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Influence of the adhesive bonding protocol on the inflammatory cellular response and gelatinolytic activity in human teeth

Influência do protocolo de união adesivo na resposta inflamatória celular e atividade gelatinolítica em dentes humanos

Influencia del protocolo de unión adhesiva en la respuesta celular inflamatoria y la actividad gelatinolítica en dientes humanos

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Abstract
The aim of this paper was to assess the biocompatibility and expression of metalloproteinase -9 of two dentin bonding agents in human teeth, using different methods of dentin pre-treatment and different time intervals. Deep Class I cavities were prepared on the occlusal surface of 18 sound molars. Restorations were performed with XP Bond™ (Dentsply) and Futurabond® DC (VOCO). After 30, 90 or 120 days, the teeth were extracted and processed for histological and immunohistochemical assessment. In XP Bond group, was observed a moderate inflammatory infiltrate and immunoreactivity of metalloproteinase -9 in all storage intervals. In Futurabond DC group, a slight inflammatory infiltrate was found in the first intervals. After 120 days, the inflammatory infiltrate was either slight or absent, while the tissue morphology remained normal. The immunoreactivity of metalloproteinase -9 was considered moderate, except for two specimen after 120 days, that demonstrated no immunoreactivity. Therefore, etch-and-rinse adhesives exhibited a more intense pulpal response than self-etching agents. When performing adhesive restorations in medium and deep cavities in human teeth, the use of a self-etching adhesive strategy should be considered in view of the lower induction of inflammatory and proteolytic activity. Further studies of molecular events should be conducted, taking into consideration the possible inflammatory pulp reactions that can contribute to the success of restorative procedures.
Keywords: Dentin; Matrix Metalloproteinase 9; Dentin-Bonding Agents.

Resumo
O objetivo deste trabalho foi avaliar a biocompatibilidade e expressão da metaloproteinase -9 de dois agentes adesivos dentinários em dentes humanos, usando diferentes métodos de pré-tratamento dentinário e diferentes intervalos de tempo. Cavidades profundas Classe I foram preparadas na superfície oclusal de 18 molares hígidos. As restaurações foram realizadas com XP Bond ™ (Dentsply) e Futurabond® DC (VOCO). Após 30, 90 ou 120 dias, os dentes foram extraídos e processados para avaliação histológica e imunohistoquímica. No grupo XP Bond, foi observado infiltrado

inflamatório moderado e imunorreatividade da metaloproteinase -9 em todos os intervalos. No grupo Futurabond DC, um leve infiltrado inflamatório foi encontrado nos primeiros intervalos. Após 120 dias, o infiltrado inflamatório era discreto ou ausente, enquanto a morfologia do tecido permanecia normal. A imunorreatividade da metaloproteinase -9 foi considerada moderada, exceto para duas amostras após 120 dias, que não apresentaram imunorreatividade. Portanto, os adesivos convencionais exibiram uma resposta pulpar mais intensa do que os agentes autocondicionantes. Na realização de restaurações adesivas em cavidades médias e profundas em dentes humanos, o uso de uma estratégia adesiva autocondicionante deve ser considerada tendo em vista a menor indução de atividade inflamatória e proteolítica. Novos estudos de eventos moleculares devem ser realizados, levando-se em consideração as possíveis reações inflamatórias pulpares que podem contribuir para o sucesso dos procedimentos restauradores.
Palavras-chave: Dentina; Metaloproteinase 9 da Matriz; Adesivos Dentinários.

Resumen
El objetivo de este trabajo fue evaluar la biocompatibilidad y expresión de la metaloproteinasa -9 de dos agentes adhesivos de dentina en dientes humanos, utilizando diferentes métodos de pretratamiento de dentina y diferentes intervalos de tiempo. Se prepararon cavidades profundas de clase I en la superficie oclusal de 18 molares sanos. Las restauraciones se realizaron con XP Bond ™ (Dentsply) y Futurabond® DC (VOCO). Después de 30, 90 o 120 días, los dientes fueron extraídos y procesados para evaluación histológica e inmunohistoquímica. En el grupo XP Bond, se observó un infiltrado inflamatorio moderado e inmunorreactividad de metaloproteinasa -9 en todos los intervalos de almacenamiento. En el grupo Futurabond DC se encontró un leve infiltrado inflamatorio en los primeros intervalos. Después de 120 días, el infiltrado inflamatorio era leve o ausente, mientras que la morfología del tejido permanecía normal. La inmunorreactividad de la metaloproteinasa -9 se consideró moderada, a excepción de dos muestras después de 120 días, que no demostraron inmunorreactividad. Por lo tanto, los adhesivos de grabado y enjuague exhibieron una respuesta pulpar más intensa que los agentes de autograbado. Al realizar restauraciones adhesivas en cavidades medias y profundas en dientes humanos, se debe considerar el uso de una estrategia adhesiva de autograbado en vista de la menor inducción de actividad inflamatoria y proteolítica. Se deben realizar más estudios de los eventos moleculares, teniendo en cuenta las posibles reacciones inflamatorias de la pulpa que pueden contribuir al éxito de los procedimientos de restauración.
Palabras clave: Dentina; Metaloproteinasa 9 de la Matriz; Recubrimientos Dentinarios.

