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Luis Gustavo Gonzalez Osuna

Influência da posição de aplicação de carga no defeito ósseo e da distância dos suportes no teste de flexão de três pontos da tíbia de rato - análise experimental e de elementos finitos

Influence of load application position on bone defect and spam distance on the three-point bending test of rat bone tibia – finite element and experimental analyses

Dissertação apresentada à Faculdade de Odontologia da Universidade de Uberlândia, como requesito parcial para obtenção do Título de Mestre em Odontologia na Área de Clínica Odontológica Integrada.

Uberlândia, 2019

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Orientadora: Prof. Dra. Priscilla Barbosa Ferreira Soares

Banca Examinadora:

Prof. Dra. Priscilla Barbosa Ferreira Soares - Universidade Federal de Uberlândia

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Timbre

Ata

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

Defesa de: Dissertação de Mestrado - COPOD

Data: 27/02/2019

Discente: **Luis Gustavo Gonzalez Osuna (11712ODO016)**

Título do Trabalho: ***Influência da posição de aplicação de carga no defeito ósseo e da distância dos suportes no teste de flexão de três pontos da tíbia de rato – análise experimental e de elementos finitos.***

Área de concentração: Clínica Odontológica Integrada.

Linha de pesquisa: Processo de Reparo.

Projeto de Pesquisa de vinculação: Processo de Reparo.

As **nove horas** do dia **vinte e sete de fevereiro de 2019** no Anfiteatro do 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 2019, assim composta: Professores Doutores: Darceny Zanetta Barbosa (UFU); Marcela Claudino da Silva Nardino (UEPG); e o orientador(a) do(a) candidato(a): **Priscilla Barbosa Ferreira Soares.** Ressalta-se que o Profa. Dra. Marcela Claudino da Silva Nardino participou da defesa por meio de web-conferência desde a cidade de Ponta Grossa UEPG (Ponta Grossa) e os demais membros da banca e o aluno participaram *in loco*.

Iniciando os trabalhos o(a) presidente da mesa **Dra. Priscilla Barbosa Ferreira Soares** 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 Dissertação de Mestrado é parte dos requisitos necessários à obtenção do título de Mestre. 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 11 horas e 01 minutos. Foi lavrada a presente ata que após lida e achada conforme foi assinada eletronicamente pela Banca Examinadora.

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DEDICATORIA

Dedico este trabalho à Luz dentro de cada um de nós, aquela voz interior que sabe que você está no mundo para cumprir um propósito único que só corresponde a você e a ninguém mais; que nos permite avançar mesmo que as circunstâncias sejam adversas, porque sabe que há um futuro melhor pela frente; que nos faze acordar todas as manhãs com a confiança de que o caminho nos guiará na busca das respostas.

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Agradeço à inteligência universal por esta experiência de vida, os milagres de todos os dias, seu cuidado, sua proteção, sua ajuda, sua orientação e acima de tudo seu Amor Incondicional e Ilimitado durante meu processo evolutivo.

Agradeço a todas as pessoas que conheci, conheço, ou nunca conheci por algum motivo, mas que contribuíram para Ser quem eu Sou hoje, do fundo do meu coração e da minha alma muito obrigado. Sou grato por ter tido a oportunidade de aprender com a luz e a escuridão de cada Ser que me tocou, assim como de ensinar, pois tenho a certeza que nada acontece por acaso, e no caminho que é a vida somos alunos e maestros de outros. Da mesma forma agradeço a quem conhecerei no futuro e me ajudará a ser uma versão melhor de mim.

Também dou agradecimentos ao Brasil que foi meu refugio e oportunidade em tempos difíceis, em especial a suas agencias de fomento CAPES, FAPEMIG e CNPq e a instituçoes como a OEA por mudar a vida de muitas pessoas como eu.

EPÍGRAFE

“Todo guerreiro da luz já ficou com medo de entrar em combate.

Todo guerreiro da luz já perdeu a fé no futuro.

Todo guerreiro da luz já trilhou um caminho que não era o dele.

Todo guerreiro da luz já sofreu por coisas sem importância.

Todo guerreiro da luz já achou que não era guerreiro da luz.

Todo guerreiro da luz já falhou em suas obrigações espirituais.

Todo guerreiro da luz já disse sim quando queria dizer não.

Todo guerreiro da luz já feriu alguém que amava.

Por isso é um guerreiro da luz; porque passou por tudo isso, e não perdeu a esperança de ser melhor do que era”.

PAULO COELHO

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

gr - gramas

mm - milímetro

MPa - Mega Pascal

N – Newton

mJ- milijoule

RESUMO

RESUMO

Diferentes métodos biomecânicos podem ser utilizados para caracterizar as propriedades estruturais do osso, sendo que o ensaio de flexão de três pontos é o método mais utilizado, mas experimenta uma variabilidade dos parâmetros durante sua execução, pelo que resulta necessário um consenso e padronização do teste. Neste sentido o objetivo de estudo foi definir, por meio de ensaios experimentais e por análise de elementos finitos parâmetros metodológicos para a avaliação do reparo ósseo de defeitos monocorticais na metáfise proximal da tíbia de ratos. Defeitos osseos foram criados em 60 tíbias de ratos Wistar machos. Os animais foram eutanasiados 7 dias após a cirurgia. Cinco espécimes foram usados para criar modelos 3D para análise de elementos finitos. Foram avaliadas pelo teste de flexão de três pontos (n = 10) e análise de elementos finitos (n = 5) duas distâncias de spam: 6 e 10mm; e tres posição do defeito ósseo em relação à ponta de aplicação de carga: para cima, frontal e para baixo. A hipostes nula testada foi que a distancia dos suportes e a posição do defeito não teveram influencia no comportamento biomecânico do osso durante a alicação do teste de flexão de três pontos. Os resultados demonstraram que as distâncias dos suportes e a variação da posição do defeito ósseo durante a aplicação da carga tiveram influência significativa no comportamento biomecânico do osso no teste de flexão de três pontos.

Palavras chaves: defeito ósseo, elemento finito, teste de flexão.

ABSTRACT

ABSTRACT

Different biomechanical methods can be used to characterize the structural properties of the bone, and the three-point bending test is the most used method, but it experiences a variability of the parameters during its execution, so a consensus and standardization of the test is necessary. In this sense, the objective of this study was to define, through experimental and finite element analysis, methodological parameters for the evaluation of bone repair of monocortical defects in the proximal metaphysis of the tibia of rats. Bone defects were created in 60 tibias of male Wistar rats. The animals were euthanized 7 days after surgery. Five specimens were used to create 3D models for finite element analysis. The three-point bending test (n = 10) and the finite element analysis (n = 5) were two spam distances: 6 and 10mm; and three position of the bone defect in relation to the load application tip: up, front and down. The null hypothesis tested was that the distance of the supports and the position of the defect had no influence on the biomechanical behavior of the bone during the presentation of the three point flexion test. The results demonstrated that the distances of the supports and the variation of the position of the bone defect during the application of the load had a significant influence on the biomechanical behavior of the bone in the three-point bending test.

Keywords: Bone, defect bone, biomechanical analysis, finite element, bending test.

Introdução e Referencial teórico

INTRODUÇÃO E REFERENCIAL TEÓRICO

A reparação óssea é um processo complexo que requer a ação coordenada de várias sinalizações bioquímicas para promover fases da angiogênese e osteogênese, as quais se distinguem por alterações moleculares, celulares e teciduais que levarão ao completo restabelecimento do tecido lesado sob condições ideais (Loi *et al*., 2016).

