FACULDADE DE ODONTOLOGIA

AVALIAÇÃO RADIOGRÁFICA E HISTOLÓGICA DO OSSO ALVEOLAR MANDIBULAR DE RATOS SUBMETIDOS À TERAPIA COM BISFOSFONATOS NITROGENADOS

JOANE WOBETO MAGLIA

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PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL

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RADIOGRAPHIC AND HISTOLOGICAL EVALUATION OF MANDIBULAR ALVEOLAR BONE OF RATS TREATED WITH NITROGEN-CONTAINING BISPHOSPHONATES

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Seja sincero em sua busca. Faça tudo por ela.

Ela é a sede de conhecer o original através do

reflexo que o torna digno do acidente final:

- A Iluminação.

Osho

(1931-1990)

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RESUMO

Os bisfosfonatos são fármacos que suprimem a reabsorção óssea, o que determina aumento da mineralização e consequente alteração do padrão ósseo típico. A presente pesquisa teve por objetivo avaliar, por meio de exame radiográfico e histológico, alterações do osso alveolar mandibular de ratos (*Rattus norvegicus*, Wistar) submetidos à administração de bisfosfonatos nitrogenados. Trinta mandíbulas de ratos foram distribuídas em 3 grupos, de acordo com o tratamento recebido: (1) 10 mandíbulas de ratos tratados com alendronato de sódio, (2) 10 mandíbulas de ratos tratados com ácido zoledrônico e (3) 10 mandíbulas de ratos sem tratamento (controle). Os espécimes foram radiografados e processados pela técnica de hematoxilina-eosina (H&E). A densidade óptica da lâmina dura, do ligamento periodontal e do osso alveolar, bem como a distância entre o limite amelocementário e a crista óssea alveolar foram mensuradas. As variáveis também foram submetidas à análise histomorfométrica. Ao exame radiográfico, (1) a densidade óptica do ligamento periodontal e da lâmina dura não diferiram entre os três grupos; (2) a densidade óptica do osso interradicular foi maior no grupo ácido zoledrônico do que no controle, enquanto o grupo alendronato não diferiu significativamente de ambos; (3) a distância entre a junção amelocementária e a crista óssea alveolar não diferiu significativamente entre os três grupos avaliados. No exame histológico, (1) não houve diferença significativa da espessura do ligamento periodontal entre os grupos; (2) o grupo ácido zoledrônico teve distância entre o limite amelocementário e a crista óssea alveolar significativamente menor que os grupos alendronato e controle, os quais não diferiram entre si; (3) a densidade de trabéculas do osso interradicular foi significativamente maior no grupo acido zoledrônico, do que no grupo-controle. Os resultados permitem concluir que (1) o ácido zoledrônico está

associado ao aumento da densidade óssea mandibular na região interradicular de molares, tanto ao exame radiográfico quanto ao histológico; (2) o uso de alendronato de sódio ou ácido zoledrônico não é fator suficiente para induzir espessamento do ligamento periodontal e da lâmina dura, ou aumento da distância entre o limite amelocementário e a crista óssea alveolar. Alterações da densidade óssea relacionadas ao alendronato de sódio devem ser investigadas em futuros estudos.

Palavras-chave: Osteonecrose dos maxilares, bisfosfonatos, ácido zoledrônico, alendronato de sódio, sinais radiográficos


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SUMMARY

Bisphosphonates are drugs that suppress bone resorption leading to increased mineralization and being able of changing the typical bone pattern. The aim of this study was to evaluate radiographic and histological changes in alveolar bone of rats (*Rattus norvegicus*, Wistar) undergone administration of nitrogen-containing bisphosphonates. The mandibles of 30 rats were allocated into three groups according to the treatment received: (1) 10 mandibles from rats treated with alendronate, (2) 10 mandibles from rats treated with zoledronic acid, and (3) 10 mandibles from rats without any treatment (control). The specimens were radiographed and processed for hematoxylin-eosin (H&E). Optical radiographic density of the lamina dura, periodontal ligament and alveolar bone, as well as the cement-enamel-junction to alveolar bone crest distance, were assessed in radiographic images. These variables were also subjected to histomorphometric analysis. On radiographic analysis, (1) the optical density of either the periodontal ligament or lamina dura did not differ between the alendronate, zoledronic acid and control groups; (2) bone interradicular optical density was higher in the zoledronic acid group than in controls, but the alendronate group did not show a significant difference relative to the zoledronic acid or control group; (3) cement-enamel-junction to alveolar bone crest distance did not differ between the three groups analyzed. On histological analysis, (1) there was no significant difference in periodontal ligament thickness between the groups; (2) the zoledronic acid group showed cement-enamel-junction to alveolar bone crest distance significantly smaller than in the alendronate and control groups, which did not differ from each other; (3) the zoledronic acid group showed bone trabecular density at interradicular space that was significantly higher than in controls. The results suggest that (1) zoledronic acid is associated with

increased trabecular density of mandibular alveolar bone as demonstrated by both radiographic and histological examination. Neither alendronate nor zoledronic acid is a sufficient factor to induce thickening of the lamina dura and periodontal ligament or increased distance between cement-enamel-junction and alveolar bone crest. The trabecular density changes associated with alendronate should be investigated in further studies.

Keywords: Osteonecrosis of the jaws, bisphosphonates, zoledronic acid, alendronate, radiographic signs

SUMÁRIO

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1 INTRODUÇÃO

Os bisfosfonatos são análogos sintéticos do pirofosfato endógeno, em que o átomo central de oxigênio é substituído por um átomo de carbono (VASCONCELLOS et al., 2004). Em função dessa característica de sua estrutura química, esses fármacos são resistentes à ação enzimática e têm sido empregados para suprimir a reabsorção óssea. Tal efeito é exercido por meio da indução dos osteoclastos à apoptose ou inibição de sua função (ANBINDER et al., 2007; SHINODA et al., 2008; VIERECK et al., 2002; WOO et al., 2006). Além de suprimirem a reabsorção óssea, também são capazes de modificar fatores angiogênicos circulantes (SANTINI et al., 2003).

A indicação terapêutica dos bisfosfonatos inclui quadros de osteopenia, osteoporose, doença de Paget, osteogênese imperfeita da infância, uso crônico de glicocorticoides, quadros de dor óssea (MARX et al. 2007; RESZKA; RODAN, 2003; RUGGIERO et al., 2009; VASCONCELLOS et al., 2004), metástases ósseas de câncer de mama, próstata e pulmão, bem como mieloma múltiplo e hipercalcemia associada ao câncer (HESS et al., 2008; RUGGIERO et al., 2009).

O aumento da mineralização óssea associado ao uso de bisfosfonatos (BADROS et al., 2006; FANTASIA 2009; MARX et al, 2007) e a consequente alteração do padrão ósseo típico (ORLANDINI et al., 2009) têm sido relatados na literatura, sendo dependentes do tempo de uso (BAGAN et al., 2006, CARMAGNOLA et al., 2008) e da potência do medicamento (O'RYAN et al., 2009; RUGGIERO et al., 2009). Esses fármacos também têm sido relacionados a casos de osteonecrose maxilo-mandibular (GUTTENBERG, 2008; RIZZOLI et al., 2008; RUGGIERO; DREW, 2007; RUGGIERO et al., 2009; ZAVRAS; ZHU, 2006), condição definida como área de osso

exposto na região maxilo-facial que não cura no prazo de oito semanas em um paciente que esteja recebendo ou foi exposto a um bisfosfonato sem, entretanto, ter sofrido radioterapia da região crânio-facial (KHOSLA et al., 2007).

Alterações radiográficas dos ossos gnáticos decorrentes do uso de bisfosfonatos têm sido relatadas. Entre elas estão: espessamento da lâmina dura, áreas de osteólise, osteogênese reacional alveolar pobre ou ausente, esclerose difusa (ORLANDINI et al., 2009; ARCE et al., 2009) envolvendo a margem alveolar (PHAL et al., 2007), perda ou reabsorção óssea alveolar não originada por doença periodontal, mudanças no padrão do trabeculado, espessamento/obscurecimento do ligamento periodontal, espessamento da lâmina dura e estreitamento do canal alveolar (RUGGIERO et al., 2009). A perda óssea entre as raízes do primeiro molar com comprometimento da furca tem sido apontada como sinal precoce da osteonecrose (MARX et al., 2005). Alterações do trabeculado ósseo por uso de bisfosfonatos podem assumir diferentes formas: desde a osteoesclerose, provavelmente ocasionada pela falta de reabsorção, até a osteólise, que ocorre em meio infectado (MARX et al., 2007).

As alterações radiográficas detectadas em região maxilo-mandibular nos pacientes que fazem uso de bisfosfonatos, tanto por via oral quanto intravenosa, são consideradas inespecíficas, uma vez que se apresentam de forma variada, com reações osteoescleróticas e líticas em diferentes sítios (ORLANDINI et al., 2009; ARCE et al., 2009; PHAL et al., 2007; RUGGIERO et al., 2009; MARX et al., 2005). Ainda, tais alterações foram observadas em pacientes, muitos deles portadores de outras enfermidades locais ou sistêmicas, que poderiam estar associadas ao padrão radiográfico alterado (DODSON, 2009; SARIN et al., 2008; WOO et al., 2006), ou mesmo, portadores de osteonecrose (MARX, 2009; WILKINSON et al., 2007).

O padrão radiográfico do tecido ósseo sob ação dos bisfosfonatos e livre de comorbidades como doença periodontal, cáries, abscessos e distúrbios sistêmicos pode revelar quais alterações estão, de fato, associadas ao uso do fármaco. Estudos experimentais padronizados capazes de minimizar os fatores de confusão e que investiguem tais alterações fazem-se necessários. A presente dissertação compreende dois artigos científicos. O primeiro deles corresponde à revisão da literatura sobre o tema em questão e tem por objetivo fundamentar, com base na literatura científica, o experimento realizado. O segundo artigo descreve a investigação das alterações radiográficas e histológicas do osso mandibular em modelo animal sob terapia com bisfosfonatos nitrogenados.

2 ARTIGO 1

O artigo a seguir intitula-se **Imaging of the jaws in patients undergoing bisphosphonate therapy** e foi formatado de acordo com as normas do periódico *The British Journal of Radiology* (Anexos A e B).

Imaging of the jaws in patients undergoing bisphosphonate therapy

REVIEW ARTICLE

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Running title

Imaging of the jaws and bisphosphonates

Imaging of the jaws in patients undergoing bisphosphonate therapy

REVIEW ARTICLE

ABSTRACT

The aim of this work was to present a literature review focusing on the imaging changes of the jaws associated with bisphosphonate therapy and the performance of imaging methods in diagnosing such changes. The lamina dura, periodontal ligament and alveolar bone are the jaw sites most reported as showing imaging alterations associated with bisphosphonates. The detection of imaging changes in bisphosphonate users is important in the patient's follow-up and early diagnosis of jaw osteonecrosis. However, this issue is still controversial with regard to the specificity of the features reported, and further controlled experimental studies are needed in this field.

Introduction

Bisphosphonates are chemical analogues of pyrophosphate, an endogenous substance responsible for inhibiting bone resorption [1]. These drugs are used to reduce bone resorption, where they act mainly on osteoclasts through inhibition of the formation, recruitment, activation and activity of these cells, as well as induction of apoptosis [2]. Bisphosphonates are indicated for treatment of bone metabolism diseases characterized by intense resorption. Among their indications are: osteopenia, osteoporosis, Paget's disease, childhood osteogenesis imperfecta, chronic use of glucocorticoid and cases of bone pain [2-5]. They are also used in the treatment of bone metastases of breast, prostate and lung cancer, as well as in patients with osteolytic lesions of multiple myeloma and hypercalcemia associated with malignancies [5, 6].

Bisphosphonates are able to change the typical bone pattern, leading to increased mineralization [7], whose intensity depends on time of use [8, 9] and drug potency [5, 10]. This may occur due to their ability to suppress bone resorption and to their antiangiogenic properties [11]. This group of drugs represents one of those most prescribed worldwide [12] and has also been linked to cases of osteonecrosis of the jaws [5, 13-16], where this disease may turn into an epidemic [17].

