UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"
FACULDADE DE ODONTOLOGIA DE ARARAQUARA

GUILHERME JOSÉ PIMENTEL LOPES DE OLIVEIRA

EFEITO DO EXTRATO INSAPONIFICÁVEL DE ABACATE E SOJA NA DOENÇA PERIODONTAL INDUZIDA, NA OSSEOINTEGRAÇÃO DE IMPLANTES E NO REPARO DE DEFEITOS CRÍTICOS DE CALVARIA DE RATOS

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Orientadora: Profª.Drª. Rosemary Adriana Chiérici Marcantonio

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Guilherme José Pimentel Lopes de Oliveira

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Dados Curriculares

Guilherme José Pimentel Lopes de Oliveira

Nascimento: 29/04/1984-Maceió-AL

Filiação: Laroni Mathias Miranda de Oliveira

Joselena Maria Pimentel Lopes de Oliveira

2002-2006-Graduação em Odontologia

Faculdade de Odontologia da Universidade Federal de Alagoas (FO-UFAL)

2007-2008-Especialização em Periodontia

Faculdade de Odontologia de Araraquara (Foar-Unesp)

2008-2010-Curso de Pós-Graduação em Odontologia

Nível de Mestrado, Área de Concentração: Periodontia

Faculdade de Odontologia de Araraquara (Foar-Unesp)

2010-2014- Curso de Pós-Graduação em Odontologia

Nível de Doutorado, Área de Concentração: Periodontia

Faculdade de Odontologia de Araraquara (Foar-Unesp)

Dedicatória

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"A ciência nunca resolve um problema sem criar pelo menos outros dez"

George Bernard Shaw

"Periodontia é show brother."

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Oliveira GJPL. Efeito do extrato insaponificável de abacate e soja na doença periodontal induzida, na osseointegração de implantes e no reparo de defeitos criticos de calvaria de ratos [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2014.

Resumo

Esse estudo teve como objetivo avaliar, em ratos, a influência da utilização do extrato de óleo insaponificável de abacate e soja (ASU) no reparo da periodontite induzida por ligaduras, na osseointegração de implantes e na integração de biomateriais osteocondutores. Para isso foram avaliadas as hipóteses de que o ASU poderia: 1)Aumentar o reparo da periodontite induzida; 2)Favorecer o reparo associado ao tratamento da periodontite induzida; 3)Acelerar a osseointegração; 4)Influenciar na integração de enxertos osteocondutores. Para a avaliação da primeira hipótese foram utilizados 84 ratos que foram submetidos a indução da periodontite por meio de ligaduras e que foram randomicamente divididos em 4 grupos: CTR: Administração do soro fisiológico(SS) no mesmo dia da indução da periodontite; ASU/-7: Administração de ASU iniciada 7 dias antes da indução de periodontite(0.6 mg/kg); ASU/0: Administração de ASU iniciada no dia da indução da periodontite; ASU/+7: Administração do ASU iniciada no dia da remoção da ligadura. As ligaduras, que foram inseridas bilateralmente nos segundos molares superiores, foram removidas após 7 dias e os medicamentos foram administrados diariamente por gavagem até o sacrifício dos animais (7, 15 e 30 dias). Foram realizadas análise microtomográfica (%volume ósseo), histomorfométricas (% osso na região da furca, distancias da junção cemento-esmalte(JCE) ao topo da crista óssea(CO) e a porção apical do epitélio juncional (aJE), imunohistoquímica (TRAP, RANKL e Fosfatase Alcalina) e de qPCR (IL1β, IL6, TNFα, RANKL e Fosfatase Alcalina). Para avaliação da segunda hipótese foram

utilizados 84 ratos que foram submetidos a indução da periodontite por meio de ligaduras e que foram randomicamente divididos em 4 grupos: SRP–Administração de SS no dia do tratamento; SRP/ASU-7: Administração do ASU iniciada 7 dias antes da indução da periodontite(0.6 mg/kg); SRP/ASU0: Administração do ASU iniciada no dia da indução da periodontite; SRP/ASU+7: Administração do ASU iniciada no dia do tratamento da periodontite. A remoção da ligadura e a raspagem foram executados 7 dias após a indução da periodontite e os medicamentos foram administrados diariamente por gavagem até o sacrifício dos animais (7,15 e 30 dias). Foram executadas as mesmas análises utilizadas para o estudo da primeira hipótese. Para a avaliação da terceira hipótese, foram instalados 1 implante por tíbia em 30 ratos que foram randomicamente divididos em 3 grupos: ASU1: Administração de ASU iniciada 7 dias antes de instalação dos implantes(0.6 mg/kg); ASU2: Administração de ASU iniciada no dia da instalação dos implantes; CTL: Administração de SS iniciada no dia da instalação do implantes. Os medicamentos foram administrados diariamente por gavagem até o sacrifício dos animais (60 dias). Foram executadas análises radiográficas, biomecânicas, histomorfométrica (Contato osso-implante(%BIC) e área de osso entre as roscas(%BBT), histológica descritiva e imunohistoquímica (BMP2, TGFβ1 e Osteocalcina). Para avaliação da quarta hipótese foram executados defeitos críticos em calotas (DCC-5mm) de 84 ratos que foram randomicamente divididos em 2 grupos: CTR-Administração de SS e ASU: Administração de ASU(0.6 mg/kg). Adicionalmente os animais foram divididos em 3 subgrupos de acordo com o biomaterial utilizado para preencher o defeito: COA-Defeito preenchido com coágulo; OBD-Defeito preenchido com osso bovino desproteinizado; TCP/HA- Defeito preenchido com β-fosfato tricálcio/Hidroxiapatita. A administração dos medicamentos foi iniciada 15 dias antes da confecção dos DCC e foram aplicados diariamente por gavagem até o sacrifício dos

animais (15 e 60 dias). Foram executados análise microtomográfica (%osso e biomaterial), histologia descritiva e histomorfométrica (%osso, biomaterial e tecido conjuntivo). No estudo I foi verificado que os grupos ASU0 e ASU+7 apresentaram maiores porcentagens de tecido ósseo na região da furca e menores distâncias JCE-CO que o grupo CTR nos períodos de 7 e 15 dias. Adicionalmente foi verificada uma maior expressão de IL-1β, RANKL e TRAP e menor expressão de fosfatase alcalina no grupo CTR em relação ao grupo ASU+7. No estudo II foi verificado que não houve diferenças entre os grupos com relação as análises histométrica e microtomográfica. Foi verificado uma maior expressão do RNAm de fosfatase alcalina no grupo SRP/ASU+7 em relação aos outros grupos aos 30 dias, e menor expressão do RNAm de RANKL nos grupos SRP/ASU0 e SRP/ASU+7 em relação aos grupos SRP e SRP/ASU-7 aos 15 dias. No estudo III os grupos de animais dos grupos ASU1 e ASU2 apresentaram maiores valores de %BIC na região cortical e maior expressão de BMP2 e TGFβ1 em relação ao grupo CTL. No estudo IV ocorreu uma maior formação óssea no grupo ASU em relação ao grupo CTR no subgrupo COA, porém não houve diferenças entre os grupos subgrupos OBD e a TCP-HA. Dessa forma pode-se concluir que o extrato de ósseo insaponificável de abacate e soja promoveu maior reparo na periodontite induzida em ratos, porém esses resultados não foram consistentes pois esse efeito não foi confirmado no modelo de tratamento da periodontite induzida. Esse medicamento induziu, de forma sutil uma aceleração da osseointegração de implantes e uma maior formação óssea associada a DCC que não foram preenchidos com biomateriais.

Palavras-chaves: Cicatrização; Implantes dentários; Fitoterapia; Materiais biocompatíveis; Mediadores da inflamação; Periodontite.

Oliveira GJPL. Effect of the avocado/soybean unsaponifiables on the induced periodontitis, on the osseointegration, and on the critical sized calvaria bone defect repair in rats [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2014.

Abstract

The aim of this study was to evaluate in rats the effect of the avocado soybean unsaponifiables (ASU) on the bone repair in induced periodontitis, on the osseointegration of dental implants, and on the repair of the critical sized calvaria defects filled or no with biomaterials. For this purpose, the following hypothesis regarding the use of ASU were tested: 1)Improve the periodontal repair in induced periodontitis; 2)Improve the periodontal repair after the treatment of the induced periodontitis; 3) Accelerate osseointegration; 4) Improve the integration of osteoconductors bone grafts in the critical size calvaria defects. For the evaluation of the first hypothesis, eighty-four animals were randomly assigned into four equally-sized groups, receiving daily by gavage either sterile saline (CTR) or ASU (0.6 mg/kg), starting either 7 days prior to- (ASU/-7), or on the day- (ASU/0), or 7 days after (ASU/+7) periodontitis induction. Periodontitis was induced by placing silk ligatures into the gingival sulcus of the second maxillary molars for 7 days; thereafter the ligatures were removed. Seven animals from each group were euthanized at 7, 15 or 30 days after ligature removal. Bone resorption was evaluated by histomorphometry and micro CT. Immunohistochemistry was used to evaluate TRAP, RANKL, Alkaline phosphatase and qPCR to evaluate IL-1β, TNF-α, IL-6, RANKL, Alkaline phosphatase (AP). For the evaluation of the second hypothesis, periodontitis was induced in 84 rats via ligature placement around the second upper molar, which was removed after 7 days,

and scaling was performed at this time. Subsequently, the rats were randomly allocated to four groups with 21 animals each: One in which saline solution (SS) was administered (SRP) and three in which ASU was administered (0.6 g/kg/day), beginning either 7 days before the induction of periodontitis (SRP/ASU-7), on the day of periodontitis induction (SRP/ASU0), or on the day of treatment (SRP/ASU+7). The ASU and SS were administered daily by gavage until the sacrifice of the animals (7, 15, and 30 days). The analysis performed was the same of the evaluation of the first hypothesis. For the evaluation of the third hypothesis, thirty rats were randomly assigned into one of three equal-sized groups: 1) ASU1; administration of ASU, starting 7 days prior to implant installation, 2) ASU2; administration of ASU, starting on the day of implant installation, and 3) CTL; administration of saline solution. In all animals, one titanium implant was placed in each tibia. All animals received ASU or saline solution by gavage daily until sacrifice 60 days post-operatively. Implant osseointegration and bone maturation were assessed by biomechanical analysis, radiographic bone density, descriptive histology, immunohistochemical analysis for bone morphogenetic protein 2 (BMP2), transforming growth factor beta 1 (TGFβ1), and osteocalcin (OCN), histomorphometric evaluation of bone-to-implant contact (BIC) and mineralized bone area fraction within the threads of the implant (BA). For the evaluation of the forthy hyphotesis, one critical sized calvaria defect (CCD-0.5 mm) was made in each of 84 rats. These defects were filled with coagulum (COA), deproteinized bovine bone (DBB), and β-tricalcium phosphate/hydroxyapatite (TCP/HA). ASU (0.6 g/kg) or saline solution (CTR) was administered daily by gavage from 15 days before surgery until the animals were euthanized 15 or 60 days after surgery (7 animals per period/group). The description and composition of the tissues that filled the CCDs were analyzed by micro CT and histomorphometry. It was showed

in the study I that the histomorphometry and micro CT showed larger bone resorption in the CTR than in the ASU/0 (15 days), and ASU/+7 (7 and 15 days). CTR presented also with a higher expression of TRAP (15 and 30 days) and RANKL (7 and 15 days) comparing to ASU/0 and ASU/+7. Similarly, qPCR showed higher levels of RANKL and IL1β and lower levels of AP in CTR comparing with all other groups (All periods). In the study II, the SRP/ASU+7 presented higher expression of the alkaline phosphatase than all the other groups at 30 days. The SRP/ASU0 and SRP/ASU+7 groups presented lower expression of RANKL mRNA than the groups SRP e SRP/ASU-7 at 15 days. However, there were no differences between the groups regarding the percentage of bone fill and in the expression of the proteins. In the study III, ASU1 and ASU2 showed a 3- and 9- times higher expression of BMP2 and TGFβ1, respectively, compared to CTL ($p<0.05$). Histomorphometric analysis, however, showed that both ASU1 and ASU2 groups presented significantly higher BIC values only in the cortical bone compartment when compared to CTL ($p<0.05$). In the study IV, the percentage of bone fill in the CCD of the COA-ASU group was significantly higher than that in the COA-CTR group at both evaluated time points ($p<0.05$). There were no differences regarding the percentage of bone between the DBB-ASU and DBB-CTR groups and the TCP/HA-ASU and TCP/HA-CTR groups at either time point ($p<0.05$). It can be concluded that the ASU can improve the repair of the induced periodontitis, but this effect was inconsistent since the ASU effects were not confirmed in the treatment of the induced periodontitis. ASU consumption has only a subtle effect on implant osseointegration and induced an enhancement in bone formation in the CCDs filled with coagulum.

Key-Words: Biocompatible materials; Dental implants; Fitotherapy; Healing; Mediators of inflammation, Periodontitis.

Lista de Abreviaturas e Nomenclaturas

PAD-Peptidilarginina deiminase

AINES- Anti-inflamatórios não esteroides

TNFα- Fator de necrose tumoral α

ASU- Extrato de óleo insaponificável de abacate e soja

PGE2- Prostaglandina E2

iNOS- Indutor de oxido nítrico sintetase

IL-Interleucina

MMP- Metaloproteinases de matriz

TGFβ- Fator transformante de crescimento β

BMP-Proteína morfogênica óssea

TIMP- Inibidor de metaloproteinases

RNA- Ácido ribonucleico

PCR- Reação em cadeia da polimerase

COX2- Cicloxigenase-2

OBD- Osso bovino desproteinizado

TCP/HA- β-fosfato tricálcio/hidroxiapatita

rhBMP2- Proteína morfogênica óssea recombinante humana

rhGDF-5- Fator de crescimento e diferenciação 5 recombinante humana

DCC- Defeitos críticos em calotas

TRAP- Fosfatase ácida tartarato resistente

RANKL- Receptor ativador do fator nuclear kappa β

AP-Fosfatase Alcalina

CTR- Controle

JCE- Junção cemento esmalte

CO- Crista óssea

BIC- Contato osso-implante

mg- miligrama

SS-Solução salina

DCC-Defeito crítico de calota

Sumário

Introdução

Introdução

A artrite reumatóide é uma doença autoimune que é caracterizada pela destruição dos tecidos conjuntivos articulares mediados por aumento da expressão de mediadores pró-inflamatórios (Detert et al.[22], 2010; Culshaw et al.[19], 2011). Apesar do agente desencadeador das respostas autoimunes que ocorrem na artrite reumatóide não serem totalmente conhecidos, tem sido destacado a importância da resposta imunológica frente a antígenos gerados pelo processo de citrunilização, que consiste na modificação da arginina em citrulina (Wegner et al.[67], 2009). Como a citrulina não é um aminoácido naturalmente sintetizado pelo organismo a sua incorporação em proteínas do hospedeiro causa uma alteração estrutural que podem converter essas moléculas em auto antígenos (Wegner et al.[67], 2009).

Como a bactéria *Porphyromonas gingivalis* é o único microorganismo capaz de produzir a enzima peptidilarginina deiminase (PAD), que converte a arginina em citrulina, e sendo essa bactéria altamente associada com a etiologia e progressão das doenças periodontais (Liao et al.[46], 2009; Smit et al.[60], 2011), tem-se pesquisado esse via de associação entre as periodontites e a artrite reumatóide (Bartold et al.[7], 2010). Adicionalmente a esse agente comum entre as duas doenças, os mecanismos patogênicos que mediam as destruições dos tecidos articulares que ocorrem na artrite reumatóide são semelhantes aos que ocorrem nas destruições do periodonto de inserção durante as periodontites (Berthelot, Le Goff [8], 2010; Detert et al[22]., 2010).

Dessa forma, terapias indicadas para o tratamento da artrite reumatóide podem beneficiar o tratamento das periodontites (Culshaw et al.[19], 2011). Algumas drogas aplicadas a pacientes com artrite tais como anti-inflamatórios não esteroides (AINES) (Emery et al.[26], 1999), bifosfonatos (Breuil, Euller-Ziegler[11], 2010) e anti-TNFα (St Clair et al.[64], 2004), também foram aplicados para o tratamento das periodontites

(Williams et al.[70], 1989; Jeffcoat et al.[37], 1995; Lane et al.[44], 2005; Ortiz et al.[56],2009). Entretanto, esses medicamentos induzem efeitos colaterais e apresentam ação apenas no bloqueio da inflamação e acredita-se que um medicamento em potencial para melhorar os resultados do tratamento periodontal também deve ativar vias de reparo tecidual (Van Dyke, Serhan[66], 2003; Kantarci et al.[41], 2006; El-Sharkawy et al.[25], 2010).

O extrato de óleo insaponificável de abacate e soja (ASU) é um medicamento a base dos frutos e das sementes do abacate e da soja na proporção de 1:2 (Cameron et al.[16], 2011). Esse medicamento tem sido indicado para o tratamento da artrite reumatoide e da osteoartrite (Appelboom et al.[4], 2001; Maheu et al.[49], 2014) e é considerado um agente de ação lenta e modificador da estrutura do tecido conjuntivo (Cake et al.[15], 2000; Boileau et al.[10], 2009). Esse efeito foi demonstrado em estudos sobre o efeito do ASU na osteoartrite induzida em cães (Boileau et al.[10], 2009) e ovelhas (Cake et al.[15], 2000) aonde foi verificado histologicamente uma menor destruição dos tecidos articulares. Estudos clínicos que avaliaram o efeito do ASU sobre o tratamento da artrite demonstraram radiograficamente uma redução no espaço intra-articular e uma melhora na cinesiologia das articulações (Appelboom et al.[4], 2001; Maheu et al.[49], 2014). Estudos in vitro demonstraram que o ASU promove uma redução na expressão de mediadores pró-inflamatórios como a PGE2, TNFα, iNOS, IL1β, -6, -8 (Henrotin et al.[35,36], 2003, 2006; Au et al.[5], 2007); e metaloproteinases da matriz como a MMP2, -3. -13 (Kut-Lasserre et al.[43], 2001; Henrotin et al.[36], 2003; Gabay et al.[29], 2008); além de promover aumento da expressão de fatores de crescimento tais como o TGFβ1, TGFβ2 e BMP2 (Kut-Lasserre et al.[43], 2001; Andriamanalijaona et al.[2], 2006; Henrotin et al.[35], 2006) e de proliferação de proteínas da matriz de tecido conjuntivo tais como a condroitina sulfato 1 e o colágeno (Henrotin et al.[35,36], 2003, 2006; Lippiello et al.[48], 2008). Adicionalmente, estudos in vivo demonstraram redução da expressão de iNOS e

MMP 13 (Boileau et al.[10], 2009) e aumento na secreção de TGF β1 e TGF β2 (Altinel et al.[1], 2007).

Apenas dois estudos in vitro avaliaram o efeito do ASU sobre células do periodonto:

Kut-Lasserre et al.[43] , 2001 avaliaram em cultura de fibroblastos gengivais a ação do extrato de óleo insaponificável de abacate e soja sobre a expressão de metaloproteinases da matriz (MMP-2 e MMP-3), bem como de seus inibidores de metaloproteinases (TIMP-1 e TIMP-2) na presença ou ausência de IL-1β. As culturas de fibroblastos foram mantidas por 72 horas em contato com o ASU ou com as frações a base de abacate ou soja nas concentrações de 0.1, 0.5, 2.5, 5.0 e 10.0 μg/ml após o tratamento ou não com IL-1β. Os níveis de MMP-2, MMP-3, TIMP-1 e TIMP-2 foram avaliados por *dot blot* e zimografia. Foi verificado que o extrato de óleo insaponificável de abacate e soja reduziu a secreção das MMPs e aumentou a secreção das TIMPs que foram alteradas pela aplicação de IL-1β nos meios de cultura. Os autores desse estudo chegaram à conclusão que a utilização deste fármaco pode inibir a ação pró-inflamatória promovida pela IL-1 β.

Adriamanalijaona et al.[2], 2006 avaliaram o efeito do ASU na expressão dos fatores transformantes de crescimento (TGF-β1 e TGF-β2) e da proteína morfogênica óssea 2 (BMP-2) em fibroblastos do ligamento periodontal e osso alveolar, na presença de IL-1β em culturas de células. As células foram incubadas por 48 horas em ASU na presença ou na ausência de IL-1β, e os níveis do RNAm e das proteínas foram avaliados por qPCR e Elisa. Os resultados desse estudo demonstraram que o ASU estimula a expressão de, TGF-β1 e BMP-2 nesses células. Os autores concluíram que esse aumento na expressão de fatores de crescimento pode explicar seus supostos efeitos no

tratamento da doença periodontal já que o TGF-β1 e a BMP-2 estimulam o reparo do periodonto.

Todos esses efeitos induzidos pelo ASU são interessantes para uma droga com potencial de ser utilizada como adjunto no tratamento das periodontites. Adicionalmente a isso, o estimulo a fatores de crescimento relacionados a formação óssea é relevante para outros procedimentos que se beneficiem desse efeito tais como a inserção de implantes de titânio e de biomateriais em defeitos ósseos. Segue abaixo uma breve justificativa de se testar o efeito do ASU no tratamento das periodontites, na osseointegração de implantes e no reparo ósseo associado ao uso de biomateriais.

Tratamento da doença periodontal

A periodontite é uma doença imunoinflamatória induzida pelo biofilme dentário que provoca uma resposta do hospedeiro contra os antígenos bacterianos e que é evidenciada clinicamente pela reabsorção do osso alveolar com concomitante perda de inserção e formação de bolsas periodontais (Van Dyke, Serhan[66], 2003). O tratamento tradicional dessa doença é executado por meio da raspagem da superfície radicular para remover o biofilme bacteriano (Heitz-Mayfield, Lang[34], 2013). Entretanto, apesar dos bons resultados clínicos que a raspagem promove no controle das periodontites (Lindhe et al.[47], 1984), sugere-se que a aplicação de medicamentos que modifiquem a resposta inflamatória do hospedeiro frente ao desafio bacteriano pode melhorar os resultados do tratamento periodontal (Kantarci et al.[41], 2006). Essa terapia, denominada de modulação da resposta do hospedeiro, tem como base a constatação de que a progressão da doença periodontal é altamente dependente da expressão de mediadores biológicos da inflamação e que a alteração da expressão dessas proteínas modificam o perfil inflamatório do paciente e auxiliam na resolução desse processo patológico (Kirkwood et al.[42], 2007).

Várias drogas imunomoduladoras da resposta inflamatória foram sugeridas como alternativas para o tratamento adjunto das periodontites (Kantarci et al.[41], 2006; Kirkwood et al.[42], 2007). Dentre elas destaca-se a aplicação de anti-inflamatórios não esteroidais (AINES) seletivos (Yen et al.[71], 2008) ou não da cicloxigenase-2 (COX2) (Williams et al.[70], 1989), bisfosfonatos (Lane et al.[44], 2005) e baixas dosagem de doxiciclina (Emingil et al.[27],2004). Em comum, todas essas drogas suprimem de alguma forma as reações inflamatórias que ocasionam a reabsorção óssea, seja reduzindo o *turnover* ósseo, a síntese de prostaglandinas E2 (PGE2) ou da metaloproteinase da matriz 8 (MMP8) (Kantarci et al.[41], 2006; Kirkwood et al.[42], 2007). Entretanto o protocolo terapêutico com esses medicamentos apresentam limitações devido aos seus efeitos colaterais. Adicionalmente a isso, todos esses medicamentos agem apenas por inibição de mediadores anti-inflamatórios da inflamação, e atualmente sugere-se que um medicamento modulador da resposta do hospedeiro deve agir também no estimulo de mediadores de reparo tecidual (Kantarci et al.[41], 2006; Bhatavadekar, Williams[9], 2009). Levando-se em consideração os efeitos anti-inflamatórios e proliferativos do ASU, e os bons resultados do mesmo no tratamento das artrites, que possuem mecanismo patogênico de destruição tecidual semelhante ao que ocorre nas periodontites, avaliamos o efeito desse medicamento sobre o reparo da periodontite induzida em ratos por dois modelos experimentais aonde no primeiro modelo foi executado a remoção da ligadura e no segundo modelo a remoção da ligadura foi associada a raspagem.

Osseointegração de implantes

A utilização de implantes osseointegrados para substituir elementos dentários que foram perdidos por diferentes motivos tem sido aplicada eficientemente no tratamento do edentulismo e tem apresentado bons índices de sucesso e sobrevivência (Buser et al.[13], 1997; Lekholm et al.[45], 1999; Jung et al.[39], 2008). Adicionalmente a isso,

fatores como a expansão de casos de edentulismo que podem ser tratados com implantes e o envelhecimento da população podem aumentar significativamente o número de pessoas aptas para receberem implantes dentários (Ellis Jr, Prince[24], 2008; Anitua, Orive[3], 2010; Collaert et al.[17], 2011; Hatano et al.[32], 2011;).

Apesar da quantificação ideal de osseointegração para o sucesso clínico dos implantes osseointegrados não ter sido ainda determinada, muitos esforços tem sido executados com o objetivo de aumentar ou acelerar o processo de osseointegração, principalmente para beneficiar os pacientes que apresentam condições que alteram o metabolismo ósseo (Schlegel et al.[58], 2013), melhorar o prognóstico de implantes instalados em osso com qualidade mecânica reduzida (Grassi et al.[30], 2007) ou para reduzir o tempo clínico de espera para iniciar dos procedimentos protéticos (Mertens, Steveling[54], 2011).

Um método comumente aplicado para acelerar a osseointegração é a modificação das superfícies dos implantes com o intuito de promover uma resposta local positiva do hospedeiro. Modificações da microtopografia da superfície do implante por meios mecânicos ou químicos executados com intuito de aumentar a rugosidade da superfície do implante demonstraram acelerar a osseointegração em comparação com implantes usinados (Buser et al.[14], 1991; Wennerberg et al.[68], 1995; Buser et al.[12], 2004; Ellingsen et al.[23], 2004; Mendes et al.[53], 2007). Além disso, o revestimento de implantes com substâncias bioativas tais como bisfosfonatos (Yoshinari et al.[72], 2002; Kajiwara et al.[40], 2005) e fatores de crescimento (Wikesjo et al.[69], 2008) também tem demonstrado promover aumento na osseointegração.

Uma outra abordagem menos explorada é a utilização sistêmica de substâncias que promovem um efeito na remodelação óssea. Foi verificado que a aplicação de sinvastatina (Ayukawa et al.[6],2004), paratormônio (Daugaard et al.[20], 2002) e ranelato

de estrôncio (Maimoun et al.[50], 2010) promoveram uma melhora no processo de osseointegração. De forma similar, a utilização de produtos "naturais" também tem sido proposta como alternativa para promover aumento na osseointegração. Um estudo pré-clínico in vivo demonstrou que a aplicação sistêmica de *Symphytum officinale* em doses homeopáticas causou um aumento nos valores de contra-torque de remoção associado a aumento na densidade radiográfica ao redor dos implantes comparado com o grupo controle (Spin Neto et al.[61], 2010).

Devido aos efeitos de proliferação de tecido conjuntivo e ao aumento na expressão de fatores de crescimento associados com a indução de formação de tecido ósseo, nos pareceu razoável avaliar se essa supra-regulação nesse fatores proporcionados pelo ASU influenciaram na osseointegração de implantes.

R***eparo ósseo associado a biomateriais***

A aplicação de biomateriais para o reparo de diferentes defeitos ósseos ou para aumento da disponibilidade desse tecido para a instalação de implantes osseointegrados tem se tornado uma prática cada vez mais corriqueira, sendo que uma grande gama de biomateriais tem sido propostos para utilização como substituto do tecido ósseo (Del Fabbro et al.[21], 2004; Ten Heggeler et al.[65], 2011).

O osso autógeno é considerado o padrão ouro entre os biomateriais propostos para regeneração/reparo ósseo, pois é o único biomaterial que apresenta concomitantemente as propriedades biológicas de osteogênese, osteoindução e osteocondução (Handschel et al.[31], 2009). Entretanto alguns fatores como a limitada disponibilidade e a morbidade relacionada ao sítio doador são algumas das limitações da utilização desse biomaterial (Nkenke et al.[55], 2001; Del Fabbro et al.[21], 2004). Dessa forma, a utilização de outros biomateriais com propriedades biológicas osteocondutoras, mas que não necessitam serem removidos do próprio paciente, tais como o osso bovino

desproteinizado(OBD) e a cerâmica bifásica a base de β-fosfato tricálcio/hidroxiapatita (TCP/HA) tem sido propostos como alternativa a utilização do enxerto de osso autógeno (Cordaro et al.[18], 2008; Mardas et al.[51], 2010).

