by nickel-nitrilotriacetic acid (Qiagen, USA) resin under native conditions and validated by SDS-PAGE.

Western blot analysis

The purified recombinant protein was confirmed by Western blot analysis using the horse anti-botulinum neurotoxin type E antibody. For this, proteins were blotted onto nitrocellulose membranes. The membrane was blocked with 5% skimmed milk at 4 °C overnight and then washed with PBS (137 mM NaCl, 2.7 mM KCl and 4.3 mM Na₂HPO₄). After decanting and discarding the blocking buffer, the membrane was incubated in a 1:5000 dilution of horse anti-botulinum toxin type E antibody in PBST (PBS containing 0.05 % Tween), with gentle shaking for 1 hr at 37 °C. After washing the membrane with PBST, blots were incubated with a 1:10000 dilution of polyclonal anti-horse HRP conjugate. The blot was washed in PBST and stained with HRP staining solution containing 3,3'-diaminobenzidine (DAB), as a substrate.

Preparation of chitosan nanoparticles containing antigen

Chitosan nanoparticles were prepared by the ionic gelation of chitosan solution with anionic tripolyphosphate. Chitosan was dissolved in 2% (v/v) acetic acid aqueous solution at the concentration of 2 mg/ml. Subsequently, 5 ml of TPP solution (1 mg/ml) was added in a dropwise manner into 7.5 ml of chitosan solution containing 0.8 mg of the recombinant protein. Chitosan colloid nanoparticles were formed under

magnetic stirring at room temperature for 60 min. Then, chitosan colloid nanoparticles were separated by centrifugation at 21,000 rpm, 4 °C, for 40 min. The supernatant was discarded and the pellet was re-dispersed in distilled water for further use.

Physical characterization of chitosan nanoparticles

The physical characteristics of nanoparticles were obtained by scanning electron microscopy (LEO-1455VP, UK). The surface zeta potential of nanoparticles was measured by a Zeta Potential Analyzer (Malvern Instruments, UK) with water as the solvent (pH=7, 25 °C). Each sample was measured in triplicate.

Determination of loading efficiency of nanoparticles

Antigen-loaded nanoparticles were separated from the solution by centrifugation at 21000 rpm at 4 °C for 40 min. All 15 ml of the supernatant was decanted and the remaining free protein in the supernatant was determined by the Bradford assay.

The loading efficiency (LE) was calculated as follows:

$$\%LE = ((A-B)/A) \times 100$$

Where A is the total amount of protein and B is the free amount of protein.

Immunization of mice

The female 6-8 week old mice, were used for the immunization programme. Animals were divided into groups of ten. Immunization was carried out through oral and intranasal routes. Mice were partially anesthetized with the ketamine and medetomidine and then gavages orally using flexible plastic feeding needle. For intranasal inoculation, mice were anesthetized and a 5 µL inoculums of nanoparticles was released into each nostril. There were six groups of mice in this study, two of which received chitosan nanoparticles containing antigen and two received just chitosan nanoparticles through oral and intranasal routes. The remaining two groups were immunized by the bare antigen (without any additions) through oral and intranasal routes (Table 1). Administrations were performed four times at intervals of 14 days.

Assessment of IgG titer

Blood was collected 8-10 days after each administration. Mice were bled periodically by retro-orbital eye bleeding. Approximately 50 µl of the sera

Table 1. Immunization groups of mice

No.	Group	Antigen (µg)	Administration route	Number of doses
1	Ag	100	Oral	4
2	Np+Ag	100	Oral	4
3	Np	0	Oral	4
4	Ag	20	Intranasal	4
5	Np+Ag	20	Intranasal	4
6	Np	0	Intranasal	4

Ag: antigen alone, Np: nanoparticles alone, Np+Ag: nanoparticles containing antigen

from each mouse were collected after centrifugation of blood, and samples were pooled and stored at -20 °C. The level of antibody was determined by indirect enzyme-linked immunosorbent assay (ELISA). Plates were coated with 5 µg of recombinant protein and blocked with 5% skimmed milk in PBST (PBS plus 0.05% Tween 20). Washed plates were incubated with serially diluted sera for 30 min at 37 °C. Then, goat anti-mouse IgG antibody (Sigma) was added to the wells. After washing as before, the plate was incubated with chromogen/substrate (100 µL/well of OPD, 0.025% H₂O₂ in 0.1 M citrate buffer, pH=5). The absorbance at 495 nm was checked with an ELISA microplate reader (17).

