UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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IMPACTO DOS BISFOSFONATOS INTRAVENOSOS NO CEMENTO E LIGAMENTO PERIODONTAL DE PACIENTES COM CÂNCER

IMPACT OF INTRAVENOUS BISPHOSPHONATES IN CEMENTUM AND PERIODONTAL LIGAMENT OF CANCER PATIENTS

Piracicaba
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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Estomatopatologia, na Área de Patologia.

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Orientador: Prof. Dr. Mario Fernando de Goes

Coorientador: Prof. Dr. Alan Roger dos Santos Silva

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The Mad Hatter: Only if you believe it is”

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RESUMO

Bisfosfonatos (BFFs) são medicamentos que reduzem a habilidade dos osteoclastos induzirem reabsorção óssea e, consequentemente, aprimoram o equilíbrio entre reabsorção e formação, sendo frequentemente prescritos por via oral (direcionados para o tratamento de doenças do metabolismo ósseo como a osteoporose e a doença de Paget) ou intravenosa (para tratamento de metástases ósseas e mieloma múltiplo). Apesar de sua comprovada eficácia no tratamento das doenças supramencionadas, o uso dos BFFs está associado a uma série de complicações, entre elas a osteonecrose induzida por BFFs. Recentemente, o uso dos BFFs também foi relacionado a alterações estruturais dos dentes, incluindo hipercementose e espessamento do espaço correspondente ao ligamento periodontal (LP). Portanto, o objetivo desse estudo foi testar a hipótese nula de que o uso intravenoso (IV) de BFFs não é capaz de alterar a micromorfologia do cemento e do ligamento periodontal de pacientes oncológicos. Com esta finalidade, foram utilizados 32 dentes; 16 oriundos de pacientes que fizeram uso de BFFs IV (grupo teste) e 16 de pacientes com câncer não tratados por BFFs (grupo controle). Os dentes foram posteriormente separados em 2 subgrupos de acordo com o processamento histológico e técnicas de investigação utilizadas: a) análise da espessura de cemento em 3 diferentes regiões (apical, média e cervical) por microscopia de luz polarizada (n=16, 8 grupo teste / 8 grupo controle) e b) análise da expressão imunoistoquímica do anticorpo anti-periostin em remanescentes do LP, contagem de fibroblastos no LP e do número de linhas incrementais de cemento (n=16, 8 grupo teste / 8 grupo controle). Não foi possível observar diferenças estatisticamente significativas entre os grupos quanto à micromorfologia do cemento [número de linhas incrementais (p=0.51)], número de fibroblastos no LP (p=0.56), espessura do cemento nas 3 regiões analisadas (p= 0.06; p=0.16 e p=0.18, respectivamente), expressão de periostin no LP entre os grupos (p=0.68) e presença de inflamação (p=0.59). A hipótese nula testada foi aceita e os resultados deste estudo sugerem que o uso de BFF IV não é capaz de causar alterações na micromorfologia do cemento ou do LP de pacientes oncológicos.

Palavras-chave: Bisfosfonato. Cemento. Ligamento periodontal. Microscopia de luz polarizada. Imunoistoquímica.

ABSTRACT

Bisphosphonates (BFFs) are medications, which reduce the ability of osteoclasts to induce bone resorption and consequently improve the balance between resorption and formation, being often prescribed orally (for the treatment of metabolic bone diseases, such as osteoporosis and Paget's disease), or intravenously (for the treatment of bone metastases and multiple myeloma). Despite its proven efficacy in the treatment of such diseases, the use of BPs is associated with a series of complications, including BP-related osteonecrosis of the jaws. Recently, the use of BPs was also associated to structural changes of teeth, including hypercementosis and thickening of the space corresponding to the periodontal ligament (PL). Therefore, the aim of this study was to test the null hypothesis that intravenous (IV) BPs are not able to change the micromorphology of cementum and PL of cancer patients. For this purpose, 32 teeth were analyzed, of which 16 were obtained from patients who received IV BPs (test group) and 16 from cancer patients naive to BPs (control group). Teeth were further divided into 2 subgroups according to histological processing and research techniques used: a) analysis of the thickness of cementum in 3 different regions (apical, middle and cervical thirds) by polarized light microscopy (n=16; 8 test group / 8 control group) and b) immunohistochemical analysis of the expression of anti-periostin antibody in the remaining of PL, number of fibroblasts in the PL and number of incremental lines of cementum (n=16; 8 test group / 8 control group). When comparing test and control group samples, it was not possible to observe statistically significant differences in the micromorphology of the cementum [number of incremental lines (p=0.51)], number of fibroblasts present in the PL (p=0.56), thickness of cement in any of the three studied areas (p=0.06; p=0.16; p=0.18, respectively), periostin expression in the PL between the groups (p=0.68) and presence of inflammation (p=0.59).The null hypothesis tested was accepted and the results of this study suggest that the use of IV BPs is not able to change the micromorphology of the cementum or PL of cancer patients.

Keywords: Bisphosphonate. Cementum. Periodontal ligament. Polarized light microscopy. Immunohistochemestry

SUMÁRIO

1.INTRODUÇÃO

Os bisfosfonatos (BFFs) fazem parte de uma classe de medicamentos análogos ao pirofosfato, apresentando, portanto, alta afinidade pela hidroxiapatita do tecido ósseo e alto potencial de inibição da função dos osteoclastos. Esta inibição acontece por vias de sinalização molecular que alteram a estrutura do citoesqueleto dos osteoclatos, prejudicando mecanismos de adesão intercelular e estimulando apoptose que, por sua vez, limita a capacidade de reabsorção óssea mediada por esta população celular (Rogers et al., 2000). Por este motivo, os BFFs são amplamente utilizados no tratamento de doenças do metabolismo ósseo, sobretudo, na osteoporose (Allen et al., 2009). Adicionalmente, os BFFs são prescritos comumente para o tratamento do mieloma múltiplo e das metástases de tumores sólidos para o esqueleto, sobretudo, no caso dos tumores de mama e próstata (Lussier et al., 2004; Maasalu et al., 2003).

A primeira geração de BFFs é composta por medicamentos como o clodronato e o etidronato, administrados por via oral e associada a poucos efeitos colaterais. As novas gerações de BFFs, que contêm nitrogênio, incluem o alendronato de sódio, o risedronato e uma série de BFFs administrados via intravenosa (IV), como o ácido zoledrônico e o pamidronato. Essa nova geração do medicamento possui alto potencial de inibição do recrutamento de osteoclastos para a superfície óssea, de inibição da atividade celular e redução do tempo de vida dos osteoblastos por meio da indução à apoptose e, finalmente, de alteração nos mecanismos de remineralização durante o processo de reabsorção óssea (Phal et al., 2007; Reszka et al., 2004).

