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Non-thermal plasma for inactivated-vaccine preparation

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ABSTRACT

Vaccines are of great importance in controlling the spread of infectious diseases in poultry farming. The safety and efficacy of vaccines are also essential. To explore the feasibility of a novel technology (non-thermal plasma) in inactivated vaccine preparation, an alternating current atmospheric pressure non-thermal plasma (NTP) jet with $Ar/O_2/N_2$ as the operating gas was used to inactivate a Newcastle disease virus (NDV, LaSota) strain and H9N2 avian influenza virus (AIV, A/Chicken/Hebei/WD/98) for vaccine preparation. The results showed that complete inactivation could be achieved with 2 min of NTP treatment for both NDV and AIV. Moreover, a proper NTP treatment time is needed for inactivation of a virus without destruction of the antigenic determinants. Compared to traditional formaldehyde-inactivated vaccine, the vaccine made from NDV treated by NTP for 2 min (NTP-2 min-NDV-vaccine) could induce a higher NDV-specific antibody titer in specific pathogen-free (SPF) chickens, and the results of a chicken challenge experiment showed that NTP-2 min-NDV-vaccine could protect SPF chickens from a lethal NDV challenge. Vaccines made from AIV treated by NTP for 2 min (NTP-2 min-AIV-vaccine) also showed a similar AIV-specific antibody titer compared with traditional AIV vaccines prepared using formaldehyde inactivation. Studies of the morphological changes of the virus, chemical analysis of NDV allantoic fluid and optical emission spectrum analysis of NTP suggested that reactive oxygen species and reactive nitrogen species produced by NTP played an important role in the virus inactivation process. All of these results demonstrated that it could be feasible to use non-thermal NTP as an alternative strategy to prepare inactivated vaccines for Newcastle disease and avian influenza.

1. Introduction

Newcastle disease virus (NDV) and avian influenza virus (AIV) are two of the most important pathogens in poultry. Outbreaks of high-pathogenicity avian influenza have been reported as a threat to both humans and animals [1,2]. Newcastle disease (ND) is usually caused by highly pathogenic NDV, which can result in 100% mortality in many species of birds worldwide [3]. Thus, severe economic loss in the poultry industry caused by NDV and AIV highlights the importance of vaccines.

Generally, there are three types of vaccines, i.e., conventional inactivated vaccines, live attenuated vaccines and gene-manipulated vaccines such as vectored viruses, subunit proteins and DNA vaccines. However, scaled-up production, cost and immune potency are issues that should be solved before commercial gene-manipulated vaccines reach the market [4–6]. Additionally, in live attenuated vaccines, there are doubts about whether virulence remains. Currently, the most popular vaccine in poultry farming is still the conventional inactivated vaccine [7]. Conventional inactivated vaccines usually rely on chemical reagents such as formaldehyde and β-propiolactone. However, the kinetics of the formaldehyde inactivation process are not first-order [8]. Formaldehyde is found to be associated with enhanced disease during subsequent infection [9,10]. Moreover, residual formaldehyde and β-propiolactone in the vaccines have mutagenic effects that are harmful to both animals and humans [11]. For both consumers and farmers, an alternative technique that is safer, more effective and lower in cost is expected for inactivated vaccine preparation.

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In recent years, non-thermal plasma (NTP) has been receiving sustained attention owing to its proved effectiveness in biomedical fields, such as bacterial sterilization [12], surface modification [13], and food preservation [14]. The advantages of NTP are that it requires a low temperature, is environment-friendly [15] and is free of toxicity [16,17]. As for the mechanism of NTP, it has been well recognized that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play key roles in its application, especially in its biomedical effects [18]. According to the literature, a few studies have demonstrated the excellent inactivation effect of NTP on viruses such as adenoviruses [19], herpes simplex virus [20] and MS2 bacteriophage [21]. Furthermore, whether NTP can inactivate a virus without damaging the antigenic determinant is deserving of investigation.

In this study, NTP was first used to inactivate NDV and AIV. Then, the inactivated vaccines were prepared by following standard procedures, and serological tests were carried out in specific pathogen-free (SPF) chickens. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to observe the morphological changes of the viruses before and after NTP treatment. Furthermore, the underlying mechanism of the NTP technology was investigated using oxidation-reduction potential (ORP) tests, pH measurements and antioxidant assays of the virus allantoic fluid, as well as optical emission spectrum (OES) analysis.

2. Materials and methods

2.1. Ethics statement

All animal experimental procedures performed in this study were reviewed, approved, and supervised by the Beijing Laboratory Animal Management Office.

