SORAYA DE AZAMBUJA BERTI COUTO

INFLUÊNCIA DO DIABETES E DA CORTICOTERAPIA NO DESENVOLVIMENTO DA OSTEONECROSE MAXILAR ASSOCIADA AO USO DE ALENDRONATO DE SÓDIO

INFLUENCE OF DIABETES AND CORTICOTHERAPY IN THE DEVELOPMENT OF OSTEONECROSIS OF THE JAWS ASSOCIATED WITH SODIUM ALENDRONATE

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Tese apresentada como requisito para obtenção do Título de Doutor pelo Programa de Pós-Graduação da Faculdade de Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul. Área de Concentração, Estomatologia Clínica.

SORAYA DE AZAMBUJA BERTI COUTO

Orientadora: Profª Drª Karen Cherubini

Porto Alegre

2011

EPÍGRAFE

Se fui capaz de ver mais longe,

é porque me apoiei em ombros de gigantes.

Isaac Newton (1643-1727)

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RESUMO

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A presente pesquisa teve por objetivo verificar a influência da corticoterapia e do diabetes mellitus no desenvolvimento da osteonecrose maxilar associada ao uso de alendronato de sódio em animais de experimentação. A amostra compreendeu 44 ratos fêmeos, da linhagem Wistar, distribuídos aleatoriamente em 4 grupos: (1) 11 ratos tratados com alendronato de sódio (grupo alendronato); (2) 11 ratos tratados com alendronato de sódio e corticoterapia sistêmica (grupo corticosteroide); (3) 11 ratos tratados com alendronato de sódio e submetidos à indução de diabetes mellitus (grupo diabetes); (4) 11 ratos aos quais foi administrada solução salina (grupo-controle). Todos os animais foram submetidos a exodontias dos molares superiores do lado direito uma vez decorridos 90 dias do início do experimento. A eutanásia foi realizada 21 dias após os procedimentos cirúrgicos. Os cortes histológicos foram corados pela técnica de hematoxilina e eosina (H&E) e, subsequentemente, submetidos a processamento imunoistoquímico empregando-se os anticorpos anti-BMP-4 e anti-MMP-13. A avaliação histológica consistiu na análise quantitativa das variáveis: epitélio, tecido conjuntivo, infiltrado inflamatório, restos radiculares, colônias microbianas, osso vital e osteonecrose pela técnica da contagem manual de pontos (*Image Pro Plus* 4.5.1). As variáveis imunoistoquímicas foram avaliadas por meio da técnica da segmentação semiautomatizada (*Image Pro Plus* 4.5.1). Os resultados foram analisados pelos testes qui-quadrado e Kruskal-Wallis complementado pelo teste de comparações múltiplas, considerando-se o nível de significância de 5%. Na análise por H&E, a proporção de infiltrado inflamatório, colônias microbianas e osteonecrose foi significativamente maior no grupo diabetes ($p<0.05$), não havendo diferença significativa para as demais variáveis entre os grupos. A expressão imunoistoquímica da BMP-4 foi significativamente maior na área de tecido conjuntivo no grupo corticosteroide, quando esse foi comparado ao alendronato ($p<0.05$), não havendo diferença significativa entre os demais grupos. A expressão imunoistoquímica da MMP-13 não diferiu entre os grupos avaliados. De acordo com os resultados, o diabetes mellitus pode ser considerado um fator de risco ao desenvolvimento da osteonecrose dos maxilares associada ao uso de alendronato de sódio. Entretanto, para a corticoterapia essa associação não foi observada.

Palavras-chave: Bisfosfonatos, BRONJ, Corticoterapia, Diabetes mellitus.

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SUMMARY

SUMMARY

This study aimed to investigate the influence of corticotherapy and diabetes mellitus in the development of the osteonecrosis of the jaws associated with sodium alendronate. The sample consisted of 44 female rats, Wistar strain, randomly allocated into 4 groups: (1) 11 rats treated with sodium alendronate (alendronate group); (2) 11 rats treated with sodium alendronate and corticotherapy (corticosteroid group); (3) 11 rats treated with sodium alendronate and subjected to diabetes induction (diabetes group); (4) 11 rats treated with saline (control group). The animals were subjected to tooth extractions 90 days after starting the bisphosphonate therapy. Euthanasia was performed 21 days after the surgical procedures. Sections were stained by hematoxylin and eosin (H&E) and by immunohistochemistry technique using anti-BMP-4 and anti-MMP-13 antibodies. Histological evaluation consisted of quantitative analysis of epithelial tissue, connective tissue, inflammatory infiltrate, root fragments, microbial colonies, vital bone and osteonecrosis using the manual point counting technique (Image Pro Plus 4.5.1). BMP-4 and MMP-13 were evaluated with the semi-automated segmentation technique (Image Pro Plus 4.5.1). The results were analyzed by chi-square and Kruskal-Wallis tests (followed by the multiple comparisons test), stating the significance level at 5%. In the H&E analysis, inflammatory infiltrate, microbial colonies and osteonecrosis proportions were significantly higher in the diabetes group ($p<0.05$), while the other variables did not differ between the groups. The immunohistochemical expression of BMP-4 in the connective tissue was significantly higher in the corticosteroid group than in the alendronate group ($p<0.05$), whereas the other groups did not differ for this variable. BMP-4 in the bone area and MMP-13 did not significantly differ between the groups. In conclusion, diabetes can be considered as a risk factor for the development of osteonecrosis of the jaw associated with alendronate therapy. However, for corticotherapy this association was not observed.

Keywords: Bisphosphonates, BRONJ, Corticotherapy, Diabetes mellitus.

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SUMÁRIO

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1 INTRODUÇÃO

Os bisfosfonatos são drogas com estrutura química semelhante à do ácido pirofosfórico que, no organismo humano, encontra-se sob a forma de pirofosfato (FERNANDES et al., 2005; FLEISCH, 1998; MELO e OBEID, 2005; MORTENSEN et al., 2007; RUSSEL et al., 1999; SARIN et al., 2008). Esses compostos têm sido amplamente aplicados na área médica nos últimos 30 anos em função de sua potente capacidade de inibir a reabsorção óssea (FARRUGIA et al., 2006; FERNANDES et al., 2005; MIGLIORATI et al., 2005). Classificam-se em não-nitrogenados e nitrogenados (FARRUGIA et al., 2006; WALTER et al., 2008), sendo o clodronato, o etidronato e o tiludronato exemplos de não-nitrogenados (MELO e OBEID, 2005), enquanto o alendronato, o pamidronato, o incandronato, o ibandronato, o risedronato e o ácido zoledrônico são representantes dos compostos nitrogenados (GREEN, 2004; WOO et al., 2006).

Embora o mecanismo de ação ainda não esteja completamente esclarecido, sabe-se que os bisfosfonatos alteram o metabolismo e a função dos osteoclastos (FLEISCH, 2002). Esses fármacos possuem alta afinidade pela hidroxiapatita presente na superfície mineral óssea, onde se fixam e, posteriormente, são liberados durante a reabsorção (GREEN, 2004), particularmente em regiões com intenso metabolismo, como a maxila e a mandíbula (FARRUGIA et al., 2006; FLEISCH, 1998; MARX et al., 2005; RUGGIERO e WOO, 2008; RUSSEL et al., 1999).

Em virtude de seu efeito sobre o metabolismo ósseo, são indicados no tratamento de metástases ósseas, principalmente do câncer de mama e de próstata, bem como do mieloma múltiplo, além de serem amplamente empregados para o tratamento da osteoporose, situações em que determinam significativa redução de complicações

como fraturas patológicas e compressão da medula espinhal (MAVROKOKKI et al., 2007; RUGGIERO et al., 2004; WOO et al., 2006). Segundo Greenberg (2004), os bisfosfonatos podem, também, ser empregados no tratamento da doença de Paget, da osteogênese imperfeita e da osteoporose juvenil idiopática ou induzida por esteroides.

Nos últimos anos, casos de osteonecrose maxilar associada ao uso de bisfosfonatos têm sido relatados na literatura mundial (GEGLER et al., 2006; FARRUGIA et al., 2006; MAVROKOKKI et al., 2007; MELO e OBEID et al., 2005; MORTENSEN et al., 2007; MURRAY et al., 2008; PARK et al., 2010; RUGGIERO et al., 2004; SANTOS et al., 2008; SHIN et al., 2010; ZUAZAGA et al., 2006). A condição é caracterizada por tecido ósseo necrótico exposto na região maxilofacial que persiste por mais de oito semanas em pacientes submetidos a terapia com bisfosfonatos e sem histórico de radioterapia prévia da região de cabeça e pescoço (RUGGIERO et al., 2009; WALTER et al., 2008).

Os bisfosfonatos mais frequentemente associados à osteonecrose maxilar são os nitrogenados, sobretudo o alendronato, o pamidronato e o ácido zoledrônico. Entre esses, os de uso intravenoso seriam responsáveis por um maior número de casos (MARX et al., 2005). Os intravenosos, em sua maioria, são indicados para o tratamento de doenças oncológicas, cujas doses são, em alguns casos, 12 vezes maiores do que aquelas administradas para o tratamento da osteoporose. Isso faz com que esses compostos liguem-se em maior quantidade à hidroxiapatita, e, consequentemente, torna a dose cumulativa no organismo elevada (WOO et al., 2006). Assim, apresentam maior meia-vida e podem provocar efeitos adversos por um longo período (BAMIAS et al., 2005; RUGGIERO e WOO, 2008). Embora seja um composto nitrogenado, o alendronato está associado a menor prevalência de osteonecrose maxilar, se comparado

ao ácido zoledrônico e ao pamidronato. Isso decorre da menor potência e menor absorção pela via oral e consequente menor dose cumulativa (WOO et al., 2006).

