PEDRO HENRIQUE JUSTINO OLIVEIRA LIMIRIO

Efeito da oxigenação hiperbárica e do laser de baixa potência no reparo e biomecânica em osso de ratos com diabetes mellitus tipo I

Effect of hyperbaric oxygen and low-level laser therapy on bone repair and biomechanics in type I diabetes mellitus rats

Tese apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito parcial para obtenção do Título de Doutor em Odontologia na Área de Clínica Odontológica Integrada.

UBERLÂNDIA, 2018

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Orientadora: Profa. Dra. Paula Dechichi, UFU

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PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

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

Defesa de: Tese de Doutorado nº 025- COPOD
Data: 12/03/2018
Discente: **Pedro Henrique Justino Oliveira Limírio (11613ODO004)**
Título do Trabalho: Efeito da oxigenação hiperbárica e do laser de baixa potência no reparo e biomecânica em osso de ratos com diabetes mellitus tipo I
Área de concentração: Clínica Odontológica Integrada.
Linha de pesquisa: Processo de Reparo
Projeto de Pesquisa de vinculação: Processo de Reparo

As **oito horas** do dia **doze de março de 2018** 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 janeiro de 2017, assim composta: Professores Doutores: Robinson Sabino da Silva (UFU); Camilla Christian Gomes Moura (UFU); Guilherme José Pimentel Lopes de Oliveira (UNESP); Mirna Scalon Cordeiro (Faculdade Pitágoras); (UFU) orientador(a) do(a) candidato(a) **Paula Dechichi.**

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 (as), que passaram a argüir o(a) candidato(a). Finalizada 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 12 horas e 30 minutos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

Profª Drª Mirna Scalon Cordeiro – Faculdade Pitágoras

Prof. Guilherme José Pimentel Lopes de Oliveira - UNESP

Prof . Dr.Robinson Sabino da Silva - UFU

Profª. Dra. Camilla Christian Gomes Moura– UFU

Profª Drª Paula Dechichi – UFU
Orientador(a)

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SUMÁRIO

RESUMO

A hiperglicemia crônica decorrente do diabetes mellitus tipo 1 afeta múltiplos órgãos interferindo na qualidade de vida dos portadores da doença. No osso, esse quadro altera o metabolismo ósseo, comprometendo o reparo e as propriedades biomecânicas ósseas. Terapias coadjuvantes como laserterapia (LT) e oxigenação hiperbárica (OH) têm sido propostas para favorecer o reparo ósseo ou melhorar as condições teciduais. O objetivo geral deste estudo foi avaliar o efeito da oxigenação hiperbárica e do laser de baixa potência no reparo, na microarquitetura e biomecânica em osso de ratos com diabetes mellitus tipo I (DMTI). Foram utilizadas análises por meio de micro-tomografia computadorizada (MicroCT), avaliação histológica qualitativa e histomorfométrica, análise biomecânica e espectroscopia no infravermelho transformada de Fourier (FTIR). Os resultados mostraram que o diabetes comprometeu significativamente o reparo ósseo, com redução de neoformação, número de trabéculas e volume ósseo. O DMTI também alterou os compostos orgânicos e inorgânicos da matriz e as propriedades mecânicas ósseas (força máxima, energia e rigidez). As terapias utilizadas, OH e LT reduziram os efeitos deletérios do diabetes, principalmente, os relativos à neoformação óssea, propriedades mecânicas e razão entre os componentes orgânico e inorgânico. Conclui-se que o diabetes mellitus tipo 1 compromete o reparo e as propriedades biomecânicas ósseas, e a oxigenação hiperbárica e a laserterapia de baixa potência reduzem os efeitos deletérios do diabetes.

Palavras chaves: Diabetes mellitus tipo 1; Oxigenação Hiperbárica; Laser de baixa potência; Osso e ossos; Biomecânica; Regeneração Óssea

ABSTRACT

Chronic hyperglycemia due to type 1 diabetes mellitus affects multiple organs interfering in the quality of patients' life with the disease. In bone, this condition changes bone metabolism, compromising regeneration and bone biomechanical properties. Supporting therapies, such as Low-Level Light (LLLT) and hyperbaric oxygen therapy (HBO) have been proposed to promote bone regeneration or improve tissue conditions. The aim of the present study was to evaluate the effect of LLLT and HBO on microarchitecture and biomechanics bone of rats with type 1 diabetes mellitus (T1DM). Analyzes were performed using computerized micro-tomography (MicroCT), histological and histomorphometric, biomechanical and Fourier transform infrared spectroscopy (FTIR). The results showed that diabetes significantly compromised the bone regeneration, with reduction bone neoformation, trabeculae number and bone volume. T1DM also change the organic and inorganic matrix compounds and mechanical bone properties (maximum strength, energy and stiffness). The therapies used, LLLT and HBO reduced the deleterious effects of diabetes, especially those related to bone neoformation, mechanical properties and ratio between organic and inorganic material. It is concluded that type 1 diabetes mellitus compromises bone regeneration and biomechanical properties; and LLLT and HBO reduce the deleterious effects of T1DM.

Keywords: Diabetes mellitus, type 1; Hyperbaric oxygenation; Low-Level Light Therapy; Bone and bones; Biomechanics; Bone Regeneration

INTRODUÇÃO E REFERENCIAL TEÓRICO

1 - INTRODUÇÃO E REFERENCIAL TEÓRICO

Diabetes mellitus tipo 1 (DMTI) corresponde ao grupo heterogêneo de doenças caracterizadas pela destruição autoimune das células β produtoras de insulina do pâncreas, normalmente, levando à deficiência absoluta de insulina (Xie *et al.*, 2014). Geralmente, ocorre como consequência de quebra na regulação imune, resultando em expansão das células T auto-reativas CD41 e CD81, linfócitos B e ativação do sistema imune inato, que colabora para destruir células β produtoras de insulina (Bluestone *et al.*, 2010). A falta de insulina provoca superprodução de glicose e diminuição da absorção celular de glicose, resultando em hiperglicemia (Kelly *et al.*, 2003). As complicações tardias da doença afetam principalmente vasos sanguíneos, nervos, rins, olhos e o tecido ósseo (Xie *et al.*, 2014).

Estudos clínicos têm associado os efeitos do DMTI aos ossos, indicando osteopenia, redução da densidade óssea, atraso na consolidação de fraturas e aumento no tempo de reparo ósseo em diabéticos, quando comparados a pacientes normoglicêmicos (Reddy *et al.*, 2001; Saller *et al.*, 2008). Estudos em ratos e camundongos com DMTI verificaram redução da densidade óssea e do conteúdo mineral, além de diminuição dos níveis séricos de osteocalcina e da resistência biomecânica (Reddy *et al.*, 2001).

O osso é um órgão do corpo humano extremamente complexo, considerado o principal reservatório de íons do organismo, como cálcio, fosfato e sódio, ele ainda armazena a medula óssea em seu interior. Além disso, está relacionado à regulação do metabolismo energético e, quando é realizado o movimento corporal, exerce funções mecânicas junto com a musculatura (Dempster, 2006; Wei e Ducy, 2010). As células do tecido ósseo têm diferentes funções, relacionadas à matriz extracelular mineralizada. Os osteoblastos são responsáveis por produzir a matriz óssea, os osteócitos mantêm a matriz e os osteoclastos reabsorvem a matriz. Apesar de sua aparência inerte, o tecido ósseo é altamente dinâmico, sendo continuamente reabsorvido pelos osteoclastos e neoformado pelos osteoblastos, caracterizando o processo de remodelação óssea (Bonewald, 2011).

Os elementos estruturais da matriz óssea são organizados de forma hierárquica desde o menor ao maior nível da escala de comprimento (Felsenberg e Boonen, 2005).

No menor nível da escala a estrutura da matriz óssea possui basicamente componentes orgânicos e inorgânicos, os quais conferem aos ossos características como flexibilidade e rigidez (Dempster, 2006). A matriz orgânica é composta por uma matriz extracelular flexível e resistente constituída por proteoglicanos, proteínas não colagenosas e, principalmente colágeno tipo I, molécula formada por três cadeias polipeptídicas organizadas em tripla hélice (Dempster, 2006).

No processo de osteogênese, a matriz orgânica incorpora em seu interior minerais, como cálcio e fosfato, que se organizam na forma de cristais de hidroxiapatita (Dempster, 2006). As moléculas de colágeno associam-se no meio extracelular formando as fibrilas, com moléculas de colágeno equidistantes, estabelecendo pequenos espaços, com cerca de 64 ηm, entre as extremidades das moléculas de colágeno (Bailey, 2001). Neste espaço, bem como nos espaços ao longo das moléculas ocorre a precipitação de cristais de hidroxiapatita (Dechichi *et al.*, 2007). A união molecular entre os componentes orgânicos e inorgânicos tem como resultado uma rede de fibrilas colágenas mineralizadas (Currey, 2009).

A conformação estrutural polipeptídica da molécula de colágeno contém principalmente amida primária (I), secundária (II) e terciária (III), compreendendo as ligações cruzadas entre as moléculas de colágeno e a interação entre estas moléculas e os nano cristais de hidroxiapatita (Chang e Tanaka, 2002). Ligações cruzadas enzimáticas maduras e imaturas entre as moléculas de colágeno têm papel fundamental na estabilidade da rede colagenosa (Barth *et al.*, 2011). As ligações cruzadas enzimáticas maduras (Piridinolina) são ligações interfibrilar, ou seja, entre as fibrilas colágenas mineralizadas e as ligações cruzadas imaturas (Dihidroxinorleucina – DHLNL) são ligações intermolecular, ou seja, entre as moléculas de colágeno (Barth *et al.*, 2011).

As ligações cruzadas enzimáticas são responsáveis pela estabilidade das fibrilas que fornecem resistência ao cisalhamento destas proteínas (Bailey, 2001; Eyre *et al.*, 2010). A extensão e o tipo da ligação cruzada variam com a idade do tecido e sua função. Durante o desenvolvimento do indivíduo, o aumento da tensão nas fibras de colágeno provoca aumento do número de ligações cruzadas com objetivo de produzir as

propriedades mecânicas ideais para dada função no organismo. Estas ligações são responsáveis pelas propriedades viscoelásticas no tecido ósseo (Barth *et al.*, 2011).

Por outro lado, ligações cruzadas não enzimáticas, como os produtos finais de glicosilação avançada (AGEs) decorrentes do quadro de hiperglicemia, associados às moléculas de colágeno, podem estabelecer conexões intra e interfibrilar fracas, aumentando a fragilidade óssea (Bailey, 2001). Em relação às células ósseas, alguns estudos mostraram que os osteoblastos no DMT1 no conjunto são submetidos a transdiferenciação, desdiferenciação e morte celular por defeitos na função da célula estromal da medula óssea (Retzepi e Donos, 2010), sendo correlacionada com a redução da densidade mineral óssea e aumento da frequência de apoptose de osteócitos (Tsentidis *et al.*, 2017).

Há evidências de que o nível de controle glicêmico está associado à saúde óssea. Um déficit de osteoblastos é um fator importante para a osteopenia em diabéticos mal compensados. A hiperglicemia prejudica a função dos osteoblastos, além disso, um deficiente controle glicêmico, também afeta a resposta de osteoclastos, prejudicando então, o reparo ósseo (Antonopoulou *et al.*, 2013).

O tecido ósseo quando lesado desencadeia um processo denominado de reparo ou reparação óssea, que consiste em quatro fases principais: 1-remoção do coágulo sanguíneo, de restos celulares e de matriz, pelos macrófagos; 2-periósteo e endósteo, próximo a área, formam um tecido rico em células osteoprogenitoras; 3-surgimento de tecido ósseo primário; 4-remodelação óssea e completa substituição do tecido primário por tecido ósseo secundário (Pinheiro *et al.*, 2009). Nas duas primeiras fases, que ocorrem imediatamente após a lesão, haverá a formação de hematoma, que acarretará em resposta inflamatória (Pinheiro *et al.*, 2009).

Em diabéticos, a fase inflamamatória do reparo ósseo é mais prolongada, o que justifica o retardo no processo de reparo tecidual em diabéticos (Al-Watban e Andres, 2006). Alguns estudos mostraram que DMT1 também tem sido associada ao desequilíbrio entre ativação e inibição de enzimas proteolíticas, inibição parcial de síntese e liberação de fatores de crescimento angiogênicos, redução nas taxas de proliferação celular e aumento de apoptose (Pacios *et al.*, 2012). A hiperglicemia

também pode influenciar a produção de citocinas pró-inflamatórias ou anti-inflamatórias, que afetam a proliferação, migração e diferenciação das células ósseas, em particular os osteoblastos (Wu *et al.*, 2008). Esses fatores comprometem eventos importantes do reparo ósseo como o reestabelecimento da rede vascular e a organização do tecido de granulação, que iniciam a fase reparadora, à qual se segue ossificação intramembranosa ou endocondral (Kanczler e Oreffo, 2008).

Algumas terapias têm sido utilizadas a fim de reduzir os efeitos do DMTI no corpo humano, como a laserterapia de baixa potência (LTBP) e a oxigenação hiperbárica (OH) (Saraiya e Martin, 2015). A OH tem acelerado a reparação de feridas e reduzido as taxas de amputação em diabéticos (Londahl, 2013). Estudos demonstraram que a OH aumenta os níveis de BMP-2, sialoproteína óssea (BSP), osteocalcina (OC) e interleucina 10 (IL-10), diminui os níveis de IL-1 e IL-6, reduz a resposta inflamatória, acelera a osteogênese, além de acelerar o processo de remodelação óssea em fraturas (Al Hadi *et al.*, 2015) e em defeitos críticos (Jan *et al.*, 2009). Pesquisadores também têm relatado a influência da OH na aceleração da síntese de colágeno estruturalmente organizado com consequente regeneração óssea em lesões ou úlceras (Al Hadi *et al.*, 2015).