1. Introduction

The vitality of dental pulp following restorative procedures depends on the degree of the inflammatory cell response, which is directly proportional to the remaining dentin thickness (RDT) (de Souza Costa *et al*. 2014, Murray *et al*. 2002), mechanical injury during tooth preparation and microleakage (Bagis *et al*. 2007). The pulpal inflammatory response can also be modified by dental materials, such as the dentin bonding agent (DBA), its components (Costa *et al*. 2021), and the technique used (etch-and-rinse or self-etching) (Alves & Sobral 2015, Elias *et al*. 2015).

Current dentin bonding agents follow either "etch-and-rinse" or "self-etching" protocols. Etch-and-rinse bonding agents contain phosphoric acid to pretreat dental tissues, followed by rinsing and the application of an adhesive. Self-etching bonding agents does not require pretreat dental tissues, but it contains acidic monomers, which simultaneously demineralize and infiltrate during the application (da Silva *et al*. 2014, Ozer & Blatz 2013), leading to the interposition of a (resin-impregnated) smear along the dentin surface (Suyama *et al*. 2013, Arrais & Giannini 2002). The composition of the material, the concentration and chemistry of the components and the tissue responsiveness influence the interaction between dental materials and tooth tissue (Ferracane *et al.* 2010). Substances commonly found in both DBA protocols have a toxic effect, i.e., Bis-GMA (bisphenol-A glycidyl methacrylate), TEGMA (triethylene glycol dimethacrylate), HEMA (hydroxyethyl methacrylate) and UDMA (urethane dimethacrylate) (da Silva *et al.* 2014, Nowicka *et al*. 2012, Tran-Hung *et al*. 2012). These substances can diffuse through dentin, reaching the pulp tissue, affecting the inflammatory cellular response and the biocompatibility of the restorative material (Machado *et al.* 2016, Elias *et al.* 2015, Shafiei *et al.* 2014, Bianchi *et al*. 2013, Nowicka *et al.* 2012, Kostoryz *et al*. 2003, Huang & Chang 2002).

When faced with an aggressor agent, an inflammatory process is initially triggered in the dentin-pulp complex. Subsequently, there is a tendency to resolve this inflammatory process in the absence of bacterial infection (Leonardi *et al*. 2011). If the offending stimulus is high intensity, pulp necrosis may occur. Chemical materials applied to the dentin-pulp

complex cause an inflammatory response (Leonardi *et al*. 2011). This response should be as minimal as possible, in order to avoid irreversible damage to the pulp tissue (de Souza Costa *et al*. 2014, Hebling *et al*. 2010). The biological response is extremely important in terms of maintaining the vitality of the dentin-pulp complex and should be carefully considered during clinical procedures (Hebling *et al.* 2010).

The inflammatory process involves inflammatory mediators (Bagis *et al*. 2007), dental fluid, odontoblasts, lymphocytes, neuropeptides, chemokines and inflammatory cytokines, which lead to a decrease in pH (Leonardi *et al.* 2011) and an increase in proteolytic activity, especially, in matrix metalloproteinases (MMPs) (Boelen *et al.* 2019). The MMPs are enzymes capable of degrading all components of the extracellular matrix (Sabatini & Pashley 2014). MMPs are believed to be involved in physiological processes, such as dentin maturation during aging, but also in pathological processes such as caries, periodontal disease, and pulpal inflammation (Boelen *et al*. 2019; Shimada *et al.* 2009). The MMPs can be activated by the drop in pH consequent to the treatment of the surface with primer and adhesive or to the biochemistry of the carious process, leading to the degradation of collagen fibrils and failure in the adhesion process (Pashley *et al*. 2004).

