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Research paper

Preparation of H1N1 microneedles by a low-temperature process without a stabilizer

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ABSTRACT

During the manufacture of H1N1 microneedles, a stabilizer is usually added to maintain the antigenicity of the vaccine. However, finding a suitable stabilizer is difficult, and the addition of a stabilizer can limit the antigen dose and the addition of an adjuvant because of the limited volume of the microneedles. In this study, the authors evaluated whether H1N1 microneedles could be fabricated without a stabilizer by keeping the production environment at a low temperature.

H1N1 microneedle patches without a stabilizer were prepared in a process that involved maintaining a low temperature of 10 °C. The protective immune response to this method of drug application was investigated by comparing it with traditional intramuscular (IM) immunization and with the use of H1N1 microneedles with a stabilizer.

A process-sensitive antigen, H1N1, was stabilized without the use of a stabilizer in a process that maintained a low temperature of 10 °C. The preparation process consisted of coating and drying processes. In animal experiments, mice were immunized using an array of low-temperature H1N1 microneedles without a stabilizer (LT-MN), and they showed strong antibody responses. Compared to three other application methods of traditional IM immunization, low-temperature H1N1 microneedles with a stabilizer (LT-MN-T), and room-temperature H1N1 microneedles with a stabilizer (RT-MN-T), LT-MN produced comparable results in inducing protective immunity. A plaque reduction neutralization test found that LT-MN and LT-MN-T provided greater immunity compared with IM and RT-MN-T.

A process in which the temperature is maintained at 10 °C can provide successful vaccination with H1N1 microneedles without the addition of a stabilizer. This process can be applied to various temperature-sensitive biologics.

1. Introduction

The microneedle system is designed to overcome the limitations of the existing transdermal drug delivery system [1–3]. Microneedles can deliver active ingredients into the skin through the stratum corneum regardless of the molecular weight and polarity of the ingredients [4,5]. The use of microneedles for vaccinations has been studied and developed because microneedles can deliver the antigen into the skin layer, where the cells that need to receive the antigen are primarily located [6–9]. Microneedles also provide the advantages of convenience and user compliance by avoiding the pain and fear of needles that patients often experience [10,11]. Vaccine microneedles have shown efficacy equivalent to intramuscular (IM) injection [12–14]. Vaccine microneedles have also shown improved storage stability compared to the conventional formulation [15,16]. However, in all previous studies, vaccine microneedles have required a stabilizer because the antigen was denatured during the process of preparation and storage [17–19]. An excessive amount of stabilizer was added to stabilize the antigen during the manufacturing process as well as during subsequent long-term storage [16,20]. When the antigen was formulated for

Abbreviations: RT-MN-T, room-temperature H1N1 microneedles with stabilizer; LT-MN-T, low-temperature H1N1 microneedles with stabilizer; LT-MN, low-temperature H1N1 microneedles without stabilizer; HA, Hemagglutinin

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microneedles, great effort was necessary to screen the optimal stabilizer [21,22]. There are a number of limitations related to the addition of the stabilizer: (1) the limitation of the appropriate co-solvent of the stabilizer and the antigen, (2) the limitation of the solubility of the stabilizer in the solvent, (3) the limitation of the amount of antigen that can be loaded into the formulation with the stabilizer, (4) the decrease in the mechanical strength of the dissolving microneedles, and (5) the phase separation of the formulation during the drying process [15,23]. In particular, in the case of influenza antigens, the amount of stabilizer was 20 times more than that of the antigen, and the addition of an excessive amount of stabilizer could cause the above-mentioned limitations [12,24].

The decrease in the stability of the antigen occurs rapidly at the stage where the liquid formulation is made into a microneedle shape and dried [17,25]. If antigenicity is maintained during the manufacturing process, the addition of a stabilizer is less constrained because the change in antigenicity was mainly caused during the manufacturing process when the vaccine microneedles were stored at room temperature and below room temperature [20,26]. Hemagglutinin (HA) activity, including H1N1, was stabilized mainly by adding a stabilizer during manufacturing, but HA activity did not change during long-term storage without a stabilizer at room temperature [16]. If HA activity can be maintained during the manufacturing process, the amount of antigen per unit area of microneedle array can be increased by minimization or elimination of the stabilizer.

The low-temperature process of producing vaccine microneedles was introduced for the first time to improve the stability of the antigen in the manufacturing process, resulting in the removal of the stabilizer during the manufacturing process. H1N1 vaccine microneedles were prepared by a low-temperature microneedle manufacturing system. To investigate the improvement in the stability of the antigen as a result of using the low-temperature process, we compared the antigenicity of low-temperature H1N1 microneedles without a stabilizer (LT-MN) to the antigenicity of IM immunization, room-temperature H1N1 microneedles with a stabilizer (RT-MN-T), and low-temperature H1N1 microneedles with a stabilizer (LT-MN-T). We then conducted animal experiments to confirm the improvement in stability by the low-temperature process.

