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Transdermal delivery of fluorescein isothiocyanate-dextrans using the combination of microneedles and low-frequency sonophoresis

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ABSTRACT

This study aimed to evaluate the patient-friendly methods that are used in the delivery of hydrophilic macromolecules into deep skin layers, in particular, the combination of microneedles patch (MNs patch) and low-frequency sonophoresis (SN). The hydrophilic macromolecule drug fluorescein isothiocyanate (FITC)-dextrans (FD-4: MW 4.4 kDa) was used as the model drug in our experimental design. In this study, excised porcine skin was used to investigate and optimize the key parameters that determine effective MNs- and SN-facilitated FD-4 delivery. *In vitro* skin permeation experiments revealed that the combination of MNs patch with SN had a superior enhancing effect of skin permeation for FD-4 compared to MNs alone, SN alone or untreated skin, respectively. The optimal parameters for the combination of MNs and SN included the following: 10 N insertion force of MNs, 4 W/cm^2 SN intensity, 6 mm radiation diameter of the SN probe, 2 min application time, and the continuous mode duty cycle of SN. In addition, vertical sections of skin, clearly observed under a confocal microscope, confirmed that the combination of MNs and SN enhanced permeation of FD-4 into the deep skin layers. These studies suggest that the combination of MNs and SN techniques could have great potential in the delivery of hydrophilic macromolecules into deep skin.

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1. Introduction

Transdermal drug delivery systems (TDDS) represent an attractive alternative form of drug delivery that minimizes and circumvents the limitations associated with the oral, parenteral, and inhalation methods of drug administration [1]. Transdermal delivery offers several potential advantages, including avoidance of hepatic first pass metabolism, reduction of the severe adverse effects on the gastrointestinal tract associated with oral drug administration, avoidance of drug level fluctuation and ease of drug delivery termination in the occurrence of toxicity. However, TDDS also face major challenges in circumventing the barrier function of the outermost layer of the skin, the stratum corneum (SC). The SC works against drug penetration into the skin and the systemic circulation of TDDS [2,3].

To overcome the SC barrier, several physical enhancement methods have been evaluated for the ability to increase skin permeation and allow for transdermal administration of water-soluble molecules and macromolecular drugs [4]. One of the interesting physical enhancement methods, the microneedles (MNs) array, is composed of small micron-sized needles which, when applied on the skin, disrupt the SC barrier by creating large aqueous microchannels that allow for the passage of molecules through the skin barrier without causing skin damage [5]. These abrasions provide physical pathways for the drug to permeate the skin in much higher concentrations than would be normally observed by topical administration. MNs design can be commonly divided into 3 types: solid MNs, hollow MNs, and biodegradable MNs. Different materials have been used in the fabrication of MNs, including silicon rubber, stainless steel, titanium, glass, polysaccharide, and numerous polymers [6,7]. Moreover, MNs are of interest primarily because they offer the promise of less invasive and painless drug delivery as MNs do not penetrate the papillary layer of dermis where nerve endings are located [7]. In addition to increasing transdermal delivery, a combination of physical enhancement methods should also reduce the level of the enhancers required to achieve the desirable drug flux [8]. Yan et al. combined the MNs array with in-skin electroporation (in-skin EP), which creates new permeation pathways in the SC, thereby enhancing skin permeability. The combination of MNs and in-skin EP was considered to have excellent synergistic effects for FD-4 skin permeation compared to MNs alone or conventional EP [9].

Recently, another physical method, sonophoresis (SN), has also been shown to effectively deliver various types of drugs regardless of their electrical characteristics [10,11]. SN is a promising approach that involves the use of ultrasound energy for delivery of molecules that are normally impermeable to deep skin layers. Several proposed mechanisms of SN have been reported; these include acoustic cavitation effects, the formation and collapse of gaseous cavities in the SC barrier [12] and thermal effects that are due to ultrasound wave attenuation. An increase in the temperature of tissues can increase skin permeability [13]. It has been suggested that SN-based enhancement in skin permeability depends on ultrasound parameters, such as frequency, intensity, duty cycle, radiating diameter of the transducer, and the duration of ultrasound application [14]. SN application, with ultrasound energy at frequencies in the range of 20 kHz–16 MHz and intensities up to 14 W/cm^2, enhances skin permeation of high molecular-weight drugs. In particular, transdermal enhancement is significantly higher at low-frequency regimens (20 kHz–100 kHz) compared to induction by high-frequency ultrasound (1 MHz–16 MHz) [15].

Despite their different mechanisms, MNs and SN can be coupled through the formation of integrated systems. The use of both methods can enhance the skin penetration of molecules by creating new permeation pathways and increasing the duration of the pore opening for higher drug permeation. Chen et al. evaluated the synergistic effect of SN combined with MNs in the delivery of calcein and bovine serum albumin. The results showed that the combination of SN and MNs greatly enhanced transdermal drug delivery rates compared to passive diffusion and either MNs or ultrasound alone [16]. Furthermore, the maximum application strength of the enhancers is typically limited by safety restrictions. The combination of two or more physical enhancement methods can achieve the desired enhancement with significantly reduced strength of the individual enhancers. Hence, a combination of physical enhancement methods not only improves the total enhancement but also increases the safety of enhancer use [8]. However, the details of factors and optimum conditions for the combination of MNs and SN application for skin permeation in TDDS are rarely studied.

