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Post-radiation MMP-20 expression and its impact on dental micromorphology and radiation-related caries

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Abstract

Recent evidence suggested that head and neck radiotherapy (HNRT) increases active forms of matrix metalloproteinase 20 (MMP-20) in human teeth, degrading the dentin-enamel junction (DEJ) and leading to enamel delamination, which is a pivotal step in the formation of radiation-related caries (RRC). Therefore, the current study tested the hypothesis that MMP-20 would be overexpressed in the DEJ and in the demineralized dentin of post-HNRT patients, leading to micromorphological changes to the DEJ components, among the other dental tissues. Thirty-six teeth were studied, including 19 post-HNRT specimens and 17 non-irradiated controls. Optical light microscopy was used to investigate the micromorphological components of the DEJ, dentin-pulp complex, periodontal ligament, and the patterns of demineralization of caries. The samples were divided into two subgroups: non-demineralized ground sections (n=20) and demineralized histological sections (n=16). In addition, immunohistochemical analysis using the immunoperoxidase technique was conducted on the samples prepared for histological analysis to semi-quantitatively assess MMP-20 expression in the DEJ, the carious dentin and the other dentin-pulp complex components. No apparent damage to the DEJ microstructure or dentin-pulp complex components was observed ($p > 0.05$), and no statistically significant differences were detected in the MMP-20 expression ($p > 0.05$) between irradiated and control groups. In conclusion, the present study rejected the hypothesis that MMP-20 would be overexpressed in the DEJ of post-HNRT patients, leading to detectable micromorphological changes. Hence, direct effects of radiation may not be regarded as an independent factor to explain the rapid onset and aggressive clinical patterns of RRC progression.

Introduction

Head and neck cancer (HNC) represents 6% of all malignancies and approximately 670,000 new cases are diagnosed annually worldwide [Argiris et al., 2008]. Treatment usually involves surgery, chemotherapy, and radiotherapy (head and neck radiotherapy - HNRT) with high doses of radiation, alone or in combination with chemotherapy [Matzinger et al., 2009]. Although effective in cancer treatment, HNRT has a negative impact on the solid tissues surrounding the tumor within the radiation field and consequently, on the patient's quality of life [Sciubba and Goldenberg, 2006].

The direct impact of radiation on the dental tissues of cancer patients remains unclear and a matter of intense academic debate. Although many studies have suggested a direct radiogenic damage to the teeth, leading to radiation-related caries (RRC) [Pioch et al., 1992; Grotz et al., 1997;], others have linked the elevated risk of caries in post-HNRT patients with the indirect effects of radiation therapy in the head and neck region [Lieshout and Bots, 2014]. These include hyposalivation, oral microbiota alterations, impaired self-cleaning properties, poor oral hygiene prior to and after treatment, increased dietary intake of carbohydrates, and insufficient fluoride exposure [Kielbassa et al., 2006; Silva et al., 2009; Faria et al., 2014]. At least 28% of these patients have been estimated to present a higher risk of these aggressive caries, but the real number may be even higher [Hong et al., 2010].

Enamel organic matrix and the dentin-enamel junction (DEJ) act in synergy to preserve the adhesion between the enamel layer and the underlying dentin [McGuire et al., 2014b]. A recent study suggested that HNRT is able to increase the active forms of matrix metalloproteinase (MMP)-20 in irradiated teeth crowns [McGuire et al., 2014a]. MMP-20 (enamelysin) is considered a tooth-specific MMP [Llano et al. 1997,] because, in addition to tooth tissues, it has been detected *in vivo* only in some odontogenic tumors [Turk et al. 2006]. MMP-20 degrades amelogenin, type IV and V collagens, aggrecan, fibronectin, laminin, and tenascin-C [Turk et al. 2006, Mazzoni et al. 2012]. It is essential for the proper junctional adherence of enamel to dentin and enamel formation, as MMP-20-/- mice showed an amelogenesis imperfect phenotype, wherein a thinner enamel layer that delaminates from dentin is observed [Caterina et al.

2002]. In irradiated teeth, MMP-20 activation has been suggested to degrade the DEJ and surrounding enamel and dentin organic matrix [McGuire et al., 2014a], eventually leading to enamel delamination, which is considered a fundamental clinical step in the onset and progression of RRC [Walker et al., 2008].

In the present study, we performed an immunohistochemical analysis of the presence and localization of MMP-20 in the DEJ and in carious lesions of teeth extracted from head and neck cancer patients who underwent radiation treatment. A micromorphological study of the dental tissues, as well as the patterns of demineralization in RRC zones, was further performed and the results were correlated with immunohistochemical findings. The hypothesis was that MMP-20 would be overexpressed in the DEJ and in the carious lesions of *in vivo* irradiated teeth, leading to micromorphological changes to the DEJ and the adjacent dental tissues and components.

Material and Methods

Patients and specimen collection

This study was approved by the Ethics Committee of the Piracicaba Dental School (protocol 012/2013), University of Campinas, Sao Paulo, Brazil and was conducted in accordance with the Declaration of Helsinki. Non-carious and carious erupted teeth (n = 36) from head and neck cancer patients were collected following the protocol of the service of origin and independently of the particulars of the present study. Dental extractions were performed due to caries or advanced periodontal disease in both teeth groups (irradiated and non-irradiated).