Estudos pré-clínicos com roedores são frequentemente utilizados para avaliar o efeito das terapias no metabolismo ósseo e correlacionar sua influência em humanos, na tentativa de melhorar o processo de reparo ósseo (Varela *et al.,* 2017; Wong *et al*., 2018), recuperar quantidade e qualidade óssea sob condições adversas, sejam estas sistêmicas ou locais como nos casos de osteoporose (Stürmer *et al*., 2006) ou osso irradiado (Merli *et al*., 2005). Outro fator importante a considerar nesses estudos é o tipo de defeito, que pode ser por perfuração, osteotomia parcial ou total (Wong *et al*., 2018), devido ao fato de apresentarem diferentes mecanismos de reparo, sendo que os defeitos de perfuração cortical ocorrem por meio da via intramembranosa (Wong *et al*., 2018), sem a formação de cartilagem e são comumente usados para testar biomateriais (Tao *et al.,* 2015) ou drogas terapêuticas (Shang *et al*., 2014).

Em relação à posição do defeito, vários estudos experimentais focaram a análise na diáfise dos ossos longos (Freidouni *et al.,* 2015; Fridoni *et al*., 2015; Varela *et al*., 2017), sendo que a diáfise do osso longo consiste apenas de osso cortical e medula óssea, e grandes alterações decorrentes de doenças que afetam a fisiologia óssea, como a osteoporose, são observadas no osso trabecular que está localizado na metáfise (Stürmer *et al*., 2006; Claes *et al.,* 2009; Prodinger *et al*., 2018).

Alterações na homeostase dos elementos minerais (Brandi, 2009) e, consequentemente, de qualquer condição sistêmica (Stürmer *at al*., 2006) ou local (Merli *et al*., 2005) que a afetem, se manifestam em alterações na massa óssea mais precocemente e em maior extensão no osso trabecular do que no osso cortical (Brandi, 2009). O osso trabecular é metabolicamente mais ativo e tem o maior turnover, com maior taxa de remodelação, pois são encontradas as trabéculas mais próximas das células da medula óssea que participam desse processo (Brandi, 2009). Ossos cortical e trabecular apresentam diferentes mecanismos reguladores devido a diferenças estruturais e processos distintos de crescimento e envelhecimento em cada região (Chen *et al*., 2015; Kelly *et al*., 2016). Assim, modelos animais que avaliam apenas a diáfise de ossos longos, considerando apenas o componente cortical, são considerados inadequados para revelar diferenças na eficácia de novos tratamentos no tecido ósseo, de modo que o nível de maturação resultante nessas duas regiões pode influenciar as propriedades estruturais do tecido (Sandberg *et al*., 2015).

Vários parâmetros são utilizados para avaliar indiretamente as propriedades estruturais do osso, como geometria, conteúdo mineral , densidade volumétrica (Van *et al*., 2001) do osso completo (tanto osso corticalquanto osso trabecular) e / ou trabéculas individuais, embora as conclusões baseadas apenas nessas características sejam inadequadas, sendo que a integridade funcional óssea só pode ser avaliada por testes de força estrutural, o que fornece evidência sólida da habilidade real do osso em resistir à fratura (Van *et al*., 2001).

Diferentes métodos biomecânicos podem ser utilizados para caracterizar as propriedades estruturais dos diferentes fenótipos ósseos como parte essencial da pesquisa óssea, entre eles ensaios de tração, compressão, torção e três ou quatro flexão de pontos (Leppänen *et al*., 2008). É necessário consenso e padronização para evitar comparações de dados obtidos a partir de diferentes estudos que não foram gerados por metodologias semelhantes, para evitar a grande variabilidade dos dados nos estudos que leva a maior parte do tempo para resultados não significativos (Stürmer *et al*., 2006; Stuermer *et al.,* 2010; Boccaccio *et al*., 2011). A este respeito, o teste de flexão de três pontos é o método mais utilizado, considerado adequado para a análise quantitativa da qualidade biomecânica de ossos longos de pequenos animais, por meio da determinação da resistência estrutural. É um teste simples, preciso e reprodutível, além de alta sensibilidade (Leppänen *et al*., 2008).

Particularmente em relação a estudos para avaliar o efeito de terapias durante o processo de reparo de defeitos ósseos por perfuração cortical na região da metáfise que incluem o teste de flexão de três pontos, a literatura é limitada e fornece informações insuficientes sobre o posicionamento do defeito durante a execução do teste (Granito *et al*., 2011; Shang *et al*., 2014; Tao *et al.,* 2015), o que pode levar a extrair dados e consequentemente conclusões que não expressam a realidade. Desta forma, para determinar o efeito de qualquer terapia durante o processo de reparo em defeitos ósseos devido à perfuração, é essencial avaliar a resistência estrutural do osso e espera-se que o teste biomecânico tenha como região de interesse o defeito ósseo criado.

Em relação às distâncias dos pontos de apoio no teste de flexão de três pontos, não há padronização na literatura (Granito *et al*., 2011; Shang *et al*., 2014; Tao *et al.,* 2015), que não permite uma estabilização adequada do osso nos suportes durante a aplicação do teste pela configuração anatômica do osso e comprimento próprio na região da metáfise, o que constitui uma limitação ao estabelecer a distância dos pontos de apoio.

Quando o teste biomecânico é realizado no osso, são geradas deformações na sua estrutura produto da aplicação da força, obtendo-se uma curva força-deslocamento cujos dados podem ser quantificados; embora também são geradas tensões que não podem ser medidas diretamente sob condições experimentais (Leppänen *et al.,* 2008). Assim, o uso de elementos finitos é essencial para viabilizar essa análise de tensão, com definição e controle de parâmetros experimentais de maneira altamente controlada, permitindo predizer mudanças na resistência óssea e possíveis padrões de comportamento das estruturas ósseas diante de estímulos biofísicos, possibilitando experimentos *in vitro* com maior confiabilidade e reprodutibilidade (Boccaccio *et al.,* 2011).

Objetivos

Objetivos

Objetivo Geral

Definir, por meio de ensaios experimentais e por análise de elementos finitos parâmetros metodológicos para a avaliação do reparo ósseo de defeitos monocorticais na metáfise proximal da tíbia de ratos.

Objetivos específicos

1. Avaliar, por meio de ensaios experimentais, a influência da distância do suporte (6,0 ou 10,0 mm) no ensaio de três pontos e a posição da lesão criada na região da metáfise em relação ao local de aplicação de carga (posicionada para frente, para cima ou para baixo) na força máxima (N), energia (mJ), relação força (N) por deslocamento (mm), e padrão de fratura de tíbia de rato com defeitos padronizados realizados na região da metáfise;

2. Avaliar, por meio de simulação pelo método de elementos finitos, a influência da distância do suporte (6,0 ou 10,0 mm) no ensaio de três pontos e a posição da lesão criada na região da metáfise em relação ao local de aplicação de carga (posicionada para frente, para cima ou para baixo) na distribuição de tensões (von Mises - MPa) e a relação força (N) por deslocamento (mm) em modelos específicos gerados por microtomografia de tíbia de rato com defeitos padronizados realizados na região da metáfise;

3. Correlacionar e validar bilateralmente os ensaios experimentais e a análise de elementos finitos pela comparação das curvas de Força X Deslocamento extraídos pelos dois métodos; e a coerência entre distribuição de tensões e o padrão de falha obtidos experimentalmente.