In this study, we examined the effect of various parameters of MNs and low-frequency SN on fluorescein isothiocyanate (FITC)-dextrans (FD-4) skin permeation. The minimum effective condition for minimal invasiveness of the combination of MN and SN was our desirable achievement, and the safety issues are important awareness for this combination study. Our evaluation included the following: force insertion of the MNs, the intensity, application time, and diameter transducer of the SN, and ultrasound duty cycle (ratio of ultrasound time duration). FD-4, a hydrophilic macromolecule with a high molecular weight (4.4 kDa), was selected as our model drug because it has characteristically poor skin penetration [9]. The effect of MNs and low-frequency SN on skin permeation enhancement was determined by *in vitro* skin permeation experiments. Skin morphology after treatment with MNs and SN was observed using confocal laser scanning microscopy.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate (FITC)-dextrans (FD-4; average molecular weight, 4.4 kDa) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and used without further purification.

2.2. Fabrication of microneedles patch

The solid MNs patch was fabricated from stainless steel, 32-gauge acupuncture needles (0.25 × 30 mm, DongBang Acupuncture Inc., Boryeong, Korea) and a silicone sheet

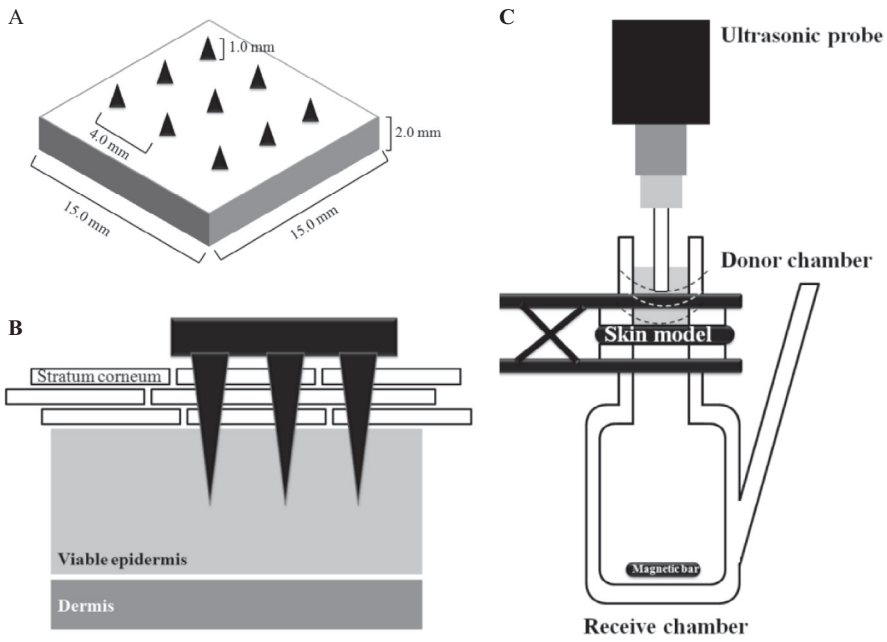

Fig. 1 – The illustration of (A) the microneedle patch used in this study, (B) the application of microneedles to skin and (C) the application of sonophoresis in the skin permeation study.

(15 × 15 × 2 mm). The acupuncture needles were cut and then used to puncture the silicone sheet vertically. The tips of acupuncture needles extended approximately 1000 μm out of the silicone sheet. The other end of acupuncture needle was bent to anchor the acupuncture needle in the silicone sheet. Each patch contained 9 acupuncture needles, with a center-to-center spacing of 4 mm. The solid MNs patch was then fixed with an adhesive tape to ensure that the acupuncture needles remained stationary (Fig. 1).

2.3. *Preparation of porcine skin*

Full thickness of abdominal adult porcine skin was obtained from the general slaughterhouse (Nakhon Pathom, Thailand). The subcutaneous fatty layer and connective tissues were carefully removed from the dermis. The hair shafts were cut off to a thickness of approximately 2.0–2.5 mm. The prepared skins were washed with phosphate buffer saline (PBS; pH 7.4), wrapped in aluminum foil, stored at –20 °C, and defrosted immediately prior to use.

2.4. In vitro *skin permeation experiments*

This experiment was performed using Franz diffusion cells after pretreatment of excised porcine skin with MNs alone, SN alone or a combination of MNs and SN. The skin samples were mounted between the donor and the receptor compartments, with the epidermis facing up toward the donor compartment, and the apparatus was affixed using clamps. The receptor compartment was filled with 6 mL of phosphate buffer saline (PBS; pH 7.4) and continuously stirred using a magnetic stirrer at 400 rpm. The temperature was maintained at 32 ± 1 °C with a water jacket. The donor compartment was filled with 1 mL of FD-4 solution (2.5 mg/mL), and the top of the donor compartment was then covered with Parafilm® for occlusion. To investigate the cumulative permeation profiles, a 500 μL aliquot of receptor was withdrawn at the time intervals of 0.08, 0.25 and 0.5 min, 1, 2, 4, 6, 8, 12, 16, 20 and 24 h and replaced with equal volumes of fresh PBS to maintain a constant volume. The skin permeation experiments were performed under various conditions.

For skin permeation experiments using the MN patch, an in-house MN system was specifically manufactured to provide a reproducible force on the MN patch for insertion into porcine skin. A plastic container filled with water was used to construct the force of our device. The amount of water was adjusted to obtain the required force: 2.5, 5.0, 10.0, 20.0 and 30.0 N. The force applied on the MN patch was conducted on porcine skin sample for 2 min *in vitro* to ascertain the amount of FD-4 able to permeate skin when the MN patch was used as a pretreatment. Untreated skins were used as the control.

In skin permeation experiments using SN, we utilized the sonicator (Vibra-cell™, VCX130 PB, Sonics and Materials, Inc., USA) with low-frequency ultrasound at 20 kHz. After placing the skin sample on the Franz diffusion cell and filling the donor compartment with the FD-4 solution as a coupling medium, the SN was activated. The effect of several parameters were investigated, including the intensity of the ultrasound (4, 10, 20 W/cm^2), radiating diameter of the transducer (3, 6 mm), application time (1, 2, 5 min), continuous (100% duty cycle) and discontinuous modes (50% duty cycle) by setting the pulse on 5 s and pulse off 5 s mode.

