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In vitro study of low-frequency ultrasound-enhanced transdermal transport of fentanyl and caffeine across human and hairless rat skin

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Abstract

The effect of low-frequency sonophoresis on fentanyl and caffeine permeation through human and hairless rat skin was studied in vitro. Experiments were performed using 20 kHz ultrasound applied at either continuous or discontinuous mode and with an average intensity of 2.5 W/cm^2. The results showed that low-frequency ultrasound enhanced the transdermal transport of both fentanyl and caffeine across human and hairless rat skin. This was explained by both increasing flux during sonication and shortening the lag time. Discontinuous mode was found to be more effective in increasing transdermal penetration of fentanyl while transdermal transport of caffeine was enhanced by both continuous and pulsed mode. Histological and electron microscopy studies showed that human and hairless rat skin was unaffected by ultrasound exposure. Further studies will be necessary to determine the relative contribution of ultrasound parameters in low-frequency ultrasound-induced percutaneous enhancement of drug transport.

Keywords: Sonophoresis; Fentanyl; Caffeine; Transdermal delivery; Human skin; Hairless rat skin

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1. Introduction

Transdermal drug delivery offers an attractive alternative to conventional oral and injection therapies as a means of achieving constant therapeutic levels of drugs. Percutaneous administration bypasses the hepatic first-pass effect and provides better compliance. However, the barrier properties of the stratum corneum, the outmost layer of the skin, prevent the percutaneous absorption of many drugs (Elias, 1996). Different approaches including chemical enhancers (Barry, 1987; Walker and Smith, 1996), iontophoresis (Prausnitz, 1999) and sonophoresis have been investigated to increase skin permeability.

The use of ultrasound as a physical enhancer for topical and systemic delivery (sonophoresis) has already been investigated using high-frequency ultrasound (Bommannan et al., 1992; Meidan et al., 1998). This ultrasound frequency range is currently used in human therapy, and is well tolerated in vivo. However, the effect of high-frequency ultrasound in increasing transdermal transport is low or even absent when heating produced by the ultrasound equipment is minimized (Machet et al., 1998).

Low-frequency ultrasound (20 kHz) seems to be more effective in increasing transdermal transport of various drugs, including large molecules such as insulin (Tachibana, 1992; Mitragotri et al., 1995a). However, the tolerance of exposed skin has not been fully studied. In a recent study (Fang et al., 1999), histological lesions were observed after 20 kHz ultrasound application on hairless rat skin in vitro.

The aim of our study was to quantify the effects of low-frequency sonophoresis on transdermal transport of a lipophilic (fentanyl) and a hydrophilic (caffeine) molecule, both already used without ultrasound in humans for pain control (fentanyl) and for treatment of lipodystrophy (caffeine). The effects of continuous and discontinuous ultrasound modes were studied in vitro on the permeation of these drugs across both hairless rat and human skin. Assessment of any possible skin changes induced by ultrasound exposure was also evaluated by analyzing sonicated skin samples with conventional electron and optical microscopy.

2. Materials and methods

2.1. Molecules

[Propionamide-1-^{14}C] fentanyl (spec. act. 55 mCi/mmol) was purchased from NEN USA, and [1-Methyl-^{14}C] caffeine (57.5 mCi/mmol) and [$^{3}H_2O$] tritiated water (5 Ci/ml) were supplied by Isotopchim (France). Caffeine and water were diluted in saline solution (NaCl 0.9%) and fentanyl in Phosphate Buffer Saline (PBS; phosphate concentration = 0.01 M; NaCl concentration = 0.1347 M) in order to obtain 5 μCi/ml (concentration of the radiolabeled permeant in the solution). High pressure liquid chromatography was performed of sonicated radiolabeled solutions (20 kHz, 2.5 W/cm^2, discontinuous mode: 10% duty cycle) and compared with a non-sonicated solution in order to assess possible structural changes in fentanyl and caffeine molecules during ultrasound exposure. This control test indicated no ultrasound-induced ^{14}C-fentanyl or ^{14}C-caffeine degradation.

