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Evaluation of safety and humoral immunogenicity of inactivated Newcastle disease virus genotype VII entrapped in calcium phosphate nanoparticles in chickens

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Abstract

Newcastle disease (ND) is a highly contagious and severe disease that affects birds, including domestic poultry, causing significant economic losses to the poultry industry. The disease is caused by the ND virus (NDV). Despite extensive vaccination efforts against NDV, controlling the disease remains challenging, primarily due to the emergence of new genotypes. Recent attention has focused on calcium phosphate nanoparticles (CaP NPs) as promising adjuvants for vaccines. This study aimed to design and construct CaP NPs containing inactivated NDV genotype VII and evaluate their safety and humoral immunogenicity in chickens. Following virus propagation in specific-pathogen-free eggs and inactivation with formalin, CaP NPs containing inactivated NDV were prepared using *in situ* and adsorption methods. The NPs were characterized for shape, size, polydispersity index and surface charge. Chickens were immunized subcutaneously with CaP NPs containing inactivated NDV and the humoral immune response against NDV was assessed using HI and enzyme-linked immunosorbent assays. Results showed a significant increase in the mean antibody titer against NDV in chickens treated with both NP structures compared to control groups. No significant difference was observed between the two NP preparation methods. The safety of CaP NPs containing inactivated NDV was confirmed. However, mucosal immunization with these NPs did not yield satisfactory results indicating the need for further research. Overall, this study confirmed the positive role of CaP as an adjuvant in enhancing the humoral immune response against NDV *via* injection and highlighted the need for continued research on mucosal immunization with the NPs.

Introduction

Newcastle disease (ND), also known as fowl plague, is a highly contagious disease that affects various types of avian species.[1] It is caused by ND virus (NDV) which belongs to the genus *Avulavirus* of the family *Paramyxoviridae* (International Committee on Taxonomy of Viruses).[2] The NDV is recognized as a significant pathogen affecting birds, leading to substantial annual economic losses in the poultry industry. The virus spreads rapidly and extensively on a global scale, originating in Indonesia in 1926 and becoming a worldwide concern within three decades.[3] The negative-stranded RNA genome of NDV encodes six proteins arranged as 5'-L, HN, F, M, P, NP-3'. The virus infiltrates the host *via* the mucosal tissues of the respiratory system and subsequently spreads to other organs.[4] Based on the pathogenicity, NDV strains are classified to five pathotypes, ranging from mild to severe in virulence: Asymptomatic enteric, lentogenic, mesogenic, viscerotropic velogenic and neurotropic velogenic.[5] Velogenic NDVs pose a significant risk to the poultry industry due to their high mortality rates and cause to decrease in egg production in laying hens. Furthermore, according to phylogenic analysis of the fusion protein of the virus, NDV strains were classified into two classes, I and II.[6] The NDV genotype VII, within class II, are greatly spread into Middle East, Asia, Europe and Africa.[7,8] Since 2011, genotype VII NDV strains have been

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common in Iranian poultry farms becoming the predominant genotype circulating among different avian species in the country.[9]

The NDV control and prevention are the strong implementation of biosecurity regulations and extensive vaccination efforts, both of which have been effectively implemented for many years around the world. Live and inactivated NDV vaccines have been widely used to control the outbreaks. However, even in vaccinated birds, the high frequency of NDV infection is not just associated with inadequate immunization or immunological suppression. It may cause from viral mutation which modifies the virus's genomic sequence and biological characteristics. Despite efforts to control the disease using vaccines, ND continues to cause problems for the poultry industry globally, leading to outbreaks and financial losses. While vaccines help prevent the disease, vaccinated birds can still carry and spread the virus to others through their feces and saliva.[8,10,11] In endemic regions, multiple lentogenic NDV strains, particularly genotype II, known for their low virulence, are used as live and inactivated vaccines for controlling ND. Evidence indicates that matched vaccines may provide a higher protection rate and result in lower viral shedding than mismatched ND vaccines.[6,12,13] The emerging NDV genotypes can complicate control strategies, making them difficult to implement, hence, there is a need to use high-quality and up-to-date vaccines.[14]