Teeth forming the irradiated group were extracted from patients subjected to clinical radiation protocols with tridimensional conformal HNRT in 6-mV linear accelerators on the Synergy Platform (Elekta AB, Stockholm, Sweden) with a cumulative dose that ranged from 40 to 70 Gy (2 Gy/day at a maximum of five days per week) 3 to 12 months after RT conclusion. All patients were diagnosed with squamous cell carcinomas, except for one who was diagnosed with non-Hodgkin's Lymphoma. Non-irradiated specimens were obtained from HNC patients before radiation treatment. The tridimensional

HNRT plan of the patients was retrieved from the CMS system XiO version 4.60 (Elekta CMS software, St. Louis, MS, USA) to study the radiation field and the total dose directed to the primary tumor and teeth. For clinical characterization of the patients in this study, the electronic medical record system Tasy (Philips Clinical informatics, Blumenau, Brazil), was consulted and data were collected. Information about age, gender, primary tumor site, alcohol abuse and smoking habit, tumor histological type, clinical cancer stage (according the American Joint Committee on Cancer - AJJC), total amount of radiation during treatment (Gy), type of radiation plan, extracted teeth, and time between the end of HNRT and teeth extraction were retrieved from the patients' charts.

Immediately after the extractions, teeth were identified, placed in plastic containers with 10% buffered formalin solution and fixed for at least 72 h at 4 °C. The specimens were divided into two groups (irradiated and non-irradiated) and further divided into two other subgroups according to the histological preparation: subgroup 1 (non-demineralized samples) and subgroup 2 (demineralized samples).

Ground Section Preparation (subgroup 1)

Twenty teeth, including irradiated (n = 11) and non-irradiated (n = 9) specimens were inspected and the dental calculus was removed with periodontal curettes. The samples were sectioned along their long axes with a diamond saw (Extec, Enfield, CT, USA) in a precision cutter (Buehler Isomet 1000- Ltda., Lake Bluff, IL, USA), passing through the center of the deepest region of caries or dividing them into two equal halves to obtain a slice with a thickness of approximately 1.0 mm. The sections were then ground to a thickness of approximately 200 µm with silicon carbide (SiC) sandpapers, following the sequence of 600, 1200, 2000, and 4000 granulation. The final thickness was verified at the end of the process using a digital caliper (Standard Gage, Poughkeepsie, NY, USA).

Demineralization and histological preparation (subgroup 2)

Sixteen teeth, including irradiated (n = 8) and non-irradiated (n = 8), were decalcified in Ana Morse's solution (equal volumes of 20% sodium citrate and 50% formic acid) at 4 °C for three weeks, with changes every two days. The

samples were embedded in Paraplast Plus® (Leica Biosystems Richmond, Inc., Richmond, IL, USA) to produce 5 µm sections on a microtome (Leica, Nussloch, Germany) in silanized slides for hematoxylin and eosin (H&E) morphological evaluation and immunohistochemical analysis.

Optical light microscopy analysis

For micromorphological study of the DEJ, enamel and dentin components, an optical light microscope (OLM) (Eclipse E200, Nikon, Tokyo, Japan) was used and one ground section of each specimen was analyzed. Tufts, lamellae, spindles, type of DEJ (smooth or scalloped) in the different areas (cervical, medium, and incisal/occlusal thirds of the dental crown), striae of Retzius, and gnarled enamel were evaluated, as well as interglobular dentin, incremental lines, Tome's granular layer and tertiary dentin in the specimens of the subgroup 1. In demineralized histological sections from specimens of subgroup 2, the dentin-pulp complex and periodontal ligament components were analyzed semi-quantitatively. Finally, the patterns of caries were analyzed in the enamel and dentin, following methods described previously [Silva et al., 2009].

Immunohistochemical analysis

Histological sections of each demineralized specimen were deparaffinized in xylene. After deparaffinization, inhibition of endogenous peroxidase was performed by immersion in 10 volumes hydrogen peroxide for three times 5 min each. The sections were washed in three successive baths of PBS (pH 7.4) for 5 min each. To better expose the epitopes, the histological sections were subjected to antigen retrieval with 0.5% trypsin for 1 h in a humid chamber at 37 °C.

Nonspecific binding was blocked with bovine serum albumin (3% BSA/PBS) for 30 min at room temperature. Each slide was then incubated with the primary anti-MMP-20 antibody (monoclonal antibody C7 for MMP-20, Fuji Chemical Industries, Toyama, Japan) diluted at 1:100 in PBS and an overnight incubation was carried out at 4 °C in a humidifier. The sections were then washed with PBS in three baths of 5 min each and incubated with biotinylated secondary antibody (LSAB-Link DAKO Corporation, Carpinteria, CA, USA) at

first and then washed with streptavidin/peroxidase system (Biotin-Labeled streptavidin, Dako Corporation, Carpinteria, CA, USA). The reaction was visualized with 3,3-deaminobenzidina (DAB Substrate Kit ®, Dako Corporation, Carpinteria, CA, USA) applied for 90 s. The sections were counterstained with Mayer's hematoxylin and mounted with coverslips. Negative controls were performed with the omission of the primary antibody and human tooth germs were used as positive controls.