To examine skin permeation using the combination of the MN patch and SN, the skin samples were punctured by the solid MN patch with 10.0 N for 2 min, the solid MN patch was then removed from the skin, and the skin was immediately mounted on the Franz diffusion cell. Before application of the ultrasound, FD-4 solutions were filled into the donor compartment.

Then, SN was applied on the skin. The parameters studied for SN-based skin permeation were the same as mentioned above.

2.5. Quantitative assay

At the end of the experiment, the concentration of the sample was analyzed by a fluorescence spectrophotometer (RF 5300PC; Shimadzu, Kyoto, Japan) at excitation and emission wavelengths of 495 and 515 nm[9], respectively. The concentrations were calculated using a calibration curve. Then, the cumulative amounts of FD-4 that permeated per unit skin surface area of the skin were plotted against time, and the slope of the linear portion of the plot was calculated as the flux value (μg/cm^2/h).

2.6. Confocal laser scanning microscope study

Skin imaging was visualized using confocal laser scanning microscopy (CLSM) to study the porcine skin distribution of FD-4. After *in vitro* skin permeation, the skins were washed with PBS. The skin was then directly mounted between a glass slide and a cover slip, and examined using an inverted Zeiss LSM 510 META microscope (Carl Zeiss, Jena, Germany). Lastly, both skin penetration depth and skin fluorescence intensity were analyzed using FV1000 Version 1 Application Software (FV10-ASW).

2.7. Statistical analysis

The data were expressed in the form of the mean and standard deviation (mean ± SD). The statistical significance of differences between groups was examined using one-way analysis of variance (ANOVA). The value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Effect of MNs on the skin permeation

3.1.1. Insertion force

Fig. 2 shows the flux of FD-4 that permeated the skin that was pretreated with different MN insertion forces (2.5, 5.0, 10.0, 20.0 and 30.0 N). The insertion force of 30.0 N revealed the most enhancing effect on the skin permeation of FD-4, followed by 20.0, 10.0, 5.0 and 2.5 N. These results indicate that insertion force could increase insertion distance; thus, an increase in insertion force significantly increases the amount of FD-4 able to permeate through porcine skin. To summarize, the values of FD-4 flux via the skin after MN pretreatment with an insertion force of 2.5, 5.0, 10.0, 20.0 and 30.0 N were 1.9-, 3.5-, 6.4-, 9.7- and 13-fold greater than that of untreated skin (passive diffusion), respectively. These results, similar to results published by Donnelly et al. [17], demonstrate that an increase in the force used for MNs application results in a significant increase in the depth of penetration achieved within neonatal porcine skin. For example, MNs with a 600 μm height penetrate to a depth of 330 μm when inserted at a force of 4.4 N/array; however, the penetration increases significantly to a depth of 520 μm when the force of application was increased to 16.4 N/array. Additionally, van der Maaden et al. [18] reported that increasing the applied force (at a constant application time of 10 s) up to 7.36 N greatly improved the penetration ability. However, FD-4 flux values caused by the MNs with an insertion force of 5.0 and 2.5 N were not significantly different from untreated skin ($P < 0.05$). This result could represent an insufficient force (5.0 and 2.5 N) to facilitate FD-4 entry through porcine skin. These low forces caused only buckled or slightly pierced skin. Olatunji et al. [19] suggested that only a proper

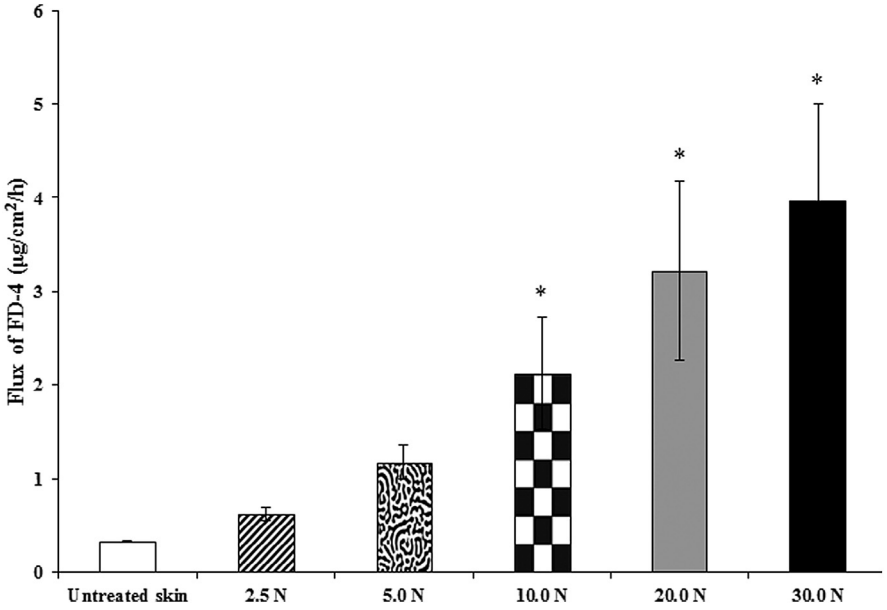

Fig. 2 – Effect of MNs insertion force (2.5, 5.0, 10.0, 20.0 and 30.0 N) on the flux of FD-4. Each value represents the mean ± SD (n = 5–7). *Compared with untreated skin ($P < 0.05$).

force can help the MNs to overcome the resistance of the skin as the skin can provide the resistance to MN insertion due to its viscoelastic properties. Cheung et al. [20] demonstrated that the SC was disrupted when insertion force of 69.1 N was applied. Therefore, the appropriate MN insertion force used should be considered. Compared to untreated skin, the 10.0 N insertion force was the minimum force that significantly enhanced the skin permeability of FD-4. Therefore, the 10.0 N skin insertion force was selected for further use in combination with low-frequency SN.

