Intranasal Immunization With O-2'-Hydroxypropyl Trimethyl Ammonium Chloride Chitosan Nanoparticles Loaded With Newcastle Disease Virus DNA Vaccine Enhance Mucosal Immune Response in Chickens

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Research

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Intranasal immunization with *O*-2′-Hydroxypropyl trimethyl ammonium chloride chitosan nanoparticles loaded with Newcastle disease virus DNA vaccine enhance mucosal immune response in chickens

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Running title: O-2′-HACC nanoparticles as adjuvant and delivery carrier for DNA vaccine

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Abstract:

Background: There is a great interest to develop strategies for enhancing antigen delivery to mucosal immune system as well as to identify mucosal active immunostimulating agents. To elevate the potential of *O*-2′-Hydroxypropyl trimethyl ammonium chloride chitosan (O-2′-HACC) nanoparticles as adjuvant and mucosal immune delivery carrier for DNA vaccine, we prepared the O-2′-HACC nanoparticles loaded with Newcastle disease virus F gene plasmid DNA with C3d6 molecular adjuvant (O-2′-HACC/pFDNA).

Results: The O-2′-HACC/pFDNA had regular spherical morphology with a particle size of 202.3±0.52 nm, zeta potential of 50.8±8.21 mV, encapsulation efficiency of 90.74±1.10 %, and loading capacity of 49.84±1.20 %. The plasmid DNA could be sustainably released from the O-2′-HACC/pFDNA after an initial burst release. Intranasal vaccination of chickens immunized with O-2′-HACC/pFDNA not only induced higher anti-NDV IgG and sIgA antibody titers, but also significantly promoted lymphocyte proliferation and produced the higher levels of IL-2, IL-4, IFN-γ, CD4+ and CD8+ T lymphocytes than the NDV commercial attenuated live vaccine. Intranasal delivery of the O-2′-HACC/pFDNA enhanced humoral, cellular and mucosal immune responses, and protected chickens from the infection of highly virulent NDV than intramuscular delivery.

Conclusions: This study indicated that the O-2′-HACC nanoparticles could be used as vaccine adjuvant and delivery system for mucosal immunity and have an immense application promise.

Keywords: Newcastle disease virus; DNA vaccine; *O*-2′-Hydroxypropyl trimethyl ammonium chloride chitosan nanoparticles; intranasal delivery; mucosal immunity.

Background

Mucosal immune system is an important part of the body's entire immune network, and it plays an active and important role in fighting infection [1]. Mucosal immune response can be improved by selecting the optimal immunization route, vaccine adjuvant and delivery system etc. [2]. Mucosal vaccination not only induces a corresponding immune response at the site of inoculation, but also produces a corresponding immune response in other distant mucosal tissues. Nasal mucosa is the first part to contact the inhaled antigen, nasal mucosal immunity can induce stronger mucosal immune response and higher systemic immune responses in the distant mucosal tissues [3, 4], thus intranasal vaccination is considered to be a more favorable mucosal immune route.

Newcastle disease (ND) is an acute and highly contagious disease caused by Newcastle disease virus (NDV) [4, 5]. The most economical and effective way to prevent ND is vaccination [6]. Compared to traditional vaccine, DNA vaccine has great advantages and potential, DNA vaccine has higher safety, better genetic stability and immune effect, simple production, convenient storage and transportation etc. However, the administration of DNA vaccine is intramuscular injection, and several studies have shown that DNA vaccines don’t effectively delivery antigen to antigen-presenting cells (APCs) after intramuscular injection, therefore, this leads to a strong immune response that can’t be induced [7, 8]. Additionally, DNA vaccine has also been limited in clinical applications due to intramuscular injection, high dose, low bioavailability and immunogenicity [9]. Various strategies have been considered for enhancing mucosal immune response by using the suitable vaccine adjuvant, specific targeting of ligands, delivery system etc. Suitable vaccine adjuvant and delivery system in DNA vaccines can improve the immunogenicity, induce stronger immune responses and reduce the dosage and production cost of vaccine in populations responding poorly to vaccination [10, 11].

Viral vectors and non-viral vectors have been used as carrier to deliver gene safely and effectively. Although viral vector has many advantages for the delivery of plasmid DNA, one of the most important issues is able to ensure that plasmid DNA is not degraded by lysosomes during transport to the host cell. And the viral vector must be non-pathogenic to the human body and will not cause proliferation and spread in the environment, high titer production and high immunogenicity safety [12]. Compared to viral vector, non-viral vector has some advantages, including no infectivity, low immunogenic response, safety, high gene capacity, stability, no carrier capacity limitation, and are easy to prepare in large quantities [13, 14]. Non-viral gene delivery system generally consists of the naked DNA delivery, lipid-based delivery and polymer-based delivery etc. Cationic polymer, which electrostatically interact with plasmid DNA to neutralize its negative charge and condense the plasmid DNA into nanosized particles, is generally served as gene delivery systems. Cationic polymer nanoparticles can protect the plasmid DNA from enzymatic degradation and facilitate cellular uptake. Intramuscularly administered polyvinyl alcohol/plasmid DNA formulation resulted in a significant increasing in the number and distribution of the reporter-gene expressing cells in rat, compared to naked plasmid DNA [15]. Biodegradable, non-antigenic polymer-based microspheres/nanoparticles have many advantages as vaccine adjuvant and delivery system. Our previous studies have shown that cellular, humoral and mucosal immune responses can be elicited to antigens encapsulated in, or conjugated onto polymer-based microspheres/nanoparticles [16, 17].

Since the particle size of nanoparticles is comparable to that of the pathogen, nanoparticles can pass through the interstitial space and capillaries to reach a site that is difficult to administer, and has the many advantages, including controlling drug release, protecting drug from degradation or leakage, and targeting administration etc., thus, nanoparticles can significantly improve the delivery efficiency of plasmid DNA. At present, biodegradable nanomaterials for preparing polymer-based nanoparticles mainly include

chitosan and its derivatives, hyaluronic acid and sodium alginate etc. Among them, chitosan and its nanoparticles have broadly used as drug/vaccine delivery vector due to their safety, non-toxicity, biocompatibility, biodegradability and sustained release in industrial and technological applications [18, 19]. However, the poor solubility of chitosan greatly restricts the application scopes and fields of chitosan. One of strategies to improve the solubility of chitosan is to modify the structure of chitosan by the addition of hydrophilic functional groups [20]. Therefore, water soluble chitosan derivatives-based nanoparticles as vaccine adjuvant and delivery vector have become novel vaccine/drug delivery system. We have synthesized the water soluble *O*-2′-Hydroxypropyl trimethyl ammonium chloride chitosan (O-2′-HACC) [21], to prove the ability of O-2′-HACC nanoparticles as DNA vaccine adjuvant and delivery vector to reach sustained release and desired mucosal immunity, we prepared the O-2′-HACC nanoparticles loaded with Newcastle disease virus F gene plasmid DNA by using the polyelectrolyte complex method, and the intranasal delivery of nano vaccine was investigated to demonstrate the potential for mucosal immunity.

Results

Characterization of the O-2'-HACC/pFDNA

O-2'-HACC/pFDNA was regular spherical morphology, smooth surface and good dispersion (Fig. 1A). The average particle size of the nanoparticles was 202.3±0.52 nm (Fig. 1B), Zeta potential was 50.8±8.21 mV (Fig. 1C), EE was 92.27±1.48%, and LC was 50.75±1.35%.

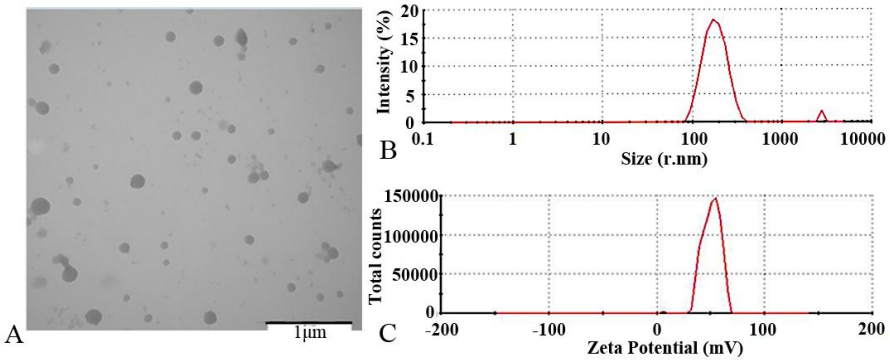


Fig. 1. Characterization of the O-2′-HACC/pFDNA. (A) Transmission electron microscope of O-2′-HACC/pFDNA; (B) Particle size of O-2′-HACC/pFDNA; (C) Zeta potential of O-2′-HACC/pFDNA.

DNase I protection assay

As shown in Fig. 2A, the integrity of plasmid DNA in O-2'-HACC/pFDNA was maintained even if the nanoparticles were treated with *DNase* I for 3 h (Lane 6, Fig. 2A), while the naked plasmid DNA was completely degraded by *DNase* I for 30 min (Lane 2, Fig. 2A). The results demonstrated that the plasmid DNA encapsulated in O-2'-HACC nanoparticles could be protected from degradation.

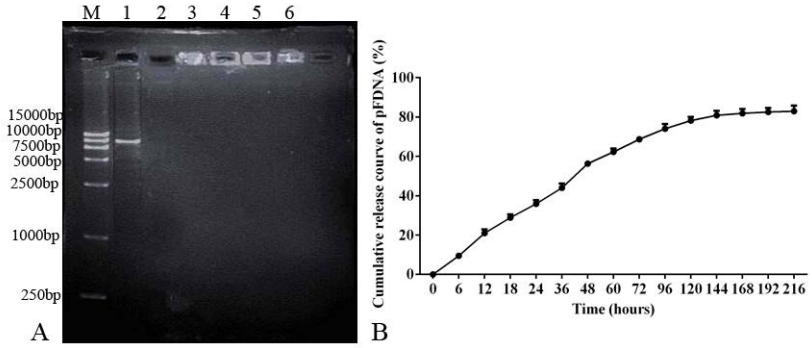


Fig. 2. Stability and *in vitro* release analysis of the plasmid pVAX I -F(o)-C3d6 after encapsulation in the O-2′-HACC nanoparticles. (A) *DNase* I protection of the pVAX I -F(o)-C3d6, M: DL 15000 Marker, Lane 1: pVAX I -F(o)-C3d6, Lane 2: *DNase* I acts on the naked DNA for 30 min, Lane 3-6: *DNase* I acts on the O-2′-HACC/pFDNA for 30, 60, 120 and 180 min; (B) *In vitro* release profiles of the O-2′-HACC/pFDNA in PBS solution (pH=7.4). Data are presented as the mean ± SD deviation (n=3).

