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WALTER ZENOBI

AVALIAÇÃO DE UM COLETOR MAGNÉTICO DE METALOPROTEINASES NA ADESÃO E ALTERACÃO DENTINÁRIA

FORTALEZA
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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para a obtenção do Título de Mestre em Odontologia. Área de concentração: Clínica Odontológica.

Orientador: Prof. Dr. Victor Pinheiro Feitosa.
Coorientador: Prof. Dr. Pierre Basílio Almeida Fechine.

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A mia madre Rosa Maria e mio padre Giovanni che da sempre hanno supportato e sopportato con immenso amore, pazienza e affetto ogni mia pazzia.

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“A herança mais linda e melhor de toda que os pais

podem deixar para seus filhos é o exemplo de uma vida honesta.”

Cicerone

RESUMO

O objetivo do presente estudo foi avaliar os efeitos da aplicação de um novo coletor magnético de metaloproteinases (MMPs) na dentina previamente à aplicação do adesivo e analisar a adesão e remoção das MMPs do colágeno dentinário. O coletor magnético (MMC) foi incorporado em um gel em concentração de 2 e 20%, além disso, um gel padronizado sem substâncias que interagem com as MMPs e um gel com digluconato de clorexidina 2% foram usados como controle negativo e positivo respectivamente. Foram preparados espécimes de dentina, restaurados com o adesivo Prime&Bond 2.1 (Dentsply), após a aplicação de gel de ácido fosfórico 37%. Os espécimes (n=5) foram cortados e avaliados pelo teste de resistência de união à microtração após 24h em água. Para a avaliação da presença/remoção de MMPs foram preparados espécimes de dentina (n=10) e aplicado o coletor com e sem o posterior uso de imã para avaliação da presença das MMPs na dentina com nanopartículas de ferrita ancoradas nas enzimas observadas em microscopia eletrônica de varredura (MEV) com confirmação por espectroscopia de energia dispersiva de raios X (EDS). Os dados foram avaliados estatisticamente por ANOVA com pós-teste de Tukey. O nível de significância adotado foi de 5%. O MMC de MMPs incorporado em um gel em concentração de 2 e 20% aplicado na dentina previamente à aplicação do adesivo demonstrou não interferir na adesão inicial (p=0,432) do sistema adesivo e as MMPs na dentina foram reduzidas de 0,3% para 0,0% somente com o uso do imã após o coletor. Conclui-se que o coletor magnético de MMPs proposto tem ação efetiva na remoção de MMPs, sem alterar a adesão à dentina.

Palavras-chave: Metaloproteinases, dentina e adesão.

ABSTRACT

The aim of the present study was to evaluate the effects of the application of a new magnetic collector of matrix metalloproteinases (MMPs) to the dentin prior to the application of the adhesive and to analyze the adhesion and removal of MMPs from dentin collagen mesh. The magnetic collector was incorporated in a gel at 2% and 20% concentration. In addition, a standardized gel without substances that interact with MMPs and a gel with 2% chlorhexidine digluconate were used as negative and positive controlrespectively. Dentin specimens were bonded with Prime & Bond 2.1 adhesive (Dentsply) after application of 37% phosphoric acid gel. Bonded teeth (n=5) were cut and evaluated by the microtensile bond strength test after 24h immersed in distilled water. To evaluate the presence / removal of MMPs, dentin specimens (n=10) were prepared and the collector was applied with and without the use of magnet to evaluate the presence of MMPs in the dentin with ferrite nanoparticles anchored in the enzymes observed in electron microscopy (SEM) with confirmation by energy dispersive X-ray spectroscopy(EDS). Data were statistically analyzed by one-way ANOVA and Tukey's post-hoc test. The level of significance was 5%. Metalloproteinases magnetic collector (MMC) incorporated in a gel at 2% and 20% concentration applied to the dentin prior to the application of the adhesive did not interfere with the initial adhesion (p=0.432) and the MMPs in the dentin were reduced from 0,3% to 0.0% only with the use of the magnet after the collector. It is concluded that the proposed MMPs magnetic collector has an effective action on the removal of MMPs, without altering the adhesion to dentin.

Keywords: Metalloproteinases, dentin and adhesion.

SUMÁRIO

1. INTRODUÇÃO GERAL

Os sistemas adesivos representam, na odontologia moderna, um dos tópicos mais discutidos tanto na esfera clínica como na esfera científica. É universalmente reconhecido o impulso significativo que esse material tem proporcionado, especialmente em termos de preservação de estruturas dentárias, reduzindo o desgaste em muitos procedimentos anteriormente estabelecidos. Da mesma forma, é muito discutida a durabilidade promovida por eles para os procedimentos restauradores.

O primeiro elemento a ser considerado é o substrato dental sobre o qual o sistema adesivo deve ser aplicado; a proporção diferente entre a matriz orgânica e inorgânica entre esmalte e dentina é tão importante que, como é amplamente demonstrado pela literatura (VAN LANDUYT et al., 2007), ainda não é possível identificar um sistema adesivo ideal para todas as situações e substratos. O princípio da adesão ao substrato dentário baseia-se no processo de troca pelo qual tecido dentário inorgânico é trocado por resina sintética. Isso acontece basicamente em duas fases: na primeira fase (condicionamento ácido), remove-se o mineral e são criadas microporosidades na superfície dentária do esmalte e da dentina; na segunda fase (hibridização), infiltram-se essas microporosidades com resina e, posteriormente, é realizada polimerização *in situ* (VAN MEERBEEK et al., 2003a). Dessa forma, se gera uma adesão micromecânica que pode também ser acompanhada por interação química adicional entre monômeros funcionais e componentes do substrato dentário para chegar realmente a poder obter resultados duráveis.

Com base nessas premissas, muitos sistemas adesivos foram desenvolvidos ao longo dos anos, que diferem entre si por vários elementos: tempos de aplicação, composição química e divisão/união dos vários componentes. O condicionamento ácido pode ser forte, médio ou fraco (pHs diferentes), incluído no primer ou usado separadamente. Tanto a técnica convencional como a técnica autocondicionante são válidas para obter uma adesão à estrutura dental satisfatória, mas em termos de durabilidade e aplicabilidade no esmalte e na dentina, ainda existem visões diferentes, sendo os adesivos convencionais a configuração mais comum utilizada nacionalmente, com um ácido (principalmente ácido fosfórico 30-40%) aplicado e lavado, seguido pelo passo de aplicação da resina adesiva (VAN MEERBEEK et al., 2003b)

De fato, a camada híbrida formada, entre o sistema resinoso e as estruturas dentárias, determina a longevidade da interface adesiva. Independentemente do tipo de condicionamento ácido, não todas as fibrilas de colágeno expostas são completamente infiltradas por monômeros resinosos, impedindo a proteção ideal contra os desafios de desnaturação e hidrólise (DE MUNCK et al., 2005). O colágeno desprotegido é mais propenso à ruptura por fadiga cíclica, após a função prolongada. Além disso, as fibrilas de colágeno desprotegidas são cercadas por água, que participa da hidrólise do colágeno, acelerada por enzimas colagenolíticas.

