

Controlled release of optimized electroporation enhances the transdermal efficiency of sinomenine hydrochloride for treating arthritis in vitro and in clinic

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Abstract: Sinomenine hydrochloride (SH) is an ideal drug for the treatment of rheumatoid arthritis and osteoarthritis. However, high plasma concentration of systemically administered SH can release histamine, which can cause rash and gastrointestinal side effects. Topical delivery can increase SH concentration in the synovial fluid without high plasma level, thus minimizing systemic side effects. However, passive diffusion of SH was found to be inefficient because of the presence of the stratum corneum layer. Therefore, an effective method is required to compensate for the low efficiency of SH passive diffusion. In this study, transdermal experiments in vitro and clinical tests were utilized to explore the optimized parameters for electroporation of topical delivery for SH. Fluorescence experiment and hematoxylin and eosin staining analysis were performed to reveal the mechanism by which electroporation promoted permeation. In vitro, optimized electroporation parameters were 3 KHz, exponential waveform, and intensity 10. Using these parameters, transdermal permeation of SH was increased by 1.9–10.1 fold in mice skin and by 1.6–47.1 fold in miniature pig skin compared with passive diffusion. After the electroporation stimulation, the intercellular intervals and epidermal cracks in the skin increased. In clinical tests, SH concentration in synovial fluid was 20.84 ng/mL after treatment with electroporation. Therefore, electroporation with optimized parameters could significantly enhance transdermal permeation of SH. The mechanism by which electroporation promoted permeation was that the electronic pulses made the skin structure looser. To summarize, electroporation may be an effective complementary method for transdermal permeation of SH. The controlled release of electroporation may be a promising clinical method for transdermal drug administration.

Keywords: electroporation, sinomenine hydrochloride, transdermal drug delivery, arthritis

Introduction

Rheumatoid arthritis (RA) is a refractory and chronic inflammatory disease, while osteoarthritis (OA) is the most common disorder of the locomotor system. RA and OA can damage joint function, induce joint injury and arthromeningitis, and lead to premature death.¹ Reports have shown that approximately 1% of the world population is suffering from RA and that OA affects mainly the elderly.^{2,3} Nonsteroidal anti-inflammatory drugs (NSAIDs) have been reported to be widely used in RA and OA therapy.^{4,5} However, NSAIDs can induce renal and gastrointestinal toxicities because they inhibit prostaglandin synthesis, thus limiting the clinical application of NSAIDs.⁶ Among the side effects, NSAID-induced enteropathy could increase small

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intestine bleeding and ulcers.⁷ Thereby, choosing a drug to reduce RA and OA symptoms and prevent future adverse effects is necessary.

Sinomenine (C₁₉H₂₃NO₄), extracted from the stems and roots of *Sinomenium acutum*, is a kind of pharmaceutically active alkaloid with few side effects.⁸ Previous pharmacological studies have demonstrated that sinomenine has analgesic,⁹ anti-inflammatory,¹⁰ cartilage protection,¹¹ and immune suppression effects.¹² For decades, sinomenine has been widely used to treat RA and OA in China.¹³ Compared with NSAIDs, sinomenine is more effective in amelioration of morning stiffness, painful joints, and erythrocyte sedimentation rate, with less adverse effects on the digestive system.¹⁴ Therefore, sinomenine is a valuable remedy to treat RA and OA in clinical practice.

Sinomenine hydrochloride (SH, Figure 1) is designed to provide better efficacy with daily application.¹⁵ SH tablets and injections have been used effectively for RA and OA therapy. The anti-RA and anti-OA effect of SH might be closely connected with its antiproliferative effect on inflammatory indexes on articular tissue and intervention of gristle degeneration and cartilage cell apoptosis.^{16,17} Hence, the articular tissue is the primary therapeutic target of SH. SH concentration within the articular tissues, such as synovial fluid (SF) or synovial membrane, is important. Nevertheless, SH tablets and injections are systemically administered. Moreover, the concentration of systemically administered SH in plasma was much higher compared with that in SF.¹⁸ Therefore, comparatively higher plasma concentration is necessary for achieving adequate amounts of SH in SF via systemic administration. Previous studies have demonstrated that high plasma concentration of SH will lead to potent release of histamine in association with the degranulation of mast cells in mammalian tissues,¹⁹ and this could cause rash and gastrointestinal side effects (eg, nausea, diarrhea, gastralgia, and occasionally vomiting). Research has also found that the adverse effects of SH appearing in some animals after dosing might be related to high plasma concentration of rapid SH distribution to the internal organs.²⁰ Undoubtedly, topical transdermal drug delivery targeting the joint tissue is a promising therapeutic method for RA and OA patients. On one hand, it decreases drug plasma concentration and SH distribution into the internal organs, which could alleviate side effects. On the other hand, it increases drug concentration in SF, which could enhance drug efficiency. Concurrently, transdermal drug delivery might enable the drug to bypass the first pass effect (oral administration) and might provide

relatively steady drug concentration in SF for long periods of time.²¹ Although transdermal delivery of SH has been reported, the application of the transdermal delivery system is often limited due to the stratum corneum (SC) layer, which is the primary barrier of the outermost skin layer.²² The transdermal delivery did not enable the passive diffusion of SH into the skin, thereby reducing SH efficacy.

At present, some physical means, such as microneedles,²³ iontophoresis,²⁴ sonophoresis,²⁵ and electroporation,²⁶ have been utilized to enhance transdermal drug delivery. Electroporation has been considered as an efficient physical technique of promoting skin permeability of small molecules and biomacromolecules.^{18,27} Electroporation can compensate for the defects of passive diffusion and significantly increase transdermal efficiency by using a series of short, high-voltage electric pulses.^{28,29} Furthermore, electroporation uses only a small instrument, is safe, and is convenient for self-administration by patients.

However, the mechanism by which electroporation improves transdermal efficiency is not clearly understood. For example, why and how could electroporation enhance permeation is yet to be determined. In this study, fluorescence method and HE staining were chosen to explore the transdermal enhancement mechanism of electroporation. The selection of appropriate parameters of electroporation is one of the keys to high transdermal enhancement effect of electroporation. Three parameters, namely, pulse frequency, pulse waveform, and pulse intensity, of electroporation were selected as the typical parameters to explore the above relationship. The transdermal enhancement effect of electroporation was confirmed by the using optimized parameters for electroporation, facilitating clinically transdermal permeation tests. Highly efficient transdermal delivery by using the optimized parameters of electroporation is recommended to enhance SH transdermal administration method in future clinical application.

Materials and methods

Materials

SH solution (25 mg/mL, injection) was provided by Zhengqing Pharmaceutical Group Co., Ltd. (Hunan, People's Republic of China). SH (Standard substance) was purchased from Dalian Meilun Biotech Co., Ltd. (Dalian, People's Republic of China). CaCl₂, KCl, NaCl, and NaHCO₃ were purchased from Sigma-Aldrich (St Louis, MO, USA). Testosterone, used as internal standard (IS), was purchased from Nacalai Tesque Company (Kyoto, Japan). Sodium fluorescein