Há relatos de casos clínicos que associam o alendronato à osteonecrose dos maxilares ou a ulcerações da mucosa oral (BEDOGNI et al., 2010; BOCANEGRA-PÉREZ et al., 2009; DEMERIJAN et al., 1999; FERNÁNDEZ et al., 2006; JUNQUERA et al., 2009; LEVIN et al., 2007; MARX et al., 2005). Entretanto, em estudo prévio *in vivo*, ratos tratados com alendronato e submetidos a exodontias não desenvolveram osteonecrose, enquanto os tratados com ácido zoledrônico desenvolveram a lesão (MAAHS et al., 2011). Estudos como os de Lobato et al. (2007) e Woo et al. (2006) salientam que alguns cofatores como corticoterapia, diabetes mellitus, exodontias e outros procedimentos odontológicos invasivos podem estar associados ao desenvolvimento da osteonecrose maxilar. Entretanto, isso ainda não está experimentalmente comprovado.

É possível que cofatores como o diabetes mellitus e a corticoterapia desempenhem papel fundamental no desenvolvimento da osteonecrose em usuários de alendronato. Há relatos de que o diabetes aumenta a atividade da colagenase, tanto na pele quanto na mucosa, o que, altera o metabolismo do colágeno nesses sítios (RAMAMURTHY e GOLUB, 1983). Por outro lado, o efeito mais significativo dos corticosteroides no tecido ósseo é a inibição da formação óssea, que ocorre em função da redução do número de osteoblastos, que são responsáveis pela síntese de colágeno tipo I da matriz óssea (CANALIS e DELANY, 2002). O metabolismo do colágeno, portanto, sofre influência direta do diabetes e da corticoterapia. Neste contexto, destacam-se as metaloproteinases de matriz (MMPs), sobretudo a MMP-13, que cliva o colágeno sintetizado e acredita-se que esteja relacionada ao processo de reabsorção óssea (GEOFFROY et al., 2004; LEONARDI et al., 2005; NANNURU et al., 2010).

Outro aspecto importante são as proteínas morfogenéticas ósseas (BMPs), que participam do processo de formação e remodelação óssea (GARIMELLA et al., 2008).

A presente tese consiste em dois artigos científicos que investigam a influência da corticoterapia e do diabetes mellitus no desenvolvimento da osteonecrose maxilar associada ao uso de alendronato de sódio. No primeiro, é feita uma revisão da literatura sobre o tema, enquanto o segundo artigo apresenta o experimento desenvolvido em modelo animal.

2 ARTIGO 1

O artigo "**Effects of diabetes mellitus, corticotherapy and bisphosphonates on bone turnover and their relationship with osteonecrosis of the jaws - An update**" foi formatado e submetido de acordo com as normas do periódico Osteoporosis International (Anexos A e B).

Effects of diabetes mellitus, corticotherapy and bisphosphonates on bone turnover and their relationship with osteonecrosis of the jaws - An update

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Abstract

Bisphosphonates are drugs widely used in the treatment of metabolic bone diseases, due to their ability to inhibit bone resorption. However, these compounds have been associated with a characteristic type of osteonecrosis of the jaws. The great predisposition of the jaws to develop osteonecrosis in bisphosphonate users could be related to a peculiar metabolism pattern of these bones. There is only one case reported in the literature of this lesion affecting skeletal bones other than the jaw. Moreover, diabetes mellitus and corticotherapy have been reported as predisposing factors for bisphosphonate-related osteonecrosis of the jaws. We present here a literature review on the effects of diabetes, corticotherapy and bisphosphonates on bone turnover and the possible relationship of these effects with osteonecrosis of the jaws. Although there are many papers focusing on this potential influence, scientific confirmation by experimental studies with standardized and well-controlled methods is still necessary.

Keywords: Bisphosphonates, Bone remodeling, Corticotherapy, Diabetes mellitus

Mini abstract

Diabetes and corticotherapy have been reported as predisposing factors for bisphosphonate-related osteonecrosis of the jaws. This paper presents a literature review on the effects of these factors on bone turnover and their possible relationship with this lesion.

Introduction

Bisphosphonates are drugs with a chemical structure similar to that of pyrophosphoric acid, which, in the human body, assumes the pyrophosphate form [1-5]. These compounds have been widely used in the last 30 years due to their potent ability to inhibit bone resorption [6,7]. The use of bisphosphonates to treat multiple myeloma and bone metastases of breast and prostate cancer, as well as osteoporosis, has shown a significant reduction in skeletal complications, including pathologic fractures, spinal cord compression and hypercalcemia of malignancies [8-10]. These drugs are also used in the treatment of Paget disease, osteogenesis imperfecta and juvenile idiopathic or steroid-induced osteoporosis [11].

The medical use of bisphosphonates has led to concern about the effects of these drugs on bone remodeling [12]. Since 2003, many cases of bisphosphonate-related osteonecrosis of the jaws have been reported in the literature [4,6,8,9,13-18]. Several hypotheses regarding the mechanisms underlying this injury have been proposed [19], but its pathogenesis is still unclear. The risk of osteonecrosis development depends on the dose, duration of treatment, administration route and type of bisphosphonate [5,10,20]. It is known that 94% of the cases are related to intravenous use, particularly of pamidronate and zoledronic acid. Some predisposing factors such as diabetes mellitus and corticotherapy can also contribute to the onset of the lesion [5,20-23].

Knowledge about bone remodeling is crucial for understanding bisphosphonate-related osteonecrosis of the jaws. In addition, a substantial number of studies have investigated the changes and mechanisms responsible for bone turnover in the axial and appendicular skeleton [24]. We present here a literature review on the effects of diabetes mellitus, corticotherapy and bisphosphonates on bone turnover and their possible relationship with osteonecrosis of the jaws.

Bone metabolism of the jaws

Bone tissue is a dynamic structure, which is constantly being remodeled by a mixed process of bone resorption and formation (bone turnover) [25,26] to maintain the integrity of the mass and microarchitecture of the skeleton [27]. Bone turnover occurs exclusively on the bone surface and is initiated by osteoclasts, which remove mineralized bone [28, 29]. Although it is known that there is a balance between these processes in physiological conditions [30,31], the exact mechanism by which this occurs is still unclear [32]. In this delicate balance between bone resorption and bone formation, the former takes 7 to 10 days, while the latter takes 2 to 3 months [29].

Although only 20% of the skeletal mass is formed by trabecular bone, the bone surface, where bone turnover occurs, is composed of 80% trabecular and 20% cortical bone. That is, trabecular bone shows metabolism and remodeling rates that are much higher compared to cortical bone [29]. Jaws, in turn, show higher turnover rates compared to other skeletal sites [19,33]. In adults, typical remodeling rates are about 3% per year for cortical and 25% per year for trabecular bone [32]. In humans, intracortical remodeling rates of the jaws are 10 to 20 times higher than within the cortex of the iliac crest [34,35]. Studies with dogs have shown that intracortical remodeling in the mandible and maxilla alveolar bone is higher than in the basal region. In the alveolar region, the cortical bone has a turnover rate of approximately 25% per year, while the basal mandible turnover rate is around 7% per year [36, 37]. The remodeling rates within the alveolar and basal regions of maxilla are heterogeneous, with higher rates in the second premolar region compared to the second molar region. In the mature skeleton, the alveolar mandible has higher turnover than the alveolar maxilla. Animal studies support the limited human data, showing that remodeling rates in the jaws are significantly higher than in long bones [24].

Changes in bone turnover occur in response to mechanical forces such as orthodontic movement and implant osteointegration [26], in bone diseases such as osteoporosis, and also during long-term use of some drugs such as bisphosphonates [19,24,37-40]. In animals treated with alendronate, the alveolar remodeling rate was 18% and 39% lower than in controls, after 3 and 6 months of treatment, respectively [37]. A long period of alendronate use (3 years) can reduce mandibular bone turnover in dogs at rates of 58% and 84%, depending on the dose administered [36].

Odvina et al. [40] reported 9 cases of patients who had spontaneous fractures while on alendronate therapy, where six out of them displayed either delayed or absent fracture healing for 3 months to 2 years during therapy for osteoporosis. Histomorphometric analysis of the trabecular bone showed markedly suppressed bone formation, with reduced or absent osteoblasts on the bone surface in most patients, lower osteoclastic surface and decreased eroded surface. The authors concluded that alendronate could cause severe suppression of bone turnover.

Bisphosphonates and osteonecrosis of the jaws

Bisphosphonates are synthetic analogues of pyrophosphate, where the oxygen atom linking two phosphate groups (P-O-P) is replaced by a carbon atom (P-C-P) with different substituents [41,42]. After administration, these drugs bind quickly to bone mineral surface and are released during resorption, especially in areas of high bone turnover such as the jaws [1,2,6,21,43].

These compounds effectively inhibit bone resorption by means of decreasing osteoclast activity [1,2]. Bisphosphonates bind to bone crystals and are internalized by osteoclasts. This disturbs the mechanism of resorption mediated by these cells, causing the suppression of bone turnover, which can sometimes result in necrosis [4].

Bisphosphonates target the osteoclasts [9] and impair the function of these cells through the inhibition of their recruitment [44], reduction of lifespan [45] and inhibition of their activity on the bone surface [46]. These drugs can also upregulate osteoprotegerin (OPG) production by osteoblasts, which contributes to the inhibition of bone resorption. This occurs because OPG neutralizes the receptor activator of nuclear factor-kB ligand (RANKL), which is essential for osteoclast formation and activation [47].

Two classes of bisphosphonates are recognized depending on the presence or absence of a nitrogen atom in the molecule [10]. The non-nitrogen-containing compounds have lower potency and induce osteoclast apoptosis through the formation of cytotoxic adenosine triphosphate (ATP) analogues, which accumulate inside the osteoclast and, consequently, impair cellular metabolism [48-50]. The nitrogen-containing compounds, in turn, inhibit farnesyl pyrophosphate synthase and geranyl geranyl pyrophosphate synthase. Both of them are prenylation proteins involved in the synthesis of cholesterol in the mevalonate biosynthetic pathway and are considered essential to osteoclast functions [43,49,50-52]. This inhibitory effect disrupts cytoskeletal function and intracellular signaling, which leads to osteoclast apoptosis and impaired osteolytic activity [42,52,53].