Assessment of IgA titer

Stool samples were collected 7 days after the 4th administration. The method used for saline extraction of fecal immunoglobulin was adapted from that used by Ferguson *et al.* (17). Approximately 1 g of feces was contained in the sample scoop of the fecal tube. 1 ml of extraction buffer (PBS, 0.5% Tween, and 0.05% sodium azide) was added to each tube and samples were thoroughly homogenized by shaking on a vortex mixer. The fecal suspensions were centrifuged. The supernatant was transferred to a sterile tube containing 20 µl of protease inhibitor cocktail, and stored at -20 °C. The level of IgA antibody was also determined by enzyme-linked immunosorbent assay (ELISA). ELISA was performed the same as the method described for the IgG assay unless the samples had been extracted from the animal stool and the applied secondary antibody was anti-IgA HRP-conjugated.

Challenge of immunized mice

Two weeks after the last administration, the immunized mice were challenged by activated botulinum neurotoxin type E through intraperitoneal injection. The challenged animals were monitored for 7 days. The number of deaths for each group was recorded.

Statistical analysis

Statistical analyses were carried out by SPSS 21.0. All data were expressed as mean±standard deviation. Comparisons among three groups were performed by one-way analysis of variance (ANOVA). For comparison between two groups, Student's t-test was applied. For both tests, *P*-value less than 0.05 was considered significant.

Results

Preparation and characterization of rBoNT/E-Hc

The BoNT/E binding domain synthetic gene was expressed in *E. coli* BL21 (DE3) and verified by SDS-PAGE. Purification of the protein was carried out under the natural conditions and SDS-PAGE analysis revealed the presence of the purified recombinant protein in the eluted fraction (Figure 1). Furthermore, the expression of the recombinant protein was confirmed by Western blot analysis (Figure 2).

Characterization of chitosan nanoparticles

As described earlier, chitosan nanoparticles containing the rBoNT/E BD protein were synthesized by ionic gelation method. Imaging by scanning electron

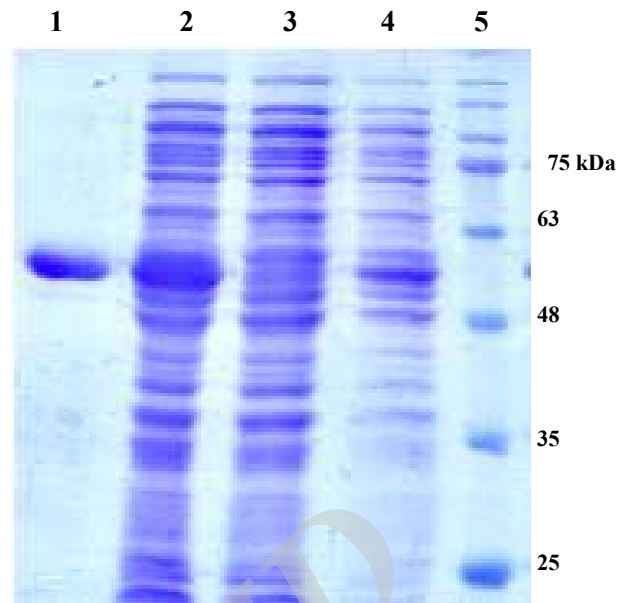


Figure 1. Purification of recombinant proteins with the nickel-nitrilotriacetic acid column. Lane 1, purified protein after elution with imidazole 250 mM; lane 2, expressed protein before purification; Lane 3, uninduced *E. coli* BL21(DE3) as control; lane 4, wash column with imidazole 40; lane 5, protein molecular marker

microscopy showed the spherical nanoparticles (Figure 3). Analyzing by PSA showed the nanoparticles with a size of 275 nm. Loading efficiency of chitosan nanoparticles was calculated as 91.24±3.5%. Zeta potential of the chitosan nanoparticles was +22.9 mV.