Bone metabolism disturbances and lesions, either neoplastic or not, cause imbalance between bone resorption and neoformation. Depending on their nature, these conditions consequently determine different degrees of demineralization or sclerosis in diagnostic imaging. In the absence of osteoblastic activity, osteolytic images can be observed [18]. On the other hand, in diseases involving the absence or reduction of osteoclast function, such as osteopetrosis, there is diffuse sclerosis and increased bone mineral density [19].

Bone tissue changes caused by bisphosphonates, either in the case of increased mineralization or induced osteonecrosis, are evident in radiographs and other imaging methods. A literature review is presented here on the spectrum of changes in diagnostic imaging of the jaws, associated with the use of bisphosphonates, also focusing on specific issues related to the available techniques.

Bisphosphonate-associated changes in diagnostic imaging of the jaws

The literature reports some effects exerted by bisphosphonates on bone tissue, which in turn appear as changes in the imaging of the jaws (Table 1). The lamina dura, periodontal ligament and alveolar bone are the jaw sites most reported as showing imaging alterations associated with bisphosphonate use [5, 11, 23].

Lamina dura

The term lamina dura, also the hard layer, defines the narrow portion of the alveolar bone that borders on the periodontal ligament, and can be seen as a well-defined, radiopaque layer on radiographs. The name is due to the fact that this structure is more radiopaque than the adjacent bone [35]. Changes in its image are generally manifested as loss of detail, loss of continuity, total or partial absence and increased thickness. The loss of detail can occur because of internal resorption in the alveolar socket, root compression, as in orthodontic movement, or inflammatory processes. The total or partial absence of this structure may result from inflammatory processes as well as advanced systemic diseases such as Paget's disease and hyperparathyroidism [36]. The thickening of the lamina dura occurs in conditions such as osteopetrosis [37] and scleroderma [36]

Table 1 – Imaging findings in patients undergoing bisphosphonate therapy

Source	Lamina dura (increased density)		Periodontal ligament (increased thickness)		Diffuse osteolysis		Diffuse sclerosis		Mandibular canal involvement		Oroantral fistula		PHES* or NHES**	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Orlandini et al. [7] n=15	4	26	-	-	6	40	4	26	-	-	1	6	-	-
Marx et al. [20] n=119	-	-	***	***	85	71.4	87	73.1	-	-	-	-	-	-
Chiandussi et al. [21] n=11	-	-	-	-	***	***	***	***	-	-	-	-	***	***
Milillo et al. [22] n=38	-	-	-	-	-	-	25	65.7	-	-	4	10.5	-	-
Phal et al. [23] n=15	7	46	4	26	-	-	15	100	3	20	2	13	5	33
Bedogni et al. [24] n=11	-	-	-	-	-	-	***	***	-	-	-	-	***	***
Bisdas et al. [25] n=32	-	-	-	-	28	87.5	32	100	14	43.7	-	-	4	12.5
Migliorati et al. [26] n=1	-	-	-	-	1	100	-	-	-	-	-	-	-	-
Arce et al. [27]	***	***	-	-	***	***	***	***	-	-	-	-	***	***
Gill et al. [28] n=19	-	-	-	-	14	73.6	15	78.9	-	-	2	10.5	-	-
Junquera et al. [29] n=21	-	-	-	-	21	100	21	100	-	-	-	-	-	-
Krishnan et al. [30] n=6	-	-	***	***	***	***	***	***	***	***	-	-	-	-
Singer; Mupparapu [31] n=1	-	-	1	100	-	-	1	100	-	-	-	-	1	100
Park et al. [32] n=5	-	-	-	-	4	75	1	33.3	-	-	-	-	1	33.3
Tong et al. [33] n=2	-	-	-	-	1	50	1	50	-	-	-	-	2	100
Treister et al. [34] n=7	-	-	-	-	1	14.2	7	100	-	-	1	14.2	2	28.5
Total of signs observed n = 428	11	2.57	5	1.16	161	37.61	209	48.83	17	3.97	10	2.33	15	3.5

*PHES= Poor healing extraction socket;
**NHES= Non-healing extraction socket;
***Finding reported without statistics

Being a site of intense bone remodeling and rich in osteoclasts, the lamina dura is capable of retaining a significant amount of bisphosphonates, and thus, it is more susceptible to their effects (Figure 1). Its thickening associated with bisphosphonate use can occur due to the lack of osteoclasia, which determines higher mineral deposition than resorption [38]. According to O'Ryan et al. [10], computed tomography scans of patients under treatment with nitrogen-containing bisphosphonates have shown persistence and sclerosis of the lamina dura in post-extraction sites, with delayed alveolar healing. Some authors report the thickening of the lamina dura in patients using these drugs [5, 7, 39], whereas others report the thickening or loss of the lamina dura as an early bone change in osteonecrosis of the jaws. Phal et al. [23] reported thickening of the lamina dura in 7 out of 15 patients with bisphosphonate-induced osteonecrosis of the jaws. Arce et al. [27] also observed this feature in conventional radiographic images of patients with osteonecrosis. According to these authors, after prolonged exposure to nitrogen-containing bisphosphonates, osteosclerosis can be seen in the radiographs, especially in the lamina dura. Fantasia [40] reported that the prominent image of the lamina dura in dentate areas might be related to the risk of osteonecrosis onset. The correlation between clinical and radiographic findings points out to the evidence that patients with mild osteonecrosis exhibit thickening of the lamina dura, which increases as the disease progresses [27].

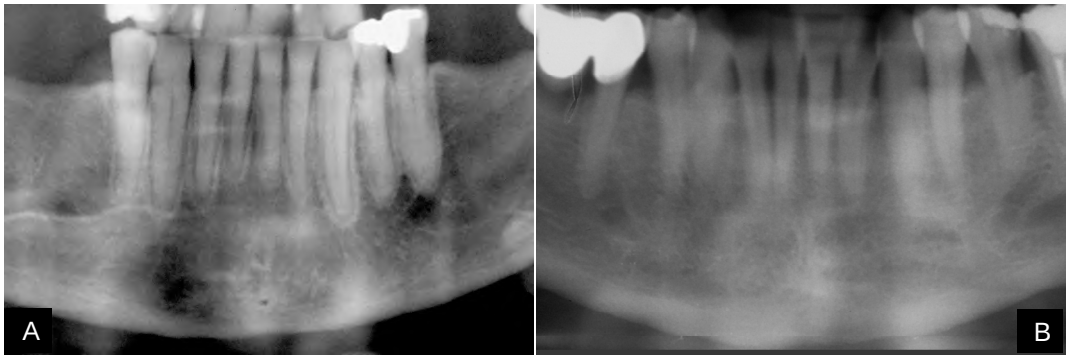

Figure 1 – (A) Thickening of the lamina dura in a female patient under intravenous bisphosphonate therapy; (B) Radiographic aspect of lamina dura in a female patient under oral bisphosphonate therapy (Panoramic radiograph zoom)

Periodontal ligament space

The periodontal ligament is composed of fibers that connect cementum to alveolar bone. In human beings, its width ranges from 0.15 to 0.38 mm, and it is a reservoir of cells required for homeostasis, regeneration and tissue repair [41]. The most common change associated with the imaging of the periodontal ligament is its thickening, i.e., increased radiolucency in the area of collagen fibers that support the tooth. This imaging finding is observed in conditions such as endodontic and periodontal lesions [36], osteonecrosis induced by bisphosphonates [30], scleroderma [42] and osteosarcoma [43]. The periodontal ligament space, observed in the radiographic examination, may undergo changes consequent to the use of nitrogen-containing bisphosphonates. Some authors noted the decrease in the periodontal ligament space with lamina dura thickening [5], whereas others reported the widening of the periodontal ligament space [11, 15, 23, 27, 39]. Phal et al. [23] reported increased periodontal ligament space in 4 out of 15 patients clinically diagnosed with bisphosphonate-induced osteonecrosis. Ruggiero and Drew [15] reported that after prolonged exposure to bisphosphonates, the enlargement of the periodontal ligament space could be seen by

radiographic examination. Krishnan et al. [30] reported increased radiolucency in the periodontal ligament space as an early sign of bisphosphonate-induced osteonecrosis, suggesting that this may be the site of origin of the lesion.

Increased density of alveolar bone

There is agreement in the literature concerning the fact that prolonged administration of bisphosphonates leads to increased alveolar bone density, and that these changes in increased bone mineral density [15] and sclerotic areas [7], to a greater or lesser degree [23], can be detected by radiographic examination. This fact is explained by the drug's inhibitory effect on bone resorption, with consequent depletion of marrow spaces [44], associated with persistence of the anabolic activity of osteoblasts [45].

Mandibular canal

Phal et al. [23] found sclerotic changes in the mandibular canal in 3 out of 15 patients clinically diagnosed as having bisphosphonate-induced osteonecrosis of the jaws, which corroborates other reports [10, 25]. The narrowing of the mandibular canal was reported as a nonspecific finding, which can be found at any stage of bisphosphonate-induced osteonecrosis [5], or in advanced disease, associated with paresthesia, which is a symptom that patients may experience [27]. The narrowing of the mandibular canal can be present in areas without clinical involvement [46]. There are also reports of effacement of the normally distinct borders of the canal as a radiographic finding in patients with multiple myeloma who developed osteonecrosis of the jaws induced by bisphosphonates [47]. Moreover, when pathological fractures develop, osteonecrosis may involve this anatomic structure [25].

Osteonecrosis of the jaws

The occurrence of bisphosphonate-induced osteonecrosis of the jaws has been reported since 2003 [5, 13-16, 48]. The condition is defined as an area of exposed bone in the maxillofacial region that does not heal within eight weeks in a patient who is receiving or was exposed to a bisphosphonate without having undergone radiation therapy of the craniofacial region [49]. Although most reported cases of this disease are associated with nitrogen-containing bisphosphonates [50], some cases have also been reported after a high cumulative dose through long-term exposure to non-nitrogen-containing ones [51].

Osteonecrosis is not a specific disease entity, but the result of some conditions that lead to deficient blood supply to bone tissue, showing evidence of changes in remodeling and weakening of its structure due to microfractures. It occurs at sites whose high rates of remodeling are suppressed by bisphosphonates. Suppression of bone remodeling is, therefore, the basic premise to explain the lesion onset [52].

Among the antiangiogenic properties of bisphosphonates, there is the significant reduction of serum vascular endothelial growth factor (VEGF) [53]. Nitrogen-containing bisphosphonates have an average half-life of ten years, a time during which the inhibition of endothelial proliferation can persist [11, 52, 54]. This effect may explain the ischemic feature of jaws in patients under treatment with these drugs, which hampers the repair of damage in the presence of normal oral microflora [39]. Such events contribute to the development of radiographic changes, and also predispose the tissue to osteonecrosis [25]. Osteonecrosis in turn results from the collapse of the bone architecture and leads to important anatomic changes. Although its anatomy and histopathology show variable characteristics [55], the histological features seem to explain the radiographic findings reported in the literature.

The histology of osteonecrosis reveals the almost complete absence of bone cells and no signs of active remodeling, giving the appearance of *frozen bone* [7]. Furthermore, the necrotic bone is associated with granulation tissue and bacterial debris, whose cultures have isolated the usual microbial flora [47]. Microbiological studies have shown *Actinomyces* sp. in most biopsy specimens [10, 25]. Specimens from osteonecrosis of jaw debridement, sequestrum and resection are similar on histological examination, showing remnants of sclerotic bone that if exposed to the oral cavity exhibits bacterial debris. The absence of osteoclasts may reflect osteoclastic apoptosis associated with bisphosphonates [40]. Biopsy specimens from 31 patients with bisphosphonate-induced osteonecrosis of the jaws exhibited areas of acute inflammation, which were more evident at the most peripheral areas of the samples from sites of extensive bone loss. Unlike other studies, there were abundant osteoclast-like cells at these sites, at the interface with the residual bone spiculae. These areas exhibited inflammatory infiltration, mostly consisting of polymorphonuclear phagocytes, plasma cells, monocytes and lymphocytes, acellular necrotic debris, dilated blood vessels with thin walls, and intense basophilic bone spiculae. Abundant bacterial colonies, some of them morphologically consistent with *Actinomyces* sp., were interspersed with necrotic debris, and almost exclusively observed at the most superficial areas, in a juxtamucosal position [56]. An experimental in vivo study inducing bisphosphonate-associated osteonecrosis in rats has also demonstrated these features [57].

