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Effect of low frequency ultrasound on the in vitro percutaneous absorption of clobetasol 17-propionate

Jia-You Fang [a,b,*], Chia-Lang Fang [c], K.C. Sung [d], Hsiang-Yin Chen [a,b]

[a] *Graduate Institute of Pharmaceutical Sciences, Taipei Medical College, 250 Wu-Hsing Street, Taipei, Taiwan*
[b] *Research Center for Clinical Pharmacy, Taipei Municipal Wanfang Hospital, Taipei, Taiwan*
[c] *Department of Pathology, Taipei Medical College, Taipei, Taiwan*
[d] *School of Pharmacy, Chia Nan College of Pharmacy and Science, Tainan Hsien, Taiwan*

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Abstract

The effect of low frequency ultrasound (20 kHz) on the permeation of clobetasol 17-propionate (CP) through skin (sonophoresis) was studied. The ultrasound was applied at either continuous or discontinuous modes and at different intensities. The results showed that low frequency ultrasound significantly enhanced the permeability of CP across hairless mouse skin in vitro. Delivering the same amount of ultrasonic energy in different modes of application markedly influenced the flux and skin residual of CP. The on/off discontinuous ultrasound had greater enhancement on CP permeation than the continuous ultrasound. The results of skin histopathology and permeation experiment using various membranes demonstrate that both disordering of stratum corneum and convective flow resulted from the cavitation effect were responsible for sonophoretic enhancement of CP. The permeation of CP through hair follicles and sweat ducts was susceptible to the application of ultrasound. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Clobetasol 17-propionate; Percutaneous absorption; Ultrasound; Sonophoresis

1. Introduction

Sonophoresis (phonophoresis), the movement of drug molecules contained in a coupling medium through the skin under the influence of ultrasound, has been studied in clinical conditions. It is well documented to practically increase skin permeation of corticosteroids (Skaun and Zentner, 1984; McElnay et al., 1987; Machet et al., 1998). The most clinically significant concern following administration topical corticosteroids is adverse effects such as pigmentation changes, skin atrophy, allergic contact dermatitis and itching (Parish et al., 1985). One of the methods to reduce the adverse effects of steroids is to enhance the permeability of corticosteroids so as to reduce the topically applied dose. Sonophoresis could thus be an aid in administration of various topical

* Corresponding author. Tel.: +886-2-27361661; fax: +886-2-27074804.
E-mail address: fajy@ms9.tisnet.net.tw (J.-Y. Fang)

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corticosteroids. Clobetasol 17-propionate (CP; $C_{25}H_{32}ClFO_5$) is considered to be the most potent of the currently available corticosteroids. The medication is approximately 1800 times more potent than hydrocortisone according to the vasoconstriction test (Harris and Hunter, 1988). Incidences of unfavorable side-effects are therefore greater than those of related compounds.

The frequency range of ultrasound used for sonophoresis is typically from 20 kHz to 10 MHz (Tyle and Agrawala, 1989). Low frequency ultrasound enhances the percutaneous absorption of drugs more effectively than therapeutic ultrasound. Low frequency ultrasound may also induce acoustic cavitation, causing several biological effects such as disordering of the structure of stratum corneum (Mitragotri et al., 1995a, 1996; Ueda et al., 1996). As a result, low frequency ultrasound (20 kHz) was utilized in this study to enhance the permeability of CP through hairless mouse skin. Despite the enhancement effect of ultrasound being reported in several publications, there are some inconsistencies in the literature on the effect of ultrasound in increasing drug flux across skin: $\sim$75% of the studies reported an enhancement effect of sonophoresis, whereas the others obtained negative results (Byl, 1995). Accordingly, in order to obtain mechanistic information and to achieve the optimum enhancement effect, the various ultrasound parameters on drug permeation have to be studied systematically.

