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PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA E
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EFEITOS DO ULTRASSOM PULSADO DE BAIXA INTENSIDADE NA PROLIFERAÇÃO E MINERALIZAÇÃO DE PRÉ-OSTEOBLASTOS *IN VITRO*

Prof. Dr. Jarbas Rodrigues de Oliveira
Orientador

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"If I have seen further it is by standing on the shoulders of Giants."

Isaac Newton

Meus pais João Caetano e
Lione Tassinary,minhas irmãs Rafaela e
Renata e a minha esposa Mariah,

DEDICO

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Durante o doutorado, aprendi que uma tese ou qualquer outro trabalho é a extensão da nossa vida. Então, para que algo de valor seja produzido, a pessoa deve primeiro criar algo de valor em si. Pessoa e obra são consistentes com o resultado. Por este motivo, agredeço de forma sincera a todas a pessoas que me encorajaram e me auxiliaram a produzir algo de valor em minha vida.

A vida não se coloca em análise de regressão e não é pelo valor que descobrimos a significância das pessoas na nossa trajetória, mas tenho certeza que este trabalho não é resultado apenas de um esforço individual. Ele nasce de significativas contribuições que recolhi durante minha trajetória profissional, acadêmica e como cidadão, ao lidar com pessoas (infelizmente algumas não estão mais entre nós) e instituições que foram fundamentais a essa construção.

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RESUMO

O ultrassom pulsado de baixa intensidade surge como recurso terapêutic de alto potencial para o tratamento de doenças osteometabolicas e fraturas com atraso de consolidação óssea. Entretanto, investigações recentes demonstram resultados controversos, sugerindo a necessidade de mais estudos acerca do entendimento das respostas biológicas e na padronização dos parâmetros das modalidades de tratamento. Sendo assim, o presente estudo teve o objetivo de avaliar o efeito do ultrassom na proliferação e mineralização de pre-ósteoblastos a partir de bioensaios *in vitro.* Foram utilizados pré-osteoblastos da linhagem MC3T3-E1, sendo estas tratadas com ultrassom terapêutico no modo pulsado a 20%, com uma frequência de 1MHz e intensidade de 0,2 W/cm^2. No estudo de proliferação celular, avaliamos cálcio intracelular, o TGF-β1, magnésio e os níveis de mRNA de osteopontina e osteocalcina, NF-κB1 e p38α. Além disso, utilizamos nifedipina e a rapamicina para investigar rotas de proliferação. Inicialmente, os resultados mostraram que o ultrassom aumenta proliferação de MC3T3-E1, diminuindo o teor de cálcio e magnésio no sobrenadante. Além disso, aumenta a concentração de cálcio intracelular, ativa NF-κB1 e o complexo mTOR via p38α e promove uma diminuição na síntese de TGF-β1, que é um inibidor do crescimento celular. Nos ensaios de mineralização celular avaliamos inicialmente a deposição nódulos de minerais na placa de cultura e a expressão gênica da osteocalcina por PCR convencional, e, posterirormente a concentração no sobrenadante celular de colágeno, fosfato, cálcio, TGF-β além de fosfatase alcalina no lizado de células. Os resultados mostraram que o ultrassom tem a capacidade de estimular a mineralização pré-osteoblastica em 192 horas após o tratamento a partir do aumento da expressão osteocalcina, captação de cálcio e fosfato e consequente formação de hidroxiapatita.

Palavras-chave: ultrassom; pré-osteoblastos; proliferação; mineralização.

ABSTRACT

Low-intensity pulsed ultrasound (LIPUS) has been proposed as a high potential therapeutic technique for the treatment of metabolic bone diseases and fractures with delayed healing. However, recent investigations have shown controversial results, suggesting the need for more studies on the understanding of biological responses and the standardization of methods and parameters. For this reason, the present study aimed to evaluate the effect of ultrasound on the proliferation and mineralization of osteoblasts using *in vitro* bioassays. Pre-osteoblastic MC3T3-E1 cells were used and treated with therapeutic pulsed ultrasound, 20%, frequency of 1MHz and 0,2W/cm^2 of intensity. In the study of cellular proliferation, intracellular calcium, TGF-β1, magnesium and osteopontin and osteocalcin mRNA levels, NF-κB1 and p38α were evaluated. In addition, nifedipine and rapamycin were used to investigate the proliferation pathways. Initially, the results showed an increase in proliferation of MC3T3-E1 and a decrease in calcium and magnesium content in supernatant with LIPUS exposure. In addition, LIPUS increased calcium deposition, activated NF-κB1 and mTOR complex via p38α and promoted a decrease in TGF-β1 synthesis, which is an inhibitor of cell growth. On cell mineralization assays, we evaluated mineral nodules deposition and expression of osteocalcin mRNA, collagen concentrations on culture supernatant, phosphate, calcium, TGF- β1 and ALP on cell lysates. The results showed that US stimulates the mineralization of preosteoblasts 192h after treatment by stimulating osteocalcin mRNA expression, calcium and phosphate uptake amd consequent formation of HA. Later, in different experimental conditions, the results showed that LIPUS had the ability to stimulate pre-osteoblastic mineralization with decreased concentration of collagen, calcium and phosphate in the cell supernatant 192 hours after treatment. It also changed the alkaline phosphatase concentration, as well as the osteocalcin gene expression.

Keywords: Ultrasound; Pre-osteoblasts; Proliferation; Mineralization

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LISTA DE ABREVIATURAS

ALP: fosfatase alcalina
BMP: proteína morfogenética óssea
DMSO - dimetilsulfóxido
EPM - erro padrão da média
FGF: fator de crescimento fibroblástico
IP3 - inositol trifosfato
IL-1β: interleucina 1 beta
IL-6: interleucina 6
IL-10: interleucina 10
IGF-1: fator de crescimento semelhante à insulina tipo 1
IGF-2: fator de crescimento semelhante à insulina tipo 2
INF-γ: interferon gama
LPS: lipopolissacarídeo
MCP-1: proteína quimiotática de monócitos 1
NO: óxido nítrico
PGE2: prostaglandina E2
SPSS - *Statistical Package for the Social Sciences*
TGF-β1: fator transformador de crescimento beta 1
TNF-α: fator de necrose tumoral alfa
US - ultrassom

SUMÁRIO

Capítulo I

Apresentação

1. Introdução

1.2. Tecido ósseo

O tecido ósseo é caracterizado por ser metabolicamente ativo, pois passa continuamente pelos processos de renovação e remodelação, que são, basicamente, produtos da atividade dos osteoblastos, osteoclastos e osteócitos[1]. Os osteoblastos são originários de proliferação de células mesenquimais osteoprogenitoras, as quais se localizam na superfície da formação óssea, sendo responsáveis pela formação dos componentes orgânicos da matriz extracelular. Os osteócitos são osteoblastos que, após a mineralização do osteóide a sua volta, ficam presos em lacunas ósseas; já os osteoclastos também são encontrados na superfície óssea em locais de reabsorção óssea[2].

O tecido esquelético é constituído de uma matriz extracelular composta por componentes inorgânicos (65%) e orgânicos (35%). A matriz orgânica é formada principalmente por colágeno (90%), glicoproteínas, mucopolissacarídeos ácidos e lipídeos. O colágeno, quando sintetizado pelos osteoblastos, dá origem paralelamente a pequenas regiões tridimensionais onde, posteriormente, acontecerá o início da mineralização óssea. Outra importanteproteína sintetizada pelos osteoblastos é a osteocalcina, que possui alta afinidade pelahidroxiapatita. Cabe descrever a importância das proteínas caracterizadas pela literatura como osteoindutoras, dentre as quais destacam-se o fator de crescimento transformante β (do inglês *tranforming*m *growth fator* TGF-β), fator de crescimento semelhante à insulina 1 e 2 (do inglês *Insulin-like growth factor* IGF-1 e IGF-2) e fator de crescimento de fibroblasto (do inglês *Fibroblast growth factor* FGF). Estas proteínas estão sendo amplamente estudadas na atualidade, sendo que existem relatos de que podem atuar tanto na proliferação como na diferenciação celular e, consequentemente, na formação óssea [3,4].

O componente inorgânico da matriz óssea é formado essencialmente por cálcio (Ca^{++}), fosfato e magnésio (Mg). O Ca^{++} é o íon mineral mais abundante no organismo e participa de inúmeros processos celulares e extracelulares, como, por exemplo, a sinalização intracelular e a

manutenção do potencial de membrana celular. Assim como o Ca^{++}, o fosfato é fundamental na formação dos cristais de hidroxiapatita [$Ca_{10}(PO_4)_6(OH)_2$] que dão resistência ao tecido ósseo e desempenham papel importante em inúmeras reações celulares, além de ser um componente de membranas e ácidos nucleicos. Outro íon importante neste processo é o Mg, que, assim como o Ca^{++}, apresenta-se de forma abundante nos tecidos mineralizados e desempenha papel em inúmeros processos metabólicos como co-fator em reações enzimáticas, incluindo a adenilciclase[3,4].

1.3. Proliferação e diferenciação óssea

A formação óssea segue uma sequência de eventos (Figura 1) que inicia a partir das células osteoprogenitoras, que se diferenciam inicialmente em pré-osteoblastos e posteriormente em osteoblastos. Este processo não é totalmente entendido, porém se sabe que glicocorticoides e certas proteínas, como as Proteínas Ósseas Morfogenéticas (do inglês *Bone morphogenetic protein* BMP), exercem papel fundamental neste processo. Uma vez diferenciada em osteoblastos, a célula é capaz de secretar vários fatores de crescimento, sendo que, vários destes fatores aumentam a velocidade de formação e regeneração de tecido, por estimularem mitose e ou/diferenciação de células osteoprogenitoras[2,5].

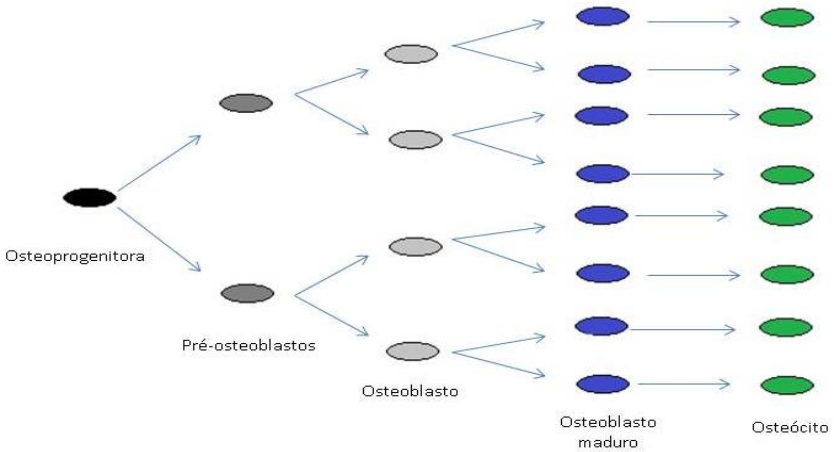

Figura 1. Sequência de eventos envolvidos na formação e diferenciação do tecido ósseo. Células osteoprogenitosras se diferenciam e pré-osteoblastos, osteoblastos, osteoblastos maduros e por fim osteócitos.
Fonte: Adaptado Raouf, 2000.

A fase proliferativa é caracterizada pela multiplicação de células osteoblásticas e pela biossíntese de colágeno tipo I da matriz óssea. Alguns genes que ativam a proliferação celular estão expressos nesta fase, além das ciclinas que atuam sobre a progressão do ciclo celular, genes que codificam moléculas de adesão celular (fibronectinas) e genes que codificam moléculas associadas à regulação da biossíntese e à constituição da matriz extracelular (TGF- β e colágeno I)[2].

