JESSYCA FIGUEIRA VENÂNCIO

Efeitos da insulinoterapia e superfícies hidrofílicas e superhidrofílicas na redução dos impactos do diabetes tipo I na osseointegração de implantes em ratos

Effects of insulin therapy and hydrophilic and superhydrophilic surfaces in reducing the impacts of type I diabetes on implant osseointegration in rats

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

UBERLÂNDIA, 2022

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Banca Examinadora:

Orientadora: Profa. Dra. Paula Dechichi - UFU

Prof. Dr. Pedro Henrique Justino Oliveira Limirio - UFU

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Prof. Dr. Luis Eduardo Carneiro Campos - UFF

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UBERLÂNDIA, 2022

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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ATA DE DEFESA - PÓS-GRADUAÇÃO

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Data:	Vinte e Sete de Julho de Dois Mil e Vinte e Dois	Hora de início:	14:00	Hora de encerramento:	17:40
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Iniciando os trabalhos o(a) presidente da mesa, Dr(a). Paula Dechichi Barbar, 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 arguição e resposta foram conforme as normas do Programa.

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DEDICATÓRIA

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"Nós somos o que fazemos repetidamente. A excelência, portanto, não é um ato, mas um hábito. "

Aristóteles

SUMÁRIO

RESUMO

A hiperglicemia crônica decorrente do diabetes mellitus tipo I (DMTI) afeta múltiplos órgãos interferindo na qualidade de vida dos portadores da doença. No osso e, consequentemente na osseointegração de implantes dentários, esse quadro altera o metabolismo ósseo, comprometendo o reparo e as propriedades biomecânicas ósseas. Terapias coadjuvantes como insulinoterapia e alterações nas superfícies dos implantes têm sido propostas para favorecer a osseointegração e melhorar as condições teciduais ao redor dos implantes. O objetivo geral deste estudo foi avaliar o efeito da insulinoterapia e de superfícies hidrofílicas e super-hidrofílicas na microarquitetura, biomecânica e osseointegração de implantes em ossos de ratos com diabetes mellitus tipo I (DMTI). Foram utilizadas análises por meio de micro-tomografia computadorizada (MicroCT), análise biomecânica, espectroscopia no infravermelho transformada de Fourier (FTIR) e microscopia eletrônica de varredura (MEV). Os resultados mostraram que o DMTI altera a microestrutura, composição e dureza da matriz óssea, pela redução da superfície óssea, espessura cortical, anisotropia, dimensão fractal, maturação e mineralização do colágeno e microdureza óssea. O DMTI causou alterações estruturais no tecido ósseo afetando a osseointegração de implantes diminuindo o processo de neoformação óssea. A insulinoterapia minimizou o efeito do DMTI na espessura cortical e matriz orgânica/mineral no osso cortical de um modelo experimental de rato. A terapia com insulina mostrou resultados favoráveis para a osseointegração, no entanto, não normalizou o reparo, permanecendo significativamente diferente dos animais normoglicêmicos. Os implantes com superfície super-hidrofílica favoreceram a osseointegração, quando associados à insulinoterapia.

Palavras-chaves: Diabetes mellitus; osseointegração; insulinoterapia

ABSTRACT

Chronic hyperglycemia resulting from type I diabetes mellitus (DMTI) affects multiple organs, interfering with the quality of life of patients with the disease. In bone and, consequently, in the osseointegration of dental implants, this condition alters bone metabolism, compromising bone repair and biomechanical properties. Adjuvant therapies such as insulin therapy and changes in implant surfaces have been proposed to favor osseointegration and improve tissue conditions around implants. The general objective of this study was to evaluate the effect of insulin therapy and hydrophilic and superhydrophilic surfaces on the microarchitecture, biomechanics and osseointegration of implants in bones of rats with type I diabetes mellitus (DMTI). Analyzes by micro-computed tomography (MicroCT), biomechanical analysis, Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) were used. The results showed that DMTI alters the microstructure, composition and hardness of the bone matrix, by reducing bone surface, cortical thickness, anisotropy, fractal dimension, collagen maturation and mineralization and bone microhardness. DMTI caused structural changes in bone tissue, affecting the osseointegration of implants, decreasing the process of bone neoformation. Insulin therapy minimized the effect of DMTI on cortical thickness and organic/mineral matrix in cortical bone in an experimental rat model. Insulin therapy showed favorable results for osseointegration, however, it did not normalize the repair, remaining significantly different from normoglycemic animals. Implants with a superhydrophilic surface favored osseointegration when associated with insulin therapy.

Keywords: Diabetes mellitus; osseointegration; insulin therapy

1 - INTRODUÇÃO E REFERENCIAL TEÓRICO

A perda de dentes permanentes, como resultado de trauma ou doença, continua sendo um problema frequente em todo o mundo. Atualmente, a reabilitação com implantes dentários osseointegrados tem sido considerada a terapia de escolha para a restauração de áreas edêntulas, pois oferece biocompatibilidade e resultados previsíveis, para a maioria dos pacientes (1,2). Os implantes dentários foram desenvolvidos para aumentar o conforto e a qualidade das reabilitações orais complexas. Nas últimas décadas houve aumento pela procura tanto dos pacientes, como dos cirurgiões dentistas, por soluções estéticas e funcionais mais adequadas, com maior durabilidade e minimamente invasivas. Porém, com o crescimento desses dispositivos instalados, as complicações têm ficado cada vez mais frequentes (3).

O conceito clássico de osseointegração, descrito anteriormente por Brånemark, como um contato direto entre osso e implante (4), foi recentemente proposto como um fenômeno de resposta de corpo estranho associado à estimulação inflamatória contínua (5). Uma resposta imune é iniciada após a inserção do implante com o objetivo de isolar a superfície de titânio, em conjunto com a inflamação crônica dos tecidos moles (6,7). A interface estabelecida entre a superfície do implante e o osso neoformado foi descrita anteriormente como sendo composta por uma camada de matriz óssea pouco mineralizada, com a presença de osteócitos em íntimo contato com o titânio, combinada com seções estreitas por onde se ancoram extensões dendríticas dos osteócitos ao implante, apoiando a osseointegração a longo prazo (8). Uma vez rompido o equilíbrio sobre esses processos, o sucesso da reabilitação com implantes pode ser comprometido.

O diabetes mellitus deve ser reconhecido como um fator de risco potencial para osseointegração, ocorrência de inflamação peri-implantar e baixa sobrevida do implante, e deve ser levado em consideração no manejo do paciente e nas decisões de tratamento, bem como nos cuidados de acompanhamento (3). Estudos mostraram que a HbA1c (Hemoglobina Glicada) mal controlada pode ter efeitos negativos na osseointegração e estabilidade primária dos implantes dentários (9).

Diabetes mellitus, bem como condições pré-diabéticas, representam um problema de saúde comum e crescente (International Diabetes Federation in IDF Diabetes Atlas, International Diabetes Federation, Bruxelas, 2019) e com extensos efeitos nocivos em todo o organismo. (10). 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 (11). 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 (12,13). Os critérios diagnósticos para diabetes mellitus são uma glicemia de jejum no plasma venoso com uma concentração de ≥126 mg/dL, uma HbA1c≥6,5%, uma medição de glicose plasmática pós-carga de 2 horas de ≥200 mg/dL ou uma glicose plasmática aleatória ≥200 mg/dL na presença de sintomas de hiperglicemia, como polidipsia ou poliúria (10).

A hiperglicemia reduz a taxa de marcadores de formação óssea, incluindo osteocalcina, fosfatase alcalina específica do osso (bALP) (14–16) e propeptídeo Nterminal do procolágeno tipo 1 (PINP) (17), bem como marcadores de reabsorção óssea, incluindo soro C-terminal telopeptídeo de colágeno tipo I (CTX), (18,19). O aumento da osteoprotegerina sérica (OPG), um inibidor da reabsorção óssea, também foi relatado após hiperglicemia (20). A hiperglicemia também resulta no acúmulo de produtos finais de glicação avançada (AGEs), que afetam a estrutura do colágeno, resultando em uma qualidade da matriz óssea orgânica comprometida (21,22). Esses AGEs também podem reduzir a proliferação e função dos osteoblastos (23) e aumentar a reabsorção óssea relacionada aos osteoclastos (24), levando a uma deterioração geral da qualidade óssea. Evidências adicionais para o efeito adverso que a hiperglicemia tem na função dos osteoblastos foram fornecidas por estudos in vitro usando osteoblastos humanos primários (HOBs), onde altos níveis de glicose demonstraram suprimir a função das células osteoblásticas (25).

Há evidências crescentes de que o DMTI prejudica o metabolismo ósseo ao redor dos implantes dentários. Apesar das taxas de falha de implante parecerem semelhantes às de indivíduos saudáveis (26–29), e a doença está claramente associada a um risco elevado de complicações periimplantares (9,30,31). Além disso, os mecanismos

subjacentes da osseointegração anormal são complexos e ainda não completamente compreendidos.

Diferentes aspectos da interação entre DMTI e o tecido ósseo ao redor dos implantes têm sido explorados. As alterações ultra estruturais da matriz óssea em condições hiperglicêmicas foram detalhadas como desorganizada, com presença de trabéculas delgadas e pontos vazios, além de conteúdo exacerbado de proteoglicanos (32). Da mesma forma, análises histomorfométricas e biomecânicas revelaram consistentemente comprometimento da reparação óssea (33,34). Também foi demonstrada limitada formação óssea primária, correlacionada com níveis elevados de fator de necrose tumoral alfa (TNF-α), um reconhecido marcador do processo inflamatório (33). Além disso, o DMTI impacta negativamente o estado ósseo de implantes já osseointegrados, embora em ritmo mais lento e em menor grau. Estudos observaram redução do contato osso-implante e menores valores de remoção de torque (35) e redução da densidade óssea (36).

O titânio e suas ligas são os materiais de implantes dentários mais utilizados devido à resistência mecânica, inércia química e biocompatibilidade (37,38). Vários estudos relataram que implantes com superfície e poros rugosos foram benéficos para a osseointegração de implantes e que a superfície do implante é um ponto chave para o sucesso da osseointegração durante o estágio inicial da reparação óssea (39). Assim, a maioria dos estudos concentraram-se nas propriedades de superfície dos implantes (40).

Atualmente, estudos têm investigado as propriedades de superfície de implantes relacionadas aos aspectos biológicos do processo de osseointegração (41,42). Diferentes abordagens de modificação da superfície têm sido exploradas para otimizar a interação entre o implante e o tecido ósseo. Ao alterar as propriedades físico-químicas, propriedades osteocondutoras da superfície podem ser melhoradas auxiliando assim a adesão e fixação da célula ao implante e, consequentemente, a proliferação celular (43–45).

As superfícies super-hidrofílicas e hidrofílicas têm mostrado melhora na molhabilidade (hidrofilicidade), aumento do contato célula-implante e maior osteogênese (46,47), auxiliando na diferenciação de células mesenquimais (48) promovendo a mineralização precoce por meio de modulação da resposta inflamatória (42,49). Implantes super-hidrofílicos com tratamento de superfície quimicamente modificada

mostraram uma maior área de contato osso implante e maior molhabilidade quando comparado a implantes hidrofílicos (50) resultando em uma osseointegração precoce (51). Assim, esse tipo de superfície tem sido avaliado para melhorar ainda mais o processo de formação óssea em condições diabéticas e osteoporóticas (52,53).

O contato osso-implante parece ser menor em ratos diabéticos (54). No entanto, estudos em animais têm demonstrado que a administração de insulina pode melhor esse contato, possivelmente, por meio da melhora na formação óssea e inibição da reabsorção devido ao controle metabólico com insulina (55–57). O processo de osseointegração dos implantes em DMTI e o efeito da insulina ainda são parcialmente compreendidos e mais estudos são necessários em situações de reparo tecidual prejudicado (45).

Embora o diabetes mellitus tenha sido considerado um fator de risco relativo para a reabilitação com implantes, várias estratégias terapêuticas são utilizadas para limitar o progresso do diabético, para melhorar a qualidade de vida, como controle dietético, exercícios físicos e terapia de reposição de insulina. A terapia com insulina acompanhada pela redução da glicemia parece ser o ponto central no tratamento e prevenção de distúrbios ósseos na TIDM (58,59). Alguns estudos mostraram que a injeção de insulina reduz a produção endógena de glicose, glicemia de jejum e hemoglobina glicada A1c (HbA1c), o que melhora o controle glicêmico corporal (60,61). Além disso, a insulina possui propriedades osteogênicas que promovem a proliferação e aumentam a atividade da fosfatase alcalina e a síntese de colágeno nas células osteoblásticas por meio de ações diretas mediadas pelo receptor de insulina (62,63).

Terapias coadjuvantes que favoreçam o restabelecimento da normalidade da morfologia e função tecidual óssea, que reduzam possíveis prejuízos ao processo natural do reparo ósseo e, consequentemente favoreçam a osseointegração de implantes dentários, seriam interessantes em diversas situações clínicas. Dessa forma, neste estudo, foi avaliada a influência da insulinoterapia e das diferentes superfícies de implantes no reparo e na qualidade do tecido ósseo normal ou comprometido pelo diabetes mellitus tipo 1.

2 – CAPÍTULOS

2.1 CAPÍTULO 1

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RESEARCH ARTICLE

Type I Diabetes Mellitus and Insulin Therapy on Bone Microarchitecture, Composition and Mechanical Properties

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Abstract: ***Background*:** The aim of this study was to evaluate the microarchitecture, composition and mechanical properties of cortical bone of rats with type I diabetes mellitus (TIDM) and submitted to insulin therapy (IT).

***Methods*:** Thirty rats were divided into three groups (n=10): non-diabetic, diabetic and diabetic+insulin. TIDM was induced by intravenous injection of streptozotocin. In diabetic+insulin group, 4IU insulin was administered twice per day (1I U at 7 am and 3I U at 7 pm). The animals were euthanized five weeks after TIDM induction; the tibiae were removed and submitted to microcomputed tomography (micro-CT, 8 µm), fourier transform infrared spectroscopy (FTIR) and dynamic microhardness indentation.

