**UNIVERSIDADE DO VALE DO PARAÍBA**
**INSTITUTO DE PESQUISA E DESENVOLVIMENTO**

**Deise Aparecida de Almeida Pires Oliveira**

# EFEITO DO LASER DE BAIXA POTÊNCIA NA ATIVIDADE CELULAR EM CULTURA OSTEOBLÁSTICA OFCOL II E FIBROBLÁSTICA L929

**São José dos Campos - SP**
**2008**

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Tese de Doutorado apresentada no Programa de Pós-Graduação em Engenharia Biomédica, como complementação dos créditos para obtenção do título de Doutor em Engenharia Biomédica.

Orientadores: Profa. Dra. Cristina Pacheco Soares
Prof. Dr. Renato Amaro Zângaro

**São José dos Campos - SP**

**2008**

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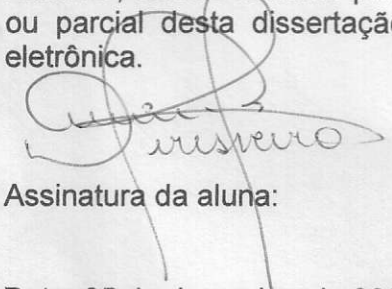

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**DEISE APARECIDA DE ALMEIDA PIRES OLIVEIRA**

**"EFEITO DO LASER DE BAIXA POTÊNCIA NA ATIVIDADE CELULAR EM CULTURA OSTEOBLÁSTICA OFCOL II E FIBROBLÁSTICA L929"**

Tese aprovada como requisito parcial à obtenção do grau de Doutor em Engenharia Biomédica, do Programa de Pós-Graduação em Engenharia Biomédica, do Instituto de Pesquisa e Desenvolvimento da Universidade do Vale do Paraíba, São José dos Campos, SP, pela seguinte banca examinadora:

Profª. Dra. **MARICÍLIA SILVA COSTA** (UNIVAP) Maricilia Costa

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Profª. Dra. Sandra Maria Fonseca da Costa

Diretora do IP&D – UniVap

São José dos Campos, 05 de dezembro de 2008.

***Dedico***

Ao meu marido e “colega” de doutorado Rodrigo, companheiro em todos os momentos, na alegria, na tristeza e no desespero, quando nos experimentos ocorriam erros , nele eu encontrava o meu porto seguro, sempre com uma força me impulsionando a persistir em busca do meu ideal. Você é parte fundamental neste trabalho.

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“... Quero a vida sempre assim, com você perto de mim, até o apagar da velha chama...”

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“Fim de caminhada
A estrada nem sempre foi de flores,
mas os espinhos do caminho
Vocês os retiraram
Obrigada amigos!...” (Autor desconhecido)

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Serei sempre grata aos meus orientadores, Profª Dra. Cristina Pacheco Soares e Prof. Dr. Renato Amaro Zângaro, os quais foram de grande acréscimo científico na minha vida acadêmica e clínica. As suas orientações e interpretações objetivas fizeram-me desenvolver um raciocínio mais lógico das situações em questão contribuindo de modo significativo para a minha formação.

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“A mente que se abre a uma nova idéia jamais voltará
ao seu tamanho original.” (Albert Einstein)

# EFEITO DO LASER DE BAIXA POTÊNCIA NA ATIVIDADE CELULAR EM CULTURA OSTEOBLÁSTICA OFCOL II E FIBROBLASTICA L929

## RESUMO

A Terapia Laser de Baixa Potencia (TLBP) tem sido usado em diferentes campos da saúde, incluindo medicina, odontologia e fisioterapia. A fotobiomodulação é um recurso terapêutico não farmacológico que contribui para acelerar a recuperação dos tecidos biológicos. O objetivo deste trabalho foi avaliar o efeito da biomodulação utilizando laser de baixa potência em células osteoblásticas e fibroblásticas e comparar o efeito das diferentes densidades de energia (dE) nestas culturas celulares. As células foram rotineiramente cultivadas em meio MEM e suplementadas com 10% SFB. Posteriormente foram sub-cultivadas em placas de cultura com 96 poços, e divididas em grupo controle (não irradiadas) e irradiadas, foram utilizadas duas linhagens de células, osteoblásticas e fibroblásticas. As culturas de células Osteoblásticas *(*OFCOL II) foram irradiadas com diodo laser Arseneto Gálio Alumínio ( AsGaAl $\lambda$=830 nm, dE 3 J/cm$^2$ e 2 J/cm$^2$) e Arseneto de Gálio ( AsGa $\lambda$=904 nm, dE 6 J/cm$^2$ e 50 mJ/cm$^2$e as células fibroblásticas (L929) foram irradiadas apenas com diodo laser Arseneto Gálio ( AsGa $\lambda$=904 nm, dE 6 J/cm$^2$ e 50 mJ/cm$^2$) e divididas em 2 grupos : Grupo 1 - células irradiadas; Grupo 2 – células não irradiadas. Irradiação foi realizada durante três dias, em intervalos 24-h. Após cada intervalo, as células foram coradas com MitoTracker Orange ™ e DioC6 para avaliar os efeitos da irradiação sobre atividade mitocondrial e as mudanças no retículo endoplasmático e utilizado teste MTT [3 - (4,5-*dimethylthiazol*-2-il) -2,5 *diphenyltetrazolium* brometo] para avaliar a proliferação celular. O laser 904nm - 50mJ/cm$^2$ apresentou-se mais efetivo 24h em relação ao grupo controle P <0,05 e a 48 horas após irradiação quando comparado com o laser 830nm - 2J/cm$^2$ P <0001. Indicando intensa atividade mitocondrial para este tipo de laser, nesta densidade de energia (904nm 50mJ/cm2). Conclui que a fotobiomodulação com 50 mJ/cm$^2$ foi ligeiramente superior ao de 6 J/cm$^2$, conforme demonstrado por microscopia de fluorescência e que o laser 904nm em emissão pulsada, com largura de pulso estreito (100ns) com densidade de energia 50mJ/cm$^2$ apresentou–se mais efetivo na proliferação celular de linhagem osteoblástica quando comparado ao laser 830nm emissão continua.

Palavras-Chaves: Células osteoblásticas, fibroblásticas bioestimulação, proliferação celular, laser de baixa potencia.

# EFFECT OF LOW-POWER LASER ON CELL ACTIVITY IN OFCOL II OSTEOBLAST AND L929 FIBROBLAST CULTURES


## ABSTRACT

The low-power laser therapy has been used in different health fields, including medicine, dentistry and physiotherapy. Photobiomodulation is a non-pharmacological therapy that contributes toward accelerating the recovery of biological tissues. The objective this work was assess the effect of biomodulation using low-power laser on osteoblast and fibroblast cells and compare the effect of different energy densities on these cell cultures. Osteoblast and fibroblast cells were routinely cultured in MEM and medium supplemented with 10% fetal bovine serum. The cells were subdivided in 96-well culture plates into control (non-irradiated) and irradiated groups. Osteoblast cell cultures (OFCOL II) were irradiated with the Arsenide Gallium Aluminum (AsGaAl) laser diode (830 nm; energy density of 3 J/cm$^2$ and 2 J/ cm$^2$) and Arsenide Gallium (AsGa) laser diode (904 nm; energy density of 6 J/cm$^2$ and 50 mJ/cm$^2$) and fibroblast cells (L929) were irradiated only with the AsGa laser diode (904 nm; energy density of 6 J/cm$^2$ and 50 mJ/cm$^2$) and subdivided into 2 groups: Group 1 - irradiated cells; Group 2 - non-irradiated cells. Irradiation was performed for three days at 24-h intervals. After each interval, cells were stained with MitoTracker Orange™ and DioC6 to assess the effects of irradiation on mitochondrial activity and changes in the endoplasmic reticulum; the MTT [3 - (4,5-dimethylthiazol-2-yl) -2, 5 diphenyltetrazolium bromide] was used to assess cell proliferation. The 904 nm laser – 50 mJ/cm$^2$ proved more effective 24 h after irradiation in comparison to the control group ($p<0.05$) and 48 hours after irradiation when compared to the 830 nm laser – 2 J/cm$^2$ ($p<0001$). This indicates intense mitochondrial activity for this type of laser at this energy density (904 nm, 50 mJ/cm$^2$). Photobiomodulation performed with 50 mJ/cm$^2$ was slightly greater than that with 6 J/cm$^2$, as demonstrated by fluorescence microscopy. Furthermore, 904 nm pulsed laser with a narrow pulse width (100 ns) and energy density of 50 mJ/cm$^2$ proved more effective in osteoblast cell proliferation when compared to the 830 nm laser in continuous emission.



Keywords: osteoblast cells, fibroblast biostimulation, cell proliferation, low-power laser.

## LISTA DE ILUSTRAÇÕES

**SUMÁRIO**

# 1 INTRODUÇÃO

A palavra Laser é o acrônimo de *Light Amplification by Stimulated Emission of Radiation* (Amplificação da Luz por Emissão Estimulada de Radiação). É uma forma de energia que se transforma em energia luminosa, visível ou não, dependendo da matéria que produz esse tipo de radiação (GENOVESE, 2007).

As propriedades terapêuticas dos lasers vêm sendo estudadas desde a sua descoberta por Einstein, em 1917.

A Terapia Laser de Baixa Potencia (TLBP) tem sido usado em diferentes campos da saúde, incluindo medicina, odontologia e fisioterapia. A fotobiomodulação é um recurso terapêutico não farmacológico que contribui para acelerar a recuperação dos tecidos biológicos. A irradiação laser é comumente associada com proliferação celular e a ação no metabolismo celular tem sido estudada por vários pesquisadores. (WERNECK et al., 2005, MOORE et al., 2005, PEREIRA et al., 2002). A ação do laser de baixa potência varia de acordo com o espectro da radiação eletromagnética, vide figura abaixo.

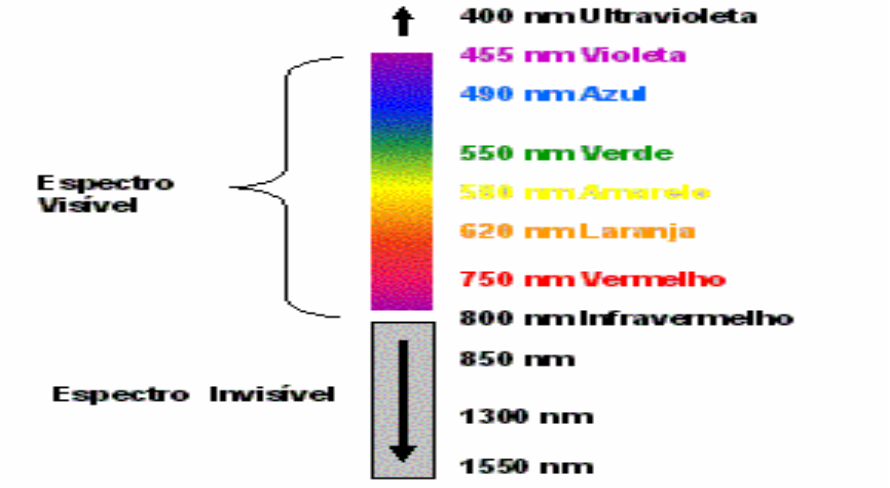


**Figura 1: Esquematização do espectro eletromagnético para comprimento de onda (λ).**
Fonte:http//images.google.com.br/imgres?imgurl=http://www.teleco.com.br/imagens/tutoriais
Acesso em 25 de novembro de 2008.