In vitro release of O-2'-HACC/pFDNA

It could be found from Fig. 2B that between 0 and 36 h, the release amount of plasmid DNA in O-2'-HACC/pFDNA reached 44.00±1.80 %, which was a process of burst release; between 36 and 120 h, the release amount reached 78.22±1.60 %; after 120 h, the release of the plasmid DNA was gentle, the release amount reached 82.97±2.30 %. *In vitro* release indicated that the O-2'-HACC nanoparticles could

serve as delivery vector for the sustained and slow release of DNA vaccine.

Safety of the O-2'-HACC/pFDNA

The survival rate of chicken embryo fibroblasts in O-2'-HACC/pFDNA was 90.48±2.14 %, and no significant change in cell morphology was observed compared to control cells (P>0.05). *In vivo* cytotoxicity analysis showed that the chickens immunized with the O-2'-HACC/pFDNA i.m. or i.n. were normal in feeding, drinking, mental state, body weight and inoculation sites, and there was no morbidity and mortality, indicating that the O-2'-HACC/pFDNA was safe. Histopathological analysis showed that glandular stomach, duodenum, quadriceps femoris and nasal mucosa were intact and no lesions as shown in Fig. 3A. These findings indicate that the O-2'-HACC NPs has little cytotoxicity as delivery vector, but has higher safety level by administration intranasal.

Stability of the O-2'-HACC/pFDNA

The O-2'-HACC/pFDNA was milky white powder, loose and spongy. The morphology of the nanoparticles didn't change after stored at room temperature, 4℃ and -20℃ for three weeks, but there was slightly shrinking at 37℃ after stored for three weeks, indicating that the O-2'-HACC/pFDNA had good storage stability and could be stored for a long period of time at the room temperature. As seen from Fig. 3B, after the O-2'-HACC/pFDNA stored for two and three months at room temperature, serum IgG antibody titers in chickens of the O-2'-HACC/pFDNA i.m. was not significantly different from the newly prepared O-2'-HACC/pFDNA i.m. (P>0.05).

In vitro expression of the O-2'-HACC/pFDNA

Fluorescence was detected in the O-2'-HACC/pFDNA and pVAXI-F(o)-C3d6 groups (Fig. 3C). No fluorescence was detected in the O-2'-HACC NPs and 293T cells groups. These results indicated that the plasmid DNA can be efficiently encapsulated by the O-2'-HACC NPs and expressed *in vitro*, indicating

that the O-2'-HACC NPs can be used for the delivery of plasmid DNA.

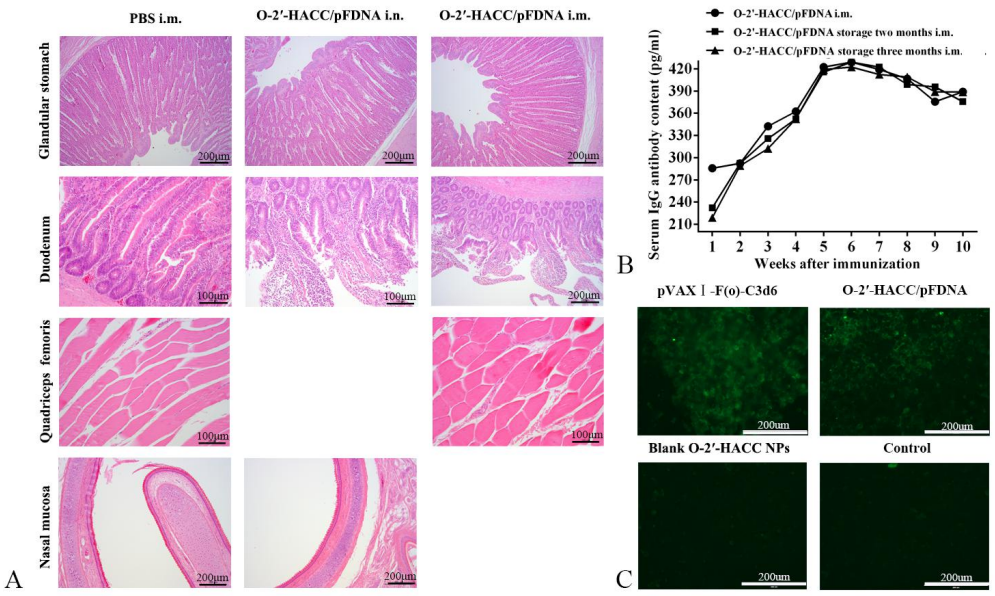


Fig. 3. Safety analysis, *in vitro* fluorescence expression and storage stability of the O-2'-HACC/pFDNA. (A) Histopathological analyses of glandular stomach, duodenum, quadriceps femoris and nasal mucosa; (B) *In vitro* expression of the O-2'-HACC/pFDNA in 293T cells assayed by indirect immunofluorescence (×40); (C) After Storage stability of the O-2'-HACC/pFDNA for two and three months at room temperature, IgG titers in serum post the immunization.

Intranasal immune response

Serum IgG antibody titers

As shown in Fig. 4A, at fifth week post the immunization, the serum antibody titers were significantly increased in pVAXI-F(o)-C3d6 i.m., O-2'-HACC/pFDNA i.m. and O-2'-HACC/pFDNA i.n. groups, and the antibody levels were higher in the O-2'-HACC/pFDNA i.m. and the O-2'-HACC/pFDNA i.n. groups. Serum IgG antibody titers in O-2'-HACC/pFDNA i.n. peaked at the 6th week and kept the higher IgG antibody levels to 10 weeks post the immunization. The differences between the O-2'-HACC/pFDNA i.n. and i.m. were not significant ($P>0.05$), but had significant difference compared to attenuated live ND vaccine i.m. group ($P<0.05$).

IgA antibody titers

IgA antibody titers in chickens immunized with the O-2'-HACC/pFDNA i.n. were significantly increased in serum (Fig. 4B), tracheal fluid (Fig. 4C), bile (Fig. 4D) and harderian gland (Fig. 4E) (P<0.01), and the time of IgA antibody secretion was also longer than the other groups (P<0.01). These results indicated that the O-2'-HACC/pFDNA i.n. induced higher IgA antibody secretion than the O-2'-HACC/pFDNA i.m, pVAX I -F(o)-C3d6 i.m. and attenuated live ND vaccine i.m. (P<0.01).

In addition, IgA antibody titers in O-2'-HACC/pFDNA i.n. was higher than that of the O-2'-HACC/pFDNA i.m, pVAXI-F(o)-C3d6 i.m. and attenuated live ND vaccine i.m. (P<0.01). The period of immunization protection in O-2'-HACC/pFDNA i.n. was longer, because the O-2'-HACC increased the contact time of antigen with the mucosal surface, thus effectively improved the antigen-associated lymphoid tissue, induced higher secretion levels of IgG and IgA in the body, indicating that the O-2'-HACC/pFDNA produced stonger humoral immune and mucosal immune responses.

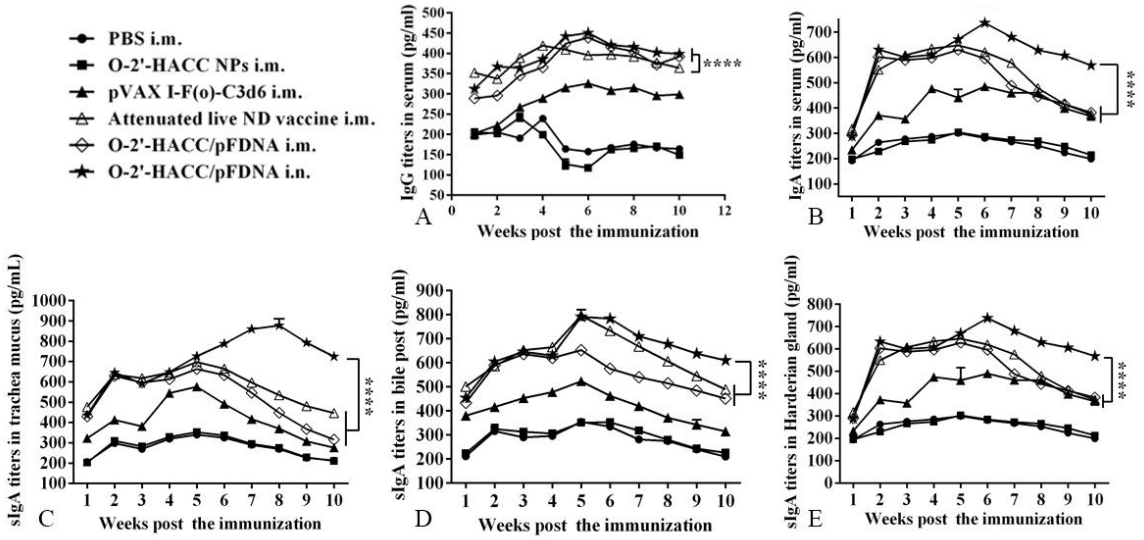


Fig. 4. IgG and IgA antibody titers in serum (A, B), trachea mucus (C), bile (D), and harderian gland (E) following administration of PBS i.m., O-2'-HACC NPs i.m., pVAX I -F(o)-C3d6 i.m., attenuated live ND vaccine i.n., O-2'-HACC/pFDNA i.m., O-2'-HACC/pFDNA i.n. Data are representative of three

independent experiments and presented as the mean ± SD (n=3). *P<0.05; **P<0.01.