Assessment of serum and mucosal antibody responses

Animals remained healthy and showed no signs of

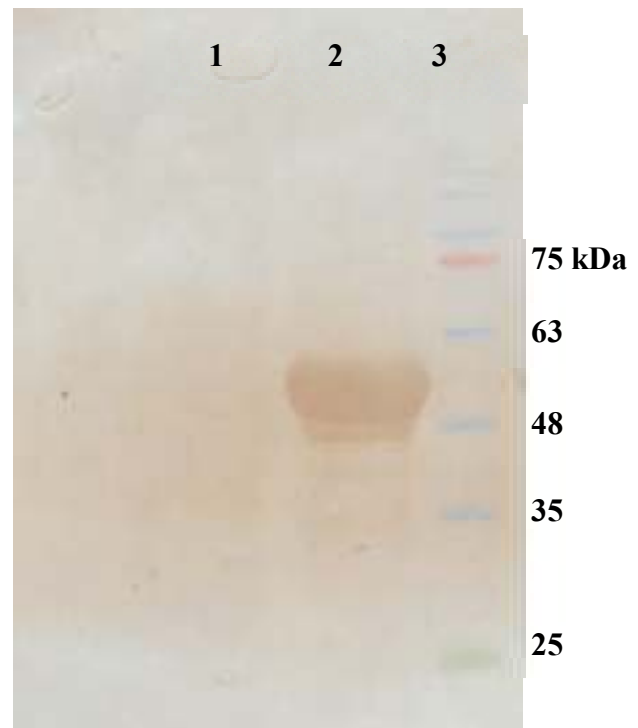


Figure 2. Western blot analysis of the rBoNT/E-Hc. Lane 1, BSA protein (as control); lane 2, rBoNT/E binding domain protein, lane 3, protein molecular weight marker

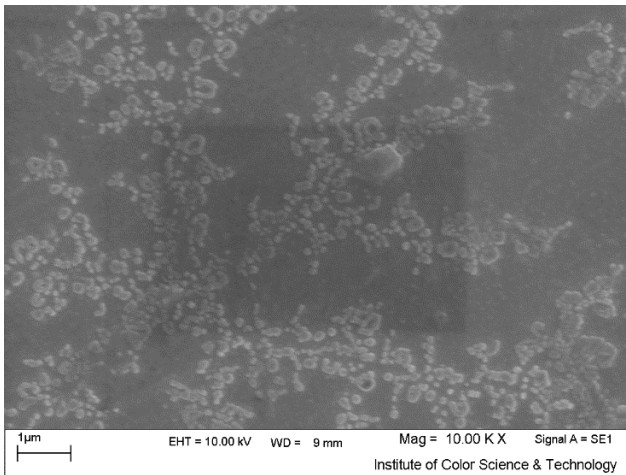


Figure 3. Scanning electron microscopy image of Chitosan nanoparticles containing rBoNT/E binding domain protein. The nanoparticles were prepared by the ionic gelation method as described in methods

abnormal behavior after each administration. In all test groups, after each administration, the amount of IgG antibody increased. The increase in antibody titer after each administration was significant ($P < 0.05$). No increase in IgG antibody titer was observed in the control groups. Statistical analysis of the results showed that in the group immunized orally by chitosan nanoparticles containing the antigen, IgG titers were significantly higher compared to the control group (which received only chitosan nanoparticles) ($P < 0.01$). The immunized mice that received intranasal nanoparticles containing antigen showed higher serum IgG titers in comparison to the control group which received only the chitosan nanoparticles, intranasally ($P < 0.05$). In orally immunized mice that received the antigen and the group that received nanoparticles containing the antigen, no significant difference in antibody titer was observed ($P > 0.05$). However, in the group that received the antigen intranasally and the group that received nanoparticles containing the antigen by the same route, a significant difference in antibody titer was observed ($P < 0.05$). Comparison of antibody levels in nasal and

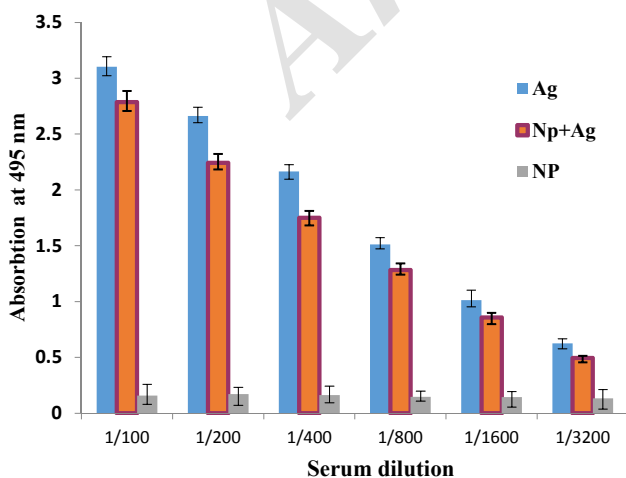


Figure 4. Evaluation of IgG antibody production. Animals were orally administered antigen (Ag), nanoparticles containing antigen (Np+Ag), and nanoparticles without antigen (Np). Immunizations were performed four times at intervals of 14 days ($P < 0.05$)