Radiographic features of jaw osteonecrosis

The radiographic features of jaw osteonecrosis range from subtle thickening of the lamina dura and alveolar crest to attenuated osteopetrosis-like sclerosis [23]. Images of osteolytic lesions with cortical bone involvement can be found [21], in addition to

areas of heterogeneous osteolysis and thickening of the cortical bone [7]. Although these radiological features are not specific for this disease [25], bone loss between the first molar roots with furcation involvement is reported to be an early sign of osteonecrosis [20].

Ruggiero et al. [5] reported the presence of alveolar bone resorption not related to periodontal disease, changes in trabecular bone pattern, dense bone pattern, thickening/obscuring of the periodontal ligament, thickening of the lamina dura and narrowing of the mandibular canal at stage 0 of bisphosphonate-induced osteonecrosis of the jaws (patients with no clinical evidence of necrotic bone, but presenting with nonspecific symptoms or clinical and radiographic findings).

According to Krishnan et al. [30], the radiographic findings are similar to those of osteoradionecrosis, which may include heterogeneous lytic areas with sites of increased density, sometimes associated with pathologic fractures. On computed tomography, these findings correspond to sclerosis, cortical thickening, loss of trabecular bone pattern, cortical disruption, pathological fractures, soft tissue swelling and edema. Early signs of osteonecrosis reported in this study were focal sclerosis adjacent to the root and thickened periodontal ligament, which progressed to cortical disruption, fragmentation, and osteolysis. Radiopaque sequestra can also be found [58].

Considerations on imaging techniques

Radiographic techniques

Because of its low cost and easy accessibility, radiography is widely used in diagnosis. Plain radiographs may be helpful in evaluating bone density [59]. Bone loss can be detected by these examinations at demineralization indices from 30% to 50%,

which represents relative sensitivity [18]. In this way, also gain of density [60] or other alterations in the alveolar bone features can be shown using these techniques [61].

The literature reports many different radiographic descriptions associated with bone necrosis either as a result of radiotherapy or use of nitrogen-containing bisphosphonates [49]. Moreover, the images of bones affected by necrotic lesions such as bisphosphonate-induced osteonecrosis of the jaws are usually obtained after the onset of clinical signs and symptoms, and demonstrate bone involvement far beyond what is assessed by clinical examination [25].

Periapical radiography

The use of periapical radiography in the detection of these changes is limited to dentate regions and the alveolar ridge. Its detail and sharpness is satisfactory, when the technique is properly applied. Changes in trabecular pattern are well detailed in this technique and may indicate bone metabolism disturbances [62], including osteoporosis and those associated with bisphosphonate use [27, 44].

Panoramic radiography

Panoramic radiography provides satisfactory visualization of the maxilla and mandible for initial assessment of the patient [27]. In cases of osteonecrosis, it has been widely used but provides no distinction between osteonecrosis and osteoblastic metastatic lesions. On the other hand, panoramic radiography is effective in detecting combinations of osteolysis and osteosclerosis, as well as woven bone formations with periosteal thickening and marrow fibrosis [14]. It is a low-cost, readily accessible procedure that provides good visualization of the entire maxillomandibular complex, where it is useful for the diagnosis of metastatic and osteonecrotic lesions when there is

a radiopaque sequestrum, but less effective in the presence of osteolytic lesions. Although early lesions may go clinically unnoticed [63], panoramic radiographs could provide early detection of asymptomatic foci of necrotic bone still not exposed to the oral environment [7]. According to Vassiliou et al. [64], bisphosphonate-induced osteonecrotic lesions have been subjected to radiographic examination only in advanced cases, in which necrotic bone tissue is already exposed. The authors recommend panoramic radiography for routine dental assessment in patients at risk for osteonecrosis, because this technique is able to detect osteolytic lesions with cortical bone involvement.

In a study by Phal et al. [23], patients with osteonecrosis of the jaws showed abnormalities in orthopantomograms, including bone sclerosis, commonly involving the alveolar margin, with lamina dura thickening. Furthermore, sclerosis of the mandibular canal, little or no healing of extraction sites, periapical radiolucency, thickening of the periodontal ligament, osteolysis, and oroantral fistula were also detected.

Multidetector computed tomography

On computed tomography, bisphosphonate-induced osteonecrosis can exhibit sclerosis, osteolysis, persistence of the lamina dura after extraction, bone sequestrum, and narrowing of bone marrow spaces [7]. Bisdas et al. [25] reported the predominance of osteolytic lesions, cortical destruction and sclerotic regions with or without periosteal proliferation. In four patients with early symptoms of tooth loosening or delayed socket healing after tooth extraction, the only striking computed tomography finding was focal sclerosis of the bone marrow in the suspected necrotic site. In early-evaluated patients with clinical symptoms, images showed subtle abnormalities, including focal sclerosis, disorganized trabeculae and poor corticomedullary differentiation. The authors believed

these to be early findings indicative of bisphosphonate-induced osteonecrosis, which should be addressed more comprehensively in future studies.

Chiandussi et al. [21] reported that among 11 patients with bisphosphonate-induced osteonecrosis, 10 showed clinical symptoms and abnormalities on computed tomography scan. The images showed moderate irregularity of the cortical margins and cortical bone destruction, as well as areas of osteolysis and osteosclerosis. Milillo et al. [22] evaluated images of 38 patients diagnosed with osteonecrosis of the jaws. All patients had the lesion developed in the alveolar ridge involved in tooth extraction, extending to adjacent structures. In tomographic images, the central portion of the lesions showed small areas of sequestration with lytic areas inside. Most patients (n = 25) had areas of increased bone density at the periphery of the lesions giving rise to intraoral bone spiculae. Oroantral fistula and discontinuity on the maxillary sinus floor appeared among the common findings of bisphosphonate-induced osteonecrosis in maxilla.

Cone beam computed tomography

Cone beam computed tomography allows the creation of images in real time, not only in axial but also in coronal and sagittal planes, as well as oblique or curved image planes - the multiplanar reformation. It also provides a reformation in volume, providing 3-dimensional information. The acquisition time of head and neck images is short (less than or equal to 10 seconds), with significant reduction in radiation compared to multidetector computed tomography [65].

A comparative study found differences in accuracy between panoramic radiography and cone beam tomography in the diagnosis of bisphosphonate-induced osteonecrosis of the jaws. Bone fragmentation, some radiolucent changes, poor healing

of extraction wounds and oroantral communication, all of them without clinical symptoms, were detected only on cone beam computed tomography images [34].

Magnetic resonance imaging (MRI)

Computed tomography scan accurately shows the extent of injury, whereas MRI reproduces the bone marrow condition [18]. Bisdas et al. [25] reported that gadolinium-enhanced magnetic resonance imaging revealed changes in the intensity of cortical and subcortical structures in 32 patients treated with nitrogen-containing bisphosphonates. These findings were homogeneous throughout the lesion or located at the periphery with a band-like pattern. In four patients with early symptoms of either tooth loss or delayed healing of extraction wounds, MRI showed subtle abnormal signal intensity extending beyond the area of the lesion, if compared to multidetector computed tomography. In patients with early or intermediate symptoms of bisphosphonate-induced ostonecrosis, T1-weighted magnetic resonance imaging exhibited hypointense regions, which corresponded to regions with normal T2-weighted signals.

Chiandussi et al. [21] found cases of bisphosphonate-induced osteonecrosis exhibiting low-signal T1-weighted images and mild hyperintensity on T2-weighted magnetic resonance imaging. After contrast injection, there was a slight enhancement of the lytic areas. In bone sequestrum zones a well-defined dark area was observed. Perilesional soft tissues, which are swollen and inflamed at clinical examination, can also be evaluated by means of this imaging method [22].

Bone scintigraphy

Bone scintigraphy has high sensitivity and is therefore capable of identifying early lesions [18]. It is routinely used in the diagnosis and management of cancer

metastases and has the potential to detect bisphosphonate-induced osteonecrosis of the jaws in early stages [10]. O'Ryan et al. [10] observed that among 35 patients under bisphosphonate therapy, 23 (65.7%) showed positive tracer uptake on scintigraphy in areas that subsequently developed osteonecrosis. Among 24 patients who underwent scintigraphy after developing osteonecrosis, 21 (87.5%) exhibited intense tracer uptake in the lesion areas.

Bone scintigraphy is very sensitive to early changes, but it is poor in distinguishing inflammatory conditions, such as osteoradionecrosis and periodontal disease, from metastases [30] or other malignant processes [66]. According to Vassiliou et al. [64], bone scintigraphy is the most sensitive examination for detection of early bisphosphonate-induced osteonecrosis of the jaws. The assessment with technetium-99m methylene is more effective for this diagnosis if compared to magnetic resonance imaging and computed tomography, these latter two being more suitable for determining the extent of the lesion.

Positron emission tomography (PET)

Morphologic imaging methods exhibit the pattern of necrotic bone, but are inadequate for assessing osteoblastic or inflammatory activity. Functional imaging methods can add information about the physiopathology of the process. Bone scintigraphy with Tc-99m identifies areas with increased osteoblastic activity, where it is more sensitive than computed tomography and magnetic resonance imaging. However, spatial resolution is limited, and the analysis of the maxillomandibular complex compromised. Improvement in spatial resolution was obtained by positron emission tomography (PET) [67]. PET scans have been used to detect bone lesions in multiple myeloma patients. As they are more sensitive than radiographs, PET scans have been

employed in the detection of occult bone disease. The major limitation of PET scanning is that small lesions may not be detected [18]. Fluorodeoxyglucose-positron emission tomography (FDG-PET) integrated with computed tomography may show focal uptake of the tracer at sites of bisphosphonate-induced osteonecrosis [68].

Final considerations

Early changes in the bisphosphonate-induced jaw osteonecrosis can be detected by imaging examinations [39]. The main objective of such detection is the early diagnosis of this disease and the understanding of the effects that these drugs exert on jaws. Actually radiographic changes induced by bisphosphonates can mimic periapical inflammatory lesions or osteomyelitis and they are not evident in the radiographic examination until there is significant bone involvement. Therefore early stages of jaw osteonecrosis may not show significant changes [54]. Some reports, however, suggest that early bone changes in bisphosphonate-induced osteonecrosis include sites of osteolysis or diffuse osteosclerosis, widening of the periodontal ligament space and thickening or loss of the lamina dura [11, 14, 23, 27].

The initial assessment of the patient can be performed using conventional radiographs, which may show those specific changes suggesting osteonecrosis [14, 23, 27]. If conventional radiographs are inconclusive or negative despite a strong clinical suspicion of a bone lesion, computed tomography, magnetic resonance imaging and positron emission tomography should be used because they are more sensitive in detecting bone changes [18]. Moreover, the screening of sites in the initial stage of the necrotic process can be made by bone scintigraphy [30]. In advanced stages of osteonecrosis, PET scans, cone beam tomography, multidetector computed tomography,

and magnetic resonance imaging show the exact extent of the injury and the involvement of adjacent structures, which is crucial in a surgical planning and in the follow-up [18, 21, 25]. Stockmann et al. [66] reported that panoramic radiographs showed signs of osteonecrosis in 13 out of 24 patients with the disease (detectability, 54.1%); multidetector computed tomography in 23 out of 24 patients (detectability, 96%); and magnetic resonance T1 signal in 22 out of 24 patients (detectability, 92%).

Knowledge of imaging changes associated with bisphosphonate use, as well as their implications is important in diagnosis. The professional must be prepared to detect them, since imaging plays a crucial role in the diagnosis of osteonecrosis, a situation in which it is preferable to avoid biopsy [23]. The detection of imaging changes in bisphosphonate users is important in the patient follow-up and early diagnosis of jaw osteonecrosis. However, it should be recalled that this issue is still controversial with regard to the specificity of the features reported. Most reports are based on clinical cases in which patients could exhibit biases such as periodontal disease, periapical lesions, diabetes and other comorbidities. Another aspect to be questioned is whether in fact some of the imaging changes reported are indicative of osteonecrosis or merely represent the effects of the drug on bone tissue with or without osteonecrosis. Still, the duration of use [8, 9] and the potency of the drug [5, 10] are important factors related to the occurrence and intensity of outbreaks. Controlled and randomized experimental studies, considering the duration of therapy and the type of bisphosphonate, are necessary to evaluate the specificity of imaging changes reported and to disclose whether they are actually associated with drug use.

