SANDRA RIBEIRO DE BARROS DA CUNHA

The Effects of Ionizing Radiation on Dentin Endogenous Proteases

São Paulo
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Corrected Version

Thesis presented to the Faculty of Dentistry of The University of São Paulo, by the Graduate Program in Restorative Dentistry to obtain the title of Doctor of Science

Supervisor: Profa. Dra. Ana Cecília Corrêa Aranha

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“The definition of insanity is doing the same thing over and over again, but expecting different results”

Albert Einstein

RESUMO

Cunha SR. Os efeitos da radiação ionizante nas proteínas endógenas da dentina [tese]. São Paulo: Universidade de São Paulo, Faculdade de Odontologia; 2018. Versão Corrigida.

A radioterapia é um dos principais tratamentos para pacientes com câncer de cabeça e pescoço e a cárie relacionada à radioterapia é um de seus efeitos colaterais, apresentando-se com alta taxa de ocorrência. Além disso, falhas precoces em restaurações realizadas em dentes de pacientes irradiados em cabeça e pescoço também são observadas. Como a degradação enzimática do colágeno ocorre principalmente através da atividade das metaloproteinases de matriz e das cisteína-catepsinas, o objetivo deste estudo foi avaliar a atividade enzimática da dentina hígida e restaurada de dentes submetidos à radioterapia *in vivo* e *in vitro*. Os dentes irradiados *in vivo* foram extraídos de pacientes submetidos à radioterapia com uma dose cumulativa que variou de 40 a 70 Gy. As extrações foram feitas de 3 a 12 meses após a RT devido a doenças periodontais. Para os dentes irradiados *in vitro*, as amostras foram submersas em água destilada com uma irradiação total e única de 70 Gy. O estudo foi dividido em 2 fases independentes: Fase 1: Dentina Não-Restaurada (avaliação de amostras não irradiadas, dentes submetidos à radioterapia *in vitro* e i*n situ*). Fase 2: Dentina Restaurada (avaliação de amostras não irradiadas e dentes submetidos à radioterapia *in vitro*) com 3 adesivos. Para o ensaio de zimografia (fase 1), os grupos irradiados *in vitro, in vivo* e não irradiados foram divididos em dois subgrupos: 1) mineralizado; 2) desmineralizado com ácido fosfórico10%. As proteínas dentinárias foram extraídas e submetidas à análise zimográfica de acordo com Mazzoni et al., 2007. Para a zimografia *in situ* (fase 2), os espécimes foram divididos em 6 grupos, de acordo com a forma de irradiação (não irradiada e irradiada *in vitro*) e o sistema adesivo testado (Adper Single Bond, 3M ESPE, ClearFil SE Bond, Kuraray ou Scotchbond Universal, 3M ESPE). Uma gelatina conjugada com fluoresceína auto-extinguível foi usada como substrato para as proteases endógenas. A atividade enzimática gelatinolítica foi observada em microscópio confocal (Zeiss LSM 780-NLO, Carl Zeiss Microscopy GmbH). Para a análise da microscopia eletrônica de varredura, amostras restauradas e hígidas foram submetidas a técnica de pré-imunomarcação

usando anticorpo monoclonal primário anti-CT-K e anti-CT-B, e anticorpo secundário conjugado com nano-partículas de ouro de 15nm. Um aumento na atividade gelatinolítica pós radioterapia para ambos os substratos (dentina restaurada e hígida) pôde ser observada. Houve uma maior expressão das formas ativas das MMP-2 e MMP-9 pós radioterapia para ambas as formas de radioterapia em dentina não restaurada. Nenhuma diferença na imuno-marcação para CT-K e CT-B entre os grupos irradiados e não irradiados foi observada. Adesivos autocondicionantes apresentaram uma imuno-marcação mais fraca para CT-K quando comparado ao adesivo de condicionamento total. Com isso, pode-se concluir que a radiação ionizante foi capaz de influenciar a atividade enzimática das proteínas endógenas da dentina restaurada e não restaurada.

Palavras-chave: Radioterapia, metaloproteinases de matriz, MMP, cisteino-catepsinas, CT, cárie relacionada à radiação.

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ABSTRACT

Cunha SR. The effects of ionizing radiation on dentin endogenous proteases. [thesis]. São Paulo: Universidade de São Paulo, Faculdade de Odontologia; 2018. Corrected Version

Radiotherapy is one of the main treatments for head and neck cancer patients. Radiation-related caries and early restorations failures are side-effects with high rate of recurrence. As enzymatic degradation of collagen occurs mainly through the activity of matrix metalloproteinases (MMPs) and cysteine-cathepsins (CTs), the objective of this study was to evaluate the influence of *in vivo* and *in vitro* radiotherapy on endogenous proteases of the restored and non-restored dentin. *In vivo* irradiated teeth were extracted from patients who underwent clinical radiation protocols with a cumulative dose of radiation that ranged from 40 to 70 Gy. Extractions were performed 3 to 12 months after radiotherapy conclusion due to periodontal reasons. For the *in vitro* irradiated teeth, samples were submerged in distilled water with a total and single irradiation dose of 70 Gy. For gelatin zymography assay, irradiated *in vivo*, *in vitro* and non-irradiated groups were divided in two subgroups: 1) mineralized or 2) demineralized with 10% phosphoric acid. Dentin proteins were extracted and submitted to zymographic analysis in accordance to Mazzoni et al., 2007. For *in situ* zymography, specimens were divided into 6 groups, according to its irradiation form (non-irradiated and irradiated *in vitro*) and the adhesive system tested (Adper Single Bond, 3M ESPE, ClearFil SE Bond, Kuraray or Scotchbond Universal, 3M ESPE) using a self-quenched fluorescein-conjugated gelatin as the endogenous proteases substrate. The endogenous gelatinolytic enzyme activity was assessed by confocal laser-scanning microscope (Zeiss LSM 780-NLO, Carl Zeiss Microscopy GmbH). For SEM analysis of the HL, restored specimens were submitted to a pre-embedding immunolabeling technique using primary monoclonal antibody anti-CT-K and anti-CT-B and a secondary antibody conjugated with 15nm gold nanoparticles. Radiotherapy groups presented increased gelatinolytic activity on both restored and non-restored dentin. MMP-2 and MMP-9 active form presented higher expression on both irradiated groups for non-restored dentin. Labeling for CT-K and CT-B did not differ from irradiated to non-irradiated groups. SE adhesives presenter weaker labeling for CT-K

when compared to the E&R adhesive. Herewith, ionizing radiation may be able to influence the enzymatic activity of the endogenous proteins of restored and unrestored dentin

Keywords: Radiotherapy, matrix metalloproteases, MMP, cysteine-cathepsins, CT, radiation-related caries.

LIST OF ABBREVIATIONS AND ACRONYMS

$CaCl^2$	Calcium chloride
CT	Cystein cathepsins
Cys	Cysteine residue
DNA	Deoxyribonucleic acid
EDTA	(Ethane-1,2-diyldinitrilo)tetraacetic acid
H^+	Hydron
H_3PO_4	Phosphoric acid
HNC	Head and neck câncer
HNCP	Head and neck cancer patient
HPV	Human Papillomavirus
IMRT	Intensity modulated radiation therapy
MMP	Matrix metalloproteinase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NRT	Non-irradiated
OH^-	Hydroxide
ORN	Osteoradionecrosis
RPM	Rotations per minute
RT	Radiotherapy
RTVI	*In vivo* radiotherapy
RTVT	*In vitro* radiotherapy
SDS-Page	Sodium dodecyl sulphate-polyacrylamide gel
TBS	Tris buffered saline
Tris HCl	Tris hydrochloride
$ZnCl_2$	Zinc chloride

SIMBOLS LIST

Gy	Grays
Wt%	Percentage by weight
h	Hours
s	Seconds
min	Minutes
nm	nanometers
L	Liters
mV	Millvolt
µl	Microliters
µg	Micrograms
%	Percentage
ºC	Celsius degree
≅	Similar
pH	Potencial of hydrogen
kDa	Kilodalton
Kv	Kilovolt
M	Molar
N	Newton
mg	Milligrams
ml	Milliliters
mM	Millimolar
mm	Millimeters

CONTENTS

1 INTRODUCTION

Over the last few decades, there has been a substantial improvement regarding head and neck cancer (HNC) treatment. Currently, radiotherapy (RT) is considered an important part of the treatment. According to the World Health Organization (WHO), two-thirds of oncological patients will have RT as a treatment option.

RT uses ionizing radiation (electromagnetic waves that carry energy) which interacts with both neoplastic and healthy tissues, ionizing the environment triggering water hydrolysis and DNA damage leading to cell apoptosis (1). For HNC, mostly treatments are based on cumulative fractionated doses that are daily delivered for up to 7 weeks, with pauses during the weekends. The standard treatment for head and neck cancer patients results in a final accumulated dose between 40 Gy and 70 Gy (2–4).

Despite the advantage of preserving the tissue structure, RT also interacts with the healthy adjacent areas that surrounds the tumor and, for HNC, this usually results in oral complications. Structural alterations in the salivary glands, decreased salivary flow, changes in salivary composition and oral microbiota, oral mucositis, muscular trismus, vascular alterations, as well as osteoradionecrosis has been known to be common side effects (3,5).

Additionally, the possibility that a direct effect of RT on the teeth may occur, promoting changes in the dentin-enamel junction, crystalline structures, acid solubility of enamel, dentin elastic modulus, matrix metalloproteinases (MMPs), and enamel and dentin microhardness have been reported (2,3,6,7).

All these structural changes cause a complex, multifactorial disease called radiation-related caries, a rapid, painless, and destructive form of tooth decay that can quickly lead to the amputation of crowns and present early restoration failures (3,8).

It is well known that endogenous proteases have an important role in caries progression and dentin bonding stability (9). The acidic environment, which is induced by the demineralization process of caries lesions and by the monomers contained in etch-and-rinse (E&R) and self-etch (SE) systems, is able to activate enzymes responsible to degrade demineralized collagen, such as matrix metalloproteinases (MMPs) and cysteine cathepsins (CTs) (9–11).

So far, no correlation between RT and endogenous proteases have been reported. Thus, this work aims to study the influence of ionizing radiation on the enzymatic activity of restored and non-restored dentin of head and neck cancer patients.

2 LITERATURE REVIEW

2.1 Head and Neck Cancer

Head and neck cancer (HNC) is a group of malignant tumors comprised at the upper aerodigestive tract anatomic sites, such as oral cavity, pharynx (oropharynx, nasopharynx and hypopharynx) and larynx. According to IARC, in 2012 approximately 530,00 new cases of oral and pharynx cases were estimated worldwide with 292,000 registered deaths. Latin America and the Caribbean accounted for 5.9% of these new cases and 5.1% of deaths (12).

Approximately 90% of all these malignant tumors are squamous cell carcinomas (13–15). These neoplasms are aggressive in their biologic behavior resulting in the early development of local lymph node metastases and evolution of distant metastases over time (16).

These cancers are strongly associated with certain environmental and lifestyle risk factors like tobacco and alcohol consumption, being responsible for the incidence of about 80 % of these cancers worldwide (17). The male to female ratio ranges from 2:1 to 4:1 and epidemiological evidence shows that the incidence of head and neck cancer increases with age being the average age 50 years (18).

More recently, patients diagnosed with oropharyngeal cancer are more likely to be younger adults in their 40’s who have never smoked or used other tobacco products (19). This could be due to a new disease that has emerged related to several strains of human papilloma virus (HPV). The relatively high rate of HPV infection in sexually active people (> 50 %) caused a significant increase in the incidence of this type of cancer over the past few decades (20,21). HPV positive head and neck cancer carries a more favorable prognosis than HPV negative cancers due to its better response to treatment (19,22,23).

Currently, there are several types of treatment for the most diverse types and locations of cancer, but we can say that the most used treatments are surgery, radiotherapy and chemotherapy, constituting the tripod of cancer treatment.

2.2 Radiotherapy

An important modality of treatment for patients with malignant head and neck neoplasms is radiotherapy (RT). This treatment can be used as a primary therapy for many tumors in the early stages, adjuvant therapy after surgical resection and palliative therapy in conjunction with chemotherapy for advanced stage tumors and cases in which it is impossible to perform a surgical resection (24).

Currently, it can be estimated that, approximately 75% of head and neck cancer patients (HNCP) will benefit from RT as part of their primary treatment or as adjuvant treatment modality after surgery (25,26). In most cases, this treatment is based on daily fractions, between 1,5 – 2 Gy, of the total dose, being the standard treatment for HNCP a final accumulated/cumulative dose between 40 Gy and 70 Gy (3,7,27).

Radiotherapy is a local therapy in which electromagnetic waves are used. These electromagnetic waves are considered as ionizing radiation, which means that the energy transported by these waves is superior to the energy that holds the electrons connected to the electrosphere, thus being able to disconnect them and creating free electrons. These free electrons are responsible for breaking the DNA molecule by direct or indirect damages, resulting in loss of physical integrity of the genetic material, preventing its duplication and followed by cellular apoptosis. The indirect damage is caused by the production of free radicals through the radiolysis of molecules, such as water, which is the most abundant molecule in the human body. Once the water molecule suffers an electronic rearrangement due to radiolysis, it produces H^+ and OH^- ions, being the latter highly unstable and reactive. H^+ and OH^- will bind to other molecules damaging and consequently making them lose function and/or produce more free radicals. The direct damage is the one caused by the direct interaction of the free electron with the DNA molecule (1,7).

The greater the DNA content in a cell population, the greater its sensitivity for radiation will be. This means that neoplastic tissues, which have a constant mitotic activity, are more prone to have a higher sensitive to radiation. Nonetheless radiosensitivity of a tissue is a complex phenomenon, and other factors such as their morphology, histogenesis and vascularization should be taken into account (28).