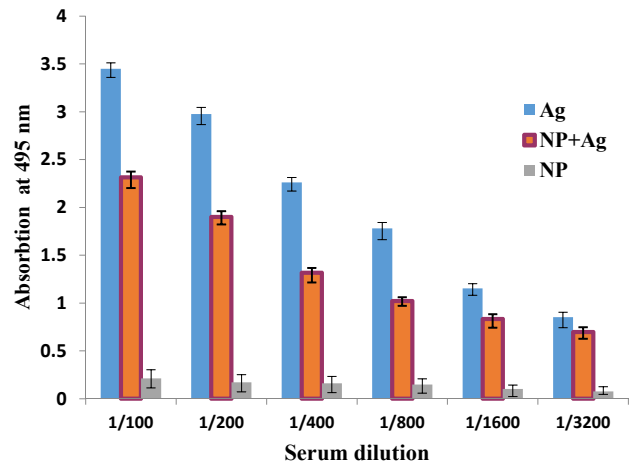


Figure 5. Evaluation of IgG antibody production. Animals were intranasally administered antigen (Ag), nanoparticles containing antigen (Np+Ag), and nanoparticles without antigen (Np). Immunizations were performed four times at intervals of 14 days ($P < 0.05$)

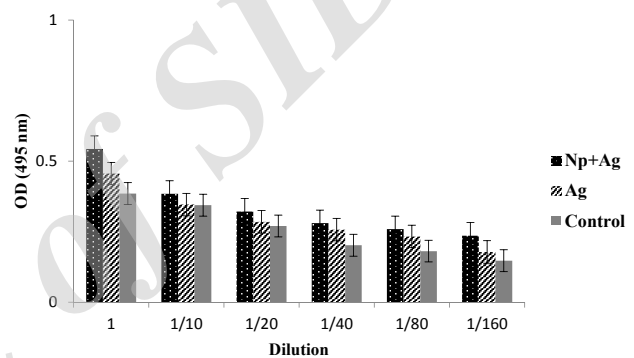


Figure 6. Evaluation of IgA antibody production. Animals were orally administered antigen (Ag), nanoparticles containing antigen (Np+Ag), and nanoparticles without antigen (Np). Immunizations were performed four times at intervals of 14 days

oral administration of nanoparticles containing the antigen showed that there is no significant difference in antibody level ($P > 0.05$) (Figures 4 and 5).

In none of the test groups, any increase in IgA antibody titer was observed. In other words, comparison of IgA antibody levels in test and control groups showed that there were no significant differences in IgA antibody level ($P > 0.05$) (Figures 6 and 7).

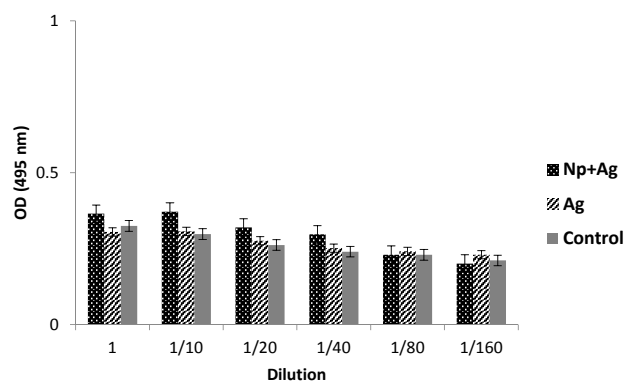


Figure 7. Evaluation of IgA antibody production. Animals were intranasally administered antigen (Ag), nanoparticles containing antigen (Np+Ag), and nanoparticles without antigen (Np). Immunizations were performed four times at intervals of 14 days

Table 2. The protection levels in the challenged mice

Mice groups	Administration type	Tolerance
Ag	Oral	5×10^2 MLD
Np+Ag	Oral	5×10^2 MLD
Ag	Intranasal	2×10^3 MLD
Np+Ag	Intranasal	5×10^2 MLD

Ag: antigen alone, Np+Ag: nanoparticles containing antigen

Challenging the immunized mice

The results of the challenge study showed that the intranasal administration of antigen protects the immunized mice from 2×10^3 MLD of botulinum neurotoxin type E. For orally immunized mice by the antigen and nanoparticle containing the antigen, and also for intranasally immunized mice by nanoparticles containing the antigen, the results were the same and they could tolerate 5×10^2 MLD of botulinum neurotoxin type E (Table 2).