***Results*:** Micro-CT analysis showed that diabetic group had lower bone surface/tissue volume ratio (BS/BV) (p=0.018), cortical thickness (Ct.Th) (p<0.001) and degree of anisotropy (Ct.DA) (p=0.034) values compared to non-diabetic group. The diabetic group showed lower Ct.Th than diabetic + insulin group (p=0.018). The non-diabetic group had lower fractal dimension (Ct.FD) values compared to diabetic groups (p<0.001). The ATR-FTIR analyses showed lower values for all measured parameters in the diabetic group than the non-diabetic group (amide I ratio: p=0.046; crystallinity index: p=0.038; matrix:mineral ratios - M:MI: p=0.006; M:MIII: p=0.028). The diabetic+insulin group showed a lower crystallinity index (p=0.022) and M:MI ratio (p=0.002) than non-diabetic and diabetic groups, respectively. The diabetic group showed lower Vickers hardness values than non-diabetic (p<0.001) and diabetic+insulin (p=0.003) groups.

***Conclusion*:** TIDM negatively affects bone microarchitecture, collagen maturation, mineralization and bone microhardness. Moreover, insulin minimized the effect of TIDM on cortical thickness and organic/mineral matrix.

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1. INTRODUCTION

Bone is a multiscale material made mostly of organic and inorganic matrix [1]. The organic matrix consists mainly (~90 %) of type I collagen, which is a triple helical molecule that is specifically arranged in hierarchical levels to provide bone elasticity and hardness [2]. The bone mineral matrix is composed of small, poorly crystalline and highly substituted apatite crystals that contribute to bone strength and stiffness [3]. The bone microarchitecture and mechanical properties depend on the specific arrangement and interaction between the organic matrix and mineral apatite crystals [2].

The substantial contribution of the collagen network and mineral crystals to the mechanical changes in bone structure was identified in several bone pathologies, such as type I diabetes mellitus (TIDM) [4, 5]. TIDM is an inflammatory autoimmune disease characterized by the destruction of pancreatic beta cells, which results in insulin deficiency and leads to chronic hyperglycemia [6]. Some studies showed that insulin deficiency in young patients with TIDM reduces peak bone mass and impairs bone formation [7-9]. Moreover, chronic hyperglycemia causes excessive formation of advanced glycation end products (AGEs) that leads to nonenzymatic crosslinking with collagen type 1 [10, 11]. Takeda *et*

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al., showed that increased AGEs levels in TIDM decreases the cortical bone surface area, volume, mineral density and reduces the bone biomechanical strength [12].

Several therapeutic strategies are used to limit diabetic progress to improve quality of life, such as dietary control, exercises, and insulin replacement therapy. Insulin therapy accompanied by reduction of glycaemia seems to be the pivotal point in the treatment and prevention of bone disorder in TIDM [13, 14]. Some studies showed that insulin injection reduces endogenous glucose production, fasting blood glucose and hemoglobin A1c (HbA1c), which improves the body glycemic control [15, 16]. Moreover, insulin has osteogenic properties that promotes proliferation and increases alkaline phosphatase activity and collagen synthesis in osteoblastic cells *via* direct actions mediated by the insulin receptor [17, 18]. However, the effects of insulin therapy on the structural cortical bone matrix of TIDM condition remains unknown.

We hypothesized that decreased hyperglycemia from insulin therapy could minimize the negative effects of TIDM on bone matrix microarchitecture, composition and mechanical properties, reestablishing the normal condition. Therefore, the aim of this study was to evaluate the effects of TIDM and insulin therapy on the rat tibia using microcomputed tomography (micro-CT), attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and dynamic microhardness indentation.

2. MATERIAL AND METHODS

2.1. Experimental Protocol

All experimental protocols with animals were approved by the Committee of the Ethics of Animal Use and Care of the Federal University of Uberlândia (permit number 026/14). All procedures were carried out in strict accordance with the recommendations in the Guide for the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Thirty male Wistar rats (*Rattus norvegicus*) weighing 240 ± 20 g (8 weeks of age) were housed in standard conditions (12 hour light/dark cycle, temperature of 22±1 °C and relative humidity of 50- 60 %), with food (composition: humidity, crude protein, ethereal extract, mineral, crude fiber, calcium and phosphorus) and water *ad libitum*. After one week of acclimatization, the animals were randomly assigned and equally distributed into the following three groups (n=10): non-diabetic, diabetic and diabetic + insulin. All animals were euthanized five weeks after the induction of diabetes.

The TIDM induction protocol began by fasting the rats for 24 hours. Anesthesia was performed *via* the intraperitoneal pathway using 7mg/kg xylazine (2 %, muscle relaxant) and 100 mg/kg ketamine hydrochloride (10 %, anesthetic and analgesic). Then, a single dose of streptozotocin (STZ, Sigma-Aldrich, Inc. St. Louis, MO, USA) was administered intravenously through a penile vein puncture at a dose of 60 mg/kg body weight, diluted in 0.1 M citrate buffer (pH 4.5) [4]. Equal protocol of anesthesia and volumes of vehicle was injected in the control rats. The hyperglycemia was confirmed by a glucometer (Accu Check Active, Roche, Jaguaré, SP, Brazil) after 24 hours of the induction, collecting a blood drop from the tail of each animal. Follow up of the glycemic rates was done one, two, three, four and five weeks after induction and animals that maintained blood glucose levels higher than 250 mg/dL were considered diabetic. Clinical diabetic signs such as polyphagia, polydipsia, polyuria, and bodyweight loss were also monitored in a qualitative analysis. The animals that did not reach the glycemic target were excluded from the study.

Thereafter, the diabetes confirmation, the animals of diabetic + insulin group received daily subcutaneous doses of 4 IU (1 IU at 7 a.m. and 3 IU at 7 p.m.) with neutral protamine Hagedorn insulin (Humulin U-100, 100 U/mL, Eli Lilly, São Paulo, Brazil) diluted in 0.9 % NaCl. The insulin doses were applied using a pen (Eli Lilly Humapen Savvio, Eli Lilly, São Paulo, Brazil) to reduce stress and performed under the same standardized conditions as previous pilot study (glucose concentrations were evaluated throughout the day over four weeks).

The animals were euthanized five weeks after diabetes induction by intraperitoneal injection with sodium thiopental and lidocaine in compliance with the principles of the Universal Declaration on Animal Welfare. The left and right tibiae were removed by disarticulation, immediately placed in gauze with physiological saline solution and kept frozen in a freezer (-20 °C). Twenty-four hours before the micro-CT analyses, the tibiae were defrosted in phosphate-buffered saline and then divided into fragments. From the tibia mid-diaphysis, 2mm was sectioned, in transversal axis, with a diamond disk under constant irrigation to ATR-FTIR analyses. The proximal and distal tibia were submitted to micro-CT and dynamic microhardness indentation test, respectively (Fig. **1**).

2.2. Micro-computed Tomography Analyses (Micro-CT)

The proximal tibia was scanned to obtain high-quality images, and the cortical bone was selected as the region of interest (ROI). All scans were performed using a micro-CT scanner (Sky-Scan 1272, Bruker, Kontich, Belgium), and the images were obtained under the following conditions: 80 kV voltage; 125 μA tube current; 1 mm aluminum filter; 180° rotation; 0.6 rotation step; and 8 μm resolution pixel size. Using a calibration scan of a hydroxyapatite (HA) phantom, the linear X-ray attenuation coefficients were converted to the volumetric tissue mineral density [19]. All of the resulting images were reconstructed using NRecon software (v.1.6.9.10, Bruker, Kontich, Belgium) [20]. For all reconstructed images, the ROI was defined as 4 mm of the tibia at 2 mm distant from the growth plate Fig. (**2**). The trabecular and cortical bone were distinguished and separated by automatic processes, and only cortical bone was analyzed (CT Analyzer, v. 1.14.4.1+(64-bit), SkyScan, Bruker, Kontich, Belgium).

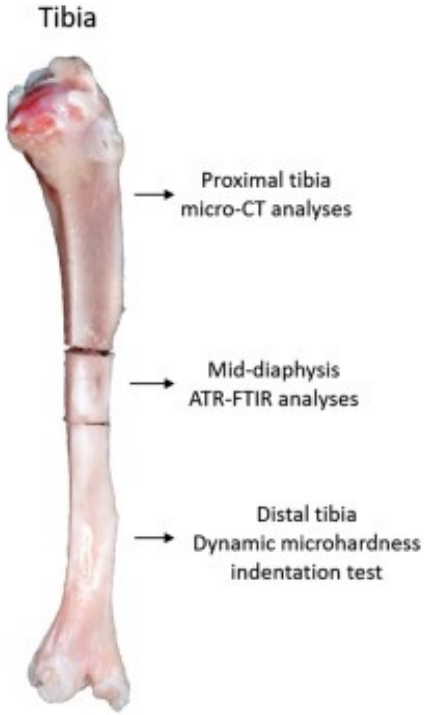

Fig. (1). Macroscopic evaluation of representative tibia specimen. Each tibia segments used in each methodology are separately represented (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

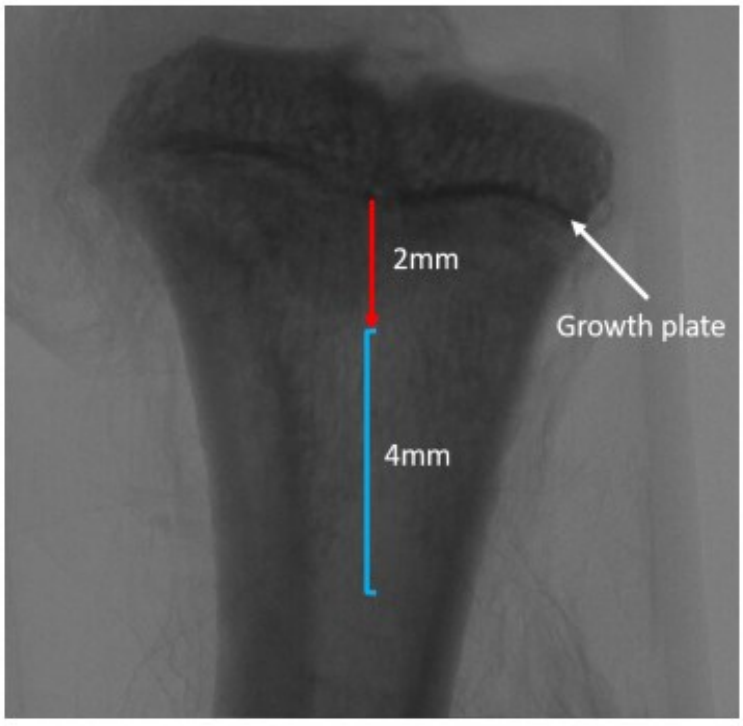

Fig. (2). Micro-CT scout-view image of the proximal tibia showed the region of interest (blue line) that included 4 mm of the tibia, at 2 mm (red arrow) from the growth plate (white arrow) (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The global threshold used for bone segmentation (0.60 g/cm^3) was chosen to differentiate the pores from bone tissue in all groups. The following parameters were measured in the cortical ROI: tissue mineral density (TMD, g/cm^3); bone surface/bone volume (BS/BV, mm^{-1}); thickness (Ct.Th, µm); porosity (Ct.Po, %); degree of anisotropy (Ct.DA); and fractal dimension (Ct.FD) [19, 20].

2.3. Attenuated Total Reflectance (ATR)-Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The fragment (2 mm) mid-diaphysis tibia was sectioned on the longitudinal axis with a diamond disk under constant irrigation in order to obtain two fragments of 2x2 mm. The bone fragments were dehydrated in an oven at 37 °C for one day, and then placed against the diamond crystal of the ATR-FTIR unit, pressed with a force gauge at a constant pressure to facilitate contact. The mean of spectra values of the cortical surface was obtained. Data were recorded and analyzed with OPUS 6.5 software (Bruker, Ettlingen, Germany). The bone composition was analyzed using Fourier transform infrared spectroscopy (FTIR, Vertex 70 Bruker, Ettlingen, Germany) equipped with an accessory that allowed spectrum acquisitions in the attenuated reflectance (ATR) mode. The spectra were recorded in the range of 400±4.000 cm^{-1} at a 4 cm^{-1} resolution, and the mean of 32 scans per fragment analyzed was used. Vector normalization and baseline correction were performed in all spectra, and these were considered absorbance height ratios.

The spectra were further analyzed by calculating the following parameters: amide I band (collagen ratio between the mature pyridinoline crosslink peaks (PYR) ± 1660 cm^{-1} and the immature crosslinking dihydroxynorleucine (DHLNL) - 1690 cm^{-1}); crystallinity Index (the intensity ratio of peaks 551 and 597 cm^{-1} for 588 cm^{-1}); and matrix-to-mineral ratios of amide I + II/hydroxyapatite (HA) (M:MI) (the ratio between the 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 the 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}) [4].

2.4. Dynamic Microhardness Indentation Test

The distal tibia diaphysis samples were stabilized in polyester resin (Instrumentos de Medição Ltda, São Paulo, SP, Brazil) using a metallic device (Metalon; Metalon Pooled Industries, Nova Iguaçu, RJ, Brazil) that measure 50 mm long, 30 mm wide and 10 mm tall. The distal diaphysis samples were positioned perpendicular to the basal surface (Fig. **3A**). After the samples were stabilized in the polyester resin, the surfaces were polished using 600, 800, 1200 and 2000 grit silicon-carbide papers (Norton, Campinas, SP, Brazil) and with metallographic diamond pastes (6, 3, 1, ¼ µm; Arotec, São Paulo, SP, Brazil). The metallic devices with the stabilized tibiae were washed in an ultrasonic bath (Cristofoli, Campo Mourão, PR, Brazil) with absolute alcohol for 10 minutes, between papers, to remove the debris [21].

Vickers hardness (VHN, expressed in N/mm^2) of the bone was assessed by using a microhardness indenter (Microhardness FM 700; Future Tech, Kawasaki, Japan). The test was performed with a load of 200 g applied for 15 s and five continuous indentations were made at a distance of 0.5 mm apart perpendicular to the cortical bone cross-section region (Fig. **3B**).