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3 ARTIGO 2

O artigo a seguir intitula-se **Radiographic and histological evaluation of mandibular alveolar bone of rats treated with bisphosphonates** e foi formatado de acordo com as normas do periódico *European Radiology* (Anexos C e D).

Radiographic and histological evaluation of mandibular alveolar bone of rats treated with bisphosphonates

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Radiographic and histological evaluation of mandibular alveolar bone of rats treated with bisphosphonates

ORIGINAL ARTICLE

Abstract

Objective: To evaluate radiographic and histological changes in mandibular bone associated with bisphosphonates.

Methods: Rats were allocated into 3 groups: alendronate-treated, zoledronic acid treated, and untreated control. The mandible specimens were radiographed and stained with hematoxylin/eosin for histology. Radiographs were assessed for density of the lamina dura, periodontal ligament space and alveolar bone as well as the cement-enamel-junction (CEJ) to alveolar bone crest distance (ABC). These variables were also histologically evaluated.

Results: Radiography: (1) optical density of either periodontal ligament or lamina dura did not differ between the groups; (2) bone optical density was higher in the zoledronic acid group than in control, but the alendronate group did not significantly differ from them; (3) CEJ-ABC distance did not differ between the groups. Histology: (1) there was no significant difference in periodontal ligament thickness between the groups; (2) zoledronic acid group showed a significantly smaller CEJ-ABC distance compared to other groups; (3) zoledronic acid group showed a bone trabecular density significantly higher than control.

Conclusion: Zoledronic acid is associated with increased trabecular density of mandibular bone. Neither alendronate nor zoledronic acid induced thickening of the lamina dura and periodontal ligament or increased the CEJ-ABC distance.

Key words Osteonecrosis – Bisphosphonates – Zoledronic acid – Alendronate - Radiography

Introduction

Bisphosphonates are drugs that suppress bone resorption by inducing apoptosis of osteoclasts or inhibiting their function [1-4]. Some therapeutic indications for bisphosphonate are: osteopenia, osteoporosis, Paget's disease, osteogenesis imperfecta in childhood, chronic use of glucocorticoids and bone pain [5-8]. They are also used in the treatment of bone metastases of breast, prostate and lung cancer, as well as in patients with lytic lesions of multiple myeloma and hypercalcemia associated with malignancies [7, 9].

Because of their suppressive effect on bone resorption and antiangiogenic properties [10], bisphosphonates are capable of modifying the typical bone pattern, leading to increased mineralization [11], whose intensity depends on the duration of use [12, 13] and the potency of the drug [7, 14]. They have also been linked to cases of jaw osteonecrosis [7, 15-18], a condition defined as an area of exposed bone in the maxillofacial region that does not heal within eight weeks in a patient who was or had been on bisphosphonate therapy and was not exposed to radiation therapy in the craniofacial region [19]. The risk of osteonecrosis is related to the route of administration [10] and to the drug potency [14]. The cases associated with alendronate have an incidence between 0.7 [7] and 1 event in every 100,000 patients per year [16]. Zoledronic acid, in turn, is ten times more potent than alendronate [20], which suggests that it also has major effects on the radiographic pattern of alveolar bone [7, 11, 21-23].

The literature indicates some radiographic changes in the maxilla and mandible, resulting from bisphosphonates. These include the following: thickening of the lamina dura; osteolysis; poor or absent reactive alveolar osteogenesis; diffuse sclerosis [11, 21] most commonly involving the alveolar margin [23]; alveolar bone resorption not related to periodontal disease; dense bone pattern; thickening/obscuring of the periodontal

ligament; thickening of the lamina dura; and narrowing of the mandibular canal [7]. Bone loss between the roots of the first molar with furcation involvement is often an early sign of osteonecrosis [22]. Because it is a site of intense bone remodeling and rich in osteoclasts, the lamina dura is able to retain significant concentrations of bisphosphonates and therefore becomes more susceptible to their effects. Its thickening due to drug use can occur as a consequence of the lack of osteoclasia, which determines higher mineral deposition than resorption [24]. Trabecular bone changes caused by bisphosphonates can assume different appearances, ranging from osteosclerosis, probably caused by lower resorption, to osteolysis, which occurs in an infected area [5].

Bisphosphonates have an average half-life of more than ten years [22], and therefore, alveolar bone changes can occur late with drug exposure. Radiographic changes detected in the maxillomandibular region in patients using bisphosphonates, either orally or intravenously, may be considered nonspecific, since they are manifested in many ways, with lytic and sclerotic bone reactions at different sites [7, 21-23]. Moreover, such changes were observed in patients, many of them with other local or systemic diseases, which may be associated with the radiographic features [4, 20, 25], or even in patients with bisphosphonate-induced osteonecrosis of the jaws already established [26, 27]. The radiographic pattern of bone tissue from the effect of bisphosphonates and free of comorbidities, such as periodontal disease, dental caries, dental abscesses, and systemic disorders, could reveal which changes are indeed associated with the drug. Experimental standardized studies capable of reducing the confounding factors and investigating such changes are necessary.

This study aimed to investigate in an animal model, some radiographic mandibular alveolar bone changes associated with the administration of oral (alendronate) and intravenous (zoledronic acid) nitrogen-containing bisphosphonates.

The optical density of the lamina dura, periodontal ligament space and alveolar bone, as well as the distance between the cement-enamel-junction and alveolar bone crest, was evaluated. Moreover, histological alterations compatible with the radiographic status were investigated by microscopic examination.

Material and Methods

Animals

This work was approved by the Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul. The sample comprised 30 female rats (*Rattus norvegicus*, Wistar strain) from the animal facility of the Federal University of Pelotas (UFPel), which had a mean age of 140 days and mean weight of 241 g. The animals were kept in plastic cages, which were placed in ventilated racks (Alesco, Monte Mor, SP, Brazil), with controlled temperature (22 ± 1°C) and 12-h light-dark cycle (lights on at 7 a.m. and off at 7 p.m.). Food (Nuvilab, Colombo, PR, Brazil) and filtered water were provided *ad libitum*.

The animals were allocated into 3 groups according to the treatment administered: (1) alendronate group: 10 rats given sodium alendronate (Galena Química & Farmacêutica, Campinas, SP, Brazil) by oral gavage at a weekly dose of 0.05 mg/kg, for 150 days; (2) zoledronic acid group: 10 rats subjected to intraperitoneal administration of zoledronic acid (Zometa®, Novartis Pharma AG, Basel, Switzerland) at 0.6 mg/kg every 28 days for 150 days; (3) control group: 10 rats subjected to the same environment as groups 1 and 2, but without bisphosphonate treatment.

Specimen Processing

The mandibles were dissected and fixed in 10% buffered formalin. Each specimen was sectioned at the midline, in the anterior region between the central incisors, using a

Bard-Parker no. 3 scalpel (Colgram, São Paulo, SP, Brazil) with a no.12 blade (Becton-Dickinson, Juiz de Fora, MG, Brazil), which resulted in two hemimandibles. This procedure was performed in such a way as to allow the right hemimandible to be subjected to histological analysis and the left hemimandible to radiographic evaluation. The left hemimandible of each specimen was dissected using a Bard-Parker no. 3 scalpel (Colgram, São Paulo, SP, Brazil) with a no.12 blade (Becton-Dickinson, Juiz de Fora, MG, Brazil) and a periotome (Ref. 12170, SSWhite, Juiz de Fora, MG, Brazil). The aim of the dissection was to totally remove the surrounding soft tissues, so they would not interfere in the radiographs. Thus, the standardization of the sample was obtained, since the soft tissues could be a variable within groups. Soft tissues in the right mandible remained intact.

Radiographic Technique

The left hemimandible was radiographed in an acrylic positioner, which allowed standardization. We used the parallelism technique, with focus/film distance of 40 cm, central beam parallel to the proximal surfaces of teeth and mandibular lingual cortical oriented to the sensitive side of the radiographic film. The film used was periapical Kodak Ultra Speed, D sensitivity (Carestream Health, Inc., Rochester, NY, USA), because it provides a better image of trabecular bone [28]. The radiographs were obtained using an X-ray Spectro II (Dabi-Atlante, Ribeirão Preto, SP, Brazil) apparatus set at 50 kVp and 8 mA, using an exposure time of 0.8 seconds. An aluminum wedge of 8 steps was placed on the film, for standardization and correction of environmental interferences (temperature, humidity, voltage). The radiographs were processed in an automatic processor (Revell, São Paulo, SP, Brazil) in dry-to-dry cycle of 4.5 minutes, with processing solutions (Kodak, Carestream Health, Inc., Rochester, NY, USA).

Digitization of Radiographs

The radiographs were digitized in a standardized manner using the Epson Perfection® 2450 scanner (Epson, Long Beach, CA, USA) with built-in transparency, at 1200 dpi (dots per inch) and 8-bit (256 grayscale) and stored in TIFF format. There was no adjustment of brightness or contrast.

Analysis of Radiographic Images

Radiographs (Fig. 1) were analyzed in Adobe Photoshop CS, version 8.0.1 (Adobe Systems, San Jose, CA, USA) by one calibrated examiner blinded to the study. After applying a virtual grid over them to guide the cut, the images were bounded on three planes: one horizontal grid line parallel and matching the occlusal plane, one vertical grid line mesial to the first molar, and one vertical grid line distal to the third molar. A 100% zoom was applied for better visualization of the structures (Fig. 2). Using the magic wand tool, points of interest were then marked in a standardized manner, regardless of the anatomical variations of each specimen.

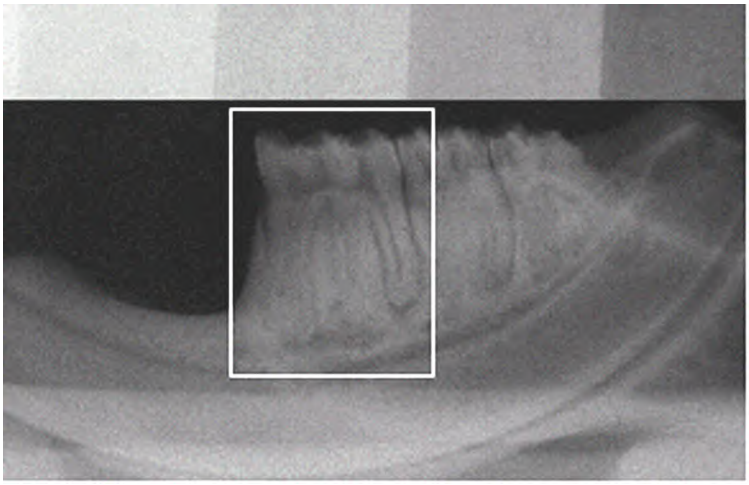

Fig. 1 – Radiograph of the mandibular first molar

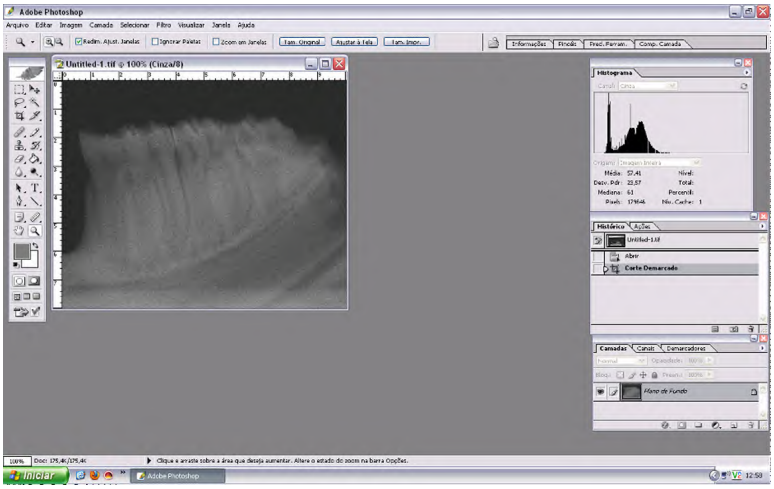

Fig. 2 – Radiographic analysis applying 100% zoom in Adobe Photoshop CS, version 8.0.1. (Adobe Systems, San Jose, CA, USA)

The optical density of the periodontal ligament, lamina dura and interradicular bone was performed using grayscale of pixels at the following sites: (1) distal periodontal ligament space of first molar at cervical, middle, and apical root sites; (2) distal lamina dura of first molar at cervical, middle and apical root sites; and (3) three sites of the interradicular space of the first molar selected in a standardized manner. The distance between cement-enamel-junction and alveolar bone crest was measured using a specific tool of the program for linear measurements.

