Development of human adenovirus type 55 vaccine using microneedle array patches and immunogenicity test in mice

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Development of human adenovirus type 55 vaccine using microneedle array patch and immunogenicity test in mice

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<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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Human adenovirus type 55 (HAdV-55) is an emerging pathogen, known to be a causative agent of acute respiratory disease (ARD), pneumonia, and bronchitis. While HAdV-55 infection results in self-limited disease in healthy adults, it has been frequently associated with large-scale outbreaks in South Korean military training camps. Therefore, the development of an effective vaccine to prevent HAdV-55 outbreaks during military training and in community outbreaks is crucial.

This study aimed to evaluate the impact of additives on vaccine stability during the production of microneedle array patches (MAP) coated with an inactivated HAdV-55 vaccine. The addition of polyethylene glycol (PEG) and Hyaluronic acid (HA) significantly improved vaccine stability during the manufacturing process and storage. Furthermore, The immunogenicity of MAP administration was compared with that of intramuscular (IM) injection. Two doses of the vaccine were administered to BALB/c mice at a two-week interval and serum samples were collected at 2 and 4 weeks post-immunization. MAP

efficacy was assessed by measuring antigen-specific IgG titers and neutralizing antibody titers. The MAP group produced antigen-specific IgG levels that were similar to those elicited by IM injection.

Considering the various advantages of using MAP for vaccination over traditional injections, this study suggests that MAP-based vaccination can be a promising alternative to IM vaccination, particularly in situations requiring mass vaccination, such as pandemics or emergencies.

Key words: human adenovirus, acute respiratory disease (ARD), inactivated vaccine, microneedle array patches

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I. INTRODUCTION

Human adenovirus (HAdV) is known to cause a broad spectrum of diseases affecting individuals of various age groups. These illnesses include acute respiratory infection, gastroenteritis, and keratoconjunctivitis.[1,2] Although HAdV infections are usually mild and self-limiting in healthy adults, they can be more severe in vulnerable populations such as children under 4 years of age, immunocompromised patients, and those living in densely populated environments like military camps, daycare centers, and schools.[2-4] HAdV can be transmitted through multiple routes including aerosols, fecal matter, and close interpersonal contact.[1,5]

Typically, symptoms appear approximately 9 days after the virus has invaded the respiratory mucosal epithelial cells. Unfortunately, there is currently no approved specific

treatment for routine adenovirus infection. However, antiviral medications like cidofovir and ribavirin can be used to treat immunosuppressed individuals infected with adenovirus.[5-7]

HAdV belongs to the *Mastadenovirus* genus within the *Adenoviridae* family.[8] It was first isolated in human adenoids in 1953, and since then, more than 100 serotypes have been identified. These serotypes are categorized into seven subgroups (designated as A to G) based on their serological characteristics. The clinical manifestations of HAdV infections can vary significantly depending on the specific serotype involved.[9,10]

HAdV is characterized as a double-strand DNA virus with a genome containing approximately 36,000 base pairs. This genome is capable of encoding 30 to 40 different genes, each of which plays a role in various aspects of the virus life cycle and pathogenicity. The virus structure of HAdV is composed of a nonenveloped icosahedral capsid, which is made up of 252 capsomeres, comprising 240 hexons and 12 pentons. The pentons are composed of a base and a fiber, with the fiber serving as the region that facilitates the attachment of the virus to host cells. This structural organization is essential for the virus ability to enter the host cells, replicate its genome, and cause infection.[1,11,12]

The replication cycle of HAdV is characterized by a duration of 32 to 36 hours during which it yields approximately 10,000 virion particles. The attachment of HAdV to the host cell is facilitated by the fiber protein, which engages various receptors, including coxsackie and adenovirus receptor (CAR), CD46, and desmoglein2 (DSG2).[6,12-14] Internalization is mediated by the interaction between the penton base of the virus and cellular integrin receptors. Subsequently, the virus is internalized through clathrin-mediated endocytosis, and the relatively high pH environment within the endosome promotes the disassembly of the viral capsid. The acidic environment of the endosome triggers the dissociation of virus particles, permitting only the viral DNA to enter the host cell's nucleus, thereby initiating the process of viral replication. Virus DNA replication occurs in a two-stage process within the host cell nucleus and is facilitated by viral-encoded DNA polymerase. The viral polymerase relies on a 55-kDa terminal protein

attached to the 5’ end of the single-strand DNA, serving as a primer for the initiation of viral DNA synthesis. HAdV is equipped with its DNA polymerase as well as proteins that play pivotal roles in promoting cell growth, suppressing apoptosis, and modulating the host's immune and inflammatory responses. The replication process occurs in two stages: Early phase and late phase. The early phase functions as a preparatory phase, during which transcription of viral DNA yields mRNA. The early transcript is subsequently translated into 20 distinct early proteins. These early proteins orchestrate a sequence of events within the host cell, inducing it to enter the s-phase of the cell cycle and creating support for viral replication. The late phase represents the stage of virus structural protein synthesis, occurring after DNA replication. A large primary RNA transcript is generated from viral DNA, which is subsequently processed into a minimum of 18 individual mRNA molecules. These mRNA molecules are transported to the cytoplasm. Capsid proteins are produced in the cytoplasm and transported back to the nucleus to form virus particles. In the beginning, empty pro-capsids are assembled, followed by the encapsulation of viral DNA and core proteins through an opening located at one of the vertices of the capsid. Subsequently, mature viruses are released from the host cell through the budding process.[6,13]

Human adenovirus type 55 (HAdV-55), belonging to the B subgroups, has emerged as a significant pathogen resulting from recombination events between HAdV types 11 and 14.[15-17] This virus is recognized as a causative agent of acute respiratory disease (ARD), pneumonia, and bronchitis. Flu-like symptoms, nasal congestion, cough, malaise, fever, and headache, are typically seen in patients with ARD, and they usually have a duration of 3 to 5 days. HAdV-55 is considered a public health concern due to its high contagiousness and causing severe respiratory illness.[17-19]

Since its initial outbreak in China in 2006, HAdV-55 has been responsible for infection cases across civilian and military populations worldwide, primarily transmitted via human to human-to-human transmission. Outbreaks of HAdV-55 associated with ARD have been reported in various regions, including Israel, Singapore, China, and the United States.[18,20-

[22] In the context of the South Korean military, it is worth emphasizing that repeated and sizable outbreaks of ARD have consistently occurred among newly recruited personnel during their initial training, with HAdV-55 frequently identified as the responsible pathogen.[20,23,24] The first HAdV-55 outbreak occurred in 2012, followed by a substantial epidemic during the winter of 2014, it has emerged as a major pathogen associated with ARD.[23] This phenomenon could potentially be attributed to the close living conditions in densely populated barracks, limited opportunities for personal hygiene, and the physical and environmental stresses experienced in training camps.[25] Repeated outbreaks raise concerns about the associated disease burdens, such as medical care costs and loss of training time. Therefore, it is crucial to develop an efficient vaccine that can prevent the transmission of HAdV-55 and thus prevent outbreaks in training camps.[19,26]

In the United States, the only licensed vaccine available for HAdV is non-attenuated live oral HAdV-4, and HAdV-7 vaccines, which have been exclusively administered within US military camps for 40 years, but have not been received approval for civilian use.[27,28] Notably, the vaccine supply faced a temporary suspension in 1999, and adenovirus-associated ARD increased to the pre-vaccine level between 1999 and 2010.[3,29,30] Subsequently, upon the resumption of vaccination efforts in 2011, there was a remarkable reduction in the adenovirus-associated disease burden, approximately 100 times among military recruits.[31,32]

Although the live adenovirus vaccine has demonstrated its effectiveness, oral vaccination faces challenges related to immune tolerance and degradation caused by mucosal enzymes in the harsh gastrointestinal tract environment. Parenteral routes, which include intramuscular (IM), subcutaneous (SC), and intradermal (ID) injections using hypodermic needles, are the main modes of vaccine administration.[33] Nevertheless, conventional vaccinations should be administered by trained health professionals and require a significant amount of manpower, time, and financial resources. The use of a syringe needle in these injections can often cause pain and can lead to poor vaccination hesitancy, especially among individuals with needle phobia who avoid getting vaccinated.