O papel das proteases naturais da dentina na degradação da adesão dentina-resina foi sugerido pela primeira vez por Pashley e colaboradores. (PASHLEY et al., 2004). Armstrong e colaboradores (ARMSTRONG et al., 2004) relataram uma perda de 70% de fibrilas de colágeno na camada híbrida, após um armazenamento de cinco anos na água, por meio da avaliação da microscopia eletrônica de transmissão (TEM). Pela primeira vez, a diminuição da resistência de união resina-dentina ao longo do tempo foi associada à degradação de fibrilas de colágeno, que formam o principal componente estrutural contínuo entre tecido mineralizado e resina adesiva. Muitos estudos subsequentes demonstraram que dentre essas proteases, as metaloproteinases de matriz (MMPs) (HEBLING et al., 2005; PASHLEY et al., 2004) e catepsinas cisteinícas (NASCIMENTO et al., 2011; TERSARIOL et al., 2010) e suas atividades são as responsáveis pela degradação hidrolítica do colágeno subjacente e na camada híbrida (HEBLING et al., 2005; MAZZONI et al., 2007; NASCIMENTO et al., 2011; PASHLEY et al., 2004; TERSARIOL et al., 2010).

As MMPs são um grupo de endopeptidases dependentes de zinco e cálcio, e são responsáveis pelo remodelamento fisiológico e patológico e pela degradação da matriz extracelular. As MMPs são ativadas por muitos processos, incluindo autoativação por outras proteases, tratamento térmico, exposição a pH baixo ou aplicação de certos reagentes químicos (KNAUPER et al., 1993). A sua ativação em dentina mineralizada está relacionada com o pH baixo de condicionadores acídicos utilizados em adesivos dentários (MAZZONI et al., 2006; NISHITANI et al., 2006). Na dentina, as MMPs participam do desenvolvimento e remodelação fisiológica dos dentes da matriz dentinária antes e durante a mineralização (TJÄDERHANE et al., 2013). As funções das MMPs podem ser controladas em várias etapas, incluindo síntese, inibição e ativação. Nos processos de remodelação do tecido fisiológico, a

inibição das MMPs é regulada por inibidores endógenos de metaloproteinases de matriz (TIMPs). No entanto, os inibidores sintéticos têm grupos funcionais específicos (por exemplo, ácido carboxílico, ácido hidroxâmico, sulfuldril, fosfonil) que podem ser usados para a inibição de MMPs devido ao mecanismo de quelação ao íon de zinco no domínio catalítico de MMPs, causando sua inativação (VISSE; NAGASE, 2003)

Estudos recentes voltados para a prevenção da perda de resistência de união entre resina-dentina, para melhorar o tempo de vida de restaurações dentárias, demostraram que a degradação maior do colágeno da dentina intacta ocorre por degradação enzimática pelas MMPS. Para tentar bloquear essa proteólise, diferentes tipos de estratégias foram investigadas, diferentes umas das outras: a remineralização do colágeno exposto, a inibição das enzimas dentinárias (MMPs e catepsinas de cisteína) e a biomodificação das matrizes orgânicas dentinárias. Embora cada estratégia tenha seus méritos, ainda existem muitas limitações, e, até hoje, ainda não existe nada que tenha sido relatado tentando remover (extrair) essas MMPs previamente à aplicação do adesivo.

2. PROPOSIÇÃO

Os objetivos deste estudo foram:

1. Objetivo geral

Avaliar a efetividade de um coletor magnético de metaloproteinases de matriz (MMPs) sintetizado com um inibidor de MMPs ancorado em nanopartículas magnéticas na degradação do colágeno dentinário, atividade de MMPs e resistência de união à dentina.

2. Objetivos específicos

- Desenvolver e caracterizar um novo coletor magnético com inibidor de metaloproteinase ancorado em nanopartículas magnéticas para a utilização em Odontologia.
- Avaliar a atividade de MMPs e degradação das fibrilas colágenas da dentina humana antes e depois do tratamento com o coletor magnético.
- Avaliar *in vitro* a adesão à dentina após o tratamento com o coletor magnético

3. CAPÍTULO

Esta dissertação está baseada no Artigo 46 do Regimento Interno do Programa de Pós-graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado e permite a inserção de artigos científicos de autoria ou coautoria do candidato (ANEXO A). Assim sendo, esta dissertação é composta por um capítulo contendo um artigo científico que será submetido ao periódico *Dental Materials*, conforme descrito abaixo:

Evaluation of a magnetic collector of metalloproteinases on dentin adhesion and alteration of dentin

Zenobi W, Andrade Neto DM, Fechine PBA, Avelino J, Lomonaco D, Mazzetto S, Sauro S, Feitosa VP.

3.1 Evaluation of a magnetic collector of metalloproteinases on dentin adhesion and alteration of dentin

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Evaluation of a magnetic collector of metalloproteinases on dentin adhesion and alteration of dentin

ABSTRACT

Objectives - The aim was to evaluate the effects of a new magnetic collector of matrix metalloproteinases (MMPs) applied to the dentin prior to the application of the adhesive and on adhesion and removal of MMPs from dentin organic matrix.

Methods – The magnetic collector was incorporated in a gel at 2% and 20% concentration. In addition, a standardized gel without substances that interact with metalloproteinases and a gel with 2% chlorhexidine digluconate were used as negative and positive control respectively. Dentin specimens were bonded with Prime & Bond 2.1 adhesive (Dentsply) after application of 37% phosphoric acid gel. Bonded teeth (n=5) were cut and evaluated by the microtensile bond strength test after 24h immersed in distilled water. To evaluate the presence/removal of MMPs, dentin specimens (n=10) were prepared and the collector was applied with and without the use of magnet to evaluate the presence of metalloproteinases in the dentin matrix anchored in the enzymes observed in SEM with energy dispersive X-ray spectroscopy (EDS). Data were statistically analyzed by one-way ANOVA and Tukey's post-hoc test ($p<0.05$).