Statistical analysis

Data was analyzed statistically with SAS software version 9.3 (SAS Institute Inc., Cary, N.C., USA) using the Cochran-Mantel-Haenszel test, with the significance level set at $\alpha = .05$.

Results

Patients and specimens features

The irradiated samples were obtained from a total of 19 post-HNRT patients (subgroup 1, n=11; subgroup 2, n=8). In subgroup 1, 10 patients were male and 1 patient was female. In subgroup 2, 5 patients were male and 3 female. The mean age was 58 (44-74) years and 60 (52-75) years in subgroups 1 and 2, respectively. Smoking habit as well as alcohol abuse was recorded in 9 and 6 patients, respectively. Tumor location was represented by tongue (3), oropharynx (3), larynx (2), base of tongue (2) and maxillary sinus (1) in the first subgroup. In the second subgroup, tumors were located in tongue (4), soft palate (2), nasopharynx (1) and one case represented an unknown primary tumor with cervical metastatic lymph node in level II. All cases of subgroup 1 presented stage IV disease. Two cases were staged III and 6 cases were staged IV in the subgroup 2. Nine patients of subgroup 1 were treated with chemoradiotherapy (CRT) protocols and 2 with exclusive RT. Seven patients of subgroup 2 were treated with CRT and 1 with isolated RT. The mean total dose of radiation delivered to the tumors was 68.6 Gy (±SD 2.4) in subgroup 1 and 66.25 Gy (±SD 10.6) in subgroup 2.

Morphological analysis

A total of 12 incisors, 10 canines, 7 premolars and 7 molars were distributed in both groups. Caries were observed in 21 specimens (15 incisal/occlusal caries, 8 proximal caries and 16 cervical caries). Fifteen specimens did not present any surface affected by caries. The characteristic brown discoloration of RRC-affected teeth was present in 10/12 (83%) irradiated teeth but only 1/9 (11%) of non-irradiated teeth presented a similar pattern. Nine specimens presented superficial filling.

Morphological analysis of ground sections (group 1) of all post-HNRT specimens showed no significant difference in the micromorphological components of dental hard tissues, including enamel and dentin, between irradiated and non-irradiated groups.

There was a trend of dominance of the conventional scalloped-pattern DEJ in irradiated teeth compared with non-irradiated teeth (10/11 vs. 5/9, respectively; $p = 0.07$). Middle and incisal/occlusal areas had a scalloped DEJ pattern in all teeth, irrespective of irradiation. None of the specimens showed gap formation, cracking, or disruption of the DEJ. The presence of tufts, spindles, lamellae (Fig. 1A vs. 1E), striae of Rezius, and gnarled enamel did not differ between the irradiated and non-irradiated teeth. In dentin, interglobular dentin was encountered in 10/11 (90.9%) of irradiated teeth, but only in 5/9 (55.6%) of non-irradiated teeth ($p = 0.07$). There were no differences between the presence of the incremental lines, Tome's granular layer, or tertiary dentin in response to caries between the irradiated and non-irradiated teeth. Also, patterns of demineralization in RRC showed half-moon-shaped lesions presenting softened dentin, superficial demineralized dentin, sclerotic dentin, and translucent zones, as would be seen in conventional caries. The summary of micromorphological analysis of specimens from group 1 is presented in Table 1.

Histological analysis

Histological components of the dentin-pulp complex and periodontal ligament of demineralized post-HNRT specimens (subgroup 2) were also apparently not changed by radiation and again, the DEJ maintained its conserved pattern (Fig. 1B-C vs. 1E-F). The pulpal components (odontoblast cell layer, extracellular matrix with fibroblasts, nerve bundles, blood vessels and

calcifications) did not differ in structure between the irradiated and non-irradiated groups. The pulp presented showed polarized odontoblastic layer arranged in palisade, sub-odontoblastic cell-poor layer of Weil and the central zone (rich in blood vessels, fibroblasts, and neural bundles) with preservation of the normal components and architecture (Fig. 2A vs. 2D). Tertiary dentine formation was present under the caries front (Fig. 2B vs. 2E). Cementum and periodontal fibers from the ligament were preserved and similar in all irradiated and non-irradiated samples (not shown). In the specimens presenting caries, an outer layer (caries-infected dentin) composed of disorganized dentin and bacterial colonies, and an inner layer with affected, but not disrupted, dentin was observed (Fig. 2C vs. 2F). No significant difference was encountered between irradiated and non-irradiated groups in any of the analyzed parameters. The summary of histological analysis of demineralized specimens (group 2) is presented in table 2.

MMP-20 expression

MMP-20 expression was pronounced and intense along the DEJ of all irradiated and non-irradiated examined specimens (Fig. 3A vs. 3D). All teeth were also highly positive for carious dentine as well as sound dentine in some focal areas. The odontoblast cell layer was positive in 9/11 teeth, showing in most of the cases a cytoplasmic dot pattern, although it was similar in both groups (Fig. 3B vs. 3E); while pre-dentin and pulp tissue demonstrated more variable staining. An intense staining was available within dilated dentinal tubules towards the pulp (Fig. 3C vs. 3F). No significant differences were detected between irradiated and non-irradiated groups (Table 3).

