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Research paper

Topically applied mesoridazine exhibits the strongest cutaneous analgesia and minimized skin disruption among tricyclic antidepressants: The skin absorption assessment

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ABSTRACT

Tricyclic antidepressants (TCAs) are found to have an analgesic action for relieving cutaneous pain associated with neuropathies. The aim of this study was to assess cutaneous absorption and analgesia of topically applied TCAs. Percutaneous delivery was investigated using nude mouse and pig skin models at both infinite and saturated doses. We evaluated the cutaneous analgesia in nude mice using the pinprick scores. Among five antidepressants tested in the in vitro experiment, mesoridazine, promazine and doxepin showed a superior total absorption percentage. The drug with the lowest total absorption percentage was found to be fluphenazine (<7%) either at an infinite dose or at saturated solubility. The follicular pathway was important for mesoridazine and promazine delivery. Mesoridazine showed stronger skin analgesia than the other TCAs although the in vivo skin absorption of mesoridazine (0.34 nmol/mg) was less than that of promazine (0.80 nmol/mg) and doxepin (0.74 nmol/mg). Mesoridazine had a prolonged duration of pain relief (165 min) compared to promazine (83 min) and doxepin (17 min). The skin irritation test demonstrated an evident barrier function deterioration and cutaneous erythema by promazine and doxepin treatment, whereas mesoridazine caused no obvious adverse effect by topical application for up to 7 days.

1. Introduction

Neuropathic pain is defined as the pain produced by a lesion or disease of the somatosensory systems [1]. The predominant occurrence of neuropathic pain is the disorder leading to a dysfunctional sensory condition, which affects >8% of the population [2]. This kind of pain largely influences the health-related quality of life with conditions such as depression, fatigue, reduced mobility, sleep disturbance, weight loss, and inability to work. It also causes substantial costs to society, raising a serious public health concern [3]. Peripheral neuropathic pain is the primary type of nerve-system injury. Damage to periphery tissues or nerves can elicit keratinocytes and vessels in the skin to release substance P, prostaglandins and calcitonin gene-related peptides. These excitatory factors bind to the receptors on nociceptive fibers, generating depolarization [4]. Several diseases, including diabetic neuropathy, postherpetic neuralgia, chemotherapy-induced peripheral neuropathy, radiation dermatitis, bullous dermatoses and hidradenitis suppurativa, can induce the skin pain. Peripheral pain is becoming the most common ailment that motivates patients to seek professional care.

Currently, the first-line treatments for peripheral neuropathic pain are tricyclic antidepressants (TCAs), serotonin-norepinephrine reuptake inhibitors, and anticonvulsants. TCAs

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have been employed as antipsychotic drugs for >60 years. They are also known to possess local anesthetic activity, showing analgesic properties when used topically [5]. The peripheral analgesic effect of TCAs can be attributed to the blockade of the sodium channel and the decrease of cyclic AMP via adenosine receptor activation [6]. An ideal aspect of topical TCAs is the maximization of the local concentration in peripheral effector sites and minimization of the serum level for providing safe administration [7]. TCAs such as amitriptyline are found to be more potent than lidocaine/prilocaine cream (EMLA) in alleviating cutaneous pain [8].

Although some therapies are used for resolving neuropathic pain clinically, patients usually do not respond adequately to drug treatment, and the pain is therefore refractory. The topical delivery is restrained due to the short duration of the analgesic effect [9]. No more than 40–60% of patients with local pain achieve lasting and partial pain relief [10]. Besides doxepin and amitriptyline, only a few options are available for clinical use in local pain control. We aimed to examine the skin absorption and local anesthetic effect of some TCAs to find new candidates for cutaneous analgesia. To our knowledge, no literature reports the permeability of TCAs into/across the skin. The three TCAs, mesoridazine, promazine, and fluphenazine structurally belonging to phenothiazines, were selected as the permeants in the present work (Fig. 1). The strong analgesic activity of phenothiazine-type TCAs was reported previously [11]. The cutaneous absorption of phenothiazines was evaluated by in vitro Franz cell for comparison with doxepin and amitriptyline, the commercially available TCAs for topical application. Different substituents are attached at the 2- and 10-position of the phenothiazine ring. Doxepin and amitriptyline are the antipsychotics with a seven-member ring. The difference in the structure between the two TCAs is an oxygen in the ring of doxepin. The skin retention of the permeants was also determined in vivo in the nude mouse. The cutaneous analgesic activity and duration of the topically applied TCAs were also evaluated in vivo. Skin irritation such as redness and burning can be observed after topical administration of TCAs at a high concentration [12]. The possible irritation produced by the drugs was explored by detecting transepidermal water loss (TEWL) and the erythema index.

2. Materials and methods

2.1. Materials

Mesoridazine besylate and promazine hydrochloride were standard references from U.S. Pharmacopeia (Rockville, MD, USA). Fluphenazine dihydrochloride, doxepin hydrochloride and amitriptyline hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). A cellulose membrane with a molecular weight (MW) cutoff of 6000–8000 Da was supplied by Membrane Filtration Products (Seguin, TX, USA).

2.2. Production of the base form from TCA salts

The base form of TCA salts was obtained by using the precipitation method. After a NH_4OH/water solution (pH = 9) was pipetted by drops into TCA salt in methanol, the base form was precipitated. This precipitate was filtered and washed with water for expelling NH_4OH. The TCA bases were verified by infrared and nuclear magnetic resonance analyses after drying.

2.3. n-Octanol/water partition coefficient (logP)

TCAs (0.5 mg) in methanol were pipetted into the test tube, followed by methanol evaporation with nitrogen gas. The *n*-octanol and water (1 ml of each) were mixed in the tube and then stoppered and agitated for 24 h. The *n*-octanol and water phases were withdrawn to quantify TCAs by high-performance liquid chromatography (HPLC). The organic phase was diluted by acetonitrile before the assay.

