H9N2 avian influenza mRNA vaccine candidate induces robust protective immunity in chickens

Soroush Sarmadi[1], Zahra Ziafati Kafi[1], Arash Ghalyanchi Langeroudi[1], Hossein Hosseini[2], Mosslim Sedghi[3], Somaye Dehghanizadeh[3], Vahid Khoddami[3], Hamideh Najafi[1*]

1- Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

2- Department of Clinical Sciences, Islamic Azad University, Karaj branch, Karaj, Iran

3- ReNAP Therapeutics, Tehran, Iran

Corresponding author:

Hamideh Najafi

Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Phone: +98 911 275 3200

Email: h_najafi@ut.ac.ir

Co – Corresponding authors:

Zahra Ziafati Kafi

Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

Phone: +98 912 412 2190

Email: zahra.ziafati@ut.ac.ir

Vahid Khoddami

ReNAP Therapeutics, Tehran, Iran

Phone: +98 912 043 6297

Email: v.khoddami@renap.ir

Preprint

Abstract

The H9N2 avian influenza remains endemic in large parts of Asia, causing substantial economic losses in poultry. Despite vaccination, continuous antigenic drift limits the effectiveness of conventional inactivated vaccines. In this study, we developed a nucleoside-modified mRNA vaccine encoding the hemagglutinin (HA) protein of H9N2 virus and formulated it into lipid nanoparticles (LNPs). The mRNA vaccine injected to chickens intramasculary and boosted 2 weeks later with the same dose (10µg). Humoral response were measured by hemagglutination inhibition (HI) assay at 14 and 28 days post-vaccination. Birds were challenged at 28 days post-vaccination with wild virus, and viral shedding was assessed at 5 and 10 days post challenge. Cytokine expression (IL-6, IFN-gamma, TNF-alpha) and histopathological lesions in the trachea and lung were examined. Chickens vaccinated with the mRNA-LNP vaccine showed a greater increase in HI antibody titers over time compared with birds receiving the inactivated vaccine. Both vaccines reduced clinical signs and viral shedding after challenge; however, consistently lower viral RNA loads and milder tracheal and pulmonary lesions were observed in the mRNA-vaccinated group. Analysis of pro-inflammatory cytokine expression revealed no significant differences between the mRNA-vaccinated and inactivated vaccine groups. However, both vaccinated groups exhibited significantly elevated cytokine levels relative to the unvaccinated control group. Results also match with earlier findings on mRNA vaccines for other influenza strains, highlighting the importance of prime – boost strategies. Overall, this platform is flexible, fast to produce, and highly immunogenic. More studies are needed to refine dosing and better understand its effects on cellular immunity.

Keywords: H9N2; mRNA vaccine; lipid nanoparticles; hemagglutinin; poultry; avian influenza; viral shedding.

Introduction

By providing an affordable source of protein, the poultry industry plays an important economic role worldwide. Approximately three-quarters of the world's poultry flocks are raised in high-density conditions, making them highly susceptible to infectious diseases, especially respiratory ones. One of these infectious diseases is avian influenza, which is among the most economically significant poultry diseases globally (1, 2). *Influenza A virus* (IAV) belongs to the *Orthomyxoviridae* family and contains a segmented (8 segments) negative-sense RNA genome encoding 10 major proteins and various accessory proteins including polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins (M1 and M2), and non-structural proteins (NS1 and NS2 or nuclear export protein (NEP)) (3, 4). The different subtypes of this virus are characterized by a combination of two major surface glycoproteins: hemagglutinin (HA) and

Preprint

neuraminidase (NA) (3, 5). Based on the amino acid sequence at the proteolytic cleavage site of the HA protein, avian influenza viruses are further classified as highly pathogenic avian influenza viruses (HPAIVs), harboring multi-basic amino acids at the HA protein cleavage site, and low pathogenic avian influenza viruses (LPAIVs) (6). Among LPAI subtypes, H9N2 is enzootic in domestic poultry across Asia and Africa (7). Infection with this subtype can increase susceptibility to other viral and bacterial infections, leading to respiratory and digestive tract diseases with high mortality. Despite its low pathogenicity, H9N2 infection can cause economic losses in the poultry farming industry and pose a potential threat to human health (7, 8). Although these viruses are considered low pathogenic because they don't cause mortality in in-vivo pathotyping test in specific pathogen-free (SPF) chickens, under field conditions, they can behave differently, causing birds to show mild to severe respiratory disease signs, decreased egg production, and in some cases up to 20% mortality (9).

To date, vaccination has proven to be the most effective strategy against H9N2 infection, and inactivated whole-virus H9N2 vaccines can protect immunized chickens by reducing clinical signs. However, challenges remain regarding the efficacy of H9N2 vaccines (10-12). The accumulation of mutations in the head of HA glycoprotein, the primary target for neutralizing antibodies, enables the virus to evade the immune system. This phenomenon can also impair the efficacy of vaccine-induced immunity. Therefore, it is necessary to develop novel vaccines and update current ones (12). Another challenge with inactivated vaccines is their insufficiency in inducing protective cellular immunity, despite the important role of this immunity in fighting influenza infection (12).

The recent success of the mRNA COVID-19 vaccine validates this platform and highlights its potential for use against other infectious diseases, including those in veterinary medicine (13). Compared to inactivated vaccines, mRNA vaccines offer key advantages, including rapid production in a cell-free environment and the ability to induce strong humoral and cellular immune responses. However, inherent mRNA instability historically limited its widespread administration. The development of lipid nanoparticles (LNPs) as a delivery system has largely addressed this, enabling effective transport of mRNA to host cells and triggering robust immune responses (12, 14, 15). mRNA vaccines have demonstrated the potential to induce prophylactic immunity in animals, as shown in studies in chickens against H9N2 influenza virus and Marek's disease virus (12, 14).

Despite extensive vaccination efforts, H9N2 avian influenza outbreaks continue to impact poultry flocks, leading to significant economic losses. The virus's capacity for rapid antigenic variation necessitates the development of novel vaccine platforms that can be quickly updated to match circulating strains. To address this challenge, we developed an mRNA-LNP vaccine candidate targeting the HA surface protein of a relevant Iranian H9N2 avian influenza virus isolate. In this study, we evaluated the vaccine candidate's

Preprint

ability to elicit robust humoral immune responses and its safety profile in vivo. Furthermore, we assessed the efficacy of the induced immunity in protecting against infection, reducing disease severity, and preventing viral shedding.

Materials and methods

Preparation of candidate virus for mRNA vaccine development

A recently isolated H9N2 virus from Iran (isolated in Iran by Ghalyanchi et al. 2022) was selected as the candidate strain for mRNA vaccine development. The complete nucleotide sequence of the gene encoding hemagglutinin of this isolate is available in NCBI GenBank under accession number ON334757.1. The virus was propagated in 9-day-old embryonated specific pathogen-free (SPF) eggs. Allantoic fluid from the inoculated eggs was harvested after 5 days, and viral load was determined by hemagglutination assay. The isolated virus was stored at -80°C for further assays.