It is still necessary to validate in vivo the activity of the collagenolytic and gelatinolytic endogenases that are stimulated by the acid (from acid attack of etch-and-rinse adhesives and acid monomers of self-etching adhesives), and it results in degradation of the hybrid layer (Hebling *et al.* 2005), comparing the two adhesive techniques over time. MMP-9 is the metalloproteinase predominantly found in the dentin matrix (Sabatini & Pashley 2014) and seems to be closely linked to pulpal pathological processes (Accorsi-Mendonça *et al.* 2013) and can serve as a biological marker related to the cellular inflammatory response.

It is believed that a group of activities at a molecular level are responsible for the structural degradation of tissue. Concerning the pulp tissue, an understanding of the inflammation stages of this tissue is essential, together with knowledge of the molecular events that can contribute to the maintenance of the vitality of the pulp, in order to preserve dental elements during restorative procedures. Therefore , the aim of the present study was to assess the biocompatibility of two dentin bonding agents in human teeth cavities based on the inflammatory cellular response (cellular response and pulp tissue organization) and the immunoexpression of MMP-9 of pulp tissue, using different methods of dentin pre-treatment (etch-and-rinse and self-etching) and different time intervals.

2. Methodology

This was a longitudinal, descriptive, and observational study. A clinical or experimental phase was followed by a laboratory phase for histopathological analysis (Costa *et al.* 2021).

Clinical procedures

This study was conducted using the same clinical methodology used in the study of Costa *et al.* (2021). The present study was approved by the Ethics Committee of the University of Pernambuco (protocol No. 297/09). Eighteen patients (age range 20-40 years) with an indication for third molar extraction were selected and randomly divided into nine groups (n = 3/group). All of the participants signed a statement of informed consent.

The third molar was anesthetized and occlusal cavities were prepared with a high-speed handpiece (under water-cooling) and a # 245 carbide bur (replaced after each preparation to avoid excessive heating). The cavity was approximately 1 - 2 mm deep from the roof of the pulp chamber. The patients were randomly assigned to groups in which they would receive either the etch-and-rinse or the self-etching dentin bonding agent. This assignment was performed using a single-blinded envelope method.

Two bonding agents were used: XP Bond – a etch-and-rinse adhesive, which involves an etching technique with 37% phosphoric acid, prior to the application of the adhesive from a single bottle containing both the primer and the adhesive; and Futurabond DC – a self-etching, one-step adhesive, which contains acid, primer and adhesive in a single bottle. In each group, the bonding procedures were carried out in accordance with the manufacturers' instructions (Table 1). Cavities were restored using the microhybrid composite resin Filtek Z250 A3 (3M ESPE Dental Products, St. Paul, MN, USA).

Table 1: Specifications of the materials tested in the present study.

Material	Classification	Manufacturers' instructions	Composition
XP Bond™ [a]	Etch-and-rinse dental adhesive system	1. Apply etching acid for 15 seconds. 2. Wash etching acid for 15 seconds. 3. Remove excess water. 4. Apply adhesive and leave for 20 seconds. 5. Apply air gently to evaporate solvent. 6. Light-cure for 10-20 seconds. 7. Apply restorative material.	Carboxylic acid modified dimethacrylate (TCB resin); Phosphoric acid modified acrylate resin (PENTA); Urethane Dimethacrylate (UDMA); Triethylene glycol dimethacrylate (TEGDMA); 2-hydroxyethylmethacrylate (HEMA); Butylated benzenediol (stabilizer); Ethyl-4-dimethylaminobenzoate; Camphorquinone; Functionalized amorphous silica; t-butanol
campherchinon			
Futurabond® DC [b]	Self-etch dental adhesive system	1. Mix equal amount of Liquid A and Liquid B for 5 seconds. 2. Apply adhesive to tooth substrates (scrubbing) for 30 seconds. 3. Air blow gently for 5 seconds. 4. Light-activate for 20 seconds. 5. Apply restorative material.	Organic acids, Bis-GMA, HEMA, TMPTMA, campherchinon, amines (DABE), BHT, catalysts, fluorides and ethanol

[a] Dentsply/De Trey GmbH, Konstanz, Germany.
[b] Voco GmbH, Cuxhaven, Germany.

Source: Authors.

After the restorative procedure, a new randomization (single-blinded envelope method) was performed for the analysis at different time intervals. For each time interval (30, 90 and 120 days), teeth extractions were performed and three specimens were taken for each material tested. The specimens were then immersed in a solution of 10% formaldehyde.