Currently, there are advanced techniques that help decrease the morbidity and toxicity to the healthy tissues surrounding the tumor. Intensity-Modulated Radiation

Therapy (IMRT) is an effective modality used for the treatment of HNC that modulates the intensity of the radiation beam, being able to reach the closest shape of the tumor, providing a more precise distribution, optimizing the distribution of the dose in the target tissue and protecting the peritumoral areas (29–31).

2.3 Radiotherapy Toxicities

Notwithstanding the advantage of preserving tissue structure, RT for HNC frequently results in a complex of oral complications affecting the oral mucosa, bone, masticatory muscles, dental structure and salivary glands (7,27). Some of these toxicities can be considered acute or chronic according to the period they arise and how long they persist after the end of treatment.

Oral mucosa responds rapidly to ionizing radiation through the appearance of mucositis, an inflammatory reaction of the mucous membranes due to loss of squamous epithelial cells, associated with the shift of the oral microbiota (32). Mucositis is the most common acute toxicity for HNCP presenting an incidence of 97% for patients receiving conventional radiotherapy (3,33–35). This toxicity usually appears at the end of the second week of RT and persists up to the third week after the end of treatment.

Dysgeusia is an alteration in taste perception and an early complication of RT. Taste buds can be found on the tongue, on the surface of the oral cavity, oropharynx and areas of the esophagus, meaning that this structure is in the radiation field of HNCP (32). It is thought that RT can cause a loss of taste buds and damages to the nerves responsible to process taste function, thus causing a reduction in taste sensitivity, an absence of taste sensation, or a distortion of normal taste (36,37). It can be seen a gradually improvement of the dysgeusia in the months after therapy, however, reduced taste is still reported as a hamper to quality of life by some patients even 1 year after the end of the treatment (32,38).

Hyposalivation and xerostomia, the subjective perception of dry mouth, are commonly reported by patients as a chronic toxicity of radiotherapy to the head and neck (39). Salivary glands, despite having a low mitotic rate, are known to be radiosensitive. A wide range of threshold mean dose for the salivary glands have been reported. Some authors also claim that with only 20Gy, approximately 80% of salivary

function is lost, after 30Gy the damage becomes permanent and, at the end of the radiotherapy treatment, the salivary flow is reduced by up to 95% (40–42). Histological changes to salivary glands comprise loss of secretory granules in acinar cells and infiltration of inflammatory cells, acinar atrophy, parenchymal loss, interstitial fibrosis, duct proliferation, dilated intercalated and striated duct (43,44). These histological changes cause not only quantitative but also several qualitative modifications in the saliva. Increased viscosity, changes in antibacterial properties with consequent alteration of bacterial flora, decrease in salivary pH (≅5), decreased buffer capacity and altered ionic concentrations were reported (3,6,39). With the decrease in pH and buffer capacity, the demineralization-remineralization system loses its equilibrium and its equation eventually moves to the demineralization side (45). Theoretically this disequilibrium should facilitate the demineralization of enamel and dentin after radiotherapy.

Until recently, hyposalivation and saliva's qualitative changes were considered the most likely cause for post-radiation dentition breakdown (3,40,46). Nevertheless, clinically it can be noticed that post-radiation dentition breakdown tend to begin, and is more severe, among teeth within the radiation field, being proposed that ionizing radiation may cause direct effects on mineralized tooth structure (47,48). It has been suggested three tiers of dose-response: Below 30Gy a minimal tooth damage can be expected; between 30 – 60Gy there is a 2 to 3x odds of increased tooth damage, probably related to the loss of saliva's protective effects; and with doses greater than 60Gy the dental tissue is 10 times more likely to develop damage by radiotherapy (47). Some studies using doses greater than 60Gy already reported changes in dentin and enamel properties including decreased hardness, elastic modulus and changes in the crystalline structure and collagen matrix (3,7,42,46,49,50).

In addition to all the toxicities mentioned, the difficulty in maintaining the oral hygiene, whether due to pain or anatomical alterations after surgery, the appearance of radiation caries in HNCP is practically unavoidable.

2.4 Radiation-Related Caries

Radiation-related caries (RRC), also known as radiation caries, is a unique disease due to its rampant onset and progression. It is a multifactorial disease presenting a highly potential for destruction of the dental element with consequent amputation of crowns and complete loss of the dentition in short periods of time without a significant painful symptomatology (3,7,42,51).

RRC is a chronic toxicity from radiotherapy which affects 90% of HNCP and tends to develop as quickly as four weeks after completion of RT presenting the highest risk of developing radiation caries in the first year following the end of the treatment (27,52–55).

Clinically, radiation-related caries normally appears as a diffuse brown discoloration on the smooth enamel surfaces, such as incisal (an area exposed to greater occlusal load), cervical (an area associated with non-axial loads causing flexion of the region) and cusps (3,6). These characteristics are unexpected, since those areas are the most resistant to caries in non-irradiated patients (3).

Until now, no histological differences between RRC and non-irradiated caries could be described. Silva et al., 2009, reported the presence of the 3 zones of non-irradiated caries in the RRC: Bacterial invasion, demineralization and translucent zones with the presence of reactionary dentin. It was also noticed the triangular pattern of demineralization in deep coronal dentin with the base at the tooth surface and the apex pointing to the pulp, thus concluding that ionizing radiation may not be essential to the microscopic progression of radiation-related caries (8).

The maintenance of the dentition for HNCP is the most appropriate decision, as removable prosthetics are not the best option since hyposalivation and anatomical changes can cause a lack of retention. Also, a rigorous oral hygiene, control of the xerostomia with artificial saliva, daily self-application of topical neutral fluoride and mouthrinses with chlorhexidine and regular appointments to the dentist can greatly help reduce the incidence of RRC, nonetheless unfortunately it does not completely prevent it (3). So, when necessary, adhesive restorations are recommended for RCC (56,57).

Restorative procedures must be kept simple for HNCP in order to preserve tooth function as well as aesthetics. There are few studies regarding adhesives protocols for HNCP. It is well known that glass ionomers can provide protection against

secondary caries. Nevertheless, in cases of xerostomic patients glass ionomers can dehydrate, causing an early erosion resulting in a rough surface and plaque retentive margins leading to a premature loss of the filling (56,58,59).

Resin based materials present greater adhesive potential and sealing ability when compared to glass ionomers. In xerostomic conditions, this material, even without fluoride release properties, presents a better marginal adaptation and structural integrity over the long term (56). Hence providing restorations with greater durability for these patients.

Studies on the effects of ionizing radiation on bond strength of composite resins are quite controversial. Several studies, both *in vitro* and *in vivo*, report no alterations on bond strength regarding irradiated teeth (2,60,61). Conversely, other authors could notice a negative effect of ionizing radiation in dentin bond strength of restorations performed after radiotherapy (5,62).

2.5 Contemporary Adhesive Systems

Bonding to dental substrate is based on the creation of micro-porosities by the removal of minerals on dental hard tissues and its replacement by resin monomers. This process comprises two phases based primarily on diffusion mechanisms: first, the removal of calcium phosphate from microporosities exposed in the enamel and dentin; second, the infiltration and subsequent in situ polymerization of the resinous monomers into microposorities created (63).

As resin monomers are not capable of infiltrating the mineralized dental tissue themselves, traditionally the adhesive bonding systems must contain an acid, so the mineral crystals can be removed and so exposing the collagen fibrils, a hydrophilic solution of resinous monomers used to displace the fluids from the dentin matrix and carry the monomers into the demineralized collagenous network called primer, and a *bond*, a mixture of monomers that can be considered the adhesive itself adhesive itself, that penetrate the surfaces treated with the *primer*, creating a mechanical adhesion to the substrate. These components can be presented in individual bottles or together, being carried out in one, two or three clinical application steps (64–66).

Adhesive systems can be classified according to the way they interact with dentin, currently presenting two categories: The etch-and-rinse (E&R), also known as total etch, and the self-etch (SE) techniques.

For the E&R strategy, an acid etchant is used to dissolve the smear layer and dentin minerals to the depth of 5-10μm deep leaving the collagen network suspended in the rinse-water. Then, the water within the collagen network must be replaced by the mixture of monomers from the adhesive system used to create the hybrid layer (HL), a structure composed of demineralized collagen fibrils reinforced by the resin matrix formed of biological polymers (64,66,67).

SE technique does not require an acid-etching step, as the adhesive systems present a hydrophilic etching primer that combines acidic resin monomers capable of simultaneously etch and prime the dental substrate (68–70). Accordingly, the difference between the depth of dentin demineralization and the capacity of resin infiltration is decreased (64,71,72). It has been reported that SE adhesives may present a better protection of collagen fibrils when compare to E&R adhesives due to a more homogenous resin infiltration, nonetheless its bond efficacy on enamel without the acid etchant is still questionable (73,74).

Regardless of the strategy used for bonding to dentin, the formation of the HL is of great importance since this connecting layer is the weakest point of the adhesive-resin bond (64,75–77).

2.6 Hybrid Layer

Bonding to dentin changes its physical and chemical properties. The tissue that previously was crystalline, hydrophilic and relatively impermeable becomes more hydrophobic, highly permeable and its organic content more notorious (78). In summary, the adhesion to dentin occurs through the infiltration of synthetic monomers within the organic scaffold in dentin, forming the base for a large variety of restorative procedures performed in dentistry (79,80).

Dentin chemical structure is based in both inorganic and organic components. Its composition consists of 70 mass% of minerals, 10 mass% of water and the remaining 20 mass% of organic components (81). Collagen is responsible for 90% of the organic matrices whereas the other 10% comprises non-collagenous proteins and

other dentin matrix proteins (82,83). Since mineralized dentin does not allow resin infiltration, to permit adhesive retention, dentin must be etched by acids or acidic monomers to remove minerals and expose collagen fibrils (84). Intra and interfibrillar spaces are created within the collagen matrix during the acid-etching, to avoid the its collapse the substrate must always be hydrated, thus allowing the hydrophilic monomers dissolved in organic solvents contained in the primer to replace the water and penetrate into and around the collagen fibrils (66).

The idea of the perfect penetration and encapsulation of the demineralized collagen fibrils by the monomeric resin underestimates the complexity of dentin supramolecular structure. It is well known that several elements can negatively interfere the arrangement of the HL, leaving exposed demineralized collagen fibrils without resin protection (79).

Customarily the acid-etching step from E&R adhesives demineralize dentin to the depth of 5-10μm, yet the monomers are not able to penetrate the entire length of the exposed collagen network, leaving on average 1μm of demineralized dentin not impregnated by the material, exposing denuded collagen fibrils not impregnated by the material, which may in the future lead to nanoinfiltrations (67,85,86).

It has already been described that although the interfibrillar space between collagen fibrils is large enough for small hydrophilic monomers (such as HEMA and TEGDMA) to penetrate completely, intrafibrillar space is smaller not allowing the material to completely infiltrate in this space, leaving collagen fibrils without the adhesive protection (79,87).

Currently, adhesive systems must be hydrophilic, so resin monomers can infiltrate the wet and demineralized dentin (65,88). However, this property causes a high sorption of water by the resin systems, making the HL behave as a permeable membrane even after polymerization. This water movement inside the adhesive facilitates the leaching of solubilized resin, thus exposing collagen fibrils and making the HL prone to enzymatic degradation (89,90).

2.7 Matrix Metalloproteinases (MMPs)

MMPs are zinc- and calcium-dependent endogenous proteases as they require these ions to maintain their proper tertiary structure and functional active sites.

These enzymes are expressed as inactive zymogens as they become trapped within the mineralized collagen matrix during dentin maturation. The Zn^{2+} ion is needed to the activation of the enzyme and Ca^{2+} is involved in the preservation of their tertiary structure.(64,91,92). Generally, MMPs comprise a hemopexin domain, a prodomain with a cysteine residue (Cys), a catalytic domain containing zinc ion, and a hinge region (93). The pro-form of the MMPs presents a bridge between the zinc ion and the cysteine residue in the pro-peptide, called the “cysteine switch”. This bridge created by the cysteine residue prevents the Zn^{2+} from interacting with water molecules, maintaining the enzyme inactive. All modes of activation lead to dissociation of the cysteine residue from the zinc atom with concomitant exposure of the active site (93–96)

While intact, meaning that no interaction between water and the Zn^{2+} of the catalytic domain occurred, it keeps the MMPs inactive.

MMPs can be activated by several processes such as heat treatment, exposure to low pH, application of certain chemical reagents and self-activation by other proteases. In dentin, their activation has been proved to be related to the low pH of organic acids produced by oral bacteria and of the acidic environment created by conditioners used in dental adhesives. Despite being activated in acidic conditions, it is after the neutralization of the pH that these enzymes reaches their highest activity (11,92,96–98).

MMPs are important components in several biological processes, since they are able to degrade almost all components of the extracellular organic dentinal matrix (ECM) (91,93). In dentin they are involved with physiological tooth development, caries evolution and degradation of the HL, participating mainly in the destruction of dentin collagen (9,99,100).

There are at least six groups of MMPs that are divided according to their properties: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other MMPs. So far only MMP-2, -3, -8, -9 and -20 have been identified in dentin (101–106). MMP-2 and -9 are the most numerous proteases of all dentin MMPs. Both MMPs are in the gelatinases group, meaning that they are capable of digest the gelatin from denatured collagen fragments. It has already been reported that both MMPs can also degrade telopeptides of collagen, making them also telopeptidases (107), facilitating and making collagen degradation by true collagenases faster.

2.8 Cysteine Cathepsins (CTs)

The cysteine cathepsins (CTs) is another group of endogenous proteases responsible for collagen degradation that have been recently reported to be present in dentin (10,108,109). As the MMPs, this family of enzymes are capable of digesting ECM proteins, specially collagen and have an important role on caries development and HL degradation (11,110,111).

Currently, 11 types of cysteine-cathepsins are known: B, C, F, H, K, L, O, S, V, X and W. CTs are synthesized in the form of pre- and pro-peptide, and after synthesis, the pre-peptide is removed during passage through the endoplasmic reticulum. Activation and maturation of the enzyme occurs when the peptide is removed through a proteolytic process, triggered by other proteases or by a mechanism of self-activation in acidic micro-environments created by lysosomes and endosomes (112,113).