Brondon et al., (2005) relatam que estudos *in vitro* e *in vivo* tem demonstrado que a TLBP exerce influência significante sobre a função celular (organização do citoesqueleto, atividade mitocondrial e estimulação da membrana plasmática).

A biomodulação desencadeada pela TLBP nas células depende da combinação de parâmetros tais como: comprimento de onda, densidade de energia e densidade de potência, podendo contribuir para aumento da síntese de colágeno, proliferação celular e respiração mitocondrial. (SILVEIRA et al., 2007, PEREIRA et al., 2002, ALMEIDA-LOPES et al., 2001, KARU 1988).

Eells et al., (2004) descrevem que a função mitocondrial é estimulada pela sua elevada fotorrecepção à luz monocromática do laser. Dessa maneira, ativando os componentes da cadeia celular.

A magnitude do efeito biomodulatório do laser depende do estado fisiológico da célula, dessa forma explica porque a biomodulação nem sempre é detectada e há variações de respostas encontradas na literatura, Karu (1988) propôs uma cascata de eventos que iniciam com a absorção da luz pelos fotorreceptores que ativam as enzimas no interior da mitocôndria, resultando em foto resposta. A absorção da luz pelos componentes da cadeia respiratória leva a mudança nas mitocôndrias e citoplasma. De acordo com Karu (1988), os mecanismos de ação do laser em relação às células na região do vermelho e infravermelho diferem, porém na clínica os resultados são similares.

Demir et al., (2004) expõe que o laser acelera reações bioquímicas, atividade fibroblástica, síntese de colágeno, neovascularização e aumento da atividade de leucócitos e fagócitos tanto em estudos *in vivo* como *in vitro.* Vários efeitos bioestimulatórios do laser foram relatados na cicatrização de feridas e síntese de colágeno tanto *in vivo* como *in vitro* (NINOMIYA, 2007).

Mecanismos físicos, tais como TLBP modula a inflamação, acelera a proliferação celular e reforça a cicatrização (NINOMIYA et al., 2007, KHADRA et al., 2005).

Entretanto, Stein et al., 2005, Ozawa et al., 1984 relatam que este tipo de terapia recebe atenção não somente ao tecido mole, mas também no metabolismo ósseo utilizada em promover a reparação óssea, formação de nódulo ósseo,

diferenciação osteoblástica, e atividade de fosfatase alcalina (ALP). Segundo Lirani-Galvão et al., (2006) a TLBP, estimula a vascularização, a organização das fibras de colágeno, os níveis de ATP, o aumento da atividade osteoblástica e a formação óssea**;** estimulando proliferação celular óssea e atividade de ALP o que reflete em atividade osteoblástica no qual pode acarretar aumento de cálcio intracelular (UEDA e SHIMIZU 2003)**.**

O mecanismo primário de interação laser-molécula foi descrito por (Karu) 1987 e encontra-se esquematizado a seguir.

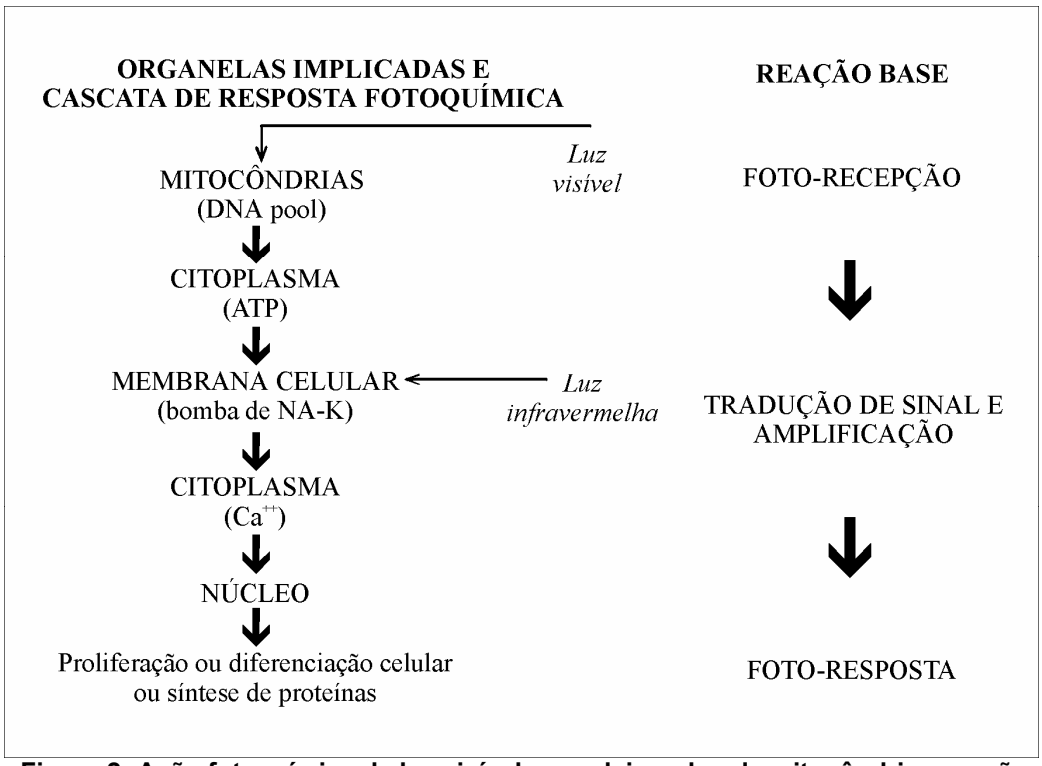


**Figura 2: Ação fotoquímica da luz visível na cadeia redox da mitocôndria e a ação da luz infravermelha na membrana celular,**
Fonte: Karu (1987).

Karu (1988) sugeriu que o mecanismo de ação da TLBP nas células eucarióticas é baseado na absorção da radiação visível monocromática e do infravermelho próximo pelos componentes da cadeia respiratória, causam um aumento no metabolismo oxidativo das mitocôndrias, devido à excitação eletrônica dos componentes da cadeia respiratória. Estas reações primárias são seguidas por modulação do estado redox intracelular (reações secundárias, sinalização

celular), sendo esta energia luminosa do laser absorvida pelos citocromos e porfirinas dentro da mitocôndria e membrana plasmática (KARU et al., 2001).

# 2 OBJETIVO

## 2.1 Objetivo Geral

Avaliar o efeito da biomodulação utilizando laser de baixa potência em células osteoblásticas e fibroblásticas.

## 2.2 Objetivos Específicos

- ✓ Comparar laser 830nm e 904nm em cultura osteoblástica.
- ✓ Comparar o efeito das diferentes densidades de energia (dE) em cultura fibroblástica e osteoblástica.

# 3 RESULTADOS

## 3.1 Artigo 1: Photomedicine and Laser Surgery

Deise A. A. Pires OLIVEIRA; Rodrigo Franco de OLIVEIRA; Renato A. ZÂNGARO; Cristina PACHECO-SOARES. Evaluation of low level laser therapy of oateoblastic cells. **Photomedicine and Laser Surgery,** v.26, p.401-404, 2008.

## 3.2 Artigo 2 : Photomedicine and Laser Surgery

Deise. A. A. Pires Oliveira, Rodrigo Franco de Oliveira, Aline Helena Araújo Machado, Renato Amaro Zângaro, Cristina Pacheco-Soares. Laser Photobiomodulation of L 929 Cell Cultures. **Photomedicine and Laser Surgery**, v .xx, n. xx, p. xx, 2008.

**Laser Photobiomodulation of L 929 Cell Cultures**

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**No competing financial interests exist.**

## ABSTRACT

Low-level laser therapy (LLLT) is a non-pharmacological resource that induces important photobiomodulation in biological tissues. The aim of the present study was to analyze the effects of photobiomodulation by a 904-nm diode laser at two energy densities, 6 J/cm$^2$ and 50 mJ/cm$^2$ on L929 fibroblast cells. The cells were routinely cultured in MEM supplemented with 10% FBS, later sub-cultivated in 96-well culture plates, and divided into three groups: Group 1, control; Group 2, irradiated at 6 J/cm$^2$, and Group 3 irradiated at 50 mJ/cm$^2$. Irradiation was performed for three days in 24-h intervals. After each interval, the cells were stained with MitoTracker Orange™ and DioC6 dyes to assess the photobiomodulatory effects of irradiation on mitochondrial activity and the changes in the endoplasmic reticulum. MTT assay [3-(4.5-dimethylthiazol-2-yl)-2.5 diphenyltetrazolium bromide] was used to evaluate cell proliferation. Results: Fluorescence microscopy assessment of mitochondria and endoplasmic reticulum of cells irradiated with 6 J/cm$^2$ and 50 mJ/cm$^2$ demonstrated intense mitochondrial activity, which was confirmed by DioC6 staining. It was observed reticular activity stemming from increased protein synthesis. Simultaneous comparison of the effects of irradiation on Groups 2 and 3 at the two different energy densities by MTT assay revealed increased cell viability with the increase in post-irradiation time relative to the control ($p \leq 0.05$). Regarding temporal evolution (24, 48, and 72 h), the cell growth rates of both irradiated groups were comparable for 24 and 72 h after irradiation within a single group ($p = 0.01$) regardless of the energy density used.

**Conclusion**: Photobiomodulation with 50 mJ/cm$^2$ was slightly higher than with 6 J/cm$^2$, as demonstrated by fluorescence microscopy results. Photobiomodulation was also time-dependent, with better results 72 h after irradiation.

**Key Words**: Cell culture, fibroblasts, low-power laser, photobiomodulation

## INTRODUCTION

Low-level laser therapy (LLLT) has been used in different healthcare fields, including medicine, physical therapy, and dentistry. Photobiomodulation is a non-pharmacological therapeutic resource that contributes to accelerate the functional recovery of biological tissues. Laser irradiation is commonly associated with cell proliferation and its action on cell metabolism has been studied by a number of researchers[1,2,3]. It is known that the action of LLLT varies according to the electromagnetic radiation spectrum.

The mechanisms of action of infrared and visible region low-level laser on cells differ[4], but their clinical results are quite similar. LLLT-triggered photobiomodulated cell response depends on a combination of parameters, such as wavelength, and energy and power density[2,4,5,6], and may contribute to increase collagen synthesis, cell proliferation, and mitochondrial respiration.

*In vitro* and *in vivo* studies have demonstrated that LLLT exerts significant influence over cell function (cytoskeleton organization, mitochondrial activity and plasma membrane stimulation)[7]. The LLLT photobiomodulatory effect on mitochondria triggers the activation of ATP production through an increase in the mitotic process due to excitation of cell respiration and endogenous porphyrins. However, the increase in mitochondrial ATP produced by the variation in the energy absorbed by the cell also affects cell metabolism[8], such as mitochondrial oxide reduction, resulting in cascaded biochemical reactions[9]. The irradiation of isolated mitochondria also affects the transcription and transduction of mitochondrial proteins, thereby increasing the reaction cascade, as well as the number of components in the respiratory chain (cytochrome oxidase and flavin dehydrogenase)[10].

MTT is one of the most frequently used assay methods for measuring cell proliferation and cytotoxicity. In living cells, tetrazolium salt MTT is reduced to formazan [1-(4.5-dimethylthiazol-2-yl)-3,5 diphenylformazan], which cannot cross the cell membranes and therefore accumulates in healthy cells and can be colorimetrically analyzed. The reduction of MTT is generally attributed to mitochondrial activity, but it has also been related to non-mitochondrial enzymes, as well as to endosomes and

lysosomes. If the MTT assay reflects mitochondrial metabolic activity precisely, it can be useful to evaluate cell viability. It has been tested for validity in various cell lines[11,12,13,14,15].

