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Research article

Assessment of the immune interference effects of multivalent vaccine for influenza epidemic strain in 2022–2023 and evaluation of its efficacy

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ABSTRACT

The various strains of influenza virus cause respiratory symptoms in humans every year and annual vaccinations are recommended. Due to its RNA-type genes and segmented state, it belongs to a virus that mutates frequently with antigenic drift and shift, giving rise to various strains. Each year, the World Health Organization identifies the epidemic strains and operates a global surveillance system to suggest the viral composition for the influenza vaccine. Influenza viruses, which have multiple viral strains, are produced in the format of multivalent vaccine. However, the multivalent vaccine has a possibility of causing immune interference by introducing multiple strain-specific antigens in a single injection. Therefore, evaluating immune interference phenomena is essential when assessing multivalent vaccines. In this study, the protective ability and immunogenicity of multivalent and monovalent vaccines were evaluated in mice to assess immune interference in the multivalent vaccine. Monovalent and multivalent vaccines were manufactured using the latest strain of the 2022–2023 seasonal influenza virus selected by the World Health Organization. The protective abilities of both types of vaccines were tested through hemagglutination inhibition test. The immunogenicity of multivalent and monovalent vaccines were tested through enzyme-linked immunosorbent assay to measure the cellular and humoral immunity expression rates. As a result of the protective ability and immunogenicity test, higher level of virus neutralizing ability and greater amount of antibodies in both IgG1 and IgG2 were

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confirmed in the multivalent vaccine. No immune interference was found to affect the protective capacity and immune responses of the multivalent vaccines.

1. Introduction

The influenza virus belongs to the Orthomyxoviridae family and causes acute respiratory infections in humans every year. Influenza viruses infect 1 billion people annually, with 3–5 million individuals experiencing severe illness and 290,000–650,000 people succumbing to the infection [1]. Influenza viruses undergo frequent mutations due to the characteristic antigenic drift of the RNA virus. Additionally, this virus is segmented, and antigenic shift occurs when genetic segments of the virus are mixed during intracellular replication. Antigenic drift entails small mutations in several gene sequences during viral replication, whereas antigenic shift involves significant mutations whereby entire segments are mixed during intracellular replication, particularly when the host becomes co-infected with viruses of different strains [2]. Influenza viruses are categorized into types A, B, C, and D [3]. Among these, types A and B are responsible for causing seasonal flu in humans every year [4]. Influenza A viruses are further classified into various strains based on the antigenic proteins on their surface, hemagglutinin and neuraminidase [5]. Influenza B viruses split into two antigenic lineages known as the Victoria and Yamagata lineages [6]. These various strains of influenza viruses cause different epidemic diseases every year.

Based on the characteristics of the influenza virus described above, the World Health Organization (WHO) collects and analyzes influenza viruses from around the world annually, selecting the predominant strain that is likely to cause an outbreak [7]. And influenza multivalent vaccines are formulated each year based on the recommendations of the WHO.

As the development of the influenza multivalent vaccine continues, instances of immune interference are consistently reported [8]. These interference phenomena like antagonistic effects, synergistic effects, antigen competition, and epitope suppression were from inappropriate immune responses or immune system overload due to the inoculation of various antigens at one time [9,10]. Therefore, it is essential to evaluate whether interference occurs, ensuring the vaccine generates a sufficient immune response. This evaluation is critical in the development and usage of multivalent vaccines.

The mechanisms governing humoral and cellular immune responses were assessed based on IgG subclasses. Helper cells, which hold a pivotal role in the immune system, differentiate into Th1 (Type 1 helper) and Th2 (Type 2 helper) cells, depending on molecular signals and antigenic stimulation presented through the major histocompatibility complex (MHC). Th1 cells primarily regulate cellular immunity and produce IgG2, whereas Th2 cells predominantly mediate humoral immunity and generate IgG1 [11]. To evaluate the dominance of the Th2 immune response versus the Th1 immune response, the IgG1/IgG2 ratio was calculated using IgG-isotyping. Notably, humoral immunity prevails in the case of inactivated vaccines [12].