Osteonecrosis of the jaws is an important complication associated with bisphosphonate use, which was first described by Marx in 2003 [13]. Since then, many cases have been reported in the literature [4,6,8,9,14-18]. This condition is characterized by exposed bone in the maxillofacial region that has persisted for more than eight weeks in a patient who was previously or is currently under treatment with bisphosphonate, and with no history of head and neck radiation therapy [54].

The sites affected by bisphosphonate-associated osteonecrosis are the mandible in 65% of cases, the maxilla in 26%, and both jaws simultaneously in 9%. Multifocal or bilateral involvement is slightly more common in the maxilla than in the mandible [10]. Some theories have tried to explain the restricted occurrence in these bones. First, it is pointed out that jaws are "protected" from the oral microflora and continuous trauma only by the periosteum and a thin mucosa. The second theory refers to the fact that the teeth, which may be associated with microorganisms that induce caries and periodontal disease, are close to the alveolar bone, which would favor its infection [8,10]. Another supported theory is that dentoalveolar surgical procedures are frequent and lead to bone exposure and bacterial colonization [8,43]. Still, the long-term use of bisphosphonates can change bone turnover, which causes the accumulation of small lesions at these sites. The result is a hypodynamic bone with reduced biomechanical properties [4,10,33]. Some contributing factors to the occurrence of jaw osteonecrosis are pointed out, such as cortico- and chemotherapy, diabetes mellitus, alcoholism, smoking, obesity, periodontal disease, denture wearing and, mainly, tooth extraction and other invasive dental procedures [5,8,20-23,55,56].

Several studies have been developed focusing on the mechanisms involved in the turnover rates of the jaws [24,57]. It has been suggested that bone turnover rates can be modified by metabolic and mechanical demands [24]. According to Allen and Burr [36], higher rates of bone metabolism in the jaws may help explain the higher prevalence of osteonecrosis in these bones. Until now, only one case of bisphosphonate-related osteonecrosis outside the oral cavity was reported in the literature. Osteonecrosis in this case occurred in the auditory canal in a patient with multiple myeloma, who was treated initially with pamidronate and, afterwards, with zoledronic acid [58].

Diabetes mellitus

Diabetes mellitus is becoming one of the leading disorders worldwide, whose estimated prevalence was 285 million adults in 2010 and which will increase to 439 million adults by 2030 [59]. This disease is a group of metabolic disorders characterized by elevated blood glucose levels, which result from insulin secretion deficiency or increased cell resistence to the insulin effects in the body [60]. Another important aspect associated with it are the changes in bone metabolism [61] that may lead to osteopenia, increased risk of fracture and osteoporosis, which has been reported in both humans [62-66] and animals [67-69].

The effects of diabetes mellitus on falls, bone mass, bone turnover and fractures have been investigated [70]. Hyperglycemia has been implicated in the pathogenesis of diabetic bone disease; however, the biologic effect of glucose on osteoclastogenesis is unclear, and the bone disease that develops in type 1 and type 2 diabetes may differ from each other. In type 1 diabetes patients, bone mineral density is reduced more than 10% compared to nondiabetics, whereas in type 2, although the bone density is often increased, it is also associated with a high risk for fractures, which could be explained by the combination of frequent falls and poor bone quality [71,72].

Diabetes mellitus can affect bone through multiple pathways, including obesity, changes in insulin levels, high concentrations of advanced glycation end products in collagen, hypercalciuria associated with glycosuria, reduced renal function, microangiopathy, and inflammation [71]. It has also been suggested that high glucose levels may alter bone turnover by decreasing osteoclast differentiation and function [72]. In premenopausal diabetic women, the disease is accompanied by high bone turnover [73-75], whereas elderly women with type 2 diabetes have decreased [76,77] or unchanged [78] bone turnover. The results are difficult to evaluate, since many of the

studies have been conducted in patients with severe kidney disease or without taking into account the patients' age or type of diabetes [79].

Streptozotocin-induced diabetes reduced bone formation rates as well as the number of osteoclasts in the alveolar wall in rats [80]. Also, mandibular osteoporosis [81] and reduced mandibular growth [61] were observed in rats with respectively alloxan-induced and streptozotocin-induced diabetes. Liu et al. [82] found a significant reduction in bone neoformation in distraction gaps of diabectic rats, which was assessed radiographically and histologically. Reinwald et al. [83] assessed bone alterations in type 2 diabetes, in two different strains of rats, using dual-energy X-ray absorptiometry, peripheral quantitative tomography, and micro-CT. The results showed lower bone mineral densities in the femoral midshaft and in the L4 vertebrae associated with diabetes.

A higher prevalence of diabetes mellitus was observed in a group of patients with cancer and bisphosphonate-related osteonecrosis of the jaws compared to the group with cancer that had not developed osteonecrosis. This finding suggests that diabetes could be a risk factor for the development of the lesion, especially during the use of intravenous bisphosphonate [55]. The microvascular changes in diabetes were pointed out as having a role in the predisposition of alveolar bone to osteonecrosis [84]. In this context, it is important to recall that diabetes is associated with decreased bone remodeling [85] and increased collagenase activity, either in skin or mucosa, which alters collagen metabolism at these sites [80].

Corticotherapy

Corticosteroids are used in the treatment of several diseases because of their potent anti-inflammatory effect and also the ability to suppress immune cell activity [86]. The long-

term use of these drugs can generate some adverse effects on bone structure, which has been recognized for more than 60 years [87]. These drugs have a significant impact on bone cells, and the continued exposure of bone tissue to them can induce osteoporosis [88]. Glucocorticoid-induced bone disease is characterized by decreased bone formation and *in situ* death of isolated segments of bone (osteonecrosis) suggesting that corticosteroid excess may affect the birth or death rate of bone cells, thereby decreasing their numbers [89]. Mankin [90] showed that patients receiving long-term corticotherapy sometimes develop a kind of collapse of the femoral head, resulting in osteonecrosis at this site. Due to the pattern of its blood supply, the femoral head is particularly vulnerable to avascular necrosis, which is a devastating disorder affecting young patients, and despite treatment it follows a progressive course toward a destructive osteoarthropathy [91].

The most significant effect of corticosteroids on bone is the inhibition of bone formation that occurs due to the reduction in osteoblast numbers. These cells are responsible for the synthesis of type I collagen, the major component of bone extracellular matrix. Glucocorticoids may also affect osteoblast function, resulting in an inhibition of the synthesis of type I collagen, and consequently decreased bone matrix available for mineralization. The effects on bone resorption include the increase of both osteoclastogenesis and activity of collagenases [92,93]. The latter are matrix metalloproteinases (MMPs) that cleave collagen fibrils [93]. The synthesis of collagen, as well as serum osteocalcin and alkaline phosphatase levels, both of them bone formation markers, was drastically reduced in a group of animals treated with methylprednisolone [94].

The effect of corticosteroids on bone surface has been suggested to be associated with the development of bisphosphonate-related osteonecrosis of the jaws [10,20]. Sonis

et al. [95] evaluated the changes in mandibular and maxillary bones of rats treated with zoledronic acid with and without corticosteroid (dexamethasone), which were subjected to tooth extractions. These authors showed that animals treated simultaneously with zoledronic acid and corticosteroid, over a one- to three-week period, developed osteonecrosis at higher rates compared to either the group treated only with zoledronic acid or the control group. These results suggest a positive correlation between corticosteroid use and osteonecrosis of the jaws associated with zoledronic acid.

Final considerations

Bisphosphonates are widely used in the treatment of bone metabolism diseases, showing for this purpose good results [6,7]. However, they are also associated with osteonecrosis of the jaws, which is an important adverse effect. The management of this condition remains a challenge [9], which implies that there is sometimes a poor patient quality of life [55]. Some predisposing factors such as diabetes mellitus, corticotherapy and tooth extraction could contribute to this injury onset [5,20-23,96].

High blood glucose levels may decrease osteoclast differentiation and function, besides increasing collagenase activity [72,80]. Moreover, long-term glucocorticoid use decreases bone formation rates, which occurs due to the reduction in osteoblast numbers. Likewise, bisphosphonates interfere with these mechanisms as well, which suggests a multiplied effect when these drugs are administered to diabetic patients or combined with corticotherapy. All these alterations impair bone formation and bone resorption, which can contribute to osteonecrosis onset in bisphosphonate users [84,97].

There seems to be evidence that either diabetes or glucocorticoids can be a risk factor for bisphosphonate-related osteonecrosis of the jaws. However, such evidence has not yet been experimentally tested. Furthermore, the degree at which these factors can

influence the development of osteonecrosis in bisphosphonate users has not been determined. Therefore, further experimental studies are necessary to elucidate the actual role of glucocorticoids and diabetes as co-factors in the development of osteonecrosis of the jaws especially in oral bisphosphonate users. These studies would improve the clinical approach for the patients and would help prevent bisphosphonate-related osteonecrosis of the jaws.

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3 ARTIGO 2

O artigo "**Diabetes mellitus and corticotherapy as risk factors for alendronate-related osteonecrosis of the jaws: a study in Wistar rats**" foi formatado e submetido de acordo com as normas do periódico Osteoporosis International (Anexos A e C).

Diabetes mellitus and corticotherapy as risk factors for alendronate-related osteonecrosis of the jaws: a study in Wistar rats

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Abstract

Purpose: This study aimed at investigating the influence of diabetes and corticotherapy on the development of osteonecrosis of the jaws associated with sodium alendronate.