The aim of the present study was to determine the action of different levels of laser photobiomodulation on L929 fibroblast cells at 904 nm and energy densities (Ed) of 6 J/cm$^2$ and 50 mJ/cm$^2$.

## MATERIALS AND METHODS

L929 cells (mouse conjunctive tissue - ATCC CCL-1 NCTC) (Cell Culture Laboratory, Instituto Adolfo Lutz, SP, Brazil) were used in *in vitro* toxicity tests, as recommended by ISO 10993-5. The cells were routinely cultivated in 25-cm$^2$ flasks (TPP, Switzerland, Europe) in Minimal Essential Medium (MEM) (Gibco™ - Invitrogen Corporation, Grand Island, USA) supplemented with 10% (vol/vol) bovine fetal serum (BFS) (Cultilab Materiais para Cultura de Células Ltda, Campinas, Brazil) 100 U/mL penicillin, 100 mM/mL streptomycin, and 0.25 μg/mL fungizone™ (GibcoBRL). The cells were cultured in an incubator (Forma USA) at 37 °C in humidified 5% $CO_2$ atmosphere. The medium was changed every 2 days. When the cells became confluent, the medium was removed and the cell layer was washed with phosphate-buffered saline and 0.25% Trypsin™ (GibcoBRL) in buffered EDTA (Carlos Erba, ABC Lab, São Paulo) was then added and incubation was continued for 2 to 4 min. A solution with proximate concentration of 1 x 10$^4$ cells/mL was prepared and poured into each well of 96-well culture plates (TPP™ Switzerland, Europe). The protocol was approved by the Research Ethics Committee of Univap, Protocol No. A061/CEP/2006.

### Laser Photobiomodulation

A gallium arsenide diode laser (GaAs, KLD™) emitting radiation at λ = 904 nm, repetition rate of 10 KHz, and output power of 50 mW was used to irradiate three groups of fibroblast cells sub-cultured in 96-well culture plates (TPP™ Switzerland, Europe) with a density of 1 x 10$^4$ cells/mL, Group 1, non-irradiated (control); Group 2,

irradiated at 6 J/cm$^2$; and Group 3, irradiated at 50 mJ/cm$^2$. The cell cultures were cultivated in a well with 0,3cm$^2$ of cross section receiving radiation perpendicularly to the plate in 24-, 48-, and 72-h intervals for 36 s at 6 J/cm$^2$ and 2 s at 50 mJ/cm$^2$.

Control cells (non-irradiated) were submitted to the same condition as the laser-irradiated cells were.

**Cell viability by MTT assay (cytotoxicity)**

For the MTT assay, 1 x $10^4$ cells/mL L929 cells were plated in each well of a 96-well plate. After each incubation period with 24-h intervals, the cytotoxic effect of laser irradiation on cell activity was analyzed by MTT assay. It measures the percent cell survival in comparison to untreated controls. It is widely used for analyzing cytotoxicity and cell viability and proliferation[15]. Each well received 20 μL of MTT to a final concentration of 0.5 mg/mL and the plate was incubated for 1 h at 37 °C in 5% $CO_2$ atmosphere. An aliquot of 100 μL of DMSO (dimethyl sulfoxide) was then added to each well and the plate was agitated for 30 min for solubilization of the formazan crystals. Crystal concentration was spectroscopically quantified by means of a microplate reader (ELISA Reader- SpectraCount - Packard Instrument, USA) at excitation wavelength of 570 nm.

**Fluorescence Microscopy Procedures**

All dyes used in the fluorescence studies were purchased from Molecular Probes (Eugene, OR, USA). Just prior to imaging, the cells were stained by incubation in fluorescent dye-containing growth medium at 37 °C as follows:

MitoTracker Orange was used as a probe for evaluation of mitochondria membrane potential changes ($\Delta\psi$m). After each incubation interval (24, 48, and 72 h), the cells were stained with: **MitoTracker Orange Dye** (CMTMros™) Molecular Probes[10]

(150 nm), incubated for 20 min in the dark, immediately washed with PHEM buffer (60 nm PIPES, 20 nm HEPES, 10 nm EGTA, 5 Mm MgCl2), and fixed in 3% PA (paraformaldehyde) (Sigma-Aldrich Chemie GmBh, Steinheim, Germany) in 0.1 m PHEM for 10 min. **DioC6** (3,3'- dihexyloxacarbocyanine iodide) was used to study endoplasmic reticulum changes. The cover slips were rinsed with PBS, next, incubated with 10 μg/mL DioC6 for 30 min, immediately rinsed twice with PBS, fixed with 4% PA in buffer PHEM (0.I M) for 10 min, and rinsed again to eliminate excess dye. Cover slips were mounted on slides with n-propyl gallate and observed through fluorescence microscopy. Observations and photographs were made in Leica microscope (DLMB). For fluorescence analysis, approximately 100 cells were observed on each plate. Plates were observed and photographed in triplicate for the increase in fluorescence intensity of organelles excitation (554 nm) and emission (576 nm) filters were used.

## STATISTICAL ANALYSIS

Three analyses were performed for each group (control and experimental), totaling 27 observations. Descriptive statistics were used to describe the results, expressed as mean and Standard Error (± SEM). Comparisons between groups were performed with two-way analysis of variance for repeated measures (ANOVA). The post hoc Tukey-HSD analysis was used to determine significant differences between groups Tukey test shows a power of contrast de 95%. Values of $p \leq 0.05$ were considered statistically significant. Statistical analysis was performed with the GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

## RESULTS

The effects induced on L929 cells by laser radiation at energy densities of 6 J/cm$^2$ and 50 mJ/cm$^2$ were determined with the aid of fluorescence staining and MTT assay in Groups 2 and 3 (irradiated) following each 24-h incubation interval. Group 1 (non-irradiated) was submitted to the same analyses.

Fluorescence microscopy demonstrated that the mitochondria of MitoTracker-stained control cells were dispersed in the periphery of the cytoplasm, with evident nucleus localization in the absence of fluorescence (Fig. 1a). The same occurred with DioC6-stained control cells, with the endoplasmic reticulum spread throughout the entire cytoplasm (Fig. 1b). However, MitoTracker was distributed throughout the cell in the groups irradiated with 6 J/cm$^2$ and 50 mJ/cm$^2$. Staining concentration in the perinuclear region suggests intense mitochondrial activity (Fig.1c-d), as confirmed by the intense reticular activity observed by DioC6-staining stemming from increased protein synthesis (Fig. 1e-f). The fluorescence intensity of all irradiated cells was larger than that of the non-irradiated cells, indicating increased ATP synthesis.

These results corroborate those of Karu's 1999[16] and Amat's 2006[17], who reported that mitochondria are sensitive to monochromatic visible and near-infrared (IR) light irradiation. Mitochondria are believed to be the primary targets when whole cells are irradiated with visible and near-infrared light and an increase in ATP synthesis is observed. Considering that the mitochondrial membrane potential is directly related to ATP production and synthesis stimulation, the cell metabolism can be significantly affected.

Simultaneous comparison by MTT assay revealed that Groups 2 and 3 exhibited an increase in cell viability with the increase in post-irradiation time under irradiation at the two different energy densities studied in relation to the control ($p \leq 0.05$) (Table 1). Concerning temporal evolution (24, 48, and 72 h), cell growth occurred with similar intensity in both irradiated groups regardless of the energy density used when comparing 24- and 72-h post-irradiation within a single group ($p = 0.01$) (Table 2).

Figure 1 Fluorescence

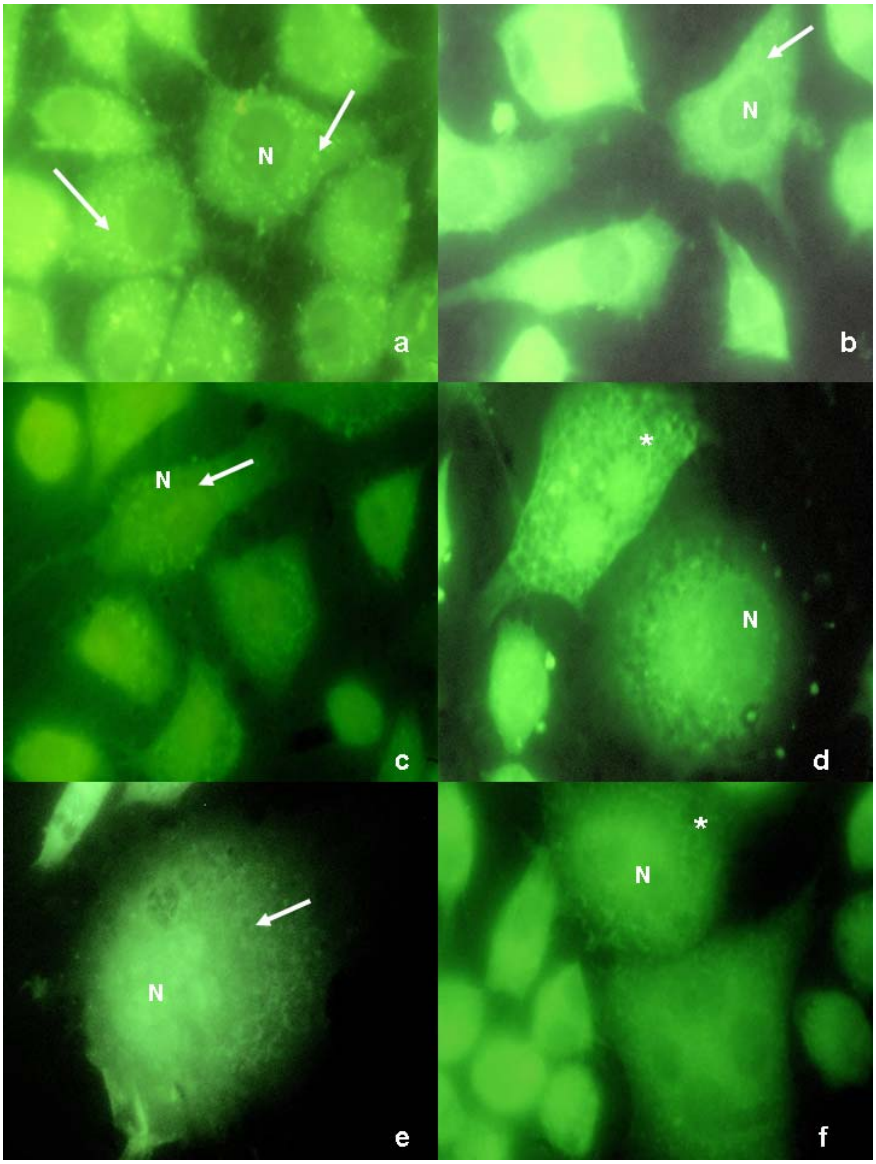


Fig 1 – Mito Tracker Orange™ and DioC6-stained L929 cells death after 72 h
**a)** Mitotracker-stained control group (non-irradiated), **b)** DioC6-stained control group (non- irradiated), **c)** Mitotracker-stained group irradiated with 6 J/cm$^2$, **d)** DioC6-stained group irradiated with 6 J/cm$^2$, **e)** Mitotracker-stained group irradiated with 50 mJ/cm$^2$, **f)** DioC6-stained group irradiated with 50 mJ/cm$^2$

