ORIGINAL ARTICLE

Effect of ultrasound treatment of the skin on activation of Langerhans cells and antibody production in rodents

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J. Adv. Pharm. Technol. Res.

ABSTRACT

In this study, we investigated whether stimulating the skin with ultrasound (US) could activate Langerhans cells (LCs) – antigen-presenting cells in the epidermis and stimulate antibody production following the subcutaneous and intradermal injection of ovalbumin (OVA) in hairless rats and BALB/c mice. Three different US frequencies (20 kHz, 1, and 3 MHz) were used and the expression of langerin was monitored as a marker for the activation of LCs in the epidermal sheet. In hairless rats, the langerin signal peaked at 12 h post-US treatment and returned to control levels at 24 h. Its expression increased with increasing irradiation time, up to 20 min, and 20 kHz US induced the highest langerin expression among the three frequencies tested. These results were reproduced in BALB/c mice. When the skin was pretreated with 20 kHz US at 0.41 W/cm^2 for 10 min, the production of OVA-specific immunoglobulin G1 in mice increased by 2.8- and 3.4-fold 28 days after subcutaneous or intradermal OVA injections, respectively. These findings indicate that stimulating the skin with US can trigger skin immune responses, leading to effective antigen-specific antibody production. US-assisted transdermal vaccine delivery delivers antigens to the skin and evokes an immune response, providing an effective noninvasive immunization strategy.

Key words: Adjuvant-like effect, immune response, Langerhans cell, langerin, ultrasound

INTRODUCTION

Viral infections including COVID-19 and influenza pose serious global health threats. Vaccination is the most cost-effective method of avoiding such infections. However, currently, most vaccination techniques are invasive and elicit pain and fear among the recipients. Thus, novel vaccination techniques using needle-free devices are desirable.[1]

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Submitted: 19-Nov-2022 **Revised:** 26-Jan-2023
Accepted: 07-Feb-2023 **Published:** 13-Apr-2023

Access this article online	
Quick Response Code:	Website: www.japtr.org
	DOI: 10.4103/japtr.japtr_647_22

Transcutaneous vaccination stimulates immunocompetent dendric cells in the skin to produce strong immune responses. Several enhancing techniques for transdermal drug delivery, including microneedles (MNs) and ultrasound (US), are potentially available for cutaneous vaccination.[2] Tezel *et al*. reported that pretreating the skin with low-frequency US (20 kHz) could evoke immune responses, evidenced by the activation of Langerhans cells (LCs).[3] Skin irritation induced by tape stripping to remove the stratum corneum (SC) activates LCs, whose dendrites then extend beyond the tight junction barrier just below the SC to capture antigens outside the barrier.[4] These findings indicate that physical stimuli used for transdermal transport enhancement can evoke cutaneous immune responses and increase antibody production.

How to cite this article: Enjo S, Hazama Y, Kimura S, Morimoto Y, Ueda H. Effect of ultrasound treatment of the skin on activation of Langerhans cells and antibody production in rodents. J Adv Pharm Technol Res 2023;14:94-8.

In our previous study, we demonstrated that physically stimulating the skin with MNs effectively activated LCs in C57BL/6 mice.[5] Given that MNs have been used for delivering drugs, including large molecules like antigens, to the skin, drug delivery techniques studied extensively enable effective vaccination to be utilized.[2,6]

We have also previously shown that low-frequency US (40 kHz)-enhanced skin permeation of large molecules, up to at least 40 kDa. The US-induced asymmetrical cavitation collapses at the skin surface are related to the mechanism governing the enhancement of skin penetration.[6] Stimuli like these cavitation collapses may have the potential to elicit immune responses in the skin, besides improving the skin penetration of applied molecules. Thus, US-assisted transdermal vaccine delivery serves both functions of delivering antigens in the skin and evoking a skin immune response, making it a potentially effective noninvasive vaccination strategy. However, the potential activation of skin immune responses after the application of US has not been well-characterized yet.

This study aimed to investigate the skin immune responses after applying US varying in its frequency, intensity, and irradiation time. The expression of langerin in the epidermal sheet was measured as an index of the potential activation of LCs after US treatment. Furthermore, potential antibody production against intradermally injected ovalbumin (OVA) was evaluated after applying US.

MATERIALS AND METHODS

Materials

OVA (albumin, chicken egg; Grade V) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade.