2. Materials and methods

2.1. Materials

Phosphate-buffered saline (PBS) and trehalose-dihydrate were obtained from Sigma-Aldrich (St. Louis, MO). Polylactic acid (PLA) was purchased from Lactel (Birmingham, AL). Carboxymethyl cellulose (CMC) was obtained from Whawon (Gyeonggi-do, South Korea). H1N1 antigen of seasonal influenza vaccine was provided by Il-Yang Pharmaceutical Co. (Yongin, Republic of Korea). The H1N1 antigen of seasonal influenza vaccine that we received from the manufacturer was composed of NaCl, $NaH_2PO_4{\cdot}nH_2O$, KH_2PO_4, distilled water and vaccine antigen, and it was confirmed that the vaccine had no other additives except the antigen and the buffering agent.

2.2. Fabrication of H1N1-coated microneedles

2.2.1. Preparation of coating formulation

The information corresponding to each sample is shown in Table 1. The influenza H1N1 monobulks were concentrated to 2 mg/ml by ultracentrifugation using Amicon Ultra-0.5 Centrifugal Filter Devices (Amicon Ultra 10 K, Billerica, MA). 500 µl of influenza monobulk was added to the Amicon Ultra filter device and centrifuged at 14,000*g* for 20 min at 4 °C. The concentrated solution was emptied by placing the filter upside down in a clean tube for 2 min at 1000*g*, 4 °C. The concentrated antigen solution was stored in a refrigerator. H1N1 microneedles and H1N1 films were prepared for animal tests and stability tests, respectively. The components of the formulation are indicated in Table 1. The coating formulations for each group were prepared by mixing three kinds of solutions—the concentrated H1N1 solution, a solution with trehalose as a stabilizer, and a CMC thickener—according to the ratio of the components as shown in Table 1.

2.2.2. Preparation of pre-coated microneedles

A polylactic acid (PLA) microneedle array had 145 pyramid-shaped microstructures 800 µm in height and 370 µm in width on a circular base 1 cm in diameter and 500 µm thick. The polydimethylsiloxane (PDMS) molds were covered with pellets of PLA and placed in a vacuum oven (VOS-301, EYELA, Tokyo) under 70 kPa vacuum for 5 min at 190 °C. The hydrophobic surface of the PLA microneedles was plasma-treated to a hydrophilic surface by using a UV/Ozone process before dipping the microneedles into the H1N1 coating solution.

2.2.3. Low-temperature dip-coating

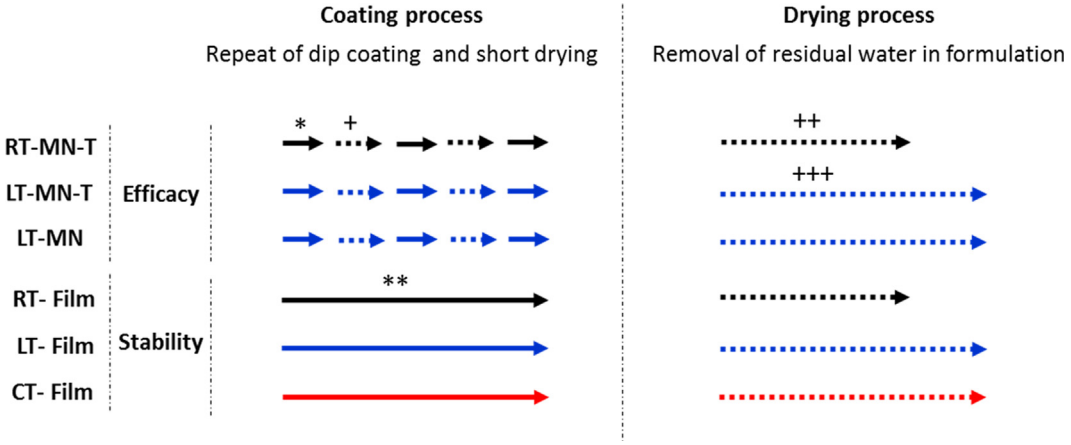
The temperature during the coating process was controlled at two points. The temperature of the atmosphere in the temperature controlled chamber was set to 10 °C, in which the coating and drying process proceeded and the temperature of the reservoir was maintained at 10 °C, in which the coating solution was placed. To maintain a constant temperature, the inside of the chamber was cooled with a heat exchanger kept at 10 °C using the refrigerant and the forced circulation of the internal air. For precise temperature control of the coating solution, a conduction cooling system was prepared and the temperature of the aluminum reservoir (1.5 cm in diameter, 500 µm in depth) containing the coating solution was maintained using a peltier device and a temperature controller. The reservoir, the dip-coating machine, and all temperature control parts are customized. All processes were carried out by setting the temperature of the dip-coating machine, the coating solution, and the chamber at 10 °C. After being coated, the microneedles were dried in a chamber maintained at 10 °C. The H1N1-coated microneedle groups (LT-MN-T and LT-MN) were produced in a chamber set at 10 °C as shown in Table 2.