Nanoparticle (NP)-based vaccines have demonstrated a highly promising approach in vaccine development over the past two decades.[15,16] Nanoparticles are solid particles with diameters between 1.00 to 1,000 nm and have proven to be effective carriers and delivery systems for vaccines.[17] Their relatively small size enables NPs to penetrate the membranes of the host cells easily. Moreover, they are readily absorbed by antigen-presenting cells, which can stimulate robust immune responses.[18,19] Currently, calcium phosphate (CaP) NPs (NPs) are recognized as one of the most promising antigen delivery systems for vaccine transport, attracting increasing interest over the past ten years.[20,21] The CaP NPs have advantageous due to their non-toxic, biodegradable properties and pH-dependent solubility.[21,22] These NPs protect the antigens from degradation, enhance targeted cell interactions and can be modified to adjuvants to improve vaccine efficacy. Despite the considerable potential of CaP, research on the application of CaP NPs in vaccine development remains limited.[17]

In this study, the role of CaP NPs in enhancing the immunogenicity of NDV was investigated. Due to the importance of matching the virus with the circulating strain in each region, an Iranian isolate of genotype VII NDV was used. The results were compared to those obtained from a conventional adjuvant vaccine.

Materials and Methods

Newcastle disease virus (NDV). In this research, the NDV of genotype VII was used. The virus was isolated in the Poultry Disease Research and Diagnosis Department of the Razi Vaccine and Serum Research Institute in Karaj. All experiments involving viruses were conducted in strict compliance with biosafety protocols and guidelines to ensure the safety of personnel and the environment. Laboratory work was performed in a certified biosafety level 2 facility, adhering to the Razi Vaccine and Serum Research Institute's biosafety standards.

Propagation and titration of NDV. The NDV was propagated according to the method described previously.[23] Briefly, 0.20 mL of the NDV was inoculated into the allantoic cavity of 9-day-old embryonated specific-pathogen-free (SPF) eggs (Vanky´s, Venkateshwara Hatcheries, Pune, India). After 48 hr incubation at 37.00 ˚C, the allantoic fluid was harvested and titrated on embryonated chicken eggs (ECE). For this purpose, different dilution of the virus (ranging from 1.00×10^1 to 1.00×10^{11}) was injected into allantoic cavity of ECE and incubated for 5 - 7 days at 37.00 ˚C. Presence of the live virus in each ECE was conformed using rapid hemagglutinin test, then, the 50.00% embryo infectious dose endpoint was calculated using the standard Reed and Muench formula.[24] Additionally, hemagglutination (HA) titration test was used to determine the amount of NDV in a suspension according to the guidelines of World Organization for Animal Health.[23] Two-fold serial dilutions of the NDV suspension were prepared in a microwell plate. Then, 1.00% of chicken red blood cells were added to each well. The agglutination result was determined after staying 45 min at room temperature. The end point of HA was the last well showing complete HA activity.

Inactivation of NDV. The infective allantoic fluid which contained NDV was treated with formalin at a final concentration of 0.50 : 1,000 for 16 hr at 37.00 ˚C. Complete inactivation of the NDV was confirmed after two serial passages in SPF embryonated eggs. No HA activity was detected in their allantoic fluid of the live embryos. The inactivated NDV in allantoic fluid was then used for following preparation of NDV entrapping in CaP.

Preparation of NDV entrapped in calcium phosphate nanoparticle (CaP). To formulate the NDV which was entrapped in CaP NP two different methods were used: Adsorption and *in situ*. In the *in situ* technique, NPs were synthesized simultaneously with virus loading. In the adsorption approach, NPs were generated first, then, the virus was adsorbed onto pre-formed particles. The main difference between these methods was in the timing of virus addition to the NPs which affected the efficiency of the virus adsorption.