3.2. *Effect of SN combined with MN on the skin permeation*

3.2.1. *Intensity of SN*

To study the effect of intensity, all other ultrasound parameters were set to remain constant. These set parameters included the diameter of the probe (6 mm), use of the continuous mode, and the application time of 2 min. Skin treated with SN alone (intensities: 4, 10, 20 W/cm^2) did not exhibit a significant elevation in the flux of FD-4 compared to the untreated skin. In contrast, skin treated with the combination of MNs and 4, 10, 20 W/cm^2 intensity of SN exhibited a significant increase in FD-4 flux when compared with either MNs alone or untreated skin (Fig. 3). FD-4 fluxes of combination treatments were 14.4-, 12.9-, and 13.3-fold higher than the untreated skin when respective intensities of 4, 10, 20 W/cm^2 were used, respectively. No significant difference was observed in the flux of FD-4 using the combination of MNs with the 3 different intensities of SN; however, the greatest enhancing effect on the permeation of FD-4 was found for the combination of MNs and 20 W/cm^2 intensity of SN, followed by 10 and 4 W/cm^2. These results suggest a great synergistic effect of MNs and SN on the skin permeation of FD-4. Correspondingly, results by Boucaud et al. [21] indicate a lack of difference in blood glucose levels at 2.5 W/cm^2. When an intensity of 5 W/cm^2 was used, only one of the four rats had a drop in blood glucose levels at the end of the ultrasound protocol. In contrast, the average blood glucose level obtained at the end of 10 W/cm^2 exposure was significantly reduced (40%, $P < 0.05$) compared to initial values. In our study, we have shown that the intensity of SN has an effect on the skin permeation of FD-4, especially when in combination with MNs. The combination of MN and SN had a synergistic effect for the transdermal delivery as SN and MN could promote different mechanisms of action for enhancing skin permeation. Thus, the combination of SN and MN was more effective than each enhancer alone. At higher intensities, SN could lead to stable cavitation, a continuous oscillation of bubbles in an acoustic field [10], transient cavitation, and the rapid growth and collapse of bubbles. When high intensities of SN encounter a gas nucleus in solution, the nucleus could rapidly expand, resulting in the implosion of the bubble at high velocity. If a bubble collapses near a surface, nonuniformity in the surrounding pressure leads to the formation of a high-velocity microjet, which could enhance the skin permeation of a compound [22]. In addition, increasing the intensity of SN increases the energy put into the skin, which might act to change the morphology of the epidermis. The enhancement of skin permeability induced by SN application depends on the strength of the application; however, the maximum level of SN that can be applied to the skin is typically limited by safety measures [8]. Thus, the combination of transdermal techniques may not only enhance the skin permeability but also increase the level of safety for the skin. In our study, the minimum effective intensity (4 W/cm^2) was used for further studies as it allowed the use of a long total application time without inducing skin damage. In addition, this intensity was selected to avoid heating of the coupling medium, which represents a major patient complaint regarding the SN regimen. During the low-frequency, 20 W/cm^2 intensity SN treatment of

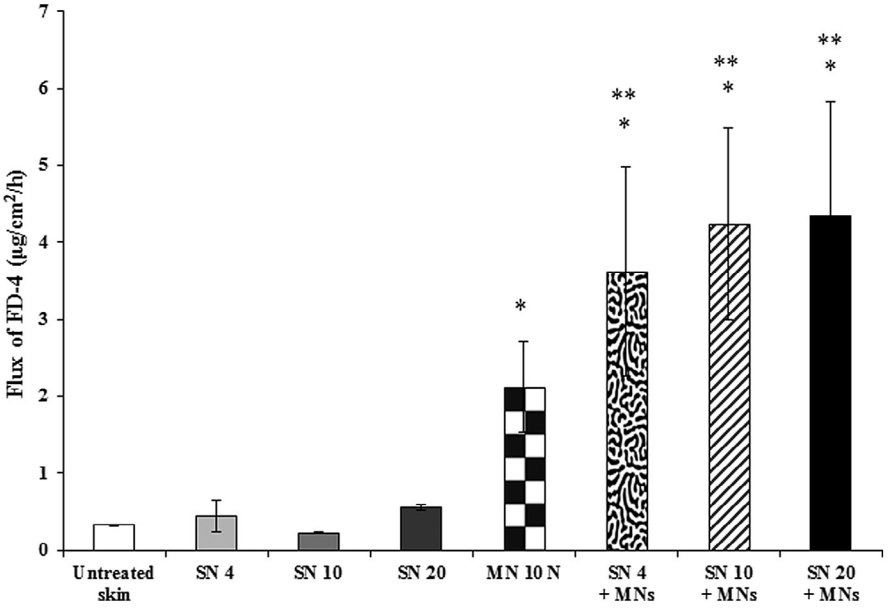

Fig. 3 – Effect of different SN intensities (4, 10 and 20 W/cm^2) on the flux of FD-4. Each value represents the mean ± SD (n = 3–9). *Compared with untreated skin ($P < 0.05$). **Compared with MNs alone ($P < 0.05$).

porcine skin, we observed both an increased temperature of the probe and an oscillation of bubbles in the medium.