Apesar de representar um grande avanço no tratamento das doenças ósseas de origem metabólica ou maligna, os BFFs também estão associados a uma miríade de efeitos colaterais sistêmicos (náusea, gastrite, hipertensão arterial sistêmica, leucopenia, anemia, letargia e deterioração da função renal, entre outras) e odontológicos, incluindo úlceras em mucosa bucal e, de modo mais significativo, a osteonecrose induzida por BFFs (ONB), que pode ser definida pela persistência de exposição óssea necrótica que não cicatriza espontaneamente até 8 semanas. A ONB foi descrita originalmente em 2003 por Marx et al. e é uma complicação com grande potencial para gerar infecção persistente, dor e morbidade; sobretudo, por

afetar pacientes oncológicos que frequentemente enfrentam condições médicas debilitantes e prognóstico oncológico desfavorável (Otto et al., 2012).

A exata incidência da ONB é desconhecida, contudo, acredita-se que pode variar de 1% a 11% para os pacientes tratados por meio de BFFs IV e 1% para pacientes que fazem uso da medicação por via oral (Bamiaset al., 2005; Durie et al., 2005; Hoff et al., 2008; Mavrokokki et al., 2007; Zavras et al., 2006; Zervas et al., 2006). A apresentação clínica da ONB é muito variada, sendo que alguns pacientes são assintomáticos, podendo ou não estar associada a dentes (que podem apresentar graus variáveis de mobilidade) e inflamação nos tecidos moles bucais adjacentes (Marx et al., 2003; Melo et al., 2005; Otto et al., 2009; Ruggiero et al., 2006). Casos de ONB em estágios iniciais geralmente são subclínicos e associados apenas a discretas áreas de osteólise, osteoesclerose ou aumento do espaço correspondente ao ligamento periodontal, eventos predominantemente assintomáticos e identificáveis, quase que exclusivamente, por meio de exames radiográficos odontológicos. Este cenário pode ocasionar diagnósticos equivocados ou tardios da ONB (Groetz et al., 2006; Markiewiczet al., 2005; Marx et al., 2005; Treister et al., 2009) e levar a sub- ou sobre-tratamentos.

Tendo em vista este desafio de padronização diagnóstica clínica, surgiram sistemas de classificação – para fins diagnósticos e terapêuticos – da ONB. Em 2014, a “American Association of Oral and Maxillofacial Surgeons” (AAOMS) desenvolveu uma classificação da ONB em 3 estágios, de acordo com as características clínicas e radiográficas encontradas (Tabela 1).

Tabela 1. Classificação dos estágios de ONB de acordo com a AAOMS, 2014.

Categoria de risco - “Estágio 0”	Pacientes que fizeram uso de bisfosfonato oral ou intravenoso e não apresentam osso necrótico exposto
Estágio 1	Osso necrótico exposto em pacientes assintomáticos e sem evidência de infecção
Estágio 2	Osso necrótico exposto, associado à infecção que é evidenciada por dor e eritema na região de osso exposto, com ou sem drenagem purulenta
Estágio 3	Osso necrótico exposto, em pacientes com dor e infecção, com uma ou mais das seguintes características: Fratura patológica, fistula extraoral ou osteólise se estendendo para a borda inferior.

O diagnóstico da ONB é realizado por meio de uma associação clinicopatológica amparada, por natureza, no histórico médico do paciente que necessariamente precisa ter sido tratado por meio de BFFs (Phal et al., 2007). É oportuno esclarecer que a biodisponibilidade dos BFFs é superior a 10 anos devido a sua capacidade de incorporação à massa óssea do paciente. Esta informação é relevante, pois entende-se que o risco para desenvolvimento da ONB é prolongado e está presente mesmo após muitos anos da interrupção do tratamento (Sparidans et al., 1998). Os principais fatores de risco para o desenvolvimento da ONB são procedimentos cirúrgicos odontológicos, incluindo extrações dentárias, cirurgias periodontais e traumas persistentes à mucosa bucal, como aqueles associados ao uso de próteses mal adaptadas. É imperioso esclarecer que a ONB também pode se desenvolver de modo espontâneo (Migliorati et al., 2005; Migliorati et al., 2008; Ruggiero et al., 2004; Wang et al., 2003). Ainda no que diz respeito aos fatores de risco para a ONB, é importante considerar que alguns fatores médicos como pacientes tratados com BFFs por períodos de tempo superiores a 3 meses, protocolos de tratamento baseados em pamidronato e ácido zoledrônico, pacientes com co-morbidades como diabetes e pacientes em tratamento para mieloma múltiplo estão sob risco aumento de desenvolver ONB (Cheng et al., 2005; Gupta et al., 2013; Marx et al., 2005).

A fisiopatologia da ONB é pouco compreendida e, por este motivo, ainda considerada controversa. Uma teoria vigente acredita que os BFFs se acumulam em quantidades maiores em maxila e mandíbula por estas áreas apresentarem maior taxa de remodelação óssea quando comparadas aos ossos longos; portanto, a inibição da remodelação óssea mediada por BFFs seria maior nesses ossos, prejudicando, desta forma, o reparo ósseo associado às microfraturas ósseas advindas da mastigação. Em casos de alterações ósseas mais importantes, como nas exodontias, este cenário prejudicaria o reparo ósseo e predisporia esta área a infecção e necrose (Borromeo et al., 2011). Outros autores defendem a ideia que ONB é de natureza multifatorial, envolvendo fatores como o efeito antiangiogênico dos BFFs sob a mucosa oral gerando isquemia dos tecidos moles que, associada à redução da remodelação óssea, geraria microfraturas, inflamação, infecção e necrose óssea (Allen et al., 2009; Fleisher et al., 2010; Fournier et al., 2002; Hoefert et al., 2010; Kumar et al., 2010; Lisclous et al., 2009; Marx et al., 2007; Takaishi et al., 2009).

Ainda no contexto da fisiopatogênese da ONB, a teoria conhecida como “inside out” sugere que a limitada capacidade de remodelação óssea induzida pelo tratamento com BFFs leva à diminuição do suprimento sanguíneo e, consequentemente, gera apoptose dos osteoblastos, infecção e necrose que facilitaria o aparecimento de uma área de osso exposto em boca (Gupta et al., 2013; Marx et al., 2003; Ruggiero et al., 2004).Outra via sugerida no desenvolvimento da ONB é conhecida como “outside in”, conjecturando que o potencial antiangiogênico dos BFFs na mucosa bucal levaria à sua fragilidade que, por sua vez, permitiria infecção do osso subjacente – já prejudicado pelo impacto negativo da medicação na remodelação óssea – e, subsequentemente, ocasionaria necrose óssea (Gupta et al., 2013).

