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Skin immunization with third-generation hepatitis B surface antigen using microneedles

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ABSTRACT

L-HBsAg is a third-generation hepatitis vaccine capable of inducing antibodies in non-responders and thus providing potentially therapeutic treatment. In this study, L-HBsAg was administered using microneedles (MN) without an adjuvant to induce intradermal (ID) immunization, and the efficacy of ID immunization was compared with that of intramuscular (IM) immunization that uses a conventional formulation with an adjuvant of aluminum hydroxide (L-HBsAg-AL-IM).

The L-HBsAg was dip-coated onto 800-μm-long microneedles made of polylactic acid (PLA). Delivery efficiency and administration time were determined through *in vitro* experiments using porcine skin. The denaturation of the formulation against sterilization by gamma rays was observed. A storage test and a freeze-thaw cycle test of the microneedles with trehalose as a stabilizer (L-HBsAg-MN-Tre) were observed. An antibody titer of L-HBsAg-MN-Tre was compared with that of the conventional IM immunization of the L-HBsAg solution with aluminum hydroxide (L-HBsAg-AL-IM).

The formulation containing L-HBsAg was located on the upper third of the microneedle tips. The formulation on the MN was dissolved and delivered within 30 min of insertion into porcine skin *in vitro*. Trehalose was selected as a stabilizer, and the stabilizing effect increased with the increase of trehalose content in the solidified formulation. L-HBsAg-MN with 15% of trehalose was stable for 7 days at 40 °C and showed increased stability compared to the conventional liquid formulations. L-HBsAg-MN-Tre showed improved stability during the freeze-thaw cycle. The antibody titer of L-HBsAg-MN-Tre at 28 days was higher than that of L-HBsAg-AL-IM.

ID administration of L-HBsAg-MN-Tre showed better efficacy and improved thermal and freeze thaw stability compared to L-HBsAg-AL-IM. Therefore, L-HBsAg-MN-Tre administration showed the possibility of ID delivery of L-HBsAg without the use of an adjuvant for the efficacy, convenience, and safety of pediatric vaccination.

1. Introduction

Hepatitis B is well known as a major global health problem caused by the hepatitis B virus (HBV), which is 7th leading cause of death worldwide. The most effective prevention currently available is immunization with the hepatitis B vaccine [1].

The first generation of HBV vaccines were used beginning in 1980, when the hepatitis B surface antigen (HBsAg) produced from the plasma of chronic HBV infected individuals [2]. The second generation of HBsAg, yeast-derived HBsAg with a small hepatitis B surface protein (S protein), was introduced to prevent the transmission of blood pathogens from human blood products of those

Abbreviations: L-HBsAg, third-generation hepatitis B surface antigen; HBV, hepatitis B virus; VLPs, virus-like particles; IM, intramuscular; ID, intradermal; MN, microneedles; Alum, aluminum hydroxide; PDMS, polydimethylsiloxane; L-HBsAg-S, L-HBsAg in aqueous solution; L-HBsAg-IM, intramuscular administration of L-HBsAg without Alum; L-HBsAg-AL-IM, intramuscular administration of L-HBsAg with Alum; L-HBsAg-MN, microneedle administration of L-HBsAg without Alum and trehalose; L-HBsAg-MN-Tre, microneedle administration of L-HBsAg with trehalose; L-HBsAg-MN-Tre(F3), microneedle administration of L-HBsAg with trehalose 15%(w/w); PLA, polylactic acid; CMC, sodium carboxymethyl cellulose.

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treated with the first generation of HBsAg [3]. The S protein of second-generation HBsAg is introduced into virus-like particles (VLPs) that are 22 nm in size, mimicking the structure of the real virus. This second generation of HBsAg is widely used for vaccination of newborns and adults in over 170 countries [4].

Second-generation HBsAg has not produced effective immunogenicity due to factors such as advanced age, renal dysfunction, liver disease, smoking, and immunosuppression [5,6]. Potential reasons for non-responsiveness are genetically induced by resistance [7] or an increase in S gene mutations [8]. In attempts to improve the effectiveness of vaccination, large- and middle-sized surface proteins (pre S1 and pre S2) were merged into S proteins to induce improved antibody response against HBV [9,10] and increase the antibody response of S gene mutants [11]. As the demand for enhanced immunogenicity to overcome the non-responsiveness to the S protein of HBsAg has increased, the third-generation HBsAg (with combination of three envelope proteins: S, pre-S1 [L protein] and pre-S2 [M protein]), has been developed and offers promising results for greatly improved vaccine efficacy [12,13].