3.2.2. *Radiating diameter of probe of SN*

To study the effect of the radiating diameter of probe, the 4 W/cm^2 intensity SN was used, and the experiment was divided into the following 4 groups: (A) continuous mode and applied for 2 min, (B) continuous mode and applied for 1 min, (C) discontinuous mode and applied for 2 min, and (D) discontinuous mode and applied for 1 min. Fig. 4 shows the flux of the diameter probe (3 and 6 mm) on *in vitro* permeation. No significant difference was found between only SN treatment (diameter probe: 3 and 6 mm) and untreated skin in all groups. However, a difference was observed in the permeation of FD-4 compared to MNs alone, and the combination of MNs and SN (diameter probe: 3 and 6 mm) resulted in a significant increase when compared with untreated skin. As shown in Figs. 4A and 3C, the flux of FD-4 through the skin treated with the combination of MNs and SN with a diameter probe of 6 mm increased approximately 1.83-fold and 1.68-fold, respectively, when compared with MNs alone. In contrast, no significant difference was observed in experiments with a diameter probe of 3 mm. However, the flux of FD-4 in Fig. 4B and D treated with a combination of MNs and SN of both diameter probes was not significantly different when compared to MNs alone. These results highlight the importance of probe diameter and application time in the use of SN. The diameter of the probe corresponds to a phenomenon referred to as microstreaming, in which bubble oscillation around asymmetric boundary conditions by stable cavitation results in generated high-velocity gradients and hydrodynamic shear stress. Stable cavitation may occur within the coupling medium between the ultrasound probe and the skin surface [11]. As a result, a larger probe diameter and longer SN application could increase skin permeability. In this experiment, we selected the SN diameter probe of 6 mm for further study.

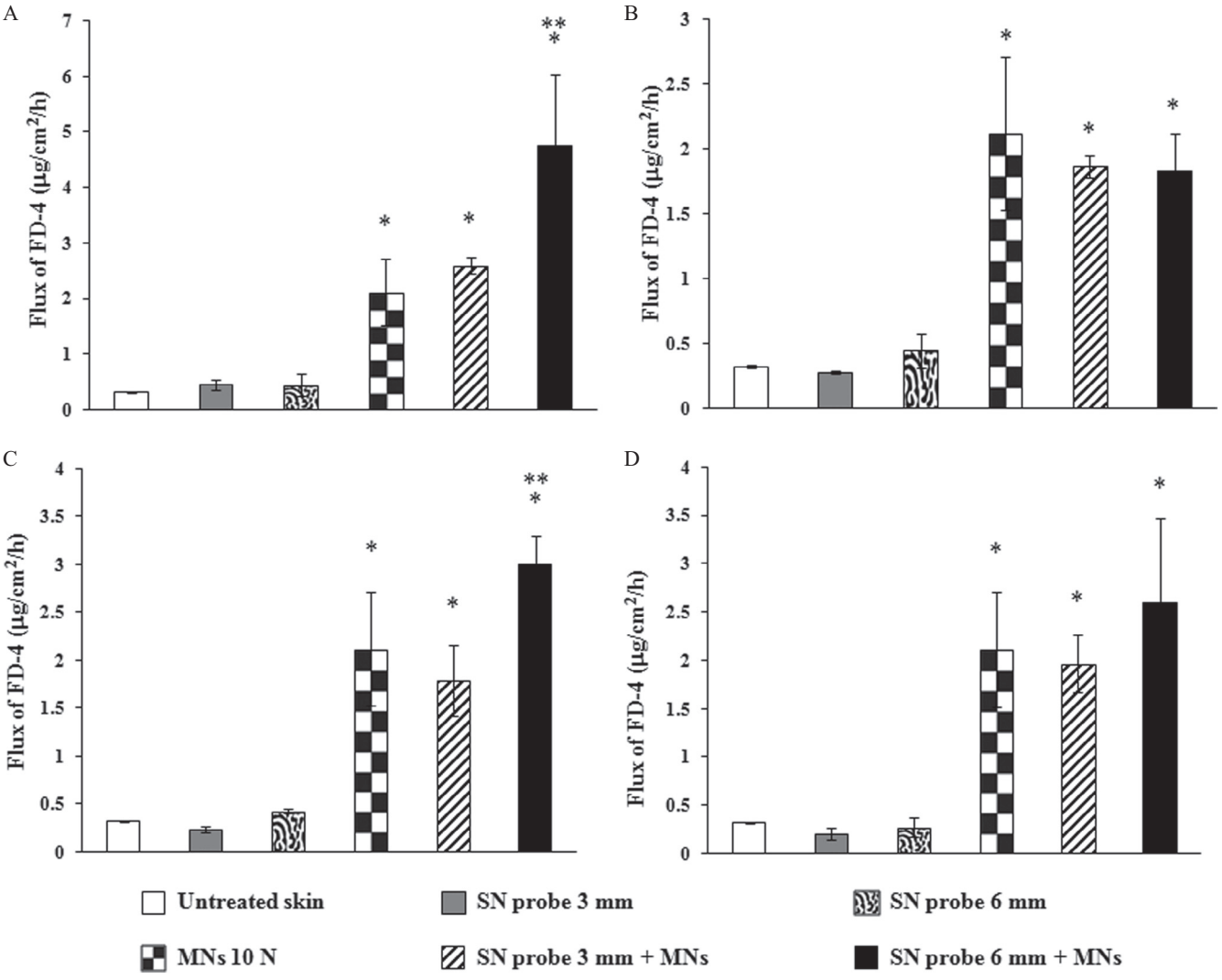

Fig. 4 – The effect of the SN probe radiating diameter (3 and 6 mm) on FD-4 flux. The treatments were divided into 4 groups: (A) continuous mode and applied for 2 min, (B) continuous mode and applied for 1 min, (C) discontinuous mode and applied for 2 min, and (D) discontinuous mode and applied for 1 min. Each value represents the mean ± SD (n = 3–11). *Compared with untreated skin ($P < 0.05$). **Compared with MNs alone ($P < 0.05$).