O OBD é um biomaterial de origem bovina produzido pela remoção do componente proteico do tecido ósseo e constituído principalmente por hidroxiapatita estruturalmente semelhante a humana (Froum et al.[28], 2008), enquanto que a TCP/HA é uma cerâmica sintética formada pela junção de 40% de β-fosfato tricálcio e 60% de hidroxiapatita (Froum et al.[28], 2008). Ambos os biomateriais tem sido aplicados clinicamente em alvéolos pós-extração (Mardas et al.[51], 2010), elevação do soalho do seio maxilar (Cordaro et al.[18], 2008) e em defeitos periodontais (Zafiropoulos et al.[73], 2007; Stavropoulos, Karring[63], 2010) apresentando bons resultados clínicos. Adicionalmente tem sido verificado que implantes inseridos nesses biomateriais apresentam taxa de sobrevivência semelhante aos implantes instalados em áreas aonde o enxerto autógeno foi aplicado (Del Fabbro et al.[21], 2004). Conceitualmente, ambos os biomateriais promovem formação óssea que ocorre ao redor de suas partículas, sendo acompanhado pela reabsorção controlada das mesmas, e que esses biomateriais inicialmente seriam mantenedores de espaço para a formação óssea (Rokn et al.[57], 2011). Entretanto, estudos histológicos demonstram que esses biomateriais não são completamente reabsorvidos e que a formação óssea ocorre ao redor de partículas que estejam perto do leito cirúrgico, enquanto que partículas que estão afastadas desse leito são encapsulas por tecido conjuntivo (Cordaro et al[18]., 2008; Mardas et al.[51], 2010), o que pode interferir na adequada regeneração/reparo do tecido ósseo.

A ausência das propriedades de osteoindução e osteogênese desses biomateriais está relacionada com essas limitação em relação a cicatrização óssea promovida pelos mesmos (Handschel et al.[31], 2009). Alternativas como a misturas desses biomateriais

com osso autógeno tem sido propostas para melhorar as propriedades biológicas (Zafiropoulos et al.[73], 2007; Handschel et al.[31], 2009; Jensen et al.[38], 2012), entretanto isso iria contra uma das principais justificativas da utilização do OBD e da TCP/HA que seria a eliminação da necessidade de captação de enxerto de um leito doador do paciente. Adicionalmente, esses biomateriais tem sido associado com fatores de crescimento tais como a proteína morfogênica óssea recombinante humana (rhBMP2) (Schwarz et al.[59], 2008), e com o fator de crescimento e diferenciação 5 recombinante humana (rhGDF-5) (Stavropoulos et al.[62], 2011), porém a aplicação terapêutica de fatores de crescimento é de alto custo (Mariner et al.[52], 2013).

Devido aos efeitos do ASU sobre a expressão de fatores de crescimento que estão relacionados a síntese de tecido ósseo e a ausência de estudos que avaliassem o efeito do ASU sobre a integração de diferentes biomateriais associado a defeitos, investigamos se esse estimulo nos fatores de crescimento e na síntese de proteínas da matriz poderiam acelerar e melhorar o padrão do reparo em defeitos críticos em calotas de ratos (DCC) associados a inserção ou não de OBD e da TCP/HA.

Proposição

Proposição

Hipótese

O extrato de óleo insaponificável de abacate e soja tem efeito na indução/tratamento da doença periodontal, na osseointegração de implantes e no reparo de defeitos críticos em calotas de ratos.

Objetivos específicos

Para avaliar a hipótese esse projeto foi divididos nos seguintes objetivos específicos:

1) Avaliar o efeito do extrato de óleo insaponificável de abacate e soja na progressão da doença periodontal induzida por ligaduras em ratos
2) Avaliar o efeito do extrato de óleo insaponificável de abacate e soja sobre o reparo após o tratamento da doença periodontal induzida por ligaduras em ratos
3) Avaliar o efeito do extrato de óleo insaponificável de abacate e soja sobre a osseointegração de implantes instalados em tíbias de ratos
4) Avaliar o efeito do extrato de óleo insaponificável de abacate e soja sobre o reparo de defeitos críticos em calotas de ratos.

Capítulo 1

Capítulo 1

Original Reseach: Avocado/Soybean Unsaponifiables (ASU) enhances bone repair after ligature-induced periodontitis in rats*

Guilherme José Pimentel Lopes de Oliveira (Oliveira GJPL) - PhD student in Periodontology[1]

Luiz Guilherme Freitas de Paula (Paula LGF) - PhD student in Implantology [1]

João Antônio Chaves de Souza (Souza JAC) - PhD in Implantology [1]

Rubens Spin-Neto (Spin-Neto R) - PhD student in Oral Radiology [2]

Andreas Stavropoulos (Stavropoulos A) - Professor of Periodontology [3]

Rosemary Adriana Chiérici Marcantonio (Marcantonio RAC) - Professor of Periodontology [1]

1 Section of Periodontology, Department of Diagnosis and Surgery, School of Dentistry of Araraquara - São Paulo State University, Araraquara, SP, Brazil.

2 Section of Oral Radiology, Department of Dentistry - Aarhus University, Aarhus, Denmark

3 Department of Periodontology, Faculty of Odontology - Malmö University, Malmö, Sweden

Author for Correspondence:

Rosemary Adriana Chiérici Marcantonio

Humaitá St, 1680. Zip Code 14801-130, Araraquara, SP.Brazil

Phone: +55(16) 3301-6376 / Fax: +55(16) 3301-6376

E-mail: adriana@foar.unesp.br

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*** A ser enviado ao periódico Plos One**

Abstract

The aim of this study was to evaluate the effect of Avocado/Soybean unsaponifiables (ASU) consumption on bone loss in association with ligature-induced periodontitis in rats. Eighty-four animals were randomly assigned into four equally-sized groups, receiving daily by gavage either sterile saline (CTR) or ASU (0.6 mg/kg), starting either 7 days prior to- (ASU/-7), or on the day- (ASU/0), or 7 days after (ASU/+7) periodontitis induction. Periodontitis was induced by placing silk ligatures into the gingival sulcus of the second maxillary molars for 7 days; thereafter the ligatures were removed. Seven animals from each group were euthanized at 7, 15 or 30 days after ligature removal. Bone resorption was evaluated by histomorphometry and micro CT. Immunohistochemistry was used to evaluate TRAP, RANKL, Alkaline phosphatase and qPCR to evaluate IL-1β, TNF-α, IL-6, RANKL, Alkaline phosphatase (AP). Statistical analysis was performed with the Shapiro-Wilk, ANOVA, and Tukey's tests for the normal data and the Kruskall Wallis, and Dunnet´s tests for the non-normal data ($p < 0.05$). Histomorphometry and micro CT showed larger bone resorption in the CTR than in the ASU/0 (15 days), and ASU/+7 (7 and 15 days). CTR presented also with a higher expression of TRAP (15 and 30 days) and RANKL (7 and 15 days) comparing to ASU/0 and ASU/+7. Similarly, qPCR showed higher levels of RANKL and IL1β and lower levels of AP in CTR comparing with all other groups (All periods). ASU exhibited a positive effect on bone repair following ligature-induced periodontitis in rats, but had no preventive effect.

Key-Words: Animal Studies, Avocado/soybean unsaponifiables, Host modulation, Periodontitis.

Introduction

During recent years, modulation of host response has been pursued as possible approach in the treatment of periodontitis[1, 2]. The rationale for such an approach is based on the known fact that, primarily responsible for attachment loss and bone resorption in periodontitis is the host immune-inflammatory response against the bacterial challenge[3], but also on the accumulating evidence that the inflammatory response may influence biofilm composition[4]. Briefly, in periodontitis susceptible individuals, excessive production of inflammatory mediators (e.g. interleukins and prostaglandins) and destructive enzymes (e.g., MMPs) – as part of the inflammatory response against infection from the oral biofilm – surpasses that of anti-inflammatory mediators and enzyme inhibitors, causing tissue breakdown. Thus, host modulation therapy aims basically at reducing (ideally avoiding) tissue breakdown either by suppressing the destructive– and⁄or enhancing the protective/reparative component of host response. For example, significant clinical improvements have been observed after long-term administration of sub-antimicrobial doses of doxycycline as adjunct to routine periodontal treatment, aiming at host modulation on the basis that doxycycline can exert an anti-MMP activity separately from its antibiotic properties[5, 6].

Host modulation is indeed employed also in the treatment of systemic diseases, where the host immune-inflammatory response consists an important aspect in their pathogenesis and related tissue breakdown like in multiple myeloma[7], osteoporosis[8], and rheumatic diseases[9, 10]. Rheumatoid arthritis (RA) is an autoimmune disease characterized primarily by inflammation in the synovial joints, which may eventually result in damage of the articular cartilage and underlying bone leading to dysfunctional or ankylotic joints[11]. Despite differences in etiology, RA and periodontitis shares common features as it regards the pro-inflammatory and anti-inflammatory cytokine

networks and proteolytic proteins in the pathogenesis of both diseases [e.g. interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-a); matrix-metalloproteinases (MMPs)[12, 13]]. Because of this fact, the use of drugs indicated for reducing immune-inflammatory response of rheumatoid arthritis may be an alternative for the modulation of host response in periodontal disease[14, 15].

Among the drugs used for palliative treatment of RA and osteoarthritis (OA), avocado and soybean unsaponifiables (ASU) has gained attention during recent years[16]. This drug has been shown to be clinically effective in the remission of the symptoms of RA, including pain reduction, and a reduction of the inter-articular space on radiographies has been observed with ASU consumption [17, 18]. Preclinical studies have indeed confirmed histologically the protective effect of ASU on intra-articular bone and cartilage[19, 20]. In vitro studies in various arthritis relevant cells, including chondroblasts, osteoblasts, and macrophages, have demonstrated that ASU reduces the secretion of a number pro-inflammatory mediators (e.g. IL- 1β, -3, -6, -8, -13, PGE-2, TNF-a)[21-25], proteolytic enzymes (e.g. MMPs)[25, 26] and enzymes that induce the synthesis of oxygen reactive specimens (e.g. iNOS)[23, 25]. Simultaneously, ASU upregulates inhibitors of metalloproteases (eg. TIMP1 and TIMP2)[25, 26], as well as of growth factors relevant for wound- and bone healing (e.g. TGF-β1 and BMP-2)[27, 28], and also components of the connective tissue matrix such as collagen and glycosaminoglycans[24, 29]. Furthermore, ASU resulted in an increase in the expression of anti-inflammatory mediators and growth factors in periodontal ligament derived fibroblasts and osteoblasts[26, 27].

It seems thus reasonable to hypothesize that ASU may also interfere with periodontitis pathogenesis. Thus, the aim of this study was to evaluate the effect of ASU consumption on bone loss in association with ligature-induced periodontitis in rats.

Material and methods (ANEXO 1)

This study was approved by the Ethical Committee for the use of animals in research of the São Paulo State University - UNESP (CEUA 17/2009) (ANEXO 3), School of Dentistry of Araraquara, Brazil. Eighty-four male rats (*Rattus norvegicus holtzman*), weighing 250 - 300 g were kept in an environment with controlled temperature (21 ± 1 ° C), humidity (65 - 70%) and light cycle (12 hours). The animals had *ad libitum* access to standard laboratory diet and water.

Groups

The animals were randomly allocated into 4 equally-sized treatment groups: a) sterile saline solution (SS) administration beginning at the day of ligature removal as control (CTR); b)ASU administration (0.6 g / kg / day)[30] (300 Piascledine, Expanscience Lab, France) beginning 7 days prior to periodontitis induction (ASU/-7); c) ASU administration beginning at the day of periodontitis induction (ASU / 0); d) ASU administration beginning at the day of ligature removal (ASU / +7). ASU and SS were administered by gavage (1.0 ml) once daily until sacrifice at 7, 15, and 30 days after ligature removal (i.e. 7 animals per treatment group per observation period).

Periodontitis induction

The animals were anesthetized with a combination of Ketamine (0.08 ml/100g; Rompum, Bayer SA, São Paulo, SP, Brazil) and Xylazine (0.04 ml/100g; Rompum, Bayer SA, São Paulo, SP, Brazil) and ligatures (cotton yarn, nº 24) were placed in the subgingival sulcus around the second upper molars on both sides of the maxilla, with the exception of 2 animals per group where the ligatures were placed on only one side. The ligatures were then removed after seven days[31].

Retrieval of samples

Euthanasia was performed by an overdose of anesthetic solution. In each of the 12 groups, the maxillae were removed in block and divided randomly by drawing a lot into seven hemi-maxillae for histomorphometric/immunohistochemistry analysis, and five hemi-maxillae for qPCR and microCT analysis; and the two hemi-maxillae without ligation were used as controls for the histology description. Hemi-maxillae aimed for histomorphometric/immunohistochemistry analysis were fixed in 4% paraformaldehyde for 48 hours. Hemi-maxillae aimed for qPCR had the gingival tissue surrounding the upper second molars removed and immediately frozen in liquid nitrogen and kept at -80 °C until extraction of mRNA; the remaining blocks containing the teeth and surrounding alveolar bone were fixed in 4% paraformaldehyde for 48 hours and subsequently scanned in micro CT scanner.

Histology - Histomorphometry

After fixation, the samples were decalcified in EDTA at 7% for 10 weeks and then processed for embedding in paraffin. Serial sections, 5 μm thick, were prepared in mesio-distal direction along the axis of the tooth and were stained with hematoxylin - eosin (HE). Histological and histomorphometric analysis was performed while viewing the images on a computer screen, via a camera connected to an incandescent and polarized light microscope (Leica - Reichert Diastar Products & Jung, Wetzlar, Germany), and using an image analysis software (Image J, Jandel Scientific, San Rafael, CA, USA), by experienced, calibrated, and masked evaluators. On three equidistant sections from each specimen, representing the entire width of the tooth (i.e. buccal, cental, and palatal aspect of the furcation), the following parameters were estimated: a)

area of mineralized bone and bone marrow within the furcation expressed as percentage of the furcation area (i.e. from the roof of the furcation to 1000μm apically[32]), and b) linear distances between the cemento-enamel junction (CEJ) to the top of alveolar crest (CEJ- CB) at a point where the periodontal ligament showed a normal width, and the CEJ to the apical termination of the junctional epithelium (CEJ- aJE).

MicroCT

The samples were scanned in a microCT scanner (Skyscan, Aartselaar, Belgium) and subsequently the images generated were reconstructed, reoriented spatially and analyzed by dedicated software (NRecon / DataViewer / CTAN, Skyscan, Aartselaar, Belgium). Briefly, the sagittal plane of the specimens was set parallel to the x-ray beam axis, and the roof of the furcation and the apices of the mesial and distal buccal roots of this tooth were used as landmarks for quantifying alveolar bone loss and regeneration within a reproducible region. The relative volume of mineralized bone between the roots of the upper second molar was estimated after delineating a region of interest (ROI) with an area of 1080x1020 μm^2 and depth of 60 sections with 18μm of thickness each (i.e., thus encompassing the entire furcation volume), by an experienced, calibrated, and masked examiner. A grayscale threshold of 55-250 the values are given as % of bone tissue in the region of interest.

Immunohistochemistry

Sections were prepared accordingly to standard protocols for identification of alkaline phosphatase (AP), tartrate resistant acid phosphatase (TRAP), and the receptor activator

of nuclear factor kappa-B ligand (RANKL). These parameters were assessed at the interproximal aspects of the tooth (Area delimitated by 1000µm below the CEJ between the mesial root the second molar and the distal root of the first molar, and between the distal root of the second molar and the mesial root the third molar), and within the furcation area (same area of evaluation of the histomorphometry analysis of the percentage of bone into the furcation area). The analysis was performed by an experienced, calibrated, and masked examiner, while viewing the sections with a similar set-up as already described with 50x magnification. Intensity of AP and RANKL was scored with a 4-grade scale: (0) no marking (i.e. 0 % of cells); (1) weak (0-25 % of cells), (2) moderate (25-50% of cells), (3) strong (50-75 % of cells)[31]. The median of the values from three regions was used to represent each sample for the RANKL and AP, while the TRAP analysis included counting labeled osteoclasts in contact with bone in the furcation area and at the top of the bone crest interproximally.

Molecular biology: Total RNA extraction, cDNA synthesis, and qPCR analysis

Total RNA was extracted from tissue samples (RNAqueous-4PCR, Ambion Inc.) and was converted into cDNA (High capacity cDNA synthesis kit, Applied Biosystems) following the instructions of the manufacturer. The qPCR reactions were performed in a 20μL volume reaction including 10 μL TaqMan qPCR master mix (Applied Biosystems), 1 μL diluted cDNA, 8μL deionized water, and 1μL rat-specific predesigned and optimized pairs of primers and probe. The pre-optimized conditions cycles were: 50 °C for 2 minutes, 95 °C for 10 minutes and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The qPCR was performed in a Real Time PCR System

7500 (Applied Biosystems). The relative levels of gene expression for IL-1β, IL-6, RANKL, TNF-α, AP were calculated using the GAPDH as normalizing gene.

Statistics

A statistical package (GraphPad Prism 5, San Diego, CA, USA) was used to perform all analyses. Since no previous studies evaluating ASU in regard with periodontology were available, sample size calculation was based on histomorphometric data of another study with similar design, but involving a different medication than ASU[32]. Thus, with seven animals in each group, a minimum average difference between groups of 16.42% with a standard deviation of 8.0% can be detected a power of 80% and an alpha error of 0.05.

Normality of the continuous variables was confirmed with the Shapiro-Wilk test ($p>0.05$) and differences among groups were evaluated with the ANOVA and the Tukey post-hoc test. Analysis of the categorical variables was performed with the Kruskal-Wallis test and the Dunns' test. The level of significance was set at $p<0.05$).

Reproducibility of examiners regarding microCT, histomorphometry (% bone fill), and imunnohistochemistry was performed by duplicate measurements of 10 samples with a 1-week interval. No differences between the evaluations were shown with the paired t-test and Wilcoxon test showed n (p>0,05). In addition, Pearson´s correlation showed an intra-examiner correlation of r=0.92 for microCT, r=0.85 for histomorphometry, and r=0.92 for TRAP. Spearman´s correlation showed an intra-examiner correlation of r=0.88 for AP and r=0.83 for RANKL.

Results

All animals tolerated well all interventions and no remarkable events were observed throughout the study period.

Histology - Histomorphometry

The results of the histological and histomorphometrical analysis are presented in figure 1. The histological evaluation showed that ligature placement induced various amounts of periodontal breakdown, both within the furcation and at the interproximal aspects of the molar. A larger variation in bone loss was observed, however, within the furcation among the groups, comparing with the bone loss in the interproximal area that was rather similar. Thus, seven days after suture removal, the major portion of the furcation space was occupied by an inflammatory cell infiltrate within a disorganized connective tissue and there were signs of ongoing bone resorption. Bone loss was largest at the CTR group with only 5.63% of the furcation area occupied with bone; comparing to CTR, bone loss in the ASU groups was less (range 13.81% - 31.83%) but with no significant differences among groups. At 15 days after ligature removal, the inflammatory cell infiltrate was remarkably reduced in all groups, and the major portion of the furcation area was occupied by a fibrous connective tissue with a high degree of organization. Bone regeneration characterized by woven bone formation was observed at the apical aspect of the furcation in all groups, but was in general limited. Comparing to CTR (7.73%), the ASU groups showed slightly larger amounts of bone regeneration – 18.76% - 35.09% of the furcation area filled with bone – with no significant differences among the three groups. Thirty days after ligature removal, connective tissue organization had further progressed in all groups and inflammatory cells could only sporadically be observed. Bone regeneration had also progressed in all groups;

27.60% - 42.43% of the furcation was occupied by mainly woven bone, with no significant differences among groups. Comparing to the 7 and 15-day observation period, the CTR and ASU/+7 groups showed a statistically significant increase ($p<0.05$), in terms of bone within the furcation area. Furthermore, no signs of root resorption could be observed at any observation period or group, while epithelial down-growth to the furcation roof was observed only in a few occasions. The control specimens of all the groups presented an organized connective tissue, without a huge inflammatory infiltrate and bone with normal shape in all the periods.

Micro CT

The microCT analysis more or less confirmed the results of the histological analysis, i.e. significant differences in terms of bone volume within the furcation area were in general scarcely observed among groups at the various observation periods. Only the CTR group presented a lower volume of mineralized bone compared with ASU/+7 at 7 and 15 days ($p <0.05$) and with ASU/0 at 15 days ($p<0.05$), Additionally, comparing to the 7-day observation period, larger volume of mineralized bone was observed at 15 days in the CTR ($p<0.01$) and at 30 days in the CTR ($p<0.001$), ASU/-7($p<0.01$), and ASU/0 ($p<0.05$). Representative images of all groups are shown in Figure 2.

Immunohistochemistry

Regarding the analysis of the proteins expression by immunochemistry it was observed at seven days a higher protein expression of RANKL in the CTR group when compared to ASU/+7 ($p<0.05$). At 15 and 30 days ($p<0.01$) after the ligature removal the CTR group presented a higher protein expression of RANKL and a higher number of osteoclasts than the groups than the ASU/0 and ASU/+7 groups ($p<0.05$). In addition

the CTR group presented a lower expression of AP than the ASU/+7 ($p<0.05$).Comparing with 7 days, it was showed a decrease in the number of osteoclasts and in the expression of the RANKL protein in the ASU/0 at 15 days and 30 days. Furthermore it was showed an increase in the number of osteoclasts in the CTR group. It was observed, at 30 days, a decrease in the RANKL expression in the CTR group compared with 15 days, and a decrease in the number of osteoclasts in the ASU/+7 comparing with 7 and 15 days. Representative images of all groups are shown in Figure 3.

qPCR

The results of the molecular biology analysis are presented in Figure 4. A significantly higher expression of IL-1β ($p<0.01$) and RANKL ($p<0.001$) was observed in the CTR group comparing to the ASU groups at all observation periods. A higher expression of IL-6 was also observed in the CTR group at 15-days comparing to ASU/0 and ASU/+7, while AP expression was always lower in the CTR group comparing to the ASU groups at all observation periods.

No significant differences were observed among the ASU groups regarding IL-1β, RANKL, and IL-6 at any observation period, while a significantly higher expression of AP was observed for ASU/+7 comparing to ASU/-7 at 7 and 15 days and with ASU/0 at all observation periods. Nevertheless, comparing to the 7-day observation period, a significant increase in AP expression was observed in the ASU/-7 group at 15 ($p<0.05$) and 30 days ($p<0.001$), and in the ASU/0 group ($p<0.05$) at 15 days.

No differences among groups were detected regarding TNFα expression at any observation periods.

Discussion

The use of ASU 7 days after removal of the ligature showed the better results of bone fill in the furcation area, connected to a higher percentage of bone, a lower distance from CEJ-CB, and a lower degree of inflammation than CTR group. The statistical difference occurred at 7 and 15 days but has not been confirmed at 30 days. This absence of difference at day 30 may be due to the experimental model of periodontitis induction used in our study since the ligature was maintained in the sub-gingival sulcus for 7 days, and it was shown that the act of the removal of the ligature incudes a periodontal repair[33]. It was confirmed in our study since, in general, all the groups showed bone tissue repair with increasing observation periods.

With the exception of the group ASU/0 at the period of 15 days, it was observed in our study that the administration of the ASU beginning 7 days before the ligature placement (ASU/-7) or beginning at the day of ligature placement (ASU/0), did not induce better results regarding the bone fill at furcation areas, % of bone tissue or CEJ-CB distance compared with the group CTR. The anti-inflammatory effects of the ASU may have caused a suppression of host defense mechanisms while the microbial / mechanical challenge induced by ligature was active. Studies that evaluated the effect of other immunomodulators of the host inflammatory response drugs showed that the use of them while the ligature was into the sub-gingival sulcus did not had effect or worsened the repair of bone tissue in the control group[34-36]. These data reaffirmed the hypothesis that use of the modulation of the host inflammatory response for periodontal treatment should always be associated with the removal of the biofilm, otherwise it may

even worsen the periodontal condition and facilitate the establishment of opportunistic infections[37].

The current observation of enhanced bone repair by ASU corroborates findings of previous preclinical *in vivo* studies on osteoarthritis showing that ASU administration had a protective effect against articular cartilage erosion[19, 20]. Studies that evaluated the effect of ASU on the treatment of rheumatoid arthritis in humans also showed that this drug induce structural changes in joint tissues due the proliferative effect on cartilage and bone tissue that was demonstrated by the reduction in the intra-articular space[17, 18]. The results presented in our study showed for the first time, *in vivo*, that the ASU improve the periodontal bone repair in an animal model of ligature induced periodontitis.

The suppression of pro-inflammatory biological mediators promoted by ASU partially explain the results in our study, which found that the group ASU/+7 had lower mRNA expression of IL-1β, RANKL, and IL-6. This effect of pre-transcriptional suppression was reflected in a lower protein expression of RANKL, and in a smaller number of osteoclasts in this group. Studies in cell cultures of chondrocytes from patients with rheumatoid arthritis[24], from healthy animals that were submitted to a challenge with IL-1β[21, 22, 25] or bacterial LPS[23] demonstrated the anti-inflammatory effects ASU due the suppressing of the expression of pro-inflammatory biological mediators such as IL-1β, RANKL, IL-6, iNOS, and MMPs[21-26]. Gabay et al., 2008[21] showed that the pre-transcriptional effect promoted by the ASU occurred due the suppression on the signaling pathways nuclear factor κβ (NF-κβ), and mitogen activated protein kinases ERK1 e ERK2 (MAPK). However it is noteworthy that this study evaluated only a cell culture of chondrocytes and this cell type is not present in the

periodontal tissues. Thus it is possible that the ASU acted by other mechanisms since different the drugs induces different effects on different cell types[24].

The lower expression of the proteins TRAP and RANKL in the group ASU/+7 compared with the group CTR occurred due the suppression that the ASU induced on the transcription on the mRNA of IL-1β, RANKL, and IL-6. This suppression had a direct influence on the pathway RANK/RANKL/OPG culminating in a lower osteoclast formation induced by osteoblasts[38], which resulted in lower bone resorption in the group ASU/+7 compared with the group CTr.

ASU is considered a structural modifier drug due the effect on the induction of new connective tissue formation that was previously lost due to the rheumatoid arthritis or osteoarthritis[18, 20]. This effect is due to the stimulation that this drug had in the release of growth factors and products of the connective tissue matrix[25, 28]. *In vitro*, ASU induced a higher expression of TGF-β1 and BMP-2 on osteoblasts and periodontal ligament fibroblasts previously challenged with IL-1β[27]. Other studies have demonstrated that ASU stimulates the synthesis of collagen and glycosaminoglycan's by chondrocytes[24, 29]. The enhance on the expression of the mRNA and the protein AP, which is a biomarker of bone formation, and the higher rates of bone in the ASU/+7 compared with the CTR found in our study corroborated with the studies that showed a proliferative effect on connective tissues induced by the ASU[19, 20, 24, 29].

Some points about ASU mechanisms of action remain unknown. Due to it being an extract, it is not clear exactly which active compound produces the effects of the ASU. One study has reported that the ASU is composed mainly of steroids from the seeds of avocado and soybeans in the ratio 1:2[29]. The evaluation of the effect of the components of the ASU individually or together showed that the effect of the avocado or soybean components individually was more subtle than the effect of these

components together[26, 29]. Other important variables regarding the application of ASU for the treatment of periodontitis is the determination of the dosage and duration of use required for the ASU appearance of the satisfactory effects still needs to be determined before the indication of this drug as an adjunct to non-surgical periodontal treatment in humans.

Conclusion

According to the results presented and the methodology applied, it was verified that the ASU consumption beginning at the day of ligature removal accelerated the periodontal bone tissue repair, and this effect may have mediated by blocking the expression pro-inflammatory biological mediators (IL-1β, RANKL) and stimulation of an expression of a biomarker of bone formation (AP).

Acknowleadgment

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Figure Legends

Figure 1: Photomicrographs illustrating the furcation areas of all the groups in all the periods stained with HE (50 and 100X), and representative graphics of the averages, standard deviation and statistical differences between the groups according the following parameters: A) % Bone fill in furcation areas; B) CEJ-CB distance; C) CEJ-aJE distance. B-Bone; T-Tooth; CT- Connective tissue; iCT-Inflammatory infiltrate; EC-Epithelial cells; FL-Furcation lesions.

Figure 2: Representative images of the micro-CT assessment of % of alveolar bone. Visualization of the 3D reconstruction and the 2D images in coronal and sagittal plane in each group at 7 days of follow up period. Note the higher bone fill in furcation area in the group ASU/+7 compared with the group CTR.

Figure 3: Panel with representative images of the immunohistochemistry analysis of TRAP, RANKL and AP in all groups ate 15 days. A stronger (brown) staining regarding TRAP and RANKL can be observed on the samples in the group CTL compared with those of the ASU groups. Additionally stronger (brown) staining regarding AP in the group ASU/+7 that in the group CTL. (X100)

Figure 4: Representative graphics of the averages, standard deviation and statistical differences between the groups according the expression of the following mRNA: IL-1β; IL-6; RANKL; TNF-α and AP.