The manufacturing process of each sample consisted of two courses, the first of short duration and the second of long duration. In the short-duration course, the coating was applied three times with 2 min of drying time for each coating. The long-duration course involved a final drying process of a few hours. The coating solution was placed in the reservoir, which was maintained at 10 °C. The ozone-treated PLA microneedles were dipped into the reservoir, with the coating solution being spread at a rate of 1 mm/s and the duration of each coating process being 30 s. After the solution was dried in a low-temperature

Table 1
Information on each sample. Hemagglutinin of H1N1, carboxymethyl cellulose (CMC), and trehalose; Hemagglutinin (HA): +, 3 µg; + +, 70 µg; Trehalose: +, 60 µg; Carboxymethyl cellulose (CMC): +, 150 µg; + +, 3527 µg; *: solution, RT: 25 °C, LT: 10 °C, CT: 4 °C.

		IM* (PBS)	IM* (H1N1)	RT-MN-T	LT-MN-T	LT-MN	RT-Film	LT-Film	CT-Film
Composition (µg)	HA	−	+	+	+	+	+ +	+ +	+ +
	Trehalose	−	−	+	+	−	−	−	−
	CMC	−	−	+	+	+	+ +	+ +	+ +

Table 2
Process flow chart for each sample. (1) Solid line: coating, dotted line: drying, (2) Black: 25 °C, Blue: 10 °C, Red: 4 °C, (3) Coating time: *: 30 s, **: 4.5 min, (4) Drying time: +: 2 min, ++: 3 hr, +++: 5 hr.



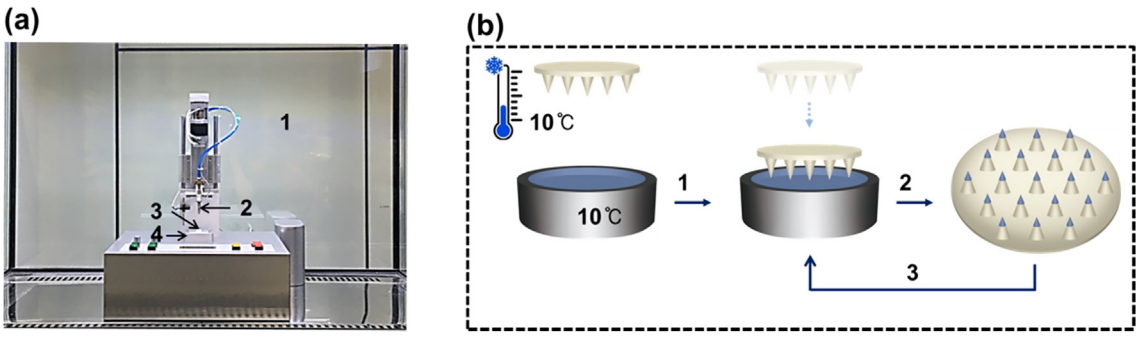


Fig. 1. (a) The photographic image of the dip-coating machine and the chamber. (1) Low-temperature–controlled chamber: The temperature of the chamber interior was maintained at 10 °C. (2) Moving actuator: Moving part that descends and rises for dip coating for 30 s. (3) Reservoir: The coating solution containing the drug is contained and the reservoir is maintained at 10 °C by the temperature controller. (4) Low-temperature controller: A peltier device and a thermo controller are used to maintain the temperature of the reservoir containing the drug at 10 °C. (b) The schematic diagram of the overall coating process: (1) dipping the ozone-treated L-PLA microneedles into the reservoir, which was maintained at 10 °C with a spreading rate of 1 mm/s, (2) drying in a low temperature chamber for 2 min, (3) repeating two more times and final drying.