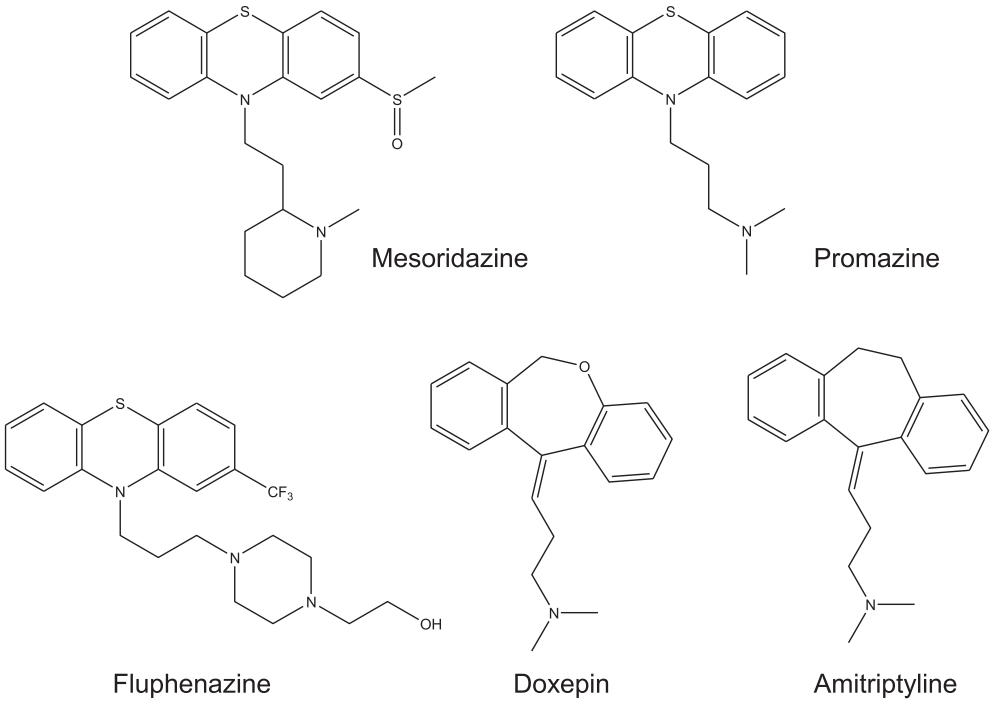

Fig. 1. The chemical structures of mesoridazine, promazine, fluphenazine, doxepin, and amitriptyline.

2.4. HPLC setup and capacity factor ($\log K'$) calculation

Chromatogram for analyzing TCAs was obtained by using a Hitachi HPLC system (7-series, Tokyo, Japan) with a LiChrospher C18 column (200 × 4.6 mm, Merck, Darmstadt, Germany). The mobile phase was a mixture of acetonitrile and double-distilled water with a pH value of 2 adjusted by phosphoric acid. The flow rate and detection wavelength were set at 1 ml/min and 254 nm, respectively. The retention time of each drug was recorded, and the capacity factor ($\log K'$) was calculated by the following equation:

$$\log K' = \log[(t_r - t_0)/t_0];$$

where t_r is the retention time of each compound; and t_0 is the retention time of the non-retained solvent peak.

2.5. Saturated solubility

The saturated solubility of TCAs was detected in a solution of propylene glycol (PG)/pH 7.4 buffer (20%). An excess amount of the drugs was loaded in the medium and shaken reciprocally at 37 °C for 24 h. This dispersion was then centrifuged at 10,000 rpm for 10 min. The supernatant was filtered with polyvinylidene fluoride (PVDF) membrane with a pore size of 0.45 μm. The resulting solution was analyzed by HPLC after an appropriate dilution with 20% PG in buffer.

2.6. Animals

Eight-week-old female nude mice (ICR-Foxn1nu strain) were supplied by National Laboratory Animal Center (Taipei, Taiwan). One-week-old specific pathogen-free pigs were purchased from Animal Technology Institute Taiwan (Miaoli, Taiwan). This study was performed in strict accordance with the recommendations set forth in the Guidelines for the Care and Use of Laboratory Animals of Chang Gung University. Food and water were given ad libitum. All efforts were made to minimize suffering.

2.7. Preparation of skin membranes

Full-thickness skin was excised from the back area of the mice and pigs after sacrifice. The skin was cleaned and stored at −20 °C till the skin was used for the permeation experiment. The skin would be used in the cutaneous absorption experiment within 2 weeks post-sacrifice. The skin was stripped by adhesive tape (Scotch®, 3M, St. Paul, MN, USA) 20 times for obtaining stratum corneum (SC)-stripped skin. Chloroform-methanol (2:1) was employed to treat the SC side of the skin for 2 h to remove the lipid bilayers. To prepare the de-sebum skin, the SC side of the skin was gently washed with cold hexane (4 °C) five times to remove the sebum on the skin surface according to the previous study [13].

2.8. In vitro cutaneous absorption

This experiment was performed using the Franz diffusion assembly (Chin-Fa Glass, Hsinchu, Taiwan). The excised skin or cellulose membrane was mounted between the donor and the receptor compartment with the SC or epidermal side facing upwards into the donor. The receptor medium consisted of 30% ethanol in pH 7.4 buffer for maintaining a sink condition (5.5 ml). The drug in 20% PG/pH 7.4 buffer was loaded in the donor compartment. Either supersaturated (15 mM) suspension or saturated solution of TCAs was filled in the donor (0.5 ml) for comparing the permeability. The TCA dose in saturated solution was the solubility in 20% PG as shown in Table 1. The valid diffusion area between the donor and receptor was 0.785 cm^2. The stirring rate of the stir bar and temperature of the receptor were kept at 600 rpm and 37 °C, respectively. At determined intervals of 0, 1, 2, 4, 6, 8, 10, 12, and 24 h, a 300-μl receptor medium was withdrawn, followed by an immediate replacement with fresh medium. The excised skin was taken from the Franz cell at the end of the experiment. The skin was washed using water and ethanol to remove the residual drug on the surface. After the skin was weighed, it was deposited in methanol (1 ml). MagNA Lyser (Roche, Indianapolis, IN, USA) was employed to homogenize the skin. The homogenates were centrifuged at 10,000*g* for 10 min. The drug amount in the supernatant was assessed by HPLC to determine the drug skin deposition.

2.9. In vivo cutaneous absorption

The nude mouse was employed as the animal model in this experiment. A glass cylinder with a hollow region of 0.785 cm^2 was fixed on the mouse's back using superglue. The glass cylinder was like the donor of Franz cell. An aliquot of 0.2 ml of 20% PG/pH 7.4 buffer containing TCAs (15 mM) was positioned in the hollow area of the cylinder. No infiltration of the drug medium outside the hollow region was observed. The mouse was sacrificed after a 6-h application. The treated skin area was excised and extracted by MagNA Lyser with the same procedure used in the in vitro permeation study.

2.10. In vivo cutaneous analgesia

The cutaneous analgesic activity of topically applied TCAs was evaluated using the cutaneous trunci muscle reflex (CTMR) [14], characterized by the reflex movement of the skin over the back produced by twitches of the lateral thoracic spinal muscle in response to a Von Frey filament (No. 15, Somedic Sales AB, Stockholm, Sweden). A 1.5 × 1.5 cm^2 area on the lumbar region of the mouse back was covered with a gauze. After a 2-h treatment, we applied six pinpricks at six different points within each treatment site with a frequency of 0.5–1.0 Hz and scored the number to which the mouse failed to respond. The cutaneous analgesia of each drug was assessed quantitatively as the number of times the pinprick failed to induce a reaction. The maximum value of % possible effect (% PE) was presented as the percent of maximum possible effect (% MPE). The duration of action was measured as the time from TCA removal to full recovery of CTMR (0% MPE recorded).

