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Combination of Chitosan and Myxovirus Resistance Oligonucleotide: An Efficient and Safe Natural Adjuvant against Influenza Virus

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A R T I C L E I N F O

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A B S T R A C T

Introduction: Avian influenza virus causes severe economic losses to the poultry industry and has a great potential for becoming a pandemic threat for humans. The application of natural adjuvants has opened up new avenues toward reaching a highly efficient and safe vaccine in recent years. In this study, we investigated the adjuvant activity of interferon-induced myxovirus resistance (Mx) protein on chitosan-based H9N2 nanoparticles in a BALB/c mouse model. **Methods:** The inactivated H9N2 virus antigen was encapsulated in chitosan nanoparticles (NPs) using gelation method. Female BALB/c mice were randomly divided into four groups (n=10). Group A received the H9N2-loaded chitosan NPs mixing with Mx intranasally and was boosted twice with a 1-week interval. Group B received the H9N2-loaded chitosan NPs in the same manner. Mice in groups C and D received the chitosan NPs and PBS, respectively as negative controls. Body weights of the mice were measured at defined times. Blood samples were collected from the animals and their influenza-specific antibody titer was determined using ELISA. **Results:** The results demonstrated a higher antibody level in treated groups A and B as compared to the control samples. We also showed that the combination of Mx and chitosan could significantly induce an influenza-specific antibody titer, indicating synergistic effects of the applied adjuvant and NPs together. **Conclusion:** The formulation of H9N2 with Mx as an adjuvant and chitosan as a nanocarrier is a promising procedure for developing an efficient vaccine against avian influenza virus.

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INTRODUCTION

Avian influenza (AI) is a worldwide contagious respiratory viral disease of poultry and wild bird species [1, 2]. Among AI virus (AIV) subtypes, H9N2 has a potential to cause a human pandemic in the future [3]. The first reported infection by this virus occurred in 1996 in Wisconsin, USA and then it was detected in several Asian countries among domestic poultry populations [4]. The extensive spread of the virus around the 1990s has led to continuous viral circulation in East Asia, the Middle East, and North Africa [5]. The inactivated H9N2 vaccines have been used for controlling the disease in most parts of Asia, especially in the endemic areas.

Due to their poor antigenicity, the inactivated vaccines are required to be formulated with an adjuvant to enhance the immune responses against the influenza virus. The activity of several potent adjuvants, including the members of interleukin family, type I interferons, CpG oligodeoxynucleotides, Mx oligodeoxynucleotides, hemokinine-1, and the ligand of TLR7/8 have been shown to trigger the innate and adaptive immune responses against influenza virus infection [6-12]. Evidence such as considerable levels of antigen-specific IgG in plasma and IgA in mucosal secretions, promotion of cytotoxic T-cell responses, and enhanced Th1- and Th2-type responses in immunized animals that receive adjuvants confirm their role in enhancing the immune responses [12].

The administration route is another critical factor in antigen uptake and presentation to the immune cells. The inactivated vaccines that are administrated by intramuscular injection do potentially fail to reach the antigen-presenting cells and to induce the immune responses [13, 14]. Over the past two decades, the development of nanoparticles (NPs) for vaccine

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delivery has been experimented. The NPs are mainly designed for enhancing antigen uptake by antigen presenting cells as well as for controlling the antigen release to promote a more rapid immune response. Chitosan is a biocompatible and biodegradable NP which has been successfully applied in a number of preclinical and clinical studies [15-19]. It has demonstrated good tolerability, positive clinical results among several infections and excellent immune stimulation. A number of studies have so far addressed the role of chitosan-based NPs on induction of humoral and cellular immune responses against influenza infection. Application of chitosan as an adjuvant has been shown to enhance Th1 and Th2 responses. Additionally, chitosan has been reported to induce strong systemic and mucosal immune responses against influenza antigens [20, 21]. In case of H9N2 virus, Khalili *et al*., have shown that H9N2 loaded into chitosan NPs causes no side-effects and strongly induces antibody titers[22]. Moreover, Sadati et al., have reported that a considerable humoral and cellular immune responses could be achieved as a results of CpG oligonucleotides and chitosan combination [9].