chamber at 10 °C for 2 min, the coating process was repeated. Dipping time was 1 s of the 30 s coating process. The actual contact time of the microneedle with the coating solution was 1 s, and 29 s was the additional processing time, including installation of microneedles on the holder, and movement and removal from the holder. After the solution was dried again in the low-temperature chamber for 2 min, the third coating process was repeated. The H1N1-coated microneedles were dried for 5 h in a chamber maintained at 10 °C. The schematic diagram of the overall coating process is shown in Fig. 1. The completion time of the final drying step was determined by measuring the change of weight using an ultramicro-balance (MSA3.6P-000-DM, Satorius, Gottingen, Germany). There was a change in the weight of the coating formulation up to 2 h at 25 °C and up to 4 h at 10 °C by microbalance. Based on these results, the drying times were set to 3 h and 5 h for 25 °C and 10 °C respectively. After final drying, the samples were stored in a refrigerator at 4 °C. In the case of RT-MN-T, both coating and drying processes were carried out at a room temperature of 25 °C and then the sample was refrigerated at 4 °C as shown in Table 2.

2.3. Determination of coated amount of H1N1

The amounts of coated H1N1 were determined by dissolving each of the H1N1-coated microneedles of each group in 1 ml of PBS for 30 min and then using the Bradford assay method.

2.4. In-vitro vaccine stability test

In order to evaluate the stability of H1N1-coated microneedles during manufacturing, the stability of HA was evaluated according to the temperature used in the process by using thin films manufactured at 4 °C, 10 °C, and 25 °C [17,27]. The formulation used for the preparation of H1N1 film was the same as the formulation used with LT-MN. For the stability test, the temperature of each film sample was 25 °C for the RT-film, 10 °C for the LT-film, and 4 °C for the CT-film. The manufacturing process of the H1N1 film is shown in Table 2. The formulations of each group were applied to the reservoir, which is 1.5 cm in diameter and 500 μm in depth and is made of aluminum for coating tips. The coating formulations of each group were dried in a chamber at 25 °C for 3 h, and at 10 °C and 4 °C for 5 h, respectively. The formulations of each group were applied to the reservoir for coating tips, and the coated formulation was dried in a chamber at 25 °C for 3 h and at 10 °C and 4 °C for 5 h. Drying time was determined as the point at which no weight change occurred when the formulation was weighed using an ultramicro-balance with 1 μg resolution (MSA3.6P-000-DM, Satorius).

The stability of HA of each film was evaluated by high-performance liquid chromatography (HPLC, Agilent 1200 series, Agilent Technologies, Santa Clara, CA). In the HPLC assay, a column Poros R1/10 (2.1 mm × 100 mm) (Applied Biosystems, CA, USA) was maintained at 60 °C, and samples were maintained at 4 °C. Mobile phase A consisted of 0.1% trifluoroacetic acid (TFA, Sigma Aldrich, St. Louis, MO) and 5%

acetonitrile (J. T. Baker, Phillipsburg, NJ) in water, and mobile phase B consisted of 0.1% TFA in 100% acetonitrile. The column was equilibrated with 80% mobile phase A and 20% mobile phase B. The flow rate was 0.8 ml/min and total run time was 14 min. Monobulks with known HA content were used as standards by diluting them with PBS ranging from 3 to 50 μg/ml of HA. To the diluted standard or sample, 500 mM dithiothreitol (DTT, Sigma Aldrich, St. Louis, MO) was added at a sample-to-DTT ratio of 100:3 by volume to disrupt the HA trimers, and the solution was incubated at 90 °C for 10 min. One hundred microliters of each standard or sample was injected, and the HA peaks were determined by UV detection at 214 nm [7,28].

2.5. Immunization and challenge of mice

BALB/c mice (female, 5 weeks old; Orient Bio, Seongnam, Republic of Korea) were immunized with a H1N1 vaccine antigen-coated microneedle patch (RT-MN-T, LT-MN-T, and LT-MN), which was applied to the clipped back skin of the mice for 30 min. The number of mice for IM, RT-MN-T and LT-MN-T was 8, and the number of mice for LT-MN was 7. After two weeks of vaccination, the mice were bled for serum collection through their retro-orbital sinus. After bleeding, the mice were challenged intranasally with 5 MLD_{50} (50% mouse lethal dose) titer of mouse-adapted 2009 pandemic H1N1 A/Korea/01/2009 virus strain, which was used in the previous study [29]. The mice were then monitored for another two weeks, and their body weight changes and survival rates were recorded every day. Mice losing more than 25% of their initial body weight were considered experimental death and humanely euthanized. PBS was used for a control group immunized through an IM route. IM immunization with H1N1 vaccine antigen was applied to another control group. Mock group mice were infected with PBS. The remaining H1N1 on the microneedle surface after vaccination was observed using the SEM images.