N - nucleus
Arrow - mitochondria
Asterisk - Intense reticular activity

Table 1 - Analysis between groups (effects of different radiation energy densities)

| LASER 904 nm - L929 | | | | | | |
|---|---|---|---|---|---|---|
| **Time (h)** | **Control** | **6J** | **50mJ** | **Control *vs.* 6J** | **Control *vs.* 50mJ** | **6J *vs.* 50mJ** |
| 24 | 94*<br>0** | 113,2*<br>17,97** | 135,3*<br>15,97** | 0,001*** | 0,001*** | 0,01*** |
| 48 | 99*<br>0** | 152,0*<br>15,68** | 179,4*<br>16,31** | 0,001*** | 0,001*** | 0,01*** |
| 72 | 96*<br>0** | 173,6*<br>18,72** | 186,5*<br>22,49** | 0,001*** | 0,001*** | 0,023*** |

[Mean]*
[SEM]** Standard Error Mean
[p≤0,05]***

Table 2: Intra-group analysis (effect of different temporal stages)

LASER 904 nm - L929

| **Time (h)** | **Control** | **6J** | **50mJ** | **6J (Time vs Time)** | **Control (Time vs Time)** | **50mJ (Time vs Time)** |
|---|---|---|---|---|---|---|
| 24 | 94*<br>0** | 113,2*<br>17,97** | 135,3*<br>15,97** | 24 vs. 48<br>0,05*** | 24 vs. 48<br>- nd | 24 vs. 48<br>0,05*** |
| 48 | 99*<br>0** | 152,0*<br>15,68** | 179,4*<br>16,31** | 24 vs. 72<br>0,01*** | 24 vs. 72<br>-nd | 24 vs. 72<br>0,01*** |
| 72 | 96*<br>0** | 173,6*<br>18,72** | 186,5*<br>22,49** | 48 vs. 72<br>0,05*** | 24 vs. 72<br>-nd | 48 vs. 72<br>0,05*** |

[Mean]*
[SEM]** Standard Error Mean
[p≤0,05]***
[Nd] For values which have variance and standard deviation equal to zero.

## DISCUSSION

In the present study, L929 cells submitted to proliferation and MitoTracker-staining exhibited mitochondria distributed throughout the cell, including in the perinuclear region, thereby suggesting intense mitochondrial activity. According to Ferreira et al.[18], the presence of mitochondria in the perinuclear region may be associated with endoplasmic reticulum with bi-directional calcium flow, as well as with acidic vesicles, which may play an important role in proton gradient regulation across the mitochondria membrane**.** Fluorescence analysis by DioC6-staining revealed intense reticular activity resulting from post-irradiation protein synthesis.

According to Vellonen et al.[13], if the concentration of MTT in the cytoplasm is increased, more formazan products are formed and higher irradiation absorption by the cells is detected, indicating higher cell viability. MTT is not permeable to the lipid plasma membrane and it has been suggested that MTT enters the cells through endocytosis. A reduction in MTT has been associated with mitochondrial activity, as well as endosome and lysosome enzymes. MTT can also be reduced by the receiving electrons in mitochondria and other cellular organelles[12], indicating that it is dependent on cellular redox activity. Therefore, MTT reduction reflects the mitochondrial function and may be used as an indicator of cellular oxidative metabolic activity.

Photobiomodulation of L929 fibroblast cells with near-infrared radiation from GaAs laser (904 nm) produces biological effects directly dependent on dosimetry[2,4,7,18]. Skinner, Gage & Wilce[19] used pulsed GaAs laser to administer different energy densities to human embryo fibroblast cell cultures and observed a significant increase in collagen levels in irradiated cells.

Both energy densities used in the present study resulted in cellular growth metabolism dependent on post-irradiation time. Moore et al.[3] and Pinheiro et al.[20] observed an inhibitory effect for high cumulative doses with energy densities above 10 J/cm$^2$ over long periods. The cumulative doses used in that study for Groups 3 and 2 of 0.15 J/cm$^2$ and 18 J/cm$^2$, respectively were not inhibitory, even at 18 J/cm$^2$. Moore et al.[3] observed different cell proliferation responses at different wavelengths (665 and 810 nm) and inhibition following fibroblast cell irradiation in the infrared band (810 nm).

Another aspect to be considered is the different wavelengths used, as the present study used 904 nm.

According to Karu[4], cell irradiation with visible light has both positive (acceleration of cell division) and negative effects (damage to the intracellular system and apoptosis). The author also states that the occurrence of cell inhibition is related to the cumulative effect of the dosage accompanied by growth inhibition, cell death, and the destruction of photoreceptors. There is accelerated electron transference in the redox pairs in some sections of the respiratory chain and transference of energy from oxygen excitation, which explains the photobiomodulatory effect of low-intensity radiation.

Karu[4] proposed a cascade of events that begins with the absorption of light by a photoreceptor that conducts photo-activated enzymes in the interior of mitochondria, with resulting photo-response. Light absorption by components of the respiratory chain leads to changes in the mitochondria and cytoplasm. The magnitude of the laser photobiomodulatory effect depends on the physiological state of the cell at irradiation. This may explain why photobiomodulation is not always detectable and the variability of results reported in the literature.

Laser therapy is commonly associated with cell stimulation by both visible and near-infrared radiation through the membrane potential or mitochondria**.** Mognato et al.[9] compared HeLa and TK6 cells irradiated with continuous 808-nm and pulsed 905-nm laser doses of 1-60 J/cm$^2$, obtaining better results and dose dependence at 905 nm. In the present study, irradiation at wavelength 904 nm increased cell proliferation at both 50 mJ/cm$^2$ and 6 J/cm$^2$ over the control group. Using the same type of pulsed laser on fibroblast cell cultures, Pereira et al.[2] also obtained better cell proliferation results at energy density of 5 J/cm$^2$. Our were results corroborate the studies by Oliveira et al.[21], who compared the effect of low-level ultrasound and low-level laser with the same laser parameters of the present study and demonstrated an effect on cell growth at 6 J/cm$^2$ and 50 mJ/cm$^2$, being higher at 50 mJ/cm$^2$.

Irradiation of L929 cells with pulsed laser in the near infrared region (904 nm) at two quite distinct energy densities allowed the observation of increased cell proliferation in both cases. The result was slightly higher for energy density of 50 mJ/cm$^2$ than for

6 J/cm$^2$, as demonstrated by fluorescence microscopy results. It was also observed that cell response is time-dependent and better 72 h after irradiation.

## ACKNOWLEDGEMENTS

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### 3.3 Artigo 3: Cell Biology International

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# Osteoblastic cell response to low-level laser irradiation

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**ABSTRACT**

The effect of low-level laser therapy (LLLT) on bone regeneration has become the focus of recent research in different healthcare fields, such as medicine, physical therapy and dentistry. The purpose of the present study was to investigate the response of osteoblastic cells derived from mouse bone marrow to LLLT (using 904 nm and 830 nm) with regard to proliferation, cytoskeleton organization and endoplasmic reticulum. Osteoblastic cell cultures (OFCOL II) were irradiated with gallium-aluminum-arsenide and gallium arsenide (GaAlAs λ=830 nm in continuous mode, 10 mW, 2 J/cm$^{2,}$ 93 s irradiation; GaAs λ=904 nm in pulsed mode, 50 mW, energy density of 6 J/cm$^2$ with 36 s irradiation and energy density of 50 mJ/cm$^2$ with 2 s irradiation) and divided into four groups: Group 1 - irradiated cells at 2J/cm$^2$; Group 2 - irradiated cells at 6J/cm$^2$; Group 3 - irradiated cells at 50mJ/cm$^2$; and Group 4 - non-irradiated cells. Irradiation occurred at 24-h intervals for a total of three days. After each interval, the cells were marked with DioC6 and Rhodamine-Phalloidin dye to assess the biostimulatory effect on mitochondrial activity and cell proliferation using an MTT assay. O laser 904nm Ed 50mJ/cm$^2$ laser was more effective at 24 h in comparison to the control group ($p<0.05$) and at 48 hours when compared to the 830 nm + 2 J/cm$^2$ laser ($p<0.001$), indicating increased mitochondrial activity for this type of laser at this energy density (904 nm 50 mJ/cm$^2$). We conclude that the 904 nm in pulsed emission, with a narrow pulse width (100 ns) and energy density of 50 mJ/cm$^2$ was more effective in promoting osteoblastic cell proliferation in comparison to the 830 nm laser in continuous emission.

Keywords: Osteoblastic cells, biostimulation, cell proliferation, low-level laser.

## INTRODUCTION

The effect of low-level laser therapy (LLLT) on bone regeneration has become a focus of recent research in different healthcare fields, such as medicine, physical therapy and dentistry. Photobiomodulation is a non-pharmacological therapeutic resource that contributes toward accelerating the functional recovery of biological tissue. Laser irradiation is commonly associated with cell proliferation and its action on cell metabolism has been studied by a number of researchers (Pereira et al., 2002; Werneck et al., 2005; Moore et al., 2005).

Regarding bone tissue, LLLT has been shown to modulate inflammation (Lizarelli et al., 1999), accelerate cell proliferation (Ueda and Shimizu, 2003) and enhance healing (Torricelli et al., 2001; Khadra et al., 2005). Ozawa et al., 1995 found that LLLT resulted in a significant increase in cell proliferation, bone nodule formation and alkaline phosphatase (ALP) activity.

Bone marrow stroma contains mesenchymal stem cells (MSCs) that have the potential to differentiate into bone, cartilage, adipocytes, muscle or connective tissue (Caplan, 1991). MSCs have been studied in sufficient detail for the definition of the mechanisms involved in normal bone remodeling and the regulation of osteogenesis under healthy and pathologic conditions, since altered dynamic responses of bone cell progenitors may be responsible for specific bone diseases (Gimble et al., 1996).

Osteoblastic cells are cells of a mesenchymal origin differentiated from fibroblastic cells only in the specific morphological characteristics of the osteoblast, which is located externally to the cell in the form of an extracellular matrix (Ducy et al., 2000). At the beginning of osteogenic differentiation, an important change occurs in the MSC morphology. The cells change from the characteristic fibroblast-like MSC phenotype to a nearly spherical form that characterizes the differentiated stage (Rodriguez et al., 2004).

There is considerable interest in the potential of MSCs in bone and cartilage tissue engineering for the treatment of musculoskeletal trauma and disease. Adult

MSCs offer certain advantages over embryonic stem cells, including their readiness and availability, as they may be obtained from the same individual (Caplan and Bruder, 2001). Potential applications of MSCs in regeneration and treatment have been reported, such as in tissue-engineered mandibular condyle, total jaw, *osteogenesis imperfecta*, heart tissue regeneration, metachromatic leukodystrophy, and Hurler syndrome (Yourek et al., 2007). During this process, the clear correlation between cell shape and differentiation supports the hypothesis that cell shape and the cytoskeleton may affect cell differentiation. The endoplasmic reticulum also contributes to the change in cell shape through protein synthesis, which affects the differentiation (Rodriguez et al., 2004).

The cytoskeleton plays an important role in cell morphology, adhesion, growth and signaling. Changes in the cytoskeleton allow the cell to migrate, divide and maintain its shape. The cytoskeleton also responds to external mechanical stimuli (Yourek et al., 2007). The cytoskeleton consists of three components: actin filaments, intermediate filaments and microtubules. The back-bone of the cytoskeleton is F-actin, which clusters to form actin filaments. The filaments are bundled and cross-linked by a network of actin-binding proteins and are most likely anchored to stable structures (anchor sites) in the cell, such as the plasma membrane (Wakatsuki et al., 2001; Mermelstein, 2003).