Lymphocyte proliferation

SI value in PBS and O-2'-HACC NPs groups was significantly lower than those of the pVAXI-F(o)-C3d6 i.m., O-2'-HACC/pFDNA i.m., attenuated live ND vaccine i.m. and O-2'-HACC/pFDNA i.n. (P>0.05). The difference between attenuated live ND vaccine i.m. and O-2'-HACC/pFDNA i.n was not significant (P>0.05), and after the 3th week, SI value in the two groups was significantly higher than the O-2'-HACC/pFDNA i.m. (P<0.05), which showed that the O-2'-HACC/pFDNA i.n. significantly stimulated the proliferation of spleen lymphocytes. Additionally, O-2'-HACC/pFDNA i.n. and attenuated live ND vaccine i.m. kept strong stimulus response to ConA until 10 weeks post the immunization and produced a longer lasting immune stimulating effect, which promoted the more lymphocytes proliferation and triggered stronger specific immune response.

Table 1 Analysis of lymphocyte proliferation in SPF chickens immunized with the O-2'-HACC/pFDNA i.n., O-2'-HACC/pFDNA i.m., pVAX I -F(o)-C3d6 i.m., attenuated live ND vaccine i.m., O-2′-HACC NPs i.m. and PBS i.m.

Groups	Weeks post the immunization									
	1	2	3	4	5	6	7	8	9	10
O-2'-HACC/pFDNA i.n.	0.604 ±0.014^{a}	2.490 ±0.038^{a}	3.213 ±0.018^{a}	4.141 ±0.012^{a}	4.410 ±0.011^{a}	4.384 ±0.056^{a}	4.360 ±0.045^{a}	3.930 ±0.054^{a}	3.228 ±0.042^{a}	2.983±0 .034^{a}
O-2'-HACC/pFDNA i.m.	0.568 ±0.003^{a}	2.350 ±0.046^{a}	3.086 ±0.047^{b}	3.705 ±0.022^{b}	4.145 ±0.110^{b}	3.756 ±0.108^{b}	2.668 ±0.032^{b}	2.345 ±0.049^{b}	1.986 ±0.019^{b}	1.639±0 .010^{b}
Attenuated live ND vaccine i.m.	0.674± 0.008^{a}	2.660 ±0.015^{a}	3.368 ±0.040^{a}	4.192±0 .016^{a}	4.488 ±0.015^{a}	4.422 ±0.012^{a}	4.439 ±0.078^{a}	3.989 ±0.014^{a}	3.162 ±0.055^{a}	2.970±0 .031^{a}
pVAX I -F(o)-C3d6 i.m.	0.435 ±0.018^{b}	2.124 ±0.006^{b}	2.779 ±0.017^{c}	3.148 ±0.047^{c}	3.598 ±0.013^{c}	3.278 ±0.017^{c}	2.440 ±0.005^{c}	1.897 ±0.009^{c}	1.810 ±0.010^{c}	1.683±0 .011^{b}
O-2′-HACC NPs i.m.	0.402± 0.004^{b}	1.058 ±0.060^{c}	1.968 ±0.019^{d}	2.156 ±0.011^{d}	2.256 ±0.038^{d}	2.171 ±0.012^{d}	2.144 ±0.010^{d}	2.023 ±0.040^{d}	1.972 ±0.017^{b}	1.581±0 .037^{c}
PBS i.m.	0.306 ±0.010^{c}	1.301 ±0.006^{d}	1.924 ±0.017^{d}	2.085 ±0.018^{d}	2.117 ±0.007^{e}	1.905 ±0.009^{e}	1.879 ±0.019^{e}	1.839 ±0.016^{e}	1.702 ±0.008^{d}	1.459±0 .022^{d}

Notes: Values represent mean ± SD (n=3). Values within the same column with the different lower case letter (a–e) in the superscript indicate statistically significant differences (P<0.05).

Cytokine levels in blood

As shown in Fig. 5, the levels of IL-2, IFN-γ and IL-4 in blood of chickens immunized with the O-2'-HACC/pFDNA i.n. and i.m. significantly increased compared with the pVAX I -F(o)-C3d6 i.m. and attenuated live ND vaccine i.m. groups (P<0.05), and the levels of IL-2 (Fig. 5A), IFN-γ (Fig. 5B) and IL-4 (Fig. 5C) in chickens from the O-2'-HACC/pFDNA i.n. group were higher (P<0.05), which indicated that the O-2'-HACC/pFDNA i.n. induced more cytokines secretion to trigger cellular immune response.

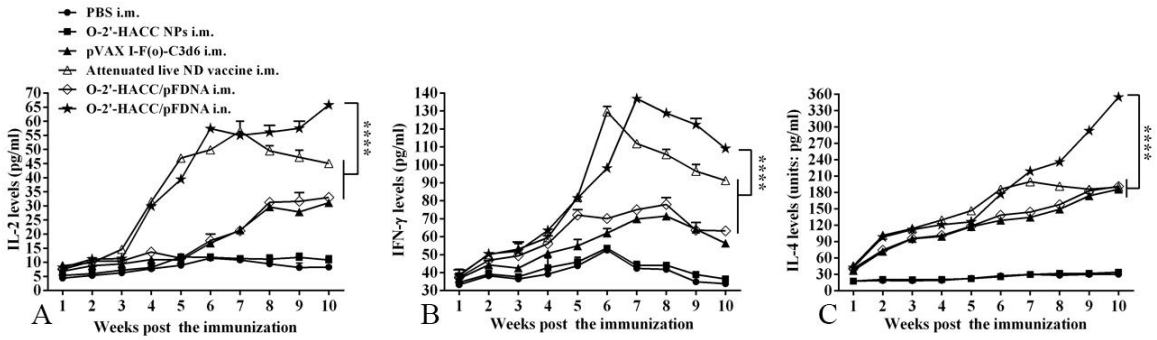
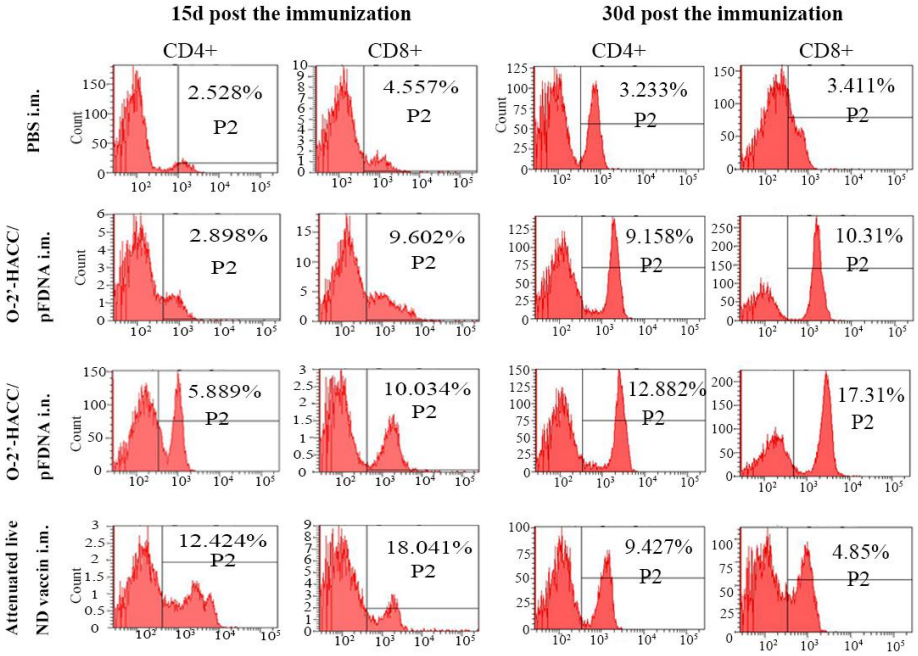


Fig. 5. IL-2 (A), IL-4 (B), and IFN-γ (C) levels in the supernatant of splenocytes harvested from the SPF chickens immunized with the PBS i.m., O-2'-HACC NPs i.m., pVAX I-F(o)-C3d6 i.m., attenuated live ND vaccine i.n., O-2'-HACC/pFDNA i.m., O-2'-HACC/pFDNA i.n. IFN-γ, IL-2, and IL-4 levels in the supernatant were analyzed in a chicken IFN-γ, IL-2, and IL-4 enzyme-linked immunosorbent assay. Results are represented as mean ± SD of three separate experiments. *P<0.05; **P<0.01.

Levels of CD4+ and CD8+ T lymphocytes in peripheral blood

At 15 days post the immunization, the levels of CD4+ and CD8+ T lymphocytes in the attenuated live ND vaccine i.m. group were significantly higher than that in PBS, O-2'-HACC/pFDNA i.n. and O-2'-HACC/pFDNA i.m. groups (P<0.05) (Fig. 6). But at 30 days post the immunization, the levels of CD4+ and CD8+ T lymphocytes in the O-2'-HACC/pFDNA i.n. were significantly higher than those in PBS, O-2'-HACC/pFDNA i.m. and attenuated live ND vaccine i.m. groups (P<0.05) (Fig. 6).



209

210 Fig. 6. Levels of CD4+ and CD8+ T lymphocytes in peripheral blood post 15 days and 30 days after the

211 immunization.

212 **Immune protective efficacy**

213 *Serum HI antibody titers*

214 Anti-NDV antibody titers in chickens immunized with the O-2'-HACC/pFDNA i.n.,

215 O-2'-HACC/pFDNA i.m. and pVAXI-F(o)-C3d6 i.m. reached peak in the third week post the immunization,

216 and the level of IgG antibody in the O-2'-HACC/pFDNA i.n. was slightly higher than that in the

217 O-2'-HACC/pFDNA i.m., but the difference between the two groups was not significant (P>0.05). IgG

218 antibody titers in the O-2'-HACC/pFDNA i.n. and i.m. were higher than those of pVAX I -F(o)-C3d6 i.m.

219 and attenuated live ND vaccine i.m. groups (P<0.05). Serum IgG antibody levels in the

220 O-2'-HACC/pFDNA i.n. decreased slowly in the 3-5 weeks after challenge and maintained a higher level

221 (Fig. 7A).

222 *Changes of cytokine levels after challenge*

223 At the second week after challenge, IL-2 content in serum in the attenuated live ND vaccine i.m.

reached the highest value (Fig. 7B), but there was no significant different between the attenuated live ND vaccine i.m. and O-2'-HACC/pFDNA i.n. (P<0.05). At the third week after challenge, IL-2 content in the O-2'-HACC/pFDNA i.n.reached the highest value, and was significantly higher compared to the other groups until the fifth week (P<0.05) (Fig. 7B).

