UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DO BIOLOGIA

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INFLUÊNCIA DO ULTRA-SOM NA PERMEAÇÃO CUTÂNEA DA CAFEÍNA: ESTUDO EM FRAGMENTOS DE PELE E EM ADIPÓCITOS ISOLADOS DE SUÍNOS

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, para a obtenção do Título de Doutor em Biologia Funcional e Molecular, área de Fisiologia.

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Campinas

2004

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"Para entender tem que se achar

Que a vida não é só isso que se vê.

É um pouco mais que os olhos não conseguem perceber,

Que as mãos não ousam tocar

e os pés recusam pisar"

(Paulinho da Viola)

DEDICATÓRIA

A meu marido, **Marcos**

Amigo e Companheiro, que sempre me apoiou, com amor, nos meus sonhos

A meus filhos, **Marcela** e **Eduardo**

Por tantas e incomparáveis alegrias e pela compreensão dos momentos ausentes

A meus **Pais**

Mestres da vida, iluminaram meu caminho para chegar até aqui.

AGRADECIMENTO ESPECIAL

Às Professoras e Amigas

Profª Drª. Dora Maria Grassi-Kassisse
Profª Drª Regina Célia Sparadari-Bratfisch

"A mortalidade de que se reveste a natureza humana faz o homem sempre presente: presente pelo conhecimento que transmitiu, pela amizade que conquistou e pelo exemplo que legou"

AGRADECIMENTOS

- A todos os amigos que conheci no LABEEST – Laboratório de Estudo do Estresse – UNICAMP, que sempre se apresentaram solícitos.
- Aos ex-alunos Carlos Eduardo Vieira e Gabriel Negretti Guirado, pela valiosa colaboração no cuidado dos animais
- À Maria Cristina Almeida Prado Ribeiro, técnica do laboratório da Universidade Metodista de Piracicaba – UNIMEP, que sempre esteve à frente de todas as nossas necessidades.
- À Profª Drª Maria Imaculada Montebello, pela valiosa colaboração estatística
- Ao LABEX – Laboratório de Bioquímica do Exercício
- Ao FAP – Fundo de Apoio à Pesquisa – UNIMEP
- Ao FAEP – Fundo de Apoio ao Ensino e à Pesquisa
- KW – Indústria Nacional de Tecnologia Ltda.
- À Fisioline Indústria de Cosméticos Ltda.

SUMÁRIO

Resumo

O ultra-som tem sido usado extensivamente nas últimas décadas para terapias físicas e como promotor de penetração cutânea de fármacos. O uso do ultra-som como facilitador de absorção cutânea é conhecido por fonoforese ou sonoforese. Os objetivos do presente trabalho foram analisar o efeito do ultra-som sobre a permeação cutânea da cafeína aplicada localmente, sobre a pele, e analisar a resposta lipolítica de adipócitos isolados de suínos após este tratamento. A cafeína inibe a fosfodiesterase, que degrada o AMPc, este efeito resulta em uma potencialização do efeito de agonistas lipolíticos. Tem sido relatado também que a cafeína tem um papel em inibir o receptor de adenosina, inibindo então seu efeito anti-lipolítico, como conseqüência, o uso da cafeína leva a facilitação da lipólise. Para este estudo, foram utilizados suínos machos Landrace x Large White com 35 dias e peso aproximado de 15 kg. Após tricotomia da região dorsal, os animais foram submetidos aos seguintes tratamentos durante quinze dias: Gel; cafeína (5%), CAF; ultra-som, US; ultra-som + cafeína (5%), US + CAF. Uma 5ª área foi submetida a infiltração intradérmica (mesoterapia) de cafeína (2%). Uma 6ª área serviu como controle e não foi submetida a tratamento. Ao final dos diferentes tratamentos os animais foram sacrificados e fragmentos de pele das diferentes áreas foram retirados para análise histológica e 15 g de tecido adiposo foi utilizado para isolamento de adipócitos. Nos adipócitos isolados foram analisadas a produção de glicerol frente ao estímulo do agonista β-adrenérgico não seletivo, a isoprenalina. A mesoterapia mostrou-se eficaz na redução da espessura da hipoderme. O ultra-som mostrou-se importante acentuador da permeação cutânea à cafeína, evidenciado tanto pelo estudo histológico, onde foram observadas significativas alterações morfológicas do tecido adiposo bem como pelo aumento da resposta lipolítica máxima, em adipócitos isolados, sem, contudo alterar a sensibilidade dos receptores β adrenérgicos. Também realizamos análise de permeação cutânea

da cafeína *in vitro* através de células de difusão vertical utilizando pele de suínos machos Landrace x Large White com 50 dias e os resultados obtidos também demonstraram uma acentuada permeação da cafeína quando associada ao ultra-som terapêutico.

Abstract

The ultrasound has been extensively used in the last decades for physical therapies and as promoter of cutaneous permeation of drugs. The use of ultrasound as facilitator of cutaneous absorption is knows for phonophoresis or sonophoresis. The aim of present research were to analyze the effect of ultrasound on the cutaneous permeation of caffeine applied locally on the skin, and to analyze the lipolytic response of isolated adipocyte of swine after this treatment. The caffeine inhibits the cAMP-phosphodiesterase which hydrolyzes cAMP, this effect result in an increase of the effect of lipolytic agonists. However, has been also mentioned that the caffeine is able to inhibit the adenosine receptors, inhibiting thus effect antilipolytic, as consequence the use of caffeine leads of the facilitation of the lipolysis. For this study, male swine were used Landrace x Large White with 35 days and approximate weight of 15 kg. After trichotomy of the dorsal area the animals were submitted to the following treatments, for fifteen days: gel; caffeine (5%); ultrasound; ultrasound + caffeine (5%). A 5^{th} area was submitted the intradermic infiltration (mesotherapy) of caffeine (2%). A 6^{th} area served as control and it was not submitted to treatment. At the end of the different treatments the animals were sacrificed and fragments of skin of the different areas were used for histological analysis and 15 g of adipose tissue for adipocytes isolation. The lipolytic activity was analyzed in the isolated adipocytes. The adipocytes were incubated with agonist β-adrenergic (isoprenaline) and the glycerol production were measured. The mesotherapy was shown effective in the reduction of the thickness of the hypodermis. The ultrasound was shown important accentuator of the cutaneous permeation to the caffeine, evidenced so much by the histological study, where it was observed significant morphologic alterations of the adipose tissue as well as for the increase of the maximal lipolytic response, in adipocytes isolated, without, however to alter the sensibility of the β adrenergic

receptors. Also accomplished analysis of cutaneous permeation of the caffeine *in vitro* through cells of vertical diffusion using skin of male swine Landrace x Large White with 50 days old and the results obtained also demonstrated an accentuated permeation of the caffeine when associated to the therapeutic ultrasound.

Lista de Abreviações

4-AAP	4-amonoantipirina
AMPc	Adenosina monofosfato cíclica
5'AMP	5' adenosina monofosfato
ANOVA	Análise de variância
ATP	Adenosina trifosfato
A_1R ou A_1	Receptor de adenosina do subtipo 1
$A_{2a}R$	Receptor de adenosina do subtipo 2 a
$A_{2b}R$	Receptor de adenosina do subtipo 2b
A_3R	Receptor de adenosina do subtipo 3
AUC	Área sob a curva
βAR	Beta-adrenoceptor
β_1- AR	Adrenoceptor do subtipo beta 1
β_2- AR	Adrenoceptor do subtipo beta 2
β_3- AR	Adrenoceptor do subtipo beta 3
CAF	Cafeína
CEEA	Comitê de Ética em Experimentação Animal
EC_{50}	Concentração do agonista que induz a 50% da resposta lípolítica máxima
ERA	Área efetiva de radiação
ESPA	socium N-ethyl-N-(3-sulfopropyl) m-anisidene
FEG	Fibro-edema-gelóide
Gi	Proteína G inibitória

GLD	Lipodistrofia ginóide
Gs	Proteína G estimulatória
HE	Hematoxilina eosina
HEPES	Ácido (N-[2-hidroxietil] piperazina-N'-[2-etanosulfonico])
LHS	Lipase-hormônio-sensível
KRBA	Krebs Ringer Bicarbonato adicionado de albumina
MHz	Mega Hertz
pD_2	Logarítmo negativo da concentração do agonista que induz 50% da resposta lipolítica máxima
PEFE	Paniculopatia edêmato-fibro-esclerótica
PKA	Proteína Kinase A
US	Ultra-som
W	Watts

Lista de Materiais

Substância	**Procedência**
Álcool etílico	Allkimia Produtos para Laboratório (São Paulo, BR)
Bálsamo do Canadá	Synth – Labsynth produtos para Laboratório (Diadema, BR)
Bicarbonato de sódio	Reagen (Quimibrás Indústrias Químicas S.A.,BR)
Cafeína Anidra	Sigma Chemical Company (St. Louis, MO, USA)
Carbopol 940®	Noveon Inc. (Bruxelas, Bélgica)
Cloreto de Cálcio	Reagen (Quimibrás Indústrias Químicas S.A., BR)
Cloreto de Potássio	Reagen (Quimibrás Indústrias Químicas S.A.,BR)
Cloreto de Sódio	Allkimia Produtos para Laboratório (São Paulo, BR)
Colagenase tipo II	Sigma Chemical Company (St. Louis, MO, USA)
Eosina	Merck SA (Rio de Janeiro, BR)
Fosfato	MERSE (São Paulo, BR)
Fosfato biácido de potássio	Reagen (Quimibrás Indústrias Químicas S.A., BR)
Glicose	Reagen (Quimibrás Indústrias Químicas S.A.,BR)
Hematoxilina	Merck S.A. (Rio de Janeiro, BR).
HEPES	Sigma Chemical Company (St. Louis, MO, USA)
(-/+)-Isoproterenol	Sigma Chemical Company (St. Louis, MO, USA)
Propilenoglicol	Ind. Química Anastácio AS (São Paulo, BR)
Soroalbumina bovina (Fração V)	Sigma Chemical Company (St. Louis, MO, USA)
Sulfato de magnésio	Reagen (Quimibrás Indústrias Químicas S.A. BR)
Acetato de Sódio Triidratado	Synth – Labsynth produtos para Laboratório (Diadema, BR)
Trietanolamina	Vital Especialidade Cosmética (São Paulo, BR)

Álcool etílico extra neutro	Álcool Moreno Lt. (Guarulhos, BR)
Xilol	Alquimia Produtos para Laboratório (São Paulo, BR)

INTRODUÇÃO

Durante as últimas décadas, muitos trabalhos têm sido realizados no sentido de esclarecer a permeabilidade cutânea a diferentes substâncias ativas. Esse tem sido um assunto de profundo interesse para os profissionais das ciências farmacêuticas e cosméticas, bem como da fisioterapia e da dermatologia.

A transmissão transdérmica de drogas oferece uma alternativa para as vias de administração oral e injetável. Entretanto, esta aplicação tem sido limitada a poucas drogas porque a pele é pouco permeável.

A propriedade de barreira da pele é atribuída à camada córnea que é formada por corneócitos, cuja bicamada lipídica aumenta a resistência a transportes de íons (KOST *et al*., 1989; MORIMOTO *et al*., 1994; BYL, 1995; MITRAGOTRI *et al*., 1996). Assim, o fluxo através da pele depende da natureza química da substância: fármacos lipofílicos são absorvidos em toda a área lipídica do estrato córneo, com coeficientes de permeação variáveis; enquanto que a absorção de fármacos hidrofílicos se dá quase que exclusivamente por poros de passagem sendo que o coeficiente de permeação é quase constante (MORIMOTO *et al*., 1994).

Devido à dificuldade na penetração de drogas pela pele, agentes químicos e físicos vêm sendo pesquisados para que as barreiras da pele sejam diminuídas e, assim, se acentue a penetração cutânea (KOST *et al*., 1989).

Uma variedade de pesquisas tem sugerido que, para aumentar o sistema de transporte de drogas pela pele, pode-se utilizar, entre outros recursos, as correntes elétricas e a aplicação de ultra-som – US – (MITRAGOTRI *et al*., 1995), porque a ação de uma força física externa melhora a permeabilidade da pele (UEDA *et al*, 1996).

O uso do US para favorecer a penetração de substâncias tópicas é denominado fonoforese ou sonoforese.

Este conceito novo de US terapêutico combinado com fármacos despertou interesse de estudos em várias áreas (TACHIBANA E TACHIBANA, 1998).

A primeira publicação sobre fonoforese data de 1954 quando se demonstrou o fluxo de hidrocortisona, através de membrana avascular. Outros estudos comprovaram a eficácia do US no aumento da absorção de lidocaína (4%), lidocaína associado ao decadron, D-manitol, dimetil sulfóxido, ácido salicílico e metil nicotinato, utilizando-se, para tanto, experimentos com coelhos, suínos e humanos (BYL, 1995).

Estudos mais recentes mostram a ação do US em aumentar a absorção de uroquinase (TACHIBANA E TACHIBANA, 1998), ácido flufenâmico (HIPPIUS *et al*, 1998), e da mesma ação com o US de baixa frequência da antipirina e dinitrato de isosorbida (UEDA *et al*., 1996), da aldosterona, butanol, corticosteróide, estradiol, ácido salicílico e sacarose (MITRAGOTRI *et al*., 1996). MONTI *et al*. (2001) e BOUCAUD *et al*. (2001) demonstraram o aumento da permeação da cafeína com US de baixa freqüência (40 e 20 KHz).

A revisão bibliográfica publicada por BYL, em 1995, afirma que em 75% dos estudos sobre fonoforese ocorre efetividade do US como acentuador da permeação de substâncias.

Um estudo de HIPPIUS (1998) verificou a eficácia da fonoforese através de técnica *in vitro,* utilizando a fluorimetria como método quantitativo. O US demonstrou acentuar o transporte transdérmico de ácido flufenâmico. Todas as intensidades usadas (0,2 à 1,5 W/cm^2) tiveram praticamente o mesmo efeito sobre a permeação. KOST *et al*. (1989),

através de uma revisão literária sobre a fonoforese, também confirmam a efetividade desta técnica.

Trabalhos de HIPPIUS *et al*. (1998) e MITRAGOTRI *et al*. (2000) afirmam que o nível de permeação promovido pela fonoforese, é diretamente proporcional ao tempo de aplicação do US.

Embora muitos estudos tenham demonstrado que o US é geralmente seguro, sem efeitos negativos a longo e curto prazo, o mecanismo pelo qual funciona como promotor da penetração ainda não está muito claro (BYL, 1995; UEDA *et al*., 1996).

Tanto o efeito térmico, como o mecânico e as alterações químicas dos tecidos biológicos, podem facilitar a difusão dos princípios ativos presentes nos medicamentos de uso tópico (HIPPIUS *et al*., 1998; BYL, 1995).

MITRAGOTRI *et al*. (1995, 1996) são da opinião que a cavitação é o principal mecanismo para a fonoforese, pois induz uma desordem na bicamada lipídica da camada córnea, aumentando o transporte através da mesma. JOHNSON *et al*. (1996) sugerem que a bicamada lipídica do estrato córneo transforma-se numa fase fluida, facilitando assim a absorção de fármacos.

Outro aspecto analisado por MITRAGOTRI *et al*. (1996) é que a bicamada lipídica produz uma alta resistência a transportes iônicos, e o US desorganizando o estrato córneo pode reduzir em 30% esta resistência.

Além da ação mencionada, o efeito mecânico difunde o princípio ativo do medicamento pela oscilação das células a uma alta velocidade, diminuindo o potencial da membrana celular, levando à quebra de ligações intercelulares e aumentando a permeabilidade da mesma (BYL, 1995; BARE *et al*., 1996).

HIPPIUS *et al*. (1998) são da opinião que o mecanismo ligado à fonoforese é o térmico. O aquecimento produzido pelo US aumenta a energia cinética das moléculas dos medicamentos e da membrana celular, dilatando os pontos de entrada como os óstios dos folículos pilossebáceos e os orifícios adenaxiais das glândulas sudoríparas, além de aumentar a circulação local, aumenta a oportunidade das moléculas difundirem-se através do estrato córneo até a rede capilar da derme (BYL, 1995).

Mudanças químicas ocorridas durante a fonoforese incluem a indução de um número aumentado de reações de oxidação, inativação de enzimas, e a cavitação. Aumento da atividade da adenosina trifosfatase e da permeabilidade da membrana celular são possíveis mecanismos (KASSAN *et al*., 1996).

O estudo de MITRAGOTRI *et al*., (1997) sobre a variação do aumento do transporte transdérmico de vários fármacos, mostra que em algumas pesquisas a fonoforese não ocorreu, o que leva à uma controvérsia sobre a eficácia de tal conduta.

Vários trabalhos tentam explicar o papel do ultra-som na permeação cutânea e a maioria deles aponta para o efeito acelerador, diminuindo a lentidão do processo, mais do que aumentar a taxa de absorção (MORIMOTO *et al*., 1994, HIPPIUS *et al*, 1998).

Numa revisão de literatura sobre a efetividade da fonoforese, BYL (1995) relata que alguns destes estudos apresentam erros metodológicos os quais limitam a sua generalização dos mesmos. Entretanto, mais recentemente estudos realizados em humanos utilizando o US para a permeação de antiinflamatório demonstraram que o medicamento foi permeado e encontrado em maior concentração no líquido sinovial do que em amostras de plasma ou tecido adiposo da região estudada, joelho (CAGNIE *et al.*, 2003).

O US é utilizado em tratamentos estéticos, mais especificamente, em casos de fibro-edema gelóide – FEG – popularmente conhecido como celulite (PIRES DE CAMPOS, 1992; ROSSI, 2000).

Diversas hipóteses sugerem a base fisiopatológica do FEG, dentre elas: o fenômeno da hiperpolimerização da substância fundamental, alterações primárias do tecido adiposo e alterações microcirculatórias com etiologia multi-fatorial (DRAELOS e MARENUS, 1997; ROSSI, 2000).

Uma das hipóteses é das alterações microcirculatórias. Estas levariam a diversas mudanças no metabolismo do tecido adiposo e conjuntivo (SEGERS *et al*., 1985; LEIBASCHOFF, 1987; RYAN e CURRI, 1989; PIRES DE CAMPOS, 1992; RYAN, 1995).