HA gene amplification and purification

Total RNA was extracted using Roche high pure viral nucleic acid kit, from the isolated virus and used for reverse transcription. For the first step of reverse transcription, 1 µL of random hexamer was added to 10 µL of template RNA, heated to 70°C for 5 minutes, and then cooled to 5°C for 1 minute. In the next step, a 9µL cDNA synthesis master mix containing 1µL of MMLV reverse transcriptase enzyme, 4 µL of enzyme buffer, and 4µL of DEPC-treated water was added to the previous reaction and incubated in a thermocycler as follows: 25°C for 5 minutes, 42°C for 1 hour, 85°C for 5 minutes, and 5°C for 1 minute. To confirm the presence of the HA gene, a pair of primers targeting the start and end of the HA open reading frame (ORF) was designed (forward 5´ATGGAAGCAATATCAC3´ and reverse 5´CTATATACAAATGTTGCAT3´) and subsequently evaluated using the Sanger sequencing method (Codon Genetic Company, Tehran, Iran). The target band was carefully excised from the gel, and the DNA was extracted using the FavorPrep™ GEL/PCR purification kit according to the manufacturer's protocol. After this step, the purified DNA was stored at -80°C.

HA Gene Cloning and In vitro mRNA transcription

The HA coding sequence was RT-PCR amplified from H9N2 viral RNA and assembled into a pUC57-based IVT plasmid containing a T7 promoter, 5′/3′ UTRs, a restriction site for linearization, and a built-in poly(A) tail sequence using Gibson Assembly Cloning Kit (NEB); plasmids were later linearized by restriction digestion. After transformation, positive clones in E. coli TOP10 competent cells were confirmed by Sanger sequencing. Verified colony was cultured in LB broth and incubated overnight at (37°C-180 rpm). Plasmid was extracted using the FavorPrep™ Plasmid DNA Extraction Kit and then linearized

Preprint

through restriction enzyme digestion. The purified linear plasmid was used as the in vitro transcription (IVT) template for co-transcriptional capping with CleanCap AG (3'O) and incorporation of m1ΨTP in place of UTP. Following transcription, the linear template was removed using RNase-free DNase I treatment, and the capped mRNA was purified using the Monarch Spin RNA Cleanup Kit (NEB). RNA yield/purity and integrity were evaluated by UV spectrophotometry and gel-based analysis.

mRNA-LNP formulation

Purified mRNA was diluted in citrate buffer (pH 4.0) to the desired concentration. A lipid mixture with a total lipid concentration of 12.5 mM was prepared by dissolving an ionizable lipid (proprietary), cholesterol, DSPC, and DMG-PEG2000 in absolute ethanol at molar ratios of 50:38.5:10:1.5, respectively. The ethanolic lipid solution and the aqueous mRNA solution were mixed using a dedicated microfluidic device at a 1:3 flow rate ratio (organic: aqueous) with a total flow rate (TFR) of 12 mL/min.

Buffer exchange and concentration

Following formulation, buffer exchange was performed by overnight dialysis using a 12 kDa MWCO dialysis membrane against a Tris–acetate buffer (pH 7.4) composed of 5.03 mM Tris, 14.91 mM Tris-HCl, 2.89 mM sodium acetate trihydrate, and 1.42 mM acetic acid, to remove residual ethanol while maintaining LNP integrity. Subsequently, the formulation was concentrated using an Amicon Ultra-15 centrifugal filter unit (100 kDa MWCO) at 1,800 ×g in a fixed-angle centrifuge, reducing the volume by 90% of the initial volume. The concentration factor (initial volume / final volume) was calculated to be about 10."

Physicochemical characterization

Particle size and polydispersity index (PDI) were measured by dynamic light scattering (DLS). Samples were diluted 10-fold in double-distilled water (ddH_2O) prior to measurement.

Encapsulation assessment

Encapsulation of mRNA within lipid nanoparticles was evaluated using an electrophoretic mobility shift assay (EMSA) based on gel retardation (15). Following electrophoresis, gel densitometry analysis was applied in a semi-quantitative manner to estimate the relative extent of mRNA encapsulation across formulations.

Chicken experiment

To evaluate the efficacy of vaccine-induced immunity, 36 one-day-old SPF chickens were randomly assigned to four groups: one group received 10μg of the mRNA-LNP vaccine, another group was vaccinated with a commercially inactivated H9N2 vaccine, the positive control group was challenged with

Preprint

the virus without prior immunization, and the negative control group received neither vaccine nor viral challenge. Chickens in the treatment groups were immunized intramuscularly on day 21 and boosted two weeks later with the same dose. The commercial inactivated vaccine also administrated according to manufacturer's protocol. At 28 days post-immunization, all birds (except those in the negative control group) were challenged with 10^9 EID_{50} of the virus. The chickens in the negative control group received only mRNA-LNP buffer (isotonic acetate buffer, pH7.4). To analyze viral replication in chickens, real-time RT-PCR was performed on tracheal and cloacal swabs collected at 5- and 10-days post-challenge. Tissue samples were also collected from the trachea and lung, and subjected to an H&E staining to examine pathological changes.

Antibody detection

To assess the humoral immune response induced by vaccination, serum samples were collected at days 14 and 28 after the first injection and evaluated using the hemagglutination inhibition (HI) assay. During viral infection, hemagglutinin (HA) proteins on the virion surface facilitate both viral entry and red blood cell agglutination. The HI test can provide a partial indication of protective efficacy. The HI test was performed according to the protocol recommended by World Organization for Animal Health (WOAH).

RNA extraction and real-time RT-PCR for viral shedding

Swab samples (collected at 5 and 10 days post challenge) were homogenized in sterile 1X PBS and extracted using the High Pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturer's protocol. Extracted RNA was eluted in 50µL of elution buffer and stored at -20°C until cDNA synthesis. For cDNA synthesis, 1 µL of random hexamer primer was added to 10 µL of total RNA. The mixture was incubated at 70 °C for 5 minutes, then immediately cooled to 5 °C for 1 minute. Next, 9µL of cDNA master mix containing 1µL of MMLV reverse transcriptase enzyme, 4µL of 5X RT-buffer (Yekta Tajhiz Azma, Iran), 1µL of dNTPs (SinaClone, Iran) and 2µL of DEPC-treated water were added, resulting in a final reaction volume of 20µL. The presence of viral RNA was measured by qRT-PCR using the specific primer pairs (F: 5'-ATG GGG TTT GCT GCC-3' , R: 5'-TTA TAT ACA AAT GTT GCA YCT G-3' and Probe: FAM-TTC TGG GCC ATG TCC AAT GG-TAMRA) (16, 17). The following thermal protocol was used for real-time RT-PCR reaction: 95°C for 10 minutes, 45 cycles of 95°C for 30 seconds and 54°C for 45 seconds.

RNA extraction and real-time RT-PCR for IL-6, IFN-Gamma and TNF-alpha

To assess the cell-mediated immune response induced by vaccination, spleen tissues were collected and homogenized under sterile conditions. Total RNA was extracted from the homogenates. The expression

Preprint

levels of immune-related genes were quantified using absolute real-time reverse transcription PCR with specific primers, enabling evaluation of vaccine-induced cellular immunity. Detection of IL-6 and IFN-gamma was performed using SYBR green based real-time RT-PCR; specific probes were used for TNF-alpha detection. Primer and probe sequences are listed in Table 1. The following thermal protocol was used for real-time RT-PCR reaction: 50°C for 12 minutes, 95°C for 3 minutes followed by 45 cycles of 95°C for 10 seconds and 60°C for 20 seconds.