At the 1-5 weeks after challenge, IL-4 levels were significantly higher in the attenuated live ND vaccine i.m. and the O-2'-HACC/pFDNA i.n. than in the pVAX I -F(o)-C3d6 i.m. and O-2'-HACC/pFDNA i.m. group (P<0.05) (Fig. 7C).

In the attenuated live ND vaccine i.m. and the O-2'-HACC/pFDNA i.n., IFN-γ content was extremely significantly higher than that of the pVAX I -F(o)-C3d6 i.m. and O-2'-HACC/pFDNA i.m. at the 3rd week after challenge (P<0.01), and IFN-γ levels in the O-2'-HACC/pFDNA i.n. continued to maintain high levels until the 5th week after challenge. From the 3rd week after challenge, serum IFN-γ levels in the O-2'-HACC/pFDNA i.n. were significantly higher than those of pVAX I -F(o)-C3d6 i.m., O-2'-HACC/pFDNA i.m. and attenuated live ND vaccine i.m. (P<0.05) (Fig. 7D).

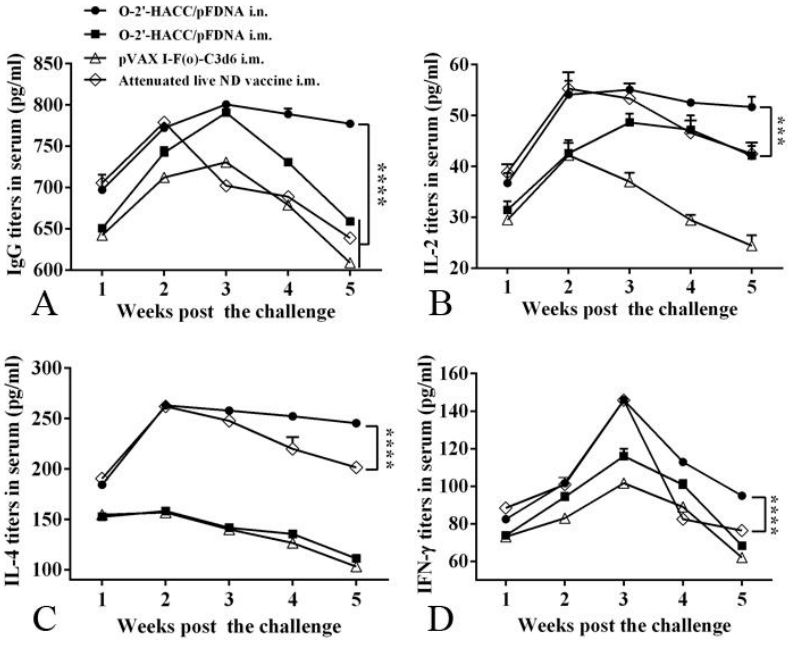


Fig. 7. Serum IgG antibody titers (A) and IL-2 (B), IL-4 (C), IFN-γ (D) levels in the supernatant of

splenocytes harvested from the immunized SPF chickens after challenge with the highly virulent NDV strain F48E9. IFN-γ, IL-2, and IL-4 levels in the supernatant were analyzed in a chicken IFN-γ, IL-2, and IL-4 enzyme-linked immunosorbent assay. Results are represented as mean ± SD of three separate experiments. *P<0.05.

Protective effect

Chickens in PBS and O-2′-HACC NPs groups died within 4-7 days after challenge. After challenge, two chickens immunized with the pVAX I -F(o)-C3d6 i.m. died, chickens in the attenuated live ND vaccine i.m., O-2'-HACC/pFDNA i.m. and the O-2'-HACC/pFDNA i.n. didn't die (Table 2). All the dead chickens showed the typical ND pathological changes, such as the severe congestion of intestinal wall and intestinal mucosa, and small bleeding spots on the surface of glandular stomach. However, these lesions didn't appear in chickens immunized with the O-2'-HACC/pFDNA i.m., i.n. and attenuated live ND vaccine i.m. (Fig. 8).

Table 2 Protection efficiency of the immunized SPF chickens after challenged with the highly virulent NDV strain F48E9

Groups	Number of dead chickens/Total number of chickens	Mortality (%)	Protection (%)
O-2'-HACC/pFDNA i.n.	0/7	0	100
O-2'-HACC/pFDNA i.m.	0/7	0	100
Attenuated live ND vaccine i.m.	0/7	0	100
pVAX I -F(o)-C3d6 i.m.	2/7	28.6	71.4
O-2′-HACC NPs i.m.	7/7	100	0
PBS i.m.	7/7	100	0

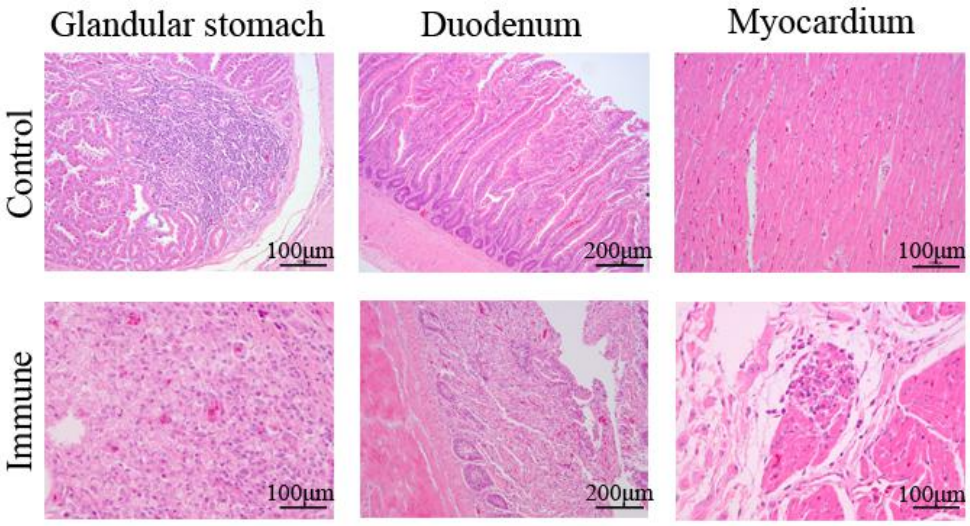


Fig. 8. Histopathological analyses of glandular stomach, duodenum, and myocardium obtained from healthy chickens and those challenged with the highly virulent NDV strain F48E9. Tissues of the glandular stomach, duodenum, and myocardium from the PBS i.m., blank O-2'-HACC nanoparticles i.n., attenuated live ND vaccine i.m., and O-2'-HACC/pFDNA i.m. and i.n. groups.

Discussion

Newcastle disease causes significant economic losses in the poultry industry every year. Traditional vaccines against ND have certain limitations, which led to the development of a new generation of vaccines. DNA vaccine is a new type of vaccines that have been intensively studied in recent years. However, compared with traditional vaccine, DNA vaccine has some disadvantages, such as potential pathogen mutation risk and lower protection [22]. Therefore, methods to improve the immune efficacy of DNA vaccine have become the focus of vaccine researches.

Biodegradable polymer-based microspheres/nanoparticles have many advantages as vaccine adjuvant and delivery system [23]. Although plasmid DNA is quite stable in vitro, it is subject to degradation by nucleases once injected *in vivo*. Encapsulation of plasmid DNA in biodegradable polymer to form nanoparticles potentially offers a way to protect plasmid DNA from degradation and control plasmid DNA release [24]. Biodegradable polymers used to encapsulate plasmid DNA mainly have poly (D,

L-lactic-co-glycolic) acid (PLGA), gelatin and chitosan. Chitosan nanoparticles have been developed for the delivery of plasmid DNA due to their cationic charge, biodegradability, biocompatibility, low toxicity, mucoadhesivity and ability to enhance the penetration of large molecules across mucosal surface. When DNA vaccine is encapsulated into chitosan nanoparticles, the integrity of plasmid DNA on the mucosal surface can be protected and the mucoadhesivity is enhanced, thereby improving its immune induction to pathogens on the mucosa [25, 26]. At present, chitosan nanoparticle adjuvant has been applied to a variety of DNA vaccines, including human and animal infectious diseases, for example reddish body iridovirus, nodavirus, foot and mouth disease virus and influenza virus [27, 28]. In order to overcome the defect that chitosan has poor water-solubility, chitosan derivative nanoparticles used in the study, O-2′-HACC nanoparticles, have better water solubility, biodegradability, biocompatibility, loading capacity and mucosal adsorption compared to chitosan. Due to the presence of negatively charged regions between the cells, thus, O-2'-HACC nanoparticles with positive charge can open cell junctions in these regions and change the shape of cytoskeleton protein, which allow the O-2'-HACC nanoparticles to pass mucosal epithelial cell barrier and be absorbed by M cells. Hence, O-2'-HACC nanoparticles can be served as vaccine adjuvant and delivery vector to improve immune effect, and the nanoparticles have many advantages than chitosan nanoparticles.

The particle size of nanoparticles is also an important quality indicator that affects transfection and the expression efficiency of target gene [29]. It is generally believed that the nanoparticles between 150-300 nm are most suitable for transfection. If the nanoparticles are too large, it is difficult to enter the target cells [30, 31]. The particle size of O-2'-HACC/pFDNA prepared in our study is about 202.3 nm, which may help the nano vaccine to enter host cells. Moreover, the level of antibodies induced by O-2'-HACC/pFDNA was significantly higher than that of commercial vaccines, which indicated that

O-2'-HACC/pFDNA induced a relatively strong immune response.

Many DNA vaccines against human and animal infectious diseases have been developed [32-34]. These vaccines provided stable and sufficient supply of antigen in transfected host cells and induced cellular immunity, mucosal immunity and long-lasting immunity [35-37], but most of DNA vaccines in use or in clinic are injected intramuscularly or subcutaneously. Thus, mucosal immune response cannot be induced. Mucosal vaccine has many advantages over injectable vaccine by being simpler to administer, less risk of transmitting infections and potentially being easier to manufacture [38, 39]. In addition, mucosal vaccination can induce humoral and cell-mediated antigen-specific immune responses, including B cell and T cell memory responses [40].