Laboratory procedures

During the laboratory phase, semi-serial sections (thickness of 5 µm for hematoxylin-eosin -H.E.- staining and 3 µm for immunostaining) were obtained in microtome (Leica RM 2125, Leica Microsystems, Nussloch GmbH, Germany). Immunohistochemical (I.H.C.) staining was performed using streptavidin-biotin optimized peroxidase, an LSAB amplification system (LSAB - Dako, Glostrup, Denmark) and the monoclonal antibody anti-MMP-9, with diaminobenzidine (DAB) used as chromogen. The primary antibody is specified in Table 2.

Table 2: Clone, specificity, source, dilution, incubation and antigen retrieval of primary antibody anti-MMP-9.

Clone	Specificity	Source	Dilution	Incubation	Antigen retrieval
2C3	MMP-9	DBS	1:125	60'	Pressure Cooking / Citrate Buffer (pH= 6.0)

Source: Authors.

Two blind observers who had experience in histological and immunohistochemical analysis using conventional light microscopy at 10X, 40X and 100X magnification (ABM-200, Alltion, Guangxi, China) analyzed the slides. A third researcher resolved disagreements when necessary.

The H.E. sections were assessed for two histologic features: the inflammatory cellular response and tissue disorganization. The degree of pulp inflammation and the organization of the pulp tissue were analyzed in accordance with the criteria proposed by de Souza Costa *et al.* (2006), as described in Table 3. The I.H.C. sections were examined considering the following histologic features: the type of immunopositive cell; the tissue location (focal or diffuse) and the intensity of the immunostaining. The immunostaining pattern was classified as positive (+) or negative (-). Positive cases were also classified quantitatively, according to the intensity of the marking: weak (+), moderate (++) and intense (+++). Subsequently, a marking score as a percentage of positive cells was assigned: score 0: no positive cell; score 1: 1-25% positive cells; score 2: 25% -50% positive cells; and score 3: > 50% of positive cells.

The negative control involved slides on which the primary antibody had been replaced by immunoglobulin G (IgG). The positive control used breast carcinoma for MMP-9.

Table 3: Inflammatory cell response (Adapted from de Souza Costa *et al.* 2006).

Score	Cellular response	Pulp tissue organization
0	None or a few scattered inflammatory cells present in the pulp area (axial wall), characteristic of normal tissue	Normal tissue morphology
1	Slight inflammatory cell infiltrate with polymorphonuclear (PMNs) or mononuclear leukocytes (MNLs)	Disorganized odontoblastic layer, but normal central portion of the dental pulp
2	Moderate inflammatory cell infiltrate involving the coronal pulp	Complete disorganization of the pulp tissue morphology
3	Severe inflammatory cell infiltrate involving the coronal pulp or characterizing an abscess	Pulp necrosis

Source: Authors.

3. Results

Analysis of cellular and morphological response and immunoexpression of MMP-9 was performed for all experimental groups. Table 4 displays the scores recorded for the inflammatory cellular response and the tissue disorganization criteria, according to group and period.

Table 4: Inflammatory cellular response and pulp tissue organization criteria score, according to the group and period.

Groups	Histopathologic event												Total Score
	Cellular Response						Pulp Tissue Organization						
	30 days			90 days			120 days			30 days			90 days			120 days			
Xp[a]	2	1	1	2	1	1	2	1	1	1	1	1	1	1	1	1	1	1	21
Fb[b]	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	0	13

[a] Xp: XP Bond™; [b] Fb: Futurabond® DC

Source: Authors.

Table 5 displays a marking score as a percentage of positive cells for immunoexpression of MMP-9.