The presence of CT-K and CT-B in sound and carious dentin has been reported (10,108,110). CT-K can be considered most important enzyme of its groups since it comprises 98% of cathepsin activity against collagen and differs from endogenous proteases due to its ability to cleave helical collagen at multiple sites generating multiple collagen fragments. CT-B is described to cleave only the non-helical telopeptide part of collagen (114,115).

It is hypothesized that CTs may be able to activate MMPs and work synergistically with those enzymes. Some studies report that both are localized very close together and in the vicinities of their target substrates, thus indicating a possible enzymatic cascade between these different classes of proteases (10,108,110,111).

Accordingly, the objective of the present study was to examine the influence of ionizing radiation and its delivery mode on the enzymatic activity of dentin. The null hypotheses tested were: 1) Radiotherapy would not influence on dentin enzymatic activity; 2) There would be no enzymatic activity difference between *in vivo* and *in vitro* radiotherapy; 3) There would be no enzymatic activity difference between within the hybrid layer regardless of the adhesive used

3 PURPOSE

The objective of the present study was to evaluate the enzymatic activity of non-restored and restored dentin with three different adhesive systems of teeth submitted to either *in vivo* or *in vitro* radiotherapy.

The following null hypotheses were tested:

1) Radiotherapy would not influence the activity of MMP-2 and MMP-9 for non-restored dentin.
2) Radiotherapy would not influence dentin gelatinolytic activity for non-restored dentin.
3) There would be no difference on enzymatic activity regarding the irradiation method, *in vitro* or *in vivo.*
4) Radiotherapy would not influence gelatinolytic activity within the hybrid layer regardless of adhesive system.
5) The distribution of CT-K and CT-B would not be altered on restored dentin after radiotherapy.

4 MATERIAL AND METHODS

This study was held at the School of Dentistry of the University of São Paulo and at the Department of Biomedical and Neuromotor Sciences of the University of Bologna as part of an international internship (BEPE – Bolsa Estágio de Pesquisa no Exterior/Research Internships Abroad – FAPESP, grant number 2017/01777-0).

All teeth were collected after approval by the Research Ethics Committee of the School of Dentistry at the University of São Paulo (CAAE: 54106616.8.0000.0075 - Report number: 1.946.959 - Attachment A) and stored at 4℃ in distilled water. The *in vivo* irradiated teeth were extracted from HNCP who underwent head and neck radiotherapy with a cumulative dose of radiation that ranged from 60 to 70 Gy (2 Gy/day at a maximum of 5 days per week), 3 to 12 months after RT conclusion due to periodontal diseases.

The study was divided into two independent phases:

- **Phase 1 – Non-restored dentin**

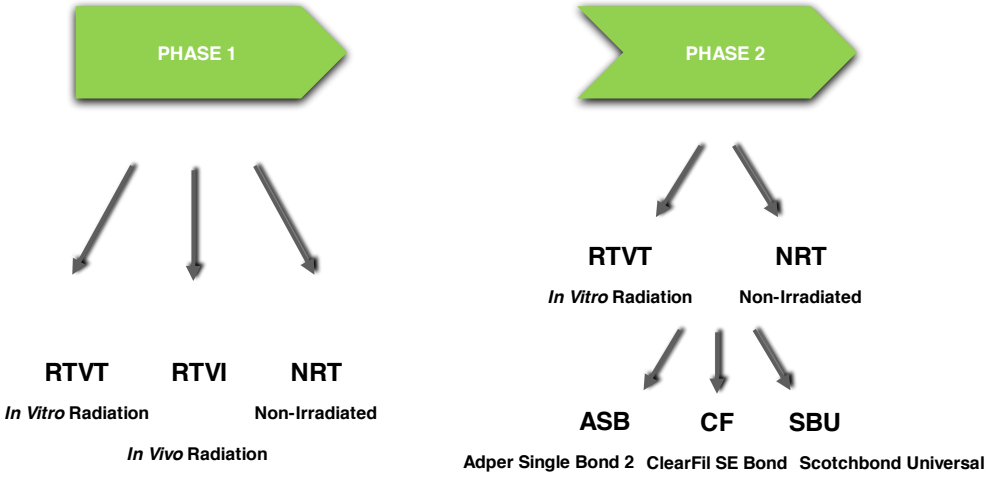
This phase had one experimental factor (n=3): 1) method of irradiation in 3 levels: Non-irradiated (NRT), *in vitro* radiotherapy (≃70 Gy) (VTRT) and *in vivo* radiotherapy (≃70 Gy) (VORT). The response variables were enzymatic activity (Densitometric evaluation of bands with gelatin zymography and integrated density of the fluorescence signals with *in situ* zymography).

- **Phase 2 – Restored dentin.**

Two experimental factors were comprised for the second phase (n=3): 1) Dentin substrate in 2 levels Non-irradiated (NRT) and *in vitro* radiotherapy (≃70 Gy) (VTRT); 2) Adhesive system used in 3 levels: Adper Single Bond 2 (ASB) (3M ESPE, Sumaré, SP, Brasil), ClearFill SE Bond (CF) (Kuraray, New York, NY, USA) and Scotchbond Universal (SBU) (3M ESPE, St. Paul., MN, USA). The response variables were enzymatic activity (Integrated density of the fluorescence signals with *in situ* zymography), localization and distribution of CT-B and CT-K (Immunolabeling with correlative field emission in-lens-scanning electron microscopy (FEI-SEM) immunohistochemical approach).

For better understanding, the division of groups for each phase can be seen at figure 4.1 and the description of the methodologies of both phases were divided into topics.

Figure 4.1 – Division of groups for both phases of this study



4.1 *In vitro* Radiotherapy

Teeth were irradiated with a total and single application of 70 Gy with X-rays from a linear accelerator (Mevatron MX2 6 mV; Siemens Healthcare, Erlangen, Germany) in the Department of Radiotherapy at Sírio-Libanês Hospital, São Paulo, SP, Brazil. Teeth were submerged in distilled water during radiotherapy. An isocentric set-up was done, so that the geometric center of the container was aligned with the isocenter of the linear accelerator. This means that from whatever angle the gantry was pointed, the distance between the “radiation source” and the container's center would be of 100 cm. For a homogeneous radiation distribution for all samples, radiation was applied in two opposing fields parallel to the incident radiation.

4.2 Evaluation of MMP-2 and MMP-9 Activity by Gelatin Zymography (Phase 1)

Gelatinase activity of dentin extracts was evaluated following the method of Mazzoni et al. 2007 (102).

Teeth had their pulp, enamel and cement removed. Dentin powder was obtained by triturating the remaining dentin with Retschmill (Reimiller, Reggio Emilia, Italy).

For each group of this phase (NRT, RTVT and RTVI) two Eppendorfs containing 100 mg of dentin powder were separated according to the treatment that dentin powder would receive, if they would be demineralized or left mineralized as a control.

For demineralized groups the dentin powder was treated with 300 µl of 10 wt% H3PO4 for 10 minutes at 4°C, and then neutralized with 70 µl of 5 N NaOH under agitation for 10 minutes. The supernatant was later removed after being centrifuged at 12000 rpm for 20 minutes at 4°C. Mineralized dentin powder served as a control.

For all groups a 1.8 ml of the extraction buffer (50 mM Tris-HCl, pH 6, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0,1% NONIDET P-40, 0.1 mM $ZnCl_2$, 0.02% NaN_3 with EDTA-free protease inhibitor cocktail) was added to the powder and was constantly agitated at 4°C for 24 hours protected from the light. Eppendorfs were then sonicated in an ultrasound bath with water and ice for 10 minutes. The vials were centrifuged at 12000 rpm for 20 min at 4°C and the supernatant transferred to a new Eppendorf. This step was repeated to guarantee that no powder was left in the liquid containing the extracted protein.

The protein content in the supernatant was concentrated using Vivaspin centrifugal concentrator (Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 minutes at 25 °C at 10000 rpm as many times needed to be concentrated up to 0.5 ml.

The total protein concentration of the dentin extracts was determined by Bradford assay and its dilution calculated. Dentin protein aliquots (60 µg) were diluted with Laemmli sample buffer in a 4:1 ratio.

Electrophoresis was performed under non-reducing conditions using 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) containing 1 mg/mL fluorescent dye- labelled gelatine. Pre-stained low-range molecular weight SDS-PAGE standards (Bio- Rad, Hercules, CA, USA) were used as reference markers. After electrophoresis, the gels were washed for 1 hour in 2% Triton X-100 and incubated in zymography activation buffer (50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, pH 7.4) for 48 hours protected from light.

Proteolytic activity was evaluated and registered with a long-wave ultraviolet light scanner (ChemiDoc Universal Hood, Bio-Rad). Densitometric evaluation of bands

obtained from zymography was performed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

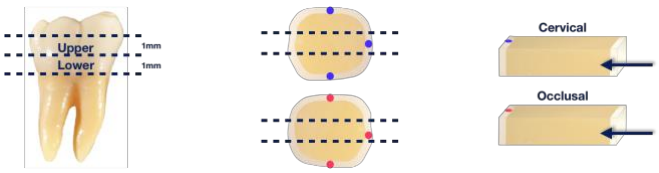
4.3 Preparation of Dentin Specimens (Phase 1 and 2 – *In situ* Zymography and Immunolabeling with FEI-SEM).

Teeth had their occlusal enamel removed with a low-speed diamond saw under water irrigation (Micromet; Remet, Bologna, Italy) to expose middle/deep dentin.

Two 1 mm-thick dentin slabs were obtained per tooth and 3 marks were made on the surface of interest of each disc. Surface of interest was always those that were in contact, which means that for the upper slice, this would be the surface facing the cervical region, and for the lower disc, the surface facing the occlusal region, this was done so the depth of the dentin was always the same for each specimen. The discs were latter cutted into 3 sticks and for all sticks, regardless of the phase, the face of interest was always the axial one (Figure 4.2).

Specimens of phase 2 had a standardized smear layer created with a 280-grit silicon-carbide paper and subsequent each one of the three pieces had the adhesive procedures done either with the Adper Single Bond 2, ClearFill SE Bond or Scotchbond Universal according to the manufacturer's instructions. A 1-mm build-up with flowable composite (Filtek 250 flow; 3M ESPE, St. Paul, USA) was build; the composite was polymerized photoactivated for 40 s using a light-emitting diode light-curing unit (Curing Light 2500; 3M ESPE, St. Paul, USA). Bonded specimens were stored at 37°C for 24 h. Later, each specimen used for Immunolabeling with FEI-SEM were sectioned into other 4 sticks.

Figure 4.2 – Scheme of the preparation and face of interest for *in situ* zymography and immunolabeling with FEI-SEM



4.4 *In situ* Zymography (Phase 1 and 2)

For the *in situ* zymography each stick obtained according to the item 4.3 was glued to a microscope slide and polished to the thickness of approximately 50 μm.

In-situ zymography was performed according to the protocol reported by Mazzoni et al. (116) using self-quenched fluorescein-conjugated gelatin as the MMP substrate (E- 12055; Molecular Probes, Eugene, OR, USA). A 1,0 mg/mL stock solution of fluorescein-labeled gelatin was prepared by the addition of 1,0 mL water to the vials containing the lyophilized substrate that was stored at -20°C until used.

The gelatin stock solution was diluted 1:8 with the dilution buffer (NaCl 150 mM, $CaCl_2$ 5 mM, Tris-HCl 50 mM, pH 8). A 50μL quantity of the fluorescent gelatin mixture was placed on top of each slab and covered with a coverslip. Slides were light-protected and incubated in humidified chambers at 37°C overnight. During incubation, the assemblies were prevented from direct contact with water, and were protected from exposure to light.

After incubation, the microscopic slides were examined using a confocal laser scanning microscope (excitation wavelength, 488 nm; emission wavelength, 530 nm; Model A1-R; Nikon, Tokyo, Japan). For each assembly, a series of 1 μm-thick two-dimensional images were made to show the hydrolysis of the quenched fluorescein-conjugated gelatin substrate, as indicator of endogenous gelatinolytic enzyme activity. Enzymatic activity was quantified on the images as the integrated density of the fluorescence signals using ImageJ software (National Institutes of Health, Bethesda, USA).

The integrated density of the fluorescence signals was quantified as indicator of the dentin enzymatic activities of the tested groups. Because the data were not normally distributed for both phases, they were statistically analyzed using the Kruskal-Wallis and Tukey test for pairwise multiple comparison, with $\alpha = 0.05$.

4.5 Immunolabeling with FEI-SEM (Phase 2).

Bonded specimens obtained according to item 4.3 were again cut in to 2 pieces according to the primary antibody that would be used. All pieces were placed into a culture cell plaque and separated according to the primary antibody that would be

used. Specimens were partially demineralized in 10% phosphoric acid for 60 seconds and then sonicated in distilled water for 1 minute to guarantee that any possible smear layer would be remove. Specimens were immediately washed in a 0.05M Tris buffer solution (TBS) at pH 7,6, with 0,15M NaCl (three rinses of 3 minutes each) and then preincubated in normal goat serum (British BioCell International; Cardiff, UK) dilution 1:20 in 0.05M TBS at pH 7.6 for 30 minutes at room temperature.

Specimens were then incubated overnight with one of the primary antibodies: rabbit IgG anti-human cathepsin B (Calbiochem; Billerica, USA) or mouse IgG anti-human cathepsin K (Biovendor; Brno, Czech Republic) (dilution 1:100 in 0.05 M TBS, pH 7.6). Three immersions of 3 minutes each in TBS 0.05M pH 7.6 were done, followed by three rinses of 3 minutes in TBS 0.02M pH 8.2. Gold labeling was performed using a secondary antibody, a goat anti- mouse or anti-rabbit IgG conjugated with 15 nm colloidal gold particles (British BioCell International, Cardiff, UK) dilution 1:20 in 0.02 M TBS (pH 8.2) for 90 minutes.

