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NARA LHAYS TEIXEIRA NUNES

EFEITOS DA ADMINISTRAÇÃO LOCAL DO ÁCIDO TILUDRÔNICO NA PERIODONTITE EXPERIMENTAL EM RATOS DIABÉTICOS

FORTALEZA

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Dissertação de Mestrado apresentada à coordenação do Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Mestre em Odontologia

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Profª. Drª. Flávia Aparecida Chaves Furlaneto
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Profª. Drª. Daniela Bazan Palioto
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RESUMO

O bisfosfonato ácido tiludrônico (TIL) apresenta propriedades antirreabsortivas e anti-inflamatórias e ainda não foi estudado na associação periodontite-*diabetes mellitus* (DM). O objetivo deste estudo foi avaliar os efeitos da administração local do TIL na periodontite experimental (PE) em ratos com DM induzido por streptozotocina (STZ). No 1º dia, trinta e dois ratos receberam injeção de STZ. Os animais foram divididos nos grupos (n = 8): DM/C (Controle), DM/PE, DM/PE/TIL1 e DM/PE/TIL3. Nos grupos PE, uma ligadura foi colocada na área cervical dos primeiros molares inferiores no 8º dia. Nos grupos DM/PE/TIL1 e DM/PE/TIL3, soluções de TIL (1 e 3 mg/kg de peso corporal, respectivamente) foram injetadas na margem gengival vestibular dos primeiros molares inferiores em dias alternados. Os animais foram submetidos à eutanásia no 18° dia. Análises histomorfométricas foram realizadas. Os dados foram estatisticamente analisados ($p<0,05$). O grupo DM/PE/TIL3 apresentou perda óssea alveolar e perda de inserção reduzidas quando comparado com o grupo DM/PE ($p<0,05$). Dentro dos limites deste estudo, pode-se concluir que i) a administração local de soluções de TIL apresentou um efeito protetor na destruição tecidual na PE em ratos diabéticos e ii) a dosagem de TIL pode influenciar seus efeitos.

Palavras-chave: Ácido Tiludrônico; Bisfosfonatos; Periodontite; Diabetes Mellitus; Reabsorção Óssea.

ABSTRACT

The bisphosphonate tiludronic acid (TIL) presents anti-resorptive and anti-inflammatory properties and it has not been evaluated in the association periodontitis-*diabetes mellitus* (DM) to date. The purpose of this study was to evaluate the effects of local administration of TIL on experimental periodontitis (EP) in rats with streptozotocin (STZ)-induced DM. On day 1, thirty two rats received STZ injection. The animals were divided into groups (n=8): DM/C (Control), DM/EP, DM/EP/TIL1 and DM/EP/TIL3. In groups EP, a ligature was placed around the cervical area of mandibular first molars at day 8. In groups DM/EP/TIL1 and DM/EP/TIL3, TIL solutions of 1 and 3 mg/kg body weight, respectively, were injected into the buccal gingival margin of mandibular first molars every other day. Animals were euthanized at day 18. Histomorphometric analyses were performed. Data were statistically analyzed ($p<0.05$). Group DM/EP/TIL3 presented reduced alveolar bone loss and attachment loss when compared with group DM/EP ($p<0.05$). Within the limits of this study, it can be concluded that i) the local administration of TIL solutions presented a protective effect on tissue destruction in EP in diabetic rats and ii) the dosage of TIL may influence its effects.

Key Words: Tiludronic Acid; Bisphosphonates; Periodontitis; Diabetes Mellitus; Bone Resorption.

SUMÁRIO

1. INTRODUÇÃO GERAL

A periodontite é uma doença multifatorial que envolve biofilmes bacterianos e a geração de respostas inflamatórias.[1,2] Ela é caracterizada principalmente pela reabsorção óssea alveolar, perda de inserção e formação de bolsas periodontais.[3] Em um recente levantamento epidemiológico realizado nos Estados Unidos, foi demonstrado que um em cada dois americanos com 30 anos de idade ou mais possui periodontite.[4] Neste estudo, 47% da amostra examinada, representando 64,7 milhões de adultos, apresentavam periodontite nas formas leve (8,7%), moderada (30%) e severa (8,5%). Para adultos com 65 anos de idade ou mais, o percentual de ocorrência de periodontite moderada ou severa foi de 64%.[4] Embora o biofilme dentário periodontopatogênico seja o fator etiológico primário da doença periodontal, existem evidências de que a resposta do hospedeiro e outras condições, incluindo o fumo e o *diabetes mellitus* (DM), estão associadas com a progressão e severidade da periodontite.[5-9]

O DM é um grupo de desordens metabólicas caracterizado pela hiperglicemia resultante de defeitos na secreção e/ou na ação da insulina.[10] A hiperglicemia crônica do DM está associada a disfunções em diferentes órgãos a longo prazo, especialmente olhos, rins, nervos, coração e vasos sanguíneos.[10] Segundo a Associação Americana de Diabetes,[10] o DM pode ser classificado em quatro tipos clínicos: (1) diabetes tipo 1, o qual resulta da destruição das células β do pâncreas, levando à deficiência absoluta de insulina; (2) diabetes tipo 2, que é o resultado de um defeito progressivo na secreção da insulina; (3) diabetes associado a defeitos genéticos na função das células β ou na ação da insulina, à insuficiência pancreática exócrina (fibrose cística) ou induzido por drogas ou outros agentes químicos e (4) diabetes gestacional, desenvolvido durante a gravidez.