In the present study, we hypothesized that the ocular administration of inactivated H9N2 antigen, encapsulated in chitosan NPs that was formulated with Mx confers systemic antibody responses in mice in a prime-boost vaccination strategy.

MATERIALS AND METHODS

Antigen Preparation and Inactivation

A locally isolated influenza virus H9N2 subtype was inoculated into embryonated eggs according to World Organization for Animal Health (OIE, 2012) protocol. The allantoic fluid was harvested after 3 days incubation at 37 ˚C and centrifuged at 1500 rpm for 15 min. The viral antigen was tittered using hemagglutination assay (HA) according to the standard protocol. The prepared H9N2 antigen was inactivated by mixing with 0.1% of the final concentration of formalin (Merck, Germany) and incubation at 37 ˚C for 16 h.

Chitosan NPs Preparation and Formulation

The NPs were initially prepared according to the ionic gelation method using chitosan and sodium tripolyphosphate (TPP) anions. Chitosan solution (0.2% w/v) was prepared by dissolving 200 mg of chitosan powder (Sigma-Aldrich, Germany) in 1 mL 1% acetic acid using magnetic mixer at 800 rpm for 24 h. The pH was adjusted to 5 by 1 M NaOH and then the solution was filtered (0.45 µm). Consequently, 0.1% TPP (Merck, Germany) dissolved in deionized water was added into the chitosan polymer solution with continuous magnetic stirring at room temperature for 1 h. Four ratios of chitosan/TPP including 1:1, 1:2, 1:3 and 1:4 were accordingly prepared. The solutions were centrifuged at 14000 rpm, 4 □C for 30 min and the upper layer containing chitosan particles was collected. The physicochemical features of the NPs such as size, surface charge and distribution index in different ratios of chitosan/TPP were measured using Malvern Zetasizer Nano ZS (Worcestershire, UK). The morphological and surface characteristics of the NPs were assessed via transmission electron microscopy (Zeiss-EM10C-100 KV, Germany), and scanning electron microscopy (FESEM ZEISS, Germany). Encapsulation efficiency and loading capacity of the NPs were calculated as follow:

Encapsulation efficiency = Total HA–Free HA/ Total HA×100%

Loading capacity = Total HA–Free HA/1 mg chitosan NPs dry weight

The same profile was utilized to prepare influenza virus chitosan-based NPs. The 1:2 ratio of chitosan-TPP was mixed by each of 0.5, 1.0, and 2.0 ml of the H9N2 inactivated antigen containing 4 HA. The physiochemical features of the prepared samples were also analyzed. Both loading capacity and encapsulation efficiency of the NPs were determined. HA assay was utilized to evaluate the stability of the NPs up to 1 month when incubated at room temperature and at 4 ˚C.

Mx Adjuvant Synthesis

The oligonucleotide coding sequence of the conserved SGKSSVLEALSGVALPR motif of Mx which was known to be effective in inducing B-cell and T-cell immune responses based on an in silico study [23] was selected. The coding sequence was constructed in pcDNA3.1 as described previously [11] and was used at final concentration of 10 µg/µl.

Mice Immunization

Healthy 6-8 week-old female BALB/c mice were considered for two replicates each containing 40 mice. The mice with an average weight of 20 g were randomly divided into 4 groups (n=10) in each trial. Group A received 20 µl of H9N2-loaded chitosan NPs in combination with Mx via the intranasal route. The mice were boosted twice at a one-week interval. Group B received H9N2-loaded chitosan NPs only and was boosted in the same manner. Mice in groups C and D were considered as negative controls by receiving plain chitosan NPs and PBS, respectively. Mx was mixed with H9N2-loaded chitosan NPs right before the administration. The body weights of the mice were measured at the time of administration and every week up to 2 months. Blood samples were collected from each group at weeks 1, 2, 3, 4, and 5, post-vaccination. The influenza specific antibody titer was determined using an ELISA assay (Influenza A virus Antibody Test Kit; IDEXX, USA) as described by the manufacturer.