Furthermore, liquid vaccine formulations typically require a large cold chain infrastructure for proper storage and distribution.[34-36] The development of a simpler and more efficient vaccine delivery method is crucial to overcome the limitations associated with conventional vaccination. Ideally, novel vaccination methods should integrate both convenience of administration and stability of the vaccine formulation during emergencies such as pandemics.

From this perspective, a microneedle array patch (MAP) is becoming a highly promising drug delivery system for the administration of active pharmaceutical ingredients (APIs) or vaccines into the skin.[37] In general, MAP typically comprises micro-sized needles designed in the shape of cones or pyramids, with an aspect ratio ranging from 1:1 to 1:3. These patches are manufactured using various micro-fabrication techniques using biocompatible materials.

The ability of the skin to detect the invasion of foreign antigens and the subsequent compromise of its physical barrier is remarkable. This attribute renders the skin an optimal site for vaccination, primarily attributed to its rich population of antigen-presenting cells.[33,38] These cells include Langerhans cells, keratinocytes in the epidermis, as well as dermal dendritic cells, plasmacytoid dendritic cells, macrophages, and monocytes within the dermis.[39] Importantly, upon encountering foreign antigens, these antigen-presenting cells become activated to eliminate invading foreign antigens, repair the skin barrier, and initiate adaptive immunity by presenting external antigens to T cells and B cells. This process primes the immune system to respond effectively in the event of potential re-exposure to external antigens.[40]

In addition to the advantage of cutaneous immunization, the utilization of MAP presents numerous advantages over the parenteral route for vaccine delivery.[33,41] These advantages include patient compatibility and the elimination of any sharp waste. Moreover, the use of solidified vaccines provides enhanced vaccine stability, prolongs shelf-life, and augments tolerance to temperature fluctuations.[42] This helps alleviate the economic burden associated with storage, transportation, and distribution.[43] The major advantage of

MAP can essentially be self-administered, offering an efficient way for large-scale immunization during pandemics and other healthcare crises. MAP has been subjected to extend research as a platform for delivering a wide range of vaccines in preclinical studies, including those targeting influenza, measles, rubella, polio, hepatitis B, and human papillomavirus (HPV) vaccines, among others.[35,44-49] Several investigations have demonstrated that MAP-based vaccination provides stronger or equivalent protective immunity in murine models, even when employing the same vaccine dosage as traditional parenteral injection.[46,47,50]

In the present study, the purpose is to develop an inactivated human adenovirus type 55 vaccine, which is fabricated with a coated-microneedle array patch (c-MAP), to examine the impact of additive on vaccine stability during the manufacturing and storage, and to evaluate immunogenicity compared to the conventional intramuscular (IM) inoculation in a mouse model.

II. MATERIALS AND METHODS

1. Cell culture

Vero cells, a monkey kidney epithelial cell, were generously provided by the International Vaccine Institute (IVI) and cultivated in Eagle's minimum essential media (MEM, Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. A549 cells, a human lung carcinoma cell, were also provided by the International Vaccine Institute (IVI) and maintained in Eagle's minimum essential media (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cell cultures were preserved within a humidified incubator set at 37℃, 5% CO_2 atmosphere to ensure optimal growth conditions.

2. Virus propagation

Human adenovirus type 55 (HAdV-55) was provided by the International Vaccine Institute (IVI). To facilitate its growth and replication, the viruses were adapted to replicate within Vero cells, which were cultured in 175T flasks under serum-free conditions at 37℃ with 5% CO_2 atmosphere. The virus solutions were harvested 24-72 hours after inoculation. Subsequently, the virus solutions underwent a process involving two rounds of freezing and thawing, followed by centrifugation at 1500 rpm for 10 min. To further eliminate any cellular debris, the virus solution was filtered through a 0.45-μm PVDF membrane filter (Merck Millipore, Tullagreen. Carrigtwohill, Ireland). The final virus bulk was stored in a deep freezer at -80℃ and retained stock material for the development of inactivated vaccine.

3. Virus titration

The determination of HAdV-55 virus infectivity was conducted through a plaque assay using A549 cells. Virus-containing samples were serially diluted in serum-free MEM at 10-fold dilutions and subsequently applied to A549 cells in a 6-well plate. After incubation, the viral solutions were then removed, and 1% low melting agarose (SeaPlaque™, Lonza, Basel, Switzerland) in 2X MEM containing 2% fetal bovine serum (FBS) was added to each well. Following a 6-day incubation period, the cells were examined for the presence of cytopathic effects (CPE) under a microscope. Subsequently, the cells were fixed with 4% paraformaldehyde (Biosesang, Seoul, Korea) and stained using a crystal violet mixture. The plaques were counted, and viral titer was calculated in PFU/ml.

4. Preparation of inactivated HAdV-55 vaccine

Virus stock solutions were purified using the Hiscreen Capto Core 700 (GE Healthcare, Chicago, USA) and an automated purification system, ÄKTA Pure (GE Healthcare). To achieve higher concentration, the purified viruses underwent centrifugation using Amicon® Ultra-15 Centrifugal Filter Unit (Millipore Sigma, MA, USA) with a 30 kDa molecular weight cut-off. Subsequently, the concentrated viruses were quantified using a plaque assay. The concentrated viruses were treated with 0.01 M formaldehyde solution and incubated at 37℃ with a 5% CO_2 humidified incubator for 24 hours. Afterward, the inactivated viruses proceeded through the Amicon® Ultra-15 Centrifugal Filter Unit, and the buffer exchange was performed 5 times by adding 5 times more PBS. The inactivated viruses were verified through a plaque assay to confirm the absence of plaque formation. 10μl aliquot of inactivated HAdV-55 was applied in A549 monolayers in 6-well plates, while 10μl pre-inactivated HAdV-55 was also treated in 6-well plates as positive control. Negative control cells were also prepared. Finally, the inactivated viruses were stored at -80℃ in a deep freezer.

