Flaviana Soares Rocha

EFEITO DO LASER DE BAIXA POTÊNCIA E DA OXIGENAÇÃO HIPERBÁRICA NO REPARO DE OSSO SUBMETIDO À RADIAÇÃO IONIZANTE.

Tese apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, para obtenção do Título de Doutor(a) em Clínica Odontológica.

Uberlândia, 2014

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Orientador(a): Profª. Drª. Paula Dechichi

Banca Examinadora:
Prof. Dr. Antônio Wilson de Almeida
Prof. Dr. Paulo Tambasco de Oliveira
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Profª. Drª. Karen Renata Nakamura Hiraki
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que tanto amo; meus pais, Gerson e Rita Nilza pelo
apoio incondicional e exemplo de coragem e determinação
que sempre busquei seguir; meus irmãos, Flávio e Fabiana,
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SUMÁRIO

RESUMO

O reparo ósseo é influenciado por vários fatores como tipo de osso, local e severidade da lesão, presença de infecção, fixação, idade, estado de saúde geral e nutricional dos indivíduos. Alguns tratamentos, como a radioterapia, também podem comprometer o tecido ósseo, limitando as possibilidades futuras de reparo. O tecido ósseo quando irradiado apresenta alterações importantes em sua morfologia e constituição. Os efeitos da radiação sobre o osso, apesar de bem descritos, ainda não têm seu mecanismo totalmente esclarecido. Além disso, considerando o grande número de procedimentos clínicos que envolvem esse tecido, o entendimento dessas alterações, bem como formas de minimizá-las, é de grande importância nas áreas médica e odontológica. O uso da terapia laser de baixa potência (LBP) em tecido ósseo tem-se mostrado efetivo na modulação da inflamação, acelerando a proliferação celular e o processo de reparo ósseo. A Oxigenação hiperbárica (OH) também tem sido utilizada para favorecer o reparo de tecidos lesados, pois ela estimula a proliferação celular e a neovascularização, o que melhora a qualidade do reparo ósseo. Considerando esse contexto, a presente tese de doutorado propôs investigar o efeito do LBP e da OH no reparo ósseo, em ratos previamente submetidos ou não à radiação ionizante. Para esse propósito, foi utilizado o modelo experimental de defeito ósseo em fêmur de rato, sendo o reparo avaliado por meio de parâmetros histológicos (qualitativos), histomorfométricos (porcentagem de neoformação óssea), densitometria óssea, bem como expressão de moléculas relacionadas à osteogênese e remodelação óssea. Os resultados indicaram que o LBP e a OH favorecem o reparo ósseo, mas esses efeitos, de forma geral, não foram suficientes para compensar os prejuízos causados pela radiação ionizante no osso.

ABSTRACT

Bone healing is influenced by several factors such as the type of bone, severity of injury, presence of infection, fixation, age, health and nutrition. Some treatments, such as radiation, may also compromise bone tissue, limiting future possibilities of repair. When submitted to radiotherapy, bone tissue presents important changes in morphology and constitution. The effects of radiation on the bone, although well described, have not been fully clarified. Moreover, considering the large number of procedures involving this tissue, the understanding of these changes and ways to minimize them is of great importance in medicine and dentistry. The use of low-power laser therapy (LLT) modulates inflammation and cell proliferation, accelerating bone repair. The hyperbaric oxygen (HBO) has also been used to improve the repair of damaged tissues since it stimulates oxygenation, cell proliferation and neovascularization, with positive results in osteogenesis. Considering this context, this thesis aimed to investigate the effect of LLT and HBO on bone healing, in rats previously submitted or not to ionizing radiation. For this purpose, the experimental model of bone defect in the rat femur was used, and bone repair was evaluated by histological (qualitative) and morphometric (percentage of bone formation) parameters, bone densitometry, as well as expression of molecules related to osteogenesis and bone remodeling. The results indicated that LLT and HBO favor bone repair, but these effects, in general, were not enough to revert the damage caused by ionizing radiation in bone.

FOLHA DE APROVAÇÃO

SERVIÇO PÚBLICO FEDERAL
MINISTÉRIO DA EDUCAÇÃO
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FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

Ata da defesa de TESE DE DOUTORADO junto ao Programa de Pós-graduação em Odontologia Faculdade de Odontologia da Universidade Federal de Uberlândia.

Defesa de: Tese de Doutorado nº 005 - COPOD
Data: 29/08/2014
Discente: Flaviana Soares Rocha; Matrícula: (11213ODO006)
Título do Trabalho: Efeito do laser de baixa potência e da oxigenação hiperbárica no reparo de osso submetido à radiação ionizante.
Área de concentração: Clínica Odontológica Integrada.
Linha de pesquisa: Processo de reparo.
Projeto de Pesquisa de vinculação: Processo de reparo.

As **treze** horas e **trinta** minutos do dia **vinte e nove de agosto do ano de 2014** no Anfiteatro Bloco 4L Anexo A, sala 23 Campus Umuarama da Universidade Federal de Uberlândia, reuniu-se a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em fevereiro de 2014, assim composta: Professores Doutores: Karen Renata Nakamura Hiraki (UFU); João César Guimarães Henriques (UFU); Antônio Wilson de Almeida (UNIPAC); Paulo Tambasco de Oliveira (FORP/USP); e Paula Dechichi (UFU) orientador(a) do(a) candidato(a) **Flaviana Soares Rocha.**

Iniciando os trabalhos o(a) presidente da mesa Dra. Paula Dechichi apresentou a Comissão Examinadora e o candidato(a), agradeceu a presença do público, e concedeu ao Discente a palavra para a exposição do seu trabalho. A duração da apresentação do Discente e o tempo de argüição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadore(a)(s), que passaram a argüir o(a) candidato(a). Ultimada a argüição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais.

Em face do resultado obtido, a Banca Examinadora considerou o(a) candidato(a) A provado(a).

Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar foram encerrados os trabalhos às 17 horas e 15 minutos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

Prof. Dr. Antônio Wilson de Almeida – UNIPAC

Prof. Dr. Paulo Tambasco de Oliveira – FORP/USP

Profa. Dra. Karen Renata Nakamura Hiraki – UFU

Prof. Dr. João César Guimarães Henriques – UFU

Profa. Dra. Paula Dechichi – UFU
Orientador(a)

INTRODUÇÃO

INTRODUÇÃO

O reparo é um fenômeno que ocorre para reconstituir áreas desorganizadas ou destruídas por trauma aos tecidos, e envolve células e inúmeros mediadores químicos. É sempre um desafio otimizar e acelerar o processo de reparo para restabelecer a fisiologia tecidual. A complexa resposta de um tecido vivo à agressão, onde houve destruição dos componentes teciduais, é denominada reparação; esta pode ocorrer por regeneração ou por cicatrização. No processo de regeneração ocorre a reconstituição da parte danificada ou perdida, resultando em estruturas com a mesma arquitetura e função teciduais originais. A cicatrização é a reparação da região lesada por tecido que não restaura completamente a arquitetura ou função da parte danificada (Schmidt-Bleek et al. 2012).

Basicamente, o processo de reparo tecidual envolve as seguintes etapas: inflamação e coagulação sanguínea; formação de tecido de granulação e; maturação e remodelação tecidual. Os eventos iniciais do processo de reparo são caracterizados pela formação do coágulo sanguíneo, com a presença de plaquetas carregadas de fatores de crescimento, além de células inflamatórias. Rapidamente, inicia-se a formação de uma rede de fibrina, que auxilia a migração das células circundantes e desenvolve-se um infiltrado inflamatório. Gradualmente, ocorre invasão de fibroblastos que têm origem a partir da diferenciação das células mesenquimais, bem como proliferação de capilares sanguíneos, originados a partir de células endoteliais, caracterizando a formação do tecido de granulação. Com o passar do tempo, o tecido de granulação origina um tecido conjuntivo fibroso, ainda não totalmente maduro e

que sofrerá processo de maturação e remodelação, com reorganização das fibras colágenas (Consolaro, 2009).

Embora o reparo do tecido ósseo apresente uma sequência de eventos semelhante, por ser um conjuntivo cuja matriz extracelular é mineralizada, ele apresenta algumas particularidades em seu reparo. Quando o osso é lesionado, ocorre rompimento do periósteo, de vasos sanguíneos, destruição de matriz, da medula óssea e morte de células ósseas. No local da lesão, ocorre hemorragia e formação de coágulo, estabelecendo um espaço hipóxico (PO_2 = 5 a 10mmHg) e acidótico (pH 4 a 6), constituído de eritrócitos, fibrina, adjacentes a células ósseas, mesenquimais e plaquetas, que liberam mediadores químicos, como PDGF, FGF, TGF-α (Lynch et al., 1999).

Na região da lesão óssea, encontra-se um tecido necrótico que estimula intensa resposta inflamatória, caracterizada por vasodilatação, exsudação do plasma, leucócitos e células mesenquimais. No início do reparo, os macrófagos removem restos celulares e matriz alterada Em seguida, ocorre resposta proliferativa intensa do periósteo e do endósteo, com neoformação de tecido conjuntivo e também de capilares, caracterizando a formação de tecido de granulação. A neovascularização também é característica do processo, sendo que novos vasos sanguíneos podem ser identificados no tecido de granulação. Com o decorrer do tempo, os osteoblastos sintetizam matriz orgânica, rica em colágeno tipo I, a qual sofrerá mineralização, posteriormente.

A extensão e a atividade do reparo ósseo dependem da quimiotaxia e ativação de macrófagos e posterior proliferação e diferenciação de células osteoprogenitoras em osteoblastos (Schmidt-Bleek et al. 2012). As células

osteoprogenitoras, apesar de serem encontradas na medula óssea, têm sua localização específica na porção do estroma, sendo células mesenquimais não-hematopoiéticas, mas de linhagem osteogênica (Bianco et al. 2011). Uma vez diferenciadas em osteoblastos, elas são responsáveis por produzir matriz orgânica e controlarem o processo de mineralização óssea. Inicialmente,os osteoblastos sintetizam matriz não mineralizada, que contém inúmeros osteócitos e fibrilas colágenas dispostas sem organização definida, caracterizando o tecido ósseo imaturo. Os osteócitos ocupam lacunas no interior desta matriz óssea, desempenhando função importante na manutenção da matriz.

O processo de reparo ósseo continua, levando a formação de tecido ósseo maduro, que apresenta organização das fibrilas colágenas em lamelas ou camadas, menor número de osteócitos incluídos na matriz mineralizada e maior conteúdo mineral. Neste momento, os osteoclastos, responsáveis pela reabsorção óssea, possuem um papel importante. Esses são células multinucleadas, que se localizam na superfície da matriz, sendo ativos no processo de remodelação óssea (Katchburian & Arana, 2004; Ross, 2012).

Mesmo após a formação do tecido ósseo maduro, o osso mantém um processo combinado e constante de formação e reabsorção. Esta remodelação é determinada pela carga genética e se mostra dependente da regulação e influências endócrinas, bioquímicas e ambientais (Dimitriou et al., 2005). Mesmo no adulto, o tecido ósseo é metabolicamente ativo e a manutenção da matriz é resultado do balanço de atividades de síntese e reabsorção, que refletem as atividades antagonistas de osteoblastos e osteoclastos.

O reparo ósseo é influenciado por vários fatores tais como o tipo de osso (cortical ou trabecular), local e severidade da lesão ou dano ao tecido ósseo, presença de infecção, fixação durante o reparo, idade, estado de saúde geral e nutricional dos indivíduos . Alguns tratamentos, como a radioterapia, também podem comprometer o tecido ósseo, limitando as possibilidades futuras de reparo (Batista et al., 2014).

O dano da radioterapia oncológica na vascularização do osso e os seus tecidos circundantes leva à hiperemia, seguido por endoarterite, trombose e uma progressiva oclusão e obliteração dos pequenos vasos. Esse fato resulta em redução do número de células e fibrose progressiva. Com o tempo, a medula apresenta acelularidade marcante e pouca ou nenhuma vascularização, com significativa fibrose e degeneração gordurosa. O endósteo atrofia, com perda significativa de osteoblastos e osteoclastos ativos. O periósteo mostra fibrose significativa, com perda de elementos celulares remodelativos (Constantino et al., 1995).

No osso irradiado pode ser observado precocemente desequilíbrio da atividade osteoblástica e osteoclástica, com favorecimento à reabsorção óssea (Maeda et al., 1988; Da Cunha et al., 2007; Pelisser et al., 2007), além de aumento da lise celular (Da Cunha et al., 2007) e redução da resistência biomecânica do osso (Maeda et al., 1988). No entanto, alterações significativas na matriz óssea após a irradiação são desenvolvidas lentamente, após dano celular. Com isso, o processo de formação da matriz óssea é paralisado, o que pode, com o tempo, levar a fraturas ósseas espontâneas e à osteorradionecrose. Algumas terapias, como laser de baixa potência (Merli et

al., 2005; Chow et al. 2011; Silva & Camilli, 2006) e oxigenação hiperbárica (Kürklü et al, 2012; Kawada et al, 2013; Neves et al, 2013), têm sido propostas para favorecer o reparo tecidual de maneira geral.

Estudos experimentais, in vitro e in vivo, têm sugerido que a laserterapia de baixa potência (LBP) modula vários processos biológicos em modelos animais, após terem sido expostos a algum tipo de trauma. A LBP atua acelerando a reparação tecidual, pois estimula a proliferação celular (Karu, 1989; Silva & Camilli, 2006); a síntese de ATP (Karu, 1989) e de colágeno, com formação das fibras colágenas tipo I e tipo III (Pinheiro & Gerbi, 2006). A LBP ainda promove o aumento na concentração de β-endorfinas e, consequentemente, desencadeia efeito analgésico (Merli et al., 2005; Chow et al. 2011).

O uso dos lasers na biomodulação do reparo ósseo por meio de suas propriedades fotoquímicas e fotobiológicas (Khadra, 2004) tem sido estudado por pesquisadores em todo o mundo, como método de estimulação da osteogênese e redução do tempo da consolidação óssea (Silva & Camilli, 2006), entretanto, há muita divergência em relação aos protocolos de uso desta terapia. A laserterapia de baixa potência tem se mostrado efetiva na modulação da inflamação, acelerando a proliferação celular e o processo de reparo (Karu, 1989; Silva & Camilli, 2006). No entanto, o mecanismo pelo qual a radiação laser interfere na formação óssea ainda não foi completamente esclarecido.