Statistical analysis

All data were analyzed using GraphPad Prism 6 (GraphPad Software Inc.). Differences between the two groups were assessed using either a t-test or a two-way ANOVA and Kruskal-Wallis test for multiple comparisons as appropriate.

Results

mRNA-LNP preparation, formulation, and quality assessment

Lipid nanoparticles have been demonstrated as an effective platform for mRNA delivery, enabling efficient intracellular expression and antigen presentation (18). In this study, nucleoside-modified HA mRNA (Figure 1a) was formulated into lipid nanoparticles using a microfluidic mixing approach as described in the Materials and Methods section. Dynamic light scattering analysis showed that the resulting HA mRNA-LNPs had an average hydrodynamic diameter of 87.05 nm with a polydispersity index (PDI) of 0.14, indicating a narrow size distribution (Figure 1b). Encapsulation of mRNA within the lipid nanoparticles was evaluated using EMSA based on gel retardation, in which encapsulated mRNA exhibited reduced electrophoretic mobility compared to free mRNA (Figure 1c). Gel densitometry analysis was subsequently applied to the EMSA results in a semi-quantitative manner to estimate the relative extent of mRNA encapsulation across formulations and to guide relative dose adjustment for subsequent in vivo experiments.

Antibody production evaluation

Blood samples collected on days 14 and 28 post-vaccination were analyzed using the hemagglutination inhibition (HI) assay. At day 14, the mean HI titers for the mRNA-vaccinated, commercial inactivated vaccine, and control groups were 6.0 ± 1.67, 7.58 ± 1.37 , and 0.11 ± 0.33, respectively. By day 28, the corresponding mean titers increased to 9.85 ± 1.09, 8.7 ± 1.05, and 0.6 ± 0.54, in mRNA-vaccinated, commercially inactivated and control groups respectively (Figure 2). The increase in HI titer between these time points was 3.85 units in the mRNA-vaccinated group and 1.1 units in the commercial inactivated vaccine group. These data demonstrate a greater rise in HI titers in the mRNA-vaccinated group over the

Preprint

study period following booster injection. Statistical analysis revealed that HI titers differed significantly among the experimental groups at both 14 and 28 days post-vaccination.

Viral RNA load in cloacal and tracheal swab

Viral shedding was assessed by determining viral titers in tracheal and cloacal swabs collected at 5- and 10-days post virus inoculation using absolute real-time RT-PCR. The results indicated that chickens receiving the mRNA vaccine exhibited reduced viral replication in both tracheal and cloacal samples at both time points, compared with those vaccinated with the commercial inactivated vaccine or inoculated with acetate buffer (Figure 3). This reduction in viral shedding highlights the mRNA vaccine's ability to elicit a protective immune response sufficient to limit viral replication following challenge. Furthermore, no statistically significant difference was observed between the mRNA-vaccinated and inactivated-vaccine groups, suggesting that the mRNA platform performs at least comparably to the established inactivated vaccine in controlling viral replication.

Clinical Observation

Following the challenge, clinical signs such as abnormal respiratory sounds and watery eyes were seen in unvaccinated chickens. In contrast, these signs were not observed in the vaccinated groups or were limited. Overall, the reduced occurrence and severity of clinical signs in the vaccinated birds suggest that both vaccination approaches provided protection against the development of clinical disase following challenge with H9N2 virus.

Evaluation of IL-6, IFN-Gamma and TNF-alpha

To further evaluate vaccine-induced cellular immune responses, the mRNA expression levels of key cytokines, including IL-6, IFN-γ, and TNF-α, were evaluated in spleen tissue at day 5 post-challenge using absolute real-time RT-PCR. As shown in figure 4, chickens vaccinated with the mRNA vaccine exhibited significantly higher expression of all three cytokines compared with the control group. However, there was no significant difference in expression of these cytokines between chickens vaccinated with mRNA vaccine and chickens immunized with commercially inactivated vaccine. These results suggest the potential ability of designed mRNA vaccine candidate in provoking cytokines associated with cell-mediated immunity.

Histopathological examination

In order to evaluate damages and lesions due to H9N2 infection, and to investigate whether mRNA vaccine has the ability to prevent virus-caused damages, tissue samples collected from lung and trachea. Pathomorphological changes were visualized by the H&E staining method (figure 5). Each sample was carefully screened for lesions and scored for lesion severity from 0 (no lesions) to 10 (severe lesions).

Preprint

Histopathological section of trachea from the mRNA group showed mild, localized epithelial degeneration with no significant necrotic changes, accompanied by sparse lymphoid infiltration limited to the superficial lamina propria. No heterophilic exudate was present in the lumen, and only minimal inflammatory cell infiltration was observed overall. In contrast, the other vaccinated group showed multifocal areas of epithelial loss and moderate epithelial degeneration. Moreover, the lamina propria contained a moderate lymphoplasmacytic infiltrate, and the lumen showed only occasional traces of mild heterophilic exudate. Quantitative analysis based on scores showed significant difference between mRNA vaccinated goup and control group. However no significant difference observed between vaccinated groups. The mean total histopathological score for trachea were: mRNA vaccinated group= 2.33 ± 1.15, commercially inactivated vaccine= 4.33 ± 0.57 and control positive group= 8.33 ± 0.57. No lesions were observed in control negative group. Histopathological analysis of mRNA group lung tissue samples showed mild focal epithelial hyperplasia, mild peribronchial mononuclear cell infiltration, and low degrees of vascular congestion. In contrast, chickens vaccinated with a commercially inactivated vaccine showed moderate epithelial proliferation and mononuclear infiltration, along with moderate vascular congestion and edema. The mean total histopathological score for lung was: mRNA vaccinated group= 3 ± 1, commercially inactivated vaccine= 5 ± 1 and control positive group= 8 ± 1. No lesions were observed in control negative group. Statistical analysis of histopathological scores is shown in Figure 6.

Discussion

Previous global experience with the COVID-19 pandemic and the successful application of mRNA vaccines to control it have accelerated efforts to design new vaccines for other diseases using this platform. Among different platforms for vaccine design, mRNA vaccines are recognized for their favorable safety profile, strong immunogenicity, high flexibility, rapid production capability, and comparatively low manufacturing requirements (19). In this study, we developed an mRNA vaccine to combat the H9N2 low-pathogenic avian influenza virus and compared its immunogenicity with that of a previously used commercially inactivated vaccine.

