Original article

CLINICAL AND EXPERIMENTAL VACCINE RESEARCH

Influence of temperature on the antigenic changes of virus-like particles

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Purpose: In this study, we investigated whether the antigenic changes of the virus-like particles (VLPs) are affected by the temperature during storage.
Materials and Methods: After exposing the recombinant influenza VLPs to various temperatures for a period, antigenic changes were examined through *in vitro* hemagglutination receptor binding assay and *in vivo* mouse experiments.
Results: Influenza VLPs were exposed at three different temperatures of low, middle, and high on a thermo-hygrostat. High temperature exposed influenza VLPs were showed significantly reduced HA activity and immunogenicity after mouse single immunization over time compared low and middle. When the VLPs exposed to the high temperature were inoculated once in the mice, it was found that the immunogenicity was significantly reduced compared to the VLPs exposed to the low temperature. However, these differences were almost neglected when mice were inoculated twice even with VLPs exposed to high temperatures.
Conclusion: This study suggests that similar protective effects can be expected by controlling the number of vaccination and storage conditions, although the antigenic change in the VLP vaccines occurred when exposed to high temperature.

Keywords: Virus-like particle vaccines, Temperature, Antigenicity

Introduction

Seasonal influenza caused by influenza virus occurs a regional epidemic every year. Because influenza vaccines have been developed, healthy adults who have been vaccinated have a relatively low risk of influenza, but when influenza in the elderly and infants has a high mortality rate [1,2]. In addition, the global pandemic caused by the emergence of new viruses threatens serious social health [3,4]. The best way to minimize the social impact of the influenza is to use a vaccine [5]. Trivalent or tetravalent influenza vaccines have been developed and are in use, but many studies are under way to make new influenza vaccines that can be produced more quickly and are effective [6]. Recombinant virus-like particles (VLPs) have an external structure very similar to that of a native virus by self-assembly, but they are safe because of their non-infectious nature [1,7]. Previous studies have revealed that VLPs produced in insect cell induce strong immune responses without adjuvants but little research has been done on antigen stability by changes in the external temperature environment [8-10]. VLPs are recombinant protein complexes that are highly influenced by temperature changes

during storage. Therefore, to use VLPs as vaccines, studies on the change in antigenicity of the VLP according to the ambient temperature are required.

In this study, to see the antigenic change according to various temperature conditions, influenza VLPs made by baculovirus-insect cell expression system were stored at low (4°C), middle (25°C), and high (42°C) temperature and compared their immunogenicity. The antigenic changes *in vitro* were measured by hemagglutination activity of the VLPs, and the changes in immunogenicity *in vivo* was confirmed by animal experiments.

Materials and Methods

Cells, viruses, and animals

Spodoptera frugiperda (Sf9) cells were obtained from the American Type Culture Collection and maintained in SF900II (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone), at 37°C in 5% CO_2. Influenza A/Puerto Rico/8/34 (H1N1) viruses for challenge experiment were inoculated in 11-day-old embryonated eggs and propagated for 3–4 days. All mice used in this study were maintained in Sungshin University animal facility and conducted with the approval of the Institutional Animal Use and Care Committee at Sungshin University (Protocol SSWIACUC-2019-003) implementing the humane care of experimental animals.

Generation and purification of influenza virus-like particles

Influenza virus-like particles (PR8 VLPs) which composed of hemagglutinin (HA) and matrix protein 1 (M1) derived from influenza A/Puerto Rico/8/34 (H1N1) were prepared by baculovirus-insect cell expression system as described previously [11]. In brief, viral HA and M1 RNA were extracted from native influenza virus and reverse transcribed by reverse transcription polymerase chain reaction (PCR). The PCR-amplified HA and M1 genes were cloned into pFastBac1 and transformed in DH10Bac cells (Invitrogen). The recombinant bacmids isolated from DH10Bac with HA and M1 genes were transfected into Sf9 cells to generate baculoviruses expressing HA and M1 proteins. Finally, PR8 VLPs were generated by co infection of baculoviruses expressing HA and M1 proteins with Sf9 cells and purified from culture supernatant using nitrocellulose membrane-based filtration system [11]. Expression of HA in PR8 VLPs was analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and western blot analysis with PR8 HA antibody followed detection by horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG; SouthernBiotech, Birmingham, AL, USA). The electron microscopic picture of PR8 VLPs was taken as described previously [12].