A prevalência do DM vem crescendo rapidamente em todo o mundo, sendo uma das principais causas de morbidade e mortalidade.[11,12] Estima-se que a prevalência do DM alcance cerca de 440 milhões de ocorrências em todo o mundo em 2030,[11] sendo esperado um aumento de 69% no número de adultos com DM em países em desenvolvimento e de 20% em países desenvolvidos. Segundo a Federação Internacional de Diabetes (IDF),[13] há 13,4 milhões de diabéticos no Brasil (tipos 1 e 2). O país ocupa o quarto lugar no mundo em número de casos, ficando atrás apenas da China, Índia e Estados Unidos. Trata-se de um número alarmante, já que a estimativa era de que teríamos cerca de 12,7 milhões de brasileiros diabéticos somente em 2030.[13]

O DM é considerado um fator de risco para a periodontite, sendo esta considerada a sexta complicação mais comum do DM.[14-16] A presença de DM está intimamente associada ao desenvolvimento, progressão e severidade da doença periodontal[17-19] e vários mecanismos tem sido estudados para compreender melhor essa associação.[20,21] A hiperglicemia pode acelerar a destruição periodontal, inibindo a função de leucócitos polimorfonucleares, alterando o metabolismo do colágeno e a permeabilidade vascular, reduzindo a viabilidade e a diferenciação celular nos tecidos periodontais e modificando a composição da microbiota bucal.[22-24] Como consequência do estado hiperglicêmico, a glicação não enzimática e a oxidação de lipídeos induzem à formação e ao acúmulo de produtos finais da glicação avançada (*advanced glycation end products* – AGEs) no plasma e tecidos.[25] AGEs podem modificar a ligação cruzada de moléculas da matriz, prejudicar a eficiência de fatores de crescimento, elevar o estresse oxidativo tecidual e intensificar a inflamação por meio de interação com receptores celulares para AGEs.[25,26] O DM pode também afetar a microarquitetura do osso.[27] A condição resultante, conhecida como osteopatia diabética, é caracterizada pelo desequilíbrio entre a síntese de matriz e formação de cristais de hidroxiapatita devido à diminuição no número e atividade de osteoblastos.[27,28] Na presença de DM, os tecidos periodontais são caracterizados pela microangiopatia, hiperplasia epitelial e acentuada inflamação.[27,29-31] Progressiva e prolongada inflamação gengival, com uma desorganização mais evidente da matriz colágena, podem ser observadas em animais com DM na presença de fatores retentivos de placa.[32]

Como indivíduos com DM e pobre controle metabólico são mais susceptíveis à periodontite, apresentando cicatrização tecidual e resposta imunoinflamatória comprometidas, o uso de terapias adjuvantes (por exemplo, antimicrobianos e moduladores da resposta do hospedeiro) pode atender às necessidades terapêuticas específicas desse grupo de pacientes.[7] Três categorias principais de modulação da resposta do hospedeiro vêm sendo estudadas na terapia periodontal: antiproteinases (representadas pelas tetraciclinas), fármacos anti-inflamatórios não esteroidais (AINEs) e fármacos que inibem a reabsorção óssea, representados por agentes antirreabsortivos, como os bisfosfonatos (BFs).[17,33]

Com o objetivo de modular a resposta do hospedeiro, doses subantimicrobianas de doxiciclina (DSD), associadas à raspagem e alisamento radicular (RAR), foram administradas em pacientes com DM e periodontite. Esse tratamento levou a melhores resultados na redução da profundidade de sondagem[34,35] e no ganho de inserção clínica[34] do que o uso de placebo associado à RAR. Foi demonstrado também que a utilização de DSD e RAR em pacientes com DM e periodontite reduziu significativamente os níveis de hemoglobina glicosilada

(HbA1c) após 3 meses, o que não ocorreu com a administração da dose antimicrobiana de doxiciclina associada à RAR.[36] Além disso, Ozdemir et al.[17] observaram que a administração local de DSD, associada ou não ao bisfosfonato (BF) clodronato, promoveu a redução da expressão de metaloproteinase de matriz (MMP)-9 e interleucina (IL)-1β em ratos com DM e periodontite experimental. Outro agente modulador em potencial para o tratamento da periodontite em pacientes diabéticos é a vitamina D3, um hormônio esteroide modulador da resposta imunológica e inflamatória.[37,38] A vitamina D3 pode influenciar o desenvolvimento de doenças inflamatórias crônicas, inibindo a via fator de transcrição nuclear kappa B[39] (NF-kB) e é capaz de inibir a expressão de fator de necrose tumoral (TNF)-α de monócitos em pacientes diabéticos.[40] Demonstrou-se também que, quando a principal forma circulante da vitamina D3 no organismo, a 25-hidroxi-vitamina D_3 ($25(OH)D_3$), foi administrada em camundongos diabéticos com periodontite experimental, houve redução nos níveis de TNF-α e diminuição da perda óssea alveolar.[41]

Os BFs são fármacos sintéticos químicos muito eficazes no tratamento de algumas patologias ósseas, como osteoporose, doença de Paget, mieloma múltiplo, hipercalcemia de malignidade e metástases ósseas, diminuindo o risco de fraturas.[42,43] Existem três gerações de BFs conhecidas, sendo que a potência dos fármacos aumenta da primeira à terceira geração.[33] A primeira geração possui cadeias laterais alquila (por exemplo, o ácido tiludrônico), a segunda geração inclui os aminobisfosfonatos com uma cadeia lateral amino-terminal (como exemplo, o alendronato) e a terceira geração possui uma cadeia lateral cíclica (por exemplo, o zoledronato).[44]

A eficácia comprovada dos BFs em inibir a reabsorção óssea osteoclástica[45] levou à utilização dos mesmos no tratamento da periodontite, agindo como um fator modulador da resposta do hospedeiro.[46,47] Alguns BFs também apresentam atividade antibacteriana[48,49] e efeito anti-inflamatório.[48,50-52] Em virtude dessas ações, os BFs parecem ser uma alternativa promissora no tratamento periodontal e tem sido avaliados em estudos em animais e em seres humanos.[17,44,47] Os BFs foram avaliados quando administrados localmente[47,53-57] e sistemicamente.[17,48,52,58-61] em vários estudos. Parecem existir diferenças quanto à ação dos BFs na periodontite dependendo do tipo BF usado, de acordo com suas cadeias laterais,[62] e também dependendo da dosagem do BF[53] e do tempo de duração da terapia.[61] Embora seja difícil comparar dados de diferentes publicações porque diversos tipos e doses de BFs foram utilizados, os estudos mostraram um benefício óbvio dos BFs na periodontite, resultando em reabsorção óssea alveolar reduzida[58,59,63] juntamente com benefícios clínicos e/ou histológicos na resolução da inflamação em tecidos periodontais.[48,50,51,64-66]