Results – Metalloproteinases magnetic collector incorporated in a gel at 2% and 20% concentration applied to the dentin prior to the application of the adhesive did not interfere with the initial adhesion ($p=0.432$) and the metalloproteinases in the dentin were reduced from 0,3% to 0.0% only with the use of the magnet after the collector.

Significance - Proposed MMPs' magnetic collector has an effective action on the removal of MMPs, without altering the adhesion to dentin.

Keywords: Metalloproteinase; dentin; adhesion.

INTRODUCTION

Physicochemical properties of dental adhesives have been improved as a result of numerous investigations into the chemical balance between their hydrophilic and hydrophobic components [1]. Although scientific research is still active, a definitive solution for the longevity of adhesive restorations has not yet been demonstrated, especially when they are encompassing dentinal tissue. Whilst high standards have been achieved on dental enamel, the same cannot be accepted to dentin. Dental adhesives, regardless their application technique, loose their bond with dentin over time, and there is consensus in the literature that the degradation of hybrid layer is related to that loss of bond strength [2,3].

After adhesive polymerization, resin-sparse collagen fibrils are encountered. Such exposed collagen is easily detected and represents a suitable area to initial degradation [4]. Non-collagen proteins, such as growth factors and matrix proteases, are also present in this unprotected zone of dentin organic matrix. These proteases are secreted by odontoblasts during dentinogenesis and remain inactive within the dentin extracellular matrix [5] as they are physiologically inactive and stable in the mineralized tissue. Upon acid etching from adhesive application or from biological carious process, different matrix metalloproteinases (MMPs) are activated [6,7]. The enzymatic degradation of the collagen matrix by enzymes has been depicted to play a significant role on the destruction of dentin bonded interface [8].

Several investigations proposed the use of specific MMP inhibitors to preserve the structural integrity of the collagen fibrils, which indeed reduces the degradation of the hybrid layer. For instance, chlorhexidine (CHX), even at low concentrations, showed striking metalloproteinase inhibition ability for MMPs 2, 8, and 9 [8,9]. Nevertheless, the treatment with MMP inhibitors and further therapeutic collagen-reinforcing strategies are reversible [10] thereby only slowing down the degradation process and allowing MMPs to act after few years. To our knowledge, no investigations tried so far to extract in situ these enzymes from dentin organic matrix, what could likely arrest definitely the issue of enzymatic degradation of collagen from dentin bonds.

Therefore, the aim of this manuscript was to synthesize a new metalloproteinase magnetic collector to remove these enzymes from etched dentin, providing the reduction of

the negative effects generated on the dentin-restoration interface and to assess its influence on dentin adhesion. The study hypotheses under investigation were that (1) the new metalloproteinase magnetic collector does remove MMPs from dentin matrix, and (2) the collector does not alter the initial bond strength to dentin.

MATERIALS AND METHODS

Synthesis of branched-polyethylenimine-coated Fe_3O_4 nanoparticles

Firstly, the Fe_3O_4 nanoparticles were functionalized by amine groups through branched-polyethylenimine (BPEI) (Fig.1). The coating process was performed using the sonochemistry approach [11]

Briefly, 1.16 g of $FeSO_4 \cdot 7H2O$ and 1.85 g of $FeCl_3 \cdot 6H_2O$ were dissolved in 15 mL of deionized water and heated at 60 ºC using water bath. Then, this iron solution was sonicated for 1 minute using ultrasonic probe. Afterwards, 7 mL of concentrated NH_4OH solution were added to the reaction medium, which remained under sonication for 4 minutes. Finally, still under sonication, 4 mL of aqueous solution containing 1.0 g of BPEI were added to the reaction medium, which remained under sonication for additional 4 minutes.

To remove the excess NH_4OH and unbounded BPEI, the resultant nanoparticles were washed several times with deionized water and precipitated with acetone. Nanoparticles were dispersed in water and centrifuged for 10 min at 3000 rpm to remove large aggregates. The remaining functionalized nanoparticles showed good colloidal stability in water. Thus, they were stored in deionized water and de-aerated with argon to remove the dissolved oxygen. This sample was labeled as Fe_3O_4@BPEI. The amount of Fe_3O_4@BPEI in the aqueous suspension was calculated through gravimetry.

Anchoring of BB94 on Fe_3O_4@BPEI

The coupling reaction of BB94 (Batimastat from Sigma Aldrich, Fig.1) on the magnetic nanoparticles was performed by an iodine-mediated oxidation. The anchoring took place through the hydroxamic functionality of BB94, and terminal amine groups of Fe_3O_4@BPEI, leading to amine bond, as shown in Fig. 1c. To the best of our knowledge, the anchoring of hydroxamic acids on amine-coated magnetic nanoparticles was not reported in the literature, and it was the key factor to synthesize the present metalloproteinase magnetic collector [12]

Initially, aqueous suspension containing 6.0 mg of Fe_3O_4@BPEI was magnetic separated and suspended in 2 mL of dimethyl sulfoxide (DMSO). Then, 2.0 mg of BB94 and 2.7 mg of iodine (I_2) were solubilized in 3 mL of DMSO and added to the Fe_3O_4@BPEI suspension. Thereafter, the reaction medium remained under stirring in room temperature for 1 hour. In the end of the reaction, BB94-modified nanoparticles were magnetic separated and washed 4 times with 5 mL of methanol. Finally, the samples were dried under vacuum. This sample was labeled as Fe_3O_4@BPEI@BB94, which represents the final metalloproteinase magnetic collector product.

Fourier transform infrared spectroscopy (FTIR)

Samples of the synthesized and isolated products were characterized by a Fourier transform infrared spectrophotometer (Spectrum Frontier, Perkin-Elmer Corp., Norwalk, United States) equipped with a crystal to perform attenuated total reflectance (ATR-FTIR) analysis. Samples were individually dispensed onto the crystal and spectra were obtained in spectral range of 4000 to 550 cm^{-1} with 4 cm^{-1} resolution in transmittance mode. FTIR spectra were obtained in triplicate for each product, using the Fe_3O_4nanoparticles as reference, and then processed for baseline correction and normalization.