Posteriormente ocorre a fase de maturação, seguindo o período inicial de proliferação, que tem inicio a partir da expressão de genes associados com a maturação dos componentes da matriz, como por exemplo, a fosfatase alcalina (do inglês *alcaline phosphatase* ALP). Este momento é fundamental para a subsequente fase de mineralização[5].

1.4. Mineralização óssea

O processo de mineralização óssea tem início a partir da secreção de vários reguladores como Interleucina 6 (IL-6), TGF-β e Interferon-γ (INF-γ), e, posteriormente, resulta na síntese e interação de inúmeros componentes, tais como: colágeno tipo I, osteocalcina, osteopontina, proteoglicanas, fosfoproteínas e citocinas, os quais formam um arcabouço onde, mais tarde, serão depositados sais minerais[6,7].

Inicialmente, os osteoblastos sintetizam a matriz orgânica óssea de onde brotam pequenas vesículas de sua superfície. Essas vesículas originárias da membrana plasmática dos osteoblastos são estruturas arredondadas chamadas de vesículas da matriz, que contêm glicoproteínas e exibem forte marcação para a ALP. Sabe-se que a ALP tem a capacidade de hidrolisar moléculas de pirofosfato, fornecendo fosfato para o interior das vesículas.

Os fosfolipídios também possuem papel importante na mineralização óssea, já que têm a capacidade de aumentar a concentração de Ca^{++} para o interior dessas vesículas. Neste sentido, o processo busca uma supersaturação de fosfato e de Ca^{++}, resultando na precipitação de fosfato de cálcio no interior das vesículas. Posteriormente, ocorre o rompimento da membrana das vesículas e a mineralização espalha-se pela matriz. Essa

interação entre fosfato e Ca^{++} forma cristais com estrutura de hidroxiapatita, que, associados às fibras colágenas, proveem a resistência e dureza características do tecido ósseo[7,8].

1.4. Ultrassom terapêutico

Atualmente, são inúmeras as modalidades de estimulação mecânica (ultrassom de baixa potência, fluxo de fluido, centrifugação, aplicação estática de carga, vibração ou campo eletromagnético) que atuam sobre as células ósseas após um processo denominado mecanotransdução. Esse processo é responsável por produzir reações bioquímicas a partir de um fenômeno mecânico (físico), determinando uma resposta celular, que pode ser de produção ou reabsorção óssea[9].

Dentre os recursos capazes de promover a estimulação mecânica, destaca-se o ultrassom, que é uma modalidade amplamente utilizada para diversas finalidades nas ciências da saúde. Sabe-se que, além de ter potencial para atuar na produção óssea[9], esse recurso, em diferentes parâmetros, pode ser utilizado como método de diagnóstico[10], na reabilitação músculo esquelética estimulando os processos de cicatrização, promovendo o alívio da dor, minimizando a rigidez articular[11] e aumentando a permeação transdérmica de fármacos[12,13].

As radiações ultrassônicas foram descobertas em 1880 por Caal Pierre e Marie Curie, ao aplicar uma corrente elétrica senoidal sobre um cristal de quartzo posicionado entre duas placas metálicas, constatando assim a geração de uma vibração de alta frequência. O aparelho de ultrassom foi inicialmente produzido através de um cristal de quartzo vibrante, submetido a uma corrente de alta frequência. Hoje em dia, os engenheiros utilizam cristal cerâmico sintético na elaboração dos aparelhos sofisticados, pois esses cristais têm maior resistência e eficiência em converter corrente elétrica em vibrações mecânicas[19]. Para aplicar as cargas elétricas no cristal é fixado um eletrodo de metal, enquanto a superfície oposta fica no ar. A maior parte da energia da vibração é transmitida do cristal para a placa, e daí para o sitio alvo[20].

Ao longo do tempo, pesquisadores mostraram que o ultrassom poderia ser utilizado de diferentes formas, a partir de seus mais variados parâmetros. Convencionalmente na área da saúde são utilizadas, frequências que variam de 0,75 MHz até 3 MHz. A unidade de frequência denomina-se hertz (Hz), que representa o número de ciclos de ondas completados a cada segundo. A frequência determina a profundidade de penetração da onda sonora, no corpo humano ou em qualquer outro meio físico. O controle da frequência de saída do ultrassom fornece aos profissionais a ideia da profundidade a ser atingida pela energia e, consequentemente, do mecanismo físico em ação[10].

O feixe de ultrassônico leva consigo uma quantidade de energia produzida pelo transdutor, sendo que essa energia é conduzida em um determinado espaço de tempo e denominada potência (Watt). No exato período em que essa potência é dividida pela área do feixe (cm^2), se define a intensidade desse feixe em W/cm^2. A intensidade ou densidade do US terapêutico pode variar de 0,1 a 3,0 Watts por centímetro quadrado (W/cm^2), sendo que o som desloca-se em forma de ondas, o que demanda um meio material para sua transmissão[14]. O profissional que manuseia o aparelho deve deliberar a intensidade terapêutica correta para cada situação clínica a ser tratada, além de ter conhecimento de qual é a dose ideal que deverá chegar até o tecido alvo, levando em consideração a atenuação das ondas sonoras nos tecidos mais superficiais[11].

Boa parte dos aparelhos terapêuticos da atualidade podem emitir dois tipos de onda sonora: contínuas e pulsadas. O dispositivo clicado no modo contínuo faz com que a intensidade do som se mantenha constante durante todo o tratamento, já no modo pulsado, a intensidade é interrompida periodicamente. Segundo a literatura a onda sonora contínua é utilizada para induzir efeitos térmicos, enquanto o pulsado apresenta uma redução do aquecimento médio do tecido, produzindo apenas o efeito mecânico[10].

Durante o processo de reabilitação, o ultrassom contínuo tem sido utilizado para o tratamento de distúrbios musculoesqueléticos, tais como espasmos musculares e quadros de rigidez articular. Já o modo pulsado, na

mesma intensidade terá uma média temporal de dose muito menor e, consequentemente, um aquecimento praticamente imperceptível, principalmente nos pulsos de 5, 10 e 20%. Neste quadro terapêutico, os efeitos serão proporcionados pelos fenômenos chamados de não-térmicos. Dentre estes efeitos destacam-se: a corrente acústica, a cavitação e as ondas estacionárias[15].

A corrente acústica é caracterizapelo fluxo circulatório invariável devido ao torque de radiação (microcorrenteza). Esse efeito desempenha sobrecarga viscosa sobre a membrana da célula e, portanto, algumas investigações sugerem que essa carga pode aumentar a permeabilidade da membrana[20].

A cavitação é descrita como a formação de pequenas bolhas gasosas, subproduto da radiação ultrassônica, que ordinariamente ocorrem com cerca de um mícron de diâmetro[15]. O terceiro fenômeno não térmico acústico é a onda estacionária, que está associado com o exato momento em que a onda ultrassônica alcança a interface de dois tecidos com distinta impedância acústica, ocorrendo reflexão de uma porcentagem da onda. As ondas refletidas podem incidir com aquelas que estão chegando para formar um campo de onda estacionária[16].

1.5. Células MC3T3 – E1

As células MC3T3-E1, foram estabelecidas a partir da calvária de camundongos recém-nascidos, os quais representam um fenótipo celular pré-osteoblástico (Figura 2) semelhante ao do homem. Mesmo que esta linhagem celular tenha uma taxa de crescimento adicional quando comparada a células humanas, existe uma grande semelhança no que diz respeito à regulação e expressão genica[17].

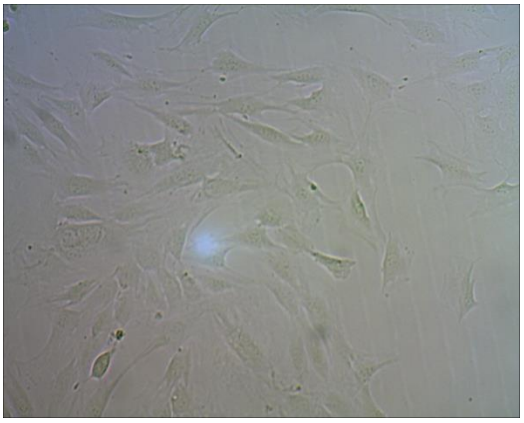

Figura 2. Imagem microscópica das células MC3T3-E1.
Fonte: Autor.

As MC3T3-E1 se proliferam e formam nódulos mineralizados de forma semelhante ao do processo de ossificação intramembranosa *in vivo*, inclusive em questões temporais. Essas células se proliferaram ativamente *in vitro* e sintetizam colágeno tipo I, a partir do terceiro dia de cultura. A atividade da enzima ALP também aumenta normalmente a partir do 3º dia e a deposição mineral é relatada a partir do 14º dia. A suplementação com ácido ascórbico e β-glicerofosfato é um pré-requisito para mineralização da matriz em células MC3T3-E1, sendo que, na ausência desses estimulantes, a atividade ALP permanece baixa e não ocorre a mineralização. Os processos de proliferação e de diferenciação das células são influenciados pelo tipo de soro utilizado no meio de cultura, citoquinas e fatores de crescimento derivados[18]. É importante frisar que se utilizou as MC3T3-E1 neste estudo, por representarem alta homologia às células osteoblásticas humanas, viabilizando o estudo *in vitro*, e serem amplamente utilizadas em investigações cientificas[18].

2. Justificativa

O ultrassom terapêutico é amplamente utilizado na prática clinica para o tratamento de lesões de tecidos moles, com finalidade anti-inflamatória e analgésica. Diversas pesquisas têm sido feitas com relação ao tratamento de doenças ósseas, entretanto, não há consenso quanto ao tempo, dose, frequência e duração de pulso de onda ultrassônica a serem utilizadas e tão pouco se conhece o seu mecanismo de ação.

Neste sentido, cabe a proposição desta pesquisa na tentativa de corroborar no entendimento dos mecanismos fisiológicos inerentes a ação do ultrassom, buscando aprimorar a eficácia e segurança de seu uso clínico para remodelação da massa óssea.

3. Objetivos

3.1. Objetivo geral

Investigar o efeito do ultrassom terapêutico pulsado de baixa potência em pré-osteblastos da linhagem MC3T3–E1 *in vitro*.

3.2. Objetivos específicos

- Avaliar os efeitos do ultrassom pulsado de baixa intensidade na proliferação de pré-osteblastos da linhagem MC3T3–E1 *in vitro*.

- Avaliar os efeitos do ultrassom pulsado de baixa intensidade na mineralização de pré-osteblastos da linhagem MC3T3–E1 *in vitro*.

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Capítulo II

Artigo Original

(Formatado de acordo com as normas da Revista Lasers in Surgery and Medicine/Anexo)

Therapeutic ultrasound stimulates MC3T3-E1 cell proliferation through the activation of NF-κB1, p38α and mTOR

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There is no conflict of interest.

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Abstract

Background and Objectives

As the population ages, osteometabolic diseases and osteoporotic fractures emerge, resulting in substantial healthcare resource utilization and impaired quality of life. Many types of mechanical stimulation have the potential of being recognized by bone cells after a mechanical sign is transformed into a biological one (a process called mechanotransduction). The therapeutic ultrasound (TU) is one of several resources capable of promoting bone cell mechanical stimulation. Therefore, the main purpose of present study was to evaluate the effect of TU on the proliferation of pre-osteoblasts using *in vitro* bioassays.