The main purpose of this study was to evaluate the effects of different ultrasound parameters on the enhancement of CP permeation and to explore the mechanisms of enhancement by using various types of skin membrane. The various ultrasound parameters examined were: ultrasound intensity, mode of ultrasound application (continuous vs. discontinuous) and pulsed length of ultrasound application. The different skin membrane types used in this study were hairless mouse skin, stratum corneum-stripped skin, ultrasound-pretreated skin, Wistar rat skin, human skin and cellulose membrane. The influence of low frequency ultrasound on the skin structure was also evaluated by histopathological examination.

2. Materials and methods

2.1. Materials

Clobetasol 17-propionate (CP) was purchased from Sigma (USA). Propylene glycol was supplied by Nihon Shiyaku (Japan). All other chemicals and solvents were of analytical grade.

2.2. In vitro permeation experiments

The in vitro permeation experiments were performed by using modified Franz diffusion cells, which allowed the application of an ultrasound probe into the donor cell. A 2-ml mixture of propylene glycol/pH 7.4 buffer (20/80, v/v) was used as the vehicle. The drug concentration in the donor was 0.05% (w/v). The receptor medium (10 ml) was composed of 30% ethanol and 70% pH 7.4 buffer. The top of the donor was covered with paraffin paper. The available diffusion area between cells was 1.77 cm^2. The receptor was kept at a constant temperature of 37°C and stirred by a magnetic stirrer at 600 rpm. At appropriate intervals, 200-μl aliquots of the receptor medium were withdrawn and immediately replaced by an equal volume of fresh receptor solution. The CP content of various samples was analyzed by the HPLC method (Fang et al., 1999). The flux of CP was determined from the slope of cumulative amount-time profiles and expressed as the amount of drug passing a unit area of skin membrane per hour (μg/cm^2 per h). Each data point represents the average determination of three experiments. The amount of CP retained in skin was also determined after the in vitro permeation experiments; the method has been described in detail elsewhere (Fang et al., 1998). Briefly, the skin was washed 20 times using a cotton cloth immersed in methanol. A 0.5-g sample of skin was cut with scissors and positioned in a glass homogenizer containing 2 ml of methanol and ground for 10 min by the electric stirrer. The resulting solution was centrifuged for 15 min at 4000 rpm and filtered through 0.2-μm pore size filter.

2.3. Application of low frequency ultrasound

Ultrasound was applied with a sonicator (VCX 600, Sonics and Materials, USA) with a transducer. The radiating diameter of transducer was 13 mm. The frequency was set at 20 kHz, and the estimated skin intensities ranged from 0.1 to 0.3 W/cm^2. The ultrasound transducer was located approximately 0.5 cm from the surface of the skin. Either continuous or discontinuous mode of ultrasound application was used in this study.

2.4. Skin temperature measurement

Skin temperature measurement was determined by a thermocouple probe assembled in the sonicator. The probe was inserted into the donor cell so as to make contact with the skin surface.

2.5. Preparation of skin membranes

The skin of female hairless mouse (7–9 weeks old) was used as the main skin barrier in this study. The mouse was killed by cervical dislocation, and full-thickness skin was excised from the dorsal region. To obtain the stripped skin, the adhesive tape was applied on the hairless mouse skin with uniform pressure and then removed. This procedure was repeated 20 times. The male Wistar rat (6–8 weeks old) was sacrificed with ether; the hair of its abdominal region was shaved with electric clippers and the full-thickness skin was then excised. Samples of whole adult human skin (40–45 years old) were obtained from breast reduction operations. Subcutaneous fat was carefully trimmed and the cadaver skin was rinsed with normal saline. The skin was then sealed in aluminum foil and a plastic bag and stored at −20°C (Fang et al., 1995). The cellulose membrane (Spectra/por® 2, Spectrum, USA) was immersed in pH 7.4 buffer for 24 h prior to the in vitro permeation experiment.

2.6. Histological examination of skin

Histological changes in the hairless mouse skin were examined after applying in vitro permeation experiments at different ultrasound modes. Each sample was fixed in 10% pH 7.4 buffered formaldehyde solution for at least 24 h. The sample was cut vertically against skin surface. Each section was dehydrated using ethanol and then embedded in paraffin wax, stained with hematoxylin and eosin. In each skin samples, three different sites were examined and evaluated under an optiphoto light microscopy.