2.2. Skin membranes

Hairless rats (males, 8–12 weeks of age, Iffa Credo) were killed by cervical dislocation. The skin from the dorsal region was removed and fat removed. Samples of whole adult human skin were obtained from freshly excised surgical specimens (abdominal and breast area). After trimming off the subcutaneous fat, the skin was stored at −20 °C and used in less than 3 months. Prior to each experiment, a 350 μm thick section was removed using an Electro-dermatome purchased from Padgett Instruments.

2.3. In vitro permeation experiments

Permeation experiments were conducted with specially modified Franz diffusion cells which allowed the introduction of an ultrasonic probe into the donor compartment. In this study, two different types of donor compartment were used: 2.27 and 3.14 cm^2. The donor compartment was filled with 3 ml of radiolabeled solution containing one of the three drugs. The receiver compartment

(dermis side) was filled with 12 ml of NaCl or PBS depending on the drug being studied, and then degassed before each new experiment. The receptor compartment was kept at a constant temperature of 37 °C and stirred with a star-head magnetic bar driven by an external magnetic stirrer at a constant speed (500 rpm). Each diffusion experiment was carried out over 8 h and repeated with four skin samples obtained from four different bodies. An appropriate sample volume (0.5 ml) was withdrawn from the receiver compartment at regular intervals and an equal volume of fresh initial receiver solution (i.e. saline solution or PBS) was replaced. Each sample collected was placed in scintillation vials filled with 6 ml of scintillation liquid (Ultimagold MV, Packard, France). Radioactivity was determined by means of a liquid scintillation counter (TR/TLL 2550, Packard, France). The area under the curve (AUC) was determined from the integral of the cumulative amount with respect to time using the trapezoidal method. The diffusion rate or flux (J) was determined from the slope of cumulative amount–time profiles and expressed as the amount of drug transported through a unit of area of skin membrane per hour (ng/cm^2 per hour). Lag time was calculated from the intersection of the steady state regression curve with the x-axis. Data are expressed as the mean $\pm$ S.D. of four experiments. Statistical analysis was performed using a Student's t-test ($P < 0.05$). When experiments were conducted with hairless rat skin samples, the amount of fentanyl and caffeine retained in skin was also determined after the experiments. Briefly, at the end of the experiment, the skin was taken and the epidermis was gently removed from the dermis using pincers. The two membranes were then placed in vials containing 1 ml of Soluene 350 (Packard, France) and kept overnight at 37 °C and 8 ml of scintillation liquid was added to the vials. Radioactivity in the resulting solution was determined in a liquid scintillation counter.

2.4. Ultrasound equipment

Ultrasound was applied using a sonicator (VCX 400, Sonics and Materials, USA) operating at a frequency of 20 kHz. The diameter of the ultrasound probe was 1.3 cm^2. The ultrasound transducer was placed in the donor compartment perpendicular to the skin surface at a distance of 1 cm and applied in continuous or in pulsed mode. Both ultrasound protocols were used at an intensity of 2.5 W/cm^2 corresponding to spatial average temporal average (I_{SATA}) i.e. indicating intensity over the pulse repetition period. This intensity was chosen since we have previously shown that 2.5 W/cm^2 was below the intensity threshold with regard to human skin safety (Boucaud et al., 2001). The temperature of the donor compartment was recorded continuously during sonication using a thin thermocouple (K-Thermometer, Bioblock Scientific, France). After ultrasound exposure, the top of the donor compartment was covered with a plastic film (Parafilm®) to prevent evaporation. Control experiments were conducted as described above but with no ultrasound.

2.5. Histology and electron microscopy of sonicated skin

Fresh skin samples (human and hairless rat) were exposed to the same ultrasound protocol used during sonophoresis experiments. The effects of low-frequency ultrasound were analyzed macroscopically by a dermatologist, and then observed under both electron and optical microscopy. Skin biopsies (4 mm diameter) were taken and fixed in Bouin's solution for optical microscopy study and in glutaraldehyde–paraformaldehyde solution for later observation under electron microscopy. In addition, immuno-cytochemistry study was performed under optical microscopy to observe the intercellular junctions within the epidermis with an *anti*-32-2B monoclonal antibody which recognizes desmosomal proteins (Vilela).