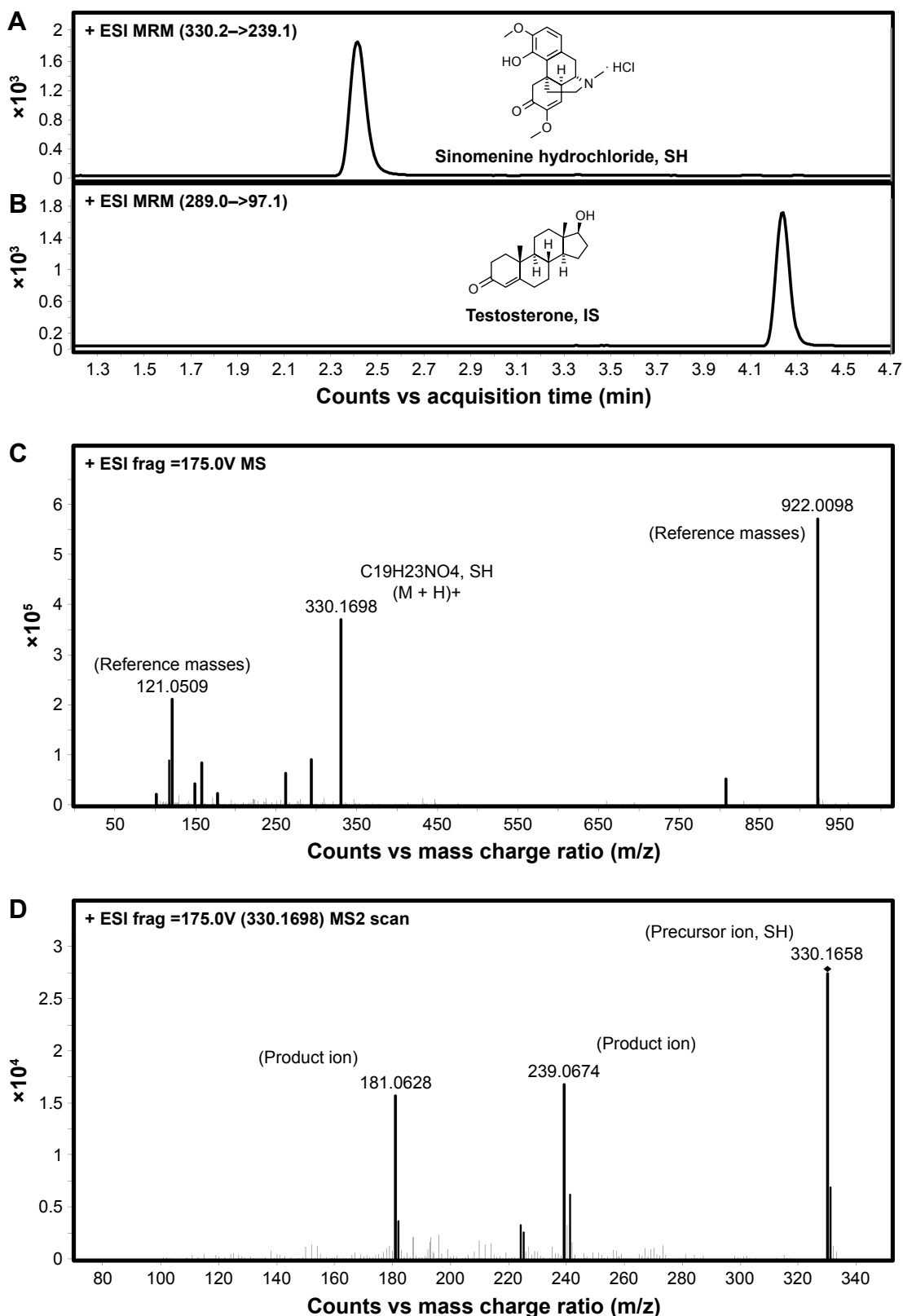


Figure 1 UHPLC–MS/MS method for analysis of SH in Ringer’s solution and SF.

Notes: The compound-dependent parameters were in MRM method. **(A)** The transition of SH was 330.2/239.1. **(B)** The transition of testosterone (IS) was 289.0/97.1. **(C)** and **(D)** were the MS spectra and the MS/MS spectra of SH, respectively.

Abbreviations: SH, sinomenine hydrochloride; IS, internal standard; MRM, multiple reaction monitoring; MS, mass; ESI, electron spray ionization; SF, synovial fluid; UHPLC, ultra high performance liquid chromatography.

(NaFluo) was from GBCBIO Biological Technology Co., Ltd. (Guangzhou, People's Republic of China). Urethane was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, People's Republic of China). The water used for the ultra-high performance liquid chromatography–mass spectroscopy/mass spectroscopy (UHPLC–MS/MS) analysis was obtained from a Milli-Q water purification system produced by Millipore (Billerica, MA, USA). HPLC-grade acetonitrile, methanol, and formic acid were acquired from Merck (Darmstadt, Germany). Other reagents were of analytical grade.

Animals

All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996). The animal protocol used in the study was approved by the Institutional Animal Care and Use Committee of the Guangzhou University of Chinese Medicine. Male Kunming mice (weighing 18–22 g) were used in the current experiments. All mice were purchased from the Laboratory Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, People's Republic of China) and were raised in the animal houses of the Laboratory Animal Center of Guangzhou University of Chinese Medicine. Four mice were housed per cage in a clean animal cabinet with ad libitum access to nutritionally adequate food and uncontaminated drinking water throughout the experimental periods. The mice were housed in an animal cabinet with unidirectional air flow under 40%–70% relative humidity, 20°C–24°C, and 12 h light/dark cycle. The abdominal skins of Bama miniature pigs (weighing 13 kg) were purchased from and prepared at Taizhou Taihe Biotech Co., Ltd. (Taizhou, People's Republic of China). All animals were killed by cervical luxation after anesthesia with an intraperitoneal injection of urethane (50%, w/v).

Skin preparation

The back skins of mice and the abdominal skins of Bama miniature pig were cut using surgical instruments and washed with Ringer's solution or normal saline. The skins were divided into 3 cm² size and stored at –80°C. The pieces of skins were used within 3 months. The skins were thawed at room temperature and then soaked in Ringer's solution (pH 7.4) for 1 h before transdermal experiments.

Passive diffusion study

Percutaneous experiments *in vitro* were done on YB-P6 intelligent transdermal diffusion instrument, which was purchased

from Tianjin Pharmacopoeia Standard Instrument Factory (Tianjin, People's Republic of China), with the perpendicular Franz-type diffusion cells. The diffusion area was 1.96 cm². The pieces of skin tissue were sandwiched between the receiver and donor compartments, and the epidermal side was facing up (Figure 2). Diffusion was continued for 8 h at 37°C±0.5°C.³⁰ A total of 2 mL SH solution was added to the donor compartment. The mouths of donor compartments were covered with parafilm (Bemis Company, Oshkosh, USA) to avoid liquid evaporation. The receiver compartments were filled with 17 mL Ringer's solution (pH 7.4) and stirred at 400 rpm. The samples (3 mL) were extracted from the receiver compartments for UHPLC–MS/MS analysis and then rapidly replaced with the same quantity (3 mL) of fresh Ringer's solution at 37°C±0.5°C at 1, 2, 3, 4, 6, and 8 h. Each experiment was repeated six times.

Electroporation study

An electroporation system (Yuandao Digital Electronics Co., Ltd., Shenzhen, People's Republic of China) was used to provide a series of electronic pulses. The electrode components, which were made of metal and shaped like a small stick, were placed in the donor and the receptor compartments (Figure 2). Other procedures were the same as described in “Passive diffusion study” section.

Transdermal enhancement regulation with different electroporation parameters and different drug concentrations

The relationship between the different frequency, waveform, and intensity of electroporation and the amount of SH that permeated in the *in vitro* transdermal experiments (mice and miniature pig skins) were studied under the fixed drug concentration.

To study the amount of SH that permeated at different drug concentrations, *in vitro* transdermal experiments (mice and miniature pig skins) were performed using fixed parameters of electroporation.

Transdermal enhancement mechanism of electroporation

Hematoxylin and eosin staining

Transdermal permeation experiments were done *in vitro*. Then, the mice and miniature pig skins were immediately collected and soaked in 4% paraformaldehyde solution for 24 h at 4°C. The skin was then dehydrated using gradient alcohol and xylene, and then embedded in paraffin according to standard processes. Serial 5 µm-thick sections of skins were