A OH consiste em saturação de 100% de O_2 enquanto exposta a aumento da pressão atmosférica (Fosen e Thom, 2014). As sessões podem ser realizadas em câmaras individuais, pressurizadas com oxigênio puro, ou ainda em câmaras que comportem várias pessoas (normalmente 2-14 pacientes), onde o paciente respira o oxigênio através de uma máscara facial, headtent ou tubo traqueal (Gill e Bell, 2004; Fosen e Thom, 2014). As pressões aplicadas na câmara são geralmente de 2 a 3 atmosferas absolutas (ATA) e o tempo de tratamento pode se estender entre 1 a 60 ou mais sessões (Jansen *et al.*, 2003), que duram cerca de 1,5-2h cada, podendo ser realizadas uma ou duas vezes ao dia (Fosen e Thom, 2014).

A OH estabiliza e ativa o fator indutor de hipóxia (HIF-1), que desempenha um importante papel no reparo de feridas por meio do aumento da proliferação celular (Sunkari *et al.*, 2015). Também exerce efeito sobre vários tipos de células e influencia tanto a angiogênese quanto a vasculogênese (Fosen e Thom, 2014). Estudos mostram que essa terapia aumenta a produção do fator de crescimento do endotélio vascular

(VEGF) em feridas feitas em modelo animal (Sheikh *et al.*, 2000), promove o aumento da angiogênese, levando a uma melhora na regeneração óssea (Grassmann *et al.*, 2015; Rocha *et al.*, 2015), aumenta a atividade bactericida dos leucócitos (Gill e Bell, 2004) e atua diretamente como modulador da proliferação fibroblástica (Kang *et al.*, 2004). Além disso, inibe a adesão de leucócitos ao endotélio, de forma a diminuir o dano tecidual; aumenta a motilidade dos leucócitos, melhorando a microcirculação e, através da vasoconstrição e mecanismos da homeostase, é também responsável por reduzir o edema (Jansen *et al.*, 2003). Em condições de hipóxia, é capaz de diminuir a infecção e morte celular e manter o tecido viável durante o reparo (Gill e Bell, 2004).

Em diabéticos, a OH reduz a hiperglicemia e a redução do nível de açúcar no sangue foi correlacionado à redução na percentagem de lesão nas células β no pâncreas. Acredita-se que a OH possa ter efeito regenerador nas células β, pois seu efeito é mantido em longo prazo (Prabowo *et al*., 2014). A OH é capaz de neutralizar os efeitos da hiperglicêmia e isquemia, e o atraso no reparo de feridas em animais diabéticos, acelerando significativamente o processo de reparo. Acredita-se que em ambientes de isquemia e hiperglicemia ocorra redução na deposição de colágeno nos estágios iniciais de reparo, e que a OH neutraliza esse efeito (Andre-Levigne *et al.*, 2016).

Além da OH, a terapia com laser de baixa potência tem sido estudada para tratamento de casos complexos. A LTBP foi desenvolvida como um tratamento promissor para a aceleração do metabolismo ósseo (Garcia *et al.*, 2013), sendo proposta para melhorar o processo de reparo ósseo (Kazem Shakouri *et al.*, 2010). Essa terapia, envolve a aplicação de radiação eletromagnética não ionizante, altamente concentrada e não invasiva, que é monocromática, coerente e direcional (Prindeze *et al.*, 2012). O método de fototerapia usando raio laser de baixa potência suporta efetivamente o tratamento convencional e traz uma melhoria significativa na qualidade de vida em pacientes portadores da diabetes (Goralczyk *et al.*, 2016).

O princípio de ação da LTBP é na sua eficácia a nível celular, melhorando processos bioquímicos e moleculares, envolvidos na reparação de tecidos. Os processos estimulados pela LTBP incluem a proliferação celular (Gao e Xing, 2009), a síntese de proteínas e colágenos (Vinck *et al.*, 2003), reparação de feridas (Hawkins *et al.*, 2005), diferenciação de osteoblastos e condrócitos (Arisu *et al.*, 2006), regeneração celular (Al-

Watban e Andres, 2006), remodelação óssea, reparo da função nervosa após lesão, equilíbrio da função hormonal, regulação da imunidade e do sistema linfático, redução da inflamação e do edema e alívio da dor (Agha-Hosseini *et al.*, 2012). Além disso, o LTBP aumenta o fluxo sanguíneo, reforça o processo de revitalização, diminui o risco de infecção, reforça as atividades metabólicas e melhora o reparo dos tecidos lesados (Freddo *et al.*, 2009).

A LTBP no osso pode aumentar a proliferação celular, devido a sua capacidade de estimulação tecidual, e ainda, acelerar a consolidação de fraturas (Renno *et al.*, 2006). Além disso, alguns estudos com LTBP mostram potencial osteogênico (Renno *et al.*, 2006; Bayat *et al.*, 2009) no tecido ósseo de ratos com osteoporose e diabetes (Renno *et al.*, 2006; Bayat *et al.*, 2009). Está bem estabelecido que, o LTBP é capaz de interagir o com tecido ósseo, modulando as reações bioquímicas das células ósseas e estimulando a atividade mitocondrial. As modificações no metabolismo celular levam a maior produção de oxigênio molecular e síntese de trifosfato de adenosina (ATP) (Patrocinio-Silva *et al.*, 2016). Além disso, evidências sugerem que LTBP aumenta a migração e diferenciação de células ósseas para o local da irradiação, culminando no aumento da produção de colágeno e mineralização da matriz extracelular (Karu, 2010; Ginani *et al.*, 2015).

A mineralização óssea também é influenciada pela LTBP: o acúmulo de cálcio pode ser aumentado em 46%, bem como os níveis séricos de fosfatase alcalina (Pinheiro *et al.*, 2009). Essa enzima é liberada pelos osteoblastos durante a osteogénese, aumentando a disponibilidade local de fosfato inorgânico necessário para mineralização óssea (Golub, 2009). Além disso, a LTBP estimula precocemente e por longo prazo a expressão de osteocalcina e osteopontina, duas proteínas associadas à formação de matriz extracelular e ativação de osteoblasto (Sella *et al.*, 2015).

Terapias coadjuvantes que favoreçam o restabelecimento da normalidade da morfologia e função tecidual, reduzam possíveis prejuízos ao processo natural do reparo ósseo, seriam interessantes em diversas situações clínicas. Dessa forma, neste estudo foi avaliada a influência do laser de baixa potência e da oxigenação hiperbárica no reparo e na qualidade do tecido ósseo normal ou comprometido pelo diabetes mellitus tipo 1.

Capítulos

2 CAPÍTULOS

2.1 CAPÍTULO 1

Limirio PHJO, da Rocha Junior HA, Morais RB, Hiraki KRN, Balbi APC, Soares PBF, Dechichi P. Influence of hyperbaric oxygen on biomechanics and structural bone matrix in type 1 diabetes mellitus rats. PloS one. 2018;13(2):e0191694.

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PLOS ONE

RESEARCH ARTICLE

Influence of hyperbaric oxygen on biomechanics and structural bone matrix in type 1 diabetes mellitus rats

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Abstract

Background

The aim of this study was to evaluate the biomechanics and structural bone matrix in diabetic rats subjected to hyperbaric oxygen therapy (HBO).

Methods

Twenty-four male rats were divided into the following groups: Control; Control + HBO; Diabetic, and Diabetic + HBO. Diabetes was induced with streptozotocin (STZ) in the diabetic Groups. After 30 days, HBO was performed every 48h in HBO groups and all animals were euthanized 60 days after diabetic induction. The femur was submitted to a biomechanical (maximum strength, energy-to-failure and stiffness) and Attenuated Total Reflectance Fourier transform infrared (ATR-FTIR) analyses (crosslink ratio, crystallinity index, matrix-to-mineral ratio: Amide I + II/Hydroxyapatite (M:MI) and Amide III + Collagen/HA (M:MIII)).

Results

In biomechanical analysis, diabetic animals showed lower values of maximum strength, energy and stiffness than non-diabetic animals. However, structural strength and stiffness were increased in groups with HBO compared with non-HBO. ATR-FTIR analysis showed decreased collagen maturity in the ratio of crosslink peaks in diabetic compared with the other groups. The bone from the diabetic groups showed decreased crystallinity compared with non-diabetic groups. M:MI showed no statistical difference between groups. However, M:MIII showed an increased matrix mineral ratio in diabetic+HBO and control+HBO compared with control and diabetic groups. Correlations between mechanical and ATR-FTIR analyses showed significant positive correlation between collagen maturity and stiffness.

Competing interests: The authors have declared that no competing interests exist.

Conclusions

Diabetes decreased collagen maturation and the mineral deposition process, thus reducing biomechanical properties. Moreover, the study showed that HBO improved crosslink maturation and increased maximum strength and stiffness in the femur of T1DM animals.

Introduction

Type 1 diabetes mellitus (T1DM) is a metabolic disorder characterized by chronic hyperglycemia that affects various human body systems [1]. Children and adolescents with T1DM are at risk for presenting a decrease in bone mass during the process of bone remodeling. This may minimize the attainment of peak bone mass and increase the fracture risk and/or osteoporosis in adulthood [2]. Some studies have suggested that T1DM negatively changed the collagen in bone matrix and reduced the necessary maximum bone fracture strength [3, 4].

T1DM has been associated with cellular and molecular changes that result in bone matrix alterations [5]. The chronic hyperglycemia has deleterious effects on structural collagen protein and this may change the biomechanical behavior of bone tissue [6]. Studies have revealed that diabetes decreased total collagen content and deteriorated pyridinoline crosslinking in fracture calluses [6, 7]. In addition, T1DM can affect bone formation and resorption, leading to several metabolic irregularities in calcium-phosphate and acid-base balances [8]. Indeed, T1DM exhibits disproportionately high fracture risk with reduced bone mass, which leads to speculation about diabetic bone having reduced maximum strength [9] and stiffness [10]. The deleterious effects of diabetes on bone response to mechanical stimuli have been shown in the literature, so it is important to investigate alternative therapy to improve bone quality [6].

Hyperbaric oxygen therapy (HBO) has been used to treat cases with impairment of repair for decades [11]. It consists of intermittent inhalation of 100% oxygen under a pressure higher than 1.5 atmospheres absolute [12]. Studies have suggested that HBO activates several mechanisms that contribute to repair [13], including increased collagen synthesis [14] and stimulation of the bone repair process [15]. Moreover, some studies have suggested that HBO induces enzymatic crosslinking, which contributes to the bone mineralization process [16]. This procedure stimulates the incorporation of mineral crystals into collagen crosslinks and increases the maximum breaking strength values in rat femurs [17]. However, the HBO effect on diabetic bone in a rat model has not been studied.

In this study, it was hypothesized that HBO would improve bone biomechanical properties (maximum strength, energy and stiffness), and the collagen and crystalline hydroxyapatite content in diabetic femurs in an animal model. Therefore, the aim of the present study was to evaluate T1DM and HBO effect on the rat femur, using biomechanics and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR—FTIR) analyses.

Material and methods

Experimental procedure

This study was approved by the Science and Ethics Committee of the Federal University of Uberlândia (026/14), Brazil, and was conducted in accordance with the Brazilian College for Animal Experimentation (COBEA) guidelines. The sample consisted of 24 male Wistar rats (*Rattus norvegicus*), weighing 240 to 280g (8 weeks of age). The animals were kept in cages, in a 12h:12h light-dark cycle, and controlled temperature conditions (22 ± 2˚C), with standard food and water *ad libitum*. The animals were randomly divided into four groups (n = 6), as

follows: Control; Control + HBO; Diabetic and Diabetic + HBO. The oxygen therapy was performed every 48h and started 30 days after streptozotocin (STZ) (Sigma Aldrich, St Louis, MO, USA) induced T1DM. All animals were euthanized 60 days after diabetic induction.

The T1DM induction protocol began by keeping the rats fasting for 24h. After this time and before T1DM induction, the mean blood glucose level of the animals was 100 mg/dL. Anesthesia was performed via the intraperitoneal pathway using 7mg/Kg xylazine 2% muscle relaxant, and 100mg/Kg ketamine hydrochloride 10% anesthetic and analgesic. Then, a single dose of STZ was administered intravenously through a penile vein puncture at a dose of 65 mg/kg body weight, diluted in citrate buffer. Hyperglycemia was confirmed by a glucometer (Accu Check Active, Roche, Jaguaré, SP, Brazil) after 24 hours; one week; 15 days, and 30 days after induction, by collecting a drop of blood from each animal's tail. Animals that maintained blood glucose levels higher than 200 mg/dL were considered diabetic. The animals that did not reach the glycemic target were excluded from the study.

HBO was performed 30 days after STZ-induction in control+HBO and diabetic+HBO groups, and was repeated every 48h, so the animals received 15 HBO sessions. The treatment 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. The animals were euthanized 60 days after diabetes induction by intraperitoneal injection with sodium thiopental and lidocaine, followed by cervical dislocation, in compliance with the principles of the Universal Declaration on Animal Welfare.