Discussion

The results of the present study are in accordance with evidence that teeth exposed to high cumulative radiation doses for HNC treatment present a particular risk of RRC [Walker et al., 2011]. Teeth that had been exposed to *in vivo* irradiation were affected by caries that varied from early stages, characterized by brown discolorations affecting non-cavitated enamel and

incisal wear, to advanced cervical an incisal/occlusal cavitated lesions [Kielbassa et al., 2006; Silva, et al., 2009].

The interesting pattern, rapid onset, and progression of RRC in post-HNRT patients, continue to reveal the nature of the effects of radiation on dental tissues in the context of cancer treatment and dental morphology [Kielbassa et al., 2002; Silva et al., 2009]. Teeth presenting cervical and incisal/occlusal caries, as presented in this study, with partial or even total delamination of the enamel and crown teeth collapse could be seen in post-HNRT patients [Kielbassa et al. 2002]. Even though these features seem to be relevant to the clinical characterization of RRC, patients with other severe xerostomia and hyposalivation conditions, such as Sjögren syndrome [Napeñas & Rouleau, 2014] and post-allogenic bone marrow transplantation [Santos-Silva et al., 2015], are susceptible to similar type of caries. This consideration undermines the idea of main direct radiation damage to the dental tissues leading to RRC [Walker et al., 2011] but does not exclude that it can overlap the hyposalivation and the other indirect effects brought by HNRT [Lieshout and Bots, 2014].

Many authors have tried to correlate this propensity for enamel loss and decreased mechanical properties with radiogenic damage to the dental collagen and DEJ constituents in order to identify the mechanisms responsible for these events [Kielbassa et al, 2002; Franzel et al, 2006]. Some have suggested that this feature is due to post-radiation instability in the organic components of the DEJ, which could reduce anchoring between enamel and dentin [Pioch et al, 1992; Grotz et al, 1997 Jansma et al., 1993; Kielbassa et al, 2002; Franzel et al., 2006]. Some of these studies have even found that the DEJ of irradiated teeth appeared blurred, damaged, and unstable [Pioch et al, 1992; Grotz et al, 1997, El-Faramawy et al, 2013]. In contrast to these observations, the present study revealed that the DEJ of *in vivo*-irradiated teeth receiving high doses of radiation retained its normal characteristics of gnarled and smooth patterns without disruptions or enamel-dentin clefts. Furthermore, morphological preservation of the enamel components including lamellae, spindles, and tufts, which are considered to play an important role in adherence capacity and mechanical strength between the enamel and the dentin, seem to be unchanged by radiation on optical light microscopy analysis, confirming the findings of some previous studies [Springer et al. 2005, Silva et al., 2009].

Also, the other dentin-pulp complex components below the DEJ appeared similar in irradiated and non-irradiated teeth. The histologically normal odontoblast morphology, the palisade construction of the odontoblast cell layer and the unaffected pulp tissue confirm the similar findings of the previous studies [Silva et al., 2009; El-Faramawy et al., 2013, Faria et al., 2014]. The formation of reparative tertiary dentin in response to caries has also been demonstrated previously [Silva et al., 2009]. Together, these findings strongly indicate that in the post-HNRT teeth, the pulp does not only remain vital but also retains its capacity to respond to external irritation, such as caries.

The micromorphological analysis demonstrated that dentin caries presented classical characteristics: an outer layer of fully demineralized and denatured collagen matrix with bacterial colonization (caries-infected layer), and partially demineralized structurally unaffected organic inner layer (caries-affected layer) [Fusayama, 1997]. The present study is the second one to demonstrate that HNRT does not affect the histopathology of dentinal caries [Silva et al., 2009]. The finding is clinically relevant, since together with previous studies of similar dentin bond strength in irradiated and non-irradiated teeth [Silva et al., 2010; Galetti et al., 2014] it indicates that HNRT patients can be subjected to normal restorative procedures.

Radiotherapy has been directly associated with the regulation and activation of MMP in the lungs, brain, and cervix [Lee et al., 2012]. Recent studies have demonstrated that endogenous dentinal and salivary MMPs can play a major role in dentinal caries and erosion pathology [Tjäderhane et al., 1998; van Strijp et al., 2003; Buzalaf et al., 2015; Tjäderhane et al., 2015]. At least MMP-2, MMP-3, MMP-8, MMP-9, and MMP-20 have been indicated to participate in the process of dentin caries [Tjäderhane et al., 1998, Sulkala et al., 2002; Vidal et al., 2014].

A recent study showed by proteomic analysis that MMP-20 was enriched in extracts from *in vitro*-irradiated crown teeth. To our knowledge, the present study is the first one to evaluate immunohistochemical expression of MMP-20 in post-HNRT teeth, in which overexpression could not be observed. Both irradiated and non-irradiated groups had apparently the same pattern of expression and no significant differences were observed in any of the analyzed patterns. However, our study does not necessarily oppose previous results [Mc-

Guire et al., 2014a], because radiation can likely affect MMP-20 activity, but not the total amount of proteins, which cannot be evaluated by conventional immunohistochemical techniques. Nevertheless, as previously reported by Sulkala et al. [2002], DEJ, dilated dentinal tubules and caries, pre-dentine and odontoblasts were positive for MMP-20, the latter with an interesting dot staining within the cytoplasm of these cells.

In summary, we conclude that the direct effects of radiation do not appear to cause morphological changes in dental tissues or generate radiogenic destruction of its components, and that neither MMP-20 expression in DEJ nor the carious front of demineralization are changed by HNRT. Therefore, any of these events alone seem to be determinants of the onset and progression of RRC and the direct effects of radiation may not be regarded as an independent factor to explain the clinical patterns of RRC.