The actin network plays a major role in the determination of the mechanical properties of living cells (Rotsch and Radmacher, 2000; Ohashi et al., 2002) by forming a direct link between the integrins and the nucleus, which mechanically stiffens the nucleus and holds it in place (Maniotis et al., 1997).

LLLT has a positive effect on the proliferation and differentiation of osteoblastic cells *in vitro* (Yamada, K. 1997). Osteoblast proliferation, in particular, is of considerable clinical interest in the regeneration of lost bone. However, as the mechanisms acting on the bone have not yet been fully elucidated, the exact mechanism involved in laser-induced cell biostimulation remains unclear and is the subject of a number of studies (Dörtbudak, Haas, Mailath-Pokorny, 2000). *In vitro* and *in vivo* studies have demonstrated that LLLT exerts significant influence over cell function, such as cellular proliferation, mitochondrial activity, plasma membrane

stimulation, collagen synthesis and the release of growth factors (Ueda and Shimizu, 2003; Brondon et al., 2005;).

The action of laser therapy is based on the absorption of light by tissue, which leads to a series of changes in cell metabolism. Upon irradiating tissue, laser light is absorbed by photoreceptor chromophores located in the cell. Once absorbed, the light may modulate biochemical reactions, stimulating mitochondrial respiration due to the production of molecular oxygen and Adenosine tryphosphate (ATP) synthesis. These effects are known to increase the synthesis of Deoxyribonucleic acid (DNA) e Ribonucleic acid (RNA) and cell cycle regulating proteins, thereby promoting cell proliferation (Renno et al., 2007).

The purpose of the present study was to investigate the response of osteoblastic cells derived from mouse bone marrow to LLLT (using 904 nm and 830 nm laser) with regard to proliferation, cytoskeleton organization and endoplasmic reticulum. We used markers for endoplasmic reticulum and cytoskeleton and tracked proliferation and key organelles to evaluate the effect of LLLT on cell function. This study is expected to increase our knowledge regarding the features of osteoblastic cells and their relationship with the pathogenesis of some diseases, providing potentially new tools to help design novel therapeutic approaches.

## MATERIALS AND METHODS

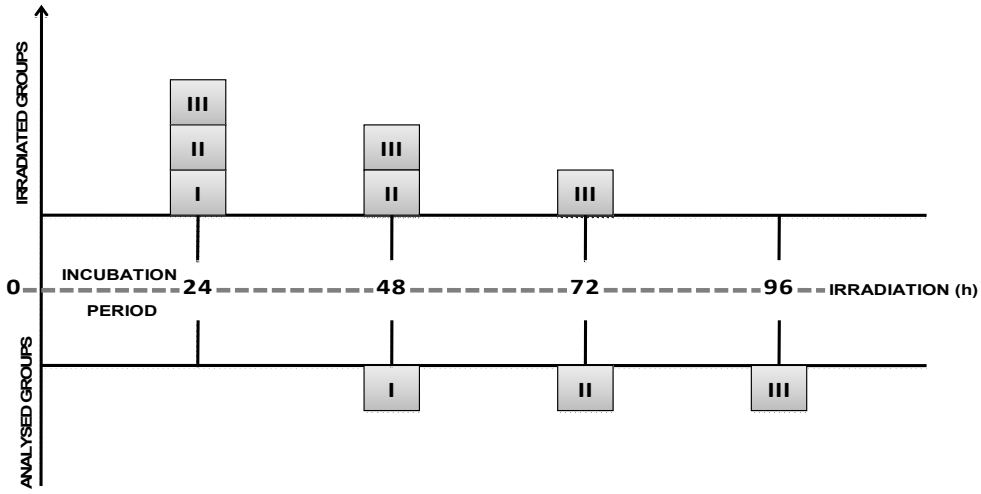
### Cell Culture

A mouse OFCOLII cell line derived from bone marrow (ATCC CCL-61; American Type Culture Collection, Rockville, MD) obtained from the Paul Ehrlich Technical Scientific Association (UFRJ RJ, Brazil) was routinely cultivated using MEM (Minimum Essential Medium) (GibcoBRL, Grand Island, NY), containing 5% (vol/vol) fetal bovine serum (FBS) (GibcoBRL), 100 U/mL penicillin, 100 mM/mL streptomycin and 0.25 µg/mL fungizone (GibcoBRL). Cells were cultured in an incubator (Forma USA) at 37°C in a humidified atmosphere containing 5% CO2.

The medium was changed every 2 days. When cells became confluent, the medium was removed and the cell layer was washed with phosphate-buffered saline. 0.25% trypsin (GibcoBRL) in buffered EDTA (Carlos Erba, ABC Lab, São Paulo) was then added and incubation continued for 2 to 4 min. A solution with a concentration of approximately 1.5 x $10^4$ cells/mL was prepared and poured into to each well of 24-well tissue culture plates (Nunc, Denmark).

**Laser Irradiation procedure**

Twenty-four hours after seeding the plates, cells were exposed to low-level laser irradiation. The wavelengths used were 830 nm in continuous mode (Thera Lase DMC Equipment Ltd., Brazil) 10 mW, energy density 2J/$cm^2$ with 93s duration irradiation; and 904 nm in pulsed mode (KLD™ Biosistemas Equipamentos Eletrônicos Ltda, Brazil) 50mW, energy density 6 J/$cm^2$ with 36s duration irradiation and energy density 50 mJ/$cm^2$ with 2 s duration irradiation. Cells were cultured in a well with 0.3 $cm^2$ of cross-section receiving radiation perpendicularly to the plate in 24-h, 48-h and 72-h. Control cells (without irradiation) were submitted to the same conditions as the irradiated cells. According to the diagram below.



**Cell Proliferation Assay**

Cell proliferation was assessed by an MTT assay. This method is based on the cellular conversion of a tetrazolium compound (MTT) into a colored formazan product that can be measured by absorbance at 570 nm, which is proportional to the number of viable cells. Each well received 20 μL of MTT for a final concentration of 0.5 mg/mL and the plate was incubated for 1 h at 37 °C in 5% $CO_2$ atmosphere. An aliquot of 100 μL of DMSO (dimethyl sulfoxide) was then added to each well and the plate was agitated for 30 min for solubilization of the formazan crystals. Crystal concentration was spectroscopically quantified using a microplate reader (ELISA Reader - SpectraCount - Packard Instrument Company, USA) at an excitation wavelength of 570 nm. Six parallels were performed on the plates and the experiments were repeated at least twice on different days.

**Procedures for Fluorescence Microscopy**

All dyes used in the fluorescence studies were purchased from Molecular Probes (Eugene, OR, USA). Just prior to imaging, the cells were stained by incubation in fluorescent dye-containing growth medium at 37 °C as follows:

DioC6 (3,3'- dihexyloxacarbocyanine iodide) was used to study changes in the endoplasmic reticulum. The cells were rinsed with PBS, for medium removal and incubated with 10 μg/mL DioC6 for 30 min, then immediately rinsed twice with PBS, fixed with 4% PA (paraformaldehyde - Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) in PHEM buffer 0.I M (60nM PIPES, 20nM HEPES, 10nM EGTA, and 5Mm $MgCl_2$) for 10 min and rinsed again to eliminate excess dye. For the cytoskeleton study based on the change in actin filaments (560 nm excitation filter and 590 nm emission filter), cells were fixed and then marked with Rhodamine-Phalloidin (1:100 – 1 hour).

Cover slips were mounted on slides with n-propyl gallate (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) and examined through fluorescence microscopy. Observations and photographs were made using a Leica microscope (DLMB - Leica Microsystems, Wetzlar, Germany). For fluorescence analysis, approximately 100 cells were examined on each plate. Plates were examined and

photographed in triplicate for the increase in fluorescence intensity of organelles. Excitation (554 nm) and emission (576 nm) filters were used.

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**Image Analysis**

Image analysis was performed using the public domain Image J software (National Institutes of Health, NIH) (Abramoff *et al.,* 2004). Laser attenuation settings were kept the same for all images in individual experiments in which fluorescence intensity was compared. Fluorescence intensity was measured as the average pixel intensity.

**Statistical Analysis**

Results are expressed as mean $\pm$ SEM. Comparisons between groups were made using two-way analysis of variance (ANOVA) and a *post hoc* test. The Bonferroni test was used to determine significant differences between groups. Values of $p \leq 0.05$ were considered statistically significant (95% confidence interval). Statistical analysis was performed with the GraphPad Prism (version 4.0) package.

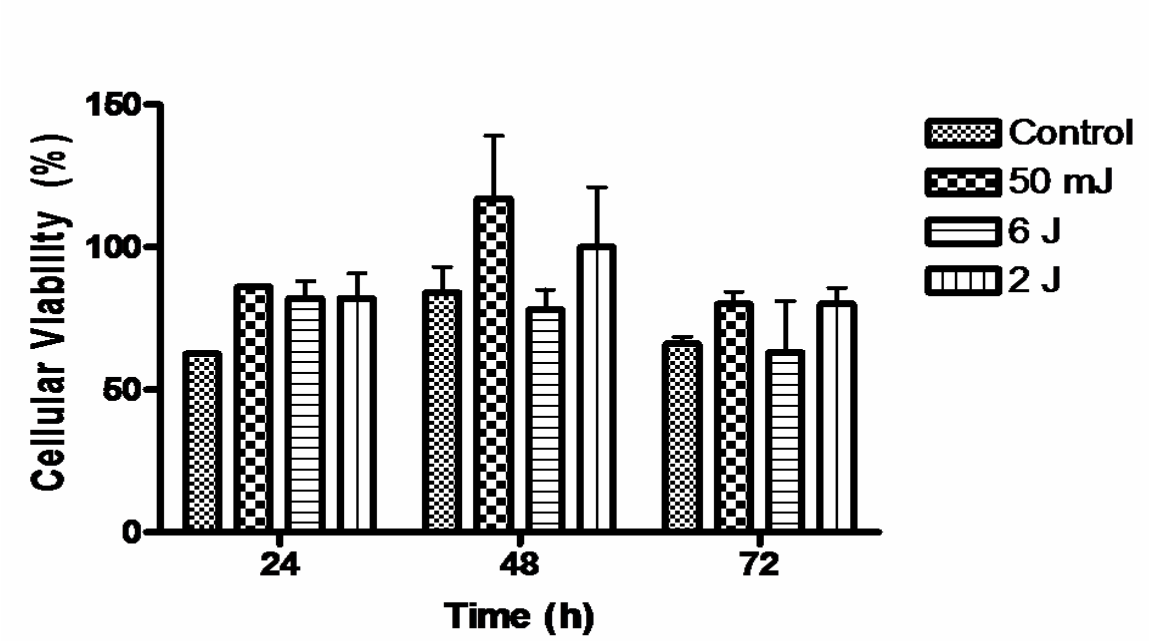


Figure 1: Cell viability based on MTT test. OFCOL II cells were irradiated with 830 nm (2 J/cm$^2$) and 904 nm (50m J cm$^2$ and 6 J cm$^2$) lasers. At 24, 48 and 72 hours following irradiation, the cells were analyzed using the MTT test to determine cell viability directly related to mitochondrial activity.