In the first method, *in situ*, CaP NPs coupled with inactivated NDV that was formulated using a method

described by He *et al.*, with some modification.[25] Briefly, 2.50 mL of inactivated NDV was added to 2.50 mL of 12.50 mM calcium chloride (Sigma-Aldrich, St. Louis, USA),. Then, 2.50 mL of 12.50 mM dibasic sodium phosphate (Merck, Darmstadt, Germany) and 500 µL 15.60 mM sodium citrate (Merck) were added. The mixture was stirred using a magnetic stirrer for 24 hr at room temperature. Afterward, the supernatant was removed, and 4.00 mL antigen was added into the sample and stirred for an additional 30 min to ensure proper mixing.

The second method, adsorption, CAP-NDV was made as described by Relyveld with some changes.[26] Briefly, 5.00 mL of 12.50 mM dibasic sodium phosphate was added to 5.00 mL of 12.50 mM calcium chloride which was continually stirred then, followed by addition of 1.00 mL of 15.60 mM sodium citrate the mixture was stirred for 48 hr. The CaP gel thus obtained was left to stand until 4.00 mL of liquid was decanted. The clear supernatant liquid was replaced by 4.00 mL of 0.90% NaCl to remove excess phosphate to avoid undesired absorption, then, 6.00 mL of inactivated NDV was added to the gel in two steps and stirred for 30 min and finally the sample was kept at 4.00 ˚C.

GaP nanoparticle characterization. The morphology, structure and composition of CaPs were characterized by scanning electron microscopy (FE-SEM S-4160; Hitachi High-Tech, Irvine, USA) and Malvern Zetasizer Nano ZS90. (Malvern Panalytical, Malvern, UK).

Antigen evaluation in CaP adjuvant. To calculate the encapsulation efficiency (EE) of antigen to the CaP gel, the prepared CaP formulated with inactive NDV antigen was centrifuged for 20 min at 8,000 RPM, the supernatant was then isolated and its protein content was measured using Lowry's protein assay method.[27] The following formula was used to calculate the amount of virus absorption:

EE%= Protein content added to the sample-protein content in eluate (not adsorbed)/ Protein content added to the sample

Antigen release from CaP nanoparticles. To monitor the release of antigen absorbed onto the CaP gel, 30.00 ml of CaP adjuvant formulated with NDV antigen was centrifuged at 10,000 RPM for 20 min. The resulting sediment was resuspended in phosphate-buffered saline and aliquoted into microtubes for the release assay. The microtubes were incubated in 37.00 ˚C and at a pre-determined point, two microtubes were retrieved and centrifuged at 10,000 RPM for 20 min. The supernatant was carefully collected and stored at – 20.00 ˚C for subsequent analysis. The amount of protein in the supernatant was quantified using a multimode microplate reader (BioTek, Winooski, USA) at a wavelength of 280 nm and used as a measure of antigen release.

Formulation of inactivated NDV with Montanide™. The inactivated NDV antigen was formulated with Montanide™ ISA71 VG oil (Seppic, Paris, France) adjuvant according to the manufacturers' instructions. The antigen was completely mixed with the adjuvant in the ratio of 30 to 70 (antigen:adjuvant).

Immunization experiment. Immunogenicity of the NDV-CaP NPs was determined in chickens (Hy-line breed) through recommended rout of vaccination (sub-cutaneously). Day-old chickens were provided and maintained for a duration of 3 weeks until the maternal antibodies were no longer detectable. A HA inhibition (HI) test was conducted on the serum of the chickens to verify the absence of NDV -specific antibodies. A total number of 60 chickens were randomly divided into six groups (G1 to G6) with 10 birds *per* group. Each chicken received a subcutaneous injection of 0.20 mL. Groups G1 and G2 were immunized with NDV-CaP NPs prepared by different methods. The G1 group received NDV-CaP NP formulated using the *in situ* method,and the G2 group was immunized with NDV-CaP prepared *via* the adsorption method. The G3 group was inoculated with Inactivated NDV antigen formulated with Montanide™ ISA71 VG oil. Groups G4, G5 and G6 were considered as control groups and received NDV inactivated antigen without adjuvant, CaP without any antigen and phosphate-buffered saline, respectively. All chickens were immunized twice at a 1-week interval. They were housed in separate rooms under consistent environmental conditions and provided with food and water *ad libitum*. Blood samples were collected from the chickens at 1-, 2-, 3- and 4-weeks after the second immunization and the sera were prepared for further analysis. All procedures involving animals were conducted in compliance with ethical principles and the national norms and standards for conducting research in Iran. Ethic approval was granted by Research Ethics Committee of the Razi Vaccine and Serum Research Institute (Approved date: October 15, 2023, Karaj, Iran).