Recentemente, em acréscimo à problemática da ONB, que já está bem estabelecida e descrita na literatura, diferentes equipes de pesquisadores descreveram alterações dentárias, que ao contrário das alterações ósseas, ainda geram muita discussão e contradição (como necrose pulpar e calcificações pulpares) e alterações radiográficas periodontais como o aumento do espaço correspondente ao ligamento periodontal, associadas ao uso dos BFFs (Correia et al., 2006; De Camargo et al., 2014; Kang et al., 2014; Phal et al., 2007). Entre estas alterações, destacam-se evidências acerca do desenvolvimento da hipercementose (deposição atípica de cemento na superfície radicular apical) em dentes humanos extraídos de ressecções cirúrgicas de pacientes tratados por meio de BFFs IV que desenvolveram ONB. Todavia, a maior parte dos trabalhos nesta linha de investigação se basearam em estudos descritivos não controlados (De Camargo et al., 2014). Complementarmente, estudos contemporâneos baseados em análises radiográficas de pacientes tratados por BFFs IV identificaram alterações no espessamento da lâmina dura que acabava por diminuir o espaço correspondente ao ligamento periodontal e, paradoxalmente, também encontraram evidências de que o tratamento com BFFs IV pode gerar o alargamento do espaço correspondente ao ligamento periodontal (1 a 2 mm de espessura entre a raiz dental e a lâmina dura) (Moeini et al., 2013; White et al., 2000). Este aumento do espaço correspondente ao ligamento periodontal associado a outras alterações radiográficas como esclerose óssea, alargamento da lâmina dura e imagens radiolúcidas na região periapical, estão entre as

principais alterações radiográficas aparentemente associadas ao risco aumentado de desenvolvimento da ONB (Phal et al., 2007). Em contradição, estudos bem controlados realizados por meio de experimentação animal sugeriram que o alargamento do espaço correspondente ao ligamento periodontal ocorreu por consequência de contaminação pulpar e não deveria ser atrelado exclusivamente ao tratamento com BFFs (Kang et al., 2014). Ainda no contexto do ligamento periodontal, um estudo baseado em cultura celular de fibroblastos humanos sugeriu que a taxa de apoptose dos fibroblastos presentes no ligamento de pacientes que fazem uso desta medicação parece não ser alterada quando comparado a um grupo controle (Jacobs et al., 2015). Em relação à expressão imunoistoquímica e aos níveis séricos de periostin (proteína encontrada em abundância no periósteo e ligamento periodontal, responsável pela manutenção e integridade do periósteo e do ligamento periodontal), estudos realizados em modelos animais não demonstraram alterações nos níveis séricos circulantes ou nos padrões de expressão imunoistoquímica em tecido mineralizado de animais tratados com BFFs (Contié et al., 2010).

Tendo em vista este cenário desafiador em termos de diagnóstico clínico e radiográfico das manifestações precoces da ONB e, sobretudo, o pouco conhecimento relacionado à capacidade dos BFFs induzirem alterações na raiz dental e no ligamento periodontal, esta dissertação se propôs a testar a hipótese nula de que o uso IV de BFFs não é capaz de alterar a micromorfologia do cemento e do ligamento periodontal de pacientes oncológicos.

2. ARTIGO: IMPACT OF INTRAVENOUS BISPHOSPHONATES IN CEMENTUM AND PERIODONTAL LIGAMENT OF CANCER PATIENTS.

Artigo submetido ao periódico Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology

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Abstract

Objective: To test the null hypothesis that intravenous (IV) bisphosphonates (BPs) therapy is not able to thicken or alter the micromorphology of cementum and periodontal ligament (PL) in cancer patients.

Study design: 32 teeth extracted from 24 cancer patients and separated in test (n=16; patients who have undergone IV BPs) and control (n=16; patients naive to BPs) groups were studied. Cementum thickness was measured in 3 different areas of the dental root through polarized light microscopy and micromorphology of cementum and PL was accessed by optical light microscopy as well as the immunohistochemical expression of periostin.

Results: No significant difference was detected in cementum thickness (apical: p=0.06; medium: p=0.16; cervical: p=0.18) between groups. The numbers of fibroblasts (p=0.56), incremental lines of cementum (p=051) and the immunohistochemical patterns of periostin expression in PL (p=0.68) did no differ between groups.

Conclusion: IV BPs therapy may not be able to thicken cementum or to change the micromorphology of LP.

Keywords: Bisphosphonates, immunohistochemistry, polarized light microscopy, cementum, periodontal ligament.

Introduction

Bisphosphonates (BPs) are one of the most important classes of antiresorptive agents that have been introduced in the clinical setting for the treatment of a series of metabolic and malignant bone diseases, including osteoporosis, bone metastases, tumor-associated hypercalcemia, and multiple myeloma[1,2].

Oral BPs are often used for benign systemic diseases, such as osteoporosis, Paget's disease, and pediatric inherited skeletal disorders, being considered one of the top prescribed drugs worldwide[2,3]. Intravenous (IV) BPs, such as pamidronate and zoledronate acid (ZA) are routinely used in patients with cancer-related conditions, such as multiple myeloma, bone metastasis from solid tumors and hypercalcemia of malignancy[4,5].

In spite of all medical benefits of these drugs, recent studies have shown a series of side effects, such as BPs-related osteonecrosis of the jaws (BRONJ)[4,6].This condition is defined by The American Association of Oral and Maxillofacial Surgeons (AAOMS) as the presence of exposed necrotic bone in the maxillofacial region that does not heal within 8 weeks after clinical identification in a patient currently or previously treated with BPs, who has never undergone head and neck radiotherapy[6,7]. Several hypotheses aimed to depicture the etiology of BRONJ, including inhibition of bone resorption by selectively affecting osteoclasts, suppression of bone remodeling, anti-angiogenic effects, infection and cytotoxicity, among others[4,8,9].

More recently, a series of dental and periapical alterations have been reported in association with the use of IV BPs, however, such results are still controversial and many studies have been conducted exclusively in animal

models[10,11] or uncontrolled studies based on human teeth extracted from BRONJ surgical specimens[12,13].In this context, the most commonly observed dental abnormality was hypercementosis, followed by pulp necrosis, pulp stones in the pulp chamber[2] and interference in root formation and tooth eruption[10]. Additionally, relatively common radiographic findings of tooth-bearing areas of people who have undergone IV BPs treatment include osteosclerosis, thickening of cementum as well as the lamina dura and widening of the periodontal ligament (PL)[12].