Animals

Female WBN/ILA-Ht strain hairless rats were obtained from Ishikawa Experimental Animal Laboratories (Saitama, Japan). Female BALB/c mice were obtained from Sankyo Labo Service Co., Ltd. (Tokyo, Japan). All animal experiments were approved by the Institutional Animal Care and Use Committee of Josai University (JU19017).

Ultrasound equipment

Three different frequencies (20 kHz, 1, and 3 MHz) were used in the present study. A custom-built US transducer generating 20 kHz waves was procured from Dai-Ichi High Frequency (Tokyo, Japan), while the ones generating 1 and 3 MHz waves were procured from Honda Electronics Co. Ltd. (Tokyo). A function synthesizer (WF1943, NF Co., Japan) along with an amplifier (HSA4012, NF Co.) was used to drive the transducers.

In vivo ultrasound treatment and subcutaneous injection

Animals were treated under anesthesia with pentobarbital. The hair of the back skin was removed using an electric shaver and Epilat (Kracie, Tokyo, Japan), after which the animals were left for 1 h to allow the irritation to quieten. For US treatment, the application chamber was attached to the hair-removal site and 5 mL phosphate-buffered saline at 32°C was applied to it. The US transducer was placed 3 mm above the skin surface. Three different frequencies of US were applied for 1–20 min. The corresponding US intensities were 0.13, 0.42, and 1.19 W/cm^2 for 20 kHz, 0.103 W/cm^2 for 1 MHz, and 0.186 W/cm^2 for 3 MHz, as calculated by the calorimetric method.[7] 1 MHz and 3 MHz intensities were maxed values. Another individual without any treatment except for the removal of hair served as a control. Skin samples were excised at 6, 12, and 24 h after each treatment to assess the immune responses. To compare with the US treatment, 0.1 mL saline was injected subcutaneously (*s.c.*) at three different points on the back skin.

Immunostaining of Langerhans cells and microscopic observation

Activation of LCs in the treated skin was determined by immunofluorescence staining according to the procedure described by Kubo *et al*.[8] Skin samples were fixed in a 95% ethanol solution for 30 min on ice and incubated in a solution of 3.8% thiocyanate ammonium for 30–60 min at 37°C to separate the epidermis from the dermis. The epidermal sheet was permeabilized with 1% Triton X-100 for 30 min, blocked in 10% skim milk solution for 1 h, and incubated with langerin/CD207 polyclonal antibody at 4°C overnight. The following day, incubated with goat anti-rabbit IgG H and L (Alexa Fluor® 568) preabsorbed at room temperature for 2 h, and mounted in Vectashield mounting medium. Immunostained langerin in the epidermal sheet was observed under a confocal laser-scanning microscope (FV1000, Olympus). Image Pro® software (Media Cybernetics, Silver Spring, USA) was used to quantify the resulting fluorescence images.

Immunization by ovalbumin with and without 20 kHz ultrasound treatment

In the US treatment group, 20 kHz US at 0.41 W/cm^2 was applied for 10 min to the skin of mice. OVA solution (20 mL, 0.1 mg/mL) was injected at the same site immediately after US application (day 0), and at days 7, 14, and 21. In the non-US group, the same volume of OVA solution was injected into the back skin *i.d.* or *s.c.* on days 0, 7, 14, and 21.

Blood samples were collected on days 14 and 28. When blood collection and OVA administration were performed on the same day, the former was executed first. Serum was separated from the collected blood.

Quantitation of ovalbumin-specific immunoglobulin G1 antibodies

OVA-specific immunoglobulin G1 (IgG_1) antibodies were measured with a mouse OVA-IgG_1 ELISA kit

(Shibayagi, Japan) following the manufacturer's protocol. The absorption at 450 nm for OVA-specific IgG_1 antibodies was measured with a SpectraMax M2e Multimode Reader (Molecular Devices).