2.11. In vivo cutaneous irritation test

TCAs (15 mM) in 20% PG/pH 7.4 buffer were applied daily on the nude mouse back for 7 consecutive days to check the possibility of eliciting irritation. A volume of 0.6 ml of TCA medium was spread on a nonwoven polyethylene cloth (1.5 × 1.5 cm^2), and then applied on the dorsal region of nude mouse. The cloth was fixed by Tegaderm® adhesive dressing (3M, St. Paul, MN, USA) and Fixomull® stretch adhesive tape (Beiersdorf AG, Hamburg, Germany). The drug medium was replaced by a new one every day for 7 days. After withdrawal of the medium, the treated skin area was detected by TEWL, erythema index (a^*), and skin surface pH. A Tewameter (TM300, Courage and Khazaka, Köln, Germany) was utilized to record TEWL. Erythema was quantified by a spectrocolorimeter (CD100, Yokozawa Electrical, Tokyo, Japan). The cutaneous surface pH was recorded by a Skin-pH-Meter 905 (Courage and Khazaka). The 20% PG/pH 7.4 buffer without TCAs was the control group.

Table 1
Physicochemical properties of tricyclic antidepressants.

Compound	Molecular formula	MW[a] (Da)	log*P*[b]	log*K*′[c]	Solubility in 20% PG[d] (mM)
Mesoridazine	$C_{21}H_{26}N_2OS_2$	386.6	0.09 ± 0.01	0.01	12.23 ± 0.18
Promazine	$C_{17}H_{20}N_2S$	284.4	0.51 ± 0.01	0.34	11.27 ± 0.57
Fluphenazine	$C_{22}H_{26}F_3N_3OS$	437.5	1.01 ± 0.0006	0.31	4.00 ± 0.18
Doxepin	$C_{19}H_{21}NO$	279.4	1.12 ± 0.01	0.25	10.87 ± 0.25
Amitriptyline	$C_{20}H_{23}N$	277.4	1.31 ± 0.05	0.52	6.54 ± 0.15

[a] MW, molecular weight.
[b] log*P*, *n*-octanol/water partition coefficient.
[c] log*K*′, logarithm of $t_r - t_0/t_0$, t_r is the retention time of compound peak, t_0 is the retention time of solvent peak.
[d] PG, propylene glycol.

2.12. Skin histopathology

The treated skin area after drug application for 7 days in the irritation test was excised after sacrifice. The skin species were immersed in a 10% buffered formaldehyde using ethanol, embedded in paraffin wax, and sliced at a thickness of 5 μm. The skin specimen was stained by hematoxylin and eosin (H&E) and monitored by a light microscopy (IX81, Olympus, Tokyo, Japan).

2.13. Data analysis

In the in vitro cutaneous absorption, the cumulative amount of TCAs permeated across the skin was plotted as a function of time. The flux value (nmol/cm^2/h) was computed by a linear regression from the slope of permeated amount-time curves. TCA deposition within the skin was measured as the molar amount per mg of skin (nmol/mg). In the case of TCA permeation from the saturated solution, the calibrated skin deposition (CSD) and permeability coefficient (K_p) were calculated from the skin deposition and flux divided by the applied dose (saturated solubility) in the donor compartment, respectively.

The data are presented as mean ± standard deviation (S.D.). The difference in the data of different experimental groups was evaluated by one-way analysis of variance (ANOVA) followed by the pairwise Tukey's honestly significant difference test. The statistical significance of the difference was set at $p < 0.05$.

3. Results

3.1. Physicochemical properties of TCAs

Table 1 summarizes the physicochemical characteristics of the five TCAs used for testing skin absorption. The MW of TCAs ranged between 277 and 438 Da, with fluphenazine showing the largest molecular size. Amitriptyline revealed the highest lipophilicity (log*P*), followed by doxepin, fluphenazine promazine, and mesoridazine. The lipophilicity also can be rated by the capacity factor (log*K*′), which demonstrates the relative retention of the chromatography. Amitriptyline and mesoridazine exhibited the greatest and least lipophilicity based on log*K*′. This was the same with log*P*. However, an opposite trend was observed between log*K*′ and log*P* for promazine, fluphenazine and doxepin although this difference was not large. The higher log*P* basically showed a lower aqueous solubility. The exception was fluphenazine, which demonstrated the lowest solubility (4.00 mM) but moderate lipophilicity.

3.2. In vitro cutaneous absorption

The in vitro cutaneous permeation of TCAs was compared using the Franz diffusion cell. Both drug deposition within the skin and the flux across the skin were determined. The skin deposition indicated drug uptake by the skin tissue, whereas the flux predicted in vivo delivery to deeper skin strata and systemic circulation. All drugs were first applied at an infinite dose (15 mM). This dose surpassed the saturated solubility, resulting in a suspension type in the donor. Table 2 shows the calculated permeation parameters. The permeation results could compare the absorption level of different TCAs under the same dose. This condition simulated the practical dose in the clinical status. Both nude mouse and pig skins were employed as the barriers for topical delivery. The mouse skin deposition was generally increased by the increase of log*P* of TCAs, except that promazine displayed a 2-fold higher deposition than fluphenazine. The same trend was found in the case of pig skin deposition. Promazine showed the greatest flux (89 nmol/cm^2/h) across mouse skin as compared to the other TCAs, followed by mesoridazine (58 nmol/cm^2/h). The trend (promazine > mesoridazine) was reversed in the pig skin flux. The drug with the least penetration through mouse and pig skins was fluphenazine. Doxepin and amitriptyline flux was comparable in mouse and pig skin. The total absorption percentage of TCAs was measured by combined skin deposition and the cumulative amount in the receptor divided by donor dose. Table 2 demonstrates the same ranking for total absorption percentage and flux, with promazine and mesoridazine showing the greatest total absorption in nude mouse and pig skin, respectively. Fluphenazine was the drug associated with the lowest absorption.

The next experiment for comparing TCA penetration used saturated solubility as the donor dose. It can assure an identical thermodynamic activity of different permeants for comparing the permeability. Table 3 represents CSD and the permeability coefficient (K_p), the skin deposition and flux calibrated by TCA concentration in the donor. With respect to nude mouse skin,

Table 2
Nude mouse and pig skin deposition (nmol/mg) and flux (nmol/cm^2/h) of tricyclic antidepressants after a 24-h in vitro percutaneous absorption from 20% PG/pH 7.4 buffer suspension at a determined concentration (15 mM).