An additional approach to improving immunogenicity against HBV is changing the administration route from intramuscular (IM) to intradermal (ID). The epidermal and dermal layers of the skin are rich in antigen-presenting cells such as Langerhans cells and dendritic cells [14]. These colonized cells have high MHC class II expression and are important mediators of antiviral immunity [15,16]. Thus an ID administration requires a lower dose of vaccine compared to an IM administration [17]. Furthermore, the safety and efficacy of ID administration of the HBV vaccine have been demonstrated by comparing ID to IM administration. [18–20], However, ID administration requires application into a skin layer at least 1 mm thick and it is not easy to administer precisely. Thus microneedles (MN) have been introduced as an easy-to-use, painless way to control injection depth and delivery of the right dose of antigen into the skin layer. Various antigens have demonstrated improved efficacy with ID administration using MN compared to IM administration [21–26]. The second-generation hepatitis B vaccine was delivered into the skin layer using MN administration, and MN encapsulating HBsAg showed effective antibody production and improved storage stability [27].

In this study, immunization through the skin using the third generation of the hepatitis vaccine with pre-S1/pre-S2/S (L-HBsAg) was performed with MN for first time, as shown in Fig. 1. Alum was not included in the MN formulation. Stability of the vaccine was observed during the harsh storage conditions and the freeze-thaw cycle. Animal experiments demonstrated immunological efficacy of the vaccine administered intradermally with MN with a stabilizer compared with IM administration.

2. Materials and method

2.1. Animals

Five-week-old female BALB/c mice were purchased from Koatech (Pyungtek, Korea). The animals were housed in standard pathogen-free facilities and maintained with free access to food and water. All studies were approved by Institutional Animal Care and Use Committees (IACUC) at the International Vaccine Institute (2017–001).

2.2. Materials

L-HBsAg was obtained from the CHA Vaccine Institute (Seongnam, Korea). PLA was purchased from LACTEL (Birmingham, AL). Sodium carboxymethyl cellulose (CMC), trehalose dihydrate, and trypan blue were purchased from Sigma-Aldrich Ltd (St. Louis, MO). Phosphate-buffered saline (PBS) and alum were purchased from Thermo Fisher Scientific (Rockford, IL). An Enzyme-linked Immunosorbent Assay (ELISA) Kit for quantification of coated L-HBsAg on MN was purchased from Alpha Diagnostic International (San Antonio, TX).

2.3. MN fabrication and coating process

Master structures of microneedles were fabricated using a micromilling process. The master structure has 145 square pyramids with a base width of 350 μm and a length of 800 μm. Liquid polydimethylsiloxane (PDMS; base: curing agent 10:1 w/w) was prepared and poured over the master structures. The PDMS was cured at 70 °C for 3 h, and the inverse-replica PDMS molds were obtained from the master structures. Pre-coated microneedles were prepared from PLA using a micromolded PDMS mold. PLA pellets were put on the mold and melted at 190 °C for 60 min in an oven (Eyela VOS 201SD, Japan). PLA MN arrays were obtained by removing cooled MN from the PDMS mold. The dimension of MN were observed using optical microscopy (Nickon Eclipse 80i, Tokyo, Japan) and a scanning electron microscope (SEM) machine (Jeol, JSM 7500F, Japan).

Before the L-HBsAg formulation was coated on the MN, PLA MN were treated with UV/O_3 irradiation for 15 min using the UV-Ozone cure system (MT-UV-O 05, Minuta Tech, Korea). CMC was dissolved in PBS for 10 min, and the solution was sterilized under autoclave at 121 °C for 15 min. The L-HBsAg solution was added to the CMC gel solution and they were mixed at room temperature. The coated L-HBsAg formulation was then loaded into the coating well to a depth of 600 μm. The coated MN were dried at 4 °C for 30 min. The sham MN (without L-HBsAg in the formulation) were prepared by the same process. The L-HBsAg solution and the

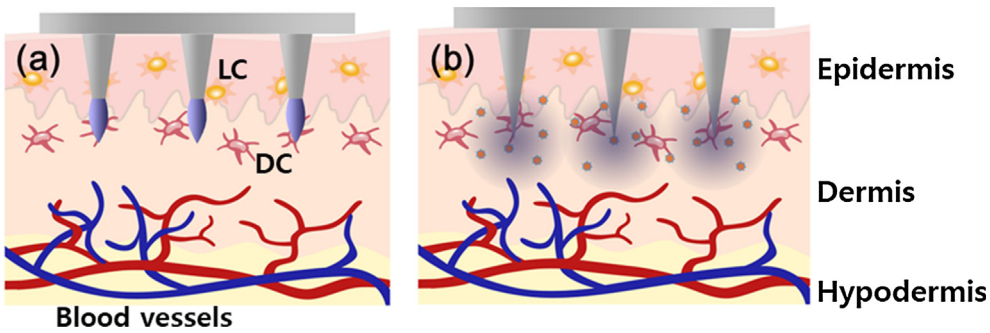

Fig. 1. Schematic description of intradermal administration of L-HBsAg using L-HBsAg–coated microneedles. (a) insertion of L-HBsAg–coated microneedles into epidermis and dermis; (b) full dissolution of coated formulation after insertion. LC: Langerhans cells. DC: Dendritic cells.

aluminum hydroxide (Alum) solution were mixed at a ratio of 1:1 (v/v) for IM administration, and then the mixture was shaken for 60 min at room temperature. These samples were stored in the refrigerator before the animal experiments.

