Immune responses following vaccination of chickens with a liposomal Newcastle disease virus HN-DNA vaccine

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Abstract

Newcastle disease virus (NDV) is an economically important infectious agent causes a respiratory and neurological disease in many species of birds. In addition to the conventional vaccines that are applied depending on the disease situation, new platforms have been investigated for the development of next-generation vaccines. The hemagglutinin-neuraminidase (HN) glycoprotein of NDV plays a crucial role in the immune response and serves as a potential target for the development of DNA vaccines. Liposomes can effectively target specific immune cells to elicit both humoral and cell-mediated immune responses. In this study, the conserved coding sequence of the HN gene extracted from the NDV Clone strain was loaded into liposomes. Physicochemical parameters such as size, charge, encapsulation efficiency, and morphology of the entrapped DNA were determined to ensure optimal vaccine efficacy. Two-week-old chickens received two doses of the vaccine with a one-week interval. The immunogenicity of the liposomal HN-DNA vaccine was evaluated using hemagglutination inhibition (HI) and cutaneous basophil hypersensitivity (CBH) assays in 2-week-old specific pathogen-free chickens over a 5-week post-vaccination period. The HN-DNA liposomes had an average size of 166.6 ± 4.65 nm, a polydispersity index (PDI) of 0.117, a positive surface charge of 13.7 ± 8.71 mV, an encapsulation efficiency of 56.4 ± 8.3%, and a homogeneous and spherical structure. A prime-boost vaccination with HN-DNA liposomes resulted in increased HI antibody titers, demonstrating a consistent immunization pattern of initial rise, peak, and decline. Furthermore, immunizing chickens with the HN-DNA vaccine significantly enhanced the CBH response, demonstrating its effectiveness in inducing cell-mediated immunity. Overall, the liposome vaccine containing HN-DNA is highly promising and should be prioritized for further investigation as an effective booster in NDV prevention strategies.

Keywords: Newcastle disease virus, cationic liposome, DNA vaccine, Hemagglutinin-Neuraminidase, immune response

1. Introduction

Newcastle disease virus (NDV) is the causative agent of a highly contagious and fatal viral disease, ND, in domestic and wild bird species. The disease is characterized by respiratory, digestive, reproductive, and nervous symptoms, which can lead to morbidity and mortality rates of up to 100%. Since the first official report of the disease in 1926 in Newcastle, England, preventing this disease has been a major interest for the poultry industry due to multiple outbreaks that have caused high mortality rates and significant economic losses in production (1). A series of vaccines against NDV strains is used in endemic areas as a control measure, either to protect chickens or to prevent the spread of the disease. Live-attenuated ND vaccines based on lentogenic NDV strains, regardless of genotype, are widely used for disease control. The primary benefit of live NDV vaccines is their mass application through drinking water or spray, which effectively stimulates both mucosal and systemic immune responses, similar to natural infection, and promotes overall herd immunity. Inactivated vaccines are given after live vaccines to enhance effectiveness against NDV; however, they typically do not induce strong mucosal or cell-mediated immune responses (2-4). The hemagglutinin-neuraminidase (HN) and fusion (F) surface glycoprotein are essential components of NDV vaccines due to multiple roles in viral tropism, virulence, producing neutralizing antibodies, and eliciting strong immune responses (5; 6).

Despite the identification of over 20 NDV genotypes, most vaccinations rely on lentogenic strains of genotypes I and II, even when the predominant virus belongs to a different genotype. There are concerns that the genetic differences between vaccine strains and field viruses may reduce the effectiveness of current vaccination strategies (3; 6; 7). Accordingly, the development of more effective vaccines is of interest to poultry disease control programs. Second and third-generation NDV vaccines have been produced using the F and HN surface glycoproteins through various production platforms. The effectiveness of the vaccines in triggering both humoral and cell-mediated immunity, as well as improving their stability, can be optimized by utilizing an appropriate delivery system (4; 8-11). Overall, the encoded antigen should be delivered to antigen-presenting cells (APCs) to trigger a robust T and B cell immune response. The selection of a delivery route, including intramuscular or *in ovo* injection, and oral administration, depends on the target pathogen, the age of the birds, and the type of immune response.

Research efforts are ongoing to improve transfection efficiency using biocompatible materials by focusing on three main areas: protecting DNA from nuclease degradation, targeting specific tissues or cells to enhance vaccine efficacy, and ensuring long-term immunity. Self-adjuvant nanomaterials are particularly promising for DNA delivery because they serve dual purposes as both delivery vehicles and adjuvants (12-14).