Especificamente em relação aos estudos clínicos em humanos, benefícios adicionais foram demonstrados quando os BFs foram associados ao debridamento mecânico, em comparação com o debridamento mecânico isoladamente.[46,54,56,64,66-68] Esses benefícios caracterizam-se principalmente pela redução da perda óssea alveolar, aumento da densidade mineral óssea e redução de profundidades de sondagem.[46,47,54,57,64,66,68]

O ácido tiludrônico (4-clorofenil tiometileno-1,1-bisfosfonato), um BF não-nitrogenado de 1ª geração, foi caracterizado por exercer atividade inibitória dose-dependente na reabsorção óssea em diversos estudos pré-clínicos *in vivo,* incluindo modelos de ratos tiroparatireoidectomizados,[69] neurectomizados[70] e ratas ovariectomizadas.[69] Estudos *in vitro* demonstraram que esse BF também possui ação anti-inflamatória, podendo inibir a liberação de IL-6 por osteoblastos[71] e a secreção de IL-1ß, IL-6, óxido nítrico (NO) e TNF-α por macrófagos ativados, de maneira dose-dependente.[72] Também foi demonstrada a ação inibitória do ácido tiludrônico (TIL) sobre enzimas importantes no processo de degradação de componentes da matriz extracelular na periodontite, a MMP-1 e a MMP-3, em cultura de células de ligamento periodontal humano.[73] Além disso, foi sugerido que o TIL suprime a síntese de fator de crescimento endotelial vascular (VEGF), fator que possui um papel importante na mediação da vasculopatia diabética e nos níveis de glicose,[74,75] a partir de osteoblastos.[76] Por não conter nitrogênio em sua formulação, o TIL não apresenta os efeitos adversos comumente associados ao uso de BFs nitrogenados, como lesões oculares,[77] irritação gastrointestinal, desenvolvimento da resposta de fase aguda[78] e osteonecrose dos maxilares.[79-83] O TIL é um composto seguro, com margens terapêuticas apreciáveis.[84] Recentemente, constatamos que a aplicação local de TIL no tecido gengival levou à diminuição da expressão de osteoclastos e da perda óssea alveolar na periodontite experimental em ratos normosistêmicos.[85] Observamos também que a terapia levou à diminuição da expressão gênica de alguns mediadores inflamatórios, como TNF-α, IL-1β, MMP-8 e ciclooxigenase (COX)-2 (dados não publicados).

O BF alendronato foi avaliado em estudos clínicos envolvendo pacientes com periodontite e DM tipo 2.[64,86] Rocha et al.[64] demonstraram que a administração sistêmica do aledronato levou a melhoras nos parâmentros clínicos periodontais e na reabsorção óssea alveolar superiores àquelas observadas em pacientes que ingeriram placebo. Constatou-se também que a aplicação local desse BF em defeitos infraósseos periodontais resultou em ganho de inserção clínica, redução da profundidade de sondagem e preenchimento ósseo superiores aos defeitos tratados com placebo.[86]

Em suma, a periodontite é considerada um problema de saúde pública em todo o mundo e as atuais abordagens preventivas/terapêuticas não tem sido totalmente efetivas para uma parcela da população que apresenta alto risco para doença e suas formas mais severas, sobretudo pacientes diabéticos. Dessa forma, é relevante o desenvolvimento de abordagens preventivas e terapêuticas mais efetivas para a periodontite, como as alicerçadas nos conceitos envolvendo a modulação da resposta imunoinflamatória do hospedeiro.[87] Considerando os efeitos antirreabsortivos e anti-inflamatórios do TIL, sua utilização no tratamento da periodontite em pacientes diabéticos parece ser uma estratégia promissora. Não existem estudos analisando os efeitos do BF TIL na associação periodontite-DM.

2. PROPOSIÇÃO

O objetivo deste estudo foi avaliar os efeitos da administração local do bisfosfonato ácido tiludrônico na periodontite experimental em ratos com *diabetes mellitus* induzido por estreptozotocina.

3. DESENVOLVIMENTO

Esta dissertação de Mestrado baseia-se no Artigo 46º do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado. Este capítulo consta de uma cópia do artigo científico de autoria da candidata, redigido de acordo com as normas da revista científica escolhida para publicação ("Journal of Periodontology").

Por se tratar de pesquisa envolvendo animais, o projeto de pesquisa referente a esta dissertação foi submetido à apreciação da Comissão de Ética no Uso de Animais (CEUA) da Faculdade de Odontologia de Ribeirão Preto (FORP) da Universidade de São Paulo (USP), tendo sido aprovado sob número de protocolo 2014.1.442.58.0 (Anexo A).

Artigo Científico:

"Effects of Local Administration of Tiludronic Acid on Experimental Periodontitis in Diabetic Rats."

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ABSTRACT

Background and Purpose: The bisphosphonate tiludronic acid (TIL) presents anti-resorptive and anti-inflammatory properties and it has not been evaluated in the association periodontitis-*diabetes mellitus* (DM) to date. The purpose of this study was to evaluate the effects of local administration of TIL on experimental periodontitis (EP) in rats with streptozotocin (STZ)-induced DM. **Methods:** On day 1, thirty two rats received STZ injection. The animals were divided into groups (n=8): DM/C (Control), DM/EP, DM/EP/TIL1 and DM/EP/TIL3. In groups EP, a ligature was placed around the cervical area of mandibular first molars at day 8. In groups DM/EP/TIL1 and DM/EP/TIL3, TIL solutions of 1 and 3 mg/kg body weight, respectively, were injected into the buccal gingival margin of mandibular first molars every other day. Animals were euthanized at day 18. Histomorphometric analyses were performed. Data were statistically analyzed ($p<0.05$). **Results**: Group DM/EP/TIL3 presented reduced alveolar bone loss and attachment loss when compared with group DM/EP ($p<0.05$). **Conclusions:** Within the limits of this study, it can be concluded that i) the local administration of TIL solutions presented a protective effect on tissue destruction in EP in diabetic rats and ii) the dosage of TIL may influence its effects.