Nasal-associated lymphoid tissue (NALT), which serves as a mucosal inductive site for immune responses against antigen stimulation in the upper respiratory tract, has an important role in the induction of mucosal immune response, including inducing the production of antigen-specific Th1 and Th2 cells and sIgA antibody [41-45]. Moreover, intranasal immunization can lead to the induction of antigen-specific protective immunity in both the mucosal and systemic immune compartments [43]. Thus, intranasal immunization is expected as a vaccine against pathogens causing upper respiratory tract infection such as NDV and influenza virus [46, 47]. Here, to evaluate the ability of mucosal immune response of O-2'-HACC/pFDNA, chickens were administered intranasal, and the content of sIgA antibody in tracheal fluid, bile and harderian gland was measured, the results demonstrated that the levels of sIgA antibody produced by the O-2'-HACC/pFDNA i.n. were higher than those of the O-2'-HACC/pFDNA i.m., and the O-2'-HACC/pFDNA i.n. had a longer immune protection period, indicating that mucosal immune response was induced in mucosal inductive site for immune responses against antigen stimulation. O-2'-HACC nanoparticles increased the contact time of antigen with mucosal inductive site, which effectively enhanced

314 the uptake rate of antigen-associated lymphoid tissue, thus, the levels of sIgA antibody were improved and
315 induced better mucosal immunity in the O-2'-HACC/pFDNA i.n.

316 T helper cells are key cells regulating humoral and cellular immunity. The functionally active region
317 of T helper cells is divided into two cell subpopulations, Th1 and Th2 cells. Cellular immunity involves
318 CD4+ and CD8+ T lymphocytes. CD4+ T lymphocytes can differentiate into Th1 cells or Th2 cells. Th1
319 cells support cellular-mediated immune responses, while Th2 cells drive humoral immune responses [48].
320 IL-2 mainly enhances cellular immunity, IL-4 mainly regulates humoral immunity, and IFN-γ mainly
321 regulates immune response by participating in Th-type cells to differentiate into Th1 type [49]. Therefore,
322 IL-2 and IFN-γ enhance the Th1 type immune response, and IL-4 can enhance the Th2 type immune
323 response [50]. The levels of IL-2, IL-4 and IFN-γ in serum of chickens immunized with the
324 O-2'-HACC/pFDNA i.n. were significantly higher, and the cytokine levels induced by the mucosal immune
325 pathway were higher than those of the non-mucosal immune pathway, which the O-2'-HACC/pFDNA i.n.
326 promoted the lymphocyte proliferation and cellular response and better induce Th1 and Th2 type responses,
327 indicating that the O-2'-HACC/pFDNA *via* the mucosal route stimulated the body to produce strong
328 cellular, humoral and local mucosal immunity.

329 After functional modification, chitosan derivatives can improve the various properties of chitosan,
330 such as water solubility, stability, membrane permeability, mucosal adhesion and controlled release, etc.
331 The study provided a theoretical basis for the application of quaternized chitosan nanoparticles as adjuvant
332 and delivery system for DNA vaccines in some *viral* infectious disease vaccines, and have the great
333 potential in the field of mucosal vaccines. Despite these advantages, chitosan derivatives nanoparticles as
334 adjuvant and delivery vector for DNA vaccine are still in its early stages, and more clinical trials are
335 needed for verification, such as irregular distribution and low physical stability etc., which hinder the

commercialization of chitosan. Therefore, it is highly desirable to study safe, efficient and targeted vaccine delivery system to prevent and control certain infectious diseases [26]. All problems will be solved in the near future with the development and application of nanotechnology, because one of the most attractive fields in nanotechnology is the use of nanomaterials as vaccine adjuvant and delivery system, and so many nanomaterials have been studied for the delivery of drugs, imaging, diagnostic and vaccines. In conclusion, the use of chitosan derivatives nanoparticles is having a significant impact on vaccinology with the perspective to obtain novel biological products to fight high pathogenic infectious diseases.

Materials and method

Animals

Two hundred and ten 1-day-old healthy SPF chickens are provided and raised by the Experimental Animal Center of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All of the animal experimental procedures were approved by the Animal Ethics Committee as stipulated in the guide to the care and use of experimental animals of Harbin Veterinary Research Institute. SPF chickens were housed in the center's negative pressure isolator during the test. The chickens were euthanized by intravenous injection of pentobarbital.

Preparation of the O-2'-HACC/pFDNA

We prepared the O-2′-HACC nanoparticles loaded with NDV F gene plasmid DNA (O-2′-HACC/pFDNA) using the polyelectrolyte complex method. The water soluble quaternized chitosan nanoparticles, O-2'-HACC nanoparticles, were synthesized as vaccine adjuvant and delivery vector according to described previously [21]. NDV F gene eukaryotic expression plasmid pVAX-optiF with C3d6 molecular adjuvant (pVAX I -F(o)-C3d6) was constructed by our group [51].

Characterization of the O-2'-HACC/pFDNA

358 To evaluate the morphological and surface characteristics of O-2'-HACC/pFDNA, the nanoparticles
359 were observed by a JEM-200EX transmission electron microscopy (Hitachi Ltd., Tokyo, Japan). Zeta
360 potential, particle size and distribution of the O-2'-HACC/pFDNA were measured by a Zeta Sizer Nano
361 ZS90 (Malvern Instruments Ltd., Southborough, MA, USA). Encapsulation efficiency (EE) and loading
362 capacity (LC) were determined by the formula, EE (%) = $(W_0-W_1)/W_0\times100\%$, LC (%) = $(W_0$
363 $-W_1)/W_N\times100\%$ [21]. In the formula, W_0 is total amount of the pVAX I -F(o)-C3d6 added, W_1 is amount of
364 the free pVAX I -F(o)-C3d6, and W_N is the weight of the O-2'-HACC/pFDNA.
365 ***DNase I protection assay***
366 To investigate the protection of O-2'-HACC/pFDNA against *DNase*, O-2'-HACC/pFDNA was
367 incubated with 10 μl of *DNase* I buffer containing 1 units *DNase* I (TaKaRa, Dalian, China) at 37℃ for 30,
368 60, 120 and 180 min, respectively. After the incubation, 5 μl of 0.5 mol/l EDTA solution was added to
369 terminate the reaction at 65℃ for 10 min. Finally, the mixture was centrifuged at 4℃, 12000 r/min for 20
370 min, and then the supernatant was taken and analyzed by 0.8% agarose gel electrophoresis at 100 V for 30
371 min.
372 ***In vitro release of the O-2'-HACC/pFDNA***
373 To test the release of the pVAX I -F(o)-C3d6 from the O-2'-HACC/pFDNA, 0.1 g of the freeze-dried
374 O-2'-HACC/pFDNA was dissolved in 2.0 ml PBS buffer (pH 7.4), then mixed fully and shaked in a shaker
375 at 37℃, 100 r/min for 0, 6, 12, 18, 24, 36, 48, 60, 72, 96, 120, 144, 168, 192 and 216 h, respectively. The
376 sample was taken and centrifuged at 4℃, 12000 r/min for 20 min. The content of plasmid DNA in the
377 supernatant was measured by UV spectrophotometry (ELX808, Bio-Tek, USA) at 260 nm. The release
378 profile was plotted using release time as the *X*-axis and cumulative release amount as the *Y*-axis.
379 ***Cytotoxicity and stability assay of the O-2'-HACC/pFDNA***

To assess the safety of O-2'-HACC nanoparticles as vaccine adjuvant and delivery system for mucosal immunity, *in vitro* and *in vivo* cytotoxicity were carried out. Any abnormal changes in the immunized chickens such as feed, water drinking, mental state, body weight, clinical symptoms, morbidity and mortality were continuously observed and recorded for 14 days, and each dead chicken was subjected to necropsy to examine the histopathological changes by histological staining.

To investigate the storage stability of the freeze-dried O-2'-HACC/pFDNA stored at room temperature for two and three months, respectively, we performed the animal experiment. Sixty 18-day-old healthy SPF chickens were randomly selected and equally divided into three groups, chickens in Group 1 were administrated with the nano vaccine no stored as control, chickens in Group 2 were administrated with the nano vaccine stored at room temperature for two months, chickens in Group 3 were administrated with the nano vaccine stored at room temperature for three months. Each chicken received 100 μl doses *via* intramuscular route. Blood samples were collected *via* heart from two chickens in each of the three groups at 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 weeks post the immunization, respectively, and then serum were obtained to measure the anti-NDV IgG antibody by hemagglutination inhibition (HI).

In vitro expression of the O-2'-HACC/pFDNA

To verify the expression of the plasmid DNA encapsulated in the O-2'-HACC nanoparticles, *in vitro* transfection was carried out by the Lipofectamine™ 2000 reagent kit (Invitrogen, USA). Group 1 was the liposome transfection group containing 4 μg of the naked pVAX I -F(o)-C3d6, Group 2 was the O-2'-HACC/pFDNA containing 4 μg of the pVAX I -F(o)-C3d6, Group 3 was the blank O-2'-HACC nanoparticles as a negative control, Group 4 was 293T cell control group. NDV positive serum obtained from Harbin Veterinary Research Institute. Epifluorescence images were obtained by a fluorescent microscopy (Zeiss, Germany).

Nasal immunization

One hundred and twenty 18-day-old healthy SPF chickens were randomly divided into six groups with twenty chickens in each group, and chickens in each group were separately housed in a stainless-steel isolator in a temperature- and light-controlled environment with free access to food and water *ad libitum*. Each chicken was given an immunization dose of 100 μl containing 200 μg of the plasmid DNA. Chickens in Group 1 were administered 100 μl PBS buffer intramuscular (i.m.), chickens in Groups 2 were administered 100 μl of O-2'-HACC NPs i.m., chickens in Groups 3 were administered 100 μl of the plasmid DNA i.m., chickens in Groups 4 were administered 100 μl of O-2'-HACC/pFDNA containing 200 μg of the plasmid DNA i.m., chickens in Group 5 were administered 100 μl of O-2'-HACC/pFDNA containing 200 μg of the plasmid DNA intranasal (i.n.), chickens in Groups 6 were administered 100 μl of attenuated live NDV vaccine i.m. The attenuated live NDV vaccine (L/N: 200805) provided by Harbin Pharmaceutical Group Bio-vaccine Co. Ltd.