Evaluation of humoral immune responses of chickens. Humoral immune responses in immunized chickens were evaluated using HI and enzyme-linked immunosorbent assay (ELISA) methods. The HI test was carried out on individual serum samples collected at 1-, 2-, 3- and 4-weeks post-immunization according to the protocol outlined in World Organization for Animal Health (WOAH) Manual.[28] The HI test was performed using 4 HA Unit of NDV antigen. The HI test was used to determine the antibody titer against the HA antigen. Serum titers were determined as the highest dilution of serum that completely inhibited the agglutination of chicken red blood cells. Specific antibody titers against (NDV were quantified using a commercial ELISA kit (Biocheck, Rotterdam, Netherlands) according to the manufacturer's instructions which specifically measured immunoglobulin (Ig) G antibodies. The geometric mean titers of antibodies were calculated for each time point of serum collection to assess the antibody response over time.

Evaluation of safety of CaP-NDV. For this purpose, a total of 30 three-week-old chicks that were seronegative for NDV antibodies were randomly assigned to three groups of 10 birds each. Two groups were inoculated sub-cutaneously with 0.40 mL (equivalent to two doses) of NDV-CaP *In situ* and NDV-CaP adsorption, respectively. The third group was considered as a negative control and received no inoculation. The chickens were monitored daily for 2 weeks for any sign of clinical symptoms of disease or the development of local lesions at the injection site.[23]

Statistical analysis. Statistical analysis was performed using GraphPad Prism (version 8.0; GraphPad Software Inc., San Diego, USA). Statistical differences were evaluated using two-way ANOVA. A p value less than 0.05 was considered statistically significant.

Results

Propagation and inactivation of NDV. A NDV, genotype VII, was propagated in the ECE of SPF eggs. The allantoic fluid contained NDV with a HA titer of 2^8 and a 50.00% embryo infectious dose per mL of 1.00×10^{10}. The virus was subsequently inactivated using formalin. The HA titer of inactivated NDV antigen was then measured and determined to be 2^7. To confirm the complete inactivation of the virus, the sample was passaged twice into embryonated SPF eggs. The absence of embryonic mortality and pathological changes in all embryos during both passages provided verification of the virus's complete inactivation.

Surface morphology of CaP particles. The size of CaP NP is shown in the diagram of Figure 1A. The diagram displays two peaks at sizes of 421.20 and 110.40 nm with an average particle size reported as 307nm. The graph in Figure 1B shows the zeta potential or surface charge of CaP NPs which was reported to be – 29.50 mV. The image in Figure 2 is the results of scanning electron microscopy imaging and show the appearance characteristics of CaP NPs which indicate a spherical shape with a smooth and uniform surface.

Assessment of Antigen in CaP Adjuvant. Protein concentration in the NDV antigen was analyzed using the Lowry method. A standard line and its corresponding equation were first generated using a 1.00 mg mL^{-1} standard solution (Fig. 3). Based on this standard line, the protein concentration of the NDV antigen was determined to be 3.60 mg mL^{-1}. To assess antigen encapsulation onto CaP NPs, protein quantification was conducted using the Lowry method, yielding a protein concentration of 0.96 mg mL^{-1}. Furthermore, the EE of the NDV antigen within the CaP NP structure was determined to be 73.00%.

Evaluation of NDV antigen release from CaP NP. The results of the evaluation of antigen release according to Figure 4 show that 80.00% of the antigen was released in the first 24 hr and the rest was released within 100 hr after the first sampling.