Comparing immune cell activity through flow cytometry is crucial for understanding immunity and evaluating immune cells within both the lungs and spleen is important when viruses predominantly target respiratory organs [13]. Representative immune cells and their markers (markers used for classifying each immune cell are enclosed in parentheses) found in the lung and spleen include T cells (TCRβ), B cells (B220), regulatory T cells ($CD4^+$ $CD25^+$ Foxp3), CD4 T cells (CD4), CD8 T cells (CD8), naïve T cells (CD62L + $CD44^-$), effector memory T cells (CD62L-$CD44^+$), as well as innate immune cells such as macrophages (F4/80+ CD11b+), neutrophils (CD11c-Ly6G+), and dendritic cells (CD11c + Ly6G-) [14–16].

In this study, we generated influenza vaccines using the H1N1(A/Victoria/2570/2019), H3N2(A/Darwin/9/2021), and BV(B/Austria/1359417/2021) strains, which are the latest epidemic strains designated by the WHO for the 2022–2023 period. Trivalent vaccines were prepared utilizing the antigens of the three viruses. A trivalent vaccine with equimolar proportions of all antigens was formulated. Furthermore, vaccines with a predominant concentration of a specific antigen were also formulated to assess immune interference with other antigens when one antigen was dominant. Safety of the antigens was assessed by administering a high-dose multivalent vaccine, followed by the monitoring side effects. The high-dose vaccine was formulated by combining each antigen at a concentration of 120 μg/ml, which is four times the dosage of the efficacy test groups.

Various methods were employed to assess immune interference through *in vivo* experiments using the latest seasonal influenza strains. Importantly, no instances of immune interference were observed in the 2022–2023 seasonal strain designated by the WHO. Nevertheless, evaluating immune interference remains a crucial step during the development of multivalent vaccines. Collectively, the procedures described in this study provide a methodological basis for the validation of multivalent vaccines intended for future development.

2. Materials & methods

2.1. Cells, viruses, and antigens

Madin-Darby canine kidney cells (MDCK, ATCC, CCL-34) were acquired from the American Type Culture Collection (ATCC). These cells were grown at 37 °C in an atmosphere enriched with 5% CO2, using Dulbecco's modified Eagle's medium (DMEM, CORNING 10-013-CV) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic combination (comprising Amphotericin B, Penicillin, Streptomycin), and 1% non-essential amino acids. The cell culture media and supplements were acquired from Thermo Fisher Scientific (Waltham, MA, USA).

Live viruses of Influenza virus A/Victoria/2570/2019 (H1N1), A/Darwin/9/2021 (H3N2), and B/Austria/1359417/2021 (B/

Victoria) were obtained from GC Biopharma Corp. These viruses were propagated within MDCK cells, which were cultured in DMEM supplemented with 1 μg/ml of TPCK-trypsin (Thermo Fisher Scientific, Waltham, MA, USA) and supplemented with 1% antibiotic-antimycotic, all maintained at 37 °C under a 5% CO_2 environment. GC Biopharma Corp. distributed the corresponding antigens used in the human multivalent vaccine produced every year. The antigens were diluted and used in accordance with the mouse experiment.

2.2. Groups

The vaccines were categorized as trivalent and monovalent. The trivalent vaccines comprised antigens from influenza virus A/Victoria/2570/2019 (H1N1), A/Darwin/9/2021 (H3N2), and B/Austria/1359417/2021 (B/Victoria). In addition, groups with twice the concentration of each antigen were also established.

In total, there were 7 experimental groups, encompassing 3 groups for each individual antigen in the monovalent vaccine category, and 4 groups for the multivalent vaccines. The multivalent vaccine groups consisted of vaccines with a uniform concentration of antigens and specific antigen dominance (Table 1).