Skin type	Compound	Skin deposition (nmol/mg)	Flux (nmol/cm^2/h)	Total absorption percentage[a] (%)
Nude mouse	Mesoridazine	2.91 ± 0.81	57.63 ± 10.55	15.94 ± 4.04
	Promazine	8.27 ± 2.56	88.92 ± 9.14	31.81 ± 3.20
	Fluphenazine	4.16 ± 0.72	2.10 ± 0.41	5.68 ± 0.80
	Doxepin	18.99 ± 3.69	14.53 ± 7.15	19.25 ± 0.92
	Amitriptyline	28.25 ± 4.15	10.22 ± 5.89	21.00 ± 1.40
Pig	Mesoridazine	1.92 ± 0.29	107.99 ± 35.60	39.24 ± 5.11
	Promazine	5.72 ± 1.94	50.64 ± 6.96	18.63 ± 1.31
	Fluphenazine	3.85 ± 0.75	10.70 ± 0.18	6.74 ± 0.80
	Doxepin	6.87 ± 1.88	20.47 ± 7.19	19.45 ± 2.99
	Amitriptyline	11.76 ± 1.77	22.53 ± 6.38	14.27 ± 1.56

The data represent the mean ± S.D. ($n = 4$).
[a] Total absorption percentage, the drug amount (nmol) absorbed in both skin reservoir and receptor/drug amount in donor.

Table 3
Calibrated skin deposition (CSD, nmol/mg/solubility) and permeability coefficient (K_p, cm/h $\times 10^{-3}$) of tricyclic antidepressants after a 24-h in vitro percutaneous absorption from 20% PG/pH 7.4 buffer solution at a saturated concentration.

Skin type	Compound	CSD[a] (nmol/mg/solubility)	K_p[b] (cm/h $\times 10^{-3}$)	Total absorption percentage[c] (%)
Nude mouse	Mesoridazine	0.22 ± 0.03	4.60 ± 0.77	17.56 ± 2.79
	Promazine	1.48 ± 0.20	7.68 ± 1.10	37.14 ± 3.25
	Fluphenazine	0.41 ± 0.09	0.29 ± 0.04	6.61 ± 0.89
	Doxepin	1.10 ± 0.18	7.84 ± 0.93	45.57 ± 8.45
	Amitriptyline	1.72 ± 0.59	1.26 ± 0.26	33.25 ± 4.17
Pig	Mesoridazine	0.14 ± 0.03	7.75 ± 1.41	27.47 ± 0.25
	Promazine	0.58 ± 0.09	5.86 ± 1.05	23.53 ± 2.22
	Fluphenazine	0.20 ± 0.02	0.66 ± 0.14	4.16 ± 0.13
	Doxepin	0.64 ± 0.10	5.89 ± 1.67	26.20 ± 7.84
	Amitriptyline	0.44 ± 0.08	4.01 ± 0.19	19.36 ± 1.21

The data represent the mean ± S.D. ($n = 4$).
[a] CSA, calibrated skin accumulation = cumulative amount in the skin/saturated solubility.
[b] PC, permeability coefficient = flux/saturated solubility.
[c] Total absorption percentage, the drug amount (nmol) absorbed in both skin reservoir and receptor/drug amount in donor.

amitriptyline and promazine exhibited the highest CSD, followed by doxepin. On the other hand, doxepin and promazine showed the highest CSD in pig skin. The K_p of doxepin across mouse skin was found to be 7.84 $\times 10^{-3}$ cm/h, which was comparable to that of promazine (7.68 $\times 10^{-3}$ cm/h). In the case of pig skin, the greatest K_p was detected in mesoridazine (7.75 $\times 10^{-3}$ cm/h), followed by doxepin and promazine. Fluphenazine always revealed the least permeability in both CSD and K_p. Doxepin indicated the greatest total absorption content in both mouse and pig skins. The total pig skin absorption of mesoridazine was comparable to that of doxepin, whereas the total nude mouse skin absorption of mesoridazine was significantly less than that of doxepin by 2.6-fold.

3.3. Cutaneous absorption via different skin types

Different skin types, including SC-stripping, de-lipid, and de-sebum skins, were used as the penetration barriers for elucidating the transport pathways of TCAs. Skin absorption of mesoridazine, promazine and doxepin at the infinite dose (15 mM) was examined in this experiment. Fig. 2 illustrates the flux of TCAs across various skin models. Stripping of the mouse SC and lipid bilayer removal resulted in a 2-fold mesoridazine flux enhancement (Fig. 2A). No significant difference of mesoridazine flux was shown after sebum removal. The flux across intact, SC-stripping, and de-lipid pig skin was comparable. Pig sebum removal even reduced the mesoridazine flux by 3-fold. Drug transport across the cellulose membrane indicated the condition of diffusion without barrier function. Mesoridazine release via the cellulose membrane exhibited a flux of 186 nmol/cm^2/h, which was much greater than that of the intact skin. As depicted in Fig. 2B, stripping and lipid removal of nude mouse skin did not promote promazine flux. Both procedures could increase promazine flux across pig skin; however, this enhancement was limited (1.4 and 1.6 times). De-sebum treatment significantly reduced promazine flux across mouse and pig skins. Promazine achieved a release rate across the cellulose membrane of 115 nmol/cm^2/h. Doxepin flux via SC-stripping and de-lipid mouse skin was about 3 times more than that via intact skin (Fig. 2C). The enhancement level (2 times) of doxepin flux by sebum removal was less than that by SC-stripping and lipid bilayer removal. On the other hand, only de-lipid pig skin showed a significantly higher doxepin flux as compared to the skin without any treatment. The release rate of doxepin via the cellulose membrane was 108 nmol/cm^2/h, showing about a 7-fold greater level than the flux via intact skin.