De acordo com PIRES DE CAMPOS (1992), no aspecto anátomo-histológico, o tecido com fibro-edema-gelóide encontra-se com aumento do número e do volume de células adiposas, lipoedema e dissociação lobular, espessamento e proliferação das fibras colágenas interadipocitárias e interlobulares, que provocam engurgitamento tecidual, rompimento das fibras elásticas, vasos linfáticos e sanguíneos ectásicos. Conseqüentemente, o tecido é mal oxigenado, desorganizado e sem elasticidade, resultante do mau funcionamento circulatório e das consecutivas transformações do tecido conjuntivo. A provável causa das alterações microcirculatórias seria uma insuficiência dos esfíncteres pré-capilares, cuja função reguladora do fluxo sangüíneo encontra-se modificada nas áreas afetadas (CURRI, 1993, CURRI e BOMBARDELLI,1994). A alteração inicial que leva à formação da celulite parece ser a deterioração da substância intersticial e rede capilar, levando à retenção excessiva de líquidos na derme e em tecidos subcutâneos. Segundo LOTTI *et al*. (1990) e DRAELOS e MARENUS (1997), há aumento de

glicosaminoglicanas na derme, levando à retenção de água nas regiões afetadas. Entretanto, estas observações têm sido objeto de grandes debates e controvérsias (RYAN, 1995).

Estudos mais atuais apontam alterações do tecido adiposo como fatores primários na fisiopatologia do FEG.

O tecido adiposo é um tipo especializado de tecido conjuntivo, sendo um grande reservatório de gordura sob a forma de triacilgliceróis. O tecido adiposo subcutâneo é importante isolante térmico e amortecedor de choques mecânicos (GARCIA *et al*., 2002). Há evidências que secrete fatores importantes na resposta imunitária, nas doenças vasculares e na regulação de apetite, funcionando também como importante órgão endócrino (BLOOM e FAWCETT, 1994; GREGORIE *et al*., 1998).

Na anatomia topográfica do tecido adiposo subcutâneo, distinguem-se duas camadas separadas por uma fáscia superficial. A camada mais externa (em contato com a derme), chamada areolar, é composta por adipócitos globulares e volumosos, em disposição verticais, onde os vasos sangüíneos são numerosos e delicados. Na camada mais profunda, camada lamelar, as células são fusiformes, menores e dispostas horizontalmente, onde os vasos são de maior calibre.

Nos mamíferos existem dois tipos de tecido adiposo: o tecido adiposo amarelo, branco ou unilocular e o tecido adiposo marrom ou pardo ou multilocular.

Praticamente todo o tecido adiposo encontrado no homem adulto é do tipo amarelo, apresentando grande capacidade de hipertrofia (GARCIA *et al.*, 2002). Aproximadamente 60 a 80% do peso deste tipo de tecido adiposo é constituído de lipídio, sendo 90 a 99% de triacilgliceróis (GREENWOOD e JOHNSON, 1993).

Este tecido forma o panículo adiposo subcutâneo do corpo humano, camada de espessura totalmente uniforme no recém-nascido. Com a idade o panículo adiposo tende a

desaparecer de certas áreas, desenvolvendo-se em outras. Esta disposição seletiva de gorduras é em parte controlada pelos hormônios sexuais e adrenocorticais, que se evidencia com a idade (GARCIA *et al*., 2002).

Neste tipo de tecido, cada adipócito mantém contato com pelo menos um capilar. O fluxo sanguíneo varia de acordo com o peso corporal e estado nutricional, aumentando no jejum. É inervado pelo sistema nervoso simpático de maneira indireta, ou seja, os vasos sangüíneos das células adiposas é que recebem as terminações nervosas, embora haja evidências de inervação direta dos adipócitos brancos (PÉNICAUD *et al*., 2000)

O tecido adiposo pardo ou marrom é encontrado em mamíferos recém nascidos de praticamente todas as espécies, sendo que em animais não hibernantes, diminui com o crescimento. Em humanos adultos, este tipo de tecido é praticamente ausente. Em recém nascidos são encontrados depósitos nas regiões cervical posterior, axilar, supra-ilíaca, peri-renal, áreas interescapulares, abdominal anterior e retropubiana, sendo substituídas por tecido adiposo branco com o crescimento (HEALTON, 1972; MERKLIN, 1973). Apresenta rica vascularização, estando mais intimamente ligada aos adipócitos. Assim como o tecido adiposo unilocular ou amarelo, é inervado pelo sistema nervoso simpático. Entretanto, neste caso, as células recebem inervação direta (GARCIA *et al*., 2002).

A principal função do tecido adiposo marrom é a termogênese, enquanto que o tecido branco apresenta um papel mais complexo e dinâmico, pois é responsável por armazenamento e balanço energético, secreção de fatores na resposta imunitária, como adipsina, Acrp30/AdipoQ (adipocyte complement-related protein), fator de necrose tumoral α (TNF-α) e fator inibidor de migração de macrófagos (MIF); em doenças vasculares pela

secreção de angiotensionogênio e PAI-I (plasminogen activator/inibitor-I), e regulação do apetite, através da leptina (FRÜHBECK *et al*., 2001).

Dentre as funções metabólicas do tecido adiposo encontramos a mobilização dos lipídeos armazenados no tecido adiposo que é iniciada pela ação de agentes lipolíticos secretados sempre que ocorre demanda por substratos energéticos, predominando sobre os processos de lipogênese (GARCIA *et al*., 2002). A regulação da lipólise depende do balanço entre mecanismos hormonais e simpáticos. É inibida predominantemente pela insulina e adenosina enquanto é promovida pelo ACTH (hormônio adrenocorticotrópico), adrenalina e noradrenalina (LAFONTAN *et al*., 1997; MORIMOTO *et al*., 1998; COMMERFORD *et al*., 2000; DODT *et al*., 2003).

A mediação das ações fisiológicas das catecolaminas, adrenalina e noradrenalina, é realizada pelos adrenoceptores. Podemos encontrar pelo menos cinco subtipos – β_1, β_2, β_3, α_{1B} e α_2 - todos coexistindo no mesmo adipócito (LAFONTAN *et al*., 1997; McNEEL e MERSSMANN, 1999; DING *et al*., 2000). A concentração destes adrenoceptores é variável de acordo com a espécie animal, como por exemplo, ratos possuem em torno de 90% de adrenoceptores β_3 enquanto que suínos possuem 70 a 80% de adrenoceptores β_1, 20% de adrenoceptores β_2 e menos que 10% de adrenoceptores β_3 (DING *et al*., 2000). A similaridade na resposta a agonistas e antagonistas beta-adrenérgicos em humanos, sugere que a proporção de β_1, β_2 e β_3 seja próxima aos suínos (McNEEL e MERSMANN, 1999).

Apesar da natureza glicoprotéica comum e de atuarem através do mesmo sistema de segundo mensageiro, que gera o monofosfato de adenosina cíclico (AMPc), os subtipos de β-adrenoceptores (βAR) apresentam especificidades estruturais e funcionais (BOWEN *et al*., 1992) relacionadas com o tipo de tecido onde aparecem e com o estado funcional do

organismo (TAOUIS *et al*., 1987, 1989; MAURIÉGE *et al*., 1987; ARNER, 1990 a e b, 1992, 1995).

A ativação dos βARs pelas catecolaminas, promove acoplamento destes receptores à proteínas Gs (estimulatória) que levam a um incremento da concentração intracelular do AMPc que ativa a proteína quinase A (PKA). Esta última produzirá fosforilação da lipase-hormônio-senível (HSL) e das perilipinas (HOLM *et al*., 2000; JOHNSON *et al*., 2000).

As perilipinas são proteínas associadas à superfície limitante das gotículas citosólicas de lipídeos que criam uma barreira limitante à ação da HSL. Sua fosforilação, pela PKA, libera este impedimento permitindo a translocação da HSL fosforilada do citosol para a gotícula lipídica, iniciando o processo de hidrólise dos triacilgliceróis em ácidos graxos (AG) e glicerol (CLIFFORD *et al*., 2000). Para impedir que o próprio aumento de AG iniba a HSL no "locus", a HSL associa-se com ALBP – "proteína ligante de adipócitos". Sua desfosforilação é induzida pela insulina que freia a lipólise (LIMA *et al*., 2002), através da fosfodiesterase (LENINGHER, 2000). Outro mecanismo de ação antilipolítico tem sido relacionado a estimulação dos α_2AR e dos receptores de adenosina que se ligam a proteína Gi (inibitória) diminuindo assim a concentração de AMPc.

Deixando as células adiposas, o glicerol e os ácidos graxos livres circulam pelo plasma ligados à albumina (LIMA *et al*., 2002).

Fatores fisiológicos como, alimentação, exercício físico e idade; bem como patológicos, entre eles, a obesidade, o diabetes e as dislipidemias; podem interferir na lipólise. Além disso, a heterogeneidade nos depósitos de gordura e as diferenças ligadas ao sexo e diversidade de hormônios (GH, cortisol, glucagon, além dos já citados) promovem uma complexidade na regulação da lipólise.

Se a celulite fosse unicamente obtida pelo volume do tecido adiposo, poder-se-ia dizer que homens e mulheres com quantidades iguais de tecido adiposo, demonstrariam celulite na mesma proporção e sua presença não se justificaria em indivíduos magros. No entanto, sua prevalência indica que a celulite está ligada à diferenças na organização do tecido conjuntivo. QUERLEUX *et al*. (2002) notaram grande porcentagem de septos perpendiculares à derme em mulheres com celulite.

A fáscia superficial do tecido adiposo dá formato às vigas ou septos que bloqueiam as zonas edemaciadas e dá o aspecto a zonas elevadas e zonas de covinhas. Além disso, as regiões de espessamento da fáscia superficial provocam a formação de aderências íntimas entre pele e tecidos profundos. Tais zonas são diferentes em ambos os sexos e são responsáveis pelo contorno corporal específico de cada um dos sexos. Nos homens, a fáscia superficial se espessa e engrossa na região da crista ilíaca. Isso provoca afundamento localizado no contorno corporal e o acúmulo de tecido adiposo. Nas mulheres, a fáscia superficial sofre as mesmas mudanças, mas vários centímetros abaixo da crista ilíaca, na altura do sub-glúteo (MORETTI,1997).

Para PIÉRARD *et al*. (2000), ROSEMBAUM *et al*. (1998) e LUCASSEN *et al*. (1997) a fáscia dérmo-hipodérmica, bem como a disposição anatômica dos septos interlobulares do tecido adiposo, são os principais responsáveis pela formação de herniações da hipoderme para a derme reticular, toda vez que o tecido subcutâneo sofrer compressão, dando o aspecto da “casca de laranja” na pele.

Tanto a variabilidade na estrutura do tecido conjuntivo, como a susceptibilidade ao aparecimento da celulite estaria, principalmente, relacionados aos hormônios sexuais e não à genética do indivíduo, pois existem evidências de estruturas de fibras subcutâneas femininas na fáscia superficial de homens hipoandrogênicos (ROSEMBAUM *et al.,* 1998).

No estudo de ROSEMBAUM *et al.* (1998) não se observou diferença no metabolismo do tecido adiposo de regiões afetadas e não afetadas por celulite, nem diferenças entre os sexos. Também não encontraram alterações no fluxo circulatório. PIÉRARD *et al.* (2000) também não observaram alterações nos capilares linfáticos, contrapondo-se com trabalhos de CURRI (1993) e CURRI e BOMBARDELI (1994) que propõe alterações linfáticas e venulares como fator etiológico para o déficit microcirculatório.

O suíno tem sido usado como modelo animal em experimentos de permeação cutânea, porque sua camada córnea apresenta semelhanças com a do homem. Segundo dados de BRONAUGH *et al*. (1989), a espessura da camada córnea dos suínos é de 26,4μm e no homem é de 16,8μm. Como a camada córnea é considerada a principal barreira à permeação, justifica-se a escolha deste animal como modelo experimental (MORIMOTO *et al*, 1994, BLANK e SCHUPLIN,1993).

As medidas lineares, morfométricas são muito utilizadas em estudos histopatológicos pois são muito mais objetivas, facilmente reproduzíveis e podem ser detectadas alterações que numa observação visual muitas vezes pode ser negligenciada (HAMILTON *et al*. 1995).

Várias modalidades terapêuticas têm sido indicadas para tratar o FEG, entre elas o US e a mesoterapia.

A mesoterapia tem a vantagem de aproximar o medicamento ao local da patologia, mediante doses intradérmicas mínimas e localizadas (POSTERNAK *et al*., 2000). As indicações desta técnica são múltiplas: para dores diversas, alopécia, celulite, entre outras. Entretanto, tendo-se em vista que a mesoterapia é uma técnica invasiva, pode trazer várias

complicações decorrentes de técnica inadequada referente à inoculação, ou da escolha dos produtos utilizados. Estes devem ser hidrossolúveis, isotônicos, com pH adequado, estáveis física e quimicamente, tolerados ao nível subepidérmico, de baixo estímulo alergênico e eficácia reconhecida (ROSSI, 2000).

Do ponto de vista farmacológico esta via utiliza, no tratamento do FEG, fármacos de ação lipolítica, vasoativa, venolinfática, eutrófica e antifibrótica (LEIBASCHOFF, 1987)

Segundo DRAELOS e MARENUS (1997) tem sido utilizadas substâncias lipolíticas, farmacologicamente ativas, por via tópica com objetivos de reduzir o tamanho das hérnias adipocitárias, diminuindo o aspecto do FEG.

O uso do US no tratamento do FEG por sua vez está vinculado a seus efeitos fisiológicos associados à sua capacidade de veiculação de substâncias (PIRES DE CAMPOS, 1992; ROSSI, 2000). São efeitos fisiológicos do US: ação tixotrópica sobre géis, despolimerização da substância fundamental; deslocamento de íons; aumento da permeabilidade das membranas; melhor reabsorção de líquidos e aperfeiçoamento da irrigação sangüínea e linfática (PIRES DE CAMPOS, 1992). Segundo YOUNG (1996) e CUNHA *et al.* (2001), o US aumenta a produção e melhora a orientação das fibras colágenas do tecido conjuntivo.

A cafeína, um derivado das metilxantinas, é largamente utilizada como um potencializador da resposta lipolítica, pois inibe a fosfodiesterase, que degrada o AMPc (SHUM *et al.*, 1997). Além de antagonizar farmacológicamente os receptores de adenosina (SATTIN e RALL, 1970).

O receptor de adenosina A_1 é responsável por inibir a lipólise e a cafeína e seus metabólitos, teofilina e teobromina, são responsáveis por inibir a ação neste receptor

(OLAH e STILES, 1995). A cafeína também exerce um papel importante no sistema de neurotransmissão/neuromodulação do sistema nervoso central, inibindo a ação da adenosina. Receptores pré-sinápticos de adenosina A_1 em ativação abrem os canais de sódio e potássio, diminuindo a liberação de neurotransmissores voltagem-dependentes (OLAH e STILES, 1995). Assim, a cafeína com sua ação bloqueadora dos receptores de adenosina pode aumentar liberação de neurotransmissores e aumenta a excitabilidade neuronal (MEEUSEN e DE MEIRLEIR, 1995).

Com o objetivo de evitar os efeitos indesejáveis da cafeína, LAMBERT (1982) e BELILOWSKY (1988), propõem a aplicação tópica de cafeína a 5%

Portanto, frente a ausência de investigações científicas quanto ao estudo da eficácia do US como acentuador da permeação cutânea da cafeína em fragmentos de pele e em adipócitos isolados de animais que sofreram este tratamento, são nossos objetivos neste trabalho:

- Analisar in vitro a permeação cutânea da cafeína através da aplicação do ultra-som (US) em fragmentos de pele isolados de suínos.
- Analisar as alterações morfológicas que ocorrem no tecido adiposo submetido a fonoforese associado ou não à cafeína e mesoterapia com cafeína.
- Analisar a resposta lipolítica de adipócitos isolados do tecido adiposo subcutâneo de suínos submetidos ao tratamento tópico, durante quinze dias, de cafeína associada ou não ao ultrassom terapêutico.

OBJETIVOS GERAIS

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Para a apresentação desta tese, os resultados obtidos foram organizados em capítulos que correspondem aos manuscritos gerados durante o seu desenvolvimento, os quais passamos a apresentar.

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

Pires-de-Campos, MSM*,[a]; Polacow, MLO[b].; Granzotto, TM[a]; Spadari-Bratfisch, RC[*]; Leonardi, GR[c]; Grassi-Kassisse, DM.[*]

Este trabalho foi redigido de acordo com as normas da revista "Journal of Controlled Release"

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Abstract

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.

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 cellulitis[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 (figure 1).

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 Student's *t* test followed by 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 2).

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 $\pm$ 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 $\pm$ 110.35 μg/ml, $p<0.01$).

Figure 3 shows that caffeine permeation gradually increased, reaching its peak 60 minutes after the beginning of the application (4.4 $\pm$ 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 $\pm$ 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 $\pm$ 0.5 μg/ml), 90th min (8.6 $\pm$ 0.6 μg/ml), 120th min (8.4 $\pm$ 0.5 μg/ml), 150th min (8.7 $\pm$ 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 $p<0.01$).

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 3). 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 2). 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.

CONCLUSIONS

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.

REFERENCES

1. C.S. ASBILL, A.F. EL-KATTAN, B. MICHNIAK; Enhancement of transdermal drug delivery:chemical and physical approaches. Crit Ver Ther Drug Carrier Syst. 17(6) (2000) 621-658.
2. N.N. BYL; The Use of ultrasound as an enhancer for transcutaneous drug delivery: Phonophoresis. Phys Ther. 75(6) (1995) 89-103.
3. S. MITRAGOTRI; Synergistic effect of enhancers for transdermal drug delivery. Pharm Res. 17(11) (2000) 1354-1359.
4. H. TANG, S. MITRAGOTRI, D. BLANKSCHTEIN, R. LANGER, Theoretical description of transdermal transport of hydrophilic permeants: Application to low-frequency sonophoresis. J Pharm Sciences. 90(5) (2001) 545-568.
5. L. MACHET, A. BOUCAUD; Phonophoresis: efficiency, mechanisms and skin tolerance. Int J Pharm 243 (2002) 1-15
6. Y, MORIMOTO, K. SUGIBAYASHI, H. NATSUME; The transdermal drug delivery system and transcutaneous absorption. Acta Derm Venered Suppl (Stockh). 185 (1994)15-17.

7. H. UEDA, M. OGIHARA, K. SUGIBAYASHI, Y. MORIMOTO; Difference in the enhancing effects of ultrasound on the skin permeation of polar and non-polar drugs. Chem Pharm Bull 44(10) (1996) 1973-1976.

8. M. HIPPIUS, U. SMOLENSKI, C. UHLEMANN, U. SCHREIBER, A. HOFFMANN; Investigations of drug release and penetration- enhancing effect of ultra-sound on transmembrane transport of fluofenamic acid. Exp Toxicol Pathol 50 (4-6) (1998) 450-452.

9. J FANG, C FANG, KC SUNG, H CHEN**;** Effect of low frequency ultrasound on the in vitro percutaneous absorption of clobetasol 17-propionate. Int J Pharm. 191(1) (1999) 33-42.