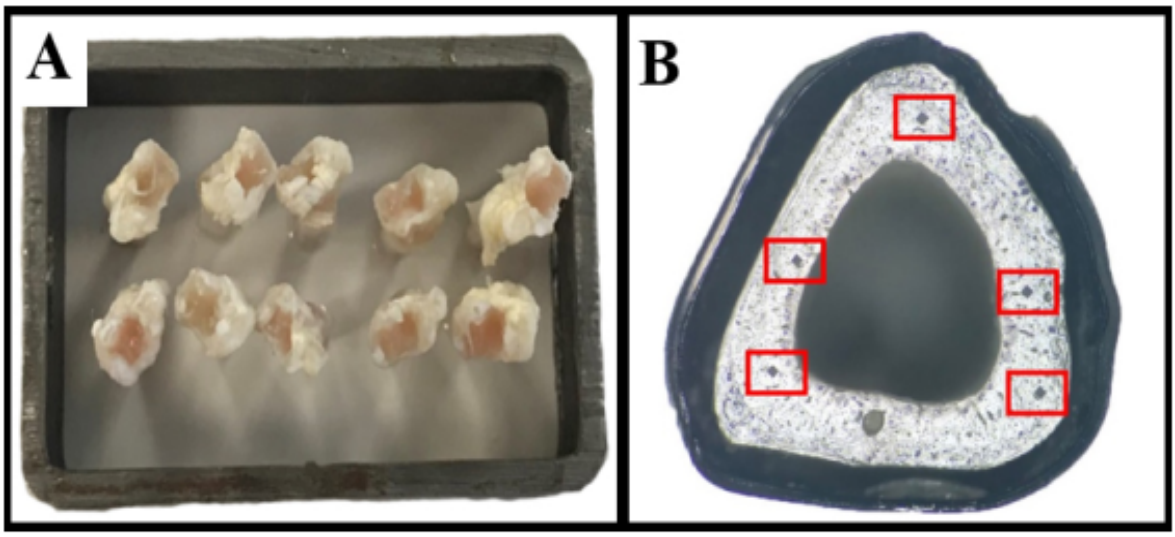

Fig. (3). (**A**) - Metallic device with tibiae embedded in polyester resin. (**B**) - Five indentations in cortical bone cross-section. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

2.5. Statistical Analysis

The data from all measured parameters were tested for normal distribution (Shapiro-Wilk) and the equality of variances (Levene's test). One-way analysis of variance (ANOVA) was performed followed by the Tukey test. All tests employed a level of significance of α=0.05 and all statistical analyses were carried out with Sigma Plot version 13.1 (Systat Software Inc., San Jose, CA, USA).

3. RESULTS

Throughout the experimental procedure it was observed that diabetic group maintained weight loss, polyphagia, polydipsia and polyuria, determined by the increased intake of feed, water and urinary excretion. The diabetic group (486.29 ± 32.12, mg/dl) showed higher glycemic rates compared to non-diabetic (106.21 ± 11.21, mg/dl) and diabetic + insulin (132.76 ± 13.57, mg/dl) groups (p<0.012). Moreover, the non-diabetic and diabetic + insulin groups showed no significant difference statistical analysis (p=1.376).

The mean and standard deviation values of all parameters calculated by using micro-CT analyses for all groups are shown in Fig. (**4**). The diabetic group showed lower values of BS/BV (p=0.018), Ct.Th (p<0.001), and Ct.DA (p=0.034) compared to non-diabetic group. The diabetic group showed lower values of Ct.Th compared to diabetic + insulin group (p=0.018). The non-diabetic group had lower Ct.FD values compared to diabetic groups (p<0.001). TMD (p=0.979) and Ct.Po (p=0.091) showed no significant differences among groups.

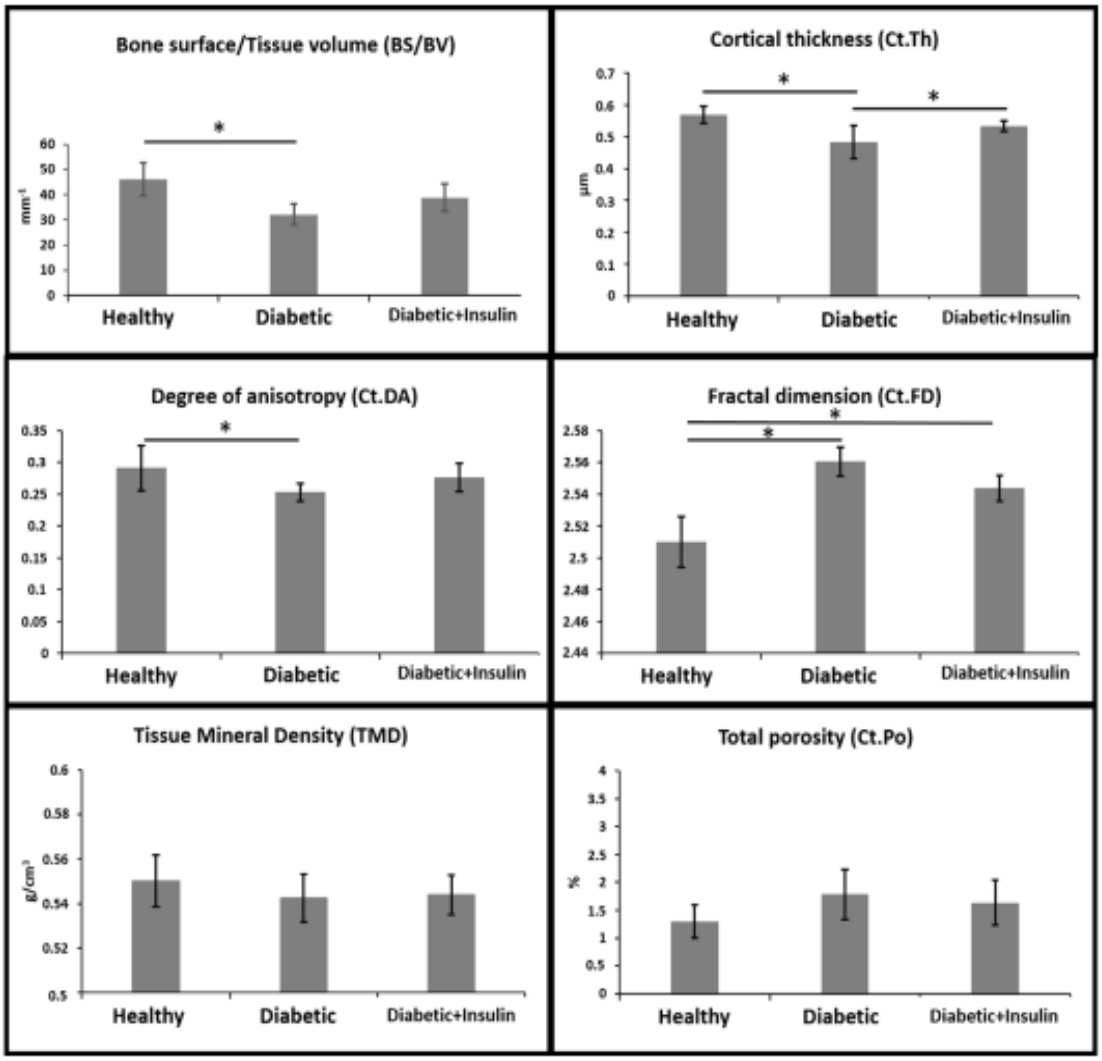

Fig. (4). The micro-CT analysis (n=10). The parameters evaluated, mean ± SD of the non-diabetic, diabetic and diabetic + insulin groups follow, respectively: BS/BV (46.0 ± 6.6, 32.1 ± 4.1 and 38.9 ± 5.5); Ct.Th (0.6 ±0.02, 0.5 ± 0.05 and 0.5 ± 0.01); Ct.Da (0.3 ± 0.03, 0.3 ± 0.01 and 0.3 ± 0.02); Ct.FD (2.5 ± 0.01, 2.6 ± 0.01 and 2.5 ± 0.01); TMD (0.6 ± 0.01, 0.5 ± 0.01 and 0.5 ± 0.01); Ct.Po (1.3 ± 0.3, 1.8 ± 0.4 and 1.6 ± 0.4). *Indicates a significant difference - Tukey's test (P<0.05).

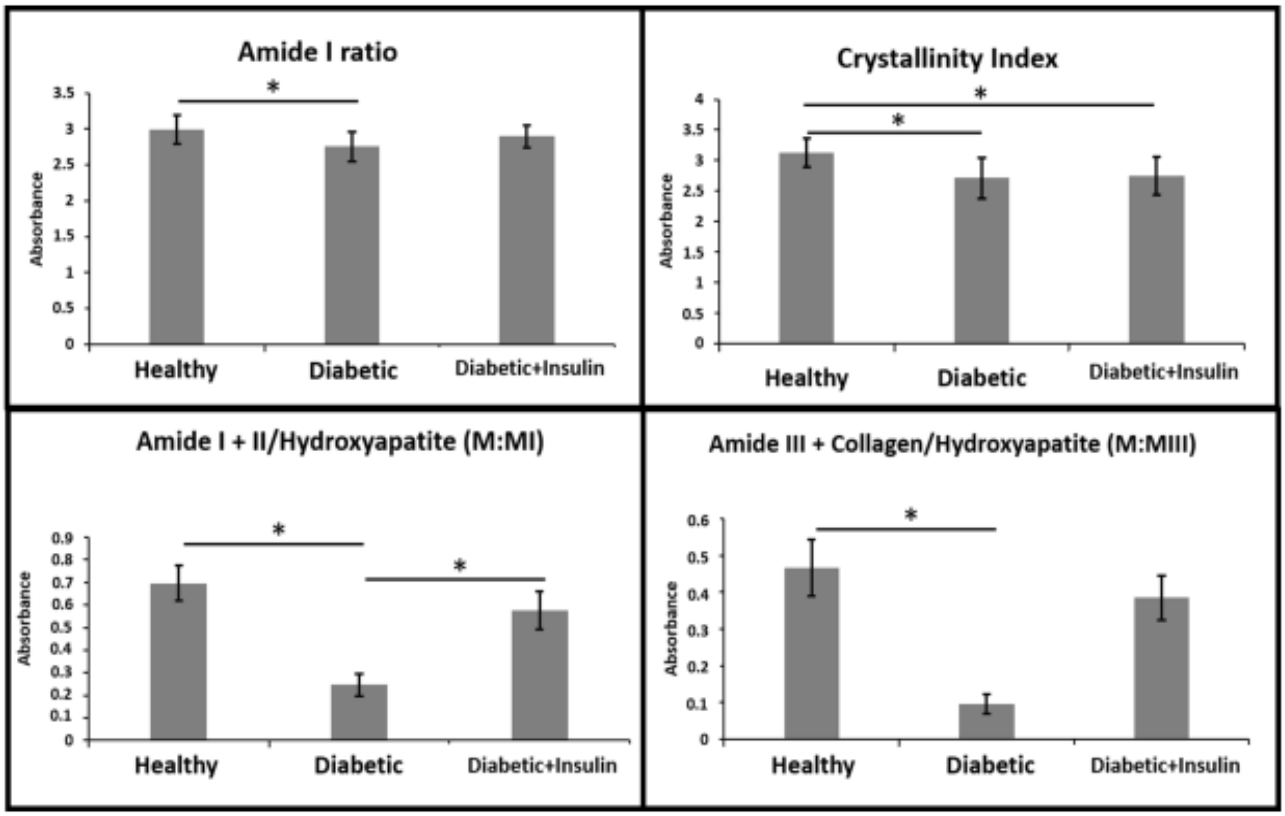

Fig. (5). ATR-FTIR analysis (n=10). The parameters evaluated, mean ± SD of the non-diabetic, diabetic and diabetic + insulin groups follow, respectively: Amide I ratio (3.0 ± 0.2, 2.8 ± 0.2 and 2.9± 0.2); Crystallinity index (3.1 ± 0.2, 2.7 ± 0.3 and 2.7 ± 0.3); M:MI (0.7 ± 0.1, 0.2 ± 0.04 and 0.6 ± 0.1); M:MIII (0.5 ± 0.1, 0.1 ± 0.02 and 0.4 ± 0.1). *Indicates a significant difference - Tukey's test (P<0.05).

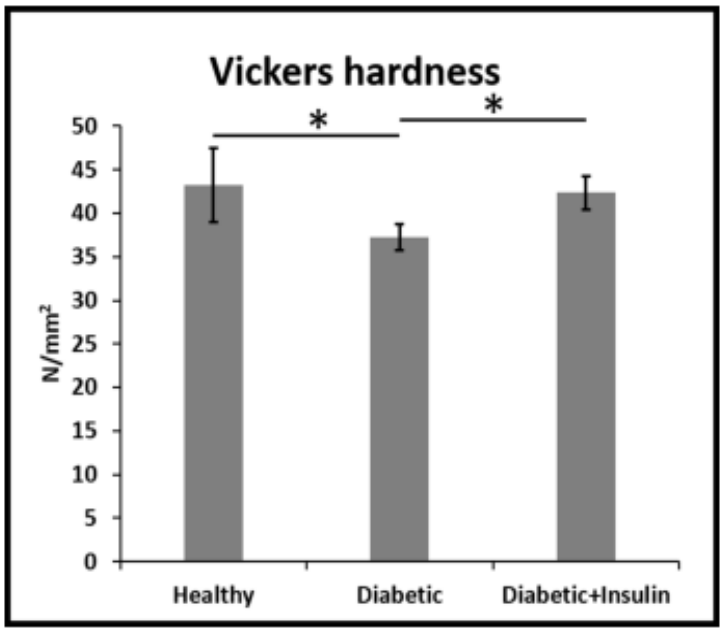

Fig. (6). Microhardness analysis (n=10). The mean ± SD of the non-diabetic, diabetic and diabetic + insulin groups follows, respectively: 43.2 ± 4.3; 37.2 ± 1.5; 42.3 ± 1.9, N/mm2. *Indicates a significant difference - Tukey's test (P<0.05).

The mean and standard deviation values of all parameters calculated by using ATR-FTIR for all groups are shown in Fig. (**5**). The spectra in ATR-FTIR analysis show the main bands that are characteristic of bone components. The diabetic group showed significantly lower values for the amide I ratio (p=0.046), crystallinity index (p=0.022), and matrix:mineral ratios (M:MI - p=0.002) and M:MIII - p=0.028) compared to non-diabetic group. In addition, the diabetic + insulin group showed lower values in the crystallinity index (p=0.038) compared to the non-diabetic group and lower values in the M:MI (p=0.006) ratio compared to the diabetic group.

In the microhardness analyses, the diabetic group showed significantly lower VHN values compared to non-diabetic and diabetic + insulin groups (p<0.001) (Fig. **6**).