Among different antigens harbored by influenza virus, immunity is mainly achieved by producing antibodies which neutralize the HA protein, the major influenza antigen, thereby preventing the virion from binding to the host cell (20). Different studies have suggested the HA protein as the main target for designing an mRNA vaccine. Xu *et al*. designed a vaccine candidate based on the HA protein of H9N2 subtypes and reported that using the HA protein can strongly activate both the humoral and cellular arms of the immune system (12). In another study, Wang *et al*. developed FLUCOV-10, a decavalent vaccine for broad, simultaneous protection against both Influenza and SARS-CoV-2 viruses. To design this vaccine, they employed mRNAs encoding full-length HAs from different influenza virus subtypes and SARS-CoV-

Preprint

2 spike proteins (19). In another similar study, Bahl et al. developed an mRNA-LNP vaccine encoding the HA proteins of the H10N8 and H7N9 subtypes, and results indicated that the vaccine can elicit rapid immune responses in models such as mice, ferrets, and non-human primates (21). Therefore, HA protein is a suitable target for vaccine design, and for this reason, the HA protein-encoding ORF was directly extracted and amplified from the virus. To reduce potential inflammatory responses associated with unmodified mRNA, all uridines in the nucleotide sequence were replaced with N1-methyl-pseudouridine during mRNA synthesis.

The present designed vaccine demonstrated that it can provoke antibody production. At 14 days post-vaccination, both vaccine groups (mRNA and inactivated) showed increases in antibody titers, with the inactivated vaccine group exhibiting a greater increase at this time point. However, following the second dose, the mRNA vaccine group demonstrated a greater increase in antibody titers at day 28 post-first injection, surpassing those induced by the inactivated vaccine. The rise in antibody titers after booster injection was also reported by Xu et al. In their study, three different doses of the mRNA vaccine (10, 15, and 20 μg) were administered. Their hemagglutination inhibition (HI) results demonstrated an increase in antibody titers following the booster, and further indicated that this rise was dose-dependent (12). In another study, Furey et al. designed an mRNA vaccine against the H5 subtype of the highly pathogenic avian influenza virus. They employed a prime-boost strategy for vaccination of ferrets in this study. Administering a second dose 28 days after the initial vaccination. This booster injection markedly enhanced the immune response, with H5-reactive antibody levels increasing approximately eightfold compared to pre-boost levels. The study confirmed that the second dose was critical for achieving robust antibody titers, thereby supporting the findings of enhanced immunogenicity following booster administration (22). In an earlier study, Wang et al developed an mRNA vaccine candidate against H5 subtype of the avian influenza virus. Using a prime boost immunization strategy in both mice and specific-pathogen-free chickens, they demonstrated that the vaccine elicited strong humoral responses. Antibody titers increased significantly after the second dose, and two-dose regimens provided complete protection against homologous strains and high levels of protection against heterologous H5N1 viruses (23). Collectively, results from different studies highlight the pivotal role of booster administration in maximizing the immunogenicity of mRNA vaccines against avian influenza. The consistent observation of significantly elevated antibody titers following the second dose underscores the need for prime-boost strategies to achieve robust humoral responses and effective protection against viral strains.

In addition to humoral immunity, we also evaluated the absolute expression of three factors related to cell-mediated immunity (IL6, IFN-gamma, and TNF-alpha). Our analysis revealed no difference between mRNA-vaccinated chickens and the group vaccinated with a commercially inactivated vaccine. However

Preprint

previous studies reported differences. For example, Xu et al. reported a higher frequency of IFN-γ-expressing cells in chickens vaccinated with the mRNA vaccine compared to those immunized with the inactivated vaccine. Their results further demonstrated that increasing the mRNA dose led to a corresponding rise in the number of IFN-γ-secreting cells (12). Wang et al. also reported increased levels of cytokines such as IFN-gamma, TNF-alpha, IL-6, and IL-13 in mice immunized with an mRNA vaccine compared to control mice. However, they indicated that the average mRNA expression levels of TNF-α, IL-6, IL-4, and IL-13 were not significantly different from those of the PBS group (23). Overall, while our data did not reveal significant differences in cytokine expression between mRNA- and inactivated-vaccine groups, previous reports suggest that mRNA vaccination can enhance cell-mediated immune responses. These contrasting findings highlight the complexity of cytokine regulation and indicate that the impact of mRNA vaccines on cellular immunity may depend on factors such as dose and possibly the experimental design.

Conclusion

In conclusion, our study demonstrates that the newly designed mRNA vaccine targeting the H9N2 subtype of avian influenza virus can induce strong humoral immune responses, particularly after booster administration, with antibody titers surpassing those elicited by the commercially available inactivated vaccine. These findings are consistent with previous reports on mRNA vaccines targeting different influenza subtypes, further underscoring the critical role of prime – boost strategies in achieving robust, durable immunity. Although our analysis did not reveal significant differences in cytokine expression between mRNA and inactivated vaccine groups, evidence from other studies suggests that mRNA vaccination can enhance cell-mediated immune responses under specific conditions. Taken together, these results highlight the promise of mRNA vaccine platforms as flexible, rapidly producible, and highly immunogenic candidates for controlling avian influenza, while also underscoring the need for further investigation into their effects on cellular immunity and optimization of dosing strategies. We also suggest additional studies to optimize dosing strategies and to better understand the efficacy of this vaccine platform in eliciting cell-mediated immunity.

Declaration of competing interest

The authors V.K. and S.D. are management board member and employees at ReNAP. M.S. is current employee at ReNAP . The authors declare no other relationships or activities that could appear to have influenced the submitted work.

Funding

Preprint

The main financial support for this research came from ReNAP Therapeutics, with additional funding provided by University of Tehran. ReNAP Therapeutics holds exclusive commercialization rights for the product developed in this study.

Complience with ethical guidelines

We confirm that the present study was conducted in accordance with all applicable ethical guidelines and standards for animal health and welfare.

Data availability

All data generated or analyzed during this study are included in present article. Further explanations or additional supporting data are available from the corresponding author upon reasonable request.

Authors contribution

SS: Writing – original draft, Visualization, Software, Project administration, Investigation, Formal analysis, Data curation.

ZZK: Writing – original draft, Visualization, Software, Project administration, Investigation, Formal analysis, Data curation.

AGL: Methodology, Study Suppervision, Project administration, Writing – original draft, Visualization, Software, Project administration, Investigation, Formal analysis, Data curation.

HH: Review & Editing, Methodology, Data Curation.

MS: Methodology, Writing – review & editing, Conceptualization, Project administration, Investigation.
SD: Supervision, Resources, Investigation, Conceptualization.

VK: Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

HN: Methodology, Study Suppervision, Writing – original draft, Visualization, Software, Project administration, Investigation, Formal analysis, Data curation.

Refrences

1. Cargnin Faccin F, Cáceres CJ, Gay LC, Seibert B, van Bentem N, Rodriguez LA, et al. Mass vaccination with reassortment-impaired live H9N2 avian influenza vaccine. npj Vaccines. 2024;9(1):136.