10. D. BOMMANNAN, H. OKUYAMA, P. STAUFFER, R.H. GUY; Sonophoresis, I: the use of high-frequency ultrasound to enhance transdermal drug delivery. Pharm Res, 9 (4) (1992) 559-564.

11. D. BOMMANNAN, G.K. MENONS, H. OKUYAMA; Sonophoresis, II: examination of the mechanisms(s) of ultrasound-enhanced transdermal drug delivery. Pharm Res, 9 (8) (1992) 1043-1047.

12. S. MITRAGOTRI, D. BLANKSCHTEIN, R. LANGER; Transdermal drug delivery using low frequency sonophoresis. Pharm Res13(3) (1996) 411-420.

13. D.G.KASSAN, A.M. LYNCH, M.J. STILLER; Physical enhancement of dermatologic drug delivery: Iontohoresis and phonophoresis. J Am Acad Dermatol 34 (4) (1996) 657-666.

14. C. SINCLAIR, J. GEIGER; Caffeine use in sports; a pharmacological review. J Sports Med Phys Fitnes, 40 (2000) 71-79.

15. M. DIAS, A. FARINHA, E. FAUSTINO, J. HADGRAFT, J. PAIS, C. TOSCANO; Topical delivery of caffeine from some commercial formulations. Int J Pharm 182 (1999) 41-47.

16. A. BOUCAUD, L. MACHET, B. ARBEILE, B. MACHET, M.C. SOURNAC, M. MAVON, F. PATAT, L VAILLANT; In vitro study of low-frequency ultrasound-enhanced transdermal transport of fentanyl and caffeine across human and hairless rat skin. Int J Pharm 228 (2001) 69-77.

17. D. MONTI, R. GIANNELLI, P. CHETONI, S. BURGALASSI; Comparison of the effect of ultrasound on transdermal permeation of caffeine and morphine through hairless mouse skin in vitro. Int J Pharm 229 (2001) 131-137.

18. L. THOLON, G. NELIAT, C. CHESNE, D. SABOREAU, E. PERRIER, J.E. BRANKA; An in vitro, ex vivo, and in vivo demonstration of the lipolytic effect of slimming liposomes: An unexpected alpha(2)-adrenergic antagonism.. J Cosmetic Sci 53 (2002) 209-218

19. M.V.L.B. BENTLEY; Desenvolvimento de produtos dermatológicos contendo corticosteróides: avaliação da liberação e penetração transcutânea por metodologia *in vitro*. **Tese de Doutorado**. Faculdade de Ciências Farmacêuticas – Universidade de São Paulo, 1994.

20. E.D. OLFERT, B.M. CROSS, A.A. McWILLIAM; Guide to the care use the experimental animals. Canadian council on animal care 213, Ottawa, Ontario, 1993.

21. M.C. FYFE; A study of the effects of different ultrasonic frequencies on experimental edema. Aust J Physiother 25 (5) (1979) 205-207.

22. A.R. WILLIAMS; Ultrasound Biologic effects and potential hazards. Academic Press, New York, 1983.

23. M.H., CAMERON, L.G. MANROE; Relative transmission of ultrasound by media customarily used by phonophoresis. Phys Ther 72 (1992) 142-148.

24. E.M.OAKLEY; Application of continuous beam of ultrasound at therapeutic levels. Physiother, 68 (8) (1978) 169-172.

25. N.N. BYL, A. MCKENZIE, B.HALLIDAY, T. WONG, J.O. O'CONNELL; The effects of phonophoresis with corticosteroids: a controlled pilot study, J Orthop Sport Phys Ther, 18 (5) (1993) 590-600.

26. R.R.J. GUIRRO, A.S. CANCELIERI, I.L. SANT'ANNA; Avaliação dos meios intermediários utilizados na aplicação do ultra-som terapêutico Rev. Bras. Fisioter., 5 (2) (2001) 49-52.

27. J.H. ZAR; Biostatistical Analysis. 2nd ed. Hall International, Upper Saddle River, NJ,1984.

28. G.W. SNEDECOR and W.G. COCHRAN; Statistical Methods. 6^{Th} Ed. Iowa State University Press. IA. 342-343, 1967.

29. J.N.S. MATTHEWS, D.G. ALTMAN, M. CAMPBELL, P. ROYSTON; Analysis of serial measurements in medical research. Brit Med J 27 (1990.) 233-235.

30. S MITRAGOTRI, D.A. EDWARDS, D. BLANKSCHTEIN, R. LANGER; A mechanistic study of ultrasonically enhanced transdermal delivery. J Pharm Sci 84 (6) (1995) 697-706.

31. R. L BRONAUGH, R. F. STEWART, E. R. CONGDON; Methods for *In Vitro* percutaneous absorption studies. II. Animal models for human skin. Toxicol Appl Pharmacol 62 (1982) 481-488.

32. M.E JOHNSON,. S MITRAGOTRI, A PATEL, D BLANKSCHTEIN, R. LANGER; Synergistic effects of chemical enhancers and therapeutic ultrasound on transdermal drug delivery. J Pharm Sci 85 (7) (1996) 670-679

33. D. EDWARDS, R. LANGER; A linear theory of transdermal phenomena. J Pharm Sci 83 (1994) 1315-1334.

34. A. BOUCAUD, J. MONTHARU, L MACHET, B ARBEILLE, M.C. MACHET, F. PATAT, L. VAILLANT; Clinical histologic and electron microscopic study of skin exposed to low-frequency ultrasound. Anat Rec, 264 (1) (2001) 114-119.

35. J.KOST, D. LEVY, R. LANGER; Ultrasound as a transdermal enhancer. *In:* R. L. BRONAUGH, H. I. MAIBACH (Ed.), Percutaneous Absorption; Mechanism – Methodology – Drug Delivery. 2.ed. New York: Marcel Dekker, 1989.

36. A.K LEVANG, K. ZHAO, J.SINGH; Effect of ethanol/propylene glycol on the in vitro percutaneous absorption of aspirin, biophysical changes and macroscopic barrier properties of the skin**.** Int J Pharm 30; 181(2) (1999) 255-263.

37. M.P. CARVER, P.L. WILLIAMS, J.E. RIVIERE, The Isolated Perfused Porcine Skin Flap. Toxicol. Ap Pharm. 97(2) (1997) 324-337.

LEGENDS

Figure 1. Cell of Bentley, composed of one piece and a PVC ring where the swine skin extracted from the back of the animal was fixed and fitted with 2 elastic bands. The cell was then placed upon a plastic cube containing 50 ml of receptor solution in constant agitation, certifying that this was in contact with the dermis. The temperature was maintained at 37ºC.

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

Figure 3. 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).

Figure 1.

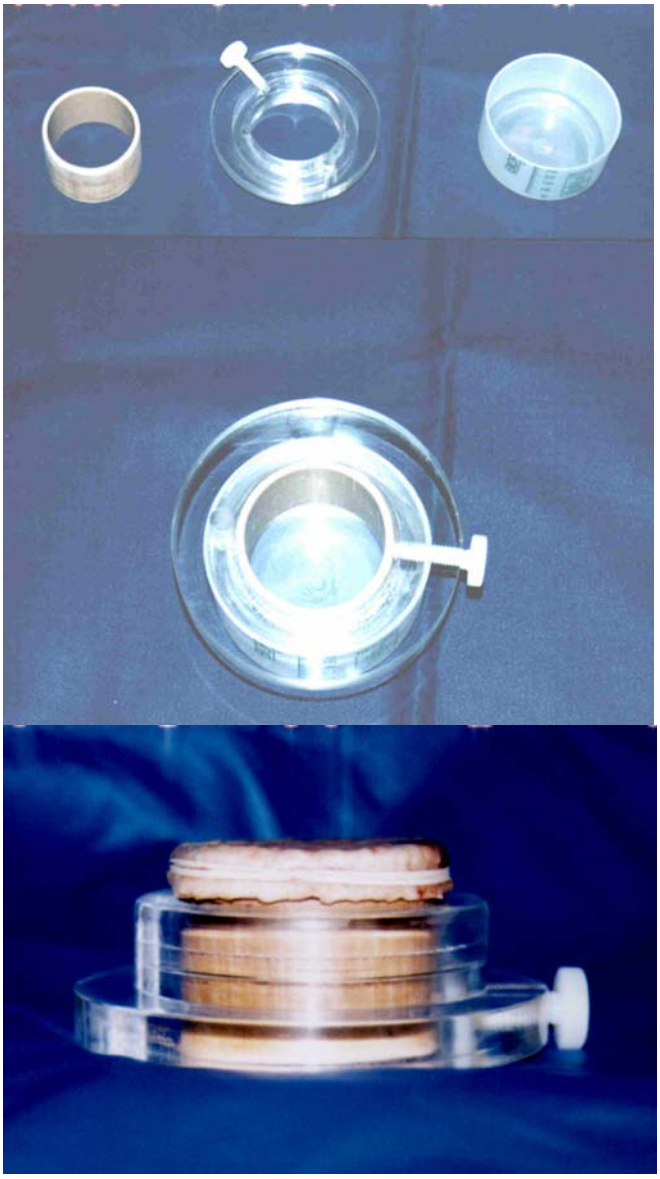


Figure 2

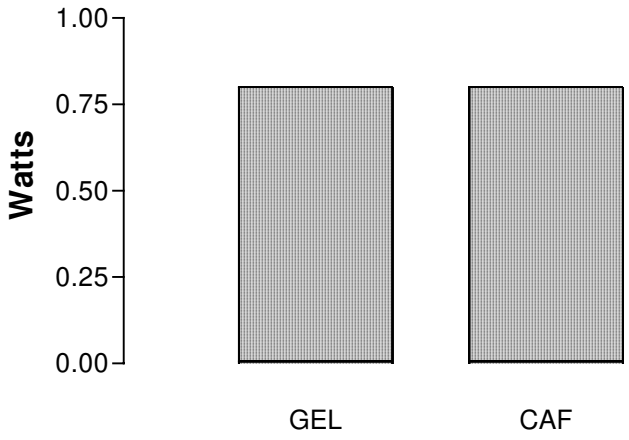
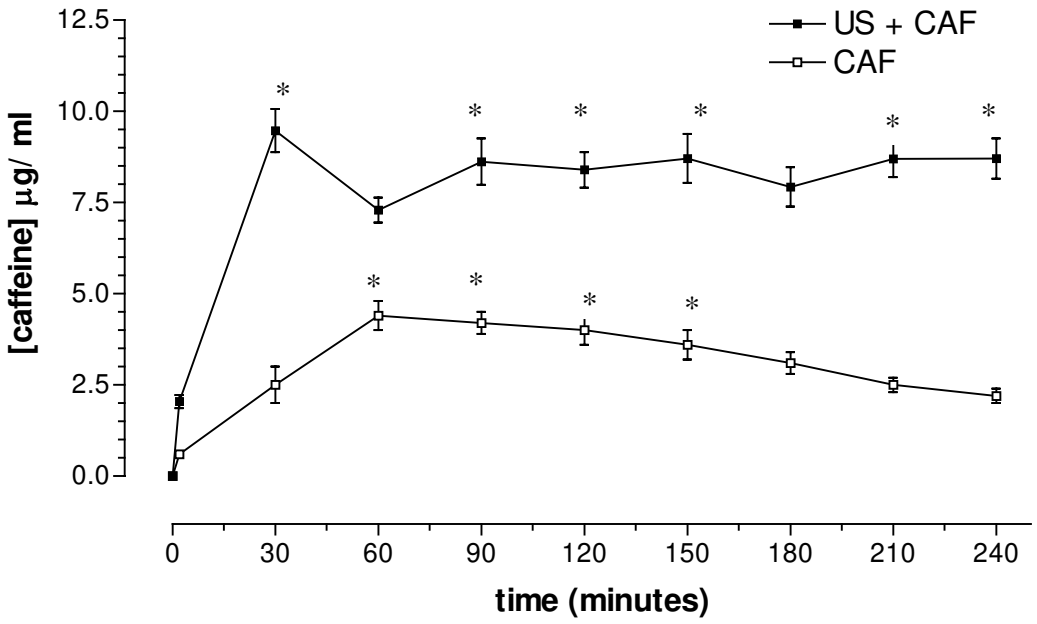


Figure 3.


US + CAF
CAF
[caffeine] µg/ ml
12.5
10.0
7.5
5.0
2.5
0.0
0
30
60
90
120
150
180
210
240
time (minutes)

EFFECT OF TOPICAL APPLICATION OF CAFFEINE ASSOCIATED OR NOT WITH THERAPEUTIC ULTRASOUND ON THE MORPHOLOGY OF SWINE HYPODERMIS.

Maria Silvia Mariani Pires-de-Campos [* a]; Gislaine Ricci Leonardi [c]; Marlus Chorilli [c]; Regina Célia Spadari-Bratfisch[*]; Maria Luiza Ozores Polacow [b]; Dora Maria Grassi-Kassisse [*#]

Este trabalho foi redigido de acordo com as normas da revista "International Journal of Dermatology"

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ABSTRACT

Background: Cellulite or lipodystrophy is a modification of the subcutaneous adipose tissue. In the wealthy diversity of topical products against cellulite, but have difficulties for absorption through the skin. The application of therapeutic ultrasound (US) is the resource for enhances transdermic drug transport. The objective of the present study was to analyze the effect of caffeine on the morphology of swine hypodermis when applied topically alone or in combination with US.

Methods: The following treatments were applied to 5 previously shaved dorsal areas of 5 swines (Landrace x Large White, 35 days old and weighing 15 kg): gel (carbopol 940), gel+US (US), gel+caffeine (5%; CAF), and gel+caffeine+US (US+CAF), daily for 15 days. A 5^{th} untreated area was used as control. Continuous US of 3 MHz with an intensity of 0.2 W/cm² was applied at a rate of 1 min/cm². After histological processing (HE), morphometric analyses were carried out to determine hypodermis thickness and numerical profile.

Results: Caffeine treatment was effective only when associated with ultrasound therapy. This treatment caused significant reduction in the subcutaneous adipose tissue thickness and, damage to the adipocytes, decreasing the cells number.

Conclusion: The US was effective in increasing the cutaneous permeation of caffeine, evidenced by the reduction of thickness of the hypodermis and adipocyte number.

Keywords: caffeine, phonophoresis, adipose tissue.

INTRODUCTION

"Miraculous" products for topical use with the intention of improving people's appearance in order to attain current beauty standards are constantly being produced. However, many of these products end up being available to the consumer without more detailed studies with regard to their real therapeutic efficiency.

Cellulite, also known as fiber edema geloid (FEG), or fibrosclerotic edematous panniculopathy (PEFE), affects 85% of women[1] after adolescence, and it is characterized by irregular dermis-hypodermic fascia, with vertical interlobular septa of the adipose tissue, favoring hernias of the areolar hypodermis in the direction of the reticular dermis[2]. The cutaneous relief thus becomes altered, leading to deterioration of the invaded tissue's capillary networks[3, 4].

Current topical treatment for esthetic purposes mainly aims to reduce cellulite. However difficulties found in the absorption of medications through the skin have led researchers to investigate the use of chemical substances in the formulation, as well as the use of physical methods such as ultrasound (phonophoresis) to aid skin penetration of different drugs[5, 6, 7, 8]. The thermal and mechanical effects, as well as the chemical alterations of the biological tissues caused by the use of ultrasound (US), accelerate the diffusion of the active ingredients present in the medications for topical use. Among the thermal effects, there is an increase in kinetic energy between the molecules of the medication, as well as dilation of the follicles and glands and an increase in peripheral

circulation [6, 9]. Among the mechanical effects, there is oscillation of the cell membrane potential, which leads to the breakdown of the intercellular links, flow of the medication's molecules in the direction of the tissue and a small oscillation in the cells position [9, 10].

Studies on cutaneous permeation have used swine skin, which is very similar to the human skin both in terms of morphological and functional aspects [8, 11, 12].

Previous investigations from our laboratory have also proved the efficiency of phonophoresis by the *in vitro* study of swine skins treated with tiratricol alone or in combination with US, reporting that US treatment led to acceleration in the diffusion of the medication[13]. POLACOW *et al.*[14] detected alterations in the morphometric measurements of swine adipose tissue *in vivo* treated with tiratricol gel and US.

In addition, the lipolytic activity of swine adipocytes, the adipose tissue functional entities, is closely similar to that of humans, with the quantity of adrenergic β_3 receptors being lower than 10% and the most predominant being the adrenoceptor subtypes β_1 and β_2[15].

The use of caffeine for the topical treatment of cellulite has been recently suggested[16]. The main action of caffeine is to antagonize the adenosine receptors. Caffeine and its metabolites, theophylline and theobromine, are responsible for blocking the adenosine receptor. In addition, caffeine also inhibits the phosphodiesterase that degrades AMPc to inactive 5'AMP, thus stimulating lipolysis[17]. The result of the sum of

these two effects is an improvement of the lipolysis, with the consequent reduction of cellulite.

On this basis, the objective of the present study was to analyze the effect on the morphology of swine hypodermis of caffeine when topically applied alone or in association with therapeutic ultrasound (US).

MATERIAL AND METHODS

1. Animals

During the experiments, the swines were cared for in accordance with the principles outlined by Olfert *et al.*[18] for the use of animals for research and education, and the experimental protocols were approved by the Committee for Ethics in Animal Research (State University of Campinas – UNICAMP – protocol nº 614-2).

Five male hybrid swine (Landrace x Large White), not castrated, 35 day old and weighing approximately 15 kg were obtained from the Alvorada's country property. The animals were fed with *"ad libitum"* and had free access to fresh water and chow composed by crushed corn, soy flour, meat flour, calcareous, salt, polinucleous Fapec. Ivomec® was applied as a prophylactic procedure care against ectoparasites.

One day before the beginning of the treatment, the animal's dorsal region was shaved with a shearing machine (comb number 0), avoiding damage to of the corneous layer, which could alter the skin permeability.

2. Topic Treatments

After trichotomy, the dorsal region of each animal was divided into five areas of 8 cm^2: control, gel (GEL), gel+caffeine (5%; CAF), gel+US (US) and gel+caffeine+US (US+CAF).

2.1. Gel Area

Gel was applied daily, during fifteen days, on the skin through out circular friction (massage therapy) until hyperemia and increase of the skin temperature were achieved. The amount of gel applied to each area was 3 g. 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).

2.2. Caffeine Gel Area

Caffeine gel was applied daily, during fifteen days, on the skin through out circular friction (massage therapy) until hyperemia and increase of the skin temperature were achieved.

The caffeine gel was prepared adding 5% of anhydrous caffeine (Sigma Chemical Company, St. Louis, MO, USA) 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.