Study Design/Materials and Methods

We used MC3T3-E1 pre-osteoblast lineage cells kept in Alpha medium. Cells were treated using pulsed mode therapeutic ultrasound, with frequency of 1 MHz, intensity of 0.2W/cm^2 (SATA), duty cycle of 20%, for 30 minutes. Nifedipine and rapamycin were used to further investigate the role of L-type Ca^{2+} channels and mTOR pathway. Intracellular calcium, TGF-β1, magnesium and the mRNA levels of osteopontin, osteonectin, NF-κB1, p38α were evaluated.

Results

The results show that TU stimulates the growth of MC3T3-E1 cells and decreases the supernatant calcium and magnesium content. Also, it increases intracellular calcium, activates NF-κB1 and mTOR complex via p38α. Moreover, TU promoted a decrease in the TGF-β1 synthesis, which is a cell growth inhibitor.

Conclusions

Therapeutic ultrasound, with frequency of 1 MHz, intensity of 0.2W/cm^2 (SATA) and pulsed mode, for 30 minutes, was able to increase the proliferation of preosteoblast-like bone cells. This effect was mediated by a calcium influx, with a consequent activation of the mTOR pathway, through increased NF-κB1 and p38α.

Key words: mechanotransduction; osteoblast; osteometabolic diseases; calcium.

1. INTRODUCTION

The last decades have been marked by numerous shifts in most of the world's population, so that the increase in life expectancy is a significant health issue. During the 20th century, the average lifespan increased by more than 30 years as a result of huge changes in demography and advances in public health. These transitions impose a huge social challenge on experts, public men, communities, and an intense demand for studies and analyses for a better definition of public health prevention policies on aging [1-3].

Aging brings with it some risks, including osteometabolic diseases, such as osteoporosis. It is estimated that in the United States approximately 38 million dollars are spent to treat osteoporosis, considering that about one million Americans suffer some form of pathological fractures each year [4]. Fractures in the elderly are also considered an important public health problem, as it may bring negative consequences for their quality of life, leading to huge costs to society that include immediate care, rehabilitation and its late effects. In Brazil, from 2005 to 2008, for example, there was an 8% increase in the number of hospitalizations due to hip fracture in the elderly. In 2008, this type of fracture was responsible for 32,908 hospitalizations in the Brazilian Unified Health System with a total cost of 152.9 million dollars [5].

The bone metabolism is regulated by various stimuli, which may be divided into mechanical and biochemical [6]. Currently, there are many types of mechanical stimulation (low level ultrasound, fluid flow, centrifugation, static load application, vibration or electromagnetic field) recognized by bone cells after a process called mechanotransduction. This process produces biochemical reactions from a mechanical phenomenon (physical), determining a cellular response, which can be new bone formation or bone resorption [7]. Among the resources capable of promoting mechanical stimulation is the therapeutic ultrasound (TU), which is widely used for several purposes in health sciences, as for example, bone production [7]. Recent studies have shown that these physical principles have the ability to produce *in vitro* positive results regarding the proliferation and differentiation of bone cells. The main cells used in these experiments were MC3T3-E1 cells, isolated from newborn rat calvaria. These cells have a pre-osteoblastic phenotype similar to those found in humans. Even though

this cell line has a faster growth rate, when compared to human cells, there is a great similarity regarding its regulation and gene expression [8].

Studies on the effects of therapeutic ultrasound in pre-osteoblasts suggest that it could be used as a possible tool for prevention and rehabilitation of common osteometabolic diseases, especially in the third age. However, recent findings demonstrate that, despite a wide range of research on the subject, the mechanism for the therapeutic effect on bone healing is still unknown [9]. It is believed that intracellular calcium concentration caused by tension is crucial to improve the osteogenesis rate [10]. Also, calcium in different experimental conditions acts on target rapamicine (mTOR) increasing proliferation [11]. Therefore, present study aimed to investigate the effects of therapeutic low-intensity pulsed ultrasound on the proliferation of *in vitro* pre-osteoblastic MC3T3-E1 cells. The role of L-type Ca^{2+} channels and mTOR pathway were also evaluated

2. MATERIALS AND METHODS

The Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul approved all experimental procedures.

2.1. Cell culture

MC3T3-E1 cells were kept in Alpha medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic-antimitotic (penicillin + streptomycin). The pre-osteoblasts were kept in an incubation cabinet at 37ºC in a humid atmosphere with 5% CO_2. The growth of cells was monitored using inverted microscope every 24 hours. Subculture was made when the cell line reached 90% confluence. The supernatant was removed, cells were washed with PBS and subsequently treated with trypsin solution 0.25% at 37°C for 1 minute. After incubation, the neutralization was performed and supplemented with 10% FBS, cells were spun at 1000 rpm at 20°C for 10 minutes with subsequent ressuspension in 1 mL using Alpha medium. The evaluation of the viability of cultured cells was analyzed by means of vital dye Trypan Blue (0.4%). For the essays, freshly obtained cells with viability higher than 95% were used .The number of viable cells was determined by using a Neubauer chamber.

2.2. Treatment with therapeutic ultrasound

For the application of ultrasound, the Sonic Digital Compact (HTM) apparatus was used, with frequency of 1 MHz, intensity of 0.2W/cm^2 (SATA), in a pulsed mode, duty cycle of 20%, for 30 minutes. The applications were performed inside a vertical laminar air flow cabinet (Telstar model AV-100). The ultrasound head was attached to a metal stand, horizontally with the crystal portion upward. The culture plate was placed on the center of the transducer with intermediate gel (Mercur) (Figure 1).

For the essays that included only cell proliferation, a total of 20000 cells per well were used for each of the experimental groups. For the remaining analyses, 70000 cells per well were used in individual plates (TTP) with 22.1 cm^2 growth area. After being plated for 24 hours, cells were subjected to ultrasonic radiation at time 0 hour. The

control group was also attached to the ultrasound, however, with the apparatus switched off.

2.3. Evaluation of ultrasonic wave

To determine the frequency and the intensity of the sound wave which effectively came into contact with the cell interface, the electrodes of an oscilloscope (Tektronix TDS 1002) were attached to a plate that received sonic therapy. Concomitantly, a multimeter (Minipa) was used to verify the value of the electrical resistance imposed by the culture plate to the sound wave. The electrodes consisted of silver wire with total area of 1.19 cm^2. To determine the intensity (in Watts), it was necessary to calculate the electric current where ampere equals the electric potential (in Volts) divided by the resistance (in Ohms) (I = E/R). After, the power is identified (in Watts) by multiplying ampere by resistance (P = I x E). The value of power obtained was divided by the total area of the electrode, resulting in Watts per cm^2. The temperature of the culture well was measured over the 30 minutes treatment with ultrasound (using a FLIR Extech IRC30 Compact InfraRed Camera).

2.4. Evaluation of calcium and magnesium concentration

For decontamination, the porcelain crucibles were placed in 10% nitric acid for 24 hours and then heated in a muffle furnace at 450ºC for 4 hours. Samples of cell supernatant were placed in the crucibles, which were weighed with and without the samples. Subsequently, samples were placed in an oven at 110ºC for 24 hours and then in a muffle furnace at 700ºC for 5 hours. The muffle furnace remained closed during cooling until the temperature reached 250ºC. The crucibles were then stored in a desiccator until the final weighing of samples. The final sample obtained in the crucible was diluted in distilled water (100 mL); 0.5 mL of each sample was taken and homogenized with 0.5 mL lanthanum oxide (0.1%) and absorbance was read in an atomic absorption spectrophotometer.

2.5. Evaluation of the intracellular calcium concentration

The intracellular calcium was evaluated using a Calcium Evaluation Assay Kit (BD Biosciences) 48 hours following the ultrasound treatment. In order to do that, the number of cells was optimized allowing the formation of a confluent monolayer. A FACS Canto Flow Cytometer (BD Bioscience) was used. Calcium inflow is proportional to the fluorescence emitted by the system.

2.6. Pre-osteoblastic proliferation with nifedipine and rapamycin

To assess the relationship between ultrasonic wave and L-type Ca^{2+} channels (long-lasting) in the pre-osteoblastic proliferation, 1 μM nifedipine treated with DMSO was added to the culture medium. In contrast, 10 μM rapamycin was added to other culture medium in order to verify the interaction between ultrasound and mTOR.

2.7 *TGF-β1* Quantification

TGF-β1 concentration was measured in cell supernatant using commercially available ELISA kit (R&D Systems). The kit contained a specific monoclonal anti-body immobilized on a 96-well microtiter plate that bound TGF-β1 in the aliquot and a second enzyme-conjugated specific polyclonal antibody. Color development was stopped by sulfuric acid and optical density was determined at 540nm with the correction wavelength set at 570 nm in an ELISA plate reader. Results were calculated on a standard curve concentration and multiplied for the dilution factor.

2.8. Real-time analysis

Cells were collected 48 hours following treatment with ultrasound. Total RNA was extracted using a Superscript Protocol III First Strand Supermix Kit (Invitrogen).

The mRNA levels of osteopontin, osteonectin, NF-κB1 and p38α were analyzed using a StepOne real-time PCR system (Applied Biosystems), applying the SYBR Green gel stain (Invitrogen), with the following cycles: initial incubation 95ºC step for 5 minutes, followed by 45 cycles with 95ºC denaturation step for 30 seconds, annealing at

with 60ºC for 30 seconds, and final 72ºC extension step of 30 seconds. To confirm the reaction specificity, a dissociation curve was performed for each primer pair using melting temperature analysis of each gene.

A standard curve was generated for each gene using StepOne software, version 2.3 (Applied Biosystems) obtained via qPCR reactions derived from a serial dilution of cDNA. Each dilution point of the curve was analyzed in triplicate, with the samples. The real-time PCR results were expressed in relative quantification of amplified cDNA with respect to the normalizer gene.

2.9. Statistical analysis

Results were expressed as means ± standard error of the mean (SEM). The Shapiro-Wilk test was applied to determine the normality of the data. Differences between samples were analyzed by using a one way analysis of variance (ANOVA), followed by the Bonferroni post-hoc test. Student's t-test was used for group comparisons. Statistical analyses were performed using Statistical Package for the Social Sciences Version 18.0 (SPSS Inc.). The level of significance was set at 5%.

3. RESULTS

In order to verify the effect of TU on the proliferation of MC3T3-E1 pre-osteoblasts, cells were attached in plates and exposed to low-intensity pulsed ultrasound. Figure 2 shows the effect of acoustic radiation on the growth of pre-osteoblasts during the culture period. It can be observed that ultrasound significantly increased the number of cells after 16, 24, 48 hours in culture.

The frequency and dose of ultrasound that have reached the cell interface were evaluated to determine the effective parameters of the sound wave that increased cell proliferation. The result showed that cells received a sound beam with frequency that ranged from 1.15 MHz to 1.38 MHz, with an average of 1.27 MHz over the 30 minute treatment (Figure 3A). As shown in Figure 3B, the effective average therapeutic dose received by cultured cells was 0.156 W/cm².

In order to disregard any thermal effect of ultrasonic wave on the cell proliferation, the culture medium temperature was measured during the sound irradiation. Low intensity ultrasound did not alter the temperature of the cell culture environment during the 30-minute treatment (Figure 3C). Experiments were performed at room temperature. The infrared thermography showed that the temperature remained the same in all culture wells (Figure 3D).

Subsequently, the concentration of Ca^{2+} in the supernatant was evaluated using atomic absorption spectroscopy. The results showed that ultrasound was able to decrease the concentration of Ca^{2+} in the extracellular medium (Figure 4A), as well as the concentration of Mg^{2+} (Figure 4B).