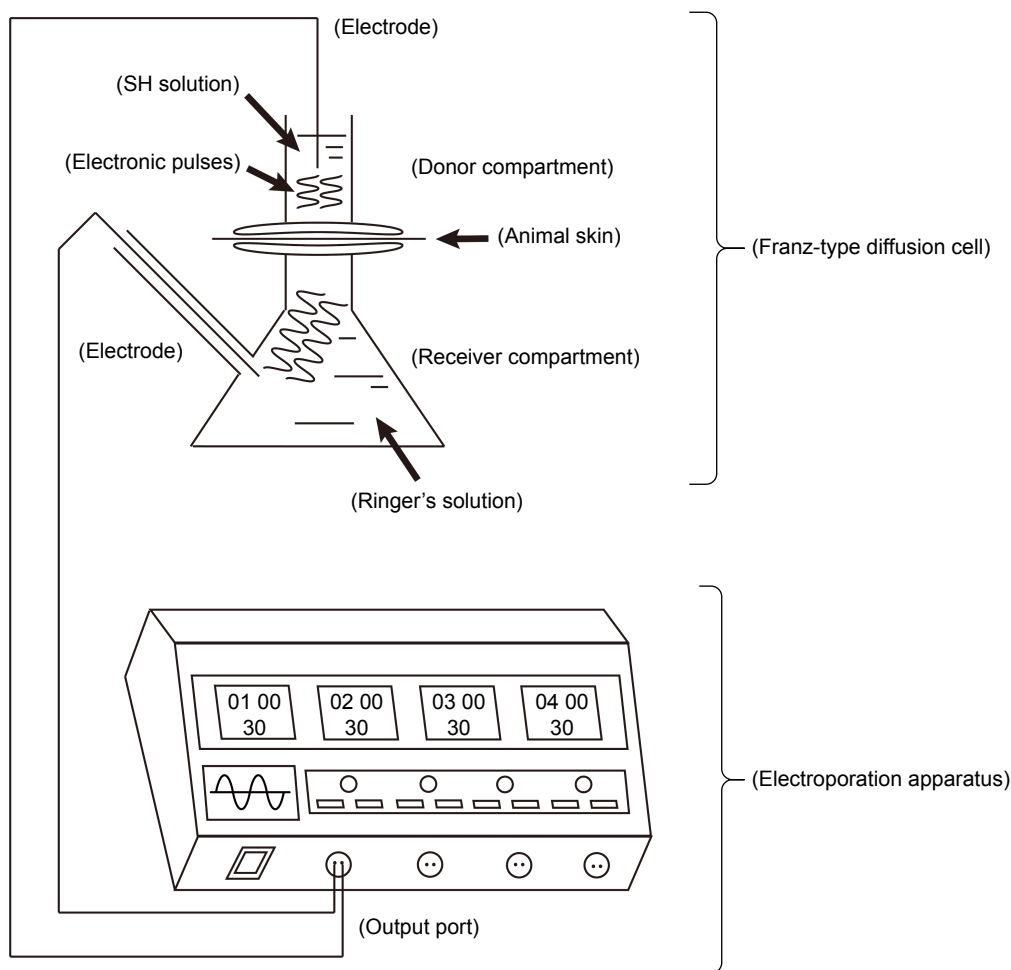


Figure 2 Flow diagram of transdermal experiment in vitro.

Notes: The skin tissues of mice and miniature pig were fixed between the donor and receiver compartments. Diffusion was continued for 8 h at $37^{\circ}\text{C}\pm 0.5^{\circ}\text{C}$.

Abbreviation: SH, sinomenine hydrochloride.

obtained using a Leica RM2235 microtome (Oberkochen, Germany). The histological properties of the skin were analyzed using hematoxylin and eosin (HE) staining and were observed with a Leica DM750 Microscopy.

Transdermal permeation experiments of sodium fluorescein

NaFluo (2 mg) was added to Ringer's solution (2 mL) and continually vortexed until it dissolved. Transdermal permeation experiments (on mice and miniature pig skins) were done using the NaFluo aqueous solution with or without electroporation following the method described in "Passive diffusion study" Section.³¹ Then, the skins were immediately collected and frozen in liquid nitrogen. The skins were then cut using a freezing microtome (Leica CM1860 UV, Leica Camera AG, Solms, Germany) into 5 μm -thick sections. The sections were washed thrice with Ringer's solution (pH 7.4) to wash off free NaFluo and observed with a Leica TSC

SP8 Laser Confocal Microscopy (Oberkochen, Germany). The conditions were as follows: 488 nm excitation wavelength, 515 nm emission wavelength, 0 to 255 fluorescence intensity.³²

Transdermal treatment of SH with optimized electroporation in clinical tests Patients and study design

The clinical tests were done at Guangzhou Hospital of Integrated Traditional Chinese and Western Medicine. These tests did not change the original treatment plan of patients. All patients in the tests were diagnosed with arthritic diseases (RA or OA) according to a detailed medical history for knee pain, a comprehensive physical exam, and imaging studies. The inclusion criteria were as follows: male or female >40 years old; presence of knee inflammation, knee pain, or any other symptom connected with knee arthritis diseases; and Kellgren–Lawrence radiological grade I–III.³³

Meanwhile, the exclusion criteria were as follows: presence of severe cardiovascular disease, immunodepression, coagulopathy, liver disease, infection, and hematological disease; and pregnancy. In patients with bilateral arthritis, only the knee that showed more advanced disease was considered. The protocol was approved by Guangzhou Hospital of Integrated Traditional Chinese and Western Medicine Ethical Committee, and informed consent was obtained from all patients. All programs were performed in conformity to the ethical standards of the institutional research committee and in accordance with the 1964 Helsinki Declaration and its latter amendments.

Evaluating the therapeutic efficacy and detecting the concentration of SH in the patient SF in clinical tests

For evaluating clinical efficacy, the test group had nine patients, and the positive control group had six patients. The therapeutic program in the test group was transdermal treatment of SH solution with optimized electroporation combined with standard therapy (eg, celecoxib). Meanwhile, the therapeutic program in the positive control group was only the standard therapy (eg, celecoxib). Clinical efficacy parameters were assessed after 8 days. The parameters for evaluating clinical efficacy were joint pain degree (Visual Analog Scale/Score [VAS]) and joint movement degree. The clinical improvement rate was calculated according to Eq (3). The transdermal treatment method using SH solution with electroporation was as follows: 4 mL SH solution (25 mg/mL, injection) was mixed with 5 mL normal saline and subsequently vortexed. After vortexing to allow homogenization, the mixture was absorbed by two pieces of cotton. The two pieces of cotton containing the liquid mixture were placed in two electrodes, and the two electrodes were subsequently placed on one knee of the patients. Clinical transdermal tests were done using the liquid mixture with electroporation for 30 minutes. The electroporation conditions were as follows: 3 KHz, exponential waveform, and intensity 10.

For detecting the concentration of SH in the patient SF, the test group had nine patients and the control group had six patients. The clinical transdermal drug delivery in the test group was done using the liquid mixture with electroporation for 30 minutes. The clinical transdermal drug delivery in the control group was done using the liquid mixture without electroporation for 30 minutes. After transdermal administration, the patient SF samples were collected by the hospital staff using the SF collection equipment. The concentration of SH in the patient SF samples were determined by an UHPLC–MS/MS system. The transdermal treatment

method using SH solution with electroporation was done as described earlier.

Sample preparation for in vitro transdermal experiments

Samples for in vitro transdermal experiments (200 μ L) were mixed with 10 μ L IS solution (0.5 μ mol/L testosterone in methanol) and subsequently vortexed for 5 min. After vortexing to allow homogenization, the mixture was centrifuged at 19,357 g for 30 min. A total of 5 μ L of the supernatant layer was injected into the UHPLC–MS/MS column for analysis.

Patient SF sample preparation

Patient SF samples (200 μ L) were mixed with 5 μ L IS solution (25 nmol/L testosterone in methanol) and 1,000 μ L methanol solution (5:1), which was added to extract the analytes and remove proteins, and subsequently vortexed for 5 min. After vortexing to homogenize, the mixture was centrifuged at 19,357 g for 30 min. The supernatant layer was transferred into a new tube and evaporated to dryness in a thermostatic vacuum drier (EYELA Vacuum oven VOS-301SD, Tokyo, Japan). The residues were redissolved using 100 μ L of 50% methanol aqueous solution and centrifuged at 19,357 g for 30 min. A total of 5 μ L of the supernatant layer was injected into the UHPLC–MS/MS column for analysis.

Analysis and identification of drugs in the receptors

UHPLC–MS/MS was performed on an Agilent 6540 accurate-mass Q-TOF MS system and 6460 Triple Quadrupole mass system with an electrospray ionization source (Agilent Technologies, Santa Clara, CA, USA). The data were recorded, and the system was controlled using the MassHunter software (version B.06.00, Agilent Technologies). The conditions were as follows: column, Zorbax SB-C18, 3.0 \times 100 mm, 1.8 μ m (Agilent Technologies), with the column temperature maintained at 25°C; mobile phase A, 0.1% formic acid water; mobile phase B, 100% acetonitrile; flow rate, 0.35 mL/min; gradient, 0–1.5 min, 15%–15% B, 1.5–2 min, 15%–90% B, 2–4.2 min, 90%–68% B, 4.2–4.7 min, 68%–15% B; and injection volume, 5 μ L.

The main MS/MS working parameters in the positive mode (1–4.7 min) were set as follows: 3.5 kV capillary voltage, 500 V nozzle voltage, 300°C gas temperature, 300°C sheath temperature, 12 L/min sheath gas flow, and 10 L/min gas flow. Quantification was performed via the multiple reaction monitoring of the transitions of m/z 330.2 \rightarrow 239.1

and m/z 330.2 \rightarrow 181.0 for SH³⁴ and m/z 289.0 \rightarrow 97.1 for IS. Collision energy was set at 26 for SH and 18 for IS. The low limit of quantitative analysis was 0.023 ng/mL for SH.