2.3. Ultrasound application

The US (Sonomaster Microcontrolado - KW Ind Nac de Tecnologia Lt, Amparo, São Paulo, Brasil) was applied daily, during fifteen days, over the skin area with gel (US) or caffeine gel (US+CAF) and it was set at 3 MHz, due to superficiality of the adipose tissue[19]; intensity of 0.2 W/cm² [20], continuous emission[21] and application time of 1 min/cm² [22], with the transducer being moved slowly and continuously until the end of the application[23].

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*.[24], 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, considerring 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. The presence of gel (carbopol

940®) or gel added of caffeine (5%) did not produce the attenuation of the US intensity evaluated by the transmissibility test (data not shown).

3. Histological Processing

After 15 days of daily treatment the animals were sacrificed. Segments of skin including hypodermis were removed from the different areas.

The skin segments were fixed in Bouin's for 24 h, processed for hematoxylin and eosin (HE) coloration and the laminas were mounted on Canada Balsam.

Nine non-serial sections (one to each 15 cuts approximately), of the 6-7 μm of thickness, per area were obtained from each animal and submitted to morphometric and numerical profile analyses. Hypodermis thickness was determined by five measurements per cut, being used objective of 40x, ocular millimetered from Zeiss adjusted with a slide object from Zeiss[25]. Hypodermis thickness was considered from the dermis-hypodermis limit up to the surface fascia; therefore the measurements obtained refer to the areolar layer. The numerical profile of adipocytes was determined in six images per section using the ProSeries Grab and Image ProPlus programs.

4. Statistical Analysis

In order to compare the effects of the different treatments in the hypodermis thickness as well as in the numerical profile one-way analysis of variance (ANOVA) followed by the Tukey test was used, with the level of significance set at 5%.

RESULTS

Morphometric analysis showed a significantly reduction in the hypodermis thickness in the area treated with caffeine in combination with ultrasound ($p < 0.05$). When the treatment was caffeine or ultrasound alone there were no significant morphometric alterations in the hypodermis thickness (Table 1).

The histological analyses of adipocytes showed that the only treatment that was effective in reduce the cell number (Table 2) and produce morphological tissue alterations (Figure 1) was the application of caffeine gel in combination with ultrasound.

DISCUSSION

Swine skin is used in this research, since its level of permeation is very similar to the permeability of the human skin. Swine skin stratum corneous presents similar thickness in relation to the human skin and smaller amount of hair compared to the skin of other animals[12]. Moreover, the β-adrenenoceptors population of the subcutaneous adipocytes is similar in swine and human beings[15].

Our results showed that the only treatment that effectively reduced the hypodermis and the adipocytes number was to the association between gel caffeine with ultrasound. These results confirm the effect of ultrasound as a promoter of cutaneous permeation of caffeine. Several studies point out to explain the role of ultrasound in cutaneous

permeation. Both the thermal and mechanical properties of US accelerate the diffusion of medications in the skin.

The thermal effects of US include increase in kinetic energy of the compound molecules of the drug, dilation of the skin follicles and glands, and vasodilation which increase local blood flow[6,9]. The mechanical effects are include oscillation in the cells position[9, 10], variation of the cell membrane potential, which leads to the breakdown of the intercellular links, and alteration of the lipid structure of the corneous layer, increase in ionic conductance, or destruction of the cell membrane[6] all these combined effects facilitates the molecules flow towards the target tissue[9, 10].

The caffeine permeation of the skin when the gel was applied in combination with US was probably due to the mechanical effect, supporting the data obtained by MITRAGOTRI[27] since topical application, even when done by massage, did not produce morphometric alterations in adipose tissue. BAAR and TASLET[28] reported that massage produces an increase in the cutaneous temperature due to direct mechanical effects and vasomotor reflexes.

ROHRICH *et al*.[29] through histological and enzymatic analyses did not observe any effect of the ultrasound and of the massage in the adipocytes.

In vitro studies have demonstrated that β-adrenergic agonists and methylxanthines stimulate lipolysis by an increase in the AMP_c intracellular concentration due to adenylilcyclase stimulation and phosphodiesterase inhibition respectively, thus reducing the adipocyte size[1]. CHEUNG *et al*.[30] as well as ZHANG and WELLS[31] observed a

decrease in adipocyte diameter and body weight, due to a decrease in the number of adipocytes, and/or decrease in adipocytes triglyceride content, in rats treated with solution of caffeine compared to control rats.

Caffeine is a competitive antagonist of the adenosine receptor. The four subtypes of adenosine receptors that have been characterized and defined are A_1, A_{2a}, A_{2b} and A_3. Activation of the A_1 receptor inhibits lipolysis and activates potassium channels[17]. Caffeine blockade of the A_1 adenosine receptor, avoid its inhibition of adenylilcyclase *via* Gi protein[32], thus increasing lipolysis.

By increasing the local concentration of AMPc, caffeine acts synergicaly with the catecholamines, potentiating the action of the already present catecholamines[36]. The action of caffeine on lipolysis may, therefore, be the result of synergism between the action of caffeine itself on phosphodiesterase and the action of catecholamines on adenylilcyclase, which contribute to increase the ratio of active AMPc and HSL, which in turn induces lipolysis[1].

In vitro[34] and *in vivo*[35] experiments demonstrated that the lipolytic action of caffeine used alone is weak.

Moreover, as is the case for all methylxanthines, caffeine may cause nervous excitation, sleep disturbances and problems with incrased diuresis and heart rate[37]. These disadvantages justify the use of a route of administration which reduces these risks to a minimum, like the cutaneous permeation route which produces lower plasma levels, and involves no risk of association of medications that can provoke serious side effects[6].

BELILOWSKY[38] and LAMBERT[33], who observed that, on applying a gel based on 5% caffeine to the skin, a localized therapeutic effect is obtained at the level of the adipose tissue, with reduced levels of caffeine in plasma. After the repeated use of the product, the serum levels obtained were 0.45 μg/ml, lower than those measured after the absorption of a cup of coffee, corresponding to a quarter of the oral absorption.

Phonophoresis has several advantages over other therapeutic modalities used for the treatment of FEG such as localization of the treated region, production of a local effect, reduced systemic effects, reduced number of sessions when the effects of medications are combined with the physiological effects of US, in addition to permitting the same penetration as obtained with mesotherapy, since US of 3 MHz can penetrate transcutaneously to a depth of 1 to 2 cm [6].

This compilation of the intrinsic effects of US and of caffeine perhaps explains the alterations seen in Figure 1, since US increases membrane permeability, increases the diffusion of the medication and potentiates its action. This may stimulate the damage to the adipocytes because when the fatty acids are released from the adipocyte by lypolisis they bind to albumin and in its absence the fatty acids forms micelles which, following LIMA *et al.* [39] are able to act as detergents, rupturing proteins and membrane structures.

By the other way LAGNEAUX *et al.*[40] observed cellular damage in human leukemia cell lines submitted to ultrasonic irradiation at low energy. However the parameters of the ultrasound, used in this healthy experiment very different from those

used by us. Though ultrasound only produces effect in the tissue that absorbs their waves. The absorption coefficient of the adipose tissue is very low, being above only of the water and of the blood[41, 42]. Besides, GLICK *et al.*[43] didn't observe significant changes in cAMP in skin, lung and peritoneal cells of mouse submitted to treatment with ultrasound.

CONCLUSION

US was effective in increasing the cutaneous permeation to facilitate the action of caffeine, resulting in a reduction of hypodermis thickness and of adipocyte number.

Acknowledgments

KW Industria Nacional de Tecnologia Ltda.

Financial Support

FAP - Fund of Support to the Research of the Methodist University of Piracicaba (UNIMEP)

FAEP - Fund of Support to the Extension and the Research of the State University of Campinas

REFERENCES

1. ROSSI, A. B. R., VERGNANINI, A. L.; Cellulite: a review. J. Eur. Acad. Dermatol. Venereol. 2000; **14** 4:251-262.

2. ROSENBAUM, M., PRIETO, V., HELLMER, J., *et al*. An exploratory Investigation of the morphology and biochemistry of cellulite. Plastic and Reconstructive Surgery 1998, **101** 7:1934-1939.

3. DRAELOS, Z. D., MARENUS, K. D. Cellulite: etiology and purported treatment. Am. Soc. Dermatol. Surg, 1997, **23** 12:1177-1181.

4. PIÉRARD, G. E., FRANCHIMONT, C. P. Cellulite: from standing fat herniation to hypodermal stretch marks. American Journal of Dermatopathology 2000, **22** 1:34-37.

5. KOST, J., LEVY, D., LANGER, R. Ultrasound as an transdermal enhancer, in: BRONAUGH, R. L., MAIBACH, H. I. Percutaneous Absorption; Mechanism – Methodology – Drug Delivery. New York: Marcel Dekker, 1989

6. BYL, Nancy. The use of ultrasound as an enhancer for transcutaneous drug delivery: Phonophoresis. Physical Therapy 1995, **75** 6:539-553.

7. MARJUKKA SUHONEN, T., BOUWSTRA, J. A., URTTI, A. Chemical enhancement of percutaneous absorption in relation to stratum corneum structural alterations. J. Control. Release 1999, **59** 2:149-161.

8. TANG, H., MITRAGOTRI, S., BLANKSCHTEIN, D., LANGER, R. Theoretical description of transdermal transport of hydrophilic permeants: application to low-frequency sonophoresis. J. Pharm. Sci., 2000, **90** 5:545-568.

9. HIPPIUS, M., SMOLENSKI, U., UHLEMANN, C., *et al*. A. *In Vitro* investigations of drug release and penetration – enhancing effect of ultrasound on transmembrane transport of flufenamic acid. Exp Toxic Pathol 1998, **50**:4-6.

10. BARE, A.C.; McANAW, M.B.; PRITCHARD, A.E.; *et al*.; Phonophoretic delivery of 10% hydrocortisone through the epidermis of humans as determined by serum cortisol concentrations. Phys. Ther. 1996, **76** 7: 738-743.

11. BRONAUGH, R. L., STEWART, R. F., CONGDON, E. R. Methods for *In Vitro* percutaneous absorption studies. II. Animal models for human skin. Toxicol. Appl. Pharmacol. 1982, **62**: 481-488.

12. WESTERN, R.C., MAIBACH, H.I. *In vivo* methods for percutaneous absorption measurements. In: BRONAUGH, R. L., MAIBACH, H. I. Percutaneous Absorption; Mechanism – Methodology – Drug Delivery. New York, Marcel Dekker, 1989. p.215-220.

13. PIRES DE CAMPOS, MSM.; POLACOW, M. L. O.; LEONARDI, G. R.; FIORI JÚNIOR, J. F.; Análise espectrofotométrica da permeação cutânea do tiratricol pelo ultra-som. *In*: Congresso Brasileiro de Cosmetologia, XV, São Paulo, 2001, Anais, Associação Brasileira de Cosmetologia

14. POLACOW, M. L. O., PIRES DE CAMPOS, M. S. M., LEONARDI, G. R., *et al.*; Efeito do ultra-som na permeação cutânea do tiratricol: análise histológica. Rev. Bras. Fisioter., 2004, **8** 1:53-60

15. DING, S.T.; McNEEL, R.L.; O'BRIAN SMITH, E.; MERSSMANN, H.J.; Modulation of porcine adipocyte β-adrenergic receptors by a β-adrenergic agonist. J. Anim. Sci, 2000, **78**:919-929

16. DI SALVO, R. M. Controlando o surgimento da celulite. Cosm. Toil. 1996, **8** 4:56-62.

17. SINCLAIR, C., GEIGER, J. Caffeine use in sports; a pharmacological review. J Sports Med Phys Fitnes 2000, **40**:71-79.

18. OLFERT, ED; CROSS, BM; McWILLIAM, AA. Guide to the care use os experimental animals. *Canadian council on animal care 213,* Ottawa, Ontario, 1993.

19. FYFE, M.C.; A study of the effects of different ultrasonic frequencies on experimental o edema. Aust. J. Physiother. 1979, **25** 5:205-207

20. WILLIAMS, A. R.; *Ultrasound:Biologic effects and potential hazards.* New York, Academic Press , 1983.

21. CAMERON, M.H., MANROE, L.G.; Relative transmission of ultrasound by media customarlly used of phonophoresis. Phys. Ther. 1992, **72**: 142-148.

22. OAKLEY, E. M.; Apllication of continuous beam ultrasound at therapeutic levels. Physiother. 1978, **68** 8:169-172

23. BYL, N. N., MCKENZIE, A., HALLIDAY, B., *et al*.; The effects of phonophoresis with corticosteroids: a controlled pilot study, JOSPT 1993, **18** 5:590-600.

24. GUIRRO, R.R.J., CANCELIERI, A.S.; SANT'ANNA, I.L.; Avaliação dos meios intermediaries usados na aplicação do ultra-som terapêutico Rev. Bras. Fisioter. 2001, **5** 2:49-52

25. DE LACERDA, C. A. M. *Manual de Quantificação Morfológica: Mofometria, alometria e Esteriologia.* Rio de Janeiro: CEBIO, 1994. p.8-9.

26. HOLLANDER D. A.; ERLI, H.J.; THEISEN, A. *et al*.; Standardized qualitative evaluation of scar tissue properties in an animal wound healing model. Wound Rep. Reg. 2003, **11** 2:150-157.

27. MITRAGOTRI, S. BLANKSCHTEIN, D.; LANGER, R.; Trasdermal drug delivery using low frequency sonophoresis. Pharmaceutical Research 1996, **13** 3: 411-420.

28. BAAR, J.; TASLET, N.; The influence of back massage on autonomic functions. Phys Ther 1970, **50**:1679-1691

29. ROHRICH, R.J., MORALES, D.E., KRUEGER, J. E., *et al*.; Comparative lipoplasty analysis of in vivo-treated adipose tissue. Plast. Reconstr. Surg. 2000, **105** 6: 2152-2158.

30. CHEUNG, W.T, LEE, C., NG, T. Potentiation of the anti-lipolytic effect of 2-chloroadenosine after chronic caffeine treatment. Pharmacology 1988, **36**: 331-339.

31. ZHANG, Y.; WELLS, J.N.; The effects of chronic caffeine administration on peripheral adenosine receptors. The Journal of Pharmacology and Experimental Therapeutics, 1990. **254** 3:757-763

32. LAFONTAN, M., BARBE, P., GALITZKY, L., *et al*.; Adrenergic regulation of adipocyte metabolism, Hum. Reprod. 1997, **12**, suppl. 1:6-20

33. LAMBERT, J. Traitemente par la percutaféine des lipodystrophies localisées. Comptes Rendus de Thérapeutiques et de Pharmacologie Clinique**.** CHU Lariboisière 1982, **1** 10:3-6.

34. BELLET, S.A.; KERSHBAUM, A.; FINCK, E.M.; Response of free fatty acids to coffee and caffeine. Metab. Clin. Exp. 1968, **17**: 702.

35. BLECHER. M.; MERLINO, N.S.; RO'ANE, J.T.; Control of the metabolism and lipolytic effects of cyclic 3', 5' adenosine monophosphate in adipose tissue by insulin, methylxanthines and nicontinic acid**.** J. Bio. Chem. 1968, **243**: 3973-3977

36. MARTIN, P., CHILDS, M., BETOURNE, C. Substances doueés d'effet lipolytique: intérêt de la caféine en thérapeutique. Comptes Rendus de Thérapeutiques et de Pharmacologie Clinique. CHU Lariboisière 1983, **1** 8:11-15.

37. MASSEY, LK.; Caffeine and the elderly. Clinical Pharmacology 1998*,* **13** 1:43-50.

38. BELILOWSKY, M.; Cellulite et caféine precutanée: études récentes sur percutaféine. Compres Rendus de Thérapeutique et de Phamacologie Clinique 1988, **6** 60:23-27

39. LIMA, B.L.; VESTRI, H.S.; VESTRI, S.; MORAES, S.M.F.; L; Lipólise. In: CURI, R.; MPOMPÉIA, C.; MIYASAKA, C.K.; PROCOPIO, J.; *Entendendo a gordura – os ácidos graxos,* São Paulo, Ed. Manole, 2002

40. LAGNEAUX, L.; de MEULENAES, E.C.; DELFORGE, A.; DEJENEFFE, M.; MASSY, M.; MOERMAN, C; HANNECART, B.; CANIVET, Y.; LEPELTIER, M.F.; BRON, D.; Ultrasonic low-energy treatment: a novel approach to induce apoptosis in human leukemic cells. Exp Hematol. 2002 **30** 11:1293-301

41. WILLIAMS, A.R., Ultrasound: Biologic effects and potential hazards. Acadmic Press, New York, 1983

42. LOW, J. and REED, A. Eletroterapia Explicada – Princícios e prática. 3rd Manole, São Paulo, Brasil, 2001

43. GLICK, D.; ADAMOVICS, A.; EDEMONDS, P.D.; TAENZER, J.C.; Search for biochemical effects in cells and tissues of ultrasonic irradiation of mice and of the in vitro irradiation of mouse peritoneal and human aminiotic cells. Ultrasound Med. Biol. 1979 **5**:23-33

Table 1. Hypodermis thickness measurements (mean ± sem, in mm) in the different treatments. Male swine were treated for 15 days. Each animal received all treatments in different areas as follows; control area, GEL; US; CAF and US+CAF area. The data were analyzed by ANOVA followed by the Tukey test (n=5).

TREATMENT AREA	mm
Control	2.81 ± 0.10
Gel	2.42 ± 0.20
US	2.40 ± 0.22
Caf	2.14 ± 0.13
US+CAF	1.95 ± 0.19*

* $p < 0.05$ compared to control

Table 2. Hypodermis numerical profile (mean ± sem) obtained in the different treatments. Five male swine were treated for 15 days. Each animal received all treatments in different areas as follows; control area, GEL area, US area, CAF area, US+CAF area. The data were analyzed by ANOVA followed by the Tukey test (n=5).