Methods: Rats were allocated into 4 groups of 11 animals each according to the treatment received: (1) alendronate; (2) alendronate and corticotherapy; (3) alendronate and induced diabetes; and (4) control. Tooth extractions were performed in all animals 90 days after starting bisphosphonate therapy. The histological features were analyzed by hematoxylin and eosin (H&E) staining and immunohistochemical assay using anti-BMP-4 and anti-MMP-13 antibodies.

Results: On H&E analysis, inflammatory infiltrate, microbial colonies and areas of osteonecrosis were significantly greater in the diabetes group, whereas the other variables did not significantly differ. BMP-4 immunohistochemical expression was higher in the connective tissue of the corticosteroid group than in alendronate group. There were no significant differences between the other groups. MMP-13 expression did not differ between the groups analyzed.

Conclusions: Diabetes acts as a risk factor in the development of jaw osteonecrosis associated with alendronate therapy. This relationship was not observed for corticotherapy.

Keywords: Bisphosphonates, BRONJ, Corticotherapy, Diabetes mellitus

Mini abstract

Some risk factors have been associated with bisphosphonate-related osteonecrosis of the jaws. This study investigated the influence of diabetes and corticotherapy on the

development of osteonecrosis of the jaws associated with sodium alendronate. The data show that diabetes may contribute to the development of this lesion

.

Introduction

Bisphosphonates are drugs that have been widely used in the last years because of their ability to inhibit bone resorption [1]. They are indicated in the treatment of bone metastases, especially in breast and prostate cancers, as well as in multiple myeloma, osteoporosis, and malignant hypercalcemia, where these agents produce a significant reduction in skeletal complications, such as pathologic fractures and spinal cord compression [2,3]. Despite the high efficacy of bisphosphonates in the treatment of several diseases, some side effects have been reported, including osteonecrosis of the jaws [3,4]. In 2003, Marx [3] reported the first cases of bisphosphonate-related osteonecrosis of the jaws (BRONJ), and since then, many other cases have also been reported [1,5].

Although the bisphosphonates most frequently associated with BRONJ are the aminobisphosphonates, which include alendronate, pamidronate and zoledronic acid, intravenous administration has been associated with the majority of cases [2,6]. This route of administration is more indicated for cancer treatment and, in some cases, the dose can be 12 times higher than that administered for osteoporosis treatment. Hence, the cumulative dose of intravenous bisphosphonate allows a progressive binding of the drug to a greater amount of hydroxyapatite in the human body [3]. In addition, bisphosphonates have a long half-life, causing side effects over an extended long period [2]. Although alendronate is an aminobisphosphonate, it is associated with a lower prevalence of BRONJ when compared to zoledronic acid and pamidronate [7]. This is due to its oral administration route, which results in lower absorption and lower cumulative dose [3].

Some cases of osteonecrosis of the jaws and oral mucosal ulcerations associated with alendronate have been reported in the literature [5,8]. However, in a previous *in*

vivo study, rats treated with alendronate and subjected to tooth extractions did not develop jaw osteonecrosis [9]. Some authors [2,3] have reported that risk factors such as chemo- and corticotherapy, diabetes, infectious diseases, malnutrition, alcoholism, smoking, periodontal disease, exostoses, denture wearing and, mainly, tooth extractions or other invasive dental procedures may be associated with the development of osteonecrosis of the jaws. However, the specific role of these risk factors has not yet been experimentally proved.

It is possible that some risk factors such as diabetes and corticotherapy play a fundamental role in the development of osteonecrosis of the jaws in patients taking alendronate. In those cases, the increased collagenase activity in skin and mucosa induced by diabetes mellitus, which changes collagen metabolism at these sites, supports this notion [10]. Diabetes is also associated with microvascular ischemia of bone [11], bone turnover and remodeling decrease [12], and induction of osteoblast and osteocyte apoptosis [13]. On the other hand, the most significant effect of corticosteroids on bone is the inhibition of its neoformation, which occurs through the reduction in osteoblast numbers. These drugs are responsible for inhibiting the synthesis of type I collagen, the main constituent of the organic bone matrix [14,15].

Collagen metabolism is therefore closely related to diabetes and corticotherapy. In this context, it is important to consider matrix metalloproteinase 13 (MMP-13), which cleaves the synthesized collagen and appears to be related to bone resorption [16,17]. Another important aspect is the role played by bone morphogenetic proteins (BMPs) in the bone remodeling process [18], especially BMP-4, which is related to bone formation.

Therefore, this work aimed at investigating the influence of diabetes and corticotherapy in the development of osteonecrosis of the jaws associated with alendronate treatment in Wistar rats subjected to tooth extractions.

Methods

Animals

This study was approved by the Ethics Committee for Animal Use of the Pontifical Catholic University of Rio Grande do Sul. During the experiment, animals were humanely treated and maintained in plastic cages, which were placed on ventilated racks (Alesco, Monte Mor, SP, Brazil), with controlled temperature (22±1^{o}C) and a 12h/12h light-dark cycle, with food (Nuvilab, Colombo, PR, Brazil) and filtered water *ad libitum*.

The sample comprised 44 female rats (*Rattus norvegicus*, Wistar strain), which were 140 days old and had a mean weight of 250 g at the beginning of the study. The animals were randomly allocated into 4 groups: (1) 11 rats treated with alendronate (alendronate group); (2) 11 rats treated with alendronate and corticosteroid (corticosteroid group); (3) 11 rats treated with alendronate and subjected to experimentally induced diabetes (diabetes group); (4) 11 rats treated with saline (control group). All animals were subjected to tooth extractions.

Experimental protocol

The drugs used were sodium alendronate (Pharma Nostra, Campinas, SP, Brazil), prednisolone sodium phosphate (Pharma Nostra, Campinas, SP, Brazil), and streptozotocin (Sigma Chemical Co., St Louis, MO, USA), which were always

administered after weighing the animals. The alendronate dose was 0.05 mg/kg [19] administered weekly to the experimental groups (1, 2 and 3) by subcutaneous injection for 111 days. The prednisolone sodium phosphate dose was 5 mg/kg/day [20] administered subcutaneously, where the steroid was given starting 30 days before and finishing 21 days after the tooth extractions.

For diabetes induction, the animals (diabetes group, n=11) received 10% D-glucose anhydrous (Dextrose, Labsynth, Diadema, SP, Brazil) in the drinking water for 11 weeks [21]. After this period, one dosage of 30 mg/kg of streptozotocin dissolved in citrate buffer (pH 4.5) was administered intraperitoneally [22]. Fasting blood samples were collected 48 hours after the administration of streptozotocin, and the glucose levels were measured using a glucometer (One Touch Ultra Mini, Johnson & Johnson, São José dos Campos, SP, Brazil). The animals were considered diabetic when blood glucose levels were equal to or greater than 150 mg/dL [22]. Two animals from the diabetes group died as a consequence of streptozotocin administration, resulting in a final sample of 9 animals in this group. Each animal from the control group received 1 mL/kg saline by subcutaneous injection, weekly, for 111 days.

Clinical evaluation

Clinical evaluation was carried out using a dental mirror, a no. 5 probe (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) and a magnifying glass (x 3), before the tooth extractions. This procedure aimed to verify the presence or absence of oral lesions prior to the surgical interventions.

Tooth extractions

The tooth extractions were performed 90 days after the beginning of the experiment. Animals were anesthetized with a mixture of ketamine 10% [100 mg/kg (Syntec, Cotia, SP, Brazil)] and xylazine 2% [10 mg/kg (Syntec, Cotia, SP, Brazil)] administered intraperitoneally. The animal was positioned in dorsal decubitus, with the mouth held opened by rubber bands anchored to the upper and lower incisors, stretched and fixed to the operating table. The three right upper molars were extracted using a 3s spatula (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) and a pediatric forceps (Edlo, Canoas, RS, Brazil) adapted to the size of the animals' teeth. After the surgical procedure, paracetamol (Medley, Campinas, SP, Brazil) was administered intraperitoneally at 50 mg/kg.

Euthanasia

After 111 days from the beginning of the experiment and 21 days from the tooth extractions, euthanasia was performed using isoflurane (Cristália, Porto Alegre, RS, Brazil) inhalation in an appropriate anesthesia chamber. Afterwards, the maxillae were dissected.

Preparation of the specimens

The maxillae were fixed for 24 hours in 10% buffered formalin (Top Glass, Porto Alegre, RS, Brazil). Afterwards, each specimen was sectioned into two fragments, in the coronal direction with a steel-sanding disk at low speed [9]. The specimens were then decalcified with formic acid solution, for 24 hours and paraffin-embedded.

Histological processing

Eighty-four paraffin blocks were prepared, 2 blocks for each animal. Three histological slides, one with 5-µm-thick sections and two with 3-µm-thick sections, were obtained from each block as follows. One slide for hematoxylin and eosin staining (H&E) and the other two for immunohistochemical processing with anti-BMP-4 and anti-MMP-13 antibodies. In the immunohistochemical technique, the slides were subjected to antigen retrieval in a 100^{o} C water bath for 40 minutes, using pH 6 Dako Target Retrieval Solution (Dako, Carpinteria, CA, USA). Endogenous peroxidase was blocked with a 3% solution of hydrogen peroxide in methanol, in two incubations of 15 minutes each. The sections were incubated with polyclonal antibody anti-BMP-4 (Novocastra, Newcastle, UK) and monoclonal antibody anti-MMP-13 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The detection system used was the Picture Max, HRP Polymer Conjugate Broad Spectrum (Invitrogen, Carlsbad, CA, USA). Color development was with the chromogen 3,30-diaminoazobenzidine and phosphate buffer solution containing 0.002% hydrogen peroxide, and slides were then stained with hematoxylin, dehydrated, cleared, and coverslipped.