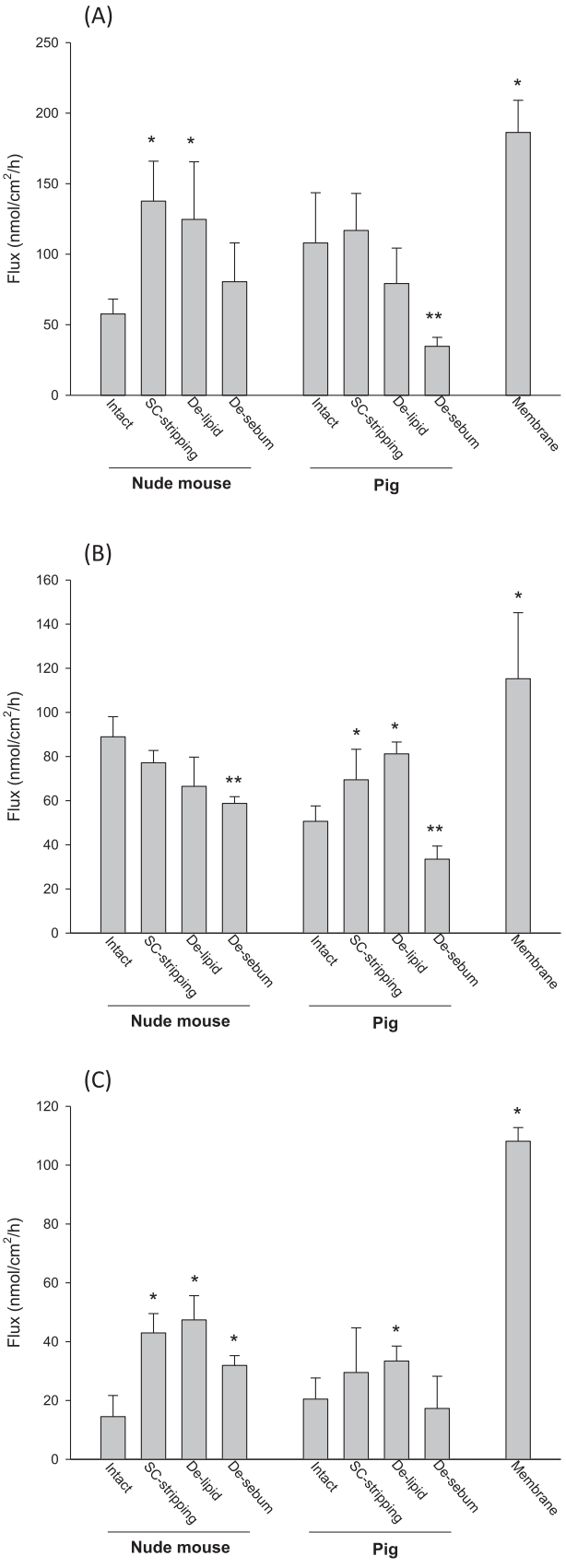

Fig. 2. Flux of tricyclic antidepressants (15 mM) across nude mouse and pig skins after different treatments including stratum corneum stripping, lipid removal, and sebum removal: (A) mesoridazine; (B) promazine; and (C) doxepin. *, higher ($p < 0.05$) as compared to intact skin; **, lower ($p < 0.05$) as compared to intact skin. All data are presented as the mean of four experiments ± SD.

3.4. In vivo cutaneous absorption

In vivo skin deposition was evaluated to compare the permeability of mesoridazine, promazine, and doxepin following topical application on nude mouse backs at an infinite dose. As shown in Fig. 3, in vivo skin uptake was higher for promazine and doxepin compared to mesoridazine. The intradermal concentration of mesoridazine, promazine, and doxepin was 0.34, 0.80 and 0.74 nmol/mg, respectively. There was no significant difference between the in vivo deposition of promazine and doxepin.

3.5. In vivo cutaneous analgesia

To survey the pharmacological activity of topically applied TCAs, we assessed the local analgesia based on the pinprick scores. The time course of cutaneous analgesia was detected after the nude mouse back had been applied with TCAs for 2 h as illustrated in Fig. 4A. The mouse topically administered with no-drug medium (20% PG in pH 7.4 buffer) served as the control group. The control medium did not reveal skin analgesia. All three TCAs tested induced cutaneous analgesic responses. Mesoridazine showed the higher analgesic effect, followed by promazine and doxepin. Mesoridazine exhibited an 86% blockade (% MPE), which was more potent than promazine (58%) and doxepin (44%) as shown in Fig. 4B. The antinociceptive activity of doxepin lasted for only 17 min after a 2-h administration (Fig. 4C). The blockade duration was prolonged in cases of the use of mesoridazine (165 min) and promazine (83 min) as compared to doxepin. All mice could recover completely after the experiment.

3.6. In vivo cutaneous irritation test

Safety is an important issue for the development of new drugs or formulations. We attempted to rate the possible skin irritation by topically applied TCAs. The change in skin physiology such as TEWL, erythema and skin surface pH was evaluated every day using a 7-day consecutive administration of the antipsychotics. TEWL is a reflection of skin barrier function, including the effect from the SC and a tight junction. The TEWL profiles showed a negligible change by the control solution application for 7 days as shown in Fig. 5A. A slight TEWL increase from 7.8 to 9.9 g/m^2/h was observed in the mesoridazine treatment up to 7 days. Promazine elicited TEWL to a significant level from 7.0 to 18.7 g/m^2/h. An increasing trend was also detected in the group of doxepin treatment (7.4–14.6 g/m^2/h). The erythema (a*) detection indicates no skin rash in the mouse treated by the control solution and mesoridazine (Fig. 5B). Both promazine and doxepin could induce cutaneous erythema to a significant level. The erythema level induced by promazine and doxepin after a 7-day treatment was similar (4.5 versus 4.0). The skin surface pH in the TCA-treated group ranged between 5.4 and 5.6 after topical application for 7 days, approaching the skin pH of the control mouse (5.5).

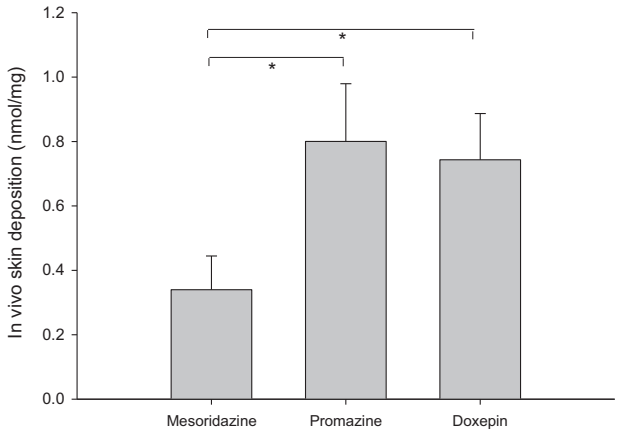

Fig. 3. In vivo skin deposition of mesoridazine, promazine, and doxepin (15 mM) after topical application on nude mouse back for 6 h. *, $p < 0.05$. All data are presented as the mean of six experiments ± SD.