4. DISCUSSION

The present study hypothesized that decreased hyperglycemia with insulin therapy minimizes the negative effects of TIDM on cortical bone microarchitecture, matrix composition and mechanical properties, maintaining the physiological conditions. In fact, our results showed that insulin minimized the deleterious effects of TIDM on bone microarchitecture, collagen maturation, crystalline HA content and mechanical properties of cortical bone. A recent study in humans showed that TIDM negatively affects the organic and mineral matrix, decreasing the mechanical properties in trabecular bone, whereas, only trends were found in cortical bone [22].

Considering the limitations in ethical guidelines in the human study, the present study used an animal model to evaluate the structural changes of the cortical bone. The present study chose TIDM rodent models due to the injection of a high dose of STZ has been described as the method to establish TIDM rodent models [23]; STZ destroys pancreatic β cells and results in typical human TIDM symptoms [24]. A significant reduction in blood glucose concentration was observed in the animals subjected to insulin therapy, confirming the efficacy of the insulin protocol [25, 26].

Micro-CT is an important methodology for analyzing bone microarchitecture and allows the characterization of changes in bone arrangement [20]. The lower BS/BV and Ct.Th values observed in the diabetic group compared to non-diabetic group suggest that TIDM decreased bone formation, leading to the loss of bone mass [27]. Some studies have shown that hyperglycemia reduces osteoblast function by oxidative stress [28, 29], decreasing bone matrix synthesis [5]. The diabetic + insulin group showed no difference in BS/BV and Ct.Th compared to non-diabetic group, which could be due to the insulin effects on bone cells. Insulin regulates the process of proliferation and differentiation of mesenchymal stem cells into osteoblasts, and these cells secrete osteocalcin [25, 30] that acts as a functional link between bone metabolism and glucose homeostasis [31].

The lower Ct.DA and higher Ct.FD in diabetic group showed that TIDM bone structure organization pattern was more isotropic and heterogeneous compared to non-diabetic bone. The present study suggests that TIDM causes significant alteration or absence of bone channels alignment along the load directional axis [32], leading to more heterogeneity on spatial structure of network bone channels. This reduces cortical complexity [33], contributing to bone fragility [32, 34].

In addition, the diabetic + insulin group showed higher Ct.FD compared to non-diabetic and no significant difference was found in Ct.DA compared to the other groups. This suggests that insulin therapy was not able to prevent changes in the channels network caused by disease, probably, it is due to partial glycemic control. Insulin therapy through injections does not allow full glycemic control, so there are periods of hypo (after the application moment) and hyperglycemia (before next application) [35]. This variation in glycemic control reduced TIDM effect on bone formation [25], leading to show a greater level of anisotropy, but not enough to maintain the Ct.FD in normal condition.

The lower VHN values of the diabetic group corroborated the findings that TIDM modifies bone microarchitecture, as shown in the Ct.DA and Ct.FD results. Also, the lower VHN values in the diabetic animals might have been induced by modifications of mineral crystal [36] and collagen [2] arrangement. In addition, our results suggest that the insulin therapy reduced TIDM effects on the mineral structure and collagen integrity, maintaining normal microhardness [37]. This could be due to normalization of matrix production (organic and inorganic) by osteoblasts [25].

Bone mechanical properties depend on the HA crystal composite, type I collagen and the interaction between them [2, 36]. Fourier transform infrared spectroscopy (FTIR) can be used to refine investigations of molecular alterations on bone matrix that affect microhardness. This method allows the bone matrix evaluation in order to characterize the mineral content, crystal size and collagen arrangement [38]. The lower amide I in the diabetic group suggests that there was a higher level of intrafibrillar (immature) dihydroxylysinonorleucine (DHLNL) than interfibrillar (mature) pyridinoline [4]. This greater immature crosslinks might have been induced by accumulate irreversible AGEs [11]. The AGEs are formed when free-floating sugars interact with exposed amino acid residues in collagen [39], impairing crosslink maturation [11], that can decrease tensile strength of collagen molecules [4, 38]. In addition, the diabetic + insulin group showed no significant difference in amide I ratio compared to the other groups. This might be due to blood glucose variation inherent to the insulin therapy, which did not prevent the changes in the collagen maturation process.

Mieczkowska *et al*., 2015 [40] showed that collagen maturity in diabetic mice increased compared to non-diabetic ones. The authors discussed their outcome supporting in reduction of immature cross-links, reported in the Saito *et al*., 2006 [41] study. However, Saito *et al*. used genetically modified WBN / kob rats, which exhibit a pre-diabetic stage, making limited comparison with the results found in STZ-induced mice used by Mieczkowska *et al.,* [40]. Throughout

bone matrix formation, in non-diabetic animals, occur increase of mature cross-links and in collagen maturity ratio, as found in the present study and in some other studies [4, 11, 38]. Furthermore, the mature collagen fibrils serve as scaffolds for bone mineral crystal growth, position and arrangement. These two processes are intimately correlated, considering the fact that collagen fibril structure and organization can limit the size of the crystals and their orientation [42]. Thus, the lower collagen crosslinks and collagen maturation disturbances can affect the mineralization process [43].

The crystallinity index lower in the diabetic groups suggest that TIDM increased the presence of large HA crystals, decreased the surface area in collagen fibrils [44] and that insulin therapy was not sufficient to minimize these deleterious effects. Studies have shown that the mineral crystallinity index (crystal size/perfection) must be separated from the mineral maturity (progressive transformation of non-apatite into poorly then crystallized apatite) [36, 44]. The present study suggests that, even though the crystallinity index was low with insulin therapy, there could be an increase in crystal maturity, contributing to the no significant difference between diabetic + insulin and non-diabetic groups in microhardness analyses.

In the diabetic group, lower M:MI and M:MIII ratios were observed, suggesting that TIDM affected collagen maturity (organic matrix) more than crystallinity (inorganic matrix), as shown in the collagen maturation analysis. A recent study used similar experimental protocols showing no difference between diabetic and non-diabetic animals in matrix:mineral analysis [4]. However, the sample size in the present study was larger than the cited article, which increases the detection of small differences between groups. The diabetic + insulin group showed a higher M:MI ratio than the diabetic group and similar values to those of the non-diabetic groups. This suggests that insulin decreases collagen degradation [25, 37], which could have inhibited the deleterious effects on bone tissue hardness.

Our results showed no differences in the TMD and Ct.Po parameters. However, it is necessary to consider that the micro-CT methodology has some limitations that could prevent accurate TMD measurements. These limitations include polychromatic X-ray spectra that could lead to beam-hardening artifacts and limited spatial resolution that could lead to partial volume effects, especially in relatively porous samples such as bone [45].

The present study suggests that TIDM had a discrete influence on bone mineralization disruption in cortical bone, as shown in the ATR-FTIR analyses, and minimal differences between groups would not be detected by the micro-CT resolution [45]. Therefore, the associate different methodologies, such as micro-CT, ATR-FTIR and Vickers microhardness analyses, are valuable tools to characterize microarchitecture, quality of mineral and organic bone matrix components and mechanical properties. This model supports the idea of a link between elastic properties, mineral content and plastic behavior with collagen maturity at the osteon level [36].

CONCLUSION

The present study demonstrated that TIDM changes the micro-structure, composition and hardness of the bone matrix, by reduced bone surface, cortical thickness, anisotropy, fractal dimension, collagen maturation and mineralization, and bone microhardness. Moreover, insulin minimized the effect of TIDM on cortical thickness and organic/mineral matrix in the cortical bone of an experimental rat model.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All experimental protocols with animals were approved by the Committee of the Ethics of Animal Use and Care of the Federal University of Uberlândia, Brazil (permit number 026/14).

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. The reported experiments on animals were performed in accordance with the recommendations in the Guide for the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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2.2 CAPÍTULO 2

Artigo a ser enviado para publicação no periódico Clinical implant dentistry and related research

The effect of diabetes mellitus on osseointegration: a review of the available strategies for better outcomes

Short title: Diabetes on osseointegration

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What is known

Diabetes mellitus impairs the osseointegration of dental implants'.

Some studies show the causes and some therapies for better results.

What this study adds

This literature review brings the different studies published in an attempt to show the ways that type 1 diabetes mellitus negatively affects osseointegration and the alternatives currently available to improve osseointegration.

Abstract

Diabetes mellitus is a metabolic disorder widely known to negatively impact bone healing, especially interfering with the osseointegration of dental implants. Several mechanisms lead to abnormal osseointegration, including overexpression of reactive oxygen species (ROS), accumulation of advanced glycation end products (AGEs), impaired angiogenesis and altered expression of proteoglycan and bone-related biomarkers. However, bone development impairment is not fully understood and requires further investigation. In order to overcome the effect of hyperglycemia in dental implant therapy, investigations have focused on experimental alternatives, such as insulin therapy, hypoglycemic agents, hyperbaric oxygen treatment, parathyroid hormone therapy, implant surface modification, naturally occurring substances, mesenchymal stem cell management, gene expression and growth factor modulation. The aim of the present review was to explore the mechanisms involved in metabolic bone changes around dental implants under high glucose levels, as well as the available therapeutic strategies to improve osseointegration.

Keywords: Diabetes Mellitus, Dental Implants, Osseointegration

Introduction

The loss of permanent teeth, weather as a result of trauma or disease, remains a frequent problem worldwide. Currently, rehabilitation with osseointegrated dental implants is considered the therapy of choice for the restoration of edentulous areas, as it offers biocompatibility and predictable outcomes for most patients [1,2]. However, local and systemic factors may limit the efficiency of the technique or even lead to failure, especially in conditions that affect the bone metabolism, for instance diabetes mellitus (DM) [3].

DM is characterized as a set of chronic metabolic disorders, subsequent to elevated plasma glucose levels resulting from complications in the mechanism of insulin production, insulin action or both situations. Type 1 diabetes mellitus is caused by autoimmune destruction of pancreatic insulin-producing β-cells, leading to total deficiency, and represents 5–10% of the diabetic population. Whereas type 2 is related to insulin resistance or partial reduction of its production, being more frequently observed (90–95% of diabetes cases) [4]. Regardless of pathophysiology, there is a straight correlation between deleterious effects of DM on the oral bone and the risk of failure in osseointegration process[5,6].

Diabetic patients, especially uncontrolled, present altered levels of a series of bone-related biomarkers, due to a state of chronic inflammation, which is mostly characterized by overproduction of reactive oxygen species (ROS) and advanced glycation end products (AGEs) [7,8]. Under these conditions, peri-implant bone tissue development and vascularization are negatively affected in terms of quantity and quality [9,10].

According to data from the International Diabetes Federation, the exponential increase in the number of DM cases is worthy of concern, accounting for 6.7 million deaths in 2021 with an updated projection of 783 million people affected worldwide by 2045 [4]. Considering the metabolic disorder of hyperglycemia in the microenvironment surrounding dental implants, it is essential to understand the mechanisms underlying the impaired osseointegration related to this patient profile. Therefore, the aim of the present review was to explore the deleterious effects of DM on bone health and provide an overview of the available adjunctive strategies to help oral health practitioners achieving successful outcomes in dental implant rehabilitation.

Understanding the effects of diabetes mellitus on osseointegration

Adequate bone remodeling conditions are necessary for primary biomechanical stability and long-term maintenance of dental implants [11]. The classic concept of osseointegration, previously described by Brånemark as a direct contact between bone and implant [12], has recently been proposed as a foreign body response phenomenon associated with continuous inflammatory stimulation [13]. An immune response is initiated after implant insertion with the purpose of isolating the titanium surface, in conjunction with chronic inflammation of soft tissues [14,15]. The interface established between the implant surface and the newly formed bone was previously described as being composed of a poorly mineralized bone matrix layer, with the presence of osteocytes in intimate contact with the titanium, combined with narrow sections through which dendritic extensions of the osteocytes anchor to the implant, supporting long-term osseointegration [16]. Once the balance over these processes is disrupted, the success of implant rehabilitation may be compromised.

There is growing evidence that DM impairs bone metabolism surrounding dental implants. Despite implant failure rates seem to be similar to those of healthy individuals [17–20], the disease is clearly associated with an elevated risk of peri-implant complications [3,5,21]. Furthermore, the underlying mechanisms of abnormal osseointegration are complex and not yet thoroughly understood. Here, we summarize the main findings described in the scientific literature regarding metabolic bone changes around dental implants in the presence of hyperglycemia.

Reactive oxygen species (ROS)

ROS overproduction and chronic inflammation have long been recognized to play a central role in the progress of metabolic disorders. Oxidative stress caused by excessive presence of these molecules in the intracellular environment leads to structural modification in numerous cell types, thus affecting their proper functioning [22,23]. This process is no different in the bone-implant interface. In vitro investigations revealed significantly heightened ROS expression, associated with impaired proliferation and calcification potential of bone marrow-derived mesenchymal stem cells cultured on titanium [24]. Likewise, it was demonstrated in an osteoblast culture model that cell exhibit

altered structure, reduced activity and elevated levels of apoptosis, correlated with increased presence of ROS, possibly due to disturbances in mitochondrial function [25]. Ma et al. (2021) demonstrated on 3D-printed titanium implants that osteoblasts display abnormal functioning upon overexpression of ROS, correlated with up-regulation of the NF-κB signaling pathway [26]. Suppression of Wnt/β-catenin signaling pathway in DM may also increase ROS levels, with a negative impact on bone formation [27]. Saito et al. (2022) observed pronounced expression of ROS in peri-implant bone, with reduced levels of proliferation and calcification in vivo [24]. Furthermore, oxidative stress apparently induces dysfunction of vascular endothelial cells subjected to hyperglycemic conditions on titanium surfaces, therefore compromising the angiogenesis process [27].

Accumulation of advanced glycation end products (AGEs)

Hyperglycemia induces intensified production of AGEs, possibly as a reflection of excessive oxidative stress. AGEs induce altered cell behavior, considerably interfering with bone metabolism, which ultimately culminates in poor quality bone tissue [28,29]. It has been reported that high concentrations of AGEs suppress bone turnover by inhibiting the differentiation and functioning of osteoblasts and osteoclasts, in addition to interfering with the collagen cross-linking process [30]. Research revealed that accumulation of AGEs restricts bone formation through impaired bone marrow mesenchymal stem cells proliferation and differentiation, mediated by reduced osteoblastic autophagy [31]. AGEs are also thought to affect osteocyte mechanosensitivity [32] and induce osteoblast apoptosis [33], interfering with bone matrix synthesis and maintenance, and also inhibit osteoblastic mineralization when associated with high glucose levels [34].