Exposure of virus-like particles and inactivated viruses on variety temperature conditions

PR8 VLPs and inactivated viruses were diluted in phosphate-buffered saline (PBS) and made to a minimum volume of 0.5 to 1 mL, and then placed in a thermo-hygrostat, kept at a predetermined temperature, low (4°C), middle (25°C), and high (42°C) with 50%–60% humidity and stored for a specified period.

Hemagglutination titer

HA titer were determined by HA assay as previously described [13]. Briefly, serially diluted VLPs were prepared in V-bottom 96 well plate with PBS and followed by addition of chicken red blood cells (RBC) at 4°C. After 1 hour, check the minimum dilution factor of the hemagglutination of RBC.

Immunization and virus challenge

BALB/c mice (female, 6–8 weeks old) were immunized on day 0 and boosted on 3 weeks with 100 μL of 2.6 μg PR8 VLPs intramuscularly exposed to different temperature conditions and control groups received PBS only. For challenge experiment, mice were intranasally injected with influenza A/PR/8 (H1N1) 10MLD_{50} in 30 μL at 3 weeks after boost immunization.

Detection of serum immunoglobulin G

To determine the systemic immune response induced by VLPs, serum was collected from groups of vaccinated mice 3 weeks after boost. Enzyme-linked immunosorbent assay (ELISA) was used to detect PR8 specific antibody response in serum as previously described [14]. Briefly, 96-well flat bottom ELISA plates were coated with coating 100 μL per well of 1 μg/mL of PR8 inactivated virus. After blocking the plate with 3% bovine serum albumin at 37°C for 2 hours, diluted serum as primary antibody followed by HRP-conjugated goat-anti-mouse IgG as secondary antibody. Tetramethylbenzidine substrate solution was used for development. Optical density was measured at optical density 450 nm.

Statistics

Statistical analyses were performed with GraphPad Prism software ver. 5.0 (GraphPad Software, San Diego, CA, USA).

All results are presented as mean±standard error of the mean. For evaluating the statistical differences among groups, Student t test was performed and p-values of less than 0.05 were considered statistically significant.

Results

Production of influenza virus-like particle and exposure to various temperature conditions

Influenza PR8 VLPs expressing HA, M1 protein derived from influenza A/Puerto Rico/8/34 (H1N1) virus were generated by baculovirus-insect cell expression system and concentrated by nitrocellulose membrane-based filtration system. VLPs were further purified through sucrose gradient ultracentrifugation, and finally PR8 VLPs were dissolved in PBS to obtain a final yield of 4,096 HAU/50 μL. Schematic diagram and negative stain electron microscopy picture of PR8 VLPs were shown in Fig. 1A and 1B. PR8 VLPs The HA and M1 proteins expressed in PR8 VLP were confirmed by Western blotting analysis, and observed with sizes of 75 kDa and 25 kDa, respectively (Fig. 1C). Experimental design to determine antigenic change according to the storage temperature of influenza VLPs. To examine the antigenic changes of influenza VLP in various temperature conditions, low (4°C), middle (25°C), and high (42°C), a certain amount of VLP was put into a micro tube and stored in a thermo-hygrostat for a given period as shown in Fig. 2. After the storage period was completed, the VLP was taken out of the thermo-hygrostat to visually inspect the properties, abnormal characteristics such as aggregation were not found (data not shown).

Changes in the properties of the influenza virus-like particle antigen after exposure to temperatures

Influenza VLPs were stored in low, middle, and high temper-

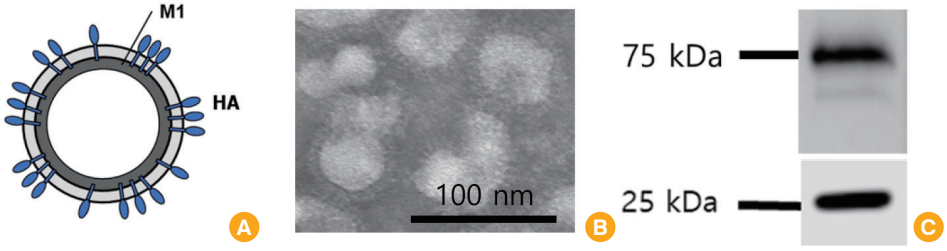

Fig. 1. Characterization of influenza PR8 VLPs. (A) Schematic diagram of influenza PR8 VLPs expressed HA and M1 proteins. (B) Negative stain electron microscopy of influenza PR8 VLPs (C) Western blot of HA (75 kDa) and M1 (25 kDa) proteins expressed in PR8 VLPs. VLPs, virus-like particles; HA, hemagglutinin.