5. Fabrication process of inactivated HAdV-55 coated MAP vaccine

The uncoated microneedle array patches (u-MAPs) made from Cyclin olefin copolymer (COC) were provided by Quad Medicine (Quad Medicine, Seongnam, South Korea). These u-MAPs are equipped with 97 obelisk-shaped microneedles with a base width of 370 μm and a length of 800 μm, arranged in a circular disk. In this study, Hyaluronic acid (HA, Sigma, St. Louis, MO) and Polyethylene glycol (PEG, Sigma, St. Louis, MO) were used as excipients. Four distinct coating solutions were prepared in phosphate-buffered saline, as summarized in Table 1. The u-MAPs were initially treated with UV/O3 irradiation for 5 min to make the MAP surface more hydrophilic. The coating solution was filled in a coating well with a depth of 300 μm, and a u-MAP attached to the linear actuator was dipped multiple times into the coating solution at a rate of 1 mm/s, pulled up at 1 mm/s, and stayed at 1.5 mm/s to achieve the target vaccine dose specified for the study. Following this, the u-MAPs were air-dried under ambient conditions for 1 h within a desiccator (24℃, 40% relative humidity). The coating microneedle array patches were coated with platinum, and the sample geometries were examined using scanning electron microscopy (SEM; Hitachi S-4700, Tokyo, Japan).

Table 1. Composition of coating solutions

Formulation	Inactivated HAdV-55 Vaccine	HA	PEG	PBS
F1	5×10^7 PFU	-	-	500 μl
F2	5×10^7 PFU	1% (w/v)		500 μl
F3	5×10^7 PFU	-	0.032% (w/v)	500 μl
F4	5×10^7 PFU	1% (w/v)	0.032% (w/v)	500 μl

6. Analysis of antigenicity of inactivated HAdV-55 coated MAP vaccine

To determine the amount of inactivated virus vaccine coated on a microneedle array patch, the inactivated HAdV-55, previously coated on the u-MAPs using the established method, was dissolved in 0.5 ml of phosphate-buffered saline (PBS) and allowed to incubate for 1 h at 4℃. Subsequently, the antigenicity of the thawed HAdV-55 solution was determined using a commercially available sandwich ELISA-based method. (Creative Diagnostics, Inc., Cat. No DEIAFY103, NY, USA). For the ELISA assay, HAdV-55 antigens, which were dissolved from c-MAP, were incubated in 96 wells pre-coated with specific anti-adenovirus antibodies. After washing the plates with a wash buffer, manufacturer-provided antibodies and horse radish peroxidase (HRP) were added to each well. Following another round of washing, a chromagen solution was treated to each well, and the reaction was stopped with the addition of a stop solution. The substrate concentration in the wells was determined using a spectrophotometric machine at 450 nm. The concentration of HAdV-55 present in the samples was then calculated using a standard linear curve. To measure the delivery rate, c-MAP after insertion into the skin of mice were collected and residual HAdV-55 antigenicity in the used patch was measured in the same manner.

7. Stability test of inactivated HAdV-55 coated MAP vaccine

Stability tests of inactivated HAdV-55 coated MAP vaccine were evaluated according to storage temperature and storage period. To examine the storage stability, MAPs were coated using each formulation, stored either at 4℃ or -20℃ for 14 and 28 days and assessed for antigenicity using a sandwich ELISA-based method.

8. Animal studies

In this study, inactivated HAdV-55 was administered to female inbred BALB/c mice aged 6 to 8 weeks (Orient Bio, Seongnam, Korea) to assess its immunogenicity. BALB/c mice were bred under specific pathogen-free conditions in the Yonsei University Medical School animal facility and provided with sterilized food and water ad libitum. All studies were conducted with the approval of the Institutional Animal Care and Use Committees (IACUC) at the Yonsei University Medical School (2022-0184). The animal study involved two experimental vaccine groups, each comprising 5 mice: intramuscular (IM) group and coated-microneedle array patch (MAP) group. Prior to vaccination and serum sample collection, all groups of mice were intraperitoneally administered with 30 mg/kg Zoletil mixed with 10 mg/kg Rampun for anesthesia. In the MAP group, the hair on the dorsal skin was removed using an electric shaver and depilatory cream. After applying the depilatory cream for 5 min, the dorsal skin was cleaned with wet tissues and allowed to dry at room temperature. The MAPs were then applied to the hair-free dorsal skin area using an adhesive patch and fixed to the skin using a clamp for 30 min to facilitate the dissolution of the inactivated HAdV-55 c-MAP. The structural characteristics of inactivated HAdV-55 c-MAP before and after insertion were examined using a scanning electron microscope (SEM). In the IM group, the vaccine was administered through injection into the quadriceps muscles of mice, with the antigen concentration being the same as that used for the MAP group. Serum samples were collected from the retro-orbital plexus of the mice at 2 and 4 weeks following immunization for subsequent analysis.

9. Measuring antibody response by enzyme-linked immunosorbent assay

To measure the HAdV-55 specific antibody response, serum samples were collected at 2 and 4 weeks post-immunization and subjected to enzyme-linked immunosorbent assay (ELISA). In this assay, inactivated HAdV-55 used for immunization was incubated on 96-well plates through an overnight incubation in 50 mM sodium bicarbonate buffer (pH 9.6)

at 4°C. After five washes with PBST (0.05% Tween 20 in 1x PBS, pH 7.4), the plates were treated with a blocking buffer (2% Skim milk in PBS) and incubated at 37°C for 2 h. Subsequently, the 96-well plates were washed 5 times with wash buffer. mouse sera, two-fold diluted in blocking buffer, were added to the plates and incubated at 37°C for 2 h. Following five more washes with wash buffer, and HRP-conjugated anti-mouse IgG, G1, or G2a antibodies, prepared at 1:3000 dilution in blocking buffer, were added and incubated at 37°C for 1 h. After a final wash with PBST, TMB (3,3',5,5'-Tetramethylbenzidine) buffer (100 μl/well) was treated, and the reaction was stopped using 2N HCL (100 μl/well 100). The optical density was measured using an ELISA reader, and all titers were expressed as the reciprocals of serum dilutions.

10. Neutralizing antibody response after immunization

The plaque reduction neutralization test (PRNT) was conducted to examine the presence of neutralizing antibodies against the inactivated HAdV-55 vaccine. Prior to use, serum samples were heat-inactivated at 56°C for 30 min. In this test, two-fold serial dilutions of the inactivated serum samples and HAdV-55 virus samples, each containing 100 plaque-forming units (PFU), were mixed in equal proportions and incubated at 37°C for 1 h. A549 cells cultured in 12-well plates were then treated with a virus-serum mixture and incubated for an additional 1 h in a rocking incubator. After removal of the virus-serum mixture, each well was overlaid with 1.5 ml of 1% low melting agarose in 2X MEM supplemented with 2% fetal bovine serum (FBS). The agarose gel in the 12-well plate was allowed to solidify at room temperature for 1 h and then further incubated at 37°C for 6 days. Following incubation, the plaques formed were fixed and stained using a crystal violet mixture, and subsequently, the plaques were counted. To evaluate neutralizing antibody (nAb) titers, the reciprocal of the serum dilution that inhibited 50% or more of the plaques relative to the number of plaques in the absence of test serum was calculated.

11. Statistical analysis

To determine statistical significance, a two-tailed Student's t-test was performed for comparing two different conditions, and two-way ANOVA was used for comparing multiple groups. Values of $p < 0.05$ were considered statistically significant.