3. Results and discussion

3.1. Effect of ultrasound intensity

Fig. 1 shows the influence of ultrasound intensity on the in vitro percutaneous delivery of CP. The ultrasound was continuously applied for 4 h with intensities ranging from 0.1 to 0.3 W/cm^2. The results demonstrate that the low frequency ultrasound was effective in enhancing the permeability of CP for all the intensities studied. However, the effect of enhancement was not proportional to the magnitude of ultrasound intensity according to the enhancement ration (ER) after ultrasound application (Table 1). The enhancement of ultrasound intensity on the flux of CP through hairless mouse skin increased in the order of $0 < 0.1 < 0.3 < 0.2$ W/cm^2.

In order to assess the safety of low frequency ultrasound on the integrity of skin structure, a histological study was performed. There was no significant change in epidermis during exposure to 0.1 W/cm^2 as compared with the control (Table 2). The only detectable change in the skin following exposure to 0.1 W/cm^2 ultrasound was the slightly chronic inflammatory cell infiltration under the epidermis as observed in Fig. 2 (A). However, while applying higher ultrasound intensities (0.2 and 0.3 W/cm^2), significant morphological alterations were observed in epidermis, dermis and subcutis (Fig. 2 (B) and (C)). Compared to the 0.2-W/cm^2 ultrasound intensity, the application of 0.3 W/cm^2 ultrasound caused more skin damage and relatively lower enhancement of CP permeability. Moreover, among three ultrasound-treated experiments (Table 1),

the application of 0.3 W/cm^2 ultrasound also showed the lowest CP amount retained in skin after 8-h permeation study. Both results indicate that the severe skin disruption caused by applying the 0.3-W/cm^2 ultrasound may result in lower amount of CP partition in skin reservoir as well as lower skin permeability. The results were consistent with previous findings in that the application of higher intensity of ultrasound showed more skin damage as well as lower permeability of indomethacin through skin tissues at therapeutic ultrasound frequency (1 MHz) (Miyazaki et al., 1992).

3.2. Effect of continuous and discontinuous modes of ultrasound

The effect of discontinuous ultrasound on CP permeation was studied by setting the on/off ratio to either long duration on/off mode (30 min/10 min, 30 min/20 min and 30 min/30 min) or pulsed on/off mode (0.3 s/0.1 s, 0.3 s/0.2 s and 0.3 s/0.3 s) at 0.1-W/cm^2 intensity. The total ultrasound application time was 4 h for all experiments. The cumulative amount-time profiles for applying the continuous ultrasound and the discontinuous ultrasound with long duration on/off modes are