2.4. Quantification of coated L-HBsAg on MN

The amount of L-HBsAg on the MN was measured using an HBsAg ELISA kit (Alpha Diagnostic Intl., San Antonio, TX). The L-HBsAg-MN was put in a 0.5 ml PBS solution for 20 min. The solution was incubated in microwells that were pre-coated with highly specific anti-HBsAg IgG. Purified antibody conjugated-horseradish peroxidase was then added. Substrate concentration was determined by a spectrophotometric machine at 450 nm. The concentration of active agents in the sample was calculated with a standard linear curve.

2.5. In vitro skin insertion

In order to demonstrate the penetration ability of coated polymer MN into a thick skin layer, trypan blue was added to the L-HBsAg formulation of and then the formulation was coated on the polymeric microneedles. The coated microneedles were applied to cadaver porcine skin with 30 N of force for 10 min. Then the punctures in the surface of the skin were counted using an optical microscope (sv-35, Sometech, Seoul, South Korea). The three separate samples were each examined. In order to determine the time required for the coated formulation to dissolve, the MN containing trypan blue were inserted into the skin for 2, 10, 20, and 30 min and the remaining coated layer on the MN was observed with an optical microscope.

2.6. Stability of antigen under sterilization process and storage condition

2.6.1. Antigenicity change during sterilization process

The formulation of L-HBsAg without a stabilizer was coated on MN and then the L-HBsAg-MN were exposed to a 15 kGy gamma irradiation for 2 h 45 min. The antigenicity of L-HBsAg on MN was measured with an ELISA kit ($n = 5$).

2.6.2. Storage stability with addition of trehalose

Trehalose has been known as a promising candidate for stabilizing a vaccine delivered with MN; therefore formulations with different concentrations of trehalose were fabricated and coated onto the MN. The ingredients in the formulations are shown in Table 1.

Microneedles were coated with formulations with different trehalose content and named F1, F2, and F3, as shown in Table 1. These MN were stored under 40 °C for 7 days to investigate the effects of trehalose on stability. After 7 days, the antigenicity of the F1, F2, and F3 antigens were determined and compared to the antigenicity of each group before being stored. These formulations were coated on PLA MN and named L-HBsAg-MNs-Tre(F1), L-HBsAg-MNs-Tre(F2), and L-HBsAg-MNs-Tre(F3). The formulation that stabilized the antigen in the MN most effectively was chosen to be used in the next step of the stability experiments.

Table 1
The ingredients of formulations with different trehalose content.

Formulation	F1	F2	F3
L-HBsAg	0.04%	0.04%	0.04%
CMC	6%	6%	6%
Trehalose	0%	4.5%	15.0%

2.6.3. Stability during storage

The F3 formulation was the most effective formulation in stabilizing the antigen in the MN. The F3 formulation was coated on MN and called L-HBsAg-MN-Tre(F3). L-HBsAg-MN-Tre(F3) and L-HBsAg-MN were stored in a sealed aluminum foil bag with a desiccant. L-HBsAg solution (L-HBsAg-S), L-HBsAg-MN, and L-HBsAg-MN-Tre(F3) were stored at three storage temperature of 4 °C, 25 °C, and 40 °C for 28 days. The antigenicity of L-HBsAg at 7 days and 28 days was measured with an ELISA kit.

2.6.4. Stability by freeze-thawing process

L-HBsAg-S, L-HBsAg-MN, and HBsAg-MN-Tre(F3) were frozen at −20 °C for 4 h and then completely thawed at 4 °C for 4 h. This freeze-thaw cycle was repeated 10 times. Antigenicity was measured at the first, third, and tenth cycles with an ELISA kit. The antigenicity of the samples was compared with the antigenicity of non-treated L-HBsAg-S.

2.7. In vivo immunization study

HBsAg-MN-Tre(F3) was administered into mice to examine its immunogenicity. Before immunization, the mice's backs were shaved using a hair clipper (Thrive, Japan). Then the skin on the back of the mice was exposed by removing the fine hair with hair removal cream (Nair, USA) and washed with 70% ethanol a day before the immunization. The day of the immunization, the animals were anesthetized by intraperitoneal injection with 100 mg/kg ketamine (Yuhan, Korea) mixed with 12.5 mg/kg rompun (Bayer, Germany). The drug was injected into the mice by the IM route (thigh muscles) and the ID route by MN. The MN were injected using a force of about 2 N and held for 30 s. A clipper was used to keep the MN attached to the skin during the treatment time (30 min). Each group is described in Table 2. Two weeks after the first immunization, the same vaccine protocol was applied for boosting immunization, and sera were collected at 2 weeks after every immunization from the retro-orbital plexus of anesthetized mice. After the animal experiment, the recovered microneedles were immersed in 0.5 ml of PBS for 30 min and the amount of L-HBsAg remaining in the solution was measured by the ELISA method.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Levels of antibody in sera were measured by enzyme-linked immunosorbent assay (ELISA). For antigen coating, 2 μg/ml of HBsAg were incubated in 50 mM sodium bicarbonate buffer (pH 9.6) on the 96-well plates (Nunc, Roskilde, Denmark) at 4 °C overnight. The coated plates were washed three times with PBST, and incubated with blocking buffer (1% Bovine serum albumin in PBS; Merck, Germany) at 37 °C for 1 hr. The blocked plates were washed three times with PBST(0.05% Tween-20 in 1xPBS, pH7.4). For the antibody reaction, primary antibody was prepared 1:30 dilution of sera with blocking buffer, added to the plate in 5-fold serial dilution and incubated at 37 °C for 1 hr. After three washes with PBST, 1:3000 diluted HRP-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA) was added to each well, and incubated at 37 °C for 1 h. After washing with PBST, TMB (3,3′5,5′-tetramethylbenzidine) solution (Millipore) was added to each well. More than 5 min of color development at RT, the reaction was stopped by adding of 0.5 N HCl (Sigma). The absorbance at wavelength 450 nm was measured by an ELISA reader Spectra Max 190 (Molecular Devices, Sunnyvale, CA, USA) and the antibody titer was calculated using Softmax program (Molecular Devies). The results were transformed into logarithmic 10 values.