Blood samples were collected *via* heart from two chickens in each of the six groups at 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 weeks post the immunization, respectively. Serum was obtained by centrifugation at 4℃, 3000 r/min for 10 min to measure the anti-NDV IgG antibody titers, and the levels of IFN-γ, IL-2 and IL-4 by ELISA kit (Thermo Fisher Scientific Inc, MA, USA) and CD4+ and CD8+ T lymphocytes distribution by FACSAria flow cytometer (BD Biosciences, San Diego, CA, USA). At the same time, to assess the mucosal immune response, sIgA antibody titers in serum, tracheal fluid, bile and harderian glands were measured using the NDV IgA ELISA Kit (Rapidbio Co. Ltd, Beijing, China). Additionally, to detect the cellular-mediated immune response, splenocytes were harvested to determine the lymphocyte proliferation by MTT colorimetric assay as previously described [21].

Protective efficacy against NDV strain F48E9

When the levels of HI antibody in serum of every immune group reached to 6.0 log2 post the immunization, seven chickens were selected randomly from each of the six groups and challenged with 100 μl of viral suspension containing $10^{4.5}$ EID_{50}/0.1ml of F48E9 *via* nasal drop. Any abnormal changes such as feed, water drinking, mental state, body weight, clinical symptoms and mortality were observed and recorded for 35 days. On day 7th, 14th, 21th, 28th, and 35th after challenge, blood samples were collected for the analysis of serum HI antibody and the contents of IFN-γ, IL-2, and IL-4. Simultaneously, the infected chickens and chickens in negative control groups were euthanized, and their glandular stomach, duodenum and myocardium were collected to examine the histopathological changes by histological staining. Chickens were sacrificed by an overdose of a mixture of isoflurane/O_2.

Statistical analysis

Data were expressed as mean value ± standard deviation (SD). All experiments were repeated for at least three times with at least triplicated samples in each experiment. Kruskal-Wallis one-way analysis of variance (ANOVA) was employed to evaluate the statistical differences among different groups with SPSS 19.0 software. The difference between groups with $P<0.05$ was considered to be statistically significant.

Declarations

Ethics approval and consent to participate

All of the animal experimental procedures were approved by the Animal Ethics Committee as stipulated in the guide to the care and use of experimental animals of Harbin Veterinary Research Institute.

Consent for publication

All authors agreed to submit this manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

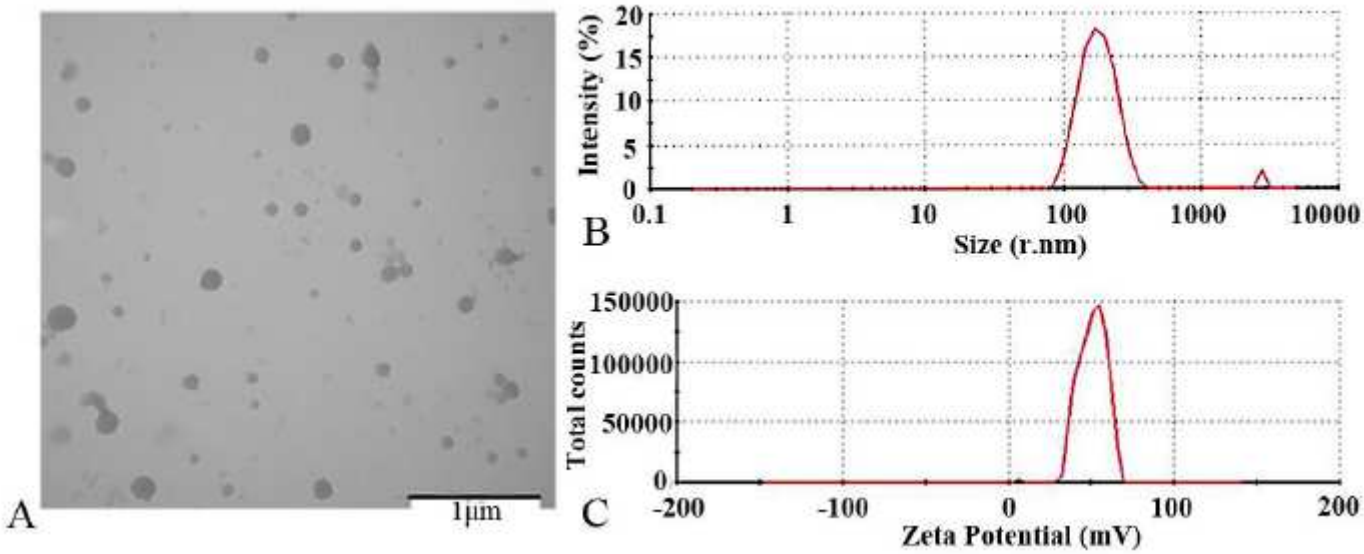

Figure 1

Characterization of the O-2'-HACC/pFDNA. (A) Transmission electron microscope of O-2'-HACC/pFDNA; (B) Particle size of O-2'-HACC/pFDNA; (C) Zeta potential of O-2'-HACC/pFDNA.

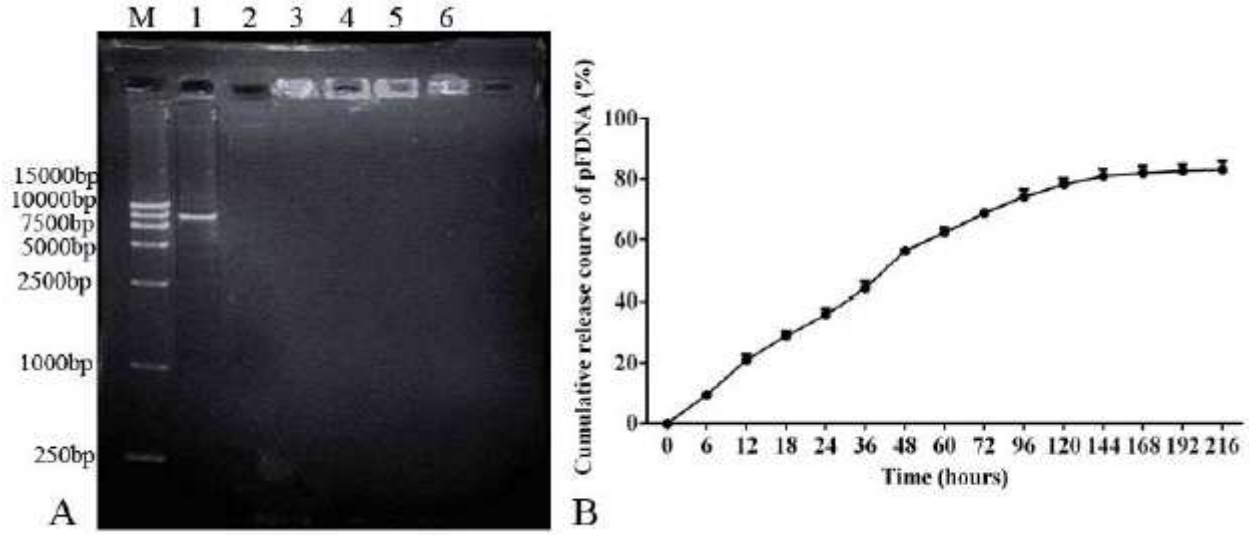

Figure 2

Stability and in vitro release analysis of the plasmid pVAX □ -F(o)-C3d6 after encapsulation in the O-2'-HACC nanoparticles. (A) DNase □ protection of the pVAX □ -F(o)-C3d6, M: DL 15000 Marker, Lane 1: pVAX □ -F(o)-C3d6, Lane 2: DNase □ acts on the naked DNA for 30 min, Lane 3-6: DNase □ acts on the O-2'-HACC/pFDNA for 30, 60, 120 and 180 min; (B) In vitro release profiles of the O-2'-HACC/pFDNA in PBS solution (pH=7.4). Data are presented as the mean ± SD deviation (n=3).