Therefore, the aim of this study was to test the null hypothesis that IV BPs therapy is not able to thicken or alter the micromorphology of cementum and PL of teeth in cancer patients. The current study combined optical light microscopy observations, polarized light microscopy analyses and the immunohistochemical expression of periostin, a protein highly expressed by PL fibroblasts, which is implicated in the maintenance of periodontal integrity.[14]

Materials and methods

Patients

This study was approved by the Ethics Committee of Piracicaba Dental School (protocol 081/2015), University of Campinas, São Paulo, Brazil. Thirty-two teeth were extracted from 24 patients and randomly separated in test (n=16) and control (n=16) groups. The test group was composed of 16 teeth extracted from 8 patients who have undergone IV BP for the treatment of multiple myeloma and bone metastases from solid tumors. Samples from test group were further divided into 2 subgroups, according to the experimental assessment they were submitted: (1) polarized light microscopy (n=8) and (2) optical light microscopy and immunohistochemistry (n=8).

All patients received IV BPs at different doses for at least 3 months. All patients underwent periodontal treatment prior to dental extraction attempting to keep it, once this procedure increases the chances to the development of ONB in patients who use IV BPs. All teeth used in this study were classified, prior to extraction, according to the clinical attachment loss (CAL)[15] as slight (1-2mm), moderate (3-4mm) or severe (>5mm), following the Periodontal Disease Classification System of the American Academy of Periodontology[15]. After extraction, all teeth were stored in 10% neutral-buffered formalin solution for 24h and further submitted to sample processing. All teeth (test and control groups) were extracted due to periodontal disease or decay before the start of cancer treatment of 16 patients who were naive to BPs therapy (and did not receive head and neck radiotherapy). Control teeth were also assembled into 2 subgroups according to the experimental assessment they were submitted: (1) polarized light microscopy (n=8) and (2) optical light microscopy and immunohistochemistry (n=8).

Micromorphological analysis

For the micromorphological study of cementum and remaining of PL, an optical light microscope (OLM) (DM 5000.B; Leica, Switzerland) was used. The specimens were decalcified in Ana Morse's solution (equal volumes of 20% sodium citrate and 50% formic acid) at 4°C for 3 weeks, with changes every two days[16]. The samples were embedded in Paraplast Plus® (Leica Biosystems Richmond, Inc., Richmond, IL, USA) to produce 5µm-thick-sections on a microtome (Leica, Nussloch, Germany). The sections were deparaffinized with xylol and hydrated in progressive concentrations of ethylic alcohol from 100 to 50%, and stained with

hematoxylin for 12 minutes. They were then rinsed under running water and counterstained in eosin for 5 minutes. The specimens were then dehydrated in ethylic alcohol (100%), cleared in xylol, and the histological glass slides were mounted for the evaluation of the following morphological structures: number of incremental lines of cementum, presence of inflammatory cells and number of fibroblasts in the remaining of PL. Hematoxylin and eosin stained slides were evaluated in a descriptive manner to classify inflammation as present or absent in the remaining of PL. In addition, the number of cementum incremental lines and the number of PL fibroblasts were counted with the use of Image J software (Image J 1.47v, Wayne Rasband, National Institute of Health, USA). Three areas (20x of magnification) of the cementum and remaining of PL of each sample were randomly selected and an average of the values obtained in these three areas was obtained, resulting in one value per sample. Two-way (test group *vs.* control group) analysis of variance (t-test) was used at the 5% level of significance.

Polarized light microscopy

Axial sections of 200-µm-thick through the middle of the teeth samples were performed with a low-speed saw (Isomet, Buehler, Lake Bluff, USA) under constant water irrigation. Sections were hand-grounded and polished by using silicon carbide paper of 600,800 and 1,200 grits to a final thickness of 100µm. Sections from each sample were investigated after immersion in water and analyzed under a polarized light microscope (DM5.000 B; Leica, Heerbrugg, Switzerland)[17]. The mean thickness was measured in 3 different areas of the dental root (apical, medium and cervical thirds) using software LAS Version 4.5 (Leica Microsystems, Switzerland). Comparisons between test and control groups were performed

among homologous teeth to a faithful comparison by using statistical analyses, which were based on the mean thickness values in the apical, medium and cervical thirds of the cementum. Two-way (test group *vs.* control group) analysis of variance (t-test) was used at the 5% level of significance.

Immunohistochemical analysis

The remaining of PL was analyzed through the immunohistochemical expression of the primary anti-periostin antibody (Rabbit Polyclonal Periostin Antibody; Novus Biologics®; dilution 1:100). Three-µm-thick histologic sections were cut from the paraffin-embedded tissue blocks and histological sections of each demineralized specimens were mounted on silane-coated glass slides, following the sample preparation stepwise described for the micromorphological analysis.

The slides were deparaffinized in xylene 2 times of 10 minutes, hydrated in decreasing ethanol solution (100 to 50%) 5 seconds each one, washed in distilled water for 3 minutes and in phosphate-buffered saline (pH=7.4), 3 times of 5 minutes each. The antigen retrieval used was proteinase K for 5 minutes and endogenous peroxidase activity was blocked using 10% hydrogen peroxide by incubation in 5 baths, for 5 minutes each. After being washed in phosphate-buffered saline (pH=7.4), slides were incubated overnight with the primary antibody. All slides were subsequently exposed to avidin-biotin complex and horseradish peroxidase reagents (LSAB Kit; Dako Cytomation, Glostrup, Denmark). Slides were further washed in phosphate-buffered saline (pH=7.4) 3 times of 5 minutes each again and in diaminobenzidine tetrahydrochloride for 5 minutes (Sigma-Aldrich, St Louis, MO, USA). The slides were washed in distilled water 3 times of 1 minute,

stained with Mayer's hematoxylin for 4 minutes, washed in running water during 5 minutes, counter-stained with eosin, dehydrated in increasing ethanol solutions (50 to 100%) 5 second each, diaphonized with 2 xylene baths of 5 minutes each and the slides were mounted with covers lips. Negative controls were performed with the omission of the primary antibody and PL from teeth samples was used as positive controls.

Two previously calibrated oral pathologists analyzed the slides. A descriptive analysis was performed for the marker, and the presence of periostin positive PL fibroblasts cells was classified as negative or positive and when positive as weak, moderate or strong. Examiners were instructed to come to a consensus in cases of disagreement. Results generated were analyzed by using descriptive statistics, absolute values, and percentages.

Results

Patients

The clinicopathologic features of the test group patients including age, gender, type and dosage of BPs, treatment length time and oncologic diagnoses, among others, are shown in table 1. The clinicopathological features of the control group patients are shown in table 2.