In grayscale, 0 (zero) represents the darker area (radiolucent), while 255 denotes the lightest area (radiopaque). The grayscale values obtained represent a greater or lesser degree of radiolucency or radiopacity. The optical density values were corrected by the optical density of the wedge by mathematic proportions. The calibration consisted of analyzing a series of 10 radiographs twice, at two different moments, 10 variables being measured in each radiographic image. The results of these two analyses were submitted

to a paired t-test and Pearson correlation test, respectively showing no significant difference (P=0.68) and a strong correlation (r=0.97).

Histological Processing

The right hemimandibles were decalcified in EDTA and embedded in paraffin. Serial 4-μm sections were obtained from the mesiodistal direction and stained with hematoxylin and eosin. From the serial sections, the first ones showing all the regions of interest were selected for analysis.

Histomorphometric Analysis

The digitization of the images was performed using a Zeiss Axioskop 40 light microscope (Zeiss, Oberkohen, Germany), connected by a Roper Scientific video camera (Media Cybernetics, Silver Spring, USA) to a Pentium IV PC 2.2 GHz with 512 MB RAM, 160 GB hard drive and Image Pro Capture Kit Platform (Media Cybernetics, Silver Spring, MD, USA). The images were captured using 5x and 10x objectives and stored in TIFF. Histological analysis was performed by a calibrated observer blinded to the study. The calibration consisted of evaluating a series of 10 histological images twice, at two different moments, with 5 variables being measured in each image. The results of these two analyses were submitted to a paired t-test and Pearson correlation test, respectively showing no significant difference between each other (P=0.33) and a strong correlation (r = 0.99).

The linear measurements were obtained by means of Image Pro Plus version 4.1 (Media Cybernetics, Silver Spring, MD, USA), using the specific tool of the program (Fig. 3): (1) thickness of periodontal ligament in the distal root of the second molar at

cervical, middle and apical root sites; and (2) distance between cement-enamel-junction and alveolar bone crest in the distal of the second molar.

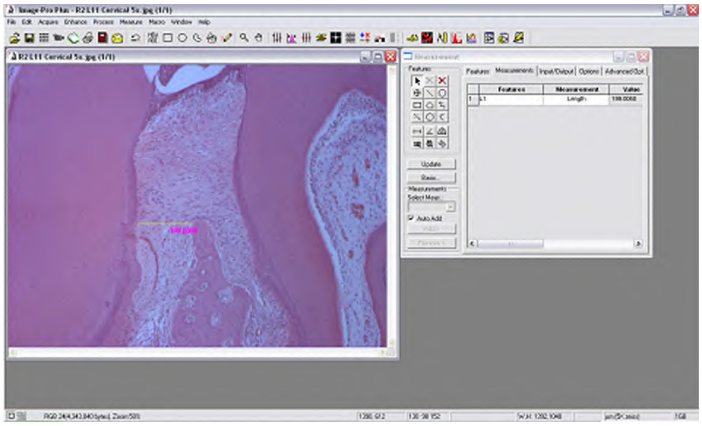

Fig. 3 – Histological analysis in Image Pro Plus version 4.1 (Media Cybernetics, Silver Spring, MD, USA): thickness of periodontal ligament at cervical site of distal root in the mandibular second molar

Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) was used to assess the trabecular bone density. Two areas (interradicular and apical) in the second molar region were selected in a standardized manner. After importing to the program, the proportion of bone trabeculae in the images was measured according to Mahl and Fontanella [29]. The *Extract* filter was applied selecting only the pixels corresponding to bone trabeculae, which allowed discarding of the images of the marrow spaces. This procedure replaced the surface not selected by the white color. Only the colored portion of the image was then obtained, and through the histogram function, the area of bone trabeculae was determined in pixels.

Unlike in the radiographic analysis, in the histological analysis, the second molar region was chosen, because this tooth had the best alignment in the images (Fig. 4).

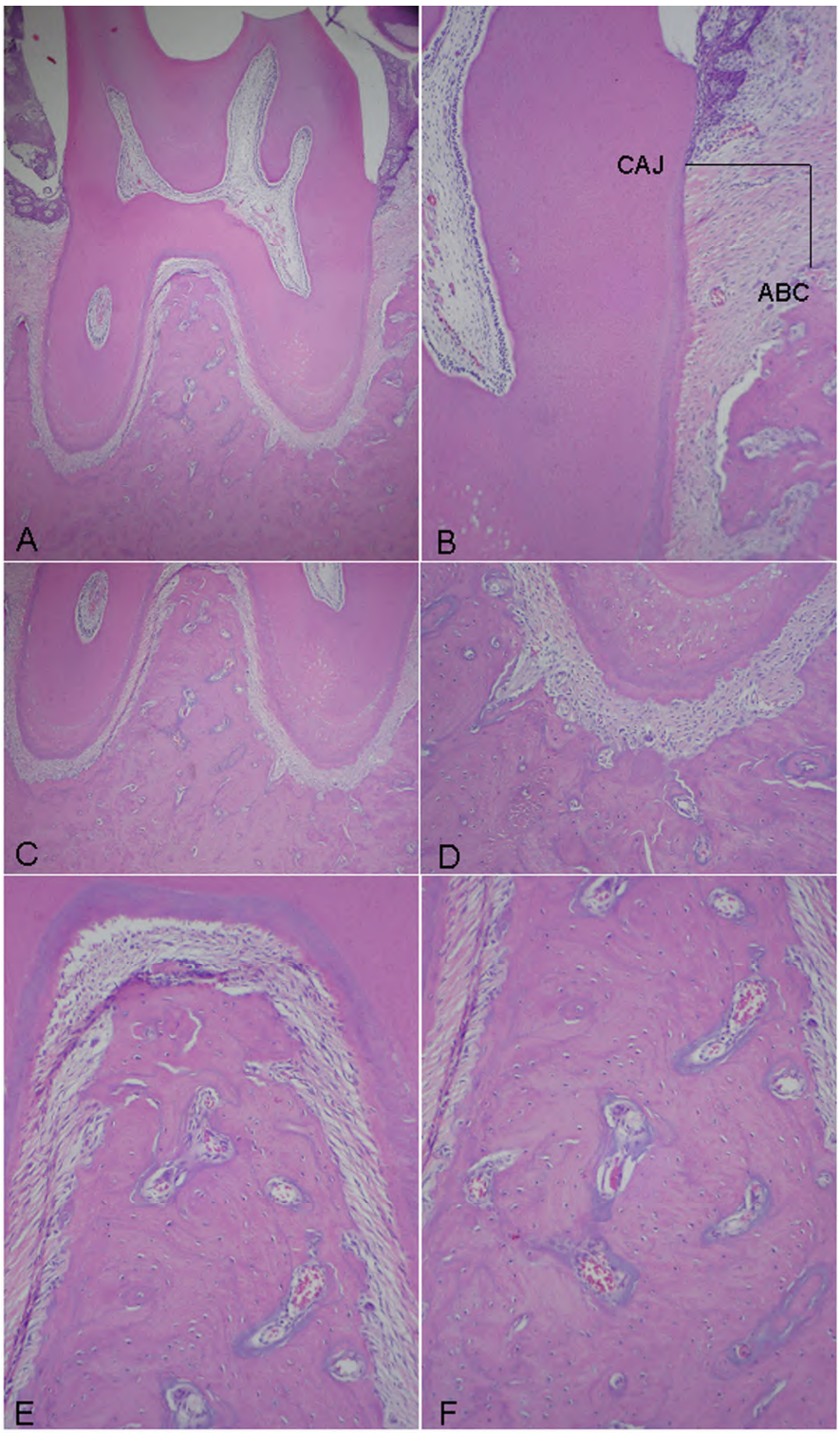

Fig. 4 - Histological analysis: (A) Mandibular second molar (HE, 100 x); (B) Cement-enamel-junction to alveolar bone crest distance (HE, 200x); (C, D) Periodontal ligament at apical site (HE, 200 x); (E, F) Interradicular alveolar bone (HE, 200 x)

Statistical Analysis

The results for radiographic and histological variables analyzed were compared between the groups (alendronate, zoledronic acid and control) by means of descriptive statistics and one-way ANOVA complemented by Tukey's test for multiple comparisons, considering a significance level of 5%.

Results

Radiographic Analysis

Radiographic optical density of periodontal ligament

Radiographic optical density of the periodontal ligament did not significantly differ between the alendronate, zoledronic acid and control groups, either when taking into account the specific sites evaluated or when the values were considered regardless of the specific sites (ANOVA, α=0.05, Table 1).

Table 1 Radiographic optical density of periodontal ligament in alendronate, zoledronic acid and control groups

Group	Radiographic optical density of periodontal ligament (pixels)							
	Cervical		Middle		Apical		Total	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Alendronate	0.230	0.032	0.334	0.016	0.326	0.024	0.297	0.016
Zoledronic acid	0.236	0.038	0.338	0.025	0.335	0.028	0.303	0.027
Control	0.235	0.035	0.353	0.029	0.321	0.017	0.303	0.010
P value	0.935		0.194		0.448		0.691	

ANOVA, significance level of 5%
SD = Standard deviation

Radiographic optical density of lamina dura

Radiographic optical density of the lamina dura did not differ between the alendronate, zoledronic acid and control groups, even when considering the specific sites evaluated (ANOVA, α=0.05, Table 2).

Table 2 Radiographic optical density of lamina dura in alendronate, zoledronic acid and control groups

Group	Radiographic optical density of lamina dura (pixels)							
	Cervical		Middle		Apical		Total	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Alendronate	0.285	0.039	0.388	0.025	0.370	0.023	0.348	0.012
Zoledronic acid	0.309	0.035	0.392	0.023	0.385	0.028	0.362	0.022
Control	0.301	0.036	0.392	0.032	0.370	0.029	0.355	0.019
P value	0.363		0.921		0.374		0.230	

ANOVA, significance level of 5%
SD = Standard deviation

Radiographic optical density of interradicular alveolar bone

Zoledronic acid group showed interradicular alveolar bone density that was significantly higher than in the control, but did not differ from the alendronate group (ANOVA, Tukey's test, α=0.05, Table 3).

Table 3 Radiographic optical density of interradicular alveolar bone in alendronate, zoledronic acid and control groups

Group	Radiographic optical density of interradicular alveolar bone (pixels)							
	Site 1		Site 2		Site 3		Total	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Alendronate	0.370	0.036	0.393 ^{AB}	0.034	0.381	0.025	0.381 ^{AB}	0.022
Zoledronic acid	0.390	0.030	0.413 ^{A}	0.030	0.411	0.035	0.405 ^{A}	0.028
Control	0.364	0.031	0.367 ^{B}	0.031	0.388	0.025	0.373 ^{B}	0.025
P value	0.216		0.015		0.077		0.032	

ANOVA, Tukey's test, significance level of 5%
Means followed by different capital letters show significant difference

Cement-enamel-junction to alveolar bone crest distance

On radiographic examination, cement-enamel-junction to alveolar bone crest distance did not differ between the alendronate, zoledronic acid and control groups (ANOVA, α=0.05, Table 4).

Table 4 Cement-enamel-junction to alveolar bone crest distance on radiographic examination in alendronate, zoledronic acid and control groups

	Cement-enamel-junction to alveolar crest distance (mm)	
Group	Mean	SD
Alendronate	0.240	0.107
Zoledronic acid	0.189	0.060
Control	0.280	0.114
P value	0.148	

ANOVA, significance level of 5%
SD = Standard deviation

Histomorphometric Analysis

Thickness of peridontal ligament

On histological examination, there was no significant difference in periodontal ligament thickness between the alendronate, zoledronic acid and control groups (ANOVA, α= 0.05, Table 5).