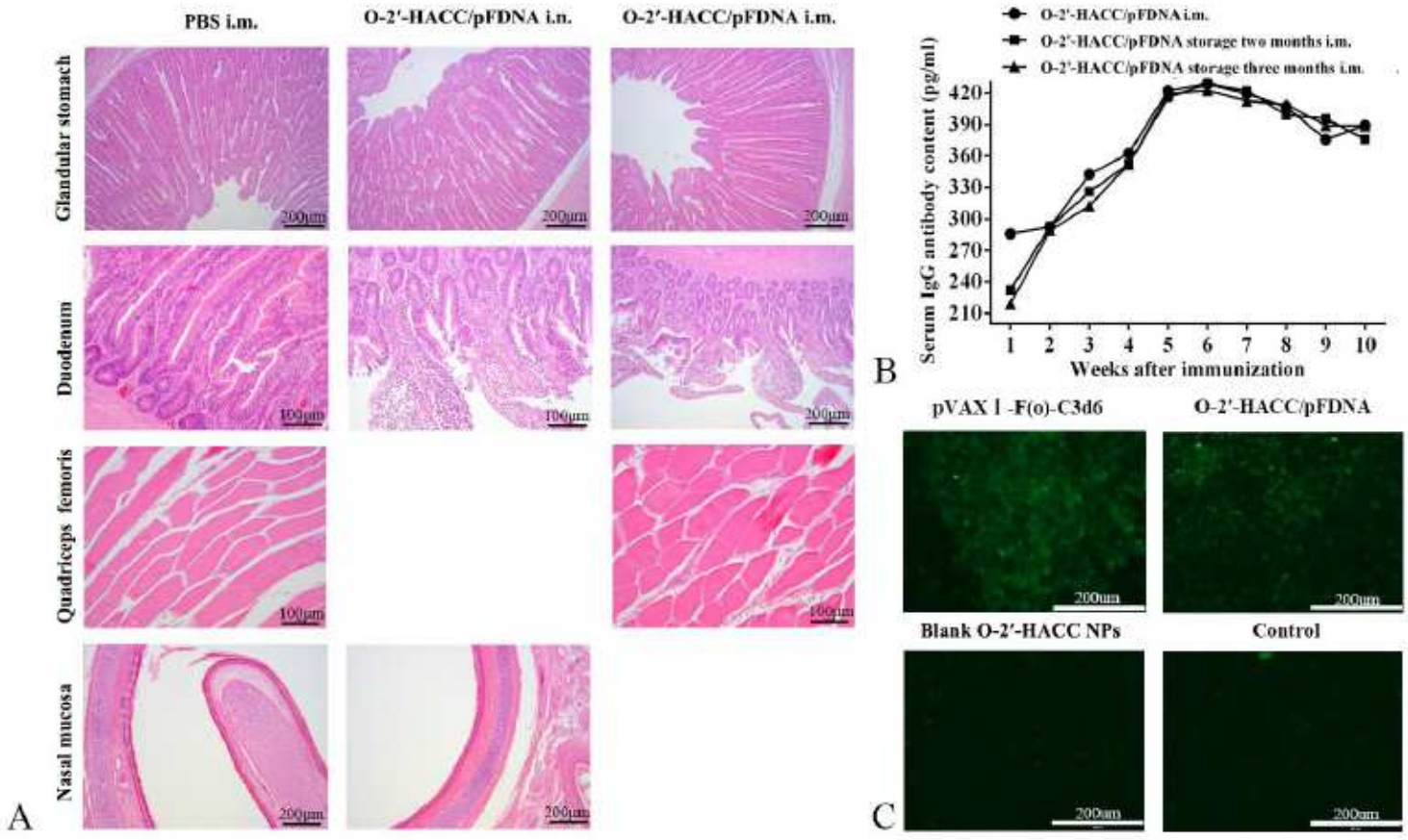

Figure 3

Safety analysis, in vitro fluorescence expression and storage stability of the O-2'-HACC/pFDNA. (A) Histopathological analyses of glandular stomach, duodenum, quadriceps femoris and nasal mucosa; (B) In vitro expression of the O-2'-HACC/pFDNA in 293T cells assayed by indirect immunofluorescence (×40); (C) After Storage stability of the O-2'-HACC/pFDNA for two and three months at room temperature, IgG titers in serum post the immunization.

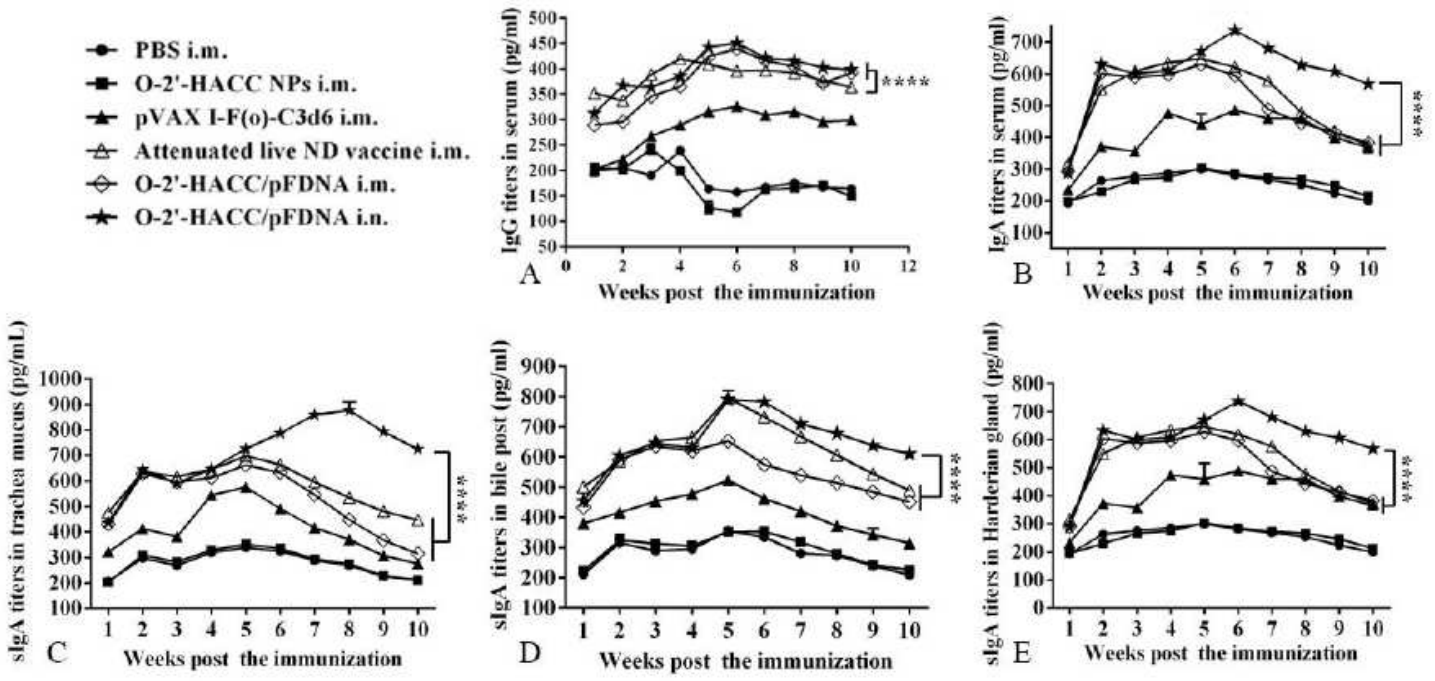

Figure 4

IgG and IgA antibody titers in serum (A, B), trachea mucus (C), bile (D), and harderian gland (E) following administration of PBS i.m., O-2'-HACC NPs i.m., pVAX I -F(o)-C3d6 i.m., attenuated live ND vaccine i.n., O-2'-HACC/pFDNA i.m., O-2'-HACC/pFDNA i.n. Data are representative of three independent experiments and presented as the mean ± SD (n=3). *P<0.05; **P<0.01.

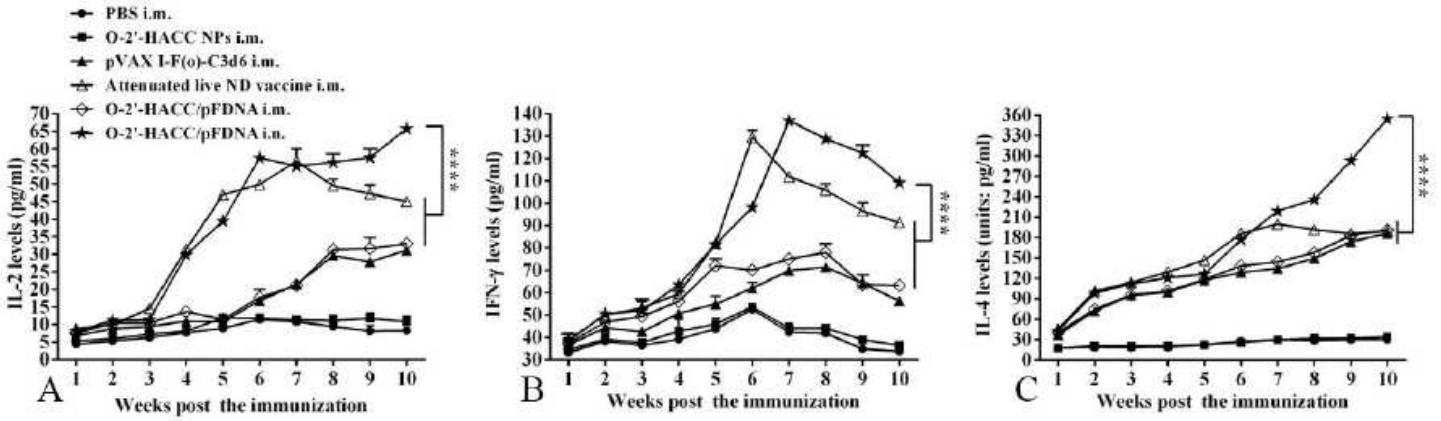

Figure 5

IL-2 (A), IL-4 (B), and IFN-γ (C) levels in the supernatant of splenocytes harvested from the SPF chickens immunized with the PBS i.m., O-2'-HACC NPs i.m., pVAX I-F(o)-C3d6 i.m., attenuated live ND vaccine i.n., O-2'-HACC/pFDNA i.m., O-2'-HACC/pFDNA i.n. IFN-γ, IL-2, and IL-4 levels in the supernatant were analyzed in a chicken IFN-γ, IL-2, and IL-4 enzyme-linked immunosorbent assay.