In order to further investigate the role Ca^{2+} plays in the process of cell growth expansion, as well as to verify possible effects of ultrasound on the L-type calcium channels, nifedipine dissolved in DMSO was used to block these channels. Low intensity ultrasound was applied as previously described in Methods. Figure 5 show that the ultrasound promoted significant cell growth when compared to control, DMSO and DMSO + nifedipine groups. However, when the DMSO + nifedipine group was treated with ultrasound significant differences were found when compared to both DMSO and DMSO + nifedipine groups. Although, no statistical difference was found when it was compared to both control and ultrasound treated groups.

The effects of ultrasound on intracellular Ca^{2+} concentration associated or not with nifedipine, were evaluated to confirm the increase in the calcium influx into the cell and its relationship with the L-type calcium channel (voltage dependent). The results indicate that low-intensity ultrasound promoted the increase of intracellular Ca^{2+} and that this effect was not blocked by nifidipine (Figure 6).

In order to determine the ultrasound involvement with the mTOR pathway, experiments using rapamycin in the culture medium were performed. Figure 7 show that rapamycin decreased cell growth and ultrasound did not reverse this effect. It is also known that there is a direct involvement of mTOR with the transformation growth factor beta (TGF-β1). Therefore, in order to investigate ultrasound effects on this pathway, TGF-β1 concentration was evaluated after ultrasound treatment. Results showed that ultrasound reduces TGF-β1 concentration in the supernatant (Figure 8).

The gene expression of two proteins that play a key role in bone mineralization was evaluated by qPCR. The results showed that ultrasound did not increase the gene expression of both osteopontin and osteonectin in the studied period (Figure 9). Also, studies have shown a cell proliferation pathway that involves mTOR, p38α and NF-κB1. Thus, the expression of p38α and NF-κB1 were evaluated by qPCR after exposure to sound irradiation (Figure 10). The results demonstrate that ultrasound increased the gene expression of both p38α and NF-κB1.

4. DISCUSSION

The results of this study showed that ultrasound treatment significantly increased the number of pre-osteoblastic cells after 16, 24 and 48 hours in culture. Both *in vitro* and *in vivo* studies suggest that ultrasound is able to promote cell proliferation, osteogenic differentiation and extracellular matrix formation [12]. It is believed that the acoustic radiation force (ARF) is capable of generating a series of biochemical events in these cells, although the mechanisms are not yet clearly understood. Some authors have hypothesized that osteoblasts may sense ARF through morphological deformation and their surface mechanosensitive structures. Studies on different isolated cells have demonstrated that the ultrasound has the ability to produce many different effects, such as damage to plasma membrane and changes in calcium channel activities [11].

The frequency and dose of ultrasound that have reached the cell interface were evaluated to determine the effective sound wave parameters that increased proliferation. The results showed that cells received the sound beam with frequency ranging from 1.15 MHz to 1.38 MHz over the 30 minutes treatment. The effective average therapeutic dose received by cultured cells was 0.156 W/cm² i.e., 22% lower intensity than the adjusted dose in the equipment panel board. Decreased intensity is related to the sound beam attenuation suffered before reaching the cells, as the acoustic force initially propagates in the culture plate. Considering that attenuation is higher with high frequency, the percentage loss of energy increases due to depth, causing a greater energy variation in the first surface layer of the material due to the sonic impact [13].

Ultrasound is widely used in medical practice, both as a diagnostic tool, as well as in therapeutic modalities. In addition to thermal effects, ultrasound introduces several non-thermal mechanical effects i.e., the use of therapeutic ultrasound can produce a variety of cellular responses *in vitro* and *in vivo* through these two acoustical phenomena [14]. Some studies have suggested that the temperature rise during ultrasound radiation may affect general metabolism and morphological structure of MC3T3-E1 osteoblasts [9]. Therefore, for the purposes of this study, the exclusion of thermal phenomena resulting from the sound wave in the cell treatment should be addressed. However, thermal mechanisms can be ruled out of present results, since it was evidenced that there was no temperature variation over the exposure time. By

providing ultrasound exposures using short pulses and low overall rate cycles of energy deposition, it is suggested that pre-osteoblast treatment is only linked to mechanical effects produced by the acoustic wave treatment.

Regarding the mechanisms involved in the proliferation of osteoblasts, it has been suggested that calcium and magnesium ions play a key role in the proliferation of this type of cells. Magnesium-deficient animals showed impaired bone growth, osteopenia and increased skeletal fragility [15]. *In vitro* studies indicate that extracellular Mg^{2+} and Ca^{2+} deficiency reduces the proliferation of human osteoblastic cells. Reduced osteoblastic proliferation would cause inadequate bone formation [16]. Our results showed that ultrasound is able to decrease the concentration of calcium and magnesium in the supernatant, whereas the proliferation mechanisms involving these ions are not fully understood. However, efforts are being made to obtain an optimal cell proliferation level [16].

Many studies report that increased intracellular calcium concentration due to strain is crucial to enhance osteogenesis [10]. Elevated calcium concentration in the extracellular medium was found to have a stimulating effect on osteoblast proliferation. It has been suggested that Ca^{2+} released from bone matrix at the resorptive sites might be linked to the coupling of osteoclast and osteoblast functions [17].

It is also important to note that the voltage-dependent L-type Ca^{2+} channels in MC3T3-E1 cells facilitate the Ca^{2+} entry under membrane depolarization circumstances [18]. Thus, one of the hypotheses is that ultrasound stimulation can increase the proliferation of this cell line through these channels, which were blocked using nifedipine (L-type voltage sensitive calcium channel blocker) that inhibits the entry of calcium through the channels [19]. The results corroborate the literature regarding the effect of nifedipine on inhibiting the proliferation of MC3T3-E1 [20]. However, our results suggest that the acoustic force does not depend entirely on (L-type) calcium channels to stimulate cell proliferation, nor has the capacity to reverse the channel blockage induced by the drug, considering that the ultrasound treated group showed no difference when compared to the group treated with nifedipine and ultrasound. In a previous study, it was reported that Ca^{2+} influx depends on the activation of stretch-stimulated calcium channels, voltage-sensitive L-type calcium channels and endoplasmic reticulum IP3 receptors. However, the same authors discuss that these

receptors are the major responsible in determining intracellular calcium concentration [10].

In order to investigate a possible increase in the Ca^{2+} influx into the cell and its relationship with the voltage-dependent L-type Ca^{2+} channel, the effects of ultrasound on intracellular Ca^{2+} concentration associated or not with nifedipine were evaluated. Results showed that low intensity ultrasound promoted either a Ca^{2+} influx or an efflux from the endoplasmic reticulum when compared to the untreated group, even when associated with nifedipine.

It is already known that intracellular calcium activates the rapamycin target mTOR [11] and, most recently, the interaction of rapamycin in mammals has been further investigated, mainly regarding its effect on growth and proliferation of several cell lines. On the other hand, its effects on osteoblasts are still poorly understood, although it is known that rapamycin is a specific inhibitor of mTOR and, consequently, has a negative effect on osteoblast proliferation and differentiation [21]. We have also aimed to evaluate a possible link between the ultrasound effects and the mTOR pathway. For that, rapamycin was used in the culture medium and results showed that the proliferation effect of ultrasound on this cell line is related to mTOR.

In order to confirm the ultrasound effect on cellular proliferation with mTOR and considering the results showed by Chatterjee [22] demonstrating the action of rapamycin on the cell cycle due to its regulatory effects on TGF-β1 signaling, we have evaluated its concentration in the supernatants. Results showed that the waveform has the ability to reduce the concentration of TGF-β1 in the supernatant, while it stimulates cell proliferation.

Recent studies have demonstrated that mTOR inhibits osteoblast proliferation and the early stage of osteoblast differentiation, although this drug treatment had no effect on osteoblast differentiation parameters when cells were completely differentiated [21]. In order to verify whether these osteoblasts were not differentiated and to confirm the involvement of ultrasound with mTOR, we evaluated the gene expression of ostepontin and osteonectin, which are directly involved in bone mineralization. Results showed that ultrasound did not increase the gene expression, suggesting that cells were not differentiated.

It is believed that there is an interaction between mTOR and p38. Recent studies reported that p38α and p38β activate mTOR under different conditions [23]. Moreover, p38 is conditioned to a subsequent activation of NF-κB1 [24]. Based on our results showing that the sound wave increased the gene expression of both proteins, it can be concluded, for these experimental conditions, that ultrasound seems to act on this pathway to increase cell proliferation.

In conclusion, the results of our study show *in vitro* effects of low-intensity therapeutic ultrasound on MC3T3-E1 cells, indicating that the treatment induced cell proliferation through an increase in NF-κB1 and p38α, via mTOR pathway. No cellular differentiation was demonstrated. These results may help in further investigations on the use of therapeutic ultrasound for the treatment of bone diseases.

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6. FIGURES

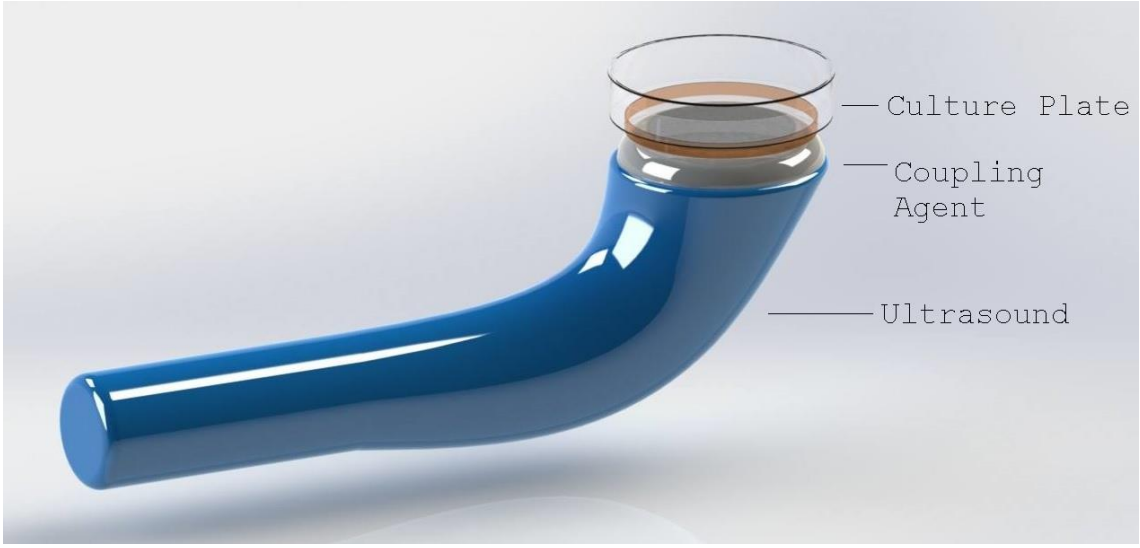

Figure 1: Schematic representation of ultrasound exposure system on the cell culture plate.

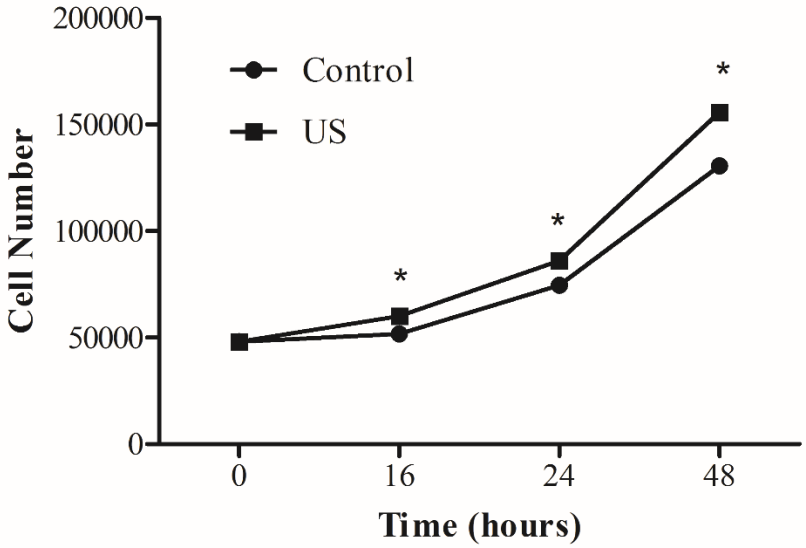

Figure 2: Effect of low intensity ultrasound (US) on pre-osteoblast proliferation considering the time of cell culture. Data expressed as means ± SEM. * $p<0.05$ vs Control.