Micromorphological analysis

Test group samples for the micromorphological analyses were composed by 4 (50%) molars, 2 (25%) premolars, 1 (12.5%) canine and 1 (12.5%) incisive. Five (62,5%) patients were female and the mean age was 59.5 (range 30-81) years.

Zoledronic acid (ZA) was exclusively used in 3 (37.5%) patients, ZA combined with pamidronate was used in 1(12.5%) patient and isolated pamidronate in 4 (50%) patients. IV BPs was used in a mean time of 14.2 months (ranging from 3 to 43 months). The control group was composed by 2 (25%) molars, 3 (37.5%) premolars, 2 (25%) canines and 1 (12.5%) incisive. Seven patients (87.5%) were male with a mean age of 60.3 years (range 52-75). All teeth (test and control groups) included in this study were vital, showing no endodontic treatment, fracture or decay. All 16 teeth samples used in this study (both test and control groups) were classified prior to extraction as slight chronic periodontitis (1-2mm CAL).

It was possible to observe the incremental lines of cement in all of the 16 (100%) teeth samples (test and control groups). They were most evident in the apical and medium thirds, where the thickness of the cement was higher (Figure 1). In the test group, it was possible to observe 3 incremental lines of cementum in the dental root of 2 (25%) samples, 4 lines in 3 (37.5%) samples, 5 lines in 2 (25%) samples and 7 incremental lines in 1 (12.5%) sample. In the control group, 1 (12.5%) sample showed 2 incremental lines, 3 (37.5%) samples showed 3 lines, 4 lines of cementum were observed in 2 samples (25%), 1 (12.5%) sample had 5 lines and 1 (12.5%) sample presented 7 lines (Table 3). T-test failed to demonstrate statistical differences among values of the number of incremental lines of cementum between test and control groups ($p=0.51$).

In the test group, 7(87.5%) samples showed evidence of inflammation in the remaining of the PL and 1 (12.5%) sample did not present inflammation at all. In the control group, the inflammation was evident in 6 (75%) samples and absent in 2 samples (25%) (Table 3). To perform the statistical analysis, the samples that showed inflammation received number 1, and the samples that were free of

inflammation received number 0. T-test failed to demonstrate statistical differences among values of inflammation between test and control groups (p=0.59).

In all studied sections of both groups, it was possible to observe variable amounts of collagen and numerous fibroblasts adhered to the surface of the cement at the PL (Figure 1). The mean number of fibroblasts per sample in the test group was 130.25 and the mean value for the control group was 117.62 (Table 4). It was not possible to observe statistically significant differences between the number of fibroblast in test and control groups (p=0.56).

Polarized light microscopy

Test group was composed by 3 (37.5%) molars, 2 (25%) incisors, 1 (12.5%) canine and 2 (25%) premolars. Five patients (62,5%) were female and the mean age was 59.5 (range 30-81) years. ZA was exclusively used in 3 patients (37.5%), the combination of ZA and pamidronate was used in 1 patient (12.5%) and pamidronate was exclusively used in 4 patients (50%). IV BPs were used in a mean time of 14.2 months (ranging from 3 to 43 months). The control group was composed by 3 (37.5%) molars, 2 (25%) incisors, 1 (12.5%) canine and 2 (25%) premolars. Seven patients (87.5%) were male, the mean age was 54.7 (range 47-66).

It was not possible to observe statistically significant differences in the thickness of the cementum between the test and control groups among the three analyzed areas (apical, medium and cervical thirds) (Figure 2). The mean values of the thickness in the apical area was: study group=733.198; control group=914.408; medium area: study group=418,273; control group=414.717; cervical area: study group=140.477; control group=170.169 (Table 5 and 6). T-test failed to

demonstrate statistical differences among mean values of cementum thickness between groups (apical area: p=0.06; medium area: p=0.16 and cervical area: p=0.18).

Immunohistochemistry

Test group was composed by 4 molars (50%), 2 premolars (25%), 1 canine (12.5%) and 1 incisive (12.5%). Five patients were female (62,5%) and the mean age was 59.5 (range 30-81) years. ZA was exclusively used in 3 (37.5%) patients, ZA combined with pamidronate were used in 1(12.5%) patient and isolated pamidronate in 4 (50%) patients. IV BPs were used in a mean time of 14.2 months (ranging from 3 to 43 months). Control group was composed by2 (25%) molars, 3 (37.5%) premolars, 2 (25%) canines and 1 (12.5%) incisive. Seven patients (87.5%) were male with mean age of 60.3 years (range 52-75). The test and control group samples showed a positive discreet granular pattern of immunohistochemical staining in the fibroblasts of the PL in all studied samples (Figure 3). In the test group, 5 (62.5%) cases were weakly positive and 3 (37.5%) moderately positive. In the control group, 4 (50%) cases were weakly positive and 4 (50%) moderately positive (Table 7). T-test failed to demonstrate statistical differences between test and control groups for the prevalence of periostin immunopositivity (p=0.51).

Discussion

BPs are structural analogs of pyrophosphates, prescribed as antiresorptives agents that inhibit osteoclast attachment to the bone matrix and enhances osteoclast apoptosis[18,19]. Despite the countless benefits obtained with the use of BPs, especially in patients with cancer, we cannot disregard a number of side

effects induced for these medicaments. The present study represents the first attempt to quantitatively characterize teeth changes related to IV BPs treatment.

BRONJ is a well-known side effect of bisphosphonate therapies in patients with multiple myeloma or other malignancies[5].The real incidence of BRONJ is currently unknown but it was estimated to range from 4.5% to 12.8% in patients with multiple myeloma and 1.2% to 12% for patients with metastatic solid carcinomas[13,20,21,22,23]. In our study, the only patient who developed BRONJ received ZA for prostate cancer metastasis. The present study was unable to assemble a well-standardized sample in terms of time of exposure to BPs and this limitation was due to the fact that tooth extraction can predispose patients to BRONJ; therefore, it is quite uncommon to have access to a large number of teeth specimens. Notwithstanding, the mean time of BPs used by the patients of the current study can be considered adequate since it was superior to 14 months.

In the present study it was not possible to observe a significant difference in the number of fibroblasts of PL when comparing patients exposed and naive to IV BPs, which corroborates to the results of Collins et al (2015), who studied human PL fibroblasts exposed to clodronate and zoledronate and showed that the fibroblast apoptosis rate was not statistically different from fibroblasts that were not exposed to BP[22].