A Oxigenação hiperbárica (OH) também tem sido utilizada para favorecer o reparo tecidos lesados, bem como para prevenção ou tratamento de necrose tecidual, por meio da inspiração de oxigênio puro sob pressão.

Acredita-se que o aumento da quantidade de oxigênio no sangue, forneça maior aporte de oxigênio para o local da ferida, estimulando a angiogênese (Jan, 2006; Fok, 2008). A alta tensão de oxigênio também tem efeitos bactericida e bacteriostático, além de potencializar a capacidade fagocitária de leucócitos, favorecer a diferenciação de fibroblastos e a síntese de colágeno.

O tecido ósseo particularmente pode se beneficiar da OH (Kürklü et al, 2012; Kawada et al, 2013; Neves et al, 2013), considerando que esse tecido tem processo de reparo lento. A hiperóxia, como ocorre na terapia hiperbárica, aumenta a quantidade de oxigênio dissolvido no plasma sanguíneo e esta condição interfere positivamente no metabolismo tecidual. Estudos têm relatado que a OH aumenta a formação óssea, a taxa de deposição mineral e angiogênese (Salgado, 2008; Muhonen, 2004), favorecendo o reparo de fraturas, provavelmente, devido ao aumento do anabolismo ósseo (Hsieh et al, 2010; Kawada et al, 2013). Esse aumento também pode ser observado na aceleração da remodelação óssea e, consequentemente, na quantidade das células envolvidas nesse processo.

Terapias coadjuvantes que favoreçam o restabelecimento da normalidade da morfologia e função tecidual, e reduzam possíveis prejuízos ao processo natural do reparo ósseo, seriam interessante em diversas situações clínicas. Dessa forma, neste estudo foi avaliada a influência do laser de baixa potência e da oxigenação hiperbárica no reparo do tecido ósseo normal ou comprometido pela radioterapia.

CAPÍTULO 1 – 1º ARTIGO

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HIGH DOSES OF IONISING RADIATION ON BONE HEALING: IS THERE ANY SYSTEMIC EFFECT?

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HIGH DOSES OF IONISING RADIATION ON BONE HEALING: IS THERE ANY SYSTEMIC EFFECT?

Running title: Systemic effect of radiotherapy

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ABSTRACT

Purpose: The aim of this study was to assess the potential systemic effects of radiotherapy in mice (*Ratthus norvergicus*) submitted to radiotherapy. Materials and Methods: The sample consisted of ten healthy male *Wistar* rats. Five animals were submitted to radiotherapy of the left femur and after 4 weeks, in all animals, bone defects were created in the right and left femurs. The femurs were randomly divided into 3 groups: Group I – Control (non-irradiated animals); Group II – Local Radiotherapy; Group III – Distant Radiotherapy. Animals were euthanized at 7 days after surgery, and bone sections were evaluated. Results: The histomorphometric analysis did not find any significant differences in the percentage of bone formation between groups I (0.24±0.07) and III (0.25±0.04). Group II did not present any bone formation. Conclusions: The results did not demonstrate any changes in bone repair after the application of radiotherapy a long distance away from the evaluated area.

Key-words: bone healing, radiotherapy, systemic effect, bystander effect, cell.

INTRODUCTION

The adverse effects of radiation therapy are well documented [1, 2, 3] and occur because of energy deposition in the cell nucleus affecting a critical target, nuclear DNA, resulting in mitotic failure and/or apoptosis [4].

As in other tissues, radiation therapy may cause changes in the skeletal system. Bone irradiation results in the depression of metabolism, decreased vascularity and remodeling alterations. Blood flow to mature bone is reduced at late times after irradiation, bone mineral density is decreased and fragility is increased [4, 5, 6, 7, 8]. The timing of these events may be critical to a full understanding of bone injury after irradiation.

Recently, evidence has indicated that radiotherapy elicits biological responses outside the treatment field, the so-called bystander effects [9, 10]. Radiation-induced bystander effects occur when an irradiated cell communicates with non-irradiated cells via secreted factors and/or the gap junction and non-irradiated cells exhibit responses that are normally characteristic of irradiated cells [10]. Another important question is whether radiotherapy to localized area exposes other distant tissues to the radiation dose. The possible systemic effects from this exposure to radiation should be distinguished from bystander effects due to cellular stress after ionizing radiation. Whereas the first type of effect depends on tissue exposure to secondary radiation, radiation-induced bystander effects are independent of direct exposure. At present, it is not known to what extent these untargeted effects contribute to overall cellular radiation responses, especially in vivo, but

they may clearly be of particular significance and may increase or decrease risk, determining overall outcome after radiation exposures [11].

These mechanisms have significant implications for a better understanding of radiation effects, but the current state of knowledge does not permit definitive statements about whether these phenomena have clinical implications on bone healing in an area that is distant from the irradiated field. Therefore, the objective of this study was to assess the potential systemic effects of radiotherapy on bone healing distant to the irradiation site.

MATERIALS AND METHODS

Animals

The sample consisted of ten healthy male *Wistar* rats (*Rattus norvergicus*), weighing 300 to 350g. Five animals were submitted to radiotherapy of the left femur and after 4 weeks, in all animals, bone defects were created in the right and left femurs. The femurs were randomly divided into 3 groups, as demonstrated in Table 1, and the irradiated femur was compared with the contralateral non-irradiated femur in the same animal, as well as with control femurs (animals not subjected to radiation). During the experiment, the animals were kept under light and temperature conditions, with standard food and water *ad libitum*. This study was previously approved by the Science and Ethics Committee from Pontifícia Universidade Católica of Rio Grande do Sul, Brazil, Protocol 037/2009, and was performed in accordance with the Brazilian College for Animal Experimentation (COBEA).

Radiotherapy

Before irradiation, animals were anaesthetized by an intraperitoneal injection of 100mg/kg ketamine, 3mg/kg xylazine hydrochloride and placed in supine position. The leg was positioned laterally and fixed in this position using a wooden stick and adhesive tape. A bolus made of wax with a thickness of 1.5cm was positioned over the leg. Both femur and tibia were irradiated by a single anterior field. The beam was collimated and irradiation was delivered using a linear accelerator (Varian Clinac® 600C S/N 0310) with a total dose of 30Gy in one session. Damage to the hair and reactions of the skin were examined twice weekly. If the animal had persistent breakdown of the skin with any sign of infection, it was excluded from the experimental groups. In Group II, the left femur was evaluated, which had been submitted to radiotherapy. In Group III, the contra-lateral femur was evaluated (radiation was performed at a distance from the evaluated area).

Surgery

Four weeks after radiotherapy, all animals were anesthetized as previously described and submitted to surgical procedure. A cephalosporin antibiotic prophylaxis (30mg/kg, IP) was used. With the animal positioned in lateral decubitus, the femur was exposed through a 2 cm longitudinal incision. Then, an osteotomy was made with a round bur creating a 2.3mm standardized bone defect. During the procedure, there was constant irrigation with saline solution. The depth of drilling was limited to cortical bone rupture (approximately 2mm). The suture was performed using nylon.

Sample evaluation

After 7 days, the animals were euthanized. The bone specimens with the defects and the attached soft tissue were removed and immediately fixed in 10% phosphate buffered formaldehyde solution for 48h. Thereafter, the tissue blocks were decalcified in 10% EDTA for 4 weeks, dehydrated with graded alcohols and embedded in paraffin. The histological semi-serial sections obtained with a thickness of 5µm were stained with Hematoxylin-Eosin and Mallory Trichrome.

Histomorphometric Analysis

In order to quantify bone formation, histological images of bone defects were captured at ×4 magnification, using an Olympus BX 40 binocular microscope (Olympus BX 40 - Shinjuku-ku, Tóquio, Japão) coupled with Olympus OLY 200 camera (OLY 200 - Center Valley, PA - USA). The histological sections of whole bone defect area were digitalized using the HL Image 2005 program (Western Vision, Salt Lake City, UT, USA). The screenshots were merged, areas of soft tissue were erased using Photoshop CS2 software (Adobe®, Adobe System Inc., San Jose, CA/EUA), and finally converted to binary images with HL Image 2005. The region of interest within the bone defect (ROI) was delineated with four straight lines from the edges of the cortical bone to the opposite cortex. With these images, the percentage of bone formation within the area of interest was calculated with the measure tool of HL Image 2005 (Figure 1).

Statistics

Analysis was performed using statistical software (GraphPad Prism version 5.0 for Windows, San Diego, CA, USA). The results obtained were

submitted to normality test and Kruskal-Wallis test (Dunn's post-test). Differences were considered statistically significant when $p<0.05$.

RESULTS

Bone neoformation was evident in Groups I and III. The ROI was occupied by primary bone with a trabecular arrangement delimiting small cavities, filled with loose connective tissue, fibroblasts, peripheral osteoblasts and blood vessels. In Group II, marrow space was mainly occupied by reticular fibrin, eventual fat cells and hemorrhage, sometimes intermingled with moderate quantities of monocytes and rare neutrophils (Figure 2).

The histomorphometric analysis did not find any significant differences in the percentage of bone formation between groups I (0.24±0.07) and III (0.25±0.04). Group II did not present any bone formation (Figure 3).

DISCUSSION

The present study evaluated the possible systemic effect of radiotherapy on bone healing, comparing the irradiated femur to the contralateral femur in an intra-group analysis, and also comparing non-irradiated animals in an inter-group analysis. The primary question is whether a possible secondary radiation distant from the primary radiation site could affect bone healing. This is important for the rehabilitation of patients cured of cancer, because these patients often undergo dental surgical procedures in areas that have not been exposed to radiation.

In the present study, the animals were subjected to a single dose of 30 Gy, which is sufficient to promote bone changes [6, 12] and bone healing was assessed by quantification of the percentage of bone formation. This parameter

indirectly reflects the proliferation and differentiation of bone cells in the repair process [13, 14]. In our study, bone healing in the irradiated femur was remarkably delayed and accompanied with poor tissue formation. In irradiated specimens, although medullary tissue was not usually maintained, in the cases where it was preserved, it was full of fat cells, with a marked reduction of cells. Knowing that the bone requires increased cell proliferation to achieve adequate healing [13], bone formation was certainly affected after radiotherapy.

Once the expected local effect of radiotherapy had been demonstrated, the next step was to verify the effect of radiotherapy on bone healing distant from the primary site of radiation. This hypothesis was not confirmed in the present study. The wound healing process in the distant irradiation femur was similar to that in the non-irradiation femur, indicating that radiotherapy did not produce systemic effects on bone healing in our experimental conditions.

Other authors found different results. Lucatto et al. (2011) [15] believe that, despite the strict delimitation of the irradiation field size, tissues far from the primary site also received ionizing radiation, but with less energy. They investigated bone neoformation in defects created on tibiae of rats previously submitted to radiotherapy (Cobalt 60) with doses of 30Gy and on the contralateral tibiae that received a secondary radiation dose calculated to be 7Gy. However, the authors did not use a control group with non-irradiated animals to compare results. In fact, the authors did not find any decrease in cell population or reduction of newly formed bone after secondary radiation. It is also interesting to consider the mechanism of action and the evolution of devices that provide radiation, as nowadays, radiotherapy is the safest and

most effective procedure. New radiotherapy regimens and modalities have been developed in human oncology and have rendered most of the experimental data available inappropriate [3].

Maeda et al. (1988) [5] used a linear accelerator as a source of radiation to evaluate the effects of high-dose irradiation on the biomechanical and morphological properties of cortical bone and their time-related changes. The authors observed a reduced number of osteocytes and bone marrow changes after radiotherapy in both the irradiated leg and the contralateral leg of mice, claiming a possible systemic effect. However, the authors did not measure whether there was any secondary radiation during the procedure. As such, the change in the contralateral non-irradiated side observed by Maeda might also be an influence of the activity-related remodeling because animals prefer to use the non-irradiated leg during activity. Additionally, Maeda evaluated morphological and biomechanical properties of the bone without any defect, for longer periods of evaluation. Considering that it is a consensus that different forms of radiation do not produce the same biological effects, even if all of the components of the irradiated bone are directly affected by radiotherapy, they would have different degrees of sensibility [3], this makes it difficult to compare the results of different experimental models.

Pacheco and Stock (2003) [4] believe that radiation effects on bone are most severe locally within the treatment field, as observed in our study. However, the same authors suggest that these effects can have a systemic reflection (bystander effects), possibly because of hormonal influences and cytokine mediators. These bystander effects occur due to cellular stress after

ionizing radiation, in which responses that are characteristically associated with directly irradiated cells are exhibited by non-irradiated cells [11, 16].

Those observations raise the issue of whether bystander responses have relevance to the clinical environment. Despite the limitations of existing experimental data, we can speculate on potential roles. It seems reasonable to believe that the environmental conditions and cell lines play an important role in the nature of the resulting bystander response due to the degrees of sensibility of the cells [3]. Some authors suggest that these non-targeted effects of radiotherapy involve the innate immune system and can occur outside the field of radiation in a paracrine way as soluble extracellular factors released by cells are involved in the process [9]. The detection of bystander effects in *in vitro* studies could point to signal-dependent processes that might have a role in late radiation-induced damage to normal tissue [16,17].

The spatial proximity of cells or direct cell-to-cell contacts may be crucial for transmitting bystander signals from irradiated cells to neighboring non-irradiated cells [17], but it remains to be determined whether similar scenarios can be envisioned *in vivo*. In our study, we did not find any systemic or bystander effects on bone healing distant to the radiation field. Considering our findings, it is possible that *in vivo*, these bystander signaling processes may have dissipated over time. Possibly, non-irradiated cells that are close to the radiation field would be sensitive to bystander effects.

Usually, in vitro studies are easier to reproduce and involve the cultivation of non-irradiated cells alone and the co-cultivation of these cells with irradiated cells [17]. In other words, these studies evaluate bystander effects of

radiation and not the possible systemic effect of secondary radiation. Even knowing that important information was obtained from in vitro studies, no definitive relationship has been established between in vitro and in vivo effects due to the difficulty in comparing these results [18].

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Tables:

Table 1 – Animal distribution according to the Groups.