Table 5 Thickness of periodontal ligament on histological examination in alendronate, zoledronic acid and control groups

Group	Thickness of periodontal ligament (μm)							
	Cervical		Middle		Apical		Total	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Alendronate	194.70	85.27	170.78	67.70	107.0	79.67	157.40	69.6
Zoledronic acid	294.80	99.07	221.82	73.06	134.64	56.90	217.00	50.8
Control	251.37	88.86	180.20	84.32	133.82	63.41	188.40	72.2
P value	0.090		0.314		0.600		0.157	

ANOVA, level of significance of 5%
SD = Standard deviation

Cement-enamel-junction to alveolar bone crest distance

On histological examination, the zoledronic acid group showed a cement-enamel-junction to alveolar bone crest distance that was significantly smaller than in the alendronate and control groups. The latter two groups did not differ from each other regarding this variable (ANOVA, Tukey's test, α=0.05, Table 6).

Table 6 Cement-enamel-junction to alveolar bone crest distance on histological examination in alendronate, zoledronic acid and control groups

Group	Cement-enamel-junction to alveolar bone crest distance (μm)	
	Mean	SD
Alendronate	618.2 [A]	299.72
Zoledronic acid	257.6 [B]	267.04
Control	698.2 [A]	344.01
P value	0.011	

Means followed by different letters differed significantly (ANOVA, Tukey's test, level of significance of 5%)
SD = Standard deviation

Alveolar bone trabecular density

On histological examination, the zoledronic acid group showed a bone trabecular density at the interradicular site that was significantly higher compared to the control, but did not differ from the alendronate group. The control and alendronate groups did not differ from each other for this site. There was no significant difference in bone trabecular density at the apical site between the groups analyzed (ANOVA, Tukey's test, α=0.05, Table 7).

Table 7 Alveolar bone trabecular density on histological examination in alendronate, zoledronic acid and control groups

Group	Alveolar bone trabecular density (pixels)			
	Apical		Interradicular	
	Mean	SD	Mean	SD
Alendronate	0.965	0.042	0.925 [AB]	0.046
Zoledronic acid	0.980	0.015	0.934 [A]	0.064
Control	0.976	0.016	0.856 [B]	0.071
P value	0.491		0.022	

Means followed by distinct letters differed significantly (ANOVA, Tukey's test, level of significance of 5%)
SD = Standard deviation

Discussion

There was no significant difference in radiographic optical density of the periodontal ligament between the groups analyzed (alendronate, zoledronic acid and control). This finding suggests that bisphosphonate used alone, either alendronate or zoledronic acid, is not associated with the thickening of the periodontal ligament. Such result was also observed with histological examination, which did not show any significant difference between the groups regarding this variable. The inflammatory process related to osteonecrosis, with the presence of microorganisms and reactive bone could be responsible for the enlargement of periodontal fibers and the increase in periodontal exudate. It is known that these two factors result in thickening of the periodontal ligament as seen on radiographic examination [30]. Therefore, the cases reported in the literature showing this feature [11, 23] might have resulted from osteonecrosis itself. Moreover, even though the literature does not associate those cases with periodontal or periapical lesions, this possibility should be taken into account [31].

The optical density of the lamina dura did not significantly differ between the groups analyzed. This finding also refutes the possibility of bisphosphonate use being capable of inducing a change in the lamina dura independent of other factors. Maybe its association with such cofactors could result in these signs. Nevertheless, it is also possible that thickening of the periodontal ligament and higher density of the lamina dura are casual findings in this population. Many patients using bisphosphonates have other comorbidities, such as advanced age and systemic diseases, e.g., diabetes, hypothyroidism, rheumatoid arthritis and cancer, which could also be responsible for the radiographic alterations reported [32-34].

The zoledronic acid group showed a significantly higher radiographic optical density of the interradicular alveolar mandibular bone compared to controls, but did not

differ from alendronate group. Also, on histological examination, the zoledronic acid group showed higher alveolar trabecular bone density. This only corroborates the evidence that bisphosphonates increase alveolar bone density and also that intravenous drugs are more efficacious in this action. It is interesting to point out that this difference occurred on histological examination for the interradicular site but not the apical site. According to Marx et al. [22], interradicular bone in the furcation area is the first site to show signs of osteonecrosis. This probably happens because this site retains higher concentrations of bisphosphonates, which would explain the higher density of trabeculae. As bisphosphonates have an affinity for sites of high remodeling rates, it appears that the interradicular site has a high rate of bone turnover [35]. Accordingly, in radiographic evaluation of patients under bisphosphonate treatment, we must pay attention to the mandibular molar interradicular space. Regarding the fact that the apical site did not show any difference concerning this variable, we should consider the anatomy of the animal model. Compared to humans, the roots of rats' molars are wider in the apical area providing the dissipation of masticatory forces toward the apical region [36]. This feature could explain the results observed for trabecular density in the apical site.

Radiographic examination showed that the cement-enamel-junction to alveolar bone crest distance did not differ between the alendronate, zoledronic acid and control groups; however, in histological analysis, the zoledronic acid group showed significantly lower values for this variable compared to the alendronate and control groups. Radiographic measurement can be less reliable in the alveolar crest because of its thinness, especially in small models such as rats, with a probable lower optical density in radiographic images, which is inherent to the technique. On the other hand, histological examination is the gold standard for this kind of measurement, since these images can

provide better sharpness of the structures being measured, despite their small size and thinness [37]. Considering histological examination as the gold standard, we can infer that zoledronic acid exposure is indeed associated with a smaller distance between cement-enamel-junction and alveolar bone crest, unlike that of alendronate, since the alendronate and control groups did not differ from each other. This means that there is no induction of alveolar bone crest resorption by bisphosphonate in dentate sites free from osteonecrosis or other disease interference. Still, as suggested by the significant lower value for this variable in the zoledronic acid group, it seems that this specific drug (zoledronic acid) may inhibit resorption of the alveolar bone crest and decrease the cement-enamel-junction distance. Similar results have been previously reported [38, 39]. Moreover, these results corroborate the report of Ruggiero et al. [7], who associated the feature of alveolar bone resorption without periodontal disease with the initial stages of bisphosphonate-induced osteonecrosis of the jaws, but did not consider it just a consequence of bisphosphonate use in spite of lesion occurrence.

Regarding the results for the alendronate group where no significant difference was observed either in comparison to the control or zoledronic acid group, it is important to consider the dose applied. We used 0.05 mg/kg/week by oral gavage [40]. To achieve a human-equivalent dose, other authors recommend the use of higher doses in animal models [41]. It is possible that the dose used was low, and if it had been increased, different results would have been found. Nevertheless, it is also important to remember that this drug is cumulative in bone, with a half-life of about 10 years, and we kept the animals under treatment for 150 days, which is a very long period. Most studies reported having maintained animal models under bisphosphonate therapy for shorter periods of time [42, 43], probably based on the premise that animal toxicology studies with up to 1 month of drug administration are able to detect 90% of the toxic effects of most drugs

[44]. Because alendronate is not metabolized and because it accumulates in the bone, long-term treatment (150 days) supported the idea that the cumulative dose would be sufficient.

The limitations of radiographic examination should also be pointed out. Conventional radiographs can detect bone density alterations at indices between 30% and 50% [45], underestimating variation in bone density. Also, regarding the ligament space, the superimposition of anatomic structures cannot be ignored, which is inherent for this technique. Computed tomography could reveal different results. Nonetheless, the digitization and the software analysis applied did improve the radiography accuracy [46], allowing the detection of minute variations in optical density. Besides, we also used histological analysis to confirm radiographic results and compensate for the limitations of radiography. These procedures reinforce the confidence in the results observed.

Alterations in normal radiographic alveolar bone pattern induced by bisphosphonates are widely reported in the literature [21, 23]. This study showed that in animal models free of the comorbidities commonly found in patients, the use of these drugs results in some expected alveolar bone alterations. Some of these changes are reported as indicative of bisphosphonate toxicity and risk of osteonecrosis onset [47]. Further prospective studies including other imaging methods could better evaluate interradicular mandibular bone density in the molar region and cement-enamel-junction to alveolar bone crest distance as such predictive factors.

Conclusions

Zoledronic acid is associated with increased trabecular density of the alveolar mandibular bone on both radiographic and histological examination. Neither alendronate nor zoledronic acid is a sufficient factor to induce thickening of the lamina dura and periodontal ligament or increase the distance between the cement-enamel-junction and alveolar bone crest. The trabecular density changes associated with alendronate should be investigated in further studies.

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4 DISCUSSÃO GERAL

Os bisfosfonatos são uma das classes de fármacos mais prescritos no mundo, sendo que o alendronato de sódio foi o 19º no *ranking* do ano de 2003 (FILLEUL et al., 2010). A prescrição desses medicamentos vem aumentando de forma expressiva. No Reino Unido, cerca de 3% das mulheres acima de 70 anos recebeu prescrição de bisfosfonato, por via oral, em 2000, sendo que essa cifra aumentou para 10% em 2005 (WATSON et al., 2007). Tal fato poderá determinar o aumento da incidência da osteonecrose maxilo-mandibular induzida pelo uso de bisfosfonatos, com o risco de tornar a enfermidade uma epidemia (HELLSTEIN; MAREK, 2005).

A osteonecrose associada aos bisfosfonatos é uma condição debilitante que exerce forte impacto na qualidade de vida dos pacientes. A prevalência estimada é de 1% a 12% entre usuários de bisfosfonatos nitrogenados por via intravenosa, dependendo da doença primária e do tipo de tratamento (MAVROKOKKI et al., 2007; ORTEGA et al., 2007). Para o uso oral, essa estimativa situa-se entre 0,7 (RUGGIERO et al., 2009) e 1 caso (RIZZOLI et al., 2008) a cada 100.000 pacientes por ano. Medidas preventivas podem e devem ser tomadas, sendo dependentes diretamente do esclarecimento dos indivíduos envolvidos e da avaliação minuciosa do paciente. A identificação precoce das lesões, embora desafiadora, é potencialmente importante para a prevenção e para o tratamento do paciente (KHOSLA et al., 2007). Nesse sentido, sinais precoces da enfermidade ou de toxicidade do fármaco ao tecido ósseo devem ser investigados e identificados.

A literatura associa o uso dos bisfosfonatos com certo grau de esclerose óssea, principalmente da margem alveolar, espessamento da lâmina dura, escassez ou ausência de osteogênese reacional alveolar, radiolucências periapicais, alargamento do espaço

periodontal, osteólise, sequestros, fístula oro-antral e formação óssea periosteal (PHAL et al., 2007). Espessamento da lâmina dura e do ligamento periodontal, aumento da densidade do osso alveolar e reabsorção do osso alveolar não atribuída à doença periodontal são interpretados como sinais radiográficos de toxicidade dos bisfosfonatos ou até mesmo manifestações que antecedem o quadro de osteonecrose (PHAL et al., 2007; RUGGIERO et al., 2009). No presente estudo, esses sinais radiográficos foram investigados em modelo animal, com o intuito de eliminarem-se variáveis intervenientes, na maioria das vezes presentes nos pacientes. De acordo com os resultados obtidos, o uso do ácido zoledrônico, na ausência de comorbidades, é capaz de induzir aumento da densidade óssea na região interradicular de molares e diminuição da distância entre o limite amelocementário e a crista óssea alveolar. Entretanto, não foram observadas alterações significativas para espessura do ligamento periodontal e densidade da lâmina dura. Já o uso do alendronato não determinou diferenças significativas para nenhuma das variáveis avaliadas.

Considerando-se a ausência de alterações significativas por ocasião do uso oral do fármaco (alendronato), é preciso lembrar que, de fato, essa via de administração está associada à menor toxicidade, e esta é diretamente proporcional ao tempo de uso (CARMAGNOLA et al., 2008). É possível que a administração por períodos mais prolongados viesse a resultar em alterações significativas. Torna-se importante, portanto, considerar o fator tempo durante a avaliação dos pacientes bem como em novos estudos.

O uso do ácido zoledrônico também não resultou em alterações significativas para aumento de espessura do ligamento periodontal e aumento de densidade da lâmina dura, o que indica a possibilidade de esses sinais radiográficos serem resultado de múltiplos fatores, como alterações locais e sistêmicas do paciente, ou da própria osteonecrose já instalada. Por outro lado, o aumento de densidade do osso interradicular

especificamente na região de molares, bem como a menor distância entre a crista óssea alveolar e o limite amelocementário verificados para o ácido zoledrônico requerem atenção intensificada para esses sítios.