Key Words: Tiludronic Acid; Bisphosphonates; Periodontitis; Diabetes Mellitus; Bone Resorption.

INTRODUCTION

Although the periodontopathogenic biofilm is considered the primary etiologic factor of periodontal diseases, there are evidences that the host response and other conditions, including smoking and *diabetes mellitus* (DM), are associated with the progression and severity of periodontitis.[1-5]

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and/or action.[6] The chronic hyperglycemia of DM is associated with long-term dysfunction of different organs, especially the eyes, kidneys, nerves, heart and blood vessels.[6] DM is a risk factor for periodontitis, which is considered the sixth most common complication of DM.[7-9] Several mechanisms have been reported to explain the greater incidence and severity of periodontal disease in patients with DM.[10,11] Diabetic periodontium is characterized by microangiopathy, increasing inflammation as well as alterations of collagen and bone metabolisms.[12-15] As a result, uncontrolled diabetes in patients with periodontitis leads to more severe bone resorption, attachment loss and impaired bone formation.[10,16-18] It is also important to emphasize that periodontitis adversely affects glycemic control in patients with DM and increases the risk of development of diabetic complications.[19]

Since poorly controlled diabetic patients are more susceptible to periodontitis, with impaired tissue healing and immuno-inflammatory response, the use of adjuvant therapies, such as antimicrobials and host response modulators, may attend to specific therapeutic needs of this group of patients.[3] Bisphosphonates (BPs) are synthetic chemical drugs very efficient in the treatment of some bone diseases.[20,21] The proven efficacy of BPs to inhibit osteoclastic bone resorption[22] has led to their use in the management of periodontitis.[23,24] Tiludronic acid (TIL; chloro-4-phenyl-thiomethylene-1,1-bisphosphonate), a non–nitrogen-containing bisphosphonate (BP) from the first generation, was characterized by dose-dependently inhibiting bone resorption in several in vivo preclinical studies.[25,26] In vitro studies demonstrated that this BP also presents anti-inflammatory actions, as it can dose-dependently inhibit interleukin (IL)-6 synthesis by osteoblasts[27] and the secretion of IL-1ß, IL-6, nitric oxide (NO) and tumor necrosis factor (TNF)-α by activated macrophages.[28] Furthermore, it was suggested that TIL suppresses the synthesis of vascular endothelial growth factor (VEGF), a protein with a major role in microvascular complications of diabetes,[29,30] from osteoblasts.[31] Recently, our group verified that the local administration of TIL in gingival

tissues decreased osteoclasts expression and alveolar bone loss in experimental periodontitis (EP) in non-diabetic rats.[32] We also observed that this therapy reduced the genic expression of some pro-inflammatory mediators, such as TNF-α, IL-1β, matrix metalloproteinases (MMP)-8 and cyclooxygenase (COX)-2 (data not published).

Considering the antiresorptive and anti-inflammatory properties of TIL, it may be a promising therapeutic strategy for periodontitis treatment in diabetic patients. To the best of the authors' knowledge, there are no studies evaluating the effects of the BP TIL in the association periodontitis-DM. The purpose of this study was to analyze the effects of local administration of TIL on EP in rats with streptozotocin (STZ)-induced DM.

METHODS

Sample

This study was conducted in compliance with the ethical principles of animal experimentation, as well as standards for the didactic-scientific practice of vivisection and the Universal Declaration of Animal Rights by the United Nations Educational, Scientific and Cultural Organization. The present study was conducted after review and approval by the Ethics Committee on Animal Experimentation at School of Dentistry of Ribeirao Preto, University of Sao Paulo – FORP/USP (protocol 2014.1.442.58.0).

A power calculation was performed to determine the sample size. The animal was considered the study unit. The sample size was determined to provide 80% power to recognize a significant difference of 20% among groups and the standard deviation of 15% with a 95% confidence interval ($\alpha = 0.05$), considering the change in the alveolar bone in the furcation area (ANBL-area of no bone or periodontal ligament) as the primary outcome variable. Therefore, a sample size of eight animals per group was required.

Experimental model

Thirty-two adult male rats (*Rattus norvegicus, albinus*, Wistar), weighing between 250 and 300 g, were used (Central Animal Facility, FORP/USP). The rats were kept in a 12-hour light/dark cycle and a temperature between 22 and 24°C. The animals were housed in plastic cages and fed with selected solid diet and water *ad libitum*. They were randomly assigned to one of four experimental groups (n=8): DM/C (Control), DM/EP (Experimental Periodontitis), DM/EP/TIL1 (EP + TIL solution at a dosage of 1 mg/kg body weight), DM/EP/TIL3 (EP + TIL solution at a dosage of 3 mg/kg body weight).

Induction of DM and evaluation of fasting plasma glucose (FPG)

On day 1, DM was induced by intraperitoneal injection of STZ[‡] (60 mg/kg body weight) dissolved in 0.2 mL citrate-buffered solution (0.01 M, pH 4.5) after 16 hours fasting.[33] Blood samples were drawn from the retro-orbital venous plexus in anesthetized animals at the day of periodontitis induction (day 8) and euthanasia (day 18) to determine FPG levels. Rats with FPG levels higher than 250 mg/dL[34] on day 8 were considered diabetic and included in the study.

Induction of periodontitis

On day 8, all animals were anesthetized by an intramuscular injection of xylazine[§] (6 mg/kg body weight) and ketamine[‖] (70 mg/kg body weight). A cotton ligature was placed around their right mandibular first molars,[35] except for the rats of group DM/C. In DM/EP/TIL1 and DM/EP/TIL3 groups, 40-µl TIL[¶] solutions (1 and 3 mg/kg body weight, respectively) were injected into the buccal gingival margin adjacent to right mandibular first molars on days 8, 10, 12, 14 and 16. Throughout the experimental period, the animals were weighed every other day, and the doses of TIL were adapted accordingly.

On day 18, the animals were anesthetized as already described and euthanized by carbon dioxide inhalation.[36] The right mandibles were excised, fixed in 4% paraformaldehyde for 24 hours and rinsed with water.