Histological evaluation

The images were captured with a Zeiss Axioskop 40 light microscope (Zeiss, Oberkochen, Germany), connected by a video camera Roper Scientific (Media Cybernetics, Silver Spring, MD, USA) to a Pentium IV 2.2 GHz with 512 MB RAM and hard disk of 160 GB and Image Pro Capture Kit Platform (Media Cybernetics, Silver Spring, MD, USA). The images were captured using 5x and 20x objectives, respectively, for H&E and immunohistochemical analyses, and stored in JPEG format. In each of the H&E and immunohistochemical slides, 4 fields were selected aiming to

cover the entire tooth extraction area. The digital images were analyzed with the Image Pro Plus 4.5.1 software (Media Cybernetics, Silver Spring, MD, USA). The quantitative analysis of epithelial tissue, connective tissue, inflammatory infiltrate, root fragments, microbial colonies, vital bone and osteonecrosis, was carried out on the H&E images using the manual point counting technique with a grid of 688 points. The quantitative analysis of immunohistochemical expression of BMP-4 and MMP-13 proteins was performed by means of semi-automated segmentation technique.

The assessment of histological images was performed by a single blinded and calibrated observer. The calibration was done by the analysis of 20 H&E images and 20 immunohistochemical images, twice and at two different times. The results of these two analyses were tested by Pearson's correlation coefficient and the paired t test, which showed, respectively, strong correlation ($r>0.8$) and no significant difference ($p>0.05$).

Statistical analysis

The results were analyzed using descriptive statistics and the qui-square and Kruskal-Wallis tests (complemented by the multiple comparisons test). The amount of epithelial tissue, connective tissue, inflammatory infiltrate, root fragments, microbial colonies, vital bone and osteonecrosis, and BMP-4 and MMP-13 expression were compared between the groups. The level of significance was set at 5% and data were processed by SPSS 17.0 (Statistical Package for the Social Sciences, Chicago, IL, USA).

Results

H&E

Table 1 shows the frequency of osteonecrosis at the tooth extraction site in the alendronate, corticosteroid, diabetes and control groups, which was significantly higher in the diabetes group (chi-square test; analysis of adjusted residuals, $p<0.001$).

Table 1 Frequency of osteonecrosis at the tooth extraction site in the alendronate, corticosteroid, diabetes and control groups

Group	Total	Present		Absent	
	N	n	%	n	%
Alendronate	11	0	0	11	100
Corticosteroid	11	3	27.3	8	72.7
Diabetes	9	7*	77.8	2	22.2
Control	11	0	0	11	100
Total	42	10	23.81	32	76.19

n = Sample size
*Chi-square test; analysis of adjusted residuals, $p <0.001$.

Table 2 shows the results for the amount of each histological variable in the H&E stained sections in the area previously submitted to tooth extractions (Fig 1). The proportion of epithelium did not differ significantly between the groups; the amount of connective tissue was significantly less in the diabetic group compared to the alendronate and corticosteroid groups; the proportion of inflammatory infiltrate, microbial colonies and osteonecrosis was significantly higher in the diabetes group compared to the others; and in the corticosteroid and diabetes groups, vital bone was significantly less than in the control (Kruskal-Wallis, multiple comparisons test, $p<0.05$).

Table 2 Quantification of histological variables (%) at the tooth extraction site in the alendronate, corticosteroid, diabetes and control groups

Histological variable	Groups											
	Alendronate (n=11)			Corticosteroid (n=11)			Diabetes (n=9)			Control (n=11)		
	Median	P25	P75	Median	P25	P75	Median	P25	P75	Median	P25	P75
Epithelium	12.12^{A}	10.48	18.57	13.55^{A}	12.02	16.02	13.91^{A}	12.28	15.45	13.37^{A}	11.5	14.84
Connective tissue	38.6^{A}	33.75	42.54	38.95^{A}	28.54	44.26	18.25^{B}	17.27	29.71	29.36^{AB}	28.17	33.3
Inflammatory infiltrate	2.53^{B}	0.9	5.2	1.83^{B}	0.29	4.5	21.24^{A}	7.07	25.24	0.95^{B}	0	2.62
Root fragments	4.01^{A}	0.92	8.09	6.04^{A}	2.93	10.17	8.16^{A}	3.28	15.78	6.11^{A}	3.93	9.42
Microbial colonies	0^{B}	0	0	0^{B}	0	0.53	2.35^{A}	0.52	3.14	0^{B}	0	0.49
Vital bone	39.86^{AB}	31.26	52.5	36.49^{B}	31.98	42.45	24.42^{B}	15.1	40.35	44.16^{A}	43.4	51.27
Osteonecrosis	0^{B}	0	0	0^{B}	0	0.36	4.6^{A}	1.23	8.02	0^{B}	0	0

n = Sample size
Different letters in the same row indicate a significant difference by the Kruskal-Wallis test complemented by the multiple comparisons test ($p \leq 0.05$).
P25 = 25 percentile
P75 = 75 percentile

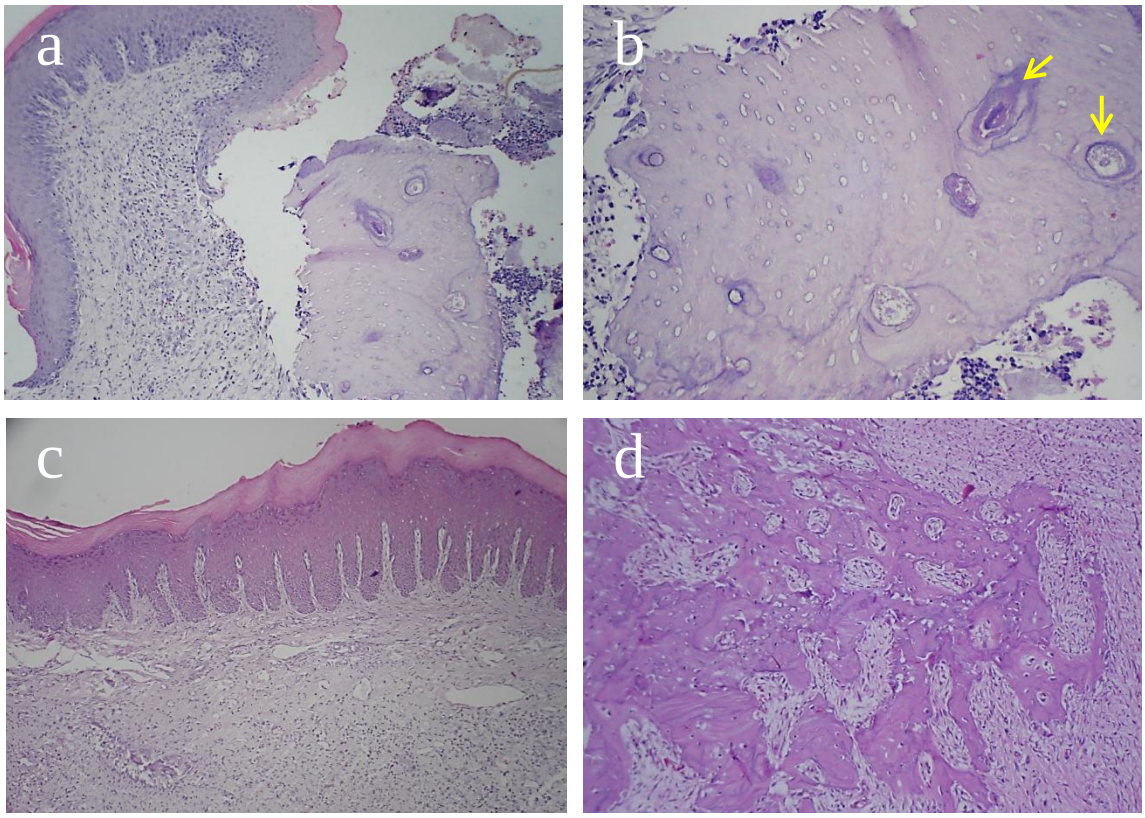

Fig 1 Site of tooth extraction after 21 days of surgical procedure. **a** Diabetes group showing necrotic bone associated with a significant amount of microorganisms and inflammatory infiltrate (H&E, 100X). **b** Osteonecrosis characterized by the presence of empty bone lacunae and bone marrow spaces infected by microorganisms in the diabetes group (arrows, H&E, 400X). **c** Control group showing stratified squamous epithelium and dense connective tissue showing normal healing (H&E, 100X). **d** Vital bone tissue and normal bone marrow in the control group (H&E, 200X).

BMP-4 and MMP-13

BMP-4 immunohistochemical expression was significantly higher in the connective tissue of the corticosteroid group than of the alendronate group, but these two groups did not differ from the diabetes and control groups (Table 3, Kruskal-Wallis, multiple comparisons test; $p<0.05$). MMP-13 immunohistochemical expression did not show significant difference, either in the area of connective tissue or bone tissue, between experimental and control groups (Table 3, Kruskal-Wallis, multiple comparisons test; $p>0.05$).