Novas pesquisas com ênfase à região de molares e de crista óssea alveolar poderão fornecer informações complementares. O emprego de metodologia padronizada com tomografia *cone beam* (TREISTER et al., 2010) e ressonância magnética pode revelar alterações precoces e subclínicas (BISDAS et al., 2008). A cintilografia óssea exerce papel importante na avaliação de disfunções metabólicas, enquanto a PET-CT pode exibir a morfologia dos sítios com função alterada. Assim, estudos prospectivos e controlados que apliquem tais exames durante todo o curso da terapia com bisfosfonatos, desde a fase inicial, podem mapear a progressão das alterações do tecido ósseo do complexo maxilo-mandibular. Há que se ressaltar a importância da inclusão do exame de pesquisa dos C-telopeptídeos (MARX et al., 2007) nessas investigações.

A Odontologia dispõe de exames complementares de diferentes níveis de complexidade, cuja escolha adequada tem papel importante na avaliação do usuário de bisfosfonatos. A solicitação desses exames por ocasião da adequação bucal dos pacientes, previamente ao início da terapia, é procedimento fundamental que deve ser adotado como rotina na prática clínica. Por outro lado, a detecção de alterações precoces nos exames imaginológicos em usuários desses fármacos pode constituir ferramenta auxiliar na prevenção da osteonecrose maxilar associada aos bisfosfonatos, bem como no controle e monitoramento das lesões. O conhecimento e a capacidade de diagnosticar tais alterações precoces é fundamental ao melhor prognóstico e dependem do desenvolvimento de novas pesquisas com exames de imagem, tanto em modelo animal, quanto clínicas, que possam fornecer os parâmetros necessários à correta avaliação do paciente e à tomada de decisões.

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ANEXO A

Karen Cherubini

De: em.bjr.0.1ee4f5.ba674e74@editorialmanager.com em nome de BJR Office **Enviada:** dom 7/11/2010 13:23

Para: Karen Cherubini

Cc:

Assunto: Submission confirmation for "Imaging of the jaws in patients undergoing bisphosphonate therapy - A Literature Review"

Anexos:

Dear Karen,

Your submission entitled "Imaging of the jaws in patients undergoing bisphosphonate therapy - A Literature Review" has been received by The British Journal of Radiology.

You will be able to check on the progress of your paper by logging on to Editorial Manager as an Author at http://bjr.edmgr.com/

You will be informed by email of the manuscript reference number in due course.

Thank you for submitting your work to the BJR.

Kind regards,
BJR Office

ANEXO B

BJR Instructions for Authors

The British Journal of Radiology
Founded 1896
http://bjr.birjournals.org

AIMS AND COVERAGE

The British Journal of Radiology (BJR) is the official peer-reviewed monthly research journal of the British Institute of Radiology (BIR). It is a multi-disciplinary journal covering all clinical and technical aspects of diagnostic imaging, radiotherapy and oncology, medical physics and radiobiology. BJR is an international journal containing papers both from the UK and overseas, and is circulated to Full Members of the BIR and to libraries worldwide.

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SUBMISSION

Manuscripts should be submitted online at
http://www.editorialmanager.com/bjr.

Online submission will expedite the peer-review process. You will also be able to check the status of your submission online.

The BJR Editorial Administrator will be pleased to update authors on the status of their manuscript. Each paper is allocated a reference number, which should be quoted in any communication with the BJR in connection with that paper.

Submission of a paper is intended to imply that it presents original unpublished work, not under consideration for publication elsewhere.

PEER-REVIEW PROCESS

All submitted manuscripts will undergo peer-review. Each manuscript is normally allocated to two reviewers from a constantly updated database containing over 800 reviewers. Reviewers receive manuscripts with blind title pages to ensure an unbiased review.

Publication times: The time from submission to first decision averages 54 days. Papers are normally published within six months of acceptance.

Reviewers are asked to provide detailed constructive criticism for transmission to the authors. BJR requests that reviewers return their reports within 3 weeks of agreeing to review a paper. All efforts are taken to provide fair and thorough reviews as speedily as possible.

Having appraised the reviewers' reports, the Editors will make a final decision on each manuscript.

Categories of decision

- Accept
- Probable acceptance following minor revision
- Possible acceptance following major revision
- Reject

When revisions are requested, all points raised by the reviewers must be answered by the authors on a separate sheet, returned with their revised manuscript. However, if the authors disagree with specific reviewers' recommendations, authors are free to explain their reasoning when resubmitting their paper.

Authors should also be aware that manuscripts may be returned without external review when the Honorary or Deputy Editor deems that the paper is of insufficient general interest for the broad readership of the BJR, or that the scientific quality is such that it is unlikely to receive favourable reviews. Editorial rejection is done to speed up the editorial process and to allow the authors' papers to be promptly submitted and reviewed elsewhere.

CATEGORIES OF PAPER

BJR includes the following categories of paper, each serving a separate purpose:

- Full papers
- Commentaries
- Review articles
- Short communications
- Case reports
- Case of the Month
- Pictorial Reviews
- Letters to the Editor
- Book reviews

In addition to the general guidelines on preparation of a manuscript, please follow the guidelines overleaf for the specific type of paper.

Revised July 2009

A TYPICAL BJR PAGE CONTAINS:

1,000 words of text, or 4 average size tables, or 6 average size figures, or 40 references.

SHORT COMMUNICATIONS

This category of paper encompasses work-in-progress, short reports, technical notes etc.

- Authors of short communications should aim to be as concise as possible and should not include too many references.

In general, a short communication should be no more than three BJR pages in length.

CASE REPORTS

Case reports should be a brief description of a case with unique features not previously reported, e.g. previously unreported:

- clinical condition;
- relevant imaging observations on recognized disease or lesion;
- interventional technique in a recognized disease;
- complication of a radiological procedure.

In general, a case report should not exceed three BJR pages in length, and have no more than four authors.

CASES OF THE MONTH

These are short papers reporting a case that illustrates a point of particular educational value.

- A case of the month should be two BJR pages in length.
- The relevant history and initial images will appear on the first page, presenting a specific problem to the reader.
- On the second page, printed overleaf, will appear the results of further investigations and a discussion of the conclusion, followed by a brief and up-to-date review of the subject, with a maximum of six references.
- A case of the month should have no more than three authors.
- Radiotherapy and oncology papers, as well as diagnostic papers, are welcomed.

PICTORIAL REVIEWS

The aim of a "pictorial review" is to provide an up-to-date visual portrayal of a topical issue, having particular educational value. The amount of text should be kept to a minimum (1,000 words maximum). The article may be based on a poster presentation at a major meeting.

- The paper should be a maximum of six BJR pages.
- No more than eight key references should be included.

FULL PAPERS

Follow the general guidelines for preparing a manuscript when submitting full papers.

REVIEW ARTICLES

Longer review articles are published in the BJR. These will usually be solicited by the Honorary Editors. However, the Editors will be happy to consider proffered review articles and suggestions for such material. All review articles will undergo peer-review.

COMMENTARIES

Commentaries cover specific, sometimes controversial, subjects that are currently of topical interest. Two types of article fall into this category.

- *Based on a current hot topic.* A "personal view" of a current important and possibly controversial topic. The author should briefly explain the current position in the topic covered, and outline the various viewpoints that exist. The author should then go on to expound his/her own particular beliefs or analysis of the situation, perhaps indicating how he/she envisages research or practice progressing in the short- or long-term.
- *Based on a scientific meeting.* A discussion or review of one or more topics raised at the meeting. Authors who wish to write such an article should initially contact the BJR Honorary Editors, to avoid several reports being submitted based on the same meeting. It is not intended that the article should be a detailed account of the events of the meeting – rather, it should summarize the status of the topic covered, making general points of clarification and perhaps raising controversial issues or pointers for the future. The author is welcome to explain his/her own personal views on the topic.

Every effort will be made to fast-track Commentaries based on meetings through the peer-review process so that their appearance in print is timely.

Commentaries should be approx. 800–2500 words, including no more than six references.

LETTERS TO THE EDITOR

Letters to the Editor intended for publication in the Correspondence section fall into two categories.

- A letter on any matter of interest to readers of the BJR.
- A letter commenting on an article that has appeared in a previous issue of BJR. These will be forwarded to the authors of that article to allow them to reply. If accepted, the letters will be published together.
- Correspondence should not, unless absolutely necessary, contain tables or figures.
- All authors to a letter must sign it.

PREPARATION OF MANUSCRIPTS

General guidelines for all manuscript types are given below.

- All papers must be written in English.
- The manuscript, including references, tables and figure legends, should be typed in double line spacing, with margins of at least 25 mm on each side.
- Manuscripts should be submitted online at http://bjr.edmgr.com. Authors unable to submit online should contact the Editorial Office, bjroffice@bir.org.uk.
- Neither authors' names nor their affiliations should appear anywhere on the manuscript pages or the images (to ensure blind peer-review).

Title page

The title page, as a separate submission item, should provide the following information:

- Title of the paper. Abbreviations other than CT or MRI should not be used in the title.
- Category of paper (unless Full Paper).
- Names of the authors, which should comprise: initials, surnames plus qualifications (not more than three qualifications per author).
- The address(es) where the work reported in the paper was carried out (do not use abbreviations), linked to the appropriate author(s) using superscript numerals.
- If the corresponding author's address has changed, the current address may be included as a footnote, linked to the relevant name by an asterisk.
- A shortened version of the title (no more than 70 characters in length, including spaces) should be provided for use as the running head. Abbreviations are permissible.
- Footnotes stating a conference or meeting where a paper was presented should not be included.
- Footnotes stating any source of funding or financial interest where relevant should be included.

Blind title page

A blind title page should be included with the full manuscript, giving only the title (i.e. without the authors' names and affiliations), for use in the peer-review process.

Abstract

The abstract should be an accurate and succinct precis of the paper, not exceeding 250 words. It should not contain references. The abstract should: indicate the specific objective or purpose of the article; describe the methods used to achieve the objective, stating what was done and how it was done; present the findings of the methods described – key statistics should be included; present the conclusion of the study, based solely on the data provided.

Main text

The main body of a paper should begin on the page following the abstract.

- There are no stringent rules regarding the use of specific headings, but the general guideline is to organize text to include the following sections: Introductory section: briefly describe the objective of the investigation and explain why it is important; Methods and materials/patients: describe the research plan, the materials or subjects, and the methods used; Results: present results in a clear logical sequence. If tables are used, do not duplicate tabular data in the text, but do describe important trends and points; Discussion; Conclusion; Acknowledgments (if relevant).
- Avoid repetition between sections.
- Abbreviations and acronyms may be used where appropriate, but must always be defined where first used.
- The names and locations (town, country) of manufacturers of all equipment and non-generic drugs must be given.
- For the purposes of clarity, up to three clearly differentiated levels of subheading may be used.
- Avoid the use of footnotes.

References

Authors are responsible for the accuracy of the references. Only papers closely related to the work should be cited; exhaustive lists should be avoided. All references must appear both in the text and the reference list.

- References should follow the Vancouver format.
- In the text, references are cited in numerical order as numerals in square brackets. Within the brackets, numerals are ¬separated by commas, and three or more consecutive references are given as ranges, e.g. [1, 2, 7, 10–12, 14].
- At the end of the paper, starting on a new page, references should be listed in numerical order corresponding to the order in which they appear in the text.
- A reference cited in a table or figure caption counts as being cited where the table or figure is first mentioned in the text.
- Papers in press may be included in the list of references.
- Do not include references to uncompleted work or work that has not yet been accepted for publication. Abstracts and/or papers presented at meetings not in the public domain should not be included as references.
- References to private communications should be given only in the text (i.e. no number allocated). The author and year should be provided.
- If there are 6 or fewer authors, list them all. If there are 7 or more, list the first 6 followed by et al.
- Abbreviations for titles of medical periodicals should conform to those used in the latest edition of Index Medicus.

Examples of references

- Journal article: Include author names and initials, paper title, abbreviated journal title, year of publication, volume number, and first and last page numbers of paper: e.g.

1. Pages J, Buls N, Osteaux M. CT doses in children: a multicentre study. Br J Radiol 2003;76:803–11.

- Complete book: Include authors'/Editors' names, title of the book, town/country of publication and publisher name, and year of publication: e.g.