Generally, liposomes are formulated from cationic lipid, cholesterol, and phospholipid to enhance the delivery of DNA into target-specific immune cells. It is expected that the lipid will bind electrostatically to the negatively charged DNA, forming the lipid-DNA complex (12; 15). Once the complex is taken up by the cell and presented to the immune system, it should trigger an immune response. These engineered nanoparticles protect DNA from degradation, facilitate cellular uptake, improve antigen delivery to APCs, and modulate immune signaling (14).

Ultimately, these functions contribute to increased immune system activation. In this study, we conducted an immunization trial in chickens using a DNA liposome vaccine targeting the HN protein. We utilized the cationic lipid D-Lin-MC3-DMA (16) as a delivery vehicle to enhance the effectiveness of our vaccine candidate. This was supported by *in vivo* experiments assessing the immunogenic responses in chickens.

2. Materials and Methods

2.1. HN plasmid construction

The RNA was extracted from the NDV Clone strain (RVSRI, Iran) using Ribospin™ (GeneAll, Korea) commercial kit. The HN gene fragment was amplified using the Next generation of premix kit (iNtRON, Korea) in a one-step RT-PCR reaction using the following target gene-specific primers: HNF: 5'-GGATCCATGGACCGCGCCCGTTAGCCA-3' (*Bam*HI G˅GATCC) and HNR: 5'-AAGCTTGCCAGACCCGGCTTCTCTAAC-3' (*Hin*dIII A˅AGCTT). The amplified products were cloned into the pcDNA3.1 vector. PCR product and plasmid were digested with the above mentioned restriction enzymes and ligated using T4 DNA ligase. The ligation product was transformed into competent *E. coli* DH5α cells. The recombinant plasmid containing the HN gene PCR product was further verified by sequencing.

2.2. Lipid-DNA complex preparation and characterization

To prepare the lipid: DNA complex, we developed liposomes by mixing the cationic lipid DLin-MC3-DMA (MC3), cholesterol, and distearoylphosphatidylcholine (DSPC) in a molar ratio of 5:4:1. The plasmid DNA was diluted in a 25 mM sodium acetate buffer (pH 4) to reach a concentration of 100 µg/ml in the aqueous phase. The ethanolic phase was added to the aqueous phase in a volume ratio of 3:1, and mixed until homogeneous. The solution was then incubated at room temperature for 15 min.

50 µl of each DNA-loaded and unloaded liposome sample was diluted with 950 µl of NaCl and characterized by dynamic light scattering (DLS) using a Zetasizer Nano ZS Plus (Malvern Instruments, Malvern, UK) at a refractive index of 1.4, absorbance of 0.01, dispersion viscosity of 0.8872 cP, refractive index of 1.330, and dielectric constant of 79 at 25 °C. Zeta potential was measured at 633 nm. The efficiency of DNA encapsulation was evaluated using a liposome lysis procedure. To distinguish encapsulated DNA from surface-absorbed DNA, liposomes were treated with 2U/100 µl of DNase I for 30 min at 37 °C, followed by enzyme inactivation with EDTA to a final concentration of 10 mM and incubation for 10 min at room temperature. Subsequently, the liposomes were lysed using Triton X-100 (0.1% v/v) for 10 min at room temperature, and the released DNA was measured at A260. Surface properties and morphology of nanoparticles were examined using a transmission electron microscope (TEM). The liposome-entrapped DNA was stored at 4 °C until use.

2.3. Chicken immunization

Thirty specific pathogen-free (SPF) chickens, each 2 weeks old, were divided into three groups of 10. The chickens were kept in separate cages with access to water and feed *ad libitum*, in accordance with the guidelines set by the Ethics Committee of the Faculty of Veterinary

Medicine at the University of Tehran (IR.UT.VETMED.REC.1404.0004). Group A received HN DNA loaded in liposomes, Group B received naked HN DNA, and Group C received PBS. Samples were administered through intramuscular injections into the chickens' pectoral muscles. One week later, the same administration schedule was repeated for all experimental groups. This study was conducted twice.

2.4. Humoral immune response

Blood was collected from the wing vein at 1, 3, and 5 weeks following the booster injection. The serum samples were kept at -20°C. The induction of specific antibodies against NDV was evaluated using the hemagglutination inhibition (HI) test.