Groups	Femur Number
Group I – Control (non-irradiated animals)	n=5 (right femur)
Group II – Local Radiotherapy	n=5 (left femur)
Group III – Distant Radiotherapy	n=5 (right femur)

Figure and Legends:

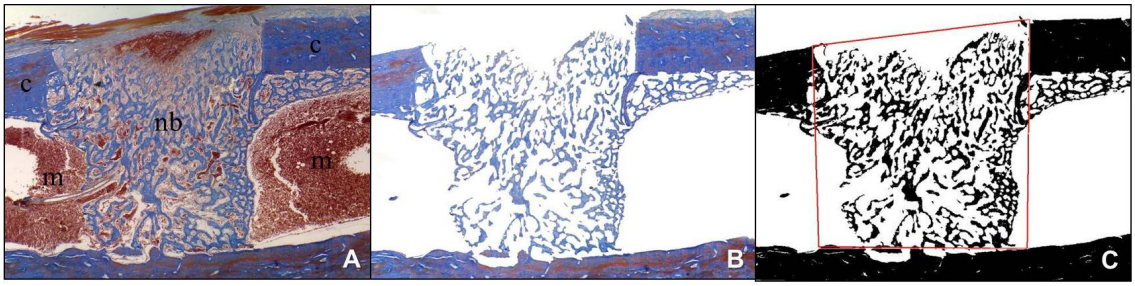

Figure 1: A- Photomicrograph of longitudinal femur section showing: cortical bone (c), bone marrow (m) and new bone (nb). B- Image after digital removal of soft tissue. C- Image after binary conversion and delimitation of region of interest (red dotted line). ×4. Mallory Trichrome.

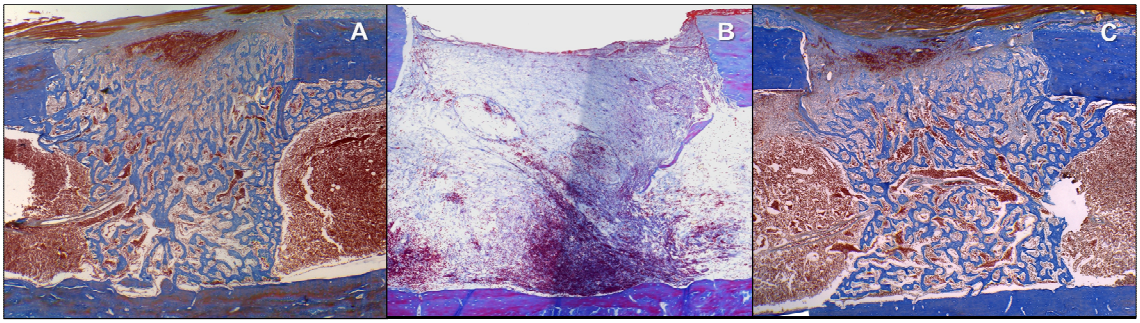

Figure 2: Photomicrograph of femur longitudinal section of all groups: A – Group I (Control); B – Group II (Local Radiotherapy); C- Group III (Distant Radiotherapy). ×4. Mallory Trichrome.

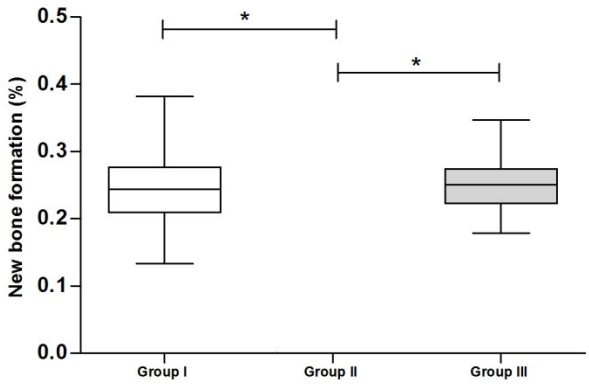

Figure 3: New bone formation in the evaluated groups (*p<0.01).

CAPÍTULO 2 – 2º ARTIGO

Comprovante de submissão – Revista: Lasers in Medical Science

Lasers in Medical Science

Effect of Low Level Laser Therapy on Repair of Bone Compromised by Radiotherapy

--Manuscript Draft--

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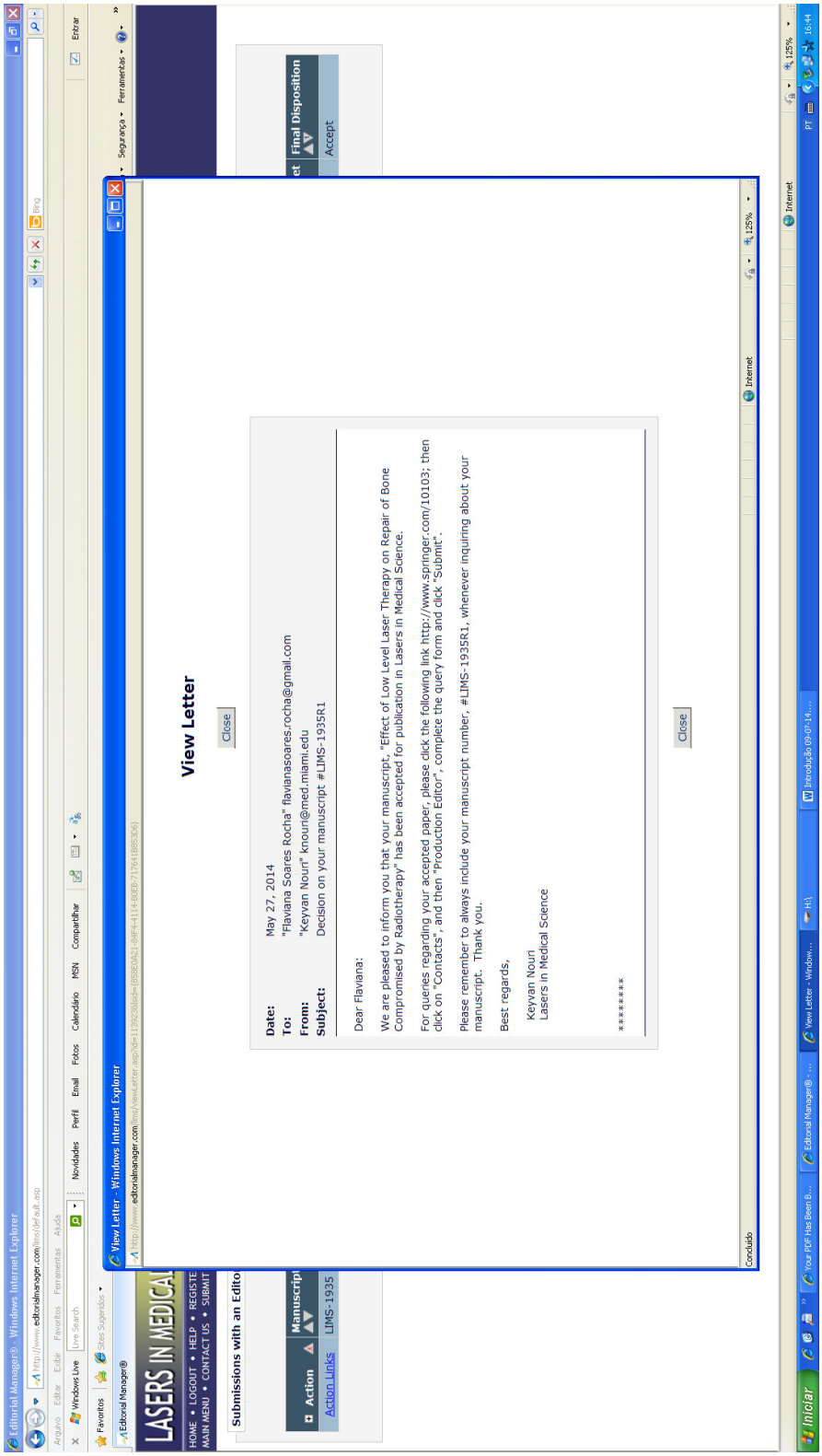
Abstract:	Radiotherapy (RDT) is commonly used for cancer treatment, but high doses of ionizing radiation can directly affect healthy tissues. Positive biological effects of low level laser therapy (LLLT) on bone repair have been demonstrated, however, this effect on surgical defects of bone previously compromised by radiotherapy has not been evaluated. The aim of this study was to investigate the influence of LLLT (λ = 830nm) in femur repair after ionizing radiation. Twenty Wistar rats were divided into four groups: GC (control, n = 5) creation of bone defects (BD) only; GL, with BD and LLLT (n = 5); GR, submitted to RDT and BD (n = 5); and GRL, submitted to RDT, BD and LLLT (n = 5). GL and GRL received punctual laser application (DE = 210J/cm², P = 50mW, t = 120s, and beam diameter of 0.04cm²) immediately after surgery, with 48h-interval during 7 days. Animals were euthanized at 7 days after surgery, and bone sections were evaluated morphometrically with conventional microscopy. Results: Bone repair was only observed in non-irradiated bone, with significantly improvement in GL in comparison to GC. GR and GRL did not present any bone neoformation. Conclusions: The result demonstrated a positive local biostimulative effect of LLLT in normal bone. However, LLLT was not able to revert the bone metabolic damage due to ionizing radiation.

Comprovante de aceite – Revista: Lasers in Medical Science



Effect of Low Level Laser Therapy on Repair of Bone Compromised by Radiotherapy

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Key-words: Bone repair, low level laser therapy, radiotherapy.

Effect of Low Level Laser Therapy on Repair of Bone Compromised by Radiotherapy

Abstract:

Radiotherapy (RDT) is commonly used for cancer treatment, but high doses of ionizing radiation can directly affect healthy tissues. Positive biological effects of low level laser therapy (LLLT) on bone repair have been demonstrated, however, this effect on surgical defects of bone previously compromised by radiotherapy has not been evaluated. The aim of this study was to investigate the influence of LLLT (λ = 830nm) in femur repair after ionizing radiation. Twenty *Wistar* rats were divided into four groups: GC (control, n = 5) creation of bone defects (BD) only; GL, with BD and LLLT (n = 5); GR, submitted to RDT and BD (n = 5); and GRL, submitted to RDT, BD and LLLT (n = 5). GL and GRL received punctual laser application (DE = 210J/cm², P = 50mW, t = 120s, and beam diameter of 0.04cm²) immediately after surgery, with 48h-interval during 7 days. Animals were euthanized at 7 days after surgery, and bone sections were evaluated morphometrically with conventional microscopy. Results: Bone repair was only observed in non-irradiated bone, with significantly improvement in GL in comparison to GC. GR and GRL did not present any bone neoformation. Conclusions: The result demonstrated a positive local biostimulative effect of LLLT in normal bone. However, LLLT was not able to revert the bone metabolic damage due to ionizing radiation.

Introduction

Radiotherapy (RDT) has been used to the treatment of cancer; however radiation can induce hypoxia, cellular depletion or hypocellularity and hypovascularity in healthy surrounding tissue [1]. In bone tissue, the radiation produces apoptosis of bone and endothelial cells, which causes decrease of blood flow, modification of channels network, reduction of matrix and relative amounts of calcium and phosphorus, leading to bone atrophy [2, 3, 4]. These alterations interfere in bone metabolism [2] and compromise the strength and healing in irradiated bone making the area susceptible to infection and necrosis, even after small trauma [5]. Even in patients cured of cancer, changes in bone tissue due to radiotherapy are still evident because the deleterious effect of ionizing radiation can extend for long periods compromising bone repair, as documented in previous studies [3, 5, 6, 7]. For this reason, surgical procedures are contraindicated up to 1 year after RDT [8].

Reconstructive surgeries and prosthetic rehabilitation are frequently associated with complications after RDT, especially the development of Osteorradionecrosis (ORN) [5, 9]. Many therapeutic options have been studied to minimize risks and achieve success in these patients, such as hyperbaric oxygenation [10], vascularized grafts [11, 12], platelet rich plasma associated with grafts [13] and low level laser therapy (LLLT) [14, 15].

Some studies indicate that LLLT stimulates cell proliferation [16], osteoblast activity, vascularization and collagen deposition [4]. During the initial stages of bone repair, LLLT increased the expression of osteogenic genes and stimulated newly bone formation [17]. However, there is lack in the literature

describing the effects of LLLT in the initial stages of bone repair after radiotherapy. In this context, the aim of the present study was to evaluate the effect of LLLT on the initial stages of bone healing after radiotherapy, in an experimental rat model.

Materials and Methods

Animals and Experimental groups

The sample consisted of twenty healthy male *Wistar* rats (*Rattus norvergicus*), weighing 300 to 350g. The animals were kept in four cages, under light-dark period of 12h and controlled temperature conditions (22±2°C), with standard food and water *ad libitum.* This study was previously submitted and approved by the Science and Ethics Committee from Pontifícia Universidade Católica of Rio Grande do Sul, Brazil, Protocol 037/2009, and was performed in accordance with the Brazilian College for Animal Experimentation (COBEA).

Each group was composed of five animals randomly divided into four groups, as follows:

- Control group (GC): Animals with bone defects (BD) only.
- Laser group (GL): Animals with BD and the application of low level laser therapy (LLLT).
- Radiotherapy group (GR): Animals previously submitted to ionizing radiation and with BD.
- Radiotherapy and Laser group (GRL): Animals previously submitted to ionizing radiation, with BD and the application of LLLT.

Radiotherapy

Before radiotherapy, the animals of GR and GRL were anesthetized by an intraperitoneal injection of 100mg/kg ketamine, 3mg/kg xylazin hydrochloride (general anesthesia) and placed in supine position. The left leg was positioned and fixed laterally using wood stick and adhesive tape. A bolus made of wax with 1.5cm of thickness, was positioned over the left leg. Both femur and tibia were irradiated by a single anterior field. The beam was collimated and irradiation was delivered using linear accelerator (*Varian* Clinac® 600C S/N 0310) with a total dose of 30Gy in one session. The interval between radiotherapy and femur surgery was 4 weeks. Damage to the hair and the reactions of the skin were examined twice weekly. If the animal had persistent breakdown of the skin with any sign of infection, it was excluded from the experimental groups.

Surgery and Laser therapy

The animals received a cephalosporin antibiotic prophylaxis (30mg/kg, IP) and subject to general anesthesia. After shaving and antisepsis, with the animal positioned in right lateral decubitus, the left femur was exposed through a 2cm longitudinal incision. Then, a full-thickness cortical bone osteotomy was made with an n°8 round bur, creating a 2.3mm bone defect.