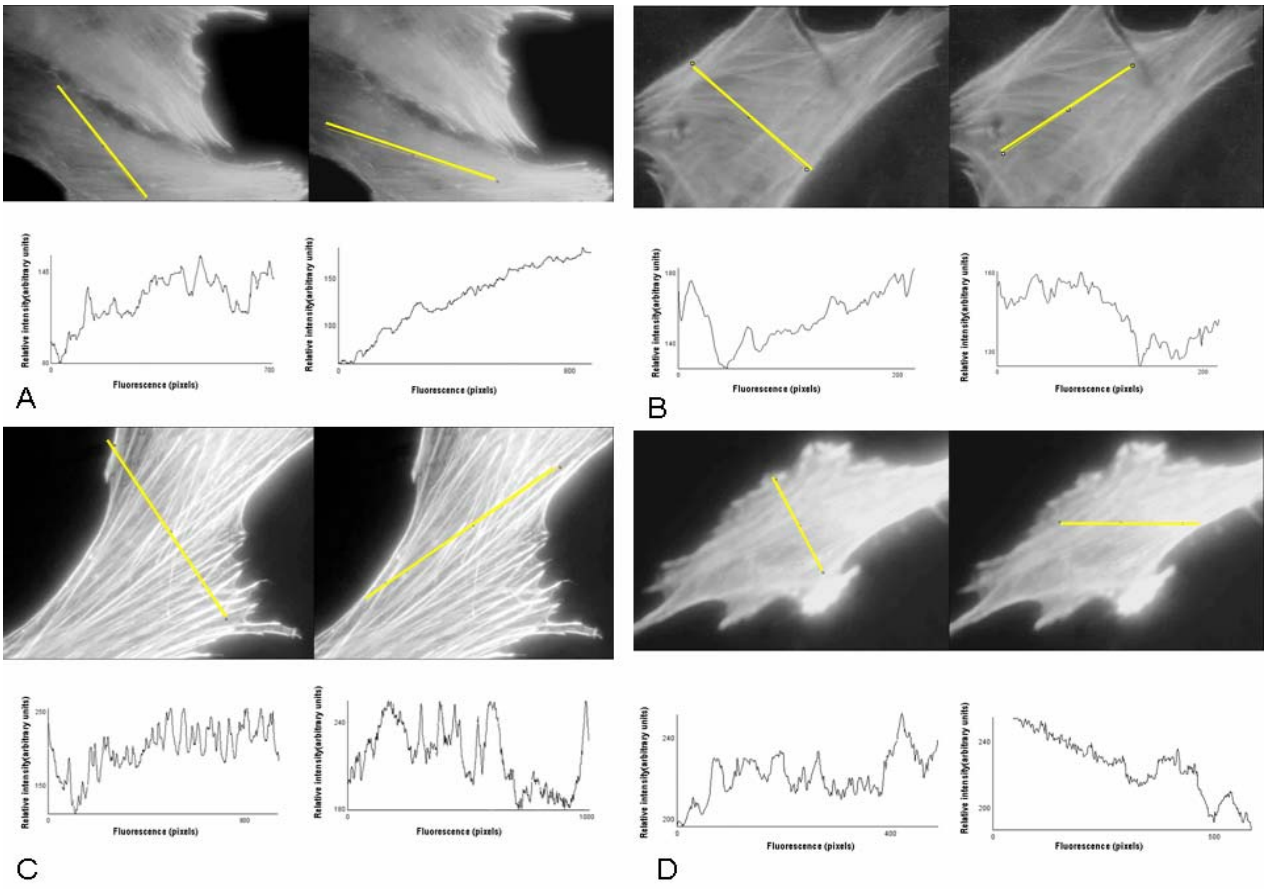


Figure 2: Densitometric profiles obtained from representative cells labeled for actin. Digital images of cells and measuring directions are shown.
(A) control; (B) 830 nm - 2 J/ $cm^2$; (C) 904 nm – 50 mJ/ $cm^2$; (D) 904 nm – 6 J/ $cm^2$.

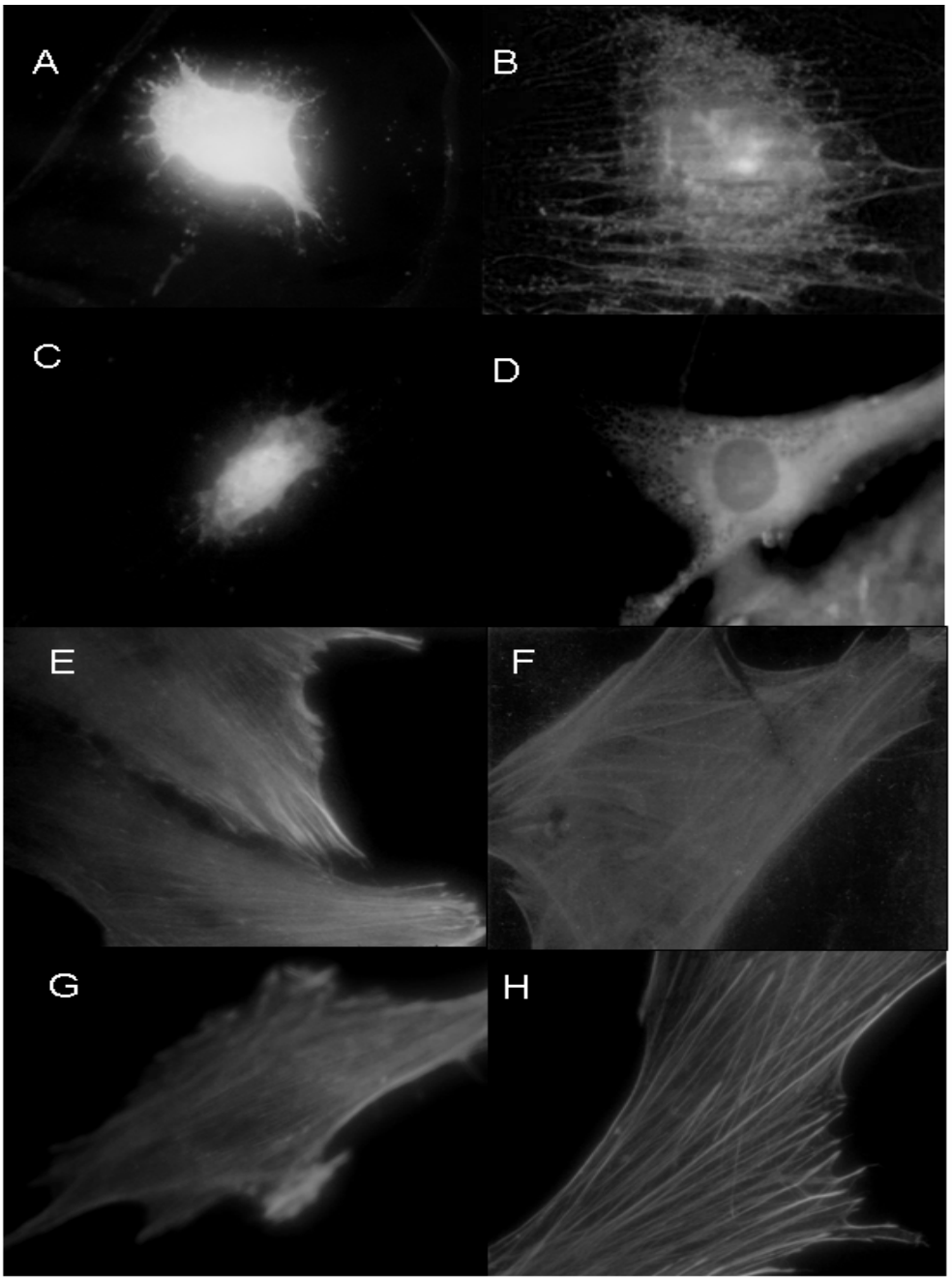


Figure 3: Fluorescence microscopy. OFCOL II cells were marked with fluorescent probes for the reticulum and cytoskeleton following irradiation with 830 nm and 904 nm laser. DioC6: control; (B) 830 nm 2 J/cm$^2$ (C) 904 nm 6 J/cm$^2$; and (D) 904 nm 50 mJ/cm$^2$. Rhodamine-Phalloidin: (E) control; (F) 830 nm 2 J/cm$^2$ (G) 904 nm 6 J/cm$^2$; and (H) 904 nm 50 mJ/cm$^2$.

## RESULTS

The comparison of the 830 nm and 904 nm lasers was carried out to determine the applicability of these lasers on cell differentiation of the osteoblast line. In the first step, the effect of laser at different wavelengths (830 nm and 904 nm) on cell viability was assessed through mitochondrial activity, determined by the MTT assay (Figure 1).

Figure 1 shows the viability of the cells submitted to irradiation. At 24 hours following irradiation, the 50 mJ/cm$^2$ energy density had a better performance than the control group ($p<0.05$) as well as the 6 J/cm$^2$ and 2 J/cm$^2$ energy densities. At 48 hours, there was an extremely significant increase in cell viability using the 904 nm laser (50 mJ/cm$^2$) when compared to the 830 nm laser (2 J/cm$^2$) ($p<0.001$), indicating intense mitochondrial activity for this type of laser at this Ed (904 nm - 50 mJ/cm$^2$). Seventy-two hours after irradiation, there was a significant drop in viability in comparison to the increase in population determined at 48 hours, thereby resembling the viability witnessed at 24 hours.

In the second step, cell structures involved in the differentiation process were evaluated, such as the cytoskeleton and endoplasmic reticulum, using fluorescence microscopy (Figure 2). The structural organization of the cytoskeleton fibers was analyzed using the densitometric profile of specifically marked cells (Figure 2). The variation in gray value (brightness) obtained throughout two perpendicular directions was different for each irradiation.

Analysis of the actin filaments in the control group with regard to fiber stress demonstrated that the filaments occurred in a nearly parallel form, converging in focal points toward the narrow cell margin (Figure 2A). The morphological characteristics revealed actin filaments in sharp contrast. For the 2 J/cm$^2$ group, a lower number of peaks was observed; tracing a transversal bundle, large peaks of poorly radiant actin bundles were found, with no occurrence of distribution as sharp as that in the control group (Figure 2B). At 50mJ/cm$^2$, image analysis revealed an actin filament pattern formed by numerous thin peaks and a low-brightness profile

in parallel and perpendicular sections, respectively (Figure 2C). In the 6J/cm$^2$ group, differences between the densitometric profiles were detected through a drop in the brightness profile throughout the main orientation network (Figure 2D).

The assessment of reticular activity in irradiation with the 830 nm (2 J/cm$^2$) and 904 nm (50 mJ/cm$^2$ and 6 J/cm$^2$) lasers suggests that protein synthesis is more accentuated with the 830 nm laser, for which a greater number of vessels was observed in the cytoplasm when compared to the control group (Figure 3B). Analysis of the 904 nm reveals that the 50mJ/cm$^2$ group had a homogeneous distribution of the reticular network (Figure 3D), but less intense than that of the control. In the 6 J/cm$^2$ group, there was a retraction in the reticular network, with a greater concentration in the perinuclear region and an absence of vessels, thereby suggesting a reduction in protein synthesis activity in comparison to the other groups (Figure 3C). In the 6 J/cm$^2$ group, there was also a de-polymerization of actin filament, which is correlated with the retraction of the endoplasmic reticulum (Figure 3G). Comparing the reticulum and cytoskeleton in the 50 mJ/cm$^2$ group, the arrangement of the actin filaments confirms the homogeneous distribution of the reticular network (Figure 3H).