Statistical analysis

Results are presented as the mean ± standard error. Statistical analysis was conducted using one-way ANOVA, followed by Dunnett's test for multiple comparisons between groups. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Effect of ultrasound frequency on Langerin expression in hairless rat skin

Figure 1 shows images of immunostained langerin in the epidermal sheet of hairless rat skin in the lateral and longitudinal directions after applying 20 kHz US at 0.41 W/cm², 1 MHz at 0.103 Wcm², and 3 MHz at 0.186 W/cm² for 10 min. In the lateral direction of the sheet, langerin fluorescence depended on the duration after US treatment for all frequencies. Figure 2 shows the quantification and comparison of the fluorescence data. For all the frequencies tested, langerin fluorescence increased with time, peaked at 12 h post-US treatment, and decreased to control levels at 24 h. The increase in langerin signal depended on the US frequency, with 20 kHz producing a notably higher effect. In the longitudinal direction, the langerin signals were localized to the SC side of the epidermal sheets. Contrastingly, *s. c.* treatment did not increase the langerin signal at 12 h.

Effect of ultrasound irradiation time and frequency on langerin expression in hairless rat skin

Figure 3 shows the quantification of langerin fluorescence at 12 h post-US application for irradiation times ranging from 1 to 20 min. The langerin signal increased with irradiation time up to 20 min. Moreover, the signal obtained after 20 kHz US treatment was higher over 10 min compared with that obtained after 1 MHz and 3 MHz US.

Figure 4 quantifies and compares the langerin signal obtained after treatment with US at different frequencies but similar intensities for 10 min. The intensities of the applied US were 0.139, 0.103, and 0.186 W/cm² for 20 kHz, 1 MHz, and 3 MHz, respectively, all of them falling in a similar range. The langerin expression induced by US followed the order 20 kHz >1 MHz >3 MHz. Low-frequency US seems to be more effective in potentially activating LCs.

Effect of ultrasound intensity on langerin expression in hairless rat skin

Using 20 kHz US, which induced relatively higher langerin expression, the effect of US intensity was examined at 0.14,

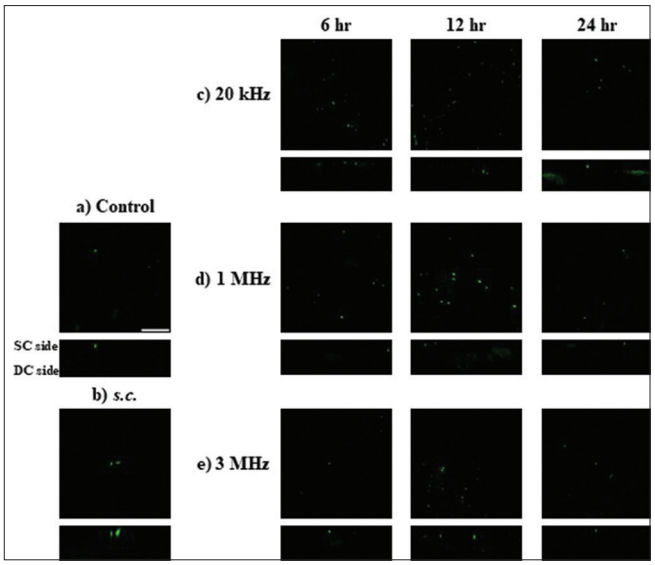

Figure 1: Immunostained langerin of the epidermal sheets. ×400 bar 30 μm. The upper/lower sides of the longitudinal direction images indicate the SC/the DC side. (a) Control, (b) 12 h post-subcutaneous treatment, (c-e) post-US treatment. SC: stratum corneum, US: Ultrasound, DC: Dermal corneum

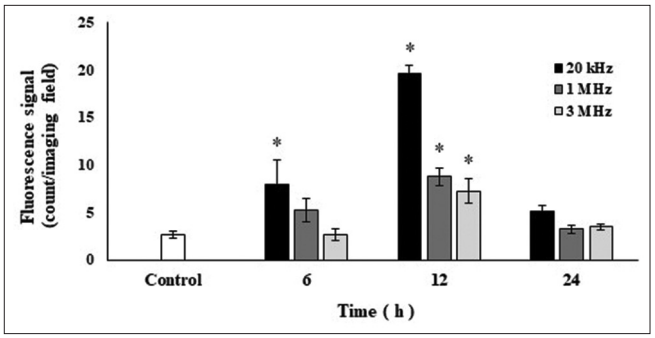

Figure 2: Time dependency of the langerin signal in epidermal sheets of hairless rats. The fluorescence signal was quantified by image analysis of Figure 1. Each data point represents the mean ± SE (n = 3–9). *$P < 0.05$ compared with the control. SE: Standard error