Statistical Analysis

The results of the experiments were statistically analyzed by Mann-Whitney using SPSS, version 22 (SPSS Inc., Chicago, IL, USA). Mean comparison was performed based on the least significant difference (LSD) test. The P-value of < 0.05 was considered statistically significant. The results were expressed as means ± standard deviation (SD).

RESULTS

Preparation and Characterization of the NPs

The chitosan NPs were successfully prepared through ionic gelation of chitosan, with TPP acting as the cross-linking moiety. To reach an optima condition of forming NPs, the size and surface charge of the prepared treatments were investigated (Fig. 1 and Fig.2). Table 1 represents quantitative analysis of 4 different ratios of chitosan/TPP, including 1:1, 1:2, 1:3 and 1:4.

According to Table 1, the largest and the smallest resulted NPs were 328 and 110.5 nm in diameter, respectively, which are generally adequate for efficient uptake by the antigen-presenting cells. Additionally, the sizes of the NPs were increased by increasing the volume of TPP. This was probably due to the molecular weight of H9N2 virus (Table 1 and Fig. 1).

Different concentrations of the antigen also changed the size and the size distribution of the NPs. All ratios of the NPs demonstrated a positive zeta potential of above + 11 mV, which was in favor of the vaccine formulation since the positively charged NPs could enhance phagocytosis.

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Table 1. Physiochemical analysis of the chitosan in different ratios of Chitosan/TPP.

Volume ratios (Chitosan/TPP)	Size of the NP[24]	Zeta potential (mV)	Distribution index	Size distribution
1:1	110.5	12.5	0.274	91.4%
2:1	159.4	11.7	0.261	100%%
3:1	281.2	16.5	0.394	96%%
4:1	328	16.8	0.462	74%%

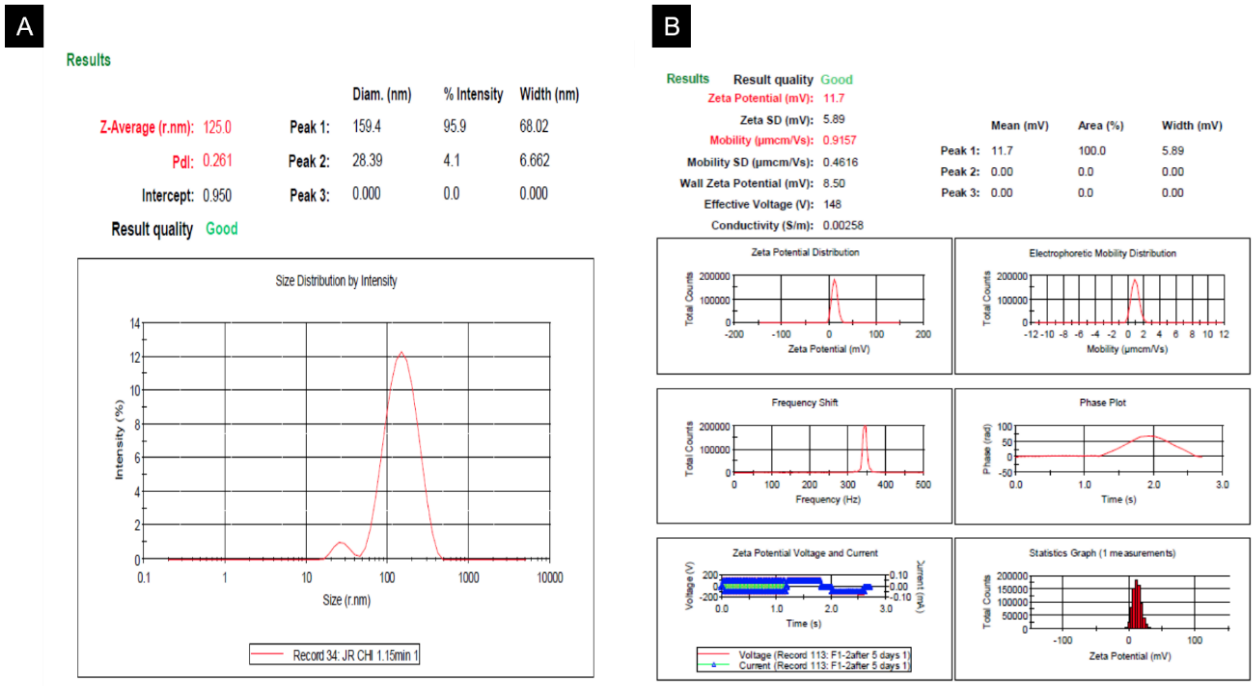

Fig. 1. (A) The size distribution of the NPs. **(B)** Surface charge of chitosan in the ratio of 1:2 chitosan/TPP.