Preprint

2. Ladman BS, Gelb Jr J, Sauble LA, Murphy MV, Spackman E. Protection afforded by avian influenza vaccination programmes consisting of a novel RNA particle and an inactivated avian influenza vaccine against a highly pathogenic avian influenza virus challenge in layer chickens up to 18 weeks post-vaccination. Avian Pathology. 2019;48(4):371-81.
3. Peacock TP, James J, Sealy JE, Iqbal M. A Global Perspective on H9N2 Avian Influenza Virus. Viruses. 2019;11(7):620.
4. Bedair NM, Sakr MA, Mourad A, Eissa N, Mostafa A, Khamiss O. Molecular characterization of the whole genome of H9N2 avian influenza virus isolated from Egyptian poultry farms. Archives of Virology. 2024;169(5):99.
5. Chatziprodromidou IP, Arvanitidou M, Guitian J, Apostolou T, Vantarakis G, Vantarakis A. Global avian influenza outbreaks 2010–2016: A systematic review of their distribution, avian species and virus subtype. Systematic reviews. 2018;7:1-12.
6. de Bruin ACM, Funk M, Spronken MI, Gultyaev AP, Fouchier RAM, Richard M. Hemagglutinin Subtype Specificity and Mechanisms of Highly Pathogenic Avian Influenza Virus Genesis. Viruses. 2022;14(7):1566.
7. Jonas M, Sahesti A, Murwijati T, Lestariningsih CL, Irine I, Ayesda CS, et al. Identification of avian influenza virus subtype H9N2 in chicken farms in Indonesia. Preventive Veterinary Medicine. 2018;159:99-105.
8. Raheel IA, Elbestawy AR, Diab MS, Abdel-Latif MA, Tag N, Orabi A. Rapid Onset of Innate Response, Cytokine Signaling and Humoral Immunity in Inactivated LPAI-H9N2-Vaccinated Broilers. Poultry. 2024;3(4):420-9.
9. Pusch EA, Suarez DL. The Multifaceted Zoonotic Risk of H9N2 Avian Influenza. Veterinary Sciences. 2018;5(4):82.
10. Liu Y, Zhao D, Zhang J, Huang X, Han K, Liu Q, et al. Development of an Inactivated Avian Influenza Virus Vaccine against Circulating H9N2 in Chickens and Ducks. Vaccines. 2023;11(3):596.
11. Wu Q, Wang W, Zhang X, Li D, Mei M. Effectively Evaluating a Novel Consensus Subunit Vaccine Candidate to Prevent the H9N2 Avian Influenza Virus. Vaccines. 2024;12(8):849.
12. Xu S, Zhang B, Yao J, Ruan W. A new H9 influenza virus mRNA vaccine elicits robust protective immunity against infection. Vaccine. 2023;41(18):2905-13.
13. Le T, Sun C, Chang J, Zhang G, Yin X. mRNA Vaccine Development for Emerging Animal and Zoonotic Diseases. Viruses. 2022;14(2):401.
14. Fazel F, Matsuyama-Kato A, Alizadeh M, Zheng J, Fletcher C, Gupta B, et al. A Marek's Disease Virus Messenger RNA-Based Vaccine Modulates Local and Systemic Immune Responses in Chickens. Viruses. 2024;16(7):1156.
15. Lee S-H, Lee JS, Kim D-E, Kim K-S. Optimization of aerosolizable messenger RNA lipid nanoparticles for pulmonary delivery. Biomedical Science Letters. 2023;29(4):231-41.
16. Monne I, Ormelli S, Salviato A, Battisti CD, Bettini F, Salomoni A, et al. Development and Validation of a One-Step Real-Time PCR Assay for Simultaneous Detection of Subtype H5, H7, and H9 Avian Influenza Viruses. Journal of Clinical Microbiology. 2008;46(5):1769-73.
17. Arafa A-S, Hagag N, Erfan A, Mady W, El-Husseiny M, Adel A, et al. Complete genome characterization of avian influenza virus subtype H9N2 from a commercial quail flock in Egypt. Virus Genes. 2012;45(2):283-94.
18. Kowalski PS, Rudra A, Miao L, Anderson DG. Delivering the messenger: advances in technologies for therapeutic mRNA delivery. Molecular Therapy. 2019;27(4):710-28.
19. Wang Y, Ma Q, Li M, Mai Q, Ma L, Zhang H, et al. A decavalent composite mRNA vaccine against both influenza and COVID-19. mBio. 2024;15(9):e00668-24.
20. Peacock TP, Harvey WT, Sadeyen J-R, Reeve R, Iqbal M. The molecular basis of antigenic variation among A(H9N2) avian influenza viruses. Emerging Microbes & Infections. 2018;7(1):1-12.

Preprint

21. Bahl K, Senn JJ, Yuzhakov O, Bulychev A, Brito LA, Hassett KJ, et al. Preclinical and clinical demonstration of immunogenicity by mRNA vaccines against H10N8 and H7N9 influenza viruses. Molecular Therapy. 2017;25(6):1316-27.
22. Furey C, Scher G, Ye N, Kercher L, DeBeauchamp J, Crumpton JC, et al. Development of a nucleoside-modified mRNA vaccine against clade 2.3.4.4b H5 highly pathogenic avian influenza virus. Nature Communications. 2024;15(1):4350.
23. Wang Z, Tian C, Zhu J, Wang S, Ao X, He Y, et al. Avian influenza mRNA vaccine encoding hemagglutinin provides complete protection against divergent H5N1 viruses in specific-pathogen-free chickens. Journal of Nanobiotechnology. 2025;23(1):55.
24. Bagheri S, Paudel S, Wijewardana V, Kangethe RT, Cattoli G, Hess M, et al. Production of interferon gamma and interleukin 17A in chicken T-cell subpopulations hallmarks the stimulation with live, irradiated and killed avian pathogenic Escherichia coli. Developmental & Comparative Immunology. 2022;133:104408.
25. Jarosinski KW, Njaa BL, O'connell PH, Schat KA. Pro-inflammatory responses in chicken spleen and brain tissues after infection with very virulent plus Marek's disease virus. Viral immunology. 2005;18(1):148-61.

Preprint

437

438 Table 1 – Primers and probes used for evaluation of IL-6, INF-Gamma and TNF-alpha

Target	Sequence	Reference
INF-Gamma	F: 5´-GTGAAGAAGGTGAAAGATAT-3´	(24)
	R: 5´-GCTTTGCGCTGGATTCTCA-3´	
	P: 5´ (HEX)-TGGCCAAGCTCCCGATGAACGA-(BHQ1) 3´	
IL-6	5´-CAGGACGAGATGTGCAAGAA-3´	(25)
	5´-CTTGGGCAGGTTGAGGTTGT-3´	
	P: 5´ (FAM)- CGTGTGCGAGAACAGCATGGAGATG -(TAMRA) 3´	
TNF-alpha	5´-CTGCTGTTACAGACACGCTT-3´	-
	5´-GTGCTGGAGGGTTCTTGTTT-3´	