Specimens were immediately rinsed three times for 3 minutes in TBS 0.02M pH 7.2 followed by a final rinse in distilled water. All specimens were fixed in 2.5% glutaraldehyde in 0.15M cacodylate buffer pH 7.2 for 4 hours, rinsed and dehydrated in graded concentrations of ethanol.

The samples were critically point dried using Balzers CPD-010 (Bal-Tec AG, Liechtenstein) and coated with carbon using a Balzers Med 010 Multicoating System (Bal-Tec AG, Liechtenstein). Observations were performed using FEI-SEM (Nova NanoSem 450; FEI, Oregon, USA) at 10 kV. Images were obtained using a combination of backscattered and secondary electron detectors (117).

5 RESULTS

5.1 Phase 1 – Non-restored dentin

5.1.1 Evaluation of MMP-2 and MMP-9 Activity by Gelatin Zymography

Mineralized NRT dentin powder, showed presence of 92 kDa MMP-9 pro-form, a vaguely fainter 72 kDa band corresponding to the pro-form of MMP-2 and an additional band around 120 kDa. NRT dentin powder demineralized with 10% phosphoric acid presented a slight decrease in the expression of MMP-9 pro-form and of the additional band at 120 kDa, but a slightly higher expression of the 72 kDa band (pro MMP-2) when compared to the mineralized NRT (Figure 5.1, Lane 1 and 2).

Radiotherapy resulted in MMP-2 and MMP-9 activation, since it was observed in all RT groups the active form of both enzymes. Mineralized and demineralized RTVI and RTVT dentin powder presented a high activity for the 86 kDa band, representing the active form of MMP-9. A strong signal for the active form of MMP-2 (66 kDa) was noticed and a milder signal for the 110 kDa could be seen.

Densitometric evaluation of the zymography bands (Figure 5.2) confirmed that radiotherapy groups presented higher enzymatic activity, specially of the active forms of MMP-2 and MMP-9 when compared to the non-irradiated group. RTVI group showed a slightly higher activity then the RTVT group.

Figure 5.1 - Gelatin zymographic analysis of proteins extracted from irradiated and non-irradiated dentin powder. Standards are reported in lane STD. Lane 1: Mineralized non-irradiated dentin. Lane 2: Demineralized non-irradiated dentin. Lane 3: Mineralized irradiated *in vivo* dentin. Lane 4: Demineralized irradiated *in vivo* dentin. Lane 5: Mineralized irradiated *in vitro* dentin. Lane 6: Demineralized irradiated *in vitro* dentin

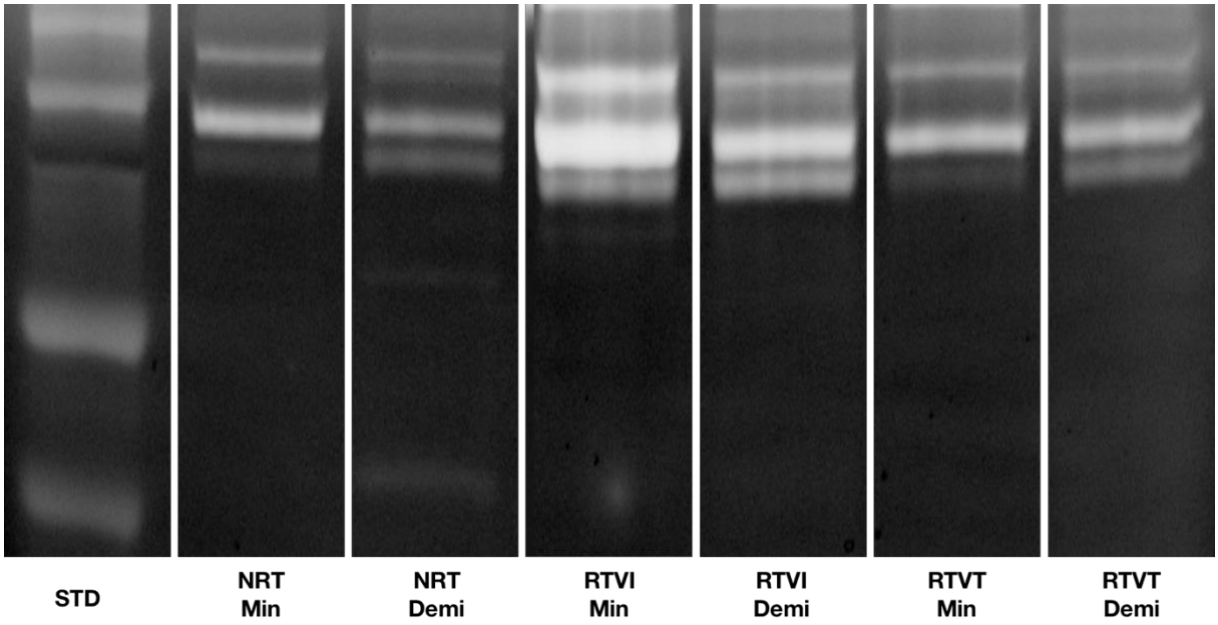
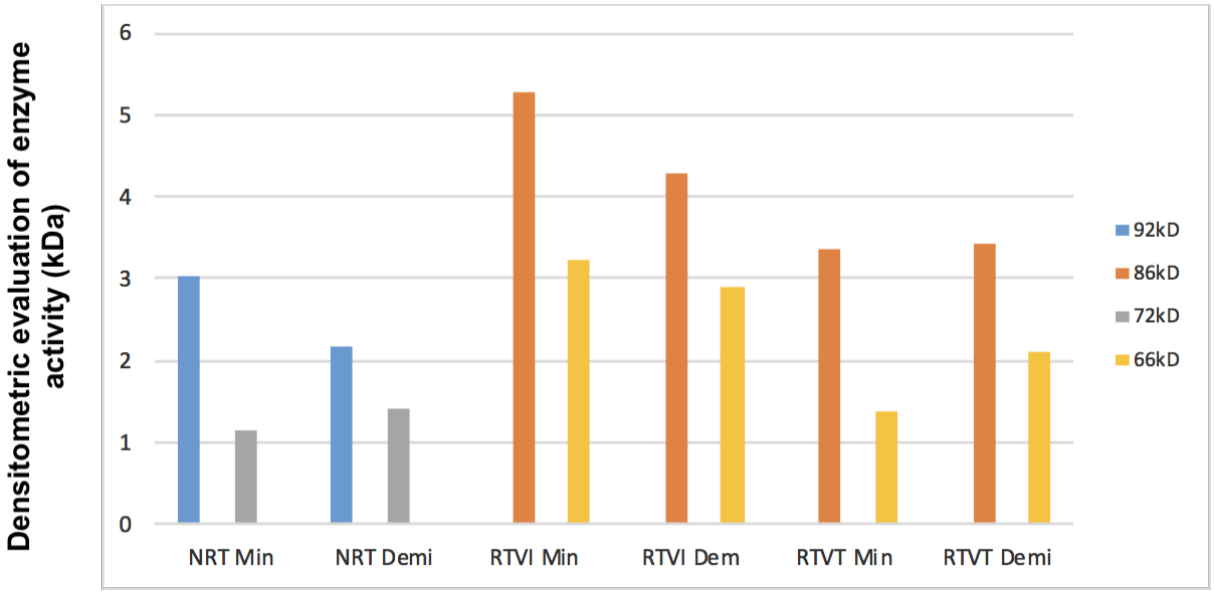


Figure 5.2 - Graphic illustrating the densitometric evaluation of bands obtained from the zymographic analysis of proteins extracted from dentin powder



5.1.2 *In situ* Zymography

Confocal laser scanning microscopy images of *in-situ* zymography and superimposition of the fluorescence with light microscopy of dentin are shown in Figure 5.3 to 5.5. Green fluorescence indicates that the fluorescein-conjugated gelatin was hydrolyzed at these sites by the presence of active dentin endogenous gelatinolytic enzymes representing gelatinolytic activity.

Non-irradiated (NRT) specimens exhibited minimal green fluorescence at dentin substrate (Figure 5.3). In contrast, specimens that underwent *in vivo* (RTVI) and *in vitro* (RTVT) radiotherapy exhibited a more intense hydrolyzation of the fluorescein-conjugated gelatin, especially inside dentin tubules (Figure 5.4 and 5.5). The quantification of the fluorescence indicates a significantly increase ($p < 0.05$) of 26% for the RTVT and 55% for the RTVI in the enzymatic activity when compared to the NRT group (Figure 6 and 7).

Figure 5.3 - Dentin slices of NRT group incubated with with quenched fluorescein-labeled gelatin. (A) Acquired image at green-channel, showing a weak fluorescence on non-irradiated dentin; (B) Differential interference contrast, showing the optical density of the dentin surface; (C) Image obtained by merging differential interference contrast image (optical density of the substrate) and the image acquired in green channel (showing enzymatic activity)

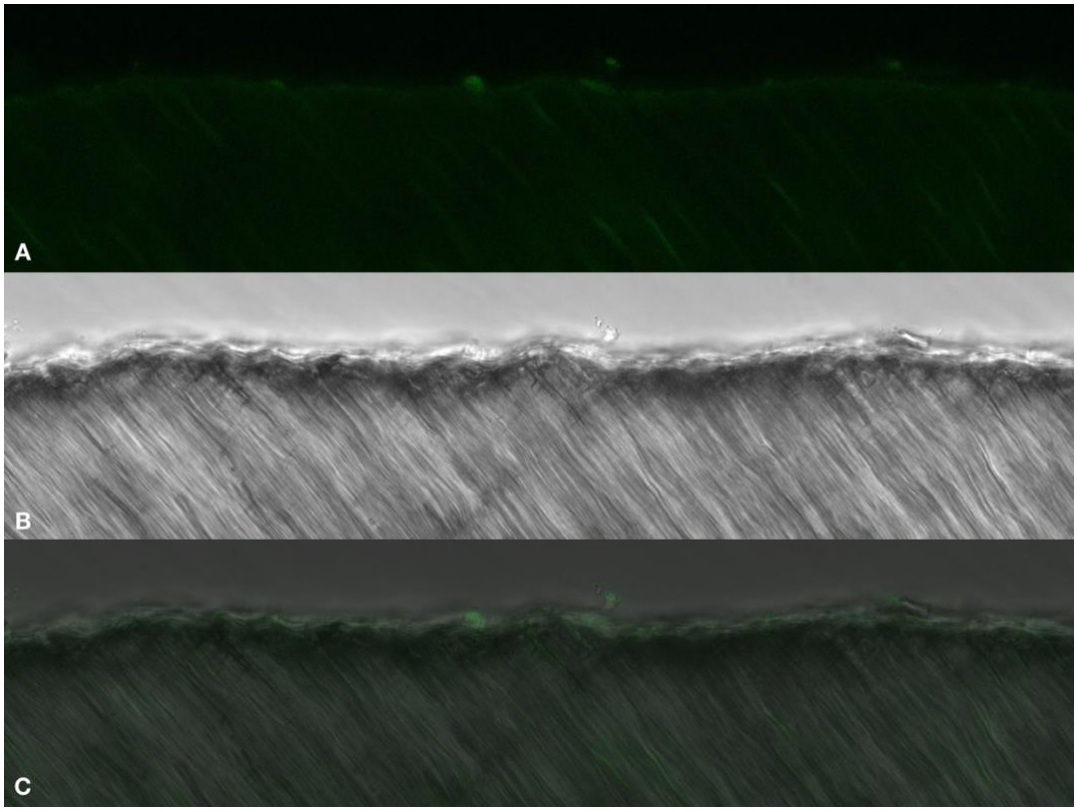


Figure 5.4 - Dentin slices of RTVT group incubated with with quenched fluorescein-labeled gelatin. (A) Acquired image at green-channel, showing a stronger fluorescence on *in vitro* irradiated dentin; (B) Differential interference contrast showing the optical density of the dentin surface; (C) Image obtained by merging differential interference contrast image (optical density of the substrate) and image acquired in green channel (showing enzymatic activity)

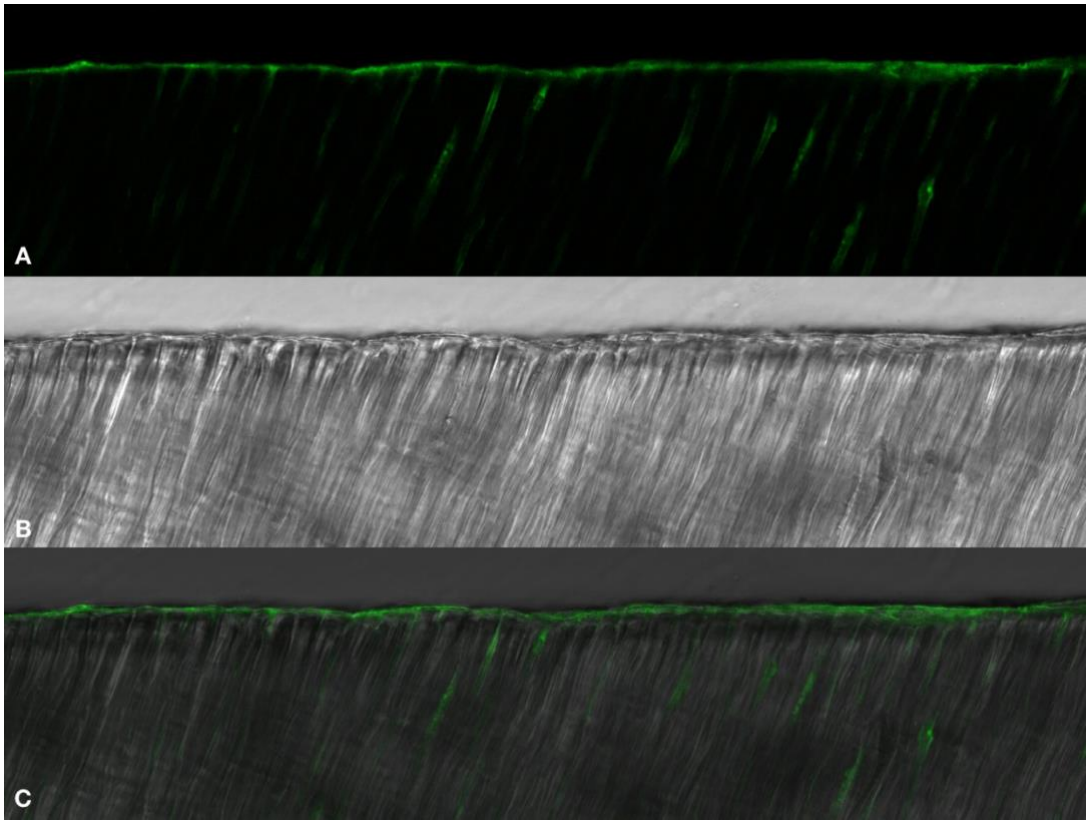


Figure 5.5 - Dentin slices of RTVI group incubated with with quenched fluorescein-labeled gelatin. (A) Acquired image at green-channel, showing a strong fluorescence on *in vivo* irradiated dentin; (B) Differential interference contrast showing the optical density of the dentin surface; (C) Image obtained by merging differential interference contrast image (optical density of the substrate) and image acquired in green channel (showing enzymatic activity)