Table 3 BMP-4 and MMP-13 immunohistochemical expression at the tooth extraction site in the alendronate, corticosteroid, diabetes and control groups

Group		BMP-4				MMP-13			
		Connective tissue		Bone tissue		Connective tissue		Bone tissue	
		μm^2	%	μm^2	%	μm^2	%	μm^2	%
Alendronate	Median	301.8^{B}	0.77^{B}	455.7^{A}	1.17^{A}	972.4^{A}	2.49^{A}	3179.5^{A}	8.15^{A}
	P25	233.2	0.6	80.6	0.21	816.9	2.09	2327.3	5.96
	P75	496.3	1.27	763.0	1.95	1438.4	3.69	4218.6	10.81
Corticosteroid	Median	1235.0^{A}	3.16^{A}	798.0^{A}	2.04^{A}	2188.0^{A}	5.61^{A}	3428.6^{A}	8.78^{A}
	P25	369.1	0.95	577.9	1.48	742.2	1.9	2570.7	6.59
	P75	1348.7	3.46	986.6	2.53	3186.6	8.16	4536.1	11.62
Diabetes	Median	626.1^{AB}	1.6^{AB}	614.1^{A}	1.57^{A}	1060.6^{A}	2.72^{A}	2625.6^{A}	6.73^{A}
	P25	339.3	0.87	407.2	1.04	612.6	1.57	1322.7	3.39
	P75	1060.1	2.72	905.4	2.32	1547.5	3.96	4172.5	10.69
Control	Median	521.2^{AB}	1.34^{AB}	957.9^{A}	2.45^{A}	1298.5^{A}	3.33^{A}	2491.3^{A}	6.38^{A}
	P25	382.3	0.98	472.7	1.21	1062.8	2.72	1799.2	4.61
	P75	691.6	1.77	1167.7	2.99	1747.3	4.48	3569.7	9.15

Different letters in the same column indicate groups that differed significantly by the Kruskal-Wallis test complemented by the multiple comparisons test (α=0.05).
P25 = 25 percentile
P75 = 75 percentile

Discussion

The role of risk factors such as diabetes and corticotherapy in the etiopathogenesis of BRONJ has been proposed by clinical studies [1,5]. Evidence suggests the possibility of these factors playing a key role in the case of lesions associated with sodium alendronate [9], however, this hypothesis needs to be experimentally proved. In this study, animals treated with sodium alendronate and subjected to diabetes induction showed a prevalence of osteonecrosis that was significantly higher than in the other groups. This finding corroborates the idea that diabetes mellitus increases the chance of lesions developing in sodium alendronate users and, therefore, acts as a risk factor, as already suggested [3].

The possibility of diabetes potentiating osteonecrosis risk during alendronate use is reinforced by the bone metabolism changes related to high blood glucose levels. These changes include inhibition of osteoclast differentiation and function, as well as induction of osteoblast and osteocyte apoptosis [13]. These effects alter neoformation and resorption rates [12,13] with consequent decrease in the production of collagen, the major constituent of bone matrix. Still, the microvascular changes in diabetes, combined with the effects exerted by bisphosphonate, could favor the development of alveolar bone necrosis [23]. It was also observed that premenopausal women with type 1 diabetes show increased bone turnover [24], whereas in elderly women with type 2 diabetes, turnover is either decreased [25] or unchanged [26]. Since bone metabolism changes can depend on the type of diabetes and because of the high prevalence of type 2 diabetes patients, we chose to induce this type of disease by adding glucose to the drinking-water [21] and intraperitoneal streptozotocin administration [22].

Bisphosphonates are potent inhibitors of bone resorption, whose effect is exerted after they bind to hydroxyapatite crystals and are internalized by osteoclasts [27]. Aminobisphosphonates, the group to which alendronate belongs, inhibit farnesyl pyrophosphate synthase (FPPS) and geranylgeranyl pyrophosphate synthase (GGPP), both prenylation proteins involved in the mevalonate pathway of cholesterol synthesis, which are essential to cell physiology. This inhibition disturbs both intracellular transport and cell proliferation resulting in osteoclast inactivation, with consequent bone turnover suppression [2]. Therefore, we can consider that diabetes potentiates the impairment of bone metabolism associated with alendronate, which increases the risk for osteonecrosis.

We did not find any significant differences in osteonecrosis occurrence when animals simultaneously treated with alendronate and corticosteroid were compared to

those from the alendronate and control groups. This result disagrees with literature reports about the development of osteonecrosis associated with concomitant use of zoledronic acid and dexamethasone [28]. However, it should be recalled that zoledronic acid combined with tooth extraction provides sufficient conditions for the development of this lesion, regardless of corticosteroid administration [9].

The corticosteroid group did not show any association with osteonecrosis, but it had a lower proportion of vital bone than did the control group, which suggests that concomitant use of this drug and alendronate led to reduced bone neoformation rates. This finding corroborates the literature reports, according to which the main effect of corticosteroid on bone tissue is reduction in osteoblast numbers [14,15], with retardation [20] and inhibition of bone formation.

Our results disagree with some studies that report corticotherapy as a risk factor for BRONJ in sodium alendronate users [2,3]. It is important to consider that even without significant difference, 3 animals from the corticosteroid group developed osteonecrosis. Maybe, if we had used a larger sample size, or a longer period of coticosteroid administration, this result could have been different. However, taking into account the risk of losing animals because of the degrading effects of the drug administration and the long-term experiment, we decided to start corticotherapy 30 days before the surgical procedures and finishing 21 days afterwards. This 21-day period was based on the fact that at this time there is bone neoformation occurring in tooth extraction wounds in rats [29]. On the other hand, type 1 collagen formation is already reduced in this period [30]. Nevertheless, even considering the possibility of the corticosteroid being a potential risk for osteonecrosis, it seems evident that this risk is significantly lower than it is in diabetes.

In the group treated only with sodium alendronate, no animal developed osteonecrosis, which indicates that the use of this bisphosphonate without any other cofactor represents a low risk for the lesion after tooth extraction. This finding corroborates literature reports [6,9,31]. Although alendronate is a nitrogen-containing bisphosphonate, it is associated with a lower prevalence of BRONJ compared to zoledronic acid and pamidronate. This is principally related to its oral route of administration, which implies lower absorption and lower cumulative dose in bone tissue [3].

We administered to rats the human equivalent dose [19], which also considers the higher elimination rates related to the accelerated metabolism of these animals [32]. Based on allometric scaling, the sodium alendronate dose used was 0.05 mg/kg/week, administered subcutaneously for 111 days [19]. Some studies have used higher doses [33] of alendronate which could be capable of inducing osteonecrosis and other adverse reactions as well [9], but we preferred the human equivalent dose [19]. Other important factors to consider are the route of administration and duration of use. Oral bioavailability of alendronate is a variable that is hard to control, especially in animal models, because of the presence of food in the stomach, which reduces these rates up to 85% [34]. Maahs et al. [9] did not find osteonecrosis in animals treated with sodium alendronate by oral gavage and subjected to tooth extractions. We avoided the oral administration bias derived from the difficulties in maintaining animals in required fasting, by using the subcutaneous route, which guarantees bioavailability. Moreover, considering that a longer time of use of oral bisphosphonate corresponds to higher risk of osteonecrosis [6], we maintained the animals under treatment for 111 days, a longer period compared to other studies using the same animal model [33].

Immunohistochemical expression of BMP-4 in the connective tissue of the tooth extraction site was significantly higher in the corticosteroid group than in the alendronate group. Nevertheless, these two groups did not significantly differ from the diabetes and control groups for this variable. On the other hand, BMP-4 in bone tissue did not show any significant differences between the groups analyzed. It is known that BMPs participate in bone remodeling [18] and repair processes [35], as they promote the differentiation of mesenchymal and medullary cells into chondroblasts and osteoblasts [29,36]. BMP-4 is directly related to bone formation by osteoblasts, whereas prolonged administration of corticosteroid can lead to bone loss and consequent osteoporosis, as reported in experimental [37] and clinical studies [38]. Considering these reports, BMP-4 expression should have been lower in the corticosteroid group, but we observed the opposite for connective tissue. It seems that the BMP-4 results for connective tissue can be explained by the fact that this protein stains positive in the mesenchymal cells found in this tissue [36]. However, this does not necessarily mean that osteoblast differentiation is actually happening or even that osteoblasts are producing bone matrix. On the other hand, although the classical reported effect of corticotherapy is the inhibition of osteoblast function [14], some *in vitro* studies report that corticosteroid increases the differentiation of osteoprogenitor bone cells [39], which agrees with the higher expression of BMP-4 in the corticosteroid group. There seems to be a positive correlation between corticotherapy and BMPs [39]. Boden et al. [40] investigated different effects of BMPs 2, 4 and 6 on osteoblast differentiation in cultures of calvarial bone cells of rats. These authors used corticosteroid as an initiator agent of osteoblast differentiation and observed that BMPs effect was increased up to 10 times with steroid use.

MMP-13 expression did not significantly differ between the groups, in connective tissue or in bone tissue. MMPs play an important role in bone resorption, because they are capable of degrading extracellular matrix components [41]. These proteins comprise an endoperoxidase family divided into the collagenases, gelatinases, stromelysins, and the membrane-type. MMP-13 is a collagenase produced by fibroblasts, epithelial cells, malignant squamous epithelial cells, plasma cells associated with destructive bone lesions, and osteoblasts [11,42]. Its expression is associated with destructive bone lesions [42] and chronic periodontitis [43]. As this protein is related to bone resorption and, consequently, to the degradation of non-mineralized bone matrix [16,17], which is mostly composed of type 1 collagen [18], and considering that diabetes and corticosteroid use interfere with collagen metabolism, we expected its significantly higher expression in these two groups. However, we did not obtain such results. This can be related to the fact that sodium alendronate, which was administered to all experimental groups, while exerting the inhibition of bone resorption, can prevent higher expression of MMP-13. That is, osteonecrotic lesions are not associated with greater degradation of bone tissue, but to its loss of vitality and turnover. Notably, CTX, an examination to evaluate collagen degradation, has shown reduced levels in bisphosphonate users [31]. Therefore, the expression of MMP-13 in the alendronate, corticosteroid and diabetes groups suggests that alendronate would be capable of neutralizing some of the diabetes and corticotherapy effects on bone metabolism. Also, it seems plausible to infer that regarding diabetes, there are other factors such as infection [44] and microvascular alterations [11,45] contributing to the risk factor role of this disease in the pathogenesis of BRONJ. Still, the classical effect of corticosteroid of inhibiting osteoblasts, which produce proteolytic proteins such as MMPs, could lead to the downregulation of MMP-13, as previously observed [16].