TREATMENT AREA	CELL NUMBER
Control	37 ± 2
Gel	35 ± 2
US	35 ± 2
Caf	36 ± 3
US+CAF	27 ± 1*

* $p < 0.05$ compared to control

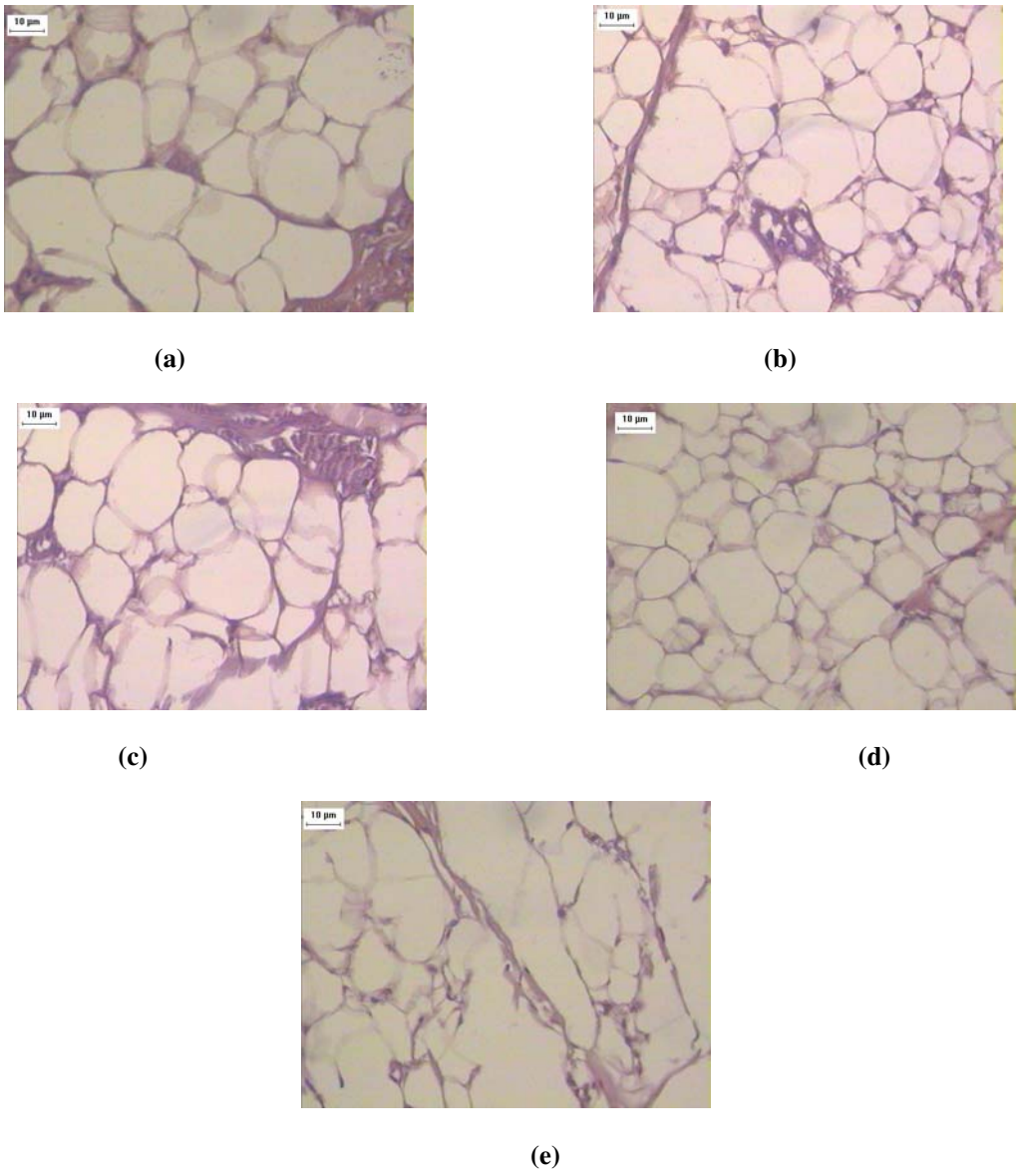

Figure 1. Photomicrographs (HE) of the hypodermis after the different treatments. Five male swine were treated for 15 days. Each animal received all treatments in different areas as follows; (a) control area, (b) gel area, (c) US area, (d) CAF area, (e) US + CAF area.

INTRADERMIC INFILTRATION OF CAFFEINE: HISTOLOGIC ANALYSIS OF HIPODERMIS.

Pires-de-Campos, M.S.M.*,[a]; Leonardi, G.R.[c]; Chorilli, M.[c]; Spadari-Bratfisch, R.C.[*]; Polacow, M.L.O.[b]; Grassi-Kassisse, D.M.[*#]

Este trabalho foi redigido de acordo com as normas da revista "Therapie"

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ABSTRACT

Mesotherapy is usually employed in cutaneous affections and esthetical conditions such as keloids and hyperthophic scar, cellulitis and lipodystrophy. Cellulitis is characterized by structural alterations in dermis and hypodermis. Caffeine has been indicated for its treatment due to lipolysis increment. The following treatments were applied to 2 previously shaved areas of the backs on each of 5 pigs (Landrace x Large White, 35 days old and weighing 15 kg): mesotherapy with caffeine (2%) area and control area. Four mesotherapy sessions were accomplished in no consecutive days (in the 3rd, 7th, 10th and 13th days of treatment). After histological processing (HE), morphometric analyses were carried out to determine hypodermis thickness of adipose tissue. The morphometric analysis showed a significant reduction in hypodermis thickness in the group treated with mesotherapy (Student *t* unpaired test; $p<0.05$).

Keywords: caffeine, intradermic infiltration, mesotherapy, adipose tissue, swine.

INTRODUCTION

Cellulites is an alteration of the topography of the skin that occurs mainly in women. It is characterized by padded or 'orange peel' appearance[1]. Although the term 'cellulites' has been used to describe an aesthetic alteration of the cutaneous surface, other names have been suggested as: nodular liposclerosis[2], oedemato-fibrosclerotic panniculpathy[3], panniculosis[4], gynoid lipodystrophy (GLD) [5], geloid fibro oedema (FEG) [6] and others.

The cellulites cannot be confused with obesity. The first is characterized by several structural alterations in dermis, in the microcirculation and within the adipocytes. These, in turn, may be associated with additional morphological, histochemical, biochemical and ultrastructural modifications[7-9]. The obesity is characterized for adipocyte hypertrophy and hyperplasia[10].

Due to multifactoral pathogenesis of GLD, there are numerous therapeutic approaches that include attenuation of aggravating factors, physical and mechanical methods, and pharmacological agents[1].

Certain drugs act on the fatty tissue and connective tissue and on the microcirculation that can be used topically, systemically or transdermally[1]. Enter the drugs used that have a lipolytic effect include the β-adrenergics agonists (isoproterenol and adrenaline), α-antagonists (yoimbine, piperoxan, etc) and methylxanthines (caffeine, theobromine, theophylline, aminophylline), which act through phosphodiesterase inhibition[5, 11].

Intradermotherapy (mesotherapy) was introduced by Pistor in 1952. This technique consists of intradermal injections of a highly diluted drugs mixture or of a single product, through syringe or apparel, with needle of 4-6 mm[12].

The 'ideal' drug used must be hydrosolluble, isotonic, have an adequate pH, be physical and chemically stable, be well tolerated after dermal administration, and have low allergenic potential[1, 5].

Although the mesotherapy be commonly used, researches controlled in this area still are poor. The aim of the present study was to investigate whether the treatment through intradermic infiltration with caffeine produce some alteration in hypodermis.

MATERIAL AND METHODS

During the experiments, the swines were cared for in accordance with the principles outlined by OLFERT *et al.*[13] for the use of animals for research and education, and the experimental protocols were approved by the Committee for Ethics in Animal Research (State University of Campinas – UNICAMP – protocol nº 614-2).

Five male hybrid swine (Landrace x Large White), not castrated, 35 days old and weighing approximately 15 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 a prophylactic measure against ectoparasites.

One day before the beginning of the treatment, two areas (2 cm²) of the dorsal region were shaved with a shearing machine (comb number 0). The mesotherapy was accomplished in four sessions (in the 3rd, 7th, 10th and 13th days of treatment). One area was used as control and the other area received intradermic infusion of 2 ml of a caffeine solution (2%).

Histological Processing

After 15 days of initiated the treatment the animals were sacrificed by infiltration of anesthetic and segments of skin were removed from the areas, with care taken to collect the underlying hypodermis as well and the skin segments were fixed in Bouin's for 24 h. Each area of treatments had obtained eight non-serial sections of 6-7 μm thickness, which are stained with hematoxylin and eosin (HE) and submitted at morphometric and histology analyzes. Hypodermis thickness was determined from the dermis-hypodermis limit up to the surface fascia; therefore the measurements obtained refer to the areolar layer. Hypodermis thickness was determined by five measurements per cut, being used objective of 40x, ocular millmetered of Zeiss adjusted with a slide object of Zeiss[14].

Statistical Analyzes

For the hypodermis thickness analysis, the Student *t* unpaired test was used, with the level of significance set at 5%[15, 16].

RESULTS AND DISCUSSION

In agreement with CIPORKIN and PASCHOAL[5], as well as KAPLAN[17] the intradermotherapy possess several advantages: loco-regional treatment, avoiding the first hepatic passage; decrease of number dose, number of sessions and side-effects. Therefore, mesotherapy treatment would be an option to reduce the side-effects of caffeine, such as: the risk of provoking nervous excitation with sleep disturbances and problems with diuresis and heart rate[18].

Data in Figure 1 show that the reduction in the hypodermis was observed in the area treated with mesotherapy of caffeine ($p < 0.05$). This treatment also provoked a flattening in the lobes of adipose tissue, taking to the reduction of the hypodermis thickness.

CHEUNG *et al*. [19] and ZHANG and WELLS[20] tested the effect of chronic treatment with caffeine on lipolysis in the adipose tissue of rats and observed a decrease in body weight and in adipocyte diameter in rats treated with caffeine compared to control rats.

The caffeine is an important antagonist of adenosine receptor and an inhibitor of the phosphodiesterase. The inhibition of the adenosine receptor maintain adenylyl cyclase stimulated, increasing synthesis of cAMP that active protein kinase A, and produce phosphorylation of hormone-sensitive lipase (HSL) and perilipin. The caffeine, also inhibit phosphodiesterase which produce cAMP degradation, maintained active the cAMP and consequently the lipolysis.

In the Figure 1 we can observe that the intradermotherapy with caffeine, besides to produce flattening of the lobules of adipose tissue, decreasing the thickness of hypodermis, also produced lyses of the adipocytes.

This damage can be occurring for mechanical forces of infiltration. Although can be observed damage cell in lipoplasty where is realized infiltration of saline, the volume is larger. Beside in previous work in our laboratory shown that does not occur this phenomena when did infiltrate amount of physiologic serum without the drug is small – 2 ml (data not shown).

When the occur lypolisis the molecules of fatty acids were mobilized for sanguine current bind to albumin. If the plasmatic concentration of albumin is low, the solubility of the fatty acids may rise above 1μM. Thus, the fatty acids form beads that act as detergents, rupturing proteins and membrane structures[21].

We should also take into account that the intradermic administration promotes an increase maximal concentration in local[22], diffusion more slow that maintaining its effect for larger time[5]. This could be contributing to a larger action of the caffeine elevating the amount of fatty acids.

Although some references in the literature indicate the caffeine as drug to be used in the mesotherapy[1, 5, 12, 17], we did not find reports demonstrating the effectiveness of this conduct, what difficult the comparison of our data.

Although our data demonstrate the efficacy of mesotherapy with caffeine decreasing the thickness of hypodermis, it should be taken into account the risks that this technique produces. Cases of systemic and local side-effects have been described, such as atypical mycobacterium infection, due to lack of aseptic conditions.

CONCLUSION

The mesotherapy with caffeine was efficient in the reduction of the adipocytes, provoking however, alterations hypodermis, with presence of adipocytes destruction.

REFERENCES

1. ROSSI, A.B. and VERGNANINI, A.L.; Cellulite: a review. **Eur Acad Dermatol Venereol** 14:251-262, 2000
2. CURRI, S.B.; Cellulite and fatty tissue microcirculation. **Cosm Toil**108:51-58, 1993.
3. BINAZZI, M., GRILLI-CICIOLONI, E.; A propósito della cosidetta cellulite e della dermao-paniculopatia edematofibroesclerotica. **Ann It Derm Clin Sper** 31:121-125, 1977
4. BINAZZI, M., Celullite, aspects cliniques et mopho-histologiques. **J Md Esth Et Chir Derm,** 10(40):229-223, 1983
5. CIPORKIN, H., PASHOAL, L.H.; **Atualização terapêutica e fisiopatogência da lipodistrofia ginóide (LDG) 'celulite'**. Livraria Editora Santos, 1992
6. PIRES-DE-CAMPOS, M.S.M.; Fibro-edema gelóide sub-cutâneo, **Ciência & Tecnologia,** 1(2):77-82, 1992.
7. ROSEMBAUM M.; PRIETO, V.; HELLMER, J.; BOSCHMANN, M.; KRUEGER, J.; RUDOLF, L.; SHIP,A.G. An exploratory investigation of the morphology and biochemistry of cellulite. **Plastic Reconst Surg** (7):1934-1939, 1998
8. RYAN, T.J.; Lymphatics and adipose tissue. **Clin Dermatol** 13:493-498, 1995.
9. _____, CURRI, S.P.; Cutaneous adipose tissue. **Clin Dermatol** 7(4), 1989
10. BRAY, G.A.; Obesity: basic consideration and clinical approaches. **Disease a Month.** 35:451-528, 1989
11. POSTERNAK, M.E., UEZEN, M.C., BUSTELO, O.J. Paniculopatia edemato-fibro esclerótica. **Mesoterapia 2000**.2(1):22-25,2000

12. ROSINA, P.; CHIREGATO, C.; MICCOLIS, D.; D'ONGHIA, F.S.; Psoriasis and side-effects of mesotherapy. **Int J Dermtal**. 40(9):581-583 , 2001.

13. OLFERT, ED; CROSS, BM; McWILLIAM, AA. Guide to the care use of experimental animals. **Canadian council on animal care 213***,* Ottawa, Ontario, 1993.

14. DE LACERDA, C.A.M. **Manual de quantificação morfológica: morfometria, alometria,** estereologia,2.ed. Rio de Janeiro, CEBIO, 1994.p.8-13

15. ZAR, **Biostatistica Analysis**. 2nd ed. Hall International, Upper Saddle River, NJ,1984

16. SNEDECOR, G.W. and COCHRAN, W.G., **Statistical Methods**. 6Th Ed. Iowa State University Press. IA. 342-343, 1967

17. KAPLAN, A.; La Mesoterapia em 1999. **Mesoterapia 2000**, 2(1):4-8, 2000

18. MASSEY, LK.; Caffeine and the elderly. **Clinical Pharmacology***,* 13 (1):43-50, 1998.

19. CHEUNG, W., LEE, C., NG, T. Potentiation of the anti-lipolytic effect of 2-chloroadenosine after chronic caffeine treatment. **Pharmacology***,* 36:331-339, 1988.

20. ZHANG, Y.; WELLS, J.N.; The effects of chronic caffeine administration on peripheral adenosine receptors. **The Journal of Pharmacology and Experimental Therapeutics,**. 254 (3):757-763, 1990

21. LIMA, B.L.; VESTRI, H.S.; VESTRI, S.; MORAES, S.M.F.; L; Lipólise. In: CURI, R.; MPOMPÉIA, C.; MIYASAKA, C.K.; PROCOPIO, J.; **Entendendo a gordura – os ácidos graxos***,* São Paulo, Ed. Manole, 2002

22. AUTRET, E.; GUILLOTEAU, D.; CORBE, D.; JONVILLE, A.P.; PEYRON, R.; GARRIGUE,M.A.; BARBIER, P.; Comparison of plasma concentration and tolerance of a single dose of human calictonin by intradermal and subcutaneous administration. **Therapie.** 46(1):5-8, 1991

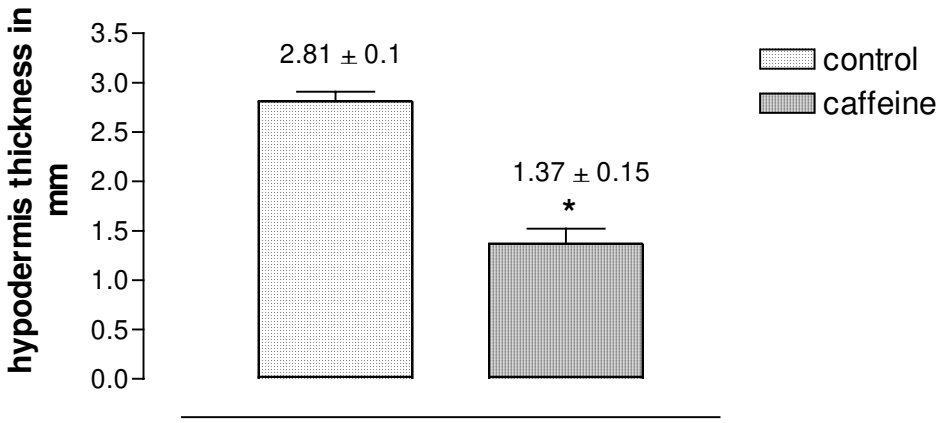
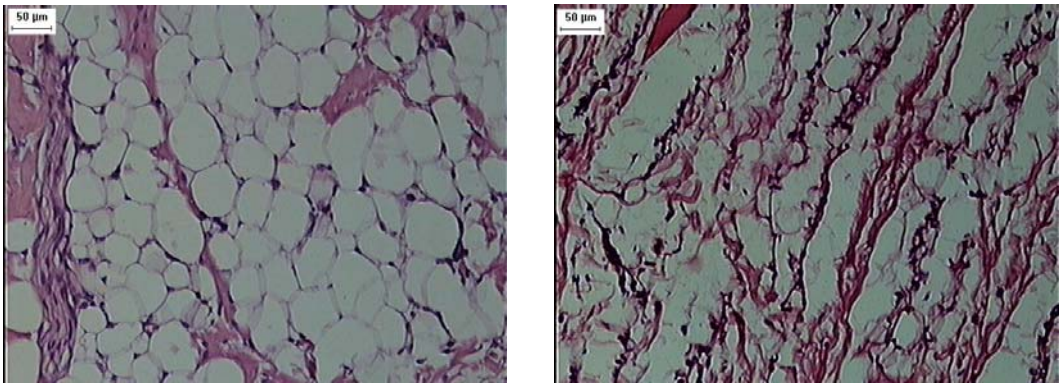


Figure 1. Hypodermis thickness measurements (mean ± SEM, in mm) in the different treatments. Male swine were treated for 15 days. Each animal received all treatments in different areas as follows; control area, mesotherapy with caffeine area. The data were analyzed by Student *t* test unpaired (n=5). * $p < 0.05$.



CONTROL CAFFEINE

Figure 2. Photomicrographs (HE) of the hypodermis after treatments with caffeine. Male swine were treated for 15 days. Each animal receive mesotherapy with caffeine in 2cm² area from the dorsal skin. Another not treated dorsal area was used as control.