Both femurs were removed by disarticulation and immediately placed in gauze impregnated with physiological saline solution and then kept frozen in a freezer (-20˚C). Twenty-four hours before the mechanical test, the femurs were defrosted and placed in phosphate buffered saline until they were analyzed.

Biomechanical and attenuated total reflectance Fourier Transform Infrared Spectroscopy (ATR—FTIR) analyses

Each femur was analyzed in a three-point bending test until failure, using universal-testing machine (EMIC DL 2000, EMIC Equipamentos e Sistemas de Ensaio Ltda, Sao José dos Pinhais, Brazil). Each specimen was placed horizontally on the two holding fixtures (16 mm) in the machine, the upper device load was applied in middle of the diaphysis at a loading rate of 1.0 mm/min. Load, displacement data were recorded, and subsequently, load vs. displacement curves were plotted. Evaluations were derived from data with maximum strength (N), energy-to-failure (mJ) and stiffness values (N/mm) and calculated as the slope of the initial linear uploading portion of the curves. Femurs fractured after the mechanical test were maintained in phosphate buffered saline until the attenuated total reflectance Fourier transform infrared spectroscopy (ATR—FTIR) analysis.

After the three-point bending test, the proximal diaphysis was sectioned with a diamond disk under constant irrigation to obtain three fragments measuring 2x2 mm, with 2 mm thick (Fig 1). The mean values of three spectrums in each femur were obtained on the external cortical surface. The bone fragment was placed against the diamond crystal of the ATR-FTIR unit and pressed with a force gauge at a constant pressure to facilitate contact. Data were recorded and analyzed with OPUS 6.5 software (Bruker, Ettlingen, Germany). The bone composition was analyzed using Fourier Transform Infrared Spectroscopy (ATR-FTIR, Vertex 70 –Bruker, Ettlingen, Germany) equipped with an accessory that allowed spectrum acquisitions in the Attenuated Reflectance (ATR) mode. The ATR spectrums were recorded in the range of 400–4,000 cm^{-1} at a 4 cm^{-1} resolution. Vector normalization and baseline correction were performed in all spectrums and these were considered absorbance height ratios.

The ATR-FTIR spectrums were further analyzed by calculating the following parameters: Amide I band (Collagen ratio between the mature pyridinoline crosslink peaks (PYR)– 1660 cm^{-1} and immature crosslinking dihydroxynorleucine (DHLNL) - 1690 cm^{-1}); Crystallinity Index (The intensity ratio of peaks 551 and 597 cm^{-1} for 588 cm^{-1}); Matrix-to-mineral ratio: Amide I + II/Hydroxyapatite (HA) (M:MI) (The ratio between integrated areas of amide I + II (1520–1720 cm^{-1}) for HA (916–1180 cm^{-1})) and Amide III + Collagen/HA (M:MIII) (The ratio between integrated areas of amide III (1210–1270 cm^{-1}) with two collagen bands (1269–1296 cm^{-1} and 1180–1213 cm^{-1}) for HA (916–1180 cm^{-1}) [18, 19] (Fig 2).

Statistical analysis

Analysis was performed using statistical software Sigma Plot 13.1® (Systat Software Inc, San Jose, CA, USA). The results obtained were submitted to the Kolmogorov-Smirnov normality test and Two-Way Anova followed by the Tukey test. Correlation between biomechanics and ATR-FTIR analysis was measured by Pearson's correlation. Differences were considered statistically significant when $\alpha<0.05$.

Results

Throughout the experimental procedure, the animals of diabetic and diabetic+HBO groups maintained hyperglycemia (glucose levels above 200 mg/dl), weight reduction, polyphagia,

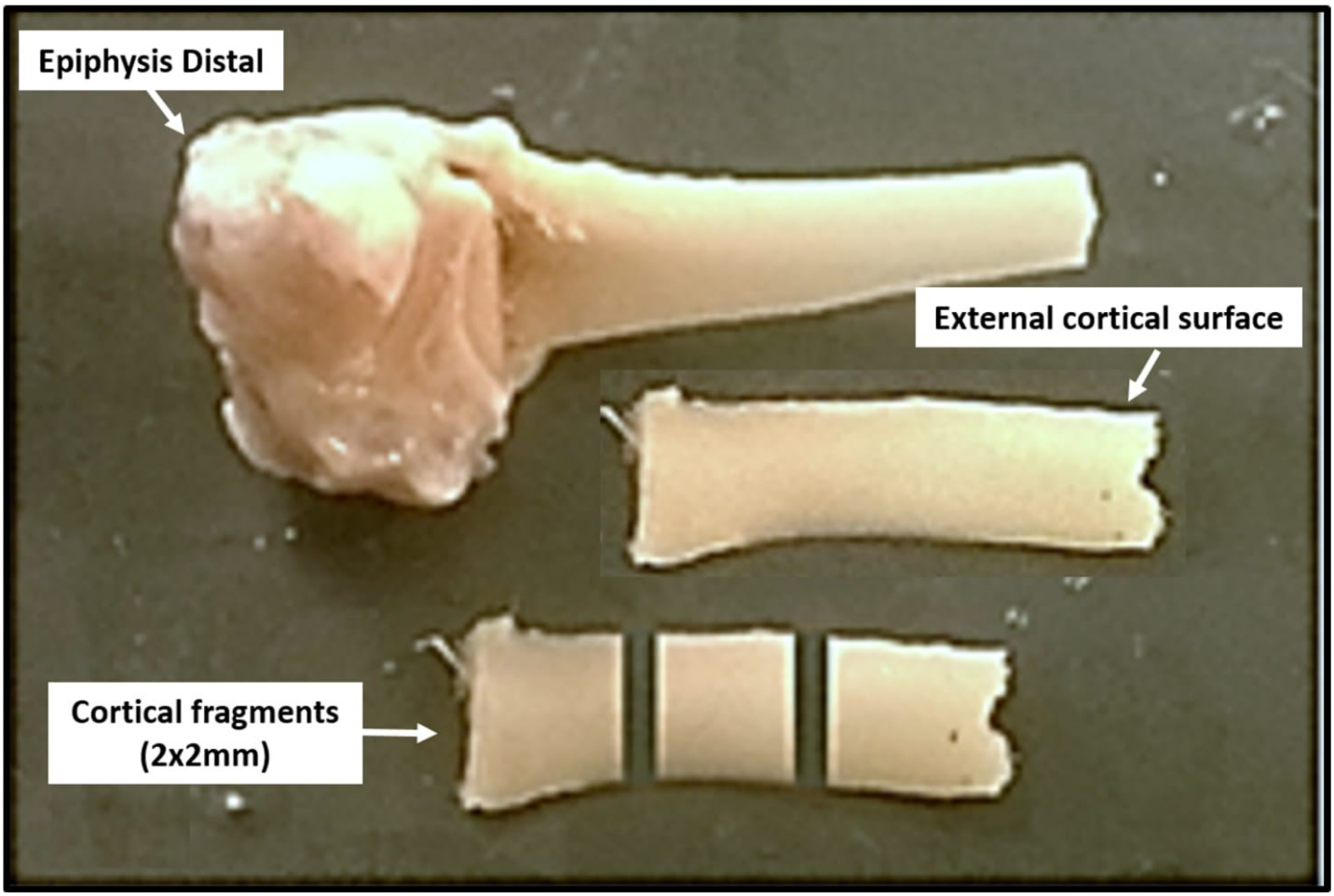

Fig 1. Cortical segments of the femur obtained to perform analysis by the ATR-FTIR.

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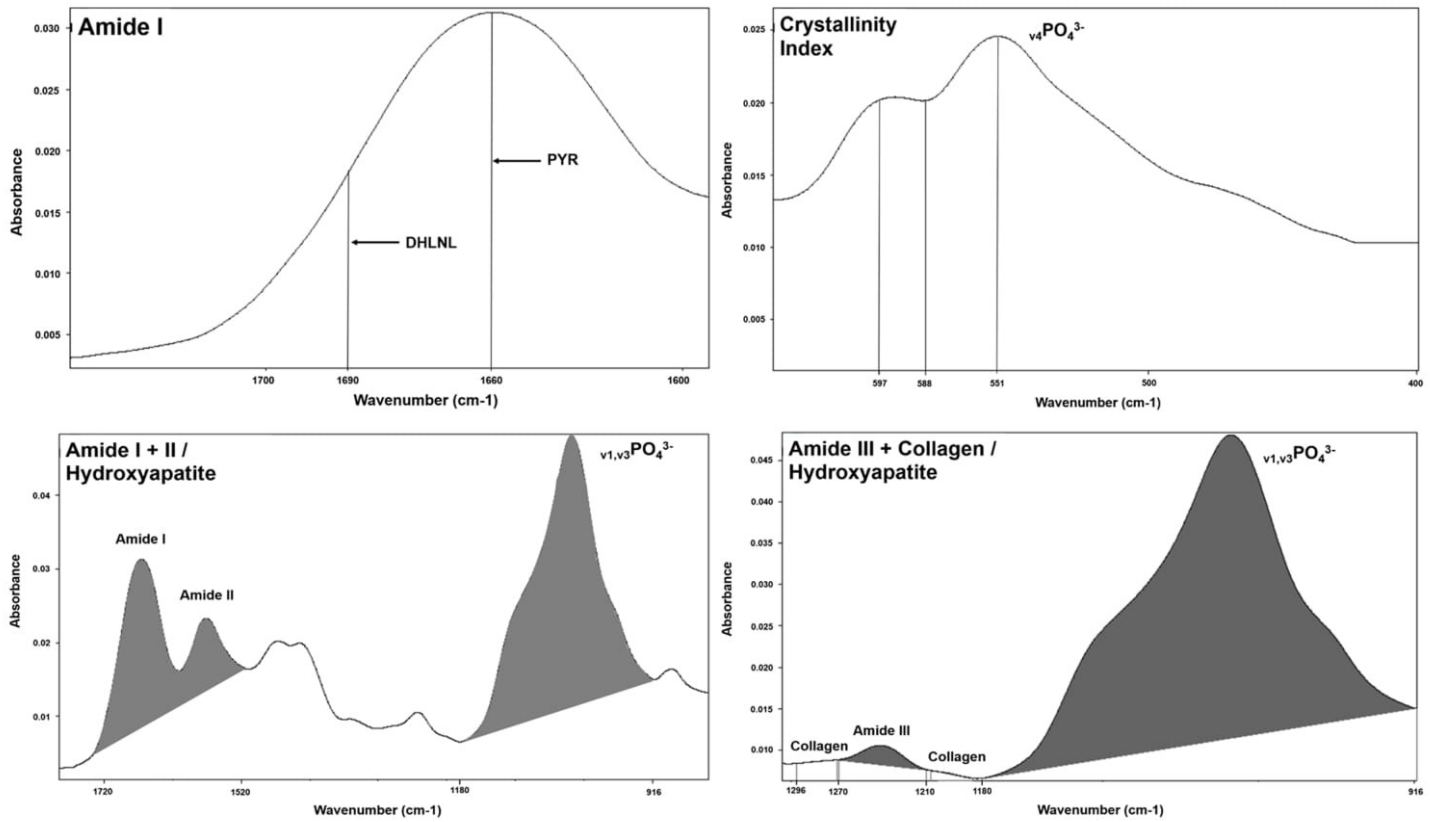

Fig 2. Parameters analyzed by means of ATR-FTIR spectrums using the program OPUS 6.5. Amide I band (Collagen ratio between the mature pyridinoline crosslink peaks (PYR)– 1660 cm-1 and immature crosslinking dihydroxinorleucina (DHLNL) - 1690 cm-1); Crystallinity Index (The intensity ratio of peaks 551 and 597 cm-1 for 588 cm-1); Matrix-to-mineral ratio: Amide I + II/Hydroxyapatite (HA) (The ratio between integrated areas of amide I + II (1520–1720 cm-1) for HA (916–1180 cm-1)) and Amide III + Collagen/HA (The ratio between integrated areas of amide III (1210–1270 cm-1) with two collagen bands (1269–1296 cm-1 and 1180–1213 cm-1) for HA (916–1180 cm-1).

https://doi.org/10.1371/journal.pone.0191694.g002

polydipsia and polyuria, observed from the increase in feed and water intake, and urinary excretion.

Mechanical analysis

In the mechanical analysis, diabetic groups showed lower values of maximum strength ((diabetic (100.5±5.6) and diabetic+HBO (107.0±8.8) vs control (117.7 ± 11.3) and control+HBO (124.2 ± 11.6)) stiffness ((diabetic (233.4 ± 33.1) and diabetic+HBO (277.9 ± 43.0) vs control (366.5 ± 37.6) and control+HBO (377.7 ± 26.0)) and energy ((diabetic (30.6 ± 3.6) and diabetic +HBO (33.0 ± 8.6) vs control (38.8 ± 8.2) and control+HBO (38.3 ± 8.3)) than non-diabetic animals ($p<0.007$). However, there were increases in the maximum strength and stiffness values in HBO groups (control+HBO and diabetic+HBO) compared with non-HBO groups (control and diabetic) ($p<0.042$) (Figs 3–5).