Figure 1:

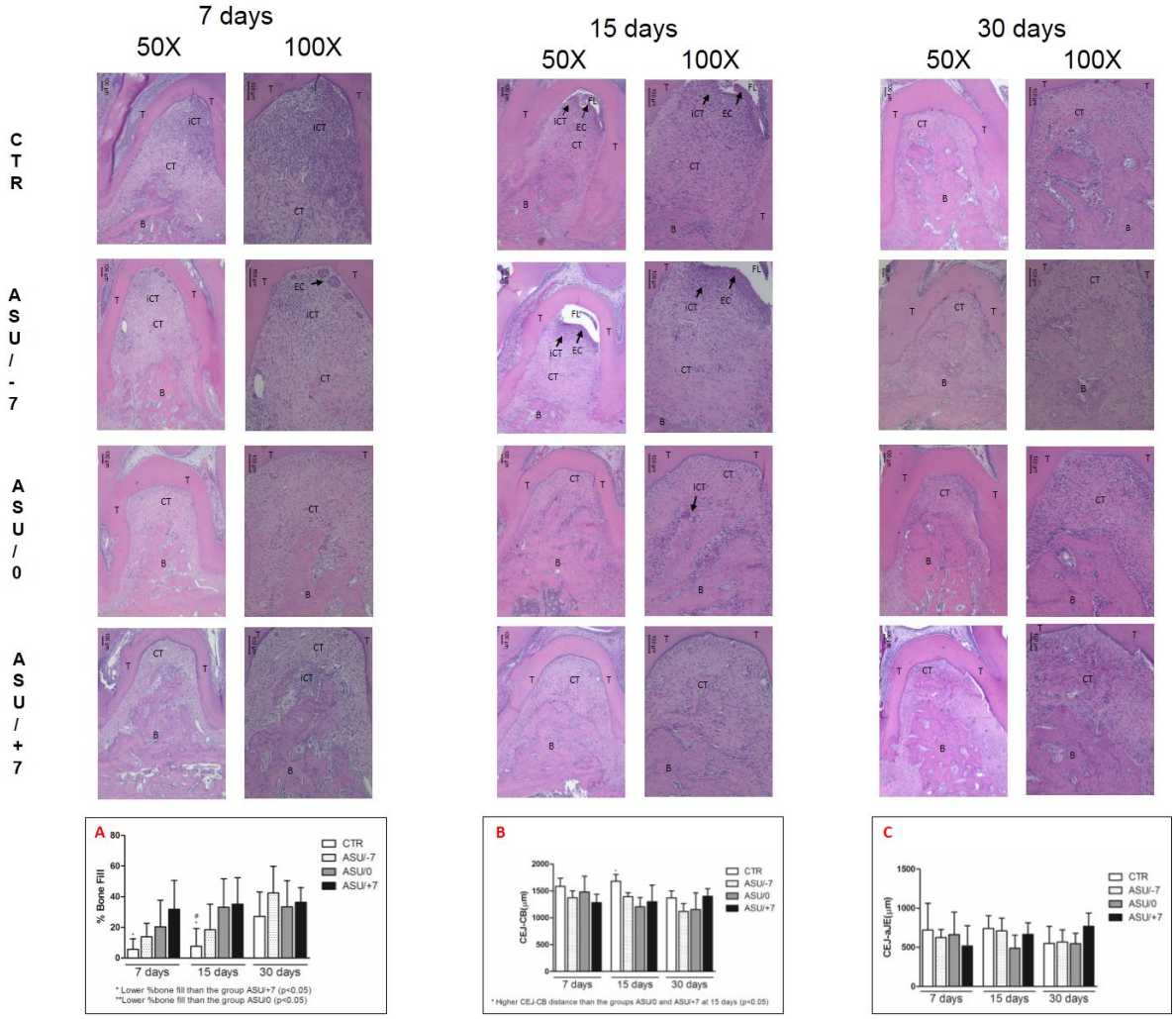
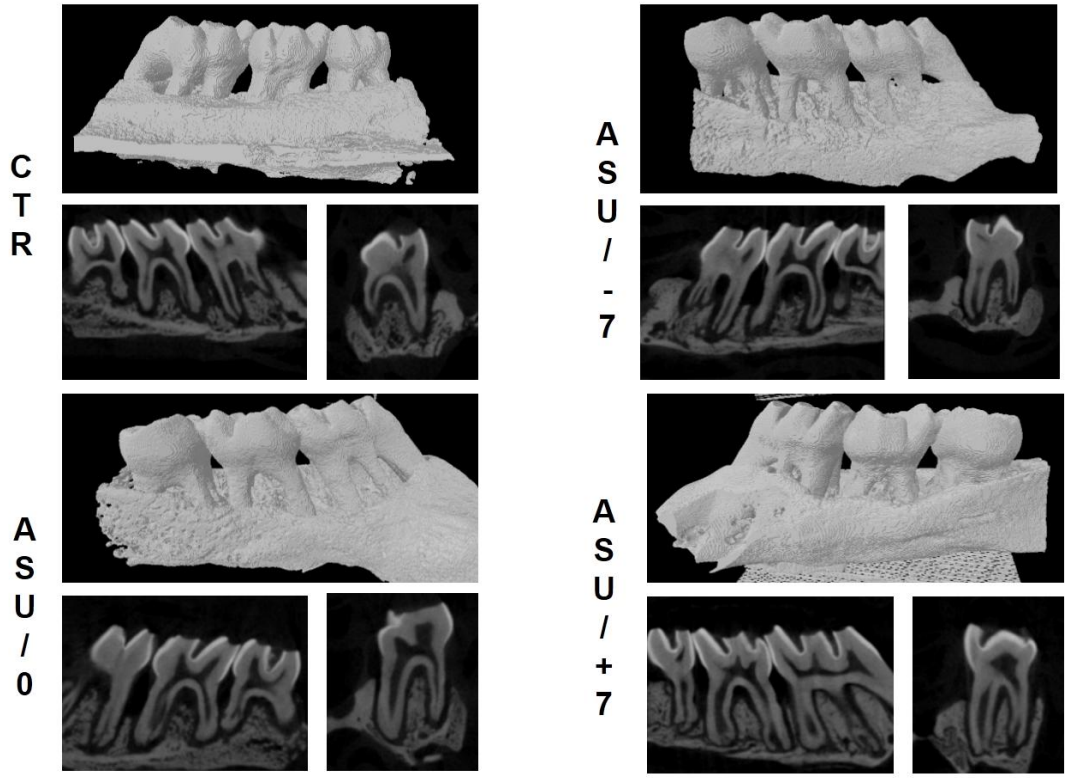

7 days
50X
100X
15 days
50X
100X
30 days
50X
100X
CTR
ASU/-7
ASU/0
ASU/+7
A
% Bone Fill
CTR
ASU/-7
ASU/0
ASU/+7
7 days
15 days
30 days
* Lower %bone fill than the group ASU/+7 (p<0.05)
**Lower %bone fill than the group ASU/0 (p<0.05)
B
CEJ-CB(µm)
* Higher CEJ-CB distance than the groups ASU/0 and ASU/+7 at 15 days (p<0.05)
C
CEJ-aJE(µm)

Figure 2:


CTR
ASU/-7
ASU/0
ASU/+7

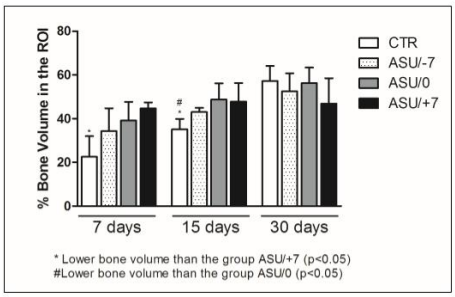

% Bone Volume in the ROI
80
60
40
20
0
7 days
15 days
30 days
CTR
ASU/-7
ASU/0
ASU/+7
* Lower bone volume than the group ASU/+7 (p<0.05)
#Lower bone volume than the group ASU/0 (p<0.05)

Figure 3:

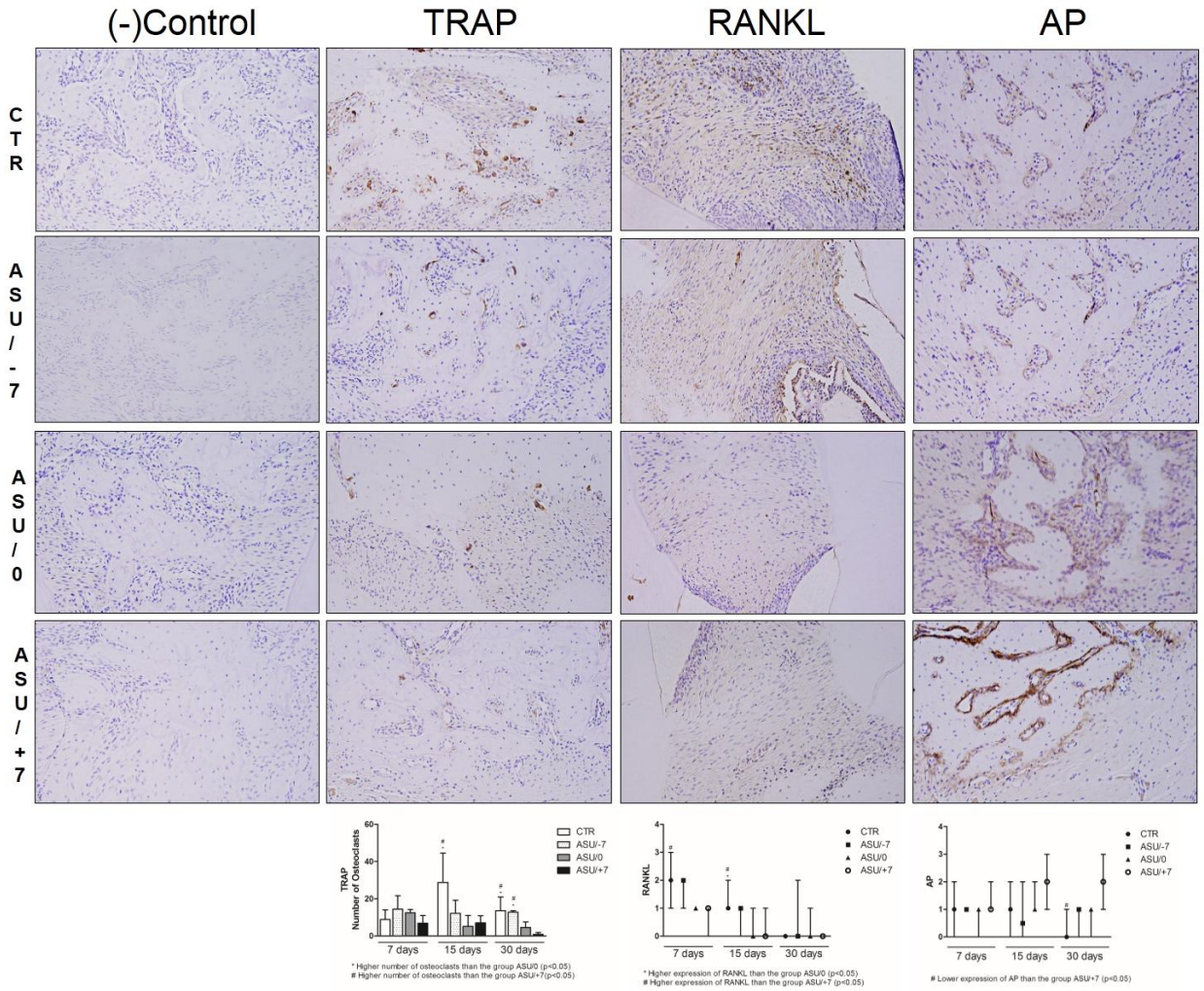
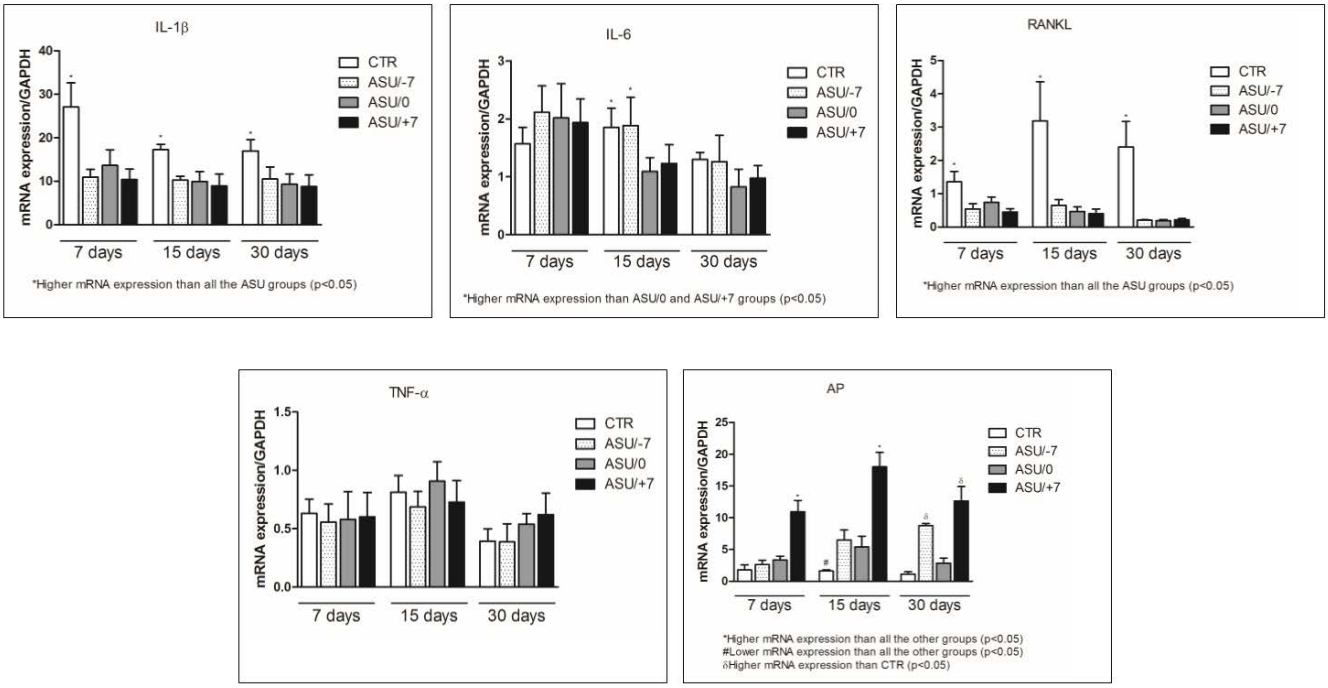

(-)Control
TRAP
RANKL
AP
CTR
ASU/-7
ASU/0
ASU/+7
TRAP Number of Osteoclasts
CTR
ASU/-7
ASU/0
ASU/+7
7 days
15 days
30 days
* Higher number of osteoclasts than the group ASU/0 (p<0.05)
# Higher number of osteoclasts than the group ASU/+7(p<0.05)
RANKL
* Higher expression of RANKL than the group ASU/0 (p<0.05)
# Higher expression of RANKL than the group ASU/+7 (p<0.05)
AP
Lower expression of AP than the group ASU/+7 (p<0.05)

Figure 4:


IL-1β
mRNA expression/GAPDH
CTR
ASU/-7
ASU/0
ASU/+7
7 days
15 days
30 days
*Higher mRNA expression than all the ASU groups (p<0.05)
IL-6
*Higher mRNA expression than ASU/0 and ASU/+7 groups (p<0.05)
RANKL
*Higher mRNA expression than all the ASU groups (p<0.05)
TNF-α
AP
*Higher mRNA expression than all the other groups (p<0.05)
#Lower mRNA expression than all the other groups (p<0.05)
δHigher mRNA expression than CTR (p<0.05)

Capítulo 2

Capítulo 2

Original Research: Effects of Avocado/Soybean Unsaponifiables (ASU) on the treatment of ligature-induced periodontitis in rats.

Guilherme José Pimentel Lopes de Oliveira (Oliveira GJPL) - PhD student in Periodontology[1]

Luiz Guilherme Freitas de Paula (Paula LGF) - PhD student in Implantology [1]

João Antônio Chaves de Souza (Souza JAC) - PhD in Implantology [1]

Rubens Spin-Neto (Spin-Neto R) - PhD student in Oral Radiology [2]

Andreas Stavropoulos (Stavropoulos A) - Professor in Periodontology [3]

Rosemary Adriana Chiérici Marcantonio (Marcantonio RAC) - Professor in Periodontology [1]

1 Section of Periodontology, Department of Diagnosis and Surgery, School of Dentistry of Araraquara-São Paulo State University, Araraquara, SP, Brazil.

2 Section of Oral Radiology, Department of Dentistry-Aarhus University, Aarhus, Denmark

3 Department of Periodontology and Community Dentistry, Faculty of Odontology- Malmo University, Malmo, Sweden

Address for Correspondence:

Rosemary Adriana Chiérici Marcantonio

Humaitá St, 1680. Zip Code 14801-130, Araraquara, SP, Brazil

Phone: +55(16) 3301-6376 / Fax: +55(16) 3301-6376

E-mail: adriana@foar.unesp.br

The authors declare that they have no conflict of interest.

A ser enviado ao período Journal Periodontal Research

Abstract

Objective: To evaluate the effect of the avocado/soybean unsaponifiables (ASU) on the treatment of induced periodontitis in rats. **Materials and methods:** Periodontitis was induced in 84 rats via ligature placement around the second upper molar, which was removed after 7 days, when scaling was performed. Subsequently, the rats were randomly allocated to four groups with 21 animals each: One in which saline solution was administered (SRP/SS), and three in which ASU was administered (0.6 g/kg/day), beginning either 7 days before the induction of periodontitis (SRP/ASU-7), on the day of periodontitis induction (SRP/ASU0), or on the day of treatment (SRP/ASU+7). ASU and SS were administered daily by gavage until the sacrifice of the animals (7, 15, and 30 days). The bone percentage in the furcation area was evaluated by histomorphometry and micro CT. The expression of proteins (TRAP, RANKL, and alkaline phosphatase) and mRNA (IL-1β, TNF-α, IL-6, RANKL, and alkaline phosphatase) were evaluated by immunohistochemistry and qPCR. The Shapiro-Wilk and the one-way ANOVA/Tukey tests were used for the statistical analysis ($p<0.05$). **Results:** The SRP/ASU+7 presented higher expression of the alkaline phosphatase than all the other groups at 30 days. The SRP/ASU0 and SRP/ASU+7 groups presented lower expression of RANKL mRNA than the groups SRP e SRP/ASU-7 at 15 days. However, there were no differences between the groups regarding the percentage of bone fill and proteins expression. **Conclusion:** ASU did not exert positive effects on periodontal repair after the treatment of induced periodontitis.

Keywords: Animal Studies, Avocado/soybean unsaponifiables, Host modulation, Periodontitis, Scaling.

Introduction

Periodontitis is an immune-inflammatory disease in which the dental biofilm induces a host response against bacterial antigens[1]. It is clinically evidenced by the resorption of alveolar bone concomitant with clinical attachment loss and the formation of periodontal pockets[2]. The traditional treatment for this disease is scaling and root planing (SRP) to remove the bacterial biofilm[3]. However, despite the good clinical outcomes promoted by SRP in the control of periodontitis[4], it has been suggested that the application of medications that modulate the host inflammatory response against bacterial challenge could improve the outcomes of periodontal treatment[5]. This therapy, termed host modulation therapy, is based on the observation that the progression of periodontal disease is highly dependent on the expression of biological mediators of inflammation, which modifies the inflammatory profile of the patients and assists in the resolution of this pathological process[1].

Several immunomodulatory drugs have been suggested as adjunctive for the treatment of periodontitis[1, 5]. Among these drugs, some are noteworthy due the positive results observed clinically, such as being selective[6] or not selective for cyclooxygenase-2 (COX-2)[7] nonsteroidal anti-inflammatory (NSAID), bisphosphonates[8], and low doses of doxycycline[9]. However, the therapeutic protocol with these drugs has limitations due to the induction of side effects[5]. Additionally, all of these drugs act only by inhibiting the anti-inflammatory mediators of inflammation, and it has been suggested that a good modulating host inflammatory response drug should also act stimulating the biological mediators of tissue repair[5, 10].

Avocado/soybean unsaponifiables (ASU) constitute a medication produced from the fruits and seeds of these two plants in a 1:2 ratio (avocado/soy), which is indicated for the treatment of rheumatoid arthritis and osteoarthritis[11]. This medication is

considered to be a structural modifier of the joint tissues that were previously destroyed by the progression of arthritis/osteoarthritis[12-15]. Considering that arthritis possess a pathogenic mechanism of tissue destruction similar to that which occurs in periodontitis[16], ASU may be applicable as a possible adjunctive agent for periodontal treatment. Clinical studies have found that ASU reduces the intra-articular space and improves joint kinesiology function[12, 15]. The effects of ASU on the protection and proliferation of intra-articular tissues was verified histologically in studies with sheep[14] and dogs[13]. *In vitro* studies showed that ASU downregulates the expression of pro-inflammatory mediators, such as PGE2, TNFα, iNOS, IL1β, IL6, and IL8[17-19], and matrix metalloproteinases, such as MMP2, MMP3, and MMP13[19-21]; it also upregulates the expression of growth factors such as TGFβ1, TGFβ2, and BMP2[18, 22, 23], and the synthesis of connective tissue matrix proteins, such as collagen and agreccans[18, 19, 24]. Furthermore, *in vivo* studies showed that ASU reduced the expression of iNOS and MMP 13[13] and increased the secretion of TGF β1 and TGF β2[25].

The objective of this study was to assess whether the use of ASU would be beneficial on the treatment of ligature-induced periodontitis in rats.

Materials and methods (ANEXO 1**)**

This study was approved by the São Paulo State University - UNESP Ethical Committee for the use of animals in research of the (CEUA 17/2009) (ANEXO 3), School of Dentistry of Araraquara, Brazil. Eighty-four male rats (*Rattus* norvegicus holtzman), weighing 250 - 300 g were kept in an environment with controlled temperature (21 ± 1 ° C), humidity (65 - 70%) and light/dark cycle (12 hours). The animals had access to a standard laboratory diet and water *ad libitum*.

Groups

The animals were randomly allocated to four equally sized groups in three follow-up periods (7, 15, and 30) with seven animals per group/period: a) Administration of saline solution (SRP/SS); Administration of ASU (0.6 g/kg/day)[26] (Piascledine 300, Expanscience Lab, France) b) (SRP/ASU-7) - 7 days before the induction of periodontitis; c) (SRP/ASU0) – on the day of the periodontitis induction; d) (SRP/ASU+7) – at the moment of the treatment of induced periodontitis. The ASU and SS were administered daily by gavage until the end of the follow-up period.

Induction and treatment of periodontitis

The animals were anesthetized with the combination of ketamine (0.08 ml/100 g; Rompum, Bayer S.A., São Paulo, SP, Brazil) and xylazine (0.04 ml/100 g; Rompum, Bayer S.A., São Paulo, SP, Brazil). Ligatures (cotton fibers, nº–24) were placed subgingivally around the upper second molar on both sides. After a period of 7 days, the ligatures were removed and scaling was performed once with manual instruments (11-12 / 13-14-Mini-Gracey, Hu-Friday, Chicago, IL, USA)[27] with the aid of a stereoscopic magnifying lens with 3.5X magnification (DMC equipamentos, São Carlos, SP, Brazil).

Retrieval of samples

Euthanasia was performed via an overdose of anesthetic solution. In each of the groups, the maxillae were removed in blocks and divided randomly into seven hemi-maxillae for histomorphometric/immunohistochemistry analysis and five hemi-maxillae for qPCR and microCT analysis; two hemi-maxillae without ligatures were used as controls for histology. The hemi-maxillae intended for

histomorphometric/immunohistochemistry analysis were fixed in 4% paraformaldehyde for 48 hours. Hemi-maxillae intended for qPCR had the gingival tissue surrounding the upper second molars removed and immediately frozen in liquid nitrogen and kept at -80 °C until the extraction of mRNA; the remaining blocks containing the teeth and surrounding alveolar bone were fixed in 4% paraformaldehyde for 48 hours and subsequently scanned in a micro CT scanner.

Micro CT

The samples were scanned by a microtomograph (Skyscan, Aartselaar, Belgium) and subsequently reconstructed, reoriented spatially and analyzed by dedicatedc software (NRecon/DataViewer/CTan, Skyscan, Aartselaar, Belgium). The bone volume in the furcation area in the second molar was measured after the delimitation of the region of interest (ROI), which presented an area of 70 x 45 pixels^2, in 60 18-µm-thick sections, and using a threshold range of 55-250 greyscale. The values were given as % bone tissue in the ROI. A blinded, trained and calibrated examiner (GJO) performed the analyses.

Histology – histomorphometric evalujation

After the fixation, the samples were decalcified in ethylenediamine tetraacetic acid (EDTA) at 7% for 10 weeks and subsequently dried in alcohol, diaphanized in xylene and embedded in paraffin. Serial sections, 5 µm thick, were prepared in a mesio-distal direction along the axis of the tooth and were stained with hematoxylin - eosin (HE). Histological and histomorphometric analysis was performed while viewing the images

on a computer screen via a camera connected to an incandescent and polarized light microscope (Leica - Reichert Diastar Products & Jung, Wetzlar, Germany), and using image analysis software (Image J, Jandel Scientific, San Rafael, CA, USA). After excluding the first and the last sections where the furcation regions were detected, three equidistant sections from each specimen block were selected. The following parameters were evaluated in the histomorphometric analysis: 1) area of mineralized bone within the furcation, expressed as percentage of the furcation area (i.e., from the roof of the furcation to 1000 μm apically [28], and b) linear distances between the cemento-enamel junction (CEJ) to the top of the alveolar crest (CEJ- CB) at a point where the periodontal ligament showed a normal width, and the CEJ to the apical termination of the junctional epithelium (CEJ- aJE). Additionally, a histological description of the locations of inflammatory reactions and bone remodeling in the furcation region was performed. Experienced, calibrated, and blinded evaluators (RSN and LGP) performed the histological description and histomorphometric analysis.

Immunohistochemistry

Sections were prepared according to standard protocols for the identification of tartrate resistant acid phosphatase (TRAP) and the receptor activator of nuclear factor kappa-B ligand (RANKL) and alkaline phosphatase (AP). Immunohistochemical staining for TRAP, RANKL and AP was performed using anti-rat antibodies (Abcam, Inc. Cambridge, MA, USA) for these proteins (Dilution: TRAP- 1:200; RANKL- 1:200; AP- 1:100). Negative control sections were incubated with PBS (omission of the primary antibody) to assess background staining. Biotinylated immunoglobulin (ABC kit Dako A/S, Denmark) was used as a secondary antibody, followed by incubation with avidin-

biotin peroxidase complex (ABC kit Dako, Glostrup, Denmark). Diaminobenzidine (DAB, Dako A/S, Denmark) was used as a chromogenic substrate. All sections were counterstained with Carrazi's hematoxylin and mounted with permount. Photomicrographs (50X) were taken using a light microscope (LEICA microsystem GmbH, Wetzlar, Germany). The analysis of TRAP was performed by counting the cells in the furcation region and on the top of the crestal bone[29]. The intensity of AP and RANKL was scored with a 4-grade scale: (0) no marking (i.e., 0 % of cells); (1) weak (0-25 % of cells), (2) moderate (25-50% of cells), (3) strong (50-75 % of cells) [27]. These parameters were assessed at the interproximal aspects of the tooth (Area delimitated by 1000μm below the CEJ between the mesial root the second molar and the distal root of the first molar, and between the distal root of the second molar and the mesial root the third molar), and within the furcation area (same area of evaluation of the histomorphometry analysis of the percentage of bone into the furcation area). The median of the values from three regions was used to represent each sample. A blinded, trained and calibrated examiner (GJO) performed the analyses.

Total RNA extraction, cDNA synthesis, and qPCR analysis

Total RNA was extracted from tissue samples using an affinity column system (RNAqueous-4PCR, Ambion Inc.) according to the manufacturer's protocol. The quantity and purity of total RNA were determined by UV spectrophotometry and 260/280 nm ratio, respectively. The RNA integrity of a subsample was confirmed by electrophoresis in formaldehyde agarose gels. Approximately 400 ng of total RNA was converted into cDNA with random hexamer primers and moloney leukemia virus reverse transcriptase in a reaction volume of 20 μL (High capacity cDNA synthesis kit, Applied Biosystems).

The qPCR reactions were performed in a 20-μL volume reaction including 10 μL TaqMan qPCR master mix (Applied Biosystems), 1 μL diluted cDNA, 8 μL deionized water, and 1 μL rat-specific predesigned and optimized pairs of primers and probe. The pre-optimized cycling conditions were: 50 °C for 2 minutes, 95 °C for 10 minutes and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The qPCR was performed in a Real Time PCR System 7500 (Applied Biosystems). The relative levels of gene expression for IL-1β, IL-6, RANKL, TNF-α, and AP were calculated using GAPDH as a normalizing gene.

Statistics

A statistical package (GraphPad Prism 5, San Diego, CA, USA) was used to perform all analyses. The sample size calculation was based on our previous study, which evaluated the effect of ASU on the periodontal repair of induced periodontitis in rats without performing scaling, with similar histomorphometric methodology (unpublished data). Thus, allocating seven animals in each group, a minimum average difference between groups of 26.20% with a standard deviation of 12.91% could be detected with a power of 80% and an alpha error of 0.05.

The parametric distribution of the continuous variables was confirmed with the Shapiro-Wilk test ($p> 0.05$), and differences among groups were evaluated with ANOVA and Tukey's post-hoc test. Analysis of the categorical variables was performed with the Kruskal-Wallis test and Dunns' test. The level of significance was set at $p\leq 0.05$.

Results

Reproducibility of the examiners

The reproducibility of the examiners' micro CT, histomorphometry (percentage of bone fill), and immunohistochemistry analyses was performed by duplicate measurements of 10 samples with a 1-week interval. No differences between the evaluations were shown with the paired t-test and Wilcoxon test ($p>0.05$). In addition, Pearson´s correlation showed high intra-examiner correlation with $r=0.94$ for micro CT $r=0.85$ for histomorphometry, and $r=0.91$ for TRAP. Spearman's correlation showed an intra-examiner correlation of $r=0.92$ for AP and $r=0.94$ for RANKL.

Micro CT

The micro CT analysis showed no statistically significant differences between groups in any of the periods. Additionally, no differences were found within each group. Table 1 shows the averages and standard deviations of the volumetric analysis for the percentage of bone fill.