Data analysis

Cumulative drug permeation amount (Q_n , $\mu\text{g}/\text{cm}^2$) was the cumulative amount of drug transdermally delivered in the receiver compartments, and it was computed according to Eq (1).

$$Q_n = \frac{C_n \times V + V_0 \sum_{i=1}^{n-1} C_i}{A} \quad (1)$$

In Eq (1), A (cm^2) is the transdermal area, V_0 (mL) is the bulk of samples extracted from the receiver compartments, V (mL) is the bulk of Ringer's solution in the receiver compartment, and C_i ($\mu\text{g}/\text{mL}$) and C_n ($\mu\text{g}/\text{mL}$) are the concentration of sample i and n .

Steady-state transdermal flux (J_{ss} , $\mu\text{g}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$) at time t represented the slope of the line graphs of cumulative drug permeation amount (Q_n) versus time (t , s). The flux enhancement ratio (ER) induced by electroporation was computed according to Eq (2).

$$\text{ER} = \left(\frac{\text{Flux with electroporation}}{\text{Flux without electroporation}} \right) = \frac{J_{ss+}}{J_{ss-}} \quad (2)$$

Clinical improvement rate was calculated according to Eq (3).

$$\text{Clinical improvement rate} = \frac{(\text{Pretreatment value} - \text{Posttreatment value})}{\text{Pretreatment value}} \times 100\% \quad (3)$$

Data were represented as mean \pm standard error of mean. One-way analysis of variance was used to evaluate statistical differences. A difference was considered statistically significant at $P < 0.05$.

Results and discussion

Analysis and identification of SH in samples

UHPLC–MS/MS was performed to identify the structure and analyze SH levels in samples. The results of UHPLC–MS/MS were as follows: in the representative MRM chromatogram, retention time of SH was 2.4 min (Figure 1A) and that of IS was 4.2 min (Figure 1B); the high-resolution mass

spectrometry of SH indicated that the $[M + H]^+ = 330.1698$ ion (Figure 1C), and the MS/MS analyses of SH product the m/z 181.0628 and 239.0674 (Figure 1D). Taken together, the data obtained for the SH in samples was in accordance with the standard substance.

Effect of electroporation parameters and drug concentration on SH penetration in mice and miniature pig skins

Transdermal enhancement by varying electroporation parameters in mice skin

1.5, 3, and 6 KHz were chosen to study the effect of pulse frequency on SH penetration in mice skins. The results demonstrated that the total Q_n of 3 KHz was $6.50 \pm 0.94 \mu\text{g}/\text{cm}^2$ (Figure 3A1). The ER of 3 KHz was 3.09 ± 0.41 , which was more than the ER seen at other pulse frequencies (Figure 3A2). Taken together, 3 KHz was found to be the optimal pulse frequency that had the most significant enhancement-promoting effect on SH penetration. Interestingly, we found total Q_n differed in the following manner: Q_n induced by 3 KHz $>$ Q_n induced by 1.5 KHz $>$ Q_n induced by 6 KHz. The situation above was exactly opposite to our primary hypothesis that 6 KHz might induce the strongest transdermal enhancement. The reason for this phenomenon might be that 6 KHz produced too many pulses, and 1.5 KHz produced too little pulses, which were not in the optimal range inducing change for skin structure. Thereby, Q_n induced by 6 KHz and 1.5 KHz were less than that induced by 3 KHz, which could produce the exact pulses required for changing the skin structure. To summarize, electroporation did enhance the SH penetration in mice skins, and 3 KHz was the optimal pulse frequency.

Different pulse waveforms produced different Q_n in mice skins. The total Q_n of exponential waveform was $10.48 \pm 1.08 \mu\text{g}/\text{cm}^2$ (Figure 3B1). The ER of the exponential waveform was 6.40 ± 0.64 (Figure 3B2). That is, exponential pulse produced more intense SH permeation. A previous study showed that exponential waveform was one of the most commonly used pulse waveforms in electroporation.³⁵ Generally, increasing pulse amplitude, duration, and number resulted in increased skin permeability, allowing for better drug transport.³⁶ The threshold-induced change in the skin structure depends on the pulse duration. An increase in the electronic pulse duration leads to a decrease in the critical threshold, and so pulse duration of exponential pulse was long.³⁷ Therefore, we concluded that the exponential waveform was the optimal pulse waveform.

The total Q_n and ER at intensity 10 were $18.32 \pm 2.98 \mu\text{g}/\text{cm}^2$ (Figure 3C1) and 9.79 ± 1.69 , respectively, (Figure 3C2), and

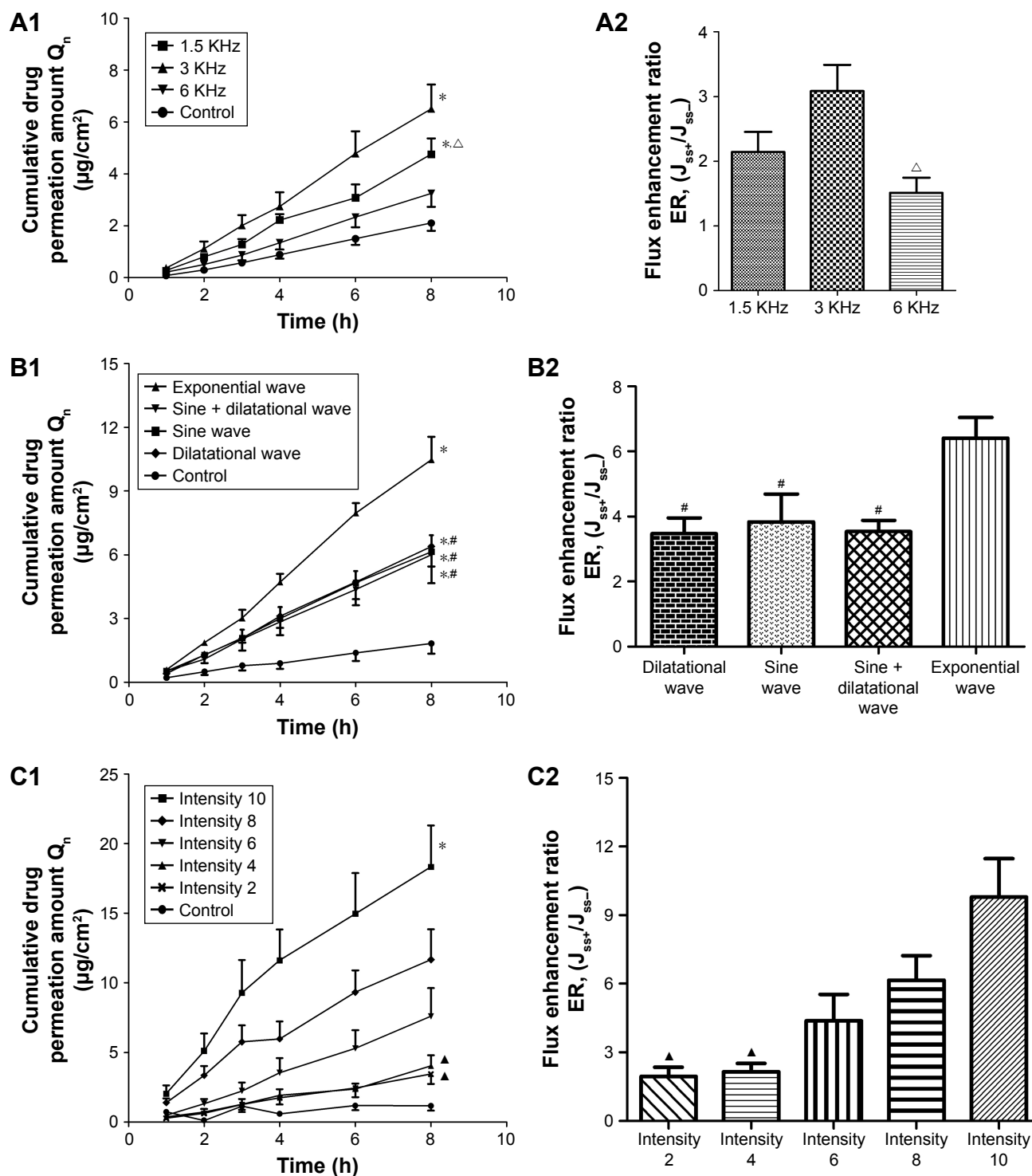


Figure 3 Transdermal enhancement of pulse frequencies, waveforms, and intensity on SH in vitro mice skins.