The animals of GL and GRL were submitted to laser therapy using an Gallium-aluminum-arsenide (GaAlAs) infrared laser diode (Flash lase III - DMC Equipamentos/SãoCarlos – SP – Brazil), λ830nm, 100mW, continuous wave, 0.028-cm^2 beam diameter. The application was punctual, with a 6 J (210J/cm^2) dose per session in the bone defect area during 2 minutes, with the laser tip positioned over and perpendicular to the long axis of the bone. The first session

was applied immediately after drilling and before soft tissue repositioning. In the postoperative period, laser was applied transcutaneously at 48-h intervals during seven days, resulting in four sessions. The animals were euthanized seven days after surgery using saturated potassium chloride associated with general anesthesia**.**

Histomorphometric Analysis

The bone defect area and attached soft tissue were removed and kept on 10% phosphate buffered formaldehyde solution during 48 hours. The samples were decalcified in 10% EDTA, dehydrated with graded ethanol and embedded in paraffin. The longitudinal 5µm histological sections obtained from the midline of the BD were stained with Hematoxiline and Eosine and Mallory Trichrome. All slides were analyzed by light microscopy by the same examiner blinded for the status of each specimen.

Histological images of the bone defect were captured ×4 magnification, using an Olympus BX 40 binocular microscope (Olympus BX 40 - Shinjuku-ku, Tóquio, Japão) coupled with Olympus OLY 200 camera (OLY 200 - Center Valley, PA - USA). The histological sections of whole bone defect area were digitalized using HL Image 2005 program (Western Vision, Salt Lake City, UT, USA). The screen shots were merged, areas of soft tissue were erased using Photoshop CS2 software (Adobe®, Adobe System Inc., San Jose, CA/EUA), and finally converted to binary images with HL Image 2005. The region of interest within the bone defect (ROI) was delineated with four straight lines as demonstrated in Figure 1. The percentage of bone neoformation within the ROI was obtained. Also, semiquantitative analysis included the evaluation of the

following parameters: 1- Presence of bone neoformation reaching the opposite cortical bone; 2- Presence of bone neoformation extending laterally beyond the ROI.

Statistical analysis

Analysis was performed using statistic software (GraphPad Prism version 5.0 for Windows, San Diego, CA, USA). Fisher exact test was performed to compare categorical parameters. Quantitative results were submitted to normality test and analyzed using Analysis of Variance (ANOVA) and Bonferroni post-hoc test. Differences were considered statistically significant if $p < 0.05$.

Results

Wound healing progressed without any signs of infection. Expected bone neoformation was evident in all animals from GC and GL, but not on those from GR and GRL (Figure 2). In these latter, bone marrow space was mainly occupied by delicate reticular fibrin network, eventual fat cells and hemorrhage. Chronic inflammatory infiltrate was observed adjacent to the BD, sometimes intermingled by moderate quantities of monocytes and rare neutrophils. In animals of GC and GL, repair was evident from the perforated cortical, extending to the opposite cortex. The area was occupied by primary bone with trabecular arrangement delimiting small cavities, filled with loose connective tissue, fibroblasts, peripheral osteoblasts and blood vessels.

The bone neoformation beyond the limits of the ROI and also the vertical extension of the bone touching the opposite cortical bone was usually more evident in GL than in GC, although such distribution was not significant (Figure 3).

The histomorphometric analysis revealed a significant increase in percentage of bone formation in GL in comparison to GC (Figure 4). GR and GRL did not present any bone matrix, as stated before, and therefore were also significantly different from GC and GL.

Discussion

The present study was performed to investigate whether low-level laser therapy was able to improve precocious healing of bone compromised by radiotherapy. The radiation resource used in our study was the linear accelerator, widely used nowadays. The buildup of 1.2 cm in this type of equipment means that in 1.2 cm of depth of tissue penetration, starts the largest concentration of energy [18]. For this reason, we used 3 slices of red wax (0.5 cm each) as bolus to guarantee the femur significant damage by ionizing radiation**.**

The complications in irradiated bone seem to be dependent on the dose [6]. This happens because bone proliferates slowly, it is less affected by radiation involving small fraction sizes or low total dose rates and is more susceptible to injury with increased doses [19]. The choice of 30 Gy for radiotherapy in the present study was based in previous studies, which showed significant alterations in bone tissue using this dose [4, 6]. This single high dose of ionizing radiation occurred mainly ensure the real damage to the bone, allowing to identify if laser therapy was beneficial to the bone in poor conditions. There are no studies comparing the exact radiotherapy dose in different animals and humans because of the variability in radiation effects from one species to

another [19]. However, according to the literature, a single 30Gy dose is approximately the equivalent of 120Gy by fractioned delivery [6].

In the present study, the damaging effect of ionizing radiation was clearly evident and the body itself cannot repair the damage. According to Hopewell (2003) [20], the primary effect of radiation on bone is atrophy, with a marked reduction in the number of tissue functional components, without a reduction in size. This could be explained the by events triggered by radiotherapy. Irradiation of tissues activates a rapid molecular response, with the production of cytokines in an attempt to heal the injury [1]. Although this was not evaluated in the present study, the immediate upregulation of proinflammatory cytokines such as TNFα, IL1 and growth factors after radiotherapy [21], affects bone healing. In our study, no bone formation was observed in both groups submitted to radiotherapy, and the defect was filled by loose poorly organized tissue. This could be due to the severe atrophy of blood vessels often results in loss of bone cells, dysregulated interactions between cell populations and hypoxia [1]. This situation results in reduction of viable undifferentiated mesenchymal cells [22] and osteoblasts [23]. For this reason, the development of radiation-induced fibrosis is frequent [21].

In our study, noxious effect of ionizing radiation leaded to an important problem related to bone resistance, since some animals submitted to ionizing radiation had to be substituted due to bone fracture during their manipulation for the LLLT sessions *(data not shown*). It seems reasonable to think that if bone repair is altered by radiotherapy, also, the whole bone structure can be compromised. It was already reported a reduction in the relative amounts of

calcium and phosphorus [20] and molecular changes in composition of apatite in irradiated bone [24], suggesting that the atrophy of this tissue is associated with bone mineral loss [18]. In addition, Wernle et al. (2010) [25], evidenced that high density irradiated bone becomes more brittle and requires less load to fracture.

The beneficial effects of LLLT on soft tissue and bone are more expressive when it is applied in the first seven post-surgical days [26, 27], so the present model evaluates bone healing in this period. During the early stages of bone repair, there is a cell proliferative phase, when the complete absorption of the laser light is more effective [28, 29], which does not occur in the later periods of bone repair [30]. However, this does not mean that LLLT is inefficient in the final stages of repair. This information suggests that the laser in the early stages stimulate bone regeneration, and may be important to maintenance of bone formation in later periods [31]. Depending on wavelength and energy density, LLLT can stimulate or inhibit biochemical, physiological, or proliferative processes in a variety of tissues. The wavelength of 830nm has a capacity to penetrate the superficial tissues, reaching the bone [32]. Laser protocols for bone healing in rat model are extremely variated in literature, even so, present positive results in bone formation.

In this study, for animals that were not submitted to radiotherapy, the positive effect of laser therapy was evidenced. This increase in bone formation may be due to the already known benefits of laser in the differentiation of mesenchymal cells and also osteoblast and fibroblast proliferation [33]. These events may explain the extensive bone neoformation, invading the medullar

area, observed in GL. Garavello-Freitas et al. (2003) [28], evaluating the influence LLLT on the repair of surgically defects in tibia of rats, also observed maximal laser-stimulated bone growth after 1 week of laser application.

On the other hand, in irradiated specimens, medullary tissue was full of fat cells, with marked reduction of bone components [4, 23], even after LLLT. Supposing that the bone requires increased cell proliferation to achieve adequate healing, bone repair seems to be affected after radiotherapy. To our knowledge, there is an experimental study that evaluated repair in bone compromised due to radiotherapy [6], as well as another one that had evaluated the use of laser in bone submitted to radiotherapy [4]. Even though, the results are not comparable because Da Cunha et al. (2007) [4] described positive effects LLLT on bone submitted to the radiotherapy, but they did not evaluate repair of surgical wounds. Also they used different laser protocols, longer period of radiotherapy with cobalt-60 as source of ionizing radiation, and sacrificed experimental animals six weeks after radiotherapy.

Unfortunately, laser therapy is still a controversial treatment because even researchers using similar protocols and laser units have reported conflicting results [34, 35]. It seems that the LLLT biostimulation effects depend upon a variety of factors including not only the laser protocol, but the type of tissue, physiological state of the cell, proliferation capacity and their changes according to the experimental model [29]. If the LLLT clearly stimulates normal bone healing, it would be expected similar effects in damaged bone, however, LLLT was not able to recover adequate healing of the bone submitted to radiotherapy (GRL). This lead us to think that positive effect of LLLT depends

on the ability of the cells to respond to the light energy, which is related to the physiological state of tissue at the time of laser application . It also explains the differences in LLLT application in hard and soft tissues. As such, if radiotherapy induces tissue fibrosis and reduces the number of viable cells [21], it could seem reasonable to believe that the irradiated tissue was not able to adequately respond to LLLT. Studies evaluating longer periods of laser treatment in irradiated bone are necessary to see if the irradiated bone recovers its properties with time and becomes able to respond to LLLT.

Once LLLT has been thought to increase cellular proliferation, it sounds questionable to employ this procedure on patients with neoplastic disease. The effect of LLLT over neoplastic cells is controversial, with evidence of different responses whether the cell is neoplastic or not [36, 37]. Anyway, LLLT should be better indicated to those patients with previously documented control of the neoplasia (e. g. surgical margins free of disease). In cases of osteorradionecrosis or even less aggressive conditions related to bone manipulation (e. g. tooth extraction and rehabilitation with implant-retained prosthesis) the immediate benefit of LLLT might be considered.

Conclusion

The hypothesized positive effect of LLLT on the repair of bone previously exposed to ionizing radiation was not confirmed by the present results, despite the evident biostimulative effect of LLLT in the repair of bone not submitted to radiotherapy. Additional studies with different LLLT protocols and its evaluation in longer periods of healing are required in order to elucidate its utility in the supportive care of oncologic patients.

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Figures and Legends

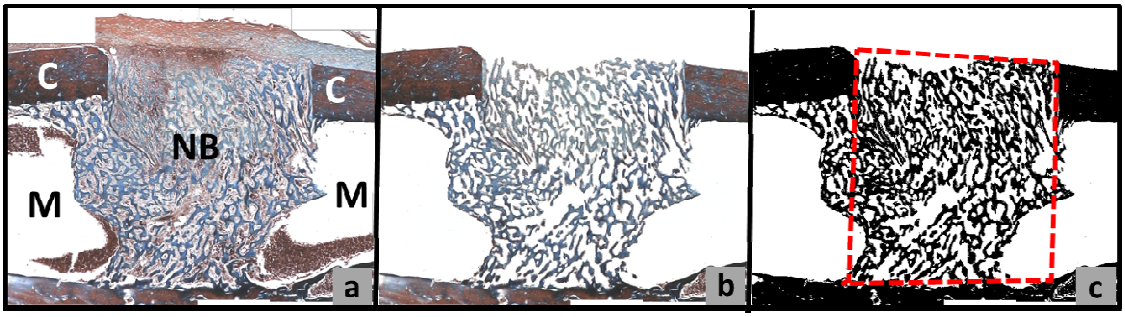

Figure 1: a- Photomicrograph of longitudinal femur section showing: cortical (C), bone marrow (M) and new bone (NB). b- Image after digital removal of soft tissue. c- Image after binary conversion and delimitation of region of interest (red dotted line). ×4. Mallory Trichrome.

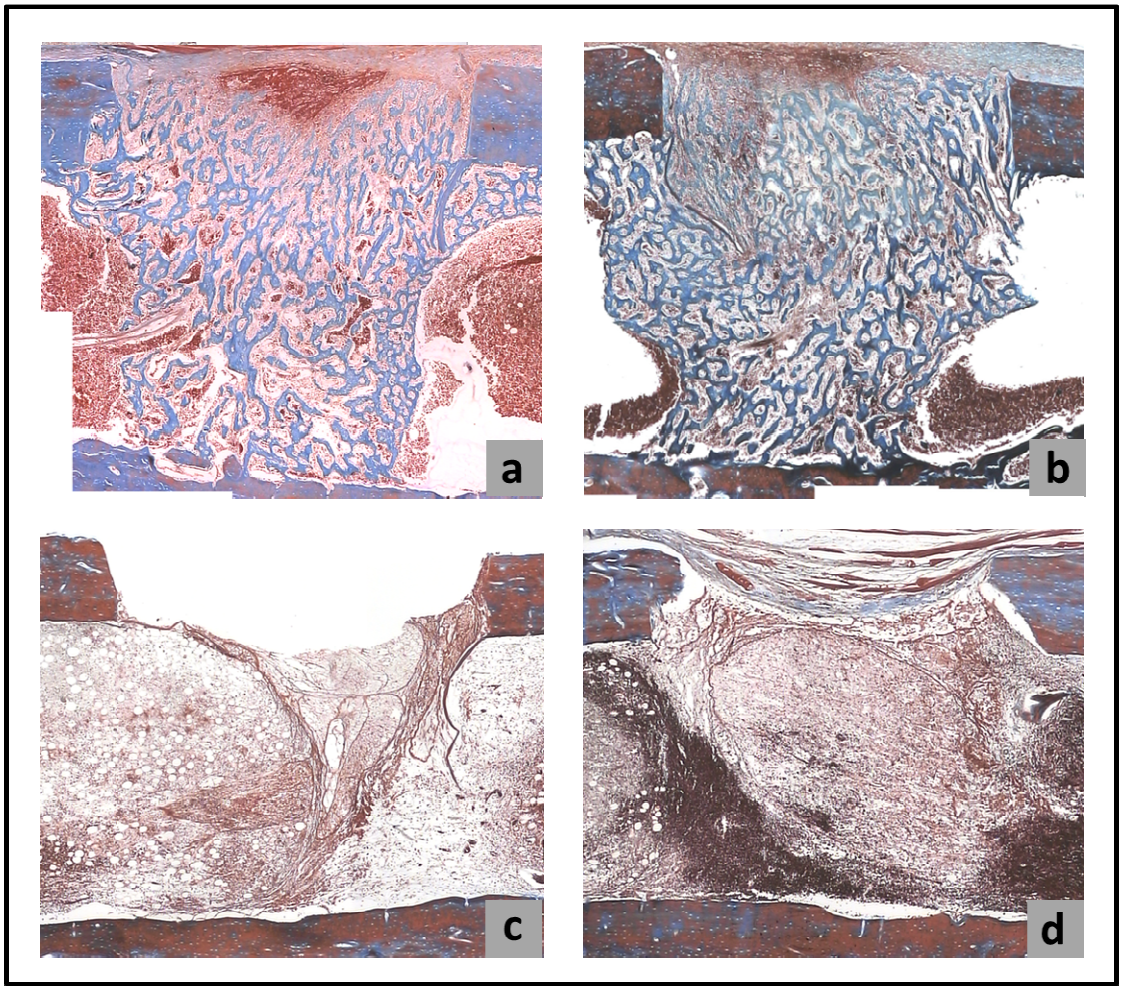

Figure 2: Photomicrograph of the femur longitudinal section of all groups: a– Control Group (GC); b– Laser Group (GL); c- Radiotherapy Group (GR) and d– Radiotherapy + Laser Group (GRL). ×4. Mallory Trichrome

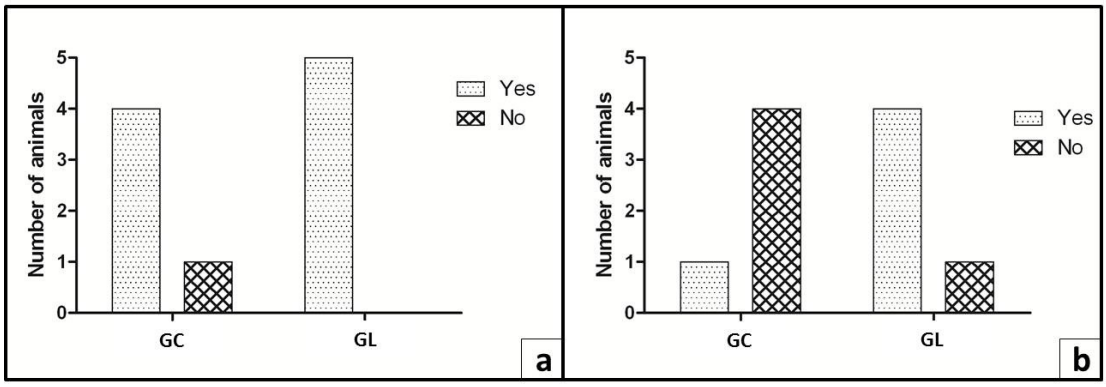

Figure 3: Histological parameters analyzed: a- Bone neoformation reaching the opposite cortical bone (p>0,05). b– Bone neoformation extending laterally beyond the ROI (p>0,05).