Preparation of specimens

Twenty extracted sound human third molars were used and they were stored in 0.1% thymol solution at 4ºC for no longer than three months after extraction. Occlusal enamel and roots were removed using a slow-speed water-cooled diamond saw (Isomet 4000; Buehler, Lake Bluff, United States) in order to obtain flat mid-coronal dentin surface from each tooth as described by Sauro et al. in 2016 [13]. These specimens were wet-abraded using a 600-grit silicon carbide paper (30s) to create standardized smear layer. The dentin specimens were randomly divided into the four treatments (n=5). In control group, a gel without addition of MMP inhibitors or nanoparticles was used after phosphoric acid etching dentin for 15s and 30s water rinsing. CHX group employed a gel with 2% chlorhexidine digluconate (Sigma Aldrich) used as positive control. Metalloproteinase magnetic collector (MMC) 2wt% group used a gel with magnetic nanoparticles attached to BB-94. MMC 20% group utilized a gel with same magnetic nanoparticles in 20wt% concentration. All gels were prepared with aerosil silica as thickener and were applied for 60s on etched dentin and rinsed for 30s prior to adhesive application. The adhesive, Prime&Bond 2.1 (Dentsply) was applied according to the manufacturers' recommendations in two coats (Table 1). After application of the pretreatment and the adhesive, a restoration with 3 increments of 2 mm was constructed with Opallis composite resin (FGM, Joinville, Brazil). Light-curing was performed with Valo LED unit (Ultradent, South Jordan, USA) with 2000mW/cm² irradiance.

Microtensile bond strength test (μTBS)

After 24-hour immersion in distilled water, the restored teeth were sectioned in resin-dentin sticks (1mm² of cross-sectional area) and tested for tensile stress in a universal test machine (DL2000, EMIC, São José do Rio Preto, Brazil). The more peripheral sticks that had residual enamel were excluded from the test, the exact cross-sectional area of each sticks was measured with a high precision digital caliper. The sticks were fixed with cyanoacrylate glue (Super Bonder gel, Loctite, Henkel Corp., Rocky Hill, USA) and tested to failure with a 500 N load cell and 0.5 mm / min crosshead speed. The results of μTBS were expressed in MPa. The μTBS values obtained from sticks of the same bonded tooth were averaged. The average bond strength of each tooth was used as a unit for statistical analysis. The μTBS data was statistically analyzed with one-way ANOVA and Tukey test with $\alpha = 5\%$ after passing

normality test (p=0.71). Fractured specimens were analyzed by stereomicroscopy (40x magnification) and failures were classified as adhesive, cohesive in dentin, cohesive in composite or mixed (partial adhesive and cohesive fracture).

Scanning electron microscopy (SEM)

To evaluate the presence/removal of MMPs, dentin specimens (n=10) were prepared and the collector was applied with and without the use of magnet to evaluate the presence of MMPs in the dentin matrix by scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (EDS). Eighteen teeth were used for this experiment, three teeth for each gel containing MMC (2% and 20%) and treatment. Each tooth was prepared as previously described and flat dentin surfaces were cut in three similar parts for different treatments.

MMP-full: Dentin surface was etched with phosphoric acid for 15s to exposed organic matrix and in the first part, MMC was applied for 60s, rinsed for 30s and processed for SEM analysis. Magnet-treated: The second third of the specimens received the same treatment, but before final rising a magnet was used for 60s at 0.5mm from dentin surface to remove magnetic nanoparticles attached with MMPs. These specimens were evaluated to survey the removal of magnetic nanoparticles from dentin. Magnet-treated +: In the final third of dentin, specimens were etched with phosphoric acid for 15s, rinsed with distilled water for 30s, MMC was applied for 60s, magnet was then employed for 60s, specimens were rinsed again with distilled water for 30s and MMC was re-applied (to assess remaining MMPs in dentin structure) for 60s before final rinsing with distilled water. Specimens were dehydrated in silica gel, mounted on stubs, gold-sputter coated and observed in SEM (Inspect S50, FEI Company, Amsterdam, Netherlands) operated at 20 kV. Representative scanning electron micrographs and EDS spectra were taken at different magnifications and were chosen by two evaluators based on the frequently observed appearance of the dentin surface and mean iron concentration from each group.

RESULTS

Synthesis of Metalloproteinase Magnetic Collector (MMC)

The synthesis of the metalloproteinase magnetic collector (MMC) proposed in the present study was successfully finalized with a final yield of 80%. Fig. 2 shows the FTIR spectra of the final product and of the intermediary products, demonstrating the presence of BB94 in the final magnetic nanoparticles.

Microtensile bond strength test (μTBS)

The results of microtensile bond strenght test of the control group, where a gel without the addition of MMP inhibitors or nanoparticles was used, achieved 32.2(±9.6) MPa. In the CHX group, mean bond strength was 26.5(±8.9) MPa. In the group using the gel with 2wt% magnetic nanoparticles connected to BB94 at, mean bond strength was 29.4(±6.4) MPa. Finally, in the group with similar gel but with 20wt% MMC, bond strength was 29.8(±7.1) MPa. Statistical analysis demonstrated no significant differences among groups ($p = 0.432$). Summary of microtensile outcomes is presented in Figure 3. Failure analysis depicted that majority of fractures were mixed.

SEM-EDS evaluation

Characteristic scanning electron microscopy (SEM) images of dentin surfaces on which MMC 2wt% was applied are shown in Figure 4, and those specimens treated with MMC 20wt% are presented in Figure 5. In the first group (MMP full) where the MMC 2wt% was applied, no magnet was used for the removal of MMPs, the energy dispersive X-ray spectroscopy (EDS) demonstrated the presence of 0.3% of Fe linked to the MMPs (Fig 4A and 4D). Such Fe undergoes a decrease to 0.1% by the use of magnet in MT2% specimens, where in addition to the MMC application a magnetical device was applied for 60s (Fig. 4B and 4E). Finally, in the last group MT2%+ where the same procedures were carried out as in

the second group (MT2%), but a new application of MMC 2% was performed, the concentration of Fe calculated was 0.0% (Fig. 4C and 4F). These results suggest significant reductions of MMPs on dentin after treatment with MMC 2wt%. Similar trend occurred by using MMC at 20wt% concentration where MMP full group (Fig .5A and 5D) attained 0.2% of Fe bound to MMP. This value was reduced, after the applying the magnet, to 0.0% (Fig.5B and 5E) and total removal of MMPs was further confirmed in the last group with final 0.0% Fe concentration (Fig 5C and 5F).

DISCUSSION

According to the results obtained herein, the first hypothesis under investigation was confirmed because the new metalloproteinase magnetic collector does remove MMPs from dentin matrix especially with higher concentration (20wt%) of MMC in the gel. Yet, the second hypothesis that the collector does not alter the initial bond strength to dentin needs to be accepted because there was no statistically significant alteration on dentin adhesion (μTBS).