2.5. Cutaneous basophil hypersensitivity response

Cutaneous basophil hypersensitivity (CBH) was employed to assess the cell-mediated immune response in the experimental chickens. Five chickens from each group at 5 weeks old were selected. The thickness of the web between the third and fourth toes was measured using a digital micrometer caliper (Vogel, Germany) with an accuracy of 0.01 mm. Then, 0.1 ml of phytohemagglutinin-P (PHA-P) at a concentration of 100 µg/ml was intradermal injected into the right foot of each chick. The left foot was injected with PBS and served as a control to evaluate any potential inflammation caused by the injection syringe. The CBH response, expressed as Foot Index (FI), was calculated by subtracting the interdigital skin thickness measured before and 24 h after the PHA-P injection: (post-PHA - pre-PHA) / (post-PBS - pre-PBS).

2.6. Statistical analysis

The data was analyzed using a one-way ANOVA and *t*-test. A p-value of less than 0.05 was considered statistically significant.

3. Results

The HN sequence of the NDV Clone strain was deposited in GenBank under accession number PV862984. The physicochemical properties of the HN DNA loaded into liposomes, as well as without loading, were evaluated (Table 1). The average particle sizes of blank and HN-loaded liposomes were measured at 110 nm and 166 nm, respectively, and both exhibited acceptable PDI values below 0.2. The zeta potential of HN-loaded liposomes was lower compared to unloaded or blank liposomes, which was attributed to the negatively charged DNA. Under the formulation condition, the encapsulation efficiency exceeded 55%, which was favorable for cationic liposomes.

Table 1. Physicochemical properties of Newcastle disease virus HN plasmid loaded into cationic liposomes

Formulation	Size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency
Unloaded Liposome	110.5±8.32	0.134	30.86 ± 5.28	-

HN-loaded liposome	166.6±4.65	0.117	13.7 ± 8.71	56.4 ± 8.3

TEM imaging data indicate the homogeneous and spherical structure of these nanoparticles after plasmid loading (Figure 1).

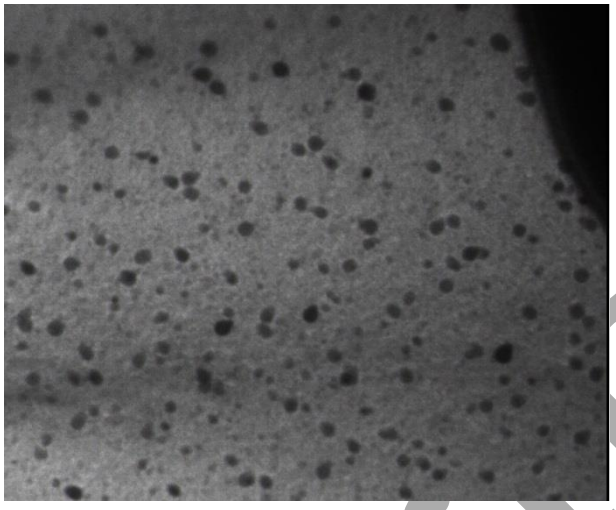

Figure 1. Morphology of the liposome-encapsulated HN DNA of the Newcastle disease virus

The DNA plasmid encapsulated in cationic liposomes was administered intramuscularly to the chickens. Then, the potential of the liposome-encapsulated HN DNA to induce NDV-specific antibodies was evaluated. Figure 2 displays the antibody titers (log_2) for all groups at weeks 1, 3, and 5 after receiving booster vaccination. Group A, which was administered HN DNA loaded in liposomes, demonstrated a significantly ($p<0.05$) higher HI titer compared to Group B. Group C did not exhibit any detectable antibody titer. The HI titers for Groups A and B began to rise in week 1 and peaked in week 3 following a decline in week 5. Group B showed a lower titer during the same time period.