Discussion

There is currently no licensed recombinant vaccine for the prevention of botulism. The most widely available botulinum vaccine is composed of formalin-inactivated crude preparations of BoNTs, in combination with aluminum phosphate, containing thimerosal as a preservative. Many studies have exploited BoNTs-Hc as a candidate vaccine against their respective toxin subtypes. The use of recombinant binding domain of BoNTs as immunogens and candidate vaccines against BoNTs has been reported by many researchers (18-23). Similarly, in this research, the binding domain of BoNT/E was used as a candidate vaccine against BoNT/E.

In recent years, the use of antigen delivery systems based on micro- and nanoparticles has been widely used by researchers (24-27). Nanoparticles, not only have potential as drug delivery carriers, which offer non-invasive routes of administration through oral and nasal routes, but also show a proper adjuvanticity effect in vaccine formulations. Among water-soluble polymers, chitosan is one of the most extensively used. This is due to some ideal properties of this nanoparticle, such as biocompatibility, biodegradability, nontoxicity, and cost-effectiveness (7). Chitosan is a cationic polysaccharide obtained by partial deacetylation of chitin, the major component of crustacean shells. This hydrophilic polymer can easily cross-link with counter polyanions, like TPP, to provide sustained release of drugs and vaccines (28). Soluble and particulate carriers based on chitosan and its derivatives have received particular interest for the delivery of subunit vaccines via the mucosal route (29-32). In one study, it was found that oral administration of chitosan containing ovalbumin is able to evoke the humoral immune system more efficiently than bare ovalbumin (33). In another study, van der Lubben *et al.* investigated the efficiency of DT-loaded chitosan microparticles in mucosal vaccination against diphtheria via oral and nasal routes in mice (34). In the oral route, they observed no detectable immune responses following the administration of the vaccine in the initial weeks, but a minor IgG titer was seen in week

6. However, they found a significant immune response following the administration of the chitosan containing DT. When they investigated the neutralization efficiency of the antibodies, they found that derived antibodies of mice that received chitosan containing DT through the oral route, were able to neutralize the toxin more efficiently than those that received the vaccine in combination with alum adjuvants. They also found that antibodies derived from the sera of mice that received the bare vaccine (DT in PBS) had no neutralization capacity (34). In the present study, we demonstrated that chitosan NPs containing the candidate vaccine and bare candidate vaccine both could stimulate the immune responses efficiently. Although, the IgG titer following the administration of the bare antigen was apparently higher than the nano-formulation, the differences were not significant. The same results were observed in the case of the challenging assay: both groups (those receiving the antigen-loaded chitosan NPs and those that received the bare antigen) were able to tolerate 2×10^2 MLD of the toxin. Differences in the findings of these studies may be due to differences in the types of antigens and the nature of the proteins. It is noteworthy that in their work Lubben *et al.* observed that following the administration of DT-loaded chitosan microparticles, secretory IgA can be detected in the feces of the immunized mice (34), however, we did not observe any increase in s-IgA in any routes or formulations. The finding may be related to the size of the particles as well as the types of the antigens; while they prepared $4.7 \mu\text{m}$ sized microparticles, our particles were considerably smaller than theirs (275 nm). However, we could not find any supportive data on the correlation of the size of NPs and their ability to evoke local immunity. In the case of nasal administration, van der Lubben *et al.* found that the DT-loaded chitosan microparticles were efficient in evoking immune responses, while the administration of the vaccine dissolved in PBS could not stimulate these responses (34). In a study by Vila *et al.* similar results were obtained; they showed that following the intranasal administration, TT-loaded nanoparticles elicited a more potent and long-lasting humoral immune response (IgG concentrations) as compared to the fluid vaccine (35). Similarly, the mucosal response (sIgA levels) at 6 months post-administration of TT-loaded CS nanoparticles was significantly higher than that obtained for the fluid vaccine (35). Similar results to Vila *et al.* were reported in another study (36). Other researchers also showed that entrapment of antigens (ovalbumin) in trimethyl chitosan resulted in increase in serum IgG and sIgA; while the nasal administration of free ovalbumin did not result in any significant increase in the mentioned antibodies (37). In one study it was demonstrated that tetanus toxoid-loaded chitosan nanoparticles that were delivered through micro-needles, induced comparable IgG and IgG1 titer, yet higher IgG2a titer than commercial TT vaccines (38). However, our findings are different. The serum IgG titer of mice that received the bare antigen nasally was significantly higher than the group that received the chitosan NPs containing the antigen. As well, challenging the animals showed that the group which received the bare antigen was able to tolerate the BoNT/E toxin