2.6. Plaque reduction neutralization test (PRNT)

Using the mouse sera collected after vaccination, neutralizing antibody titers of the immunized mouse sera were determined. Briefly, the mouse sera were first treated with a receptor-destroying enzyme (RDE; Denka Seiken, Tokyo, Japan) according to the manufacturer's protocol. The RDE-treated mouse sera were incubated with 100 plaque-forming units (PFU) of virus at 37 °C for 1 h. Monolayered Madin-Darby canine kidney (MDCK) cells were then inoculated with the serially two-fold diluted mouse sera-virus mixture at 37 °C for 1 h. Non-immunized mouse sera were used as a control. After decanting the mouse sera-virus mixture, the MDCK cells were incubated with overlay media at 37 °C for 3 days. Viral plaque formation was then observed with crystal violet staining, and 50% reduction dilution of viral plaque titers compared to the controls was determined as a neutralization titer of the immunized mouse sera.

2.7. Statistical method

The arithmetic mean and standard error of the mean were calculated. A two-tailed Student's t test ($\alpha = 0.05$) was performed when comparing two different conditions, and ANOVA was used when comparing multiple groups. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Fabrication of H1N1-coated microneedles

Each microneedle group was coated with each component in the ratio of the components shown in Table 1. The targeted amount, 3 μg of H1N1, was loaded on the surface of the microneedle structure using the dip-coating method at 25 °C for RT-MN-T and at 10 °C for LT-MN-T and LT-MN. The coating and drying process for LT-MN-T and LT-MN was performed in the 10 °C chamber of the open system. If the temperature inside the chamber and the reservoir was as low as 4 °C, condensation of water occurred on the surface of the reservoir and the samples during the process. Viscosity was an important variable in determining the coated amount. In this study, the viscosity of the coating solution was 4500 cps at 10 °C and 3000 cps at 25 °C. As the temperature of the system decreased, the viscosity of the coating solution rapidly increased [30] and there was a small increase in viscosity because we selected a process temperature of 10 °C instead of 4 °C. Another variable was the elastic property of the coating solution. The elastic modulus of the CMC solution increased as the temperature decreased [31]. As the elastic modulus of the coating solution increased, elastic deformation of coating solution occurred when the microneedle tips were inserted, resulting in the reduction of the coating area. By increasing both the viscosity and the elastic modulus of the coating solution, a consistent amount of coating solution was maintained when the temperature was decreased from room temperature to 10 °C. Thus, a process temperature of 10 °C instead of 4 °C was selected. If the process could be performed in a closed system, a lower temperature of 4 °C was possible. However, even though the process can be conducted by an automated system, a semi-manual operation was necessary because of the accessibility of equipment and samples. Therefore, an operating temperature of 10 °C was chosen to ensure process accessibility, to control the viscosity of the coating solution, and to eliminate the effects of water condensation.

After coating and drying at each process temperature, the final coated shape of the microneedles was observed using an optical microscope as shown in Fig. 2(a). The antigen-containing formulations are locally distributed within the upper 400 μm of the microneedles, which is half of the entire tip length of 800 μm as shown in Fig. 2(b). The

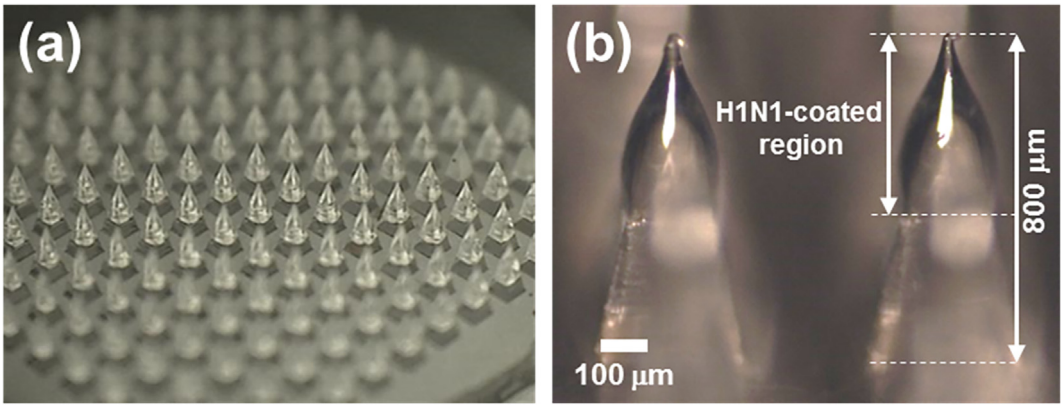


Fig. 2. Optical microscopic images of H1N1-coated microneedles fabricated at 10 °C (a) at low magnification and (b) high magnification.

