INFLUENCE OF THE ULTRASOUND IN CUTANEOUS PERMEATION OF THE CAFFEINE: *IN VITRO* STUDY

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Summary

The aim of the study was to analyse, *in vitro*, the effect of ultrasound application (US) on cutaneous permeation of caffeine (phonophoresis). This evaluation was carried out using diffusion cells, as proposed by BENTLEY. Four pieces of skin excluding hypodermis were extracted from swine dorsal region. The skin was attached to the diffusion cells, maintained in contact with a receptor solution at 37ºC, and each one was submitted to one of the following treatments: gel, caffeine (5%) gel, gel plus US, and caffeine (5%) gel plus US. The US was applied in the frequency of 3 MHz, with intensity of 0.2W/cm², and continuous emission mode. Receptor solution was collected in different time (0-240 min) after gel/US administration. The quantification of the drug that crossed the barrier was taken through the spectrophotometer (λ = 273 nm). We are able to conclude with our results that the US was effective as an accentuator (gel caffeine: 767.70 ± 55.8 μg/ml to US+gel caffeine: 1925.4 ± 110.35 μg/ml AUC in 240 min, $p<0.01$) and accelerator (peak gel caffeine 4.4 $\pm$ 0.4 μg/ml in 60 min and peak US plus gel caffeine 9.5 $\pm$ 0.6 μg/ml in 30 min after the beginning of the application) of cutaneous caffeine permeation.

Keywords: caffeine, ultrasound, phonophoresis, cutaneous permeation, skin swine.

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Introduction

Transdermic transport has been intensely studied during the last decades by virtue of several advantages that this route of drug application(1) offers, due to its being a non-invasive technique, thus avoiding both the effect of passing first through the liver and the degradation of peptide and protein drugs (2-5). However, this transport is limited by the most external epidermis layer, the stratum corneous (6), due to its structural and biochemical characteristics. Therefore, chemical and physical accentuator have been used, which aim at increasing cutaneous permeation. Among the chemical accentuator we may mention ethanol, and among the physical ones, ultrasound (US). The use of US with this purpose is named phonophoresis or sonophoresis (3, 4).

Many studies have demonstrated that US is usually safe and presents no negative effects at long or short term. The enhancement of drug penetration in the skin by phonophoresis is due to its thermal, mechanical and chemical properties (2, 7-9).

The US mechanical effect produces cell oscillation, thus promoting the enlargement of intercellular spaces, modifying the lipidic structure of the cell membrane, increasing cell permeability and ionic conductance, and reducing the rest membrane potential, or destroying the cells (2, 5, 13). According to MITRAGOTRI *et al*.(12) phonophoresis disorganizes the cell membrane lipidic bilayer of the skin corneous layer, reducing in 30% its resistance to permeation. Due to the cavitation mechanism, gaseous micro blisters are produced, which allow the passage of the drug, as they violently burst. The increase of membrane permeability also facilitates the penetration (2, 5, 13). In addition, the higher temperature produced by the ultrasound increases the kinetic energy of the medicament molecules and of the cell membrane, expands the hair sebaceous follicle ostium and the sweat glands, in addition to increasing the local circulation (2, 8).

Chemical changes reported to occur during phonophoresis include the induction of an increased number of oxidation reactions, inactivation of enzymes, and formation of small gaseous bubbles induced by molecular splitting within cells, known as cavitations. Increased adenosine triphosphatase activity and increased cell membrane permeability are possible mechanisms (13).

Although phonophoresis offers one advantage in relation to other types of drug administration (2), according to MITRAGOTRI et al (12) phonophoresis varies according to the drug and the US frequency used. Therefore, the US may not enhance permeation, which leads to controversy about the efficacy of such procedure.

Several authors have proposed the administration of caffeine in the lipodystrophy treatment (14-17), by topical or endogenous (i.v.) administration, associated or not to US. The main action of caffeine is to antagonize the adenosine receptors. Once activated, the type A1 adenosine receptor inhibits lypolisis. Caffeine and its metabolites, theophylline and theobromine, block the reversible mode of this receptor. In addition, caffeine also inhibits the phosphodiesterase that degrades cAMP to the inactive form 5'AMP, thus stimulating lypolisis. The result of the sum of these two effects of caffeine is an increase of the lipolysis induced by lipolytic agents and reduction of cellulites (18).

The aim of this study was to analyze, *in vitro*, the effect of the US application on fragments of swine skin on the cutaneous permeation of caffeine, using BENTLEY's (19) diffusion cells.

Materials and Methods

1. Animals

During the experiments, the swines were cared for in accordance with the principles outlined by OLFERT *et al.*(20) for the use of animals for research and education, and the experimental protocols were approved by the Committee for Ethics in Animal Experimentation of the Institute of Biology (UNICAMP nº 614-2).