Histopathological and histometric analyses

The specimens were decalcified in 10% EDTA solution. After complete decalcification, they were processed and embedded in paraffin. Serial sections, 5 µm thick, were obtained in a mesio-distal direction. The sections were stained with hematoxylin and eosin. Sections representing the most central buccal-lingual portion in the furcation area of right mandibular first molars were selected for histopathological and histometric analyses. The histopathological analysis was performed by a certified histologist using a light microscope.[#] The parameters analyzed and the scores were based on the study by Lisboa et al.[35] (Table 1).

For histometric analysis, photomicrographs were captured using a digital camera[**] connected to a light microscope[††] with an original magnification of x40. The images were analyzed using appropriated software[‡‡]. The furcation area not filled with bone or periodontal ligament (ANBL) was measured by outlining the region surrounded by the roof of the furcation, the most coronal portion of the ABC in furcation, the mesial and the distal roots of

the first molar. In order to assess the attachment loss (AL), the linear distance between the CEJ and the epithelial attachment was measured on the distal root of the mandibular first molar. Histometric analysis was performed by one blinded and calibrated examiner (N.L.T.N.).

Examiner calibration

To estimate the intra-examiner error, the same sample was measured again one week after the first measurement. Examiner calibrations were assessed by Intraclass Correlation Coefficient (ICC >0.8).

Statistical analysis

Data were grouped and presented as means and standard deviations. Normality and homoscedasticity of the data were verified. The significance of differences among groups were assessed by analysis of variance (ANOVA) followed by *post-hoc* Tukey test. Paired t-tests were used for intra-group comparisons. The significance level was set at 5% in all tests.

RESULTS

All animals tolerated the experimental procedures well. No significant differences in relation to body weight were observed among groups (ANOVA, Tukey; $p>0.05$; Fig. 1A). When intra-groups comparisons regarding body weight were performed, a significant difference between the beginning and the end of the experiment was observed in all groups (paired t-test; $p<0.05$). All animals presented FPG levels higher than 250 mg/dL throughout the study. No significant differences regarding FPG levels were observed in intra or inter-group comparisons (ANOVA, Tukey; paired t-test; $p>0.05$; Fig. 1B).

Histopathological analysis

The results of the histopathological analysis based on scores are depicted in Table 1. In group DM/C, periodontal ligament (PL) presented intense vascularization and a great amount of fibroblasts and collagen fibers. These fibers were inserted in both cementum and alveolar bone. Cementum outer surface was sound and covered with a large number of cementoblasts. Alveolar bone presented a regular outer contour and was composed of thick trabeculae covered with active osteoblasts or bone lining cells. Osteoclasts were sparsely

distributed in the alveolar bone. The little medullary spaces harbored a loose and vascularized connective tissue and/or bone marrow.

The destruction of all periodontal tissues prevailed in group DM/EP. The connective tissue presented a severe inflammatory infiltrate extending to the boundaries of the bone tissue. A great number of neutrophils and macrophages and a few lymphocytes and fibroblasts were sustained by a poorly organized extracellular matrix. The cementum presented many areas of active resorption. The alveolar bone presented thin trabeculae and a very irregular contour due to the presence of many resorption lacunae with a great number of active osteoclasts. In most of the specimens, the inflammatory infiltrate reached bone medullary spaces. Some specimens presented necrotic bone spicules surrounded by inflammatory cells, specially in the furcation region.

Animals of group DM/EP/TIL1 presented less intense inflammatory response and better periodontal repair when compared with the ones of group DM/EP. The inflammatory process was restricted to the connective tissue and did not extend to the bone tissue. The cementum presented areas with resorption in some specimens. The bone tissue of the interradicular septum presented thin bone trabeculae and irregularities on its surface. Besides, trabeculae were coated with a great number of active osteoclasts.

In group DM/EP/TIL3, although some inflammation was present in the connective tissues, they were clearly restructured. These tissues presented a moderate amount of fibroblasts and collagen fibers. Cementum resorption level was very discreet. The bone tissue presented trabeculae of moderate thickness and few active osteoclasts. The contour of the alveolar bone was more regular than that present in the animals of group DM/EP/TIL1.

Histometric analysis

Group DM/EP/TIL3 presented lower ANBL than group DM/EP ($p<0.05$; Figs. 2A, 2C-F). There were no differences in ANBL when groups DM/C and DM/EP/TIL3 were compared ($p>0.05$). Group DM/EP/TIL3 presented reduced AL when compared with groups DM/EP ($p<0.001$) and DM/EP/TIL1 ($p<0.05$; Figs. 2B, 2G-J).

DISCUSSION

The model of EP used in the present study, already reported previously,[35] allowed a successful induction of the disease. Significant alveolar bone loss, attachment loss and considerable inflammatory infiltrate were observed in group DM/EP, but not in group DM/C.

The diabetogenic agent used, streptozotocin, causes selective destruction of pancreatic beta cells[37] and, thereby, decreases insulin secretion.[38,39] In fact, the general metabolism of STZ-induced diabetic rats is similar to that in human DM.[40] In this study, the FPG levels remained elevated throughout the experimental period and there was a significant reduction in body weight.

Since we have previously investigated the effects of TIL in normoglycemic rats,[32] that was not the purpose of the present study. Besides, it is well established that DM increases the severity of periodontitis in the experimental model used.[14,41] Diabetic rats with EP present abscess formations[14,42] and augmented alveolar bone loss,[14,39,41-45] higher degree of inflammation[14,36,39,41,42,46] and worsen organization of the gingival connective tissue[14,36] when compared with normoglycemic rats with periodontitis. Furthermore, the level of pro-inflammatory mediators is increased[41,45,46] and the reparative capacity of periodontal tissues is reduced in the diabetic animals.[33]