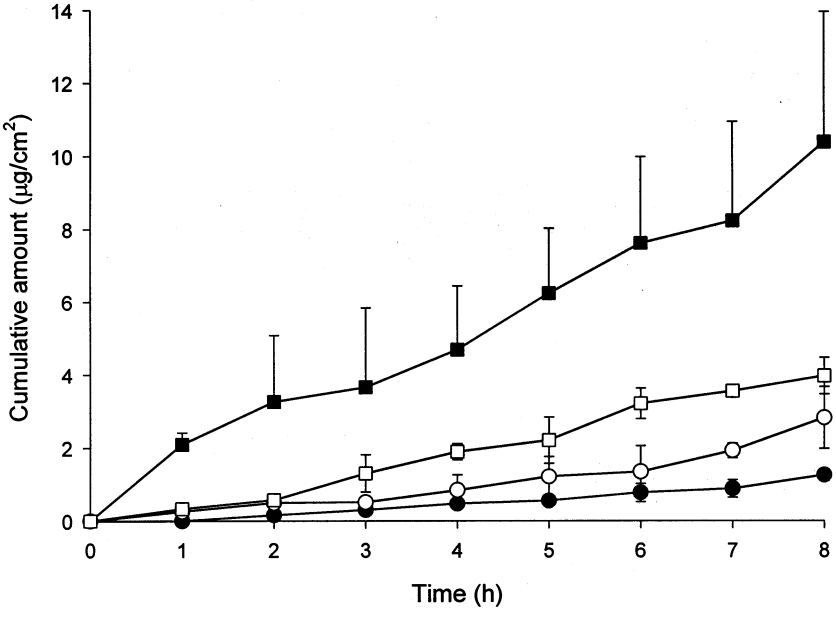


Fig. 1. Cumulative amount-time profiles of CP across hairless mouse skin under continuous application of various ultrasound intensities: 0 W/cm^2 (●); 0.1 W/cm^2 (○); 0.2 W/cm^2 (■); 0.3 W/cm^2 (□). Each value represents the mean ± S.D. ($n = 3$).

Table 1
The permeation parameters of CP under the application of various ultrasound intensities[a]

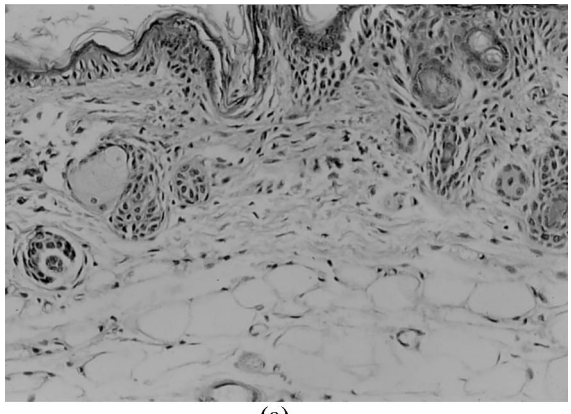
Intensity (W/cm^2)	CP in skin at 8 h (μg/g)	Flux (μg/cm^2 per h)	ER
0	581.21 ± 146.98	0.20 ± 0.05	–
0.1	980.32 ± 98.15	0.31 ± 0.13	1.55
0.2	1124.47 ± 144.97	1.18 ± 0.49	5.90
0.3	738.15 ± 243.57	0.53 ± 0.02	2.65

[a] Each value represents the mean ± S.D. ($n = 3$). ER, enhancement ratio; US, ultrasound. ER = flux with US/flux without US.

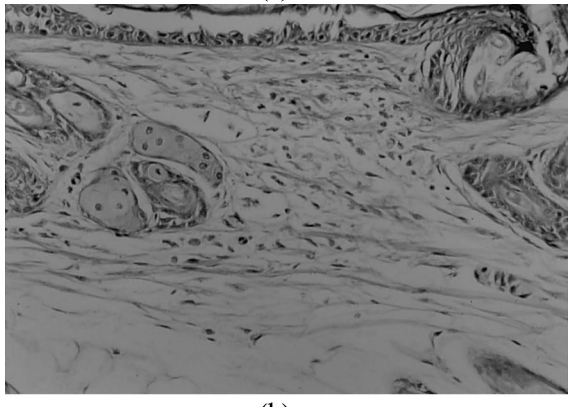
Table 2
Histopathological findings of hairless mouse skin at 8 h after application of various modes ultrasound

	Control	0.1 W/cm^2	0.2 W/cm^2	0.3 W/cm^2	30 min/10 min[a]	30 min/20 min[a]	30 min/30 min[a]	0.3 s/0.1 s[a]	0.3 s/0.2 s[a]	0.3 s/0.3 s[a]
Epidermis										
Liquefaction (acanthlysis, intraepidermal cleft)	–[b]	–	+	+ +	+ + +	+ +	+	+ +	+ +	+ + +
Dermis										
Upper dermis										
Neutrophil infiltration	–	–	–	–	+	–	–	±	+	–
Chronic inflammatory cell infiltration	–	+	+	+ +	+	+	+ +	+ +	+	±
Edema	–	–	–	+	+	–	+	+	+	–
Reticular dermis										
Neutrophil infiltration	–	–	–	–	+	–	–	–	+	–
Chronic inflammatory cell infiltration	–	+	+	+ +	+	+	+ +	+ +	+	±
Edema	–	–	–	+	+	–	+	+	+	–
Subcutis										
Neutrophil infiltration	–	–	–	–	–	–	–	–	–	–
Chronic inflammatory cell infiltration	–	+	–	+	–	–	+	+	+	–