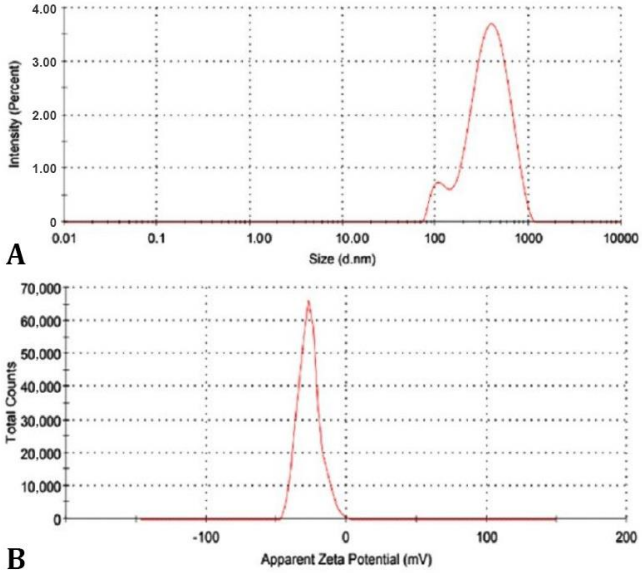

Fig. 1. Surface morphology of calcium phosphate particles. **A)** Size diagram of calcium phosphate nanoparticles. **B)** Zeta potential diagram of calcium phosphate nanoparticles.

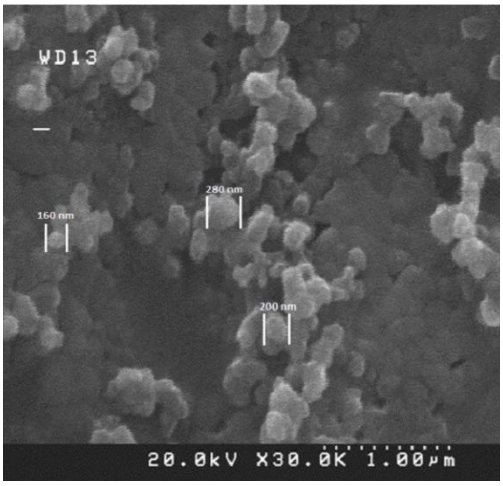

Fig. 2. Scanning electron microscopy images of calcium phosphate nanoparticles.

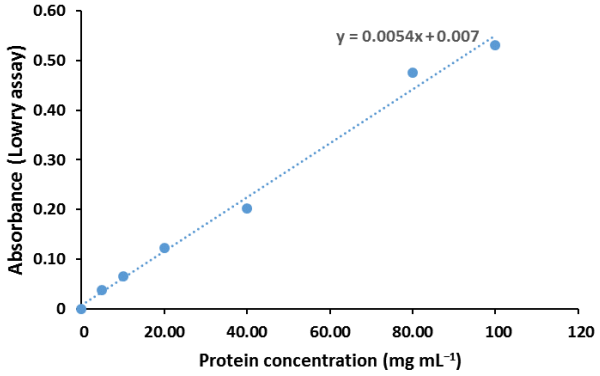

Fig. 3. Standard sample diagram and equation.

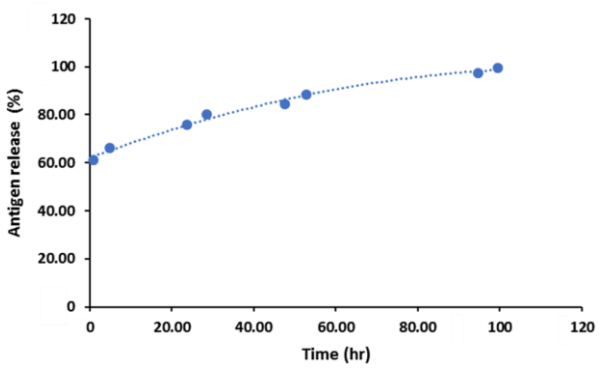

Fig. 4. Diagram of Newcastle disease virus antigen release from calcium phosphate nanoparticles.