3. Results

3.1. Effect of low-frequency ultrasound on transdermal transport of water, fentanyl and caffeine across human skin

In this set of experiments, the diffusion cell area was 3.14 cm^2. Ultrasound was applied in pulsed

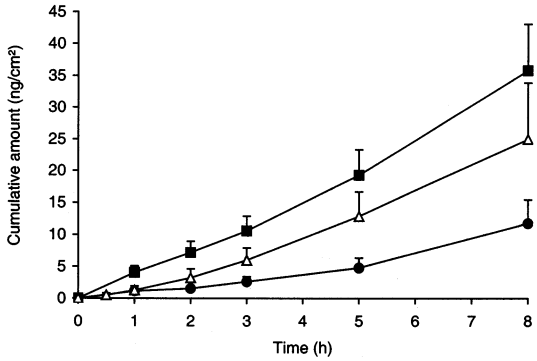

Fig. 1. Cumulative amount–time profiles of caffeine across human skin under continuous mode (2.5 W/cm^2, 10 min) (△) and pulsed mode (10% duty cycle, 2.5 W/cm^2, 1 h) (■) and without ultrasound (●). Each value represents the mean ± S.D. ($n = 4$).

mode (0.1 s on/0.9 s off) over 1 h or continuously for 10 min, both with an average ultrasound intensity of 2.5 W/cm^2. The increase in temperature in the donor compartment was 7 °C thus reaching a final temperature 40 °C. Fig. 1 shows the caffeine transport through human skin as an example. As a result, continuous mode ultrasound was found to be more effective on the diffusion kinetics of water while the transdermal transport of caffeine and fentanyl across human skin was more highly increased by pulse mode treatment. The area under the curve was significantly greater for fentanyl and caffeine when ultrasound was applied whereas it remained unchanged for water (Table 1). The diffusion flux of the three substances calculated at the end of ultrasound exposure was much greater than that of controls. When ultrasound was used in pulsed mode, the diffusion flux of fentanyl was 35-fold greater than controls. However, diffusion flux calculated 7 h after the end of ultrasound exposure was not significantly different from controls. The lag time of caffeine and fentanyl was shortened when ultrasound was used in continuous mode.

3.2. Comparison of the effect of low-frequency sonophoresis on the transdermal transport of caffeine and fentanyl across human and hairless rat skin

In this set of experiments, the diffusion cell area was 2.27 cm^2. Ultrasound was applied in continuous mode over a period of 2.5 min, and then repeated once again after 2.5 min with an intensity of 2.5 W/cm^2. The increase in temperature in the donor compartment was about 5 °C, thus reaching a final temperature of 38 °C. The enhancement effect of ultrasound on the permeation of fentanyl and caffeine is shown in Fig. 2a and b, respectively, as the cumulative amount–time profiles. Values obtained for caffeine and fentanyl across human skin samples were comparable to

Table 1
Area under the curve (AUC), flux and lag time of fentanyl, caffeine and tritiated water across human skin (diffusion surface: 3.14 cm^2) after application of continuous and pulsed mode ultrasound

Drug	US mode	AUC (μg/cm^2)	Flux (ng/cm^2 per hour)	Lag time (h)
Fentanyl	Controls	0.86 ± 0.25	1.6 ± 0.6	1.05 ± 0.14
	Continuous	1.22 ± 0.47	5.9 ± 4.5	0.06 ± 0.01[a]
	Pulsed	2.03 ± 0.35[a]	55.3 ± 20.4[a]	0.8 ± 0.3
Caffeine	Controls	0.03 ± 0.02	1.1 ± 0.3	0.57 ± 0.12
	Continuous	0.08 ± 0.03[a]	1.2 ± 0.5	0.26 ± 0.08
	Pulsed	0.13 ± 0.03[a]	4.1 ± 1.0	0.54 ± 0.32
Tritiated water	Controls	54.99 ± 26.16	1.5 ± 0.5	0.17 ± 0.05
	Continuous	84.92 ± 47.41	2.2 ± 1.3	0.12 ± 0.03
	Pulsed	84.89 ± 44.91	3.7 ± 1.6	0.22 ± 0.11

Each value represents the mean ± S.D. ($n = 4$).
[a] Means data are statistically different ($P < 0.05$ using Student t-test) from control data.