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Author Contributions

W.G.S., M.R.M. and A.R.S.-S. conceived of and designed the experiments. T.B.B. and A.C.P.R. obtained the samples. W.G.S., T.S. and M.R.M. performed the experiments. W.G.S., M.F.G., G.C.Jr. and A.R.S.-S. analyzed the data. W.G.S., M.A.L. and A.R.S.-S. wrote the paper. M.P.M., T.S. and L.T. reviewed the paper.

Disclosure Statement

We declare that this study comprises original results and that there are no conflicts of interest for any of the authors.

References

Argiris A, Karamouzis MV, Raben D, Ferris RL: Head and neck cancer. Lancet 2008;371:1695–1709.

Buzalaf MA, Charone S, Tjäderhane L: Role of host-derived proteinases in dentine caries and erosion. Caries Research 2015;49 (Suppl 1):30–37.

Caterina JJ, Skobe Z, Shi J, Ding Y, Simmer JP, Birkedal-Hansen H, Bartlett JD: Enamelysin (matrix metalloproteinase 20)-deficient mice display an amelogenesis imperfecta phenotype. J Biol Chem 2002;277:49598–49604

El-Faramawy N, Ameen R, El-Haddad K, El-Zainy M. Effects of gamma radiation on hard dental tissues of albino rats: investigation by light microscopy: Radiat Environ Biophys 2013;52:375–387.

Faria KM, Brandão TB, Ribeiro AC, Vasconcellos AF, de Carvalho IT, de Arruda FF, Castro Junior G, Gross VC, Almeida OP, Lopes MA, Santos-Silva AR: Micromorphology of the dental pulp is highly preserved in cancer patients who underwent head and neck radiotherapy. J Endod 2014;40:1553–1559.

Fränzel W, Gerlach R, Hein HJ, Schaller HG: Effect of tumour therapeutic irradiation on the mechanical properties of teeth tissue. Z Med Phys 2006;16:148–154.

Fusayama, T: Two layers of carious dentin; diagnosis and treatment. Oper Dent 1997;4:63–70.

Galetti R, Santos-Silva AR, Antunes A, Alves FA, Lopes MA, Goes MF: Radiotherapy does not impair dentin adhesive properties in head and neck cancer patients. Clin Oral Invest 2014;18:1771–1778.

Grötz KA, Duschner H, Kutzner J, Thelen M, Wagner W: New evidence for the etiology of the so-called radiation caries. Proof for directed radiogenic damage od the enamel-dentin junction. Strahlenther Onkol 1997;173:668–676.

Hong CH, Napeñas JJ, Hodgson BD, Stokman MA, Mathers-Stauffer V, Elting LS, Spijkervet FK, Brennan MT: A systematic review of dental disease in patients undergoing cancer therapy. Support Care Cancer 2010;18:1007–1021.

Jansma J, Vissink A, Jongebloed WL, Retief DH, Johannes ‘s-Gravenmade E: Natural and induced radiation caries. A SEM study. Am J Dent 1993; 6:130–136.

462 Kielbassa AM, Hinkelbein W, Hellwig E, Meyer-Lueckel H: Radiation-related
463 damage to dentition. Lancet Oncol 2006;7:326–335.
464 Kielbassa AM, Muntz I, Bruggmoser G, Schulte-Mönting J: Effect of
465 demineralization and remineralization on microhardness of irradiated dentin. J
466 Clin Dent 2002;13:104–110.
467 Llano E, Pendás AM, Knäuper V, Sorsa T, Salo T, Salido E, Murphy G, Simmer
468 JP, Bartlett JD, López-Otín C: Identification and structural and functional
469 characterization of human enamelysin (MMP-20). Biochemistry 1997;36:15101–
470 15108.
471 Lee WH, Warrington JP, Sonntag WE, Lee YW: Irradiation Alters MMP-2/TIMP-
472 2 System and Collagen Type IV Degradation in Brain. Int J Radiation Oncol Biol
473 Phys 2012;82:1559–1566.
474 Lieshout HF, Bots CP: The effect of radiotherapy on dental hard tissues – a
475 systematic review. Clin Oral Investig 2014;18:17–24.
476 Matzinger O, Zouhair A, Mirimanoff RO, Ozsahin M: Radiochemotherapy in
477 locally advanced squamous cell carcinomas of the head and neck. Clin Oncol
478 2009;21:525–531.
479 Mazzoni A, Breschi L, Carrilho M, Nascimento FD, Orsini G, Ruggeri A, Gobbi
480 P, Mazzotti G, Tay FR, Pashley DH, Tjäderhane L: A review on nature, role and
481 functions of dentin non-collagenous proteins. Part II: enzymes, serum proteins
482 and growth factors. Endod Topics 2012;21:19–40.
483 McGuire JD, Mousa AA, Zhang BJ, Todoki LS, Huffman NT, Chandrababu KB,
484 Moradian-Oldak J, Keightley A, Wang Y, Walker MP, Gorski JP: Extracts of
485 irradiated mature human tooth crowns contain MMP-20 protein and activity. J
486 Dent 2014a;42:626–635.
487 McGuire JD, Walker MP, Dusevich V, Wang Y, Gorski JP: Enamel organic
488 matrix: potential structural role in enamel and relationship to residual basement
489 membrane constituents at the dentin enamel junction. Connect Tissue Res
490 2014b;55(Suppl 1):33–37.
491 Napeñas JJ, Rouleau TS: Oral complications of Sjögren's syndrome. Oral
492 Maxillofacial Surg Clin N Am 2014;26:55–62.
493 Pioch T, Gofels D, Staehle HJ: An experimental study of the stability of
494 irradiated teeth in the region of the dento-enamel junction. Endod Dent
495 Traumatol 1992;8:241–244.