Table 2
The composition of L-HBsAg, Aluminum hydroxide (Alum), carboxy methyl cellulose (CMC) and trehalose in formulation of samples for animal test.

Group	PBS (IM)	Sham (MN)	Sham (MN-Tre)	L-HBsAg-IM	L-HBsAg-Al-IM	L-HBsAg-MN	L-HBsAg-MN-Tre(F3)
Route	IM	MN	MN	IM	IM	MN	IM
L-HBsAg	–	–	–	0.15	0.15	0.15	0.15
Alum	–	–	–	–	+	–	–
CMC	–	+	+	–	–	+	+
Trehalose	–	–	+	–	–	–	+

+: with, –: without, 0.15: 0.15-μg dose.

2.9. 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 $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Characteristics of L-HBsAg microneedles

L-HBsAg MN were coated with three envelope proteins, L-protein, M-protein, and S-protein, with the amount of the three proteins in the coating formulation measuring about 8%, 17%, and 75%, respectively. L-HBsAg contained virus-like particles with sizes ranging from 20 nm to 40 nm [13].

L-HBsAg has been developed to induce antibodies in non-responding hepatitis B patients and to provide therapeutic treatment for these patients [12]. Formulations consist of a combination of a CMC thickening agent, trehalose stabilizer, an L-HBsAg antigen, and an Alum adjuvant. CMC was added as a thickening agent for uniform coating to prevent excessive moisture on an array. Trehalose was selected as an additive to improve stability of L-HBsAg because trehalose is an effective stabilizer for other vaccines [28,29] and for second-generation HBsAg consisting of S-protein [30]. In our study, Alum was excluded from the microneedle formulation because, although it has been used as a safe adjuvant for IM administration for a long period of time, the safety of ID administration that includes Alum is still unclear. Especially in case of pediatric vaccination with L-HBsAg, ID vaccination without an adjuvant is intended to reduce toxicity and pain.

The microneedle array contained 145 needles 800 μm in length, with a base diameter of 350 μm as shown in Fig. 2(a) and (b). The needles were spaced 300 μm apart within the 1 cm diameter circle. The measured value of L-HBsAg content per array was 0.151 μg (average, $n = 5$). The intended 0.15 μg amount of antigen could be obtained by dip coating. The formulation was located within 300 μm from the end of the microneedle tip, and SEM images showed the L-HBsAg formulation located uniformly on the upper third of the microneedles, as shown in Fig. 2(c). The local distribution of the L-HBsAg formulation was intended to deliver a predetermined dose of L-HBsAg into the skin where antigen-presenting cells were mainly distributed [31].

3.2. In vitro skin insertion

When trypan blue MN were administered into cadaver porcine skin for 30 min, the MN left visible blue dots, as shown in Fig. 3(a). This result demonstrated that all microneedles were inserted and that the coating formulation was delivered successfully. Pre-coated microneedles were prepared using poly lactic acid because this material has sufficient mechanical strength for successful insertion [32].

The dissolution rate of the coated formulation was also investigated by insertion and removal of trypan blue MN into the cadaver porcine skin for a predetermined time. As shown in Fig. 3(b), the coated layer dissolved gradually from the time of insertion and was totally dissolved in the skin after 30 min. Therefore, MN insertion time was set at 30 min for the *in vivo* animal experiment.

3.3. Stability study

3.3.1. Stability during sterilization process

Sterilization was performed to determine if gamma ray sterilization was appropriate for L-HBsAg-MN. In this study, the antigenicity of L-HBsAg on MN was measured after exposure to gamma rays (15 kGy) for 2 h 45 min. The antigenicity of L-HBsAg was reduced to 29% ± 2% of original antigenicity after exposure. The average intensity of gamma radiation used in previous studies was used in this study. Previous studies reported that gamma sterilization could affect the stability of an antigen [33] on MN. MN containing ovalbumin were exposed to gamma radiation (25 kGy) for 14 h, after which antigenicity was reduced to 41.7% of original activity [33]. Therefore, it is more appropriate to prepare L-HBsAg MN in an aseptic environment instead of using gamma ray sterilization.