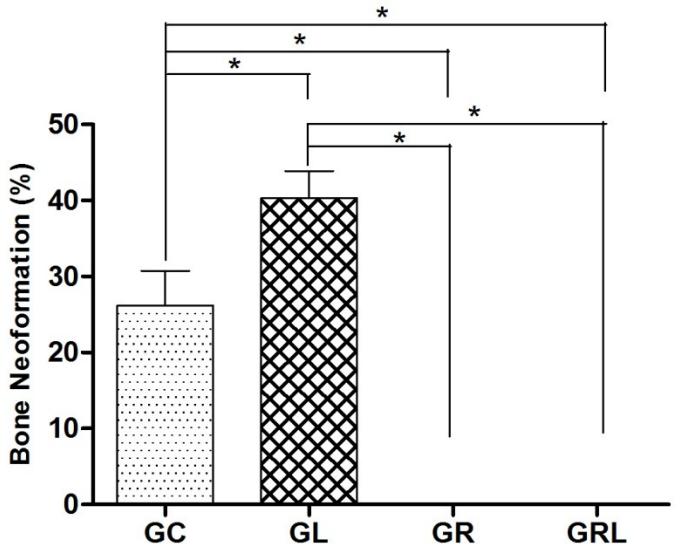

Figura 4 - Percentage of bone neoformation within the ROI, evidencing bone increase in Group GL (*p<0.05.)

CAPÍTULO 3 – 3º ARTIGO

Comprovante de submissão – Revista: Lasers in Medical Science

Lasers in Medical Science

Systemic Effect of Low Level Laser Therapy on Bone Repair

--Manuscript Draft--

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Abstract:	The biological effects of local therapy with laser on bone repair have been well demonstrated, however, this possible systemic effect on bone repair has not been evaluated. The aim of this study was to investigate the systemic effect of low level laser therapy (λ = 830 nm) on repair of surgical bone defects in rats. Methods: Forty-Five Wistar rats were submitted to osteotomy on left femur and randomly separated into three groups (n=15): group I, control, bone defect only; group II, laser applied on the right femur (systemic dose); group III, laser applied locally on bone defect and also on the right femur (local and systemic doses). Laser groups (II and III) received applications within a 48-h interval in one point per session of DE = 210 J/cm², P = 50 mW, t = 120 sec, and beam diameter of 0.04 cm². Five animals of each group were euthanized 7, 15 and 21 days after surgery. Results: Histologic analysis in all groups showed new bone formation in the ROI area at 7 days. After 15 days, it was observed bone remodeling with a decrease of bone neoformation in marrow area in all groups. After 21 days, it was observed advanced bone remodeling with new bone mostly located in cortical area. The histomorphometric analysis showed at 7 days a significant increase of bone formation in group III compared to group I and II. At day 15 and 21, histomorphometric analysis showed no significant differences between them. Conclusion: Laser therapy presented a positive local biostimulative effect in early stage of bone healing, but it was not observed LLLT effect a long distance away from the evaluated area.

Systemic Effect of Low Level Laser Therapy on Bone Repair

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ABSTRACT

The biological effects of local therapy with laser on bone repair have been well demonstrated, however, this possible systemic effect on bone repair has not been evaluated. The aim of this study was to investigate the systemic effect of low level laser therapy (λ = 830 nm) on repair of surgical bone defects in rats. *Methods:* Forty-Five *Wistar* rats were submitted to osteotomy on left femur and randomly separated into three groups (n=15): group I, control, bone defect only; group II, laser applied on the right femur (systemic dose); group III, laser applied locally on bone defect and also on the right femur (local and systemic doses). Laser groups (II and III) received applications within a 48-h interval in one point per session of DE = 210 J/cm², P = 50 mW, t = 120 sec, and beam diameter of 0.04 cm². Five animals of each group were euthanized 7, 15 and 21 days after surgery. *Results:* Histologic analysis in all groups showed new bone formation in the ROI area at 7 days. After 15 days, it was observed bone remodeling with a decrease of bone neoformation in marrow area in all groups. After 21 days, it was observed advanced bone remodeling with new bone mostly located in cortical area. The histomorphometric analysis showed at 7 days a significant increase of bone formation in group III compared to group I and II. At day 15 and 21, histomorphometric analysis showed no significant differences between them. *Conclusion:* Laser therapy presented a positive local biostimulative effect in early stage of bone healing, but it was not observed LLLT effect a long distance away from the evaluated area.

Key-words: low level laser therapy, bone healing

INTRODUCTION

The low level laser therapy (LLLT) is used in medical and dental treatments due to its therapeutic action on different tissues. The use of lasers in biomodulation of bone repair has been studied in bone defects [1], associated with biomaterials [2,3] or with bone morphogenetic protein (BMP) [4]. Some authors affirm that LLLT can accelerate bone formation by increasing osteoblast activity [5,6,7], vascularization [8], organization of collagen fibers [9], and ATP levels [10], accelerating bone repair [1,11,12]. However, in addition to local effects, other important question is if LLLT to localized area exposes other distant tissues to some radiation dose. These possible systemic effects from LLLT has been reported[13,14,15,16].

The reported systemic effect of laser was observed in soft tissue healing [13,15,16]. Based on these data, most of the studies evaluating LLLT on bone healing, used different animals for experimental and control groups because of the possibility of systemic effects [2,8,17,18,19,20,21]. Some authors have suggested that systemic effects may explain the absence of laser biomodulator effects in studies that used the same animal as experimental and control subject [13,15]. However there are studies that used internal control and also had positive results of LLLT on bone [1,11] and cutaneous healing [22]. Considering these conflicting results, the aim of this study was to investigate the systemic effect of low level laser therapy (λ = 830 nm) on bone healing distant to the irradiation site.

MATERIAL AND METHODS

Animals

Forty five male healthy *Wistar* rats, weighing 300 to 400 grams, were randomly selected and distributed into three groups of fifteen animals (Table 1): Control Group (GI), Systemic LLLT Group (GII) and Local and Systemic LLLT Group (GIII). Animals were maintained under light-dark period of 12h and controlled temperature conditions (22±2°C), with balanced diet and water drinking *ad libitum*. This study was approved by Science and Ethics Committee from Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Brazil (Protocol 037/2009).

Surgery

All animals were submitted to osteotomy on left femur for bone defect creation. All of them were anaesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 3 mg/kg xylazin hydrochloride. They were positioned on its right lateral decubitus, and the bone access was achieved through a 2 cm continuous longitudinal incision exposing the mid-diaphysis. A standardized 2.3 mm diameter osteotomy was performed with a round bur under saline solution irrigation. The depth of drilling limit was the disruption of femur cortical bone. Then, soft tissues were repositioned and the suture was performed in muscular and cutaneous layer using nylon 4-0.

Laser protocol

The animals of Group II and III were submitted to sessions of laser therapy. The animals of group II were irradiated on the right femur (systemic dose), and group III in both femurs (local and systemic doses). The equipment used was Gallium-aluminum-arsenide (GaAlAs) infrared laser diode (Flash Lase III - DMC Equipamentos/SãoCarlos – SP – Brazil) with a continuous wavelength

of 830nm, 100mW of potency, and 0.028-cm² beam diameter. The application was punctual, with a 6 J (210J/cm²) dose per session during 2 minutes. The first session was applied immediately after drilling and before soft tissue repositioning in the defect area (local dose). The systemic dose was applied in the sequence in the contralateral femur with the laser tip positioned over and perpendicular to the long axis of the bone. In the postoperative period, applications were taken every 48 hours for 7, 15 and 21 days, resulting in 4, 8 and 11 sessions, according to each subgroup (Table 2.). Animals were euthanatized at 7, 15 and 21 postoperative using saturated potassium chloride associated with general anesthesia.

Histological procedure

The bone defect area and the attached soft tissue were removed and immediately fixed in 10% phosphate buffered formaldehyde solution during 48 hours. Thereafter, the tissue blocks were decalcified in EDTA 10% along 4 weeks, dehydrated with graded alcohols and embedded in paraffin. From the central region of the defect, histological sections of 5µm were obtained and stained in Hematoxiline and Eosine and Mallory Trichrome.

Histomorphometric Analysis

The percentage of bone neoformation was quantified by the same examiner in a blind study. Histological images of the bone defect were captured ×4 magnification, using an Olympus BX 40 binocular microscope (Olympus BX 40 - Shinjuku-ku, Tóquio, Japão) coupled with Olympus OLY 200 camera (OLY 200 - Center Valley, PA - USA). The histological sections of whole bone defect area were digitalized using HL Image 2005 program (Western Vision, Salt Lake

City, UT, USA). The screen shots were merged, areas of soft tissue were erased using Photoshop CS2 software (Adobe®, Adobe System Inc., San Jose, CA/EUA), and finally converted to binary images with HL Image 2005. The region of interest within the bone defect (ROI) was delineated with four straight lines (Figure 1). The percentage of bone formation within the ROI was obtained.

Statistical analysis

Analysis was performed using statistic software (GraphPad Prism version 5.0 for Windows, San Diego, CA, USA). The results were submitted to normality test and analyzed using Analysis of Variance (ANOVA) and Bonferroni. Differences were considered statistically significant if $p<0,05$.

RESULTS

Histological results

Histologic analysis in all groups showed new bone formation in the ROI area extending through medullar until the opposite cortical. The new bone tissue was primary type with trabecular arrangement delimiting small cavities, filled with cells, blood vessels and collagen fibers. After 15 days, it was observed bone remodeling with a decrease of bone neoformation in marrow area, when compared with 7 days period in all groups. After 21 days, it was observed advanced bone remodeling with new bone mostly located in cortical area. The cortical bone defect was almost filled by secondary bone. Little or no bone was observed in marrow area (Figure 2).

The histomorphometric analysis revealed a significant increase in percentage of bone formation in group III in comparison to group II ($p<0.005$)

and control group ($p<0.05$), at 7 days. There was no difference between Group II and Control in this period (Figure 3). At 15 and 21 days, the histomorphometric analysis revealed no significant differences of new bone formation between group I, group II and group III (Figures 4 and 5).

Regarding bone healing in the course of time, histomorphometric analysis showed a significant decrease of bone percentage in all groups when compared 7 and 21 days ($p<0.05$), being more evident in Group III ($p<0.005$). Group III also showed a significant decrease of bone when compared 7 and 15 days analysis ($p<0.005$) (Figure 6).

DISCUSSION

This study evaluated the possible induction of systemic effects of LLLT on bone repair distant to the irradiation site in an animal model. The study of laser therapy should consider aspects such as wavelength and radiation dose. The wavelength of 830nm (used in the present study) penetrates the tissue surface (skin) reaching the underlying bone (femur), thus it is more appropriate for bone related applications as in the present case. The application of 6J ($210J/cm^2$), although considered a high dose, is similar to previous studies that found positive results on bone healing with doses of$178\ J/cm^2$ per session [23]. In our study, the local positive effect of laser therapy was well evidenced in animals with laser applied directly over the bone defect area (Group III) at 7days.

The observed increase in bone formation at this period may be due to local effects of laser stimulating the differentiation of mesenchymal cells and also osteoblasts and fibroblasts proliferation [3]. These events may explain the

extensive bone neoformation, invading the medullar area and extending beyond the defect, which was frequently observed at 7 days in group III. This corroborates with other studies like Gál et al. (2006) [22] that evaluated the effect of laser in wound repair and reported that the most significant morphological changes occurred during the first 7 days of healing. Also, Garavello-Freitas et al. (2003) [24] found maximal laser-stimulated bone growth after 7 days of irradiation.

An interesting fact observed was that the bone remodeling in group III was faster than groups I and II. The greater initial bone formation (7 days) was followed by accelerated bone resorption and remodeling, so no difference was found in the percentage of bone between all the groups at 15 and 21 days. This interpretation is also based on the significantly reduction ($p<0,005$) in the percentage of bone in group III between 7 and 15 days and also between 7 and 21 days. These findings suggest that low-level laser irradiation would stimulate osteoclast activity to promote bone resorption and remodeling. Garavello-Freitas et al. (2003) [24] that evaluated LLLT in rat model, also observed smaller area of trabeculae in the 14 days group compared to the 7-day-irradiated group rats submitted to the same dose of laser.