Artigo

Artigo

Artigo a ser enviado para publicação no periódico Plos One

Influence of load application position on bone defect and spam distance on the 3-point bending test of rat bone tibia – finite element and experimental analyses

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Running title: Effect of the parameters on bending bone test.

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Influence of load application position on bone defect and spam distance on the 3-point bending test of rat bone tibia – finite element and experimental analyses

Abstract

Three-point bending test is the most commonly mechanical test used for quantifing the biomechanical quality of bone tissue and bone healing of small animals under several conditions. However, there is a lack of standardization when bone repair evaluation by cortical perforation is the focus. Therefore, the aim of this study was to determine the influence of load application position on bone defect created in the proximal metaphysis of tibias rats and the spam distance on the 3-point bending test to provide a methodological protocol. Cortical defects of 1.6 mm diameter were created at standardized location of medial surface of 60 tibias of male *Wistar* rats. The animals were euthanized 7 days after surgery. Five specimens were used to create 3D models for finite element analysis using high-resolution micro-CT images. Two distances of spam: 6 and 10mm; and the position of the bone defect in relation to the load tip: upward, frontal and downward; were evaluated by three-point bending test (n=10) and finite element analysis (n=5). Data of maximum load (N) and Energy (mJ) were analyzed by using 2-way ANOVA and Tukey test (α=0.05). The results demonstrated that the distances of the supports and variation of bone defect position during the load application had a significant influence on the fracture pattern, stress distribution and force *versus* displacement relation. Therefore, from the results we can recommend the following: creation of the bone defect located at 8mm from the extremity of the proximal epiphysis; establishment of 10 mm of distance between the supports and downward defect positioning, since this scenario presents a better distribution of stress with more evident fracture patterns that reached the target area represented by bone defect and also showed less intra-group variability.

Keywords Bone, defect bone, biomechanical analysis, finite element, bending test.

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Introduction

Development of new therapeutic strategies and biomaterials to improve bone repair process, or to recover bone quality under systemic or local condition (e.g.: osteoporosis or irradiated bone) required standardized methodological approaches that play a pivotal role in this research field (1,2). An important factor to consider in these studies is the type of defect, which may be by perforation of the cortical, partial osteotomy or total fracture. Each experimental model present different mechanisms, once bone repair of cortical perforation defects occurs by intramembranous ossification, whereas bone repair by fracture takes place as a result of abundant cartilaginous callus formation followed by endochondral ossification (3). Regarding defect area, various experimental studies focused their analysis on the diaphysis of long bones (1,2,4). However, to evaluate mechanical bone alterations integrally, it is indicated to consider the region of metaphysis of the femur or the tibia (5). The reason is that diaphysis of the long bone consists only by cortical bone and bone marrow, whicj major changes result by bone degenerative diseases, are also observed in the trabecular bone that is located in the metaphysis (3,6–9).

Several structural parameters such as geometry, mineral content and volumetric density are used to indirectly evaluate the biomechanical properties of bone. Nonetheless, bone ability to resist the fracture can only be assessed by biomechanical strength tests (10), which conclusions are based mainly by structural characteristics. Different biomechanical methods can be used to characterize the structural properties of the bone, among them are tensile tests, compression loading, torsion test and three- or four-point bending tests (11). Three-point bending test is the most commonly used mechanical test to characterize bone material and biomechanical properties of long bones, and also allows to analyze varied sample shape and minimal mechanical processing (12,13). In order to determine the effect of any therapy during the repair process on bone defects, it is essential to evaluate the structural resistance of the bone and it is expected that the biomechanical data represent the analysis of the bone defect area. Standardization of three-point bending test on the methaphysis of long bone without bone defect has been reported (3,7,14). However, there is a lack in the literature regarding parameters definitions of the test, such as spam distance and bone defect positioning during the stabilization of the bone over the supports and the determination of the local of loading application (12–14). Adequate stability of the bone between the supports during the test must be determined by the length of metaphysis region, which constitutes a limitation to establish the spam distance. It is necessary a consensus and standardized protocol to avoid comparisons of data obtained from different methodological parameters and to evaluate biomechanical properties exactly in the targed area of the bone defect (3,7,14).

During the 3-bending test with the bone tissue, deformations are generated towards the load application and a force-displacement curve is possible to be obtained. The parameters like maximum load and energy is possible to be calculated by using the curves. However, if the parameter of the test is modified, different stress distribution is generated. However, stress distribuction can not be measured under experimental conditions (11). Consequently, finite elements method is essential to quantify the stress concentration locations allowing to predict subtle changes in bone resistance and possible behavior of inner bone structures during biophysical stimuli, enabling greater reliability and reproducibility (15,16). Considering to better explain the mechanism involved in bone response for mechanical stimules, the combination of experimental mechanical test and finite element analysis should be used. On author's knowledge, no study has analysed the effect of the parameter of the 3-bending test used. Therefore, the aim of this study was to determine the influence of load application position on bone defect created in the proximal metaphysis of tibias rats and the spam distance on the 3-point bending test to provide a methodological protocol. The null hypothesis tested was that the variation of bone defect position and the distances of the base supports during the load application has no influence on the biomechanical properties of bone tissues.

Materials and Methods

Characterization of the sample

This study was carried out in strict compliance with the ethical principles for the care and use of laboratory animals and according to the ARRIVE guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Uberlândia (Protocol Number: 178/17, CEUA-UFU). Thirty male Wistar rats, clinically healthy at 8 weeks of age (300-320g) were included. The animals were kept at the Animal Experimentation Center of the Federal University of Uberlândia in closed plastic cages with a temperature of 22 ° C and a light-dark cycle of 12 hours. Diet consisted of standard laboratory pellets (Labina, Purina, Paulinia, SP, Brazil) and water *ad libitum*.

Surgical procedure of bone defect creation

The animals were submitted to anesthesia intraperitoneally, using 0.07 ml / 100 g of muscle relaxant xylazine hydrochloride 2%, and 0.1 ml / 100 g of anesthetic and analgesic 10% ketamine hydrochloride. After trichotomy (Fig. 1A), antissepsia was performed with 2% chlorhexidine solution and the operative area was delimited with sterilized fenestrated surgical field adapted to the procedure. Surgical access to the tibia methaphysis was obtained by means of a continuous longitudinal standardized incision of 2 cm in length. After that, the musculature was divulsed until the periosteum, which was exposed and incised. The tibia was delimited in three portions (upper, middle and lower), aiming to standardize the area to be manipulated, thus half of the medial face of the proximal metaphysis region was the chosen area to the creation of the bone defect. A bone defect was performed in standardized location with a drill measuring 1.6mm diameter (Neodent, Brazil) at 15000 rpm, under irrigation with sterile saline solution of 0.9% sodium chloride (Fig. 1B). The depth reference of the perforation was the rupture of the cortical bone to the bone marrow (Fig. 1C). The suture was performed with 4-0 nylon surgical monofilament. All procedures were performed by a single operator (IM).

Sample collection

The animals were submitted to euthanasia after 7 days of surgery, by intraperitoneal overdose of thiopental (150mg/kg). A longitudinal incision was performed following the existing cutaneous scar, the tissues were divulsed and the tibias were removed by disarticulation (Fig 1D). The metaphysis length (from the crest of the tibia to the proximal extremity of the epiphysis) and the distance from the center of the defect to the proximal extremity of the epiphysis of each specimen were measured using a pachymeter (Mitutoyo, Japan) at a precision of 0.01 mm. Then each tibia was covered with moist gauze containing sterile saline solution of 0.9% sodium chloride, stored in plastic tubes and frozen at -20 °C until the time of analysis.