Santos-Silva AR, Feio PDSQ, Vargas PA, Correa MEP, Lopes MA: cGVHD-related caries and its shared features with other 'dry-mouth'-related caries. Braz Dent J 2015;26:435–440.

Sciubba JJ, Goldenberg D: Oral complications of radiotherapy. Lancet Oncol 2006;7:175–183.

Silva AR, Alves FA, Antunes A, Goes MF, Lopes MA: Patterns of demineralization and dentin reactions in radiation-related caries. Caries Res 2009;43:43–49.

Silva AR, Alves FA, Berger SB, Giannini M, Goes MF, Lopes MA: Radiation-related caries and early restoration failure in head and neck cancer patients. A polarized light microscopy and scanning electron microscopy study. Support Care Cancer 2010;18:83–87.

Springer IN, Niehoff P, Warnke PH, Bocek G, Kovacs G, Suhr M, Wiltfang J, Açil Y: Radiation caries–radiogenic destruction of dental collagen. Oral Oncol 2005;41:723–728.

Sulkala M, Larmas M, Sorsa T, Salo T, Tjäderhane L: The localization of matrix metalloproteinase-20 (MMP-20, enamelysin) in mature human teeth. Journal of Dental Research 2002;81:603–607.

Tjäderhane L, Buzalaf MAR, Carrilho M, Chaussain C: Matrix metalloproteinases and other matrix proteinases in relation to cariology: the era of “Dentin Degradomics”. Caries Research 2015;49:193–208.

Tjäderhane L, Larjava H, Sorsa T, Uitto VJ, Larmas M, Salo T: The activation and function of host matrix metalloproteinases in dentin matrix breakdown in caries lesions. J Dent Research 1998;77:1622–1629.

Turk BE, Lee DH, Yamakoshi Y, Klingenhoff A, Reichenberger E, Wright JT, Simmer JP, Komisarof JA, Cantley LC, Bartlett JD: MMP-20 is predominately a tooth-specific enzyme with a deep catalytic pocket that hydrolyzes type V collagen. Biochemistry 2006;45:3863–3874.

van Strijp AJ, Jansen DC, DeGroot J, Ten Cate JM, Everts V: Host-derived proteinases and degradation of dentine collagen in situ. Caries Res 2003;37:58–v65.

Vidal CM, Tjäderhane L, Scaffa PM, Tersariol IL, Pashley DH, Nader HB, Nascimento FD, Carrilho MR: Abundance of MMPs and cysteine-cathepsins in carious dentin. J Dent Res 2014;93:269–274.

530 Walker MP, Wichman B, Cheng AL, Coster J, Williams KB: Impact of
531 radiotherapy dose on dentition breakdown in head and neck cancer patients.
532 Pract Radiat Oncol 2011;1:142–148.
533 Walker MP, Williams KB, Wichman B: Post-radiation dental index: development
534 and reliability. Support Care Cancer 2008;16:525–530.

Legends

Table 1. Micromorphological analysis of preserved DEJ, enamel, and dentin components of non-demineralized specimens (subgroup 1).

Table 2. Micromorphological analysis of preserved DEJ, dentin-pulp complex, and periodontal ligament components of demineralized specimens (subgroup 2).

Table 3. Immunohistochemical analysis of MMP-20 in demineralized specimens (subgroup 2)

Fig. 1. Optical light micrographs of enamel and DEJ morphology of irradiated (group.1; A-C) and non-irradiated (group 2; D-F) specimens. (A) Detail of preserved tufts (arrowhead), spindles (arrow) and lamellae (*) in an irradiated specimen. Preservation of smooth (B) and gnarled (C) patterns of DEJ in both areas, showing no disruption or changes. (D) Micromorphological components of enamel with clear visualization of spindles (arrows). Smooth (B) and gnarled (C) patterns of DEJ representing cervical and incisal areas respectively.

Fig. 2. Morphological analysis of dentin-pulp complex components and caries of irradiated (group.1; A-C) and non-irradiated (group 2; D-F) specimens. (A) Preservation of the morphological dentin-pulp complex hierarchy in an irradiated sample. (B) Tertiary dentin formation with a poorly organized replacement odontoblast layer and mild pulp inflammation. (C) RRC showing an outer layer composed by a disorganized dentin and bacterial colonies, followed by an inner layer of affected dentin and dilated dentinal tubules underneath the demineralization front. (D) From the top, in sequence, the secondary dentin, predentin, odontoblast layer, cell-poor Weil zone, and a neurovascular bundle in the inner region of the pulp with a dystrophic calcification (arrow) are seen. (E) Tertiary dentin formation. (F) Representation of an occlusal conventional caries showing similar dentin breakdown.