Descriptive Histology

The control specimens of all the groups presented an organized connective tissue, without a huge inflammatory infiltrate and bone with normal shape in all the periods. In the teeth in which periodontitis was induced, all the groups presented a similar histological pattern, in which the presence of inflammatory infiltrate in the roof of the furcation, the presence of blood vessels associated with this infiltrate and surrounding bone tissue, and disorganization of collagen fibers were found at 7 days. At 15 and 30 days, a reduction in the inflammatory infiltrate, which was replaced by organized collagen fibers, associated with a reduction of the presence of blood vessels and improved bone tissue organization was observed. Furthermore, the presence of

reversion lines on the bone tissue demonstrated bone formation in all groups. Representative images of the descriptive histological analysis are shown in Figure 1.

Histomorphometry

It was shown that the SRP/ASU+7 group presented a higher percentage of bone fill in the furcation area than in the SRP group after a period of 7 days ($p<0.05$). An increase in that parameter in the SRP/SS group at 30 days relative to 7 days ($p<0.05$) was also shown in the intragroup comparison. Table 2 shows the averages and standard deviations of the percentage of bone fill in the furcation regions.

Regarding the analysis of the CEJ-CB distance, there were no differences between the groups in all the periods evaluated. In the intragroup analysis, a reduction in the CEJ-CB in the SRP/ASU-7 group at the period of 30 days was shown compared with the periods of 7 and 15 days ($p<0.05$). Furthermore, in the SRP/ASU0 group, a reduction in the CEJ-CB was shown at the period of 30 days compared with the period of 15 days. Table 3 shows the average and standard deviation of the CEJ-CB distance.

Regarding the CEJ-aJE distance, there were no differences between the groups in all the follow-up periods. In the intragroup analysis, a reduction in the CEJ-aJE distance at 30 days compared with 15 days was shown in the SRP/ASU-7 group ($p<0.05$). Table 4 shows the average and standard deviation of the CEJ-aJE distance.

Immunohistochemistry

No statistically significant differences were detected between groups in any of the proteins analyzed. Figure 2 shows representative images of all groups with respect to the immunohistochemical labeling of the evaluated proteins.

qPCR Analysis

No differences between the groups in all the periods of evaluation regarding the analysis of the expression of the mRNA of IL1β, IL6, and TNF***α*** were observed.A higher expression of RANKL mRNA was shown in the SRP and SRP/ASU-7 groups compared with the SRP/ASU0 and SRP/ASU+7 groups at 15 days ($p<0.05$). A higher expression of the AP mRNA was shown in the SRP/ASU+7 relative to all the other groups at 30 days ($p<0.001$). Figure 3 shows a graphic representation of the averages and standard deviations of the mRNA expression of AP, RANKL, IL1β, IL6, and TNFα in relation to GAPDH gene expression.

Discussion

The results of this study showed that in general, the adjunctive use of ASU did not exert an additional effect on periodontal repair after the treatment of induced periodontitis, as demonstrated by histology, histomorphometric and micro CT analysis. Furthermore, despite the higher expression of AP in the SRP/ASU+7 group than in all the other groups, and a lower expression of RANKL in the SRP/ASU0 and SRP/ASU+7 groups than in the SRP and SRP/ASU-7 groups, these differences in gene expression were not detected by immunohistochemistry analysis.

At 7 days follow-up, it was shown that the animals of the SRP/ASU+7 group presented a higher percentage of bone fill in the furcation regions than the SRP/SS group. It can be assumed that this fact must be due to anti-inflammatory and proliferative effects in the connective tissue that were induced by the ASU[13-15, 18, 21, 22]. However, these results were not maintained in the other periods or confirmed by the other analyses, such as the CEJ-CB and the volumetric percentage of bone fill analysis

performed by the micro CT. The discrepancy between the data obtained from the analysis of the percentage of bone fill in the furcation with data of the distance between the CEJ-CB and CEJ-aJE at 7 days might be due to the different areas of assessment: while the first analysis was performed at the furcation, the other analyses were performed in the interproximal region. The discrepancy between the results of the histomorphometric and micro CT analyses in the furcation regions might be due to the extension of the analysis, since histomorphometric evaluation was used to analyze only three sections in two-dimensional images, while micro CT performed a three-dimensional analysis of the entire furcation region[30].

Comparing the histomorphometric and the micro CT data of this study with those of a previous study performed by our research group (unpublished data), distinct results were detected. The use of the ASU beginning at the day of the ligature removal induced a lower degree of bone resorption, a lower number of osteoclasts, lower expression of RANKL and IL1β, and a higher expression of AP than in the control group (unpublished data), but these results were not confirmed by this study. The different models for the evaluation of the periodontal repair after induced periodontitis in rats could explain the distinct results presented in these studies. In our previous study, the model of periodontal repair consisted of the removal of the ligatures 7 days after their insertion[29, 31], while in this study, scaling with manual instruments was performed at the time of ligature removal[27, 32]. Despite the fact that the depths of the periodontal pockets were not measured in this study, it was shown that the attachment loss verified by the linear measures of the CEL-CB distance varied in the range of 1.27-1.59 mm, and it can be assumed that the depth of the periodontal pockets formed by the induction of periodontitis was less than 1.5 mm. Although the manual instruments used to perform the scaling had a tip with reduced size (mini-five), the active tip of this curette was

relatively large in relation to the periodontal pockets that were present in the animals of the present study. It has been shown that the trauma caused by scaling in shallow periodontal pockets induced clinical attachment loss in humans[33, 34]. The additional trauma in the dentogingival insertion caused by scaling may have impaired the possible positive effects of the ASU on the periodontal repair.

It was shown in this study that the SRP/ASU0 and SRP/ASU+7 groups presented lower expression RANKL mRNA of than the SRP/SS and SRP/ASU-7 groups at 15 days. Additionally, it was shown that the SRP/ASU+7 group presented a higher expression of the mRNA of AP than all the other groups at 30 days. These results showed that the ASU produced subtle anti-inflammatory and proliferative effects, which did not elicit changes in protein synthesis and in periodontal repair. The ASU is considered to be like a medication that modifies the structure of the connective tissue; however, these effects occurred at a slow rate,[12, 13] and the increase in the expression of the mRNA of AP at 30 days could represent a possible enhancement of the periodontal repair in longer periods of evaluation, though this fact cannot be proven by this study.

When evaluating the results presented in this study, some drawbacks should be considered. Only one concentration of ASU was tested, and despite the fact that clinical and pre-clinical studies showed that the increase of the dosage did not produce different effects regarding the clinical and histological characteristics of rheumatoid arthritis and osteoarthritis[12, 14], in vitro studies showed that ASU produces effects in a dose-dependent manner[17, 21]. Therefore, the impact of different dosages of ASU on periodontal repair remains unknown. Another limitation of this study is the fact that ASU is a slow-acting drug, and this may mean that the effect of this drug will be better detected in longer evaluation periods. Indeed, previous pre-clinical studies made by our research group detected the acceleration of the osseointegration of implants inserted in

the tibia and greater bone formation in critically sized calvaria defects after using ASU for periods between 60-75 days (unpublished data), and this effect was associated with increased expression of the growth factors BMP2 and TGFβ1 (unpublished data). Furthermore, due to the inconsistent effects of ASU in the different models of the evaluation of the periodontal repair after inducing periodontitis with ligatures, additional studies about the effect of ASU in other models of induced periodontitis (e.g., maintenance of the ligature in position or the injection of lipopolysaccharide) and studies with animals with conditions which predispose to higher bone resorption (e.g., diabetes, smoking) will be necessary to elicit a better understanding of the real effect of ASU on induced periodontitis and in which situations this drug would produce more significant effects.

Conclusion

The use of ASU did not promote beneficial effects on periodontal repair following the treatment of induced periodontitis.

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Tables: Effects of Avocado/Soybean Unsaponifiables (ASU) on the treatment of ligature-induced periodontitis in rats.

Table 1: Average and standard deviation of the volumetric analysis of the percentage of bone detected by micro CT in all the groups/periods

Group/Period	7 days	15 days	30 days
SRP	19.71±6.82	22.22±9.34	21.15±4.30
SRP+ASU/-7	18.48±6.16	21.58±5.28	19.40±6.37
SRP+ASU/0	16.73±3.70	18.11±5.24	17.14±7.22
SRP+ASU/+7	17.32±3.70	18.31±4.65	15.02±6.70

Table 2: Average and standard deviation of the percentage of bone fill in the furcation in all the groups/periods

Group/Period	7 days	15 days	30 days
SRP/SS	9.90±10.65*	17.57±10.68	35.05±18.48
SRP+ASU/-7	20.30±15.51	24.34±18.75	33.38±21.08
SRP+ASU/0	24.27±14.78	26.60±17.89	32.71±16.76
SRP+ASU/+7	29.84±16.34*	25.12±12.07	38.25±19.15

*$p<0.05$-One way Anova and post test Tukey

Table 3: Average and standard deviation of the CEJ-CB (mm) in all the groups/periods

Groups/Periods	7 days	15 days	30 days
SRP/SS	1.59±0.27	1.53±0.15	1.30±0.171
SRP+ASU/-7	1.27±0.18	1.54±0.31	1.04±0.14
SRP+ASU/0	1.33±0.29	1.48±0.29	1.15±0.18
SRP+ASU/+7	1.38±0.28	1.49±0.31	1.29±0.27

Table 4: Average and standard deviation of the CEJ-aJE(mm) in all the groups/periods

Groups/Periods	7 days	15 days	30 days
SRP/SS	0.81±0.36	0.87±0.22	0.61±0.10
SRP+ASU/-7	0.57±0.17	0.83±0.30	0.64±0.12
SRP+ASU/0	0.59±0.20	0.75±0.33	0.55±0.15
SRP+ASU/+7	0.49±0.20	0.72±0.18	0.68±0.28

Figure Captions

Figure 1: Representative images of the histological analysis in all the groups/periods of evaluation. The healing pattern was similar in all groups, where it was observed a reduction of the inflammatory infiltrate, an increase in the organization of the connective tissue and the formation of osteoid tissue in all the groups with increased evaluation periods. B-Bone; T-Teeth; CT-Connective tissue, iCT- Inflammatory infiltration in the connective tissue. (50x and 100X) HE.

Figure 2: Representative images of the groups of the immunohistochemical analysis of TRAP, RANKL and AP. It can be observed that there were no differences between the groups regarding the intensity of the brown label of RANKL and AP proteins, as well as no difference was observed between the groups regarding the number of osteoclasts associated with bone tissue (100X)

Figure 3: Representative graphics of the average, standard deviation, and statistical differences among the groups in the expression of mRNA for IL1β, IL6, TNF, RANKL, and AP. The data were normalized with the housekeeping gene (GAPDH).

Figure 1:

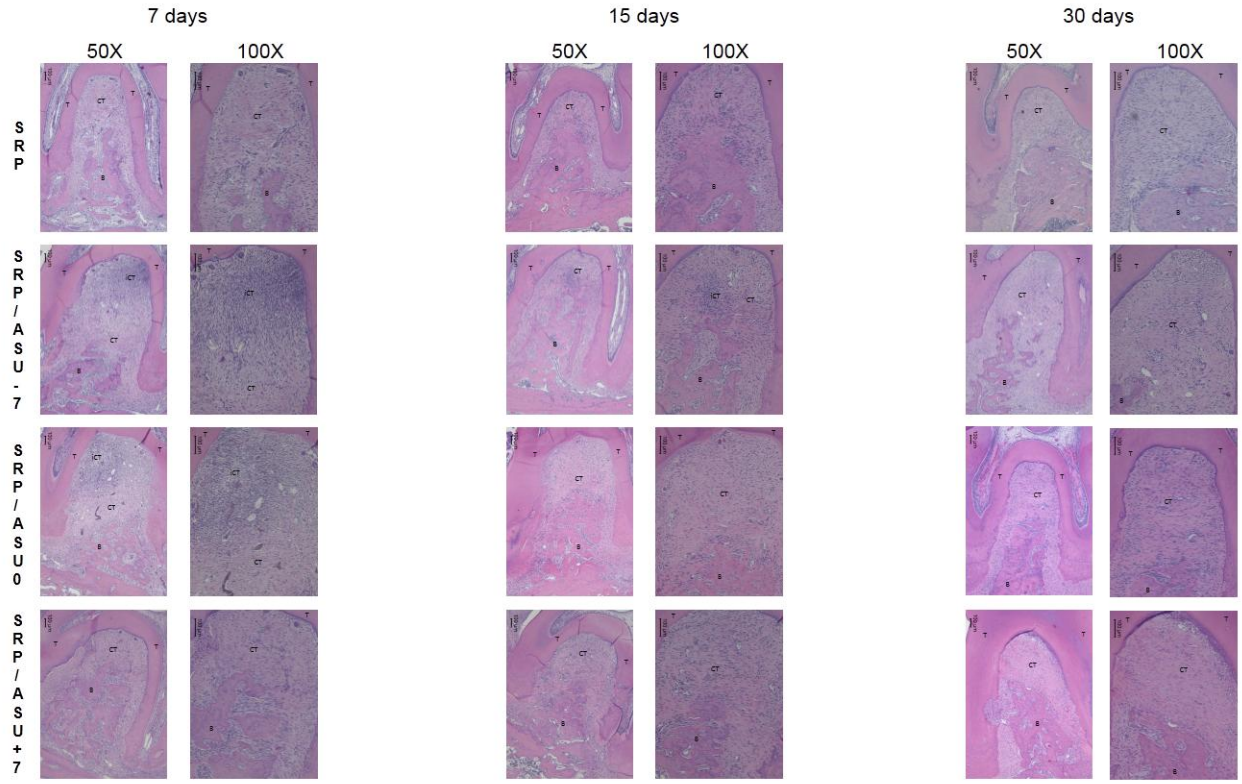


Figure 2:

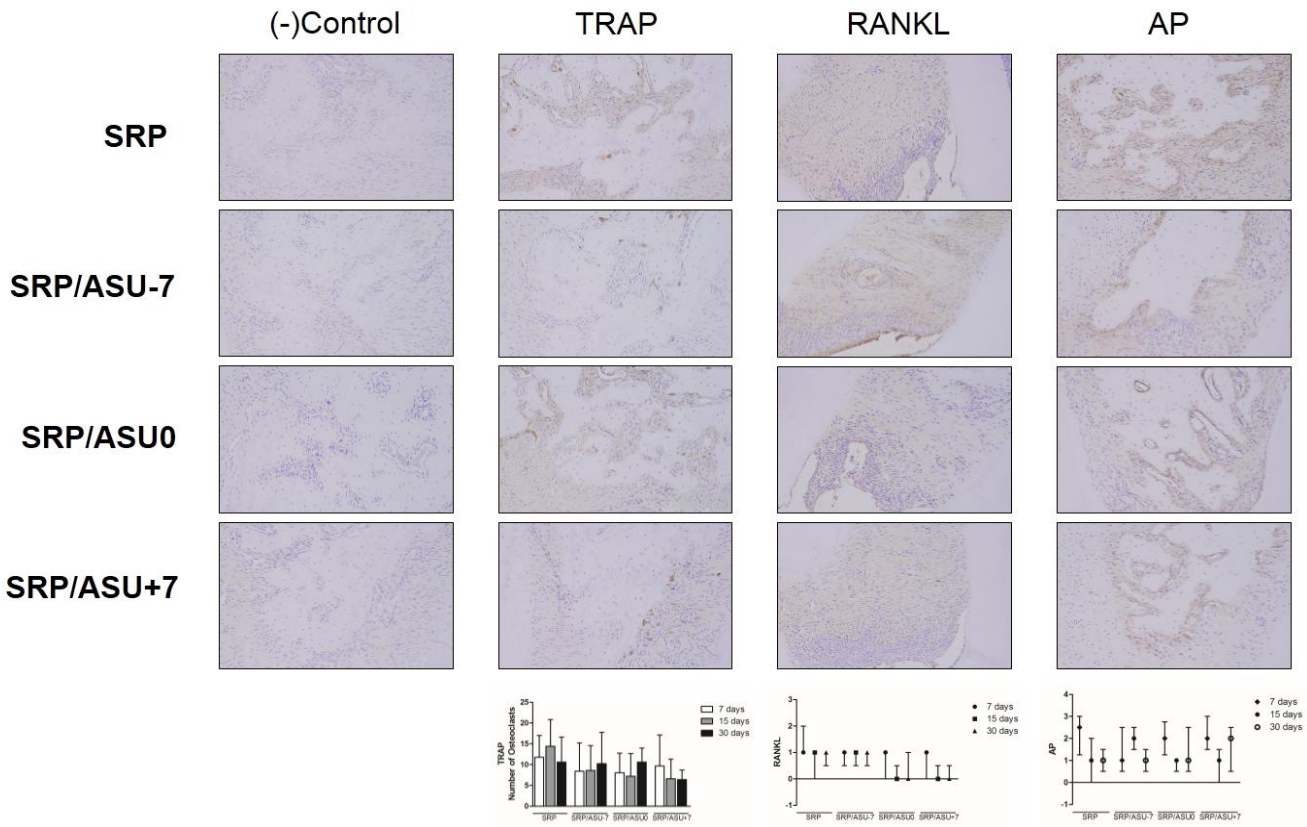
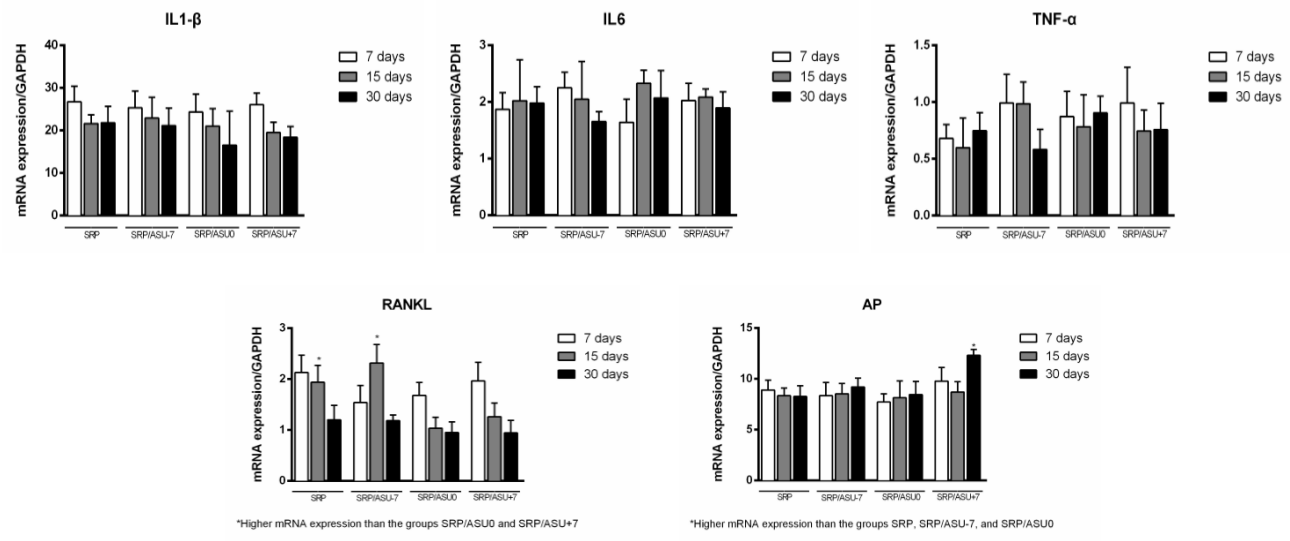

(-)Control
TRAP
RANKL
AP
SRP
SRP/ASU-7
SRP/ASU0
SRP/ASU+7
TRAP
Number of Osteoclasts
7 days
15 days
30 days
RANKL
AP
SRP
SRP/ASU-7
SRP/ASU0
SRP/ASU+7

Figure 3:


IL1-β
IL6
TNF-α
RANKL
AP
mRNA expression/GAPDH
7 days
15 days
30 days
SRP
SRP/ASU-7
SRP/ASU0
SRP/ASU+7
*Higher mRNA expression than the groups SRP/ASU0 and SRP/ASU+7
*Higher mRNA expression than the groups SRP, SRP/ASU-7, and SRP/ASU0

Capítulo 3

Capitulo 3

Effect of Avocado / Soy Unsaponifiables (ASU) on osseointegration: A proof-of-principle preclinical *in vivo* study*

***Guilherme José Pimentel Lopes de Oliveira (Oliveira GJPL),** PhD Student[1];

***Luiz Guilherme Freitas de Paula (Paula LGF),** PhD Student[1];

Rubens Spin-Neto (Spin-Neto R), PhD Student[2];

Andreas Stavropoulos (Stavropoulos A), Professor in Periodontology[3];

Luis Carlos Spolidório (Spolidório LC), Professor in Oral Patology[1];

Elcio Marcantonio Junior (Marcantonio Jr, E), Professor in Periodontology[1];

Rosemary Adriana Chiérici Marcantonio (Marcantonio RAC), Professor in Periodontology[1].

[1]Foar-UNESP, Araraquara Dental School, Universidade Estadual Paulista, Araraquara, SP, Brasil.

[2]Section of Oral Radiology, Department of Dentistry – Aarhus University, Aarhus, Denmark.

[3] Dept. of Periodontology and Community Dentistry, Faculty of Odontology, Malmö University, Malmö, Sweden.

*Both authors contributed equally to this article.

Corresponding author

Rosemary Adriana Chiérici Marcantonio

Humaitá St, 1680. Zip code: 14801-130, Araraquara, SP, Brazil

Telefone:+55 (16) 33016376 / Fax: +55(16) 33016376

e-mail: adriana@foar.unesp.br

Conflict of interest

The authors declare to have no other conflict of interest regarding this study.

*** Artigo aceito pelo periódico IJOMI (**ANEXO 2**)**

Effect of Avocado / Soy Unsaponifiables (ASU) on osseointegration: A proof-of-principle preclinical *in vivo* study

ABSTRACT

Purpose: To evaluate the influence of Avocado / Soy Unsaponifiables (ASU) administration on the osseointegration of implants installed in rat tibiae. **Materials and Methods:** Thirty rats were randomly assigned into one of three equal-sized groups: 1) ASU1; administration of ASU, starting 7 days prior to implant installation, 2) ASU2; administration of ASU, starting on the day of implant installation, and 3) CTL; administration of saline solution. In all animals, one titanium implant was installed in each tibia. All animals received ASU or saline solution by gavage daily until sacrifice 60 days post-operatively. Implant osseointegration and bone maturation were assessed by biomechanical analysis, radiographic bone density, descriptive histology, immunohistochemical analysis for bone morphogenetic protein 2 (BMP2), transforming growth factor beta 1 (TGFβ1), and osteocalcin (OCN), histomorphometric evaluation of bone-to-implant contact (BIC) and mineralized bone area fraction within the threads of the implant (BA). **Results:** ASU1 and ASU2 showed a 3- and 9- times higher expression of BMP2 and TGFβ1, respectively, compared to CTL ($p<0.05$). Histomorphometric analysis, however, showed that both ASU1 and ASU2 groups presented significantly higher BIC values only in the cortical bone compartment when compared to CTL ($p<0.05$). **Conclusion:** ASU consumption has only a subtle effect on implant osseointegration.

Key-Words: Avocado-soybean unsaponifiables, implant osseointegration, histomorphometry, animal study.

INTRODUCTION

Although the optimal amount of osseointegration (i.e. bone-to-implant contact; BIC) for the clinical success of oral implants is yet to be determined, much research efforts are still directed towards accelerating and/or enhancing BIC. One common approach is by altering the implant itself, which in turn provokes a positive response from the host. Modifications of the implant surface microtopography (i.e. increasing implant surface roughness) and/or implant surface chemistry have indeed been shown to enhance osseointegration comparing to control implants (e.g. "machined implants)[1-5]. Similarly, functionalization of the implant by adding bioactive substances to its surface, e.g. bisphosphonates[6, 7] and growth factors[8] has also been shown to enhance osseointegration.

An approach, less explored, to accelerate osseointegration is by administration of substances that exert an effect on bone remodeling. For example, systemic administration of simvastatin[9], strondium ranelate[10], or parathyroid hormone[11] have been shown to enhance osseointegration. Similarly, the use of "natural" products has also been proposed as an alternative way to improve osseointegration. For example, a recent preclinical *in vivo* study demonstrated that systemic consumption of comfrey, a common plant, in homeopatheic concetrations resulted in increased implant removal torque values and peri-implant radiographic bone density compared with placebo treatment[12].

Another plant-derived medicine that might have a positive effect on implant osseointegration is the avocado/soybean unsaponifiable (ASU) extract. ASU is produced from the fruits and seeds of these two plants in a 1:2 ratio (avocado/soy) and is indicated as supplement for the palliative treatment of rheumatoid arthritis and osteoarthritis[13, 14] The beneficial effect of ASU is attributed to controlling the action of

several pro-inflammatory mediators such as Interleukin-1β (IL-1β), -6 (IL-6), -8 (IL-8), Tumor Necrosis Factor-α (TNF-α)[15-19], Prostaglandin E2 (PGE 2)[16, 17, 19], inducible Nitric Oxide Synthase (iNOS)[18-20], Metalloproteinase-2(MMP2), -3 (MMP3)[21]. Indeed, one pre-clinical *in vivo* study observed a reduction in iNOS and MMP-13 after treatment of induced arthritis in dogs with ASU compared with placebo[20]. In vitro studies have also shown that ASU enhances Transforming Grown Factor- β1 (TGFβ1), and bone morphogenetic protein-2 (BMP2) expression[15, 22]. TGFβ1 and BMP2 are important factors for wound and bone healing[23, 24], regulating primarily later stages of osteogenesis[25]. It seems thus reasonable to evaluate whether upregulation of these two growth factors relevant for wound- and bone healing, by means of ASU, may also have a positive effect on osseointegration. The aim of the present study was to evaluate whether systemic administration of ASU may enhance osseointegration of titanium implants.

MATERIALS AND METHODS (ANEXO 1)

The present study was approved by the Committee of Animal Experiments Ethics Committee of the Sao Paulo State University - UNESP (CEEA 17/2009) **(**ANEXO 3), Araraquara, Brazil.

Experimental groups

Thirty male Hotzman rats (weight 200–250 gr) were randomly divided into three equal-sized groups: 1) ASU1; animals receiving – by gavage – daily administration of ASU (Piascledine ® 300, Expanscience Lab, France) at a doses of 0.6 gr per kg body weight[26], starting seven days prior to implant placement, 2) ASU2; animals receiving daily administration of ASU at a dose of 0.6 gr per kg body weight starting at the same

day of implant placement, and 3) CTL; animals receiving daily administration of physiologic saline solution starting at the same day of implant placement to serve as controls.

The rats were kept in a room with controlled temperature (21±1°C) and humidity (65-70%), and a 12-hour light-dark cycle. The animals had throughout the experiment ad libitum access to standard rat chow and water.

Surgical Procedures

The animals were anesthetized by a combination of xylazine 2% (Rompum, Bayer S.A., São Paulo, SP, Brazil; 0.04 mL/100 gr body weight) and ketamine 10% (Ketamine Agener, Agener União Ltda, São Paulo, SP, Brazil; 0.08mL/100gr body weight). In addition, mepivacaine hydrochloride 2% with epinephrine 1:100,000 (Mepíadre, DFL Industria e Comercio SA, Rio de Janeiro, RJ, Brazil) was administered locally to the inner region of the leg to improve hemostasis during surgery. By means of single incisions, approximately 10 mm long, each tibia was exposed. One implant site was prepared in proximal epiphysis of each tibia with a twist drill (2.0 mm in Ø) in a low speed hand-piece under copious sterile saline irrigation. A titanium implant (4.0 x 2.2 mm, L x Ø; Conexão, São Paulo, SP, Brazil) with a machined surface was inserted using 15 rpm and 10 N torque until the shoulder of the implant was in contact with the bone surface. The tissues were then sutured in layers with 5.0 bioabsorbable (Vicryl, ETHICON, J&J, São José dos Campos, Brazil) and 4.0 silk (ETHICON, J&J, São José dos Campos, Brazil) sutures.

After surgery the animals received a single intramuscular injection of a combination of penicillin and streptomycin (0.1 ml/kg) (Multibiótico Small, Vitalfarma, São Sebastião do Paraíso, MG, Brazil) for infection control, and a single gavage of

dipyrone (0.1 ml/kg) (Dipirona Ibasa 50% - Ibasa, Porto Alegre, RS, Brazil) for pain control. All animals were euthanized sixty days after surgery by an overdose of ketamine. One of the tibias was used for radiographic, biomechanical, histological, and immunohistochemical analysis (n=10), while the other tibia was used for histomorphometric analysis(n=10).

Radiographic analysis

Digital radiographs were taken using a CDR sensor (Schick CDR, Schick Technologies Inc, City Island, NY, USA) on the day of sacrifice. After flap elevation, the tibias were fixed in a customized fixing device assuring that the long axis of the implant was perpendicular to the X-ray beam and parallel to the sensor, and with a 40 cm spot-to-object distance. The X-ray unit (GE 1000, General Electric, Milwaukee, WI, USA) was operated with standardized parameters: 70 kVp, 10 mA, and exposure time of 0.3 seconds.