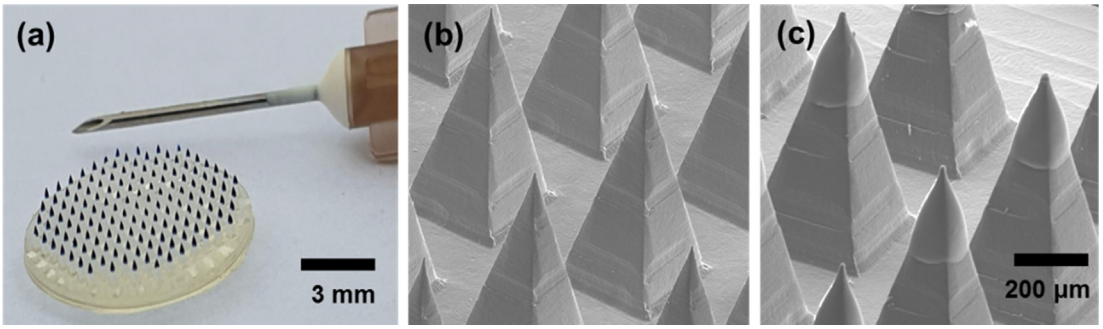

Fig. 2. (a) Comparison images of a trypan blue microneedle array adjacent to a 26-gauge needle to illustrate their relative sizes. (b) SEM image of pre-coated PLA microneedles. (c) SEM image of microneedles coated with L-HBsAg formulation (L-HBsAg-MN-Tre(F3)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

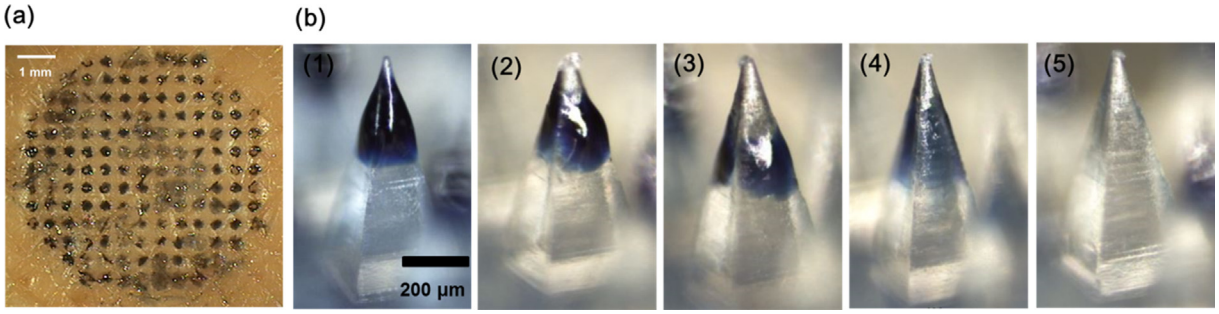

Fig. 3. (a) Optical image of blue dots on treated skin after insertion and removal of trypan blue–coated microneedles into porcine skin *in vitro*. (b) Optical images of gradual dissolution of trypan blue formulation from microneedles at (1) 0, (2) 2, (3) 10, (4) 20, and (5) 30 min of insertion into porcine skin *in vitro*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.2. Stability during storage

The antigenicity of vaccine products can be damaged during the storage test, so a stabilizer was added to improve the stability of the vaccine. Trehalose has been used to increase the stability of the antigen during the storage of microneedle products [34,35], and it has been used to increase the stability of second-generation HBsAg [30]. The percentage of antigenicity of L-HBsAg remaining after L-HBsAg-MNs-Tre(F1), L-HBsAg-MNs-Tre(F2), and L-HBsAg-MNs-Tre(F3) were stored at 40 °C for 7 days was measured and is displayed in Fig. 4. As shown, when the content of trehalose was higher in the formulation, the antigenicity of L-HBsAg was higher. The F3 formulation containing 15% trehalose showed the greatest stability without change of antigenicity at 40 °C for 7 days, so this formulation was chosen for the storage test and the freeze–thaw cycle test.

The change in antigenicity of the F3 formulation was observed according to storage temperature. L-HBsAg-MN-Tre(F3) was stored at three different temperatures (4 °C, 25 °C, and 40 °C). The changes in antigenicity of L-HBsAg-MN-Tre(F3), L-HBsAg-MN (MN without trehalose), and L-HBsAg-S (L-HBsAg solution without trehalose) were compared during storage. As shown in Fig. 5, antigenicity did not change in any of the samples for 28 days at 4 °C and 25 °C. However, there was difference in antigenicity at 40 °C. The percentages of antigenicity of L-HBsAg-S and L-HBsAg-MN were 42% and 1.3% at 40 °C at Day 28 respectively, as shown as the black solid circle in Fig. 5(a) and (b). The antigenicity of L-HBsAg-MN-Tre(F3) did not change during 7 days of storage at 40 °C and decreased slowly to 79% of initial antigenicity at 40 °C on Day 28, as shown as the black solid circle in Fig. 5(c). This result demonstrated that trehalose was a suitable stabilizer for L-HBsAg. However, solidified L-HBsAg without trehalose shows easier denaturation than liquid L-HBsAg. Usually, solid formulations provide better stability than liquid formulations, whereas solid L-HBsAg showed lower temperature stability than liquid formulations. Therefore, the addition of trehalose in the formulation was necessary for improved storage stability and distribution stability without a cold chain.