Fig. 3. Immunohistochemical analysis of MMP-20 of irradiated (group.1; A-C) and non-irradiated (group 2; D-F) specimens. (A) Expression along DEJ of an irradiated specimen. (B) MMP-20 expression in pre-dentin, odontoblasts and pulpal fibroblasts with a remarkable dot-pattern cytoplasmic staining within the mature odontoblasts (C). Intense staining corresponding to the front of RRC demineralization in cervical caries and within dilated dentinal tubules towards the pulp (D). Same pattern of DEJ positivity in non-irradiated teeth. (E) Corresponding predentin, odontoblasts and pulp immunostainig. (F) MMP-20 positivity of superficial caries, and peri and intratubular staining of dilated dentinal tubules.

Table 1. Micromorphological analysis of preserved DEJ, enamel and dentin components of non-demineralized specimens (subgroup 1).

	Irradiated	Non-irradiated	*p*
DEJ			
Cervical	10/11 SC (91%) - 1/11 SM (9%)	5/9 SC (57%) - 4/9 SM (44%)	0.07
Middle	11/11 SC (100%) - 0/0 SM (0%)	9/9 SC (100%) - 0/0 SM (0%)	NA
Incisal/occlusal	9/9 SC (100%) - 0/0 SM (0%)	8/8 SC (100%) - 0/0 SM (0%)	NA
Tufts			
Cervical	7/9 (78%)	8/8 (100%)	0.16
Middle	9/11 (89%)	9/9 (100%)	0.18
Incisal/occlusal	10/11 (91%)	8/9 (90%)	0.88
Spindles			
Cervical	8/9 (89%)	5/8 (62%)	0.21
Middle	8/11 (73%)	8/9 (89%)	0.38
Incisal/occlusal	9/9 (100%)	8/9 (89%)	0.31
Lamella			
Cervical	8/9 (89%)	8/8 (100%)	0.34
Middle	7/11 (64%)	7/9 (78%)	0.50
Incisal/occlusal	7/10 (70%)	8/9 (89%)	0.32
Enamel	5/10 (50%)	7/9 (78%)	0.22
Striae of Retzius			
Gnarled enamel	6/11 (54%)	5/9 (55%)	0.96
Dentin	10/11 (91%)	5/9 (55%)	0.07
Interglobular dentin			
Incremental lines	3/11 (27%)	5/9 (55%)	0.21
Tomes' granular layer	8/11 (73%)	7/9 (78%)	0.80
Tertiary dentin	3/11 (27%)	3/9 (33%)	0.77
Caries (I/O)	8/11 (73%)	6/9 (67%)	0.80
Caries (C)	8/11 (73%)	7/9 (78%)	0.77

DEJ = dentin-enamel junction, SC = scalloped, SM = smooth, NA = not available, I = incisal, O = occlusal, C = cervical.

Table 2. Micromorphological analysis of preserved DEJ, dentin-pulp complex and periodontal ligament components of demineralized specimens (subgroup 2).

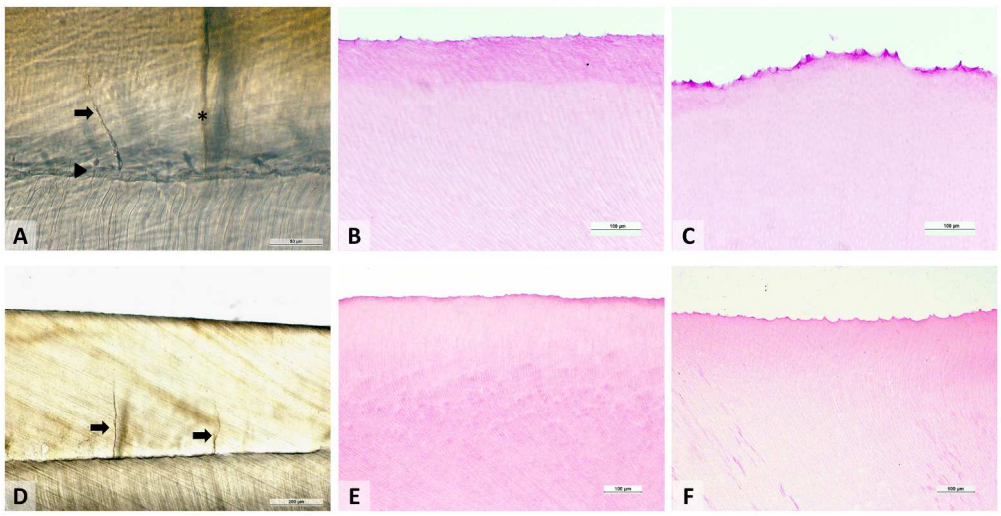
	Irradiated	Non-irradiated	*p*
DEJ	8/8 (100%)	8/8 (100%)	NA
Dentin			
Carious dentin	8/8 (100%)	5/7 (71%)	0.11
Tertiary dentin	3/8 (37%)	3/7 (43%)	0.77
Pulp			
Odontontoblastic layer	5/5 (100%)	4/6 (67%)	0.17
Extracellular matrix and fibroblasts	3/5 (60%)	3/5 (60%)	1.00
Calcifications	3/8 (37%)	3/7 (43%)	0.77
Nerve bundles	5/6 (83%)	6/7 (86%)	0.77
Blood vessels	6/6 (100%)	7/7 (100%)	0.54
Periodontal ligament			
Cementum	8/8 (100%)	8/8 (100%)	NA
Periodontal fibers	8/8 (100%)	8/8 (100%)	NA

NA = not available.