Three-point bending biomechanical test

Each sample was submitted to a 3-point bending tests to failure using a universal testing machine (EMIC DL 2000, EMIC Equipamentos e Sistemas de Ensaio Ltda, São José dos Pinhais, Brazil). In order to avoid bias during the allocation of the experimental groups, the specimens were systematically coded by a third blinded person and randomly distributed into six groups (n=10) generated by combination of the two study factors, 1) bone defect position duing load application (to upward, to frontal and to downward) and 2) spam distances: with 6.0mm and 10.0mm between base supports.

The bone specimen was positioned horizontally under two base supports. The distance between the two lower supports (spam) was set at 6.0 or 10.0 mm. The metallic divice attached to the load cell 50kgf applied the load to the metaphysis with reference to the center of the bone repair area (bone defect), with the defect positioned upward, frontal and downward through at a crosshead speed of 1.00mm/min (Fig. 1E-J). The upper device and the two supports had 2.0 mm rounded surface to avoid shear load and cutting of bone tissue. The load and displacement data were recorded, and subsequently, the load versus displacement curves were plotted. The maximum load values (N) were derived from the experimental data and the energy (mJ) were calculated as the slope of the initial linear uploading portion of the curves.

3D finite element model generation

Five representative specimens were scanned by a high resolution micro-CT scanner (SkyScan 1272, Bruker Corporation Billerica, MA, EUA), (Fig. 2A-B). The images had approximately 880 slices with image pixel size of 16.31 um, obtained with exposure parameters of 80 kV and 125 uA, using filter aluminum of 1mm. The micro-CT projection data were exported in Device Independent Bitmap (BMP) file format and imported into an interactive medical imaging software (Mimics 21.0, Materialize, Leuven, Belgium). The segmentation of the different structures (compact bone, cancellous marrow and bone defect) was accomplished using image density thresholding (Fig. 2C-D). After segmentation, the 3D triangle-based surface of each bone structure (Fig. 2E) was exported in stereo lithography (STL) format, which is a file format that stores information about the external topography of the bone specimens. For the optimization of the mesh was use the software 3-Matic 8.0 (Materialize) (Fig. 2 F-G). The STL surface models were imported and meshed in MSC.Patran® 2010 (MSC.Software, MSC software, Santa Ana, CA, USA) with tetrahedral elements, which is element number 134. The created volumetric element mesh was imported in a FEA software package (MSC.Marc/Mentat; MSC. Software) to perform the structural analysis. All materials were considered linear-elastic, isotropic, and homogeneous(17). The applied properties of bone tissues and bone deffect (elastic modulus, Poisson's ratio) were obtained from the literature (Table 1).

To simulate the 3-point bending test, three triangular surfaces with rounded corners, similar to experimental conditions were constructed, two at lower position of the bone and one at upper position, equidistants from each other. All bone models (n=5) were positioned in three different test conditions, changing the position of the bone defect (upward, frontal and downward) and evaluating two distances between the lower surfaces (6 and 10mm), defining 6 conditions for each bone molde (Fig. 2H-M), resulting in 30 models. The interfaces between the different tissues were considered bonded (except for the bone surfaces to the metallic supports, which were represented by frictional contact – 0.5 fricctional coefficient). The tibia was fixed to prevent translation in the x-, y- and z-directions, although the boundary conditions allow the tibia to adapt to the supports during the application of the loading force perpendicular to the tibia. The models were loaded simulating the three-point bending tests with a load of 50 N in 11 increments and the displacement was recorded (Fig. 2N). The first and the second increment of the pre-load were of 0.1 N and 4.9 N respectively to allow the adaptation of the bone to the surfaces and the 9 remaining increments of 5 N. The von Mises equivalent stress and displacement ratio were recorded for all finite element models.

Radiographic analysis

Digital radiography images were obtained before and after the 3-point bending test with the dental x-ray unit (Gnatus, Brazil) at 70 kV, 7 mA, and 0.12 seconds in combination with VistaScan phosphor plates (3x4cm) (Dürr Dental, Bietigheim-Bissingen, Germany). Phosphor plates were scanned by using a VistaScan Mini View (Dürr Dental). The digital radiography images were analyzed using the software DBSWIN 5.2.0 supplied with the VistaScan system (D 'urr Dental), in order to evaluate the fracture pattern (Fig. 1K-L).

Statistical analysis

The maximum force (N) and energy (mJ) data were tested for normal distribution (Shapiro–Wilk's test) and equality of variances (Levene's test), followed by parametric statistical tests. Two-way analysis of variance (2-way ANOVA) was performed to analyze the position of the defect (3 levels – downward, frontal and upward) and spam during (2 levels – 6.0 mm and 10.0 mm). Multiple comparisons were made using Tukey's test. All tests were performed using a significance level of $\alpha = 0.05$, and all analyses were performed using the Sigma Plot version 13.1 statistical package (Systat Software Inc., San Jose, CA, USA). The metaphysis length, the distance from the defect to proximal epiphysis and the stress distribution using the finite element method and was analyzed descriptively.

Results

Location parameters of the bone defect

The measurement of metaphysis of *Wistar* rat tibiae analyzed had a length of the 15.3 ± 0.7mm. The distance between the bone defect and the proximal epiphysis corresponds to 8.2 ± 0.9mm.

Three-point bending test – maximum force (N), energy (mJ) and failure mode.

The maximum force (N) mean and standard deviation values calculated by 3-point bending test of the rat bone tibia with the defect positioned to down, frontal and up are shown on Fig. 3A. Two-way ANOVA demonstrated significant effect for defect position (P = 0.03), however no significance was found for spam distance (P = 0.173) and interaction of defect position and spam distance (P = 0.913). The 3-point bending test performed with the defect positioned to frontal resulted in significant higher maximum force than when the bone defect was positioned to up and down.

The energy (mJ) mean and standard deviation values for the rat bone tibia with the defect positioned downward, frontal and upward are shown in Fig. 3B. Two-way ANOVA demonstrated significant effect for defect position ($P < 0.001$), however no significance was found for spam distance (P = 0.110) and interaction of defect position and spam distance (P = 0.406). The 3- point bending test performed with the defect positioned to frontal resulted in significant higher energy than when the defect was positioned to up and down.

The failure mode distributions for the tested samples are shown on Table 2. When the defect was positioned to down, the failure mode involved more the tested area of the prepared defect for both spam distance, with the 10.0mm of the spam distance resulting in more evident fracture line. When the defect was positioned to frontal, the most of the failure modes did not involve the defect region. When the defect was positioned to up, the most of the failure modes demonstrated crushing of the defect region and the separation of the epiphysis.

Stress distribution - Finite element analysis

The von Mises stress distribution for the rat bone tibia at the defect positioned to downward, frontal and upward using 6.0 and 10.0mm conditions for one representative model are summarized in Fig. 4. The use of 10.0 mm of spam distance resulted in higher stress concentration on the bone area located between load point and inferior supports, mainly in the region opposite the application of load, than when the 6.0mm of spam distance, irrespective of the defect position. When the defect was positioned to downward, the stress concentrated at the inferior surface close to the bone defect limit and at frontal surface (Fig. 4A). When the defect was positioned to frontal, the stress concentrated cortical integra of the tibia positioned at inferior surface and at the center of the posterior surface (Fig. 4B). When the defect was positioned to upward, the stress concentrated at the inferior surface, crest of the tibia and at the center of the posterior surface (Fig. 4C).