III. RESULTS

1. HAdV-55 virus production

To optimize the production of a viral stock with highly efficient manufacturing capabilities, the growth kinetics of HAdV-55 was evaluated based on the Multiplicity of Infection (MOI) and hours post-infection (hpi). HAdV-55 was cultured and propagated within Vero cells in a 175T flask, and they were infected with three distinct multiplicity of infection (MOI): 0.1, 1, and 10. Subsequently, the infected cells were maintained in culture for varying durations of 24, 48, and 72 hours.

The growth kinetic analysis of HAdV-55 in Vero cells revealed that this viral stock exhibited efficient replication capabilities, reaching a peak titer exceeding 8.0 log10 PFU/ml at 72 hours post-infection (hpi) across MOI of 10 (Fig.1A, B). Consequently, the optimized condition for efficient virus production was achieved by treating with 10 MOI and culturing for 72 hours.

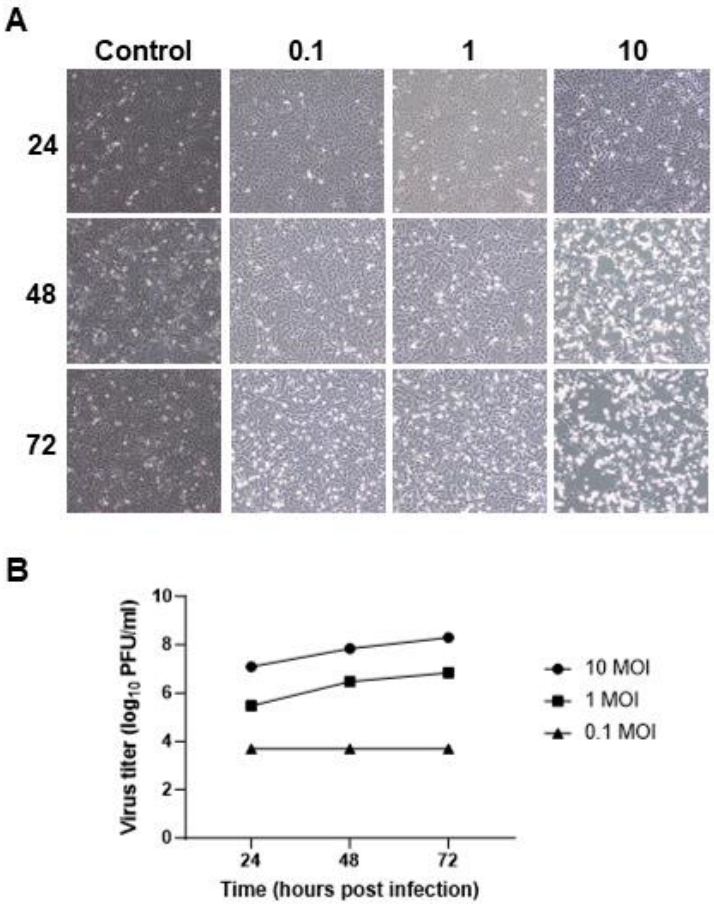

Figure 1. The comparison of HAdV-55 growth rates is based on a multiplicity of Infection (MOI) and hours post-infection (hpi). A comparison of the growth rates of HAdV-55 was conducted following infection of Vero cells. Vero cells were infected with a virus stock solution that had been diluted 10-fold, and subsequent incubation was carried out for 24, 48, and 72 hours. (A) The evaluation of the viral growth rate was determined by monitoring the development of cytopathic effects in the infected cells under a microscope. (B) Vero cells treated with a multiplicity of infection (MOI) of 10 exhibited significantly higher growth rates when compared to cells treated with MOIs of 0.1 and 1.

2. Verification of the inactivated HAdV-55

To obtain a viral stock suitable for high productivity, HAdV-55 was purified and concentrated. The final formulation was subjected to a 10-fold dilution, and each dilution was applied to A549 cells cultured in 12-well plates. After 6 days, the number of plaque formations was counted to determine the concentration of the infectious virus. The viral titer in the final stock was found to be 1.04×10^9 PFU/ml (Fig. 2A).

To inactivate virus production, HAdV-55 was purified and thoroughly mixed with 0.01 M formaldehyde solution. The virus-formaldehyde mixture was incubated for 24 h at 37℃ with 5% CO_2 humidified incubator.[51] Following the formalin-inactivation process, the absence of viral infectivity was thoroughly validated through a plaque assay. This process aimed to confirm whether the virus has been completely inactivated. A comparison of plaque-forming activity was carried out between the pre-inactivated virus and the formalin-inactivated virus. In direct comparison to the pre-inactivated virus, the formalin-inactivated virus did not exhibit any plaque formation (Fig. 2B). These findings provide evidence that the formalin-inactivated virus exhibits no infectious activity, thereby establishing its lack of toxicity as an inactivated vaccine.

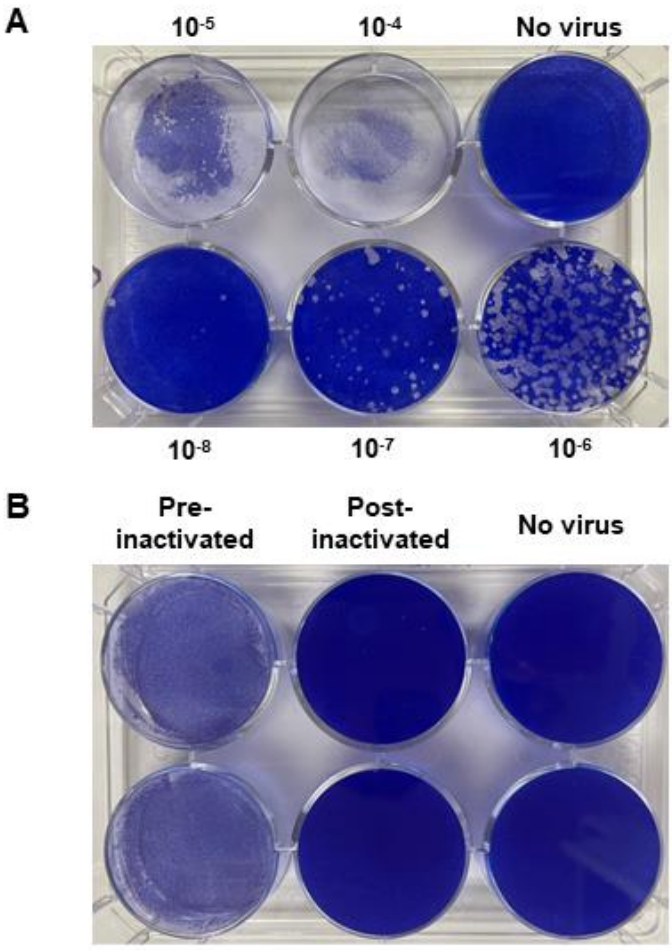

Figure 2. Plaque forming activity of HAdV-55. (A) The final pre-inactivated HAdV-55 was serially diluted on A549 cells. After being cultured for 6 days, A549 cells were fixed and stained, and the number of plaque formations was counted. Using the proposed formula (PFU = $N \times I \times 1/10^d$), the viral titer of the final solution was estimated to be approximately 1×10^9 PFU/ml. (B) Following the formalin-inactivation process, the inactivated virus was subjected to a plaque assay to confirm the absence of plaque formation in comparison to the wells treated with the pre-inactivated virus. No plaque formation was detected in the post-inactivated virus when compared to the pre-inactivated virus.