4-times higher than the other group. Ravichandran *et al.* also showed that the immunoglobulin responses and the levels of resistance to a bare candidate vaccine against botulinum neurotoxins A, B, and E are high so that the immunized animals were able to tolerate the active toxins (5). In another study, it was shown that the oral administration of a candidate vaccine against *Clostridium botulinum* type C (heavy chain of BoNT/C) can provoke the mice immune responses as well as immunize the animals against the BoNT/C toxin. As these studies show, apparently the nature of the antigen plays an important role in mucosal immunization (39). Although there are many studies on the usage of chitosan as a drug or vaccine carrier, it is the first study that exploits these nanoparticles for the delivery of the binding domain of BoNT/E through oral and intranasal routes. However, this protein has previously been delivered by other nanoparticles for immunization of animal models (40, 41).

In the oral administration route, IgG assay through ELISA showed that the level of the antibody has been increased after each administration. Despite our expectation, the level of serum IgG in the mice that had received the bare antigen was higher compared with the group that received the antigen-loaded nanoparticles, though this difference was not significant ($P>0.05$).

Through the intranasal administration route, in both groups, the group that had received the antigen-loaded nanoparticles and the group that had received the bare antigen, the level of serum IgG had been increased. The increase in the IgG level in the group that had received the bare antigen intranasally was in agreement with the results of Ravichandran *et al.* study (5). As in the oral route, the serum IgG level in mice that had received the bare antigen was higher compared with the mice that received antigen-loaded nanoparticles. However, in the intranasal route, this increase was statistically significant ($P<0.05$).

The challenge results in the groups that had been orally immunized, showed that these mice were able to tolerate 500 MLD. In the group that had been immunized intranasally by chitosan nanoparticles containing the antigen, the mice were also able to tolerate 500 MLD. The mice that received the bare antigen intranasally, were able to tolerate 2000 MLD, and this was in agreement with the average antibody level. In the study conducted by Ravichandran *et al.*, both groups had tolerated the same amount of the toxin (5).

All and all, the results showed that the rBoNT/E binding domain can be a good candidate for using as a vaccine against BoNT/E. By analyzing the results of the challenge, it can be concluded that the intranasal and oral administration of the bare rBoNT/E binding domain, can effectively stimulate the immune system and the use of chitosan nanoparticles has not any significant effects on increasing antibody production.

Indeed, our results showed that the use of various administration routes by chitosan nanoparticles, has no effect on the immunogenicity of an antigen, even, it leads to immunogenicity reduction. One reason for this finding could be the loss of antigen conformational epitopes during the synthesis of the nanoparticles. Of course, this issue should be studied with other procedures, such as

CD, FTIR, or NMR.

Conclusion

Altogether, the results of this study suggest that the intranasal administration of bare antigen is the best way to create protective immunity against botulinum neurotoxin type E in mice.

Acknowledgment

We thank Biology Research Centre of Imam Hossein University (Tehran, Iran) for support. The results presented in this paper were part of a student thesis.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