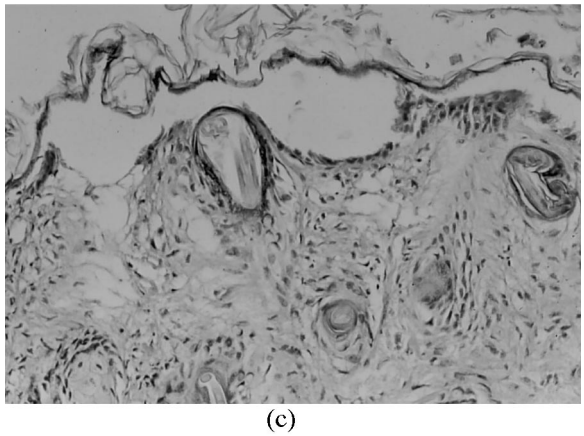
[a] On/off time.
[b] Score: –, no change; +, very slight; +, slight; + +, moderate; + + +, marked.



(a)



(b)



(c)

Fig. 2. Microscopic photographs of hairless mouse skin after continuous ultrasound application for 4 h at: (A) 0.1 W/cm^2; (B) 0.2 W/cm^2; (C) 0.3 W/cm^2.

shown in Fig. 3. In general, the application of discontinuous ultrasound is more effective in enhancing CP permeation. Fig. 3 also demonstrates that delivering the same amount of ultrasonic energy in different modes markedly influences the permeation of CP. According to the ER value (Table 3), discontinuous ultrasound with higher on/off ratio is more effective in enhancing CP permeation than the discontinuous mode with lower on/off ratio. The flux obtained by applying discontinuous ultrasound of 30 min/10 min and 30 min/20 min was higher than that of continuous mode (Table 3). According to the literature, the cell damage after ultrasound irradiation may be reversible (Tyle and Agrawala, 1989). The 10- and 20-min ultrasound-off durations may not be enough to allow the skin to recover from abnormal status, which may explain the higher CP permeability in these two modes. Indeed, the histological findings in Table 2 demonstrate that the shorter ultrasound-off period of the discontinuous mode produces greater epidermis liquefaction. Moreover, the higher enhancing effect by applying ultrasound with higher on/off ratio correlated well with the amount of CP in the skin. Table 3 shows that, with the three on/off application modes, the skin residuals of CP showed a trend of 30 min/30 min $<$ 30 min/20 min $<$ 30 min/10 min, which is consistent with the order of CP flux and ER. The permeation and partition results both suggest that the higher on/off ratio of ultrasound may cause the higher amount of CP in the skin and therefore the higher enhancing effect of CP permeation.

Fig. 4 depicts the profiles of CP permeation under the application of three modes of short duration on/off (pulsed) ultrasound. The skin residual and flux of CP after applying pulsed ultrasound are shown in Table 4. No significant difference ($P > 0.05$, ANOVA test) was observed for the amount of CP inside skin among the studies applying continuous ultrasound and the three pulsed modes. However, there was a significant difference in the CP flux. The short-pulsed ultrasound provided more effective enhancement of the CP permeation than the continuous mode. The results are in accordance with previous studies using pulsed ultrasound to improve permeabilities of lignocain, prilocain