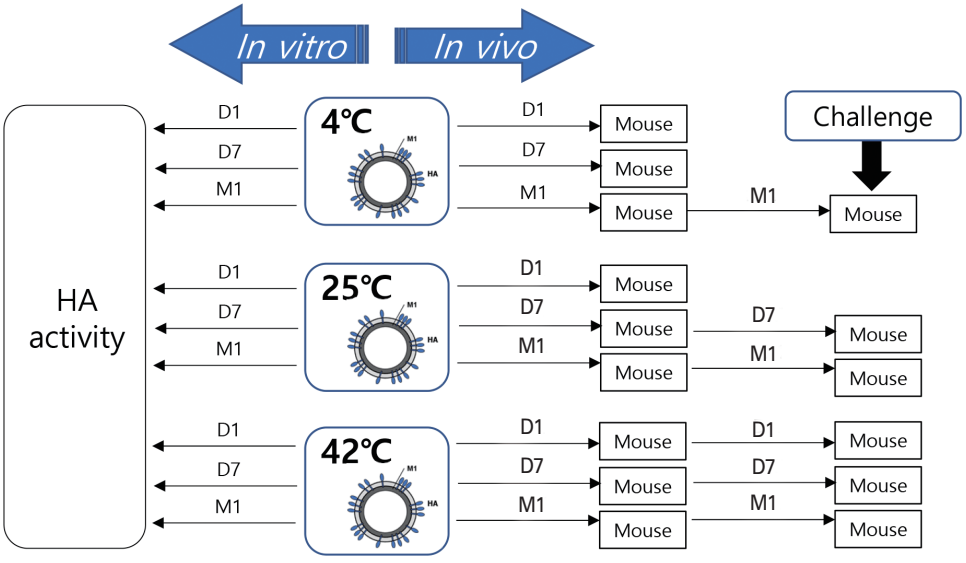

Fig. 2. Experimental design to determine antigenic change according to the storage temperature of influenza VLPs. Influenza VLPs were stored in low, middle, and high temperature conditions, and after 1 day (D1), 7 days (D7), and 1 month (M1), the *in vitro* antigenicity was measured by HA assay, and *in vivo* immunogenicity was measured through animal experiments. VLPs, virus-like particles; HA, hemagglutinin.

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ature, and after 1 day (D1), 7 days (D7), and 1 month (M1), the HA and changes in the binding ability of HA present in influenza VLPs to red blood cells were confirmed through HA assay. As shown in Fig. 3, influenza VLPs stored for 1 day at three temperature conditions did not differ in HA activity (Fig. 3A). However, when stored for 7 days, HA activity was lost in VLPs stored at high temperature (Fig. 3B). In the case of the samples stored for 1 month, the low temperature sample still had HA activity similar to the beginning, whereas the VLP stored at the intermediate temperature showed a significant decrease in activity. VLPs stored at medium temperature showed a significant decrease in activity (Fig. 3C). Through this result, it can be seen that HA protein, which plays a role in binding to the cell receptor, has a significant decrease in binding ability when stored at room temperature for more than 1 month.

Antigenic changes of influenza virus-like particles exposed to temperatures

To examine the antigenic change of influenza VLP vaccine exposed to various temperatures, PR8 VLPs after storage in different temperatures were immunized with the mice once or twice in 3-week interval. Three weeks after the last vaccination, blood was collected to measure the virus-specific antibody titer present in the serum. As shown in Fig. 4A and 4B, there was no significant difference in the antibody titer induced by the VLP vaccine stored at low and middle temperatures regardless of the storage period. However, VLP vaccine stored at a high temperature did not show a significant change in antigenicity during the 1-day and 7-day periods, but when stored for 1 month, immunogenicity was significantly reduced (Fig. 4C).