3. Optimization of coating formulation

The coating formulation consists of a vaccine and excipients designed to control the loading amount and stabilize the antigen during manufacturing and storage. The design of these coating formulations takes into account three key factors: transparency, dispersion stability, and immunological stability. It is important to note that the HAdV vaccine is an inactivated whole virus, and there have been no previous studies on microneedle coating.

In the preparation of the microneedle coating formulation, excipients should be chosen with consideration for the dispersion stability of the antigen and its immunological stability. Initially, sucrose and hyaluronic acid (HA) were used, which are commonly used excipients in coating formulations. Sucrose, a widely used stabilizer, is a disaccharide with a fast dissolution rate. HA was selected as a viscosity enhancer to facilitate uniform coating. HA has a well-established history of use as a drug delivery carrier and has received approval from the Food and Drug Administration (FDA).

The combination of sucrose and HA resulted in aggregated inactivated HAdV-55 in the solution, making it appear opaque (Table 2, F5). However, inactivated HAdV-55 showed good dispersibility when HA was added to the formulation alone (Table 2, F2). Sucrose as an excipient was found to be unsuitable for our experimental purposes because aggregation negatively affected the dispersion stability and antigen stability during the manufacturing process.

Table 2. The dispersion stability of coating formulations regarding excipients

Formulation	Vaccine	Excipient	Transparency	Dispersion	Viscosity for coating
F1	Inactivated HAdV-55	-	Clear	Monolithic	Not suitable
F2	Inactivated HAdV-55	HA	Clear	Monolithic	Suitable
F3	Inactivated HAdV-55	PEG	Clear	Monolithic	Not suitable
F4	Inactivated HAdV-55	PEG HA	Clear	Monolithic	Suitable
F5	Inactivated HAdV-55	Sucrose HA	Opaque	Aggregate	Not Suitable

Subsequently, Antigen stability was evaluated by reconstituting each formulation after the solidification process. The assessment of the antigenicity between the formulation with and without HA revealed a significant stability decline of approximately 80% in the group without HA, whereas the HA group sd a mild decrease of around 20%. (Fig.3A F1, F2). To improve vaccine stability during the manufacturing process, we needed to consider alternative excipients, excluding disaccharides like sucrose. We examined the effect of PEG, which serves as a water-soluble matrix, making it easier to dissolve and separate the coated solution when inserted into the skin. The addition of PEG exhibited better stability than the group without it, reaching a level similar to the antigenicity in the liquid state (Fig.3A F3).

The antigenicity of c-MAP can be impaired during storage. We conducted additional tests to assess the vaccine stability during storage. As shown in Figure 3, we categorized the vaccines into four groups based on the coating formulations and stored each group of vaccines at 4°C and 25°C for 14 and 28 days to assess changes in vaccine stability. The antigenicity of F4 (inactivated HAdV-55, HA, and PEG) did not change for 14 days at 4°C and 25°C. When stored at 4°C and 25°C for 28 days, F4 showed a 10% reduction compared to the liquid formulation. However, it exhibited less antigenicity reduction compared to the

formulation containing PEG alone (Fig. 3A F3, F4). Therefore, the combination of PEG and HA as excipients was selected as the final coating formulation for uniform coating and stability during manufacturing and storage.

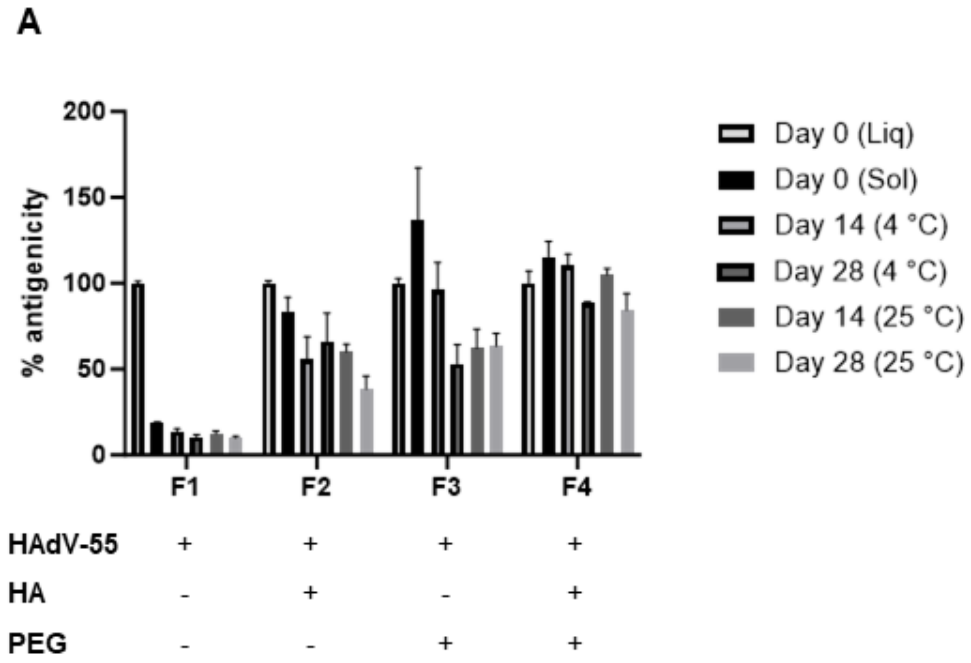

Figure 3. Influence of excipients on vaccine stability during manufacturing and storage. The antigenicity of (F1) inactivated HAdV-55 vaccine only, (F2) inactivated HAdV-55 + HA, (F3) inactivated HAdV-55 + PEG, and (F4) inactivated HAdV-55 + PEG + HA. Solidified samples from each group were reconstituted in 0.5 ml of PBS, and antigenicity was measured using a sandwich ELISA-based kit. The combination of HA and PEG exhibited better stability during manufacturing and storage in comparison to the group without PEG or HA, while maintaining a similar antigenicity level to the liquid formulation. (Liq: liquid formulation, Sol: solidification)

4. Characteristics of inactivated HAdV-55 coated MAP vaccine

To carry out this study, the microneedle array comprised 97 needles, each 800 μm in length, spaced with a base diameter of 370 μm. These microneedles were mounted on an adhesive backing (Fig 4A). The coating formulation was applied within 300 μm from the tip of each microneedle to facilitate antigen delivery to antigen, as shown in the enlarged image (Fig 4B). Trypan blue-coated microneedles were inserted into porcine skin and subsequently removed after 30 min. Successful vaccine delivery through c-MAP was confirmed by examining the insertion sites after the removal of c-MAP. Puncture performance was evaluated by the number of stained holes relative to the number of microneedles (No. of stained holes / No. of microneedles). The puncture performance was 100%, with all microneedles successfully generating holes in the porcine skin (Fig. 4C). These microneedles have sufficient mechanical strength for successful insertion.