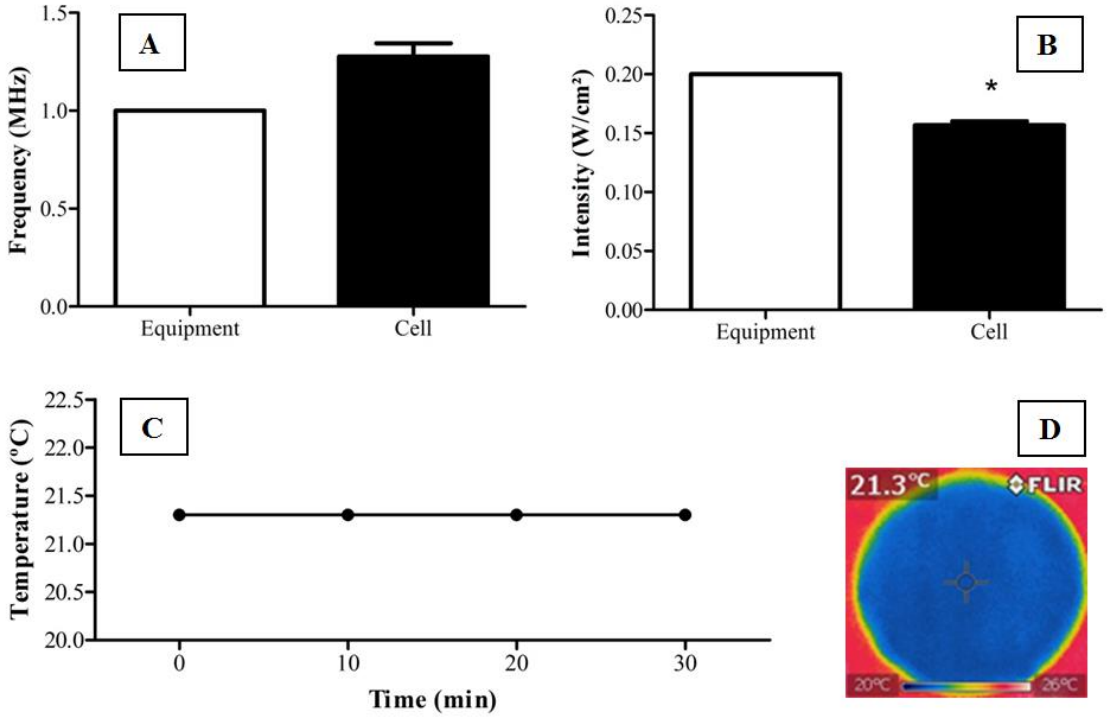

Figure 3: *A)* Frequency of the sound wave programmed in the equipment and wave frequency received by the cells at the time of treatment. Data expressed as means ± SEM. *B)* Intensity of the sound beam adjusted in the equipment and the wave dose received by cells during treatment. Data expressed as means ± SEM. * $p<0.05$ vs. Equipment. *C)* Ultrasound-induced temperature in different time periods (in minutes) of pulsed acoustic wave. Data expressed as means ± SEM. *D)* Infrared thermography of a cell culture well.

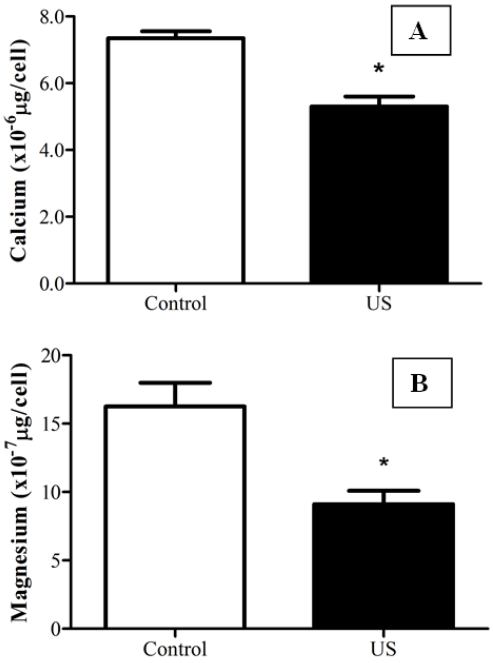

Figure 4: *A)* Effect of low-intensity pulsed ultrasound (US) on calcium concentration in extracellular medium in 48 hours. Data expressed as means ± SEM. * p<0.05 vs. Control. *B)* Effect of low-intensity pulsed ultrasound (US) on magnesium concentration in extracellular medium after 48 hours in culture. Data expressed as means ± SEM. * p<0.05 vs. Control.

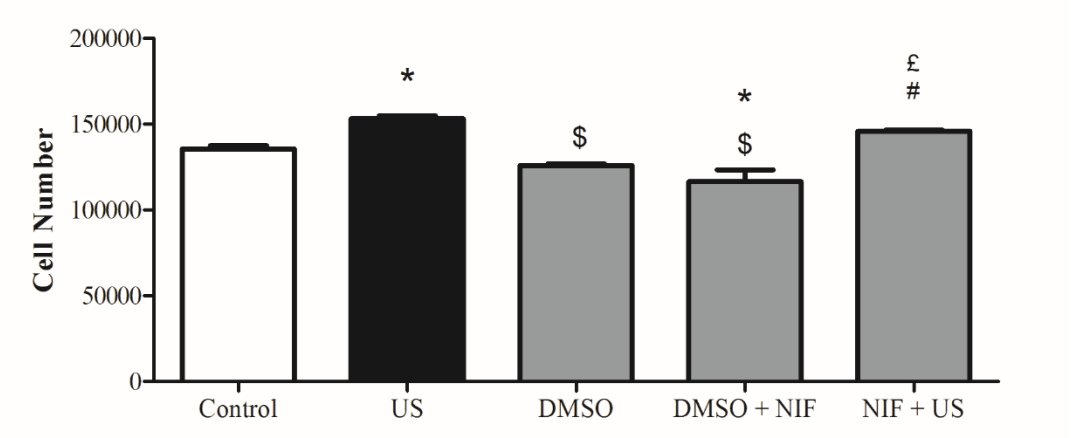

Figure 5: Number of cells after 48 hours in culture in the following groups: control, after the application of ultrasound (US), in medium with DMSO, in medium with DMSO plus nifedipine (DMSO + NIF), with DMSO and nifedipine treated with ultrasound (DMSO + NIF + US). Data expressed as means ± SEM. * p <0.05 vs Control. $ p<0.05 vs US. # p<0.05 vs DMSO. £ p<0.05 vs DMSO + NIF.

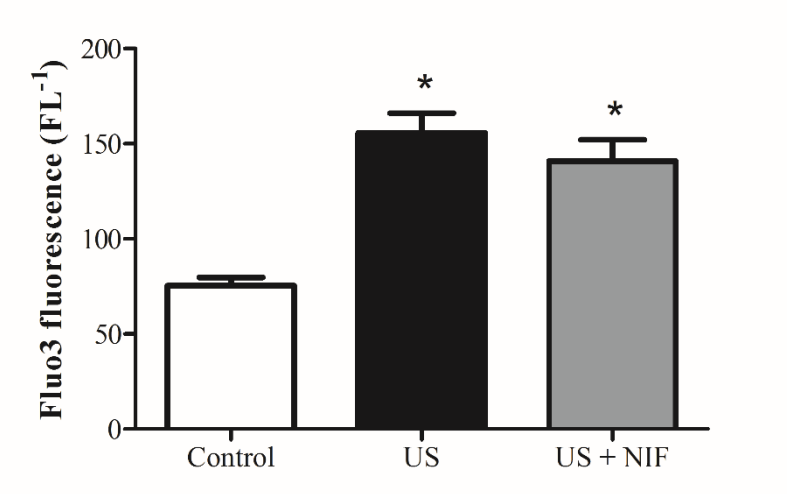

Figure 6: Effect of low-intensity pulsed ultrasound (US) on intracellular Ca^{2+} concentration in Control, ultrasound (US) and ultrasound plus nifedipine (US + NIF) experimental groups in 48 hours. Data expressed as means ± SEM. * $p<0.05$ vs Control.

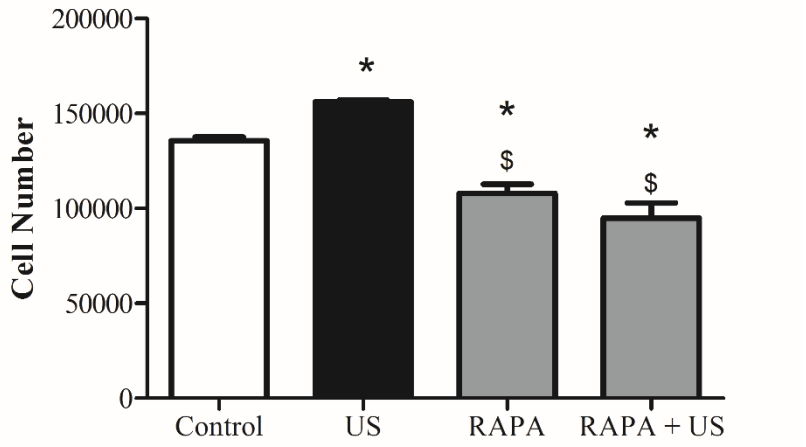

Figure 7: Number of cells after 48 hours in culture in the following groups: Control, after the application of ultrasound (US), in medium with rapamycin (RAPA) and in medium with rapamycin treated with ultrasound (RAPA + US). Data expressed as means ± SEM. * $p<0.05$ vs Control. $ $p<0.05$ vs US.

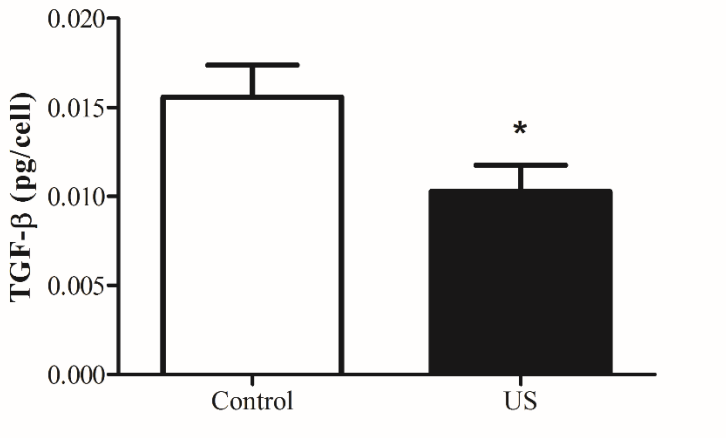

Figure 8: Effect of low-intensity pulsed ultrasound (US) on TGF-β concentration in extracellular medium in 48 hours. Data expressed as means ± SEM. * $p<0.05$ vs Control.