ATR-FTIR analysis

In the spectrums, main bands, characteristic of bone components were observed. The collagen maturity analysis showed a decreased ratio of crosslink peaks in diabetic (1.72±1.12) compared with the other groups (control (4.23±0.88), control+HBO (3.83±1.37) and diabetic+HBO (3.83±1.58) ($p = 0.003$) (Fig 6). The bone from the non-diabetic groups presented increased

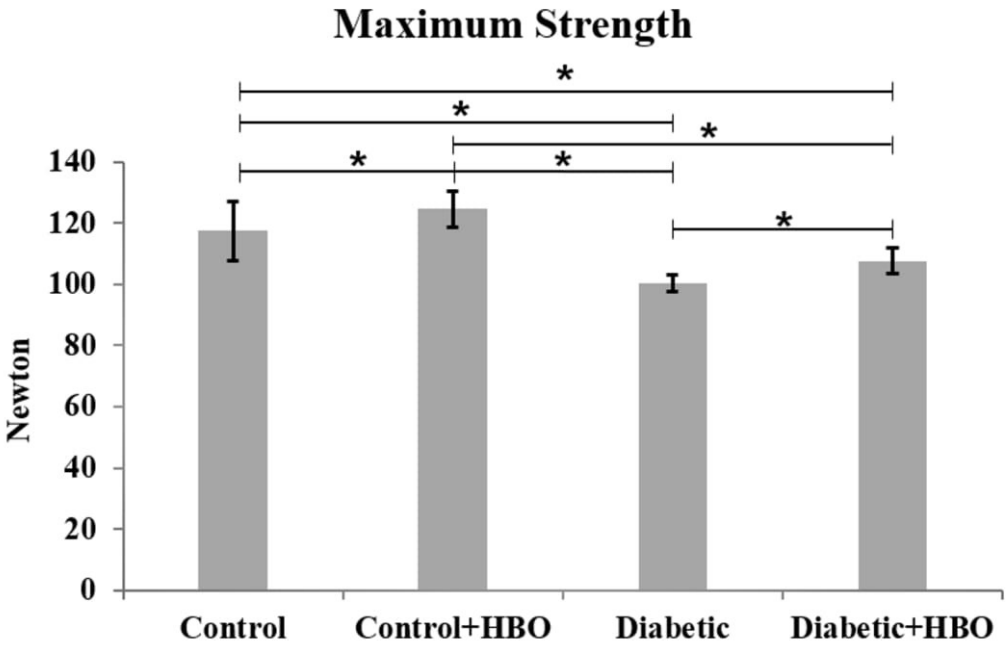

Fig 3. Maximum strength of biomechanical analyses in different groups (*p<0.05).

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crystallinity compared with those from the diabetic groups (control (3.01±0.30) and control +HBO (3.02±0.38) vs diabetic (2.48±0.38) and diabetic+HBO (2.90±0.33)) (p<0.034) (Fig 7). Matrix-to-mineral ratio evaluation of M:MI showed no statistical difference between groups control (0.62±0.38), control+HBO (0.60±0.40), diabetic (0.51±0.28) and diabetic+HBO (0.82 ±0.37) (p>0.278) (Fig 8). For the parameter, M:MIII there was an increase in matrix mineral ratio in diabetic+HBO (0.06±0.04) and control+HBO (0.04±0.04) compared with diabetic

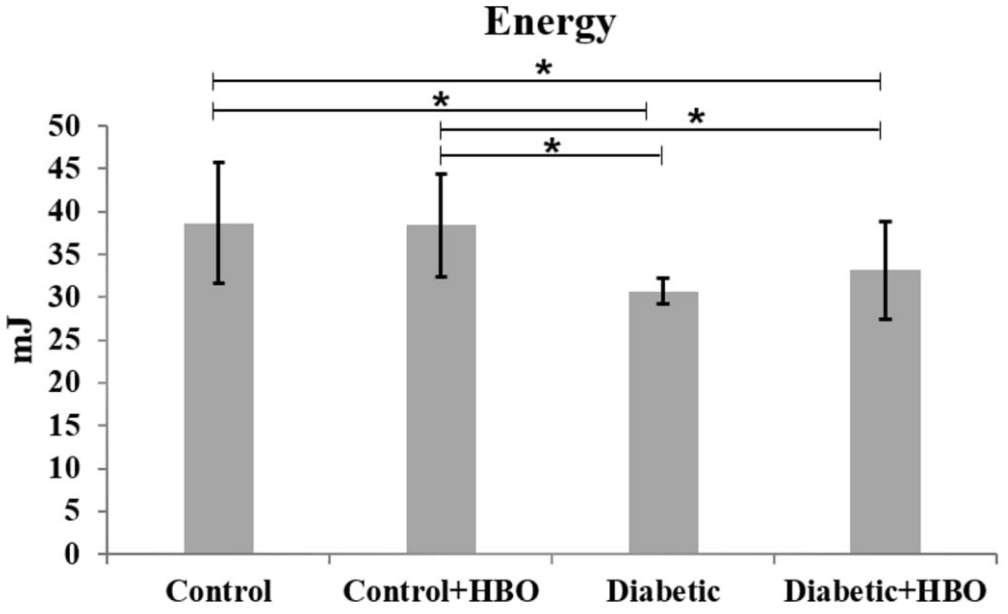

Fig 4. Energy of biomechanical analyses in different groups (*p<0.05).

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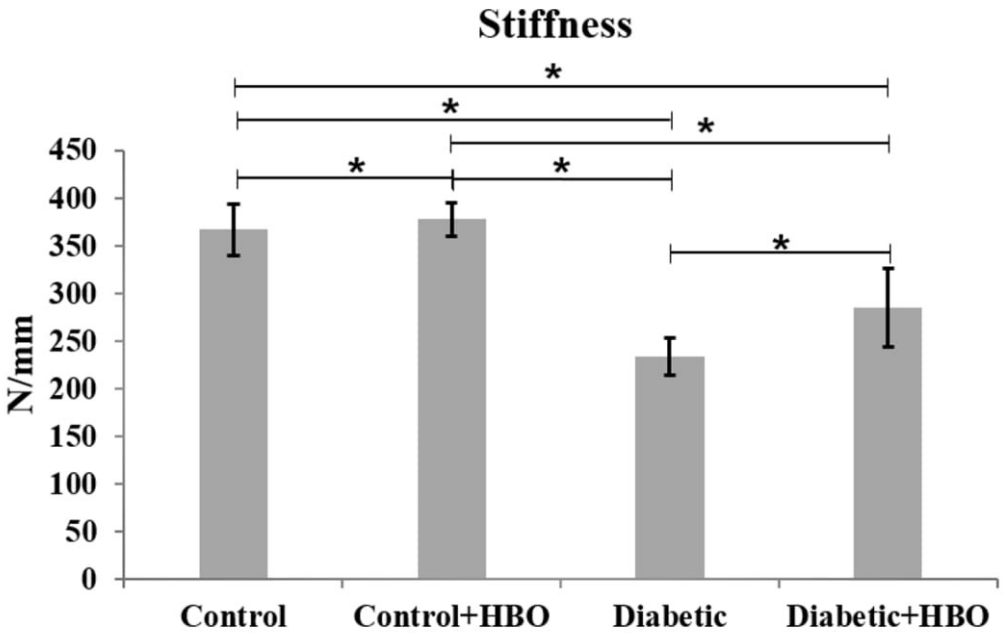

Fig 5. Stiffness of biomechanical analyses in different groups (*p<0.05).

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(0.03±0.01) and control (0.02±0.02), respectively (p = 0.035) (Fig 9). Correlations between mechanical tests and ATR-FTIR analyses showed significant positive correlation between collagen maturity and stiffness (r = 0.56, p = 0.02).

Discussion

The present study hypothesized that HBO would improve the bone matrix composition and mechanical properties in diabetic rats. In fact, our results showed that HBO minimized the deleterious effect of T1DM on collagen maturation and increased maximum strength and stiffness in diabetic rat femurs.

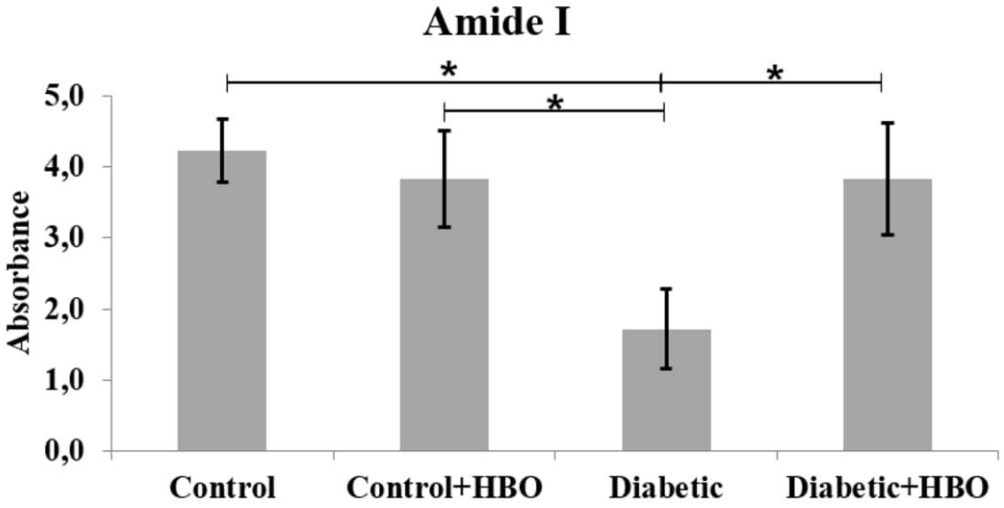

Fig 6. Amide I of ATR-FTIR analyses in different groups (*p<0.05).

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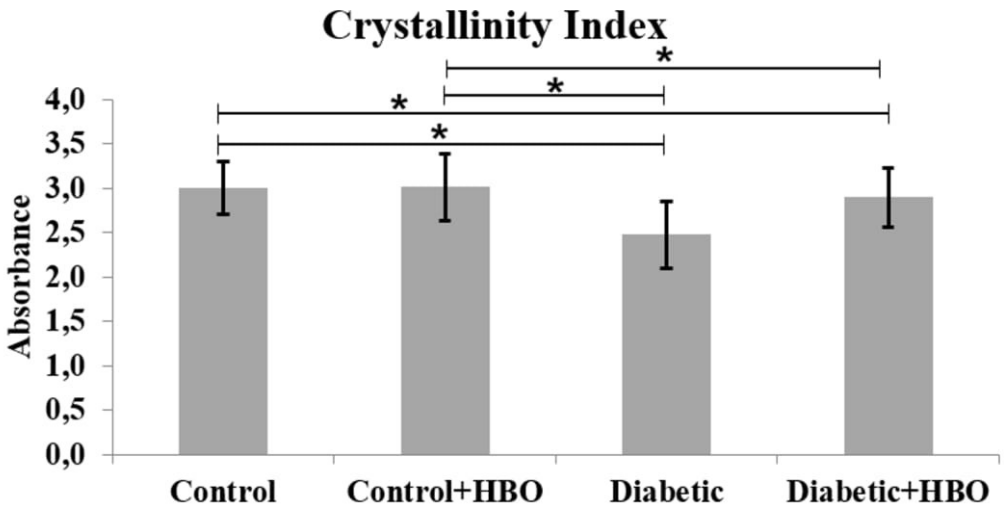

Fig 7. Crystallinity index of ATR-FTIR analyses in different groups (*p<0.05).

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T1DM is an autoimmune insulin-dependent disease characterized by remarkable reduction in insulin production and chronic hyperglycemia, and accounts for approximately 10% of all diabetes cases. T1DM is associated with younger people [20], and due to longer exposure time to the disease, it generally has more serious repercussions on tissues, compared with diabetes mellitus type 2 [21].

The most common methods for establishing T1DM rodent models [22] is by means of a high-dose injection with STZ, which rapidly destroys pancreatic β cells and results in typical human T1DM symptoms [23]. Some studies have shown that STZ-induction decreased bone formation, deteriorated bone architecture, and compromised skeletal health, quality and strength, which revealed similarity to the bone phenotype of T1DM in human patients [23, 24].

The decreased maximum biomechanical strength, energy and stiffness in diabetic groups suggest that T1DM increased the fracture risk, which may be due to the structural changes in bone. Consistent with these finding, studies have shown that diabetes decreased bone strength, energy absorption [9, 25], and mineral content in diabetic rats [8].