Table 3. Immunohistochemical analysis of MMP-20 in the demineralized specimens (subgroup 2).

	Irradiated	Non-irradiated	*p*
DEJ	8/8(100%)	8/8(100%)	NA
Carious dentin	8/8(100%)	5/7 (71%)	0.10
Sound dentin	8/8(100%)	8/8(100%)	NA
Tertiary dentin	1/4 (25%)	4/5 (80%)	0.11
Pre-dentin	3/6 (50%)	4/7 (57 %)	0.80
Odontoblastic layer	5/5 (100%)	4/6 (67%)	0.17
Pulp (ECM)	3/5 (60%)	3/5 (60%)	1.00

ECM = extracellular matrix, NA = not available.



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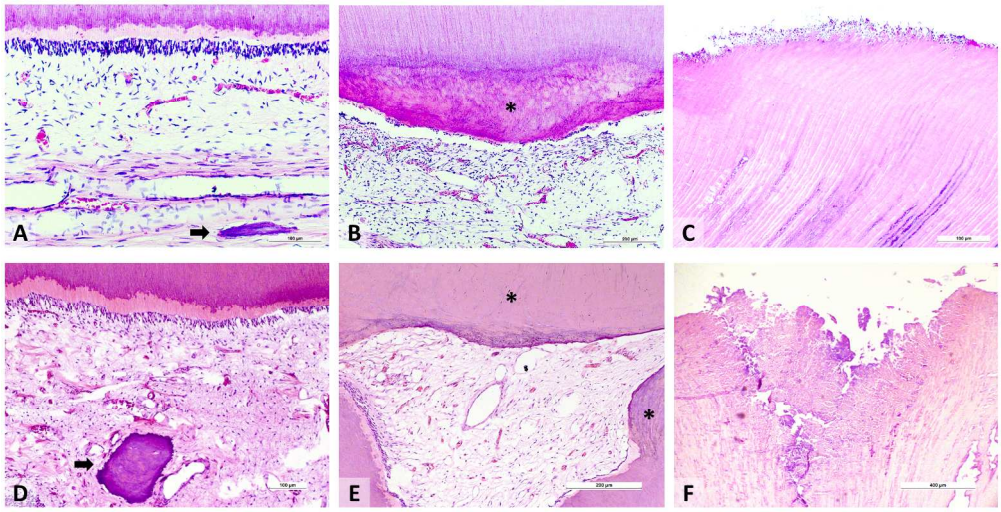

Fig. 2. Morphological analysis of dentin-pulp complex components and caries of irradiated (group.1; A-C) and non-irradiated (group 2; D-F) specimens. (A) Preservation of the morphological dentin-pulp complex hierarchy in an irradiated sample. (B) Tertiary dentin formation with a poorly organized replacement odontoblast layer and mild pulp inflammation. (C) RRC showing an outer layer composed by a disorganized dentin and bacterial colonies, followed by an inner layer of affected dentin and dilated dentinal tubules underneath the demineralization front. (D) From the top, in sequence, the secondary dentin, predentin, odontoblast layer, cell-poor Weil zone, and a neurovascular bundle in the inner region of the pulp with a dystrophic calcification (arrow) are seen. (E) Tertiary dentin formation. (F) Representation of an occlusal conventional caries showing similar dentin breakdown.

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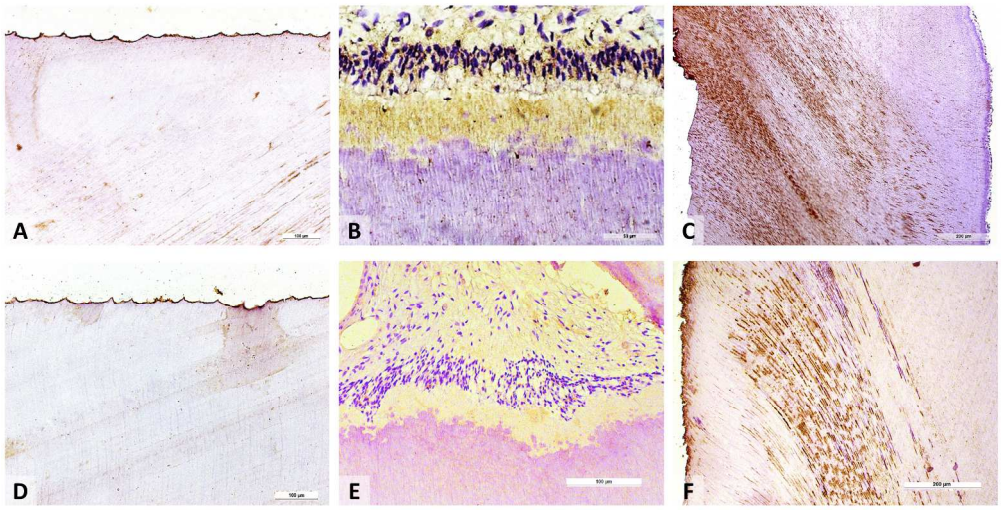

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