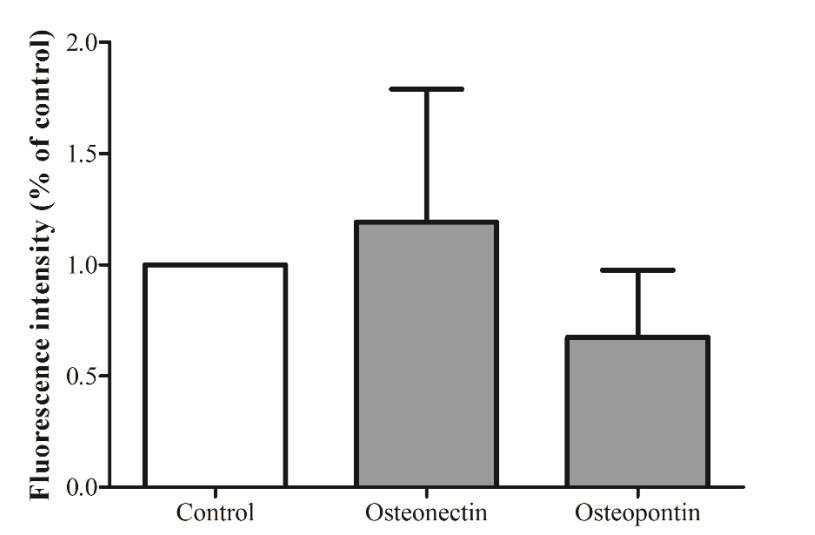

Figure 9: Fluorescence intensity of osteonectin and osteopontin mRNA levels compared to Control group. Data expressed as means ± SEM. * $p<0.05$ vs Control.

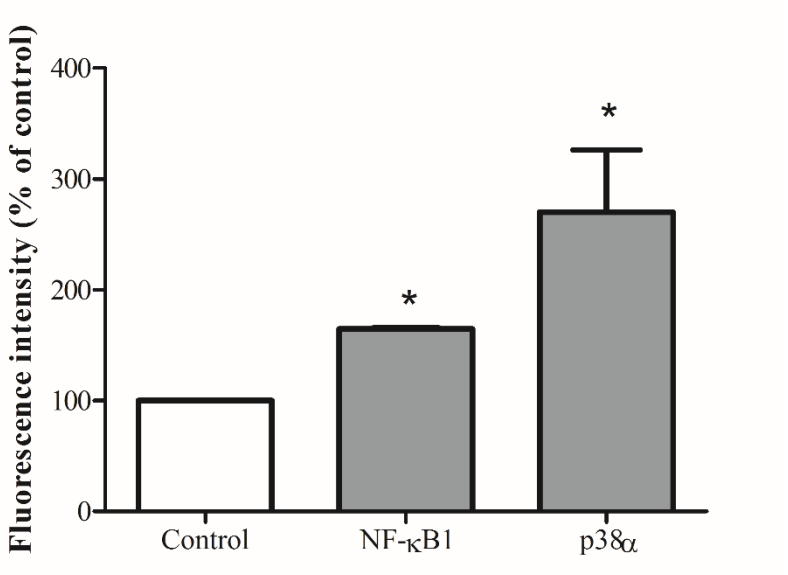

Figure 10: Fluorescence intensity of NF-κB1 and p38α mRNA levels compared to Control group. Data expressed as means ± SEM. * $p<0.05$ vs Control.

Capítulo III

Artigo original

(Formatado de acordo com as normas da Revista Ultrasound in Medicine and Biology/Anexo)

Low-intensity pulsed ultrasound (LIPUS) stimulates mineralization of MC3T3-E1 cells through osteocalcin activation

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Abstract

The present study aimed to evaluate the effect of low-intensity pulsed ultrasound (LIPUS) on pre-osteoblast mineralization using *in vitro* bioassays. Pre-osteoblastic MC3T3-E1 cells were exposed to LIPUS at 1 MHz frequency, 0.2W/cm^2 (SATA) intensity and 20% duty cycle for 30 minutes. The analyses were carried out at 48, 96 and 192 hours after exposure. The concentration of collagen, phosphate, calcium, and TGF-β in cell supernatant and the presence of calcium deposits in the cells were analyzed. Osteocalcin gene expression was analyzed by RT-PCR. Our results showed that LIPUS promotes mineralized nodules formation and increases osteocalcin gene expression. Total collagen, phosphate, and calcium levels were decreased in cell supernatant at 192 hours after LIPUS exposure. However, alkaline phosphatase and TGF-β concentrations remained unchanged. Therapeutic pulsed ultrasound is capable of stimulating differentiation and mineralization of pre-osteoblastic MC3T3-E1 cells by increasing osteocalcin gene expression, calcium and phosphate uptake with consequent hydroxyapatite formation.

Key Words: Low-intensity pulsed ultrasound, Osteoblast, Osteocalcin, Mineralization.

INTRODUCTION

The use of low-intensity pulsed ultrasound (LIPUS) is widespread in clinical practice, mainly for soft tissue rehabilitation (Khanna, et al. 2009). Most of the knowledge on the effects of therapeutical ultrasonic waves is focused on skin wounds, tendons, and muscles (Speed 2001). Currently, the effect of ultrasound (US) on bone rehabilitation is being extensively investigated (Lim, et al. 2013). It is known that the maintenance of bone mass is regulated by multiple stimuli, divided into biochemical (hormones, and growth factors) and mechanic stimulus. Regarding mechanic stimulus, it is well described in the literature that prolonged immobilization, as well as situations of low gravity, induce bone loss (Dauty, et al. 2000). In contrast, the impact on bone tissue increases mass density, a phenomenon described as mechanotransduction (Spyropoulou, et al. 2015). This process occurs when cells sense and respond to mechanical stimuli. The biochemical chain reactions induced by mechanical stimuli act at cellular level and can cause numerous effects, including increased proliferation and bone mineralization (Gusmão and Belangero 2009).

Bone mineralization process starts with the secretion of various regulators, including interleukin-6 (IL-6), transforming growth factor-β (TGF-β) and interferon-γ (INF-γ), and subsequently passes through the synthesis and interaction of several components such as type I collagen, osteocalcin, osteopontin, proteoglycan and phosphoproteins, which form hole-zones where minerals are deposited (Andia, et al. 2006, Koeppen, et al. 2009). Initially, osteoblasts synthesize new collagenous organic matrix and regulate mineralization of the matrix by releasing small, membrane-bound matrix vesicles (MV) that contain glycoproteins and exhibit strong marking for alkaline

phosphatase (ALP). ALP catalyzes the hydrolysis of phosphate ions, releasing them into the vesicles, as well as phospholipids increase calcium concentration into the MV. In this regard, the process goes up to calcium phosphate supersaturation, resulting in the precipitation of calcium phosphate within vesicles. Subsequently, a disruption of membrane vesicles occurs and mineralization spreads by the matrix. The concentration of phosphate and calcium ions forms hydroxyapatite crystals that deposit in and around the collagen fibers and provides strength and hardness to bone tissue (Andia, et al. 2006, Aranachavez, et al. 1995)

MC3T3-E1 cells are rat osteoblasts isolated from neonatal calvaria and represent a pre-osteoblastic phenotype similar to human osteoblasts. Although this cell line has an additional growth rate when compared to human cells, there is a great similarity as to regulation and gene expression (Kang, et al. 2006).

Different treatment parameters and methods have been proposed for the use of US to stimulate bone formation. However, the mechanisms of how US improves bone healing are not well understood. Moreover, the clinical results on bone healing are still controversial, suggesting the need to further investigate its biological responses and the standardization of treatment methods in terms of duty cycle, pulsed mode, frequency, intensity, among others (Padilla, et al. 2014). Therefore, the present study aimed to evaluate the effect of 20% pulsed US at 1 MHz frequency, 0.2W/cm^2 (SATA) intensity for 30 minutes on the mineralization of pre-osteoblastic MC3T3-E1 cells cultured in osteogenic medium.

MATERIALS AND METHODS

Cell culture

Pre-osteoblastic MC3T3-E1 cell line was used in this study. Cell were cultured in alpha medium (Gibco) containing 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Gibco), supplemented with osteogenic medium containing 50 µg/mL ascorbic acid and 5 µg/mL β-glycerophosphate. Cells were kept in an incubator at 37°C in a humidified atmosphere of 5% CO_2 and analyzed daily with an inverted microscope. The subculture was performed when cells reached 90% confluence. Cell viability analysis was performed using trypan blue staining.

LIPUS treatment

Cells were treated using a digital Sonic Compact (HTM) ultrasound apparatus at 1 MHz frequency, 0.2W/cm^2 (SATA) intensity and duty cycle of 20% for 30 minutes. Irradiation of sound waves in MC3T3-E1 was performed inside a laminar air flow cabinet (Telstar AV-100). The ultrasound head was attached to a metal stand with the crystal upwards; the culture dish was placed on the center of the transducer with conductive gel (Hydrogel, Mercur).

For the experimental tests, cells were seeded at 70000 cells per well with a growth area of 22.1 cm². Twenty-four hours after plating, cells supplemented with osteogenic medium were subjected to 30 minutes of US exposure. The control samples were exposed to switched-off US. Analyses were performed 48, 96 and 192 hours after US exposure.

Collagen quantification

The amount of total collagen in culture supernatants was determined using picro-sirius red staining. The absorbance was measured at 540 nm in an ELISA plate reader. Each sample was normalized to the relative amount of total protein. Results were presented as microgram of collagen per milligram of protein.

Analysis of phosphate and calcium content

The calcium and phosphate levels were determined in culture supernatants and specific colorimetric methods (Labtest Diagnóstica) were used for the analyses. The manufacturer's instructions were followed and the results were corrected by the amount of protein. Phosphate and calcium levels were presented as milligram per milligram of total protein.

TGF-β1 quantification

TGF-β1 levels in the cell supernatant were measured by ELISA (R&D Systems). The kit contained an immobilized specific monoclonal antibody on 96-well microplate that binds to TGF-β1. The color development was stopped with sulfuric acid, and the optical density was set to 540 nm with wavelength correction set to 570 nm in an ELISA plate reader. The results obtained were calculated using a standard curve concentration and multiplied by the dilution factor. TGF-β1 levels were presented as picograms per milliliter.

Analysis of alkaline phosphatase activity

The analysis of ALP activity was performed using a commercial method (Labtest Diagnóstica) and US-treated group was compared to controls after 48, 96 and 192 hours. Cell lysates were incubated in a buffer glycylglycine, p-nitrophenyl phosphate, at 37ºC for 2 hours (Vinals, et al. 2002). This methodology is based on the hydrolysis of p-nitrophenyl that releases p-nitrophenol and inorganic phosphate. Data obtained were expressed as ALP activity normalized by total protein content.

Polymerase chain reaction (PCR)

Conventional PCR was performed to analyze the osteocalcin expression. β-actin gene expression was used as an internal control. The PCR products were mixed with bromophenol blue solution, glycerol, and water (6X buffer) and subjected to electrophoresis in 2% agarose gel containing ethidium bromide (EtBr), 1x TAE buffer and after visualized by ultraviolet-induced fluorescence. The bands were analyzed using the Image J software.

Von Kossa staining

The von Kossa staining method (Wang, et al. 2006) was used to quantify mineralization of cell culture 192 hours after US exposure. Cells were fixed with 400 µL 3.7% paraformaldehyde for 10 minutes and then washed twice with distilled water; 400 µL 5% silver nitrate was added and exposed to UV light during 60 minutes. Fixed and stained samples were washed with distilled water and then examined using an inverted microscope.

Statistical analysis

The results were expressed as means ± standard error of the mean (SEM). The Shapiro-Wilk test was applied to determine the normality of the data. The samples were compared using One Way ANOVA, followed by the Bonferroni post-hoc test. Student's t-test was used for group comparison. Statistical analyses were performed using Statistical Package for the Social Sciences Version 18.0 (SPSS Inc.). The level of significance was set at 5%.