439

440

441

442

443

444

445

Preprint

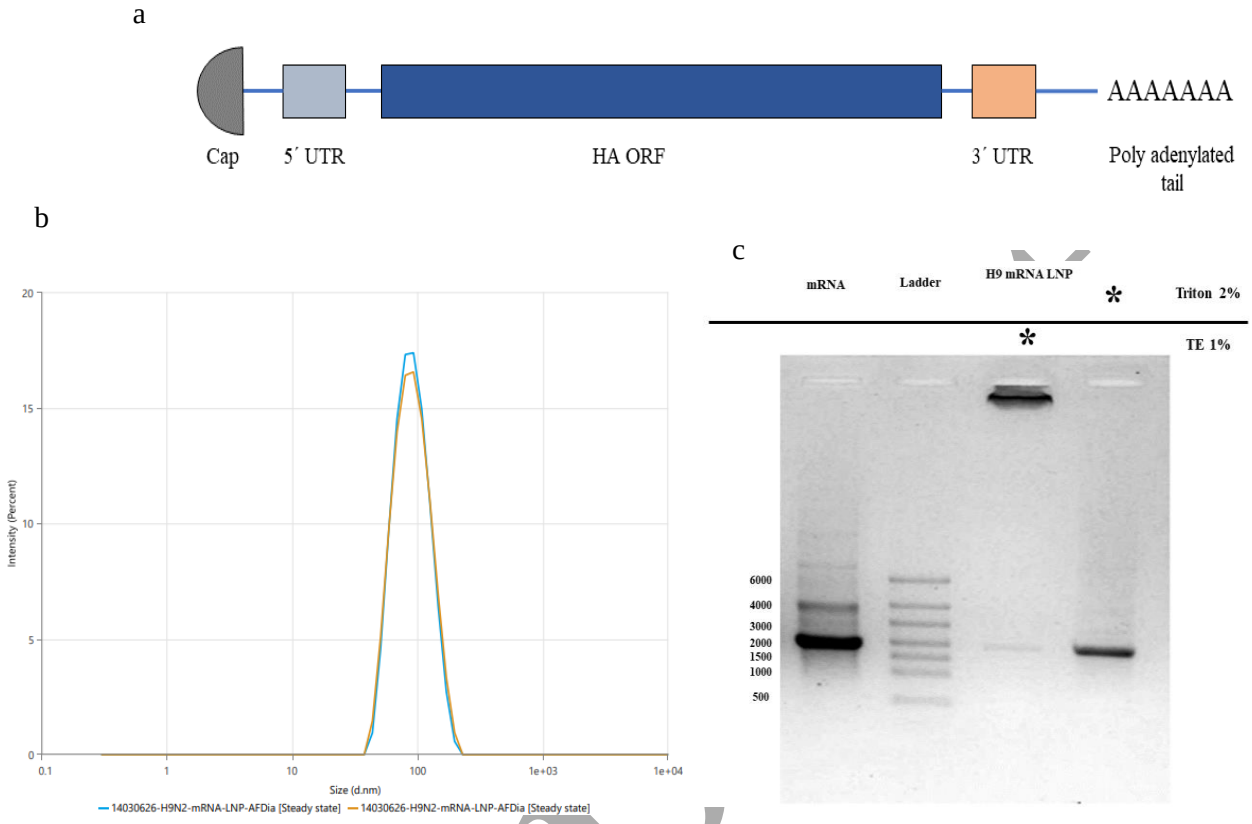
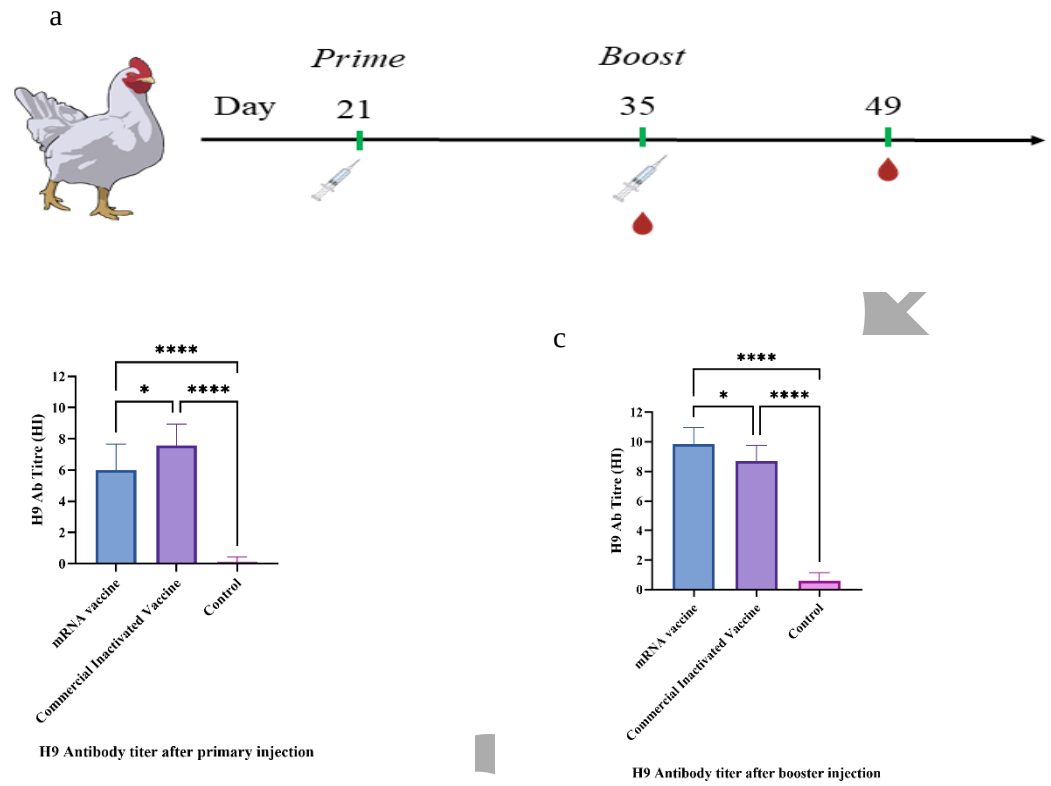


Figure 1 – HA mRNA LNP qualification test. Schematic diagram of designed mRNA (a). characterization of LNP size using Dynamic Light Scattering (DLS) granulometry (b). mRNA-LNP encapsulation rate assessment by Electrophoretic Mobility Shift Assay (Gel retardation assay) (c).

Prep



	mRNA vaccine	Commercial Inactivated Vaccine	Control
Mean	6.000	7.583	0.1111
Std. Deviation	1.673	1.379	0.3333
Std. Error of Mean	0.5045	0.3981	0.1111

	mRNA vaccine	Commercial Inactivated Vaccine	Control
Mean	9.857	8.700	0.6000
Std. Deviation	1.099	1.059	0.5477
Std. Error of Mean	0.2938	0.3350	0.2449

Figure 2. SPF chcikens were immunized with mRNA-LNP and commercial inactivated vaccines. Chickens in control group received only mRNA-LNP buffer (acetate buffer) (a). Hemagglutination inhibition (HI) antibody titers were measured in chickens vaccinated with an mRNA vaccine, a commercial inactivated vaccine, or left unvaccinated (control). (b) HI titers at 14 days post-vaccination. (c) HI titers at 28 days post-vaccination. Statistical significance was determined using one-way ANOVA followed by multiple comparisons. Asterisks (*) indicate statistically significant differences ($p<0.05$), with an increasing number of asterisks representating stronger statistical significance.