3.2.3. *Application times of SN*

To evaluate the effect of 3 different application times (1, 2 and 5 min), the sonication device was set with the 6 mm probe diameter and the continuous mode for all studies. Under treatment with only SN, the flux of FD-4 through the skin for the different application times (1, 2 and 5 min) was not significantly different when compared with untreated skin. Nevertheless, the permeation of FD-4 from MNs alone and the combination with MNs and SN resulted in a significant increase when compared to untreated skin (Fig. 5). Very little FD-4 permeated intact skin or skin treated only with SN, whereas MNs alone and the combination of MNs and SN treatment with an application time of 1 min enhanced permeability by approximately 6.24-fold and 5.06-fold, respectively. Furthermore, the combination of MNs and SN with application times of 2 and 5 min showed respective increases of 14.39-fold and 19.21-fold permeability compared to the control. The results demonstrated that the FD-4 flux increases with regard to increasing application time. The application time of 2 min was selected for further experiments because this application time represented the least amount of time that caused a significant enhancement in skin permeability of FD-4 compared to untreated skin. These results correspond to those by Herwadkar et al. [14]. These authors reported that SN application for 2 min significantly increased ketoprofen permeation by approximately 6.56-fold, while no significant enhancement was observed in the application times of 0.5 and 1 min. In addition, the amount of ketoprofen accumulation in the skin increased significantly from 35 μg (passive diffusion) to 212 μg (ultrasound application for 5 min). These results might be caused by a thermal effect due to the duration of ultrasound energy. When ultrasound passes through a coupling medium, ultrasound energy is partially absorbed. The increase in the temperature of the coupling medium may enhance permeability due to an increase in diffusion of the skin. Merino et al. [23] reported that increased temperature enhanced the transdermal permeability of mannitol. Skin temperature was increased to approximately 20 °C with low-frequency ultrasound (frequency 20 kHz, intensity 15 W/cm^2), applied for 2 h, and resulted in a significant enhancement of skin permeability of approximately 35-fold. Boucaud et al. [21] reported that skin permeability to insulin was enhanced with increased total application time. The thermal effect may not be the main factor in the enhancement of skin permeability, even though the increased temperature may affect skin permeability. Merino et al. [23] found that enhancement of mannitol increased only 25% when using simple heating of the diffusion cell. The cavitation effect may synergize with the thermal effect to enhance skin permeation.

3.2.4. *Duty cycle modes of SN*

The effect of the continuous (100% duty cycle) and discontinuous (50% duty cycle) duty cycle modes were investigated. Throughout the experiment, the SN intensity was fixed at 4 W/cm^2, and the experiment was divided into 4 groups: (A) probe diameter of 6 mm applied for 2 min, (B) probe diameter of 6 mm applied for 1 min, (C) probe diameter of 3 mm applied for 2 min, and (D) probe diameter of 3 mm applied for 1 min. To set SN to 50% duty cycle, the pulsed treatment was carried out for a total time of 2 and 4 min, with the ultrasound being active for 1 and 2 min, respectively, with a pulse of 5 s. Fig. 6 shows the flux of FD-4 that permeated skin treated with SN (duty cycle of 50% and 100%). This graph shows that no significant difference was observed between only SN (duty cycle of 50% and 100%) in all groups compared to untreated skin. However, the FD-4 flux was significantly increased in the skin treated with both MNs and SN. Compared to MN treatment alone, a significant increase in the flux of FD-4 was observed only in

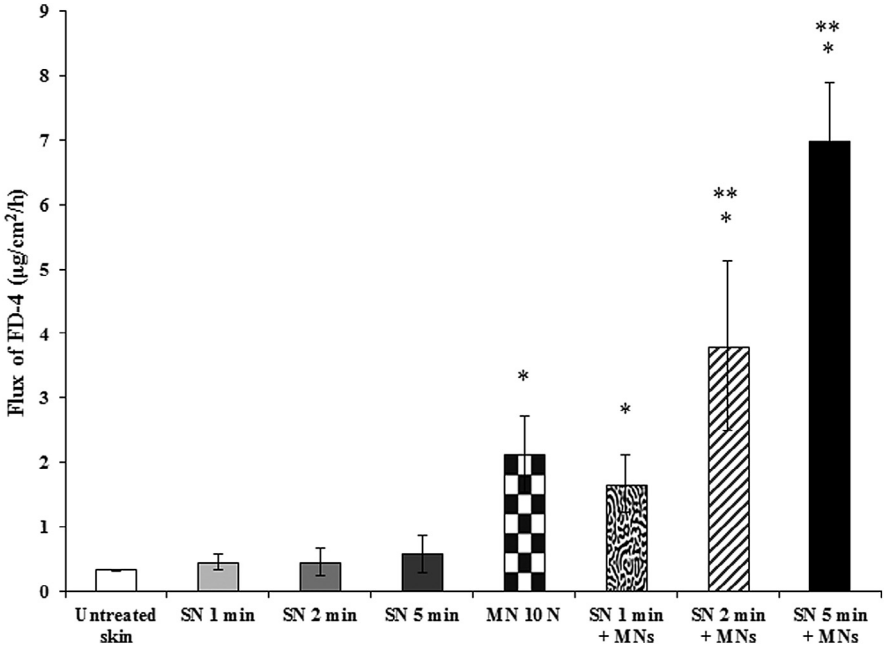

Fig. 5 – Effect of different SN application times (1, 2 and 5 min) on the flux of FD-4. Each value represents the mean ± SD (n = 3–11). *Compared with untreated skin ($P < 0.05$). **Compared with MNs alone ($P < 0.05$).