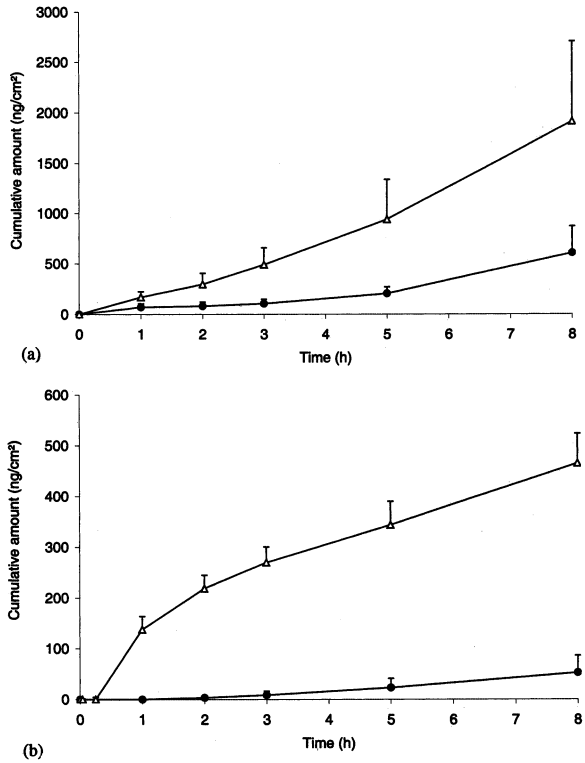

Fig. 2. Cumulative amount–time profiles of fentanyl (a) and caffeine (b) across hairless rat skin under continuous mode (2.5 W/cm^2, 2×2.5 min) (△) vs. controls (●). Each value represents the mean ± S.D. ($n = 4$).

those obtained with the 3.14 cm^2 diffusion cell reported in the previous section. The use of low-frequency ultrasound in our conditions significantly enhanced the amount of both drugs in the receiver compartment, with a much more pronounced effect on the transdermal transport of caffeine across hairless rat skin. Moreover, the flux calculated 1 h after ultrasound exposure was 75-fold greater for caffeine compared to controls (Table 2). At the end of the experiment, no statistical difference was found between the amount of fentanyl in the whole hairless rat skin and controls (Fig. 3a). On the other hand, the amount of caffeine in the hairless rat dermis was significantly higher ($P < 0.05$) after ultrasound exposure than controls (Fig. 3b).

3.3. Tolerance of ultrasound exposed skin

Microscopy study of sonicated skin (human and hairless rat) demonstrated that ultrasound exposure did not induce changes in skin structure. Scanning electron microscopy of fresh human skin samples exposed to 2.5 W/cm^2 (continuous or pulsed mode) revealed no modification of the skin surface (i.e. the stratum corneum). Moreover, transmission electron microscopy of sonicated human skin (Fig. 4b and c) showed a normal appearance compared to controls (Fig. 4a). Microscopy study of HES-stained human skin sections did not reveal any lesions. The stratum corneum and viable epidermis were of normal appearance. No enlargement of inter-keratinocyte spaces was observed. Staining of desmosomes with *anti*-32-2B monoclonal antibody was normal.

4. Discussion

This study demonstrated that low-frequency ultrasound enhanced the percutaneous transport of water, caffeine and fentanyl across human and hairless rat skin in vitro. Discontinuous mode ultrasound (1 h, 10% duty cycle) was found to be more effective than short continuous mode ultrasound when experiments were performed with human skin, and low-frequency seemed to be more effective in enhancing the transdermal transport of fentanyl and caffeine across hairless rat skin than human skin. Moreover, in our conditions, ultrasound protocols seemed to be safe for human and hairless rat skin since no morphological or ultrastructural changes were observed. Fentanyl is used in transdermal patches (Duragesic®, Janssen-Cilag) for the treatment of chronic pain, especially in cancer (Caplan and Southam, 1990). Pain control must be achieved as rapidly as possible, and ideally should be modulated to increase efficacy and tolerance. This is currently achieved with subcutaneous injections using an external pump monitored by the patient. Using transdermal drug delivery, control of pain is regulated by the permeability of the stratum corneum, and there is a 24 h delay thus necessitating oral mor-