To confirm the antigenic change according to the temperature conditions in the boost immune effect of the VLP vaccine, the second vaccination was performed after storing the influenza VLPs at the same temperature and time conditions. Mice inoculated twice with VLP vaccine stored at low and middle temperatures were found to induce a robust immune response regardless of the storage period, as in the case of the primary vaccination (Fig. 5A, B). On the other hand, in the influenza VLP vaccine group stored for 1 month at high temperature, which showed low immunogenicity in primary vaccination, high antibody titers were identified (Fig. 5C). In oth-

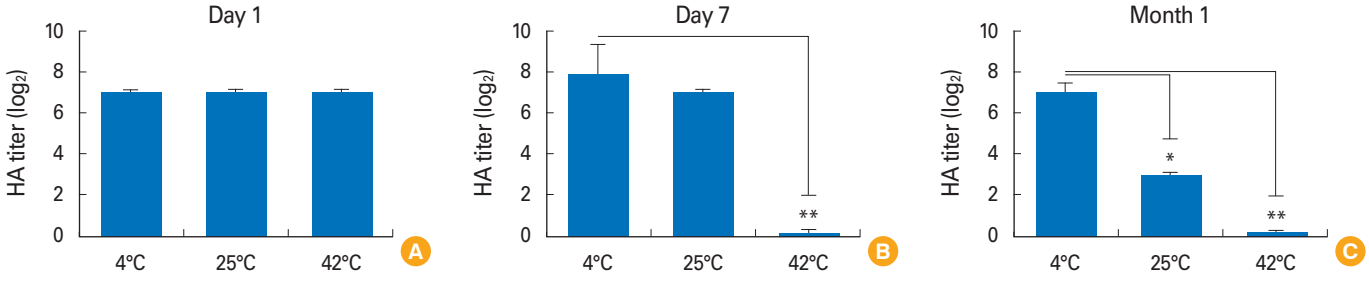

Fig. 3. Measurement of HA activity change of influenza VLPs stored at different temperature conditions. PR8 VLPs of the same amount and volume were stored for 1 day (A), 7 days (B), and 1 month (C) under low (4°C), middle (25°C), and high (42°C) temperature conditions and HA activity was measured by HA assay. VLPs, virus-like particles; HA, hemagglutinin. *$p<0.05$ and **$p<0.01$; significant differences (Student t test).

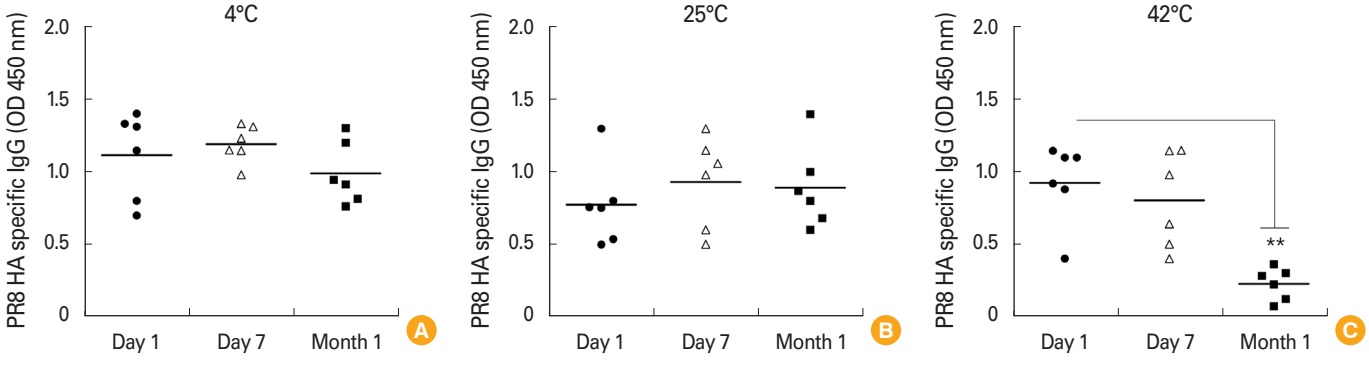

Fig. 4. Influenza A/PR8 virus specific IgG responses. Group of mice (n=6) were intramuscularly immunized with 2.6 μg of PR8 VLPs stored at low (A), middle (B), and high (C) temperature conditions. Blood sample were collected from PR8 VLP immunized mice at 3 weeks after immunization. VLPs, virus-like particles; HA, hemagglutinin; IgG, immunoglobulin G; OD, optical density. **$p<0.01$; significant differences (Student t test).

er words, antibody formation was restored due to two immunizations.

Protective immunity of influenza virus-like particles exposed to temperatures

To compare the protective immunity-inducing effects of VLP vaccines exposed to various temperature conditions, mouse adapted lethal influenza A/PR/8 virus (H1N1, 10MLD$_{50}$) was challenged at 3 weeks after boost vaccination.