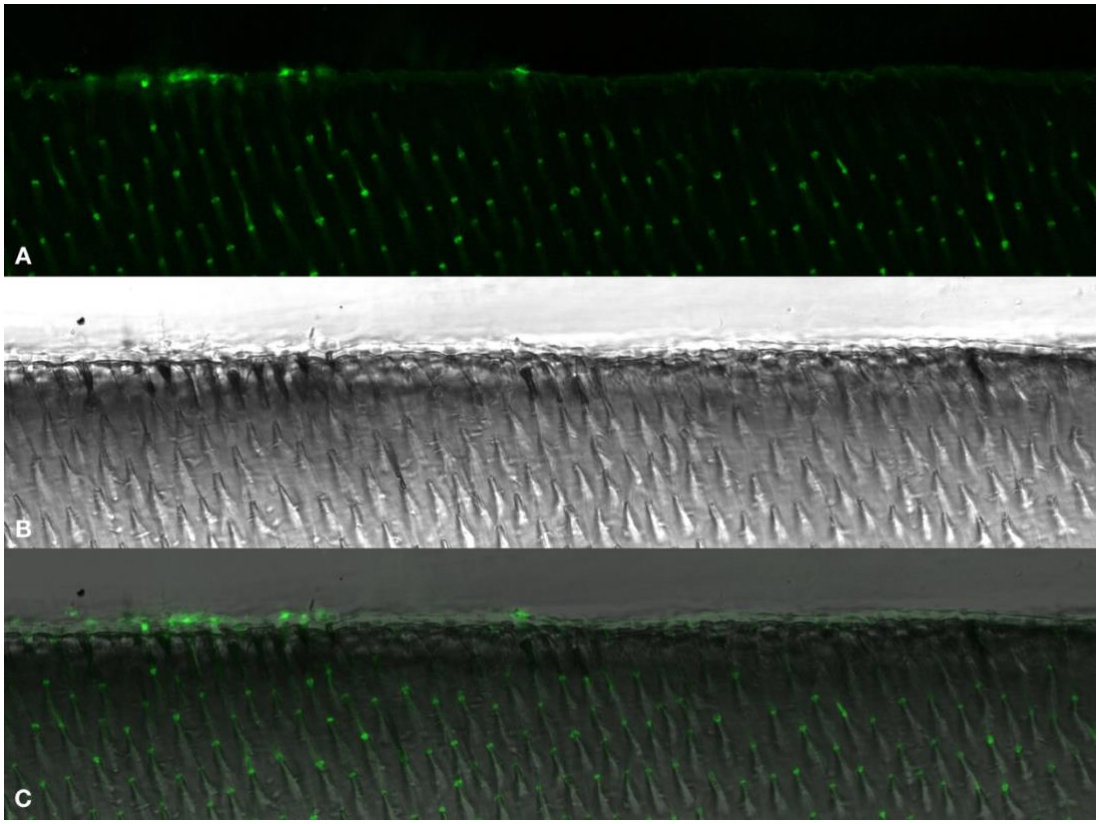


Figure 5.6 - Graph illustrating the median and intervals (25% and 75%) of the quantification of the enzymatic activity measured as integrated density of fluorescence signal for the tested groups from phase 1. Different letters denote significant difference among groups ($p < 0.05$)

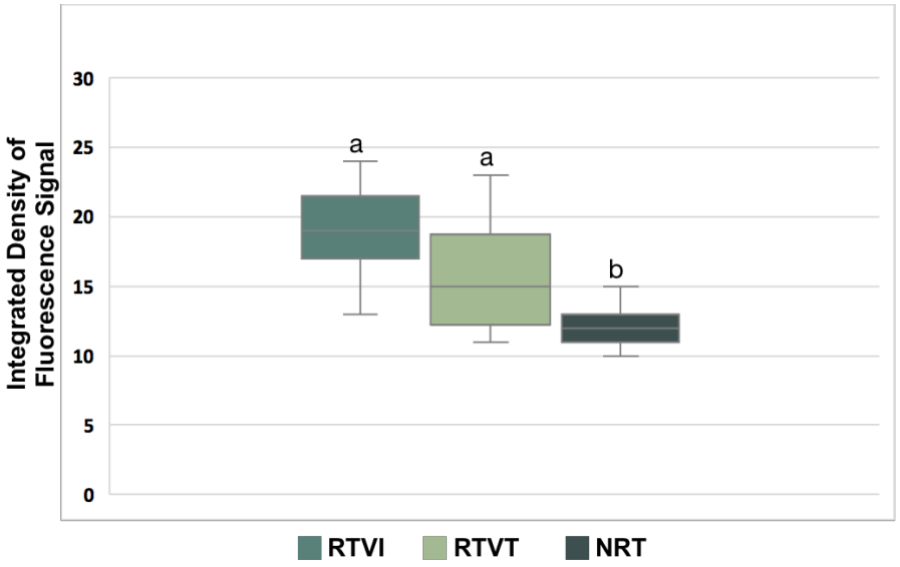
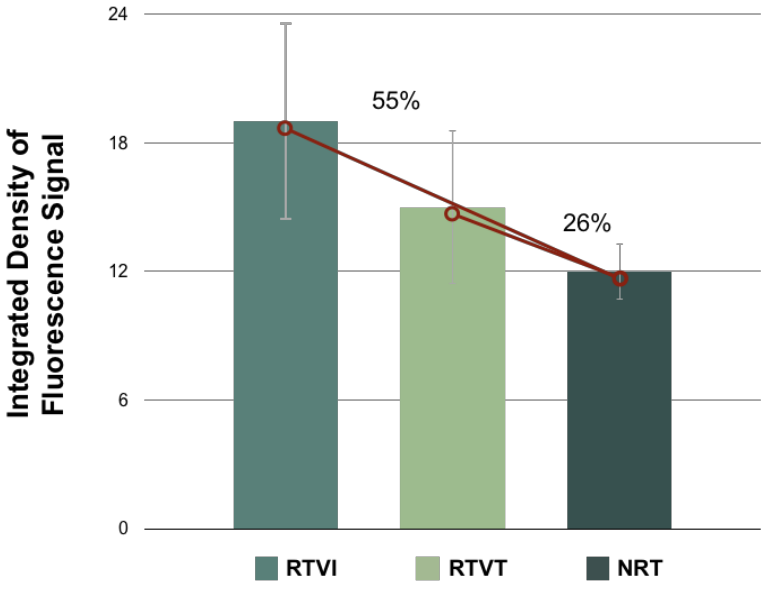


Figure 5.7 - Graph illustrating the mean and standard deviation of the quantification of the enzymatic activity for the tested groups from phase 1. Red bars represent the percentage of the increased enzymatic activity of the irradiated groups when compared to the NRT group



5.2 Phase 2 – Restored dentin

5.2.1 *In situ* Zymography

Representative images obtained by confocal laser scanning microscopy of the adhesive-dentin interface of irradiated and non-irradiated specimens restored with ASB, CF and SBU are presented in Figures 5. 8 to 5.10.

A significant difference of enzymatic activity between irradiated and non-irradiated groups could be seen for the adhesives used with the SE technique. Quantification of the fluorescence of the specimens indicates a substantial increase ($p < 0.05$) of 149% of the enzymatic activity for the RTVT groups when compared to the NRT groups using the CF adhesive. As for the SBU adhesive, an increase of 162% ($p < 0.05$) on the hydrolysis of the quenched fluorescein-conjugated gelatin could be seen for the RTVT group when compared to the NRT group. For the E&R adhesive (ASB) an increase of 29% with no statistical difference ($p > 0.05$) between RTVT and NRT groups was noted. No statistical difference was detected ($p> 0.05$) when comparing the enzymatic activity among all adhesives for the RTVT treatment. For the NRT groups, the SBU adhesive presented a statistically lower endogenous protease activation than the ASB adhesive, while the CF adhesive did not differ statistically from both. Median and intervals (25% and 75%) of HLs exhibiting hydrolysis of the quenched fluorescein-conjugated gelatin in the 6 subgroups are shown in figure 5.11. Mean, standard deviation and increased percentage are shown in figure 5.12.

Figure 5.8 - Resin-bonded dentin interfaces prepared with ASB for NRT (A, B, C) and RTVT (D, E, F) groups incubated with quenched fluorescein-labeled gelatin. (A, D) Acquired image at green-channel, showing fluorescence in dentinal tubules and within the HL created with ASB; (B, E) Differential interference contrast showing the optical density of the resin-dentin interface; (C, F) Image obtained by merging differential interference contrast image (optical density of the substrate) and image acquired in green channel (showing enzymatic activity)

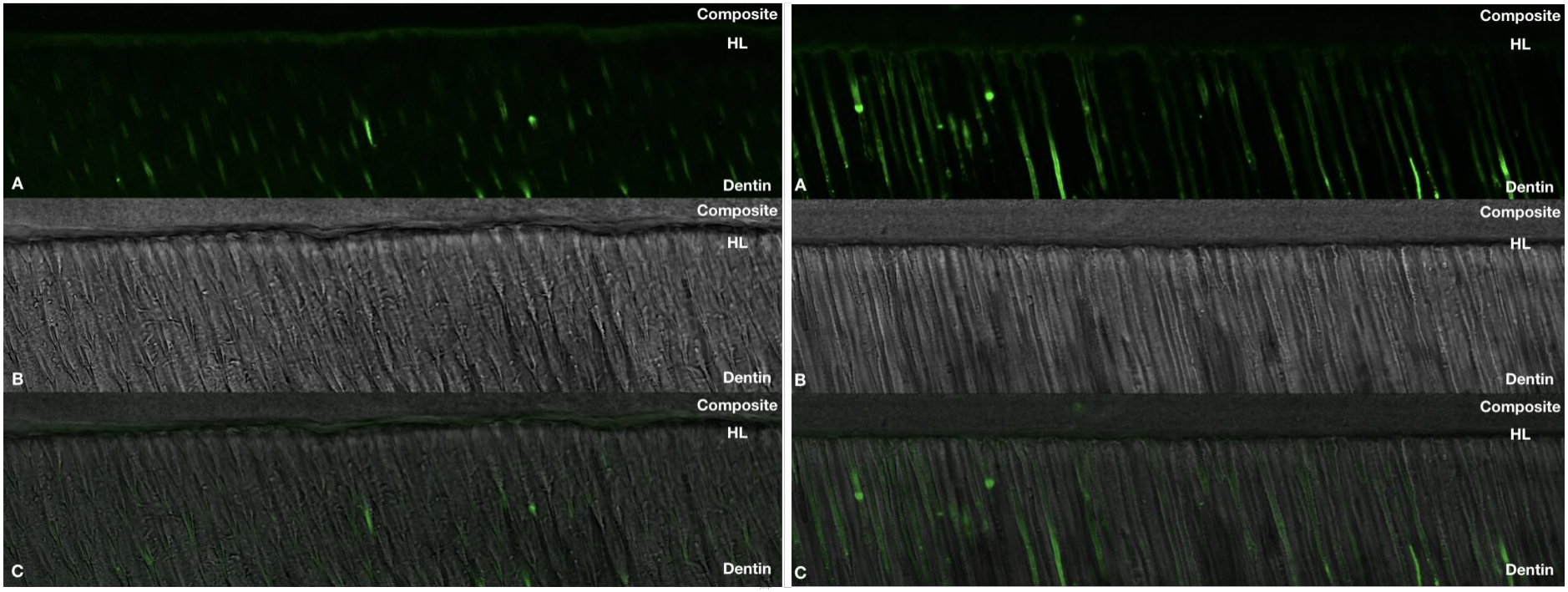


Figure 5.9 – Resin-bonded dentin interfaces prepared with CF for NRT (A, B, C) and RTVT (D, E, F) groups incubated with quenched fluorescein-labeled gelatin. (A, D) Acquired image at green-channel, showing fluorescence in dentinal tubules and within the HL created with ASB; (B, E) Differential interference contrast showing the optical density of the resin-dentin interface; (C, F) Image obtained by merging differential interference contrast image (optical density of the substrate) and image acquired in green channel (showing enzymatic activity)