With the purpose of evaluating the effects of TIL on periodontal bone loss, the alveolar bone level was measured in diabetic animals' first molars. The animals treated with TIL 3mg/kg presented less alveolar bone resorption than the animals not treated (group DM/EP). In this context, it is important to emphasize that DM can affect the bone microarchitecture.[12] The resultant condition, known as diabetic osteopathy, is characterized by the disruption of matrix synthesis and hydroxyapatite crystal formation, presumably due to the decreased numbers and activity of osteoblasts.[12,47] There is also a delay in protein synthesis and collagen metabolism[13,48] implying that the bone apposition may be slower in DM.[14] Therefore, the treatment with TIL may be a promising approach for minimizing the effects of DM associated with periodontitis, since calcified tissues appear to be the main target for deposition of TIL.[49] TIL can dose-dependently inhibit osteoblasts-derived IL-6,[27] which induces osteoclast formation[50] and therefore stimulates bone resorption.[53~~51~~] Other factors that favor the choice for this BP is that it presents a long skeletal-retention time, leading to a persistent biologic effect,[52] and its anti-inflammatory properties.[27] Actually, the animals treated in this study presented reduced intensity and extension of inflammatory infiltrates. In fact, other types of BPs were evaluated in the association DM-periodontitis with favorable results.[53-55] Ozdemir et al.[53] observed that the administration of mono and combined clodronate and low-dose doxycycline may significantly reduce MMP-9 and IL-1β expressions in rats with DM and EP. Clinical trials also demonstrated promising results with BPs administration in type-2 diabetic patients with periodontitis[54,55] Rocha et al.[54] showed that systemic administration of alendronate induced more improvements in periodontal clinical

parameters and alveolar bone resorption than the placebo therapy. It has been also verified that the local treatment of periodontal intrabony defects with alendronate resulted in significant clinical attachment gain, reduction of probing depths and improved bone fill compared to the defects treated with placebo.[55]

An interesting finding of this study is that, when the parameters of area of no bone or periodontal ligament and attachment loss were analyzed, group DM/EP/TIL3 presented results statistically different from group DM/EP while group DM/EP/TIL1 did not show significant difference when compared with the animals not treated. Furthermore, group DM/EP/TIL3 was the only one that did not present significant difference in relation to group DM/C in histometric analysis in the furcation area. These data may suggest that the dosages of TIL influenced its properties, indicating a dose-dependent effect. In fact, some studies demonstrated that the effects of BPs on decreasing cellular infiltration, number of osteoclasts and alveolar bone loss in periodontal tissues are dose-dependent.[56,57] The dose-dependent effect of TIL was observed in a previous study of our group, when it was locally administered in non-diabetic rats.[32] In this preceding study, dosages of 0.1, 0.3 and 1 mg/kg body weight were evaluated and the last was the only one to provide a significant decrease in alveolar bone loss. In the present study, since the dosage of 3 mg/kg presented a trend towards better results than the dosage of 1 mg/kg in some parameters, it is possible that, depending on the effects on the periodontium to be considered, greater dosages are required in diabetic than in non-diabetic animals. It might occur due to the more pronounced periodontal inflammatory process usually observed in diabetic when compared with normoglycemic rats.[14,36,39,41,42,46]

There are few studies[32,58-60] demonstrating the effects of local administration of BPs on periodontitis, an approach that presents the advantage of avoiding possible systemic adverse effects of the drugs. The findings of the present study corroborate previous studies in which other types of BPs were locally applied.[58,60] Mitsuta et al.[58] administered the BP clodronate into the palatal gingiva of rats and demonstrated its ability to prevent bone resorption and decrease the number of osteoclasts in EP. Goya et al.[60] applied olpadronate solutions in the depth of the gingival sulcus at the level of the furcation around rat molars with periodontitis. This treatment inhibited alveolar bone loss and caused marked morphologic changes in the cytoplasm of osteoclasts, which indicated apoptosis.[60] It is important to emphasize that, since TIL is a non–nitrogen-containing BP, the possibility of occurrence of undesirable effects usually associated with nitrogen-containing BPs, such as ocular lesions,[61] gastrointestinal disorders,[62] acute-phase reactions[61] and osteonecrosis of the jaw,[63-67] is minimized. TIL is a safe compound with an appreciable therapeutic margin.[68]

It is essential that the effects of TIL in EP in diabetic rats be studied in cellular and molecular levels, in order to evaluate the inflammatory mediators that might be influenced by the drug and its pathways of action. The findings of the present study need to be confirmed with more advanced experimental models in the phylogenetic scale and in clinical trials, including type-2 diabetic patients. It is also mandatory to analyze if the local administration of TIL could provide additional benefits to scaling and root planing, which is the conventional periodontal therapy currently. More studies are required also to generate dose-response curves and evaluate different therapeutic regimens.

CONCLUSIONS

Within the limits of this study, it can be concluded that i) the local administration of TIL solutions presented a protective effect on tissue destruction in EP in diabetic rats and ii) the dosage of TIL may influence its effects.

FOOTNOTES

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[‡]Streptozotocin, Amresco® Life Science Research, Solon, OH, USA.

[§]Rompum®, Bayer Saude Animal, Sao Paulo, SP, Brazil.

[‖]Dopalen®, Agribands, Paulinia, SP, Brazil.

[¶]Tiludronic acid, Tildren®, Ceva Saude Animal Ltda, Paulinia, SP, Brazil.

[#]Axiovision 4.8.2, Carl Zeiss MicroImaging GmbH, Jena, Germany.

[**]DC300F, Leica Microsystems, Wetzlar, Germany.

[††]DMLB, Leica Microsystems, Wetzlar, Germany.

[‡‡]ImageJ®, National Institutes of Health, Washington, DC, USA.

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FIGURE LEGENDS

Figure 1. Means and standard deviations of body weight (g) **(A)** and fasting plasma glucose (mg/dL) **(B)** in groups DM/C, DM/EP, DM/EP/TIL1 and DM/EP/TIL3; *p= 0.0098; **p< 0.0001; ***p= 0.0018.

Figure 2. Histomorphometric analysis of periodontal tissues. Means and standard deviations of area of no bone or periodontal ligament (mm^2) **(A; furcation area)** and attachment loss (mm) **(B; interproximal area)** of the specimens, with comparisons among groups. Photomicrographs of periodontal tissues in the furcation **(C - F)** and interproximal **(G - J)** areas of mandibular first molars: group DM/C **(C** and **G)**; group DM/EP **(D** and **H)**; group DM/EP/TIL1 **(E** and **I)**; group DM/EP/TIL3 **(F** and **J)**.