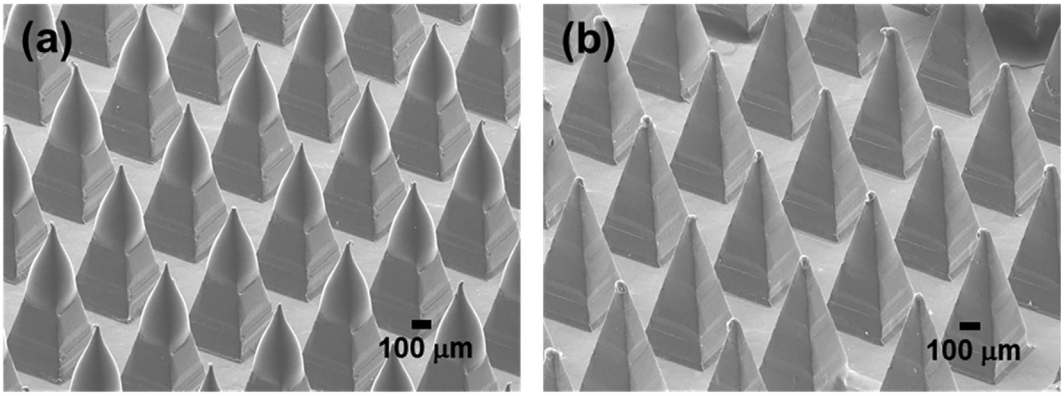


Fig. 3. SEM image of H1N1-coated microneedles (a) before insertion and (b) after 30 min of insertion into the mouse *in vivo*.

means and standard deviations between arrays of coated H1N1 were 3.1 ± 0.2 μg, 3.1 ± 0.1 μg, and 3 ± 0.2 μg for RT-MN-T, LT-MN-T, and LT-MN, respectively. The uniformity of the coated amount of H1N1 on the microneedles at low temperature was about 7% of relative standard deviation. Thus, H1N1 microneedles with low-dose error between samples can be economically prepared using standardized dip-coating processes.

3.2. Determination of H1N1 delivered into the skin

The dissolution of H1N1 microneedles was observed *in vivo*. The H1N1-coated microneedles shown in Fig. 3(a) were administered to the mouse for 30 min, and the sample was collected to check the existence of remaining formulation on the surface using a scanning electron microscope (SEM; JSM-7001F, JEOL Ltd, Tokyo, Japan). As shown in Fig. 3(b), the H1N1 formulation on the microneedles dissolved rapidly (within 30 min of administration), and no residual formulation was found on the microneedle surfaces. H1N1 was 1.4% (w/w) of the dried coating formulation, and the formulation was distributed only on the upper tips of the microneedles. The formulation dissolved quickly (within 15 min) into the skin layer [32], and the predetermined amount of H1N1 could then be delivered into the skin layer. No residual H1N1 was detected by HPLC, and no residual formulation was measured using the microbalance measurement.

3.3. Vaccine stability testing on films

Half of the film solution was exposed to air, and the rest was in contact with the reservoir. The air was hydrophobic and the reservoir was made of aluminum. The contact angle of water to reservoir was 65 degrees, which was similar to that of a surface-treated PLA surface. Thus, the condition of the drying process was similar to that of the coating process. During the preparation of most vaccine microneedles, the process of loading the antigen-containing formulation on the microneedles took several hours and all procedures were conducted at room temperature [33–35]. The drying process was also carried out at room temperature and usually took several hours to a day or longer [36–39]. Vaccine microneedles showed improved stability compared to the conventional liquid formulation [40]. However, antigens were sensitive to temperature during the preparation process because the preparation of vaccine microneedles was conducted on a microscale and a larger area of the volume of sample was exposed to the environment. Thus, the antigen reached the environmental temperature quickly. Therefore, a stabilizer was added to the solution to inhibit denaturation of the antigen during the preparation and drying process [17]. Although the addition of stabilizers could provide a solution to denaturation, it was difficult to find a suitable stabilizer for each antigen and to add an appropriate amount of stabilizer because of the limited volume of the microneedles. Therefore, this study suggested controlling the temperature of the process. A low-temperature process could eliminate the need of a stabilizer for the preparation of vaccine microneedles.

Previous studies reported that H1N1 could not be stable without the addition of a stabilizer when H1N1 was formulated and loaded into microneedles [20,27,41,42]. As a result of the *in vitro* stability test, the activity of H1N1 present in the film was studied in terms of process temperature, and the experiment was conducted with a formulation without an additional stabilizer. As a result, the stability of the H1N1 antigen was maintained at 94 ± 1.1% in the case of the film manufactured at 10 °C, while the stability of the H1N1 antigen was 83 ± 1.2% when it was manufactured at 25 °C. It was difficult to maintain the antigenicity of H1N1 without the addition of the stabilizer during the process conducted at room temperature. The antigenicity in the film was maintained at 4 °C and 10 °C without the addition of a stabilizer. A relatively large amount of stabilizer is added to the antigen in most microneedle manufacturing processes. Therefore, minimizing or eliminating the use of a stabilizer enables more antigen to be loaded into drug formulations.