Although being bone markers, in this study, BMP-4 and MMP-13 did not show the expected changes. Anyway, the H&E results evidenced diabetes as a risk factor for osteonecrosis. Moreover, the finding that alendronate is not associated with osteonecrotic lesions, if used either alone or with corticotherapy, suggests that factors related to diabetes other than bone metabolism should also be investigated to determine the role of this disease in the etiopathogenesis of osteonecrosis. Among these factors are the higher susceptibility to infections and microvascular alterations. Therefore, further studies qualitatively evaluating the microflora involved in alendronate-associated osteonecrosis in diabetic patients would be helpful. It is also important to consider the evaluation of other types of BMPs and MMPs in the same investigation comparing them in the different phases of wound healing.

Conclusion

Diabetes is a risk factor for alendronate-associated osteonecrosis of the jaws in experimental animals subjected to tooth extractions. In contrast, corticotherapy does not seem to be an effective risk factor for osteonecrosis in this same situation. Further studies investigating microbiological aspects related to the involvement of diabetes in alendronate-associated jaw osteonecrosis are necessary.

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DISCUSSÃO GERAL

4 DISCUSSÃO GERAL

Os bisfosfonatos têm sido amplamente empregados no tratamento de doenças que alteram o metabolismo ósseo, para o que apresentam resultados satisfatórios (FARRUGIA et al., 2006; MIGLIORATI et al., 2005). Entretanto, também estão associados a efeitos adversos, dentre os quais destaca-se a osteonecrose dos maxilares (MARX, 2003), condição que reduz significativamente a qualidade de vida do paciente (RUGGIERO et al., 2009).

A literatura tem relatado a possibilidade de que fatores como cortico- e quimioterapia, diabetes mellitus, doenças infecciosas, malnutrição, etilismo, tabagismo, doença periodontal, exostoses, uso de próteses dentárias, exodontias e outros procedimentos odontológicos invasivos exerçam importante papel no desenvolvimento da lesão (ASSAEL, 2009; HAMADA, 2007; LOBATO et al., 2007; RUGGIERO e WOO, 2008; WOO et al., 2006). O risco representado pelos procedimentos odontológicos invasivos dos ossos maxilares em usuários de bisfosfonatos é evidente e pode aumentar de acordo com a dose, o tempo de uso, a via de administração e o tipo de bisfosfonato (LOBATO et al., 2007; SARIN et al., 2008; WOO et al., 2006), sendo 94% dos casos relacionados ao uso intravenoso do fármaco, sobretudo pamidronato e ácido zoledrônico. Já a osteonecrose dos maxilares associada ao alendronato de sódio, apesar de menos prevalente, também tem sido relatada (BEDOGNI et al., 2010; BOCANEGRA-PÉREZ et al., 2009; CONTE-NETO et al., 2011; JUNQUERA et al., 2009; KUIJPERS et al., 2011; MARX et al., 2007; PARK et al., 2010).

De acordo com a literatura, em usuários de alendronato de sódio, um ou mais fatores de risco, como o diabetes mellitus (KHAMAISI et al., 2007), a corticoterapia (RUGGIERO et al., 2009) e a realização de exodontias (MAVROKOKKI et al., 2007)

poderiam contribuir significativamente para o desenvolvimento da osteonecrose. Entretanto, a rotina clínica mostra que, em alguns desses pacientes, o procedimento odontológico invasivo dos ossos maxilares não constitui fator suficiente para a ocorrência da lesão. Isto é, alguns pacientes usuários de alendronato de sódio submetidos a exodontias ou outros procedimentos cirúrgicos dos ossos maxilares não desenvolvem a osteonecrose, enquanto outros, uma vez submetidos ao mesmo tipo de procedimento e mesmo tempo de uso do fármaco, são capazes de desenvolvê-la. Hamada (2007) relatou três casos de pacientes que usavam bisfosfonatos orais e desenvolveram a lesão após exodontias. Um deles usava risedronato e altas doses de corticosteroide para o tratamento de artrite reumatoide; o outro era portador de diabetes mellitus e usava alendronato de sódio, além de referir histórico de doença cardíaca; e o terceiro apresentava histórico de carcinoma de estômago e uso de alendronato de sódio. Tais relatos, entre outros, sustentam a hipótese de que o diabetes mellitus e a corticoterapia favorecem a instalação da osteonecrose maxilar associada ao bisfosfonato oral. Entretanto, o efetivo papel de cada um desses cofatores na etiopatogenia da lesão requer investigações que contemplem o isolamento e o controle das variáveis envolvidas.

Na presente pesquisa, a influência do diabetes e da corticoterapia foi avaliada em animais de experimentação tratados com alendronato de sódio e submetidos a exodontias. O grupo de animais tratados com alendronato de sódio e submetidos à indução de diabetes exibiu maior prevalência de osteonecrose quando comparado aos demais grupos. Esse resultado corrobora a ideia de que o diabetes aumenta significativamente o risco de desenvolvimento da lesão em usuários de alendronato de sódio, constituindo, de fato, um fator de risco como sugerido previamente (LOBATO et al., 2007; MARX et al., 2005; MIGLIORATI et al., 2006; PURCELL e BOYD, 2005;

SARIN et al.2008). Tal achado parece estar associado aos efeitos que as taxas glicêmicas elevadas exercem sobre o metabolismo ósseo (KUMEDA, 2006), bem como à susceptibilidade a infecções e às alterações microvasculares provocadas pelo diabetes (FAVUS, 2007). O grupo corticosteroide, por sua vez, não exibiu diferença significativa de osteonecrose quando comparado aos grupos alendronato e controle. Isso sugere que o uso do corticosteroide não constituiria um fator de risco para o desenvolvimento da lesão, o que discorda de alguns estudos (ASSAEL, 2009; LOBATO et al., 2007; WOO et al., 2006; RUGGIERO e WOO, 2008). Quanto à avaliação imunoistoquímica, não foram observados aumento da expressão da BMP-4 e diminuição da MMP-13 nos grupos corticosteroide e diabetes como era esperado. Nesse contexto, deve-se considerar que o alendronato de sódio, que foi administrado a todos os grupos experimentais, ao inibir a reabsorção óssea, tenha inibido também a expressão das proteínas investigadas.

Os resultados da presente pesquisa tornam evidente o risco representado pelo diabetes em usuários de bisfosfonato oral submetidos a procedimento cirúrgico dos ossos maxilares. Embora não tenha sido observado o mesmo resultado para a corticoterapia, novas investigações que empreguem outros corticosteroides em distintas posologias ainda devem ser exploradas. Os resultados obtidos para o uso isolado de alendronato de sódio seguido de exodontias também não evidenciaram associação deste com a osteonecrose. É preciso considerar, entretanto, que a amostra empregada no presente estudo (modelo animal) caracteriza-se pela padronização das condições individuais e ambientais, o que não acontece com os pacientes. Estes exibem intensa especificidade e variabilidade de características individuais e ambientais, o que pode significar participação de inúmeras variáveis intervenientes. Ou seja, em pacientes, o risco de osteonecrose associada ao uso de alendronato, acompanhado ou não da

corticoterapia, após procedimentos cirúrgicos dos ossos maxilares, não pode ser descartado e constitui fator de difícil avaliação. Por outro lado, o risco da combinação alendronato e corticoterapia parece ser menor do que o risco da combinação alendronato e diabetes. Assim, ressalta-se a importância de futuras pesquisas que contribuam com a elucidação e compreensão do real papel dos fatores de risco enunciados pela literatura no desenvolvimento da osteonecrose dos maxilares associada ao bisfosfonato oral. Nesse ínterim, pacientes usuários de alendronato de sódio, especialmente os portadores de diabetes mellitus ou que estejam sob corticoterapia, requerem avaliação cautelosa e individualizada diante da necessidade de procedimentos odontológicos invasivos dos ossos maxilares a fim de prevenir-se a osteonecrose.

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ANEXOS

ANEXO A

Normas para a submissão de artigos científicos no periódico Osteoporosis International

ANEXO B

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Title:	Effects of diabetes mellitus, corticotherapy and bisphosphonates on bone turnover and their relationship with osteonecrosis of the jaws - An update
Authors:	Couto, Soraya Vasconcelos, Ana Carolina Lopes, Tiago Figueiredo, Maria Antonia Salum, Fernanda Cherubini, Karen
Date Submitted:	11-Nov-2011

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Manuscript ID:	OI-2011-11-0786
Title:	Diabetes mellitus and corticotherapy as risk factors for alendronate-related osteonecrosis of the jaws: a study in Wistar rats
Authors:	Couto, Soraya Vasconcelos, Ana Carolina Iglesias, Júlia Figueiredo, Maria Antonia Salum, Fernanda Cherubini, Karen
Date Submitted:	11-Nov-2011

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ANEXO D

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 022/10 – CEUA

Porto Alegre, 11 de março de 2010.