LIPOLYTIC RESPONSE OF SUBCUTANEOUS ADIPOCYTES ISOLATED FROM SWINES TREATED DURING FIFTEEN DAYS WITH TOPIC APPLICATION OF CAFFEINE ASSOCIATED OR NOT WITH THERAPEUTIC ULTRASOUND

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ABSTRACT

Cellulites is characterized by structural alterations in dermis and hypodermis. Topical use of caffeine has been indicating for his treatment by being an increase of the lipolysis. Ultrasound is usually employed in cutaneous affections and esthetical conditions such as keloids and hyperthophic scar, cellulites and lipodystrophy and as an accentuator of cutaneous permeation. The aim of this work was verify the lipolytic response of subcutaneous adipocytes isolated from swines treated during fifteen days with topic application of caffeine associated or not with therapeutic ultrasound. For this purpose the following treatments were performed, during fifteen days, to five previously shaved areas of the backs on each of 5 pigs (Landrace x Large White, 35 days old and weighing 15 kg): gel (carbopol 940), gel+US (US), gel+caffeine (5%; CAF), and gel+caffeine+US (US+CAF), daily for 15 days. A 5^{th} untreated area was used as control. Continuous US of 3 MHz with an intensity of 0.2 W/cm² was applied at a rate of 1 min/cm². After the end of treatments adipocytes from each area were isolated and a lipolytic assay was carried out to analyze the sensitivity to isoprenaline. Our results showed that the adipocytes isolated from US+CAF area presented an increase on lipolysis production when submitted to ice melting, and basal incubation. The maximal lipolysis induced by isoprenaline was also increased in adipocytes isolated from US+CAF treated area. Our results allowed us conclude that the US and caffeine application was not effective when applied isolated but the US was effective as an accentuator of caffeine permeation due to a lipolytic effects observed in adipocytes isolated from this area.

Key word: subcutaneous adipocytes, caffeine, phonophoresis, lipolytic response.

INTRODUCTION

The measurement of lipolysis can be used to assess the response of adipose tissue to β-adrenergic agonists and antagonists, or to other physiologically or pharmacologically active compounds[1]. It is well established that at least three β-adrenoceptor subtypes (β_1,β_2 and β_3) coexist in the fat cells of the white adipose tissue of swine[2] or other mammalian species[3,4]. Lipolysis is stimulated when one of several different agonist types interacts with a specific cell membrane receptor the β-adrenoceptors (β-AR), and stimulates Gs proteins with sequential activation of adenylyl cyclase, increases synthesis of cAMP, activation of protein kinase A (PKA), produces phosphorylation of hormone-sensitive lipase (HSL) and perilipins[5-7]. This initiates the lipolytic process, which releases fatty acids and glycerol[1,8-10]. Endogenously, the β-AR are activated by norepinephrine and epinephrine which are the endogenous lipolytic agents. Conversely, A_1 adenosine receptors (A_1R) are cell surface receptors coupled to Gi proteins that inhibit adenylyl cyclase and reduce intracellular cAMP levels. Therefore, the stimulation of A_1R opposes the action of the βAR[11]. Lipolysis may also be inhibited by an increase in activity of the enzyme cAMP-phosphodiesterase which hydrolyze cAMP to AMP and adenosine[1,12].

Caffeine, a xanthine derivative, is an inhibitor of cAMP-phosphodiesterase and act as an antagonist of adenosine receptors[13-15]. For these reasons, caffeine is largely used as agent that potenciate the lipolytic response and it can be found in a number of topic cosmetic preparations.

In order, to reduce the barriers of the skin and improve permeation some chemical and physical agents had been use. A physical agent commonly used as accelerator of the

transdermic transport is the ultrasound, in a process called phonophoresis or sonophoresis[16-18].

The aim of the present study was to investigate whether the treatment during fifteen days with topic application of caffeine, associated or not with ultrasound, in swine would induce any alteration in the basal or stimulated lipolytic response in subcutaneous adipocytes.

MATERIAL AND METHODS

1. Animals

During the experiments, swine were cared for in accordance with the principles outlined by OLFERT *et al.*[19] for the use of animals for research and education, and the experimental protocols were approved by the Committee for Ethics in Animal Research (State University of Campinas – UNICAMP – protocol nº 614-2).

Five male hybrid swine (Landrace x Large White), not castrated, 35 days old and weighing approximately 15 kg were used and obtained from the Alvorada's country property. The animals were fed with water and chow composed by crushed corn, soy flour, meat flour, calcareous, salt, polinucleous Fapec *"ad libitum"*. Ivomec® was applied as a prophylactic measure against ectoparasites.

One day before the beginning of the treatment, the dorsal region areas was shaved with a shearing machine (comb number 0), avoiding the removal of the corneous layer, which could alter the skin permeability.

2. Topic Treatments

After trichotomy, the dorsal region of each animal was divided into five areas of 8 cm^2: control, gel (GEL), caffeine gel (CAF), ultrasound application plus gel (US) and US application plus caffeine gel (US+CAF).

2.1. Gel Area

Gel was applied daily, during fifteen days, on the skin through out circular friction (massage therapy) until hyperemia and increases of the temperature of the skin were achieved. The amount of gel applied to each area was 3 g.

The gel was prepared with 1% Carbopol 940®, 10% propyleneglycol 5%,0.1 M sodium acetate buffer, pH 7.1, 25% absolute ethyl alcohol, and triethanolamine (qsp, pH 7.0).

2.2. Caffeine Gel Area

Caffeine gel was applied daily, during fifteen days, on the skin through out circular friction (massage therapy) until hyperemia and increases of the temperature of the skin were achieved. The amount of gel applied to each area was 3 g.

The caffeine gel was prepared as follows: 5% of anhydrous caffeine (Sigma Chemical Company, St. Louis, MO, USA) was pre solubilized in sodium acetate buffer 0.1 M pH 7.1 containing ethyl alcohol absolute (25%) and propyleneglycol (10%); final solution revealed a pH around 7.0 – 7.5 and was incorporate to gel formulation upper described.

2.3. Ultrasound application

The US (Sonomaster Microcontrolado - KW Ind Nac de Tecnologia Lt, Amparo, São Paulo, Brasil) was applied daily, during fifteen days, over the skin area with gel (US) or caffeine gel (US+CAF) and it was set at 3 MHz, due to superficiality of the adipose tissue[20]; intensity of 0.2 W/cm² [21], continuous emission[22] and application time of 1 min/cm² [23], with the transducer being moved slowly and continuously until the end of the application[24].

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*.[25], 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. 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 a frequency of 3 MHz and a power of 0.8 W (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, pondering the variation of 10% in the US apparatus and 0.07% in the balance. The procedure was realized five times for each kind of gel. The presence of gel (carbopol 940®) or gel added of caffeine (5%) did not produce the attenuation of the US intensity evaluated by the transmissibility test (data not shown).

3- Adipocytes isolation and lipolysis measurements

The animals after being exposed to the treatment for 15 days were sacrificed by infiltration of anesthetic and the adipocytes of the different areas were isolated for the lipolytic activity analysis. Adipocytes were isolated from subcutaneous fat pads based on modifications of RODBELL's original procedures[26]. Adipose tissues from each area were weighed, divided in 5 samples of 2-3g, then each sample was minced and digested in five 20 ml polyethylene vials containing 6 ml of Krebs Ringer bicarbonate buffer, 25 mM HEPES, 6mM glucose pH 7,4 (KRB buffer) with 1 mg/ml collagenase (type 2, *Clostridium histoliticum,* Sigma), 3% bovine serum albumin (BSA, fraction 5, fatty acid-free, Sigma, Chemical.Co., St. Louis, MO, USA) - KRBA. The vials were shaken at 60 cycles/min at 37ºC during 45 min. The resulting cell suspension was filtered through a nylon mesh (200 µm) and washed tree times with 10 ml of KRBA. The cell suspensions from five different vials were then mixed and washed. Each wash had an interval of 3 minutes. An aliquot of the final cellular suspension was counted in Mallasez chamber.

Aliquots of the cell suspension (100,000 cells) were distributed in polyethylene vials with capacity for 2 ml containing fresh KRBA with 10 µl of isoprenaline (10 nM to 10 µM) for 60 min with gentle shaking of 60 cycles/min) in water bath, at 37^{o}C. The final volume was of 1 ml. Isoprenaline at suitable dilution was added to the cell suspension just before the beginning of the assay. Tubes containing cells and KRBA buffer, without agonist, were carried out to obtain basal glycerol production. After incubation, the tubes were placed in melting ice, the floating adipocytes were discarded and the infranatant was assayed for glycerol measuring.

The lipolysis produced by technical handle manipulation was analyzed preparing tubes containing cells (100,000 cells) and KRBA buffer which were immediately placed in melting ice. This procedure has the objective to stop all enzymatic reactions[27]. These tubes were maintained in melting ice during 75 min, after this time floating adipocytes were discarded and the infranatant was assayed for glycerol measuring.

Lipolysis was quantified by measuring the release of extra cellular glycerol. Aliquots of the infranatant (100 μl) were used for glycerol determination. Each incubation vial was run in triplicate, and the results are the means of experiments from different animals performed on different days.

The glycerol produced is measured by coupled enzyme reactions catalyzed by glycerol kinase, glycerol phosphate oxidase and peroxidase. The enzymatic reactions involved in the assay are as follows[28, 29]. Glycerol is phosphorylated by adenosine triphosphate (ATP) forming glycerol-1-phosphate and ADP in the reaction catalyzed by glycerol kinase. Glycerol-1-phosphate is then oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. A quinoneimine dye is produced by the peroxidase catalyzed coupling of 4-amonoantipyrine (4-AAP) and sodium N-ethyl-N-(3-sulfopropyl)m-anisidene (ESPA) with hydrogen peroxide**,** which shows maximum absorbance at 540 nm. The increase in absorbance at 540 nm is directly proportional to the glycerol concentration of the sample. (Catalog number 02700-LABORLAB AS, Guarulhos, SP-BRASIL).

Half-maximal effective isoprenaline concentration values (EC_{50}) were obtained and expressed as pD_2 values (- log EC_{50}). The results are expressed as means of μmol glycerol

produced by 10^6 cells during 60 min of incubation time. Concentration-curve effects were plotted as percentage of isoprenaline maximal effect in control group.

4. Statistical analysis

Data were analyzed statistically by one-way analyses of variance followed by the Newman-Keuls test[30, 31] or by Stundent´s *t* test for unpaired samples[32, 33]. Differences were considered significant at $p<0.05$.

RESULTS

Melting ice induced a significant increase on glycerol production by adipocytes isolated from the skin area submitted to caffeine associated to US treatment (control: 0.40 $\pm$ 0.03, GEL: 0.39 $\pm$ 0.04, US: 0.57 $\pm$ 0.08, CAF: 0.47 $\pm$ 0.05, US + CAF: 0.94 $\pm$ 0.25 μmol glycerol.10^6 cells/75 min; Figure 1). None of the other treatments induced any significant alteration in the glycerol release from adipocytes maintained in melting ice.

The basal glycerol production was also significantly higher in adipocytes isolated from the area submitted to caffeine associated to US treatment (control: 0.49 $\pm$ 0.02, GEL: 0.61 $\pm$ 0.04. US: 0.48 $\pm$ 0.08; CAF: 0.51 $\pm$ 0.05; US+CAF: 0.76 $\pm$ 0.11 μmol glycerol.10^6 cells/60 min; Figure 2).

None of the treatments altered the sensitivity of adipocytes to isoprenaline. However the maximal lipolytic response induced by isoprenaline was significantly increased in adipocytes isolated from the skin area submitted to caffeine associate with therapeutic ultrasound (Table 1).

The analysis of the dose-response curves show that adipocytes isolated from GEL and from CAF area treatments present a significant reduction of lipolysis induced by 0.1 and 1 μM isoprenaline (Figure 3 A and C), whereas the treatment of ultrasound associated to caffeine produces a significant increase of lipolysis induced by 0.1 and 1 μM to isoprenaline (Figure 3 D).

DISCUSSION

In the last several years, body-contouring management has been the subject of many innovative ideas. Numerous publicities promise decreases the local fat through physical treatment[34, 35]. However this effect remain controversial because exist few research with scientific validation[36].

Researches on this field are performed in swine skins since there are anatomical and physiologic similarities between human and swine skin [37]. The similarity in the response to β−adrenergic agonists and antagonist in human suggests that the proportion of β_1, β_2 and β_3 is similar to the swine[2].

The figure 1 shows that the phonophoresis of caffeine produce raised glycerol production in the aliquots submitted in melting ice. However this procedure has the objective to stop all enzymatic reactions, we cannot say that this didn't occurred, since in other groups there was not increase in the glycerol production. WATSON and MORRIS[38] mention that damage to cell structure and function arising from a sudden reduction in temperature depends on cell-type, but reflects the underlying structural and biochemical damage which has been inflicted by rapid cooling. In particular, membranes lose their selective permeability with the result that many cellular components are released including

lipids, proteins and ions. Additionally, sodium and calcium gain access to the interior of the cell[39]. Consequent upon this initial disruption, metabolic activities are diminished and further secondary changes ensue. This is influenced by membrane composition, and much experimental evidence points to particular involvement of membrane lipids. One hypothesis implicates lipid phase changes in a cooling rate dependent loss of membrane integrity.

As the melting ice effects on lipolysis were observed only on adipocytes isolated from US+CAF area probably this association induces fragility on membrane cells adipocytes. This phenomenon occurred, probably, for the effect cytotoxic of the fatty acids liberated by the lipolysis induced by the caffeine. Works previous in our laboratory demonstrated that the treatment of ultrasound with caffeine gel produced lysis in the adipocytes due to the effect cytotoxic the fatty acids. We should take into account that the number of incubated cells was the same for all of the experimental groups, discarding the hypothesis of have incubated destroyed cells. However, the adipocytes of the group US + CAF incubate in ice could be with their fragile membranes due to the large amount of fatty acids.

Our results also showed that the adipocytes isolated from area submitted the US + CAF presented a basal glycerol production increased significant (figure 2). The hormones that affect the lipolysis acutely in adipocytes are the catecholamines (epinephrine and norepinephrine) and the insulin[40]. The regulation of the lipolysis for the catecholamines involves the stimulation of the adenylate cyclase for the subtypes β-AR and the inhibition for the subtype α_2-AR[41]. Besides, paracrine mediators, such as the adenosine, they present potent action antilipolytic[42]. The adenosine liberated endogenously if it links to A_1R of the plasmatic membrane of the adipocytes and they inhibit the adenylate cyclase, taking to a

tonic inhibition of the lipolysis[43,44]. The deamination of the adenosine in iosine, for the enzyme adenosine deaminase, increases the lipolysis in adipocytes[43].

The basal lipolysis is inhibited preferentially by the adenosine. The insulin, same being a potent substance anti-lipolytic doesn't affect the basal lipolysis. Morimoto studying isolated adipocytes of mice demonstrated that the insulin inhibits only the lipolysis induced by the agents lipolytics, such like the noradrenaline and ACTH, however no the basal lipolysis that it happens in the absence of agents lipolytics. As already mentioned above, the adipocytes of swine submitted to the treatment of US + CAF presented an expressive increase in the basal lipolysis, what can be explained by the antagonistic action of the caffeine on the adenosine receptors.

PEDINI and ZAIETTA[45] observe increase of plasmatic glycerol and fatty acids in individuals submitted to the local massage by 20 minutes. LUCASSEN *et al.*[46] through measure by ultrasound imaging verify decrease in the adipose tissue of the thigh submitted to mechanical massage. CHANG *et al.*[35] through corporal circumference analyses observed that the massage is an effective method for fat mobilization and body contouring. However other researches observed no results using this treatment[34, 36, 47].

The analysis of dose-response curves showed that adipocytes isolated from GEL and from CAF area treatments present a significantly reduction of lipolysis induced by 0.1 and 1 μM to isoprenaline (Figure 3 A and C). The manipulation, induced by massage (GEL and CAF area) of the skin and of the fascia exerts an effect reflex on the muscles of the arterioles, what activates a contraction additional reflex, following by dilatation[48]. This arterial dilation is predominately mediated via the activation of adenosine receptors[49]. Being the adenosine an agent anti-lipolytic, the reduction of response to isoprenaline can

be explained. This result reinforces the evidence that caffeine is not easily permeate through the skin and needs US association to exert its effects.

The massage as promoter of the cutaneous permeation to caffeine cannot be observed, since there is no alteration on basal lipolysis in adipocytes isolated from CAF area. Besides, the maximal response induced by isoprenaline in adipocytes isolated from this treated area was significantly reduced when compared to control area adipocytes. Several authors mentions that the increase of temperature is a promoter of the permeation due to enlarger the pores and intercellular spaces[16, 50, 51]. The concentration-response curve to isoprenaline in adipocytes isolated from GEL and CAF area showed the same profile (figure 3A and C). These results, in this time studied, contradict LAMBERT[52] that obtained decrease of circumference of knee and perithrocanter region with topical application of caffeine gel (5%).

Our results showed that the ultrasound treatment did not produce effect lipolytic, stimulate or not by isoprenaline, produced by adipocytes isolated from this area. However several authors mention the use of ultrasound for body-contouring[53-55]. The defenders of the ultrasound application as a reducer of the corporal fatty justify these effects due to cavitations mechanism. Cavitations can easily be observed in liquid media due to local variations in acoustic pressure induced by the ultrasound wave generate small bubbles. The cavitation can be of two types: stable in response to the regularity of the pressure changes and unstable when there is a violent implosion of bubbles if the peack of the intensity goes. The unstable cavitation loud provokes the production of free radicals[51, 56]. ROHRICH *et al.*[57] through histological and enzymatic analyses didn't observe any effect of the ultrasound and of the massage in the adipocytes cellular destruction. Though the ultrasound only produces effect in the tissue that absorbs their waves. The absorption

coefficient of the adipose tissue is very low, being above only of the water and of the blood[21, 58]. Besides, GLICK *et al*.[59] didn't observe significant changes in cAMP in skin, lung and peritoneal cells of mouse submitted to treatment with ultrasound.

The increase on basal glycerol production (155% to control) and maximal response to isoprenaline (152%) in the area submitted to caffeine associated to US treatment demonstrates the effectiveness of the ultrasound in increase the permeation cutaneous of drugs. The promotion of the permeation occur mainly mechanic effect. The mechanisms involved with the mechanical effects are cavitation, radiation fork and acoustic microflow[60].

The concentration-response curve show an shift to the right, demonstrating a tendency of increasing the sensibility of the receptors, although it has not had significant differences in the pD_2 value for this time of treatment. The increase on basal glycerol production and maximal response to isoprenaline is clearly an effect induced by caffeine, as an inhibitor of adenosine receptors (basal glycerol increase) and as an phosphodiesterase inhibitor (increasing the isoprenaline lipolytic effects).

With our protocol treatment (based on clinical ones) we can concluded that caffeine exerts its effect on lipolysis only when associated with US application and also that this effect is local because no alteration on lipolysis is observed in any other area.