In previous studies, when a stabilizer was not added in a formulation, antigenicity was reduced as a result of the preparation process and long-term storage at a high temperature of 40 °C [43]. However, antigenicity was maintained during long-term storage at 4 °C and 25 °C without the addition of a stabilizer [16]. Therefore, if the vaccine can be stored at room temperature or below room temperature and antigenicity can be maintained during storage, the addition of a stabilizer was only necessary to maintain antigenicity during the preparation process, including drying. For vaccine microneedles, various excipients are added in the formulation. However, because of the limited amount and space in microneedles, the increased amount of excipients could limit the dose of antigen. In cases when more than 10 times the amount of stabilizer and adjuvant was added to the formulation, only a small portion of antigen was allowed in the formulation. In this study, a small amount of stabilizer (60 μg) was added for RT-MN-T and LT-MN-T because the amount of antigen (3 μg) was for vaccination of mice. However, in the case of humans, it is necessary to use an H1N1 dose of more than 10 μg as well as 200 μg of stabilizer. That is, much more stabilizer is required for human vaccination. Therefore, the development of a process that does not require the addition of stabilizers makes possible the addition of adjuvants and can be used to manufacture more effective vaccine microneedles.

3.4. Immunogenicity of vaccine administered to mice using microneedles

3.4.1. Body weight changes and survival rates

In the animal experiments, the vaccinated mice were completely protected against lethal virus challenge. There was no significant difference in rate of body weight in all groups except for IM administration of PBS (Fig. 4a). When H1N1 was administered through the

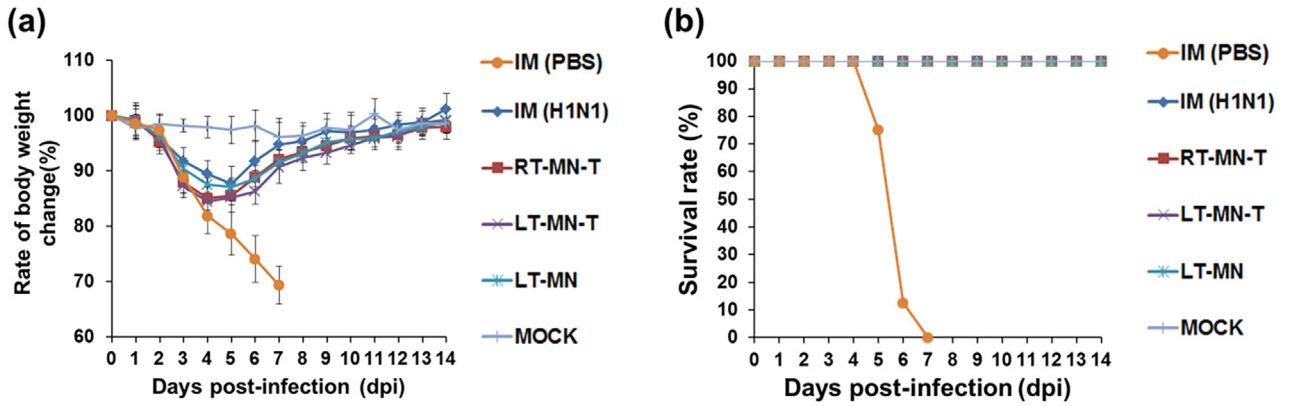


Fig. 4. Protective immunity after lethal challenge with homologous virus of immunized mice. Immunized groups were challenged with mouse-adapted H1N1 virus 2 weeks after immunization. (a) Rate of body weight changes and (b) survival rate after challenge with 5xLD50 of virus were monitored for 14 days. IM (H1N1): intramuscular administration, RT-MN-T: room-temperature H1N1 microneedles with stabilizer, LT-MN-T: low-temperature H1N1 microneedles with stabilizer, LT-MN: low-temperature H1N1 microneedles without stabilizer.

microneedles, the survival rate of the mice was 100% regardless of the addition of trehalose and the process temperature (Fig. 4b). H1N1 vaccination with microneedles was not different from the conventional IM injection method in survival rate or body weight change rate. The removal of the stabilizer and preparation at room temperature did not cause a significant difference.