Notes: The effect of pulse frequencies on Q_n -time curves (**A1**) and ER (**A2**). The other fixed parameters of electroporation: sine wave and intensity 4. The effect of pulse waveforms on Q_n -time curves (**B1**) and ER (**B2**). The other fixed parameters of electroporation: 1.5 KHz and intensity 4. The effect of pulse intensities on Q_n -time curves (**C1**) and ER (**C2**). The other fixed parameters of electroporation: sine wave and 1.5 KHz. * $P < 0.05$ versus the control groups. $\Delta P < 0.05$ versus the 3 KHz groups. # $P < 0.05$ versus the exponential waveform groups. $\Delta P < 0.05$ versus the intensity 10 groups. Data are represented as mean \pm SEM. $n = 6$.

Abbreviations: SH, sinomenine hydrochloride; Q_n , cumulative drug permeation amount; ER, enhancement ratio; SEM, standard error of mean.

Q_n increased in an intensity-dependent manner in the intensity range 2–10 (Figure 3C1 and C2). Therefore, enhanced pulse intensity could enhance skin permeability. For our electroporation equipment, intensity 10 was the maximum intensity that

could be given to humans without harm, and for animals it was 4. Using higher intensity might result in excessive stimulation of the animal and make them feel uncomfortable. The total Q_n and ER of intensity 10 was more than those seen at

other pulse intensities. As expected, intensity 10 was found to be the optimal pulse intensity for humans. Above all, pulse frequency, waveforms, and intensity could affect the SH penetration in mice skins, and the optimal parameters were 3 KHz, exponential waveform, and intensity 10 for humans.

Transdermal enhancement of electroporation in miniature pig skins by varying the parameters

The total Q_n of 3 KHz, exponential waveform, and intensity 10 were 8.67 ± 0.87 , 8.67 ± 0.87 , and 53.92 ± 5.07 $\mu\text{g}/\text{cm}^2$, respectively (Figure 4A1, B1, and C1). Meanwhile, ER for 3 KHz, exponential waveform, and intensity 10 were 8.12 ± 0.92 , 8.12 ± 0.92 , and 48.17 ± 3.93 , respectively (Figure 4A2, B2, and C2). These results showed that Q_n and ER induced by electroporation parameters 3 KHz, exponential waveform, and intensity 10 were higher compared with that induced by other electroporation parameters. On analysing the overall situation, the results in miniature pig skins were in accordance with that in mice skins, and the optimal parameters were also same in both mice and miniature pig skins, and the optimal electroporation parameters were 3 KHz, exponential waveform, and intensity 10 for humans. In practical clinical application, we could choose different parameters of electroporation to achieve different kinds of enhancement in transdermal efficiency so as to achieve the purpose of controlled release drugs.

Effect of different drug concentration of SH on cumulative drug permeation amount in mice and miniature pig skins

The total Q_n of different groups were 0.73 ± 0.08 , 2.22 ± 0.67 , 5.41 ± 1.21 , and 10.57 ± 2.80 $\mu\text{g}/\text{cm}^2$ in mice skins (Figure 5A2), and 1.28 ± 0.35 , 2.71 ± 0.71 , 8.67 ± 0.87 , and 15.50 ± 1.01 $\mu\text{g}/\text{cm}^2$ in miniature pig skins (Figure 5B2). We also found that Q_n increased gradually with the increase of the drug concentration in vitro in mice and miniature pig skin permeation experiments with electroporation (Figure 5A1 and 5B1). According to these results, the total Q_n of 3.04 mmol/L was more apparent than that of other drug concentrations in in vitro mice and miniature pig skins. In brief, drug concentration was one of the key factors for electroporation to promote transdermal penetration of SH, and Q_n values increased in a drug-dependent manner.

Drug penetration routes include intercellular route, intracellular route, and eccrine route (eg, sweat glands).³⁸ Cell gap fillers and skin are usually hydrophobic;³⁹ therefore, penetrating through the skin into the blood circulation is difficult for the hydrophilic SH. As shown above, the Q_n of the passive diffusion transdermal experiments without electroporation were all so low that concentration of SH might not reach the

targeted therapeutic concentration. Through inducing the change of skin structure, electroporation could enable SH to overcome transdermal defects caused by its hydrophilic property. All Q_n values in the four drug concentrations with electroporation were more than that in control without electroporation, and this was a strong proof that electroporation significantly enhanced the transdermal permeation of SH.

Transdermal enhancement mechanism of electroporation in mice and miniature pig skins

The epidermis is the outermost skin layer that consists of that hypodermis, hydrophilic dermis, and epidermis.⁴⁰ The layers of the epidermis are stratum spinosum, stratum granulosum, and SC, which protects the skin from damage and is the main transdermal barrier of the skin.⁴¹ On HE staining analysis, the nucleus was blue and the cytoplasm was red (Figures 6A and B, 7A and B). In normal skin tissues, the skin structures were tight and integrated, and the flattened cells of the epidermis were tightly arranged, and the edge of cells was difficult to distinguish (Figures 6A and 7A). After electroporation stimulation, the skin structures were loose, the intercellular intervals and epidermal cracks of skin increased, and the distribution of stratified cells in SC was chaotic (Figures 6B and 7B). Taken together, electroporation did change the skin structure, increased the skin permeability, and enhanced transdermal drug delivery.

NaFluo is a fluorescent agent with a similar structure to SH. The molecular weight of NaFluo is 376.27. SH and NaFluo are both hydrophilic. NaFluo is only distributed in the outmost SC after passive diffusion without electroporation (Figures 6C and 7C). However, when electroporation was applied to the skins, the stratum spinosum, stratum granulosum, and SC were observed to have very strong fluorescence of NaFluo (Figures 6D and 7D), and this meant that NaFluo deeply permeated into epidermis.⁴² Therefore, electroporation could significantly promote NaFluo transdermal permeation. In summary, the mechanism by which electroporation promoted permeation was that electronic pulses made the skin looser and increased the intercellular spaces in the skin. Also, a schematic mechanism of electroporation enhancing SH transdermal delivery is shown in Figure 8. In addition, we found that the skin permeability enhanced by electroporation in miniature pig skin was greater than that in the mice skin in this study. This might be because the electroporation thresholds and electrical resistance of miniature pig abdomen skin was smaller than that of skin taken from the backs of mice; thus, the electronic pulse could better penetrate the miniature pig skin and cause the skin structure to change.⁴³