Changes in the peri-implant microenvironment leading to inadequate osseointegration are also expected. It was identified in animal model that the presence of AGEs reduces bone to implant contact, hindering the stability of dental implants [35]. Clinically, through peri-implant sulcular fluid analysis, elevated levels of AGEs in diabetic patients were correlated with greater probing depth and marginal bone loss [36,37]. Fiorellini et al. (2020) reported that osteoblast adhesion to titanium implant surfaces is limited under hyperglycemic conditions, mediated by AGEs production, thus reflecting the impact of host serum quality during the early stages of bone healing [38].

Impaired angiogenesis

Adequate blood supply is critical during bone tissue neoformation and maintenance. Blood vessels actively participate in the cross-talk between bone and adjacent tissues to deliver nutrients, cells, cytokines, growth factors, and other molecules required for osteogenesis [39]. However, the mechanisms by which impaired angiogenesis occurs under hyperglycemic conditions, specifically at the bone-implant interface, have not yet been fully understood. Vascular endothelial cell dysfunction due to oxidative stress mediated by excessive production of AGEs and ROS overexpression has been proposed as a central pathway of compromised bone healing [27]. In addition, Xiang et al. (2020) recently identified that DM-induced M1 macrophage polarization negatively affects neovascularization around titanium implants, which was indirectly reversed with M2 macrophage polarization stimulation by sitagliptin, a dipeptidyl peptidase-4 suppressing agent which is used to control blood glucose levels [40]

Proteoglycan expression

Extracellular matrix proteoglycans are glycosaminoglycan binding protein compounds, which are essential in maintaining the balance of homeostatic functions by controlling different cellular mechanisms [41]. With regard to bone tissue, these biomolecules play an important role in modulating osteogenesis and bone remodeling, by regulating the formation and development of collagen fibrils and directly interacting with a series of cytokines and growth factors [42]. In fact, up-regulated expression of proteoglycans is expected during the initial phases of bone healing, reaching the highest values within 7-14 days [43]. In contrast, proteoglycan deficiency can be observed in hyperglycemic circumstances, either by reduced synthesis or increased elimination, although the exact mechanisms are not yet fully understood [44].

It has long been recognized that a slender layer of proteoglycans is formed at the bone-implant interface, supporting the inflammatory response modulation after implant placement [45]. Interestingly, Sousa et al. (2020) observed large amounts of proteoglycans correlated with disorganized peri-implant bone tissue under hyperglycemic conditions. Further observation was made that proteoglycans were present to a lower extent upon insulin therapy, suggesting a mechanism of delayed osseointegration [46].

Bone-related biomarkers expression

The gene expression of several modulators of bone activity is altered in diabetic peri-implant domains. Receptor activator of nuclear factor-kappa B ligand (RANKL) together with osteoprotegerin (OPG) are well known to have a regulatory role in osteoclast activity. OPG prevents the RANK-RANKL receptor interplay, limiting osteoclastogenesis and subsequent bone resorption [47,48]. In a study that evaluated bone biopsies from diabetic patients receiving dental implants, up-regulated RANKL expression, higher RANKL/OPG ratio and a trend towards reduced OPG were associated with an osteoclastic profile [49]. Likewise, Correa et al. (2020) found increased expression of RANKL, in contrast to reduced levels of OPG, associated with compromised bone-implant contact and lower counter-torque parameters in diabetic animals compared to healthy animals [50].

Another factor altered by DM is the Runt-related transcription factor 2 (Runx2), an imperative transcription factor for satisfactory bone development, which controls the differentiation of osteoblast progenitor cells [51]. Studies have confirmed that DM induces significant reduction in Runx2 gene expression, thus affecting dental implant osseointegration [50,52]. Downregulation of other molecules that actively participate in the bone tissue formation and mineralization process has been observed, such as osteocalcin, osteopontin, transforming growth factor-β1, bone morphogenetic protein-252 [53], bone morphogenetic protein-455 and microRNA-491-5p [54]. In addition, Smpd3 and Itga10 hub genes, along with rno-mir-207 microRNA, have been identified as possible biomarkers of impaired osseointegration in DM [55].

Additionally, atypical peri-implant protein content limits cell adhesion to the implant surface. Liu et al. (2015) identified diabetes-induced downregulated expression of fibronectin and integrin α5β1, its primary receptor, which play an important role in stimulating binding action of osteoblasts and subsequent bone neoformation [56].

Implant osseointegration in experimental models of diabetes mellitus

Different aspects of the interplay between DM and the bone tissue around implants have been explored. The ultrastructural changes of the bone matrix under hyperglycemic conditions were detailed in a recent study by scanning electron microscopy, which described it as disorganized, with the presence of slender trabeculae and empty spots, in addition to an exacerbated content of proteoglycans [46]. Similarly, histomorphometric and

biomechanical analyzes consistently revealed bone healing impairment [57,58]. Coelho et al. (2018), demonstrated limited primary bone formation in a diabetic minipig model, correlated with elevated levels of tumor necrosis factor-alpha (TNF-α), an acknowledged marker of the inflammatory process [57].

In addition, DM negatively impacts the bone status of already osseointegrated implants, though at a slower pace and to a minor extent. In the study by de Molon et al. (2013), reduced bone-to-implant contact and lower torque removal values were observed in a 4-month follow-up period in rats, with diabetes induction 60 days after implant placement [59]. Likewise, de Morais et al. (2009) identified reduced bone density by means of a digital subtraction method [60].

Therapeutic approaches to assist dental implant osseointegration

Several investigations have focused on therapies in an attempt to minimize or avoid the effect of hyperglycemia in dental implant therapy, such as insulin therapy, hypoglycemic agents, hyperbaric oxygen treatment, parathyroid hormone therapy, implant modification, naturally occurring substances, mesenchymal stem cell management, gene expression and growth factor modulation [61–65]. However, these therapies are still seen as experimental methods, requiring further research and clinical validation [61].

Insulin therapy

Insulin remains the mainstay therapy for type 1 diabetic patients. Several treatment regimens are used for glucose level management, including daily injections of rapid-action insulin combined with basal insulin, as well as continuous subcutaneous infusion [66]. Maintaining strict glycemic control has been shown to reduce the long-term risk of macro and microvascular disease in both type 1 and type 2 diabetes [67]. Survival of implants could be improved in patients with diabetes if the plasma glucose concentration was controlled [56]. However, normal glucose levels obtained by insulin therapy might not restore all alterations yielded by diabetes [58].

In diabetic animals, insulin therapy prevented the occurrence of bone abnormalities [59], was able to maintain bone density [60] and osseointegration was not compromised [59], although it was not possible to reach the results obtained in the control group [68]. Upon

insulin therapy, diabetic rats presented blood glucose levels reduced to normal, elevated body weight, slightly increased implant stability [69], and increased implant fixation in 12 weeks after implantation [70]. However, micro-CT and histomorphometry indicated impaired implant osseointegration and peri-implant trabecular microstructure not as well organized as control groups [71].

Naturally occurring substances

Investigations have focused on more predictable therapeutic alternatives with fewer side effects for use in diabetic patients in need of bone repair, benefiting both the treatment and prevention of related complications [72]. Resveratrol is one of these active substances derived from plants and food with numerous pharmacological activities [73], including potential to prevent islet β-cell apoptosis, improve insulin action, regulate glucose metabolism [74,75], and inhibitory impact on osteoclast differentiation [76]. It has been shown to increase peri-implant bone density, improve trabecular architecture and enhance biomechanical fixation [50,77]. However, the level of osseointegration was lower than that observed in control groups, according to histological and micro-CT analyses [50,77].

Berberine, the main component of Rhizoma Coptidis (of Chinese herbal medicine), promotes β-cell regeneration [78], regulates the release of insulin-like peptide 1 [79], inhibits inflammation and exhibits hypoglycemic effect [80]. Berberine has been shown to act as an efficient agent to osseointegration in diabetes, which indicates it might be a good strategy for dental implants in diabetic patients [81]. In addition, its combination with insulin was more effective than when administrated as monotherapy [82].

Curcumin has been used as a spice, herbal supplement, and traditional medicine in Asia for more than 4000 years. It has been widely studied with respect to many diseases and is considered to have potential medical benefit [83]. The potential effects of this nutraceutical with regard to its anti-bacterial, anti-inflammatory, and anti-oxidant properties have been studied *in vitro* and *in vivo* [83–86]. Curcumin reverses the harmful effects of diabetes in bone healing, contributing to the modulation of bone-related markers, especially in association with insulin therapy. Additional use of curcumin, could represent an interesting therapeutic for diabetic patients undergoing dental implants [87].

Genipin is an active constituent isolated from the fruit of Gardenia Jasminoides, which is widely used in traditional oriental medicine as an anti-inflammatory [88], antiangiogenic [89],

antioxidant [90], antidiabetic agent [70,91,92]. It has been suggested that genipin in combination with insulin could be an effective method for promoting implant osseointegration in type 2 diabetes rats [69].

Mesenchymal stem cell management, gene expression and growth factor modulation

Bone marrow is a potential source of multipotent adult stem cells, which are known to have high osteogenic ability [93,94]. Stem cell therapy with osteoinductive bone marrow mesenchymal stromal cells and platelet-rich plasma may offer a novel approach to enhance the osseointegration of dental implants in uncontrolled diabetic patients. Micro-CT scan analysis revealed improved osseointegration around implants in diabetic rabbits [95].

Studies have demonstrated that a chronic high glucose level results in defective response of tissues to hypoxic conditions by impairing the function of hypoxia-inducible factor 1 alpha [96], and influences numerous target genes, such as vascular endothelial growth factor and Runx2, which is associated with angiogenesis and osteogenesis [97]. Oh et al. (2019) showed that local administration of hypoxia-inducible factor 1 alpha via protein transduction domain-mediated DNA delivery system may boost bone formation around implants and induce gene expression favorable to bone formation in diabetic mice [98].

Basic fibroblast growth factor (bFGF) plays an important role in bone healing as a potent stimulator of osteoblastic proliferation [99]. Studies have affirmed that bFGF regulates extracellular matrix production of osteoblastic cells *in vitro* and, when systemically administered *in vivo*, increased endosteal bone formation in rats [100]. Therefore, local delivery of bFGF from poly (lactide-co-glycolide) microspheres to areas around titanium implants may improve osseointegration in diabetic rats [101].

Parathyroid hormone (PTH) has significant effects in regulating bone metabolism [102]. The synthesis and secretion of PTH are sensitively controlled by the calcium concentration detection mechanism. PTH exerts anabolic effects on both osteoblasts and osteocytes by regulating bone remodeling [103], and mesenchymal stem cell differentiation fluctuates following PTH changes [104]. PTH has been shown to promote the osteogenic potential of mesenchymal stem cells from ovariectomized rats [105], which provides new insights into a potential strategy for managing diabetic bone loss [106]. However, metabolic

characteristics of the diabetic rats produced a condition that was unable to respond to PTH treatment, with or without associated insulin [62,107].

Hypoglycemic agents

Metformin is one of the most used pharmacological means to control blood glucose levels. Its action occurs in fasting and postprandial state, acting to reduce gluconeogenesis and hepatic glucose production and/or increase glucose uptake in skeletal muscle [108]. Improvements in blood glucose levels and healing around implants were observed in diabetic rats using metformin [109], in addition to increased OPG expression and decreased RANKL/OPG ratio in the medullary área [64]. Despite this, negative results were also found where there was no modulation of the harmful effect of hyperglycemia on bone healing [64] or reduced percentage of bone to implant contact and increased expression of RANKL around implants [110]. Therefore, this drug may be insufficient to reverse the negative influence of hyperglycemia around bone implants.

Hyperbaric oxygen treatment

Hyperbaric oxygen therapy is a treatment with inhalation of 100% oxygen in a closed air chamber, where the atmospheric pressure is increased and controlled [61]. It stimulates angiogenesis, fibroblast activity and collagen synthesis [111]. Oxygen levels are increased along the periphery of ischemic wounds, promoting the formation of oxygen-dependent collagen matrix necessary for angiogenesis, thus improving wound healing [112].

Hyperbaric oxygen therapy, either before or after the installation of implants, increased the bone-to-implant contact in diabetic rats to the level of healthy rats [113]. Histomorphometry findings suggest that hyperbaric oxygen therapy has positive effect on implant osseointegration in the early healing period in diabetic rabbits. However, in clinical repercussion, the improvements on osseointegration are not enough to increase implant mechanical stability [61].

Implant modification

Titanium and its alloys are the most used dental implants materials due to mechanical strength, chemical inertness and biocompatibility [114]. Several studies have reported that implants with rough surface and pores were beneficial for osseointegration and that

implant surface is a key point during the early stages of bone healing [115]. Therefore, a large number of studies have focused on the surface properties of implants [116].

Implants with hydrophilic surfaces have been utilized to improve osseointegration in challenging scenarios, such as patients with diabetes. It is suggested that hydrophilic surfaces have the potential to modulate the osseointegration process yielding more predictable results [117,118]. Schuster et al. (2021) compared the bone neoformation of a hydrophilic surface (Acqua®) and a hydrophobic surface (Neoporos®) in diabetics rats. The diabetic group, after a 7-day healing period, yielded with the Acqua implants presented significantly higher total bone-implant contact and trabecular bone-implant contact values in comparison to the Neoporos implants. The positive effects of the Acqua surface were able to counteract the adverse impact of uncontrolled diabetes at early osseointegration periods. However, after 28 days *in vivo*, the metabolic systemic impairment caused by diabetes overcame the surface treatment effect [119].

SLA implants are sandblasted, large grain, acid etched implants and SLActive additionally feature hydrophilic surfaces. Experimental studies revealed that SLActive implants led to significantly higher bone-implant contact compared with SLA in diabetic animals [117], and bone-implant contact comparable to that observed in healthy animals [120,121]. Hydrophilic surfaces resulted in positive effects in healthy and especially in diabetic animals, which demonstrates that it could improve the osseointegration progress in diabetic humans [117,120]. Furthermore, SLActive titanium implants showed a trend of promoting superior total bone formation at the early osseointegration [121], suggesting that a better prognosis is possible for implant treatment of diabetic patients.

TiO2 nanotubes prepared by the anodic oxidation technique mimic the fundamental nanoscale structure of the bone [122,123]. Compared to SLA surfaces, implants with TiO2 nanotube surface reduced the osteogenetic inhibition induced by high-glucose states by reversing ROS overproduction *in vitro*. Micro-CT scan analysis further confirmed, *in vivo*, the better osteogenetic ability of TiO2 nanotube surface implants in diabetic rats. This strategy may provide more favorable implant surfaces than mechanically polished and SLA surfaces for patients with diabetes [124].