As shown in Fig. 6, all mice survived except PBS control group (Fig. 6D). Mice vaccinated with the influenza VLPs exposed to low and middle temperatures, recovered after a

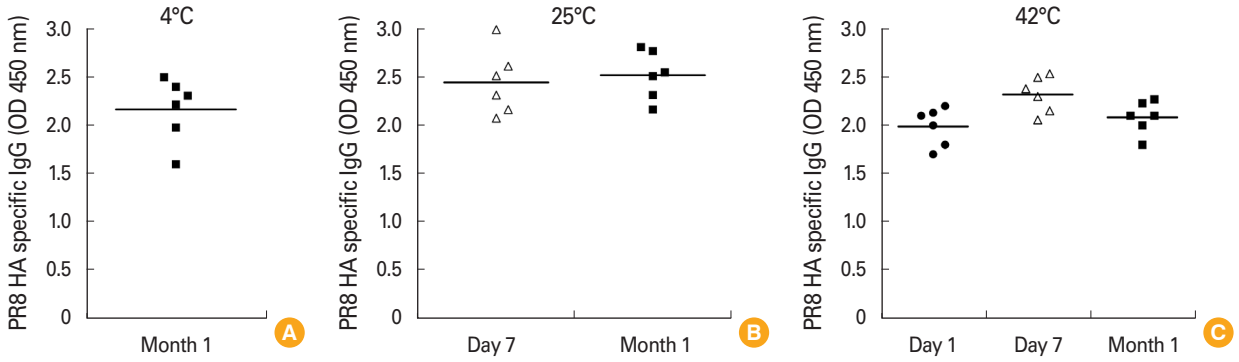

Fig. 5. Influenza A/PR8 virus specific IgG responses after boost immunization with PR8 VLPs stored at different temperature conditions. Mice were intramuscularly immunized on days 0 and 21 with PR8 VLPs stored at low (A), middle (B), and high (C) temperature conditions. Sera were collected from PR8 VLP immunized mice at 3 weeks after last immunization. VLPs, virus-like particles; HA, hemagglutinin; IgG, immunoglobulin G; OD, optical density.