The images were imported to dedicated software (Image J 1.32j, National Institutes of health, USA) and displayed on a 15-inch WLED flat screen monitor (1336×768 pixel resolution) (Inspiron 15R, Dell Computer Corporation, Minneapolis, MN, USA). Using a 300% zoom, 4 regions of interest (4 x 4 pixels = 1 mm^2) were drawn juxtaposed to the crest of the threads – one in the cortical (at the level of the implant platform) and one in the cancellous bone (at the level of the top of the second thread), at both sides of the implant (Figure 1). Bone density was expressed as a ratio of the average gray values (range 0-255) in the cortical and cancellous compartment of the bone, respectively, to the gray value obtained from a similar sized region of interest drawn over a 10 mm thick aluminum scale, which was included in each radiograph as a reference[12]. Comparisons among groups were performed separately for the cortical and

cancellous compartment and also for both compartments combined. A trained examiner, masked in regard with the treatment group, performed the analysis.

Biomechanical analysis

After registration of the radiographs, the tibia was dissected to expose the implant head; a torque meter with a scale range from 0.1 to 10 N/cm (Tohnichi, Shimizu, Tokyo, Japan) was attached to the head of the implant through a hexagonal wrench and applied a counterclockwise force until disrupting the bone/implant interface. Thereafter, the implant was carefully unscrewed and the tibia was prepared for histological and immunohistochemical analysis.

Histology, immunohistochemistry, and histomorphometry

Blocks with the portion of the tibias, previously containing the implant, were prepared and fixed in 4% paraformaldehyde for 48 hours, then decalcified in 7% EDTA solution (pH 7.2) at 4 ^{o}C for approximately 2-3 months, and embedded in paraffin. Serial sections 5 μm thick were obtained parallel to the long axis of the implant site. Some sections were subsequently stained with hematoxylin and eosin, while others were accordingly prepared for immunohistochemical evaluation for transforming growth factor-β1 (TGFβ1), bone morphogenetic protein 2 (BMP2), and osteocalcin (OCN) expression. The intensity of labeling of antibodies on the bone between the first and second thread (on both sides of the implant in the cortical compartment) was scored with a 4-grade scale: negative (-), positive (+), superpositive (++), and hyperpositive (+++). To facilitate comparisons, the scores were converted into percentile average

frequencies of 0%, 20%, 60%, and 90%, respectively[27]. The average of the right and left measurements was used as the value to represent each animal.

Blocks from the remaining tibias containing the implants and surrounding tissues were immersed in 4% paraformaldehyde for 48 hours, dehydrated in an ascending series of ethanol (60-100%), and then embedded in light-curing resin (Technovit 7200 VLC, Kultzer heraus GmbH & CO., Wehrheim, Germany). One longitudinal section through the middle of the implant was cut, ground, and polished (Exakt Apparatebeau, Hamburg, Germany) to a final section thickness of ca. 45 μm, and then stained with Stevenel's blue in combination with acid fuchsine. Mineralized bone-to-implant contact expressed as percentage of the threaded implant perimeter (%BIC) and the relative amounts of mineralized bone within the implant thread area (%BA) were estimated separately for the part of the implant in the cortical and cancellous bone compartments, respectively. Comparisons among groups were performed both separately for the cortical and cancellous compartment and also for both compartments combined.

All histological, immunohistochemichal, and histomporphometrical analyses were performed by experienced examiners, masked regarding the treatment group. A light microscope with incandescent light (Leica-Reichert Diastar Products & Jung, Wetzlar, Germany) and connected to a pc running an image analysis program (Image J, Jandel Scientific, San Rafael, CA, USA) was used during the analyses.

Statistical analysis

Sample size calculation was based on BIC data from a previous study with a similar design (but not involving ASU) showing a minimum average difference between groups of 15.94±11.13%[28]. With ten animals in each group and an alpha error of 0.05, the power of the study was calculated to be 75%. Continuous data herein were tested for

normality with the Shapiro-Wilk test, and the one-way ANOVA followed by the Tukey post-hoc test was used for evaluating differences among groups regarding the radiographic, biomechanical, and histomorphometric data. Significance of differences among groups regarding the data from the immunohistochemical analysis was tested with the Kruskal-Wallis and Dunnet´s tests. Reproducibility of the radiographic, histomorphometric, and immunohistochemical measurements was evaluated with the Kendall's concordance index on the basis of duplicate measurements of 30% of the sections, taken within at least 2 weeks interval. The Graphpad Prism 5.0 software (GraphPad Software, Inc. La Jolla, CA, USA) was used and the significance level was set to 0.05.

RESULTS

No animal showed changes in the soft tissues overlying the implants or experienced tibia fracture, during the entire study period. Evaluation of reproducibility showed a high concordance index ($r^2 > 0.90$) for all assessed parameters.

The results of the radiographic, biomechanical, histomorphometrical, and immunohistocemical analysis are presented in Table 1.

No statistically significant differences were found among the three groups regarding the average gray values at the cortical or cancellous compartments of the bone, or when these regions were combined (Table 1). Similarly, no significant differences among the groups were found regarding removal torque values (Table 1).

During histological examination of the decalcified sections, the bone surrounding the implants in the ASU1 and ASU2 groups appeared denser compared to that around the implants in the CTL sites. Moreover, the bone in the ASU groups showed a single row of flattened osteoblasts (lining cells) on its surface, included large

numbers of rounded osteocytes, and generally presented with fewer Haversian systems and lacked reversal lines, i.e., appeared somehow less mature comparing to the CTL group (Figure 2).

The histomorphometric analysis showed that within the cortical region of the bone, BIC in the ASU1 and ASU2 groups (71.95 ± 13.01 and 73.81 ± 9.01, respectively) was significantly higher comparing to that in the CTL group (59.51 ± 9.68) ($p < 0.05$). In the cancellous compartment of the bone, BIC in the ASU1, ASU2 and CTL groups was 46.69±15.60, 44.87± 18.52, and 39.71±19.00, respectively, but the differences were not significant. Similarly, no significant differences among the three groups were found when evaluating both the cortical and cancellous bone compartments together. No significant differences were also observed regarding BA, irrespective if the evaluation regarded the cortical or cancellous compartment, separately or combined (Figure 3) (Table 1).

Immunohistochemichal analysis revealed that both ASU 1 and ASU2 groups exhibited a higher expression of BMP2 and TGF-β1 compared to the CTL group ($p < 0.05$). However, the expression of OCN was similar among the groups. Representative images of each biomarker and the results are illustrated in Figure 4.

DISCUSSION

The present study showed that systemic administration of ASU, starting either shortly before or immediately after implant installation in the tibia of healthy rats, resulted in larger amounts of bone-to-implant-contact (BIC) and less mature bone in the cortical compartment, comparing to implants installed in control animals that did not receive ASU. No differences among groups could be observed regarding BIC in the cancellous compartment.

At the time the present study was conceived, the hypothesis was that ASUs' anabolic effect on TGFβ1 and BMP2, as observed in *in vitro* studies[15, 22], might also enhance osseointegration *in vivo*, and was based on the known fact that TGFβ1 and BMP2 are important factors for bone healing[23, 24]. The present study was therefore designed such as to evaluate whether a possible enhancement of osseointegration through continuous ASU administration would have a significant long lasting effect, thus the 60-day observation period. Indeed, a 9- to 3- fold higher expression of TGFβ1 and BMP-2, respectively, was observed in the ASU treated groups comparing to controls. Despite this finding, the only significant difference among groups was observed in regard with BIC in the cortical compartment of the ASU treated animals, comparing to controls and it may reflect the slower healing property of the cortical bone compared with the cancellous bone. This fact was highlighted in a review about the healing events that occur in the bone tissue during the fracture repair, and it was described that the healing of the cancellous bone occurs early compared to cortical bone due to higher presence of undifferentiated mesenchymal cells in the cancellous bone due the presence of the bone marrow[29]. It is possibly that the enhance in osseointegration observed in the ASU groups on the cortical bone could be also detect in cancellous bone at early healing periods of evaluation. Furthermore, the results of the radiographic and biomechanical analysis herein, showed no significant differences among the ASU1, ASU2 and CTL groups. Analysis of grey values on digital radiographs, as performed in the present study, can only capture rather large true differences in bone density among groups[30]. Similarly, analysis of removal torque forces, which evaluates the three-dimensional shear strength of the implant-to-bone connection, is a test with limited ability to detect small quantitative differences in osseointegration[31, 32]. Thus, it is

reasonable to state that ASU administration, as delivered in the present study, had only a subtle effect on implant osseointegration.

On the other hand, the possibility that, the lack of statistically significant differences among groups in the present study – in terms of BIC and bone area in the cancellous compartment, radiographic bone density, and removal torque values – could be explained by the late observation period (60 days) used herein cannot be excluded. Studies evaluating the influence of different types of implant surfaces or the effects of drugs, using long-bone models in small animal platforms, have detected statistically significant differences in BIC only at early stages of wound healing, while at later stages of healing osseointegration in control sites "caught up" with that in test sites[33, 34].

A recent in vivo study has demonstrated that oxidized implant surfaces reveal a weaker and shorter inflammatory response during the very early stages of wound healing, comparing to machined implant surfaces[35]. In this context, it could be suggested that ASU might have downregulated several inflammatory cytokines (e.g. IL-1β, TNF-α, MMP-2, -3)[15-19, 21] and indeed enhanced osseointegration during the early stages of healing in the ASU1 and ASU2 groups. This assumption may also be supported by the apparent reduced bone maturation observed in the two test groups comparing to the CTR group, which is indicative of fast bone formation[36, 37]. It can be further suggested, that this enhancement might have "faded away" during later stages of healing, while it would have been possible to capture it better during the early stages of healing, i.e. if an earlier observation period had been used.

From the discussion above it becomes obvious that the present study has some limitations. Thus, the use of only one late observation period, not allowing evaluation of the possible effect of ASU during the early stages of osseointegration should be considered as the major drawback of the current study design. Another limitation of the

present study is the fact that only one dose of ASU was used. This dose was calculated on the basis of the dose usually administered in the clinic[26, 38] during rheumatoid arthritis treatment, and it cannot be excluded that a different dose of ASU might have exerted a more notable effect in terms of osseointegration. Different doses of systemically administered medications have indeed resulted in significant differences in terms of osseointegration in the same preclinical model as herein, e.g. simvastatin[39], strontium ranelate[40]. In addition, in the present experiment, ASU was administered throughout the study period; whether the enhanced benefit in BIC would remain without concomitant ASU consumption remains obscure.

Another possible explanation for the lack of a more pronounced effect in terms of BIC after ASU administration may be the fact that all animals in the present study were systemically healthy. In previous studies using the same animal model and evaluating the effect of systemic administration of medications (e.g., strontium ranelate, simvastatin) on implant osseointegration in systemically compromised (e.g. osteoporotic-like) animals comparing to healthy conditions, the difference in terms of enhanced osseointegration in the treated animals comparing to controls was much more pronounced in the systemically compromised animals rather than in the healthy conditions[28, 41]. For example, in previous studies using the same animal model and evaluating the implant osseointegration in osteoporotic-like conditions showed that these animals had a poor BIC than the controls, and the consumption of medications like strontium ranelate or simvastatin caused an enhance on implant osseointegration in osteoporotic-like conditions, showed that these medications can equalize the degree of BIC between the systemic disease animals comparing to controls animals[28, 41] . Thus, the possibility that a given positive effect of ASU would have become more tangible if a

systemic disease model interfering with bone healing was used herein remains to be explored.

In conclusion, the results of the present study suggest that ASU administration may only subtly enhance osseointegration of implants in healthy animals, at least regarding late stages of healing. Thus, further studies should aim to elucidate the possible effect of ASU administration during earlier stages of osseointegration and in cases of systemically compromised individuals (i.e. suffering from rheumatoid arthritis, osteoporosis, diabetes), as well as the possibility of dose and duration of ASU administration optimization.

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Tables

Table 1: Results of the radiographic, biomechanical, histomorphometric and immunohistochemical analysis in the three groups.

Analysis		ASU1	ASU2	CTL
Radiographic (Ratio at ROI : Al scale)	Cortical	0.43±0.05	0.31±0.15	0.35±0.09
	Cancellous	0.31±0.10	0.36±0.11	0.25±0.09
	Combined	0.37±0.05	0.33±0.11	0.30±0.08
Biomechanical (N/cm)		18.35 ± 4.2	20.67 ± 1.43	19.5 ± 1.42
Histomorphometry (%)	BIC			
	Cortical	71.95 ± 13.01*	73.81 ± 9.01#	59.51 ± 9.68*#
	Cancellous	46.69±15.60	44.87± 18.52	39.71±19.00
	Combined	59.32±9.42	59.34±9.76	49.61±11.70
	BA			
	Cortical	74.77 ± 17:26	78.90 ± 9.94	70.24 ± 14:28
	Cancellous	46.84±14.13	47.77±14.83	38.71±17.97
	Combined	60.80±9.69	63.33±8.67	54.48±14.58
Immunohistochemistry (%)	BMP2	68.00±22.50*	61.11±27.13#	20.00±21.90*#
	TGF-β1	52.50±29.64*	60.00±34.64#	6.66±10.32*#
	OCN	52.22±32.70	77.14±16.03	63.33±25.81

Similar symbols denote statistical significant difference between groups.

Figures

Figure 1

Four regions of interest (4 x 4 pixels = 1 mm^2) were drawn juxtaposed to the crest of the threads – in the cortical and in the cancellous bone (B and C, respectively) at both sides of the implant. Bone density was expressed as a ratio of the average gray values (range 0-255) in B and C to the gray value obtained from a similar sized region of interest drawn over a 10 mm thick aluminum scale (A).

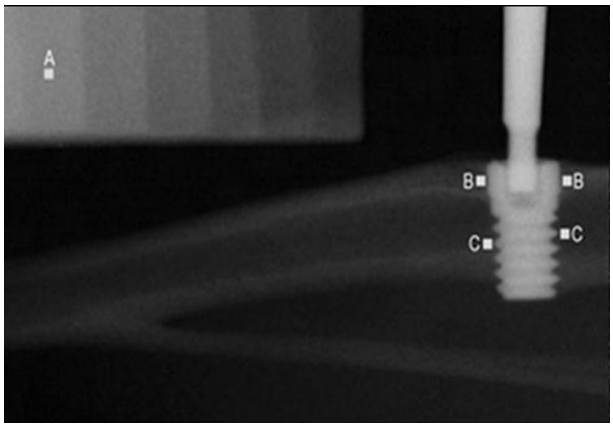


Figure 2

Panel with representative histomicrographs from decalcified sections used in the histological evaluation. The bone surrounding the implants in the ASU1 and ASU2 groups appeared denser compared to that around the implants in the CTL sites, but generally presented with fewer Haversian systems and reversal lines and appeared somehow less mature comparing to the CTL group.

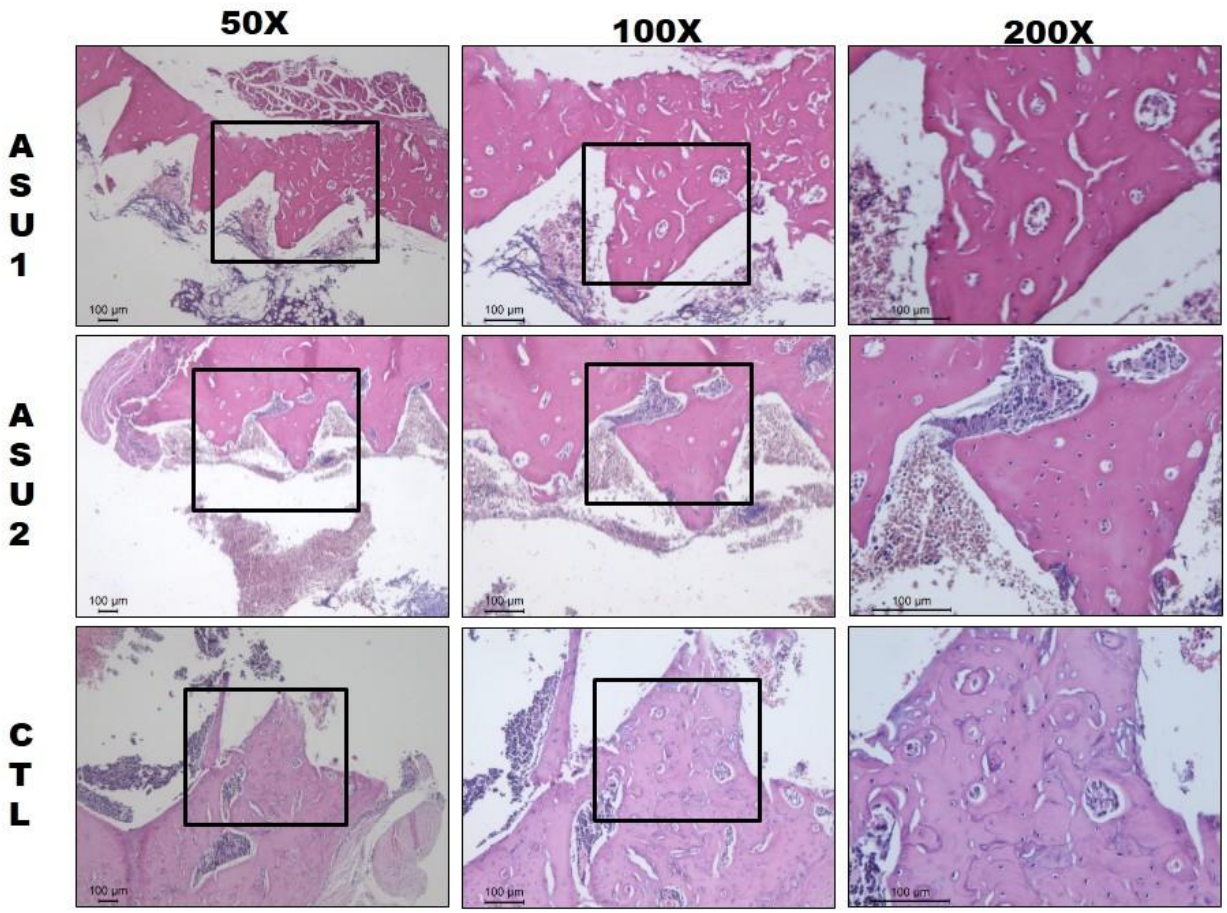


Figure 3

Panel with representative histomicrographs from non-decalcified sections used in the histomorphometric evaluation. A subtle enhancement in bone-to-implant contact (BIC) can be observed in the ASU1 and ASU2 groups comparing to controls in the cortical compartment. No remarkable differences among groups can be seen regarding BIC in the cancellous compartment. (x100) Stevenel's blue /acid fuchsine.

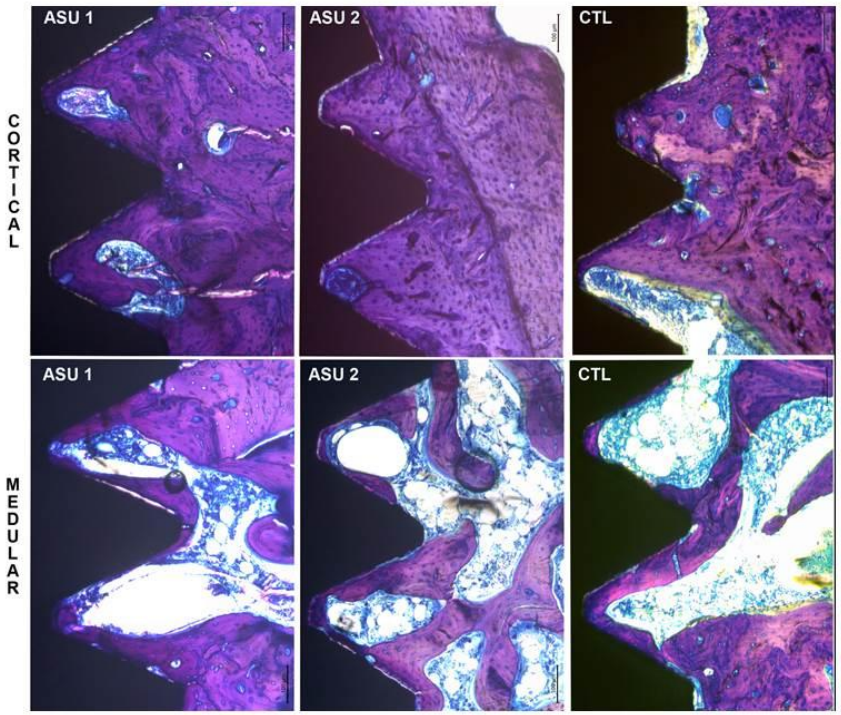
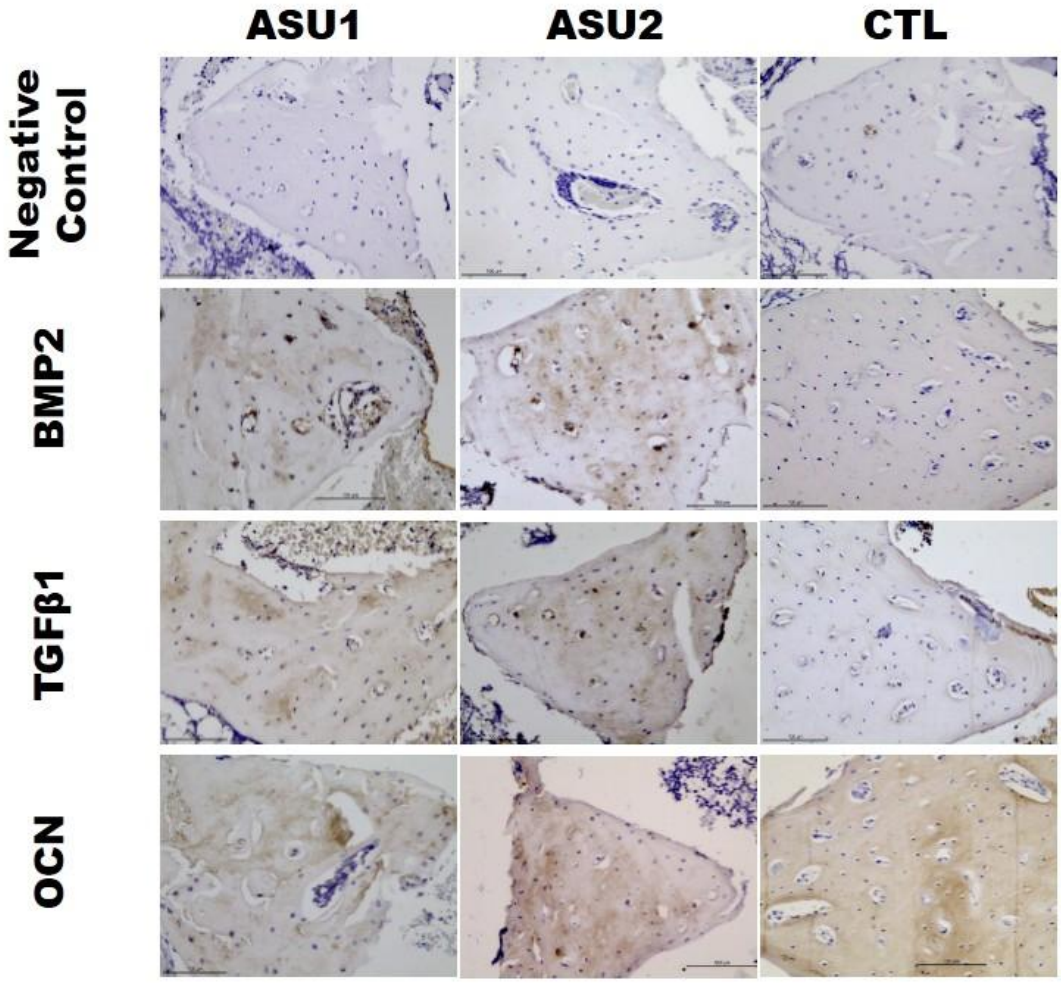


Figure 4

Panel with representative histomicrographs from decalcified sections used in the immunohistochemical analysis of BMP2, TGFβ1 and OCN in all groups. A stronger (brown) staining regarding BMP2 and TGFβ1 can be observed on the samples of the ASU groups compared with those of the CTL group (x200).



Capítulo 4

Capítulo 4

Original Research: Effects of Avocado/Soybean Unsaponifiables (ASU) on the bone repair of critical-sized calvarial defects with different biomaterials*

Guilherme José Pimentel Lopes de Oliveira (Oliveira GJPL) - PhD student in Periodontology[1]

Cássio Rocha Scardueli (Scardueli CR) - PhD student in Implantodontology [1]

Luiz Guilherme Freitas de Paula (Paula LGF) - PhD student in Implantodontology [1]

Rubens Spin-Neto (Spin-Neto R) - PhD student in Oral Radiology [2]

Andreas Stavropoulos (Stavropoulos A) - Professor in Periodontology [3]

Rosemary Adriana Chiérici Marcantonio (Marcantonio RAC) - Professor in Periodontology [1]

1 Section of Periodontology, Department of Diagnosis and Surgery, School of Dentistry at Araraquara-Univ Est Paulista, Araraquara, SP, Brazil.

2 Section of Oral Radiology, Department of Dentistry-Aarhus University, Aarhus, Denmark

3 Department of Periodontology and Community Dentistry, Faculty of Odontology- Malmo University, Malmo, Sweden

Corresponding Author:

Rosemary Adriana Chiérici Marcantonio

Humaitá St, 1680. Zip Code 14801-130, Araraquara, SP.Brazil

Phone: +55(16) 3301-6376 / Fax: +55(16) 3301-6376

E-mail: adriana@foar.unesp.br

The authors declare that they have no conflicts of interest.

***A ser enviado para a Clinical Oral Implants Research**

Abstract

Objective: To evaluate the effect of avocado/soybean unsaponifiables (ASU) on the repair of critical sized calvarial defects (CCDs) filled with biomaterials or unfilled. **Materials and Methods:** One CCD (0.5 mm) was made in each of 84 rats. These defects were filled with coagulum (COA), deproteinized bovine bone (DBB), and β-tricalcium phosphate/hydroxyapatite (TCP/HA). ASU (0.6 g/kg) or saline solution (CTR) was administered daily by gavage from 15 days before surgery until the animals were euthanized 15 or 60 days after surgery (7 animals per period/group). The description and composition of the tissues that filled the CCDs were analyzed by micro CT and histomorphometry. Unpaired *t*-tests and one-way ANOVA/Tukey tests were used for the statistical analyses ($p<0.05$). **Results:** The percentage of bone fill (%bone fill) in the CCD of the COA-ASU group was significantly higher than that in the COA-CTR group at both evaluated time points ($p<0.05$). There were no differences regarding the %bone fill between the DBB-ASU and DBB-CTR groups and the TCP/HA-ASU and TCP/HA-CTR groups at either time point ($p<0.05$). The %bone fill was higher in the COA-ASU group than in the DBB-ASU and TCP/HA-ASU groups at 60 days ($p<0.05$). **Conclusion:** ASU induced an enhancement in %bone fill in the CCDs filled with coagulum; however, this positive effect was not sufficient to induce an enhancement in %bone fill associated with the use of DBB and TCP/HA.

Key Words: ASU, biomaterial, bone healing, critical-sized calvarial defects, histomorphometry, micro CT.

Introduction

The use of biomaterials for the repair of bone defects or to increase the amount of bone to enable the placement of dental implants has become an increasingly common practice, and a large range of biomaterials have been proposed for use as bone substitutes[1, 2].

Autogenous bone grafts are considered the gold standard among biomaterials proposed for the regeneration/repair of bone tissue because this type of graft is the only biomaterial that simultaneously presents the biological properties of osteogenesis, osteoinduction, and osteoconduction[3]. However, the use of this biomaterial has several limitations, such as limited availability and donor site morbidity[1, 4]. Thus, the use of biomaterials with biologically osteoconductive properties that are not taken from the patient), such as deproteinized bovine bone (DBB) and biphasic ceramics based on β-tricalcium phosphate/hydroxyapatite (TCP/HA), has been proposed as an alternative to the use of autogenous bone grafts[5, 6].

DBB is a biomaterial of bovine origin that is produced by removing the protein components of the bone tissue and is composed of the remaining inorganic portion of the bone, which has hydroxyapatite structurally similar to that in human bone as its main component[7]. TCP/HA is a biphasic synthetic ceramic formed by mixing 40% β-tricalcium phosphate and 60% hydroxyapatite[7]. Both biomaterials have been used and have presented good clinical outcomes in post-extraction sockets[6], sinus floor augmentation[5], and intrabony periodontal defects[8, 9]. Additionally, it has been shown that implants placed in sites grafted with these biomaterials have similar survival rates to implants placed in areas grafted with autogenous bone[1]. Conceptually, both biomaterials promote bone formation around their particles, accompanied by controlled resorption; thus, these biomaterials are initially space maintainers to guide bone

formation[10]. However, histological studies have shown that these biomaterials are not completely resorbed, and bone formation occurs around the particles close to the native bone, while particles that are far from the native bone are encapsulated by connective tissue[5, 6], which may impair the bone tissue regeneration/repair.

The lack of osteoinductive and osteogenic properties of these biomaterials is related to limitations with respect to the bone regeneration / repair that they promote[3]. Alternatives, such as mixtures of these biomaterials with autogenous bone, have been proposed to improve their biological properties[3, 9, 11]; however, this would defeat one of the major purposes for the use of DBB and TCP/HA, which is the elimination of the need to remove a graft from a donor area on the patient. Additionally, these biomaterials have been coated with growth factors, such as recombinant human bone morphogenic protein 2 (rhBMP2)[12] and recombinant human growth and differentiation factor-5 (rhGDF-5)[13]; however, the coating of biomaterials with growth factors is a high-cost procedure[14].