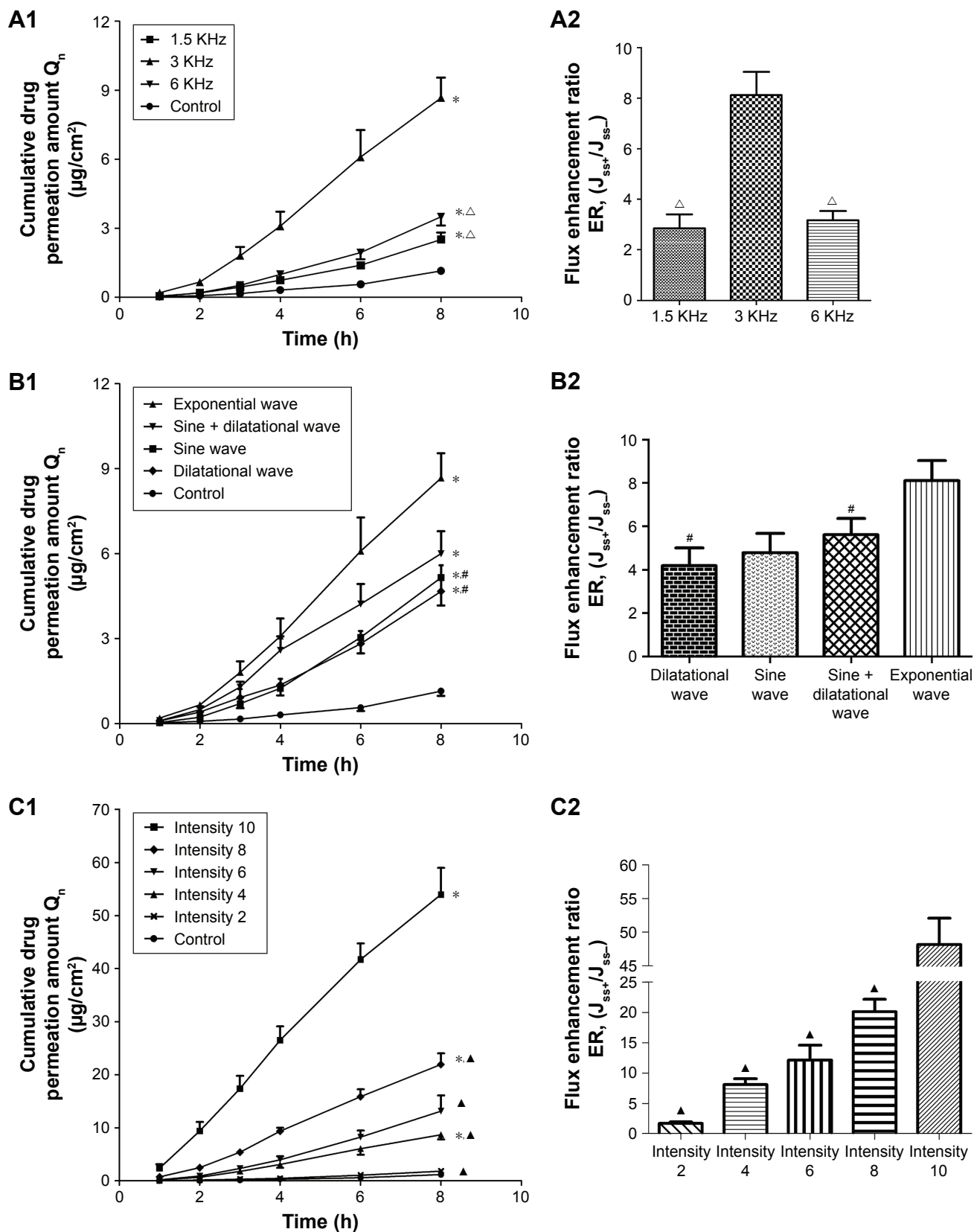


Figure 4 Transdermal enhancement of pulse frequencies, waveforms, and intensity on SH in vitro miniature pig skins. **Notes:** The effect of pulse frequencies on Q_n-time curves (A1) and ER (A2). The other fixed parameters of electroporation are as follows: exponential wave and intensity 4. The effect of pulse waveforms on Q_n-time curves (B1) and ER (B2). The other fixed parameters of electroporation: 3 KHz and intensity 4. The effect of pulse intensities on Q_n-time curves (C1) and ER (C2). The other fixed parameters of electroporation: exponential wave and 3 KHz. *P<0.05 versus the control groups. #P<0.05 versus the 3 KHz groups. ΔP<0.05 versus the exponential waveform groups. Data are represented as mean ± SEM. n=6. **Abbreviations:** SH, sinomenine hydrochloride; Q_n, cumulative drug permeation amount; ER, enhancement ratio; SEM, standard error of mean.

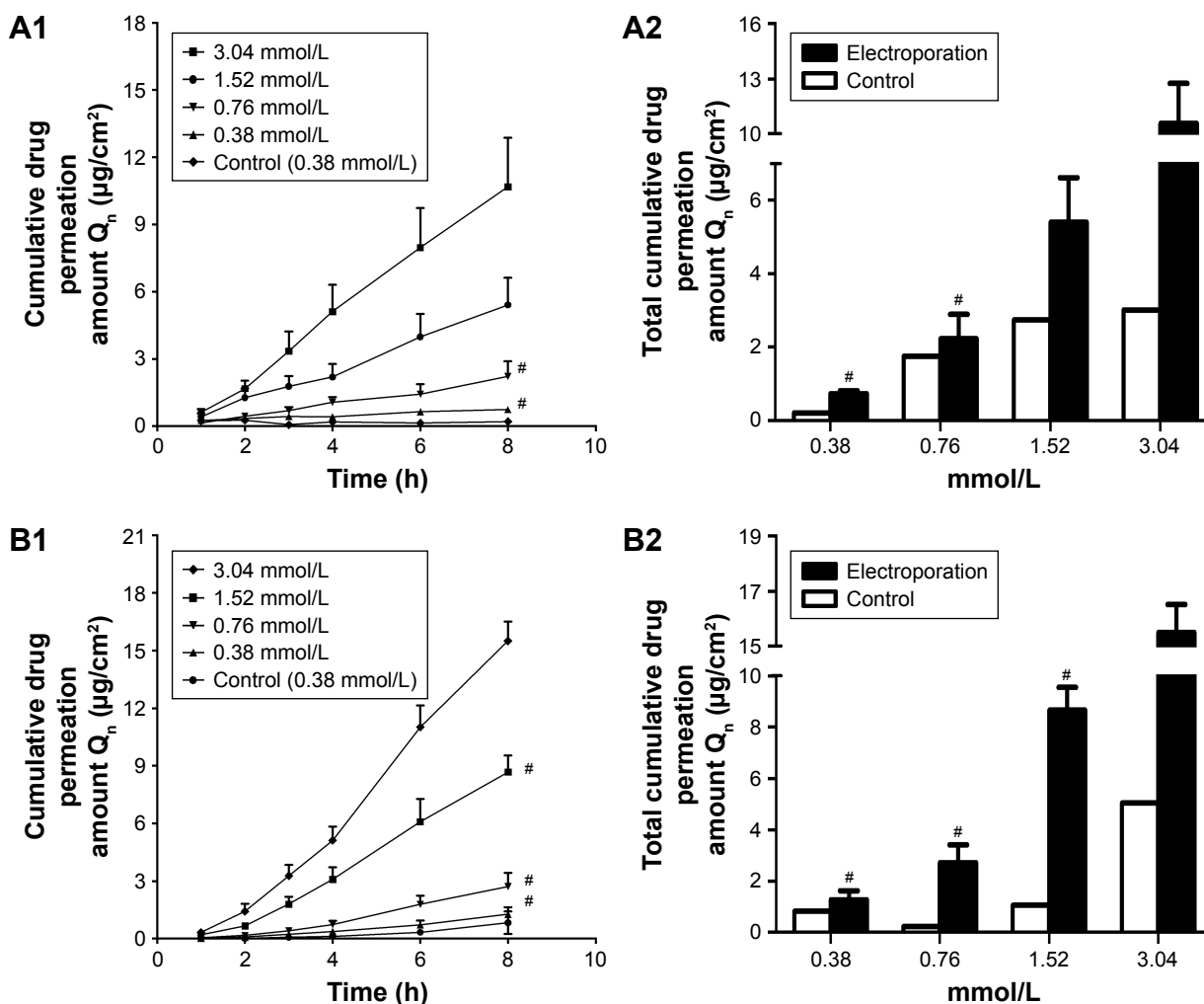


Figure 5 Relationship between drug concentration and transdermal amount of SH.

Notes: The Q_n increased as drug concentration rose in the skin taken from the back of mice (A1) and in miniature pig abdominal skins (B1). Total Q_n for different drug concentrations in mice skins (A2) and in miniature pig abdominal skins (B2). Electroporation condition: exponential wave, 3 KHz, and intensity 4. # $P < 0.05$ versus the 3.04 mmol/L groups. Data are represented as mean \pm SEM. Electroporation groups, $n=6$. Control groups, $n=1$.

Abbreviations: SH, sinomenine hydrochloride; Q_n , cumulative drug permeation amount; SEM, standard error of mean.

Effect of optimized parameter electroporation in clinical tests

In the test group, the improvement percentage in joint movement degree was $94.75\% \pm 6.84\%$ (Figure 9B), and the effective percentage of joint pain relief (VAS) was $79.39\% \pm 4.63\%$ (Figure 9C). Compared with the test group, the above two parameters were lower in positive control group, with the improvement percentage of joint movement degree being $52.41\% \pm 12.51\%$ (Figure 9D) and the effective percentage of joint pain relief (VAS) being $62.40\% \pm 7.54\%$ (Figure 9E). Moreover, the improvement percentage of joint movement degree was statistically significant ($P < 0.05$).

The concentration of SH in the patient SF of the test group and the control group was examined. In the test group, the maximum and minimum concentrations of SH were approximately 20.84 and 0.39 ng/mL, respectively (Figure 9A). In

the test group, we found that the concentration fluctuation of SH between different individuals was a little high due to the existence of high personal differences. The concentration of SH in patient SF samples of the control group without electroporation was below limit of quantification, so the results of the control group are not shown in this paper.