The von Mises stress distribution for the defect area for the five bone tibia models simulatin all defect positions and spam distances are summarized in Fig 5. When the defect was positioned to downward demonstrated better homogeneity of the stress distribution than the defects positioned to frontal and upward (Fig. 5A-C). The stresses are more concentrated at the target area, expressed by defect interface, when the defect was positioned to down (Fig. 5A). When the defect was positioned to frontal, no stress was observed on the target area (Fig. 5B). High stress concentrated are located in small area of the load application for the models where the defects were positioned to up (Fig. 5C). The stress distribution demonstrated great correlation with the failure mode distributions for all experimental conditions.

Force (N) X Displacement (mm) relation

The mean values of the relation between force (N) and displacement (mm) obtained from 0 to 50N by finite element analysis and experimentally for 3-point bending test of the rat bone tibia with the defect positioned to downward, frontal and upward with spam distance of 6.0 mm and 10.0mm are shown on Fig. 6A and 6B, respectively. The relation curves calculated by FEA and experimentally showed similar behavior, however the values calculated experimentally were always higher than FEA values. When the defect was positioned to frontal, the displacement values were lower, followed by defect positioned to downward, and to upward.

Discussion

Bone repair process might be influenced by many conditions, as chronic diseases or therapeutics approaches. Several studies have taken advantage of animal models by using long bones to evaluate such events (5,21). Three-point bending test is one of the main biomechanical test that provides reliable data of bone fracture resistance, being methodological standardization fundamental to the test (22). The results of the present study demonstrated that the variation of bone defect position during the positioning of bone on support device and the distances of the supports during the load application in 3-point bending test had a significant influence on the fracture pattern, stress distribution and force *versus* displacement relation. Additionally, the spam distance influenced the maximum force and energy of bone tissue. Therefore, the null hypothesis was rejected.

Bone exhibits a varied arrangement of material structures at different length scale and this entire scenario perform mechanical, biological and chemical functions. Each methodological approach for bone tissue evaluation can provide its own resolution, therefore, a combination of techniques is required to access the bone behaviour under diverse conditions (23). We evaluate bone repair after 7 days as long as it is a critical defect under healing process. We evaluate bone repair after 7 days as long as it is a critical defect under healing process. Taken this into account, biomechanical testing of structural properties is an important field of basic bone research. Long bones, as tibia and femur, are widely used to investigate bone repair characteristics. They present 3 parts: the diaphysis, formed by cortical bone; the metaphysis, consisted of cortical and trabecular bone; and the epiphysis containing the joint structures. Although 3-point bending test is the most suitable and established method to quantify the bone resistance to fracture (24), there is still a lack of standardization when metaphysis region is analyzed. This area plays an important role in bone biology researchs, particularly when alterations in trabecular bone architecture is the primary focus. Cortical defects surgically created by using round burs are commonly used to evaluate the efficacy of biomaterials and novel therapies in bone repair(25–27).

Surprisingly, few (28–30) have investigated biomechanical analysis in metaphysis area. It might be due to the lack of standardization protocols in 3-point bending test regarding defect position and spam distance. It is imposed by limitations such as the length of the metaphysis and the proximity to the articular surface, in association with the absence of parameters definitions for the load application location. In this context, we evaluated the effect of bone defect position and the spam distance on the stress distribution. The effect of bone defect position is possible to be observed since divergence during the sample placement is susceptible to occur. It was observed that stress concentration is higher in the target area (bone defect) in the group of defects positioned downward. Consequently, the fracture pattern crossed through the bone defect in this group. This aspect is explained by the fact that during the test, the sample is subjected to a combination of load-side compression forces and tensile forces on the opposite side where the defect is situated. As bone is less resistant to tensile, the fracture starts at the surface undergoing tensile forces and spreads toward the compression surface until its complete rupture (11). Taking this into account, in the upward position, the bone defect was the side underwent compression forces followed by crushing of this region of interest, whereas the cortical (non-target area) that resisted the tensile forces. It can explain the higher values of load displacement in this group. When analyzing the frontal positioning group, both compressive and tensile forces are located in cortical and did not comprised the defect region. As a consequence, it was observed a neutral area of stress in the bone defect leading to higher failure force and less load displacement entailing into accumulation of greater amount of energy until bone fracture.

Another fundamental aspect of our findings was the greater data homogeneity in the group of downward defect position when compared to others groups regardless of the distance of the supports. It is important to highlight this factor since high standard deviation values in studies might be observed conferred by the difficulty in the sample positioning that might suppress possible correlations to other methodological analysis regarding bone mineral density or bone formation with bone fragility (3).

Regarding the spam distance, it is fundamental to calculate bone structural properties such as stress, strain, moment of inercia and elasticity modulus (11). Variation of this value lead to non-comparability of data. The distance is usually fixed in 20mm and load applied perpendicularly in the middle of the bone. However, when the target area is the metaphysis, this protocol is not appropriate explained by the short dimension of the metaphysis that does not allow to fix the spam distance in 20mm. Some authors (17–19), employed 15 - 25mm spam distance to evaluate bone repair in metaphysis. In addition, description regarding lesion position during the biomechanical test is not specified in the aforementioned studies. Our study showed higher tension values in models with 10mm spam distance compared to 6mm, concentrated mainly in the opposite side to the load application resulting in failures modes with more evident fracture lines. (31) pointed to a more consistent and reliable results by reducing shear stress and increasing flexural ones. For this, supports distance were maximized as much as bone length allowed. Furthermore, the authors suggest that tibia is more indicated for 3-point bending test than other long bones due to triangular cross-sectional shape enabling reproducible sample positioning during the test. On the other hand, this triangular shape is also considered a limitation in terms of the Euler–Bernoulli beam theory (17,31).

The 3D finite elements models of 3-point bending test created from 5 tibias provided desirable data to analyze the stress distribution during the experiment, excluded possible methodological bias and considered possible anatomical variations of the samples. The stress concentration in the internal structure of bone can not be directly obtained from experimental tests (11), neither from the distincts bone parts constituents. For this reason, finite elements analysis exerts an essential role in the stress distribution assessment in the same model to the different load application position on bone defect and variation of spam distances which is not possible to perform on three-point bending test once it is a destructive method. Force and displacement response can be obtained by experimental method such as FEA analysis. Thus, the covalidation of both methods was performed using force and displacement data. The experimental design of the present study showed consistency of the results between the methodologies applied. The FEA models explained the fractures pattern that took place in the samples after the load. Greater concentration of tensile tensions was shown where the fracture occurs reinforcing the need to associate different methodologies to establish biomechanical protocols. Furthermore, FEA also minimize the number of animals included in the experiment, which is in agreement with the Guidelines for Ethical Conduct in the Care and Use of Animals (32).

Among the limitations of the present study, it is important to mention that FEA models were created considering the materials as linear, isotropic and homogeneous, even though biomechanical behaviour of the bone is considered complex due to its viscoelastic, anisotropic and heterogeneous characteristic attributed to macro-, micro- e nanostructure of the bone (11, 23). However, to minimize this bias, the results were validated by laboratorial experiment. We must also point out that if the distance between the bone defect and the limit of the epiphysis was shorter than 8.0mm and did not allow a correct standardization of the test with a spam distance of 10 mm, this sample was not used because it did not allow a centralized and stable placement of the defect bone between the supports.