1. Kobayashi R, Kohda T, Kataoka K, Ihara H, Kozaki S, Pascual DW, *et al.* A novel neurotoxicoid vaccine prevents mucosal botulism. *J Immunol* 2005; 174:2190-2195.
2. Shone C, Agostini H, Clancy J, Gu M, Yang HH, Chu Y, *et al.* Bivalent recombinant vaccine for botulinum neurotoxin types A and B based on a polypeptide comprising their effector and translocation domains that is protective against the predominant A and B subtypes. *Infect Immun* 2009; 77:2795-2801.
3. Byrne MP, Smith LA. Development of vaccines for prevention of botulism. *Biochimie* 2000; 82:955-966.
4. Henkel JS, Tepp WH, Przedpelski A, Fritz RB, Johnson EA, Barbieri JT. Subunit vaccine efficacy against Botulinum neurotoxin subtypes. *Vaccine* 2011; 29:7688-7695.
5. Ravichandran E, Al-Saleem FH, Ancharski DM, Elias MD, Singh AK, Shamim M, *et al.* Trivalent vaccine against botulinum toxin serotypes A, B, and E that can be administered by the mucosal route. *Infect Immun* 2007; 75:3043-3054.
6. Slütter B. Challenges and opportunities in nasal subunit vaccine delivery: mechanistic studies using ovalbumin as a model antigen. Thesis to obtain a PhD at Leiden University, 2011.
7. Tiyaabonchai W. Chitosan nanoparticles: a promising system for drug delivery. *Naresuan Univ J* 2003; 11:51-66.
8. Baudner BC, Balland O, Giuliani MM, Hoegen PV, Rappuoli R, Betbeder D, *et al.* Enhancement of protective efficacy following intranasal immunization with vaccine plus a nontoxic LTK63 mutant delivered with nanoparticles. *Infect Immun* 2002; 70:4785-4790.
9. Shahiwala A, Vyas TK, Amiji MM. Nanocarriers for systemic and mucosal vaccine delivery. recent patents on drug delivery & formulation 2007; 1:1-9.
10. Slütter B, Bal SM, Que I, Kaijzel E, Löwik C, Bouwstra J, *et al.* Antigen-adjutant nano conjugates for nasal vaccination: an improvement over the use of nanoparticles? *Mol Pharmaceutics* 2010; 7:2207-2215.
11. Raut S, Sutar M, Singh S. Nasal vaccine: a novel approach in nasal drug delivery system. *World J Pharm Pharm Sci* 2015;4:1520-1536.
12. Hajizade A, Ebrahimi F, Salmanian AH, Arpanaei A, Amani J. Nanoparticles in vaccine development. *J Appl Biotechnol Rep* 2014; 1:125-134.
13. Ariful Islam M, Firdous J, Choi YJ, Yun CH, Cho CS. Design and application of chitosan microspheres as oral and nasal vaccine carriers: an updated review. *Int J Nanomed* 2012; 7:6077-6093.
14. Wang JJ, Zeng ZW, Xiao RZ, Xie T, Zhou GL, Zhan XR, *et al.* Recent advances of chitosan nanoparticles as drug carriers. *Int*