According to the Zetasizer, the chitosan and Mx, individually and in combination demonstrated spherical shape with diameters in the nanometer scale and cationic surface charge (a range from +11.8 mV to +16.8 mV), suggesting that the prepared NPs had a suitable size and charge. The zeta potential of the developed NPs were slightly increased after loading the H9N2-inactivated antigen by +22.4 mV (Table 2 and Fig. 2), which was probably due to the load of virus surface glycoprotein that processes the positive charge. Encapsulation efficiency of H9N2 on chitosan NPs was estimated as 88.62% which indicated that the antigen was sufficiently encapsulated.

Table 2. Physiochemical analysis of the influenza virus antigen loaded on different ratios of chitosan/TPP.

Volume ratios (Chitosan/TPP/Virus)	Size of the NP [24]	Zeta potential(mV)	Distribution index	Size distribution	Loading efficiency
1:2:2	231.6	19.8	0.215	96.6%	40%
1:2:1	328.6	23.5	0.251	100%	52%
1:2:0.5	310.6	22.4	0.334	95.7%	48%

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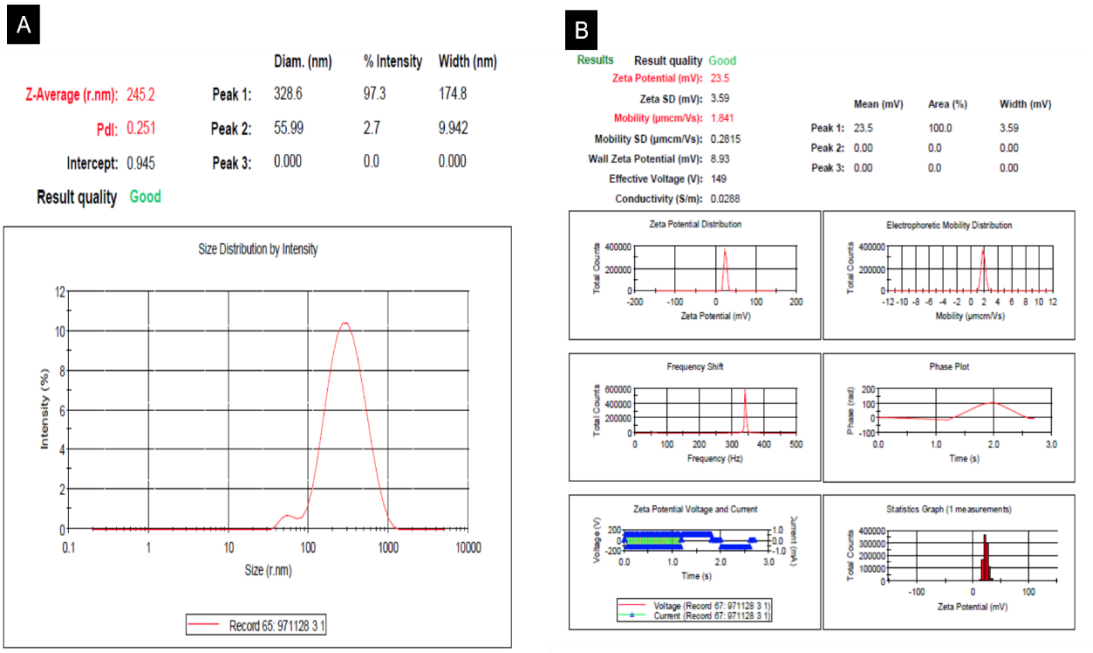

Fig. 2. (A) The size distribution and **(B)** the zeta potential of influenza antigen loaded into chitosan.