RESULTS

To determine the effect of therapeutical LIPUS on the mineralization of MC3T3-E1 pre-osteoblasts, the cells were plated, supplemented with osteogenic medium and stimulated with pulsed US as previously described. Since von Kossa staining is used to demonstrate possible calcium deposits in tissue, we investigated whether LIPUS had an effect on the cell cultures 48, 96 and 192 hours after exposure. In this method, silver salts are formed and later reduced to metallic black silver, and thereby visualized as calcium salts that are often stained black. As shown in Figure 1, mineralization nodules were observed after 192h of treatment. However, this effect was not seen in 48 and 96h.

We have also evaluated the concentration of total collagen in the cell supernatant at 48, 96 and 192 hours after US exposure. Figure 2 shows that at 48 and 96 hours there was no difference between the control group and the US-treated group, however, at 192 hours, the latter presented a lower concentration of collagen when compared to control.

When analyzing the phosphate concentration in the supernatant at the same time points mentioned above, it was observed that the US-treated group showed no difference compared to the control group at 48 and 96 hours, respectively. However, the use of LIPUS decreased the concentration of phosphate at 192 hours after treatment (Figure 3). Subsequently, the concentration of calcium in cell supernatant was analyzed in the same experimental design used for collagen and phosphate measurements. The results showed that the LIPUS decreased the concentration of calcium ion in the supernatant 192 hours after US treatment (Figure 4).

To confirm the effect of LIPUS on mineralization at 192 hours, we also evaluated the gene expression of osteocalcin, which plays a key role in bone

mineralization. The presence of osteocalcin transcripts was observed only in the group treated with LIPUS at 192 hours, as shown in Figure 5.

TGF-β plays a distinct role at each stage of the osteoblast life cycle (Raisz and Rodan 1998). Therefore, we evaluated the concentration of TGF-β1 in the supernatant at 48, 96 and 192 hours after LIPUS treatment. The results showed that there was no difference between the treated and control groups regardless of the time points, as shown in Figure 6.

Since ALP has an important role during pre-osteoblast mineralization, we also evaluated the ALP activity under the same experimental conditions. The results showed that LIPUS had no effect on alkaline phosphatase production, as shown in Figure 7.

DISCUSSION

The present study demonstrates that LIPUS promoted mineral nodules formation 192 hours (8 days) after treatment and that these nodules were not observed in the control group. Usually, these nodules are stimulated in MC3T3-E1 supplemented with osteogenic medium in 336 hours (14 days) (Barbara, et al. 2004). That way, US diminished near 57% the formation time of mineralized nodules.

In vitro and *in vivo* studies have discussed that therapeutic ultrasound, depending on the parameters used, can promote cell proliferation, osteogenic differentiation, and also the formation of extracellular matrix (Man, et al. 2012). It is believed that acoustic radiation forces can generate biochemical signals in MC3T3-E1 cell line, however, the molecular mechanism of such mechanotransduction processes is still elusive. It has been hypothesized that, initially, these acoustic radiation forces can sensitize the osteoblasts through its mechanosensitive surface structure and consequently, obtain a morphological deformation (Zhang, et al. 2012).

The formation of bone tissue depends on the production of bone matrix, which is essentially constituted by collagen and deposition of hydroxyapatite crystals, containing calcium and phosphorus (inorganic phosphate) (Mechica 1999). In this respect, the concentration of collagen, phosphate and calcium were analyzed.

We suggest that US effects on the process of mineralization happens when the extracellular matrix composed of collagen serves as site for calcium (Ca^{2+}) and inorganic phosphate (Pi) accumulation, creating a specific environment where hydroxyapatite (HA) is produced. These needle-like crystals form on the inner surface of the vesicle membrane. The extracellular matrix contains sufficiently high levels of

Ca^{2+} and Pi concentrations to sustain the nucleation process of HA and to propagate mineralization. Then, HA crystals are released into the extracellular matrix after reaching a certain thickness, leading to calcification. The release of Pi into the vesicles is strictly regulated and involves many enzymes and proteins (Anderson 2003, Unsworth, et al. 2007). Among these proteins, Osteocalcin is one of the most important. It is expressed by osteoblasts, odontoblasts and hypertrophic chondrocytes during the development of mineralized tissues and presents a main function of accumulatting bone extracellular matrix (Lian, et al. 1998). Osteocalcin is the second most abundant bone protein, composing 1-2% of the total. It is closely involved in the bone mineralization process and binds to the collagen (Collins, et al. 2002). Based on this, we evaluated the osteocalcin mRNA expression by conventional PCR. LIPUS was effective in stimulating the gene expression at 192 hours.

It has been described that mechanical stimulation, e.g. ultrasound, activates and stimulates the expression of integrins in the cellular surface of MC3T3-E1 cells, the release of anabolic factors and osteoblasts maturation and differentiation (Tang, et al. 2007). Mature osteoblasts produce osteocalcin, which is related to specific signs in bone matrix. Studies showed that osteocalcin induces the formation of HA, especially during Ca^{2+} influx through plasma membrane and from intracellular compartments release (Chenu, et al. 1994).

It is known that osteoblasts secrete TGF-β, which is a local factor and has multiple controversial effects on the osteoblastic activity of cell proliferation and differentiation (Raisz and Rodan 1998). Our results showed that LIPUS has not changed the concentration of TGF-β1 in the supernatant of MC3T3-E1. These cells normally present well-defined sequence of events, including cell proliferation, matrix formation

and its subsequent mineralization. Researches showed that TGF-β1 exerts irreversible effects at a specific stage of osteoblast phenotype development resulting in a potent inhibition of osteoblast differentiation. However, studies have showed that TGF-β1 have paradoxical effects on differentiation of osteoprogenitors cells and may not only stimulate osteoblastic differentiation but also inhibit it. It depends of several factors, such as cell type, cell maturation stage and culture conditions (Breen, et al. 1994, Lieb, et al. 2004). As US accelerated the mineralization rates, we expected a decrease on TGF-β1 levels. However, our results revealed no differences on TGF-β1 concentrations when compared to control group and this leads us to believe that the mineralization occurs through the expression of osteocalcin and does not involve TGF-β1 signaling.

ALP is an important component in hard tissue formation, highly expressed in differentiated tissue cells (Raouf and Seth 2000). According to the studies on the role of ALP in the differentiation of osteoblasts, this enzyme acts both increasing local concentration of Pi, a mineralization promoter, and decreasing concentrations of extracellular pyrophosphate, an inhibitor of mineral formation. For this reason, the increased level of ALP during differentiation process is very important to the development of mineralization. The enzyme is localized in the outside of the cell plasma membrane and in the membrane of MV, attached by a glycophosphatidylinositol anchor (Golub and Boesze-Battaglia 2007). These MV contain high levels of ALP, accumulate high levels of Ca^{2+} and Pi and induce mineralization by compartmentalization of these ions to an extent where crystal nucleation and mineral deposition can occur within membrane-enclosed extracellular microstructures (Bellows, et al. 1991). In the process of bone formation, the matrix collagen production precedes mineralization, therefore, it can be suggested that the production of collagen matrix coincides with a greater

production of ALP, whereas the mineralization coincides with a greater production of osteocalcin (Stein and Lian 1993). Our results showed that US did not change ALP levels on cell lysates in any analyzed time. This suggests that US stimulates mineralization independently of ALP synthesis.

Based on the results obtained, it can be concluded that LIPUS (1 MHz frequency, 0.2W/cm^2 [SATA] intensity and 20% duty cycle for 30 minutes) can stimulate the pre-osteoblast bone cell mineralization 57% faster. We can suggest that LIPUS stimulates the differentiation and mineralization of MC3T3-E1 cells through osteocalcin mRNA expression, calcium and phosphate uptake and consequently HA formation deposited in a framework of collagen fibers. It is important to highlight that US, on these experimental conditions, has not changed ALP and TGF-β1 levels.

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FIGURES

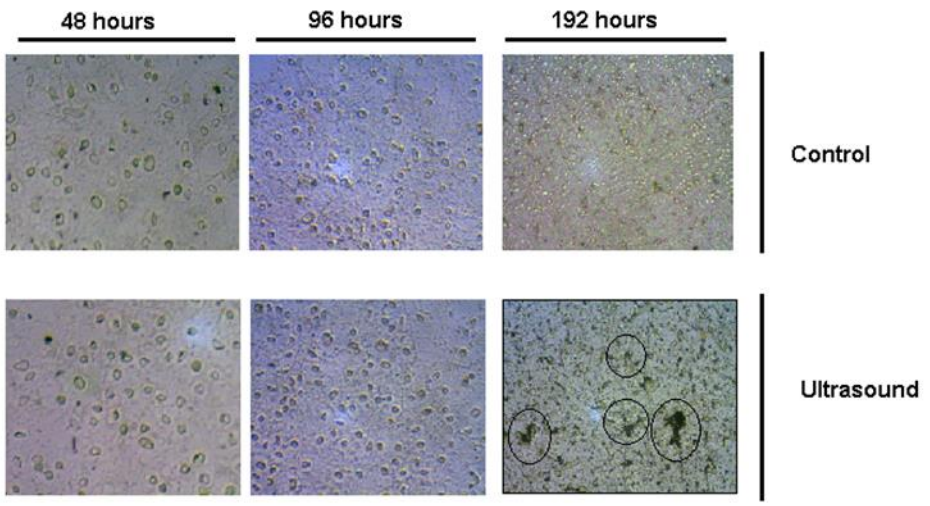

Figure 1. Images of cells submitted or not to LIPUS and stained using the von Kossa method. Black circles show mineralization nodules. Magnification 100x.

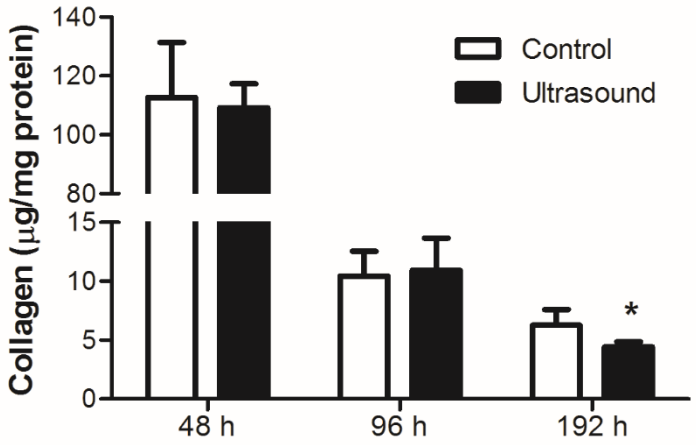

Figure 2. Concentration of total collagen in the extracellular medium at 48, 96 and 192 hours after LIPUS exposure. Data are expressed as mean ± SEM. * $p < 0.05$ versus control.

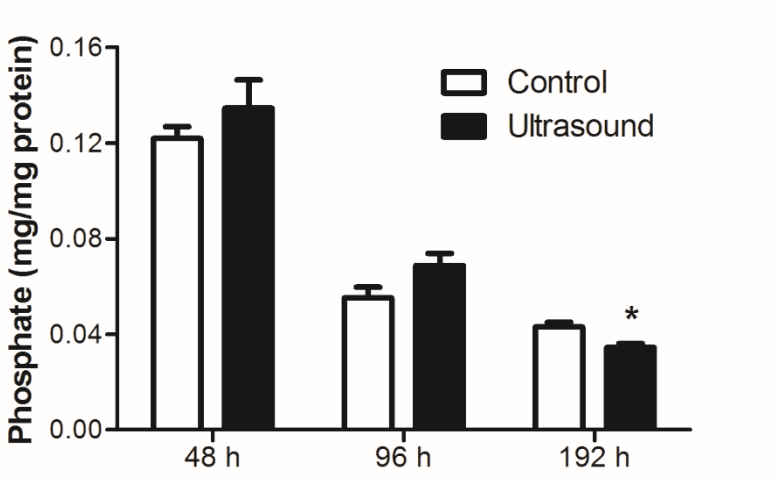

Figure 3. Phosphate levels in the extracellular medium at 48, 96 and 192 hours after LIPUS treatment. Data are expressed as mean ± SEM. * $p < 0.05$ versus control.