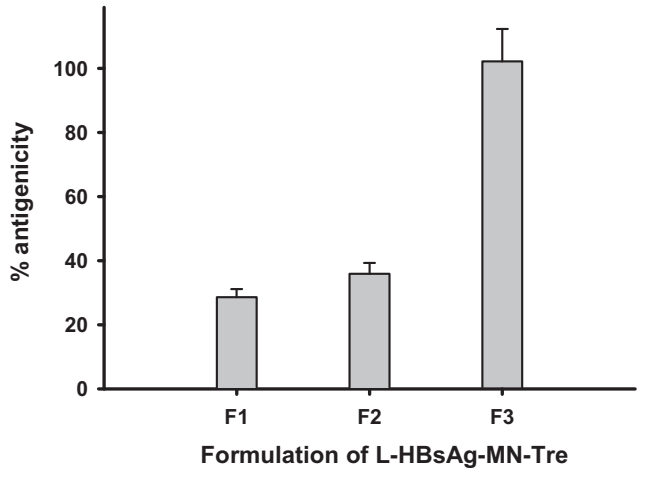

Fig. 4. Percentage of remaining antigenicity of microneedles coated with F1, F2, and F3 formulations at 40 °C for 7 days. Data presented as average ± *SD* ($n = 3$).

The reduction of antigenicity in the liquid group was comparable with findings in other studies when the samples are exposed to high temperature [36]; the commercial product of second-generation HBV vaccine is also less stable at high temperature [37]. A decrease of antigenicity in the coated formulation without stabilizer at high temperature was also observed, and the high molecular weight of CMC could induce the aggregation of antigen during the process [38,39]. Therefore, the addition of trehalose to the formulation improved stability by lowering membrane phase transition and inhibiting aggregation between adjacent vesicles [39,40].

3.3.3. Storage during freeze-thaw cycle

Not only is high temperature a barrier to vaccine distribution, but low temperature (below the freezing point of water) also causes vaccine deformation. Previous studies have demonstrated that freeze-thaw cycles reduce the antigenicity of vaccine products [41,42]. Moreover, the HBsAg particle vaccine can be damaged as a result of freezing conditions [43]. Therefore, the stability of the thermal cycle is also important when the vaccine is exposed to the environment during delivery in cold regions. In this study, we examined the stability of LHBsAg-S, L-HBsAg-MN and L-HBsAg-MN-Tre(F3), during 10 freeze-thaw cycles. The results showed that there was no reduction of antigenicity L-HBsAg-MN-Tre(F3) through the 10 cycles as shown in Fig. 6. In contrast, L-HBsAg-MN showed a significant decrease of antigenicity over 3 cycles and most antigenicity was lost at 10 cycles. A reduction of antigenicity also was investigated in the L-HBsAg-S group at 3 cycles, and it was found that a decrease of antigenicity was slow until the 10th cycle (Fig. 6).

Recent results have demonstrated that addition of sugar can prevent the reduction of antigenicity as a result of freezing [44]. This result shows that the addition of trehalose increases the stability of third-generation hepatitis B in a solidified MN formulation through repeated freeze-thaw cycles.

3.4. Immunization study

As shown in Fig. 7(a), on Day 13 after the primary immunization, HBsAg-specific IgG in sera was detected in all samples (via both ID and IM administration) with L-HBsAg and IgG was not