The present study did not observe systemic effect of low level laser therapy in bone healing, considering that laser application distant from the defect did not interfere on bone wound healing. The assessment of the systemic effect on bone tissue is not well described in literature because most studies only assessed this effect in the healing of soft tissue wounds [13,16] and with conflicting results [13]. Braverman et al. (1989) [13] that evaluated the systemic

effect of laser in cutaneous wound repair in rabbits showed significant effect only on tensile strength evaluation of wounds; however no statistically significant difference was observed in the histological evaluation of samples.

Schindler et al. (2002) [16] reported that the systemic effect of LLLT is due to the release of cytokines and growth factors into the systemic blood stream, causing vasodilation and neoangiogenesis. In our current study, the laser application point was the contralateral leg and possibly, the release cytokines and growth factors did not reach the bone defect in sufficient concentration to interfere on the repair of bone tissue distant to the irradiation site. This leads us to think that spatial proximity of cells or direct cell-to-cell contacts may be crucial for transmitting the signals from irradiated cells to neighboring non-irradiated cells. Another important issue is that the laser scope is wider than the application site, causing what could be called *regional effect*. In fact, many some studies that evaluated the systemic effect of LLLT on soft tissues, used relatively close cutaneous injuries (control and experimental) [13,25] what could interfere in the results.

To date, in bone Coelho et al. (2014) [26] found systemic effects of LLLT, however the authors used different experiment design and laser protocols. Additionally, the implantation of PLLA-PGA screws modifies the evaluated microenvironment. Considering that it is a consensus that different forms of LLLT do not produce the same biological effects, even if all of the components of the irradiated bone are directly affected by laser, they would have different degrees of sensibility, and this makes it difficult to compare the results of different experimental models. Our present work provides relevant data about

the potential efficacy of local LLLT, but not systemic, even with higher doses. However, the reasons for the laser stimulatory effect and also the absence of define parameters to clinical use warrant further investigation.

CONCLUSION

In the present study, LLLT was locally effective at the early stages of bone repair. The results did not demonstrate any changes in bone repair after the application of LLLT a long distance away from the evaluated area.

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Tables

Table 1. Animal distribution in control and experimental groups

Groups	*Animals*	*Description*	*Evaluation periods (n=5)*
I	15	Surgery (control)	7 days , 15 days, 21 days
II	15	Surgery + Systemic laser	7 days , 15 days , 21 days
III	15	Surgery + Local and systemic laser	7 days, 15 days , 21 days

Table 2. Dose of LLLT in each group.

TIME / GROUP	7 DAYS		15 DAYS		21 DAYS	
	Local	Systemic	Local	Systemic	Local	Systemic
GROUP II	0	24J	0	48J	0	66J
TOTAL DOSE	24J		48J		66J	
GROUP III	24J	24J	48J	48J	66J	66J
TOTAL DOSE	48J		96J		132J	

Figures and Legends

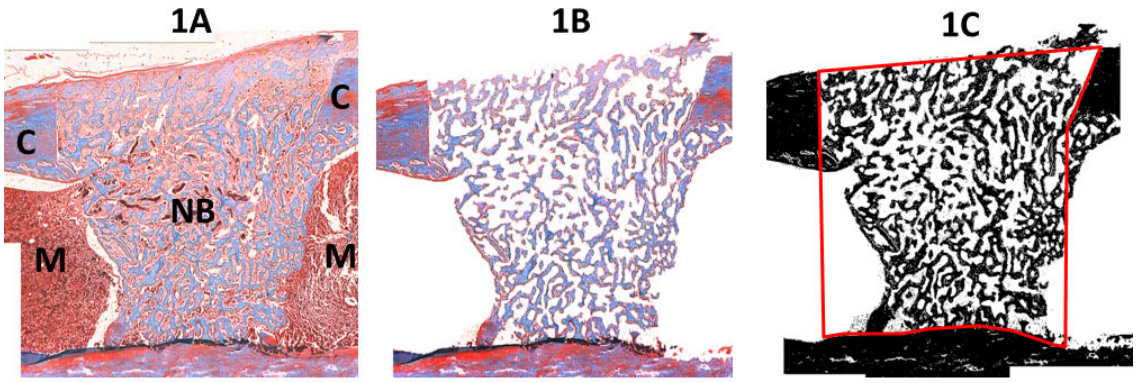

FIGURE 1: A- Photomicrograph of femur longitudinal section showing: cortical (C), marrow (M), area of new bone formation (NB); **B -** Image after the removal of soft tissue; **C -** Image after conversion to binary image and delimitation of bone defect area (red line). Mallory Trichrome, X4 magnification.

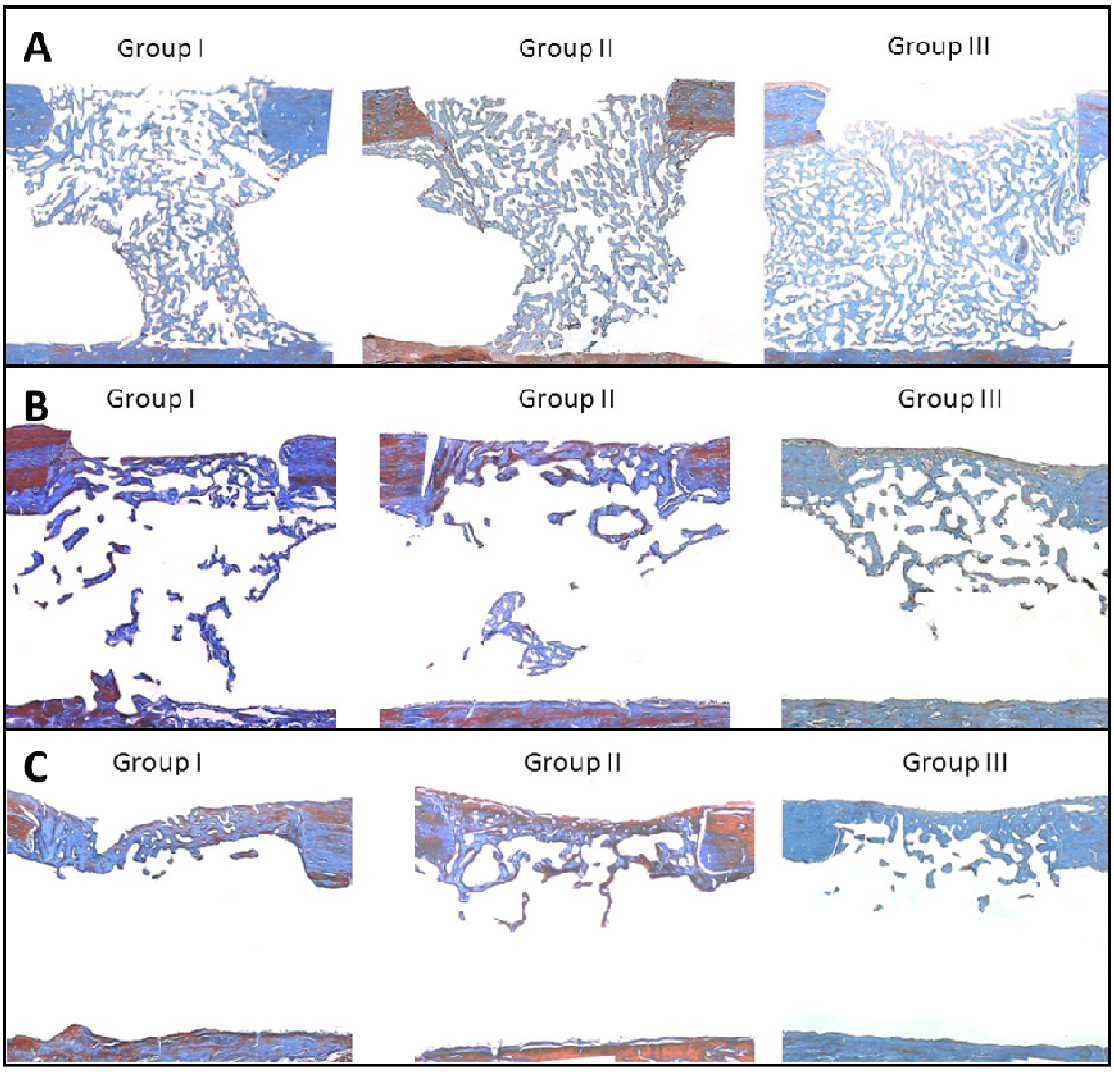

FIGURE 2: Photomicrograph of the evaluated groups at 7 (A), 15 (B) and 21 (C) days.

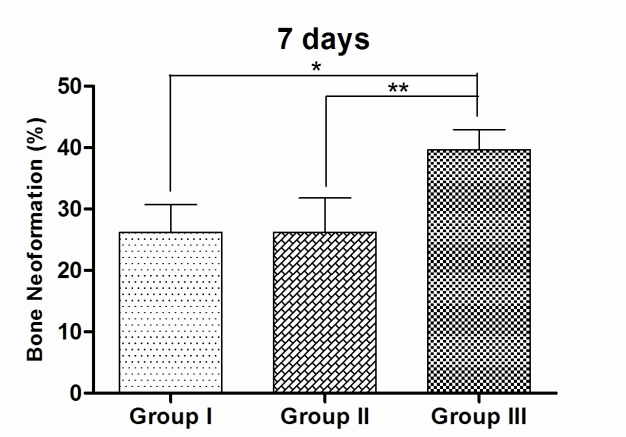

FIGURE 3: New bone formation in the evaluated groups at 7 days (*p<0.05; ** p<0.005).

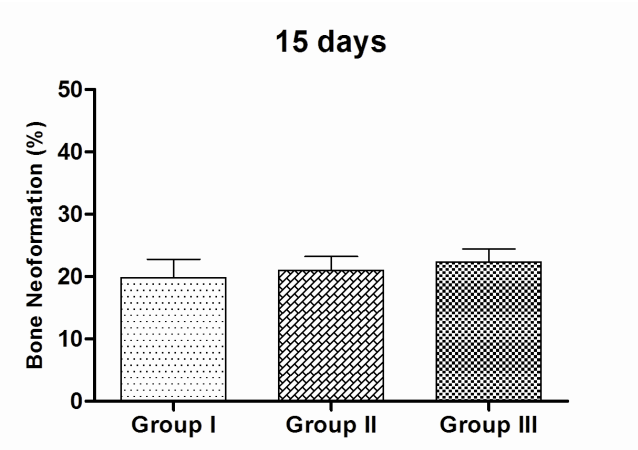

FIGURE 4: New bone formation in the evaluated groups at 15 days (p>0.05).

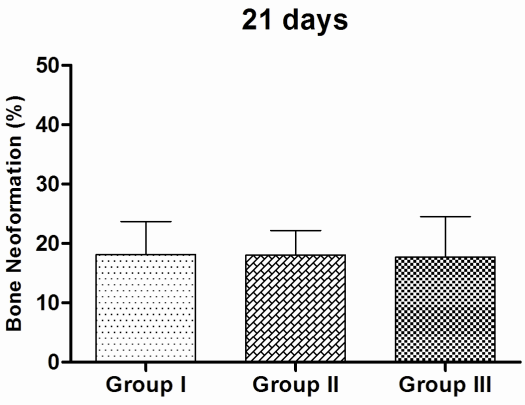

FIGURE 5: New bone formation in the evaluated groups at 21 days (p>0.05).

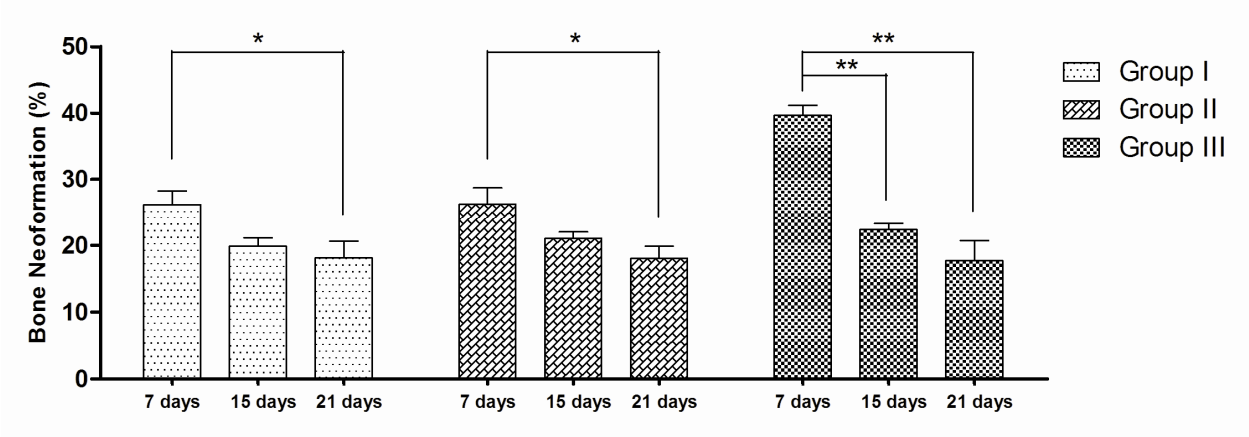

FIGURE 6. Bone formation percentage during evaluation periods (*p<0.05; ** p<0.001).

CAPÍTULO 4 – 4º ARTIGO

Influence of Hyperbaric Oxygen on the initial stages of bone healing.

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ABSTRACT

The objective of this study was to evaluate the effect of HBO on the healing of experimental bone defects at the first 7 days. Forty male rats were used, divided into two equal groups according to the treatment and time of sacrifice: Group Control: creation of bone defects (BD); or Group HBO: with BD and HBO. HBO sessions were realized daily, at 2.5 ATA for 90 min and the animals were euthanized after 1, 3, 5 or 7 days. Bone density, bone neorformation and expression of Runx2 and TRAP were evaluated. Computed Tomographic analysis revealed significant differences only at 3 days ($p=0.01$) between control and HBO groups. HBO treatment accelerated the initial events of bone repair, resulting in improved bone neoformation. It was observed increased expression of Runx-2 especially at 5 and 7 days in HBO group, although not significantly. There was no significant difference ($p=0.09$) in the number of TRAP-positive osteoclasts between control and HBO groups at 7 days. These results suggest that exposure to HBO enhances bone anabolism, reduce inflammation and accelerates bone healing, with positive results is bone neoformation.

Key-words: Hyperbaric oxygen, bone healing, osteogenesis, Runx2.

Introduction

Hyperbaric oxygen (HBO) is a treatment in which 100% oxygen is delivered to a patient at greater than the normal atmospheric pressure at sea level. The mechanism of HBO action is that it increases the amount of oxygen dissolved in the blood (oxygen tension), which in turn can increase the amount of oxygen delivered to hypoxic sites [1]. This may stimulate cellular proliferation and collagen synthesis with positive effects on healing [2,3].