Based on the above results, high SH concentration in SF might provide better joint pain relief and joint mobility improvement. Therefore, to ensure therapeutic efficacy, we predicted that the minimum therapeutic concentration of SH in SF should be 20.84 ng/mL. As previously reported, the maximum concentration (C_{max}) of transdermally delivered sinomenine (50 mg/kg) with electroporation was 29.33 ± 4.87 ng/mL in rabbit plasma and 58.87 ± 7.50 ng/mL in rabbit SF, and these were lower compared with oral and injection administration.¹⁸ However, the C_{SF}/C_{Plasma}

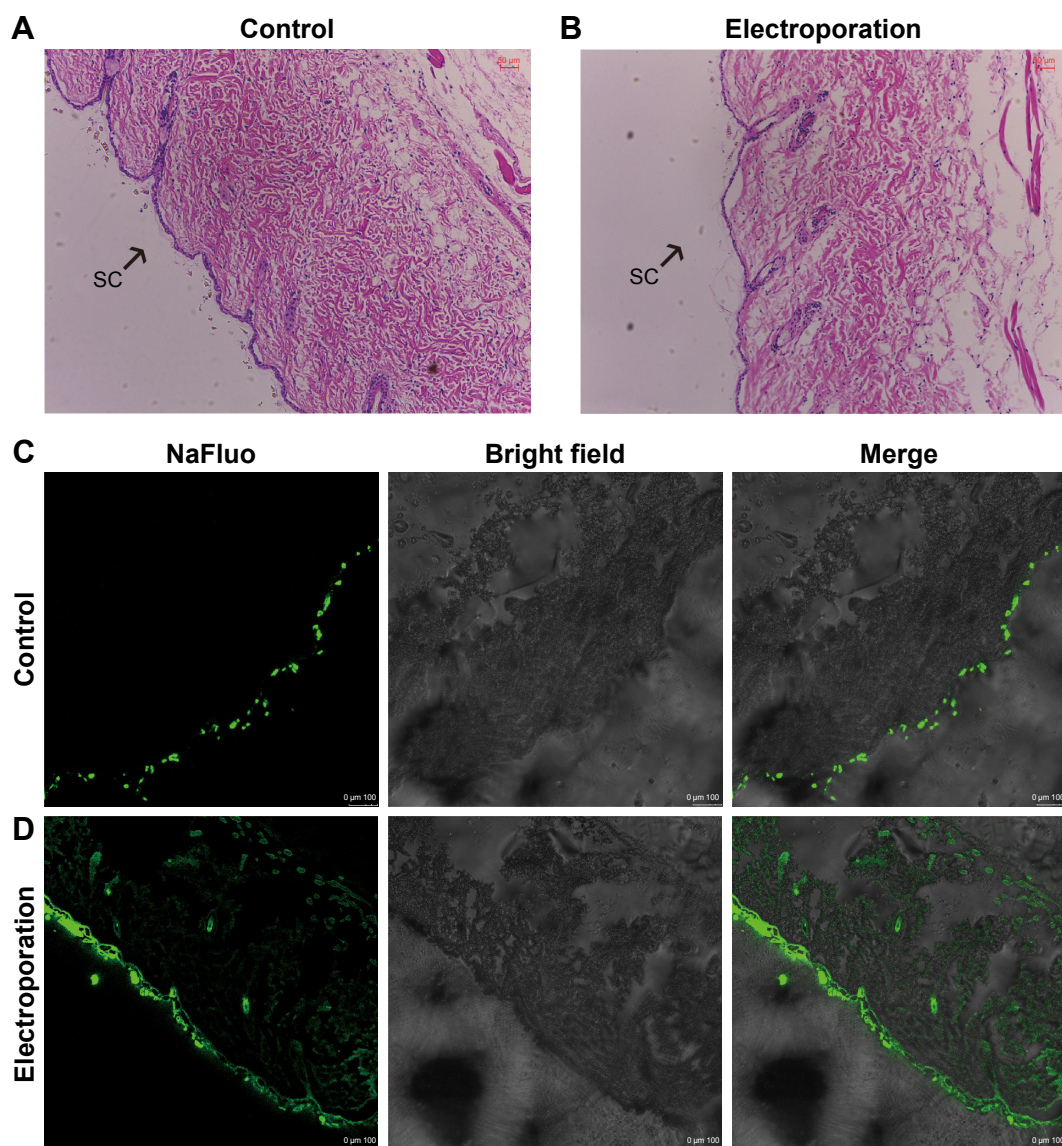


Figure 6 Effect of electroporation on mice skin structure.

Notes: The analysis of HE staining in control groups (A) and in electroporation groups (B). Distribution of NaFluo was in mice skins, and the control and electroporation groups are displayed in (C) and (D), respectively. All experiments were performed in triplicate. Magnification $\times 100$.

Abbreviations: NaFluo, sodium fluorescein; HE, hematoxylin and eosin; SC, stratum corneum.

(concentration of sinomenine in SF versus that in plasma) by electroporation was 3:1, and the C_{SF}/C_{Plasma} of systemic administration routes was approximately 1:3. In this study, the C_{max} of transdermally delivered SH (11.11 mg/mL, 9 mL, approximately 1.67 mg/kg) with electroporation was 20.84 ng/mL in patient SF. The result revealed that the C_{max} of SH in patient plasma that produced systemic side effects might be low. Hence, the side effects might easily be relieved. Concurrently, high SH concentration in SF could enhance the therapeutic effect of the target joint. In short, transdermal delivery of SH with electroporation could be selected as a promising treatment regimen of RA and OA to improve efficacy and reduce side effects.

Electroporation has some advantages compared with other transdermal enhancing methods. The chemical transdermal enhancers have some side effects, such as irritation and irreversible skin damage. However, no skin damage is caused in transdermal treatment with electroporation. Another widely used physical means, ie, sonophoresis, is not convenient due to large machines needed and the necessity for skilled nurses. Electroporation is excellent because it uses small machines and is convenient. In general, the major advantages of electroporation on improving transdermal drug delivery include high transdermal efficiency, simple performance, safety, and the small instrument needed. Therefore, electroporation could be

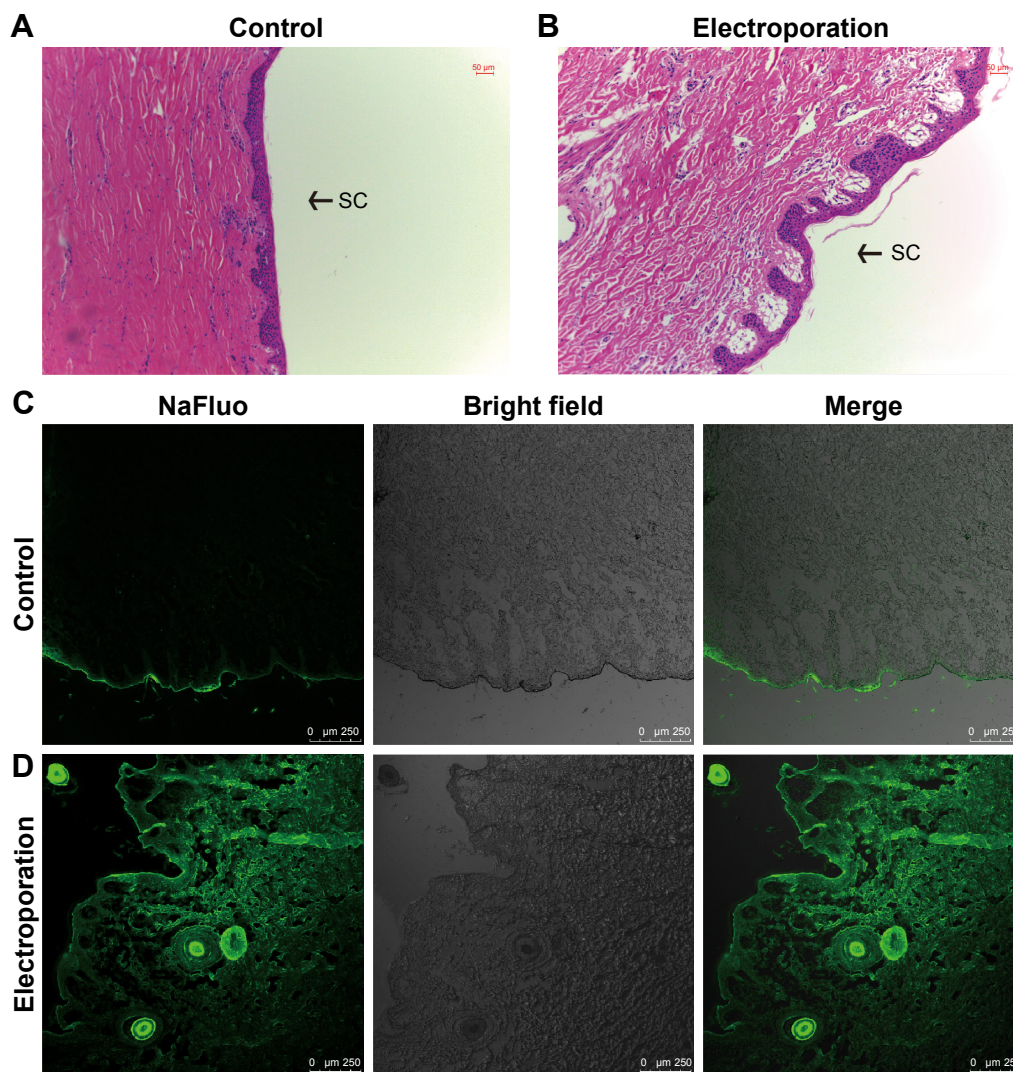


Figure 7 Effect of electroporation on miniature pig skin structure.