- J Nanomed 2011; 6:765-774.
15. Nagpal K, Singh SK, Mishra DN. Chitosan nanoparticles: a promising system in novel drug delivery. *Chem. Pharm. Bull* 2010; 58:1423-1430.
 16. Zhao LM, Shi LE, Zhang ZL, Chen JM, Shi DD, Yang J, et al. Preparation and application of chitosan nanoparticles and nanofibers. *Braz J Chem Eng* 2011; 28:353-362.
 17. Ferguson A, Humphreys KA, Croft NM. Technical report: results of immunological tests on fecal extracts are likely to be extremely misleading. *Clin Exp Immunol* 1995;99:70-75.
 18. Valipour E, Moosavi ML, Amani J, Nazarian S. High level expression, purification and immunogenicity analysis of a protective recombinant protein against botulinum neurotoxin type E. *World J Microbiol Biotechnol* 2014; 30:1861-1867.
 19. Zhao H, Nakamura K, Kohda T, Mukamoto M, Kozaki S. Characterization of the monoclonal antibody response to botulinum neurotoxin type A in the complexed and uncomplexed forms. *Jpn J Infect Dis* 2012; 65:138-145.
 20. Baldwin MR, Tepp WH, Przedpelski A, Pier CL, Bradshaw M, Johnson EA, et al. Subunit vaccine against the seven serotypes of botulism. *Infect Immun* 2008; 76:1314-1318.
 21. Mansour AA, Mousavi SL, Rasooli I, Nazarian S, Amani J, Farhadi N. Cloning, high level expression and immunogenicity of 1163-1256 residues of C-terminal heavy chain of *C. botulinum* neurotoxin type E. *Biologicals* 2010; 38:260-264.
 22. Yu YZ, Sun ZW, Wang S, Yu WY. High-level expression of the Hc domain of *Clostridium botulinum* neurotoxin serotype A in *Escherichia coli* and its immunogenicity as an antigen. *Sheng Wu Gong Cheng Xue Bao* 2007; 23:812-817.
 23. Boles J, West M, Montgomery V, Tammariello R, Pitt ML, Gibbs P, et al. Recombinant C fragment of botulinum neurotoxin B serotype (rBoNT/B (HC)) immune response and protection in the rhesus monkey. *Toxicon* 2006; 47:877-884.
 24. Nazarian S, Gargari SL, Rasooli I, Hasannia S, Pirooznia N. A PLGA-encapsulated chimeric protein protects against adherence and toxicity of enterotoxigenic *Escherichia coli*. *Microbiol Res* 2014;169:205-212.
 25. Jahantigh D, Saadati M, Fasihi Ramandi M, Mousavi M, Zand AM. Novel intranasal vaccine delivery system by chitosan nanofibrous membrane containing N-terminal region of Ipad antigen as a nasal shigellosis vaccine, studies in guinea pigs. *J of Drug Delivery Sci Technol* 2014; 24:33-39.
 26. Bret D, Ulery BD, Kumar D, Ramer-Tait AE, Metzger DW, Wannemuehler MJ, et al. Design of a protective single-dose intranasal nanoparticle-based vaccine platform for respiratory infectious diseases. *PLoS ONE*. 2011; 6: e17642.
 27. Nnamani PO, Scoles G, Kröl S. Preliminary characterization of N-trimethyl chitosan as a nanocarrier for malaria vaccine. *J Vector Borne Dis* 2011; 48:224-230.
 28. Jadhav SS, Bhalerao AV. Formulation and characterization of chitosan nanoparticles loaded with rizatriptan benzoate. *Der Pharmacia Lettre* 2013; 5:218-223.
 29. Bento D, Staats HF, Gonçalves T, Borges O. Development of a novel adjuvanted nasal vaccine: C48/80 associated with chitosan nanoparticles as a path to enhance mucosal immunity. *Eur J Pharm Biopharm* 2015; 93:143-164.
 30. Biswas S, Chattopadhyay M, Sen KK, Saha MK. Development and characterization of alginate coated low molecular weight chitosan nanoparticles as new carriers for oral vaccine delivery in mice. *Carbohydr Polym* 2015;121:403-410.
 31. Xu JH, Dai WJ, Chen B, Fan XY. Mucosal immunization with PsaA protein, using chitosan as a delivery system, increases protection against acute otitis media and invasive infection by *Streptococcus pneumoniae*. *Scand J Immunol* 2015;81:177-185.
 32. Islam MA, Firdous J, Choi YJ, Yun CH, Cho CS. Design and application of chitosan microspheres as oral and nasal vaccine carriers: an updated review. *Int J Nanomed* 2012; 7:6077-6093.
 33. Slütter B, Plapied L, Fievez V, Sande MA, des Rieux A, Schneider YJ, et al. Mechanistic study of the adjuvant effect of biodegradable nanoparticles in mucosal vaccination. *J Controlled Release* 2009; 138:113-121.
 34. Van der Lubben IM, Kersten G, Fretz MM, Beuvery C, Verhoef JC, Junginger HE. Chitosan microparticles for mucosal vaccination against diphtheria: oral and nasal efficacy studies in mice. *Vaccine* 2003; 21:1400-1408.
 35. Vila A, Sa'nchez A, Janesa K, Behrens I, Kisselb T, Vila Jato JL, et al. Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice. *Eur J Pharm Biopharm* 2004; 57:123-131.
 36. Rezaei Mokarram A, Alonso MJ. Preparation and evaluation of chitosan nanoparticles containing diphtheria toxoid as new carriers for nasal vaccine delivery in mice. *Arch Razi Inst* 2006; 61:13-25.
 37. Slütter B, Bala S, Keijzerb C, Mallantsc R, Hagenarsd N, Que I. Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: Nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen. *Vaccine* 2010; 28:6282-6291.
 38. Siddhapura K, Harde H, Jain S. Immunostimulatory effect of tetanus toxoid loaded chitosan nanoparticles following microneedles assisted immunization. *Nanomedicine* 2016;12:213-222.
 39. Kiyatkin N, Maksymowych AB, Simpson LL. Induction of an immune response by oral administration of recombinant botulinum toxin. *Infect Immun* 1997; 65:4586-4591.
 40. Bagheripour MJ, Ebrahimi F, Hajzadeh A, Nazarian Sh. Immunogenicity effect of chitosan nanoparticles containing botulinum neurotoxin E binding domain recombinant protein in mice. *J Mazandaran Univ Med Sci* 2015; 25:37-47. (in Persian)
 41. RabieeRudsari A, Ebrahimi F, ArefpourTorabi MA. Study of adjuvant capability of the gold nanoparticles on the immunity of botulinum neurotoxin serotype E in mouse. *Sci J Adv Defence Sci Technol* 2013; 4:87-92. (in Persian)