Strontium (Sr) has been widely studied in bone tissue engineering because it can not only stimulate bone formation but also inhibit bone resorption [125]. *In vivo* it could prevent bone

structural mechanical changes as a consequence of diabetes [126]. Strontium may also stimulate osteoblasts to osteogenesis [127]. SLA-Sr surfaces showed significantly higher bone-implant contact at 4 and 8 weeks and upregulated osteoprotegerin expression at 4 weeks in diabetic rats. Besides, it displayed higher bone-implant contact at 4 weeks in normoglycemic rats. It is suggested that strontium-incorporated titanium implant surfaces could enhance implant osseointegration in diabetic rats [128].

Large-grit sandblasting with micro-arc oxidation implants (SL-MAO) and implants with interconnected 3D tubulous structures (I3D) have the potential to be tested under hyperglycemic conditions. SL-MAO surface modification created a topographic morphology characterized by both micron-sized craters and sub-micron-scale pits. This surface resulted in superior chemical composition, which promoted cell adhesion, proliferation, and osteogenic differentiation. SL-MAO modified titanium implants osseointegrated more efficiently than SLA or MAO controls, with significantly higher bone-area ratio and bone-implant contact in the peri-implant region [129]. The tube-shaped structure of the I3D implants allows the storage of chemoattractants to mobilize stem cells, improving osseointegration. It was reported greater calcium deposition and torque force required to remove I3D implants compared to solid implants [130].

Conclusion

In conclusion, this review highlights the range of factors that negatively influence bone neoformation under hyperglycemic conditions and elucidates possible strategies to improve osseointegration. Although promising, it is worth noting the experimental nature of these therapies and the need for additional research and clinical validation.

Author Contributions

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Statements and Declarations

Ethics approval and consent to participate

Not applicable.

Competing interest

All authors declare that there are no conflicts of interest.

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2.3 CAPÍTULO 3

Artigo a ser enviado para publicação no periódico Clinical implant dentistry and related research

Impact of superhydrophilic dental implants associated with glycemic control by insulin therapy to minimize the deleterious effects of type 1 diabetes mellitus in osseointegration process: A animal pre-clinical study

Short title: Superhydrophilic dental implants

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What is known

A large number of studies have focused on increasing dental implants' stability.

Some studies showed that hydrophilicity surface improves osseointegration at various levels.

What this study adds

This study is showed that type 1 diabetes mellitus impaired bone formation around the dental implant surface. The insulin therapy associated with superhydrophilic surface minimized the diabetes effects on the early stage of the osseointegration process.

Abstract

The aim of present study was to evaluate the stability, morphology and chemical components on osseointegration process of dental implants with superhydrophilic (Acqua®) and hydrophilic (NeoPoros®) surface in diabetic rats, submitted to insulin therapy (IT). Thirty male rats were randomly assigned into the following three groups (n=10): non-diabetic, diabetic and diabetic+insulin. Type 1 diabetes mellitus (TIDM) was induced by intravenous injection of streptozotocin. In diabetic+insulin group, 4IU insulin was administered twice per day. After 1 week of T1DM induction, all animals were submitted to implant placement, with superhydrophilic and hydrophilic surface in left and right tibiae, respectively. The animals were euthanized two weeks after surgical procedure and the samples were submitted to removal torque test (N/cm), energy-dispersive X-ray spectroscopy (%, EDS) and scanning electron microscopy (SEM). The analysis of maximum torque removal force showed that diabetic group had lower values on hydrophilic and superhydrophilic surfaces compared non-diabetic and diabetic+insulin groups. The non-diabetic group showed higher values compared to the others groups. The EDS showed that diabetic group had lower values of calcium compared to non-diabetic and diabetic+insulin groups. In oxygen analysis, the non-diabetic group showed lower values compared to diabetic and diabetic + insulin groups. In addition, the superhydrophilic surface showed higher values in diabetic group compared to diabetic+insulin. The superhydrophilic surface showed higher value of calcium and lower values of oxygen compared to hydrophilic surface in diabetic+insulin

group. In SEM analysis, the diabetic group showed bone structure loose-looking bone matrix, irregular arrangement, thin trabeculae and more empty spaces compared to non-diabetic and diabetic+insulin. Moreover, the superhydrophilic surface showed more bone distribution along the implant surface and intimate contact with grooves. The present study showed that TIDM impaired bone formation around dental implant surface and the insulin therapy associated to superhydrophilic surface minimized the diabetes effects on early stage of osseointegration process.

Keywords: Dental implants; ossseointegration; diabetes mellitus, type 1; hydrophilic surface; insulin

Introduction

The rehabilitations with dental implants for replacing missing teeth may be considered an important therapeutic alternative to adequate masticatory and aesthetics function, and prevent atrophy alveolar bone, improving the life quality of patients [1, 2]. The long-term clinical success of dental implantation depends on the degree of osseointegration [3] that is defined as a direct bone-to-implant contact without interposition of any other tissue [4]. According to Albrektson *et al*., 1981, some requisites are important for achieving osseointegration, including the material biocompatibility, surgical technique, implant design, condition of applied loads after implant placement, implant surface quality, site of installation and host systemic condition [5].

Some studies showed that type 1 diabetes mellitus (T1DM) have a deleterious effect on the success of osseointegration process [6, 7]. T1DM is an inflammatory autoimmune disease characterized by the destruction of pancreatic beta cells, which results in insulin deficiency and leads to chronic hyperglycemia [8]. The hyperglycemic microenvironment reduces angiogenesis process [9] and increases the production of advanced glycation end products (AGEs) that, by interacting with specific osteoblastic receptors (RAGEs), damage the proliferation, differentiation, and activity of osteoblasts [10], impaired bone formation around dental implants [11, 12].

Insulin therapy accompanied by reduction of glycaemia has been to be the pivotal point in treatment and prevention of TIDM effects [13, 14]. Insulin injection reduces endogenous glucose production, fasting blood glucose and hemoglobin A1c (HbA1c), which that improves the body glycemic control [15, 16]. Recent findings suggest that dental implant treatment can be safely carried out in diabetic patients with well-controlled blood glucose [17, 18]. However, it has been shown that maintenance of excellent glycemic stability is difficult to achieve, and hyperglycemia impairs bone healing and osseointegration [18, 19]. Consequently, therapies to improve the early stages of osseointegration in diabetic patients are demanding.

Considering that the implant surface is a key point of successful osseointegration at the early stage of bone healing, a large number of studies have focused on increasing dental implants' stability. The strategy for this has been managing the surface properties of the implants, to reduce the failure rate and recovery time after implantation [20, 21]. Some studies showed that hydrophilicity surface improves osseointegration at various levels, directly promoting early expression of the pathways involved in cell proliferation and differentiation of osteoblast precursors, alongside regulation of angiogenesis, bone mineralization, and bone remodeling [22, 23].

Therefore, the present study hypothesized that decrease hyperglycemia from insulin therapy associated to hydrophilic surface accelerate the osseointegration in the early stages, reducing the negative effects of TIDM in dental implants installation. The aim of present study was to evaluate the stability, morphology and chemical components on osseointegration process of dental implants with superhydrophilic (Acqua®) and hydrophilic (NeoPoros®) surface in diabetic rats submitted to insulin therapy (IT), using removal torque test, scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS).

Material and Methods

Experimental protocol

All experimental protocols with animals were approved by the Committee of the Ethics of Animal Use and Care of the Federal University of Uberlândia (permit number 022/17). All procedures were carried out in strict accordance with the recommendations

in the Guide for the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Thirty male Wistar rats (*Rattus norvegicus*) weighing 240 ± 20 g (8 weeks of age) were housed in standard conditions (12 hour light/dark cycle, temperature of 22±1°C and relative humidity of 50– 60%), with food (composition: humidity, crude protein, ethereal extract, mineral, crude fiber, calcium and phosphorus) and water *ad libitum*. After one week of acclimatization, the animals were randomly assigned and equally distributed into the following three groups (n=10): non-diabetic, diabetic and diabetic + insulin therapy. After 1 week of T1DM induction in diabetics groups, all animals were submitted to implant placement, with superhydrophilic (Acqua®) and hydrophilic (NeoPoros®) surface in left and right tibiae, respectively. All the animals were euthanized two weeks after the surgical procedure.

Type 1 diabetes mellitus (T1DM) induction and Insulin therapy

The T1DM induction and insulin therapy protocol were performed following previously described methodology [24]. A single dose of streptozotocin (STZ, Sigma-Aldrich, Inc. St. Louis, MO, USA) was administered in diabetic and diabetic + insulin groups by intravenously through a penile vein puncture at a dose of 45 mg/kg body weight, diluted in 0.1 M citrate buffer (pH 4.5). Equal protocol of anesthesia and volumes of vehicle were injected in the control rats (non-diabetic group). The hyperglycemia was confirmed by a glucometer (Accu Check Active, Roche, Jaguaré, SP, Brazil) after 48 hours of the induction, collecting a blood drop from the tail of each animal. Follow up of the glycemic rates was done one, two and three weeks after induction and animals that maintained blood glucose levels higher than 200 mg/dL were considered diabetic. Clinical diabetic signs such as polyphagia, polydipsia, polyuria, and bodyweight loss were also monitored in a qualitative analysis. The animals that did not reach the glycemic target were excluded from the study. Thereafter the diabetes confirmation, the animals of diabetic + insulin group received daily subcutaneous doses of 4 IU (1 IU at 7 a.m. and 3 IU at 7 p.m.) with neutral protamine Hagedorn insulin (Humulin U-100, 100 U/mL, Eli Lilly, São Paulo, Brazil) diluted in 0.9% NaCl.

Implant placement surgery

One week after the animals submitted to diabetes induction or receive vehicle injection, the dental implants were placement. Initially, general anesthesia was done by intraperitoneal injections of 7 mg/kg xylazine (2%, muscle relaxant) and 100 mg/kg ketamine hydrochloride (10%, anesthetic and analgesic). The animals were submitted to a trichotomy in the inner leg and after disinfection with iodine solution, a 2 cm incision was performed on the internal side of the right hind leg, just below the knee, and the tibial metaphysis was exposed by blunt dissection. Then, the osteotomy was performed using a progressive sequence of drills under profuse saline irrigation. The implants measuring 4.0 mm length and 2.2 mm diameter (Neodent®, Curitiba, PR, Brasil) were placement until the screw thread had been completely introduced into the cortical bone, with superhydrophilic (Acqua®) and hydrophilic (NeoPoros®) surface in left and right tibiae, respectively [25]. Incisions were then closed in layers. The fascia and skin were sutured separately using nylon sutures. Analgesics (tramadol 1 mg/kg) and antibiotics (cefazolin 25 mg/ kg) were administered via the intramuscular route and twice per day for 3 days after the operation. All implants at the end procedure were in good stability and without mobility signs.

The animals were euthanized two weeks after surgical procedure by intraperitoneal injection with sodium thiopental and lidocaine in compliance with the principles of the Universal Declaration on Animal Welfare. The removal torque test was made after the animals' sacrifices and the implants collect were submitted to a scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS).

Removal torque testing

The tibiae without disarticulating were dissected to expose the implant and the cover screws and attach to a suitable device. A torque meter (Tohnichi, Model STC400, Tokyo, Japan) with a scale range of 3–24 N/cm and divisions of 0.05 N/cm was used for the test (Figure 1A). A wrench was inserted to the implant head to apply torque in the reverse direction of implant placement until rupture of the bone-implant interface was signaled by rotation of the implant (Figure 1B). The torque force value (N/cm) achieved was considered as the torque required for the interruption of osseointegration [26].

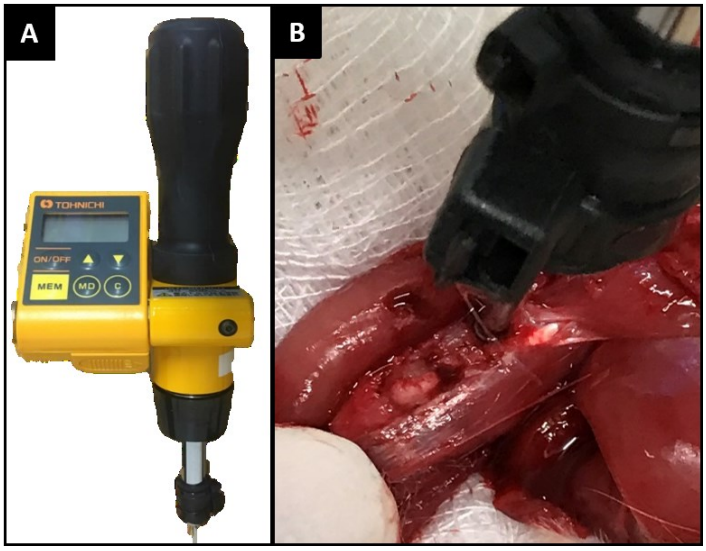


Figure 1. A- Torque meter with attached digital wrench. B- Device attached to the implant rat tibia to perform reverse torque until interrupted osseointegration.

Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS) analysis

The implants obtained from removal torque test were immersed in a 3% sodium hypochlorite solution for 20 minutes, followed by washing in running water for 10 minutes, dehydrated an increasing series of alcohol (50; 75; 90; 100%) and dried in a device (critical point device) (Balzers CPD -300 - Leica Microsystems Vienna, Austria). Then, the samples were pulverized with gold in a vacuum metallizing machine (Bal-Tec SCD-050, Leica Microsystems; Wetzlar, Germany). High resolution micrographs obtained in SEM (LEO-1430; Carl Zeiss, BW, Oberkochen, Germany) operated at 15 kV were performed on two selected regions of each implant (apex and cervical) at magnifications of 50x to 50,000x in the vertical and horizontal position. In the qualitative analyzed was observed the characteristics of the newly formed peri-implant bone [27].

The chemical composition of the bone remnant on the surface of the implants was analyzed by EDS (Oxford Instruments, England) coupled to the SEM. The implants were positioned horizontally and the regions of interest were magnified at 90x. The composition and distribution of organic and inorganic elements were analyzed by weight concentrations of the following chemical elements: Ca (calcium), O (oxygen), and C

(carbon) on the surface of each implant. Results were expressed as the mean value of all measurements and performed in triplicate [28].