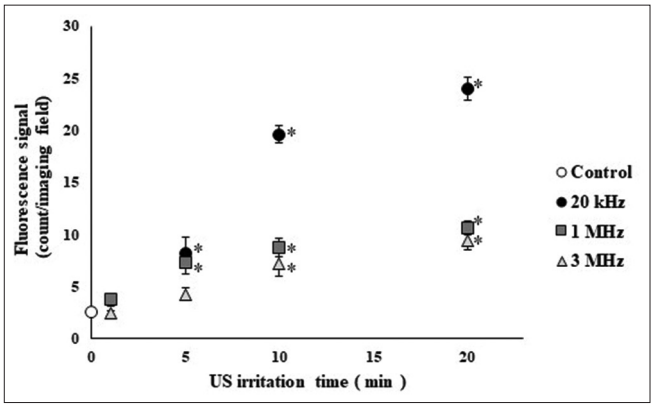

Figure 3: Effect of US irradiation time on the expression of langerin in the epidermal sheets of hairless rats. Each data point represents the mean ± SE (n = 3–9). *$P < 0.05$ compared with the control. (○, control; ●, 20 kHz; □, 1 MHz; ▲, 3 MHz). US: Ultrasound, SE: Standard error

0.41, and 1.19 W/cm^2 [Figure 5]. The skin was irradiated with US for 10 min and excised 12 h later. The langerin signal increased with increasing intensity, although the signal at 0.41 and 1.19 W/cm^2 did not differ significantly.

Effect of ultrasound pretreatment on ovalbumin-specific immunoglobulin G1 antibody production in BALB/c mice

OVA-specific IgG_1 antibody production was measured after *i.d.* or *s.c.* administration of OVA in mice either with or without 20 kHz US pretreatment at 0.41 W/cm^2 for 10 min. Because BALB/c mice were used instead of hairless rats in this experiment, the langerin signal was observed to confirm the activation of LCs [Figure 6]. The time-dependent increase in the langerin signal was like that observed in hairless rats, indicating that the US-induced activation of LCs was similar in BALB/c mice.

In both *i.d.* and *s.c.* injection groups, the amount of OVA-specific IgG_1 antibody was higher in US-pretreated mice than in untreated mice by 2.3- and 1.3-fold at day 14, 3.4-, and 2.8-fold at day 28, respectively [Figure 7]. Therefore, pretreatment with 20 kHz US could stimulate the skin immune response to increase antibody production.

DISCUSSION

Our results demonstrated that low-frequency US could activate epidermal LCs and increase antibody production against intradermally administered OVA.

LCs were shown to be activated within 12 h after the physical stimulus of tape stripping, and a number of them emigrated from the epidermis after 48 h.[4] In the case of US treatment, the langerin signal peaked at 12 h and returned to control levels at 24 h after treatment with all US frequencies [Figures 1, 2, and 6], indicating that LCs were maximally activated around 12 h. A similar time-dependent activation of LCs was recorded after applying 500 mm MNs to the skin in our previous study.[5] Thus, the 12 h time point was used as the default in the following experiments.

The activation of LCs was dependent on US conditions, including irradiation time, frequency, and intensity [Figures 3-5]. The increase in langerin signal was dependent on the irradiation time of US treatment at all frequencies, although distinctly higher signals were observed in the case of 20 kHz US, especially after 10 and 20 min of treatment. In terms of frequencies, 20 kHz US induced the highest activation of LCs, followed by 1 and 3 MHz [Figure 4]. These findings suggest that low-frequency US can trigger skin immune responses through the activation of LCs. Compared with therapeutic frequencies such as 1–3 MHz, low-frequency US generates strong mechanical effects on the skin due to cavitation. Asymmetric cavitation collapses at the skin surface generated by

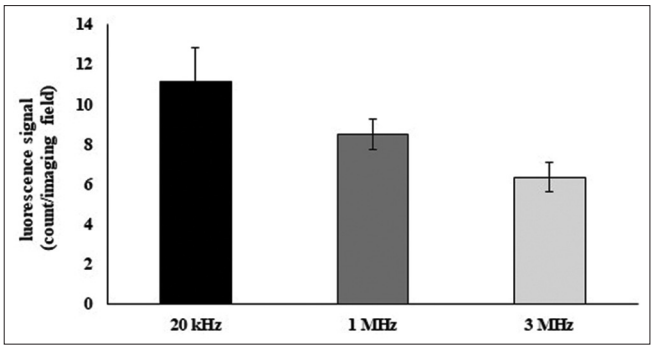

Figure 4: Expression of langerin in the epidermal sheets of hairless rats after treatment with US of different frequencies. Each data point represents the mean ± SE (n = 3–9). US: Ultrasound, SE: Standard error