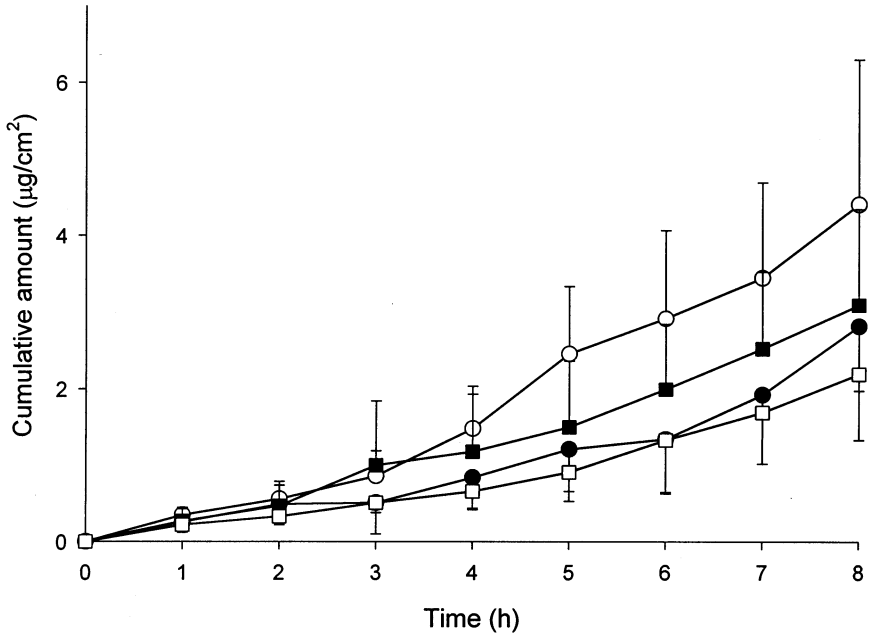


Fig. 3. Cumulative amount-time profiles of CP across hairless mouse skin with various on/off discontinuous modes of ultrasound application: continuous mode for 4 h (●); 30-min/10-min mode (○); 30-min/20-min mode (■); 30-min/30-min mode (□). Each value represents the mean ± S.D. ($n = 3$).

and indomethacin (Benson et al., 1988; Asano et al., 1997).

The pulsed ultrasound with lower on/off ratio showed higher enhancing effect on CP permeability than the pulsed ultrasound with higher on/off ratio. Interestingly, this observation was exactly opposite to the results when applying long duration on/off mode (Tables 3 and 4). The discrepancy may be explained by the following inferences: the ultrasound-off periods in these studies were short ($0.1 \sim 0.3$ s), no damaged cell may recover within this short period of time. As a result, the total application time of ultrasound may be regarded as the sum of times with and without ultrasound application; the longer application time (0.3/0.3 mode) corresponds to higher enhancement effect. The histological observations (Table 2) also demonstrate that the irradiation of smaller on/off ratio ultrasound on skin induces greater liquefaction in epidermis, which also contribute to its higher enhancement effect. Further investigation is needed and is in progress to elucidate the mechanism of pulsed output sonophoresis.

3.3. Permeation of CP across various types of skin membrane with ultrasound irradiation

Various mechanisms have been suggested to explain the enhancement of drug permeability via sonophoresis. Those mechanisms include: temperature increase, induction of convective flow, and

Table 3
The permeation parameters of CP under the application of various modes of on/off discontinuous ultrasound[a]

US mode	CP in skin at 8 h (μg/g)	Flux (μg/cm² per h)	ER[c]
Without US	581.21 ± 146.98	0.20 ± 0.05	–
Continuous 4 h	980.32 ± 98.15	0.31 ± 0.13	1.55
Pulse 30/10 min[b]	1321.42 ± 336.63	0.55 ± 0.08	2.75
Pulse 30/20 min	782.64 ± 230.64	0.38 ± 0.08	1.90
Pulse 30/30 min	443.56 ± 127.39	0.26 ± 0.09	1.30

[a] Each value represents the mean ± S.D. ($n = 3$).
[b] On/off time.
[c] ER, enhancement ratio = flux with US/flux without US.