The fabrication process of c-MAP involved repetitively coating a liquid formulation containing inactivated HAdV-55 using a dip coating method and subsequently drying it to solidify the coating formulation. The c-MAP was produced by coating the final formulation containing PEG and HA with the inactivated HAdV-55 vaccine. Each microneedle SEM image showed that the coating formulation was evenly distributed on the top half of the microneedles for high delivery efficiency (Fig. 4D, E). This uniform distribution is crucial for ensuring effective antigen delivery during vaccination. The coated formulation was successfully delivered into the skin following mouse immunization, and no residue was found on the microneedles (Fig. 4F).

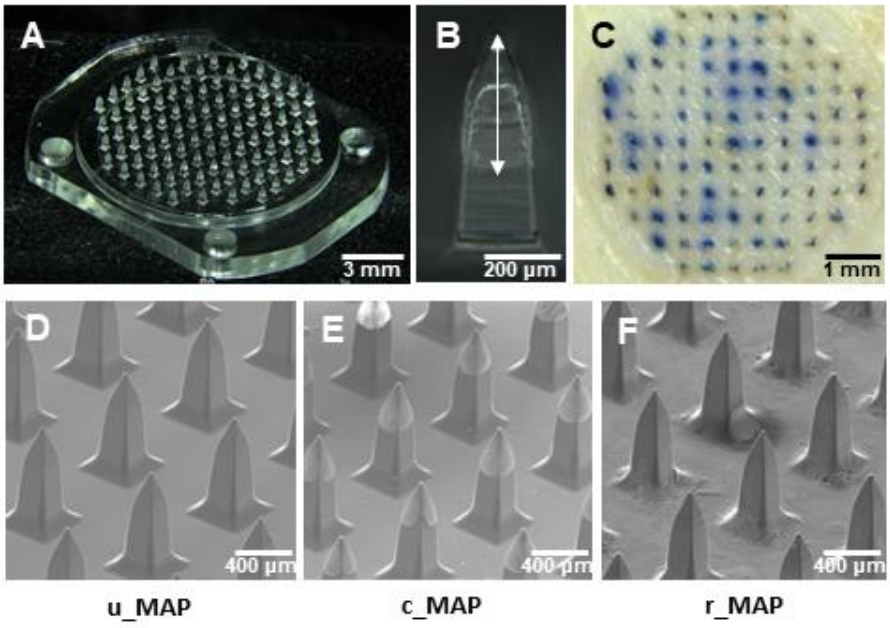

Figure 4. Image of coated-microneedle array patches. (A) Optical microscopic image of inactivated HAdV-55 coated microneedle array patches with 97 needles (800 μm height, 370 μm base) (B) Magnified image of inactivated HAdV-55 coated microneedle (300 μm coating height, white double arrow) (C) Optical microscopic image showing blue dots on treated porcine skin after the insertion and removal of trypan blue coated-microneedle array patches, illustrating the mechanical performance of the microneedle array patches. (D) SEM image of uncoated microneedles (E) SEM image of microneedles coated with inactivated HAdV-55 coating formulation. (F) SEM image of microneedles after insertion into the skin of mice, revealing that the coating formulations were dissolved.

5. Delivery efficiency of inactivated HAdV-55 coated MAP vaccine

For this study, c-MAPs were coated with 1.2×10^6 PFU of inactivated HAdV-55 virus (Table 3). To evaluate the delivery efficacy, the inactivated HAdV-55 c-MAPs were applied with thumb pressure for 30 seconds to the back of mice and fixed to the skin using a clamp for 30 min to facilitate the dissolution. After application, the inserted c-MAPs were dissolved in PBS for 1 hour and the antigenicity of the dissolved solution was assessed using a sandwich ELISA-based method. Delivery efficiency was calculated as follows: [(initial dose – residual dose)/initial dose × 100%]. During the first immunization, an average delivery rate of 73.4% was observed, ranging from 71-86% (Fig 5A). However, the average delivery rate was 49.7%, ranging from 45-56% for the second immunization. Although the delivery rate of c-MAP was slightly lower compared to the typical 80% delivery rate of coated microneedles, inactivated HAdV-55 c-MAP appeared WHO's recommended delivery threshold of 50% for microneedle vaccine administration.[44,52]

Table 3. Antigenicity loaded on MAP and delivery rate before and after vaccination

1st Sample No.	A	B	C	D	E
Initial HAdV-55 c-MAP (PFU)	1.32×10^6	1.38×10^6	1.43×10^6	1.32×10^6	1.48×10^6
Residual HAdV-55 r-MAP (PFU)	1.83×10^5	5.68×10^5	4.03×10^5	2.84×10^5	4.26×10^5
Delivery rate (%)	86	59	72	79	71
2nd Sample No.	A	B	C	D	E
Initial HAdV-55 c-MAP (PFU)	1.18×10^6	1.25×10^6	1.18×10^6	1.32×10^6	1.10×10^6
Residual HAdV-55 r-MAP (PFU)	6.37×10^5	6.79×10^5	5.54×10^5	6.91×10^5	4.87×10^5
Delivery rate (%)	46	45	53	48	56

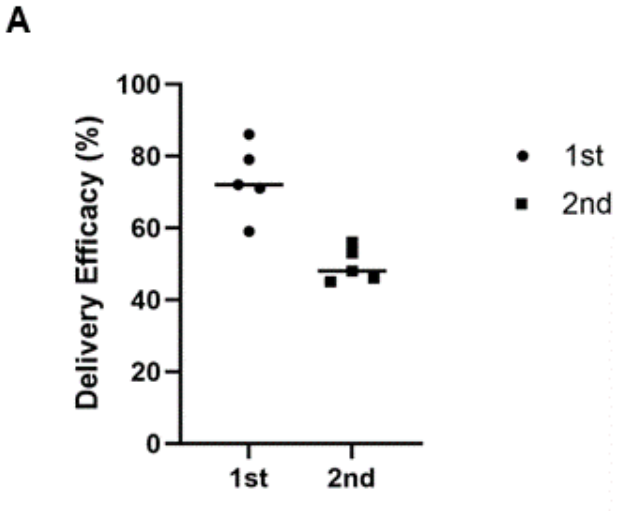

Figure 5. Delivery efficiency of inactivated HAdV-55 coated MAP vaccine. (A) MAPs were designed to deliver approximately 1×10^6 PFU of inactivated HAdV-55. Residual antigen on the c-MAP was measured after two immunizations. Delivery efficiency was calculated using the following formula: [(initial dose – residual dose]/initial dose × 100%]. The delivery rates were 73.4% after primary immunization and 49.7% after boosting immunization. (n=5)

6. Antibody response after delivery of inactivated HAdV-55 coated MAP vaccine

To assess vaccine efficacy, two groups of mice (n=5 BALB/c mice per group) were given two doses of immunizations, at a two-week interval. One group received the inactivated HAdV-55 c-MAP applied to dorsal skin, while the other group received an intramuscular injection into the quadriceps. Serum samples were collected at 2 and 4 weeks after the first immunization, and the level of total serum antibodies (IgG) and antibody subtypes (IgG1, IgG2a) were measured (Fig 6A). Both IM and c-MAP groups exhibited an increase in virus-specific antibody levels over 4 weeks following two rounds of immunization (Fig 6B). The c-MAP group induced lower virus-specific antibody responses compared to the IM group for the prime vaccination, however, following boost vaccination, the MAP group showed a comparable antigen-specific IgG level to the IM group. (Fig 6C). This suggests that two doses of vaccination with c-MAP are sufficient to provide protection.