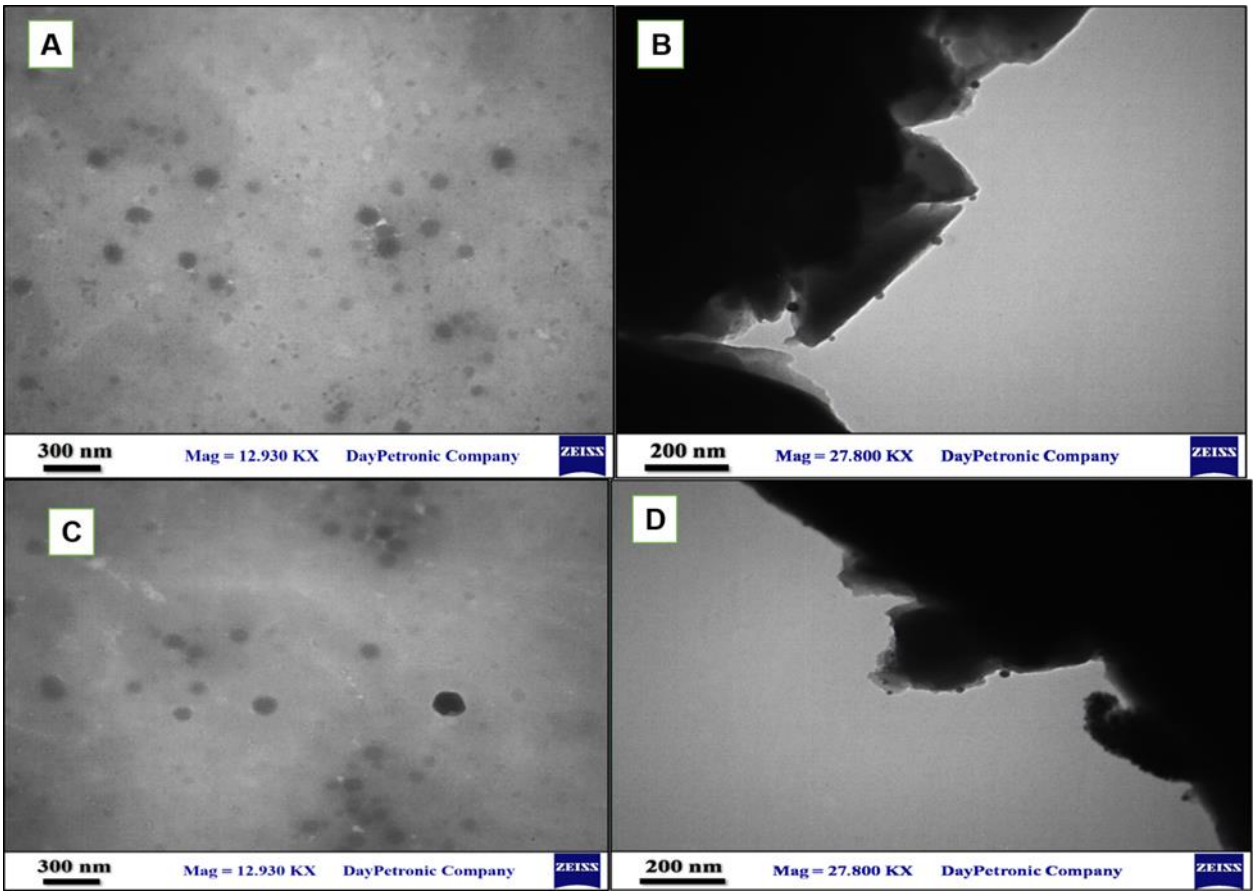

Fig. 3. Transmission electron microscope and Scanning electron microscopy (SEM) images of chitosan NP **(A and B)** and influenza antigen loaded onto chitosan **(C and D)** showing morphological features of the NP in the ratio of 1:2 chitosan/TPP. The scale bars are presented in each panel.

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Morphological analysis of the chitosan examined by electron microscopy showed that they were approximately uniform spheres as shown in Fig. 3.