In previous H1N1 microneedle studies involving experiments with mice, the activity of H1N1 decreased after the process without the addition of a stabilizer [20]. H1N1 was abruptly deactivated during the drying process at 25 °C, and it was difficult to maintain its activity without the addition of trehalose during the 24-h observation period [16]. HA activity decreased by 50% when vaccine was dried without stabilizer in formulation [16]. Even when trehalose was added, H1N1 activity decreased by 36% after the manufacturing process [44]. H1N1 was degraded during the microneedle processing within 24 h at 25 °C, and this change in activity affected the survival rate and change of body weight *in vivo* of the mice. However, if there was no significant change in antigenicity, it was difficult to obtain a large difference in survival rate and weight change. Therefore, a neutralization test was performed to observe in detail the effect of the process temperature on antigenicity.

3.4.2. Plaque reduction neutralization test

The amount of neutralization antibody produced from sera of the immunized mice was evaluated by PRNT. Mouse sera immunized with the microneedles prepared at 10 °C (LT-MN-T and LT-MN) showed a higher percentage of plaque reduction than those prepared at the RT condition with a stabilizer (RT-MN-T). When microneedles were prepared at 10 °C, there was no significant difference in percentage of plaque reduction between LT-MN-T and LT-MN by addition of a stabilizer (Fig. 5). Higher amounts of neutralizing antibodies in the sera of mice immunized with the microneedles prepared at a low temperature suggested better preservation of the active form of the vaccine antigen. The preparation process of the H1N1 microneedle vaccines under a 10 °C condition appears to have played a significant role in preserving the antigenicity of the vaccine. This result was consistent with the evaluation of the stability of the antigen according to the manufacturing process temperature, which was verified *in vitro* (see Section 3.3). Two conclusions follow: (1) The IM administration group had a lower neutralizing antibody than the microneedle group, which delivered the antigen into the skin layer where immune cells are distributed in a large amount. Thus, the effectiveness of inducing immunogenicity using microneedles can be verified. (2) The microneedle group prepared at low temperature showed a similar antibody titer regardless of the addition of a stabilizer. Therefore, when the whole microneedle production process is performed at a low temperature, the influence of the stabilizer and the denaturation of the antigen can both be minimized, and as a result, immunogenicity can be induced effectively.

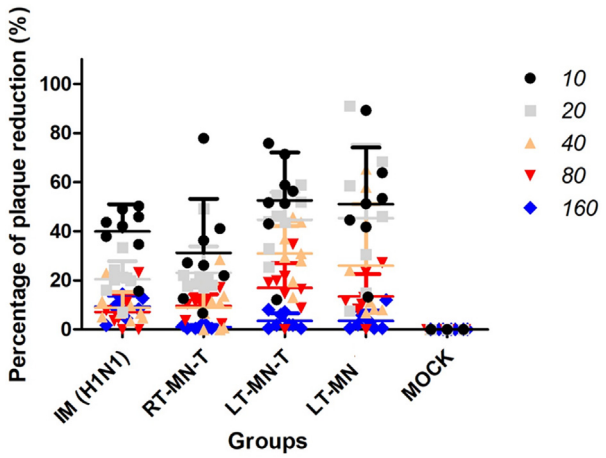


Fig. 5. Plaque reduction neutralization test (PRNT) for virus neutralization. IM (H1N1): intramuscular administration, RT-MN-T: room-temperature H1N1 microneedles with stabilizer, LT-MN-T: low-temperature H1N1 microneedles with stabilizer, LT-MN: low-temperature H1N1 microneedles without stabilizer.

4. Conclusions

In the case of the temperature-sensitive H1N1 antigen, denaturation of the antigen occurred during the manufacturing process, and the previous solution for inhibiting denaturation was the addition of stabilizers to the formulation. In this study, conducting the manufacturing process at a low temperature without a stabilizer was suggested. According to the results of the *in vitro* test, the antigenicity of H1N1 could be preserved by maintaining the temperature of the process at 10 °C without adding a stabilizer. In animal experiments, H1N1 microneedles were equivalent to IM administration of H1N1 in body weight changes and survival rates and antibody neutralization was better than IM administration of H1N1.

H1N1 microneedles were successfully prepared without a stabilizer by keeping the samples and the environment at a low temperature of 10 °C. Vaccine microneedles produced by this process can have the following advantages: (1) Human H1N1 vaccine requires a greater amount of antigen, so more stabilizer is needed. Because no stabilizer is added, more antigen can be loaded on an array of microneedles; (2) it is possible to reduce the difficulty of selecting the appropriate stabilizer depending on the antigen that is used; and (3) removing the stabilizer

can allow for the inclusion of other additives such as an adjuvant. This process can be applied to various temperature-sensitive biologics.

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