To further understand the type of immune response to HAdV-55, serum IgG isotypes (IgG1, IgG2a) were analyzed after booster vaccination. IgG1 and IgG2a antibody titers were used as indicators to assess the nature of the T-helper (Th) response. IgG1 serves as a marker for Th2-type immune response while IgG2a is a marker for Th1-type immune response.[53] Each group was capable of generating IgG1 and IgG2a subclass antibodies (Fig 6D) and c-MAP administration induced a higher level of IgG1 antibodies compared to IgG2a antibodies. In contrast, IM route showed higher levels of antigen-specific IgG2a antibodies compared to IgG1 isotype antibodies (Fig 6E). These results provide evidence that inactivated HAdV-55 vaccine delivered to the skin through c-MAP can efficiently induce T helper type 2 (Th2) immune responses to viral antigens.

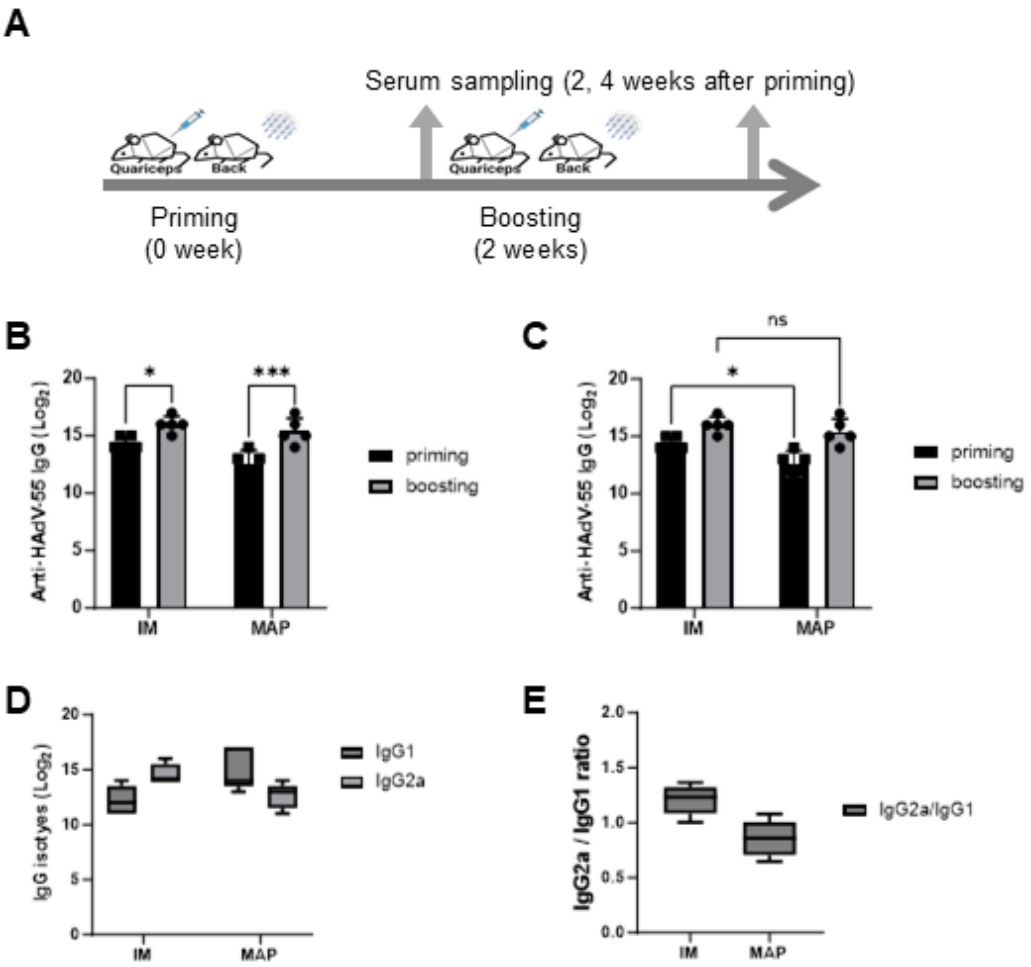

Figure 6. Evaluation of the immunogenicity of inactivated HAdV-55 vaccine with different injection routes in mice. (A) Immunization schedule: BALB/c mice were immunized with inactivated HAdV-55 c-MAP or intramuscular (IM) injection twice in two-week intervals. Blood samples were collected through orbital plexus blood sampling on days 1 and 28 after the first immunization. (B), (C) Serum samples from mice immunized with inactivated HAdV-55 vaccine via both IM and MAP methods. Vaccine efficacy was assessed by measuring HAdV-55 specific IgG titer using ELISA. Both vaccination methods successfully induced immune responses leading to the production of IgG antibodies. The

c-MAP group initially induced lower virus-specific antibody responses compared to the IM group after the prime vaccination. Following the boost vaccination, MAP groups exhibited a comparable immune response to the IM group. (D) Anti-HAdV-55 IgG1 and IgG2a isotypes were measured through ELISA. (E) Ratios of IgG2a/IgG1 isotype levels were analyzed by ELISA. Statistical significance was performed by two-way ANOVA in GraphPad Prism (*** $p<0.001$, ** $P<0.01$, * $p<0.05$).

7. Evaluation of neutralizing activity after delivery of inactivated HAdV-55 coated MAP vaccine

The PRNT50 was conducted to evaluate viral neutralizing activity, offering a direct and sensitive measurement for functional antibodies. The humoral immune response can block infection through neutralizing antibodies, which bind to the virus in a way that prevents host cell infection. Mouse sera were collected two weeks after boosting immunization with c-MAP or IM injection, and neutralizing antibodies against HAdV-55 were analyzed by $PRNT_{50}$. While IM groups showed slightly higher titers of plaque reduction neutralizing antibodies compared to the MAP group, the MAP vaccination groups also exhibited a sufficient level of neutralizing antibody titers. (Fig. 7A).

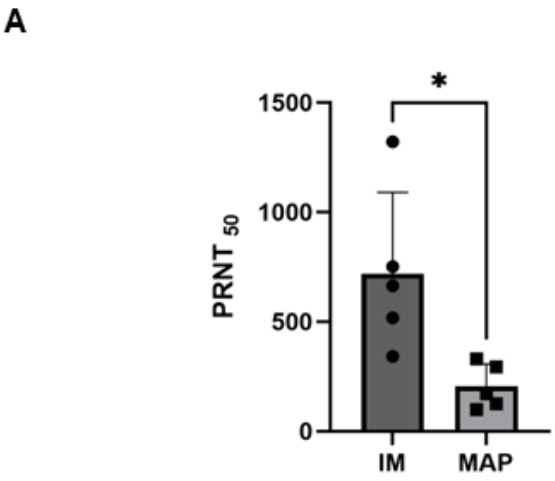

Figure 7. Anti-HAdV-55 neutralizing antibody titer in sera after IM and MAP boosting immunization. Plaque reduction neutralization test 50% ($PRNT_{50}$) titers of inactivated HAdV-55 microneedle array patches (MAP) and IM groups mouse sera at 14 days after boosting immunization. Statistical significance was determined by a t-test (* $p < 0.05$).