Mice Body Weight and Safety Evaluation

During the experiment, the bodyweights of the mice in the vaccinated and the unvaccinated groups reached from 24.5 to 43.8 g, indicating that the NPs did not have any adverse effect on the weight gaining and growth of the animals (Fig. 4).

Humoral Response Evaluation

Efficacy of H9N2-loaded chitosan NPs with and without Mx adjuvant was investigated. The levels of specific influenza titers in mice receiving the H9N2-loaded chitosan NPs plus Mx, H9N2-loaded chitosan NPs alone, plain chitosan-TPP, and PBS evaluated as Geometric Mean Titer (GMT) are shown in Table 3.

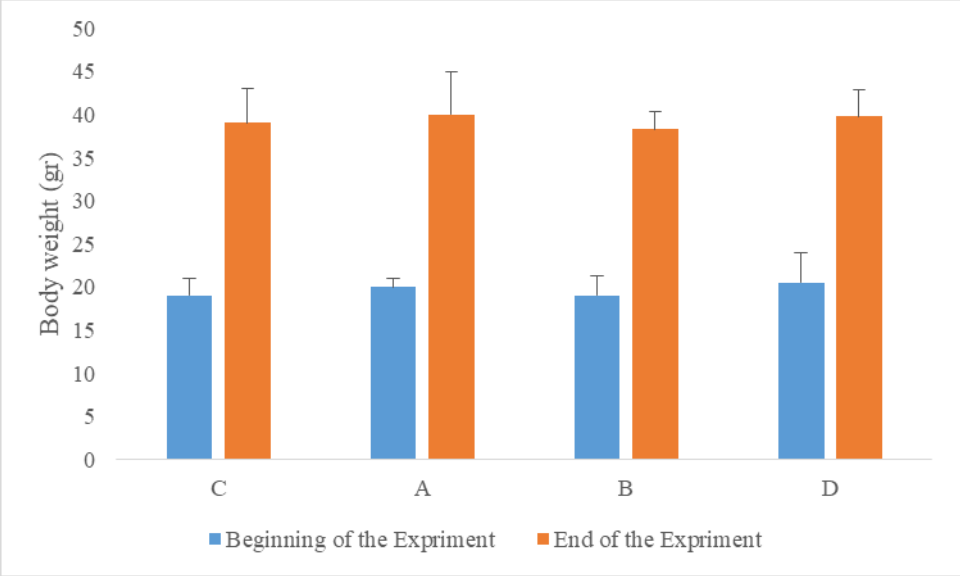

Fig. 4. Measurement of body weights of the mice after treatment with H9N2-loaded chitosan NPs plus Mx adjuvant. (A) H9N2-loaded chitosan (B) Plain chitosan-TPP (C) PBS (D) Control samples.

Table 3. The mean of antibody production level against influenza virus in mice vaccinated in different groups in 5 weeks following the boosting.

Groups and treatments		Blood samplings (Weeks after boosting)				
		1	2	3	4	5
A	H9N2-loaded chitosan and Mx	2886±12.12	3465±19.09	3897±11.03	4733±12.02	4936±19.11
B	H9N2-loaded chitosan	2764±85.11	3036 ±85.10	3692 ± 86.09	3672±889.10	3468 ±65.09
C	PBS	241±21.10	267± 21.08	237 ± 20.07	270± 22.08	224 ± 21.08
D	plain chitosan-TPP	225±24.09	314 ±24.07	256± 21.09	246 ±24.08	258 ± 27.10

The specific antibody titers were significantly increased in groups of mice that received H9N2-loaded NPs with or without adjuvant compared to the control groups. However, the antibody level was significantly higher (4936 unit) in mice immunized with NPs plus adjuvant compared to without the adjuvant (3468 unit), five weeks after the boosting. Given these results, the mean immunogenicity was significantly induced in H9N2-loaded chitosan plus Mx treatment compared to the other groups and particularly the controls during each blood sampling after boosting. Therefore, combining the Mx adjuvant significantly improved the vaccine efficiency even for H9N2-loaded NPs.