Abbreviations and symbols: ab= alveolar bone; CEJ= cementoenamel junction; ct= connective tissue; pl= periodontal ligament; black arrows= cementoenamel junction; white arrows= epithelial attachment; *p<0.05; **p<0.01; ***p<0.001. Scale bars: **C - J** = 200 µm. (Hematoxylin and Eosin stain).

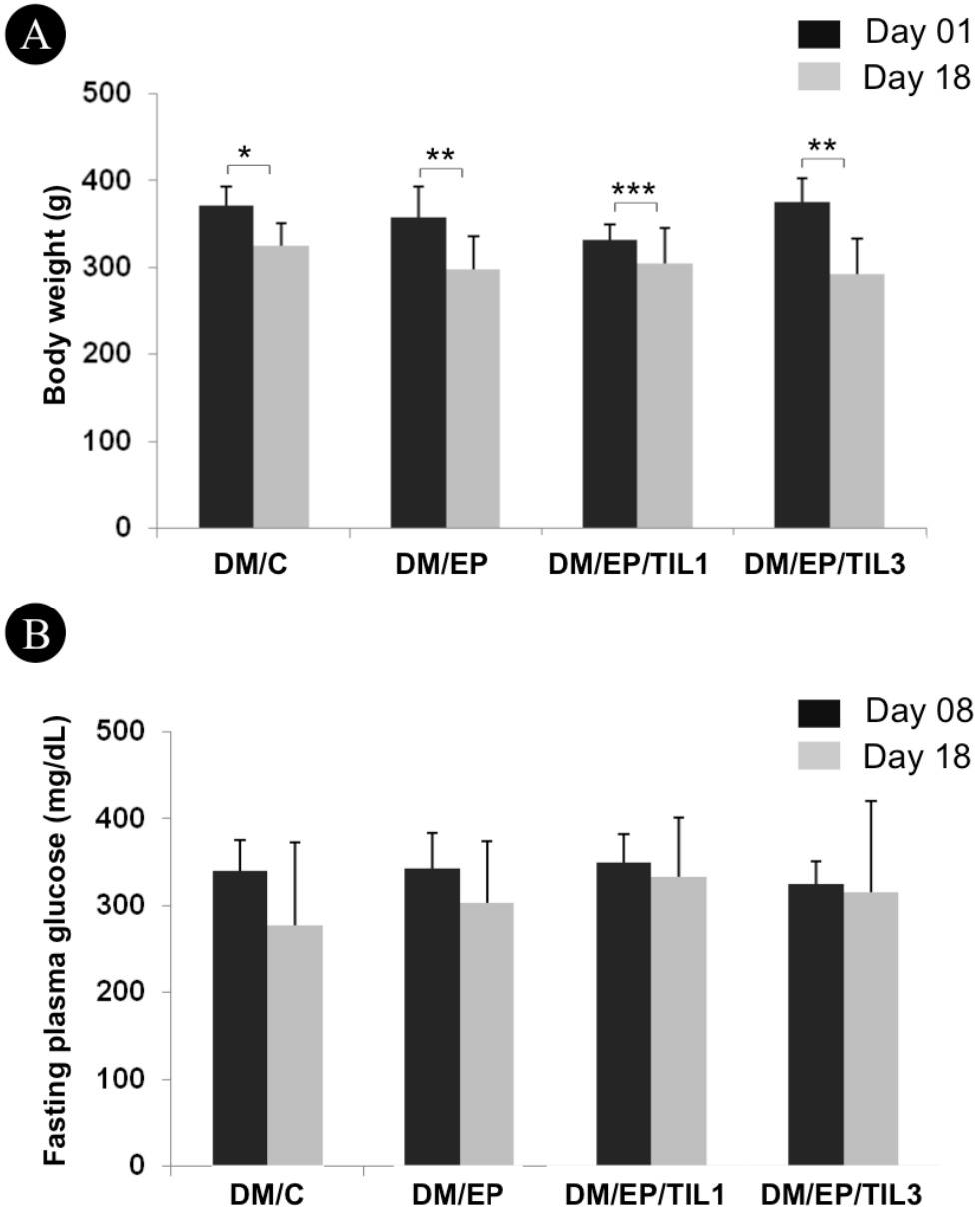
TABLE

Table 1. Histopathological analysis of the furcation and interproximal areas of mandibular first molars: percentage of animals per score evaluated.

HISTOPATHOLOGICAL ANALYSIS				
PARAMETERS AND RESPECTIVE SCORES	**EXPERIMENTAL GROUPS**			
	DM/C	**DM/EP**	**DM/EP/TIL1**	**DM/EP/L3**
	n=8	**n=8**	**n=8**	**n=8**
	% animals/ Score	% animals/ score	% animals/ score	% animals /score
Intensity of local inflammatory infiltrate				
(0) Absence of inflammation	100	0	0	0
(1) Small amount of inflammatory cells	0	0	50	100
(2) Moderate amount of inflammatory cells	0	0	50	0
(3) Large amount of inflammatory cells	0	100	0	0
Extension of inflammatory infiltrate				
(0) Absence of inflammation	100	0	0	0
(1) Extending to part of the connective tissue of the furcation/interproximal areas	0	0	50	100
(2) Extending to the whole connective tissue of the furcation/interproximal areas	0	0	50	0
(3) Extending to the whole connective tissue and to the bone tissue of the furcation/interproximal areas	0	100	0	0
External root resorption (cementum e dentin)				
(0) Absent	100	0	0	0
(1) Only inactive resorption areas	0	0	50	50
(2) Few active resorption areas	0	0	50	50
(3) Many active resorption areas	0	100	0	0