Avocado/soybean unsaponifiables (ASU) are used as a medication for rheumatoid arthritis and osteoarthritis[15, 16]. Studies have shown that ASU modifies the structures of joint tissues damaged by the progression of arthritis[16-18]. This phenomenon occurs due to the stimulatory effect of the ASU on the expression of growth factors such as TGFβ1, TGFβ2, and BMP2[19-21], as well as on the synthesis of proteins of the connective tissue matrix (e.g., collagen and agrecans)[19-23]. Clinical studies have shown through X-ray analysis that ASU induced a reduction in the interarticular space in patients with rheumatoid arthritis[16, 17]; furthermore, it was shown in histological studies with osteoarthritis induced in model animals (sheep[24] and dogs[18]) that ASU induced a reduction in the destruction of joint tissues. Additionally, one study by our group showed that the systemic administration of ASU promoted a higher degree of

osseointegration of dental implants placed in the tibiae of rats associated with higher expression of the growth factors TBG β1 and BMP2 (unpublished data).

Because the effects of ASU on the integration of different biomaterials in bone defects have not been previously investigated, the aim of this study was to evaluate whether this drug can enhance bone repair of critical sized calvaria defects (CCDs) filled or unfilled with DBB and TCP/HA.

Material and methods (ANEXO 1)

Distribution of the animals and groups

The study was approved by the ethics committee for animal research of the School of Dentistry of Araraquara (FOAr-UNESP, CEUA nº 01/12) (ANEXO 4). Eighty-four adult (3 months of age) male rats (*Rattus norvegicus*, var. Holtzman) weighing between 300-350 g were used in this study. The rats were kept in a room with controlled temperature (21±1ºC) and humidity (65-70%) and a 12-hour light-dark cycle. The animals had access ad libitum to standard rat chow and water throughout the experiment.

The animals were randomly allocated into 2 groups with 3 subgroups per group that were followed for 15 and 60 days; there were 7 animals per subgroup/period. The groups were divided according to the drug administered to the animals. Control (CTR): saline solution was administered to the animals daily; ASU group (ASU): ASU (Piascledine 300®, Expanscience Lab, France) was administered to the animals daily at a dosage of 0.6 mg/kg/day[25]. The ASU and saline solutions were administered daily by gavage, beginning 15 days before the surgical procedures until the end of the experimental period (15 or 60 days). The subgroups were divided according to the type of biomaterial used to fill the CCDs. COA subgroup: The CCDs were filled with coagulum; DBB subgroup: The CCDs were filled with DBB (Bio-Oss®, Geistlich AG,

Wolhusen, Switzerland); TCP/HA subgroup: The CCDs were filled with a biphasic TCP/HA ceramic (Straumann® Bone Ceramic, Straumann AG, Basel, Switzerland).

Surgical procedure

The animals were anesthetized with a combination ketamine (0.08 ml/100 g; Rompum, Bayer S.A., São Paulo, SP, Brazil) and xylazine (0.04 ml/100 g; Rompum, Bayer S.A., São Paulo, SP, Brazil). Subsequently, the calvarial region of the animals was shaved after the surgical field was sterilized with sterile gauze soaked in a solution of povidone, and the animal was placed in the prone position on the operating table.

A surgical access to the anterolateral portion of the calvaria was created through a bicoronal skin and muscle incision, with dimensions of approximately 3x2cm in the anterior and the lateral portions, respectively. Then, the scalp tissues were divulsed using small scissors with blunt ends and dissecting tweezers until the periosteum was exposed; it was then incised and detached to expose the bone. Then, CCDs were made in the parietal bone of the rats immediately under the apex of the coronal suture opposite the lateral incision. The CCDs were 5mm in diameter and 1.5mm thick, and they were created by removal of the bone tissue by a trephine drill (3i – 3i implantes, Brasil), mounted on a low-speed hand-piece (Anthogyr – Injecta – Diadema, Brasil) under copious irrigation with sterile saline. Care was taken to avoid any injury to the dura mater. The biomaterials were implanted in the bone defects on the dura mater without extravasation. Then, the tissues were then sutured in layers with 5.0 bioabsorbable (Vicryl, (ETHICON, J&J, São José dos Campos, Brazil) and 4.0 silk (ETHICON, J&J, São José dos Campos, Brazil) sutures. After surgery, the animals received a single intramuscular injection of a combination of penicillin and streptomycin (0.1 ml/kg) (Multibiótico Small, Vitalfarma, São Sebastião do Paraíso,

MG, Brazil) for infection control and 3 days of dipyrone by gavage (0.1 ml/kg) (Dipirona Ibasa 50% - Ibasa, Porto Alegre, RS, Brazil) for pain control.

Micro-CT analysis

Five animals from each subgroup were positioned in a supine position and had their calvarias scanned by a microtomography machine (Skyscan, Aartselaar, Belgium). The images generated were then reconstructed, spatially oriented, and analyzed by specialized software (NRecon/DataViewer/CTan, Skyscan, Aartselaar, Belgium). For delimitation of regions of interest (ROIs), the images were save in the transaxial plane as a reference and then, 40 sections that encompassed the whole defect were selected (section thickness=35 μm; 40 sections approx.=1.5 mm). The ROIs selected in the CTan software had a rounded shape and were similar in all animals (5x5mm). The results were expressed as percentage of bone filling the CCD, and in the subgroups treated with the biomaterials, the analysis was performed considering a separate evaluation of the percentage of biomaterial and bone that filled the CCD (Figure 1). A *threshold* range between 55 and 250 in grayscale was used to evaluate the percentage of biomaterial plus bone, and a threshold of 55-90 was used to evaluate the percentage of bone. A blinded, trained and calibrated examiner (GJO) performed the analyses.

Obtaining the samples, histomorphometric analysis and histological description

After scanning the calvarias, 7 animals per subgroup were euthanized through an overdose of anesthetic. Subsequently, a bicoronal incision was made in the scalp of the animal, and the entire top portion of the calvaria was removed. The samples were fixed in 4% paraformaldehyde for 48 hours and then decalcified in 7% EDTA solution for 90

days; after this period, the samples were histologically processed, embedded in paraffin, sectioned, and stained (HE, Masson's trichrome, Goldner's trichrome).

Sections were cut from each sample beginning at the edge and continuing to the middle of the CDDs. Twenty histological slides with four sections each were prepared from each sample. For every section, five captured sections were excluded, which provided a distance of 25 μm between each section captured. The linear cross-sectional area of evaluation for each sample was 2500 μm^2 from the defect edge. For each sample, a number between 1 and 6 was drawn to determine the first glass that was stained. From the selected number, a semi-graded staining of the slides was performed, where three slides were stained and the following 3 were not stained, giving nine stained slides per sample. The third section of the first and third slides in each cluster was selected for analysis, giving six sections per sample analyzed.

The images were captured by optical microscopy (DIASTAR; Leica Reichert & Jung products, Wetzlar, Germany), with an original magnification of 25X for the histomorphometric analysis and 50X and 100X for the histological description. Because it was not possible to capture the entire CCD in a single image, it was necessary to join 2-4 images using Windows Photo Gallery software (Microsoft, Redmond, WAUSA) to include the entire defect in a single image for analysis. Determination of the different tissues that filled the CCDs (%biomaterial, %bone, %connective tissue) was performed using an image analysis program (Image J, Jandel Scientific, San Rafael, CA, USA). A blinded, trained and calibrated examiner (LGP) performed the histomorphometric analyses. Furthermore, a histological description of the samples in each group was made according to the characteristics of the newly formed tissues, the presence of inflammatory cells and the relationship between the particles of biomaterials and the new bone. These analyses were performed for five animals per group in two sections

close to the middle of the defect that were stained with HE and Masson's trichrome. A blinded, trained and calibrated examiner (GJO) performed the histological descriptions.

Statistical Analyses

The GraphPad Prism 5.0 (San Diego, CA, USA) software package was used to perform the statistical analyses. The sample size calculation of this study was based on a study that evaluated the effects of different biomaterials on the repair of CCDs in rats by applying a similar histomorphometric analysis to that used in this study[26]. It was shown that the minimum difference between the treatments regarding the average %bone fill in the CCDs was 7.3% with a standard deviation of 3.75%. Therefore, when applying ANOVA, it was determined that with seven animals in each subgroup and an alpha error of 0.05, the power of the study was 80%.

To assess the calibration of the examiners, 10 samples from the micro CT and histomorphometric analyses were re-examined after a 1-week interval. When applying the paired *t*-test, it was verified that there were no statistically significant differences between the evaluations ($p > 0.05$). When the Pearson linear correlation was applied, an index of intra-examiner agreement of $r = 0.99$ was found for the micro CT analysis, and an index of intra-examiner agreement of $r = 0.82$ was found for the histomorphometric analysis.

The data generated by the micro CT and histomorphometric analyses were numerical, and the Shapiro-Wilk test showed that the data were normally distributed ($p > 0.05$). ANOVA, complemented by a post-hoc Tukey test, was used for intergroup analysis for each time point. An unpaired *t*-test was used for intragroup evaluation to

verify the effect of time. All tests in this study were performed with a significance level of 95% ($p < 0.05$).

Results

Micro CT analysis

It was shown that within the COA subgroups, the ASU groups presented a higher %bone fill in the CCDs than did the CTR group at 60 days ($p<0.05$). Additionally, it was observed that the COA subgroup presented a higher %bone fill in the CCDs than did the DBB and TCP/HA subgroups at 60 days ($p<0.05$). It was observed that the ASU group had a higher %bone fill in the CCDs in the COA subgroup than did the the DBB and TCP/HA subgroups at 15 and 60 days ($p<0.05$).

Regarding the %biomaterial that filled the CCDs, it was found in both the CTR and ASU groups that the DBB showed a higher %biomaterial than did the TCP-HA group at 15 and 60 days ($p < 0.05$). Table 1 shows the mean and standard deviation of the %bone and %biomaterial that filled the CCDs in all of the groups.

Histological descriptions

At 15 days

No histological differences were observed between the CTR and ASU groups regarding the DDB and TCP/HA subgroups. In the subgroups treated with biomaterials, immature bone was found between and in contact with the particles, especially those close to the edge of the CCDs. The presence of inflammatory infiltrates was not observed, and the presence of osteoclasts was rarely observed in the DDB subgroup, while osteoclasts were not observed in the TCP / HA subgroup. In the center of the CCDs, an extremely large quantity of biomaterial particles was observed in contact with disorganized connective tissue. It was also observed that when a biomaterial was used to fill the

CCD, this grafted area had similar thickness to that of the native bone. When evaluating the COA subgroup, bone formation was observed at the edges of the defect in both groups, but it was observed in the ASU group that some samples presented bone formation in the central region of the defect. The presence of a few inflammatory cells and disorganized connective tissue were also observed. Representative images from 15 days are shown in figure 2.

At 60 days

After 60 days, no differences were observed between the histological patterns of the CCDs filled with DDB and TCP/HA in the CTR and ASU groups. The subgroups that were filled with biomaterials presented bone formation at the edges of the CCDs and between the particles of the biomaterials, and in some samples, the bone was in direct contact with the particles. The particles of the biomaterials located in the center of the defects were in contact with the connective tissue, which at this point was more organized and mature compared with the tissue at 15 days. Additionally, the particles of the biomaterials that were close to the edges of the defects were smaller than the particles in the center of the CCDs. No inflammatory infiltrate or osteoclasts were present. It was also observed that when a biomaterial was used to fill the CCD, this grafted area had a similar thickness to that of the native bone. In the COA subgroup, the ASU group showed bone formation in the center of the CCD that almost completely occluded the defect in some samples, but the thickness of the bone formed was thinner than that of the native bone. This pattern of bone formation was not observed in the CTR group, where bone formation was confined to the peripheral region of the CCDs. In addition, in the COA subgroups, mature connective tissue with organized collagen fibers with well-defined long axes perpendicular to the edges of the CCD was observed.

Additionally, no inflammatory infiltrate was observed in these subgroups. Representative images from 60 days are shown in figure 3.

Histomorphometric analysis

The COA subgroup treated with ASU presented a higher %bone and a lower %connective tissue than did the CTR group at the time points of 15 and 60 days ($p<0.05$). A higher %bone was also verified in the COA subgroups of the ASU group compared with the DDB and TCP/HA subgroups at the time points of 15 and 60 days ($p<0.05$). In the CTR group, it was observed that the COA subgroup presented a higher %connective tissue than did the DDB and TCP/HA subgroups at the time points of 15 and 60 days ($p<0.05$). Furthermore, it was observed in both groups that the %biomaterial was higher in the DDB subgroups than in the TCP/HA subgroups at 60 days ($p<0.05$).

Discussion

The results of the present study demonstrate that ASU enhanced the bone formation in the CCDs in the COA subgroups compared with the bone formation in the same subgroups of the CTR group at the both of the evaluated time points. These results confirm the findings of other studies that reported that ASU stimulated the formation of connective tissues due to up-regulation of the expression of growth factors related to bone formation, such as TGFβ1 and BMP2[19-21], and induced the synthesis of components of the connective tissue matrix[22, 23].

However, when biomaterials were placed in the CCDs, differences between the ASU and CTR groups related to bone formation were not verified. Furthermore, it was shown that the COA subgroup of the ASU group presented a higher %bone in the CCDs

than did the DBB and TCP/HA subgroups. The lower amounts of bone formation in the DBB and TCP / HA subgroups may be due to the slow resorption rates of these biomaterials, which occupy the space that would eventually potentially be occupied by regenerated bone. A study that evaluated the use of bioactive glass in CCDs also detected higher bone formation in the COA group, and these authors suggested that biomaterials, which require long periods for complete resorption, will reduce new bone formation[27]. This increased bone formation in the COA group in relation to the DBB and TCP / HA groups was also observed in the CTL group at 60 days with the micro CT analysis, but these results were not confirmed by histomorphometric analysis.

The discrepancy between the results from the micro CT and histomorphometric analyses followed a pattern wherein the micro CT analysis underestimated the presence of bone tissue and overestimated the presence of biomaterial. No studies have correlated the data obtained by micro CT and histomorphometric analyses regarding the composition of tissue when biomaterials have been placed to fill CCDs. It is likely that the radiopacity of these biomaterials produces artifacts that hinder the correct identification of the bone and the biomaterial[28]. Metallic compounds that exhibit high radiopacity, such as titanium, produce artifacts that interfere with the measurement of bone tissue formation[29]. Additional tests will be necessary to evaluate the different micro CT parameters that can be modified to promote increased agreement with the histomorphometric data associated with the repair of CCDs after the placement of different biomaterials.

With further regard to the histomorphometric analysis, it was verified that the COA subgroup of the CTR group presented a higher %connective tissue in the CCDs than did the DBB and TCP/HA subgroups. This occurs because the presence of the biomaterials maintains the space and prevents the connective tissue and soft tissue from

invading the CCD[30, 31]. However, this difference was not detected in the ASU groups. It is unlikely that accelerated bone formation in the COA subgroup treated with ASU prevented the proliferation of connective tissue into the CCD.

Regarding the biological behavior of the biomaterials evaluated in our study, the presence of biomaterial particles remaining inside the CCDs in the DBB and TCP/HA subgroups was verified after 60 days, which is in agreement with results from histological studies that found particles of DBB and TCP / HA remaining in grafted sites at the time of reopening for implant placement[5-7]. Additionally, the micro CT and histomorphometric analyses showed that the DDB subgroup in both the CTR and ASU groups presented a higher %biomaterial in the CCD than did the TCP/HA subgroups at the 60-day time point. These results confirm the results of another study that showed a higher amount of particles in sites grafted with DDB than in sites grafted with TCP/HA [5]. Both biomaterials used in this study were reabsorbed by osteoclasts[32, 33]; however, the variation in the speed of reabsorption between the DBB and TCP/HA is due to the different degrees of solubility of the components of these biomaterials in the acids produced by the osteoclasts[34]. The 40% of TCP/HA corresponding to β-tricalcium phosphate resorbed faster than the 60% corresponding to hydroxyapatite[34]. Furthermore, in addition to osteoclasts, other cells such as macrophages also induce the resorption of β-tricalcium phosphate[35]. Regarding the DBB and the hydroxyapatite portion of the TCP/HA, in addition to the low solubility, the absence of protein from these biomaterials, which directs the function of resorption by osteoclasts in bone, decreases the resorption rates of these biomaterials, as observed in this and in other studies[32, 33]. In this study, neither histologically significant amounts of osteoclasts nor an abundant inflammatory infiltrate was observed to be associated with DBB and TCP/HA, and these findings corroborate the histological data reported in other studies[10, 36].

Regarding the histological descriptions of the DBB and TCP/HA subgroups, the presence of bone formation between and in contact with the biomaterial particles was observed in both groups (ASU and CTR), and the size of the particles close to the edges of the CCDs was smaller than the size of the particles in the center. Furthermore, the majority of the particles in the center of the CCDs were surrounded by connective tissue. These findings confirm the osteoconductive potential of DBB and TCP/HA, which formed frameworks that guided the formation of bone around the biomaterial particles[5-7, 10, 12]. However, for the reduction of the volume of biomaterials to allow concomitant bone formation to occur, it is necessary for the biomaterial particles to have a close relationship with the blood supply provided by the receptor site. Two studies that compared the histology of areas grafted with DBB and TCP/HA in post-extraction sockets[6] and sinus floor augmentation[5] showed that particles of these biomaterials located far from the native bone were surrounded by connective tissue. Additionally, in one study that assessed the repair of CCDs with bioactive glass/calcium sulfate, greater contact was observed between the biomaterial particles and the connective tissue in the center of the defect [27].

The small amount of bone formation promoted by the DBB and TCP / HA does not mean that it is clinically disadvantageous to use these biomaterials for the treatment of bone defects or to increase bone availability. Although the COA subgroups presented a higher %bone in the CCDs, the regenerated region was thinner than the native bone that was not involved in the CCD. This finding demonstrates that the DDB and TCP/HA were more effective in maintaining the shape of the native bone, and this fact has been demonstrated in a study where DDB and TCP/HA promoted a good outcome in the preservation of bone walls[6, 37]. In addition, the placement of implants in areas grafted

with osteoconductive biomaterials has demonstrated survival rates similar to implants placed in autogenous bone[1].

When analyzing the data obtained in this study, some obvious limitations must be considered. ASU is a drug that alters the structure of connective tissues (e.g., bone and cartilage) at a slow rate; thus, it is not known whether the evaluation time was sufficient to identify differences in bone repair associated with DBB and TCP / HA. Factors related to dose-response effects (application of higher doses) and routes of administration (local or systemic) that may also interfere with the effects of the drug were not evaluated in this study, nor was the concentration of ASU that acted directly on the CCD. Finally, the use of membranes could have interfered with the differences between the ASU and CTR groups with respect to the bone repair in the COA subgroup.

Thus, according to the results shown, and taking into account the methodology used, it can be concluded that ASU increased the bone repair of CCDs in the COA group compared with that in the CTR group, but this effect was not sufficient to accelerate the bone formation associated with the use of DBB and TCP/HA.

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Tables

Table 1: Average and Standard deviation of the % of bone and biomaterial that filled the CCD in all the groups evaluated by micro CT.

Group	Biomaterial	15 days		60 days	
		%Bone	%Biomaterial	%Bone	%Biomaterial
CTR	COA	28.04±3.90	-	34.77±9.02*#	-
	DBB	21.15±8.89	50.89±13.83#	14.97±3.08#	44.05±12.24#
	TCP/HÁ	20.93±3.39	32.23±10.52#	23.14±4.29#	19.60±9.81#
ASU	COA	27.95±4.13#	-	48.97±8.10*#	-
	DBB	15.58±1.99#	36.67±8.28#	13.58±0.71#	37.62±5.43#
	TCP/HA	16.47±6.13#	17.48±8.71#	20.27±1.82#	20.33±3.33#

*Differences between the CTR and ASU groups- Unpaired t-test
#Differences between the biomaterials into each group- One-way Anova and Tukey

Table 2: Average and standard deviation of the % of bone, biomaterial, and connective tissue (CT) into de CCD in all the groups evaluated by the histomorphometric analysis.

Group	Biomaterial	15 days			60 days		
		%Bone	%Biomaterial	%CT	%Bone	%Biomaterial	%CT
CTR	COA	29.00±8.81*	-	71.00±8.81*#	42.71±5.21*	-	58.71±6.62*#
	DBB	31.43±7.54	20.57±7.02	48.00±10.41#	39.86±10.45	18.57±5.65#	41.57±8.84#
	TCP/HA	33.00±6.48	15.86±8.91	51.14±7.38#	39.57±8.69	11.14±5.17#	49.29±4.34#
ASU	COA	46.40±10.41*#	-	53.60±10.41*	52.14±6.12*#	-	47.57±5.99*
	DBB	29.29±4.53#	18.71±3.25	52.00±5.85	33.14±4.59#	18.00±3.41#	51.14±3.80
	TCP/HA	33.33±6.68#	15.86±4.59	56.33±5.68	31.71±4.99#	10.33±5.75#	52.43±3.45

*Differences between the CTR and ASU groups- Unpaired t-test
#Differences between the biomaterials into each group- One-way Anova and Tukey

Figure Captions

Figure 1: Evaluation of the %bone and %biomaterials by the micro CT A) ROI delimitation; B) % biomaterial + % bone; C) % bone.

Figure 2: Representative images of the histological analysis in all the groups at the period at 15 days. B-Bone; NB-New bone; BM-Biomaterial; CT-Connective tissue; M-Middle of the defect; E-Edge of the defect.

Figure 3: Representative images of the histological analysis in all the groups at the period at 60 days. B-Bone; NB-New bone; BM-Biomaterial; CT-Connective tissue; M-Middle of the defect; E-Edge of the defect.

Figure 1:

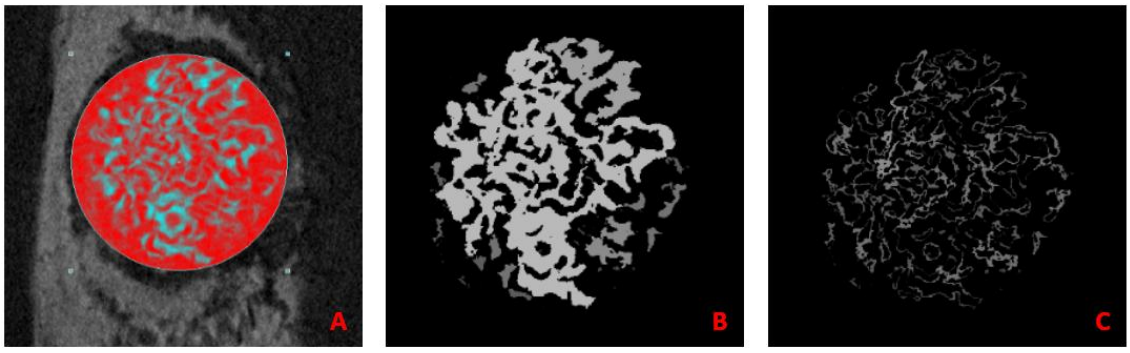


Figure 2:

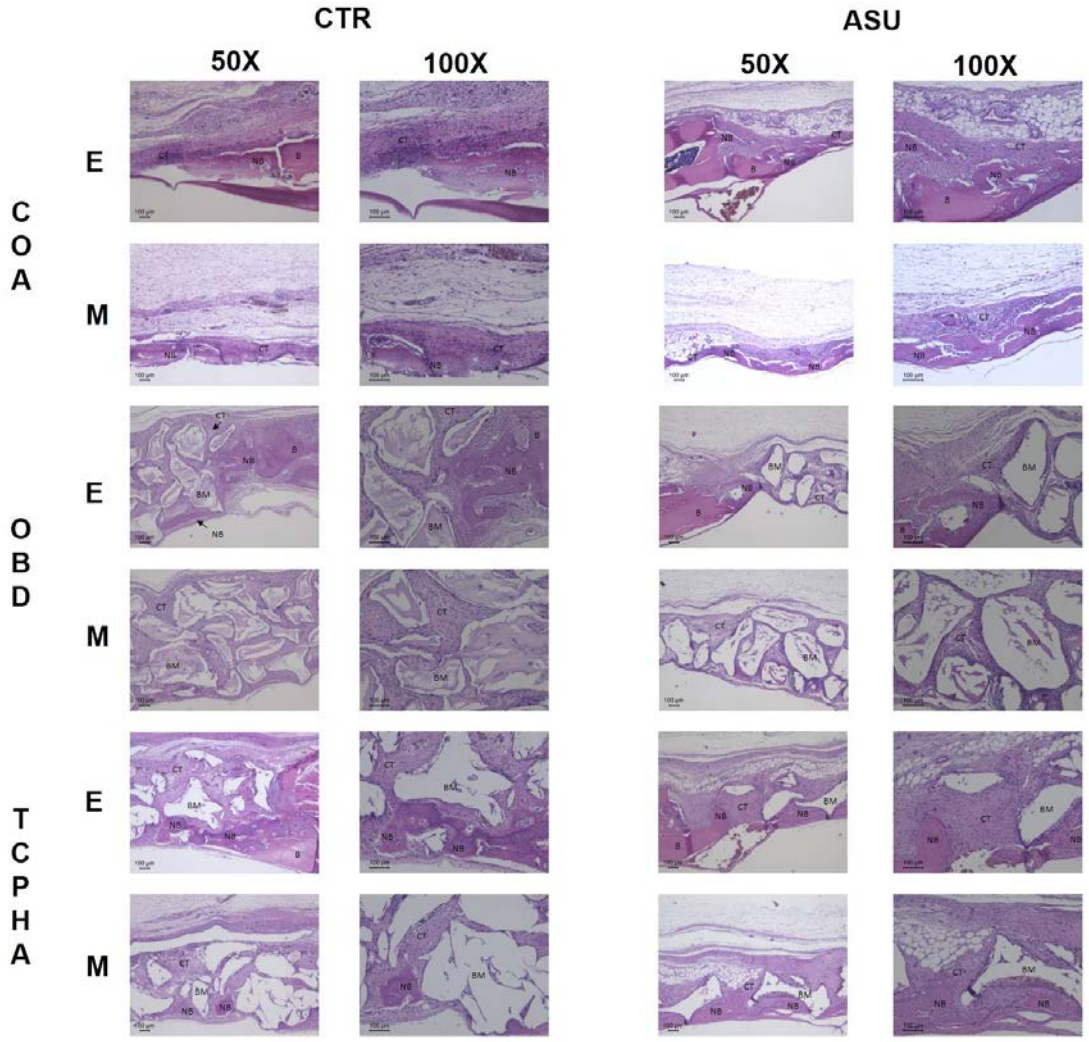
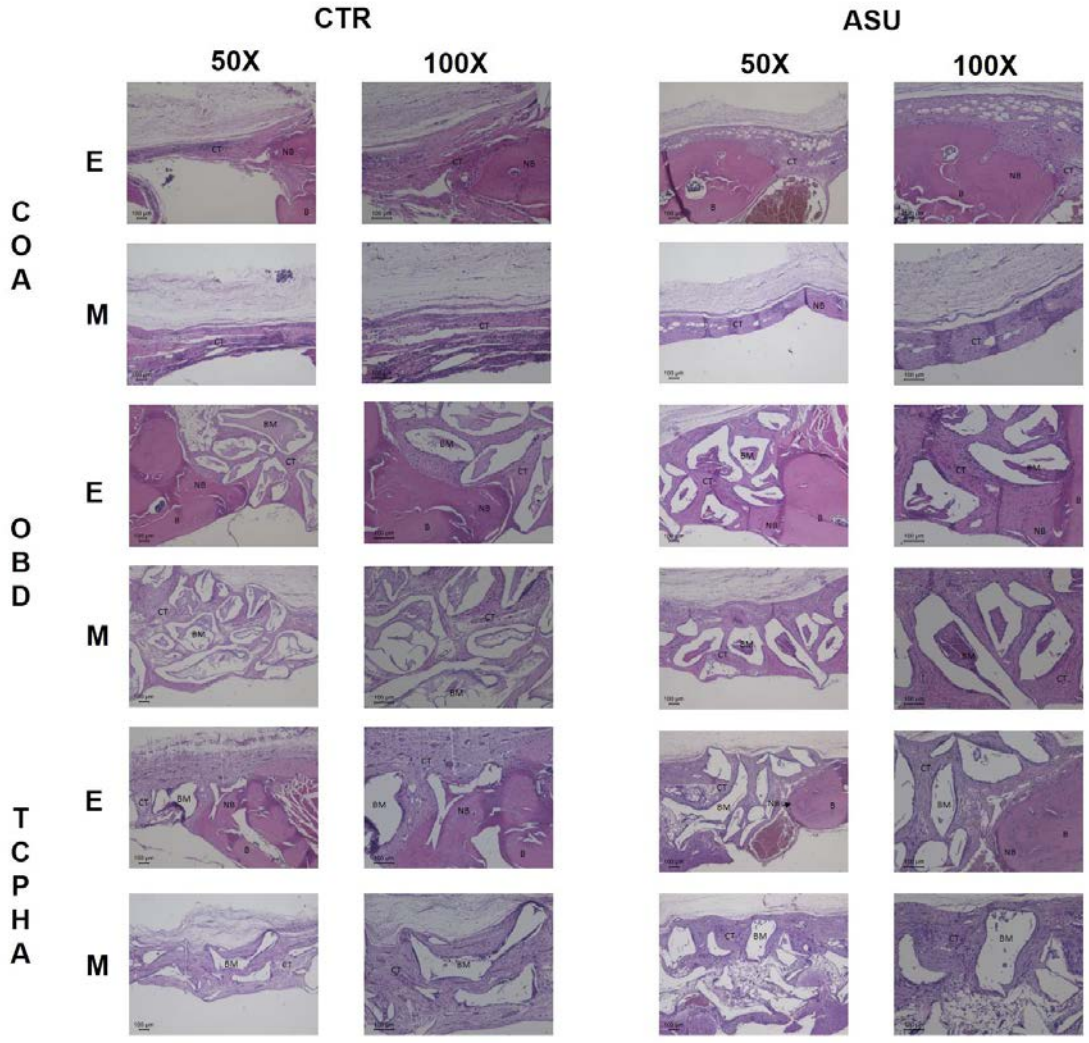


Figure 3:



Considerações Finais

Considerações Finais

Esse projeto teve como objetivo avaliar o efeito do ASU sob diversas condições pré-clínicas que pudessem ser beneficiadas por um aumento na síntese de matriz de tecido conjuntivo ósseo e redução do processo inflamatório. Dentre estas condições foram avaliadas o efeito desse medicamento sobre: 1) A periodontite induzida por ligaduras em ratos; 2) Tratamento por raspagem da periodontite induzida; 3)Osseointegração de implantes instalados em tíbias de ratos; 4) Reparo de defeitos críticos de calvárias de ratos associados ao preenchimento com biomateriais.