We used male, non-castrated, 50 days swines (Landrace x Large White), old, weighing from 23.5±3.2 kg, were used and obtained from the Alvorada's country property. The animals were fed with water and ration composed by crushed corn, soy flour, meat flour, calcareous, salt, polinucleous Fapec "*ad libitum*". Ivomec® was applied as prophylactic measure against ectoparasites. One day before sacrifice by infiltration of anesthesic, the dorsal area of the animals was shaved with a shearing machine (comb nº 0), avoiding damage to the corneous layer, which could alter the skin permeability.

2. Experimental Groups

The animals (n=5) were sacrificed and four 10 cm² rectangle of the dorsal skin without the hypodermis were removed. Each skin sample was used for one of the following treatments: gel (GEL), caffeine (5%) gel (CAF), gel plus US (US), and caffeine (5%) gel plus US (US+CAF).

3. Drug and gel preparation

The gel was prepared with 1% Carbopol 940®, 10% propyleneglycol, 0.1 M sodium acetate buffer, pH 7.1, 25% absolute ethyl alcohol, and triethanolamine (qsp, pH 7.0).

The caffeine gel was prepared adding 5% of anhydrous caffeine (Sigma Chemical Company, St. Louis, MO, USA). Caffeine was pre solubilized in sodium acetate buffer 0.1M, pH 7.1 containing 25% ethyl alcohol absolute and 10% propyleneglycol. Final solution revealed a pH between 7.0 and 7.5 and this solution was incorporate to the gel formulation above described.

4. Ultrasound

The US (Sonomaster Microcontrolado, KW Ind. Nac. de Technology Lt, Amparo, São Paulo, Brazil) was applied over the skin area of the groups gel (US) or caffeine gel (US+CAF), at 3 MHz, due to the superficiality of the adipose tissue (21), 0.2 W/cm² (22), continuous emission mode (23) and application time of 1 min/cm² (24), with the transducer being moved slowly and continuously until the end of the application (25).

The calibration of US intensity set at a frequency of 3 MHz in the US scale OHMIC CS Instruments Co (Easton, USA) was performed with degassed and distilled water. The measurement of US transmission in gel was performed**.** The methodology adopted was the one proposed by GUIRRO *et al.*(26), which consists of the use of a US scale composed of a conical metal target inside a rubber reservoir containing degassed distilled water. An acrylic ring was fitted to the US transducer and the gel was added to this ring, which was then covered with a

PVC film so that the gel would not dissolve in the scale's water, and fixed with elastic bands. The transducer was immersed 1 cm below the water surface, directly above the conical metal target. The US waves release energy on this target, which is triggered by the scale. The US was regulated to supply frequency of 3 MHz and 0.8 W power (0,2W/cm², ERA 4 cm²). This assay was carried out using gel and caffeine gel, and its transmission percentage was verified in relation to water, considering the variation of 10% in the US apparatus and 0.07% in the balance. The procedure was repeated five times for each kind of gel.

5. Permeation Analysis

For the study of chemical substances absorption through the skin, the *in vitro* technique proposed by BENTLEY (19) was used. The diffusion cells are composed of one plate and a PVC ring where the swine skin (8 cm²) was fixed. The cell was then placed on a plastic cube containing 50 ml of a receptor solution in constant agitation, at 37ºC certifying that the solution was in contact with the dermis.

The receptor solution composition was the same of the solution used for caffeine solubilization, since it did not show absorbance in the wavelength used in the experiment (data not shown).

After setting up the skin in the diffusion cells, 3 g of the appropriated gel was applied on the epidermis as upper described. The diffusion cells were maintained with PVC film after the treatments.

From all of the samples, 2 ml of the receptor solution were collected and replaced at 0, 2, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after gel administration and/or US application. The caffeine concentrations of the samples were determined by spectrophotometry as described below.

5.1. Determination of caffeine

A UV – Visible spectrophotometer (1601PC, Shimadzu, Sidney, Australia) was used, adjusted at 273 nm wavelength. Before the sample readings, the spectrophotometer was set at zero with receptor solution.

In order to eliminate the interference of other skin substances, which could have been diffused in the receptor solution data are expressed as, the difference between the caffeine concentration in the receptor solution of the skin treated with gel plus caffeine plus US and that treated with gel plus US, and the difference between skin treated with gel plus caffeine and gel.

6. Statistical Analysis

The results are presented as mean $\pm$ standard error mean (SEM) of the values obtained in mg/ml of caffeine present in the receptor solution at the different times. To analyze the transmissibility of US through the gels, Student's *t* test was used (27, 28).

The area under the curve (AUC) was determined by the trapezoidal method (29) and used to compare data obtained by application of caffeine and with that obtained by association of US by using Mann-Whitney test because the data do not obey the Gauss curve.