2. Peters AM, editor. Nuclear medicine in radiological dinosis. London, UK: Martin Dunitz, 2003.

- Chapter in book: Include authors of the relevant chapter, title of chapter, followed by "In: " and editors' names, title of book, town/country of publication and publisher name, year of publication, and first and last page numbers of material cited: e.g.

3. Brooks DJ. Functional imaging of movement disorders. In: Peters AM, editor. Nuclear medicine in radiological diag¬nosis. London,

UK: Martin Dunitz, 2003: 449–66.

- Conference proceedings: Include names of editors, title of publication, title of meeting, date and location of meeting, town/country of publication and publisher name, and year of publication: e.g.

4. Ring EFJ, Elvins DM, Bhalla AK, editors. Current research in osteoporosis and bone mineral measurement IV: 1996. Proceedings of the 1996 Bath Conference on Osteoporosis and Bone Mineral Measurement; 1996 June 24–26; Bath, UK. London, UK: British Institute of Radiology, 1996.

- Conference paper: Include author(s) and title of paper followed by "In:" and the details of the Conference Proceedings in which it appears – see above.

- Journal article on the internet:

5. Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12];102(6):[about 3 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

- Homepage/Web site:

6. Cancer-Pain.org [homepage on the Internet]. New York: Association of Cancer Online Resources, Inc.; c2000-01 [updated 2002 May 16; cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/.

Tables

- Tables should be referred to specifically in the text of the paper but provided as separate files.
- Number tables consecutively with Arabic numerals (1, 2, 3, etc.), in the order in which they appear in the text.
- Each table should have a short descriptive title.
- Tables should be self-explanatory and not duplicate data given in the text or figures.
- Aim for maximum clarity when arranging data in tables. Where practicable, entries in tables of figures should be confined to one line (row) in the table, e.g. "value (±sd) (range)" on a single line is preferred to stacking each entry on three separate lines.
- Ensure that all columns and rows are properly aligned.
- Include horizontal rules at the top and bottom of a table and one below the column headings. If a column heading encompasses two or more subheadings, then the main headings and subheadings should be separated by a single short rule. No other rules should be included, neither horizontal nor vertical.
- Appropriate space should be used to separate columns. Rows should be double-spaced.
- A table may have footnotes if necessary. These should be referred to within the table by superscript letters, which will then also be given at the beginning of the relevant footnote. Each footnote should begin on a new line. A general footnote referring to the whole table does not require a superscript letter.
- Abbreviations in tables should be defined in footnotes even if defined in the text or a previous table.

Figures

Figures should be referred to specifically in the text of the paper. Number figures consecutively using Arabic numerals. Concise, numbered legend(s) should be listed on a separate sheet. Avoid repeating material from the text. Abbreviations used in figures should be defined in the caption.

Files

- Image files should be supplied in EPS, TIFF or JPEG format.
- TIFF is preferred for halftones, i.e. medical images such as radiographs, MR scans etc.
- EPS is preferred for drawn artwork (line drawings and graphs).
- For JPEG files, it is essential to save at maximum quality, i.e. "10", to ensure that quality is satisfactory when the files are eventually decompressed.
- DO NOT supply PowerPoint files as these may be problematic with respect to quality rendering. Files supplied in Word or Excel may prove acceptable, but please supply in EPS, TIFF or JPEG if practicable. Other formats will not be usable.
- DO NOT supply GIF files – GIF is a compressed format that can cause quality problems when printed.
- Each figure should be uploaded separately and numbered.

Colour

- Unless essential to the content of the article, all illustrations should be supplied in black and white with no colour (RGB, CMYK or Pantone references) contained within them.
- If a paper contains colour figures, authors should state in the notes section of the submission page whether or not they feel it is vital that these figures are printed in colour if the paper is accepted. If the Editors agree that it is important for the figures to be in colour, they will be reproduced in colour AT NO COST TO THE AUTHOR. The Editors reserve the right to ask for a colour illustration to be converted to black and white
- Images that do need to be reproduced in colour should be saved in CMYK, with no RGB or Pantone references contained within them.

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- Files should be saved at the appropriate dpi (dots per inch) for the type of graphic (the typical screen value of 72 dpi will not yield satisfactory printed results). Lower resolutions will not be usable.
- Line drawings – save at 800 dpi (or 1200 dpi for fine line work).
- Halftone and colour work – save at 300 dpi.

Composition

- The image should be cropped to show just the relevant area (i.e. no more than is necessary to illustrate the points made by the author whilst retaining sufficient anatomical landmarks). The amount of white space around the illustration should be kept to a minimum.
- Supply illustrations at the size they are to be printed, usually 76 mm wide (single column of text) or for especially large figures 161 mm (two columns of text). The intermediate width of 100 mm is also available should neither of these suffice.
- Annotations, e.g. arrows, should be used to indicate subtle but salient points. All annotations should be included within the

images supplied.
• Patient identification must be obscured.
Additional points to note
• Do not put a box around graphs, diagrams or other artwork.
• Avoid background gridlines unless these are essential (e.g. confidence limits).
• Fonts should be Adobe Type 1 standard – Helvetica or Times are preferred.
• Ensure that lettering is appropriately sized – should correspond to 8 or 9 pt when printed.
• Include all units of measurement on axes.
• All lines (e.g. graph axes) should have a minimum width of ¼ pt (0.1 mm) otherwise they will not print; 1 pt weight is preferable.
• Avoid using tints (solid black and white or variations of crosshatching are preferred), but any tints that are used must be at a minimum 5% level to print (but do not use too high a tint as it may print too dark).
• Do not use three-dimensional histograms when the addition of a third dimension gives no further information.

Appendices

Authors are discouraged from including appendices if the material can be included in the main text. If an appendix is necessary, e.g. mathematical calculations that would disrupt the text, it should be supplied as a separate file. If more than one appendix is included, these should be identified using different letters.

- An appendix may contain references, but these should be listed separately and numbered A1, A2, etc.
- Appendices must be referred to in the main text.

ETHICS

When reporting experiments on human or animal subjects, the authors must indicate that the procedures followed were in accordance with the ethical standards of the responsible committee on human or animal experimentation (institutional or regional) or with the Helsinki Declaration of 1975, as revised in 1983. Patients' names, initials or hospital numbers should not be used, especially in illustrative material [3]. Papers submitted from overseas should adhere to UK ethical requirements.

Patient consent

Patient anonymity must be maintained. If there is any possibility that the patient can be identified in an illustration, written consent must be obtained from the patient/parent/guardian by the author, and a line stating that this has been received must be included in the figure caption.

BJR authors are of course aware that trust between doctor and patient is of paramount importance: the informed consent of all patients participating in reported trials must be obtained and a statement to this effect must be included in submitted manuscripts, when relevant.

Original data

The Editors reserve the right to ask to inspect the raw data on which the results of a submitted article are based.

UNITS, SYMBOLS AND MATHEMATICS

Authors should use the International System of Units (SI) [1]. Units of radiation should be given in SI, e.g. 1 Sv, 1 Gy, 1 MBq. Exceptions are mmHg for blood pressure and g dl–1 for haemoglobin. For guidance, authors can refer to the publication Units, Symbols and Abbreviations. A guide for medical and scientific authors [2].

- All radiation factors (dose/time/fractionation) must be listed.
- Equations should be numbered (1), (2) etc. to the right of the equation. Do not use punctuation after equations.
- Do not include dots to signify multiplication – parameters should simply be typed closed up, or with a multiplication sign if necessary to avoid ambiguity.

STATISTICAL GUIDELINES

The aim of the study should be clearly described and a suitable design, incorporating an appropriate number of subjects, should be used to accomplish the aim. It is frequently beneficial to consult a professional statistician before undertaking a study to confirm it has adequate power, and presentation of a power calculation within the paper demonstrates the ability of the study to detect clinically or biologically meaningful effects.

Details should be provided on selection criteria, whether data were collected prospectively or retrospectively, and any exclusions or losses to follow-up that might affect the study population. Information on subject characteristics in groups being compared should be given for any factors that could potentially bias the comparison of the groups; such information is often best presented in a tabular format in which the groups are in adjacent columns. If the study was randomized, details of the randomization procedure should be included.

Measures of variation should be included for all important results. When means are presented, the standard deviation or the standard error of the mean should also be given, and it should be clear which of these two measures is being quoted. When medians are given, measures of variation such as the interquartile range or overall range should also be included. Estimates of differences, e.g. between two means being compared, should be provided with 95% confidence limits to aid the reader and author to interpret the results correctly. Note that estimation of the size of effects, e.g. treatment or prognostic factor effects, is as important as hypothesis testing.

Statistical procedures should be described and referenced for all p-values given, and the values from which they were derived should be included. The validity of statistical procedures should also be confirmed, e.g. the t-test requires normal distribution(s) in the basic data and the 2 test is not valid when the expected numbers in cells are less than 5. Data may sometimes be transformed, e.g. using a log or square root transformation, to achieve normality. Non-parametric tests should be used when the conditions for normality are not met. It should be noted, however, that the Wilcoxon signed rank test (the non-parametric equivalent of the paired t-test) is semi-quantitative. If more than two groups are being compared then an analysis of variance should be performed before undertaking comparisons of pairs of groups. You are advised to seek the help of a professional statistician if you are uncertain of the appropriateness or interpretation of statistical methods.

Analysis of repeated measurements on the same subject can give

rise to spurious results if comparisons are made at a large number of different time points. It is frequently preferable to represent each subject's outcome by a single summary measure chosen for its appropriateness. Examples of such measures are the area under the curve, the overall mean, the maximum or minimum, and the time to reach a given value. Simple statistics can then be applied to these summary measures.

The results of the evaluation of a test procedure should state clearly the criteria used to define positivity, and the sensitivity, specificity, positive predictive value and negative predictive value should all be quoted together with their 95% confidence limits.

AUTHORSHIP

All authors are required to identify their manuscript contributions for which they are responsible. The author(s) responsible for the integrity of the entire study should also be identified. To be listed as an author, an individual should have made substantial contributions to all three categories established by the International Committee of Medical Journal Editors (ICMJE): (a) "conception and design, or acquisition of data, or analysis and interpretation of data"; and (b) "drafting the article or revising it critically for important intellectual content"; and (c) "final approval of the version to be published" (www.icmje.org) [3]. The ICMJE further states that "Acquisition of funding, the collection of data, or general supervision of the research group, by themselves, do not justify authorship." BJR asks that authors fulfill the ICMJE requirements to be so listed.

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ANEXO C

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Para: Karen Cherubini; kebini.ez@terra.com.br
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ANEXO D

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(last updated: March 2010)

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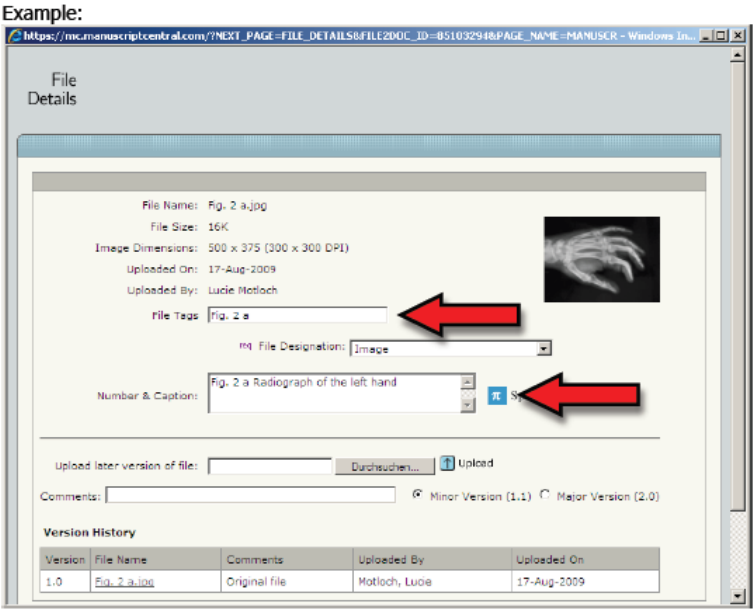

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2. Ward J, Robinson PJ (2002) How to detect hepatocellular carcinoma in cirrhosis. Eur Radiol. doi:10.1007/s00330-002-1450-y

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