Therefore, from our results is it possible to recommend the following: create the bone defect located at 8mm from the extremity of the proximal epiphysis to allow a correct positioning of the defect and to make sure trabecular bone in the repair area and also providing greater stability by placing supports in a flat area avoiding the condyle; use a 10.0 mm spam distance with the defect position facing down, so it is necessary to mark the perimeter where the defect is located in order to apply the load in the same direction of the defect; stabilize the bone in the support before load application, which is easily handling considering the stability provided by the triangular morphology of the tibia. Finally, try to associate biomechanical analysis with finite element simulation models to better explain the performance of the factors under study.

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Figures

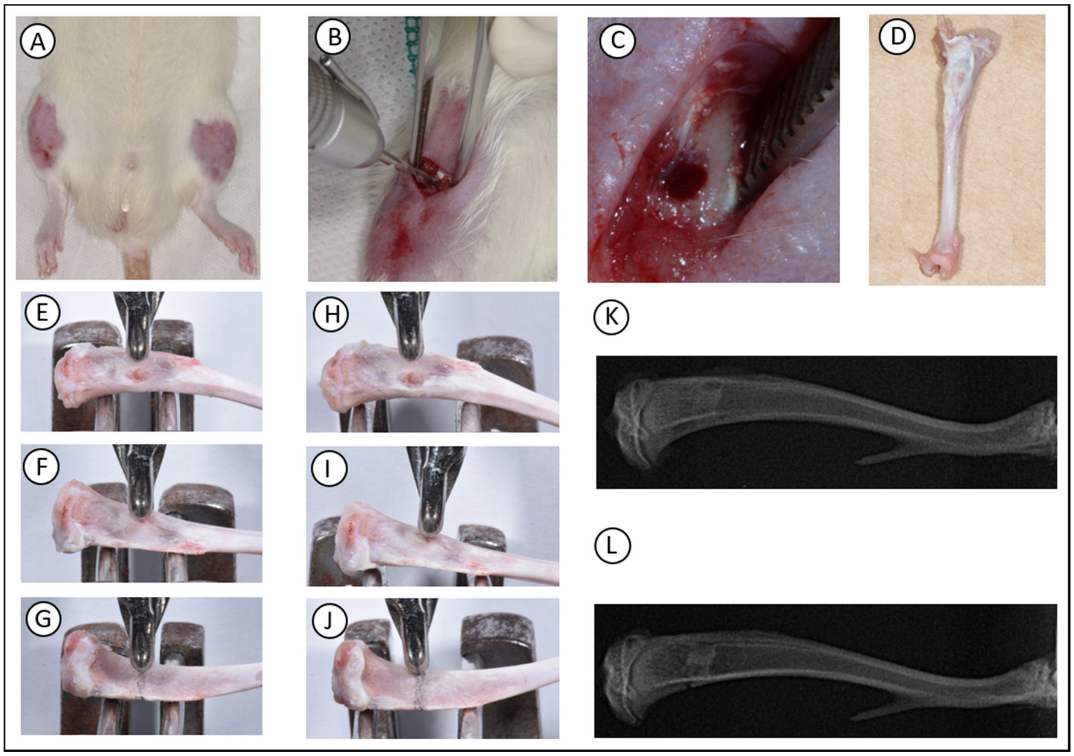

Fig. 1. Surgical procedure and positioning of the tibia during three-point bending test on the rat tibia. Radiographics shows the tibia before and after the test. A.Trichotomy on the tibia; B. Creation of the bone defect in standardized location on the tibia methaphisis; C. Bone defect of 1.6 mm in diameter; D. Removal of the tibia by disarticulation; Positioning of the load application on relation to the defect bone: E. frontal; F. upward; G. and downward with 6 mm of distance between the supports; H. frontal; I. upward; J. and downward with 10 mm of distance between the supports; K. Radiographic image of the tibia before 3-point bending test; L. Radiographic image of the tibia after 3-point bending test showing crack propagation on the bone defect area.

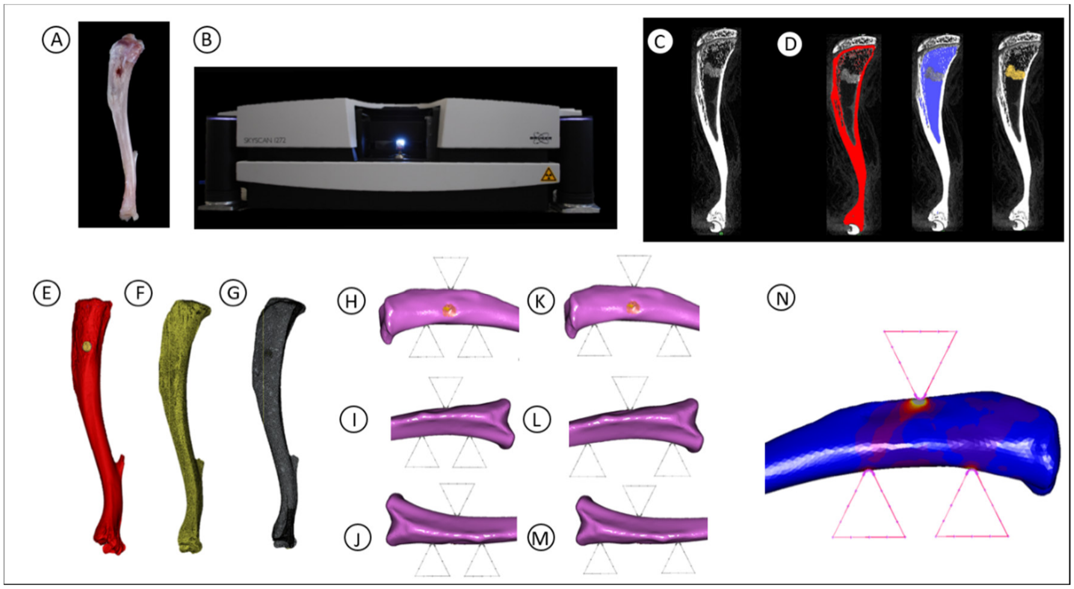

Fig. 2. Sequence of 3D models creation for simulation of the three-point bending test on the rat tibia for FEA, positioning the bone defect downward, frontal and upward in relation to the load application. A. Representative specimen of tibia with defect bone; B. Micro-Ct Scanner high resolution, SkyScan Bruker 1272; C. BMP file format for bone specimen with defect on metaphisis; D. Segmentation of the cortical, cancellous marrow and defect bone tissues; E. 3D model generated by the Mimics 21.0 software; F. STL-stereo lithography format file before optimization of the mesh; G. STL format file after optimization of the mesh by using Patran and 3Metric softwares; Positioning of the load application on relation to the defect bone: H. frontal; I. upward; J. and downward with 6 mm of distance between the supports; K. frontal; L. upward; M. and downward with 10 mm of distance between the supports; N. Post-processing image of the three-point bending tests simulating dynamic incremental loading of 50 N.