Notes: The analysis of HE staining in control groups (A) and in electroporation groups (B). Distribution of NaFluo was in miniature pig skins, and the control and electroporation groups are displayed in (C) and (D), respectively. All experiments were performed in triplicate. Magnification $\times 100$.

Abbreviations: NaFluo, sodium fluorescein; HE, hematoxylin and eosin; SC, stratum corneum.

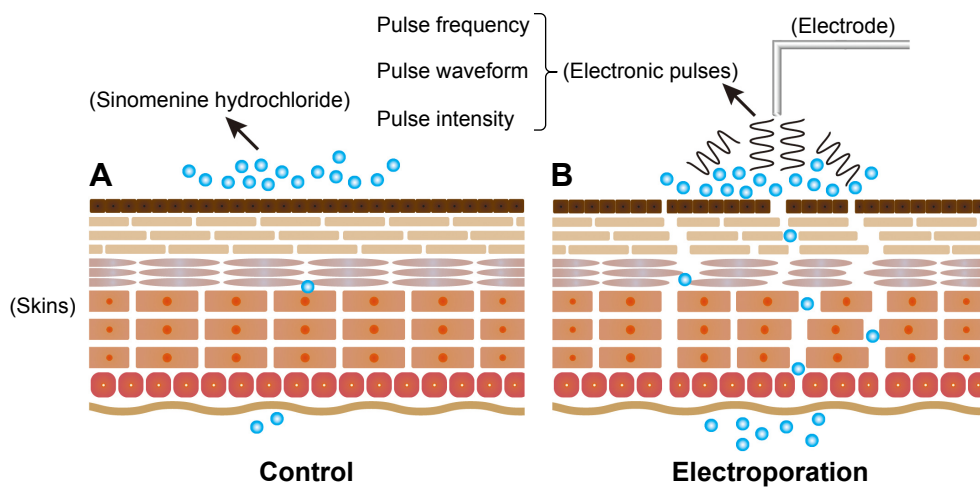


Figure 8 The mechanism of electroporation enhancing SH transdermal delivery.

Notes: (A) In control, the overall skin was tight and integrated. (B) The intercellular intervals and epidermal cracks of skin were increased after the electroporation stimulation, which would result in that SH had an enhanced permeation in the skin.

Abbreviation: SH, sinomenine hydrochloride.

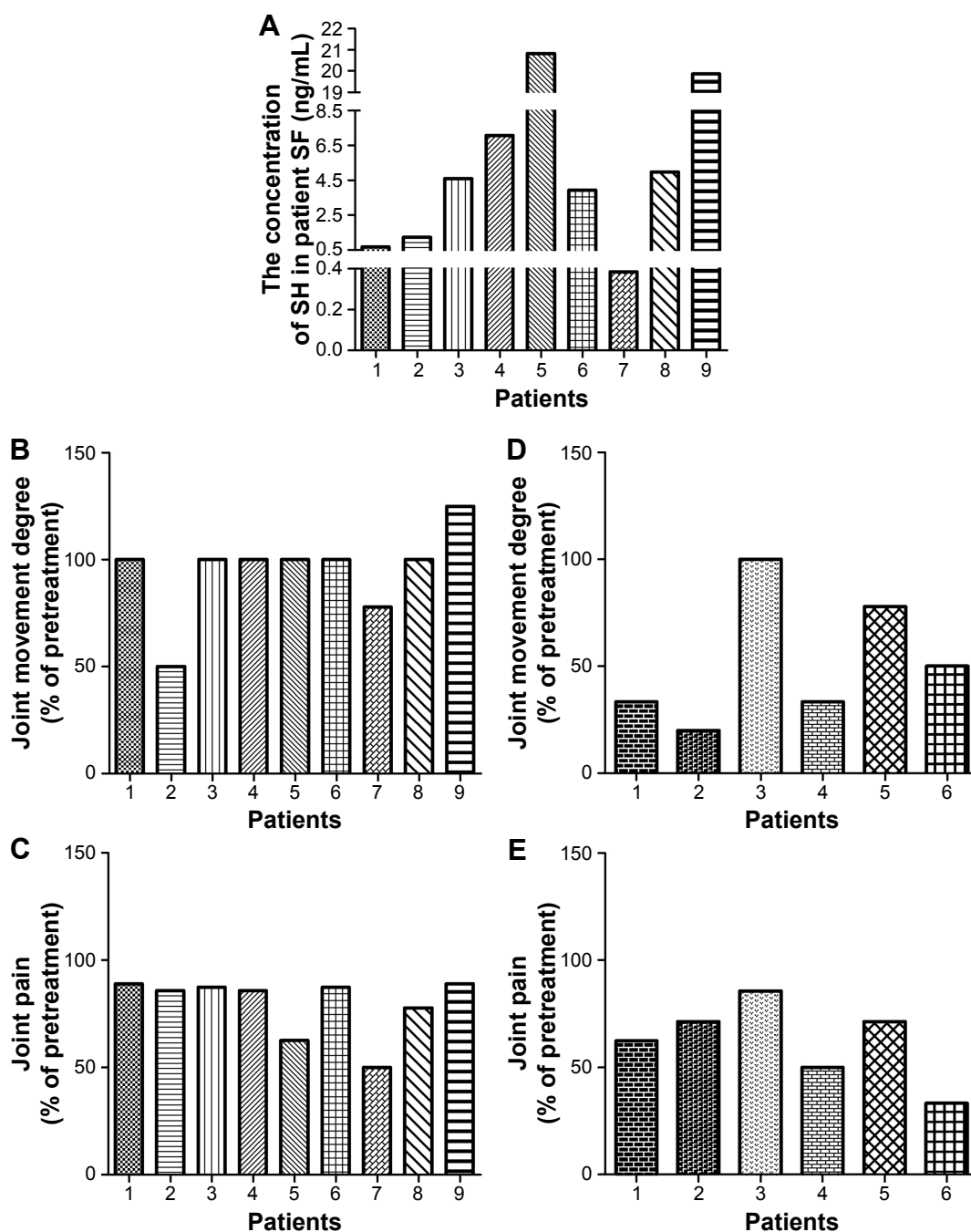


Figure 9 Optimized electroporation increased SH concentration in patient SF and improved the therapeutic effect of SH against arthritis.

Notes: (A) The SH concentration in patient SF of the test group that tested the combination of celecoxib with electroporation. (B) The improvement percentage of joint movement degree in test group. (C) The effective percentage of joint pain relief in test group. (D) The improvement percentage of joint movement degree in the positive control group that was treated with celecoxib without electroporation. (E) The effective percentage of joint pain relief in the positive control group. (B–E) the percentage of clinical symptom improvement is shown as patient self-control. Electroporation condition: 3 KHz, exponential waveform, and intensity 10.

Abbreviations: SH, sinomenine hydrochloride; SF, synovial fluid.

selected for promoting transdermal drug delivery of SH in clinical applications.

Conclusion

One of the most important factors determining the transdermal enhancement effect of electroporation is the

parameters of electroporation. The relationship between electroporation parameters (pulse frequency, waveform, and intensity) and the transdermal enhancement effect of electroporation was deeply investigated in this study. We found that exponential waveform, 3 KHz, and intensity 10 were the optimum parameters for electroporation in

humans. The results of our study demonstrated that with optimized parameters, electroporation exhibited significant enhancing effects on percutaneous permeation of SH. In vitro electroporation-promoting permeation experiments showed that the Q_n values increased gradually as the drug concentration (0.38–3.04 mmol/L) increased. Therefore, the drug concentration was also one of the key factors that promoted transdermal penetration of SH by electroporation. The mechanism by which electroporation promoted permeation was that electronic pulses made the SC looser and increased the intercellular spaces in the skins. The electroporation-induced change of skin structure reduced skin barrier properties and promoted SH permeation. In clinical transdermal test, SH combined with the optimized parameter electroporation produced high SH concentration in SF. The optimized parameters of electroporation improved the efficacy of SH through enhancing efficiency of SH transdermal delivery. At the same time, electroporation is safe, uses only small instruments, and is convenient for self-administration by patients. In general, electroporation is an ideal technology for enhancing the transdermal permeation of SH. Electroporation using optimized parameters can be a basis for further improvement of the clinical transdermal application of SH.

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Disclosure

The authors report no conflicts of interest in this work.

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