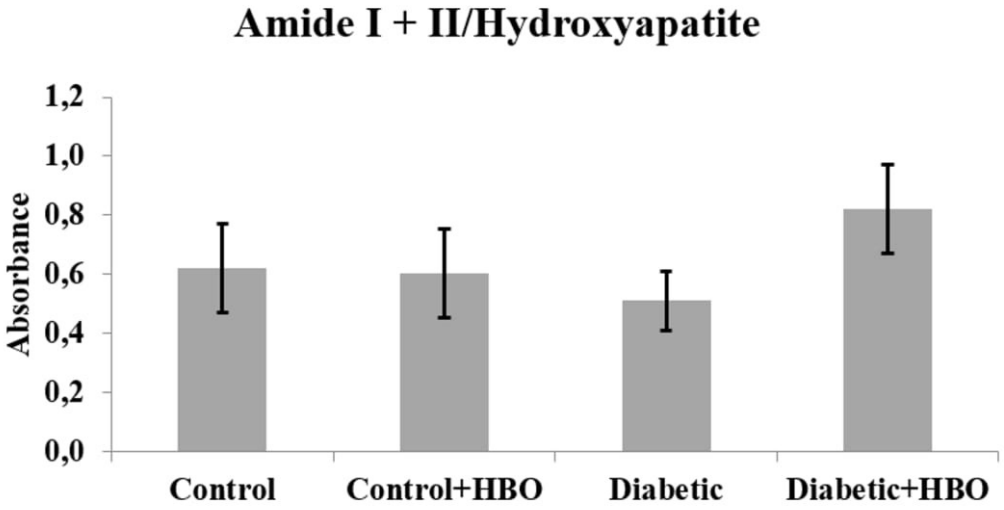

Fig 8. Matrix: Mineral ratio (Amide I+II/Hydroxyapatite) of ATR-FTIR analyses in different groups (*p<0.05).

https://doi.org/10.1371/journal.pone.0191694.g008

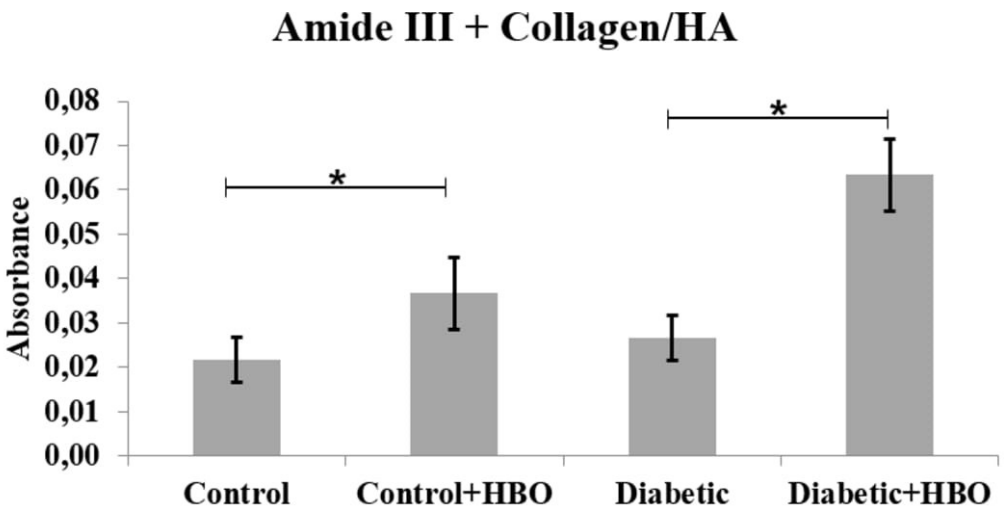

Fig 9. Matrix: Mineral ratio (Amide III+Collagen/Hydroxyapatite) of ATR-FTIR analyses in different groups ($^{*}p<0.05$).

https://doi.org/10.1371/journal.pone.0191694.g009

Bone fracture resistance depends on several bone characteristics, and has been described as a multiple-scale process, the scale of which has a level within the structural hierarchy [6]. It is likely that T1DM, in some way, compromises the bone hierarchical structure, reducing its resistance, which could explain our results. The macroscopic structure (size and shape), architecture (cortical and cancellous components) and the bone substance (organic and inorganic components) are also influenced by T1DM [25].

Bone is a two-phase composite material in which the mineral phase provides stiffness and the collagen provides strength and the post-yield property of ductility [17]. Bone matrix development starts when the collagen fibrils appear and follows a process of enzymatically induced cross-linking that stabilizes the fibrils [17]. The collagen fibrils serve as scaffolds on which nucleation and growth of the mineral crystals will take place. These two processes are intimately correlated as shown by the similar trend between the crystallinity and the collagen crosslink ratio pattern across the osteons [26].

In the present study, the collagen maturity analysis showed decreased enzymatic crosslink peak ratios in diabetic when compared with the other groups. This decrease suggested that there was a higher proportion of immature crosslinks compared with the mature crosslinks in diabetic animals. Indeed, either an increase in immature DHLNL crosslinks (intrafibrillar) or a reduction in mature PYR crosslinks (interfibrillar) could disrupt the mature crosslink integrity, leading to decreased energy and premature bone failure [27]. The collagen crosslink ratio indicates the state of maturity of the crosslinking network in the bone collagen fibrils, which is important for the structural and mechanical properties of bone [26].

The degree in collagen crosslink formation is regulated by the extent of glycation [17]. Experimental studies have shown that advanced glycation end products (AGEs) are formed when free-floating sugars interact with exposed amino acid residues on collagen, resulting in a reversible Amadori intermediate that ultimately undergoes oxidation to form irreversible AGEs [28, 29]. These compounds accumulate and affect cross-links within type 1 collagen [29, 30]. The AGEs impair immature and mature crosslinks in the collagen matrix contributing to bone fragility [30, 31], which could be associated with the results of the present study.

Diabetic animals submitted to HBO showed an increased PYR/DHLNL crosslink ratio. Intermolecular collagen crosslinking is important for development of the underlying matrices

that are essential for initial mineral formation and crystal growth [26]. According to a previous study, a specific PYR induces the type of enzymatic crosslinking pattern that influences bone matrix mineralization, increasing bone maximum strength and stiffness [17], as shown in our study. However, the mechanism of how HBO changes the crosslinks in diabetes is unknown [32].

In the present study, crystallinity decreased in diabetic compared with non-diabetic animals. This result suggested that TIDM increased the presence of large HA crystals and decreased the surface area in collagen fibrils [33]. Boyar et al showed that crystallite sizes were changed in the bone mineral matrix of diabetic rats [8]. The highly ordered location and orientation of very small crystals within the collagen fibrils contribute to the bone rigidity and strength. In addition, their small size allows an acceptable range of flexibility without fracture or disruption of the bone substance [34]. Recent studies have suggested that increased bone mineral particle size was associated with increased bone fragility [26].

The positive correlation between collagen maturity and stiffness showed that both parameters decreased in animals with TIDM. This could be due changes in the enzymatic process that induces fibril stabilization by collagen crosslinking [17], leading to deterioration in mineralization [35] and decreased bone stiffness [6]. Recently, some studies on diabetic bone showed that hyperglycemia affected the type I collagen and compromised the mineralization process [17, 26, 27].

M:MI showed no statistical difference between the groups. This suggested that TIDM reduced collagen maturity and crystallinity in the same proportions, thus without change in the ratio between the organic and inorganic matrix. Some studies in humans and animals have shown that diabetes impaired bone metabolism, leading to decreased bone mass [4]. However, M:MIII showed that the matrix mineral ratio increased in control+HBO and diabetic+HBO compared with control and diabetic groups. Our results suggested that HBO increased intermolecular interactions (by hydrogen bonds) in the collagen, followed by induced cross-linking that stabilized the fibrils [36], which explained the increase maximum strength and stiffness in HBO groups.

Although there was no statistical difference between groups, DH showed higher values in M:MI and M:MIII. It could be that the effect of T1DM [26] and HBO [36] on collagen crosslinks increased the interaction with fibrils, increasing the matrix:mineral ratio. However, how the mechanisms of T1DM with HBO therapy affect the organic matrix is unknown.

Therefore, the present study suggested that fracture risk was increased in STZ-induced diabetic rats due to the reduced bone strength, energy and stiffness characterized by changes in collagen crosslinks [6, 27]. Our findings confirmed those of previous studies and increased the knowledge of how the mechanisms of HBO increase the stability of enzymatic crosslinks and may change organic and mineral bone matrix.

Conclusion

The results showed that diabetes decreased collagen maturation and the mineral deposition process, reducing the bone capacity to absorb energy, maximum strength and stiffness. Moreover, the study showed that HBO improved the crosslink maturation and increased maximum strength and stiffness in the femur of animals with STZ-induced diabetes.

Author Contributions

Conceptualization: Pedro Henrique Justino Oliveira Limirio, Huberth Alexandre da Rocha Junior, Richarlisson Borges de Morais, Karen Renata Nakamura Hiraki, Ana Paula Coelho Balbi, Priscilla Barbosa Ferreira Soares, Paula Dechichi.

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Formal analysis: Pedro Henrique Justino Oliveira Limirio, Huberth Alexandre da Rocha Junior, Priscilla Barbosa Ferreira Soares, Paula Dechichi.

Funding acquisition: Paula Dechichi.

Investigation: Pedro Henrique Justino Oliveira Limirio, Huberth Alexandre da Rocha Junior, Richarlisson Borges de Morais, Karen Renata Nakamura Hiraki, Ana Paula Coelho Balbi, Priscilla Barbosa Ferreira Soares, Paula Dechichi.

Methodology: Pedro Henrique Justino Oliveira Limirio, Huberth Alexandre da Rocha Junior, Richarlisson Borges de Morais, Karen Renata Nakamura Hiraki, Ana Paula Coelho Balbi, Priscilla Barbosa Ferreira Soares, Paula Dechichi.

Project administration: Paula Dechichi.

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2.2 CAPÍTULO 2

Dias PC, Limirio PHJO, Linhares CRB, Bergamini ML, Rocha FS, Morais RB, Balbi APC, Hiraki KRN, Dechichi P. Hyperbaric Oxygen therapy effects on bone regeneration in Type 1 diabetes mellitus in rats. Connective tissue research. 2018 Jan 29:1-7.

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Hyperbaric Oxygen therapy effects on bone regeneration in Type 1 diabetes mellitus in rats

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ABSTRACT

Purpose: The aim of this study was evaluate the effect of HBO on diabetic rats.

Materials and Methods: Twenty rats were distributed into four groups ($n = 5$): Control (C); Control + HBO (CH); Diabetes (D) and Diabetes + HBO (DH). Diabetes was induced by streptozotocin, and bone defects were created in both femurs in all animals. HBO therapy began immediately after surgery and was performed daily in the CH and DH groups. After 7 days, the animals were euthanized. The femurs were removed, demineralized, embedded in paraffin, and histologic images were analyzed.

Results: Qualitative histologic analyses showed more advanced stage bone regeneration in control groups (C and CH) compared with diabetic groups (D and DH). Histomorphometric analysis showed significantly increased bone neoformation in CH compared with the other groups ($p < 0.001$). Diabetic Group (D) showed decreased bone neoformation compared with non-diabetic groups (C and CH) ($p < 0.001$); however DH did not differ from C Group ($p > 0.05$). The mast cell population increased in CH compared with the other groups (C, D, and DH) ($p < 0.05$). The mast cell population did not differ between D and DH Groups.

Conclusions: This study showed that HBO therapy improved early bone regeneration in diabetic rats and increased the mast cell population only in non-diabetic animals. HBO was shown to be important treatment for minimizing deleterious effects of diabetes on bone regeneration.

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1. Introduction

Type 1 diabetes mellitus (T1DM) is a metabolic disorder characterized by absolute deficiency of insulin secretion resulting from autoimmune destruction of insulin producing pancreas β cells (1). The chronic hyperglycemia in T1DM has been associated with the occurrence of complications involving the skeletal system (1). Some studies have shown that diabetes may negatively affect bone health by unbalancing several processes: bone resorption, collagen formation, secretion of inflammatory cytokines, calcium metabolism, and bone regeneration (2).

Bone regeneration is a complex process that involves the coordination of multiple events. The interaction between bone cells, signaling molecules and constituents of the immune system, such as mast cells, continues to be of great scientific interest (3,4). Some studies have shown important participation by mast cells in vascular endothelial cell recruitment (4) and bone remodeling (3,5). However, the participation of these cells in bone regeneration, especially in diabetics, is still not entirely understood.

Several mechanisms have been proposed to explain bone repair abnormalities in diabetics, including reduction in inflammatory mediators, delay in or compromise of the angiogenic process (2,6,7), inhibition of osteoblast differentiation, and induction of osteoblast apoptosis (2,7,8). Some studies have shown that diabetes increased the release of advanced glycation end products and decreased osteoblast activity (2,7,8). The deleterious effects of diabetes on bone formation have been shown in the literature, thus it is important to investigate alternative therapy to minimize these effects.

Hyperbaric oxygen therapy (HBO) consists of intermittent inhalation of 100% oxygen under a pressure

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higher than 1.5 atmospheres absolute (ATA) (9). This therapy has been associated with accelerated tissue repair, especially in adverse conditions. Studies have shown that HBO increased collagen synthesis (10), accelerated osteoblast differentiation (11), and stimulated the bone repair process (11–13). However, studies that investigated the effects of HBO on diabetes were performed only in skin wounds healing (9,10,14).

Therefore, the aim of present study was to evaluate the effects of HBO on bone regeneration and mast cell population in diabetic rats. We hypothesize that HBO would improve early bone regeneration and increase the mast cell population in diabetic rats.

2. Material and methods

2.1. Experimental procedure

Twenty male *Wistar* rats, weighting 240–280 g (2 months of age), were kept in cages, under a 12-h light-dark cycle, and controlled temperature conditions (22 ± 2°C), with standard food and water *ad libitum*. The animals were randomly divided into four groups (n = 5), as follows: Control (C); Control + HBO (CH); Diabetes (D); and Diabetes + HBO (DH). Thirty days after T1DM induction by streptozotocin (STZ) (Sigma Aldrich, St Louis, MO, USA), bone defects were created in both femurs of all animals. The oxygen therapy was performed once a day and started after the surgery. The Control group received no treatment other than the surgical procedure. Seven days after the surgery, all animals were euthanized and the femurs were removed for analysis (Figure 1). This study was previously approved by the Science and Ethics Committee (Protocol 026/14) and was conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA).