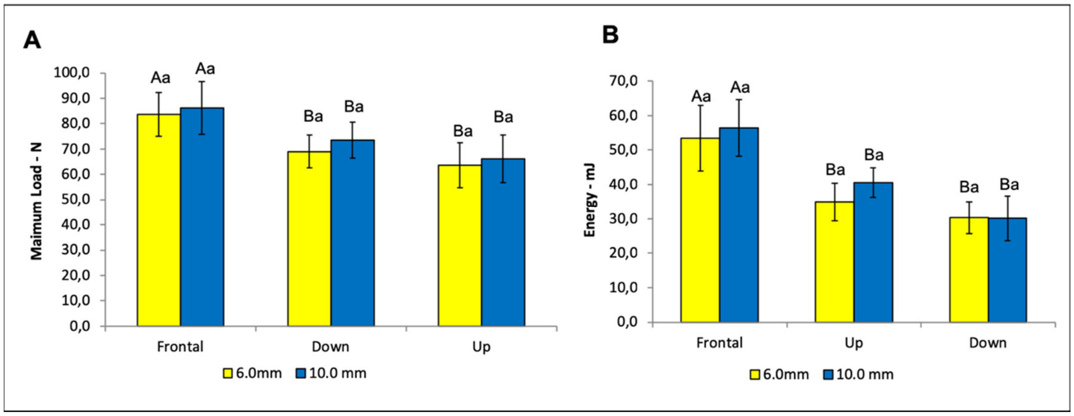

Fig. 3. A. Maximum force (N), **B.** Energy (mJ) calculated experimentally by three-bedding test with the rat bone tibia (n=10) with the defect positioned to down, frontal and upward position and spam distance of 6.0 and 10.0mm.

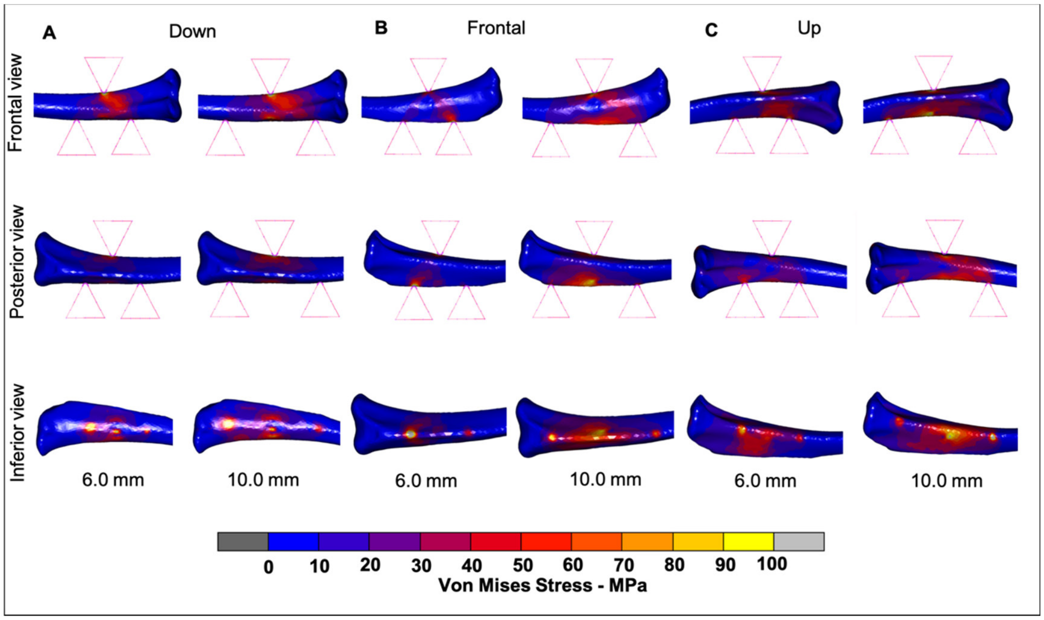

Fig. 4. Von Mises stress distributions on frontal, posterior and inferior surfaces of rat bone tibia during three-point bending test when the defect is positioned to down, frontal and up.

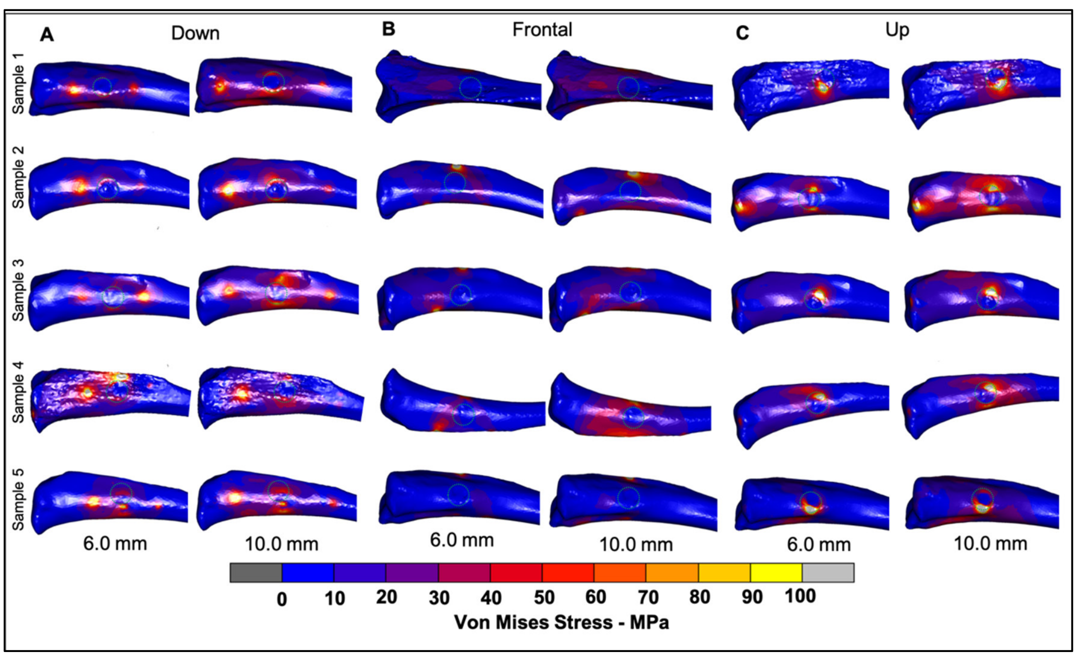

Fig. 5. Von Mises stress distributions of the surface of the rat bone tibia where the defect was made when the defect is positioned to down, frontal and up during three-point bending test.

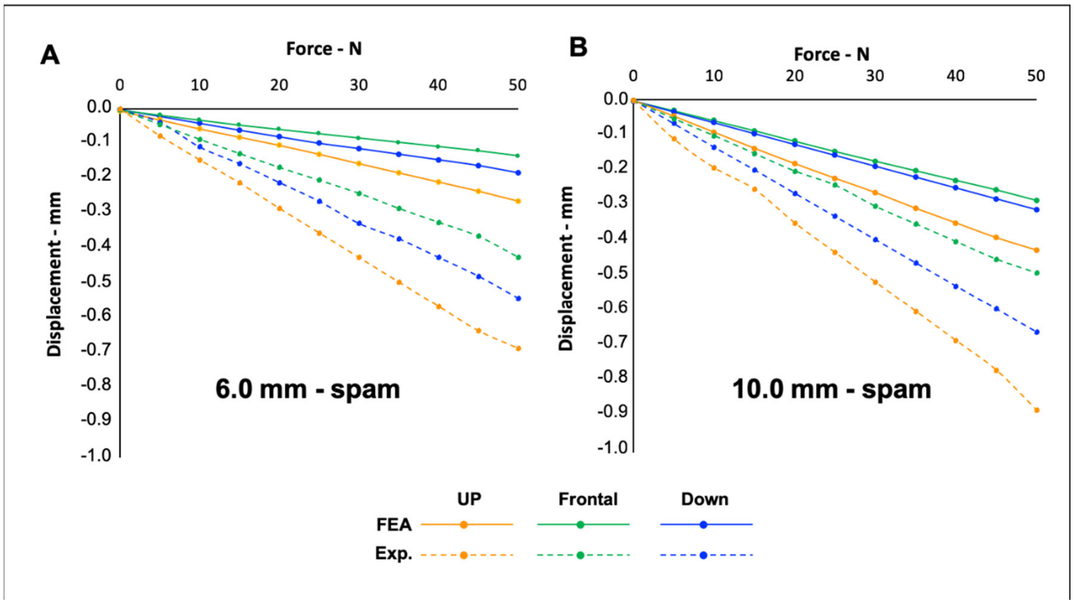

Fig. 6. Force (N) X displacement (mm) relation obtained for finite element analysis - FEA (n=5), and experimentally – Exp. (n=10) of the three- point bending test with the rat bone tibia where the defect was positioned to down, frontal and up position.