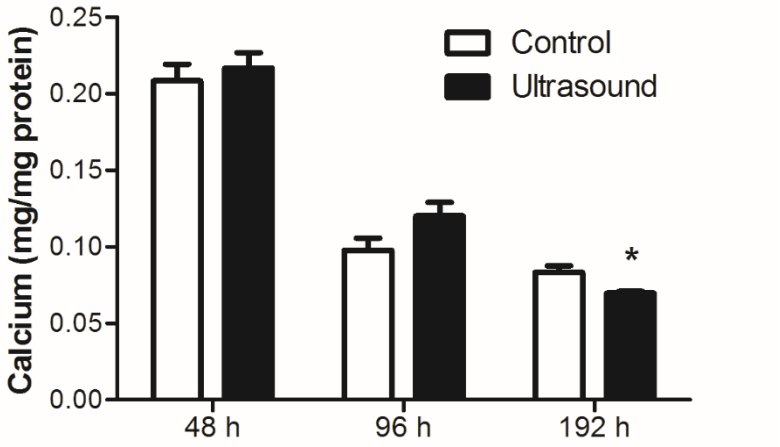

Figure 4. Concentration of calcium in the extracellular medium at 48, 96 and 192 hours after US treatment. Data are expressed as mean ± SEM. * $p < 0.05$ versus control.

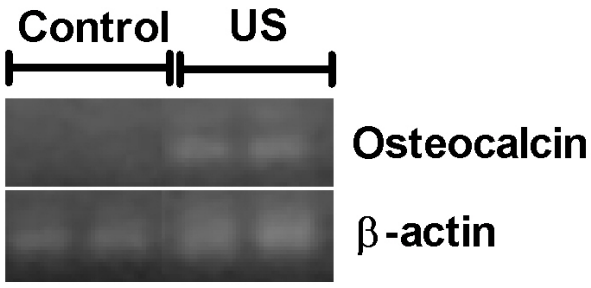

Figure 5. mRNA levels of osteocalcin in MC3T3-E1 cells at 192h after LIPUS exposure.

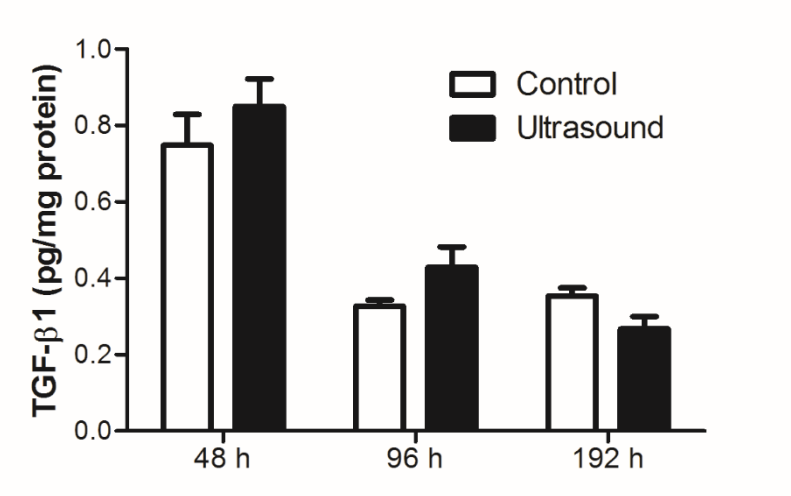

Figure 6. ELISA assay of TGF-β1 in cell supernatant at 48, 96 and 192 hours after LIPUS treatment. Data are expressed as mean ± SEM.

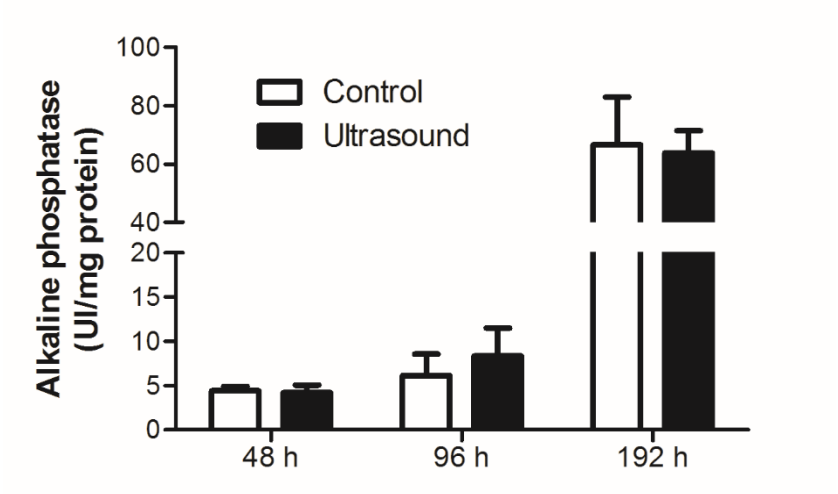

Figure 7. Alkaline phosphatase activity in cell lysate at 48, 96 and 192 hours after LIPUS treatment. Data are expressed as mean ± SEM.

Capítulo IV

Considerações Finais

1. Considerações Finais

Em conjunto, este trabalho verificou o efeito do ultrassom terapêutico no modo pulsado 20%, com uma frequência de 1 MHz, intensidade de 0,2 W/cm^2 (SATA), durante 30 minutos, sobre a proliferação e mineralização de pré-osteoblastos da linhagem MC3T3-E1 *in vitro.* Entretanto, tendo conhecimento que a onda sonora sofre modificações em função do meio em que se propaga, iniciou-se o trabalho avaliando os parâmetros de ultrassom que afetivamente atingiriam a interface das células, e os resultados mostraram que as MC3T3-E1, durante todos os ensaios experimentais, receberam um feixe ultrassônico com uma frequência que variou de 1,15 MHz a 1,38 MHz, ao longo dos trinta minutos de tratamento, com uma dose média de 0,156 W/cm^2, e que não houve aumento de temperatura da placa de cultura. Desta forma, atribuiu-se todos os resultados subsequentes aos princípios físicos do ultrassom e não aos térmicos.

Posteriormente, buscou-se avaliar o efeito do ultrassom na proliferação dos pré-osteoblastos. Nesse sentido, fizemos uma curva de crescimento e os resultados mostram que o ultrassom estimula a proliferação de MC3T3-E1 em 16, 24 e 48 horas após o tratamento. Na busca dos mecanismos que levaram o ultrassom a aumentar a proliferação celular, realizou-se ensaios utilizando o sobrenadante e, igualmente, as células, e sugeriu-se, na primeira parte do trabalho, que o aumento na proliferação celular está diretamente relacionado ao incremento da concentração intracelular de Ca^{++}, ativação do NF-κB1 e do complexo mTOR via p38α, e ainda, por uma diminuição na síntese de TGF-β1, um inibidor do crescimento celular.

É importante frisar, que alguns resultados não foram adicionados à tese, como, por exemplo, o ciclo celular, que mostrou resultados inconclusivos, e as Interleucinas inflamatórias, que foram avaliadas por citometria de fluxo. O método mostrou-se impreciso e com sensibilidade incapaz de detectar concentrações das interleucinas nos sobrenadantes das culturas celulares.

Considerando que os pré-osteoblastos seguem uma sequência de eventos até formar o tecido ósseo, buscou-se averiguar se, nestas mesmas condições experimentais, o ultrassom terapêutico teria a capacidade de acelerar a mineralização.

Sendo assim, suplementamos as células com meio osteogênico, tratamos por 30 minutos e avaliamos uma possível formação de nódulos mineralizados em 48, 96 e 192 horas. Os resultados mostraram que a onda sonora têm efeitos positivos no que diz respeito à mineralização de MC3T3-E1 em 192 horas após o tratamento. Estes efeitos foram inicialmente evidenciados pela técnica de von kossa, que mostrou nódulos de mineralização nas placas de cultura nos grupos tratados com ultrassom, ao contrário do grupo controle. O ultrassom parece estimular a diferenciação dos osteoblastos e consequente provocar o aumento da expressão osteocalcina, captação de cálcio e fosfato o que levaria a formação de cristais de hidroxiapatita, sendo que este processo não parece estar as associado à produção de ALP e TGF-β. Os resultados obtidos nesta tese nos conduziram a um racional que pode explicar o mecanismo de ação do ultrassom nas células MC3T3-E1, que tentamos sumarizar na figura 1.

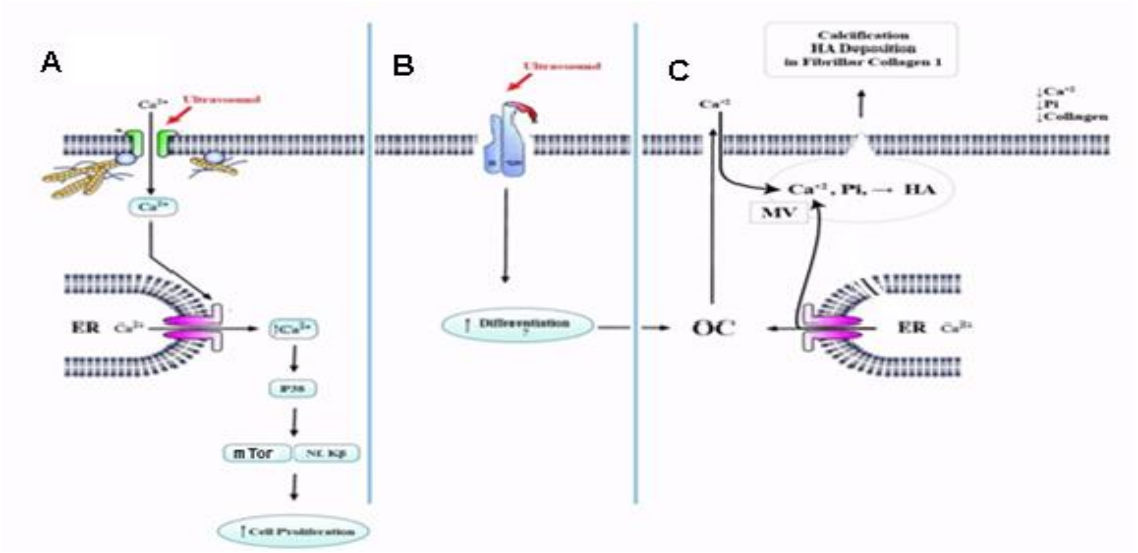

Figura 1. Efeito do ultrassom na proliferação, deferenciação e mineralização de MC3T3-E1. (A) O ultrassom promove um incremento da concentração intracelular de cálcio, ativação do NF-κB1 e do complexo mTOR via p38α, estimulando a proliferação celular. (B) O ultrassom atua sobre as integrinas promovendo diferenciação osteblastica. (C) As células já diferenciadas aumentam a expressão osteocalcina, captação de cálcio e fosfato o que levaria a formação de cristais de hidroxiapatita inicialmente na membrana da vesícula (MV) que posteriormente vai se depositar sobre fibras de colágeno na matriz extracelular.