It has been widely demonstrated in the literature that among the various methods used to block the action of MMPs, chlorhexidine (CHX) currently represents the only really feasible in daily clinical practice. Since the first studies, in fact, chlorhexidine has shown to exert great inhibition on MMPs [9]. The use of CHX to inhibit the degradation of unprotected dentin collagen fibrils was first suggested by Pashley et al. (2004) [14]. Recent studies reported improvement in the durability of resin-dentin bonds by using CHX-incorporated adhesives [15,16]. However, others studies [17,18] showed no significant improvements on long-term adhesion after CHX application. Sadek et al. (2010) [19] showed that, after 18 months of incubation, tensile bond strength of CHX-treated samples was no longer stable. Despite the optimal initial enzymatic inhibition effect of CHX, water-soluble CHX can leach out from dentin due to CHX substantivity and fluid replacement by dentinal fluid-containing competing cations [20,21].

A meta-regression analysis indicated that the addition of chlorhexidine based on results from only in vitro studies should be carefully analyzed before implementing new protocols for clinical adhesive procedures, as the association between the concentration of chlorhexidine and bond strength is not linear, and many other factors affect bond strength [10]. Compliant with the currently available literature, the results of this study obtained in the μTBS test demonstrate that the pre-treatment with 2% digluconate chlorhexidine does not demonstrate any alteration on initial dentin adhesion as described at Figure 3.

Regarding these same results, it is also possible to highlight how the new pre-treatment proposed in this study with MMC used both in 2wt% and 20wt% concentrations did not promote any alteration on μTBS. Considering that enzymatic structures of MMPs are attached to dentin collagen [22], one of the major concerns in using an extractor of these MMPs is possible aggressive treatment likely with alteration/cleavage of collagen. Indeed, this would yield consequent alteration of the substrate essential for the dentinal adhesion thereby reducing bond strength. Also in this case, the results obtained in μTBS test denies the possibility that this happened, indicating that pre-treatment with MMC along with magnet removal is totally compatible and provides similar dentin adhesion to control group (Figure 3).

A further concern was related to possible negative antioxidant effect of BB94 on adhesive polymerization and consequent reduction of bond strength. Indeed, the similar bond strength (Fig. 3) attained after removal of magnetic nanoparticles from dentin reinforces the idea that BB94 was also removed bridging magnetic nanoparticles and MMPs. Furthermore, concerning some residual BB94 remaining on dentin, a previous investigation [23] with modified adhesives containing BB94 showed high affinity for both synthetic and dentin powder substrates, but with minor alteration on initial bond strength. Therefore, it is possible to state that the use of MMC (at 2wt% and 20wt%) does not hinder and does not alter the formation of the hybrid layer, does not cause negative effects on adhesive infiltration/polymerization, and it likely managed to remove the MMPs as demonstrated by EDS analysis. In a long-term investigation, MMC might lead to an increase on the durability dentin bonds. Clearly, further studies are needed to confirm this, with 6-24

months aging time and/or thermo-mechanical cycling to achieve better degradation of the hybrid layer.

By evaluating the results obtained in the SEM-EDS, it is possible to state that by comparing Figures 4 and 5, the percentage of iron decreased with the increase of MMC. This represents optimal extraction of MMC anchored to the MMPs, as reduced to values of 0.1% in the MT 2% (Fig. 4E) and to 0.0% in MT 20% (Fig. 5E). The success of the synthesis of this new product (MMC) represents a new strategy to overcome the issue of rapid enzymatic degradation resin-sparse demineralized dentin collagen.Cysteine cathepsins (CTPs) are a further class of endogenous proteases that may be activated during demineralization of sound and carious dentin [24] but the percentage of protonation (enzymatic properties) of MMPs plays a major role in enzymatic activity [25]. The outcomes of Tezvergil-Mutluay *et al*. (2014) [26] demonstrated that collagen degradation promoted by MMPs is remarkably higher (67-fold) than that attained with cathepsins, thus demonstrating that overall proteolysis in neutral and mildly acidic environments is accomplished mainly by MMPs.

A further proof-of-the-concept in the present study was related to the real effectiveness of MMC. In fact, the ability of BB94 to rapidly bind MMPs was previously demonstrated [23]. The results obtained in FTIR (Fig. 2) demonstrated strong anchoring of BB94 onto magnetic nanoparticles surfaces. We evidenced this by splitting the set of bands in the region between 1250 and 1750 cm^{-1} for the sample Fe_3O_4@BPEI@BB, in comparison to the spectrum of the sample Fe_3O_4@BPEI (Figure 2a). This change in the set bands is probably due to the decreasing amount of primary amines, which possess a broad vibrational mode in this region due to bending of N–H bond (δ_{N-H}). Further evidence of the anchoring of BB94 is the increasing in the relative intensity of the bands in the region between 2700 and 3000 cm^{-1}, which are attributed to the stretching of the CH_2 group (ν_{CH2}) that increase its amount as BB94 was attached (Figure 2b).

However, we can confirm the synthesis by the set of bands in the range of 1250-1750 cm^{-1} of the spectrum of the sample Fe_3O_4@BPEI@BB. The vibrational mode in 1625 cm^{-1} can be attributed to the stretching of carbonyl ($\nu_{C=O}$) of amides that are generated by the anchoring between BB94 and BPEI molecules, and to the amide groups present in BB94 molecules. The band in 1527 cm^{-1} can be attributed for the secondary amides, which are present in BB94 and also formed by the reaction of primary amines of BPEI and BB94. This

vibrational mode is characteristic of secondary amides, and it is due to the coupling between δ_{N-H} and stretching of the bond C–N (ν_{C-N}). Additionally, the band in 1460 cm^{-1} can be assigned to stretching of the bonds C=C ($\nu_{C=C}$) present in aromatic and thiophene rings. All attributions are summarized in the Table 2, thereby confirming the synthesis of final metalloproteinase magnetic collector with high yield (80%).

Although there are some relative limitations in this study, the primary, but positive, results obtained herein might be a threshold for an innovative and decisive method of treatment of dentinal surfaces to extract instead of inhibiting the enzymatic degradation promoted by MMPs. In support of the results obtained and with the aim to confirm the data obtained, it would be interesting to analyze Raman or FTIR spectroscopy of dentin after treatment with the MMC trying to detect the presence of the peak of BB94 in order to confirm that bond between magnetic nanoparticles and BB94 is stronger than that between MMPs and collagen fibrils. Indeed, this would confirm the removal of MMPs after the application of the magnetic device. Nevertheless, the present outcomes provide initial direct evidence of this anchoring and MMPs removal, opening a scientific field for possible therapeutic effects with the use of MMC to improve the performance of adhesive restoratives.