Table 1. Mechanical properties used in the FE model

Structure	Elastic modulus (MPa)	Poisson's ratio	References
Cortical bone(18)	20000	0.3	18
Cancellous marrow(19)	50	0.3	19
Bone defect (18,20)	2100	0.3	18,20

Table 2. Classification of the fracture mode for bone tibias after three bedding tests.

Position of the defect during three bedding tests.								
Down			Frontal			UP		
Spam distance		Fracture modes	Spam distance		Fracture modes	Spam distance		Fracture modes
6.0mm (n=10)	10.0mm (n=10)		6.0mm (n=10)	10.0mm (n=10)		6.0mm (n=10)	10.0mm (n=10)	
4	3	Starting at defect surface involving frontal surface reaching to superior loading point	6	3	Staring at loading point involving posterior surface reaching the inferior surface at the center of spam - no involvement of defect	5	5	Crushing the defect area with fracture line at superior involving the initial of the anterior and posterior surfaces.
1	4	Starting at defect surface involving all surfaces dividing the sample at defect line	2	4	Staring at loading point involving posterior surface reaching in diagonal line the condyle - no involvement of defect	1	0	Crushing the defect area with fracture line involving posterior surface.
4	2	Starting at defect involving inferior surface only	2	3	Staring at loading point involving posterior and inferior surfaces at the center of spam reaching the base of defect	1	3	Crushing the defect with total fracture of the sample in diagonal line to the inferior support close to the condyle
1	1	starting at load area involving frontal surface reaching condyle at inferior support area				3	2	Crushing he defect area, with no evident fracture in this area. Fracture starting at inferior support area involving posterior surface.

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Anexo

Anexo

Anexo 1. Parecer do Comitê de Ética

Universidade Federal de Uberlândia
Pró-Reitoria de Pesquisa e Pós-Graduação
Comissão de Ética na Utilização de Animais (CEUA)
Rua Ceará, S/N - Bloco 2D, sala 02 – CEP 38405-315
Campus Umuarama – Uberlândia/MG – Ramal (VoIP) 3423;
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ANÁLISE FINAL Nº 178/17 DA COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS PARA O PROTOCOLO REGISTRO CEUA/UFU 076/17

Projeto Pesquisa: "Efeito da suplementação de micronutrientes no reparo ósseo em ratos diabéticos".

Pesquisador Responsável: Priscilla Barbosa Ferreira Soares

O protocolo não apresenta problemas de ética nas condutas de pesquisa com animais nos limites da redação e da metodologia apresentadas. Ao final da pesquisa deverá encaminhar para a CEUA um relatório final.

Situação: PROTOCOLO DE PESQUISA APROVADO.

OBS: A CEUA/UFU LEMBRA QUE QUALQUER MUDANÇA NO PROTOCOLO DEVE SER INFORMADA IMEDIATAMENTE AO CEUA PARA FINS DE ANÁLISE E APROVAÇÃO DA MESMA.

Uberlândia, 18 de outubro de 2017.

Prof. Dr. Lúcio Vilela Carneiro Girão
Coordenador da CEUA/UFU
Portaria nº 665/17

Anexo 2. Release para imprensa

Modalidade: Pesquisa Científica.

Assunto: Dissertação defendida no Programa de Pós-Graduação em Odontologia – Faculdade de Odontologia – UFU.

Autores: Osuna LGG, Soares CJ, Rodrigues MP, Vilela ABF, Irie MS, Soares PBS.

São várias as condições que podem afetar negativamente o osso, tais como a osteoporose, a diabetes, a radiação, entre outras doenças. Muitas pesquisas são realizadas com o objetivo de avaliar estas condições e avaliar a efetividade dos novos tratamentos para melhorar o processo de reparação óssea. Muitos estudos fazem uma lesão em ossos longos, como a tíbia e o fêmur de ratos, para avaliar a cicatrização do defeito criado, porém, as análises geralmente são feitas na parte central do osso, que é denominada de diáfise. No entanto, as principais alterações que afetam o tecido ósseo têm um maior efeito em outra região situada na extremidade do osso, chamada de metáfise.

Testes biomecânicos são comumente utilizados em pesquisas para mensurar a resistência do osso à fratura. Dentre os testes, o mais utilizado é o teste de flexão de 3 pontos onde se tem dois suportes inferiores e uma carga que é aplicada de cima para baixo na metade da distância entre esses 2 suportes. No entanto, somente com esta metodologia, não é possível saber como as tensões ocorrem no interior do osso durante a aplicação de força, portanto, nós utilizamos uma simulação por computador através de um método denominado análise por elementos finitos que calcula e mostra por mudanças da cor, a distribuição de tensões quando uma força é aplicada. Diversos estudos científicos avaliam o reparo do osso por meio do teste de flexão de 3 pontos, porém, cada grupo de pesquisa realiza o experimento utilizando diferentes parâmetros de uma forma não padronizada, o que impede a comparação dos dados entre os estudos. Desta forma, o objetivo deste trabalho foi avaliar quais os melhores parâmetros para se realizar o teste de flexão de 3 pontos quando a lesão está localizada na região da metáfise de ossos longos. Foi avaliada a distância entre os suportes (6 ou 10 mm de distancia) e a posição da lesão em relação à aplicação de força (lesão posicionada para acima, para frente e para baixo). Da mesma forma, foi feita a simulação por meio de elemento finitos para testar as distancias e as posições da lesão.

Os resultados demonstraram que estes fatores influenciaram o teste, sendo que a posição da lesão voltada para baixo, em relação à aplicação da carga, apresentou melhores resultados, uma vez que, neste grupo, foi observada maior concentração de tensão ao redor da lesão, que é a região que se deseja avaliar e onde deve iniciar a fratura do osso, enquanto que, nos demais grupos, a fratura se inicia fora da lesão. Em relação a distância, melhores resultados foram demonstrados no grupo que se utilizou 10 mm de distância entre os suportes, já que fraturas muito mais evidentes foram observadas quando comparado com o grupo que utilizou a distância de 6mm. Portanto, com os resultados de nosso estudo, sugerimos que para futuras pesquisas avaliando o processo de reparo na metáfise o teste biomecânico de 3 pontos seja realizado posicionando a lesão voltada para baixo em relação à aplicação de carga com uma distancia entre os suportes de 10 mm, para que o teste tenha resultados mais confiáveis e para que haja uma padronização entre os estudos.

Contato: Secretaria PPG Odontologia, Sra. Maria das Graças, fone: 3225-8108.