To compare the permeation of caffeine with and without US in the different time periods, the on parametric test of Kruskall-Wallis was used, followed by Dunn's test of multiple comparison (27, 28).

Results

The presence of gel (carbopol 940®) or caffeine gel (5%) did not produce any attenuation of the US intensity evaluated by the transmissibility test (figure 1).

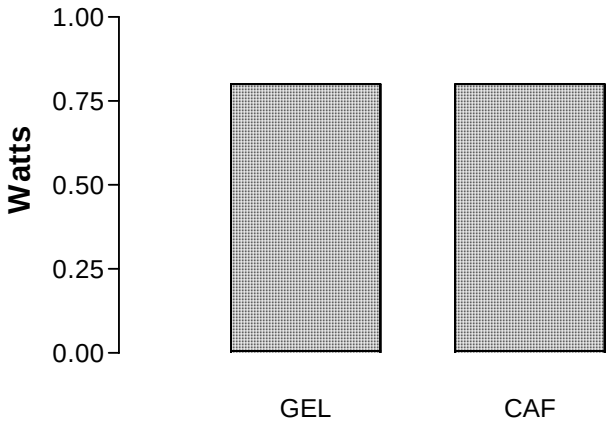

Figure 1. Transmissibility of ultrasound in the frequency of 3 MHz, intensity 0,2W/cm², continuous emission mode through gel (GEL) or through gel containing 5% caffeine (CAF).

The application of 3 g of caffeine (5%) gel upon the skin fragment fitted in the diffusion cell led to a caffeine concentration of 767.70 ± 55.85 μg/ml (AUC) of in the receptor solution in 240 minutes. The use of US added to caffeine (5%) gel significantly potentiated the caffeine permeation through the skin, significantly increasing its concentration in the receptor solution during a 240 min period (1925.4 ± 110.35 μg/ml, $p<0.01$).

Figure 2 shows that caffeine permeation gradually increased, reaching its peak 60 minutes after the beginning of the application (4.4 ± 0.4 μg/ml; $p<0.05$), in the skin where US was not used. US application induced an increase on caffeine permeation (9.5 ± 0.6 μg/ml, $p<0.001$) and accelerated it since the maximal concentration was reached at 30 minutes, remaining unchanged until the end of the experiment at 60th min (7.3 ± 0.5 μg/ml), 90th min (8.6 ± 0.6 μg/ml), 120th min (8.4 ± 0.5 μg/ml), 150th min (8.7 ± 0.7 μg/ml), 180th min (7.9 ± 0.5 μg/ml), 210th min (8.7 ± 0.5 μg/ml), and at the 240th min (8.7 ± 0.6 μg/ml $p<0.01$).

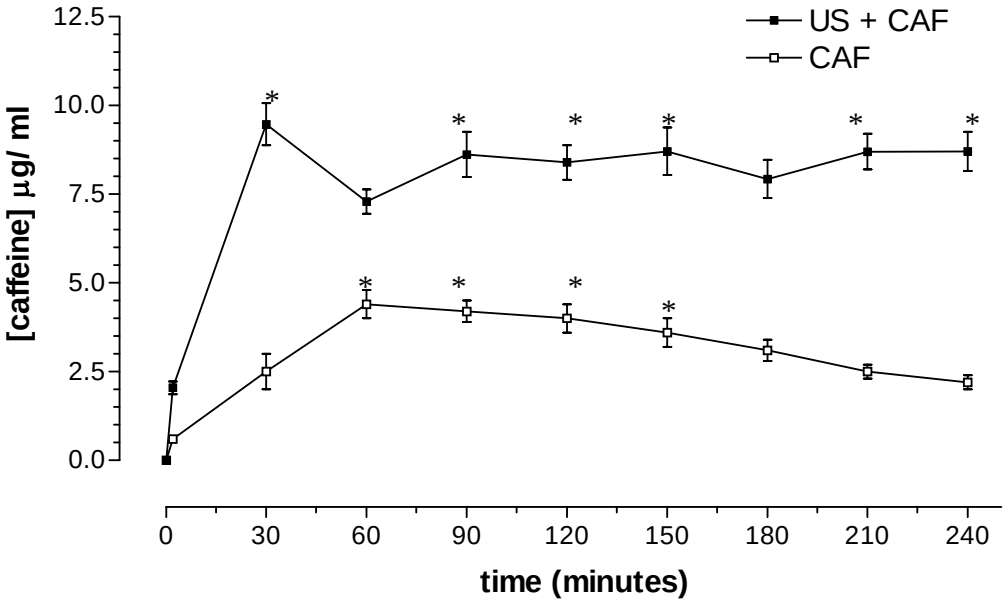

Figure 2. Caffeine concentration (mg/ml) in the receptor solution at different times, with and without the use of US. The gel containing 5% caffeine (US+CAF) was applied on the skin at time zero. US of 3 MHz was applied soon after the administration of the gel, in a 0,2W/cm² intensity, continuous emission mode for 2 minutes (1min/cm²). The areas under the curves for CAF and US + CAF were statistically different (Mann-Whitney test; $p < 0.01$). *$p<0.05$ compared with the time 0 (Kruskall-Wallis followed by Dunn's test).