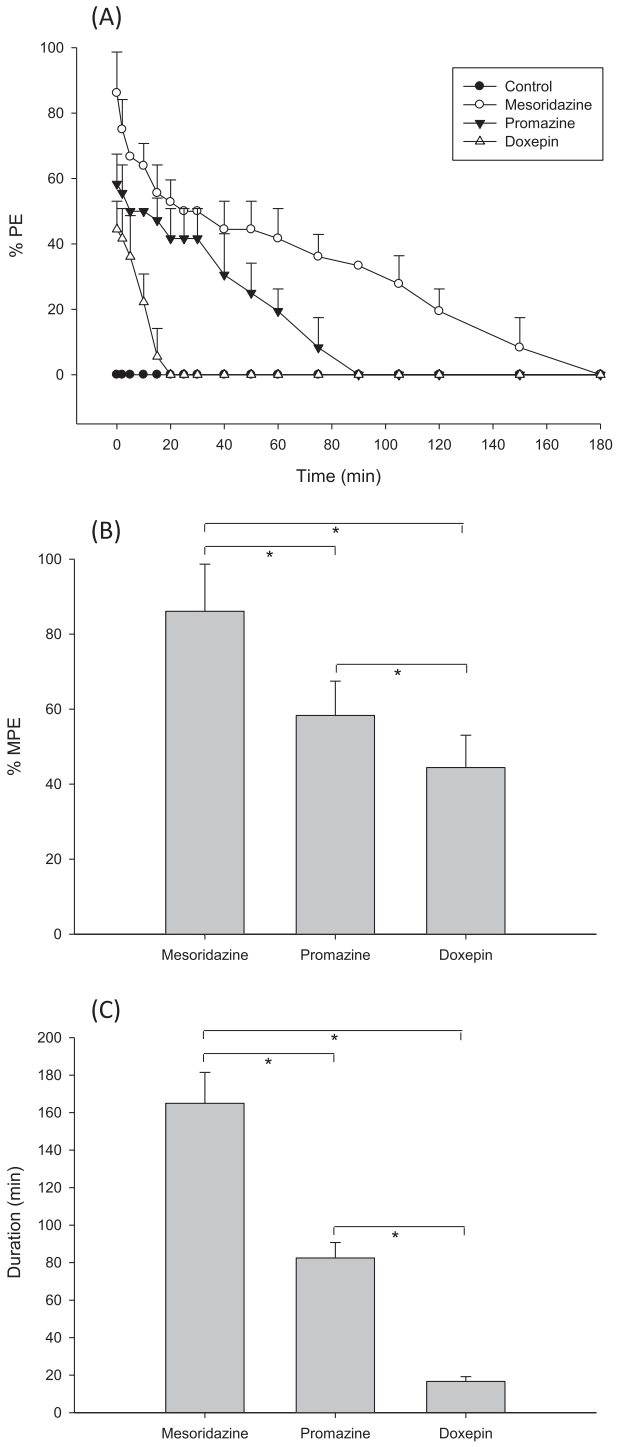

Fig. 4. In vivo cutaneous analgesia of mesoridazine, promazine, and doxepin (15 mM) after topical application on nude mouse back for 2 h: (A) time-course of cutaneous analgesia (% PE); (B) percentage of the maximal possible effect (% MPE); and (C) time required for full recovery from cutaneous analgesia. *, $p < 0.05$. All data are presented as the mean of six experiments ± SD.

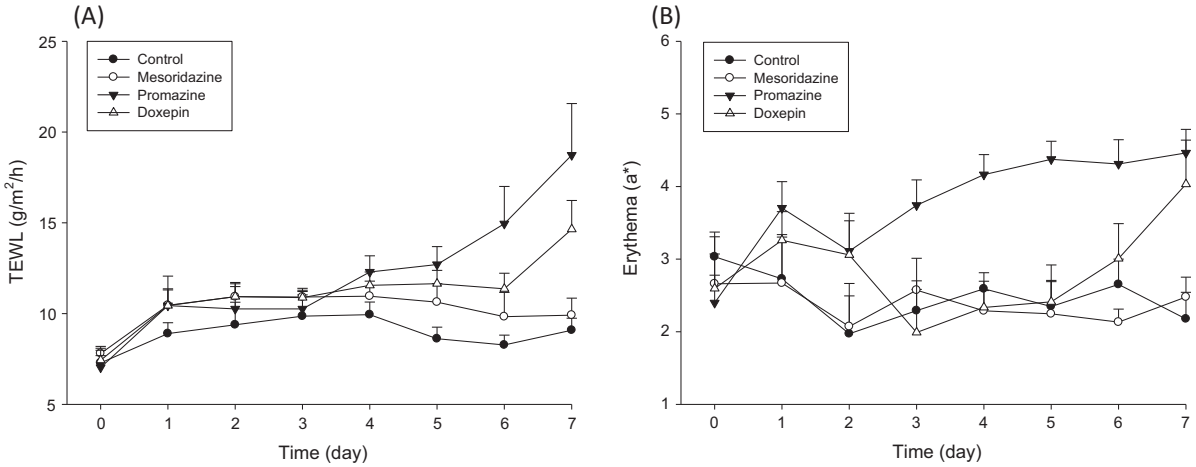

Fig. 5. In vivo skin irritation examination after a 7-day application of topically applied mesoridazine, promazine, and doxepin (15 mM): (A) transepidermal water loss (TEWL) and (B) erythema (Δa^*). All data are presented as the mean of six experiments ± SD.

3.7. Skin histopathology

The skin damage or irritation was also visualized by H&E staining of morphology as shown in Fig. 6. The histology indicated no observable disruption in the intact skin without any treatment (Fig. 6A). The skin treated with mesoridazine demonstrated some inflammatory cell infiltrations in the dermis (arrows in Fig. 6B). Fibrosis and congestion were also observed in the dermis. No significant change could be detected in the epidermis. In the promazine-treated skin, the acute inflammatory cells such as neutrophils were observed in the dermis (arrows in Fig. 6C). Fibrosis and mild spongiosis could be visualized in the dermis after promazine treatment for 7 days. There was an increase in the thickness of the promazine-treated epidermis, indicating a phenomenon of hyperproliferation. A focal hyperkeratosis was noted in superficial skin treated with doxepin (arrows in Fig. 6D). Hypergranulosis and hyperproliferation in the epidermis were exhibited in the skin exposed to doxepin (circles in Fig. 6D). In the dermal layer, spongiosis, congestion, and chronic inflammatory cell infiltration could be detected.

4. Discussion

Antidepressants are currently the first-line therapy for neuropathic pain. Nevertheless, the treatment efficiency is only moderate with many patients failing to achieve the benefits [2]. The development of novel and improved management is urgent.