CONCLUSION

Proposed magnetic collector of metalloproteinases encompassing ferrite nanoparticles with batimastat has an effective ability to remove MMPs from demineralized dentin matrix, without jeopardizing initial dentin bonds.

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TABLES

Table 1– Commercial adhesive composition and application procedure.

Adhesive	Components	Procedure	Manufacturer
Primer&Bond 2.1 (Dentsply)	Uretane-dimethacrylate, Penta-P, Camphorquinone, ethyl-dimethyl-amine-benzoate, BHT, BisGMA, Cetilamine fluoro-hydrate e acetone.	Apply after etching the dentin for 15 sec. Remove excesses brief air jet (5 sec). Photopolymerize for 10 sec.	DENTSPLYCaulk (USA)

Table 2 - Assigns for the main bands relatives to the anchoring of BB94 in the Fe_3O_4@BPEI nanoparticles.

Wavenumber (cm^{-1})	Attribution
2915	ν_{CH2} asymmetric
2848	ν_{CH2} symmetric
1625	$\nu_{C=O}$ of amides
1527	Coupling between δ_{N-H} and ν_{C-N} of secondary amides
1460	$\nu_{C=C}$ of the benzene and thiophene ring of BB94

FIGURES

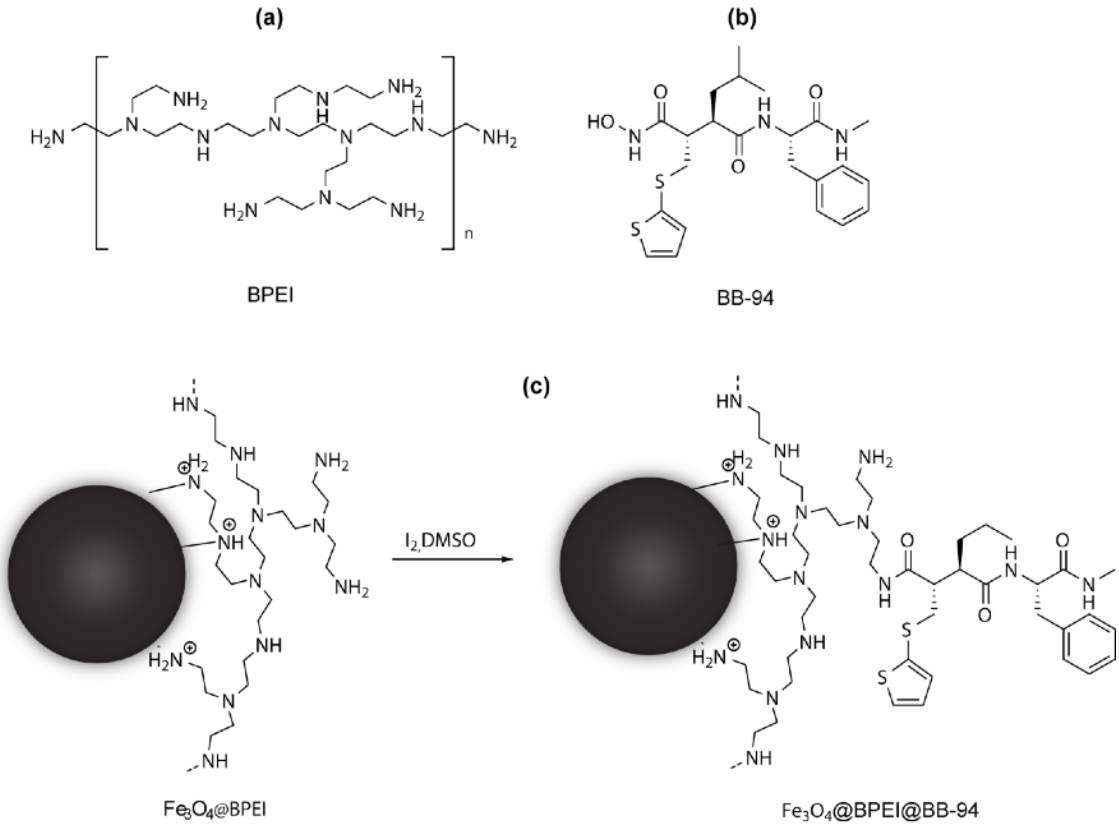

Figure 1 – Scheme of the synthesis of metalloproteinase magnetic collector. Chemical structure of BPEI and BB94 are depicted in (a) and (b) respectively. (c) Chemical strategy to anchor BB94 on the surface of pre-synthesized Fe_3O_4@BPEI nanoparticles to produce metalloproteinase magnetic collector.