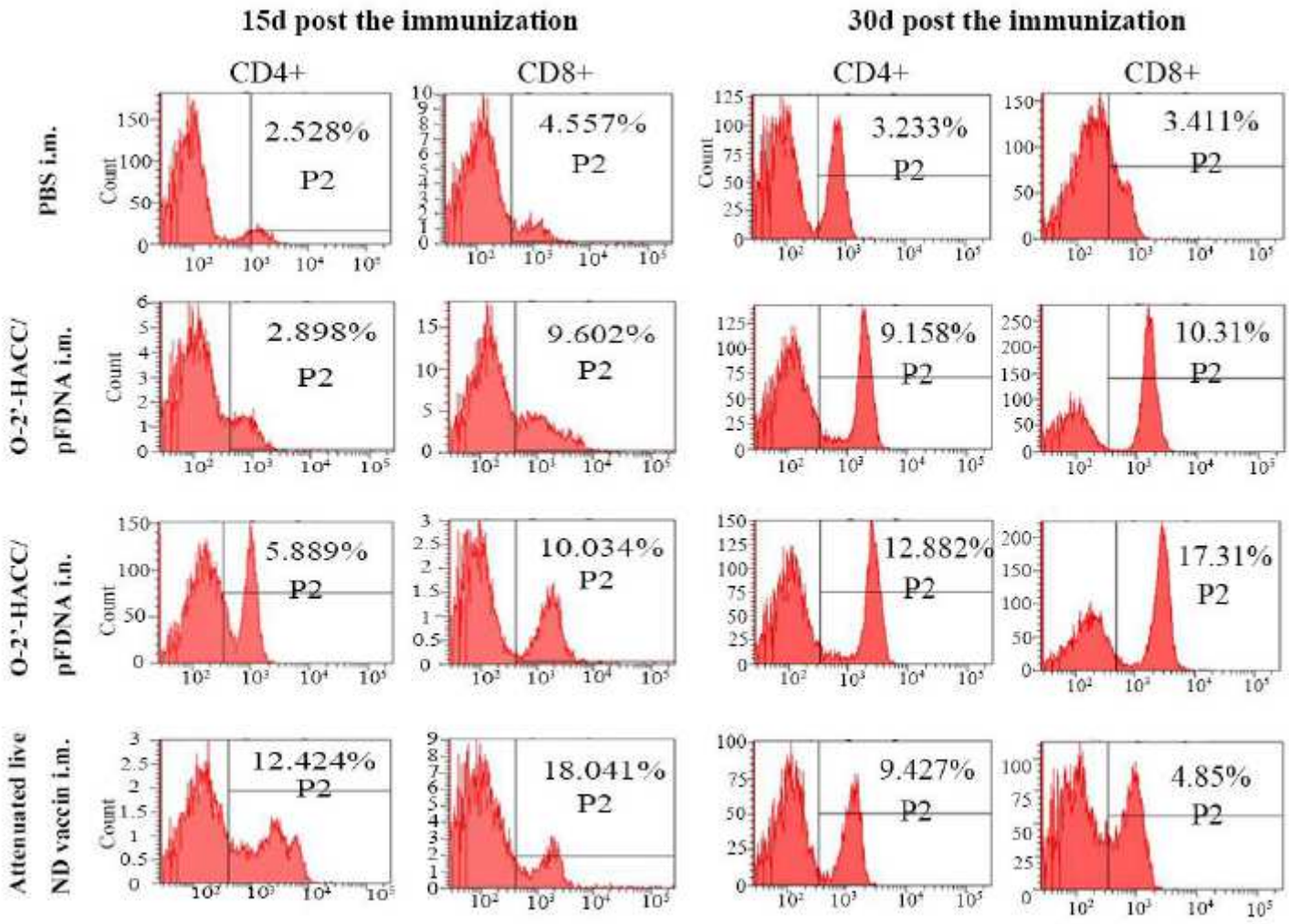

Figure 6

Levels of CD4+ and CD8+ T lymphocytes in peripheral blood post 15 days and 30 days after the immunization.

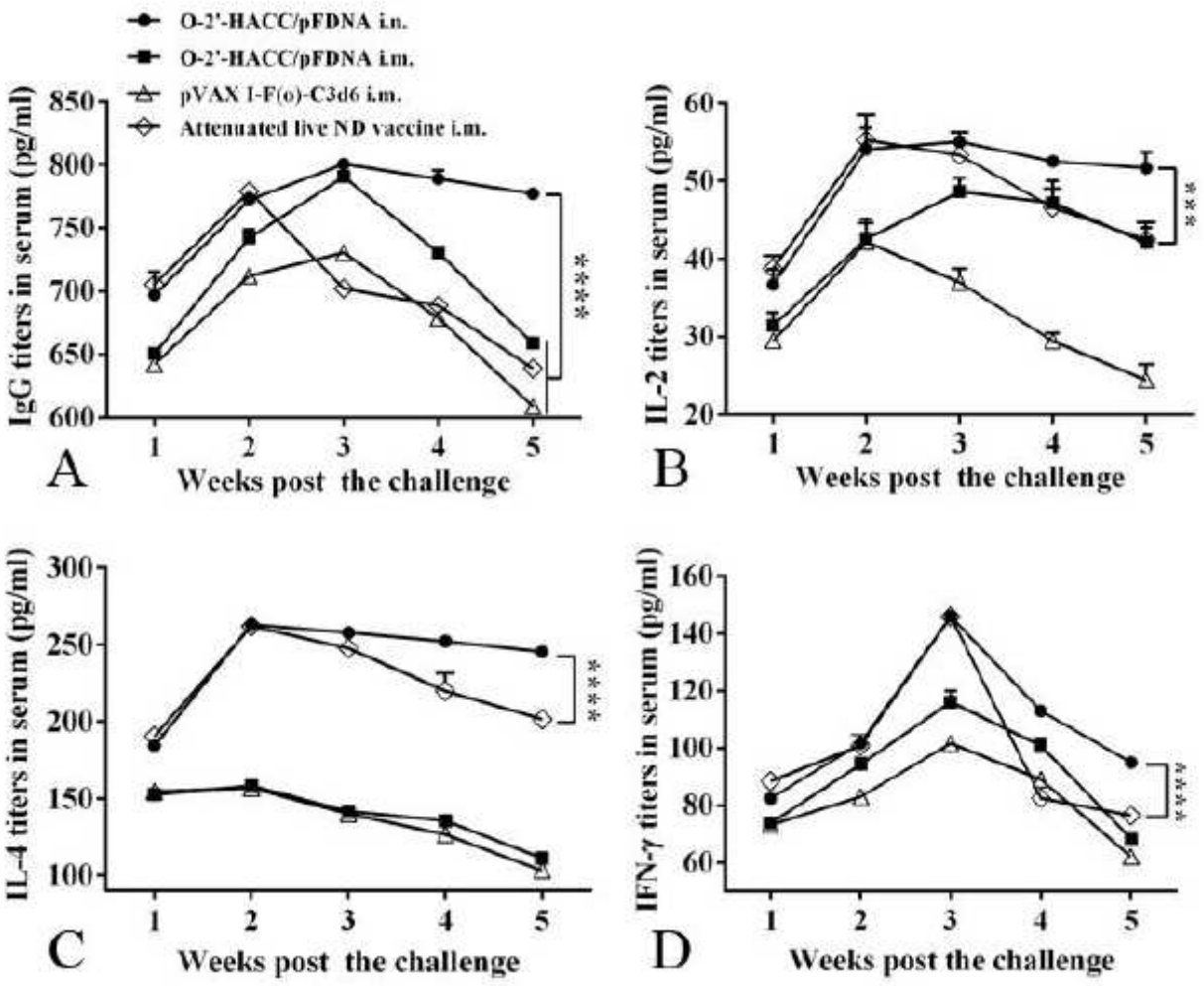

Figure 7

Serum IgG antibody titers (A) and IL-2 (B), IL-4 (C), IFN-γ (D) levels in the supernatant of splenocytes harvested from the immunized SPF chickens after challenge with the highly virulent NDV strain F48E9. IFN-γ, IL-2, and IL-4 levels in the supernatant were analyzed in a chicken IFN-γ, IL-2, and IL-4 enzyme-linked immunosorbent assay. Results are represented as mean ± SD of three separate experiments. *P<0.05.

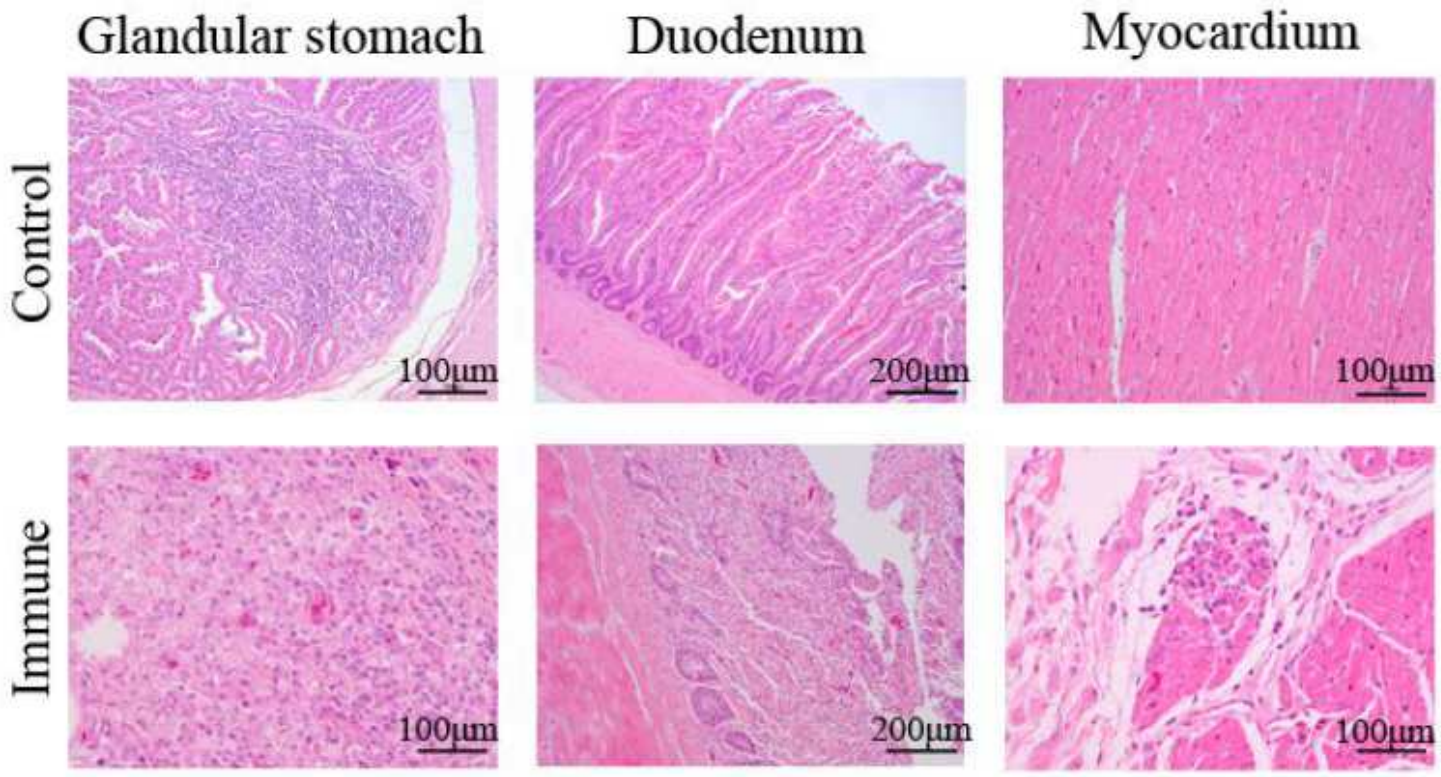

Figure 8

Histopathological analyses of glandular stomach, duodenum, and myocardium obtained from healthy chickens and those challenged with the highly virulent NDV strain F48E9. Tissues of the glandular stomach, duodenum, and myocardium from the PBS i.m., blank O-2’-HACC nanoparticles i.n., attenuated live ND vaccine i.m., and O-2'-HACC/pFDNA i.m. and i.n. groups.

Supplementary Files

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