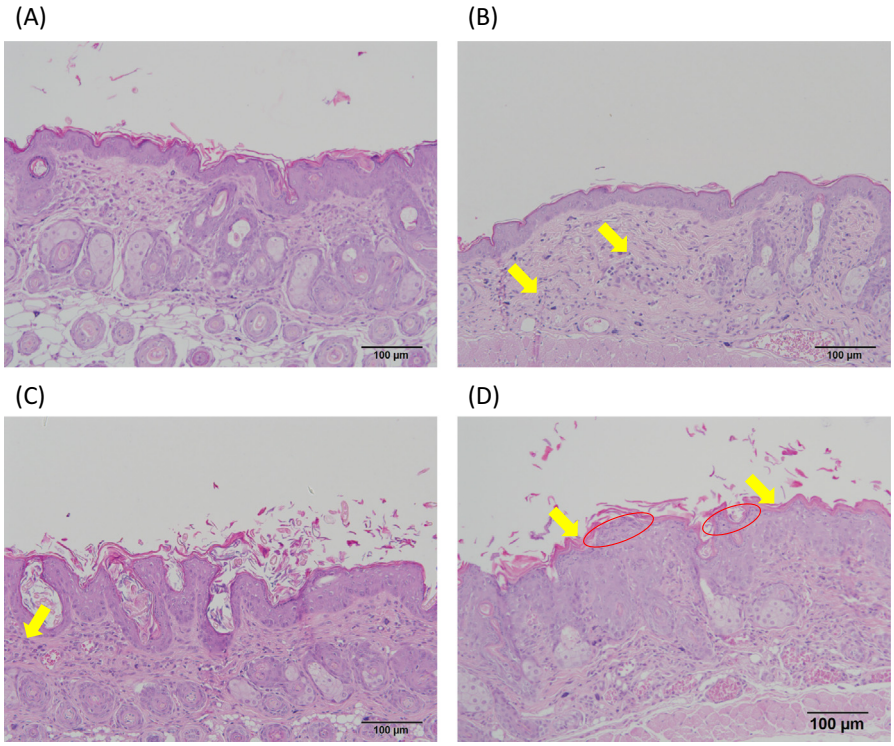

Fig. 6. Histological examination of nude mouse skin stained with hematoxylin and eosin after a 7-day application of topically applied mesoridazine, promazine, and doxepin (15 mM): (A) non-treatment skin; (B) mesoridazine; (C) promazine; and (D) doxepin.

Despite many investigators reporting the pharmacological activity of TCAs for cutaneous use, no study has been conducted to evaluate the skin permeability of these drugs. The goal of this work was to compare the skin absorption of TCAs for searching the pain-relieving candidates with superior permeation and acceptable tolerance for clinical application. TCA salts were transformed into base form for topical application in this study since the non-ionic permeants usually show better skin absorption than the ionic form due to the higher lipophilicity for partitioning into lipid bilayers [15]. The physicochemical characterization suggests that the higher log$P$ was not necessary to correlate well with the higher logK' and lower aqueous solubility of TCAs. The retention of the compound in HPLC is not only influenced by lipophilicity but also by the molecular size and steric structure [16]. That is why doxepin showed a higher log$P$ but a short retention in the C18 column. The aqueous solubility of doxepin was also high, which was confirmed by the previous study [9].

We selected nude mouse and pig skins as the barriers in the in vitro absorption study. Both skins are good models for substituting human skin in terms of similar SC morphology and hair sparseness [17,18]. According to our previous study [19], the SC and epidermis of nude mouse skin were about 11 and 18 μm, respectively. The SC and epidermal thickness of neonatal pig skin used in this study were 10 and 38 μm, respectively [20]. Though nude mouse and neonatal pig skin are more permeable than human skin, they are still an acceptable alternative due to the limited intersubject variation compared to human skin. The in vitro skin deposition and flux offer an anticipation of the delivery into the skin reservoir and deeper skin strata/circulation, respectively. Both parameters are important for realizing the drug input into the skin where the cutaneous pain occurs. The total absorption amount demonstrated a similar trend of TCA delivery either at the infinite dose or at the dose of saturated concentration. A small difference was observed between mouse and pig skins. Mesoridazine exhibited the greatest total absorption in pig skin, whereas it displayed only a moderate absorption in nude mouse skin. Doxepin and amitriptyline, the two most lipophilic permeants, are the TCAs with superior skin deposition. A permeant in aqueous medium first partitioned and accumulated into the SC, which is rich in lipids [21]. The high log$P$ of doxepin and amitriptyline may contribute to the high deposition in the skin. Amitriptyline has been proved to be highly lipophilic for its easy storage in lipids and fats [22].

Both the SC and epidermal junction give the principal barriers for skin permeation [23]. Viable epidermis is regarded as a more hydrophilic layer than SC. The highly lipophilic drugs are partitioned with difficulty from the SC to the epidermis since the diffusion resistance shifts from the SC to viable strata [24], leading to the limited flux of doxepin and amitriptyline through the skin. It can be expected that the flux, which would be diminished as the permeant is largely kept in the SC layer. Previous studies [8,22] also suggest a negligible systemic absorption of amitriptyline after topical administration. Doxepin penetration across the skin was not very low, especially in the case of a saturated dose. This could be due to the high aqueous solubility of doxepin for showing some partitioning to viable skin. Fluphenazine was the least permeable drug tested in this report. The permeant should be in the solubilized form for penetrating into the skin. The aqueous solubility of fluphenazine was the lowest among TCAs examined. Most of the fluphenazine molecules in dispersion were insoluble at the infinite dose, resulting in minimal diffusion. Moreover, the production of precipitate film on the surface of the skin also led to the creation of the barrier for fluphenazine transport. The ceramide monolayers in the SC lipid bilayers were found to be stereoselective when the permeants penetrated into the skin [25,26]. Fluphenazine shows a complex steric structure [27]. This may cause the difficulty of fluphenazine passage via the SC layer. Although the size of the fluphenazine molecule can fit the criterion of MW < 500 Da for feasible skin transport, the largest MW (438 Da) of this drug among the TCAs tested may also impede the facile penetration into the skin.

Mesoridazine revealed a significant flux because the hydrophilic permeants generally escape from the SC easily and then continue to the deeper strata and receptor medium [28]. This effect contributed to the high total absorption percentage of mesoridazine from aqueous dispersion. The high level of mesoridazine resided in deeper strata was beneficial for skin pain control since most of the pain-related nociceptors such as Meissner's corpuscle, Merkel's disk, Pacinian corpuscle, and Ruffini corpuscle are located in dermis and subcutis [29]. Another drug with a high level of total absorption was promazine. The permeants with balanced lipophilicity and solubility are ideal for achieving sufficient skin absorption. Promazine showed a comparable solubility but greater lipophilicity as compared to mesoridazine. It can be supposed that promazine was forced into the SC via intercellular lipids and then continued to viable skin and the receptor. Another explanation of the facile absorption of mesoridazine and promazine may be that phenothiazine derivatives are amphiphilic in nature. The high surface active feature of phenothiazine contributes to a strong interaction with lipid bilayers [27]. The surfactant effect can increase the biomembrane permeability by lipid bilayer structure disruption [30]. Although fluphenazine possesses an amphiphilic nature, the negative effects such as low solubility, steric structure, and large molecular size had offset the surface's active property for pushing it into the skin.