Immunogenicity assessment of NDV-CaP NPs. The level of HI and ELISA titers in immunized and control groups of chickens were measured at regular intervals throughout the vaccination schedules. As shown in Figure 5, the amount of HI- specific antibodies against the NDV in chickens administered NDV-CaPs (G1 and G2 groups) as well as the NDV-Montanide™ (G3 group) group exhibited a statistically significant increase in antibody response when compared to the three control groups (G4, G5 and G6 groups; $p < 0.0001$). The findings indicated that HI antibody titers in chickens treated with NDV-Cap (using both *in situ* and Adsorption method preparation) were significantly increased at 2- and 3-weeks post-immunization relative to the preceding week within the same group. An increasing trend in antibody titers was observed until 3 weeks post-immunization followed by a gradual decline. Four weeks after immunization, a slight decrease in HI antibody titers was found compared to the previous week within the same group, however, this decrease was not statistically significant. No significant differences were observed in HI antibody titers between the two groups of chickens treated with NDV-CaPs at any of the times pointed assessed. Conversely, the group inoculated with NDV-Montanide™ demonstrated a significantly higher antibody titer than the other groups. Furthermore, the serum ELISA antibody titers were assessed at 1-, 2-, 3- and 4-weeks post-immunization in the study groups (Fig. 6). The specific ELISA antibody titers in the groups treated with ND-CaP (both G1 & G2) exhibited a significant increase at all blood sampling time points compared to the control groups ($p < 0.0001$). At week two post-immunization, an increase in antibody titers was observed in the groups immunized with NDV-CaPs, however, this increase was not statistically significant. In the 3rd week of post-immunization, the ELISA antibody titers in chickens treated with NDV-CaP-Adsorption were significantly increased compared to the 2nd week of blood sampling. However, in the 4th week post-immunization, a decrease in ELISA antibody titers was observed in the groups treated with NDV-CaP, although this decrease was not statistically significant. Additionally, the results indicated that the group immunized with NDV-Montanide™ exhibited significantly higher ELISA antibody titers at various blood sampling points compared to the other immunized groups.

Safety of NDV-CaP NPs. In the safety test, chickens were inoculated subcutaneously with two doses of the NDV-CaP NPs (prepared using both methods). The NDV-CaP NPs were confirmed to be safe for the birds. During the 14-day observation period, there were no mortalities among the chickens, and no abnormal clinical signs were observed in the immunized birds indicating that the NDV-CaP NPs were safe for use.

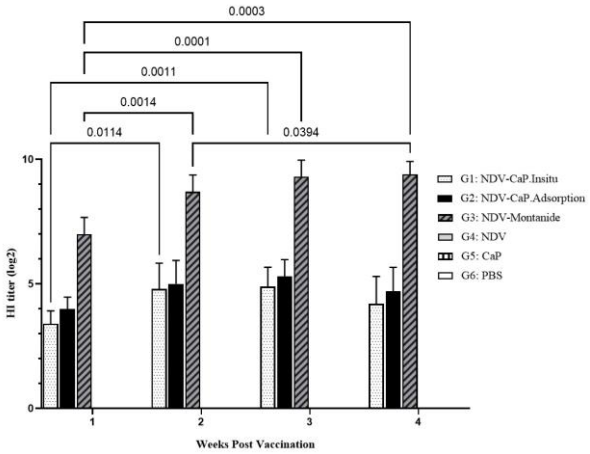

Fig. 5. Hemagglutination inhibition (HI) antibody levels in the groups of chickens inoculated subcutaneously with Newcastle disease virus (NDV) genotype VII antigen entrapped in calcium phosphate (CaP) nanoparticles and control groups. The bar shows the mean value and standard errors of the mean. PBS: Phosphate-buffered saline. The numbers above the bar indicate *p* values.