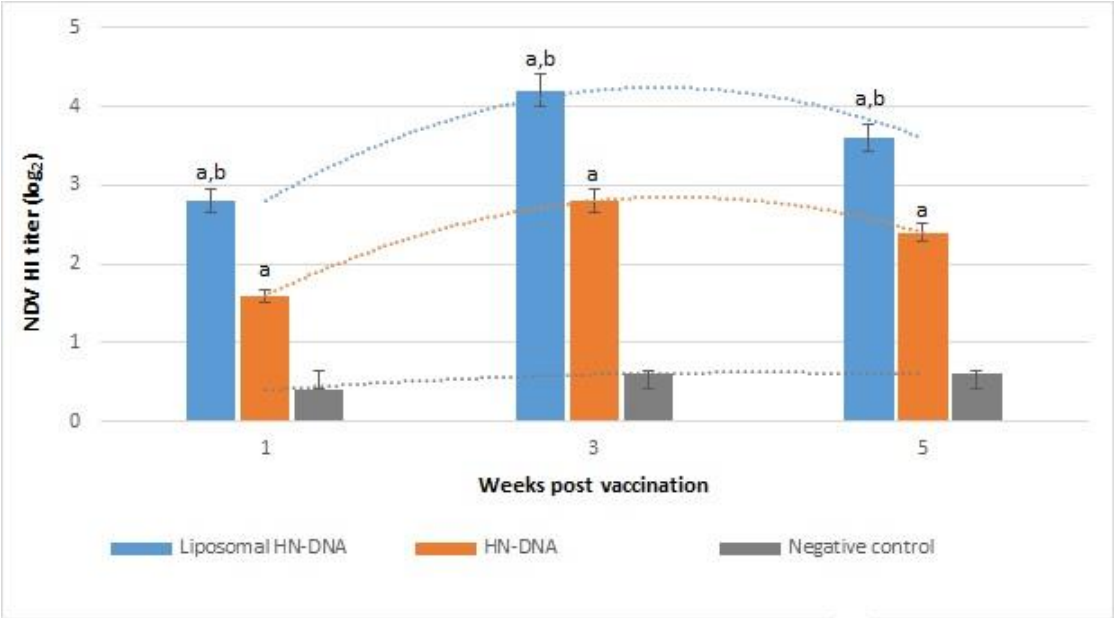

Figure 2. The hemagglutination inhibition (HI) titer following various vaccination profiles against Newcastle disease virus, values followed by different lowercase superscripts are significantly different ($p < 0.05$). A second-order polynomial trend line was fitted to the viral titer data, showing an initial rise in NDV titer followed by a decline after reaching a peak. Lowercase letters indicate significant differences ($p<0.05$) among the experimental groups.

CBH was assessed in order to evaluate the development of cell-mediated immunity following immunization with the liposomal HN-DNA vaccine. The increase in skin thickness, ranging from 1.72±0.06 to 1.86±0.07, observed in the vaccinated group, was statistically significant ($p<0.05$) compared to the other groups (Figure 3). The significant CBH reaction indicates that the liposomal HN-DNA vaccine can elicit a relatively robust cell-mediated immune response in chickens.

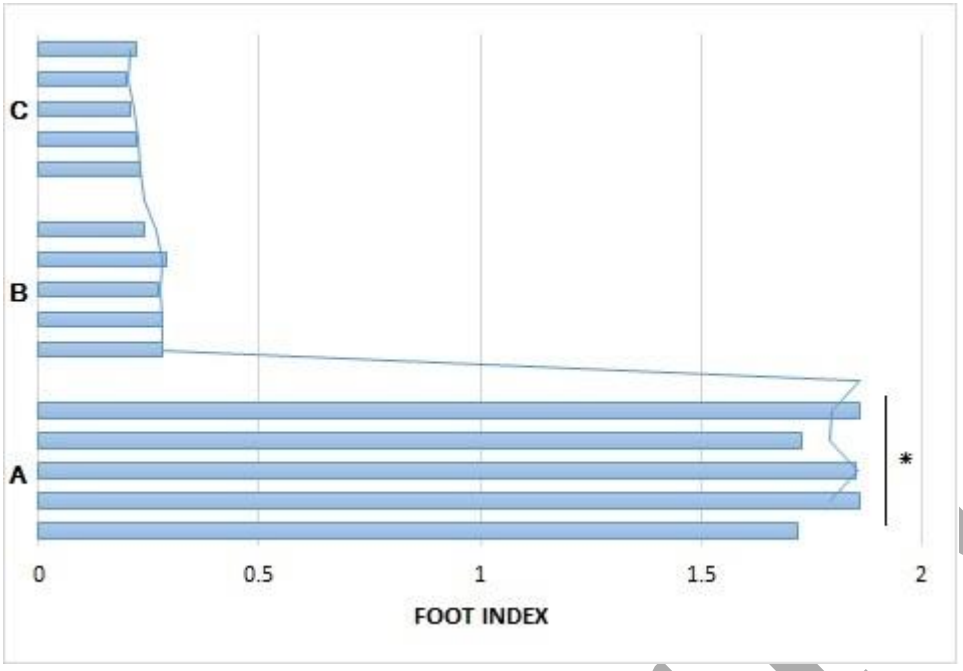

Figure 3. Cutaneous basophil hypersensitivity response following various vaccination profiles against Newcastle disease virus. * Significant differences ($p<0.05$) among the experimental groups.