The rats were kept on 24-h fasting before the T1DM induction. The animals were anesthetized by an intraperitoneal injection of 100 mg/Kg ketamine 10% and 7 mg/Kg xylazine 2%. Afterwards, STZ (65 mg/kg), freshly dissolved in 0.01 M citrate buffer, pH 4.5, was injected into the penile vein (2 mL/kg). At time intervals of 24 h, 7, 15, 30, and 37 days after diabetes induction, blood glucose was determined with a glucometer (Accu Check Active®, Roche, Jaguaré, São Paulo, Brazil) using blood from the tail vein. The animals with blood glucose levels higher than 200 mg/dL were considered diabetic, and those that did not reach the glycemic target were excluded from the study. Each group had five animals.

Thirty days after the diabetes induction, bone defects were created in both femurs of all the animals. After being anesthetized, they were submitted to a surgical procedure as described by Batista et al. (15). With the animal positioned in lateral decubitus, the femur was exposed by means of a 2 cm longitudinal incision. A standardized 2.3 mm bone defect was created using a round bur, and the drilling depth was limited to cortical bone rupture (approximately 2 mm). Subsequently, the muscle and cutaneous layers were sutured with nylon 4–0. The HBO sessions started immediately after the surgical procedure, with therapy carried out in a cylindrical pressure chamber (Ecobar 400, Ecotec Equipamentos e Sistemas Ltda®, Mogi das Cruzes, SP, Brazil) at 2.5 ATA, in 90-minute sessions, conducted daily for 7 days, as described by Rocha et al. (13).

The animals were euthanized 7 days after surgery. The femurs were disarticulated, the epiphyses were removed, and the diaphyses were immediately fixed in 4% paraformaldehyde solution in phosphate buffered for 48 h. After this, the diaphyses were decalcified in 4.13% EDTA for 5 weeks, and embedded in paraffin. From each animal were obtained 12 semi-serial histological sections (5μm) stained: 3 in Hematoxylin-Eosin (HE), 3 in Mallory Trichrome, and 6 in Toluidine Blue.

2.2. Histological and histomorphometric analyses

Qualitative histological analysis (HE sections) was performed in three sections per animal totaling 15 histological sections per group. The bone regeneration of the injured area was evaluated in the qualitative form, considering type of bone tissue, bone cell morphology, presence of clot, and bone trabecula thickness in a comparative analysis (thicker or thinner).

Bone neoformation was quantified (Mallory Trichrome sections) in 3 sections per animal totaling 15

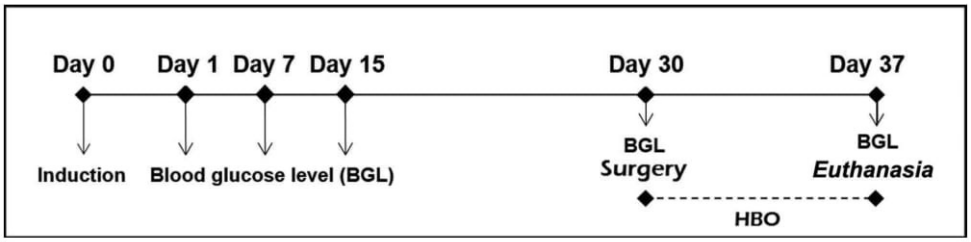

Figure 1. Experimental design in the groups evaluated. BGL—blood glucose level.

histological sections per group, as described by Batista et al. (15). Briefly, histological images of the bone defects were captured, using a digital microscopic camera (Leica ICC50, Leica Microsystems. Heerbrugg, Switzerland) coupled to a microscope (Leica DM500, Leica Microsystems®, Heerbrugg, Switzerland). The screenshots were merged, the areas of soft tissue were erased using Photoshop CS6 software (Adobe®, Adobe System Inc., San Jose, CA/EUA), and converted to binary images with HL Image 2005 ++ (Western Vision®, Salt Lake City, UT, USA). The region of interest (ROI) (corresponding to the bone defect area) was delineated with four lines from the cortical bone edges to the opposite cortical and the bone neoformation percentage was calculated from the box area filled with trabeculae bone, using measurement tool of HL Image 2005++.

For mast cell quantification (Toluidine Blue sections), 6 sections per animal totaling 30 histological sections per group were analyzed with an optical microscope (Olympus BX 50, Shinjuku-ku, Tokyo, Japan) at ×40 magnification. Single blinded examiners counted all the mast cells present in the ROI considering cells that presented metachromatic granule staining with crystal violet.

2.3. Statistical analysis

Histomorphometric analyses (bone neoformation and mast cells) were performed using statistical software Sigma Plot 13.1® (Systat Software Inc, San Jose, CA, USA). The results obtained were submitted to the Kolmogorov-Smirnov normality test and Two-Way ANOVA followed by the Tukey test, and presented in mean and standard deviation (SD). Differences were considered statistically significant when $\alpha < 0.05$.

3. Results

Throughout the experimental procedure, the diabetic animals (D and DH) maintained hyperglycemia (glucose levels above 200 mg/dl), weight reduction, polyphagia, polydipsia, and polyuria, observed from the increase in feed and water intake, and urinary excretion.

3.1 Histological analysis

In C Group, histological analysis showed ROI partially filled with primary bone, many osteoblasts, and few osteoclasts. The following were also shown: a foci of hemorrhage, richly cellularized and vascularized tissue, with young osteoblasts associated with osteoid matrix. The CH group also showed primary bone filling almost all of the ROI. The trabeculae were thicker compared to other groups, with many areas of osteoclastic activity. There were few red blood cells and many young osteoblasts associated with osteoid matrix. Bone regeneration in CH Group was at a more advanced stage compared with C Group. Histological morphology in D and DH Groups was similar to that of C Group, but in the diabetic were observed thinner trabecular with few osteoclasts, and a larger clot area. However, the diabetic group with HBO (DH) showed increase in the bone regeneration process when compared with the diabetic group (D), and apparently similar to C group (Figure 2).

3.2 Histomorphometric analysis

The histomorphometric analysis showed increased bone matrix neoformation percentage in CH (38.08 ± 4.05) in comparison with the other groups (C (32.05 ± 5.51), D (24.62 ± 2.28), and DH (27.14 ± 4.21)) ($p < 0.001$). The diabetic group (D) showed less bone matrix neoformation percentage compared with the nondiabetic groups ($p < 0.001$), however DH did not differ from the C Group ($p > 0.05$) (Figure 3).

Mast cells were located mainly at the periphery of the ROI and were shown to be large cells with a central nucleus. There was an increase in the mast cell number in CH (21.06 ± 4.91) in comparison with the other groups (C (8.06 ± 5.15), D (9.00 ± 4.99), and DH (9.60 ± 7.18)) ($p < 0.05$) (Figure 4).

4. Discussion

The present study showed that HBO minimized the deleterious effects of diabetes on bone regeneration. STZ-induced T1DM via intravenous injection, used in present study, allowed a stable and reproducible experimental model (16) and has been widely used as a T1DM study model (16–18). The experimental period of 30 days after diabetes induction allowed the authors to evaluate the late deleterious effects of diabetes on early bone regeneration.

The presence of a wide clot area in the diabetic group (D) showed that T1DM delayed the initial inflammatory phase and compromised bone regeneration. These changes could have been due to diabetes damaging cell migration, cell/tissue differentiation, growth factors, organic matrix synthesis (4) and delay in, or compromised angiogenesis during bone regeneration (18). Some studies have shown that T1DM reduced FGF-2 (19), impairing angiogenesis (2,6,7,18) and may affect osteoblast migration during the regeneration process (2).

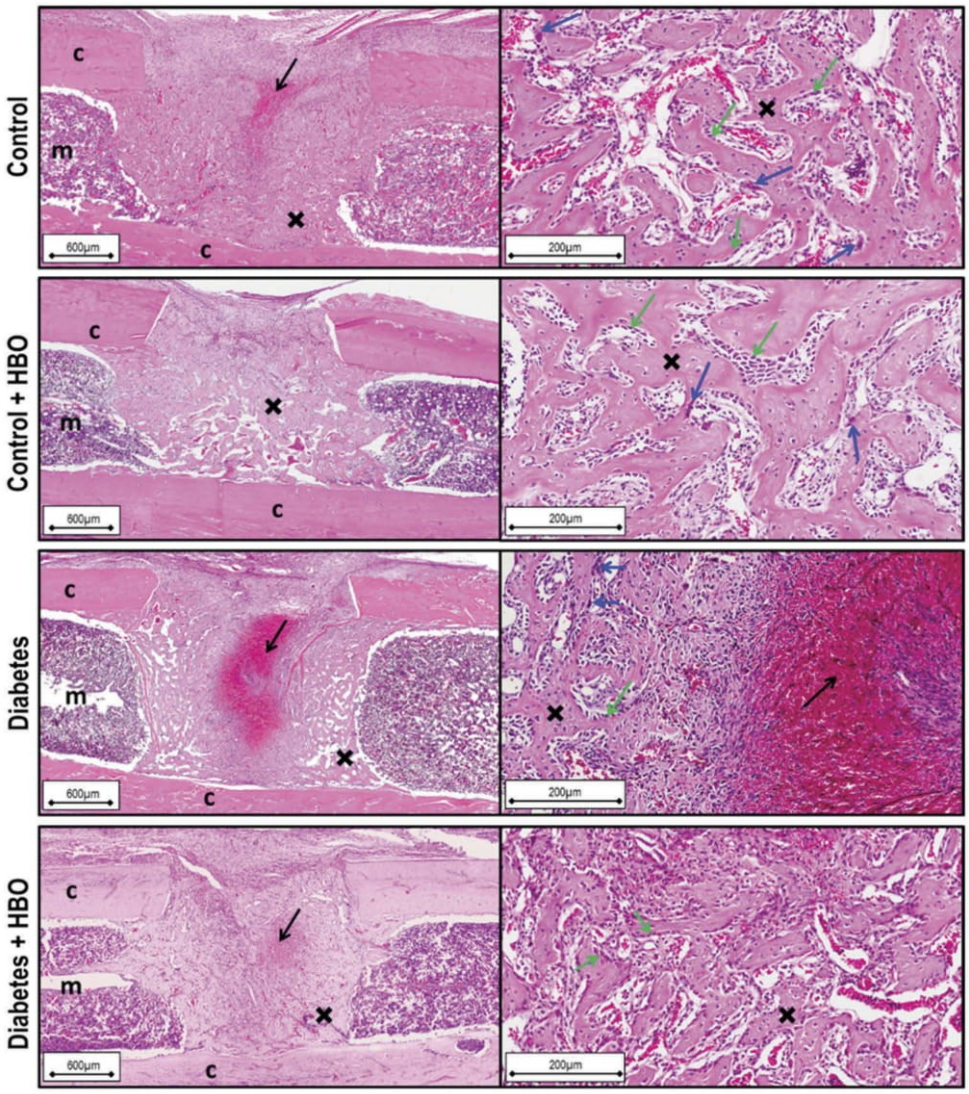

Figure 2. Longitudinal femur sections showing cortical bone (c), bone marrow (m), new bone formation (×), remnant clot (black arrow), osteoblasts (green arrows), and osteoclasts (blue arrows). Hematoxylin-Eosin.

In addition, studies have shown that chronic elevation of extracellular glucose levels could lead to glycosylation of protein and other cell components, releasing advanced glycation end products (AGE). These AGEs inhibit osteoblast differentiation and induce osteoblast apoptosis (2,7,8). Our results suggested that diabetes compromised osteoblast function and decreased bone neoformation, because in the diabetic groups, trabecular bone was thinner compared with nondiabetic groups.

HBO increased bone neoformation in nondiabetic animals (CH) in the present study. This was in accordance with our qualitative histological analysis, showing thicker trabeculae and many areas of osteoclastic activity, suggesting accelerated bone regeneration. In the normoglycemia condition, studies have shown that HBO accelerated osteoblast differentiation (11) stimulated cell proliferation, reduced edema, and inflammatory damage, resulting in acceleration of bone neoformation (12,13). The positive findings after HBO in patients damaged by diabetes have been demonstrated in wound (9,10) and peri-implant bone healing (20). However, the effect of HBO on diabetic bone tissue had not yet been described.

The present study showed that HBO minimized the deleterious effect of T1DM on bone regeneration in the Diabetic Group (DH). Indeed, HBO accelerated the initial bone regeneration processes in the histological aspects similar to those of C Group. Recent evidence in healing diabetic wounds has indicated that tissue-level hyperoxia achieved by HBO can influence the distribution of progenitor cells within the wound microenvironment (10). This also increased the mobilization of endothelial progenitor cells (EPC), from the bone marrow into peripheral blood, exhibiting better growth potential (10).