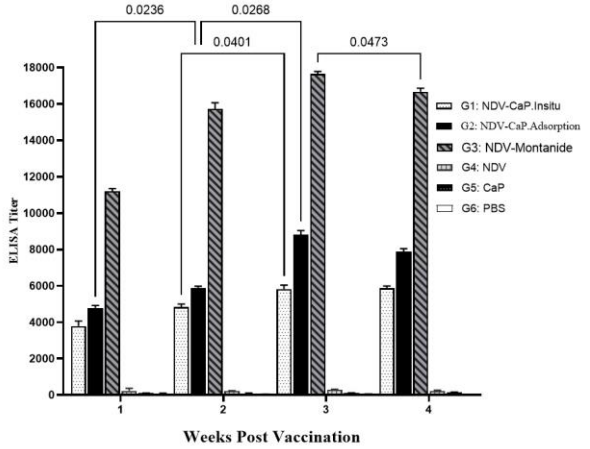

Fig. 6. Enzyme-linked immunosorbent assay (ELISA) specific-antibody levels in the groups of chickens inoculated subcutaneously with Newcastle disease virus (NDV) genotype VII antigen entrapped in calcium phosphate (CaP) nanoparticles and control groups. The bar shows the mean value and standard errors of the mean. PBS: Phosphate-buffered saline. The numbers above the bar indicate *p* values.

Discussion

In recent years, the design and manufacturing of inactivated vaccines have increasingly explored the use of micro- and NP as both carriers and adjuvants to enhance antigen delivery to the host. Nanoparticle delivery systems present several advantages over conventional vaccines including an extended duration of immune responses, induction of cellular immunity and improved antigen uptake by cells. These NPs efficiently entrap antigens, regulating their release, facilitating targeted delivery to antigen-presenting cells and functioning as adjuvants to optimize the immune response.[29] Among the various antigen delivery carriers, CaP NPs have gained significant attention over the past decade as a promising vaccine carrier. Calcium phosphate is a biodegradable inorganic material with low toxicity and cost-effectiveness, and it can be easily converted into nanoscale. Since the 1970s, CaP NPs have been investigated in various biomedical applications including DNA/gene silencing, drug delivery, protein/peptide delivery, dentistry and bone tissue engineering.[17,20] They have also been used to link various viruses such as Epstein-Barr virus and Herpes simplex virus-2.[25] In the present study, inactivated NDV was entrapped in CaP NPs. Given the importance of utilizing antigenically matched vaccines with circulating viruses, this study incorporated an Iranian isolate of genotype VII of NDV with CaP to create a NP formulation.

Calcium phosphate nanoparticles were prepared using the method described in a U.S. patent,[30] which involves washing the CaP solution to eliminate free phosphate ions and forming undesired bonding. A study by Hayashi *et al*., highlighted the importance of particle size and shape in inducing antibody responses. Their findings indicated that particles in the size range of 100 to 400 nm produced significantly higher antibody responses compared to smaller or larger particles. Additionally, rod-shaped particles induced stronger inflammation related to IL-1β production than spherical particles.[31] In our study, the average size of the particles was 307 nm and imaging results revealed that the CaP NPs had a homogeneous, spherical structure with a smooth surface, indicating successful particle formation.

The Zeta potential or surface electric charge of NPs was evaluated. Higher zeta potential values, whether positive or negative, are essential for ensuring stability and preventing particle aggregation. Nanoparticles exhibiting a zeta potential greater than +30.00 mV or less than –30.00 mV are considered stable in aqueous dispersion.[32] In the present study, the zeta potential of CaP particles was measured at – 29.50 mV which was nearly within the stable range.

In a study by Koppad *et al*., allantoic fluid containing inactivated NDV directly for conjugation with CaP was used and resulted in an antigen EE of 10.00%.[33] In contrast, our study achieved 70.00% antigen entrapment in CaP. Bisht *et al.,* reported an antigen EE of 99.00% using highly purified plasmids, highlighting that higher purification can increase antigen entrapment efficiency.[34] Indeed, it is possible to increase the efficiency of antigen entrapment in NP through the purification of the antigen.

Our findings showed that CaP NPs were able to induce immune responses against NDV and were safe. We evaluated the humoral immune response in chickens immunized with inactivated NDV genotype VII entrapped in CaP NPs via subcutaneous injection. Results revealed that the average titer of specific antibodies against NDV was significantly higher ($p < 0.0001$) in chickens treated with two NDV-CaP NP formulations compared to control groups (CaP, NDV, and phosphate-buffered saline). Antibody titers increased up to 3 weeks following the second immunization indicating the stability of the immune response. No significant differences were observed between the two NDV-CaP groups (NDV-CaP. *In situ* and NDV-CaP-Adsorption), although the NDV-CaP-Adsorption group showed a slightly higher immune response.