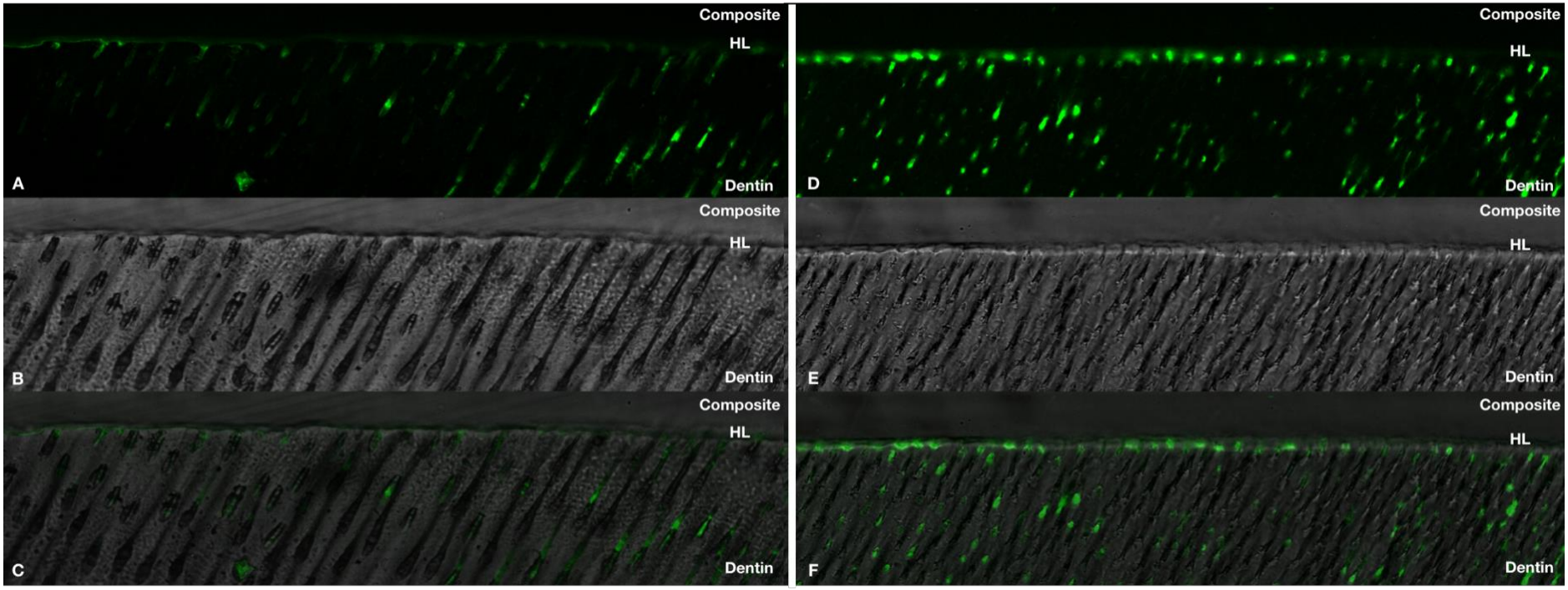


Figure 5.10 - Resin-bonded dentin interfaces prepared with SBU for NRT (A, B, C) and RTVT (D, E, F) groups incubated with quenched fluorescein-labeled gelatin. (A, D) Acquired image at green-channel, showing fluorescence in dentinal tubules and within the HL created with ASB; (B, E) Differential interference contrast showing the optical density of the resin-dentin interface; (C, F) Image obtained by merging differential interference contrast image (optical density of the substrate) and image acquired in green channel (showing enzymatic activity)

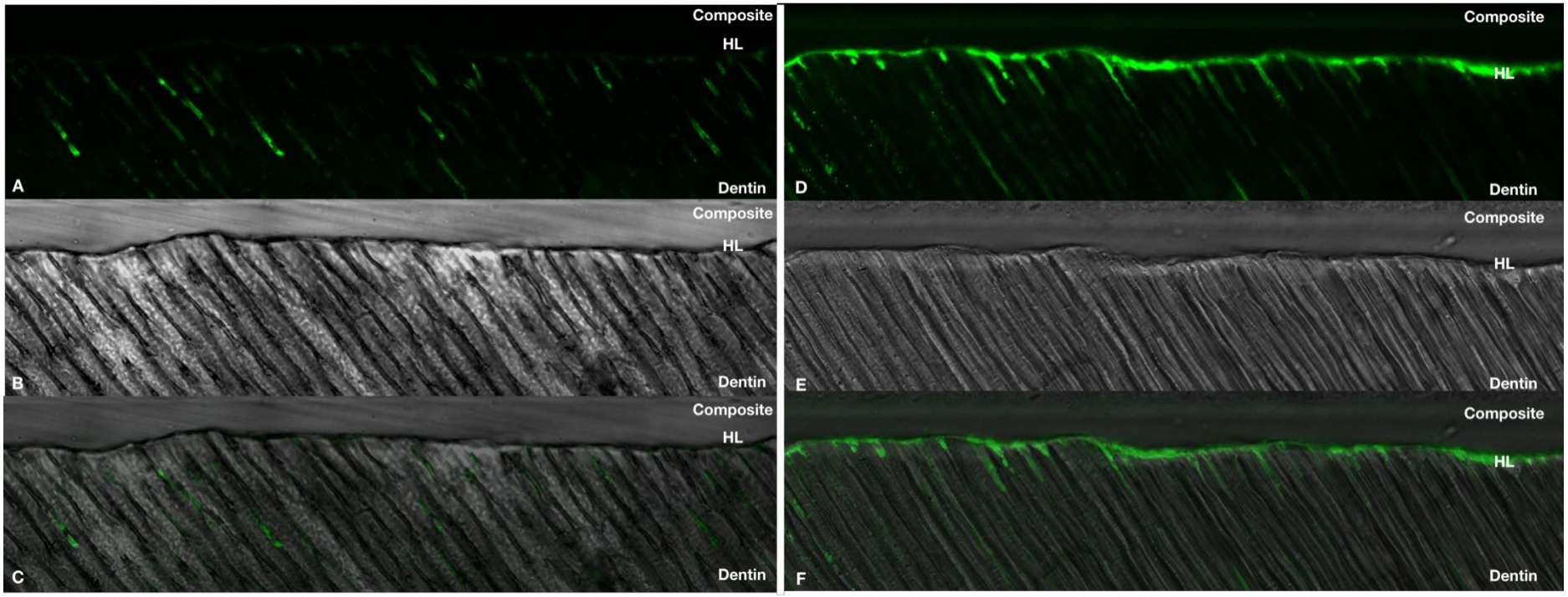


Figure 5.11 - Graph illustrating the median and intervals (25% and 75%) of the quantification of the enzymatic activity measured as integrated density of fluorescence signal for the tested groups from phase 2. Different letters denote significant difference among groups ($p < 0.05$)

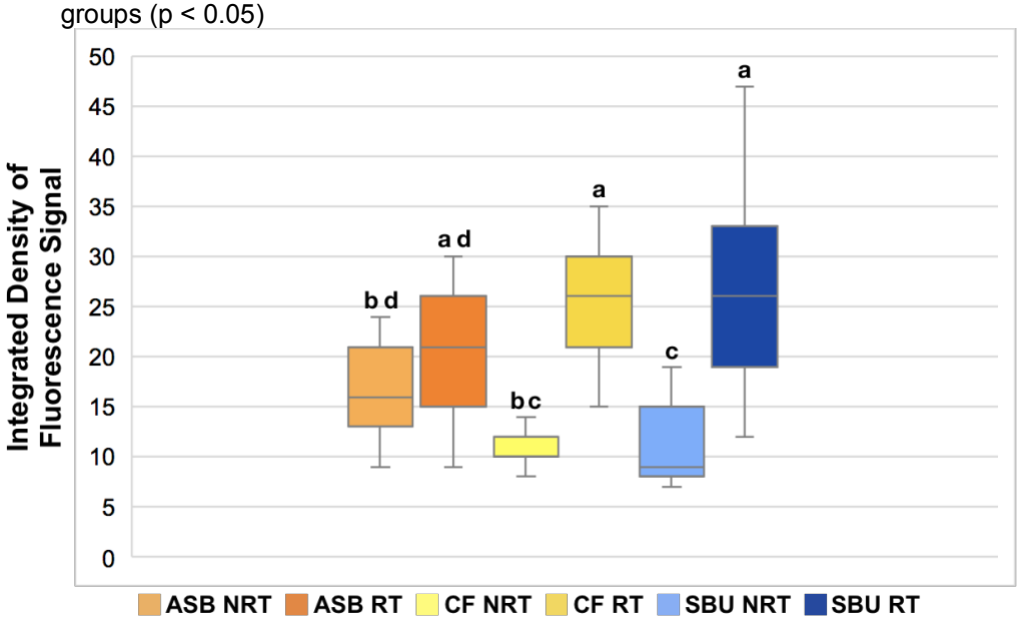
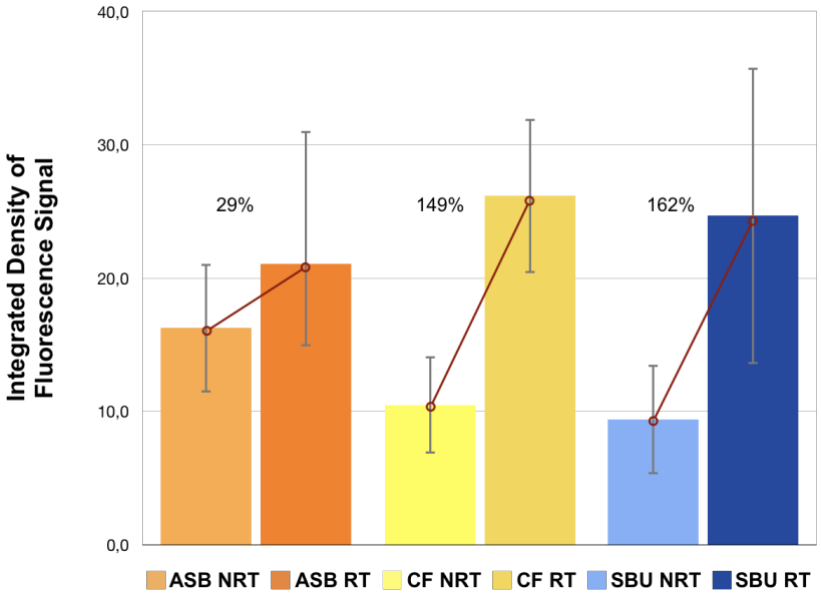


Figure 5.12 - Graph illustrating the mean and standard deviation of the integrated density of fluorescence signal for the tested groups from phase 2. Red bars represent the percentage of the increased enzymatic activity of the irradiated groups when compared to the NRT groups with the same adhesive.



5.2.2 Immunolabeling with FEI-SEM

Positive immunolabeling patterns for CT-B and CT-K were observed in partially demineralized dentin surfaces and HL under FEI-SEM examination for all groups. Labeling is indicated by the presence of the gold nanoparticles which appear as spherical spots of around 15 nm in diameter (Figures 5.14 – 5.16). Images of bonded interfaces for all groups can be seen in Figure 5.13.

Both CT-B and CT-K were localized along the collagen fibrils with labeling anchored to fibrillar structures, regardless of the radiation or adhesive used, suggesting that gelatinases may be tightly bound, directly or indirectly, to the collagen. Overall, CT-K seems to present a more globular aggregation by clusters up to 8 gold nanoparticles pattern along the branching points on collagen, while CT-B shows more scattered and individual labeling

ASB presented the strongest CT-K labeling among the adhesives with a similar pattern between NRT and RTVT groups. Immunoreaction for CT-K seems to present a stronger labeling when compared to CT-B.

CF and SBU also demonstrated a similar labeling between RT and NRT groups. A weaker labeling for CT-K between the SE adhesives groups was noticed when compared to the ASB adhesive, while CT-B immunoreaction apparently presents a comparable pattern among all groups. A similar ratio between CT-K and CT-B for CF and SBU groups could be noticed.

Figure 5.13 - FEI-SEM micrographs of adhesive interfaces created by ASB, CF and SBU. (A) CF infiltrated no further than the smear layer and smear plugs; (B) SBU present characteristics as CF adhesive, showing no adhesive tags inside the dentin tubules within the HL; (C) Long resin tags clearly detectable in the ASB adhesive HL due to the acid-etching step