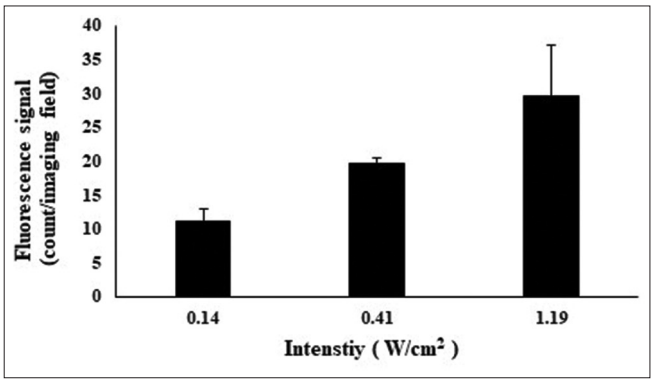

Figure 5: Effect of US intensity on expression of langerin in the epidermal sheets of hairless rats treated with 20 kHz US. Each data point represents the mean ± SE (n = 3–9). SE: Standard error

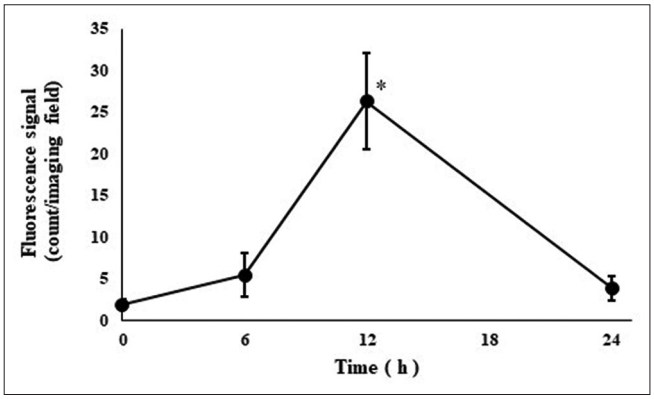

Figure 6: Time dependency of the langerin signal in epidermal sheets of BALB/c mice. Each data point represents the mean ± SE (n = 4). *P < 0.05 compared with the control. SE: Standard error

low-frequency US (<100 kHz) could induce microjets into the skin. Such a mechanical effect was not induced when US frequencies of 158 and 450 kHz, much lower than the therapeutic US frequency range, were used.[6,9] Thus, mechanical effects produced by low-frequency US can serve as physical stimuli to the skin. The effect of 20 kHz US on the langerin signal depended on the applied intensity, up to 1.19 W/cm^2 [Figure 5]. The langerin signal obtained

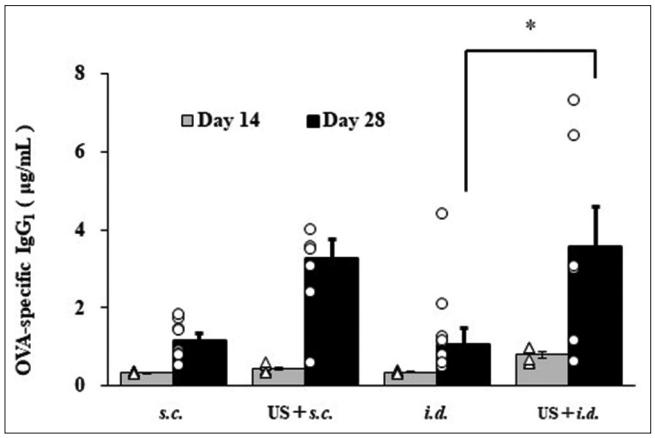

Figure 7: OVA-specific IgG_1 antibody levels in the serum of BALB/c mice either pretreated with or without 20 kHz US.Each data point represents respective OVA-specific IgG_1 antibody levels in 5–8 mice. *$P < 0.05$ compared with *i. d.* OVA: Ovalbumin, IgG_1: Immunoglobulin G1. Δ indicated individual data of OVA-specific IgG1 antibody at day 14. ○ indicated individual data of OVA-specific IgG1 antibody at day 28

under such conditions was comparable to that induced by applying 500 mm MNs (data not shown), which activated in mouse LCs.[5] Hence, low-frequency US could be used as a physical stimulus for activating skin immune responses.