2.3. Immunization of BALB/c mice

Six-week-old female BALB/c mice were purchased from CLSbio (Bucheon, South Korea) and were housed in isolated cages within the BSL-2 facility at Chonnam National University (Gwangju, South Korea) for the duration of the study. The study complied with all current laws of South Korea regarding animal experimentation. The care and treatment of animals followed the guidelines established by the Chonnam National University Institutional Animal Care and Use Committee (CNU-IACUC-YB-2023-64). For the immune interference evaluation experiments, mice [n = 7 per group, comprising 4 mice for the hemagglutination inhibition (HI) test, and 3 mice for the in-direct enzyme-linked immunosorbent assay (ELISA) and flow cytometry assay] were immunized through intraperitoneal injection at weeks 0 and 2, with serum samples collected at week 3. In the vaccine safety test, the high-dose vaccine groups were monitored for body weight changes and observed for clinical signs over a period of 7 days following vaccination. The high-dose vaccine was formulated by combining each antigen at a concentration of 120 μg/ml, which is four times the dosage of the monovalent vaccines (Fig. 1).

2.4. Hemagglutination inhibition test

Fresh serum was collected from mice to conduct the HI test. Chicken red blood cells were prepared and suspended in Alsever's solution. To ensure specificity, all sera were treated with receptor destroying enzyme prior to the commencement of the test. A series of two-fold serial dilutions of serum (ranging from $1:2^2$ to $1:2^{12}$) were combined in a 1:1 ratio with 25 μl of virus. This mixture was then incubated at 37 °C for 1 h, after which 50 μl of chicken red blood cells were mixed, and subjected to a 1-h incubation at 4 °C. Agglutination patterns were observed and recorded within a span of 10 min. Throughout the test, chicken red blood cells at a concentration of 0.5% and 4 hemagglutinating units (HAU) of the virus were utilized. The HI titer was defined as the reciprocal of the final serum dilution that completely inhibited hemagglutination [17]. In the graph, the HI titer was expressed on a modified log 2 scale to compare each group.

2.5. In-direct ELISA (IgG)

Immunized mouse serum was analyzed using commercially available immune assays according to the manufacturer's instructions. Briefly, IgG1-target specific antibody and IgG2a-target specific antibody (88-50410-88, 88-50420-88, Thermo Fisher Scientific, Waltham, MA, USA) coated 96-well ELISA plates were used. They were blocked using PBS supplemented with 1% bovine serum albumin (BSA) at 37 °C for 2 h. The plates were then washed three times with PBS supplemented with 0.05% Tween 20. After the washing step, the collected sera were added to the plates. The plates were then incubated at 37 °C for 2 h and washed. Next, HRP-conjugated anti-mouse IgG1 or IgG2a antibodies were added to each well, and the plates were incubated at 37 °C for 2 h and washed. The HRP substrate, 3,3′,5,5′-tetramethylbenzidine, was subsequently added to each plate. The enzyme-substrate reaction was terminated by adding a stop solution (2 N sulfuric acid), after which the absorbance values of the wells were determined using a VICTOR microplate reader at optical densities of 620 nm and 450 nm. The multiple values compared to the absorbance value of the

Table 1
Vaccines of 2022–2023 WHO recommended influenza viruses.

	Vaccine type	H1N1	H3N2	B/Victoria
Group 1	Monovalent	30 μg/ml	–	–
Group 2		–	30 μg/ml	–
Group 3		–	–	30 μg/ml
Group 4	Trivalent	30 μg/ml	30 μg/ml	30 μg/ml
Group 5		60 μg/ml	30 μg/ml	30 μg/ml
Group 6		30 μg/ml	60 μg/ml	30 μg/ml
Group 7		30 μg/ml	30 μg/ml	60 μg/ml

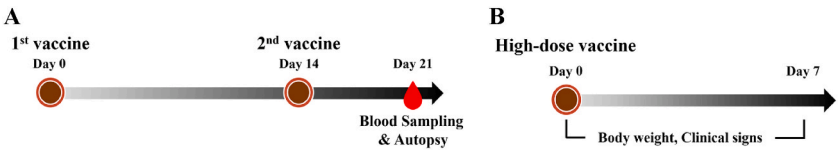

Fig. 1. Diagrams of mice immunization schedules. *In vivo* experiments were conducted to assess the immune interference phenomenon and safety, utilizing six-week-old female BALB/c mice. (A) The mice received vaccinations at 2-week intervals. Serum and organs for testing were collected 7 days after the second vaccination. (B) For the vaccine safety assessment, the high-dose vaccine groups were monitored for changes in body weight and observed for clinical signs over a period of 7 days post-vaccination. The high-dose vaccine was formulated by combining each antigen at a concentration of 120 μg/ml, which corresponds to a four-fold dosage compared to the monovalent vaccines.