A plausibilidade biológica desse estudo foi o fato de que este medicamento proporcionou bons resultados na prevenção e no reparo das destruição intra-articulares que ocorrem devido a progressão da artrite reumatoide e da osteoartrite (Cake et al.[15], 2000; Appelbomm et al.[4], 2001; Boileau et al.[10],2009; Maheu et al.[49], 2013). Estudos in vitro em condroblastos, osteoblastos e macrófagos demonstraram que o ASU suprime a secreção de mediadores pró-inflamatórios como a IL-1β, -6, -8, TNF-α, iNOS (Herontin et al.[35,36], 2003, 2006; Au et al.[5], 2007), MMP2,-3, -13(Kut-Lasserre et al.[43], 2001, Henrotin et al.[36], 2003; Gabay et al[29], 2008; Lippielo et al.[48], 2008), PGE-2 (Lippielo et al.[48], 2008; Heinecke et al.[33], 2010), e induzem a produção de fatores de crescimento como o TGF-β1, TGF-β2 e BMP2 (Andriamanalijaona et al.[2], 2006), moléculas componentes da matriz do tecido conjuntivo como o colágeno e as glicosaminoglicanas (Herotin et al.[35], 2006; Lippielo et al.[48], 2008) e dos inibidores de metaloproteinases (TIMP1 e TIMP2) (Kut-Lasserre et al.[43], 2001).

Esse mecanismo de ação foi confirmado em nosso projeto. No estudo I foi verificado que os animais do grupo ASU+7 apresentaram maiores expressões de fosfatase alcalina e menores expressões de IL1β, TRAP e RANKL em relação ao grupo CTR. No estudo III foi verificado que os animais que fizeram uso dos grupos ASU 1 e

ASU2 apresentaram uma maior expressão das proteínas TGBβ1 e BMP2 em comparação ao grupo CTL. Esses efeitos podem explicar os melhores resultados com relação aos resultados histométricos nos estudos I e III. No estudo I foi verificado que houve uma maior %osso na região da furca e uma menor distância entre a JCE-CO no grupo ASU+7 em relação ao grupo CTR. No estudo III foi verificado que os grupos ASU1 e ASU2 apresentaram maiores valores de BIC na região cortical do que o grupo CTL.

Após esses resultados, foi formulada a hipótese de que o ASU poderia também influenciar o reparo de defeitos críticos em calotas de ratos e a integração de diferentes biomateriais, já que esse processo também é altamente dependente do metabolismo ósseo, tal como nos processos de osseointegração. Nesse estudo ao qual denominamos de estudo IV houve uma maior formação óssea associado ao reparo de defeitos críticos em calotas no grupo ASU do que no grupo CTR quando não foi inserido biomaterial dentro do defeito. É provável que os efeitos que o medicamento promove aumentando a expressão das proteínas TGBβ1 e BMP2 durante os processos de osseointegração também tenha interferido no processo de reparo dos defeitos críticos nas calvárias dos animais.

Algumas informações sobre a utilização do ASU permanecem desconhecidas. Por se tratar de um extrato, não se sabe bem ao certo qual composto ativo que produz os efeitos do ASU. Um estudo descreveu que o ASU é composto principalmente de esteróides proveniente das sementes do abacate e dos grãos de soja na proporção 1:2 (Lipiello et al.[48], 2008). A avaliação do efeito do componentes do ASU individualmente e em conjunto demonstraram que o efeito dos diferentes componentes é mais sutil do que o efeito dos componentes em conjunto (Kut Lasserre et al.[43], 2001; Lipiello et al.[48], 2008). Outras variáveis importantes em relação a aplicação do ASU como a dose e o

tempo de utilização necessário para que o ASU produza seus efeitos satisfatórios ainda necessita ser determinado para cada tipo de indicação. Estudos in vitro demonstraram que o efeito do ASU depende da dose aplicada (Kut Lasserre et al.[43], 2001; Herotin et al.[36], 2003) e que diferentes tipos celulares respondem de forma diferente ao ASU (Herotin et al.[35], 2006). Um ensaio clínico controlado e randomizado que avaliou o efeito do ASU na redução dos sintomas da osteoartrite verificou que a dose de 600mg/dia não foi mais efetiva na redução do consumo de analgésicos que a aplicação do ASU na dose de 300mg/dia (Appelboom et al.[4], 2001), porém os indivíduos que fizeram uso de maiores doses do ASU apresentaram melhoras na função das articulações em relação ao grupo controle e do que o grupo que utilizou a menor dose de ASU. Dessa forma, alterações na dose podem produzir efeitos mais consistentes no tratamento da doença periodontal induzida e nos processos de osseointegração e integração de biomateriais.

Em resumo, foi verificado nesse projeto que o ASU promoveu maior reparo na doença periodontal induzida em ratos, porém esses resultados não foram consistentes pois esse efeito não foi confirmado no modelo de tratamento da doença periodontal induzida. Esse medicamento induziu, de forma sutil um melhor padrão de osseointegração de implantes e uma maior formação óssea associada a defeitos críticos em calotas que não foram preenchidos com biomateriais.

Conclusão

Conclusão

De acordo com os resultados obtidos e levando-se em consideração a metodologia aplicada pode-se concluir que:

1) ASU acelerou o reparo do tecido ósseo periodontal após a remoção das ligaduras, e esse efeito foi mediado pelo bloqueio de mediadores pró-inflamatórios (IL-1β; RANKL) e estimulo da expressão de um marcador de formação óssea (Fosfatase Alcalina);
2) A utilização do ASU não promoveu efeitos benéficos sobre o reparo periodontal após tratamento da periodontite induzida;
3) O ASU promoveu maior osseointegração em implantes instalados em tibias de ratos, porém esse efeito só foi verificado na região cortical e parece ter sido mediado pela maior expressão de TGFβ1 e BMP2;
4) O ASU aumentou a formação de óssea em defeitos críticos de calotas em relação ao grupo CTR, entretanto esse efeito não foi o suficiente para acelerar a formação óssea associada a utilização de biomateriais.

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Anexo 1

Material e métodos

ANEXO 1 Material e métodos

Cada objetivo específico dessa tese foi avaliado nos seguintes estudos:

Estudo I: Efeito do extrato de óleo insaponificável de abacate e soja sobre o reparo da periodontite induzida por ligaduras.

Estudo II: Efeito do extrato de óleo insaponificável de abacate e soja sobre o reparo associado ao tratamento da periodontite induzida.

Estudo III: Avaliação da influência do extrato de óleo insaponificável de abacate e soja na osseointegração de implantes instalados em tíbia de ratos.

Estudo IV: Efeito do extrato de óleo insaponificável de abacate e soja sobre o reparo de defeitos críticos em calotas de ratos associado a inserção de biomateriais.

As metodologias de cada estudo serão descritas separadamente com exceção dos estudos I e II que tiveram metodologia semelhante.

ESTUDO I Efeito do extrato de óleo insaponificável de abacate e soja sobre o reparo da periodontite induzida por ligaduras.

ESTUDO II Efeito do extrato de óleo insaponificável de abacate e soja sobre o reparo associado ao tratamento da periodontite induzida.

Os estudos I e II foram aprovados pelo Comitê de Ética de Utilização de Animais da Universidade Estadual Paulista - UNESP (CEUA 17/2009), Faculdade de Odontologia de Araraquara, Brasil.

Indução da doença periodontal

Os animais foram anestesiados por uma combinação de Quetamina (Agener União Ltda, São Paulo, SP, Brasil) na dosagem de 0,08 ml/100g de massa corporal com

Xilazina (Rompum, Bayer S.A., São Paulo, SP, Brasil) na dosagem de 0,08:0,04 ml/100g de massa corporal. Posteriormente, os ratos foram colocados em posição supina na mesa operatória e tiveram a boca retraída por um retrator para afastar mandíbula e língua, facilitando a abertura bucal. Ligaduras (fios de algodão, nº24) foram inseridas através de sonda e pinça específicas na região subgengival em volta dos segundos molares superiores de ambos os lados, para que fosse induzida a doença periodontal através do acúmulo de biofilme bacteriano. Após um período de sete dias, as ligaduras foram removidas.

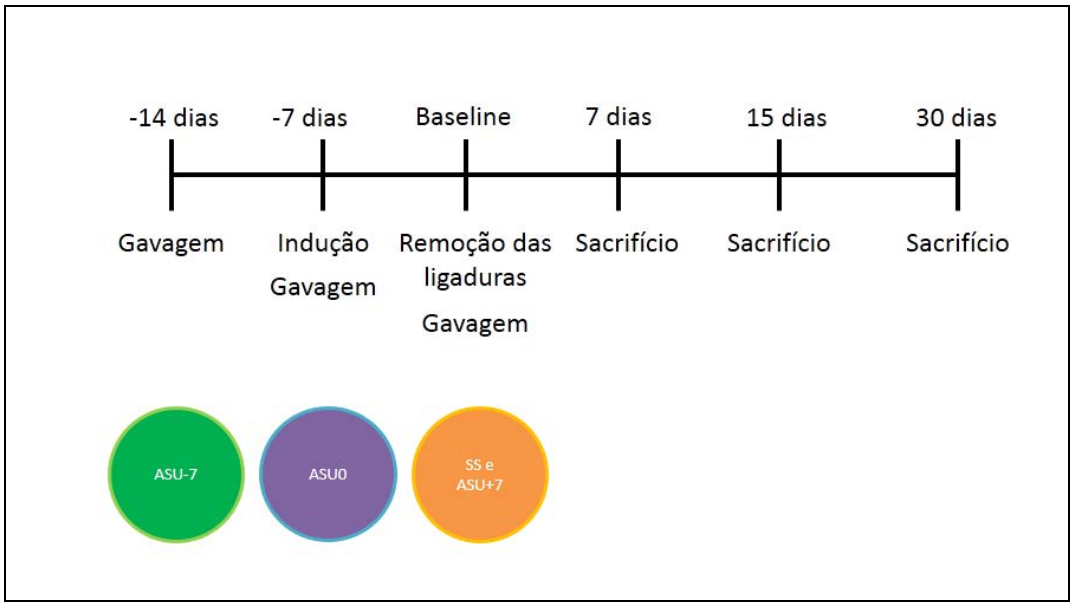
Grupos Experimentais

Os grupos experimentais foram divididos em 2 estudos com objetivos distintos. O estudo I teve como objetivo avaliar o efeito do ASU no reparo da periodontite induzida e o estudo II teve como objetivo avaliar o efeito do ASU no reparo associado ao tratamento da periodontite induzida.

Estudo I

84 animais foram distribuídos randomicamente em 4 grupos avaliados em três períodos experimentais (7, 15 e 30 dias) com 7 animais em cada grupo/período: **CTR**: Administração de soro fisiológico (SS) iniciada no dia da indução da periodontite; **ASU-7**: Administração do ASU (Piascledine 300, Expanscience Lab, França) iniciada 7 dias antes da indução da periodontite; **ASU0:** Administração do ASU iniciada no dia da indução da periodontite; **ASU+7:** Administração do ASU iniciada no dia da remoção da ligadura. A figura 1 expõe o fluxograma desse estudo. O ASU (0.6 g/kg/dia) (Yaman et al, 2007) e o SS foram administrados por gavagem diariamente até o sacrifício dos animais.

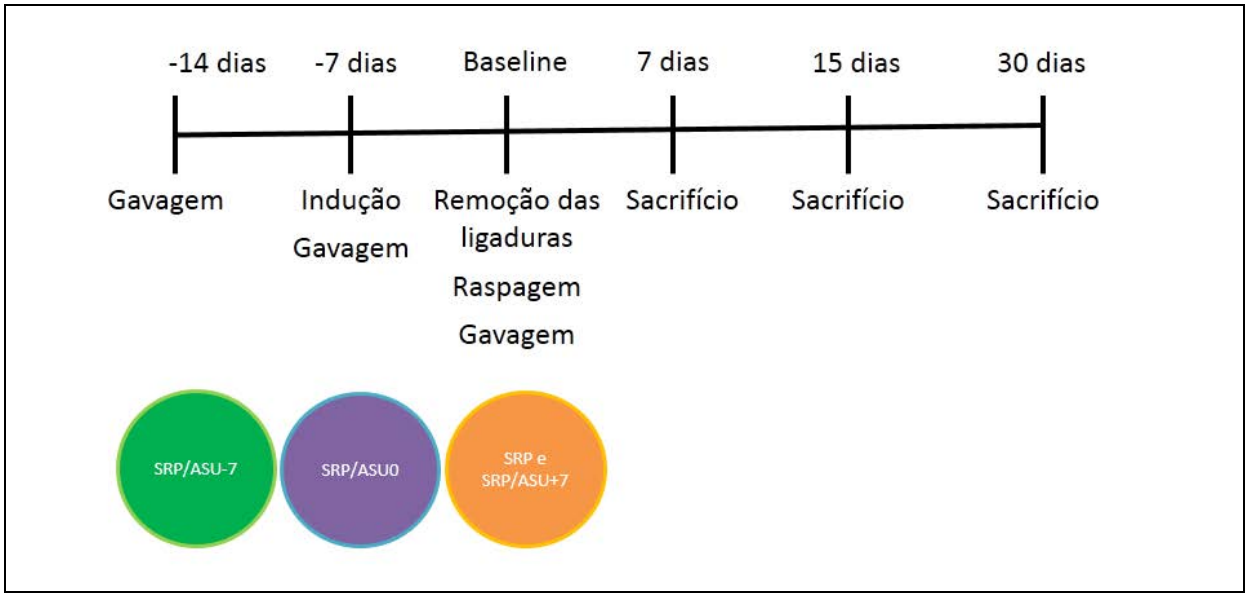
Figura 1: Fluxograma dos procedimentos realizados no estudo I



Estudo II

84 animais foram distribuídos randomicamente em 4 grupos avaliados em três períodos experimentais (7, 15 e 30 dias) com 7 animais em cada grupo/período: **SRP:** Administração de SS iniciada no dia da indução da periodontites; **SRP/ASU-7:** Administração do ASU iniciada 7 dias antes da indução da periodontite; **SRP/ASU0:** Administração do ASU iniciada no dia da indução da periodontite; **SRP/ASU-7:** Administração do ASU iniciada no dia do tratamento da periodontite induzida. O tratamento foi executado por raspagem com curetas Gracey 11-12 e 13-14 do tipo Mine Five (Hu-Friedy, Chicago, IL, EUA) com auxílio de uma lupa estereoscópica com aumento de 3.5X (DMC equipamentos, São Carlos, SP, Brasil) para melhorar a visualização. A figura 2 expõe o fluxograma dos estudo II.

Figura 2: Fluxograma dos procedimentos realizados no estudo II



Obtenção das peças cirúrgicas de todos os grupos

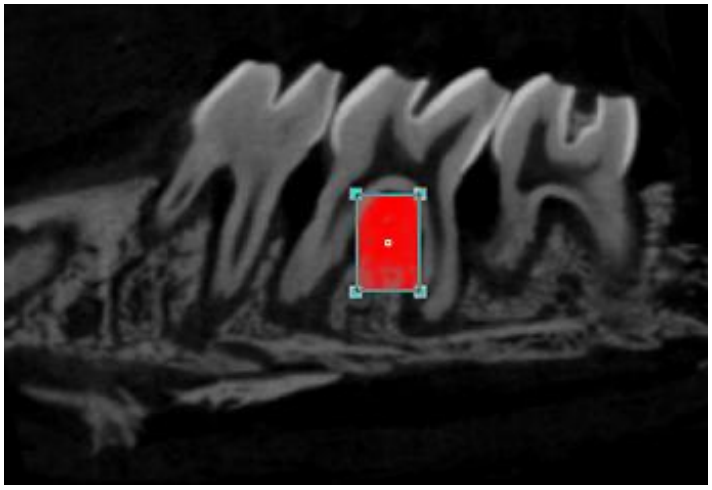
Decorridos os períodos de avaliação, os animais foram sacrificados por sobredose anestésica. As hemimaxilas foram removidas e divididas aleatoriamente em: 7 hemimaxilas foram coletadas para realização das análises histomorfométrica/imunohistoquímica, 5 foram coletadas para análise de qPCR e microCT e as 2 hemimaxilas que foram mantidas sem ligadura foram utilizadas como controle para análise histológica descritiva. As hemimaxilas que foram utilizadas para análise histomorfométrica/imunohistoquimica foram fixadas em paraformaldeído a 4 % por um período de 48 horas. As hemimaxilas utilizadas para análise de qPCR tiveram o tecido gengival circunvizinho aos segundos molares superior removidos e imediatamente congelados em nitrogênio líquido e mantidos em freezer -80ºC até o

momento da extração do mRNA e foram posteriormente escaneadas no microtomógráfo.

Análise microtomográfica

As amostras foram escaneadas em um microtomógrafo (Skyscan, Aartselaar, Bélgica) e posteriormente reconstruídas, reorientadas espacialmente e analisadas por softwares específicos (NRecon/DataViewer/CTan, Skyscan, Aartselaar, Bélgica). O volume do tecido ósseo entre as raízes do segundo molar superior foram mensurados após delimitação da região de interesse (ROI) que apresentou uma área de 70 x 45 pixels^2 e profundidade de 60 secção com 18µm de espessura cada (Figura 3) sendo que foi utilizado uma escala de tons de cinza (*threshold*) de 55-250 tons de cinza para avaliação do volume do osso. Os valores foram fornecidos como % de tecido óssea na região de interesse. A seleção do ROI foi realizada por um examinador cego, treinado e calibrado**.**

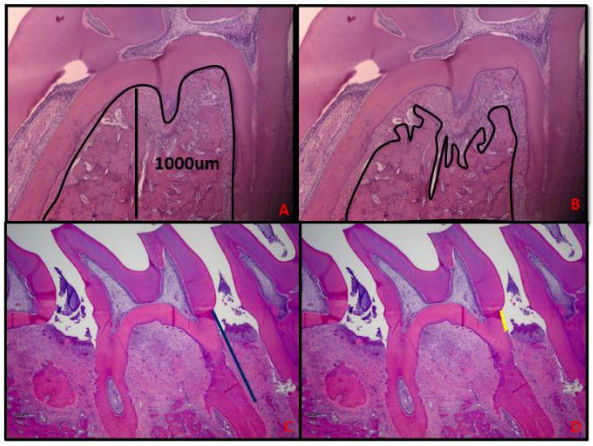
Figura 3: Esquema para análise do volume ósseo por Micro Ct. O volume do tecido ósseo entre as raízes do segundo molar superior foi mensurado após delimitação da região de interesse (ROI) que apresentou uma área de 70 x 45 pixels^2 e profundidade de 60 secção com 18µm de espessura cada.



Avaliação e análise histomorfométrica

Após a fixação, as peças foram descalcificadas em ethylenediamine tetraacetic acid (EDTA) a 7% por 10 semanas e posteriormente foram desidratadas em álcool, diafinizadas em xilol e incluídas em parafina. Os cortes tiveram 5µm de espessura e foram realizados no sentido mésio-distal ao longo eixo do dente, sendo posteriormente corados pela técnica de hematoxilina-eosina (HE). As imagens (50X e 100X) foram obtidas através de uma câmera acoplada a um microscópio de luz (Leica-Reichert Diastar Products & Jung, Wetzlar, Alemanha) e foram avaliadas em um software para análise de imagens (Image J, San Rafael, CA, EUA). O primeiro e o último corte que detectaram a região da furca foram eliminados e 3 cortes equidistantes foram utilizados para análise histomorfométrica. A análise histomorfométrica foi dividida em 2 partes (Figura 4): 1) Análise da porcentagem do tecido ósseo na região da furca que englobou toda a região a partir do teto da furca até 1000µm apicalmente (Duarte et al., 2006), 2) Análises lineares interproximais da junção cemento-esmalte ao topo de crista óssea (ECJ-CB) e da junção cemento-esmalte a porção apical do epitélio juncional (ECJ-aJE). Adicionalmente foi realizada uma análise histológica na região da furca aonde foram descritas as reações inflamatórias e o processo de remodelação óssea nessa região. Essas análises foram executadas por um examinador cego, treinado e calibrado.

Figura 4: Análise histométrica. Análise do preenchimento da região da furca (A e B); Mensurações nas regiões interproximais: C) Distância JCE-CO; D) Distância JCE-aJE.



Análise imuno-histoquímica

A análise imuno-histoquímica foi realizada para identificar e localizar a expressão das proteínas fosfatase ácida tartarato resistente (TRAP), do ligante do ativador do receptor de fator kappa B nuclear (RANKL) e da fosfatase alcalina(AP). Cortes histológicos com 5 µm de espessura foram montados em lâminas silanizadas (Fisher Scientific Superfrost Plus, Fisher, New York, NY, EUA), seguido de tramitação laboratorial de rotina para deparafinização e rehidratação. Em seguida os cortes foram submetidos ao bloqueio dos epítopos inespecíficos através da aplicação de peróxido de hidrogênio 3% por 30 minutos e da proteína bovina albumina (BSA) a 3% por 120 minutos. Logo após, os cortes foram incubados por 16 horas nos anticorpos primários nas seguintes concentrações: TRAP- 1:200; RANKL- 1:200; AP-1:100 (Abcam, Inc. Cambridge, MA, EUA). Como controle negativo, cortes histológicos foram tratados por IgG normal (R & D Systems, Minneapolis, MN, EUA), em substituição aos anticorpos

primários nas mesmas diluições aplicadas aos mesmos. Posteriormente os cortes foram tratados pelo método do complexo avidina-biotina-peroxidase (ABC) com a utilização do kit ABC Staining System (ABC kit DAKO A/S, Copenhagen, Dinamarca) segundo as instruções do fabricante. Os cortes foram contra-corados com solução de hematoxilina de Carrazi para visualização dos núcleos celulares. As imagens (50 e 100X) foram obtidas através de uma câmera acoplada a um microscópio de luz (Leica-Reichert Diastar Products & Jung, Wetzlar, Alemanha). A análise de TRAP foi realizada através da contagem dos osteoclastos marcados que estivessem em contato com o tecido ósseo da região da furca ao redor do segundo molar (Pavone et al., 2014). A análise de RANKL e AP foram executadas através um indice de extensão de marcação proteica utilizado por Garcia et al. 2011: (0) sem marcação (0% das células); (1) marcação fraca (<25% das células); (2) marcação moderada (<50% das células); (3) marcação forte (<75% das células). Esses parâmetros foram avaliados nas regiões interproximais (área delimitada por 1000µm da JCE entre a raiz mesial do segundo molar e distal do primeiro molar e entre a raiz distal do segundo molar e a mesial do terceiro molar) e na região da furca (mesma área de avaliação da análise histomorfomérica de preenchimento ósseo da furca), e a mediana desses valores foi utilizada para a representar o resultado de cada amostra. Enquanto que para a análise do TRAP os resultado de cada amostra foi a soma dos valores dos osteoclastos nas três regiões de interesse. As análises foram realizadas por um examinador cego, treinado e calibrado.

Extração de RNA total, Transcrição reversa e qPCR

O RNA total dos tecidos gengivais coletados foi extraído com o kit RNAqueous-4PCR, segundo o protocolo do fabricante (Life Technologies, Carlsbad, CA, EUA). A

quantidade e pureza do RNA foram determinadas em espectrofotômetro de luz UV por meio da avaliação das absorbâncias a 260nm e da relação entre as absorbâncias a 260/280nm, respectivamente. A integridade do RNA total foi avaliada por meio de resolução de 0,5µg do RNA purificado em eletroforese em gel de agarose a 1%. 400ng do RNA total foi convertido em cDNA com iniciadores aleatórios de hexâmero e a enzima transcriptase reversa do vírus da leucemia de Moloney em um volume de reação de 20 μL (High capacity cDNA synthesis kit, Life Technologies, Carlsbad, CA, EUA ms).

Para a reação de qPCR foi utilizado 20µL de solução que foi composta de 1 µL de cDNA, 8µL água livre de nucleases, 10µL TaqMan gene expression master mix e 1µL TaqMan gene expression assays (Life Technologies, Carlsbad, CA, EUA) para os genes alvo de rato. As condições pré-otimizadas de ciclagem utilizadas foram: 50ºC por 2 minutos, 95ºC por 10 minutos e 40 ciclos de 95ºC por 15 segundos e 60ºC por 1 minuto. O qPCR foi realizado em um equipamento Real Time PCR System 7500 (Life Technologies, Carlsbad, CA, EUA). Os níveis relativos da expressão dos genes para IL-1β, IL-6, RANKL, TNF-α, Fosfatase Alcalina foram calculados utilizando a GAPDH como gene normalizador.

Análise Estatística

O software Graphpad Prism 5 (San Diego, CA, EUA) foi utilizado para execução da análise estatística. Para o cálculo do tamanho da amostra do estudo I foram utilizados os dados do estudo de Duarte et al., 2006 que utilizou metodologia semelhante a nossa com relação a análise do preenchimento da região da furca. Foi verificado que a diferença mínima entre as médias da preenchimento ósseo na região da furca de 16.42% com desvio padrão aproximado de 8.00. Portanto, ao se aplicar o teste ANOVA

levando-se em consideração um poder β do estudo de 0.8 e o poder α de 0.05, determinou-se uma amostra mínima de 7 animais por subgrupo. Para o cálculo do tamanho da amostra do estudo II, foram utilizados os dados do estudo I. Foi verificado que a diferença mínima entre as médias do preenchimento ósseo na região da furca foi de 26.2% com desvio padrão de 12.91. Portanto, ao se aplicar o teste ANOVA levando-se em consideração um poder β do estudo de 0.8 e o poder α de 0.05, determinou-se uma amostra mínima de 7 animais por subgrupo para avaliação dos diferentes tratamentos.

Para avaliação da calibração dos examinadores, 10 amostras de cada estudo foram reexaminadas com intervalo de 1 semana em relação as análises microtomográficas, histomorfométricas do preenchimento da furca e imuno-histoquimicas das 3 proteínas avaliadas. Ao se aplicar o teste t-pareado e o teste de Wilcoxon foi verificado que não houve diferenças estatisticamente significativas entre os resultados das avaliações ($p<0.05$). Ao se aplicar a correlação linear de Pearson foi verificado um índice de correlação intra-examinador de $r>0.92$ para análise microtomográfica, de $r>0.85$ para a análise histomorfométrica e de $r>0.91$ para análise de TRAP. Ao se aplicar a correlação linear de Spearman foi verificado um índice de correlação intra-examinador de $r>0.83$ para as análises imuno-histoquimicas de RANKL e Fosfatase Alcalina.

Todos os dados desses estudos, com exceção da expressão das proteínas RANKL e AP obtidas por imuno-histoquimica, foram numéricos e desta forma o teste de normalidade de Shapiro-Wilk foi aplicado para verificar se os dados se distribuíram de acordo com o teorema da distribuição central. Foi verificado que todos os dados se distribuíram de acordo com a normalidade ($p>0.05$), e dessa forma, testes paramétrios foram utilizados para a análise inferencial dos dados. O teste de Anova complementado

pelo teste de Tukey foi utilizado para a análise intergrupos dentro de cada período de avaliação. As proteínas RANKL e AP, analisadas por imuno-histoquimica, foram estatisticamente avaliadas através do teste não-paramétrico de Kruskall-Wallis complementado pelo teste de Dunn. Todos os testes estatísticos foram aplicados com nível de confiança de 95%($p<0.05$).

ESTUDO III: Avaliação da influência do extrato de óleo insaponificável de abacate e soja na osseointegração de implantes instalados em tíbia de ratos.

Esse estudo foi aprovado pelo Comitê de Ética de Utilização de Animais da Universidade Estadual Paulista - UNESP (CEUA 17/2009), Faculdade de Odontologia de Araraquara, Brasil.

Distribuição dos animais e grupos

Foram instalados implantes bilateralmente nas tíbias de 30 ratos que foram randomicamente divididos em 3 grupos: **ASU 1:** Administração do ASU iniciada 7 dias antes da instalação dos implantes; **ASU 2:** Administração do ASU iniciada no dia da instalação dos implantes; **CTR:** Administração de SS iniciada no dia da inserção dos implantes. O ASU (0.6 g/kg/dia) (Yaman et al, 2007) e a SS foram administrados por gavagem diariamente até o sacrifício dos animais (60 dias).