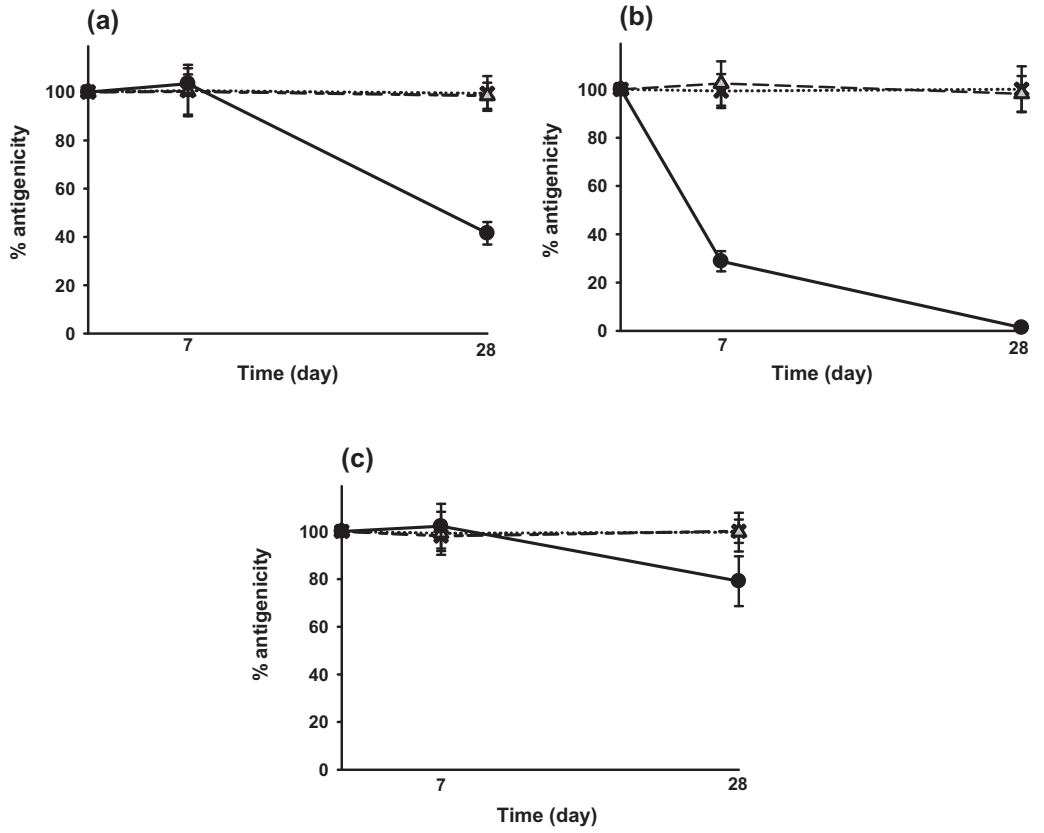

Fig. 5. Percentage of antigenicity of L-HBsAg remaining after storage at different temperatures at 7 days and 28 days. Three groups were (a) L-HBsAg-S, (b) L-HBsAg-MN and (c) L-HBsAg-MN-Tre(F3) respectively. Data presented as average ± *SD* ($n = 3$), (···✖··· 4 °C, –△– 25 °C, —●— 40 °C).

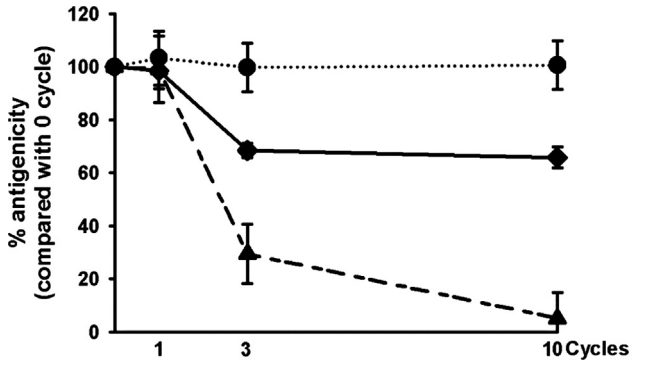

Fig. 6. Percentage of antigenicity of L-HBsAg of L-HBsAg-S (—◆—), L-HBsAg-MN (–▲–), and L-HBsAg-MN-Tre(F3) (··●··) during freeze–thaw cycles. Data presented as average ± *SD* ($n = 3$).

detected in the sample without LHBsAg. The antibody titers of L-HBsAg-MN and LHBsAg-MN-Tre were higher than those of L-HBsAg-IM and L-HBsAg-Al-IM on Day 27 after primary and secondary immunization ($p < .002$ and $p < .003$, respectively). There was no difference in the antibody titers of L-HBsAg-MN and LHBsAg-MN-Tre on Day 27 ($p = .43$). The addition of trehalose to the formulation did not affect the antibody titer. When the third generation of L-HBsAg was delivered intradermally using microneedles without an adjuvant, more antibodies were produced compared to that produced following IM administration of a conventional formulation with Alum. Previous studies showed that MN loaded with the second generation of HBsAg produced antibody lower than or comparable to the immune response produced by IM administration of HBsAg without an adjuvant [30,36].

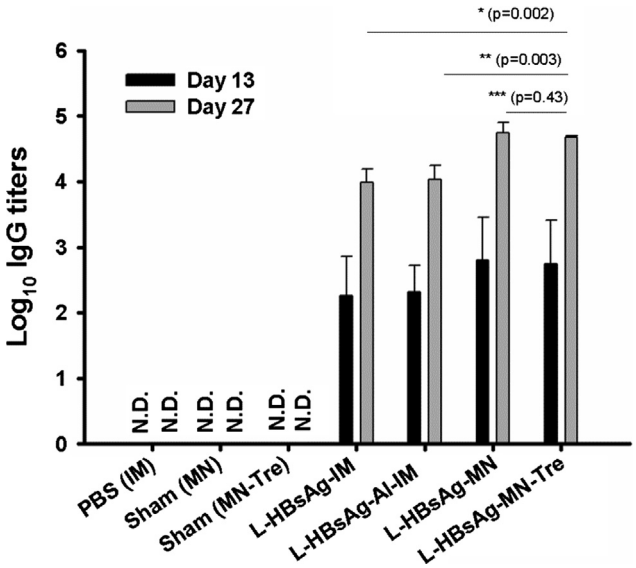

Fig. 7a. IgG titer after primary (Day 13) and boost immunizations (Day 27); sham (MN-Tre) was MN without L-HBsAg and trehalose; *, **, *** were compared between L-HBsAg-MN-Tre to IM injection of L-HBsAg solution, L-HBsAg plus Alum, and L-HBsAg-MN; N.D. = was not detected. Data represented average ± *SD* ($n = 5$).