Previous studies have demonstrated that CaP NPs can induce stronger and more prolonged antibody responses (IgG, IgG1) compared to traditional adjuvants such as aluminum salts.[17] In a study conducted by Joyappa *et al*., CaP NPs were utilized to encapsulate a foot-and-mouth disease virus DNA vaccine, demonstrating that these NPs elicited a robust humoral immune response characterized by the production of neutralizing antibodies in mice as well as providing protection to guinea pig during infection with the live virus.[35] Another study showed that inactivated human enterovirus 71 encapsulated in CaP NPs resulted in a significantly higher antibody response (both IgM and IgG) compared to the unabsorbed vaccine alone or CaP NPs with micro size in rabbits.[36] It should be noted that a strong T-cell immune response plays a crucial role in clearance and recovery from infectious diseases. Several studies have demonstrated that CaP NPs loaded with viral and bacterial antigens can activate protective T-cell responses against these infections.[17] For instance, one study found that hemagglutinin-loaded CaP NPs functionalized with a Toll-like receptor9 ligand (CpG; Cytosine-Phosphorothioate-Guanine) induced dendritic cell maturation and antigen-specific CD4+ T cell proliferation.[37] Another study indicated that immunization of mice with Toll-like receptor -functionalized CaP NPs effectively induced dendritic cells maturation as well as the induction of both CD4+ and CD8+ T cells.[38] Another study compared the adjuvant properties of CaP and alum using herpes simplex virus type 2 and Epstein-Barr virus antigens in mice. Calcium phosphate proved to be a more effective adjuvant, causing minimal inflammation and inducing high levels of IgG2a antibodies, resulting in substantial protection against herpes simplex virus.[25] However, to date, no comparison has been made between

the immune responses induced by CaP NPs and those elicited by oil adjuvants. Our study demonstrated that the CaP -adjuvanted NDV vaccine could induce lower antibody responses than the group receiving the oil adjuvanted vaccine. Furthermore, an evaluation of the safety of CaP NPs containing NDV in chickens indicated no adverse effects or mortality.

Koppad *et al*., evaluated the humoral and cellular immunity elicited by a CaP NP vaccine containing inactivated Newcastle virus, administered intraocularly and intranasally. They showed the CaP -loaded vaccine maintained high antibody HI titers until the 5th week indicating stronger and more prolonged immune responses compared to commercial vaccine.[33] In our study, we investigated the mucosal immunization of chickens treated with NDV entrapped in CaP NPs, however, no significant increase was observed in antibody titers against the Newcastle virus (data not shown). Further investigations are needed to determine the cause of this discrepancy observed in relation to the findings of other researchers. In a study by Jayawardane *et al*., it was stated that chickens vaccinated mucosally with the V4 strain of NDV and having low or undetectable levels of anti-Newcastle serum HA inhibitory antibodies may be resistant in the challenge test with the pathogenic virus.[39] In the present research, due to the limitations of using the pathogenic virus, it was not possible to challenge the vaccinated chickens that will be done in the future study. Moreover, by evaluating cellular immunity in vaccinated chickens with NDV-CaPs, more comprehensive information about immune status of chickens can be accessible which will be discussed in future studies.

In conclusion, this study effectively demonstrated that CaP NPs loaded with inactivated Newcastle virus were completely safe. The results of the present research confirmed the role of CaP particles as good carriers for antigens in the structure of protein vaccines. The role of CaP in increasing the induction of humoral immune response as an adjuvant was determined. In this study, it was shown that CaP NPs with NDV antigen were able to induce humoral immune response and increase antibody titer against NDV in vaccinated chickens by subcutaneous inoculation method. Although the partial protection and efficacy of these NPs were not evaluated using the challenge test with the pathogenic virus, their partial protection was largely related to the HI antibody titer. More research is needed to investigate the mucosal immunogenic effect of these NP structures.

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Conflict of interest

The authors declare that there is no conflict of interest**.**

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