phine over this period. The administration of ultrasound to a transdermal patch might allow self regulation of pain by the patient. Though steady state flux was not changed by ultrasound in the present study, shortening of lag time resulted in an increase in the total amount of fentanyl in the receiver compartment. Sonophoresis of caffeine across human epidermis has previously been reported with 1 MHz ultrasound (2 W/cm^2, continuous mode) over 20 h (Mitragotri et al., 1995b). While significant enhancement has been found with some drugs such as estradiol and testosterone, no enhancement of the diffusion rate across the human epidermis was observed with caffeine in this study. In our study the percutaneous flux of caffeine across 350 μm thick human skin was slightly increased by ultrasound (pulsed and continuous modes) compared to controls. However, the mean AUC value was four times greater with pulsed ultrasound. We believe that shortening the lag time of caffeine and fentanyl is of importance and should be taken into account in ultrasound-induced enhancement of drug transport.

Several mechanisms, including thermal (i.e. heating of the coupling medium) and non-thermal effects, are involved in sonophoresis. We previously observed an increase in temperature of the donor compartment when experiments were carried out with high-frequency ultrasound (Machet et al., 1996). Heating of the skin surface with electrical resistance resulted in enhancement of the transdermal digoxin transport similar to that observed with high-frequency ultrasound. Suppression of the heating of the skin surface with a cooling system resulted in no enhancement of the transdermal transport of mannitol, estradiol or hydrocortisone (Machet et al., 1998). In the present study, using low-frequency ultrasound raised the temperature in the donor compartment 5–7 °C. However, the 75-fold flux enhancement of caffeine across hairless rat skin samples suggests that the ultrasound-enhancement was induced by factors other than heating of the donor compartment. Other factors have previously been reviewed (Simonin, 1995). Cavitation, which is the generation, oscillation and subsequent violent collapse of gaseous micro-bubbles within the coupling medium and/or within the skin, is probably the main mechanism that could explain the enhancement of percutaneous absorption. At a constant intensity, the cavitation threshold decreases with lower frequency while micro-bubble size increases with low ultrasound frequency. Microbubble size is about 150 μm at 20 kHz and the occurrence of cavitation within the donor compartment solution can easily be observed macroscopically during sonication. It has been

Table 2
Area under the curve (AUC), flux and lag time of fentanyl and caffeine across hairless rat and human skin (diffusion surface: 2.27 cm^2) after application of continuous and pulsed mode ultrasound

Skin membrane	Drug	US mode	AUC (μg/cm^2)	Flux (ng/cm^2 per hour)	
				At the end of sonication	7 h after sonication
Human	Fentanyl	Controls	0.8 ± 0.4	2.60 ± 0.81	44.54 ± 16.34
		Ultrasound	1.48 ± 0.43[a]	11.80 ± 8.87	46.77 ± 11.59
	Caffeine	Controls	0.08 ± 0.03	1.57 ± 0.52	3.20 ± 1.2
		Ultrasound	0.11 ± 0.06	3.70 ± 1.34	4.22 ± 2.62
Hairless rat	Fentanyl	Controls	1.72 ± 0.62	7.03 ± 1.49	55.00 ± 7.22
		Ultrasound	6.42 ± 2.51[a]	16.08 ± 5.53[a]	64.08 ± 25.30
	Caffeine	Controls	0.16 ± 0.05	0.20 ± 0.05	0.60 ± 0.12
		Ultrasound	2.51 ± 0.27[a]	15 ± 2.40[a]	5.04 ± 0.48

Each value represents the mean ± S.D. ($n = 4$).
[a] Means data are statistically different ($P < 0.05$ using Student t-test) from control data.