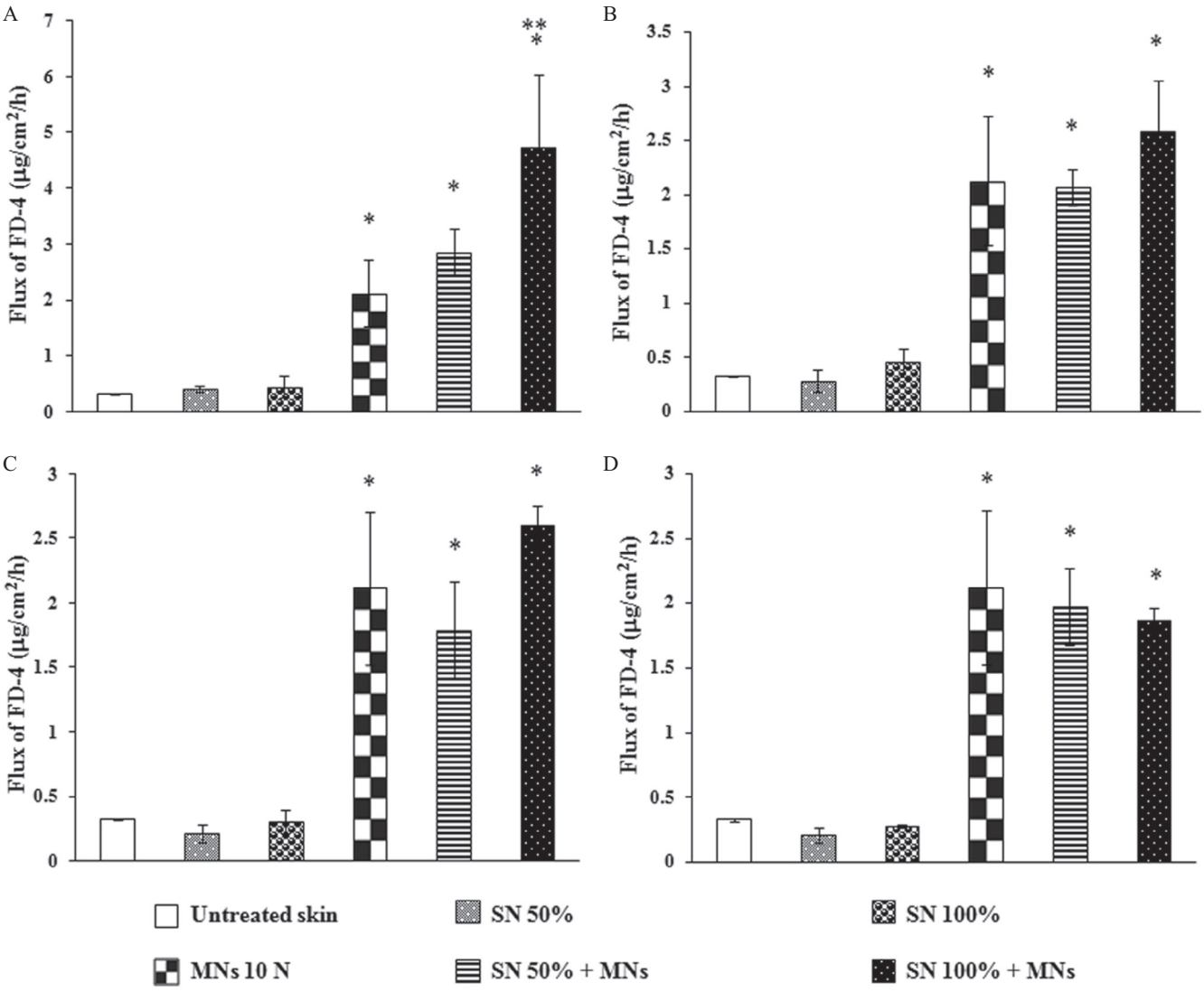

Fig. 6 – The effect of duty cycle modes of SN (100% duty cycle and 5% duty cycle) on the flux of FD-4. SN treatments were divided into 4 groups with the following characteristics: (A) diameter probe of 6 mm and applied for 2 min, (B) diameter probe of 6 mm and applied for 1 min, (C) probe diameter of 3 mm applied for 2 min and (D) probe diameter of 3 mm applied for 1 min. Each value represents the mean ± SD (n = 3–11). *Compared with untreated skin ($P < 0.05$). **Compared with MNs alone ($P < 0.05$).

group A (the skin treated both MNs and SN under continuous mode). Han et al. [24] found that SN alone could not provide sufficient permeability enhancement in the delivery of large molecules such as bovine serum albumin (BSA), and the combination of MNs and SN resulted in higher skin permeation of BSA than SN treatment alone. In groups A and C, the 100% duty cycle significantly increased the FD-4 flux over the 50% duty cycle. A possible explanation for this result could be the thermal effect. A faster increase in the temperature of the coupling medium was observed with continuous ultrasound treatment compared to discontinuous treatment [13,23]. In contrast, we observed no significant difference in the permeation of FD-4 between groups B and D, which were treated under the discontinuous and continuous mode. These results indicate that cavitation and the thermal effect of SN may be reduced when using shorter application times. The application time represents an important factor that enhances skin permeation by SN [13]. In this study, the continuous mode (100% duty cycle) was selected for further use in our studies because this mode more effectively enhanced the skin permeability of FD-4 compared to the discontinuous mode (50% duty cycle).