The antibody titers of IgG1 and IgG2a were used to evaluate the type of Th response. It was demonstrated that all groups were capable of eliciting both IgG1 and IgG2a responses. In the IgG1 result, there was the same trend with a total titer of IgG at Day 27, as L-HBsAg-MN administration by microneedles induced a higher antibody level compared to IM injection of L-HBsAg and L-HBsAg with

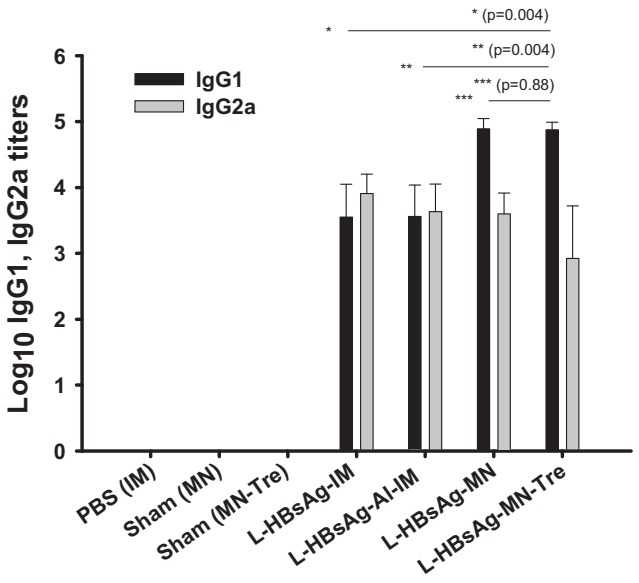

Fig. 7b. IgG1 and IgG2a titers after boost immunization (Day 27); *, **, *** were compared between L-HBsAg-MN-Tre to IM injection of L-HBsAg solution, L-HBsAg plus Alum, and L-HBsAg-MN, respectively; N.D. = was not detected. Data represented average ± *SD* (n = 5).

Alum, as shown in Fig. 7(b). The IgG1 titers of L-HBsAg-MN and LHBsAg-MN-Tre were similar to each other (p = .88). There was no difference in the IgG2a titers of L-HBsAg-MN, L-HBsAg-MN-Tre, and L-HBs-IM-Al. In the mouse's antibody response pattern, IgG1 is a marker for Th2-type immune response and IgG2a is a marker for Th1-type immune response [45,46]. The IM administration group induced a balanced subtype of IgG level. In contrast, in the ID administration group, the type of immune response was Th2 predominant; this predominant Th2 response might be caused by the type of antigen-presenting cells of Langerhans cells and dendritic cells in the epidermis and dermal layers [36,47–49].

Compared to previous results, a higher value of antibody titer was obtained with the L-HBsAg–coated microneedle system. In this study, antigens without Alum were administered using microneedles. The aim of this study was to use microneedles for pediatric immunization to avoid pain and also to avoid the use of an adjuvant for children's vaccination. Alum is an adjuvant approved for IM administration of vaccine, but ID administration of Alum has not yet been proven to be stable [12]. As shown in the results, ID administration of L-HBsAg produced a higher antibody titer than IM administration of L-HBsAg both with and without Alum. Thus L-HBsAg-MN-Tre is an advantageous administration method of L-HBsAg for antibody production without the use of Alum or other adjuvants.

The amount of HBsAg remaining on microneedles was 10 ± 0.7% of the coated HBsAg. Thus, about 90% of the coated HBsAg (specifically, 0.135 μg) was delivered into the skin. The 10% loss was attributed to the adhesion of a small amount of the dissolved formulation to the surface of the microneedle tips after 30 min of insertion and removal.

4. Conclusion

The third generation of hepatitis B antigen (L-HBsAg) was formulated without an adjuvant for microneedles, which were delivered into the skin. Microneedle injection (ID administration) induced superior antibody production compared to IM injections of L-HBsAg with an Alum adjuvant. The antigenicity of L-HBsAg-MN was reduced by gamma ray irradiation. When 15% (w/w) of trehalose was added as a stabilizer in a solidified formulation, the antigenicity of L-HBsAg-MN-Tre(F3) did not change even at a high temperature of 40 °C for 7 days and after 10 freeze–thaw cycles. Thus ID injection using microneedles loaded with L-HBsAg is an attractive delivery method to replace IM injection because it provides increased storage stability, increased delivery stability, and improved antibody-inducing effect. L-HBsAg-MN without an adjuvant can reduce pain as well as even avoid toxicity for pediatric immunization.

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.

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