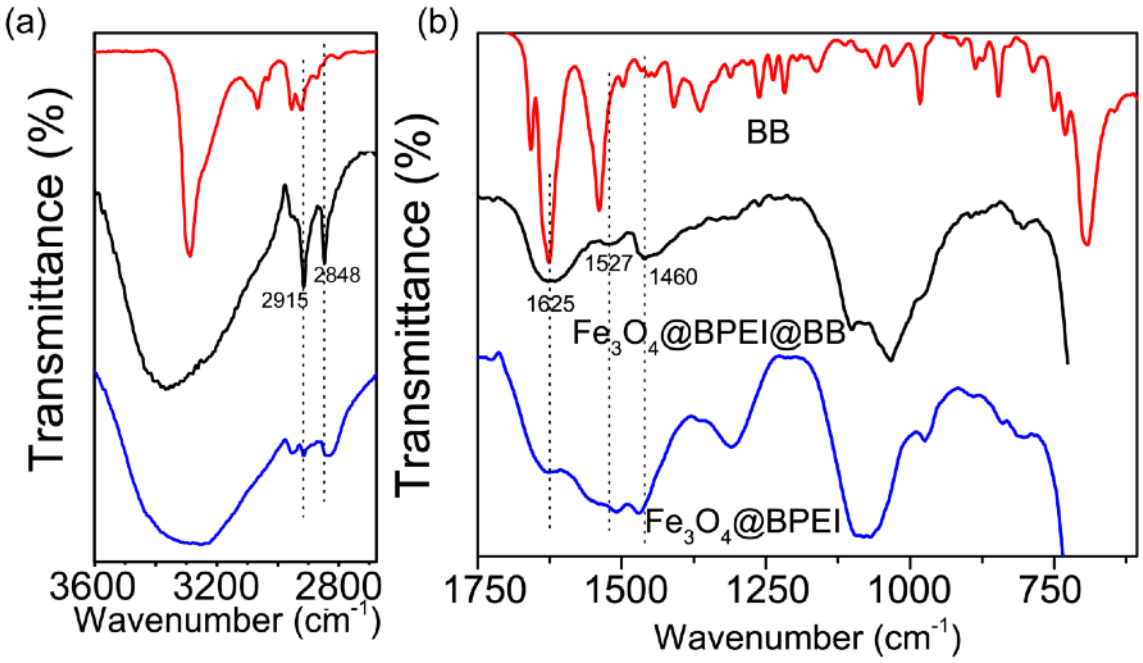

Figure 2 – FTIR spectra of the BB94 (batimastat), Fe_3O_4@BPEI@BB and Fe_3O_4@BPEI separated in the two main ranges to observe the synthesis. (a) 2860-3600 cm^{-1} range and (b) 615-1750 cm^{-1} range. Dotted lines indicate peaks of BB94 presented/modified in metalloproteinase magnetic collector (Fe_3O_4@BPEI@BB), the final product (middle spectrum).

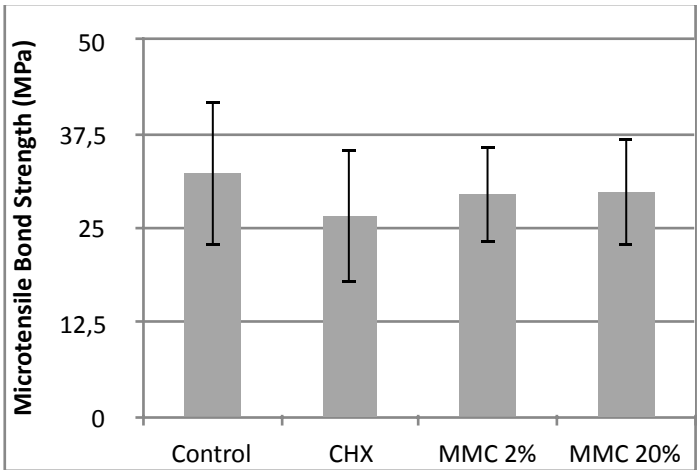

Figure 3 – Outcomes of microtensile bond strength test. No difference was found (p=0.432) among groups in one-way ANOVA. CHX means chlorhexidine and MMC is the acronym of metalloproteinase magnetic collector.

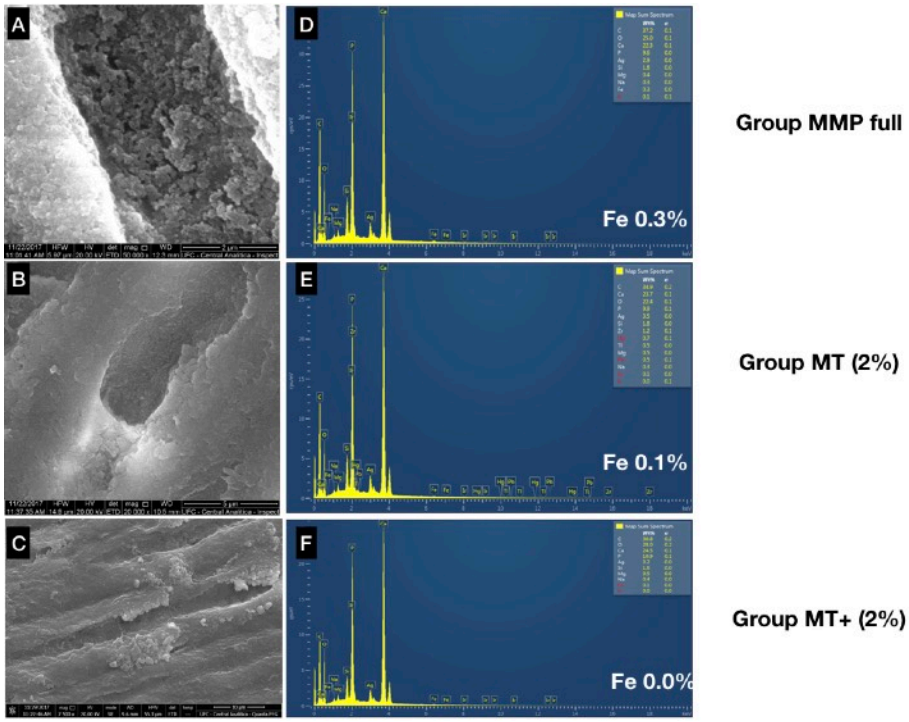

Fig. 4 - SEM micrographs of dentin specimens treated with MMC at 2wt%. The different groups are divided as follows: (A) MMP-full: dentin surface was etched with phosphoric acid for 15s to expose organic matrix, MMC was applied for 60s, rinsed for 30s and processed for SEM analysis to identify the amount of MMPs in partially demineralized dentin matrix. (B) Magnet-treated (MT 2%): the specimens received the same treatment, but before final rising a magnet was used for 60s at 0.5mm from dentin surface to remove magnetic nanoparticles attached with MMPs. After such treatment, the concentration of Fe and MMPs was reduced to 0.1% as identified by EDS. (C) Magnet-treated + (MT 2%+): the specimens were etched with phosphoric acid for 15s, rinsed with distilled water for 30s, MMC was applied for 60s, magnet was then employed for 60s, specimens were rinsed again with distilled water for 30s and MMC was re-applied for 60s (to assess remaining MMPs in dentin structure) prior to final rinsing with distilled water. (D) EDS spectrum of the first group (MMP full) showing the presence of 0.3% of Fe bound to MMP, (E) EDS spectrum of the second group (MT 2%)

showing the presence of 0.1% of Fe bound to MMP and last image (F) EDS spectrum of the last group (MT 2% +) showing the presence of 0.0% of Fe bound to MMP.