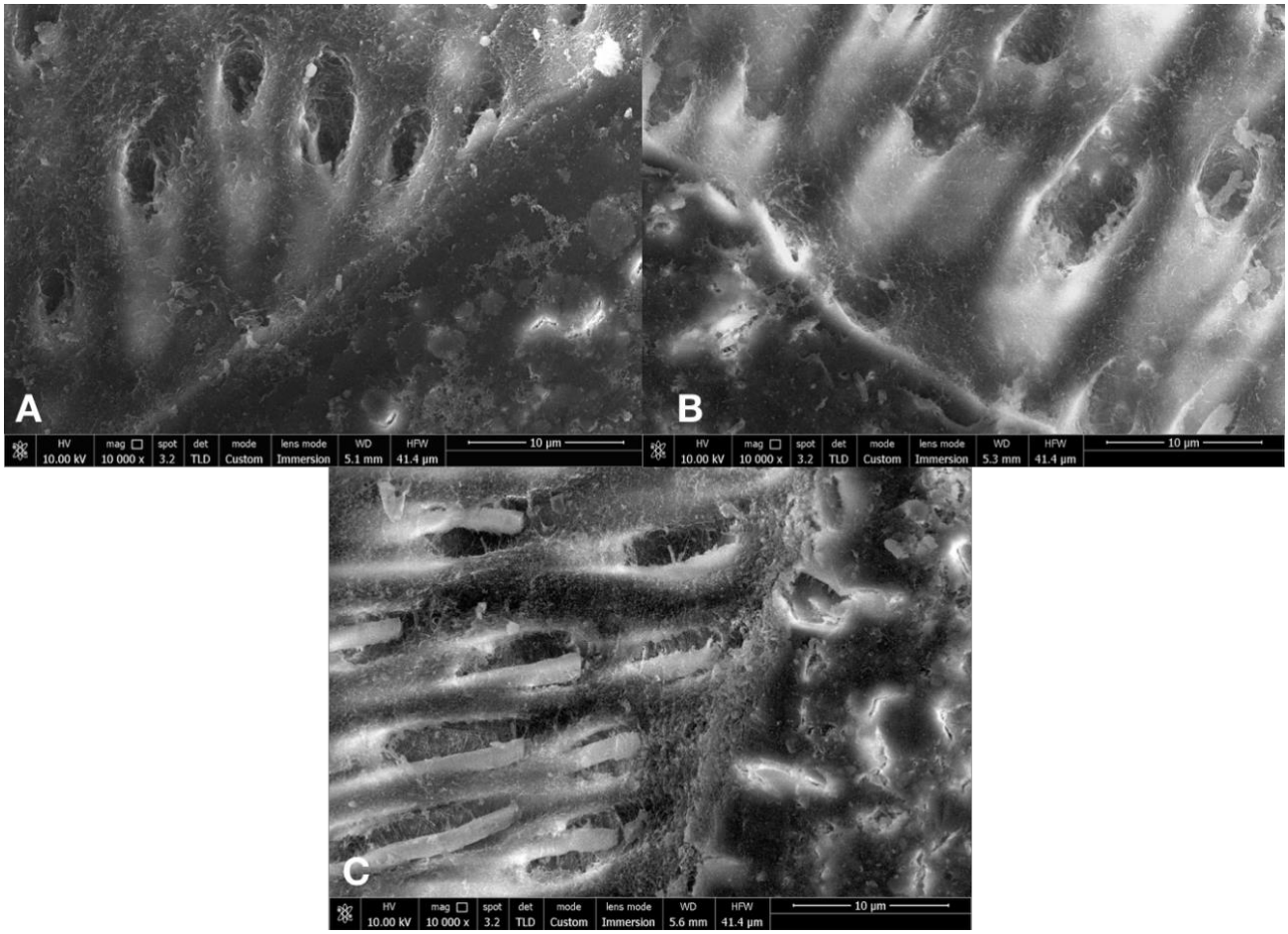


Figure 5.14 - FEI-SEM images of of partially demineralized dentin after a pre-embedding immunolabeling procedure restored with ASB adhesive. Labeling can be identified as electron-dense white spots under the electron beam. (A) NRT dentin presenting a strong labeling with several clusters for CT-K along the collagen fibrils; (B) RTVT dentin a presenting similar pattern for CT-K labeling as the NRT group; (C) A weak labeling for CT-B with individual and sparse white spots distributed along the collagen fibrils can be seen for NRT group. (D) RTVT group with a weak to mild labeling presenting rare small clusters of CT-B

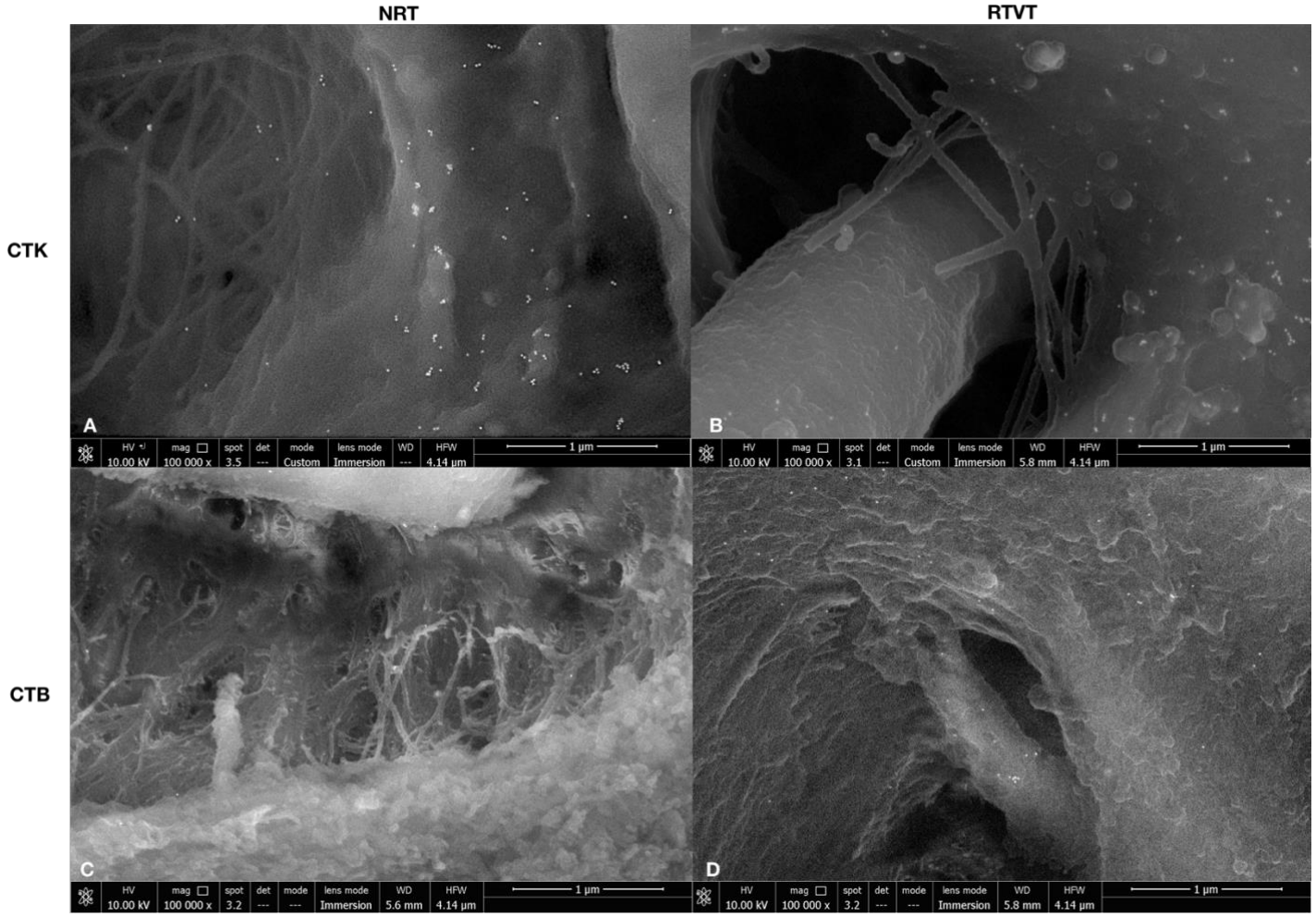


Figure 5.15 - FEI-SEM images of partially demineralized dentin after a pre-embedding immunolabeling procedure restored with CF adhesive. Labeling can be identified as electron-dense white spots under the electron beam. (A) NRT dentin presenting a mild labeling with some clusters for CT-K along the collagen fibrils; (B) RTVT dentin a presenting similar pattern for CT-K labeling as the NRT group; (C,D) A weak labeling for CT-B with individual and sparse white spots distributed along the collagen fibrils can be seen for NRT and RTVT group.

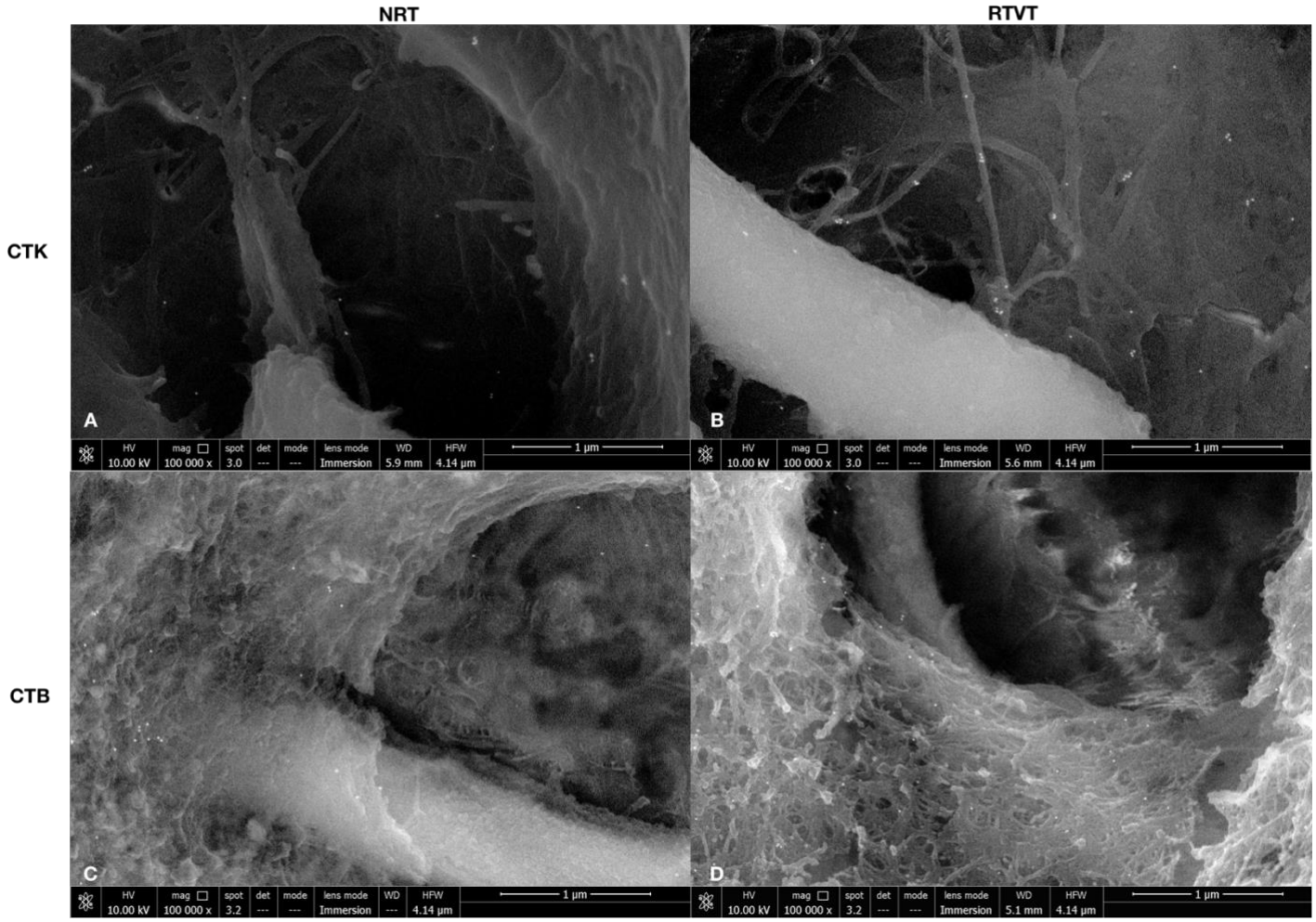
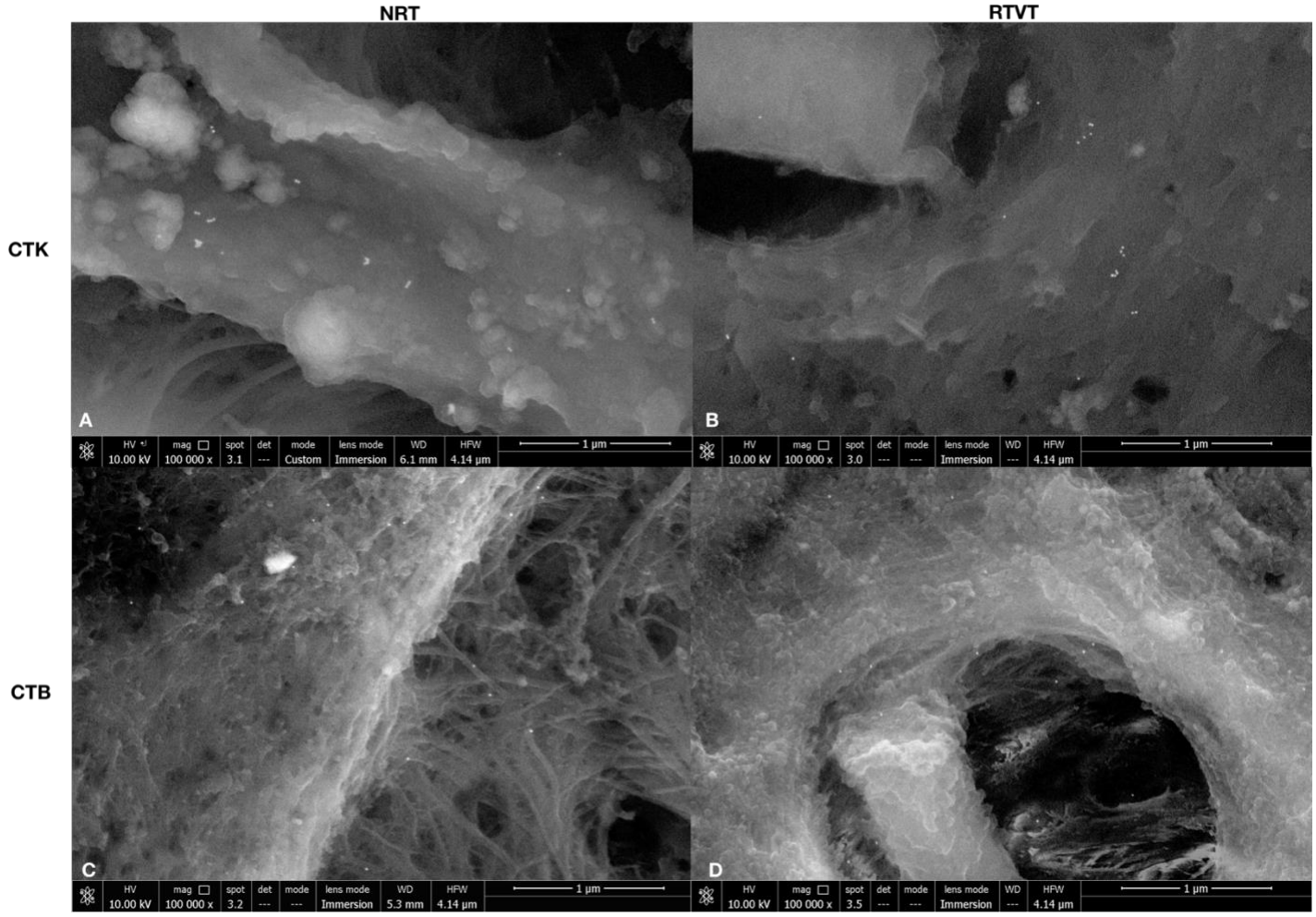


Figure 5.16 - FEI-SEM images of partially demineralized dentin after a pre-embedding immunolabeling procedure restored with SBU adhesive. Labeling can be identified as electron-dense white spots under the electron beam. (A,B) NRT dentin presenting a mild to weak labeling for CT-K along the collagen fibrils; (C,D) A weak labeling with individual and sparse white spots distributed along the collagen fibrils can be seen for NRT and RTVT groups for CT-B.