Alveolar bone resorption				
(0) Within normality patterns	100	0	0	75
(1) Small amount of resorption areas	0	0	100	25
(2) Moderate amount of resorption areas	0	0	0	0
(3) Large amount of resorption areas	0	100	0	0
Connective tissue pattern				
(0) Moderate amount of fibroblasts and large amount of collagen fibers (dense connective tissue)	100	0	0	0
(1) Moderate amount of both fibroblasts and collagen fibers	0	0	50	100
(2) Small amount of both fibroblasts and collagen fibers; presence of interstitial edema	0	37.5	50	0
(3) Severe tissue destruction with interstitial edema and necrotic areas	0	62.5	0	0
Alveolar bone pattern				
(0) Bone trabeculae with regular contour coated with active osteoblasts, including areas of new bone formation	100	0	0	0
(1) Bone trabeculae with irregular contour coated with active osteoblasts and osteoclasts	0	0	50	100
(2) Bone trabeculae with irregular contour coated with active osteoclasts	0	62.5	50	0
(3) Areas of necrotic bone and bone trabeculae with irregular contour coated with active osteoclasts	0	37.5	0	0

FIGURES



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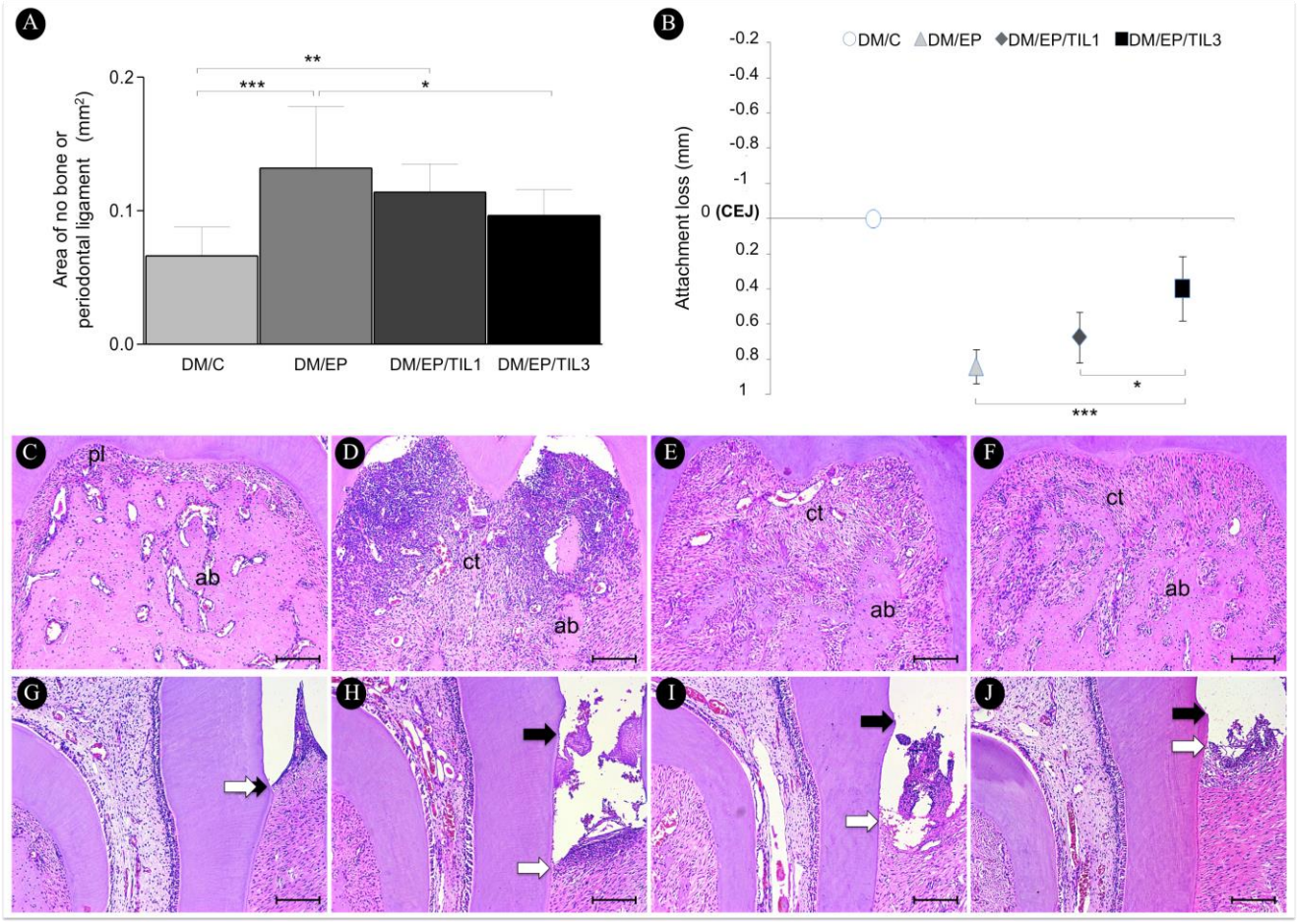

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4. CONCLUSÕES GERAIS

Dentro dos limites deste estudo, pode ser concluído que i) a administração local de soluções de ácido tiludrônico (1 e 3 mg/kg de peso corporal) apresentou um efeito protetor na destruição tecidual na periodontite experimental em ratos diabéticos e ii) a dosagem de ácido tiludrônico pode influenciar seus efeitos.

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ANEXO A

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE RIBEIRÃO PRETO
CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE RIBEIRÃO PRETO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO CEUA – FORP/USP

Certificamos que o Protocolo nº 2014.1.442.58.0 sobre a pesquisa intitulada **"Efeitos da administração local do Tiludronato na Periodontite Experimental em ratos diabéticos"**, sob a responsabilidade do Prof. Dr. Arthur Belém Novaes Junior, está de acordo com os Princípios Éticos na Experimentação Animal adotados pela Comissão de Ética no Uso de Animais da Faculdade de Odontologia de Ribeirão Preto, USP, foi APROVADO em reunião da CEUA de 15/07/2014 (totalizando 76 animais).

We hereby certify that the protocol nº 2014.1.442.58.0 regarding the research entitled **"Effects of local administration of Tiludronate on experimental periodontitis in diabetic rats"**, under the responsibility of Prof. Dr. Arthur Belém Novaes Junior, is in accordance with the Ethical principles in animal research adopted by the Animal Research Ethics Committee of the School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil, and was approved in 15/07/2014 (totalizing 76 animals).

Ribeirão Preto, 18 de julho de 2014.

Profa. Dra. Andiara De Rossi Daldegan
Coordenadora da CEUA – FORP/USP