MOCK group, administered the same amount of PBS instead of vaccine, were graphed for each group (Fig. 3).

2.6. Flow cytometry assay

The isolated lung tissues were sectioned into smaller pieces and incubated in a digestion buffer containing collagenase IV and DNase I, after which they were incubated at 37 °C for 30 min. The digested tissue fragments were pressed through a cell strainer and subsequently suspended in PBS supplemented with 2% FBS. Lymphocytes in spleen tissues were isolated using Red Blood Cell (RBC) lysis buffer. The cells were washed by centrifugation (4 °C, 600×*g*, 5 min), and re-suspended at 1×10^6 cells/ml in PBS supplemented with 2% FBS. The positive control group was administered a commercially available trivalent vaccine with the same antigen concentration as the multivalent test group 4 (Table 1).

The expression of cell surface markers was determined by staining the cells with antibodies specific for TCRβ, B220, CD4, CD8, CD62L, CD44, Ly6G, F4/80, CD11b, and CD11C. Regulatory T cells (Treg) were assessed using CD4, CD25, and Foxp3. Intracellular staining was performed after permeabilization using the Foxp3 intracellular staining kit (00-5523-00, Thermo Fisher Scientific, Waltham, MA, USA). The cells were analyzed using a BD VERSETM flow cytometer using the FlowJo software. Cell numbers were calculated based on live cell yields and the percentages of gated live cells.

3. Results

3.1. Analysis of immune interference for the assessment of the vaccine's protective effects and safety

The protective efficacy was evaluated through the hemagglutination inhibition (HI) test, assessing the neutralizing capability against viruses. Upon comparing the immunogenicity of the monovalent and multivalent vaccines, our findings indicated that the antibody production of the multivalent vaccine was higher than that of the monovalent vaccine. Furthermore, when the concentration of each antigen in the multivalent vaccine doubled, there was no significant difference in antibody production (Fig. 2 A). Next, tests were conducted to evaluate the safety of the multivalent vaccine at high concentrations. Clinical signs, such as hair loss or shivering, were not observed in mice following the administration of the high-concentration multivalent vaccine. Moreover, weight changes were measured for 7 days after vaccination and there was no significant difference between the mock group and the safety test group (Fig. 2 B).