In order to further explore the transport pathways of TCAs, mesoridazine, promazine, and doxepin were selected as the model permeants to examine flux via different skin types. Fluphenazine was withdrawn in this experiment due to its low cutaneous absorption and relatively high toxicity compared to promazine (10 times) [31]. Although amitriptyline expressed the largest skin reservoir after topical application, the possible neurotoxicity and less-effective skin pain relief than doxepin have limited its practical use [4,32]. Hence, amitriptyline was ignored in further experiments. The SC stripping and lipid removal of nude mouse skin led to a significant increase of mesoridazine flux, indicating that the passage of lipid bilayers in the SC was the rate-limiting process for this antidepressant. In the case of pig skin, the SC and lipid removal did not affect mesoridazine penetration across the skin because of the facile entrance into the skin and the receptor. Promazine revealed a contrary result as compared to mesoridazine, with the SC-stripping and de-lipid pig skin but not mouse skin showing a greater flux than intact skin. This result could explain mesoridazine and promazine demonstrating the highest total absorption percentage in pig and mouse skin, respectively.

Sebum produced by the sebaceous glands spreads on the skin surface and inside the hair follicles. Removal of sebum generally decreased mesoridazine and promazine flux compared with intact skin. This indicates the need for sebum for both drugs to result in high absorption. The follicular route may be essential for the permeation of mesoridazine and promazine. Contrary to this effect, doxepin flux was enhanced by sebum removal. This suggests the hindrance of promazine uptake into sebum-containing follicles. The cellulose membrane allows the free molecules to diffuse through. The release rate of the permeant via cellulose membrane is a function of the diffusion coefficient. Only the viable skin is present in the SC-stripping skin. The difference in flux between the cellulose membrane and the SC-stripping skin can be considered as the barrier effect on the viable skin. The enhancement level of cellulose membrane diffusion compared to the SC-stripping skin was greater for doxepin than for phenothiazine TCAs, suggesting that the main barrier of doxepin absorption was the deeper skin strata but not the SC layer in superficial skin. This is reasonable

since the hydrophilic viable skin is formidable to lipophilic permeant transport, although doxepin showed a high aqueous solubility.

The in vivo cutaneous absorption of TCAs was significantly lower than the in vitro skin uptake. This could be due to the shorter exposure time for in vivo study (6 h) compared to in vitro permeation (24 h). In vivo skin deposition of promazine and doxepin was approximately 2-fold greater than that of mesoridazine. This discrepancy was smaller than that in the in vitro mouse skin deposition at an infinite dose. Doxepin presented an in vitro skin uptake 6.5-fold higher than mesoridazine. This indicates that the flux level played a role in the assessment of in vivo TCA absorption within the skin, since mesoridazine showed greater flux as compared to doxepin. Drug flux across mouse or neonatal pig skin, which is thinner than human skin, can act as a detector of systemic absorption and delivery to deeper skin strata in the in vivo or clinical condition.

The control group demonstrated no pain blockade in the in vivo cutaneous analgesia study, verifying that these TCAs were directly responsible for pain control. We found that topically applied mesoridazine was more potent and long-acting in analgesia than promazine and doxepin. Since the in vivo skin deposition of mesoridazine was lower than the other two drugs, the strong analgesic effect of mesoridazine could be due to the slow dissociation of mesoridazine molecules from the binding site. The strong binding of mesoridazine to the sodium channel is proved by the previous study [11]. Mesoridazine treatment for 2 h could maintain the analgesic duration to 165 min. This compound was likely to be retained within the skin relatively longer than the others. It is meaningful to provide patients who have neuropathic pain with a prolonged and noninvasive analgesia for improving compliance [33]. Topically applied mesoridazine may accomplish this aim. The local neuropathic pain always accompanied skin damage and impaired barrier function [34]. Mesoridazine may display a greater absorption and cutaneous analgesia in practice. The analgesic effect of local anesthetic EMLA cream persists for only 1–2 h after occlusive dressing removal [35]. Mesoridazine surpassed EMLA by the analgesic duration after topical application. Although caution should be taken in comparing the studies with different setups, mesoridazine could be a potential candidate for local pain relief.

The major limitation in using topically applied TCAs to reduce pain sensation is the risk of adverse drug reactions such as rash, eczema, and urticaria [12]. It is reported that the cutaneous side effects produced by TCAs are twice as frequent as those elicited by other drugs [36]. Although mesoridazine was proved to exhibit a strong and sustained analgesic effect, the assurance of its safety is important as well. According to the results of the in vivo skin irritation test, a negligible increase of TEWL, erythema, and cutaneous pH was found to be associated with mesoridazine application. The histology showed some inflammatory infiltrations after the administration of promazine and doxepin, which could also be detected by colorimetry (a^*) since inflammation can induce erythema and edema. Promazine and doxepin caused some disruptions on both the epidermal and dermal layers. The damage of skin morphology was slighter for mesoridazine than for promazine and doxepin. Morphological change was observed in the dermis but not in the epidermis after mesoridazine treatment, confirming a facile entrance of this compound to deeper skin strata. TEWL was significantly elevated by the other TCAs, especially promazine. The lipid bilayer structure would be destroyed at a high concentration of phenothiazine TCAs [37]. This could be the occasion of promazine-induced skin barrier impairment although this impact was not detected for mesoridazine. The capability of lipid bilayer disruption by mesoridazine might be reversed to intact status after a determined period. Cutaneous erythema was seen in the group of promazine and doxepin. TCAs have the ability to induce vasodilation, correlating well with the skin's redness [35]. Some cases of allergic contact dermatitis caused by topically applied doxepin have been reported earlier [38].

5. Conclusion and future perspective

Our work represented the first investigation of the cutaneous absorption of TCAs when applied topically. The experimental results demonstrated that different antipsychotics showed different delivery levels, with amitriptyline exhibiting the greatest deposition within the skin. The total absorption percentage (skin deposition + cumulative amount in receptor) was the highest for mesoridazine and promazine. Fluphenazine permeation was the lowest at both infinite and saturated doses. Lipophilicity, aqueous solubility, and the steric structure of TCAs could influence skin absorption. Mesoridazine displayed more potent activity and a longer period of analgesia than the other drugs at blocking cutaneous pain. This drug can be chosen as the most potent candidate for cutaneous analgesia due to its high skin absorption, impressive pharmacodynamic effect, and insignificant skin irritancy. Topically applied mesoridazine may provide an efficient management of neuropathic pain. The results of this study will help us to understand cutaneous delivery of TCAs and to plan further animal and clinical research. Before proceeding to further work, it is essential to clarify some issues. In addition to peripheral ectopic discharge, a central mechanism also showed an important role in the production of neuropathic pain. An overdose of mesoridazine can cause cardiac toxicity. Systematic side effects should be taken into account when using topical mesoridazine. Modulation of the applied dose and creation of the acceptable formulation for mesoridazine are required for future development.

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