## DISCUSSION

According to Vellonen (2006), when the concentration of MTT in the cytoplasm is increased, more formazan products are formed and higher irradiation absorption by the cells is detected, indicating higher cell viability. MTT is not permeable to the lipid plasma membrane and it has been suggested that MTT enters the cells through endocytosis. A reduction in MTT has been associated with mitochondrial activity as well as endosome and lysosome enzymes. MTT can also be reduced by the receiving electrons in mitochondria and other cellular organelles, indicating that it is dependent on cellular redox activity. Therefore, MTT reduction reflects mitochondrial function and may be used as an indicator of

cellular oxidative metabolic activity (Takahashi, 2002). The results obtained in the present study demonstrate that the lasers influenced cell proliferation. The 904 nm laser (Ed 50 mJ/cm$^2$) proved more effective than the 830 nm laser (2 J/cm$^2$). According to Karu (2005), the action spectra in the visible-to-near infrared (IR) region measured for biological responses of cultured cells demonstrated that red light was not the only wavelength suitable for laser biostimulation. Irradiation of cells and modification of light effects with chemicals demonstrated that laser biostimulation is a photobiological phenomenon. This indicates that, due to its power of depth, the 904 nm laser acts on the plasma membrane, indicating that the target in this sense is the inner mitochondrial membrane, thereby triggering an increase in enzyme activity (succinate dehydrogenase).

The high resolution and sensitivity of fluorescence microscopy combined with specific markers and associated to image analysis allows a better understanding of the functional and structural aspects of the cytoskeleton. Fluorescence imaging analysis is a suitable method for a more precise investigation of the normal architecture of the components of the cytoskeleton as well as their possible changes in the experimental setting or pathological condition (Buño et al., 1999). We therefore used this method to understand the action of laser light on actin filaments.

At 72 hours, there was a reduction in mitochondrial activity stemming from the change in the actin filaments, as illustrated in the graph on cell viability. This event suggests that the cell respiration process of the mitochondria was compromised due to the beginning of de-polymerization of the actin filaments, which give sustenance to the mitochondria. With the 50 mJ/cm$^2$ laser, mitochondrial activity was active, as confirmed by densitometric analysis, revealing a characterization of the cytoskeleton of the actin networks, with high orientation of the contour and direction of the fibers.

In order to understand the role of the cytoskeleton in the mechanisms involved in normal bone remodeling, Rodriguez et al. (2004) studied the importance of the microfilaments and microtubules in osteogenic differentiation. In particular, the evident correlation between cell shape and differentiation supports

the hypothesis that the cytoskeleton may affect cell differentiation (Rodriguez et al., 2004). It is important to mention that the alterations in actin cytoskeletal organization in cells derived from osteopetrotic and osteoporotic bones have been previously reported (Watanabe et al., 1998; Perinpanayagam et al., 2001). In addition, MSCs derived from healthy control and postmenopausal osteoporotic women share some functional characteristics, but differ importantly with regard to others (Rodrıguez et al., 2000). These same authors report a correlation between the abnormal organization of actin in the cytoskeleton with some human bone diseases.

Mermelstein et al. (2003) report that, although the involvement of microfilaments in connections between the cytoskeleton and extracellular matrix has been intensively studied, microtubules are also implicated in the process of cell adhesion to a substrate and the maintenance of cell shape.

In the present study, there was organization of cytoskeleton fibers in terms of structural parameters, as revealed by densitometric profiles, characterized by a higher orientation than cytokeratin fibers at the 50mJ/cm$^2$ energy density. The high resolution and sensitivity of fluorescence microscopy, coupled with specific labeling, use of drugs or experimental treatments, and the application of the densitometric profile methodology could aid in the further understanding of structural and functional aspects of the cytoskeleton. It is clear that the accuracy and versatility offered by the orthogonal densitometric profile can provide a suitable method for a more precise investigation into the normal architecture of cytoskeletal components and their possible modifications under experimental or pathological conditions (Buño, 1998).

From the data obtained in the densitometric analysis of the cytoskeleton, we can see that the 50 mJ/cm$^2$ density performed better than the 2 J/cm$^2$ and 6 J/cm$^2$ for osteoblastic cells. The graphs demonstrated the marking of actin filaments in all groups, but the distribution and organization of the filaments was clearer in the 50 mJ/cm$^2$ group. This reveals the influence of the 904 nm laser at this energy density on the polymerization of the actin molecules. The images of

the marked cells demonstrate the arrangement of the filaments and the orthogonal densitometry graphs reflect the fluorescence intensity in each group.

In previous studies, we assessed the cytoskeleton and endoplasmic reticulum submitted to low-level laser therapy (LLLT) and low-level pulsed ultrasound (LLPUS) using the same parameters for the 904 nm laser on fibroblastic cells and found an increase in protein synthesis (Pires Oliveira et al, 2008). Irradiation with 904 nm led to an increase in cell proliferation at both the 50 $mJ/cm^2$ and 6 $J/cm^2$ energy densities when compared to the control group, but the 50 $mJ/cm^2$ energy density demonstrated greater effectiveness. Regarding the evaluation of the cytoskeleton, the 50 $mJ/cm^2$ laser demonstrated a better organization of the actin filaments, with no changes in reticular activity when compared with the cells marked for the endoplasmic reticulum. Our results corroborate studies by Oliveira et al. (2008), who compared the effect of LLLT and LLPUS on fibroblast cells using the same laser parameters as in the present study and demonstrated an effect on cell growth at 6 $J/cm^2$ as well as at 50 $mJ/cm^2$, with a higher growth rate at 50 $mJ/cm^2$.

We conclude that the 904 nm laser in pulsed e emission with a narrow pulse width (100 ns) and 50 $mJ/cm^2$ density was more effective on osteoblastic cell proliferation in comparison to the 830 nm laser in continuous emission.

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# 4 DISCUSSÃO

A Terapia Laser de Baixa Potência (TLBP) tem sido usado em diferentes campos da saúde, incluindo medicina, odontologia e fisioterapia. A TLBP vem ganhando considerável reconhecimento nas últimas décadas. Suas possíveis aplicações clínicas, como modalidade terapêutica, em lesões de partes moles e de tecido ósseo, têm sido amplamente estudadas na contribuição para acelerar a recuperação dos tecidos biológicos (MOORE et al., 2005). A irradiação laser é comumente associada com proliferação celular e a ação no metabolismo celular tem sido estudada por vários pesquisadores. (WERNECK et al., 2005, MOORE et al., 2005, PEREIRA et al., 2002).

Diferentes tipos de lasers têm sido utilizados para obter diferentes efeitos bioestimulatórios: Rubi, Hélio Neônio (He-Ne), Arseneto de Gálio e Alumínio e Arseneto de Gálio (AsGaAl, AsGa) entre outros; administrada como contínuos e pulsados. Entretanto o efeito biológico da irradiação laser depende de suas especificações como: comprimento de onda, potência e densidade energia. (SATO et al., 2001, NINOMIYA et al., 2007).

Estudos *in vitro* e *in vivo* tem demonstrado que o laser de baixa potência exerce influência significativa sobre a função celular, como a proliferação celular, atividade mitocondrial, ativação de proteínas de membrana plasmática, síntese de colágeno e liberação de fatores de crescimento (UEDA; SHIMIZU, 2003; BRONDON et al., 2005).

Atualmente, atenção tem sido focada nos efeitos da irradiação no tecido ósseo, sendo esta terapia reportada nos efeitos bioestimulatórios nas diferentes células e organelas, apresentando efetividade na estimulação e proliferação celular óssea, aumentando a atividade osteoblástica no local da fratura e contribuindo para a aceleração da consolidação de fraturas. Além disso, favorece a angiogênese e aumenta a deposição de fibras colágenas (DINIZ et al. 2008, OBRADOVIÉ et al. 2008, RENNO et al, 2006; KADRA, 2005, BARBER, 2001).

Pinheiro et al., (2006) relatam que em vários estudos sobre laser os

mesmos apresentam efeitos positivos sobre o tecido ósseo, sendo que estes estudos refletem a idéia de que células mesenquimais não diferenciadas podem ser biomoduladas ativando para osteoblastos**.**

De acordo com Yamada (1991), o laser 632nm Hélio Neônio (HeNe), apresenta um efeito positivo na proliferação e diferenciação em células osteoblásticas *in vitro*. A proliferação osteoblástica é considerada de interesse clínico pela regeneração de perda óssea, entretanto o mecanismo de indução laser e células osteoblásticas não estão bem elucidados (DÖRTBUDAK et al., 2000).

Medrado et al., (2003) e Reddy et al., (2003) relatam que o efeito biomodulador do laser ocorre por aumento do metabolismo celular e por redução da resposta inflamatória, diretamente associado ao estímulo da função mitocondrial e celular.

Segundo Karu (1995), a resposta biológica de células irradiadas com laser de baixa potência revela alteração na atividade mitocondrial, quando o laser é irradiado na faixa visível do espectro eletromagnético, ocorrendo uma primeira bioestimulação em nível de mitocôndrial. No entanto, quando a radiação é na faixa do espectro infravermelho, o laser estimula a membrana plasmática.

Karu (1988) propôs uma cascata de eventos que começa com a absorção de luz por um fotorreceptor que conduz a foto-ativação das enzimas no interior da mitocôndria, resultando com a foto-resposta. Absorção da luz pelos componentes da cadeia respiratória leva a mudança nas mitocôndrias e citoplasma. A magnitude do efeito biomodulatório do laser depende do estado fisiológico da célula, dessa forma explica porque a biomodulação nem sempre é detectada e há variações de respostas encontradas na literatura.

Entretanto esta mesma autora em (1999), relata que a estimulação é um efeito do laser observado em estruturas celulares principalmente na membrana plasmática e membrana mitocondrial interna, observados após a irradiação com alguns tipos de lasers, no qual induz reações que interferem no metabolismo, decorrente da energia absorvida estimulando funções celulares.

De acordo com Vellonen et al., (2006) se a concentração de MTT no citoplasma esta aumentada, mais cristais de formazan são formados e maior absorção é detectada, indicando alta viabilidade celular. O MTT não é permeável à membrana plasmática e lipídios sugerindo que as células podem entrar em endocitose através de MTT. A redução de MTT tem sido associada à atividade mitocondrial, bem como enzimas de endosoma e lisossoma. A redução do MTT reflete a função mitocondrial e pode ser utilizado como um indicador da atividade celular oxidativa.

No presente estudo, as células (OFCOL II e L929) submetidos à proliferação e marcadas com MitroTracker exibiu mitocôndrias distribuídas em toda a célula, inclusive na região perinuclear, assim, sugerindo intensa atividade mitocondrial durante a divisão celular e/ou síntese protéica. De acordo com Ferreira et al., (2004), a presença de mitocôndrias na região perinuclear podem estar associados com retículo endoplasmático devido ao fluxo bidirecional de cálcio, mas também com vesículas ácidas que pode desempenhar um papel importante na regulação de gradiente de próton através da membrana mitocondrial. Análise por fluorescência com marcação de DioC6 revelou intensa atividade reticular resultante de pós-irradiação.

A partir dos dados obtidos na análise do perfil densitométrico do citoesqueleto, podemos ver que as densidades de energia 50 mJ/cm$^2$ e 6J/cm$^2$ foram melhor do que dE 2 J/cm$^2$ em células osteoblásticas. Os gráficos demonstram a marcação dos filamentos de actina, em todos os grupos, mas a distribuição e organização dos filamentos foi mais clara no grupo 50 mJ/cm$^2$. Isso revela a influência do laser 904 nm, nesta densidade de energia sobre a polimerização das moléculas de actina.