6 DISCUSSION

Results of the present study indicates that RT may be associated with the activation of endogenous proteases in dentin. Phase 1 of this study analyzed the enzymatic activity of *in vivo* and *in vitro* irradiated non-restored dentin. An increased activity of MMP-2 and MMP-9 active form was observed with gelatin zymography assay for RTVI and RTVT groups. *In situ* zymography complemented the results demonstrating that a higher gelatinolytic activity could also be observed for both RT groups. Thus, the null hypothesis that radiotherapy would not influence the activity of MMP-2 and MMP-9 for non-restored dentin and that radiotherapy would not influence dentin gelatinolytic activity for non-restored dentin had to be rejected.

It is well described in the literature that MMPs are activated by the “cysteine-switch” mechanism. This mechanism consists of removing the residual cysteine (Cys) present on the enzymes exposing the Zn^{2+} at the active site. The removal of the Cys has been described to occur in several ways, such as interaction with chaotropic ions and exposure to oxidizing conditions (95,96,118). Radiotherapy interacts with the tissue when an incident photon or an electron released into the environment by ionization, interacts with water molecules causing its disintegration, also known as radiolysis. The radiolysis of water commonly occurs during RT creating extremely reactive free radicals, such as e^-_{aq}, $HO^\bullet$, $H^\bullet$, $HO^\bullet_2$, H_3O^+, OH^-, H_2O_2 and H_2 (119–121). The activation of MMPs by the removal of the cysteine-residual by oxidation has been already described (122,123). It can be hypothesized that water radiolysis products, specially the oxidant agents, such as hydrogen peroxide and hydronium, created after the RT due to the radiolysis could have interacted with the Cys of MMPs molecules, activating these proteases and increasing the gelatinolytic activity as seen on the results of the present study.

Some studies have already provide evidence that RT can be directly associated with the regulation and activation of MMPs in several tissues (124–127). More recently, the only study analyzing the influence of ionizing radiation on dentin endogenous proteases reported no changes in gelatinase expression and activity (128). This divergent result could be due the storage solution used to store teeth immediately after extraction by the authors. The sample was fixed at 10% buffered formalin solution for at least 72 h. It is well reported in the literature that aldehydes group, such as formaldehyde, the basic chemical compound of the formalin solution, can enhance

collagen stability and decrease enzymatic activity (129,130). Thus, the non-alteration of the enzymatic activity of the irradiated group might be due to the crosslinking and inhibition effects of the formaldehyde used as a storage solution. The storage solution for this study, regardless of the group or methodology used was distilled water.

There is no histological difference between RRC and non-irradiated caries, yet the rapid onset and progression are features that only radiation caries present (8,131). It has already been proved that cariogenic bacteria are not capable of degrading dentin matrix even after they have demineralized it and that protease activity is necessary for cavity formation (99,132,133). MMPs have been suggested to contribute to dentin caries progression and their inhibition to significantly decrease caries development (134). Thus, the increased expression of the active form of MMP-2 and MMP-9 in addition with the higher gelatinase activity on non-restored dentin found in this study, could justify the faster development of RRC without interfering with the histological pattern of the disease. Early restoration failures for HNCP is another recurrent side-effect of RT (135). The participation of endogenous protease in the degradation of the HL has been first suggested by Pashley et al., in 2004 (100), since then several other studies confirmed the degradation of collagen matrix by MMPs and CTs (78,136–138). Since irradiated restored dentin also presented a higher enzymatic activity within their HL when compared to non-irradiated specimens, the early adhesive failures seen on HNCP that underwent radiation therapy could be correlated to this increased collagen degradation.

It could also be notice that no difference on dentin enzymatic activity were detected between RTVT and RTVI, thus the third null hypotheses that there would be no difference on dentin enzymatic activity regarding the irradiation method, *in vitro* or *in vivo* was rejected. This confirms that *in vitro* radiotherapy can be considered a valid method to study the effects of radiotherapy on dentin proteins. Nonetheless it should be noticed that still not statistically significant, the RTVI group presented a slightly higher activation of endogenous enzymes than the RTVT group for both methodologies used in phase 1. This could be due the exposure of the teeth from RTVI group to the other side-effects of the oral cavity of HNCP, such as the decreased in pH of the oral cavity (≅5), changes on saliva's buffer capacity and ionic concentrations (3,6,39), thus all these oral alterations may also influence the enzymatic activity on dentin,

emphasizing the multifactorial cause in the development of the RRC and the limitations of an *in vitro* study.

Dental maintenance is the most appropriated decision for HNCP. Nevertheless, RCC cannot be completely prevented after RT and adhesive restorations are the optimal choice for these patients (3,56,57). There are few studies on the effects of ionizing radiation on bond strength of composite resins, and results are still controversial. Nonetheless, some authors affirm that a negative effect of ionizing radiation in dentin bond strength of restorations performed after radiotherapy could be seen and early restorations fails are recurrent at the clinic (5,62).

It is known that regardless of adhesive technique used, the acidity of the adhesive systems is capable of activating endogenous proteases playing a direct role on the degradation of the hybrid layer and loss of bond strength over time (66,98,139).

The results of phase 2 of the present study are in agreement with the literature. A lower activation of gelatinolytic activity for the non-irradiated groups using SE adhesives could be seen, while Adper Single Bond 2, an E&R adhesive, presented a slightly higher collagen degradation. Nonetheless, RTVT group using ClearFil SE Bond and Scotch Bond Universal adhesives showed a significant increase of 149% and 162% of gelatinolytic activity, respectively. Generally, a higher level of enzymatic activity for E&R adhesives with a faster degradation of the HL have been reported in the literature (11). This difference in the enzymatic activation between adhesives might be due to the higher exposure of dentin matrix caused by the additional acid-etching step required by the E&R adhesives. Since ASB NRT group already presented a high enzymatic activity due to the acid-etching step, the possible influence of RT on the activation of endogenous proteases was not enough to differentiate NRT and RTVT groups for this adhesive. It can be notice that, irrespective of the adhesive used, radiotherapy was able to increase the enzymatic activity within the hybrid layer, rejecting the fourth null hypotheses that radiotherapy would not influence gelatinolytic activity within the hybrid layer regardless of adhesive system.

The last null hypotheses that the distribution of CT-K and CT-B would not be altered on restored dentin after radiotherapy was accepted since no alterations on their distribution between RTVT and NRT groups were seen. The lack of alterations on CT-K and CT-B could imply that ionizing radiation may influence only on the activation of

these enzymes, preserving their localization along the collagen fibrils and anchored to fibrillar structures.

Despite the implementation of multidisciplinary treatment in HNCP, their oral environment still represents a challenge for restorative dentistry. The early participation of the dental team in the development of preventive and therapeutic strategies and rehabilitation of the patient is of great importance. With increasing life expectancy of the world population, longevity of the teeth in the oral cavity, and an increase in neoplastic diseases, it is essential for the dentist to be aware of the changes in the oral cavity produced by radiation of the head and neck, and to act correctly in both the pre and post cancer treatment period.

Furthermore, it is important to know the effects of ionizing radiation on dental tissues. Knowing the particular modifications of the irradiated substrate could lead to more appropriate approaches and strategies for the patient. Thus, the findings presented in this study may help us to understand some unique dental characteristics presented on these patients after RT and find solutions to improve longevity of dental restorative procedures through better quality of bonding interfaces and, so quality of life.

7 CONCLUSION

7.1 Phase 1 – Non-restored dentin

- In vitro and in vivo radiotherapy influenced the activity of MMP-2 and MMP-9 active form.
- A higher gelatinolytic activity was detected for both radiation groups.
- No difference for *in vitro* and *in vivo* radiotherapy was seen regarding enzymatic activity.

7.2 Phase 2 – Restored dentin

- In vitro radiotherapy increases the gelatinolytic activity within the hybrid layer, especially when self-etch adhesives were used.
- CT-K and CT-B distribution were not affected by the radiation.

7.3 General Conclusion

Radiotherapy is capable of stimulate the enzymatic activity on non-restored and restored dentin without influencing on the distribution of CT-K and CT-B.

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APPENDIX A – Ethics Committee appraisement

USP - FACULDADE DE ODONTOLOGIA DA UNIVERSIDADE DE SÃO

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Os efeitos da radiação ionizante nas proteases endógenas em dentina hígida e restaurada.

Pesquisador: Sandra Ribeiro de Barros da Cunha

Área Temática:

Versão: 2

CAAE: 54106616.8.0000.0075

Instituição Proponente: Universidade de São Paulo - Faculdade de Odontologia

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.946.959

Apresentação do Projeto:

Este trabalho tem como objetivo analisar a localização e a atividade das proteases endógenas, MMP-2, MMP-9, CT-B e CT-K em dentina hígida, irradiada in vitro e in vivo, restauradas com diferentes adesivos e suas influências na propriedade de nanodureza dos materiais restauradores e dentina subjacente em três diferentes tempos.

Objetivo da Pesquisa:

Objetivo 1ª Fase: Possível diferença da localização e atividade das MMP-2 e -9 e CT-B e -K e possíveis alterações de dureza entre dentes hígidos, irradiados in vitro e in vivo.Delineamento experimental: Inteiramente ao acaso com 1 fator - Substrato; e 3 níveis - Dentina hígida, dentina irradiada in vivo (70Gy) e dentina irradiada in vitro (70Gy). Variáveis de resposta: Localização das proteases através de Imuno-histoquímica, atividade das proteases a partir de zimografia in situ e alteração
da dureza com teste de nanodureza em esmalte, dentina e junção amelo-dentinária.
2ª Fase: Objetivo Principal: Possível diferença da localização e atividade das MMP-2 e -9 em restaurações adesivas realizadas pré e pós radioterapia in vitro
(70Gy) em 3 diferentes tempos. Objetivo Secundário: Relação entre as atividades das MMPs durante os 3 diferentes tempos e a influência na nanodureza dos adesivos, resina

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composta e camada híbrida.
Delineamento experimental: Inteiramente ao acaso em esquema fatorial 3x2x3.
Fator 1: Momento da restauração em 3 níveis - Controle (dentina hígida), restauração pré-radioterapia e restauração pós-radioterapia.
Fator 2: Sistema adesivo em 2 níveis: Adper Single Bond 2 (condicionamento total) e Clear Fill SE Bond (auto-condicionante).
Fator 3: Tempo de armazenamento em 3 níveis: 24hrs, 6 meses e 12 meses após o procedimento restaurador.
Variáveis de resposta: Localização das proteases através de Imunocitoquímica por MEV, atividade das proteases a partir de zimografia in situ e
alteração da dureza com teste de nanodureza em resina composta, adesivo e camada híbrida.

Avaliação dos Riscos e Benefícios:
Não existem riscos aos pacientes já que os dentes utilizados nesta pesquisa serão provenientes do Banco de Dentes Humanos da FOUSP (DIVISÃO DENTES PERMANENTES)(n=190) e dentes (n=5) provenientes do Serviço de Odontologia Oncológica do Instituto de Cancer de São Paulo (ICESP), cedidos por autorização da coordenadora do serviço.
Como benefícios é citado o auxílio da escolha do melhor material restaurador para pacientes que passam por radioterapia em região de cabeça e pescoço e Saber se a radiação in vitro de fato mimetiza o que ocorre com as MMPs e CTs na clínica.

Comentários e Considerações sobre a Pesquisa:
Pesquisa bem estruturada com metodologia sofisticada e pertinente à área de conhecimento.

Considerações sobre os Termos de apresentação obrigatória:
São apresentados todos os Termos obrigatórios ao desenvolvimento da pesquisa.

Recomendações:
Tendo em vista a legislação vigente, devem ser encaminhados ao CEP-FOUSP relatórios parciais anuais referentes ao andamento da pesquisa e relatório final, utilizando-se da opção "Enviar Notificação" (descrita no Manual "Submeter Notificação", disponível na Central de Suporte - canto superior direito do site www.saude.gov.br/plataformabrasil).
Qualquer alteração no projeto original deve ser apresentada "emenda" a este CEP, de forma objetiva e com justificativas para nova apreciação.

Conclusões ou Pendências e Lista de Inadequações:
Não constam pendências ou inadequações.

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Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_665859.pdf	02/02/2017 18:14:59		Aceito
Projeto Detalhado / Brochura Investigador	projeto.pdf	02/02/2017 18:13:48	Sandra Ribeiro de Barros da Cunha	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	BDHICESP.pdf	02/02/2017 17:56:17	Sandra Ribeiro de Barros da Cunha	Aceito
Declaração de Manuseio Material Biológico / Biorepositório / Biobanco	BDH.pdf	11/03/2016 22:12:22	Sandra Ribeiro de Barros da Cunha	Aceito
Folha de Rosto	FDR.pdf	10/03/2016 16:41:58	Sandra Ribeiro de Barros da Cunha	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 03 de Março de 2017

Assinado por:
Maria Gabriela Haye Biazevic
(Coordenador)