Discussion

The results presented here showed that ultrasound increased significantly the permeation of caffeine in the fragments of swine skin. The concentration of caffeine in the receptor solution immediately after application of US and in later analyzed times was considerably greater than in the skin treated just with gel containing caffeine. Also there was permeation acceleration inasmuch as the peak concentration was reached 30 minutes after application of caffeine associated with US whereas without this physical resource the caffeine maximum concentration was reached in 60 min. This result confirms the effectiveness of US as accentuator of the cutaneous drugs permeation and thwarts results of MITRAGOTRI et al (30) that there was no observed increase in the caffeine diffusion through human skin with high frequency application of US.

The swine skin is used as a model for experiments of drug permeation because the thickness of the corneous layer is similar to the human skin (26.4µm in swines and 16.8µm in human). This layer is the main barrier for permeation (6, 31).

The increase of drug diffusion through the skin occurs due to US thermal and mechanical effects (2, 8). The disorder of the cells lipidic bilayer (12), the transformation of this to a fluid phase (32) and the oscillatory movement provoked by the US longitudinal waves (33) are the main mechanisms responsible for the increased diffusion.

Although mechanical and thermal factors exist capable of allowing phonophoresis, in our study it was not observed that the latter factor accentuates drug diffusion, since the temperature remained constant at 37°C during every experiment.

Our results show that cutaneous permeation of caffeine increased gradually, reaching its peak in 60 min and decreasing from then until 240 min after application of just caffeine.

In addition, the level of absorption of the drug continued increasing considerably even after the end of the application of US, reaching its peak in 30 min and persisting high in every other analyzed time (figure 2). Although BOUCAUD *et al*.(34) demonstrated the opinion that the increase in the permeability of the skin for drugs with the use of US does not persist after termination of the US application, our results do corroborate KOST *et al*.(35) that demonstrated constant penetration of drugs after US was turned off. On the other hand TANG *et al*.(4) relate that the waves of US induce convection and increase the coefficient of diffusion of the skin by altering its structure, and that this alteration persists after the end of the treatment.

Although BOUCAUD *et al*.(16) observed a discreet increase in the diffusion of caffeine when applied with US of high frequency and significantly increased permeability after US of low frequency (17), we verified that US of high frequency significantly the speeded the diffusion of caffeine in the skin. These data agree with those reported by BYL (2) where the effectiveness of US is verified as accentuator of substances permeation in 75% of the phonophoresis related reports.

According with BYL (2) and MITRAGOTRI *et al* (12), some pharmaceutical preparation could prevent the transmission of the ultrasonic wave, thus decreasing the phonophoresis effectiveness. Such effect was not observed in this research (figure 1). In previous studies of our laboratory about the transmissibility of the ultrasonic wave through some pharmaceutical preparations, it was verified that hydrosoluble drugs in several concentrations did not impede the transmission since the preparation were homogeneous and with formation of bubbles.

Caffeine is a hydrophilic drug and, therefore, of low permeability. The application of US has a great effect in the permeation of drugs with these physicochemical characteristics (16).

It should also be considered that the permeation of caffeine by the skin of swine was probably possible due to the use of etanol in the gel formulation used. Ethanol is a chemical permeation accentuator that, according to LEVANG *et al* (36), increases the drug absorption by the skin while disturbing the integrity of the corneous stratum, causing destruction of its lipidic layer. The combination of chemical and physical permeants is commonly used, due to the synergism of their actions, to increase the concentration and the depth of penetration of the drug in the skin (3). Our results clearly showed this synergism, since the use of US produced an increase in the permeation of 150%, when we analyzed the area under the curve of both treatments. Although MONTI *et al*.(17) compared the effect of phonophoresis with some chemical permeants, realizing pretreatment of mice skin with the same permeants, they observed just a slight superiority of the combination of acid oleic and propyleneglycol on US of low frequency (20 KHz).

Although experiments *in vitro* are considered valuable for the study of the mechanisms of percutaneous absorption and they have shown similar correlations (37), caution should be observed when extrapolating the results for *in vivo* situations.

We concluded that US significantly accentuates and accelerates the permeation of caffeine through the skin, thus allowing the accomplishment of the phonophoresis with this drug in the lipodystrophy treatment.

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