3.3. *Confocal laser scanning microscopic study (CLSM)*

Confocal laser scanning microscopy (CLSM) is used for visualization of fluorescent compounds and permits simultaneous viewing of multiple fluorophores, increasing the simplicity and accuracy of location identification of fluorescent compounds in the skin [25]. Fig. 7A–D shows images of the skin vertical depth sections observed under CLSM after the permeation of FD-4. It was known from the above-described *in vitro* skin permeation experiments that the flux of FD-4 permeation is very

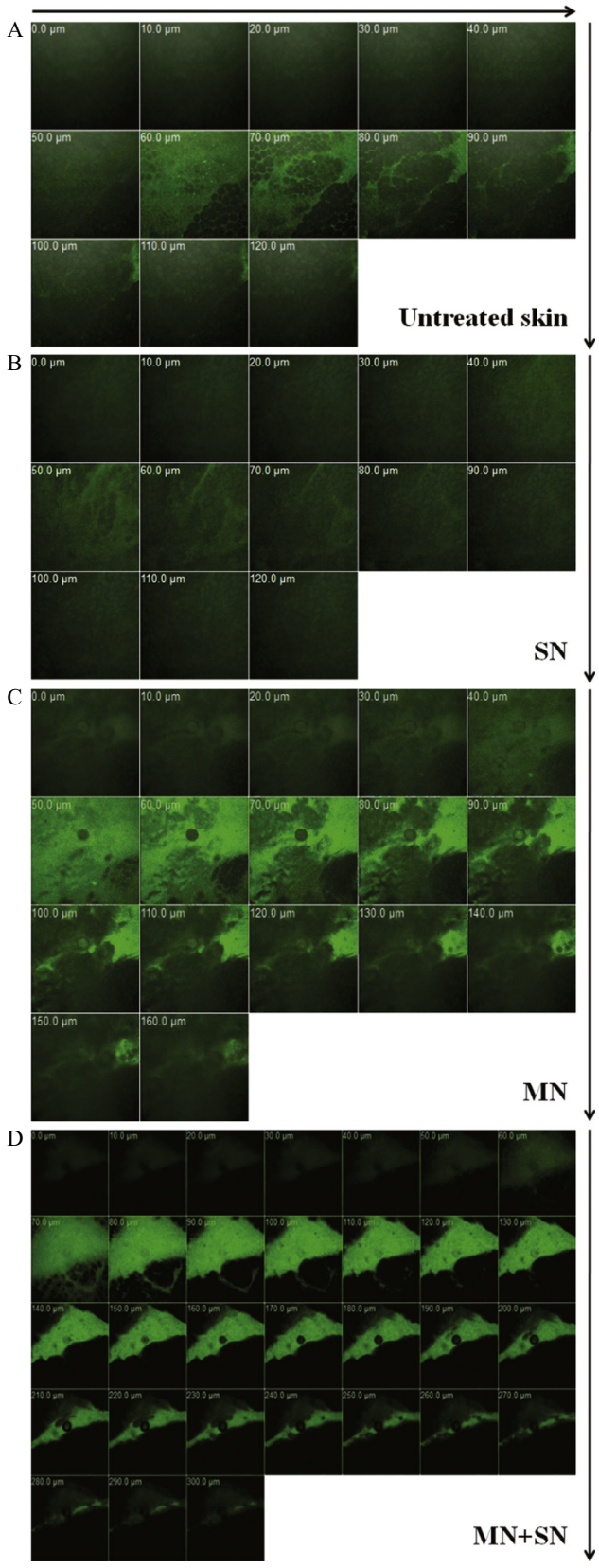

Fig. 7 – Fluorescence confocal laser scanning microscopy (CLSM) images of FD-4 distribution after *in vitro* permeation of intact and pretreated skin. (A) untreated skin, (B) only low-frequency SN, (C) only MNs, (D) combination of MNs and low-frequency SN.

limited in untreated skin and in skin treated with only SN (Fig. 7A and B). Green fluorescence was observed up to 120 μm, and intense fluorescence was found in the range between 50 and 90 μm. Compared to the control, a greater level of FD-4 was clearly visible in skin treated with MNs alone, up to a depth of 160 μm. In addition, strong fluorescence was detected in the range of 50–150 μm (Fig. 7C). In contrast, the fluorescent signal from the skin treated with the combination of MNs and SN was observed deep into the dermal region, at a depth of 300 μm, with higher intensity green fluorescence detected in the range of 70–270 μm (Fig. 7D). The pore channel beneath the skin surface produced by MNs is shown in Fig. 7C and D, where the zone of the puncture periphery is represented by a dark circular region. Likewise, this zone is also considered to be an effect of MNs breaching the SC barrier and acts to enhance drug permeation. It is obviously seen in Fig. 7 that the fluorescence intensity of FD-4 in skin treated with the combination of MNs and SN was the highest followed by MNs alone, SN alone, and untreated skin. These results verified the influence of MNs, which create holes in the skin, and low-frequency SN, which results in a thermal and cavitation effect, on skin permeation enhancement of FD-4. Our results support the idea of a synergistic effect in the combination of MNs and low-frequency SN for the skin permeation enhancement of hydrophilic macromolecules.

4. Conclusion

In this study, the following parameters were investigated: insertion force of MNs, intensity, radiation diameter of the probe, application time, and duty cycle of SN. SN or MNs alone increased the permeation of FD-4 through porcine skin; however, the combination of MNs and SN significantly increased skin permeation. This result is considered to be an excellent synergistic effect due to the combination of MNs and SN. The use of 10 N insertion forces of MNs, minimum intensity (4 W/cm^2), a larger radiation diameter of probe (6 mm), an application time of 2 min and continuous mode duty cycle of SN resulted in satisfactory permeation with acceptable safety. In summary, the combination of MNs and SN techniques provides an effective method for the delivery of hydrophilic macromolecule. This method represents a patient-friendly alternative to hypodermis injections for the delivery of large molecule drugs. In addition, the optimal conditions for this combination method may be applied in the clinic after further safety analysis studies.

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