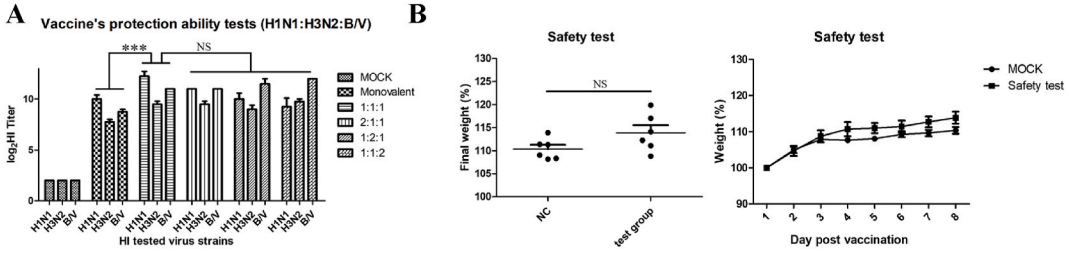

Fig. 2. Hemagglutination Inhibition (HI) titers of sera from vaccinated mice and safety assessment. (A) Female BALB/c mice were vaccinated twice at 2-week intervals (n = 4). Serum was collected 7 days after the second vaccination. HI tests were performed using 4 hemagglutinating units (HAU) of influenza virus. The mock group received PBS instead of vaccines. The viral strains on the X-axis refer to the strains used in the HI test. The sera from the monovalent vaccine groups were tested against the corresponding viral strains. The sera from the trivalent vaccine groups were tested against all three strains individually. The ratios in the legend represent the ratios used in the formulation of the multivalent vaccines for the H1N1, H3N2, and Flu B/Victoria lineages. A synergistic effect was observed when comparing the monovalent and multivalent vaccines with equal antigen concentrations (***: $P < 0.001$). No significant difference in efficacy was noted among multivalent vaccines when a specific antigen was dominant. (B) For the safety test, female BALB/c mice received a single administration of a high-dose vaccine (n = 6). The high-dose multivalent vaccine was prepared with a four-fold quantity of each antigen compared to the tested monovalent vaccines. Monitoring of body weight for 7 days post high-dose vaccination revealed no significant difference between the mock group and the test group in terms of the weight change trend and the change rate in final body weight compared to the beginning of the experiment. The error bars represent the standard error of the mean of the measurements.

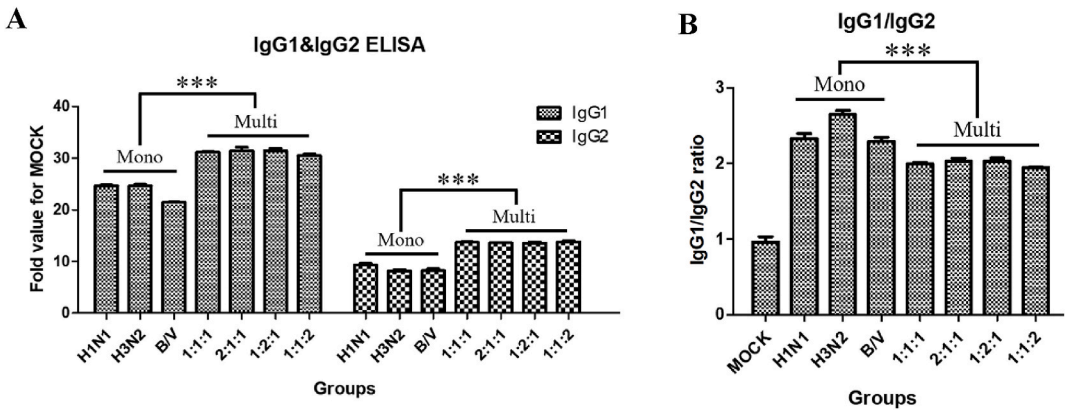

Fig. 3. Indirect ELISA for mouse Immunoglobulin G1 & G2 (IgG1 & IgG2). Female BALB/c mice were vaccinated twice at 2-week intervals. Serum was collected 7 days after the second vaccination. Indirect ELISA was performed to measure the amount of immunoglobulins in the sera of vaccinated mice. The mock group was administered with PBS instead of the vaccines. The ratios in the X-axis of the graph represent the ratios used in the formulation of the multivalent vaccines for the H1N1, H3N2, and Flu B/Victoria lineages. (A) The Y-axis shows the fold value of the experimental groups compared to the mock group. This value represents the fold change of the OD relative to the control. For both IgG1 and IgG2, the amount of detected immunoglobulin in the multivalent vaccine was higher than that of the monovalent vaccine (***: $P < 0.001$). (B) To determine the dominant immune reaction between Th1 and Th2, the IgG1/IgG2 ratio was examined. The IgG1/IgG2 values exhibited a reduction in the multivalent vaccine groups compared to the monovalent vaccine groups (***: $P < 0.001$). The error bar represents the standard error of the mean of the measurements.

3.2. Confirmation of immune interference in cellular and humoral immunity through immunoglobulin analysis through ELISA

Typically, mice respond to inactivated influenza vaccines by eliciting a Th2-type immune response, which is correlated with the stimulation of IgG1 antibodies. The ratio of antigen-specific IgG1 to IgG2a antibodies serves as an indicator to characterize immune response biases. An IgG1:IgG2a ratio of <1 indicates a Th1-biased immune response, whereas a ratio >1 indicates a Th2-biased immune response [18,19].