The results presented here suggest that the effect lipolytic of the caffeine only occur with association of the ultrasound, demonstrated that the ultrasound is important promoter of the cutaneous permeation. The treatment of caffeine isolated or associated with ultrasound, as well as the massage and ultrasound no produce alterations in sensitivity of tissues to isoprenaline in adipocytes isolated of swines.

REFERENCES

1. MERSMANN, H.J. 1992. Adenosine-theophylline enteractions on the lipolytic response to beta-adrenergic agonists in porcine adipose tissue. *Com. Biochem. Physiol*., **103**C: 541-547.

2. McNEEL, R.L.; H.J. MERSMANN. 1999. Distribution and quantification of $Beta_1$, $Beta_2$ and $Beta_3$ receptor subtypes transcripts in porcine tissues. *J. Anim. Sci.,* **77**:611-621.

3. LANGIN, D., M PORTILLO; J.S. SAULNIER-BLANCHE and M LAFONTAN. 1991. Coexistence of three beta-adrenergic receptor subtypes in white fat cells of various mammalian species. *Eur. J. Pharmacol*. **199**:191-301.

4. CARPÉNÉ, C.; I CASTAN, P. COLLON, J. GALITZKY, J. MORATINOS and M. LAFONTAN. 1994. Adrenergic lipolysis in guinea pig is not a β_3 adrenergic response: comparison with human adipocytes. *Am. J. Physiol*., **266**:R905-R913.

5. LONDOS, C., D.L.BRASAEMLE, C.J. SCHULTZ, J.P. SEGREST, A.R. KIMMEL. 1999. Perilipin, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Cell & Developmental Biology*. **10**:51-58.

6. CLIFFORD, G.M.; C.LONDOS, F.B. KRAEMER, R.G. VERNON. 2000. Translocation of homone-sensitive lipase and perilipin upon lipolytic stimulation of rat adipocytes. *J Biol. Chemistry*. **275**:5011-5015.

7. HOLM, C.; T. OSTERLUND, H. LAUREL, J.A. CONTRERAS. 2000. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Annu Rev Nutr* **20**:365-93.

8. AKANBI, K.; H.J. MERSMANN. 1996. β-Adrenergic receptors in porcine adipocyte membranes: modification by animal age, depot site, and dietary protein deficiency. *J Anim Sci*. **74**:551-561

9. DOLE, V.P.; and H. MEINERTZ. 1960. Microdetermination of long-chain fatty cells in plasma and tisues. *J. Biol. Chem.* **235**:2595-2599.

10. FARIAS-SILVA, E.; D.M. GRASSI-KASSISSE, V. WOLF-NUNES and R.C. SPADARI-BRATFISCH. 1999. Stress-induced alteration in the lipolytic response to β-adrenoceptor agonists in rat white adipocytes. *J Lipid Res.* **40**:1719-1727.

11. MERSMANN, H.J.; G.B. CAREY, E. O'BRIAN SMITH. 1997. Adipose tissue β-adrenergic and A_1 adenosine receptors in suckling pigs. *J. Anim. Sci*., **75**:3161-3168.

12. ZHANG, X. and G. B. CAREY. 2004. Plasma membrane-bound cyclic AMP phosphodiesterase activity in 3T3-L1 adipocytes. *Comp Biochem Physiol Part B* **137**: 309-316.

13. CHEUNG, W.T.; C.M. LEE, NG, T.B. 1988. Potentiation of the anti-lipolytic effect of 2-chloroadenossine after chronic caffeine treatement. *Pharmacology* **36**: 331-339.

14. MASSEY, L.K. 1998 Caffeine and the elderly. *Drugs & Aging* **13**: 43-50.

15. SINCLAIR, C.J.D. and J.D. GEIGER. 2000. Caffeine use in sports. *J Sports Med Phys Fitness*, **40**:71-79.

16. BYL, N.N. 1995. The use of ultrasound as na enchancer of transcutaneous drug Delivery phonophoresis. *Phys. Ther*., **75**: 539/89-553/103.

17. A. BOUCAUD, L. MACHET, B. ARBEILE, B. MACHET, M.C. SOURNAC, M. MAVON, F. PATAT, L VAILLANT. 2001 In vitro study of low-frequency ultrasound-enhanced transdermal transport of fentanyl and caffeine across human and hair less rat skin. *Int J Pharm* **228**:69-77.

18. D. MONTI, R. GIANNELLI, P. CHETONI, S. BURGALASSI. 2001 Comparison of the effect of ultrasoud on transdermal permeation of caffeine and morphine through hairless mouse skin in vitro. *Int J Pharm* **229**:131-137

19. OLFERT, ED, B.M. CROSS, A.A. McWILLIAM. 1993. Guide to the care use os experimental animals. *Canadian council on animal care 213,* Ottawa, Ontario.

20. FYFE, M.C. 1979 A study of the effects of different ultrasonic frequencies on experimental o edema. *Aust. J. Physiother*. **25** 5:205-207

21. WILLIAMS, A. R. 1983 *Ultrasound:Biologic effects and potential hazards.* Academic Press, New York.

22. CAMERON, M.H., L.G. MANROE. 1992 Relative transmission of ultrasound by media customarlly used of phonophoresis. *Phys. Ther.*, **72**:142-148.

23. OAKLEY, E. M. 1978 Apllication of continuous beam ultrasound at therapeutic levels. *Physiother.*, **68** 8:169-172

24. BYL, N. N., A. McKENZIE, B. HALLIDAY, B., T. WONG, J.O. O'CONNELL. 1993. The effects of phonophoresis with corticosteroids: a controlled pilot study, *J Orthop Sport Phys Ther*, **18** 5:590-600.

25. GUIRRO, R.R.J., CANCELIERI, A.S.; SANT'ANNA, I.L. 2001. Avaliação de meios intermediários usados na aplicação do ultra-som terapêutico. *Rev. Bras. Fisioter.*, **5**: 49-52

26. RODBEL , M. 1964 Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239**: 375-380.

27. SAITO, Y., N. MATSUOKA, A. KUMAGAI, H OKUDA, S. FUJII. 1978. Studies on reduciton of lipolysis in adipose tissue on freezing and thawing. *Endocrinl. Jpn*. **25**: 13-18

28. McGOWAN, M.W.; J.D. ARTISS, D.R. STRANDBERGH, B. ZAK. 1983 A peroxidase-coupled method for the colorimetric determination of serum triglycerides. *Clin Chem*. **29**: 538-542

29. FOSSATI, P. and L. PRENCIPE. 1982. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clin Chem*. **28**: 2077-2080.

30. RIBEIRO, G.A.; L.O.RODRIGUES, M.C. MOREIRA, E. SILAMI-GARCIA, M.R. PASCOA, F.F. CAMARGO. 2004. Thermoregulation in hypertensive men exercising in the heat with water ingestion. *Braz J Med Biol Res*. **37**: 409-417, 2004

31. GODFREY, K.; Comparing the means of several groups. *New Eng J Med*., **313**: 1450-1456, 1985

32. ZAR, J.H. 1984. *Biostatistical Analysis*. 2nd ed. Hall International, Upper Saddle River, NJ.

33. SNEDECOR, G.W. and W.G. COCHRAN. 1967. *Statistical Methods*. 6Th Ed. Iowa State University Press. IA. 342-343.

34. HEYTERS, C. and S. SPIERCKEL. 1987. Effets du massage, de l'exercice et de l'électrositmulation sur la graisse sous-cutanée locale. *Kinésth. Sci*. **261**:45-54

35. CHANG, P., J. WISEMAN, T. JACOBY, A.V. SALIBURY and R.A. ERSEK. 1998. Noninvasive mechanical body contouring: (endermologie) A one-year clinical outcome study update. *Aesth. Plast. Surg*. **22**:145-153

36. COLLIS, N., E.A. ELLIOT, C. SHARPE and D. SHARPE. 1999. Cellulite treatment: a myth or reality: a prospective randomized, controlled trial of two therapies, endermologie and aminophylline cream. *Plast Reconstr Surg*. **104**: 1110-1114

37. HOLLANDER D. A.; H.J. ERLI, A. THEISEN, S. FALK, T. KRECK, S. MULLER. 2003. Standardized qualitative evaluation of scar tissue properties in an animal wound healing model. *Wound Rep. Reg.* **11** 2:150-157.

38. WATSON, P.F. and G.F. MORRIS. 1987. Cold shock injury in animal cells. *Symp. Soc. Exp. Biol.*, **41**:311-40

39. ASLANDI, K.B., G.V.ASLANDI, D.M. VACHADZE, V.P. ZINCHENKO, Yua LAVAS, T.V. POTAPOVA. 1997. A possible role of cold-induced ionic stress in cold-induced cell death. Membr. *Cell Biol.* **11**: 57-76

40. LARGE, V., P. ARNER. 1998. Regulation of lipolysis in humans; pathophysiological modulation in obesity, diabetes and hyperlipidaemia. *Diabetes metab.* **24**:409-418

41. LEIBEL, R.L., N.K. EDENS, S.K. FRIED. 1989. Physiological basis for the control of body fat distribution in humans. *Annu Rev Nutr* **9**:417-443, 1989

42. CASTAN, I. 1994. Antilipolytic effects of $alpha_2$-adrenergic agonists, neuropeptide Y, adenosine, and PGE_1 in mamml adipocytes. *Am. J. Physiol* **266**:R1141-1147

43. HONOR, R.C.; G.S. DHILLON, C. LONDOS. 1985a. cAMP-dependent protein kinase and lipolysis in rat adipocytes. I. Cell preparation. Manipulation, and predictability in behavior. *J Biol Chem* **260**:15122-15129

44. HONOR, R.C.; G.S. DHILLON, C. LONDOS. 1985b. cAMP-dependent protein kinase and lipolysis in rat adipocytes. II. Definition of steady-state relationship with lipolytic and antilipolytic modulators. *J Biol Chem* **260**:15130-15138

45. PEDINI G. and P. ZAIETTA. 1975. Su alcuni aspetti della attivazione della lipolisi tessutale da parte di fattori meccanici. *Min Med.* **66**:318-323

46. LUCASSEN, G.W.; W.L.N. VAN DER SLUYS, J.J. VAN HERK, A.M. NUIJS, P.E. WIERENGA, A.O. BAREL, R. LAMBRECHT, R. 1997. The effectiveness of

massage treatment on cellulite as monitored by ultrasound imaging. *Skin Research and Technology*, **3**: 154-160.

47. BENELLI, L., J.L. BERTA, C. CANNISTRA, P. AMRAM, G. BENHAMOU. 1999. Endermologie: humoral repercurrions and estrogne interaction. *Aesth Plast Surg*. **23**:312-315

48. CASSAR, M.P., 2001. *Manual de massagem terapêutica*. Manole, São Paulo, Brasil,

49. TABRIZHI, R. and S. BEDI. 2001. Pharmacology of adenosine receptors in the vasculature. *Pharmacol and Therapeutics*. **71**:113-147

50. KOST, J., D. LEVY, R. LANGER. 1989. Ultrasound as an transdermal enhancer, in: BRONAUGH, R. L. and H.I. MAIBACH. Percutaneous Absorption; Mechanism – Methodology – Drug Delivery. Marcel Dekker. New York

51. MITRAGOTRI, S; D. BLANKSCHTEIN, R. LANGER. 1997. An explanation for the variation of the sonophoretic transdermal transport enhancement from drug to drug. *J. Pharm. Sci.*, **86**: 1190-2.

52. LAMBERT, J. 1982. Traitment par la percutaféine des lipodystrophis localisées. *Comptes Rendus de Thérpeutiques et de Pharmacologie Clinique* **1**:3-6

53. MENDES, F.H.M. 2000. External ultrasound-assisted lipoplasty from our own experience. *Aesth Plast Surg*. **24**:270-274

54. LA TRENDA, G.S. 1999. Endermology versus liposuction with external ultrasound assist. *Aesth Plast Surg.* **16**:124-129

55. KINNEY, B.M. 1999. Body contouring with external ultrasound. *Plast Reconstr Surg.* **103**:728-729

56. SIMONIN, J.P. 1995. On the mechanisms of in vitro and in vivo phonophoresis. *J. Control Release* **33**:125-141

57. ROHRICH, R.J., D.E. MORALES, J.E. KRUEGER, M. ANSARI, O. OCHOA, J. Jr ROBINSON, S.J.BERAN. 2000; Comparative lipoplasty analysis of in vivo-treated adipose tissue. *Plast. Reconstr. Surg*. **105**: 2152-2158.

58. LOW, J. and A. REED. 2001. *Eletroterapia Explicada – Princípios e prática.* 3rd Manole, São Paulo, Brasil.

59. GLICK, D., A. ADAMOVICS, P.D. EDMONDS, J.C. TAENZER. 1979. Search for biochemical effects in cells and tissues of ultrasonic irradiation of mice and of the in vitro irradiation of mouse peritoneal and human aminiotic cells. *Ultrasound Med. Biol*. **5**:23-33

60. YOUNG, S. 1996. Terapia por Ultra-som; In: KITCHEN,S. and S. BAZIN. *Eletroterapia de Clayton*, 10th, Manole, São Paulo, Brasil

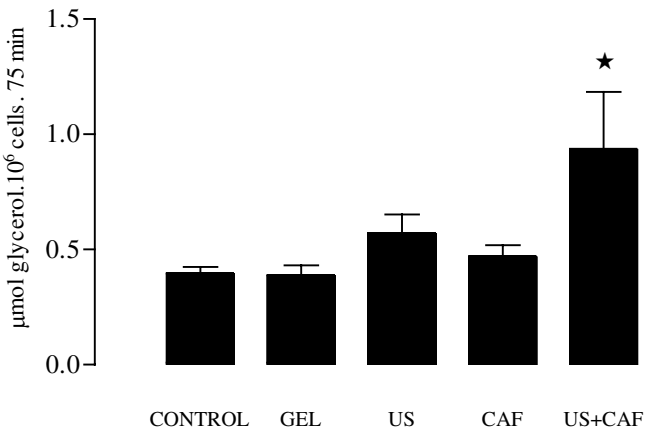

Figure 1. Glycerol production by adipocytes isolated from subcutaneous area submitted to different treatments and after 75 min in melting ice. Each animal received all treatments in different areas as follows; control, US, CAF, US+CAF. Values are means $\pm$ SEM of experiments performed in triplicate. Statistical analysis was performed using ANOVA plus Newman-Keuls tests. * Significantly different from the other groups, at $p < 0.005$.

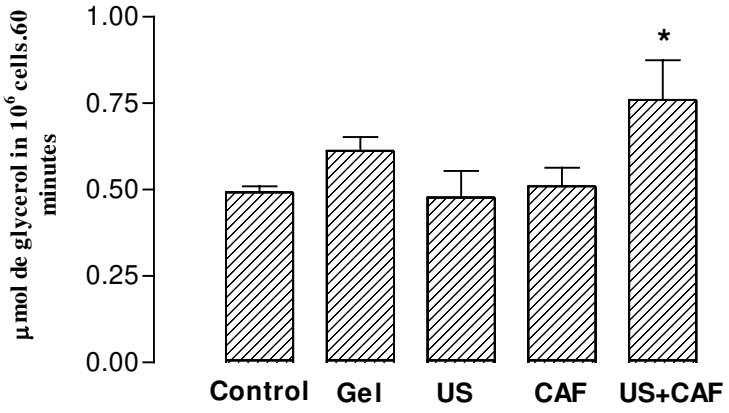

Figure 2. Basal glycerol production by adipocytes isolated from subcutaneous area submitted to different treatments. Each animal received all treatments in different areas as follows; control area, gel + US area, gel + caffeine area, gel + caffeine + US area. Values are means $\pm$ SEM of experiments performed in triplicate. Statistical analysis was performed using ANOVA plus Newman-Keuls tests.

* Significantly different from control, US and CAF the other groups, at $p < 0.005$.

Table 1- Lipolytic potency and maximal lipolytic responses to isoprenaline in subcutaneous adipocytes isolated from swines.

Treatments Area	Isoprenaline	
	pD_2	E_{max}
Control	7.74±0.34	0.25±0.01[a]
Gel	7.39±0.41	0.19±0.03[a]
Ultrasound	7.95±0.24	0.20±0.03[a]
Caffeine	7.62±0.31	0.18±0.02[a]
Caffeine plus ultrasound	7.35±0.20	0.38±0.06[b]

The values are means ± SEM of 5 experiments performed in triplicate. The potencies of the different treatments were evaluated by their EC_{50}, which corresponds to the concentration of agonist inducing 50% of maximal lipolysis, expressed as pD_2 (- log EC_{50}). E_{max} is the maximal responsiveness minus basal lipolysis (control: 0.49 ± 0.01, gel: 0.61 ± 0.04. ultrasound: 0.48 ± 0.08; caffeine 0.51 ± 0.05; caffeine plus ultrasound: 0.76 ± 0.11 μmol glycerol.10^6 cells/60 min). Statistical analysis was performed using ANOVA plus Newman-Keuls tests. Values followed by different letters are significantly different ($p<0.05$).