De acordo com Buño et al., (1999), a alta resolução e sensibilidade combinada com microscopia de fluorescência e de marcadores específicos associados à análise da imagem permitem uma melhor compreensão dos aspectos funcionais e estruturais do citoesqueleto. Análise do perfil densitométrico é um método adequado para uma investigação mais precisa da arquitetura normal dos componentes do citoesqueleto, bem como suas possíveis alterações na

configuração experimental ou patológico. Portanto, utilizamos este método para entender a ação do laser em filamentos de actina. As imagens das células marcadas demonstram a disposição dos filamentos e os gráficos do perfil densitométrico refletem a intensidade de fluorescência de cada grupo.

A irradiação de células L929 com laser pulsado na região do infravermelho próximo (904 nm) em duas densidades de energias muito diferente permitiu a observação de um aumento da proliferação celular em ambos os casos. O resultado foi melhor para densidade de energia de 50 mJ/cm$^2$ do que para 6 J/cm$^2$, conforme demonstrado nos resultados por microscopia de fluorescência e MTT. De acordo com Ozawa et al., (1984) e Ninomiya et al., (2007) o comprimento de onda é um fator importante, que diz respeito à penetração do laser através do tecido biológico. Segundo estes autores o laser pulsado pode ser capaz de aumentar a resistência óssea, aumentar o volume ósseo e promover a mineralização óssea.

As densidades de energia (6 J/cm$^2$ e 50 mJ/cm$^2$) utilizadas no estudo resultou no crescimento celular dependente do tempo pós-irradiação. Moore et al. (2005) e Pinheiro et al. (2005) observaram um efeito inibitório de altas doses cumulativas com densidade energética acima de 10 J/cm$^2$ durante longos períodos. Nossos resultados corroboram com os estudos de Oliveira et al., 2008 que compararam o efeito do ultra-som de baixa intensidade e laser de baixa potência em cultura celular fibroblástica L929 utilizando os mesmos parâmetros do laser do presente estudo no qual demonstraram um efeito sobre o crescimento celular em 6 J/cm$^2$ e 50 mJ /cm$^2$, sendo superior a 50 mJ/cm$^2$.

Em estudos anteriores Pires Oliveira, et al., (2008) compararam o laser de baixa potência e o ultra-som de baixa intensidade avaliando as estruturas celulares como citoesqueleto e retículo endoplasmático em cultura celular fibroblásticas L929, no qual observaram um aumento na síntese protéica para o ultra-som. Usando os mesmos parâmetros para a irradiação laser este apresentou um aumento na proliferação celular, tanto a densidade de energia de 50 mJ/cm$^2$ e 6 J/cm$^2$ quando comparados ao grupo controle, entretanto a dE 50 mJ/cm$^2$ demonstrou maior eficácia. No que diz respeito à avaliação do citoesqueleto, a dE

50 mJ/cm$^2$ demonstrou uma melhor organização dos filamentos de actina, sem alterações na atividade reticular, quando comparado com as células marcadas para o retículo endoplasmático.

Os resultados obtidos no presente estudo demonstram que o laser influenciou a proliferação celular e o laser 904 nm pulsado (dE 50 mJ/cm2) revelou maior eficácia do que o laser 830 nm contínuo (2 J/cm$^2$) em cultura osteoblástica, estes dados corroboram com Sato et al., (2001) e Ninomyia et al., (2007) no qual relatam que o mecanismo de formação óssea por irradiação laser de baixa potência pulsado pode ter resposta diferente do que pela irradiação com laser de baixa potência do tipo contínuo; isto porque o laser pulsado apresenta alto pico de potência; sugerindo que o tratamento com laser pulsado pode ser eficaz para pacientes com doenças ósseas e consolidação de fratura.

O mecanismo preciso de estimulação de células ósseas por irradiação de um laser pulsado, é mais eficaz nas condições para a formação óssea. De acordo com Nissan et al., (2006), o uso do laser Arseneto de Gálio $\lambda$=904nm (AsGa) tem aumentado nos últimos 10 anos o qual apresenta um maior poder de penetração, quando comparado com outros tipos de lasers, tornando-se assim um recurso indispensável na terapia de reabilitação.

## 5 CONCLUSÕES

- O Laser de baixa potência mostrou eficácia para proliferação de células osteoblásticas e fibroblásticas.

- O Laser 904nm – 50 mJ/cm$^2$ mostrou-se ser mais efetivo em culturas celulares em relação ao 6 J/cm² e ao 830nm nas densidades de energia 2J/ cm² e 3 J/ cm².

- Em relação aos filamentos de actina o laser 904nm 50 mJ/cm² propiciou um melhor arranjo dos filamentos de actina.

## 5.1 TRABALHO EM ANDAMENTO

Laser 904 nm action on bone repair in rats with osteoporosis

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## 5.2 PROJETOS FUTUROS

Avaliação da expressão de proteínas decorrentes de biomodulação.

## 5.3 ARTIGOS PUBLICADOS

### 5.3.1 Cell Biology International, v.32, p.1329-1335, 2008.

Rodrigo Franco de Oliveira, Deise A. A. Pires Oliveira, Aline H. A. Machado, Newton Soares da Silva, Marcio Magini, Cristina Pacheco-Soares. Assessment of fibroblast cells submitted to ultrasonic irradiation. **Cell Biology International.** v.32, p.1329-1335, 2008.

## ABSTRACT

Physiotherapists consider ultrasound an indispensable tool, which is commonly employed in clinical practice as a treatment aid for musculoskeletal dysfunctions. The aim of our study has been to analyze fibroblast cell structures following low-intensity pulsed ultrasonic irradiation. Fibroblast cell cultures irradiated with ultrasound were analyzed through electron microscopy to determine an ideal irradiation beam that preserved cell morphology and integrity. Analysis by fluorescence microscopy and transmission electron microscopy was used to follow morphological changes of the nucleus and cytoskeleton following different ultrasound irradiation intensities. According to the parameters used in the pulsed irradiation of fibroblast cultures, control over the intensity employed is fundamental to the optimal use of therapeutic ultrasound. Cell cultures submitted to low-intensity pulsed ultrasonic irradiation (0.2 e 0.6 W/cm2) at 10% (1:9 duty cycle) and 20% (2:8 duty cycle) maintained shape and cellular integrity, with little damage. In the group irradiated with an intensity of 0.8 W/cm2, a loss of adhesion was observed along with an alteration in the morphology of some cells at an intensity of 1.0 W/cm2, which resulted in the presence of cellular fragments and a decrease of adhering cells. In cells irradiated at 2.0 W/cm2, there was a complete loss of adhesion and aggregation of cellular fragments. The present study confirms that biophysical properties of pulsed ultrasound may accelerate proliferation processes in different biological tissues.


Keywords: Therapeutic ultrasound; Repair; Fibroblasts cell; Proliferation; L929

### 5.3.2 Photomedicine and Laser Surgery. v.26, v.1, p.6-9, 2008.

Rodrigo Franco de Oliveira, Deise A. A. Pires Oliveira, Wagner Monteiro, Renato Amaro Zângaro, Márcio Magini, Cristina Pacheco Soares. Comparison Between the Effect of Low-Level Laser Therapy and Low-Intensity Pulsed Ultrasonic Irradiation *in Vitro.* ***Photomedicine and Laser Surgery.*** v.26, v.1, p.6-9, 2008.


**ABSTRACT**

*Objective:* The objective of this study was to compare the effect of low-level laser therapy (LLLT) and low intensity pulsed ultrasound (LIPUS) on fibroblast cell culture. Several methods, including ultrasound treatment and LLLT, are being used to facilitate tissue repair and healing processes. *Materials and Methods:* L 929 fibroblast cell cultures were irradiated with low-level laser energy and LIPUS. Cultures irradiated with ultrasound were divided into five groups: group 1: control (did not receive irradiation); group 2: 0.2 W/cm2 in pulsed mode at 10% (1:9 duty cycle); group 3: 0.6 W/cm2 in pulsed mode at 10% (1:9 duty cycle); group 4: 0.2 W/cm2 in pulsed mode at 20% (2:8 duty cycle); and group 5: 0.6 W/cm2 in pulsed mode at 20% (2:8 duty cycle). Cultures irradiated with laser energy were divided into three groups: group 1: control (did not receive irradiation); group 2: 6 J/cm2; and group 3: 50 mJ/cm2. Each group was irradiated at 24-h intervals, with the following incubation periods post-irradiation: 24, 48, and 72 h; after each irradiation cycle the cultures were analyzed using MTT [3-(4.5-dimethylthiazol-2-yl)-2.5 diphenyltetrazolium bromide]. *Results:* Analysis of results after LLLT and LIPUS demonstrated that the effect of laser therapy on fibroblast cell culture was greater than that of LIPUS ($p < 0.05$). *Conclusion:* Results demonstrated that LLLT significantly increased fibroblastic activity more than LIPUS. Therefore, in the first and second phases of tissue repair, laser treatment may be more effective than ultrasound treatment.

### 5.3.3 Photomedicine and Laser Surgery. In Press

Deise A. A. Pires Oliveira, Rodrigo Franco de Oliveira, Renato Amaro Zângaro, Márcio Magini, Cristina Pacheco Soares. Assessment of Cytoskeleton and Endoplasmatic Reticulum of Fibroblast Cells Submitted to Low-level Laser Therapy and Low-intensity Pulsed Ultrasound. **Photomedicine and Laser Surgery.** In Press


**ABSTRACT**

Objective: The aim of the present study was to compare the effect of low-level laser therapy (LLLT) and low-intensity pulsed ultrasound (LIPUS) on the cytoskeleton and endoplasmatic reticulum of L929 cells. Thermal and non-thermal physical mechanisms, such as LLLT and LIPUS, induce clinically significant responses in cells, tissues, organs and the organism itself. Materials and Methods: L929 fibroblast cell cultures were irradiated with LLLT and LIPUS. Cultures irradiated with laser $\lambda$=904 nm were divided into 3 groups: Group I - control (without radiation); Group II – irradiated at 6 J/cm$^2$; and Group III – irradiated at 50 mJ/cm$^2$. Cultures irradiated with ultrasound were divided into five groups: Group I - control (without radiation); Group II – irradiated at 0.2 W/cm$^2$ in pulsed mode at 10% (1:9 duty cycle); Group III – irradiated at 0.6 W/cm$^2$ in pulsed mode at 10% (1:9 duty cycle); Group IV – irradiated at 0.2 W/cm$^2$ in pulsed mode at 20% (2:8 duty cycle) and Group V – irradiated at 0.6 W/cm$^2$ in pulsed mode at 20% (2:8 duty cycle). Each group was irradiated with 24-hour intervals, observing the following post-irradiation incubation times: 24, 48 and 72 hours. The action of LLLT and LIPUS on cytoskeleton and endoplasmatic reticulum was evaluated through the use of fluorescent probes and fluorescence microscopy analysis. Results: The results following LLLT and LIPUS demonstrate that ultrasound was more effective than the laser on fibroblast cell cultures when the endoplasmatic reticulum was assessed,

whereas there was a better distribution of the filaments of the cytoskeleton in the cells submitted to laser radiation.

Key words**:** Cell culture, fibroblast, LLLT, LIPUS, L929, cytoskeleton, endoplasmatic reticulum.

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