More recently, dental abnormalities[2,12,10] have been described in patients treated with IV BPs (regardless of the presence of BRONJ), which could possibly be related to the pharmacodynamics of the drug, but most of these studies were conducted in animal models and when applied to human teeth, did not use control group for comparison of the results. For instance, De Camargo et al. (2014) performed a study with human teeth extracted from patients who underwent IV BPs

treatment and describe hypercementosis as one of the most commonly found change. Other reported BPs-related dental changes were ankylosis, pulp necrosis, pulp calcification and obliteration of the pulp chamber[2]. However, this study did not use a control group and performed only a descriptive and subjective analysis. In addition, most of the participants were elderly patients and it is widely accepted that the thickness of the cement increases with age, so that the evaluation of the thickness of the cementum – as performed in the current study – is a more accurate method for cementum thickness estimation[24]. Therefore, the increase in the thickness of the cementum could only be considered a physiological event, rather than a change genuinely caused by the use of BPs. In this present study, the mean age of the patients of the test and control groups of the polarized microscopy group were very similar and no statistically significant difference was detected between test and control groups in terms of cementum thickness, suggesting that hypercementosis may represent merely a physiological event in such patients and not a true BPs- related dental change. In support of these findings, the lack of detectable differences concerning the number of cementum incremental lines, PL inflammation and PL collagen distribution between the two studied groups suggest that IV BPs is not able to change the micromorphology of the cementum and the PL in cancer patients.

Periostin was initially reported as osteoblast-specific factor-2 (OSF-2), it was renamed ‘‘periostin’’ due to its preferential location in the periosteum and the periodontal ligaments[11]. It is a matricellular adapter protein highly expressed by PL fibroblasts and is implicated in the maintenance of periodontal integrity, which is compromised during periodontal diseases[17,14]. Its immunolocation is accepted to be between the cytoplasmic processes of PL fibroblasts and cementoblasts and the

adjacent collagen fibrils[25]. This antibody has been extensively characterized in immunohistochemical mouse studies[26,27] and has been used as a marker to investigate the functional integrity of a regenerated PL interface[28]. Periostin was used in this study to check if the use of IV BPs would be able to promote a change in the metabolism of the PL, which could be the basis of the so-called widening of the PL reported in patients undergoing IV BPs. However, the present study failed to identify any differences in the frequency and patterns of periostin immunoexpression in control and study group samples, which were all similarly positive. Such result confirms previous data suggesting that the intensity of periostin immunostaining in the tibiae from mice treated with zoledronate did not differ from those of untreated animals, a finding which was consistent with the absence of changes in periostin circulating levels[11].

A previously published study performed in rat teeth treated with IV BPs and placebo exposed the pulp to the oral environment and evaluated the PL space was observed. The widening of PL space occurred only in drilled teeth in the both groups, confirming our results that such changes of the PL in BPs patients are more likely to develop by the pulp contamination rather than by the action of BPs[23]. In addition, another *in vitro* study analyzed the effects of IV alendronate in rat teeth and observed that BPs generally are not toxic to the cells of the PL[24]. Okamoto et al., (2013) suggested that periodontal space narrowing was induced by ZA administration, in a dose-dependent manner. However, a significant increase in the periodontal space was observed in animals treated with very high doses of ZA (500µg/kg), which are much higher than the doses prescribed for human patients with cancer (50-80 µg/kg).[29]

Another fact that should be taken into consideration is that the teeth used in the current study were extracted due to the presence of periodontal disease, which explains why most of the teeth samples presented PL inflammation. Previous evidence pointed out that inflammation may compromise the integrity of PL[17], however, even in the presence of inflammation, all studied samples preserved the expression of periostin, suggesting the maintenance of the PL homeostasis in spite of the IV BPs treatment. Periostin tissue levels significantly decrease under chronic inflammatory response and correlate with the detrimental changes to the PL over time[17]**.** All teeth used in this study were extracted from patients with slight chronic periodontitis[15] (test and control groups) and this might explain why the immunohistochemical staining pattern was very discreet in all samples of the current study.

Variables such as the number of doses of the BPs and treatment time may have influenced in the present results, however, due to the difficulty of having the standardization of these factors and, most importantly, to have access to teeth extracted from patients who underwent IV BPs, a more accurate comparison is hard to get. Therefore, the null hypothesis of this study was confirmed and it could be concluded that IV BP treatment may not be able to increase cementum thickness or change the PL micromorphology and metabolism.

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TABLES

Table 1. Clinicopathological features of patients from study group.

Patient	Gender	Age	BPs	Diagnosis	BPs time used(months)
1	F	68	Pamidronate (90mg)	Metastatic breast cancer	6
2	F	53	Pamidronate (90mg)	Metastatic breast cancer	22
3	F	48	Pamidronate (90mg)	Metastatic breast cancer	9
4	M	61	Pamidronate (90mg)	Multiple myeloma	15
5	M	81	Pamidronate (90mg) + Zoledronic acid (4mg)	Metastatic thyroid cancer	43
6	F	30	Zoledronic acid (4mg)	Metastatic breast cancer	8
7	F	74	Zoledronic acid (4mg)	Metastatic lung cancer	7
8*	M	61	Zoledronic acid(4mg)	Metastatic prostate cancer	3

F=female; M=male; BPs=bisphosphonates; *=bisphosphonate-related osteonecrosis of the jaw.

Table 2. Clinicopathological features of patients from control group.

Patient	Gender	Age	Diagnosis
1	M	47	Laryngeal SCC
2	M	47	Nasopharyngeal carcinoma
3	F	57	Lymphoepithelioma
4	M	48	Tongue SCC
5	M	66	Multiple myeloma
6	M	61	Laryngeal SCC
7	M	60	Sarcomatoid carcinoma
8	M	52	Hypopharyngeal SCC
9	M	52	Multiple myeloma
10	M	58	Multiple myeloma
11	M	63	Multiple myeloma
12	M	58	Tongue SCC
13	F	59	Tongue SCC
14	M	60	Multiple myeloma
15	M	58	Oropharyngeal SCC
16	M	75	Cervical SCC

SCC = squamous cell carcinoma.

Table 3. Number of incremental lines of cementum and presence of PL inflammation.

Test group	Incremental lines of cementum *	Presence of inflammation†	Control Group	Incremental lines of cementum *	Presence of inflammation†
1	4	+	1	4	+
2	4	-	2	2	+
3	5	+	3	3	+
4	7	+	4	3	-
5	3	+	5	5	-
6	3	+	6	3	+
7	5	+	7	7	+
8	4	+	8	4	+

+= presence of inflammation; - = absence of inflammation; *p value=0.51; †p value=0.59
.