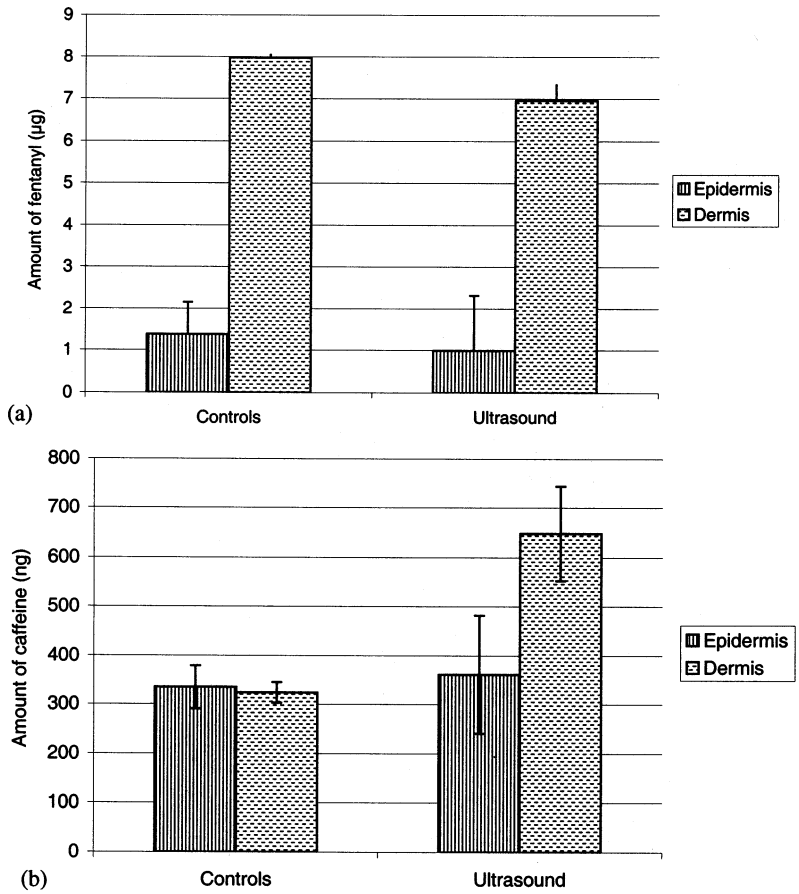

Fig. 3. Amount of fentanyl (a) and caffeine (b) in hairless rat skin at the end of ultrasound exposure (2.5 W/cm^2, 2 × 2.5 min).

hypothesized that cavitation occurs near the surface of the stratum corneum and hence temporarily disorganizes intercellular phospholipid bilayers and/or corneocytes by creating a transient aqueous channel in the stratum corneum, thus making transdermal absorption easier (Ueda et al., 1995; Mitragotri et al., 1996). In the present study, the results obtained with hairless rat skin samples support this hypothesis since the skin permeability of caffeine (hydrophilic drug) was significantly increased by low-frequency ultrasound while that of fentanyl (hydrophobic drug) was slightly increased. Hence, in view of the physicochemical parameters of these drugs, our results suggest that sonication has a greater effect on the skin permeation of hydrophilic drugs, which usually have low permeability. Previous studies have demonstrated size dependence of the permeability enhancement through the disorganized bilayer of the stratum corneum (Johnson et al., 1996). The exact localization of cavitation in the skin during ultrasound exposure is not fully understood. Using high-frequency ultrasound in vitro (1.1 MHz, 1.5 W/cm^2), we observed small holes (1–2 μm diameter) in the surface of human skin that we assumed to be a consequence of cavitation activity at the skin–medium interface (Machet et al., 1998). Such disruption of the lipids at the surface of the stratum corneum has also been demonstrated in vitro with low-frequency ultrasound (Malghani et al., 1998). The occurrence of pits on aluminum foil surface exposed to 20 kHz ultrasound (Mitragotri et al., 2000) similarly demonstrated the occurrence of cavitation in