Procedimentos cirúrgicos

Os animais foram anestesiados por uma combinação de Quetamina (Agener União Ltda, São Paulo, SP, Brasil) na dosagem de 0,08 ml/100g de massa corporal com Xilazina (Rompum, Bayer S.A., São Paulo, SP, Brasil) na dosagem de 0,08:0,04 ml/100g de massa corporal. Posteriormente, foram submetidos à tricotomia da região

interna das pernas direita e esquerda e realizada a antissepsia com gaze estéril embebida em solução de iodopovidina.

Uma incisão de aproximadamente 10 mm foi realizada, em planos, sobre a metáfise tibial. Após uma dissecção delicada, o tecido ósseo foi submetido à osteotomia, realizada por meio de uma sequência progressiva de fresas (fresa lança; fresa espiral de 2.0 mm – Conexão®; São Paulo, SP, Brasil) para acomodar um implante de titânio com 4 mm de comprimento por 2.2 mm de espessura (Conexão ®; São Paulo, SP, Brasil). Todas as perfurações foram realizadas com auxílio de um motor elétrico (BLM 600 – Driller, São Paulo, SP, Brasil), ajustado a 1200 rpm, sob abundante irrigação com solução salina estéril. O implante foi instalado com a ajuda de uma chave digital e todo o acesso cirúrgico foi suturado por planos, internamente com fio reabsorvível 5.0 (Vicryl Ethicon, Johnson & Johnson, São José dos Campos, Brasil) e externamente com fio de seda 4.0 (Ethicon, Johnson & Johnson, São José dos Campos, Brasil). Os animais receberam, em dose única, penicilina associada à estreptomicina na dosagem 0,1 ml/kg de peso (Multibiótico Small, Vitalfarma, São Sebastião do Paraíso, MG, Brasil) e 0.1 ml/kg de peso (Dipirona Ibasa 50% - Ibasa, Porto Alegre, RS, Brasil)

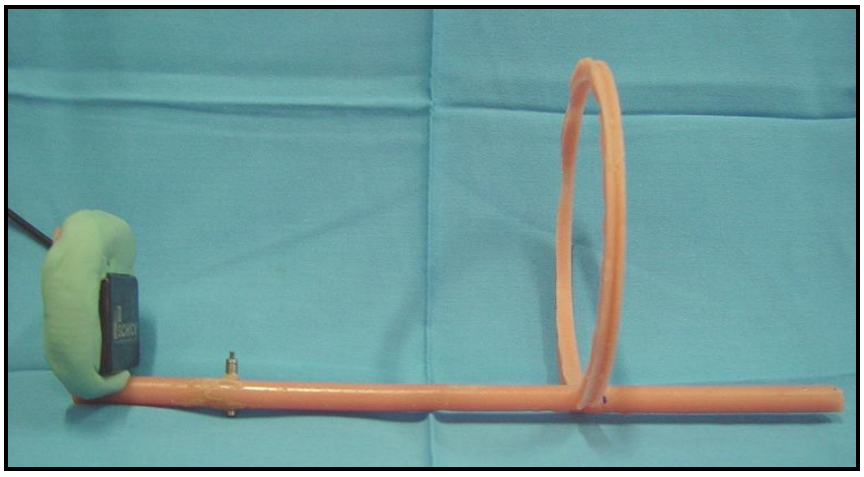
60 dias após os procedimentos cirúrgicos, todos os animais foram sacrificados por aprofundamento da dose de anestésico. As tíbias foram separadas de acordo com as análises. A tíbia direita foi utilizada para as análises radiográficas, biomecânicas, descrição histológica e imuno-histoquimica, enquanto que a tíbia esquerda foi utilizada para a análise histomorfométrica.

Análise Radiográfica

Imagens radiográficas digitais foram obtidas no dia do sacrifício dos animais através de um sensor CDR (Schick CDR, Schick Technologies Inc, City Island, NY,

USA). Após a abertura dos retalhos, os implantes inseridos nas tíbias foram fixados em um posicionador (Figura 5), com o longo eixo vertical do implante posicionado perpendicularmente ao feixe central de raio X e paralelo ao sensor, numa distância focal de 40cm do objeto. Com um aparelho de raio-X GE 100 (Fairfield, CT, EUA) o sensor foi exposto à tomada radiográfica ajustado em 70KVp e 10mA, com tempo de exposição de 0,3 segundos.

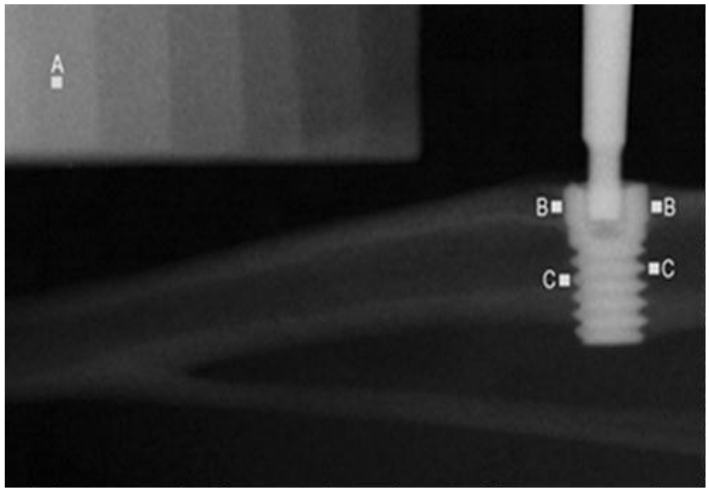
Figura 5: Modelo do posicionador utilizado para a padronização das radiografias



As imagens foram importadas para o software de imagem digital (Image J, San Rafael, CA, EUA) e exibidas em um monitor de tela plana de 15 polegadas S-VGA (1280 x 960 pixels de resolução). A densidade mineral óssea foi avaliada utilizando análise de histograma dos tons de cinza em uma área de 4 x 4 pixels ($1mm^2$) para as regiões de interesse (RI), ou seja, osso cortical e osso medular de ambos os lados do implante. Como pontos de referência, o ponto médio da plataforma do implante e o topo da segunda rosca do implante foram utilizados. Um zoom de 300% foi usado para facilitar o desenho da área. Os cálculos da densidade óssea foram realizadas pelo primeiro ponto a obter os tons de cinza da RI e depois dividindo-os pelos tons de cinza de uma escala de alumínio, usado como um RI de referência. O RI de referência

também foi medido em uma área de 4 x 4 pixels, colocado sobre uma etapa da divisão correspondente a uma profundidade de 10 mm de alumínio, a fim de compensar as diferenças entre as exposições (Figura 6).

Figura 6 : Modelo para análise radiográfica. A) escala de alumínio; B) Referência do osso cortical; C) Referência do osso medular.



Análise Biomecânica

Após o sacrifício, as tíbias foram removidas e estabilizadas em uma pequena morsa. Uma chave hexagonal de 1.17mm (Conexão®, São Paulo, SP, Brasil) foi conectada tanto no implante como no torquímetro (Tohnich, Tóquio, Japão). Realizou-se um movimento anti-horário para que a força necessária para o rompimento da interface osso/implante fosse obtida e registrada. Depois de gravar os valores de torque, os implantes foram completamente desaparafusados e as tíbias separadas para análise histológica. As avaliações desses valores foram realizadas por um examinador treinado e cego para os grupos experimentais.

Análise histomorfométrica

As tíbias que não passaram pela análise biomecânica foram utilizadas para análise histomorfométrica. As peças foram imersas em paraformaldeido 4% por 48

horas e foram posteriormente foram desidratadas em uma série crescente de etanóis (60 – 100%) e infiltradas e polimerizados em resina fotopolimerizável (Technovit 7200 VLC, Kultzer Heraus GmbH & CO, Wehrheim, Alemanha). Os blocos contendo o implante e o tecido ósseo foram cortados em um ponto central usando um sistema de corte e desgaste (Exakt Apparatebeau, Hamburgo, Alemanha). As seções finais que compuseram as lâminas apresentaram aproximadamente 45 μm de espessura e foram corados com azul de Stevenel associado à fucsina ácida e analisadas em um microscópio óptico (DIASTAR – Leica Reichert & Jung products, Wetzlar, Alemanha) com o aumento de 100X. As avaliações histomorfométricas foram realizadas com o software para análise de imagem (Image J, San Rafael, CA, EUA). As porcentagens de contato osso-implante (%BIC) e de área óssea entre espiras (%BBT) foram avaliadas separadamente para a região cortical e medular. Essa análise foi realizada por um examinador cego, treinado e calibrado.

Análise histológica descritiva

As tíbias que tiveram os implantes removidos foram fixadas em paraformaldeido 4 % (48 horas), lavadas em água corrente por 12 horas e colocadas em solução de EDTA para descalcificação por um período de 8 semanas. Posteriormente as amostras foram lavadas e desidratadas em álcool, diafanizadas em xilol e incluídas em parafina. As secções foram realizadas paralelas ao longo eixo do sítio ao qual o implante estava inserido. Os cortes com espessura de 4μm foram corados com hematoxilina-eosina (HE).

Foi avaliado na descrição histológica o aspecto do tecido ósseo, com destaque para o processo de remodelação e maturação óssea. As avaliações foram realizadas por

um examinador treinado e cego para os grupos experimentais com auxílio de um microscópio óptico DIASTAR (Leica Reichert & Jung products, Wetzlar, Alemanha) no aumento de 100X e 200X.

Análise imuno-histoquimica

Essa análise foi executada com o intuito de identificar e localizar a expressão dos fatores de crescimento fator transformante de crescimento β1 (TGFβ1), proteína morfogênica óssea 2 (BMP2) e da proteína osteocalcina(OCN). Cortes das amostras embebidos em parafina com 5 μm de espessura foram montados em lâminas silanizadas (Fisher Superfrost plus- Fisher Scientific, New York, NY, EUA), seguido de tramitação laboratorial de rotina para deparafinização e rehidratação. Em seguida, as amostras foram submetidas a recuperação antigênica realizada através da aplicação de tripsina pancreática de origem suína (R&D Systems, Minneapolis, MN, EUA) a 0.05% em tampão fosfato(PBS) a 37ºC por 20 minutos em estufa e resfriadas à temperatura ambiente. Em seguida os cortes foram submetidos ao bloqueio dos epítopos inespecíficos através da aplicação de peróxido de hidrogênio 3% em metanol por 30 minutos e da proteína bovina albumina (BSA) a 3% em PBS também por 30 minutos. Logo após os cortes foram incubados por 16 horas nos anticorpos primários nas seguintes concentrações: TGFβ1:100; BMP2-1:100; OCN-1:200 (Abcam, Inc. Cambridge, MA, EUA). Como controle negativo, cortes histológicos foram tratados por IgG normal (R&D Systems, Minneapolis, MN, EUA), em substituição aos anticorpos primários, nas mesmas diluições destes. Posteriormente os cortes foram tratados pelo método do complexo avidina-biotina-peroxidase (ABC kit DAKO A/S, Copenhagen, Dinamarca) com a utilização do kit ABC Staining System (ABC kit DAKO A/S, Copenhagen, Dinamarca) seguindo as instruções do fabricante. Os cortes foram contra-

corados com solução de hematoxilina. A análise das imagens obtidas foi executada por um avaliador treinado, calibrado e cego para os tratamentos de acordo com a intensidade da marcação dos anticorpos na matriz através dos seguintes scores: (-)Negativo; positivo(+), superpositivo(++), e hiperpositivo(+++)(Queiroz et al.,2008). O osso que estava inserido entre a primeira e a segunda rosca foi avaliado em ambos os lados e uma média entre essas avaliações foi considerada como o valor de cada animal.

Análise estatística

O software Graphpad Prism 5 (San Diego, CA, EUA) foi utilizado para execução da análise estatística. Para o cálculo do tamanho da amostra desse estudo foram utilizados os dados de %BIC do estudo de Du et al., 2009 que avaliou esse parâmetro de forma semelhante a que fizemos nesse estudo. Foi verificado que a diferença mínima entre as médias da preenchimento ósseo na região da furca de 15.94% com desvio padrão aproximado de 11.13. Portanto, ao se aplicar o teste ANOVA levando-se em consideração um poder β do estudo de 0.75 e o poder α de 0.05, determinou-se uma amostra mínima de 10 animais por grupo.

Para avaliação de reprodutibilidade dos dados radiográficos, histomorfométricos e da imuno-histoquimica das 3 proteínas avaliadas, 10 amostras de cada estudo foram reexaminadas com intervalo de 2 semanas. Ao se aplicar o teste t-pareado e o teste de Wilcoxon foi verificado que não houve diferenças estatisticamente significativas entre os resultados as avaliações ($p<0.05$). Ao se aplicar a correlação linear de Pearson foi verificado um índice de correlação intra-examinador de $r=0.86$ para a análise radiográfica e de $r=0.88$ para a análise histomorfométrica. Ao se aplicar a correlação linear de Spearman foi verificado um índice de correlação intra-examinador de $r>0.81$ para as análises imuno-histoquimicas de TGFβ1, BMP2 e OCN.

Todos os dados desse estudo, com exceção da análise imuno-histoquimica, foram submetidos ao teste de normalidade de Shapiro-Wilk para verificar se os dados se distribuíram de acordo com o teorema da distribuição central. Todos os se distribuíram de acordo com a normalidade ($p>0.05$), e dessa forma, testes paramétricos foram utilizados para a análise inferencial dos dados, enquanto que a análise dos dados da análise imuno-histoquímica foi realizada através de testes não paramétricos. Para a análise dos dados da análise radiográfica, biomecânica e histomorfométrica foi utilizado o teste de ANOVA complementado pelo teste de Tukey. Para a análise imunohistoquímica foram utilizados os testes não-paramétricos de Kruskall-Wallis complementado pelo teste de Dunn. Todos os testes desse estudo foram aplicados com nível de confiança de 95%.

ESTUDO IV: Efeito do extrato de óleo insaponificável de abacate e soja sobre o reparo de defeitos críticos em calotas de ratos associado a inserção de biomateriais.

Distribuição dos animais e grupos

Neste estudo foram utilizados 84 ratos (*Rattus norvegicus,* variação *Holtzman*), adultos do gênero masculino, com aproximadamente 3 meses de idade e massa corporal entre 300-350g. Esses animais foram mantidos no Biotério da Faculdade de Odontologia da UNESP de Araraquara (FOAr-UNESP), com acesso a ração específica a água ad libitum durante todo o período experimental, em ambiente luz (ciclos de 12 h), temperatura(21±1ºC) e humidade (65-70%) controladas.

Os animais foram selecionados aleatoriamente e divididos em 2 grupos com 3 subgrupos cada, que foram avaliados em dois períodos experimentais (15 e 60 dias), com 7 animais em cada subgrupo. Os grupos foram divididos de acordo com o medicamento que foi aplicado nos animais: Grupo controle (CTR):Os animais foram

submetidos a administração diária de solução salina; Grupo Teste (ASU): Os animais foram submetidos a administração de ASU aplicado 0.6 mg/kg/dia (Yaman et al., 2007). Ambos os medicamentos foram aplicados por gavagem diariamente com início 15 dias antes da cirurgia para a confecção dos defeitos até o dia do sacrifício dos animais. Os subgrupos foram divididos de acordo com o tipo de biomaterial que foi aplicado no defeito crítico na calvaria DCC executados nos mesmos: Subgrupo COA: Defeito preenchido por coágulo sanguíneo; Subgrupo OBD: Defeito preenchido por osso bovino desproteinizado (Bio-Oss®, Geistlich AG, Wolhusen, Suiça); Subgrupo TCP/HA: Defeito preenchido por biomaterial a base de β-fosfato tricálcio e hidroxiapatita (Straumann® Bone Ceramic , Straumann AG, Basel, Suiça).

Procedimento Cirúrgico

Os animais foram anestesiados por uma combinação de Quetamina (0,08 ml/100g- Cloridrato de Quetamina – Francotar – Virbac do Brasil Ind. Com. Ltda, São Paulo, SP, Brasil) com Xilazina (0,04 ml/100g-Cloridrato de Xilazina - Virbaxyl 2% - Virbac do Brasil Ind. E Com. Ltda, São Paulo, SP, Brasil). Posteriormente os animais foram submetidos à tricotomia na região da calvária e foi realizada antissepsia do campo cirúrgico com gaze estéril, embebida em solução de povidine, com o animal sendo então posicionado em decúbito ventral sobre a mesa cirúrgica.

Foi executado um acesso cirúrgico à porção antero-lateral da calvária aonde foi executado uma incisão bicoronal cutânea e muscular, com aproximadamente 3cm na porção anterior e 2 cm na porção lateral. Em seguida, os tecidos foram divulsionados com o auxílio de uma tesoura pequena com a extremidade romba e uma pinça de dissecção, até a exposição do periósteo, sendo este incisado e descolado para expor o tecido ósseo. Foram então confeccionados defeitos ósseos críticos circulares (DCC), na

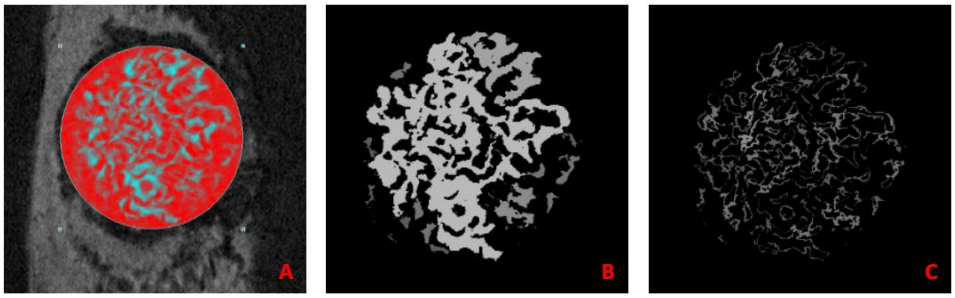
porção no osso parietal dos animais, imediatamente após o vértice da sutura coronal. O DCC apresentou 5 mm de diâmetro e aproximadamente 1,5 mm de espessura dada pela remoção total do tecido ósseo que foi executado por meio de uma fresa trefina (3i – 3i implantes, Brasil), montada em contra-ângulo (Anthogyr – Injecta – Diadema,SP, Brasil) com redução de 16:1 acoplado em motor para implante (BML 600 Plus, CK Driller, São Paulo, SP, Brasil), com 1500 rpm, sob irrigação constante com solução salina. Todos os cuidados foram mantidos para evitar qualquer lesão à dura máter. Cada biomaterial foi implantado e acomodado no defeito ósseo sobre a dura máter com auxílio de uma espátula nº 7 preenchendo completamente o defeito, sem extravasamento. Em seguida, todos os defeitos foram suturados em planos com fio de sutura Vycril 4.0 (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brasil) para sutura interna e fio de seda 4.00 (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brasil) para sutura externa. Os animais foram medicados com uma dose única intramuscular de uma combinação de Penicilina com Estreptomicina (0.1 ml/kg) (Multibiótico Small, Vitalfarma, São Sebastião do Paraíso, MG, Brazil) para controle de infecções e de dipirona sódica por gavagem durante 3 dias (0.1 ml/kg) (Dipirona Ibasa 50% - Ibasa, Porto Alegre, RS, Brazil).

Avaliação por Micro-CT

Cinco animais de cada subgrupo foram posicionados em posição de decúbito dorsal e tiveram suas calvárias *scaneadas* através de um microtomógrafo (Skyscan, Aartselaar, Bélgica). As imagens geradas foram posteriormente reconstruídas, reorientadas espacialmente e analisadas por softwares específicos (NRecon/DataViewer/CTan, Skyscan, Aartselaar, Bélgica). A análise do micro-CT foi

realizada através do programa CTAN aonde foram selecionados 40 cortes que englobavam o defeito criado pela norma axial do crânio (espessura de corte=35µm; 40 cortes aprox.=1.5mm). A região de interesse selecionada teve formato circular e foi a mesma em todos os animais (5x5mm). Os resultados foram expressos em relação a porcentagem de preenchimento do defeito de calota e nos grupos com biomateriais foi executado uma avaliação separada da porcentagem de biomaterial e de tecido ósseo que estavam preenchendo o defeito da calota (Figura 7), sendo que foi utilizada uma escala de tons de cinza (*threshold*) de 55-250 tons de cinza para avaliação do volume do osso somado ao biomaterial e um *threshold* de 55-90 para avaliação do volume ósseo. As análises foram executadas por um examinador cego, treinado e calibrado.

Figura 7: Análise de Micro Ct. A)Delimitação do ROI; B)Tecidos mineralizados que preencheram o defeito (*threshold* 55-250); C)Tecido ósseo (threshold 55-90).



Obtenção das peças Cirúrgicas, análise histométrica e descrição histológica

Após a realização das microtomografias, os 7 animais de cada subgrupo foram sacrificados por sobredosagem anestésica. Logo após, foi realizada uma incisão bicoronal no animal, removendo toda a porção superior da calvária em bloco. As peças foram fixadas em paraformaldeido 4% por 48 horas e foram posteriormente descalcificadas em EDTA 7% por 90 dias sendo subsequentemente submetidas para

processamento laboratorial, realização de cortes histológicos e coloração específica (HE, Tricrômio de Mason, Tricrômio de Goldner).

De cada peça foram executados cortes que iniciaram-se da margem do defeito até a região mediana do mesmo. Foram removidas 20 lâminas com 4 cortes cada, sendo que para cada corte captado 5 eram excluídos, o que forneceu uma distância de 25μm entre os cortes. A área linear de secção de cada amostra foi de 2500μm^2 a partir da identificação do defeito. De cada amostra, foi sorteado um número de 1 a 6 para determinar qual seria a primeira lamina corada. A partir do número selecionado fez-se a coloração semi-seriada das laminas, nas quais 3 laminas eram coradas e as 3 subsequentes não eram coradas de tal forma que 9 laminas foram coradas de cada amostra. O terceiro corte da primeira e da terceira lâmina foram selecionados para a análise dando um total de 6 cortes analisados por amostra.

As imagens das amostras foram captadas por meio do microscópio óptico DIASTAR (Leica Reichert & Jung products, Wetzlar, Alemanha), em um aumento de 25X para a análise histomorfométrica e de 100X para a análise de descrição histológica. Posteriormente as imagens foram enviadas para um microcomputador. Como não foi possível incluir todo o defeito em uma imagem, foram executadas entre 2-4 imagens de cada amostra que foram posteriormente agrupadas através do software Adobe Photoshop CS5.1(Adobe Systems Ltda, San Jose, CA, EUA). A determinação dos valores de porcentagem de preenchimento do defeito crítico com osso, tecido conjuntivo ou biomaterial foi executada empregando-se um software analisador de imagens (Image J, San Rafael, CA, EUA). Essa análise foi executada por um examinador cego, treinado e calibrado. Adicionalmente foi executada uma descrição histológica de cada grupo de acordo com a característica dos tecidos neoformados, presença de células inflamatórias e relação entre as partículas dos biomateriais com o osso neoformado. Essas análises

foram executadas em duas lâminas próximas da região central do defeito coradas com HE e Tricrômio de Mason em 5 animais por grupo. Essa descrição foi executada por um examinador cego, treinado e calibrado.

Análise Estatística

O software GraphPad Prim 5.0(San Diego, CA, EUA) foi utilizado para execução das análises estatísticas desse estudo. Para o cálculo do tamanho da amostra foi utilizado um estudo que avaliou o efeito de diferentes biomateriais sobre o reparo de DCC de ratos e que utilizou metodologia semelhante para análise histométrica (Park et. 2009). Foi verificado que a diferença mínima entre as médias da formação óssea dentro do DCC de 7.3% com desvio padrão de 3.75. Portanto, ao se aplicar o teste ANOVA levando-se em consideração um poder β do estudo de 0.8 e o poder α de 0.05, determinou-se uma amostra mínima de 7 animais por subgrupo para avaliação dos diferentes tratamentos.

Para avaliação da calibração dos examinadores, 10 amostras foram reexaminadas com intervalo de 1 semana tanto para a análise microtomográfica como para a análise histomorfométrica. Ao se aplicar o teste t-pareado foi verificado que não houve diferenças estatisticamente significativas entre os resultados as avaliações ($p<0.05$). Ao se aplicar a correlação linear de Pearson foi verificado um índice de concordância intra-examinador de $r=0.99$ para análise microtomográfica e de $r=0.82$ para a análise histomorfométrica.

Os dados gerados pelas análises histométricas e tomográficas foram numéricos e dessa forma o teste de normalidade de Shapiro-Wilk foi aplicado para verificar se os dados se distribuíram de acordo com o teorema da distribuição central. Foi verificado que os dados se distribuíram de acordo com a normalidade($p>0.05$), e dessa forma,

testes paramétricos foram utilizados para a análise inferencial dos dados. O teste de ANOVA complementado pelo teste de Tukey foi utilizado para a análise intergrupos dentro de cada período de avaliação. O teste t-não pareado foi utilizado para avaliação intragrupos para verificar o efeito do tempo. Todos os testes desse estudo foram aplicados com nível de significância de 95% ($p<0.05$).

Anexo 2

ANEXO 2 Carta de Aceite Estudo III

manuscript 3498 - Decision - The International Journal of Oral & Maxillofacial Implants

Manuscript title: Effect of Avocado / Soy Unsaponifiables (ASU) on

osseointegration: A proof-of-principle preclinical in vivo study

Dear Dr Oliveira

It is a pleasure to inform you that the above article is now acceptable for publication.

The authors will receive a revised manuscript in the galley proof stage. They are asked to carefully evaluate any changes that were made to ensure that the meaning of their article has not been changed. The galley proofs will be provided to the authors electronically shortly before the time that the manuscript goes to final press.

We thank you very much for submitting this valuable information and hope that you will continue to consider The International Journal of Oral & Maxillofacial Implants the primary journal of publication for your most interesting and important studies.

Sincerely,

The Editorial Staff

The International Journal of Oral & Maxillofacial Implants

Anexo 3

ANEXO 3 CEUA Estudos I, II e III

UNIVERSIDADE ESTADUAL PAULISTA
Faculdade de Odontologia
Câmpus de Araraquara
Comissão de Ética no Uso de Animais - CEUA

C E R T I F I C A D O

Certificamos que o protocolo nº **17/2009** referente à pesquisa "**Utilização do extrato de óleo insaponificável de abacate e soja na doença periodontal induzida e na osseointegração. Avaliação histológica, radiográfica e imuno-inflamatória em ratos**" sob a responsabilidade da **Profa. Dra. Rosemary Adriana Chiérici Marcantonio**, **Prof. Dr. Elcio Marcantonio Junior**, **Prof. Dr. Luiz Carlos Spolidorio**, **Guilherme José Pimentel Lopes de Oliveira**, **Luiz Guilherme Freitas de Paula** e **Dr. Rubens Spin Neto** está de acordo com os Princípios Éticos em Experimentação Animal adotado pela legislação brasileira atualmente em vigor, tendo sido aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Faculdade de Odontologia de Araraquara-UNESP.

C E R T I F I C A T E

We certify that the protocolo **17/2009** referring to the research "**Influence of avocado and soybean unsaponifiables in the induced periodontal disease and bone around integrated dental implants. Histological, radiographic and immuno-inflammatory analysis in rats**" under responsibility **Profa. Dra. Rosemary Adriana Chiérici Marcantonio**, **Prof. Dr. Elcio Marcantonio Junior**, **Prof. Dr. Luiz Carlos Spolidorio**, **Guilherme José Pimentel Lopes de Oliveira**, **Luiz Guilherme Freitas de Paula** and **Dr. Rubens Spin Neto** in agreement with the nowadays specific brazilian laws and was approved by the Araraquara Dental School-UNESP Ethical Committee for Animal Research (CEUA).

Araraquara, 03 de Julho de 2013.

Prof. Dr. PAULO SÉRGIO CERRI
Coordenador da CEUA/FOAr/UNESP

Anexo 4

Anexo 4 CEUA Estudo IV

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara

FACULDADE DE ODONTOLOGIA

Proc. CEUA nº 01/2012

Araraquara, 13 de Abril de 2012

Senhores Pesquisadores:

A Comissão de Ética no Uso de Animal - CEUA desta Faculdade reunida em 03/04/2012, após a avaliação do projeto de sua responsabilidade intitulado "Efeitos de diferentes medicamentos sobre a integração de biomateriais em defeitos críticos de calota em ratos" (Proc. CEUA nº 01/2012) AUTORIZA a realização da pesquisa, ficando a apresentação do RELATÓRIO FINAL para JULHO/2014.

Atenciosamente.

Prof. Dr. PAULO SÉRGIO CERRI
Coordenador da CEUA

À
Profª Drª ROSEMARY ADRIANA CHIÉRICI MARCANTONIO
DD. Pesquisadora Responsável
a/c Guilherme José Pimentel Lopes de Oliveira
Departamento de Diagnóstico e Cirurgia

Comitê de Ética em Experimentação Animal - CEEA
Rua Humaitá nº 1.680 – Centro – CEP 14801-903 – Caixa Postal nº 331 - ARARAQUARA – SP
5º andar – fone (16) 3301-6431/6432 / fax (16) 3301-6433 / e-mail: diretor@foar.unesp.br - home page: http://www.foar.unesp.br

Não Autorizo a publicação total dessa tese até o dia 12 de Março de 2017

Guilherme José Pimentel Lopes de Oliveira

Araraquara, 12 de Março de 2014