Senhora Pesquisadora:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 09/00139, intitulado: **"Influência do diabetes e da corticoterapia no desenvolvimento da osteonecrose maxiliar associada ao uso de alendronato de sódio: Estudo *in vivo*".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora do CEUA – PUCRS

Ilma. Sra.
Profa. Dra. Karen Cherubini
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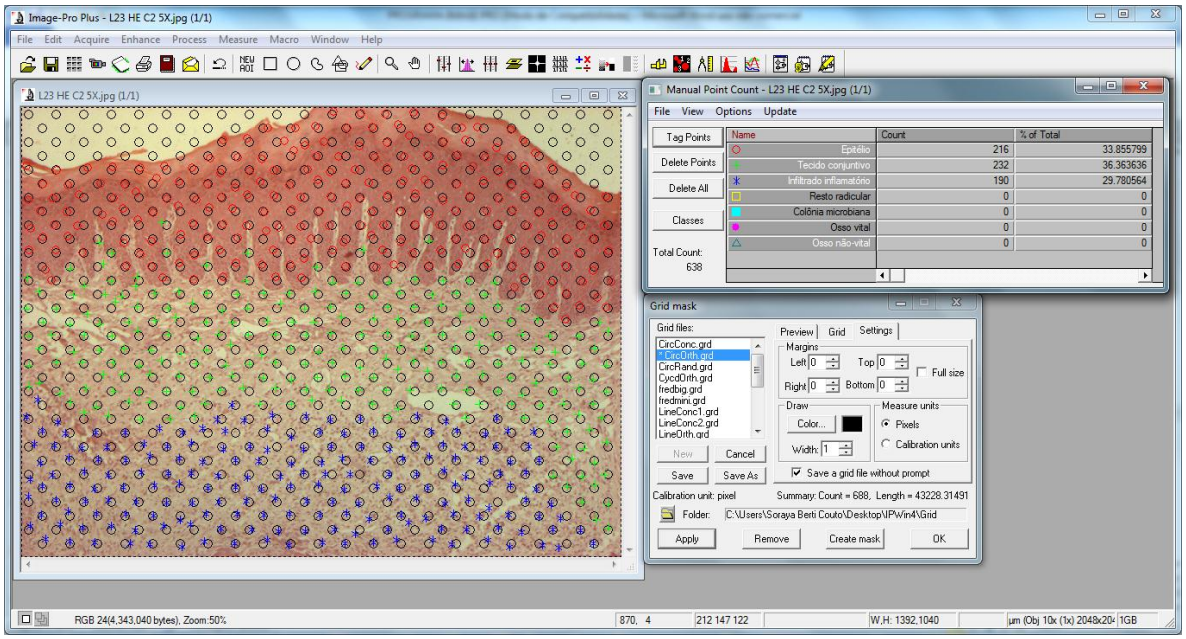
APÊNDICES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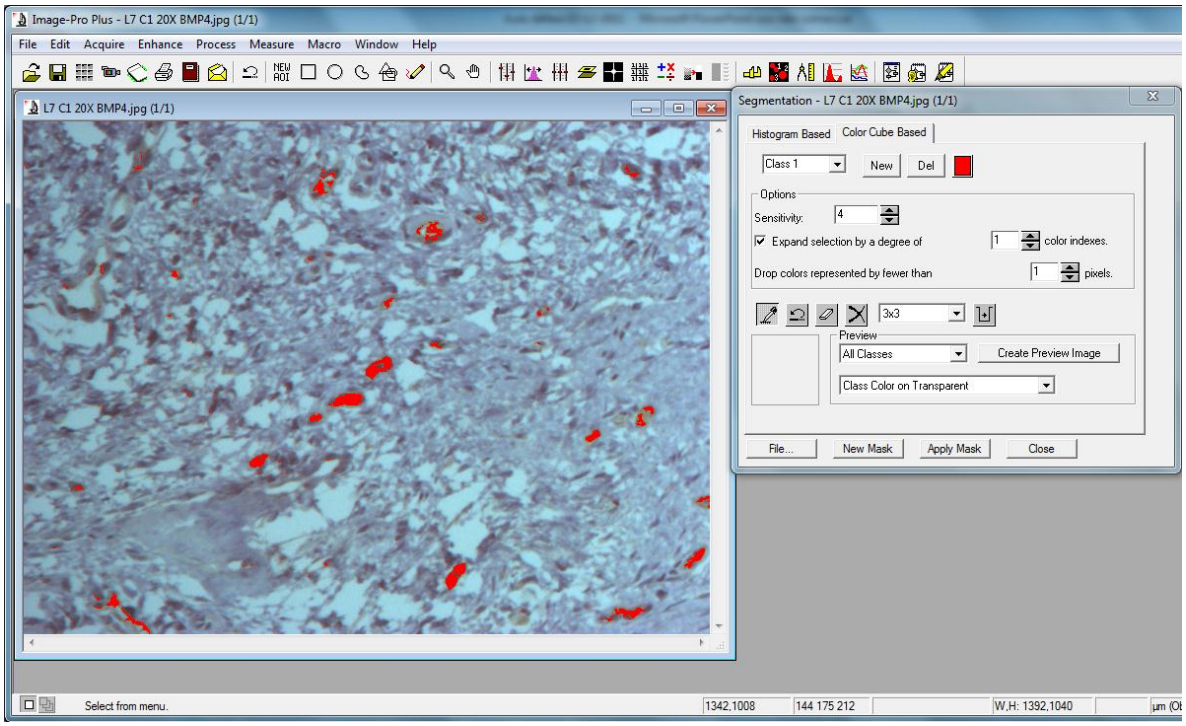
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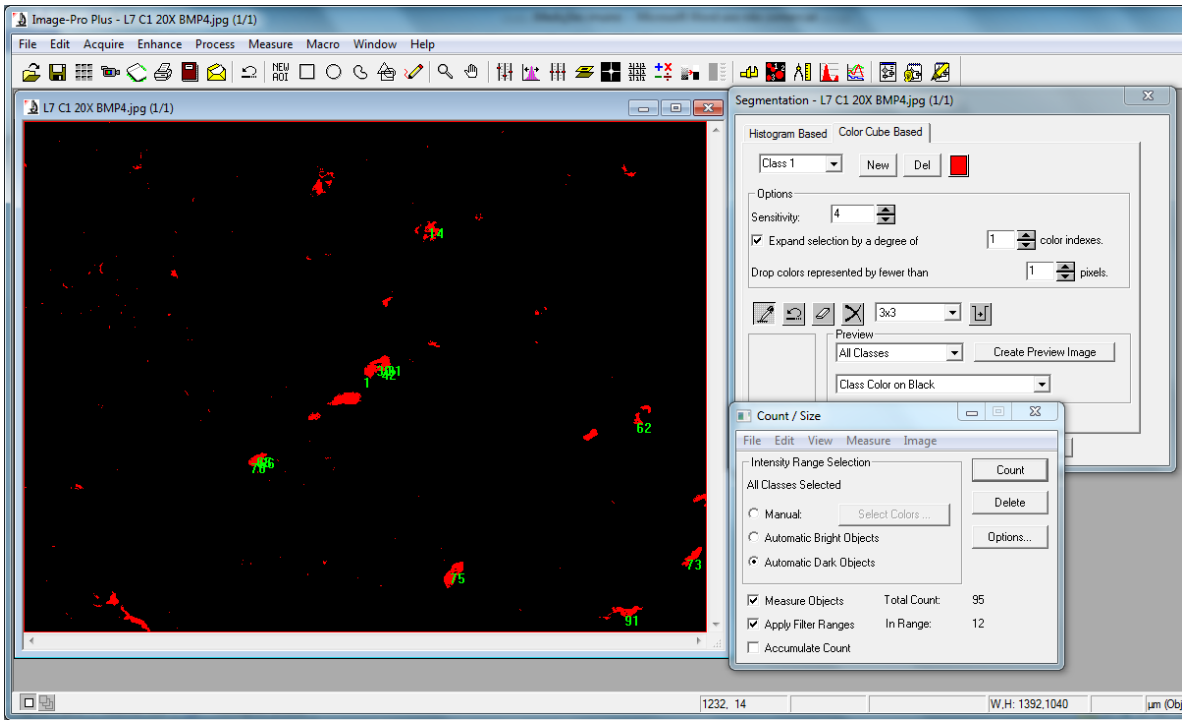
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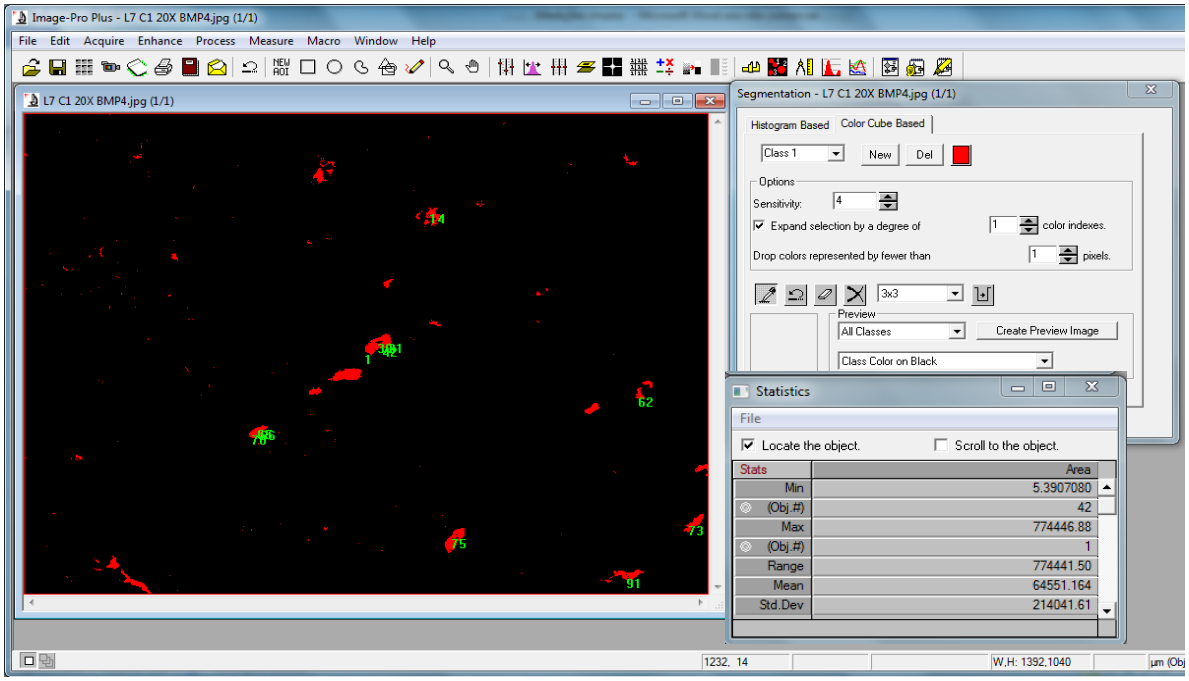
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