the medium exposed to ultrasound and hence the creation of pathways in the skin. Using the same frequency range (48 kHz, 0.5 W/cm^2, continuous mode: 10 min), 100–150 μm diameter crater-like lesions were observed on the surface of hairless mice skin after sonication (Yamashita et al., 1997). These lesions were not observed on human skin using the same ultrasound conditions. This result strongly suggests that hairless mice skin is more sensitive to ultrasound than human skin. We demonstrated in our study that applying the same ultrasound conditions resulted in greater permeation of the hairless rat skin than human skin. Using our ultrasound conditions, cavitation was macroscopically visible in the donor compartment and percutaneous transport of caffeine and fentanyl was enhanced across hairless rat skin. However, study of skin tolerance to ultrasound showed no disruption or disorganization of the stratum corneum. Moreover, the other skin layers were of normal appearance. Thus, if cavitation plays an important role in enhancing percutaneous transport by inducing disorganization of bilayers, it may be assumed that it is a transient and/or reversible phenomenon that no longer exists after ultrasound exposure. This result is supported by similar flux of sonicated and control skin 7 h after ultrasound exposure. The occurrence of cavitation within the skin is not well defined with the techniques currently used in sonophoresis. Tissue necrosis has been observed in the 400–1000 W/cm^2 intensity range both around the area of the sonicated surface and deep in the tissue, probably because of blood vessel damage induced by cavitation (Fry et al., 1995). Few articles have addressed the question of the cavitation activity within the skin at the much lower intensities currently used in sonophoresis. Indirect evidence of cavitation within living tissue is supported by the production of free radicals during ultrasound exposure (Mitragotri et al., 1995b). However, the skin needs to be systematically checked for possible damage induced by ultrasound, since obvious and delayed lesions can appear, depending on the intensity, duration and duty cycle of the ultrasound protocol (Boucaud et al., 2001).

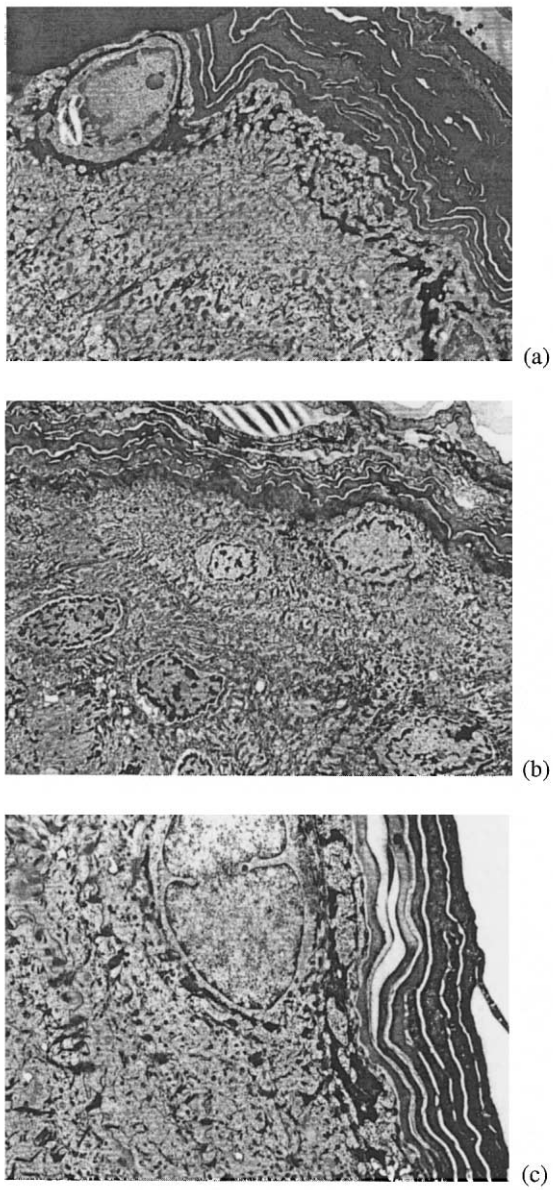

Fig. 4. Transmission electron microscopy micrographs of human skin samples without ultrasound (a), after continuous ultrasound (2.5 W/cm^2, 10 min) (b) and pulsed ultrasound (2.5 W/cm^2, 1 h 10% duty cycle) (c).

5. Conclusion

The aim of the present work was to study the potential of low-frequency ultrasound in transder-

mal delivery of fentanyl and caffeine across human and hairless rat skin. Using discontinuous ultrasound across hairless rat skin resulted in greater enhancement of percutaneous permeability. The absence of skin modification after ultrasound exposure and the similar permeability of sonicated and control skin 7 h after ultrasound exposure indicate that sonophoresis is a safe and reversible phenomenon. Future works should be conducted in order to better understand the mechanism of ultrasound-enhanced transdermal drug transport.

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