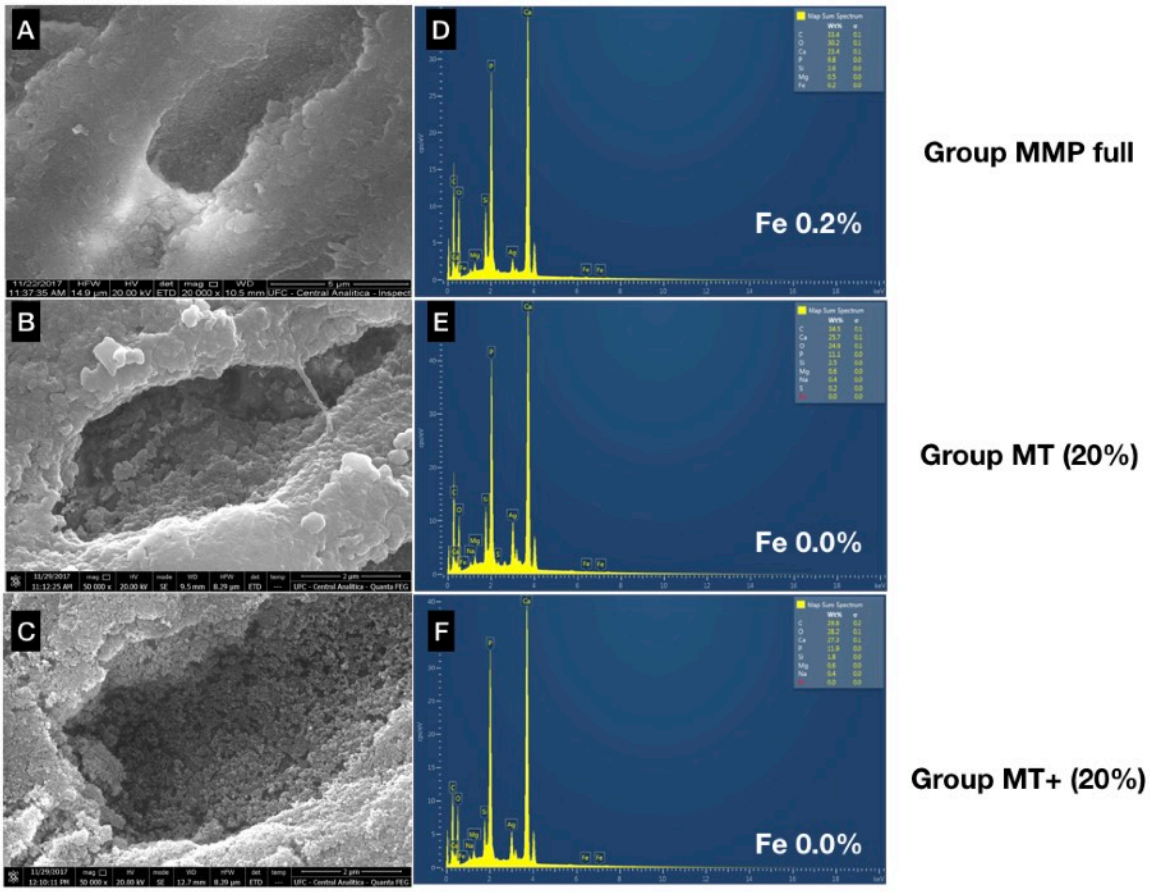

Fig. 5 - SEM micrographs of dentin specimens treated with 20wt% MMC. The different groups are divided similarly to Fig.4 as follows: (A) MMP-full: dentin surface was etched with phosphoric acid for 15s to expose organic matrix, MMC was applied for 60s, rinsed for 30s to identify the amount of MMPs in dentin matrix. (B) Magnet-treated (MT 20%): the specimens received the same treatment, but prior to final rising a magnet was used for 60s at 0.5mm from dentin surface to remove MMPslinked to magnetic nanoparticles. After this treatment, the concentration of Fe and MMPs was reduced to 0.0% as identified by EDS. (C) Magnet-treated + (MT 20%+): the specimens were etched with phosphoric acid for 15s, rinsed with distilled water for 30s, MMC 20% was applied for 60s, magnet was then employed for 60s, specimens were rinsed again with distilled water for 30s and MMC was re-

applied for 60s (to assess remaining MMPs in dentin structure) prior to final rinsing with distilled water. (D) EDS spectrum of the first group (MMP full) showing the presence of 0.2% of Fe bound to MMP, (E) EDS spectrum of the second group (MT 20%) showing the presence of 0.0% of Fe bound to MMP and last image (F) EDS spectrum of the last group (MT 20% +) showing the presence of 0.0% of Fe bound to MMP.

4. CONCLUSÃO GERAL

Diante dos resultados obtidos neste estudo, pode-se concluir que:

1. O coletor magnético de metaloproteinases foi sintetizado com sucesso, ancorando de forma eficiente o batimastat nas nanopartículas magnéticas.

2. O tratamento da dentina condicionada com o coletor magnético não altera a resistência de união inicial de um adesivo convencional de dois passos à dentina.

3. O coletor magnético remove as metaloproteinases da matriz de dentina, sendo mais eficiente em concentração de 20%.

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6. ANEXO A – SEGUIMENTO DO REGIMENTO INTERNO

CAPÍTULO VI

DOS EXAMES E DA DEFESA DE DISSERTAÇÃO E TESE

Art. 45 - O Exame Geral de Qualificação de que trata o *Artigo 50 das Normas para os Cursos de Pós-Graduação da UFC* deverá ser realizado perante uma comissão julgadora composta de no mínimo 03 (três) membros efetivos e um suplente, tendo o orientador como seu presidente.

§1º - O Exame Geral de Qualificação deverá ser realizado antes da matrícula na atividade acadêmica dissertação ou tese e será composto por duas fases. A primeira constará da defesa do projeto de pesquisa, a qual deverá ser realizada até seis meses após o ingresso no curso (nível Mestrado) ou até 12 meses (nível Doutorado). A segunda fase constará da defesa da pesquisa (uma pré-defesa) e deverá ser realizada até 45 dias antes da defesa da dissertação ou da tese.

§2º - As duas fases do Exame Geral de Qualificação constarão de sessão pública com: (1) aula expositiva com duração de 30 a 40 minutos; (2) arguição pelos membros da banca avaliadora com duração de 20 minutos para cada componente desta, bem como 20 minutos destinados às respostas do aluno para cada avaliador.

§3º - As bancas das duas fases do Exame Geral de Qualificação serão compostas por 2 (dois) avaliadores e pelo orientador.

§4º - No caso de não cumprimento do prazo estipulado no §1º, o orientador deverá encaminhar à coordenação do PPGO, antes de seu vencimento e ouvido o aluno, solicitação de ampliação do prazo, mediante justificativa e descrição da etapa de desenvolvimento do projeto.

§5º - O aluno que não obtiver aprovação no Exame Geral de Qualificação terá direito à nova oportunidade, com data a ser definida pela Coordenação do PPGO.

§6º - O aluno só poderá defender a dissertação ou tese após aprovação no Exame Geral de Qualificação de que trata este artigo.

Art. 46 – As dissertações e as teses apresentadas ao Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará poderão ser produzidas em formato alternativo ou tradicional. O formato