Statistical analysis

The data from all measured parameters were tested for normal distribution (Shapiro-Wilk) and the equality of variances (Levene's test). Two-way analysis of variance (ANOVA) was performed followed by the Tukey test. All tests employed a level of significance of $\alpha=0.05$ and all statistical analyses were carried out with Sigma Plot version 13.1 (Systat Software Inc., San Jose, CA, USA).

Results

Throughout the experimental procedure it was observed in qualitative analysis that diabetic group maintained weight loss, polyphagia, polydipsia and polyuria, determined by the increased intake of feed, water and urinary excretion. The diabetic group (486.29 ± 32.12, mg/dl) showed higher glycemic rates compared to non-diabetic (106.21 ± 11.21, mg/dl) and diabetic + insulin (132.76 ± 13.57, mg/dl) groups ($p<0.012$). Moreover, the non-diabetic and diabetic + insulin groups showed no significant difference statistical analysis ($p=1.376$).

The analysis of maximum torque removal force (N/cm) showed that diabetic group had lower values of hydrophilic (8.38 ± 3.33) and superhydrophilic (8.98 ± 2.73) surfaces compared non-diabetic (hydrophilic: 17.64 ± 1.80, $p<0.001$; superhydrophilic: 19.32 ± 2.20, $p<0001$) and diabetic + insulin (hydrophilic: 12.35 ± 2.71), $p=0.008$; superhydrophilic: 12.70 ± 3.07, $p=0.014$) groups, among the respective surfaces. The non-diabetic group showed significant higher values compared to diabetic + insulin group, among the respective surfaces (hydrophilic: $p=0.004$; superhydrophilic: $p<0001$) (Figure 2).

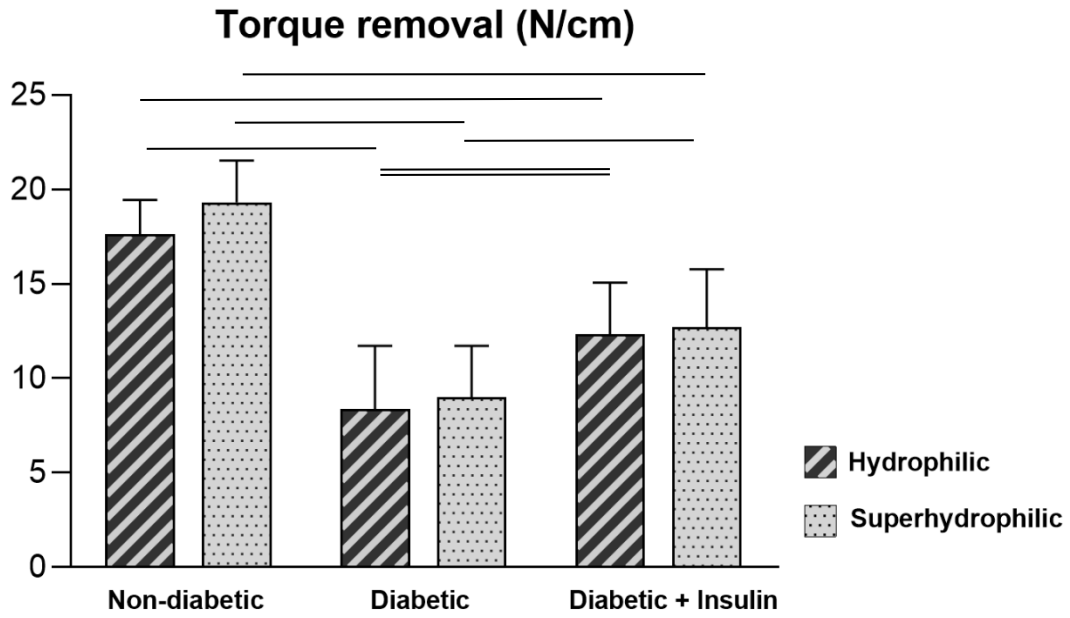
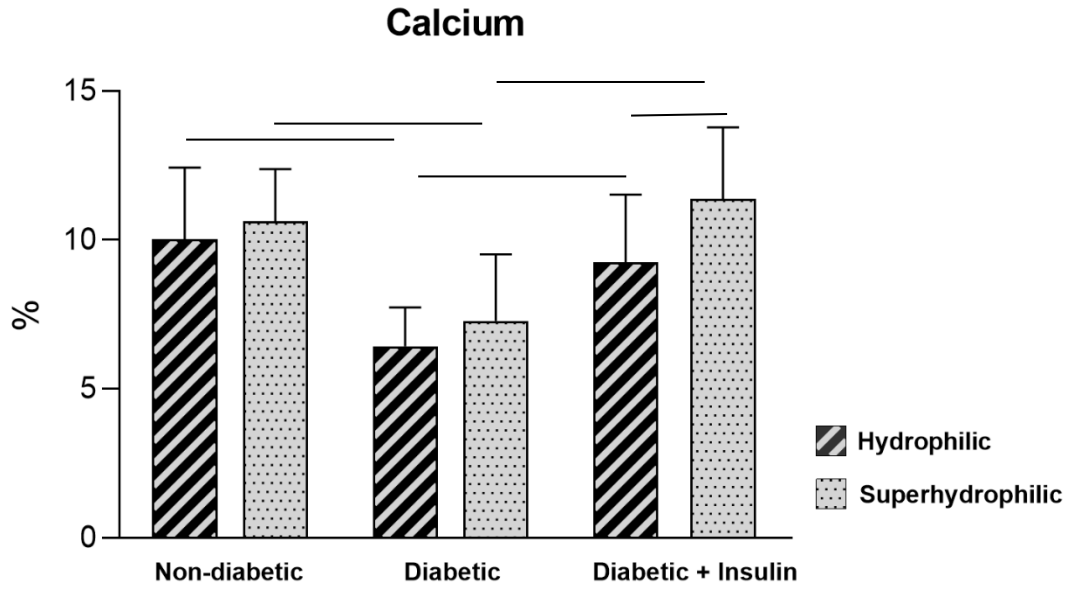


Figure 2. Results of removal torque testing. —— ($p<0.05$)

Chemical composition analysis (EDS) showed that hydrophilic and superhydrophilic surface of diabetic group had lower values of calcium compared to non-diabetic (hydrophilic: p=0.009; superhydrophilic: p=0.023) and diabetic + insulin (hydrophilic: p=0.038; superhydrophilic: p=0.008) groups, among the respective surfaces. In oxygen analysis, the non-diabetic group showed that hydrophilic and superhydrophilic surface had lower values compared to diabetic (hydrophilic: p=0.038; superhydrophilic: p=0.021) and diabetic + insulin groups (hydrophilic: p=0.043; superhydrophilic: p=0.033), among the respective surfaces. In addition, the superhydrophilic surface showed higher values in diabetic group compared to diabetic + insulin group (p=0.041). The superhydrophilic surface showed higher value of calcium (p=0.047) and lower values of O (p=0.012) compared to hydrophilic surface in diabetic + insulin group. In carbon analysis, no statistical difference was observed between the groups and surfaces (p>0.514) (Figure 3). The mean and standard deviation were showed in Table 1.


Calcium
15
10
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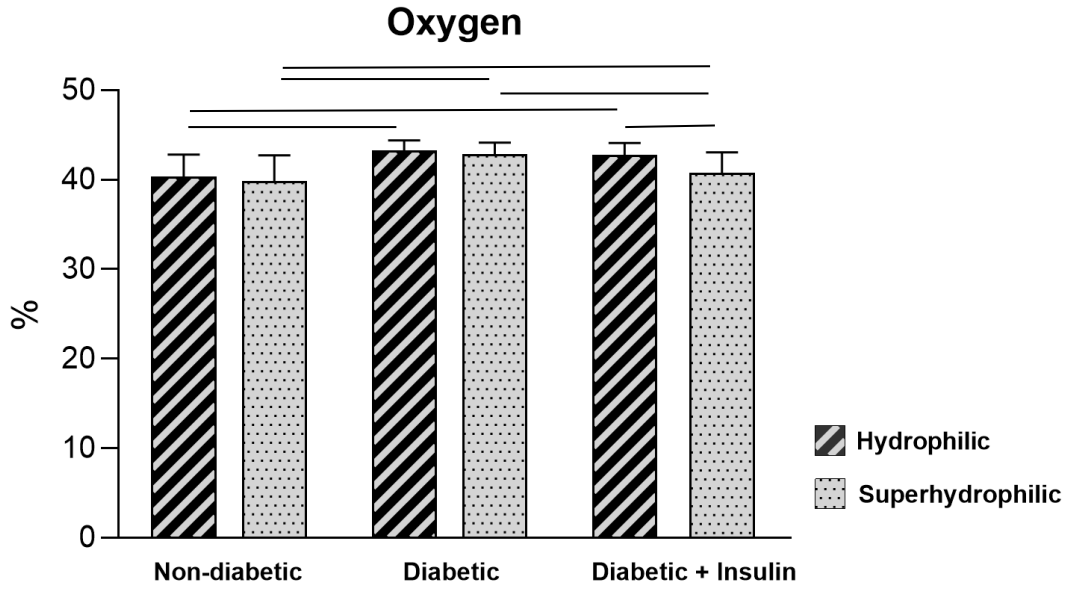
%
Non-diabetic
Diabetic
Diabetic + Insulin
Hydrophilic
Superhydrophilic


Oxygen
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%
Non-diabetic
Diabetic
Diabetic + Insulin
Hydrophilic
Superhydrophilic

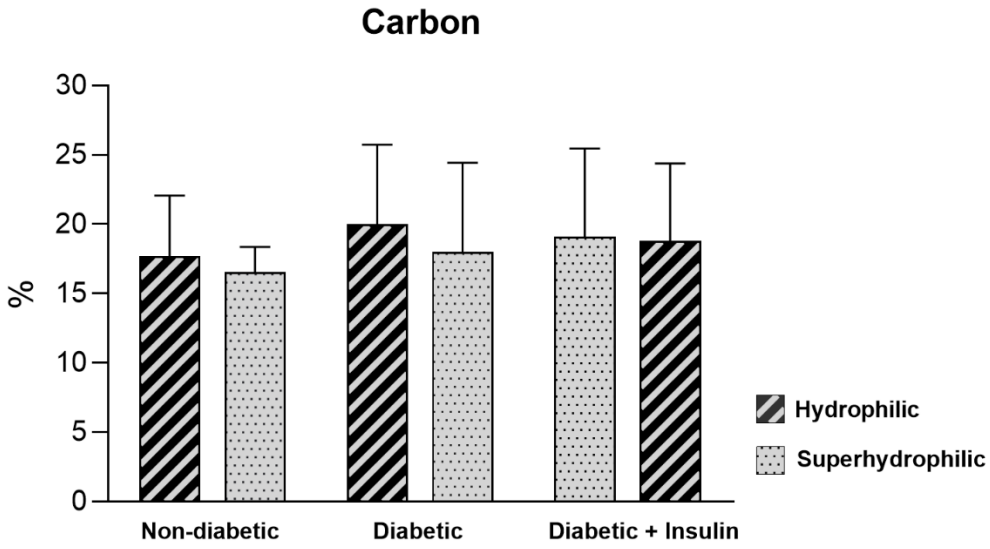

Figure 3. Results of chemical composition analysis (EDS). —— ($p<0.05$)

Table 1: The mean and standard deviation of chemical composition analysis (EDS).

	Non-diabetic		Diabetic		Diabetic + Insulin	
Measures/ Groups	**Hydrophilic**	**Superhydrophilic**	**Hydrophilic**	**Superhydrophilic**	**Hydrophilic**	**Superhydrophilic**
Calcium	10.02 ± 2.40	10.61 ± 1.76	6.42 ± 1.29	7.28 ± 2.24	9.25 ± 2.26	11.38 ± 2.40
Oxygen	40.33 ± 2.49	39.86 ± 2.84	43.26 ± 1.13	42.86 ± 1.29	42.75 ± 1.34	40.75 ± 2.30
Carbon	17.72 ± 4.33	16.54 ± 1.82	19.99 ± 5.74	18.01 ± 6.41	19.10 ± 6.36	18.82 ± 5.56

In the ultrastructural qualitative analyzes of SEM, differences matrix composition were more evident between diabetic and non-diabetic groups. In diabetic group, the bone structure presented loose-looking bone matrix, irregular arrangement, thin trabeculae and more empty spaces compared to non-diabetic and diabetic + insulin groups. The non-diabetic group presenting a denser bone structure, with few empty spaces and; the diabetic + insulin group showed intermediate morphological aspects between these two groups comparing the same surface. The superhydrophilic surface showed more bone distribution along the implant surface and intimate contact with grooves. At a small magnification (70x) that allowing a more panoramic view of the implants, the non-diabetic and diabetic + insulin groups showed, a bone tissue formed along the entire surface with more distribution (Figure 4).