Table 4. Mean number of LP fibroblasts per area in which sample

Patients	Test group	Control group	p value
1	134	72	
2	155	157	
3	166	69	
4	119	101	
5	110	186	
6	172	115	
7	66	60	
8	120	181	
Mean value	130.25	117.62	0.56

Table 5. Thickness of cementum at the apical, medium and cervical thirds

Patient (test group)	Thickness apical area(µm) *	Thickness medium area(µm) †	Thickness cervical area(µm) ᵉ
1	472,75	292,36	81,76
2	738,63	451,32	137,58
3	1057,01	154,77	141,81
4	523,54	171,95	86,05
5	1527,20	1271,32	130,38
6	432,69	234,87	181,09
7	791,42	582,79	192,67
8	322,31	186,78	90,65
Mean value	**733.19**	**418.27**	**130,24**

Table 6. Thickness of cementum at the apical, medium and cervical thirds.

Patient (control group)	Thickness apical area (µm) *	Thickness medium area (µm) †	Thickness cervical area(µm) ᵉ
1	763,04	164,92	217,71
2	509,52	201,98	167,82
3	1539,87	326,64	201,98
4	915,47	176,25	52,28
5	1583,96	1203,59	266,43
6	512,67	192,54	102,65
7	820,84	209,87	197,68
8	669,86	148,36	154,77
Mean value	**914,40**	**328,01**	**170,16**

*p value=0.06; †p value=0.16; ᵉ p value=0.18

Table 7. Periostin immunohistochemical expression

Patients	Test group	Control group	p value
1	+	++	
2	++	++	
3	+	+	
4	++	+	
5	+	++	
6	+	++	
7	++	+	
8	+	+	
			0.68

FIGURES

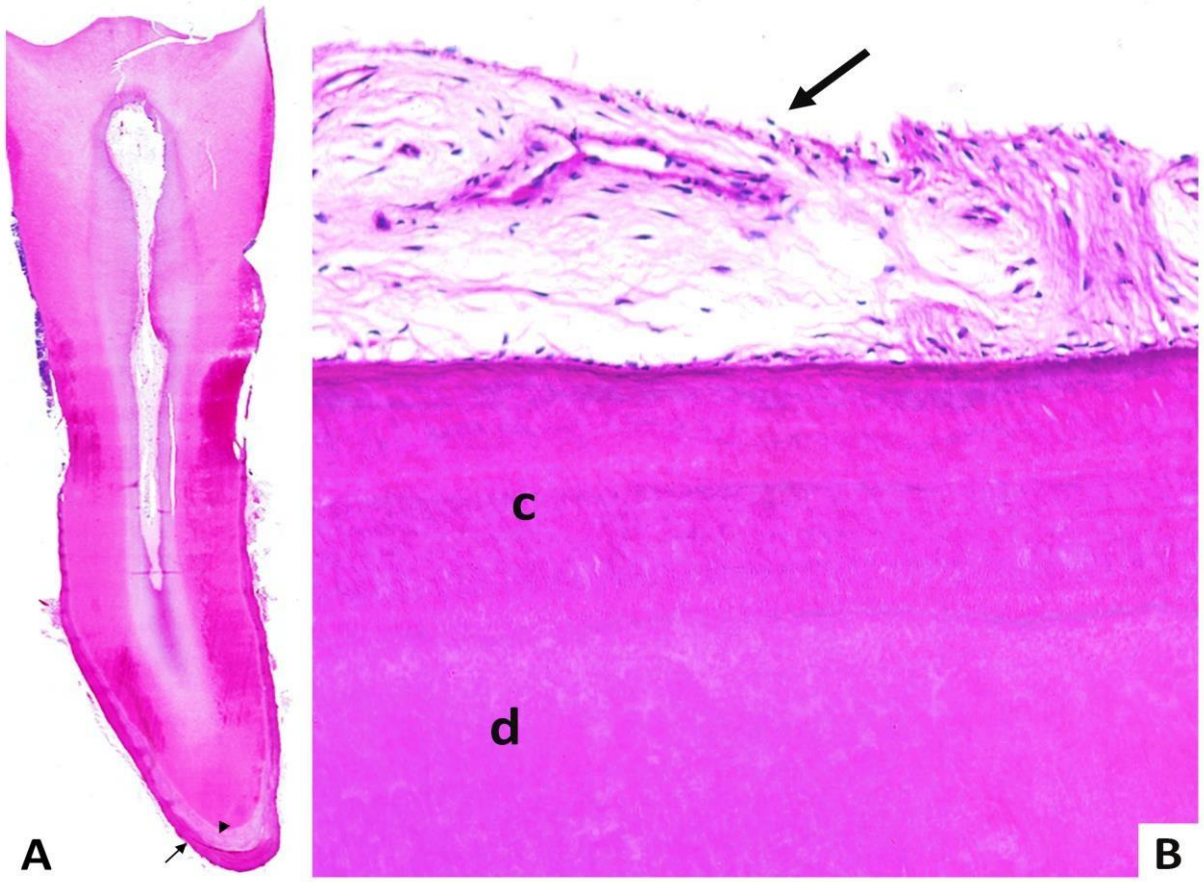

Figure 1. Morphological analysis of the cementum and periodontal ligament. **A.** Panoramic view of a study group sample. It is possible to observe the incremental lines of the cementum (arrow and arrowhead), which is more evident in the apical area of the tooth (H&E, 2x). **B.** Microscopic view of the periodontal ligament (arrow) of a test group sample (H&E, 40x). It is possible to observe preserved micromorphology of the fibroblasts, cementum (c) and dentin (d).