Preprint

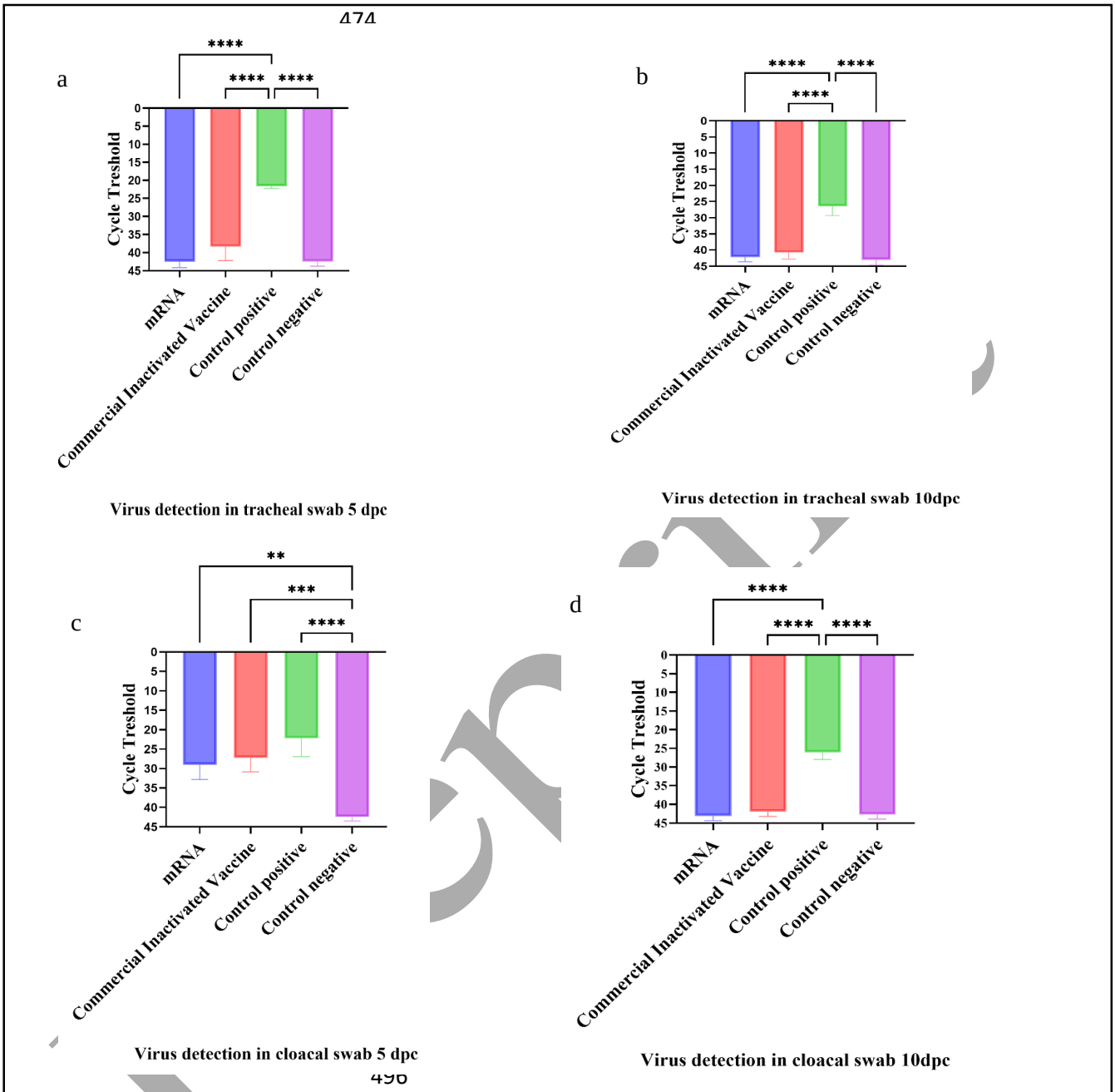


Figure 3. Evaluation of virus shedding in tracheal and cloacal swabs collected at 5- and 10-day post-challenge using absolute real time RT PCR. Tracheal swabs from the mRNA-vaccinated group showed lower viral titers than those from chickens receiving the commercial inactivated vaccine at both day 5 (a) and day 10 (b). Similarly, cloacal swabs revealed reduced viral shedding in the mRNA-vaccinated group relative to the inactivated-vaccine group at day 5 (c) and day 10 (d). Although the differences between the two vaccine groups were not statistically significant, both vaccines showed significantly lower viral titers than the control group. Asterisks (*) indicate statistically significant differences ($p<0.05$), with an increasing number of asterisks representating stronger statistical significance.