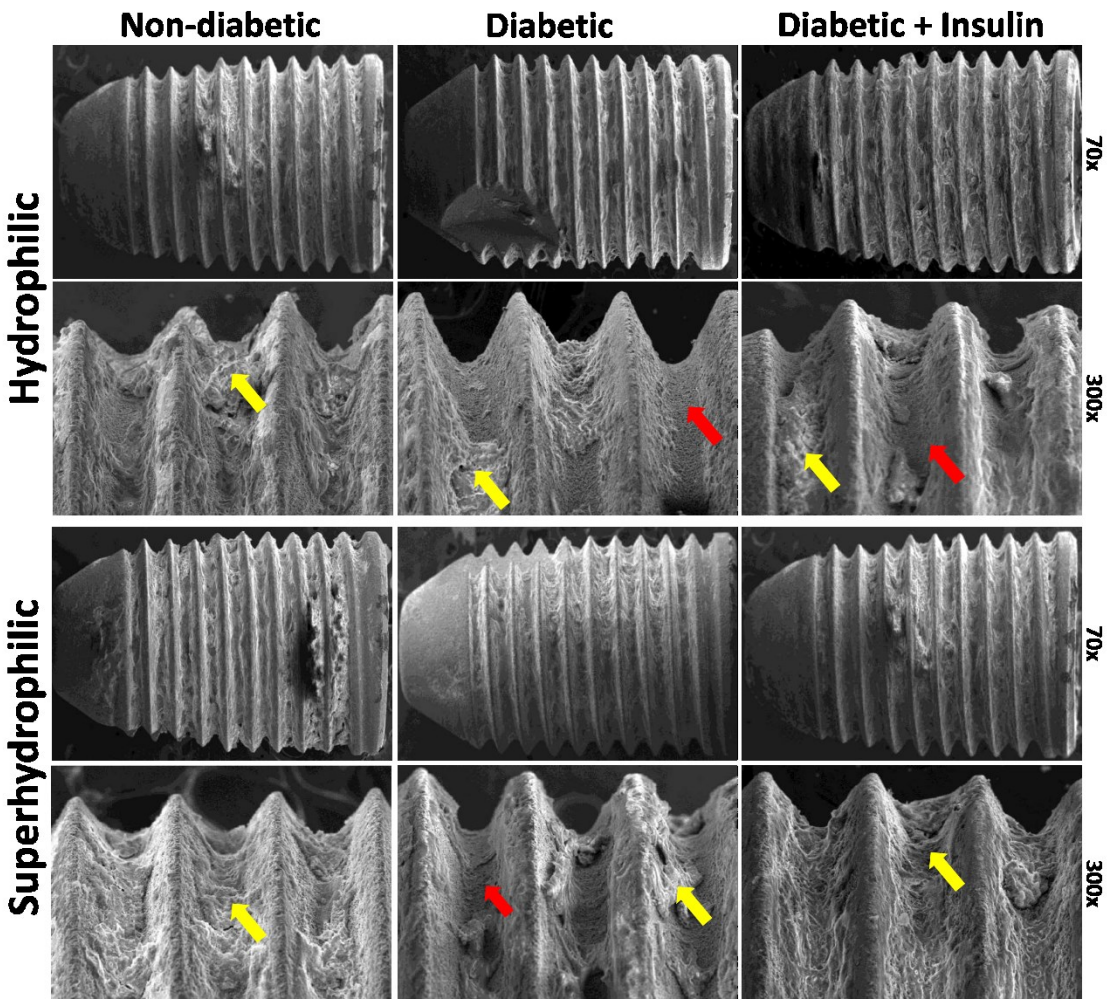

Figure 4. 70x - Low-magnification scanning electron microscopy shows the panoramic viewer of dental implants. 300x – High-magnification images shows the dental implant surface with the remaining osseointegrated bone tissue from the torque removal test. The red arrows showed the dental implant surface and yellow arrows were the bone tissue.

Discussion

The present study showed that decrease hyperglycemia with insulin therapy associated to superhydrophilic surface, improve the osseointegration process in TIDM condition. The frequency of opportunities regarding perioperative systemic management for medically compromised patients with diabetes mellitus is steadily increasing in dental implantology. It is essential to understand the pathologic mechanism of the disease on osseointegration to determine the ideal method of treatment for these underrepresented patients [23].

Considering the limitations of ethical guidelines in human study, the present study used animal model to improving our understanding of treatments to minimize the hyperglycemia effects on osseointegration process. The induction hyperglycemia by destroys pancreatic β cells with injection a single high dose of STZ, have resemblance characteristic to pathogenesis and natural disease progression of TIDM in human population [29, 30]. A significant reduction blood glucose concentration was observed in the animals received insulin therapy, confirming the efficacy of the protocol [31, 32]. Moreover, the proximal tibia was used to implant placement that surgery access was easier and the more trabecular with limited cortical bone provides a better environment for evaluate vascularization response of hyperglycemia condition [33]. The evaluation time of 2 weeks after implant placement was used to analyze the early stage that characterized to osteoid matrix formation and integration around the implant surface [34].

The biomechanical analysis showed that diabetic group has lower values of torque removal compared to other groups. This study suggest that hyperglycemia decreases mineralization process of osteoid matrix, compromising the mechanical retention of bone-implant surface [25, 35]. Some studies showed that free-floating sugars create irreversible compounds of advanced glycation end products (AGEs) [36, 37], which that reduce collagen formation and affect the differentiation and function of osteoblastic cells [38, 39].

Indeed, the EDS analysis showed that diabetic group has lower values of calcium and higher oxygen compared to other groups. These results associated to empty bone lacunae around the implants surface shown in diabetic group at qualitative SEM analysis, suggest that a delay organization and maturation of osseointegration process [40, 41]. The early process of osseointegration was characterization that cellular and plasmatic hemostasis lead to fibrin polymerization and the formation a blood clot, which serves as a matrix for neoangiogenesis, extracellular matrix deposition, and invasion of bone forming cells [42, 43]. Some studies showed that diabetes induce vascular endothelial cells dysfunction and angiogenesis impairment by increase cellular oxidative stress and decrease vascular endothelial growth factor (VEGF) [44], which plays a critical role of recruitment osteogenic cells around dental implant [45, 46], showed in our results.

Therefore, identification of new management strategies to minimize TIDM impaired osseointegration process is a major focus in the prevention dental implants loss

[47]. Our results showed that insulin therapy (diabetic+insulin group) was able to keep decrease the deleterious effects of diabetes, but not similar to non-diabetic group. The osseointegration is a relatively long healing process, maintaining the well-controlled glycemic status might not be possible during all phases [48]. This suggests that insulin was not able to prevent damages in the osseointegration process caused by disease, probably, it is due the partial glycemic control. Some studies showed that insulin therapy through injections does not allow full glycemic control, so there are periods of hypo (after the application moment) and hyperglycemia (before next application) [49].

In addition, the association of superhydrophilic surface with insulin therapy has shown higher potential to minimize the deleterious effects of TIDM in early stage of osseointegration. The period immediately following installation is an increased involvement of genes linked to the inflammatory response, blood vessel development, coagulation, angiogenesis, complex interaction, and cell adhesion on the implant surface [22]. This study suggest that superhydrophilic surface associate with insulin therapy enhances bone formation compared to diabetic group at multiple levels by directly promoting an earlier expression of pathways involved in cell proliferation and osteoblast precursor differentiation but also by positively regulating angiogenesis, bone mineralization, and bone remodeling [50].

The superhydrophilic surface had a higher affinity of the initial blood clot, an enhanced neoangiogenesis, increased bone-to-implant contact, and greater bone density were described within the first 2 weeks of bone healing [51]. Some studies showed that storage implants in isotonic NaCl solution contribute to the high surface energy is sustained by a hydroxylated/hydrated surface that minimizes the absorption of contaminating hydrocarbons and carbonates from air [52]. In addition, the important step in the wound healing process around the implant is the formation of a fibrin blood clot that serves as a bridging scaffold for migrating cells. The moderate immune response [53] and lower activation of thrombocytes [54], found on superhydrophilic surfaces compared to hydrophilic can facilitate the invasion and mobilization of the blood clot by mesenchymal stem cells (MSCs) [55], considered as one of the initial non-hematopoietic cell types to colonize an implantation site [56, 57].

Thus, despite the positive findings observed in this study, the effects of the insulin and superhydrophilic surface on TIDM implant osseointegration was evaluate in other

factors specific to the intraoral environment, such as the presence of biofilm, implant loading in secondary stability, and masticatory function could influence this process [58]. Thus, it is important to consider intraoral clinical situations to evaluate the osseointegration process in future studies [23]. Moreover, the patients with diabetes who are undergoing surgery, appropriate glycemic control throughout the perioperative period needs to be maintained to conserve the endocrine-metabolic balance between insulin and hyperglycemia-promoting hormones [59].

Conclusion

The present study showed that TIDM impaired bone formation around dental implant surface and; the insulin therapy associated to superhydrophilic surface minimized the diabetes effects on early stage of osseointegration process.

Author Contributions

Jessyca F Venâncio: Conceptualization, Methodology, Investigation, Formal analysis, Writing - Original Draft

Pedro HJO Limirio: Methodology, Investigation, Writing - Review & Editing

Camila RB Linhares: Methodology, Writing - Review & Editing

Priscilla BF Soares: Supervision, Writing - Review & Editing

Darceny Zanetta-Barbosa: Supervision, Writing - Review & Editing

Paula Dechichi: Supervision, Writing - Review & Editing

Statements and Declarations

Ethics approval and consent to participate

Not applicable.

Competing interest

All authors declare that there are no conflicts of interest.

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3 – CONCLUSÃO

Pode-se concluir do presente estudo que:

• Os resultados mostraram que o diabetes mellitus tipo 1 altera a microestrutura, composição e dureza da matriz óssea, pela redução da superfície óssea, espessura cortical, anisotropia, dimensão fractal, maturação e mineralização do colágeno e microdureza óssea. Causou, ainda, alterações estruturais no tecido ósseo afetando a osseointegração de implantes em tíbia de ratos diabéticos diminuindo o processo de neoformação óssea.

• A insulina minimizou o efeito do diabetes mellitus tipo 1 na espessura cortical e matriz orgânica/mineral no osso cortical de um modelo experimental de rato. Além disso, a terapia com insulina mostrou resultados favoráveis para a osseointegração, no entanto, não chegou próximo as condições encontradas no grupo controle.

• Os implantes com superfície super-hidrofílica mostraram uma melhor neoformação óssea comparado a superfície hidrofílica em condições de DMT1 submetidos a insulina.

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4 – ANEXOS

1 – Certificado do parecer de ética em utilização de animais

Universidade Federal de Uberlândia

– Comissão de Ética na Utilização de Animais –

Comissão de Ética na Utilização de Animais CEUA

CERTIFICADO

Certificamos que o projeto intitulado "Efeito da oxigenoterapia hiperbárica e insulinoterapia na osseointegração em ratos diabéticos", protocolo nº 022/17, sob a responsabilidade de **Paula Dechichi** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS (CEUA) da UNIVERSIDADE FEDERAL DE UBERLÂNDIA, em reunião de **26 de maio de 2017.**

(We certify that the project entitled "Efeito da oxigenoterapia hiperbárica e insulinoterapia na osseointegração em ratos diabéticos", protocol 022/17, under the responsibility of Paula Dechichi - involving the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata, for purposes of scientific research - is in accordance with the provisions of Law nº 11.794, of October 8th, 2008, of Decree nº 6.899 of July 15th, 2009, and the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and it was approved for ETHICS COMMISSION ON ANIMAL USE (CEUA) from FEDERAL UNIVERSITY OF UBERLÂNDIA, in meeting of May 26th, 2017).

Vigência do Projeto	Início: 01/06/2017 Término: 01/04/2019
Espécie / Linhagem / Grupos Taxonômicos	Rato heterogênico Wistar
Número de animais	120
Peso / Idade	300 g / 18 semanas
Sexo	Machos
Origem / Local	CBEA
Número da Autorização SISBIO	-
Atividade(s)	-

Uberlândia, 30 de maio de 2017.

Prof. Dr. Lúcio Vilela Carneiro Girão
Coordenador da CEUA/UFU

2 – Normas da revista a serem publicados os artigos dos capitulos 2.2 e 2.3.

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New submissions should be made via the Research Exchange submission portal https://wiley.atyponrex.com/journal/CID. Should your manuscript proceed to the revision stage, you will be directed to make your revisions via the same submission portal. You may check the status of your submission at anytime by logging on to submission.wiley.com and clicking the "My Submissions" button. For technical help with the submission system, please review our FAQs or contact submissionhelp@wiley.com.

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ii. A short running title of less than 40 characters;
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iv. The authors' institutional affiliations where the work was conducted, with a footnote for the author's present address if different from where the work was conducted;
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vi. A conflict of interest statement. Please note the any funding received to conduct a study needs to be reported. If authors have no conflict of interest relevant to the content of the submission, please state "The authors declare no conflict of interest";
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- A first header with what is known on the topic, followed by 1-3 bullet points.
- A second header with what the submitted study adds, followed by 1-2 bullet points.

Example:

> **What is known:**
> A recent systematic review suggested that short and long dental implants have the same survival probability.
> Most of the studies in this systematic review were observational studies and suffered from several biases.
>
> **What this study adds**:
> This study is the 3rd registered randomized controlled trial on this topic and suggests that long dental implants have a better survival probability.

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Review	**Yes**	New developments in basic sciences related to implant dentistry and clinically applied concepts	
Systematic Review and Meta-analysis	**Yes**	Systematic reviews and meta-analyses	**PRISMA**
Case Reports	**Yes**	Preliminary findings of research in progress providing or documenting new fundamental knowledge in language understandable to the clinician	**CARE**
Commentaries	**No**	Evidence-based opinion pieces involving areas of broad interest and invited commentaries.	
Letter to the Editor	**No**	Comments on published articles or current implant dentistry topics are welcome and will be published if appropriate.	

All submitted clinical studies should have a minimum of 20 patients with complete observations, regardless of sample size calculations. Studies submitted with a smaller sample size will only rarely be considered and should provide an explicit justification for sample size smaller than 20 in the cover letter.

It was reported in 2012 that 98.6% of studies employed the implant as the unit of analysis with little consideration of clustering within patients and that the periodontal and general health of study groups was unclear for more than 80% studies[1]. We aim to improve these statistics. Studies with more than one observation per patient (e.g., multiple dental implants, multiple measures on one dental implant, etc.) need to consider the within-patient correlation of such measures. Authors are recommended to follow the guidelines laid out in the following report: "Statistical Analyses and Methods in the Published Literature: The SAMPL Guidelines*" (**https://www.equator-network.org/wp-content/uploads/2013/03/SAMPL-Guidelines-3-13-13.pdf**). Studies should describe the patient population in terms of the prevalence of systemic diseases, nutrition, and smoking.

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1st Priority: Randomized Controlled Clinical Trials with minimal follow-up of 1 year for all enrolled trial participants. Trials which were registered in a readily accessible database *prior to* the first patient enrollment will receive priority. Please note, trial registration is different from obtaining human subjects' approval.

2nd Priority: Systematic reviews of randomized controlled trials. Please note that the conduct of systematic reviews in dental implant research is complex as most studies fail to consider the within-patient correlation of dental implant observations. Submitted systematic reviews need to report on which of the included studies reported results based on inappropriate analyses and report how inappropriate analyses were adjusted for by means of imputed within-patient correlation coefficients. Submitted systematic reviews should also report whether trials were registered prior to patient enrollment.

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- Future of Research Communications and e-Scholarship (FORCE11)
- National Research Council's Institute for Laboratory Animal Research guidelines
- The Gold Standard Publication Checklist from Hooijmans and colleagues
- Minimum Information Guidelines from Diverse Bioscience Communities (MIBBI) website
- FAIRsharing website

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