HBO therapy has already been successfully used to improve bone healing [4, 5, 6] because it also stimulates angiogenesis and osteogenesis [4, 7].The molecular mechanisms of HBO on bone formation may be mediated via increased osteogenic differentiation, which is related to Runx2 production [8]. Runx2 triggers the expression of major bone matrix protein genes; including osteopontin and osteocalcin genes at an early stage of osteoblast differentiation and its actions sustain a supply of preosteoblasts [8]. Although HBO is considered a valuable adjunct on the healing of bone defects [4, 5, 6], the current knowledge about its influence on initial stages of bone healing is limited. Therefore, the aim of this study was to evaluate the effect of HBO on the healing of experimental bone defects at the first 7 days.

Materials and Methods

Animals and Experimental groups

The sample consisted of 40 healthy male *Wistar* rats (*Rattus norvergicus*), weighing 250 to 300g. The animals were kept in cages under light-dark period of 12h and controlled temperature conditions (22±2°C), with standard food and water *ad libitum.* This study was approved by the Science

and Ethics Committee from Federal University of Uberlândia, Brazil, Protocol 028/12, and was performed in accordance with the Brazilian College for Animal Experimentation (COBEA). Each group was composed of five animals randomly divided into groups, as described in Table 1.

Surgery and Hyperbaric Oxygen

The animals received a cephalosporin antibiotic prophylaxis (30mg/kg, IP) and subject to general anesthesia. After shaving and antisepsis, with the animal positioned in right lateral decubitus, the right femur was exposed through a 2cm longitudinal incision. Then, a full-thickness cortical bone osteotomy was made with round bur, creating a 2.3mm bone defect. During the procedure, there was constant irrigation with saline solution and the suture was performed using nylon 4.0. Treatment with HBO was realized in a cylindrical pressure chamber Ecobar 400 (Ecotec Equipamentos e Sistemas Ltda®, Mogi das Cruzes, SP, Brasil) at 2.5 ATA for 90 min. HBO sessions started immediately after surgical procedure and was performed daily according to the subgroup which belong each animal. After 1, 3, 5 or 7 days, the animals were euthanized. The femurs were removed, fixed in 4% paraformaldehyde solution during 48h and maintained in phosphate buffered solution.

Computed Tomographic (CT) Analysis

The specimens were positioned in a standard device and scanned in a Cone-Beam 3D scanner (Gendex, GX-CB500-ICAT) at 7mA, 120kvp and 0,125 voxel of resolution. From each specimen, 3 tomographic images from the central region of the bone defect were selected. On these images, for Image-specific calibration it was selected one rectangle mark of $1mm^2$ (at a distance of

3mm from the defect) of the bone medullar region and one from the bone cortical region. After calibration, the region of interest (ROI) within the bone defect was delineated with a rectangle mark from the defect edges on cortical bone until the inner surface of opposite cortex (Figure 1C). The Hounsfield scale within these regions was obtained using specific software (i-CAT ® Vision, Imaging Sciences International, Penn Road, Hatfield, PA).

Histomorphometric Analysis

After CT analysis, the specimens were decalcified in 10% EDTA, dehydrated with graded ethanol and embedded in paraffin. The longitudinal 5µm histological sections obtained were stained with Hematoxiline and Eosine for morphological analysis and Mallory Trichrome for histomorphometric analysis. Mallory histological images of bone defect were captured at ×4 magnification, using an Olympus BX 40 binocular microscope Nikon Eclipse E2000 (Nikon®, Nikon do Brasil, São Paulo, Brasil) coupled with Moticam Pro 252B (Motic®, British Columbia, Canada) and the software Motic Live Imaging Module (Motic®, British Columbia, Canada). The screen shots were merged and the soft tissue erased using Photoshop CS2 software (Adobe®, Adobe System Inc., San Jose, CA/EUA), and finally converted to binary images with HL Image 2005++ (Western Vision, Salt Lake City, UT, USA). The region of interest (ROI) within the bone defect was delineated with four straight lines from the defect edges on cortical bone until the inner surface of opposite cortex. The percentage of bone neoformation within the ROI was calculated with the measure tool of HL Image 2005, as previously described by Batista et al. (2014) [9]. Also, semi quantitative analysis included the evaluation of the following

parameters: 1- Presence of bone neoformation reaching the opposite cortical bone; 2- Presence of bone neoformation extending beyond the ROI.

Runx-2 Immunohistochemistry

After deparaffination in xylene and rehydration in graded ethanol solutions, sections of 5 µm were pre-treated with 10% ammonium hydroxide for 10 minutes to remove formolic pigment and antigen retrieval was performed with citrate buffer for 30 minutes at 90℃. The material was incubated in 30% hydrogen peroxide with methanol (1:1 proportion) for 2 cycles of 15 min for inactivation of endogenous peroxidase and then permeabilized with Triton for 5 minutes. The sections were blocked with 5% Bovine serum albumin in PBS solution for 5 min. The specimens were then incubated overnight at 4 ℃ with anti-Runx2 monoclonal primary antibody at 1:100 dilution (Invitrogen, CA, USA). The sections were washed twice with PBS followed by the application of preformed universal immune-alkaline-phosphstase polymer (Histofine, Nichirei Biosciences, Tokyo, Japan) for 30 min. The bound complexes were visualized by the application of DAB solution (DAKO, Carpinteira, California, EUA) and counterstained with Harris Hematoxylin. Positively stained cells in ROI area were evaluated by two blinded examiners and semiquantitative analysis was determined as described by Detre et al. (1995) [10]. Briefly, the Quickscore index was obtained by multiplying the staining intensity by the frequency of reactive cells in a predetermined score (Table 2).

TRAP Staining at 7 days

The sections were stained for tartrate-resistant acid phosphatase (TRAP) activities using a commercially available Acid Phosphatase, Leukocyte

(TRAP) Kit (Sigma-Aldrich, St. Louis, USA) according to manufactures instructions. The ROI was evaluated by two blinded examiners and TRAP-positive multinucleated cells attached to the bone were identified as osteoclasts.

Statistical analysis

Analysis was performed using statistic software (GraphPad Prism version 5.0 for Windows, San Diego, CA, USA). The normality of all variables distribution was verified using D'Agostinho & Pearson test. CT, percentage of bone neoformation and TRAP staining results were analyzed using Unpaired t test. Fisher exact test was performed to compare the categorical parameters and Chi-square to compare Runx2 Quickscore. Differences were considered statistically significant if $p<0.05$.

Results

Computed Tomographic analysis of the ROI reveled significant differences only at 3 days ($p=0.01$) between control (-102.40 ± 32.76) and HBO (-68 ± 18.15) groups. (Figure 1).

In Control Group at day 1, histological analysis demonstrated the region of the defect totally filled with blood clot, with few inflammatory cells and discrete fibrin mesh. At day 3, blood clot was still predominant, with increased inflammatory infiltrate surrounding the clot, increased exudate, and initial fibrin mesh organization. There were few areas with initial granulation tissue at blood clot periphery. At day 5, the blood clot is reduced and granulation tissue becomes predominant. At day 7, the defect region is filled with primary bone tissue delimitating small cavities with new vessels, with few areas of granulation tissue (Figure 2).

In Experimental Group at day 1, histological analysis also demonstrated the region of the defect filled with blood clot; however, intense inflammatory infiltrate surrounding and invading the clot was evident. It was observed a fibrin mesh and few exudate spaces. At day 3, the blood clot was highly reduced and the inflammatory infiltrate was intense, extending laterally, beyond the defect area, into the medullar channel. Granulation tissue is already seen in the blood clot periphery. At day 5, it was observed bone neoformation filling the defect area, and also extending into the medullar channel. The primary bone tissue presented osteoblasts with marked cytoplasmic basophilia indicating intense protein synthesis activity and many osteocytes recently enclosed in the new bone matrix. Among the new bone trabeculas, in the defect area was observed the development of blood vessels. Organized granulation tissue formation, with many fusiform cells was also seen. At day 7, the primary bone tissue totally filled the defect area and extended largely into the medullar channel. At this period, the bone trabeculas were well defined, and delimitated small cavities with medullar tissue (Figure 2).

The histomophometric analysis showed, there was no bone neoformation at 1 and 3 days in Control and HBO groups. Statistical analysis showed a significant increase in percentage of bone neoformation in HBO (15.95 ± 7.68) group when compared with Control (7.06 ± 3.71) at 5 days ($p=0.005$). There was no significant difference in the percentage of bone neoformation between the control (23.10 ± 7.4) and HBO (28.10 ± 5.4) groups at 7 days ($p=0.10$) (Figure 3). The bone neoformation reaching the opposite cortical bone was significantly more evident in HBO than Control group at 5

days (Figure 4). The bone neoformation extending beyond the limits of the ROI was usually more frequent in HBO than in control groups at 5 and 7 days, although such distribution was not significant.

After immunohistochemical analysis, higher percentage of labeled cells (osteoblasts, osteocytes and osteoprogenitor cells) was observed in HBO groups. It was observed increased expression of Runx-2 especially at 5 and 7 days in HBO group, although not significantly (Figure 5A-B). Both Groups presented TRAP-positive osteoclasts but this amount was low. There was no significant difference ($p=0.09$) in the number of TRAP-positive osteoclasts between control (3.20 ± 1.64) and HBO (5.0 ± 0.80) groups at 7 days (Figure 5C-D).

Discussion

Although HBO is an established modality in the treatment of many disorders and has been successfully used in clinical conditions related to ischemia and/or hypoxia [4, 5, 6], there is still no consensus regarding the protocol for the use of HBO. This diversity occurs because often the choice of HBO protocols is performed considering the experience of the authors, since there are no universally accepted parameters. Unfortunately, the wide variation of HBO protocols, both in humans and in animals, limits full understanding of the biological effects and mechanism of HBO in tissue repair. Whereas bone healing is a complex process, this study proposes to evaluate the effect of HBO in the early stages of bone repair, contributing to the better understanding of the mechanism of action of this therapy.

In all groups, bone repair occurred as intended, but always with a tendency of better results in the experimental groups. HBO treatment clearly accelerated the histological processes underlying the initial events of bone repair. Animal studies have shown that HBO contributes to dissociation of acute inflammation [11, 12], controls vascular permeability, decreasing tissue edema, and reducing inflammatory damage from tissue ischemia [12]. Considering that persistent inflammation inhibits tissue formation, the rapid resolution of the initial inflammation enhanced by HBO might therefore be beneficial for osteogenesis, as observed in the present study. Corroborating with our results, Kawada et al. (2013) [6] conducted a study to evaluate the effect of HBO on fracture healing in mice and the results indicate that HBO enhances bone anabolism and accelerates fracture healing.

Additionally, the molecular mechanisms of HBO action might be of particular importance. Although this was not evaluated, HBO treatment results in increased vascular endothelial growth factor expression [7, 13] and acts as a direct modulator of fibroblast proliferation [13], which are essential for tissue repair. It also elevates alkaline phosphatase activity, a marker of bone formation [14] and stimulates differentiation of osteogenic cells by up-regulating Runx2 expression [8].

Our study indicates that HBO stimulates initial cell proliferation and directly enhances osteogenic differentiation of osteoblasts because of the increased expression of Runx2 in experimental groups, although not significantly. This protein is essential for osteoblastic differentiation and skeletal morphogenesis [8]. It is already demonstrated that bone formation is totally

absent in Runx2-null mice [15] as such, its high levels is related to the presence of bone forming cells [8]. In fact, the periods of 5 and 7 days of healing are characterized for active osteogenesis, with increased osteoblast activity and the deposition of bone matrix. Wu et al. (2007) [3] already demonstrated that HBO enhanced differentiation of osteoblasts, with increased bone nodule formation, calcium deposition, and alkaline phosphatase activity indicating that HBO therapy enhances regenerative bone formation.

However, it important to emphasize even with evident acceleration of bone repair after HBO treatment, the lack of statistical difference in histomorphometric analysis at 7 days may be due to the size of the defect in our study. Despite the defect diameter is compatible with the size of the femur of the rat, it continues to be relatively small, favoring a rapid bone healing in both groups. To our knowledge, only two studies have investigated the ability of HBO to promote bony healing of critical sized defects, demonstrating that HBO enabled the healing of critical- and supra critical–sized calvarial defects in rabbits [4, 16].

At 7 days, the formation of immature bone takes plane in the ROI. During these periods, formative events are predominant with intense deposition of bone matrix by osteoblasts. Thus, the number of osteoclasts observed was small as expected. Probably, during the advanced stages of bone repair, when the remodeling process becomes driven by a coupled process of orderly bone resorption followed by the formation of lamellar bone, osteoclasts would be more frequently seen [17].

In this study, the CT findings demonstrated a greater radiodensity only at 3 days in the HBO group. Despite the CT scans resolution limitation that may contribute to degradation of the grayscale fidelity of the final displayed image [18] it still provided important information about density in our study [18]. The results demonstrated greater radiodensity in HBO group at 3 days indicating an early granulation tissue organization [19]. HBO can raise the normal tissue oxygen level, stimulate tissue growth in wound healing, and enhance granulation tissue formation as already demonstrated [19].

However, CT did not show any significant differences in bone density in the ROI between the HBO and non-HBO groups at 5 and 7 days. This could be related to the limited resolution of CT scans. In general, smaller irradiated volumes are less prone to inaccurate CT numbers/Hounsfield Units, caused by scattered radiation and by non-ideal geometry, however, even with appropriate image acquisition, cone beam tomography has limitations when evaluation bone density [18]. It is interesting to note that only at 7 days the HU reach higher density values when compared to the other groups suggesting that only at this period, the newly formed tissue present more expressive mineralization. In fact, must enter the late stage of osteogenesis to become able to deposit calcium to mineralized bone [20].

In conclusion, this study indicates a mechanism for improved wound healing with HBO that includes controlling vascular permeability, decreasing tissue edema, and reducing inflammatory damage with positive results is bone neoformation. Therefore, HBO might be valuable for bone regeneration.

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Conflict of Interest

The authors declare no conflict of interest.

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Tables

Table 1 – Animal distribution according to the Groups.

Groups	Day 1	Day 3	Day 5	Day 7
Control Group (only bone defect)	n=5	n=5	n=5	n=5
HBO Group (bone defect and HBO)	n=5	n=5	n=5	n=5

Table 2 – Method of determination of the Quickscore index.