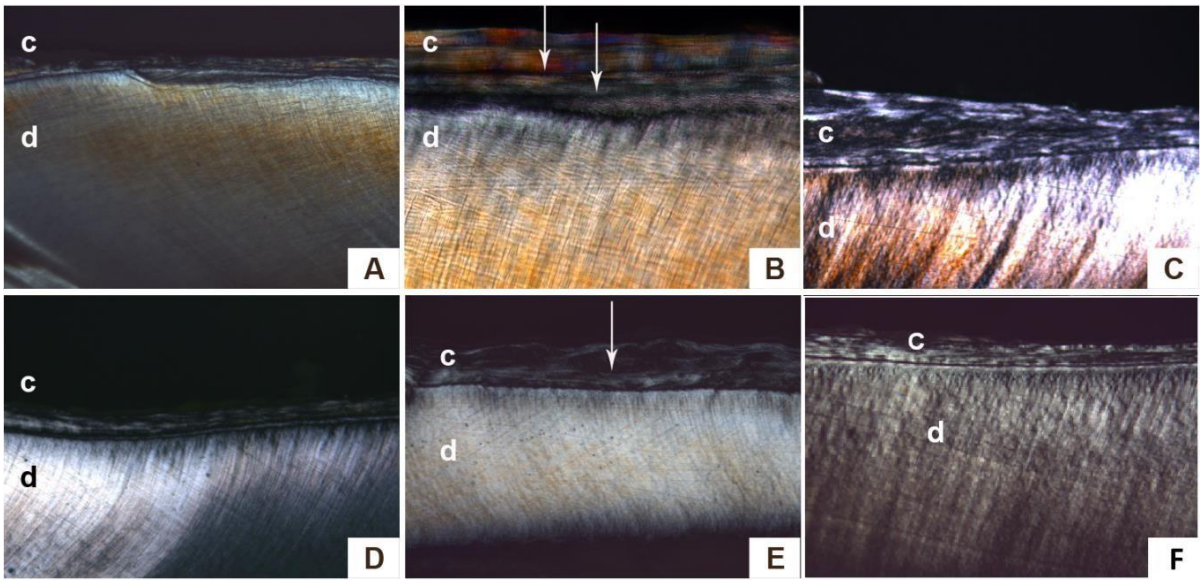

Figure 2. Polarized light microscopy images. **A.** Cervical area of the cementum of an upper molar of the study group (5x). **B.** Medium third of the same sample observed on figure A (5x). It is possible to observe incremental lines of the cementum (arrows). **C.** Apical area of the same sample seen on images A and B (5x). **D.** Cervical area of the cementum of an upper molar of the control group (5x). **E.** Medium area of the cementum of the same sample seen on image D (5x). **F.** Apical area of the same sample seen on D and E (5x). C=cementum and d=dentin.

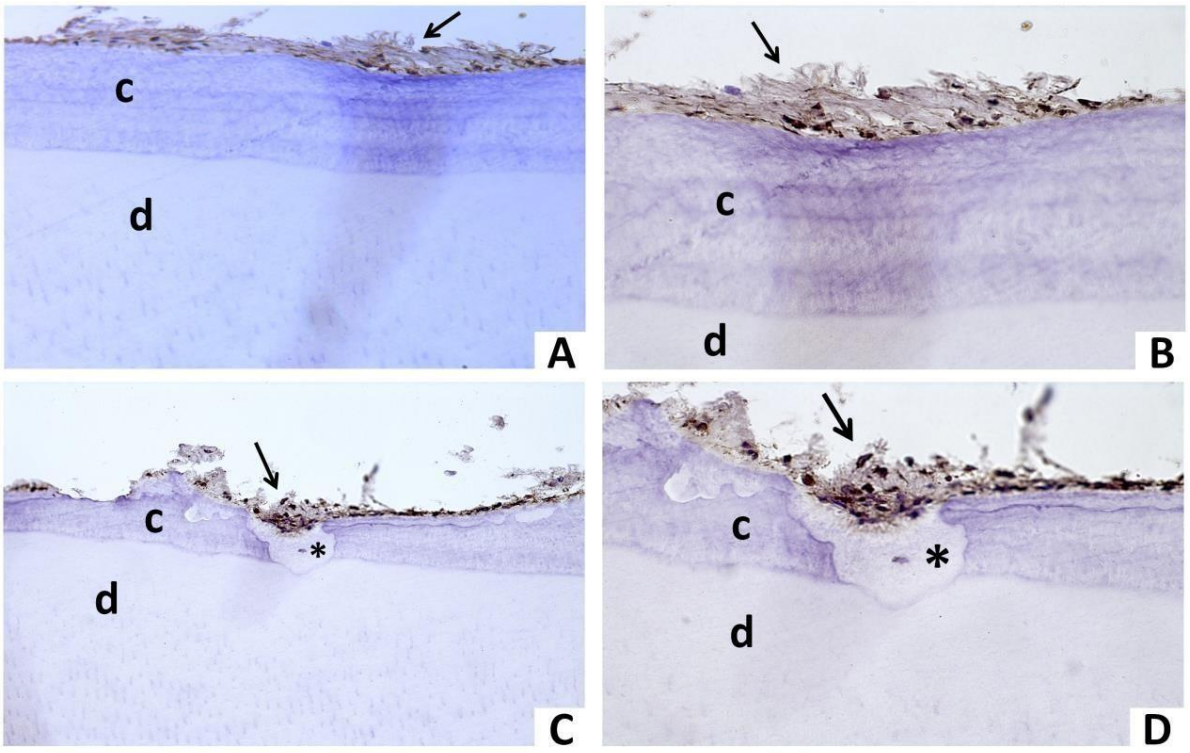

Figure 3. Immunohistochemical expression of periostin in remaining of periodontal ligament. **A.** Periodontal ligament fibroblasts (arrow) attached to the cementum showing positivity (control group, 20x). **B.** Higher magnification (40x) of same area on image A highlighting positivity on periodontal ligament fibroblasts. **C.** Test group sample with focal area of root reabsorption (asterisk) and periostin positivity on periodontal ligament fibroblasts attached to the cementum (20x). **D.** Higher magnification of the same area seen on figure (40x) with arrow identifying periostin positivity on periodontal ligament fibroblasts. C= cementum and d= dentin.

3.CONCLUSÃO

Não foi possível observar alterações significativas na micromorfologia do cemento ou do ligamento periodontal nos dentes do grupo estudo quando comparado ao grupo controle;

Não foi possível observar diferenças estatisticamente significantes na contagem de fibroblastos do ligamento periodontal do grupo estudo quando comparado ao grupo controle;

Não foi possível observar diferenças estatisticamente significativas na espessura do cemento entre o grupo estudo e grupo controle nas 3 diferentes regiões analisadas;

Não foi possível observar diferenças estatisticamente significativas na frequência ou no padrão de expressão imunoistoquímica do anticorpo anti-periostin no ligamento periodontal entre o grupo teste e grupo controle.

A consecução desta dissertação aceitou a hipótese nula de que o uso IV de BFFs não parece ser capaz de alterar a micromorfologia de cemento e do ligamento periodontal de pacientes com câncer.

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COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Análise morfológica e imunoistoquímica do cemento em dentes de pacientes que realizaram tratamento com bisfosfonatos** ", protocolo nº **081/2015**, dos pesquisadores **MARIANA DE PAULI PAGLIONI e ALAN ROGER DOS SANTOS SILVA**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 09/12/2015.

The Ethics Committee in Research of the Piracicaba Dental School, University of Campinas, certify that the project "**Morphological and immunohistochemical evaluation of cementum in theeth of patients who underwent treatment with bisphophonate**", register number **134/2015**, of **MARIANA DE PAULI PAGLIONI and ALAN ROGER DOS SANTOS SILVA**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on Dec 09, 2015.

Prof. Jacks Jorge Junior

Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.