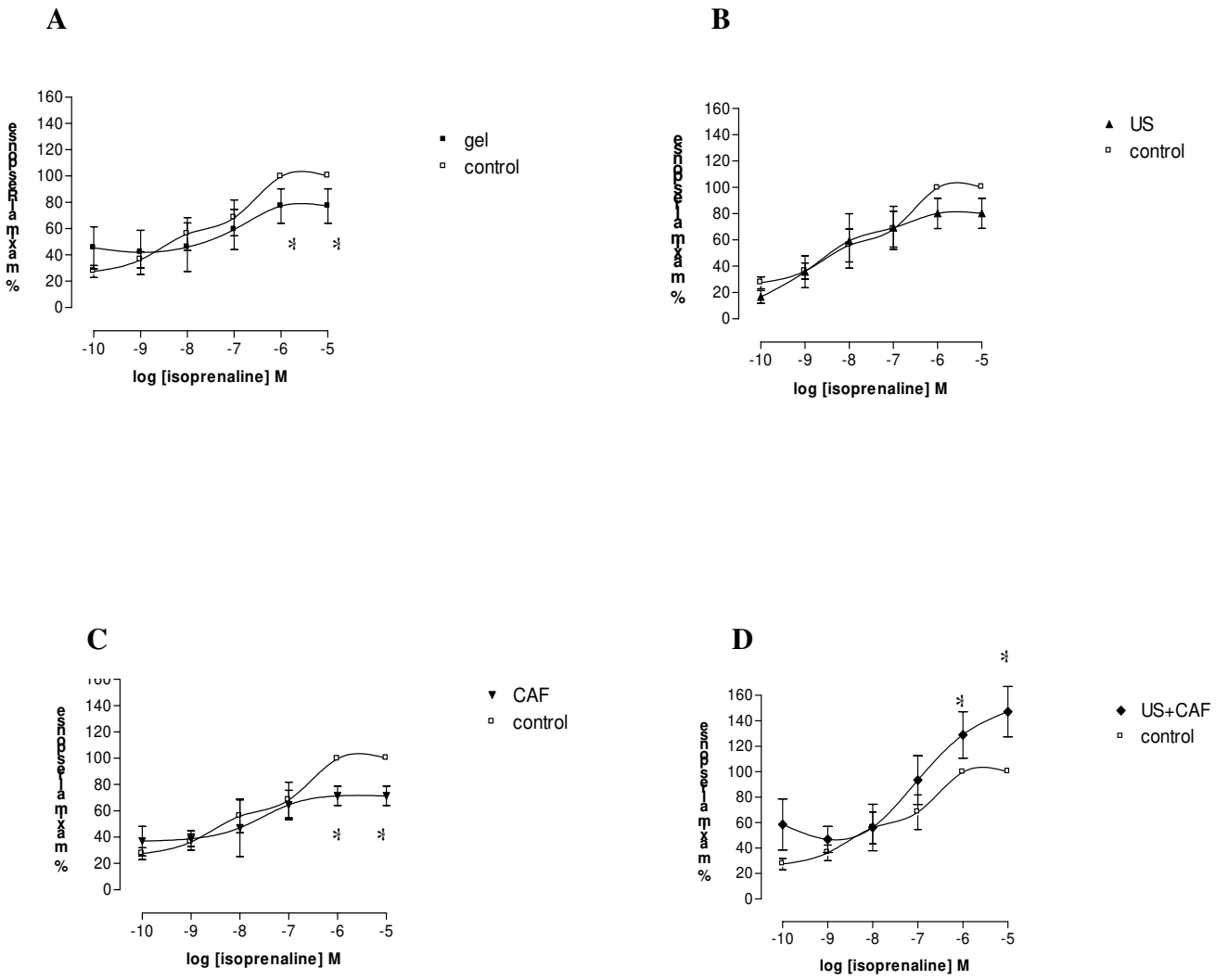

Fig. 3. Concentration-response curves for stimulation of glycerol release subcutaneous adipocytes of control (□) and submitted to different treatments, gel (■), ultrasound (▲), caffeine (▼) and ultrasound plus caffeine ( ) . For the several treatments the values were normalized by assuming the maximal effect of treatment in the control groups to be 100%. Data are means and vertical bars represent the SEM. * $p<0.05$ when compared with control values in the same agonist concentration.

CONCLUSÕES

- Os resultados obtidos a partir do estudo *in vitro*, utilizando célula de difusão nos levam a conclusão de que o ultra-som acentua e acelera significativamente a permeação da cafeína através da pele, permitindo então a realização da fonoforese com este fármaco no tratamento de lipodistrofias.
- As análises histológicas da pele de suínos submetidas aos diferentes tratamentos demonstraram que o ultra-som foi eficaz em aumentar a permeação cutânea à cafeína, facilitando assim sua ação. Os resultados obtidos demonstraram que a cafeína aplicada desta forma levou a uma redução significativa da espessura da hipoderme e que esta redução está relacionada a uma redução significativa no número de adipócitos presentes nesta região.
- A aplicação intradérmica de cafeína produz redução na espessura do tecido adiposo subcutâneo de suínos promovendo lise dos adipócitos.
- O estudo dos adipócitos isolados do tecido adiposo subcutâneo de suínos evidenciou, mais uma vez, a eficácia do ultra-som em promover a permeação cutânea, demonstrado pelo aumento da lipólise basal e da resposta máxima estimulada pela isoprenalina.
- A redução drástica da temperatura, associada à ação da fonoforese com cafeína produz, provavelmente, lise dos adipócitos.
- A aplicação tópica da cafeína, no tempo de tratamento estudado, não produz nenhuma alteração no processo de lipólise.

- A aplicação do ultra-som, sem a utilização de fármacos, não leva a nenhum efeito sobre os parâmetros analisados provavelmente devido a baixa absorção do ultra-som pelo tecido adiposo.

BIBLIOGRAFIA

ARNER, P.; KRIEGHOLM, E.; ENGFELDT, P. & BOLINDER, J. Adrenergic regulation of lipolysis in situ at rest and during exercise. **J. Clin. Invest.**, 85: 893-898, 1990a.

ARNER, P; HELLSTROM, L.; WAHRENBERG, H. & BRONNEGARD, M. Beta-adrenoceptor expression in human fat cells from different regions. **J. Clin. Invest.**, 86: 1595-1600, 1990b.

ARNER, P. Adrenergic receptor function in fat cells. **Am. J. Clin. Nutritional**, 55: 2285-36S, 1992.

ARNER, P. Differences in lipolysis between human subcutaneous and omental adipose tissues. **Ann. Med**. 27, 435-438, 1995

BARE, A.C.; McANAW, M.B.; PRITCHARD, A.E.; STRUEBING, J.G.; SMUTOK, M.A.; CHRISTIE, D.S.; DOMENECH, M.A.; BARE, M.A.; BLOODWORTH, M.L.; SEAL, L.A.; Phonophoretic delivery of 10% hidrocortisone through the epidermis of humans as determined by serum cortisol concentrations. **Physical Therapy,** 76(7):738-743, 1996.

BELILOWSKY, M.; Cellulite et caféine precutanée: études récentes sur percutaféine. **Compres Rendus de Thérapeutique et de Phamacologie Clinique**. 6(60):23-27, 1988

BLANK, I.H, SCHUPLIN, R.J. Radionale and approachesto skin permeation. In: ZATZ,J.L. **Skin permeation fundamentals and application**, 1993.p.11-125.

BLOOM, W.; FAWCETT, D.W.; **A textbook of histology**. Cap. 6., 12ª ed., New York, Ed. Chapman & Hall, 1994

BRONAUGH, R. *In vitro* tecnics for percutaneous absorption. In: BROUNAUGH, R.L & MAIBACH, H.I.; **Percutaneus Absorption,** 2ª ed., New York, Ed. Dekker, 1989.

BOWEN, W.P.; FLINT, D.J. & VERNON, R.C. Regional and interspecific diferences in the ligand binding properties of adrenergic receptors of individual white adipose tissue depots in the sheep and rat. **Biochem, Pharmacol.**, 44(4): 681-686, 1992.

BYL, N.N.; The use of ultrasound as na enhancer for transcutaneous drug delivery: Phonophoresis. **Physical Therapy**, 75(6):539/89-553/103, 1995.

BOUCAUD, A.; MACHET, L.; ARBIELLE, A.; MACHET, M.C.; SOURNAC, M.; MAVON, A.; PATAT, F.; VAILLANT, L.; In vitro study of low-frequency ultrasound-enhanced transderam transport of fentanyl and caffeine across human and hairless rat skin. **Int J Pharm**., 228:69-77, 2001

CAGNIE, B.; VINCK, E.; RIMBAUT, S. et al.; Phonophoresis versus topical application of ketoprofen: comparison between tissue and plasma levels. **Physical Therapy,** 83(8):707-712, 2003.

CLIFFORD, G.M.; LONDOS, C.; KRAEMER, F.B. et al.; Translocation of hormone-sensitive lipase and perilipin upon lipolytic stimulation of rat adipocytes. **The Journal of Biol. Chemistry.** 275(7):5011-5015, 2000

COMMERFORD, S.R.; PAGLIASSOTTI, M.J. et al., Fat oxidation, lipolysis, and free fatty acid cycling in obesity-prone and obesity-resistant rats. **Am. J. Physiol. Endocrinol. Metab.**, 279:E875-885, 2000

CURRI, S.B. Cellulite and fatty tissue microcirculation. **Cosm. Toil.**108:51-58, 1993.

_____, BOMBARDELLI, E. Proposed etiology and therapeutic management of local lipodystrophy and districtual microcirculation. **Cosm. Toil.** 109:51-62,1994.

DING, S.T.; McNEEL, R.L.; O'BRIAN SMITH, E. et al., Modulation of porcine adipocyte β-adrenergic receptors by an β-adrenergic agonist. **J. Anim Sci.,** 78:919-926, 2000

DODT, C.; LÖNNROTH, P.; WELLHÖNER, J.P. et al.; Sympathetic control of white adipose tissue in lean and obese humans. **Acta Physiol Scand.**, 177:351-357, 2003

DRAELOS, Z.D, MARENUS, K.D. Cellulite: etiology and purported tratment. **Am. Soc. Dermatol. Surg.** 23(12)1177-1181, 1997.

FRÜHBECK, G.; GÓMEZ-AMBROSI, J.; MURUZÁBAL, F.J. et al., The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. **Am. J. Physiol. Endocrinol. Metab.**, 280:E827 – E847, 2001

GARCIA, M.P.; CHAVES, S.B.; AZEVEDO, R.B.; O tecido adipose. **In:** CURI, R. et al., Entendendo a Gordura. São Paulo, Ed. Manole, 2002

GREENWOOD, M.R.C.; JOHNSON, P.R.; Genetic differences in adipose tissue metabolism and regulation. **Ann NY Acad Sci.** 676:378-3883, 1993

GREGORIE, F.M.; SMAS, C.M.; SUL, H.S.; Undestarding adipocyte differentiation. **Physiol. Rev.**, 78:783-809, 1998

HAMILTON, P.W., ALLEN, D.C. Morphometry in histopathology. **J. Pathol.** 175:369-379,1995.

HEALTON, JM.; The distribution of brown adipose tissue in the human. **J Anat.** 112:65-41, 1972

HIPPIUS, M. et al.; *In vitro* investigations of drug release and penetration – enhancin effect of ultrasound on transmembrane transport of flufenamic acid. **Exp. Toxic. Pathol**. 50: 4-6, 1998.

HOLM, C.; OSTERLUND, T.; LAURELL, H.; et al., Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. **Annu Rev Nutr** 20:365-393, 2000

JOHNSON, M.E. et al.; Synergistic effects of chemical enhancers and therapeutic ultrasound on transdermal drug delilvery. **J. Pharm. Sci**., 85(7): 670-679, 1996.

JOHNSON, W.J.; JANNE, S.Y.; BERNARD, D.W.; Hormone sensitivite lipase mRNA in both monocyte and macrophage forms of the human THP-1 cell line. **Comp Biochem Physiol B. Biochem Mol Biol**, 126;543-552, 2000

KASSAN, D. G.; LYNCH, A. M.; STILLER, M.J.; Physical enhancement of dermatologic drug delivery: Iontohoresis and phonophoresis. **J Am Acad Dermatol** 34 (4):657-666, 1996

KOST,J. *et al.*; Ultrasound as a transdermal enhacer. In: BROUNAUGH, R.L & MAIBACH, H.I.; **Percutaneus Absorption,** 2ª ed., New York, Ed. Dekker, 1989.

LAFONTAN, M.; BARBE, J.; GALIZKY, J. et al.; Adrenergic regulation of adipocyte metabolism. **Human Reproduction**. 12 Suppl 1:6-20, 1997

LAMBERT, J. Traitemente par la percutaféine des lipodystrophies localisées. **Comptes Rendus de Thérapeutiques et de Pharmacologie Clinique.** CHU Lariboisière, Paris, 1(10):3-6, 1982.

LEHNINGER, A., NELSON, D., COX, M. **Princípios de Bioquímica.** 2.ed. São Paulo: Sarvier, 839p., p.356-358, 2000.

LEIBASCHOFF, G.H.; Uso de la electroforese y mesoterapia en um seguimiento de um año en el tratamiento de la dermatopaniculopatia edematosa, fibrosa y esclerosa: "celulitis". **Associación Argentina de Medicina Estetica**, 1(1):19-23, 1987.

LIMA, F.B.; VESTRI, H.S.; VESTRI, S. MORAES, S.M.F.; Lipólise. **In:** CURI, R. et al., Entendendo a Gordura. São Paulo, Ed. Manole, 2002

LOTTI, T.,GHERSETICH, I.,GRAPPONE, C. Proteoglycans in so-called cellulite. **Int. J. Dermatol.** 29:272-274,1990.

LUCASSEN, G.W.; VAN DER SLUYS, W.L.N.; VAN HERK, J.J.; NUIJS, A.M.; WIERENGA, P.E.; BAREL, A.O.; LAMBRECHT, R.; The effectiveness of massage treatment on cellulite as monitored by ultrasound imaging. **Skin Research and Technology**, 3(3):154-160, 1997

MAURIEGE, P.; GALITZKY, J.; BERLAN, M.; LAFONTAN, M. Heterogeneous distribution of beta and alpha-2 adrenoceptor binding sites in human fal cells from various fat deposits: functional consequences. **Eur. J. Clin. Invest**. 17:156-65, 1987

McNEEL, R.L.; MERSMANN, H.J.; Distribution and quantification of beta $_{1}$-, beta $_{2}$-, and beta $_{3}$-adrenergic receptor subtype transcripts in porcine tissues. **J. Anim. Sci.**, 77:611-621, 1999

MEEUSEN, R.; DE MEIRLEIR, K.; Exercise and brain neurotransmission. **Sports Med.,** 20(3):106-188, 1995

MERKLIN, R.; Growth and distribution of human fetal brown fat. Anat. Rec. 178:637-46, 1973

MITRAGOTRI, S. et al.; A mechanistic study of ultrasonically enchanced transdermal delivery. **J. Pharm. Sci**, 84:697-706, 1995.

_____; BLANKSCHTEIN, D.; LANGER, R.; Transdermal drug delivery using low-frequency sonophoresis. **Pharmaceutical Research,** 13(3): 411-420, 1996.

_____; BLANKSCHTEIN, D.; LANGER, R.; Na explanation for the variation of the sonophoretic transdermal transport enhancement from drug to drug. **J. Pharm. Sci.,** 86(1): 1190-2, 1997.

_____ ; FARRELL, J.; TANG, H.; TERAHARA, T.; KOST, J.; LANGER, R.; Determination of threshold energy dose for ultrasound-induced transdermal drug transport. **J Control Release**,3;63(1-2):41-52, 2000

MONTI, D.; GIANNELLI, R.; CHETONI, P.; BURGALASSI, S.; Comparison of the effect of ultrasound and of chemical enhancers on transdermal permeation of caffeine and morphine through hairless mouse skin in vitro. **Int J Pharm.**, 229: 131-137, 2001

MORETTI, E.A. Estudo macro e microscópico da fáscia superficial em membros inferiores: um conceito diferente sobre a celulite. **Mesoterapia Atual,**1997.p.9-12.

MORIMOTO, C.; TSUJITA, T.; OKUDA, H.; Antilipolytic actions of insulin on basal and hormone-induced lipolysis in rat adipocytes. **J. Lipid Res.**, 39:957-962, 1998

MORIMOTO, Y.; SUGIBAYASHI, K.; NATSUME, H.; The transdermal drug delivery system and transcutaneous absorption. **Acta Derm Venerol. Suppl.** 185: 15-17, 1994

OLAH, M.E.; STILES, G.L.; Adenosine receptor subtypes: Characterization and therapeutic regulation. **Annu Ver Pharmacol Toxicol.**, 35:581-606, 1995

PÉNICAUD, L.; COUSIN, B.; LELOOUP, C. et al.; The autonomic nervous system, adipose tissue plasticity, and energy balance. **Nutrition** 16:903-908, 2000

PIÉRARD, G.D., NIZET, J.L., PIÉRARD-FRANCHIMONT, C. Cellulite fron stading fat herniation to hypodermal stretch marks. **Am. J. Dermatol.**22(1):34-37,2000.

PIRES DE CAMPOS, M.S.M.; Fibro-edema gelóide sub-cutâneo, **Ciência & Tecnologia,** 1(2):77-82, 1992.

POSTERNAK, M.E., UEZEN, M.C., BUSTELO, O.J. Paniculopatia edemato-fibro esclerótica. **Mesoterapia 2000**.2(1):22-25,2000

QUERLEUX, B.; CORNILLON, C.; JOLIVET, O.; BITTOUN, J.; anatomy And physiology of subcutaneous adipose tissue by in vivo magnetic resonance imaging and spectroscopy: Relationships with sex and presence of cellulite. **Skin Research and Technology,** 8:118-124, 2002

ROSENBAUM, M.; PRIETO, V.; HELLMER, J.; BOSCHMANN, M.; KRUEGER, J.; RUDOLF, L.; SHIP,A.G. An exploratory investigation of the morphology and biochemistry of cellulite. **Plastic and Reconstructive Surgery**. (7):1934-1939, 1998

ROSSI, A.B.; VERGNANINI, A.L.; Cellulite: a review. **European Ac Derm and Venereology**. 14:251-262, 2000

RYAN, T.J.; Lymphatics and adipose tissue. **Clinics in Dermatology**, 13:493-498, 1995.

_____, CURRI, S.P.; Cutaneous adipose tissue. **Clinics in Dermatology**, 7(4), 1989

SATTIN, A.; RALL, T.W.; The effect of adenosine and adenine nucleotides on the cyclic adenosine 3' 5' –phosphate content of guinea pig cerebral cortex slices. **Mol. Pharmacol.**, 6:13-23, 1970

SEGERS, A.M.,FORTEZA,I.E., CONFORTI, F.Y., ABUFALIA,J. Celulitis, estudio de la microangiopatia de 254 casos. **Med. Cut.** I.L.A.v.13,.p385-390, 1985.

SHUM, S.; SEALE, C.; HATHAWAY, D.; CHUCOVICH, V.; BEARD, D.; Acute caffeine ingestion fatalities: Management issues. **Vet. Hum. Toxicol**., 39(4):228-230, 1997

TACHIBANA, K & TACHIBANA, S; Application of ultrasound energy as a new drug delivery system. **Nippon Rinsho**, 56(3): 584-8, 1998.

TAOUIS, M.; BERLAN, M. MONTASTRUC, P. & LAFONTAN, M. Characterization of dog fat cell adrenoceptors: variations in alpha-2 and beta adrenergic receptors distribuition according to the extent of the fat deposits and the anatomical location. **J. Pharmacol. Exp. Ther.,** 242(3): 1041-1049, 1987.

TAOUIS, M.; VALET, P.; ESTAN, L.; LAFONTAN, M.; MONTASTRUC, P. & BERLAN, M. Obesity modifies the adrenergic status of dog adipose tissue. *J.* **Pharmacol. Exp. Ther**., 250(3): 1061-1066, 1989.

UEDA, H. et al.; Difference in the enhancing effects of ultrasound on the skin permeation of polar and non-polar drugs. **Chem. Pharm. Bull**., 44(10): 1973-76, 1996.

YOUNG, S. Terapia por Ultra-som; In: KITCHEN,S. & BAZIN,S.; **Eletroterapia de Clayton,** 10ª ed., São Paulo, Ed. Manole, p. 235-260, 1996.