Next, we investigated whether activation of the immune response, evidenced by the activation of LCs in response to US, could induce antigen-specific antibody production. OVA-specific IgG_1 production, measured on day 28, was evidently higher when the skin was pretreated with 20 kHz US in both *i.d.* and *s.c.* OVA administration groups [Figure 7]. Since 20 kHz US activates LCs, which are responsible for capturing antigens, the activation of skin immune responses in the early stages may be involved in US-induced IgG_1 antibody production. Antibody production was also shown to be enhanced by applying plastic MNs to the skin near the sites of *s.c.* or *i.d.* OVA injections.[10] Therefore, the application of physical stimuli to the skin can enhance IgG_1 antibody production through the skin immune responses.

The mechanism by which physical stimuli trigger skin immune responses to increase antibody production is yet unclear. Disrupting the skin barrier function can cause the release of cytokines, such as interleukin-1 and tumor necrosis factor-α.[11] Moreover, applying US to the skin induces cytokine secretion, leading to the migration of LCs.[12] Therefore, these cytokines would most likely be involved in the enhanced antibody production orchestrated by the skin immune responses.

CONCLUSION

Physical stimulation of the skin using US can activate skin immune responses and enhance antibody production. Activation of the immune responses depended on US application conditions, including frequency, intensity, and irradiation time. Low-frequency US, such as 20 kHz, may be more effective in activating immune responses. Because low-frequency sonophoresis assists the transdermal transport of large molecules like antigens;[2,6] this technique holds promise in the development of transcutaneous vaccine systems.

Acknowledgment

This work was supported by JSPS KAKENHI Grant Numbers 17K08246,22K06748. The authors would like to thank Enago (www.enago.jp) for the English language review.

Financial support and sponsorship

JSPS KAKENHI Grant Numbers 17K08246, 22K06748.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Franjić S. Briefly about the immune system: A review article. EC Clin Med Case Rep 2022;5:1-7.
2. Gao Y, Du L, Li Q, Li Q, Zhu L, Yang M, *et al.* How physical techniques improve the transdermal permeation of therapeutics: A review. Medicine (Baltimore) 2022;101:e29314.
3. Tezel A, Paliwal S, Shen Z, Mitragotri S. Low-frequency ultrasound as a transcutaneous immunization adjuvant. Vaccine 2005;23:3800-7.
4. Kubo A, Nagao K, Yokouchi M, Sasaki H, Amagai M. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. J Exp Med 2009;206:2937-46.
5. Takeuchi A, Nomoto Y, Watanabe M, Kimura S, Morimoto Y, Ueda H. Application of microneedles to skin induces activation of epidermal Langerhans cells and dermal dendritic cells in mice. Biol Pharm Bull 2016;39:1309-18.
6. Polat BE, Hart D, Langer R, Blankschtein D. Ultrasound-mediated transdermal drug delivery: Mechanisms, scope, and emerging trends. J Control Release 2011;152:330-48.
7. Mitragotri S, Farrell J, Tang H, Terahara T, Kost J, Langer R. Determination of threshold energy dose for ultrasound-induced transdermal drug transport. J Control Release 2000;63:41-52.
8. Kubo A, Nagao K, Amagai M. 3D visualization of epidermal Langerhans cells. Methods Mol Biol 2013;961:119-27.
9. Park D, Park H, Seo J, Lee S. Sonophoresis in transdermal drug deliverys. Ultrasonics 2014;54:56-65.
10. Ito S, Hirobe S, Yamashita R, Sugiyama A, Takeuchi H, Eguchi R, *et al.* Analysis of immune response induction mechanisms implicating the dose-sparing effect of transcutaneous immunization using a self-dissolving microneedle patch. Vaccine 2022;40:862-72.
11. Chopra A, Gupta A. Skin as an immune organ and the site of biomimetic, non-invasive vaccination. Med Nov Technol Devices 2022;16:100196.
12. Choi EH, Kim MJ, Yeh BI, Ahn SK, Lee SH. Iontophoresis and sonophoresis stimulate epidermal cytokine expression at energies that do not provoke a barrier abnormality: Lamellar body secretion and cytokine expression are linked to altered epidermal calcium levels. J Invest Dermatol 2003;121:1138-44.