4. Discussion

ND remains a major concern for breeding herds. The consequences of this disease extend beyond individual farms and affect production stability, supply chains, and ultimately the market economy. Vaccination plays a crucial role in controlling ND by reducing or eliminating clinical disease. There is concern that antigenic diversity between vaccine viruses and circulating viruses may lead to immune evasion and inadequate protection , particularly in enzootic regions where virulent genotype XIII strains are predominant (17; 18).

The HN glycoprotein is the main antigenic target of the host immune response against NDV (5). Before constructing the HN plasmid, our focus was on the genetic divergence of proteins between genotypes II and XIII in relation to immunity against NDV. This was done to select an appropriate candidate for a vaccine virus (Data not shown). Overall, no structural changes were detected in the sialic acid receptor recognition and binding sites, antigenic sites, catalytic regions, and glycosylation of the HN protein in the Clone strain and genotype XIII, which lead to immune evasion. Next, we developed a liposome-encapsulated DNA vaccine, which encodes the HN as a key antigenic component of the NDV. Our findings reveal its ability to enhance both humoral and cellular immune responses. While the rise in HI antibody titer following the administration of the liposomal vaccine suggests that a humoral immune response has been activated, it is important to highlight that this response is lower compared to the antibody titers observed after immunization with conventional vaccines.

Liposomal delivery systems are known to protect antigens from premature degradation and facilitate efficient uptake by antigen-presenting cells, thereby prolonging antigen availability and enhancing B-cell activation. The ability of liposomes to enhance antigen presentation via both MHC class I and II pathways likely contributed to the simultaneous activation of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells (12; 19). Such coordinated activation is crucial for effective viral clearance, as cell-mediated immunity plays a pivotal role in limiting the intracellular replication of NDV and reducing viral shedding following exposure. The CBH test provided evidence of effective activation of cell-mediated immune components. Basophils play a crucial role in early immune signaling through the release of cytokines and their interaction with T lymphocytes (20). Therefore, the CBH reaction is recognized as an indicator of T-cell-mediated (Th1-type) immunity in poultry (21). In the present study, the liposomal HN-DNA vaccine elicited a marked and statistically significant CBH response, as reflected by FI values exceeding 1.80 at peak activity. This strong response indicates effective recruitment and activation of basophils and mononuclear inflammatory cells at the site of HN antigen challenge, directing the Th1-mediated response.

The HI and CBH findings comparison supports the concept that DNA vaccination preferentially promotes cell-mediated immunity, as opposed to inactivated vaccines, which mainly stimulate humoral responses. The use of a cationic liposomal carrier likely enhanced plasmid, improved antigen presentation, and effectively activated T lymphocytes (22). This may explain the strong and sustained CBH reactions recorded in the vaccinated group. Overall, liposomal DNA vaccines effectively induce strong Th1 and cytotoxic T-cell responses, which are crucial for protection against intracellular pathogens. This delivery system improves the uptake of cellular DNA, protects plasmid constructs from degradation, and promotes sustained expression of antigens in host cells (12; 14). As a result, it leads to a more robust and long-lasting immune memory compared to the transient antigen exposure seen with inactivated vaccines. Furthermore, the adjuvant properties of liposomes reduce the need for chemical adjuvants that are typically required in inactivated vaccine formulations.

One of the main advantages of liposomal DNA vaccines over whole-virus inactivated vaccines is their effectiveness against the extensive genetic diversity of NDV. The modular structure of DNA constructs allows quick redesign and adaptation to emerging viral strains, whereas traditional inactivated vaccines may offer limited cross-protection against heterologous genotypes (23; 24). This applies only to inactivated vaccines, as lentogenic live vaccines can provide protection against virulent NDVs and help control viral shedding (25). A key finding from the pre-study is the absence of significant mutations in the HN gene sequence used in the liposomal vaccine. Because the HN sequence is conserved across different genotypes, the DNA vaccine could enhance immunity and extend protection against circulating virulent NDV strains. This is particularly important when the vaccine is used as a booster in regions where genotype XIII is prevalent. Further challenge studies and long-term immunity assessments are warranted to confirm its protective efficacy under field conditions.

The ethics section

The animal trial was conducted following the guidelines established by the Ethics Committee of the Faculty of Veterinary Medicine at the University of Tehran (IR.UT.VETMED.REC.1404.0004).

Conflict of Interest

The authors declare that they have no conflict of interest.

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