IV. DISCUSSION

Although HAdV-55 infection typically results in mild and self-limiting symptoms in healthy adults, HAdV-55 has been recognized as a significant pathogen contributing to acute respiratory disease (ARD). Outbreaks of respiratory diseases related to HAdV-55 have been reported in several regions and HAdV-55 infections have continued in Korea following a significant outbreak within the South Korean military in 2014. As a result, it is now recognized as a public health concern within a military population. These outbreaks lead to increased administrative burdens and healthcare costs, affecting the operation of units, training, and the combat readiness of military personnel. Therefore, the development of an effective vaccine to prevent HAdV-55 outbreaks during military training is crucial.[26]

Microneedle array patches (MAP) offer an appealing approach for vaccine delivery and play a crucial role in mass vaccinations. Antigens delivered into the skin through MAP activate adaptive immune cells, such as antigen-presenting cells, which in turn activate T and B cells. This activation leads to a stronger immune response upon re-exposure to external antigens. Additionally, microneedles offer advantages over conventional needle-and-syringe methods, allowing for self-administration, easier storage, and distribution. Considering situations requiring mass vaccination, vaccination via MAP could effectively prevent infectious diseases among military personnel, potentially resulting in cost savings in vaccination efforts.

In this study, the inactivated HAdV-55 coated microneedle array patch (c-MAP) was developed and vaccine stability was evaluated concerning the manufacturing process, storage temperature, and duration. The solidification of the liquid coating formulation during the manufacturing process decreased vaccine stability. The drying process can affect the antigen stability of the vaccine due to stresses such as changes in pH, ionic strength, and osmolytes.[54] To enhance viscosity and vaccine stability, four coating formulations were prepared using PEG and HA as excipients. The combination of PEG

and HA showed higher vaccine stability during the solidification process compared to formulations containing PEG or HA alone. In addition, a combination of PEG and HA maintained vaccine stability at levels similar to the liquid formulation when stored at 4℃ for 2 and 4 weeks, while exhibiting approximately a 10% reduction in antigenicity when stored at 25℃ during the same period, showing the least reduction compared to PEG or HA alone.

The average delivery rate was 73.4% and 49.7% after two rounds of skin immunization in mice. Although the WHO-recommended delivery threshold of 50% has been achieved, considering various references indicating microneedle delivery rates of around 80%, it seems necessary to enhance the delivery rate.[52] The delivery rate can be influenced by factors such as characteristics of MAP itself — like shape, size, and type of needles — as well as the force applied during administration and coating formulation.[52,55] It is essential to verify the diffusion profile to confirm the correlation with excipients. Exploring different combinations of excipients facilitates enhancements in both vaccine delivery and efficacy.

The immunogenicity of MAP administration was compared with that of intramuscular (IM) injection. The MAP group showed a similar virus-specific IgG level as the IM injection group. Neutralizing antibody titers in the MAP group were slightly lower compared to the IM group, however, MAP exhibited sufficient levels of neutralizing antibody titers. Our results suggest that antibody responses induced by microneedles would be comparable to those induced by IM injection when the inactivated HdV-55 vaccine is administered to mice.

The present study is subjected to some limitations. First, it is necessary to verify the differences in immune responses generated by varying antigen concentrations. Several studies have compared immune response between IM and MAP immunization by setting antigen concentration as low or high doses and found stronger or similar immunogenicity, which suggested a dose-sparing effect.[48,56,57] Second, this study primarily evaluated humoral immune responses to compare two vaccination methods. Considering that

intradermal vaccination directly targets epidermal Langerhans cells and dermal dendritic cells, essential for effective cellular and humoral response, further assessment of cellular immune response is needed.

Unlike IM injections, MAP offers several advantages including self-applicable, minimizing vaccine wastage and contamination, better patient compliance, and reducing the economic burden associated with vaccine storage and distribution. In conclusion, MAP can serve as a potential alternative method that can be quickly used in mass vaccination such as pandemics or emergencies, replacing traditional vaccine administration.

V. CONCLUSION

In this study, the feasibility of inactivated HAdV-55 coated microneedle array patches (MAP) was explored by evaluating the antigen stability and comparing their immunogenicity with the intramuscular (IM) injection in a mouse model. The addition of polyethylene glycol (PEG) and Hyaluronic acid (HA) significantly improved vaccine stability during the manufacturing process and storage. MAP efficacy was assessed by measuring antigen-specific IgG titers and neutralizing antibody titers. MAP produced similar levels of antigen-specific IgG compared to IM injection. Although the neutralizing antibody titers in the MAP group were slightly lower in comparison to the IM group, the MAP approach induced sufficient levels of neutralizing antibody titers. Considering the various advantages of MAP, it could serve as a potential alternative method that is rapidly used in emergencies replacing traditional vaccine administration.

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ABSTRACT(IN KOREAN)

마이크로니들 어레이 패치를 적용한 인간 아데노바이러스 55형 백신 개발 및 마우스에서의 면역원성 평가

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한 겨 레

인간 아데노바이러스 55형 (HAdV-55)은 급성 호흡기 질환 (ARD), 폐렴 및 기관지염의 원인체로 알려진 신생 병원균이다. 건강한 성인에서는 HAdV-55 감염이 제한적인 질환을 유발하나, 한국군의 신병 훈련소에서는 대규모의 ARD 발병이 빈번하게 발생한다. 따라서 군과 지역사회 발병 예방을 위한 효과적인 백신 개발이 필요하다. 본 연구에서는 불활성 HAdV-55 백신으로 코팅된 마이크로니들 어레이 패치 (MAP)를 제작해서, 제작과정 중 백신 안정성에 대한 첨가제의 영향을 평가하였다. 폴리에틸렌 글리콜 (PEG)과 히알루론산 (HA)의 첨가는 제조 및 저장 중 백신 안정성을 향상시켰다. 더불어 MAP 투여와 근육 내 주입 (IM)을 비교하여 면역원성을 확인했다. 각 종류의 백신을 두 번의 접종으로 2주 간격을 두고 BALB/c 쥐에게 투여한 후, 2주와 4주 후에 혈청 샘플을 수집했다. MAP의 효능은 항원 특이적인 IgG 수치와 중화항체 수준을 측정하여 평가하였다. MAP의 항원 특이적인 IgG와 중화항체 역가는 근육주사와 비슷한 수준의 면역반응을 형성하였다. 따라서 마이크로니들 백신 접종의 다양한 장점을 고려할 때, 마이크로니들을 활용한 백신 접종은 팬데믹과 같은 비상 상황과 같이 대규모 백신 접종이 필요한 상황에서 IM 백신 접종을 대체할 수 있는 대안으로 활용될 수 있다는 것을 제시하는 바이다.

핵심되는 말 : 인간 아데노바이러스, 급성 호흡기 질환, 불활화 백신, 마이크로니들 어레이 패치