To assess this dominance, both IgG1 and IgG2a were quantified using sera obtained from immunized mice through enzyme-linked immunosorbent assay. Our findings revealed that in the multivalent vaccine groups, the quantity of both IgG1 and IgG2a increased compared to the monovalent vaccine groups, indicating an augmentation of both Th1 and Th2 immune responses. Moreover, even if the amount of a specific antigen within the multivalent vaccine was increased, it did not affect the immune response (Fig. 3 A). Notably, due to differing rate increases in IgG1 and IgG2a, the ratio of IgG1/IgG2a changed. This observation suggests that the augmentation in IgG2a occurred more prominently within the multivalent vaccine groups, while maintaining the dominance of the Th2 immune response (Fig. 3 B).

4. Discussion

Based on our experimental findings, no discernible differences in protective capability were observed between the monovalent and multivalent vaccines. Notably, there were no significant differences among the various multivalent vaccine formulations, regardless of whether antigens were mixed in equal proportions or with a specific antigen dominant proportion. All vaccines demonstrated sufficiently robust levels of protection against infection [20]. Furthermore, the results of our immunoglobulin analyses revealed that the expression of immunoglobulins increased in the experimental groups administered with the multivalent vaccines compared to the monovalent vaccines. Moreover, based on the IgG1 and IgG2 ratio, both vaccine types elicited a dominant humoral immune response. However, the multivalent vaccine groups induced a relatively stronger cellular immune response compared to the monovalent vaccine groups. We speculated that the simultaneous presence of various antibody types in substantial quantities stimulates cellular immune cells synergistically. Hence, it can be inferred that the multivalent vaccines exhibited no immune interference due to factors like antagonistic effects, synergistic effects, antigen competition, or epitope suppression among antigens. Additionally, no adverse effects were observed when antigens were combined at a four-fold concentration.

Furthermore, an immune mechanism analysis was conducted comparing monovalent, laboratory-combined multivalent, and commercially licensed multivalent vaccines against influenza antigens. The composition of immune cells was assessed using flow cytometry (Supplementary data 1). The functionality of T cells and B cells, crucial to the immune response, was examined, with additional analysis on CD4, CD8, memory, and regulatory T cells among T cells. Although the activities of macrophages and neutrophils responsible for innate immunity were analyzed, no significant variations were observed across the test and control groups. The results indicated an absence of immune interference based on the activity levels of immune cells.

Remarkably, the multivalent vaccines for the circulating influenza virus strains of 2022–2023 demonstrated satisfactory levels of protection and immune response without encountering any immune interference phenomena. Nevertheless, the immune response remained biased toward humoral immunity due to the characteristics of inactivated vaccines. The activation of cellular immunity

enhances protective capacity and prolongs immune memory. These observations highlight the need for the development of multivalent vaccines that can stimulate cellular immunity without resulting in immune interference.

Although no instances of interference were observed with the prevalent influenza strains in 2022–2023, the exact triggers or preventive strategies for immune interference in multivalent vaccinations have not been identified. In the case of vaccines encompassing various strains administered to a global population, such as influenza vaccines, the verification of immune interference must be an integral part of development. In this context, our findings provide a valuable basis for future studies on immune interference.

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Institutional review board statement

The study was conducted in accordance with the Declaration of Republic of Korea and approved by the Ethics Committee of Chonnam National University (Yong-bong campus) Institutional animal care and use Committee (Approval code: CNU-IACUC-YB-2023-64, Approval date: May 22, 2023).

Informed consent statement

Not applicable.

CRediT authorship contribution statement

Eulhae Ga: Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization. **Jung-Ah Kang:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Jaehyun Hwang:** Methodology, Formal analysis, Data curation. **Suyun Moon:** Methodology, Formal analysis, Data curation. **Jaeseok Choi:** Methodology, Formal analysis, Data curation. **Eunseo Bae:** Methodology, Formal analysis, Data curation. **Hyein Seol:** Methodology, Formal analysis, Data curation, Conceptualization. **Yubin Mun:** Methodology, Formal analysis, Data curation, Conceptualization. **Daesub Song:** Supervision, Software, Resources, Project administration, Formal analysis, Conceptualization. **Dae Gwin Jeong:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis. **Woonsung Na:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28326.

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