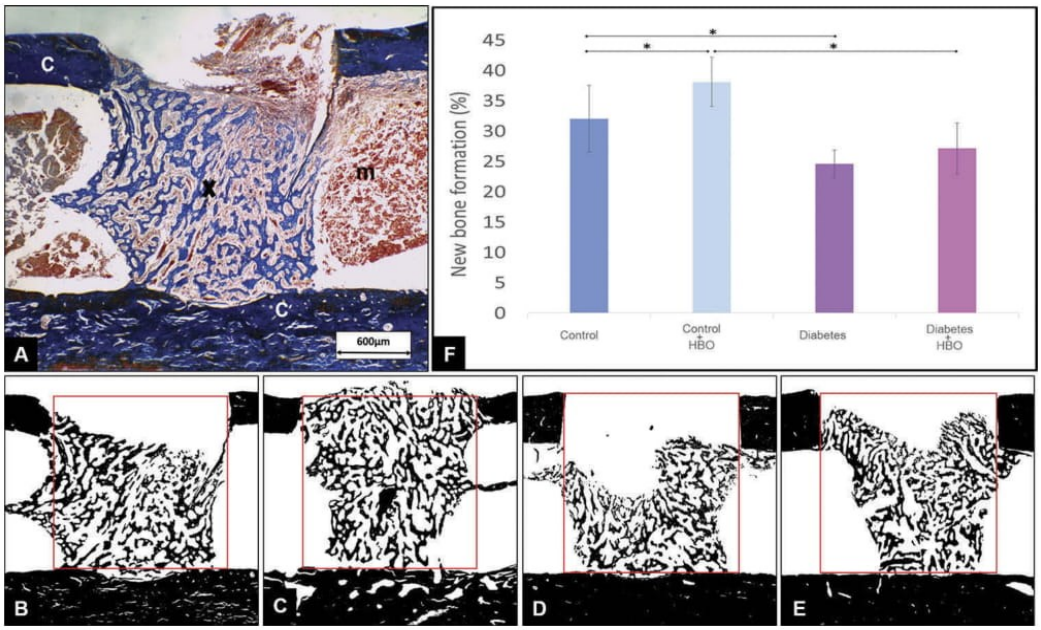

Figure 3. **A**—Histological image of bone defect in Control group. Cortical bone (c), bone marrow (m), and new bone formation (×). **B** (Control), **C** (Control + HBO), **D** (Diabetes), and **E** (Diabetes + HBO)—Image of the experimental groups after soft tissue removal, binary conversion, and ROI delimitation (red lines). The bone neoformation percentage was calculated from the red box area filled with trabeculae bone. Mallory Trichrome. **F**—Bone matrix neoformation percentage represented in the evaluated groups (15 histological sections), follows the Mean ± SD: C (32.05 ± 5.51), CH (38.08 ± 4.05), D (24.62 ± 2.28), and DH (27.14 ± 4.21) (*$p < 0.05$).

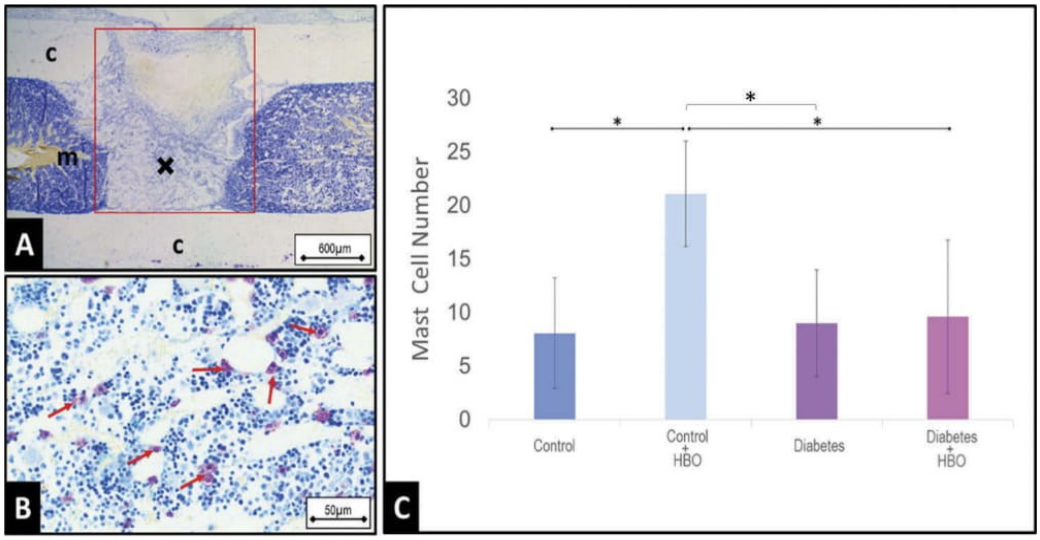

Figure 4. **A**—Histological image of bone defect in the Control + HBO (CH) group showing the ROI (red lines) for mast cell counting. Cortical bone (c), bone marrow (m), and new bone formation (×). **B**—Mast cells (arrows). Toluidine Blue. **C**—Number of mast cells in the groups evaluated (30 histological sections), follows the Mean ± SD: C (8.06 ± 5.15), CH (21.06 ± 4.91), D (9.00 ± 4.99), and DH (9.60 ± 7.18) (*$p < 0.05$).

In the present study, C, D, and DH Groups showed few mast cells in the granulation tissue during the proliferative phase. Some studies have shown that absence of mast cell mediators could compromise bone repair with reduced vascularization and mesenchymal stem cell differentiation into osteoblasts (3,4). It is therefore reasonable to propose that activation of mast cells might increase the speed and quality of the repair process (4,5).

However, HBO was not able to increase the number of mast cells in diabetic rats, as observed in the non-diabetic groups. The findings of the present study suggested that diabetic animals were at an initial stage of repair, when the mast cell appeared to be less active. The mast cells may not play an important role in neovascularization, especially in the proliferative phase (21).

Our findings suggested that hyperglycemia may also have interfered in mast cell activity. Nishikori et al. (17) showed that diabetes delayed mast cell proliferation in the remodeling phase and compromised neovascularization and vascular regression in proliferative and remodeling phases, respectively. This indicated that the reduction in mast cells in diabetes observed in our study might be directly linked to these events. Increase in the number of mast cells and more advanced bone regeneration in CH group indicated that these cells participated in the bone regeneration process, but the mechanism of how this occurs is unknown.

5. Conclusion

The present study showed that HBO therapy improved early bone regeneration in diabetic rats and increased the mast cell population only in nondiabetic animals. HBO was shown to be an important treatment for minimizing the deleterious effects of diabetes on bone regeneration.

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Declaration of interest

The authors declare that they have no conflicts of interest.

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Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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2.3 CAPÍTULO 3

Artigo a ser enviado para publicação no periódico Lasers in medical science

Low-Level laser therapy effects on bone regeneration in type 1 diabetes mellitus rats.

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Abstract

Low-level laser therapy is a highly concentrated non-ionizing radiation that has been used for therapeutic purposes. The present study evaluated the bone repair in femurs rats submitted to low power laser therapy. Five animals were subject to T1DM induction and the other five animals remained healthy. After 4 weeks, it was created bone defects in the femurs and the left femurs were received Laser therapy in all animals. Seven days after surgery, animals were euthanized, the femurs were removed and divided into 4 groups (n=5), as follows: Healthy (right femur of the healthy animals); Healthy+Laser (left femur of the healthy animals subject to Laser therapy); Diabetic (right femur of the diabetic animals); Diabetic+Laser (left femur of the diabetic animals subject to Laser therapy). The femur was scanned using micro-computerized tomography and, in qualitative histological analysis, the injured bone area was evaluated in a comparative analysis. In the Micro-CT analyses, diabetic groups showed lower values of BV/TV, Tb.Th, Tb.N, Tb.Sp, Conn.Dn and SMI than non-diabetic animals ($p<0.05$). However, there was increase BS/BV values in diabetic groups compared to non-diabetic groups ($p<0.05$). The Diabetic+Laser showed increase Tb.Sp and decrease Conn.Dn compared to Diabetic group ($p<0.05$). In Healthy+Laser, SMI increase compare to Healthy group. In histological analyses, all groups showed primary bone tissue in defect area. Healthy group presented more residual clot and granulation tissue compared to the Healthy+Laser. In the healthy groups, the neoformation bone tissue presented thicker trabeculae in relation to the diabetic groups. In Diabetic group, large areas of clot and granulation tissue were observed, as well as few trabeculae in relation to the other groups. In the Healthy+Laser group, there was less clot extension and amount of granulation tissue compared to Diabetic. The bone repair of Diabetic+Laser was apparently similar to Healthy group. The authors concluded that diabetes decreased bone regeneration process and Low-Level Laser Therapy was important treatment for minimizing the deleterious effects of diabetes on bone regeneration.

Keywords: Diabetes Mellitus, Type 1; Low-Level Light Therapy; Femur; Rats

Introduction

The increase in the Type 1 Diabetes Mellitus (T1DM) occurrence has become a worldwide concern, mainly because this disease presents significant morbidity and mortality [1]. Diabetes mellitus (DM) is chronic disease characterized by hyperglycemia resulting from deficits of insulin secretion, insulin action, or both [2]. Chronic hyperglycemia leads to long-term damage, dysfunction, and failure of various organs.

In bone, T1DM has been associated with delay repair; bone remodeling and microarchitecture altered; reduced bone mass, osteopenia, osteoporosis, and increased bone fractures risk [3]. Studies have been reported that T1DM causes an imbalance between activation and inhibition of proteolytic enzymes, partial inhibition of synthesis and release of angiogenic growth factors, reduced rates of cell proliferation and increased apoptosis [4]. The decrease in collagen synthesis has been suggested the main contributing factor for bone repair reduction in diabetics. [5]. The decrease in collagen production and the increase in collagenase levels lead to degradation of newly synthesized collagen with low levels of cross-links, defective bone remodeling and bone repair delay [6].

Therapies have been proposed to reduce the deleterious T1DM effects on bone metabolism. Among these, low level laser therapy (LLLT) has gained attention due to its biostimulating effect [2,7,8]. Studies have shown several biological effects of LLLT, such as anti-inflammatory activity, proliferation stimulation of the osteoblast precursor, increase collagen synthesis, and increase bone formation[9]. Studies showed that the biostimulatory effect of LLLT is given by light energy that is converted into chemical

energy inside the cell, stimulating cellular activity, acting in the repair, besides the analgesics, disinfectant and anti-inflammatory effects [3].

The absorption of laser light of a specific wavelength by target tissue enhanced fibroblast proliferation and promoted collagen metabolism and granulation tissue formation during wound healing in diabetic rats [9]. LLLT reduces the inflammatory phase, promote vascularization and new bone formation, to a limited extent, and accelerates bone repair stages [10,11]

Although the exact mechanism LLLT action in the repair process has not yet been fully elucidated, studies suggest that this therapy stimulates mitochondrial metabolism, leading to increased proliferation and differentiation of osteoblasts, and increased bone matrix deposition[12]. We hypothesize that LLLT improves quality and accelerates bone repair in diabetic animals. Therefore, the objective of this study was to evaluate the effect of LLLT on bone repair of diabetic rats.

Material and methods

Experimental Procedure

Ten male Wistar rats, weighing 220 to 250 g (8 weeks of age), were kept in cages with a 12-hour light-dark cycle, and controlled temperature conditions (22 ± 2°C), with standard food and water *ad libitum*. This study was approved by the Science and Ethics Committee of the Federal University of Uberlândia, Brazil, and was performed in accordance with the provisions of Law No. 11,794, Decree No. 6.899 and Complementary legislation of the Brazilian National Council for the Control of Animal Experimentation (CONCEA) guidelines.

Five animals were subject to T1DM induction and the other five animals remained healthy. After 4 weeks, it was created bone defects in the femurs and the left femurs were received LLLT in all animals. Seven days after surgery, animals were euthanized, the femurs were removed and divided into 4 groups (n=5), as follows: Healthy (right femur of the healthy animals); Healthy+Laser (left femur of the healthy animals subject to Laser therapy); Diabetic (right femur of the diabetic animals); Diabetic+Laser (left femur of the diabetic animals subject to Laser therapy). The femurs were fixed in 4% paraformaldehyde solution in phosphate buffered for 48h and subject to micro-computerized tomography and histologic analysis.

T1DM Induction

The T1DM induction protocol began by keeping the rats fasting for 24h. Anesthesia was performed via the intraperitoneal pathway using 7mg/Kg xylazine 2% muscle relaxant, and 100mg/Kg ketamine hydrochloride 10% anesthetic and analgesic. Then, a single dose of streptozotocin (STZ) was administered intravenously through a penile vein puncture at a dose of 60 mg/Kg body weight, diluted in citrate buffer 0.01M. Hyperglycemia was confirmed by a glucometer (Accu Check Active, Roche, Jaguaré, SP, Brazil) after 24 hours; one week; 15 days, 30 days and 37 days after induction, by collecting a drop of blood from each animal's tail. Animals that maintained blood glucose levels higher than 200 mg/dL were considered diabetic. The animals that did not reach the glycemic target were excluded from the study.

Surgery and Laser therapy

Thirty days after the diabetes induction, bone defects were created in both femurs of all the animals. After being anesthetized, they were submitted to a surgical

procedure as described by Batista et al., 2014. With the animal positioned in lateral decubitus, the femur was exposed by means of a 2cm longitudinal incision. A standardized 2.3mm bone defect was created using a round bur, and the drilling depth was limited to cortical bone rupture (approximately 2mm). Subsequently, the muscle and cutaneous layers were sutured with nylon 4-0.

The animals of Healthy+Laser and Diabetic+Laser were submitted to laser therapy in left leg (Batista, 2015) using an Gallium-aluminum-arsenide (GaAlAs) infrared laser diode (Flash lase III – DMC Equipamentos/SãoCarlos – SP – Brazil), λ808nm, 64mW, continuous wave, 0.028-cm^2 beam diameter. The application was punctual, with a 4J/cm^2 dose per point and 16J/cm^2 dose per session in the bone defect area for 4 min (1min per point), with the laser tip positioned over and perpendicular to the long axis of the bone. The first session was applied immediately after the 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 7 days after surgery by intraperitoneal injection with sodium thiopental and lidocaine, followed by cervical dislocation, in compliance with the principles of the Universal Declaration on Animal Welfare.