2.2. NTP device description

The NTP device used in this experiment is shown in Fig. 1(a). The NTP jet was composed of a Teflon tube (Daxiang Inc, Beijing, China) whose inner and outer diameters were 7 mm and 10 mm, respectively, and a high-potential electrode made of a copper foil with a thickness of 2 mm surrounded the Teflon tube 5 cm from the end of the tube. The plasma was generated by an 18 kV (peak-to-peak) sinusoidal alternative voltage source with a frequency of 10 kHz. The input current was set at ~2–2.5 A. A mixture of argon (Ar), oxygen (O_2) and nitrogen (N_2) containing 88% Ar, 2% O_2 and 10% N_2 at a flow rate of 5 L/min was used as the working gas. The plasma plume was approximately 5 cm from the end of the nozzle, with a temperature of 28 °C ± 3 °C. The distance from the end of the nozzle to the virus allantoic fluid was 1 cm. The optical emission spectrum of the plasma from 200 to 850 nm was detected using a multi-channel fiber optic spectrometer (AvaSpec-2048-8-USB2, Avantes, Eerbeek, Netherlands) with a fiber optic cable placed along the nozzle axis with an end-on view. Representative species were assigned by referring to the database from the National Institute of Standards and Technology. The detailed geometry and operating parameters of the device are illustrated in a previous work [22]. The overall experimental procedure is shown in Fig. 1(b).

2.3. Preparation of the inactivated vaccine

NDV (LaSota strain) and AIV (H9N2, A/Chicken/Hebei/WD/98 strain) were propagated in 11-day-old SPF chicken embryos. The allantoic fluid was harvested, clarified by low-speed centrifugation and stored at −70 °C before use. To inactivate the virus by NTP, 10 mL of NDV-allantoic fluid was thawed at 37 °C and treated with NTP for different amounts of time (2 min, 4 min and 6 min), and the same process was applied to 10 mL of AIV-allantoic fluid with NTP for 1 min, 2 min, 3 min and 4 min. Allantoic fluid containing NDV or AIV without NTP treatment was used as the control. A conventional embryo lethality assay (ELA) and a hemagglutination (HA) test were performed to determine the viability of the viruses after NTP treatment. Subsequently, using Drakeol 6-VR (Penreco, Calumet, USA) as an oil adjuvant [23], inactivated oil-emulsified vaccines were prepared with NDV or AIV antigens treated by NTP and 0.1% formaldehyde [23]. The SPF chicken embryos were supplied by Beijing Merial Vital Laboratory Animal Technology Co., Ltd.

2.4. Embryo lethality assay (ELA)

Viruses treated by different methods and the control sample were injected into 11-day-old SPF chicken embryos, with five embryos in each group. Based on previous studies by other groups [24–26] and our group, 0–120 h was chosen as the monitoring time. The eggs were candled every 24 h, and the death number beyond 24 h was recorded until 120 h. Dead eggs were chilled at 4 °C. Then, the ELA results were summarized.

2.5. Hemagglutination (HA) and hemagglutination inhibition (HI) tests

The HA and HI assays were performed in accordance with OIE standard procedures [27]. The HA titer was read as $\log_2$ of the highest dilution of antigen giving complete HA (no streaming). The HI titer was read as $\log_2$ of the reciprocal of the highest dilution of serum causing inhibition of HA. The samples with HA or HI titers of 4 $\log_2$ or higher were considered HA or HI positive.

2.6. Assessment of the vaccine efficacy

Vaccination. A total of 165 28-day-old SPF chickens (Beijing Merial Vital Laboratory Animal Technology Co., Ltd, China) were tagged and divided randomly into 10 groups (saline, formaldehyde-NDV-vaccine, NTP-2 min-NDV-vaccine, NTP-4 min-NDV-vaccine, NTP-6 min-NDV-vaccine, formaldehyde-AIV-vaccine, NTP-1 min-AIV-vaccine, NTP-2 min-AIV-vaccine, NTP-3 min-AIV-vaccine and NTP-4 min-AIV-vaccine), with 30 chickens in the saline group and 15 chickens in each of the other groups. Each chicken in the vaccinated groups was inoculated subcutaneously with 0.2 mL of vaccine. Chicken sera were collected from the wing vein on day 0 (pre-vaccination) and on days 7, 14, and 21 (post-vaccination) and were tested for the presence of NDV-specific or AIV-specific antibodies using the HI test. The chickens were maintained in negative pressure isolators (Mordun, Edinburgh, UK) throughout the experiments.

Challenge test. The chickens were challenged according to a previous study [28]. Three weeks after vaccination, the chickens in the NDV groups were subcutaneously injected with 0.5 mL of 10^5 embryo lethality dose (ELD_{50}) velogenic NDV according to previous studies [29–31]. Pictures were taken every 24 h, and the dead chickens were stored at 4 °C. Clinical signs and deaths were monitored for 10 days post challenge. Sections of the trachea and glandular stomach were collected during necropsy and inspected. Chickens in AIV groups were injected with 0.5 mL of 10^8 ELD_{50} virulent AIV. Cloacal swabs and throat swabs were collected into PBS, centrifuged and filtered before injection into 11-day-old chicken embryos for HA testing. Ten days later, all live birds were killed by intravenous pentobarbital sodium (Merck, Germany) overdose.

2.7. ORP and pH test

ORP and pH assessment of allantoic fluid containing NDV during NTP treatment was carried out using an ORP

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