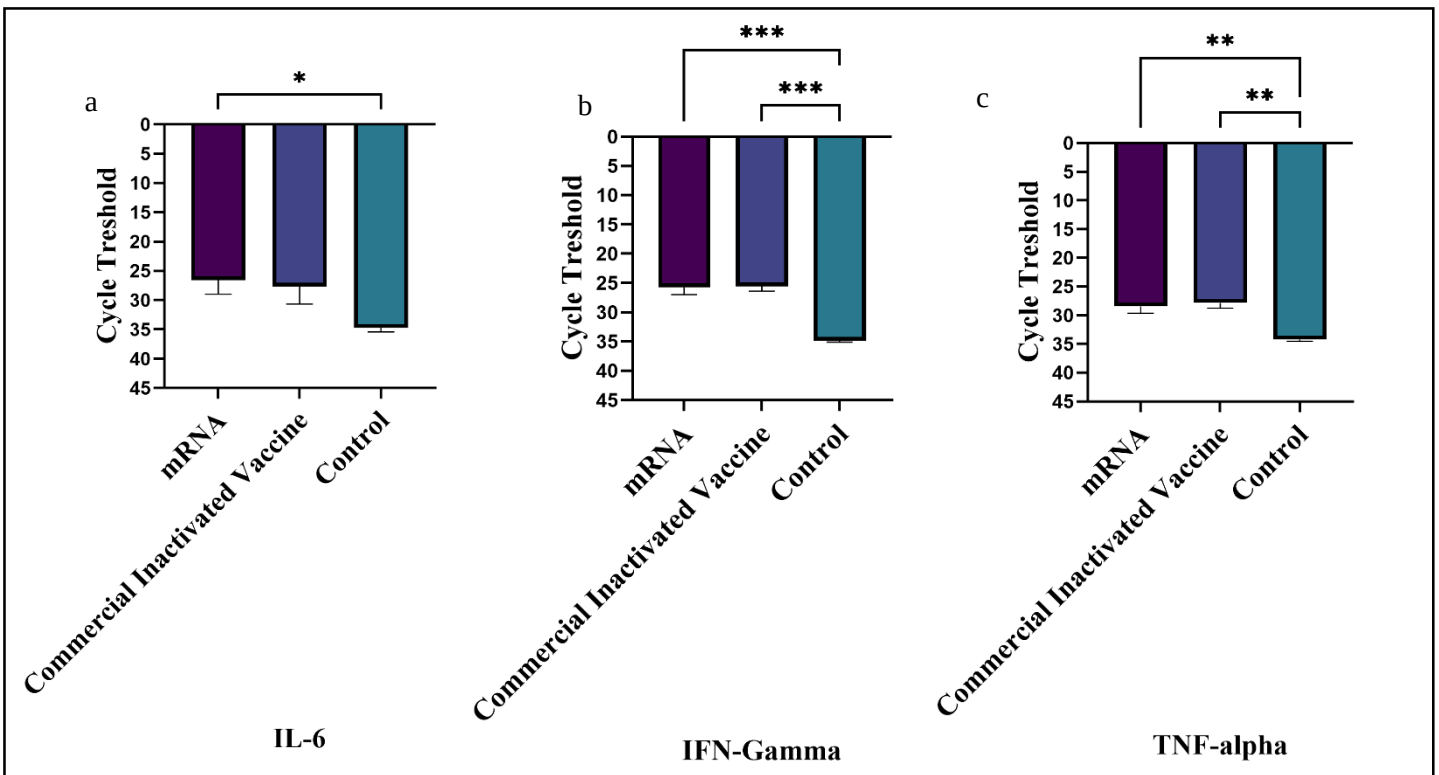


Figure 4. Absolute real-time RT-PCR evaluation of IL-6 (a), IFN-Gamma (b), and TNF-alpha (c) expression in spleen tissue samples collected 5 days post challenge. Asterisks (*) indicate statistically significant differences ($p<0.05$), with an increasing number of asterisks representating stronger statistical significance.

Prep

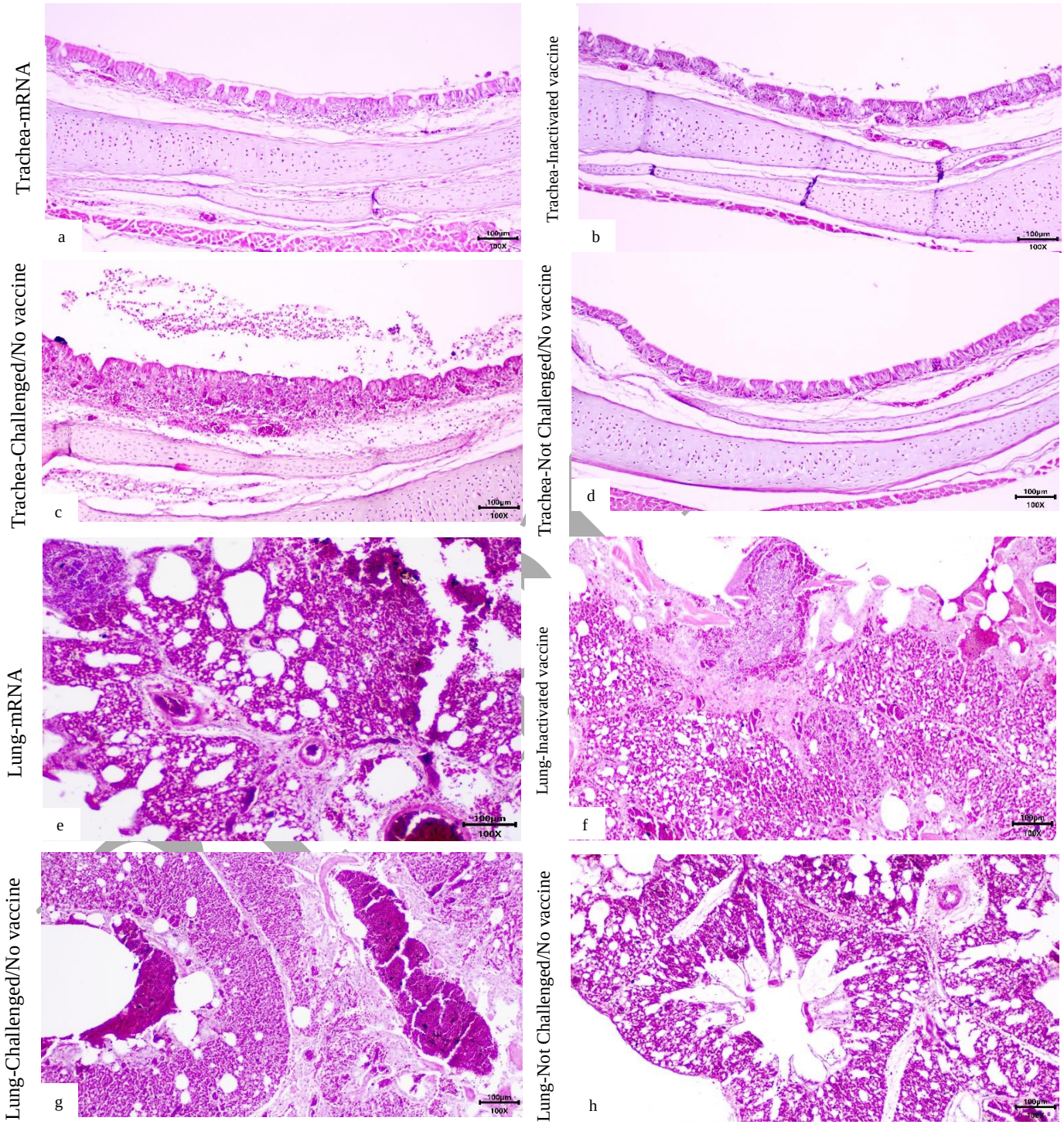
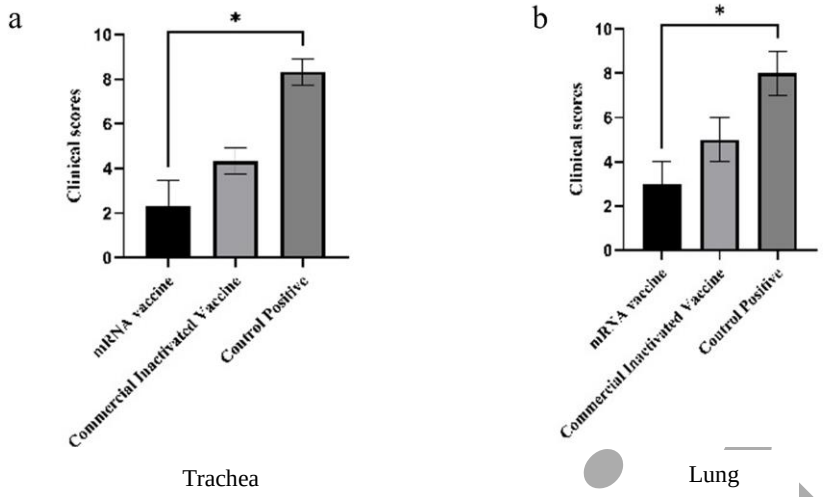


Figure 5. The histopathological examination of trachea (a-d) and lung (e-h). a and e: mRNA vaccinated group, b and f: commercially inactivated vaccine group, c and g: control positive group, d and h: control negative group.



Trachea

Lung

Figure 6. Analysis of histopathological scores of trachea (a) and lung samples (b). According to the results, the mRNA-vaccinated group had a lower average score than other vaccinated groups, indicating the potential of the mRNA vaccine to help mitigate histopathological lesions. Asterisks (*) indicate statistically significant differences ($p<0.05$).

Preprint