DISCUSSION

Influenza is a global health concern and one of the most common viruses among human beings. To overcome influenza infection, researchers are inclined to integrate novel vaccines using potential adjuvants, thereby boosting mucosal and systemic immunity. It is well-established that antigen contents, the immunological aspects and the formulation and type of the applied adjuvant, are among the most determinative factors for protection against a virus [25, 26]. Therefore, in this study, we examined the potential impact of loading H9N2 antigen into chitosan along with Mx adjuvant, alone and in combination, on the antibody titer and general health, in a BALB/c mouse model.

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Analyzing the body weight of the mice pointed out that neither chitosan nor Mx adjuvant were toxic for the health of the animals, confirming the safety of this potential nanovaccince. Mx, is a member of dynamin-like large guanosine triphosphatases (GTPases), and has been repeatedly shown antiviral activities [27, 11, 28]. Soleimani and co-workers have demonstrated no side-effects regarding the application of Mx as a bioadjuvant on mice body weight [11]. In addition, chitosan is a natural polysaccharide, nontoxic, biodegradable, biocompatible NP, which has been successfully utilized as an efficient carrier in drug delivery in previous studies [17, 19, 29]. Chitosan also increases antigen size [30], and strongly activates the immune system by being produced as NPs, indicating that it would be a promising substitute for a simple antigenic vaccine, either purified or recombinant. Characterization of the developed NPs also suggested that the size of antigen-containing chitosan is within the range of 231.6 to 328.6 nm. In fact, it has been previously reported that the size of antigen-containing NPs which are greater than 225 nm in diameter are able to induce Th1 cytokines; however, NPs less than 155 nm tend to induce Th2-cytokines [31, 32]. A suitable size can efficiently facilitate the phagocytosis mechanism by macrophages after the administration, and their migration to the target (i.e. lysosome of the macrophage) to strongly stimulate the immune system against the antigen. Consistent with our findings, Dehghan and co-workers have been reported that influenza virus antigen encapsulated onto chitosan NPs with 338 nm in size could be more efficient in the vaccine formulation [9]. According to zeta sizer and morphological analysis, our prepared H9N2-loaded chitosan NPs not only possessed a suitable size which is crucial for being up-taken by the APCs, but also had positive charges cause mucoadhesion by interacting with the negative charges on the cell surfaces.

Herein, by comparing between the treated groups, we showed an increase in the antibody titers against H9N2 antigen, confirming the capability of Mx as a bioadjuvant to improve the immune responses. Chitosan is known as a superior adjuvant in mediating the cell-mediated immune responses. The polymer promotes maturation and activation of dendritic cells via cGAS-STING signaling pathway, which finally enhances Th1 cellular immune responses [33]. This signaling pathway also improves the production of type I interferons, which promote the migration of matured dendritic cells. The induction of interferon genes and activation of dendritic cells trigger innate and adaptive immune responses. Therefore, it would be suggested that the increased humoral immunity in mice that received NPs is resulting from engaging the STING-cGAS pathway by chitosan.

As show in table 3, group B (immunized with H9N2-loaded chitosan) produced specific antibodies against the influenza which were significantly improved in the combination of Mx and chitosan-based NPs. Recently, Soleimani et al., have been demonstrated that application of Mx, as a bio adjuvant, with HA2 DNA vaccine can markedly effective against influenza virus infection. They revealed that administration of two doses of the adjuvanted vaccine can successfully induce both cellular and humoral immune responses [11]. In addition, Dehghan et al. have reported that encapsulation of H9N2 antigen/HK-1 into chitosan NPs resulted in higher level of specific-influenza antibodies [9].

Consistent with the previous studies [9, 22], our data indicate that the groups of mice vaccinated with H9N2-loaded chitosan NPs induced detectable antibody titers in the experimental animals. The increase in antibody levels clearly shows the potential role of the adjuvant in the effectiveness of a nanovaccine. Taken together, our results indicated that application of natural adjuvants such as a combination of chitosan and Mx is a promising approach to produce a safe, efficient and affordable vaccine against influenza virus which can efficiently improve the humoral immune response.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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