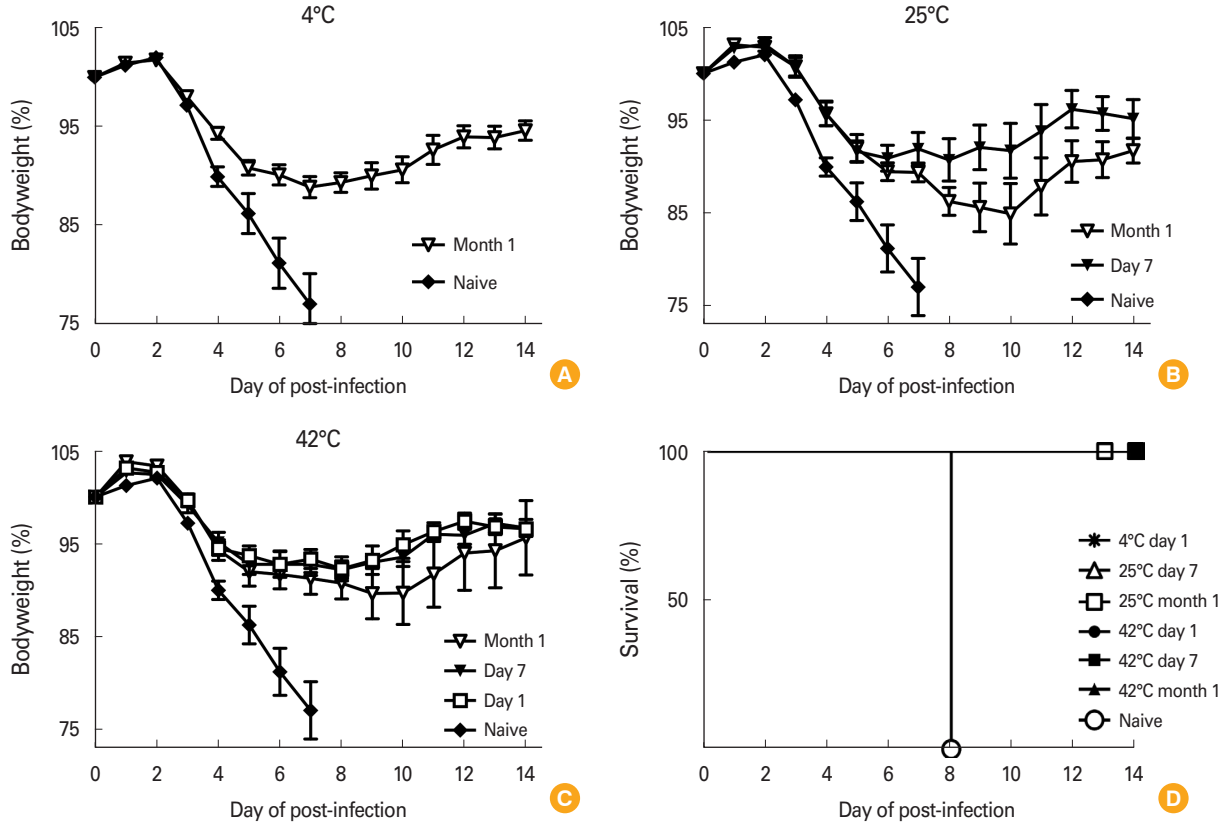

Fig. 6. Influenza A/PR8 virus specific immunoglobulin G responses after boost immunization with PR8 VLPs stored at different temperature conditions. Mice were intramuscularly immunized on days 0 and 21 with PR8 VLPs stored at low (A), middle (B), and high (C) temperature conditions. Sera were collected from PR8 VLP immunized mice at 3 weeks after last immunization. VLPs, virus-like particles.

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weight loss of about 7% to 14%, with some differences depending on the duration of exposure (Fig. 6A, B). In addition, the mice vaccinated with VLPs exposed to high temperature twice, also recovered after a weight loss of about 8% to 9%, thereby inducing a sufficient level of protective immunity (Fig. 6C).

Overall, the influenza VLP vaccines showed a decrease in HA activity when stored for 1 month or more at room temperature or higher, and the reduced immunogenicity was confirmed when immunized once. However, most of these differences were recovered by boost immunization.

Discussion

In 2009, many countries experienced a shortage of vaccines caused by the pandemic influenza [15-18]. To overcome these limitations, in several studies, a platform technology is being developed to utilize VLP as a vaccine that is highly productive and effective in inducing neutralizing antibodies [8,13]. Since most VLPs are composed of recombinant proteins, if VLPs are used as vaccines, their efficacy may vary greatly depending on the temperature at which they are stored. However, many studies have not been conducted on the difference in the effect of inducing immune responses *in vivo* according to the storage temperature of VLPs [19,20]. Influenza VLPs, which has undergone many studies as VLP vaccine candidates, are usually composed of HA and NA which are the major antigens, and these proteins are affected by the temperature at which they are stored [21].

In this study, influenza PR8 VLPs were prepared using the baculovirus-insect cell expression system and the thermal stability of VLPs was investigated. HA expression on VLPs were confirmed by Western blot analysis and HA assay, then VLPs were preserved in a thermo-hygrostat to expose the VLPs to various temperature conditions, low, middle, and high for 1, 7 days, and 1 month. *In vitro*, antigenicity was confirmed by HA analysis. The group stored at low temperature conditions did not show any decrease in HA titer over time, whereas the group stored at middle temperature conditions gradually decreased after 7 days and 1 month. The group stored under high temperature conditions remained unchanged until day 1, but no titer was found on days 7 and month 1.

Animal experiments were carried out to confirm whether these changes in HA activity under different temperature conditions have a correlation in inducing immune responses *in vivo*. As a result of measuring the antibody titer after single immunization of each VLPs, the middle temperature group showed the same levels of IgG titer regardless of the storage period, whereas the IgG titer was significantly decreased in high temperature group as the storage period increased. On the other hand, these results were quite different after boost immunization. Even in the group immunized with the VLP vaccine stored at high temperature, although the antibody titer was lower than other temperature groups, it was found that a significantly increased level of antibody was confirmed by the second vaccine. That is, it was found that even when the VLP vaccine was stored at high temperature for 1 month and had no HA activity *in vitro*, a sufficient level of antigen-specific antibody was induced when inoculated twice [22].

In the challenge experiment, all mice inoculated with PBS failed to survive on the 7 days after live virus infection, while all VLP-vaccinated mice lost some weight but subsequently recovered health conditions. This study showed that the VLP vaccine maintains a high immunogenicity even at middle temperature close to room temperature. Even the group of mice inoculated with VLPs stored at high temperatures had different recovery points depending on how long the VLPs were exposed to high temperatures, but thy all survived and recovered.

As a result, we conclude that even when the VLP vaccine is not refrigerated, similar protective effects can be expected by controlling the number of vaccinations and storage conditions.

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