Score / Parameter	0	1	2	3	4	5	6
Proportion of positively stained cells in ROI area (PSC)	-	0-4%	5-19%	20-39%	40-59%	60-79%	80-100%
Staining intensity (SI)	-	weak	moderate	high			

* The Quickscore index is obtained by multiplying PSC by SI.

Figures and Legends

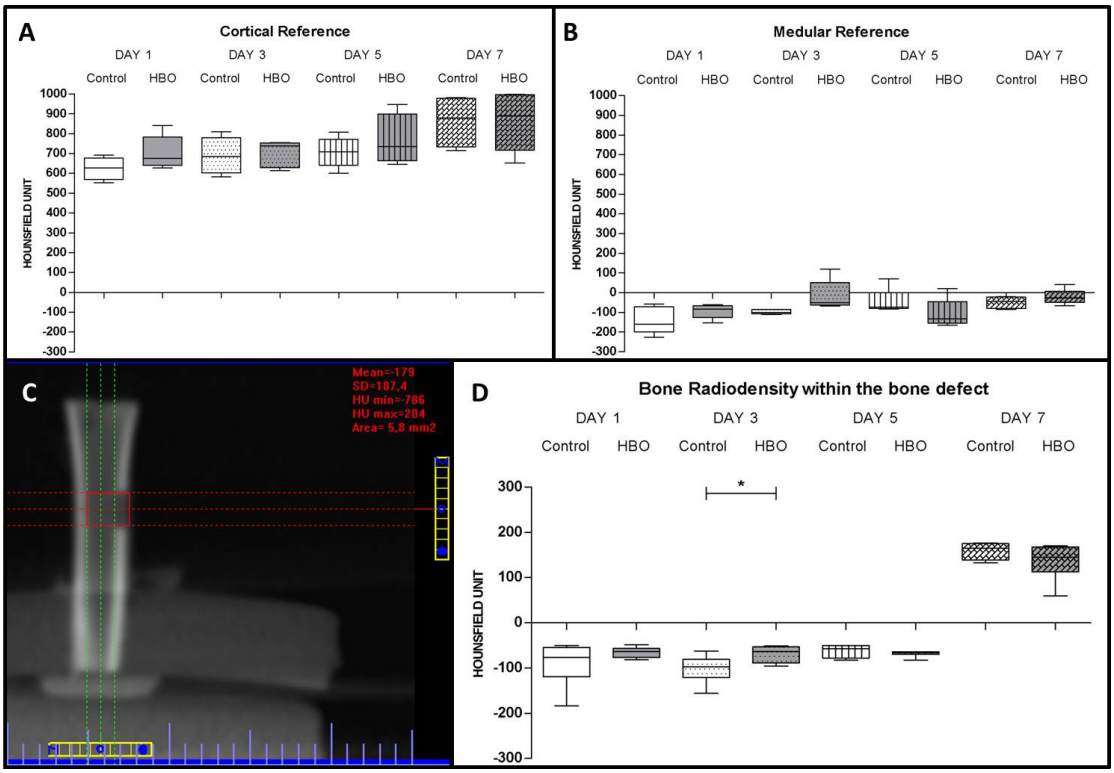

Figure 1: Bone radiodensity references in cortical (A) and medullar (B) areas. CT Image demonstrating the ROI delineation (C). Bone radiodensity in ROI in all evaluated groups (D).

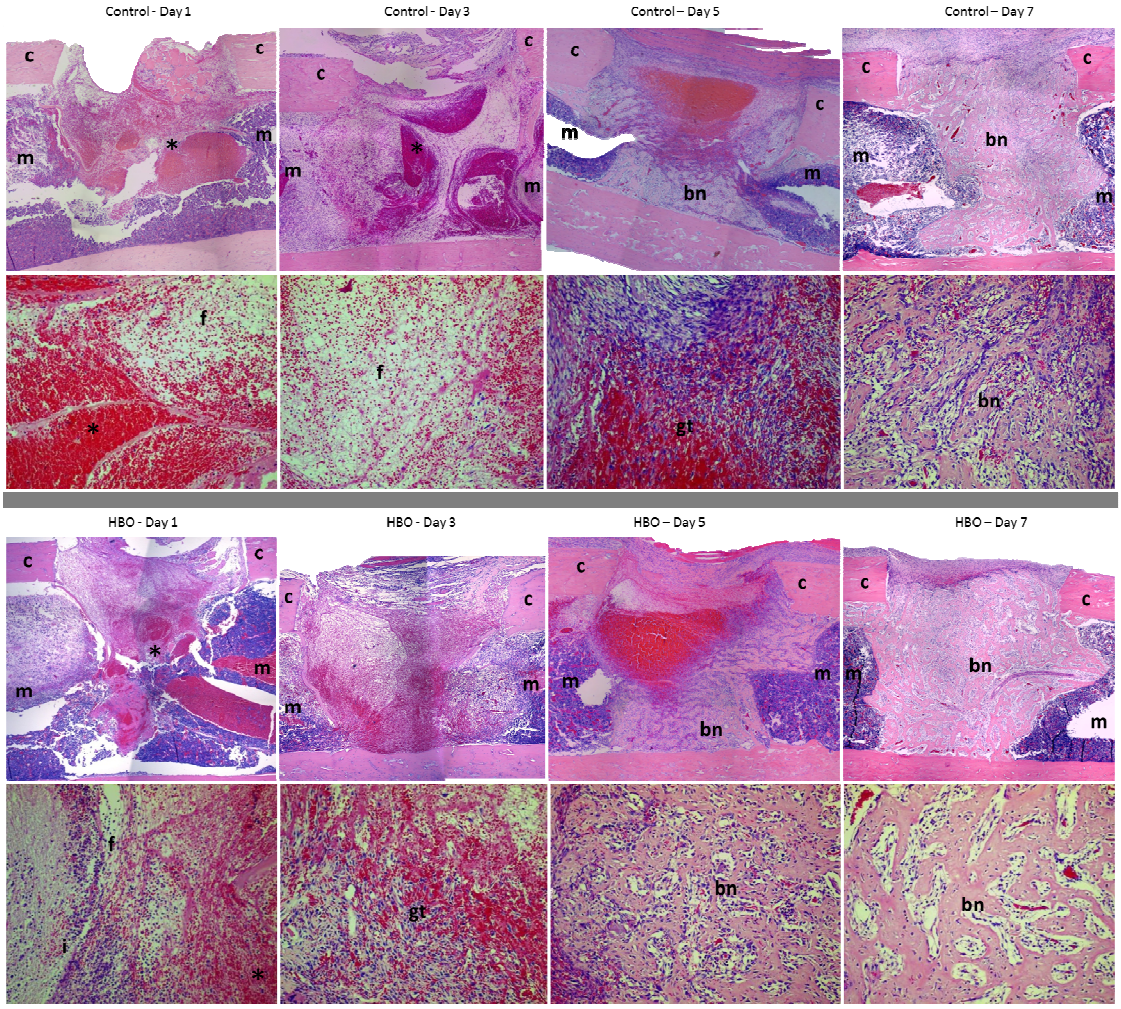

Figure 2: Photomicrograph of the bone defect showing evaluated of control and HBO groups at different experimental periods. Bone cortical (c); Medullar channel (m); Blood clot (*); Granulation tissue (gt); Fibrin mesh (f); Inflamatory infiltrate (i); Bone neofomation (bn). HE, X4 and X10.

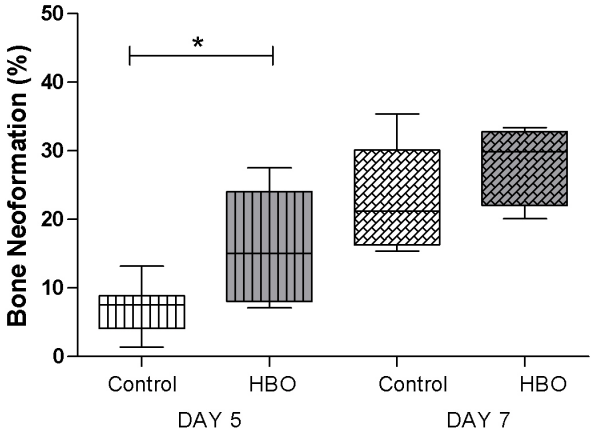

Figure 3: Porcentage of bone neoformation in Control and HBO Groups at 5 and 7 days (*p<0.05).

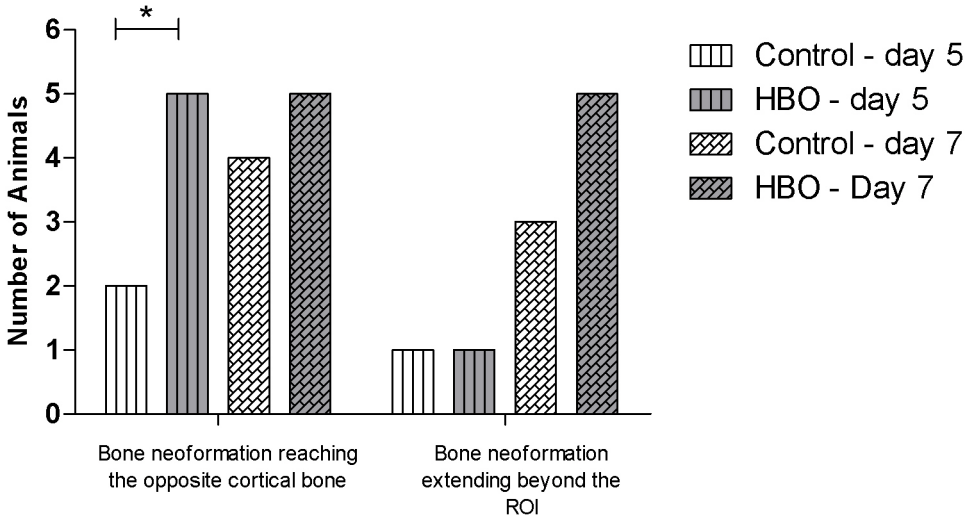

Figure 4: Histological parameters analyzed: Bone neoformation reaching the opposite cortical bone and Bone neoformation extending laterally beyond the ROI (*p<0.05).

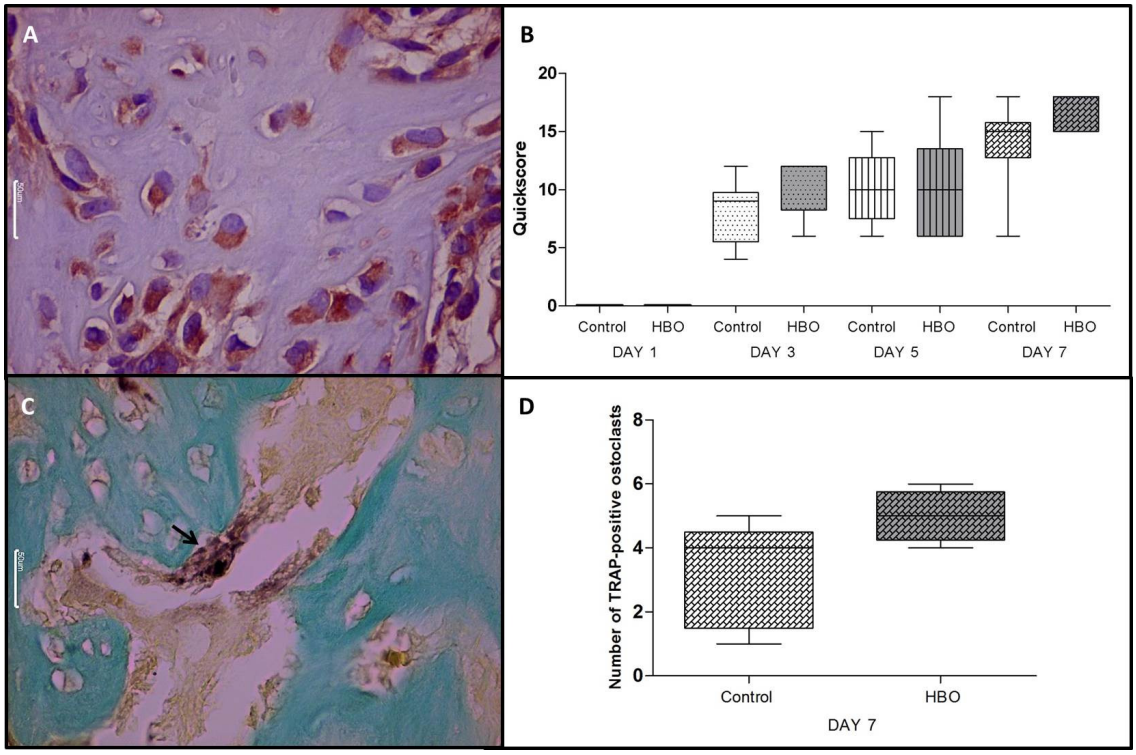

Figure 5: A- Runx2 expression (brown staining) on osteogenic cells. B- Quickscore of Run-2 in all evaluated groups ($p>0.05$). C- Black arrow indicates positive TRAP-positive osteoclasts. D- TRAP-positive osteoclasts in Control and HBO Groups at 7 days ($p>0.05$).

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ANEXOS

Universidade Federal de Uberlândia

– Comissão de Ética na Utilização de Animais –

Comissão de Ética na Utilização de Animais CEUA

CERTIFICADO

Certificamos que o protocolo para uso de animais em experimentação nº 028/12, sobre o projeto de pesquisa intitulado "Efeito da laserterapia e oxigenoterapia hiperbárica no reparo, microestrutura e resistência biomecânica do osso submetido à radiação ionizante.", sob a responsabilidade da **Profa. Dra. Paula Dechichi,** está de acordo com os princípios éticos na experimentação animal conforme regulamentações do Conselho Nacional de Controle e Experimentação Animal (CONCEA) e foi **APROVADO** pela Comissão de Ética na Utilização de Animais (CEUA) – UFU em reunião de **29 de Maio de 2012.**

(We certify that the protocol nº 028/12, about "Effect of laserthe rapy and hyperbaric oxygenotherapy in bone repair, microstructure and resistance after ionizing radiation", agrees with the ETHICAL PRINCIPLES ON ANIMAL RESEARCH as regulations of National Advice of Control and Animal Experimentation (CONCEA) and approved by Ethics Commission on Use of Animals (CEUA) – Federal University of Uberlândia in 29/05/2012).

Uberlândia, 04 de Junho de 2012.

Universidade Federal de Uberlândia

Profa. Dra. Ana Elizabeth Iannini Custódio
Vice Coordenadora *Pro Tempore* da Comissão de Ética
Na utilização de animais