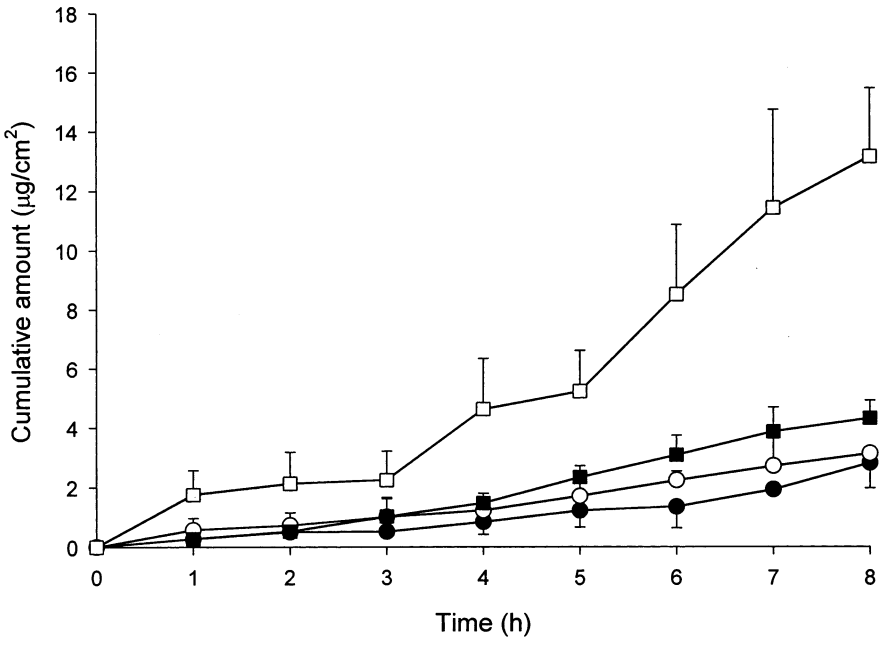


Fig. 4. Cumulative amount-time profiles of CP across hairless mouse skin with various on/off discontinuous modes of ultrasound application: continuous mode for 4 h (●); 0.3-s/0.1-s mode (○); 0.3-s/0.2-s mode (■); 0.3-s/0.3-s mode (□). Each value represents the mean ± S.D. ($n = 3$).

cavitation as well as radiation pressure (Tyle and Agrawala, 1989). Recently, Simonin has ruled out the involvement of radiation pressure after using mathematical calculations (Simonin, 1995). Ultrasonic heating can be minimized by applying the mode of millisecond on/off pulses (Meidan et al., 1995). In the present study, a less than 1°C (0.5 ± 0.2°C) skin temperature increase was observed while applying the pulsed ultrasound; the skin temperatures after applying continuous ultrasound with 0.1 W/cm^2 were increased no more than 5°C (4.2 ± 1.1°C), which is not high enough to cause reasonable increase in skin permeability (Levy et al., 1989; Bommannan et al., 1992). Accordingly, the enhancement of CP permeation in this study can be attributed to the effect of cavitation and the induction of convective flow.

The effect of cavitation involves generation and oscillation of gaseous bubbles in a medium, and can be induced by exposure to ultrasound. Cavitation may occur inside as well as outside the skin. These oscillations may disorganize the lipid bilayers of stratum corneum (Mitragotri et al., 1995a). In order to determine whether ultrasound affects the permeation by directly acting on the skin or outside the skin, cellulose membranes were used as the barrier in the in vitro permeation experiment. The advantage of using cellulose membrane is that its permeability characteristics would not be changed before and after applying

Table 4
The permeation parameters of CP under the application of various modes of on/off pulsed ultrasound[a]

US mode	CP in skin at 8 h (μg/g)	Flux (μg/cm^2 per h)	ER[c]
Continuous 4 h	980.32 ± 98.15	0.31 ± 0.13	1.55
Pulse 0.3/0.1 s[b]	981.61 ± 208.09	0.38 ± 0.04	1.90
Pulse 0.3/0.2 s	970.26 ± 315.31	0.57 ± 0.11	2.85
Pulse 0.3/0.3 s	999.34 ± 375.73	0.82 ± 0.15	4.10

[a] Each value represents the mean ± S.D. ($n = 3$).
[b] On/off time.
[c] ER, enhancement ratio = flux with US/flux without US.