Micro-computerized tomography

The femurs were disarticulated; the epiphyses were removed and the femur were immediately fixed in 4% paraformaldehyde solution in phosphate buffered for 48h. After this, the femur was scanned using micro-computerized tomography (μCT—SkyScan 1272, Bruker, Kontich, Belgium), with a nominal isotropic voxel size of 8μm (X-ray source 90 kVp, 111 μA). The reconstruction was made in 3D by software

nRecon (version 1.6.10.1, SkyScan, Bruker, Belgium), smoothing 1 and a ring artifact correction 2. After that, they were analyzed in CTAn software (version 1.14.4.1, SkyScan, Bruker, Belgium), using a standard threshold (upper 255 and lower 65) and the parameters used to analyze the area of bone repair were: bone volume fraction (BV / TV, %); bone surface density (BS/BV, 1/mm); Trabecular thickness (Tb.Th, mm); Trabecular number (Tb.N, 1/mm); Trabecular separation (Tb.Sp, mm); Connectivity density (Conn.Dn, $1/mm^3$); Structure Model Index (SMI, WU).

Histological analyses

After micro-Ct analyses, the femurs were decalcified in 4.13% EDTA for 5 weeks and processed for paraffin embedded. From each femur were obtained 4 semi-serial histological sections (5μm) that were stain in Hematoxylin-Eosin (HE). The histological images of the bone defects were scanned, using a digital scanner ScanScope AT Turbo® (Leica Biosystems Nussloch, Shinjuku-ku, Nußloch/Alemanha). In qualitative histological analysis, the injured bone area was evaluated considering type of bone tissue, bone cell morphology, presence of clot and thickness bone trabecula, in a comparative analysis.

Statistical analysis

Analysis was performed using statistical software Sigma Plot 13.1® (Systat Software Inc, San Jose, CA, USA). The results obtained in Micro-CT analyses were submitted to the Kolmogorov-Smirnov normality test and Two-Way Repeated Measures Anova followed by the Tukey test. Differences were considered statistically significant when $\alpha<0.05$.

Results

Throughout the experimental procedure, the diabetic animals maintained hyperglycemia (glucose levels mean 486,29 mg/dl), weight reduction, polyphagia, polydipsia and polyuria, observed from the increase in feed and water intake, and urinary excretion.

Micro-CT analyses

In the Micro-CT analyses, diabetic groups showed lower values of BV/TV, Tb.Th, Tb.N, Tb.Sp, Conn.Dn and SMI than non-diabetic animals ($p<0.05$). However, there was increase BS/BV values in diabetic groups compared to non-diabetic groups ($p<0.05$). The Diabetic+Laser showed increase Tb.Sp and decrease Conn.Dn compared to Diabetic group ($p<0.05$). In Healthy+Laser, SMI increase compare to Healthy group. Table 1.

Histological analyses

In histological analyses, all groups showed primary bone tissue in defect area. Healthy group presented more residual clot and granulation tissue compared to the Healthy+Laser. In the healthy groups, the neoformation bone tissue presented thicker trabeculae in relation to the diabetic groups. In Diabetic group, large areas of clot and granulation tissue were observed, as well as few trabeculae in relation to the other groups. In the Healthy+Laser group, there was less clot extension and amount of granulation tissue compared to Diabetic. The bone repair of Diabetic+Laser was apparently similar to Healthy group. Figure 1.

Discussion

The present study evaluated the Low-Level Laser Therapy (LLLT) effects on bone regeneration in diabetic animals. STZ-induced T1DM via intravenous injection, used in present study, allowed a stable and reproducible experimental model (16) and has been widely used as a T1DM study model STZ causes alkylation of β-pancreatic cell DNA, leading to cell necrosis, irreversibly inhibiting insulin production [15].

The reduced in BV/TV, Tb.Th, Tb.N, Tb.Sp, Conn.Dn in diabectis group was confirmed by and histological analyses where it was observed thinner bone trabeculae, lower bone neoformation, greater amount of granulation tissue, and clot characterizing impairment in bone repair. T1DM has been showed delay bone regeneration process in diabetic patients[16], that is attributed to the dysfunction of polymorphonuclear leukocytes, macrophages and fibroblasts, longer inflammatory phase, decrease collagen and glycosaminoglycan biosynthesis[17]. Studies have shown that diabetes decreases proliferation and differentiation of osteoblastic and chondrocyte cells, leads to formation of smaller callus, with decrease cartilage and bone formation [18]. Park & Kang (2012) also observed lower bone matrix formation in the diabetic group when compared to the healthy and diabetic group submitted to laser therapy. In addition, diabetics had a greater amount of inflammatory infiltrate and clot formation.

The deficiency of bone formation in the repair early stages in diabetic animals can also be explained by the possible increase in the osteoblasts number in apoptosis [19]. In addition, the increase osteoclastic activity may also contributed to the delayed bone repair observed in diabetic animals [20].

The present study showed in histological analyses that Diabetic+Laser bone repair was apparently similar to Healthy group. LLLT is able to improve bone

regeneration through the direct stimulation of osteoblastic metabolism, with consequent increase in the proteins synthesis, mainly collagen, increasing bone matrix deposition [21]. LLLT also appears to increase expression of Runx-2, a transcription gene involved the osteoblasts differentiation [22]. In addition, laser therapy promotes important changes in the chemical mediators directly involved repair, such as the reduction of cyclooxygenase-2 (COX-2) expression and increased expression of VEGF, a growth factor essential for granulation tissue formation [23]. The same findings were reported by Nascimento et al. (2005), which observed similar histological pattern of bone regeneration in healthy, healthy group submitted to laser therapy and diabetic submitted to laser therapy. There was extensive formation of thick bone trabeculae with marrow intertrabecular spaces. However, in the Diabetic group, the trabecular bone was thinner, irregular, with intertrabecular large spaces (Tb.Sp) and the large amount of granulation tissue.

In the present study, Healthy+Laser showed that trabeculae bone slightly thicker when compared to Healthy group, but did not present significant results in the Micro-CT analysis. LLLT presented positive results in bone repair of healthy animals in studies conducted by Batista et al. (2013). In this study, healthy animals with LLLT showed acceleration regeneration in bone defect and significant increase the percentage of bone neoformation when compared to the healthy group. The area was occupied by primary bone tissue, delimiting small cavities filled by connective tissue, fibroblasts, peripheral osteoblasts and blood vessels.

LLLT is still a controversial treatment, and the effects seem to depend not only on the protocol, but on several factors, such as tissue type, physiological state and

cellular proliferation capacity [24]. Further studies relating diabetes and LLLT should be performed in order to clarify the effects of this therapy in the hyperglycemia condition.

Conclusion

The results showed that diabetes decreased bone regeneration process and Low-Level Laser Therapy was important treatment for minimizing the deleterious effects of diabetes on bone regeneration.

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Table 1: Mean and standard deviation of the parameters analyzed in microCT

Mensures	Health	Health + Laser	Diabetic	Diabetic + Laser
BV/TV (%)	16.37 ± 5.04^{Aa}	18.73 ± 4.25^{Aa}	5.26 ± 2.91^{Ba}	2.88 ± 1.39^{Ba}
Tb.Th (mm)	0.030 ± 0.004^{Aa}	0.030 ± 0.002^{Aa}	0.027 ± 0.002^{Ba}	0.026 ± 0.004^{Ba}
Tb.N (1/mm)	5.43 ± 1.61^{Aa}	6.22 ± 1.27^{Aa}	1.90 ± 0.96^{Ba}	1.08 ± 0.45^{Ba}
Tb.Sp (mm)	0.08 ± 0.03^{Aa}	0.07 ± 0.01^{Aa}	0.18 ± 0.04^{Bb}	0.25 ± 0.06^{Bc}
BS/TV (1/mm)	148.47 ± 15.42^{Aa}	140.41 ± 12.05^{Aa}	175.34 ± 15.40^{Ba}	194.89 ± 25.27^{Ba}
SMI (wu)	2.62 ± 0.26^{Ab}	2.45 ± 0.23^{Aa}	3.04 ± 0.21^{Bc}	3.17 ± 0.11^{Bc}
Conn.Dn ($1/mm^3$)	3847.60 ± 1232.17^{Aa}	4209.69 ± 792.03^{Aa}	1143.94 ± 500.35^{Bb}	686.88 ± 252.75^{Bc}

Capital letters represent important difference between conditions. Lowercase letters represent important difference between treatments.

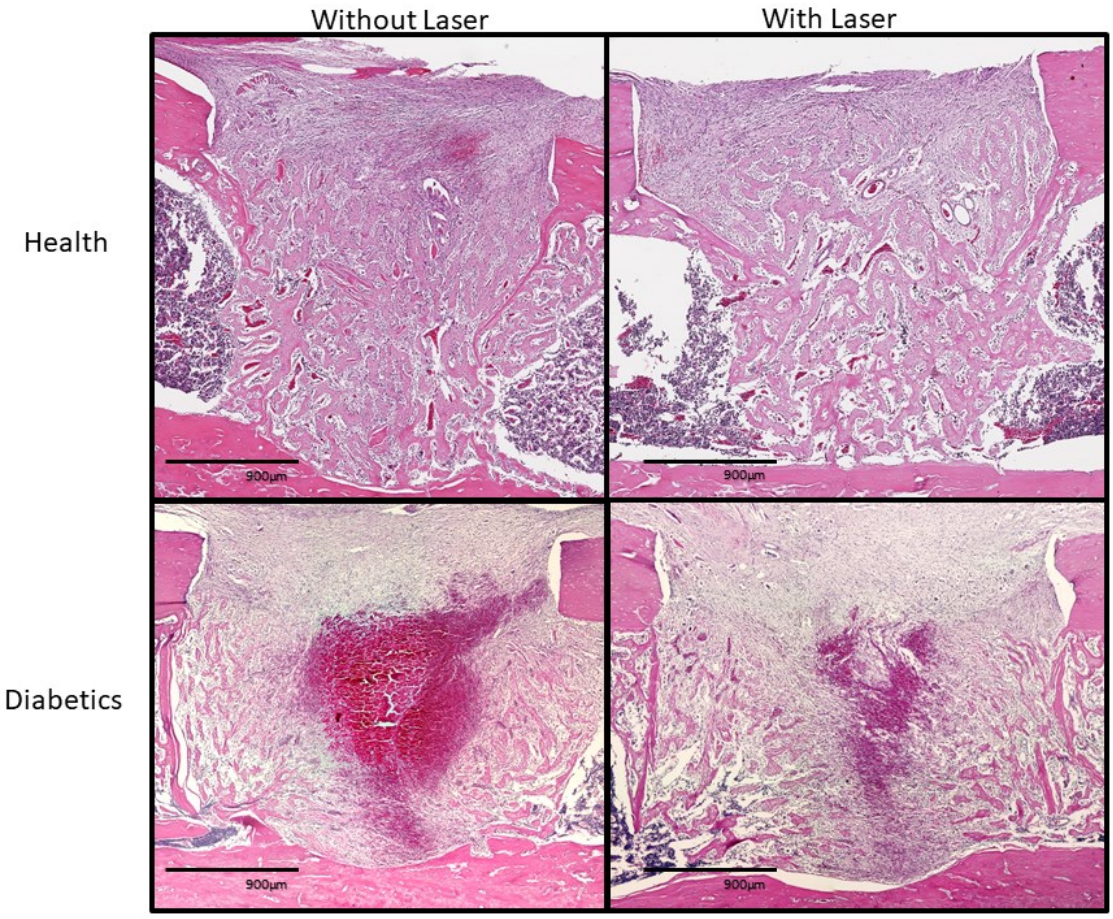

Figure 1: Longitudinal femur sections showing cortical bone (C), bone marrow (M), new bone formation (N), remnant clot (X),. Hematoxylin-Eosin.

Conclusões

3 - CONCLUSÕES

Pode-se concluir do presente estudo que:

- Os resultados mostraram que o diabetes mellitus tipo 1 altera a arquitetura estrutural da hidroxiapatita e do colágeno, efeito caracterizado pela redução na capacidade em absorver energia, na força máxima à fratura e na rigidez, bem como aumento do risco de fratura.
- A oxigenação hiperbárica mostrou ser um importante tratamento ao melhorar propriedades mecânicas e neoformação óssea em ratos diabéticos.
- A laserterapia de baixa potência foi um tratamento importante para minimizar os efeitos inflamatórios, durante o processo de reparação óssea. E, apesar de não ter aumentado o volume ósseo, a laserterapia de baixa potência interferiu positivamente na estrutura morfológica do tecido ósseo neoformado, tanto no grupo saudável quanto no diabético.

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ANEXO

Universidade Federal de Uberlândia

– Comissão de Ética na Utilização de Animais –

CERTIFICADO

Certificamos que o protocolo para uso de animais em experimentação nº 026/14, sobre o projeto de pesquisa intitulado "Efeito da oxigenação hiperbárica e do laser de baixa potência no reparo e biomecânica em osso de ratos diabéticos", 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 Agosto de 2014.**

(We certify that the protocol nº 026/14, about "Efeito da oxigenação hiperbárica e do laser de baixa potência no reparo e biomecânica em osso de ratos diabéticos", 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 29/08/2014).

Uberlândia, 29 de Agosto de 2014.

Prof. Dr. César Augusto Garcia
Coordenador da CEUA/UFU

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