Table 5
The permeation parameters of CP across various types of skin membrane with the application of 0.1 W/cm^2 ultrasound for 4 h[a]

Membrane type		CP in skin (μg/g)	Flux (μg/cm^2 per h)	ER[d]
Cellulose membrane	Without US[b]	–[c]	2.50 ± 0.19	–
	With US	–	3.03 ± 0.31	1.21
Pretreatment with US for 4 h	Without US	510.59 ± 130.16	0.33 ± 0.07	–
	With US	741.94 ± 100.02	0.40 ± 0.08	1.21
Stratum corneum stripped	Without US	1135.63 ± 317.58	1.21 ± 0.30	–
	With US	2122.10 ± 561.28	1.20 ± 0.16	0.99
Wistar rat skin	Without US	1382.01 ± 246.44	0.08 ± 0.02	–
	With US	2900.81 ± 704.57	0.26 ± 0.05	3.10
Human skin	Without US	102.26 ± 68.71	0.04 ± 0.01	–
	With US	219.39 ± 72.88	0.05 ± 0.01	1.37

[a] Each value represents the mean $\pm$ S.D. ($n = 3$).
[b] US, ultrasound.
[c] No data.
[d] ER, enhancement ratio = flux with US/flux without US.

ultrasound (Julian and Zentner, 1986; Levy et al., 1989); thus it can be utilized to assess the enhancing mechanism of cavitation.

Table 5 shows that the permeabilities of CP through hairless mouse skin and cellulose membrane were increased by 55 and 21% over control after 4 h of ultrasound application at 0.1-W/cm^2 intensity. These findings suggest that the effect of convective flow occurred outside the skin and may influence the permeation of CP with the ultrasound conditions examined. The discrepancy of enhancement (55 vs. 21%) suggests that cavitation inside the skin may also contribute to the enhancement effect. To verify this point, the effect of 4-h ultrasound pretreatment on skin permeability was examined (Table 5). The results indicate that the amount of CP in the skin as well as the flux were increased by the pretreatment with 4-h ultrasound, suggesting that the cavitation may affect the structure of skin and thus increase the drug permeability.

The effect of cavitation in the skin may enhance drug permeability via two routes: ultrasound may (i) cause structural changes in the stratum corneum, and (ii) enhance the permeant transport through hair follicles and sweat ducts of the skin (Mitragotri et al., 1995b). Table 5 shows the permeation of CP through stratum corneum-stripped skin with or without applying ultrasound. There was no significant difference ($P > 0.05$, t-test) between CP flux in the presence or absence of ultrasound, suggesting the enhancement of CP permeation by low frequency ultrasound is primarily due to structural or conformational changes of stratum corneum. Wistar rat skin was used as the model membrane to perform the in vitro sonophoretic study since Wistar rat has a greater number of hair follicles compared to hairless mouse (Bronaugh et al., 1982). Both the amount of CP in the skin and CP flux were enhanced significantly (Table 5). The results suggest that the appendageal pathways are more susceptible to ultrasonic enhancement than the transcellular pathway. The above results are in accordance with the previous study which demonstrated that CP penetrates skin more readily through follicles than epidermis (Osamura, 1982; Fang et al., 1998, 1999). Furthermore, the results are consistent with the literature in that the convective transport of permeant, which is one of the factors in enhancing CP permeation, occurred predominantly through appendageal routes (Mitragotri et al., 1995b).

Table 5 also demonstrates the influence of low frequency ultrasound on CP permeation through the human skin. The data show that the excised human skin was the least permeable of all the skin membranes studied. It is generally known that

rodent skins are generally more permeable than full-thickness human skin (Catz and Friend, 1990). In addition, the human breast skin used in this study often shows a relatively lower permeability than that from other anatomic sites (Harada et al., 1993).

In conclusion, the percutaneous absorption of CP was significantly enhanced by applying low frequency ultrasound. The application of discontinuous on/off ultrasound showed greater enhancement on CP flux than the application of continuous ultrasound. The permeation studies using cellulose membrane and various skins indicated that cavitation both outside and inside the skin were important in enhancing the CP permeation by ultrasound. The appendageal routes were also found to be important in affecting permeation of CP during sonophoresis.

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