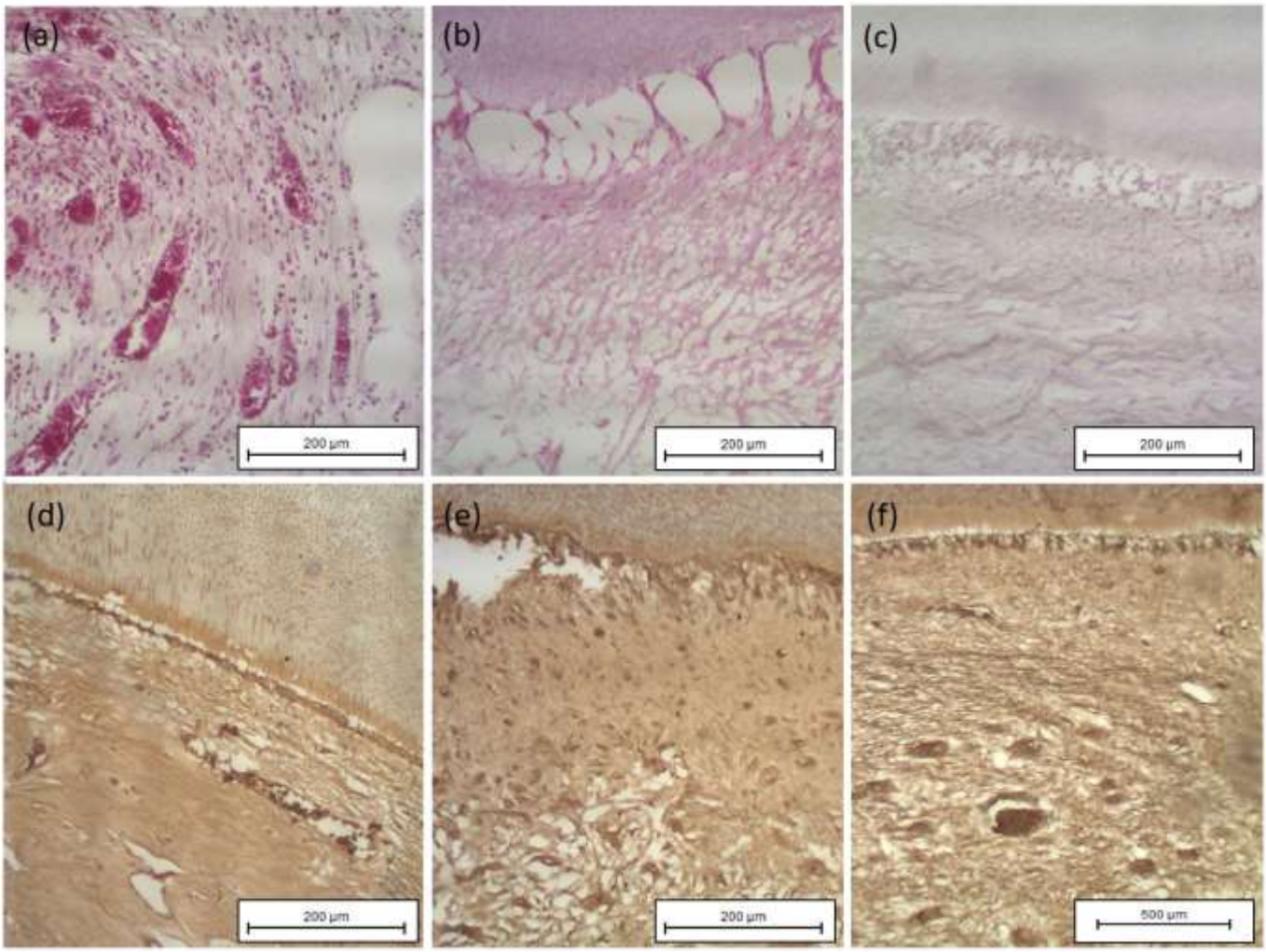
Table 5: Immunostaining pattern of MMP-9 at different time intervals and DBA.

Antibody/ Time interval	**Specimens**					
	1	**2**	**3**	**1**	**2**	**3**
	XP Bond			**Futurabond DC**		
MMP-9 30 days	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal
90 days	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal
120 days	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal	++/ Score 2/ Focal	-/ Score 0	-/ Score 0

Source: Authors.

Histological features for XP Bond bonding agent in each interval group can be exemplified in Figure 1. For the 30-, 90- and 120-day intervals in the XP Bond dentin bonding agent group, a moderate inflammatory infiltrate (score 2) was observed in three specimens (Fig. 1(a)). Partial disorganization of the pulp tissue morphology and odontoblastic layer (score 1) was found for all specimens (Fig. 1(b)/1(c)). Regarding MMP-9 immunoexpression, the three intervals presented moderate and focal expression for all specimens (Fig. 1(d)). Each time interval presented a specimen with localized expression in the odontoblastic layer, close to the congested vessels, and in the inflammatory cells in the center of the pulp. For the other two specimens of each time interval, the expression was evidenced in the odontoblastic layer and in the inflammatory infiltrate (Fig. 1(e)/1(f)).

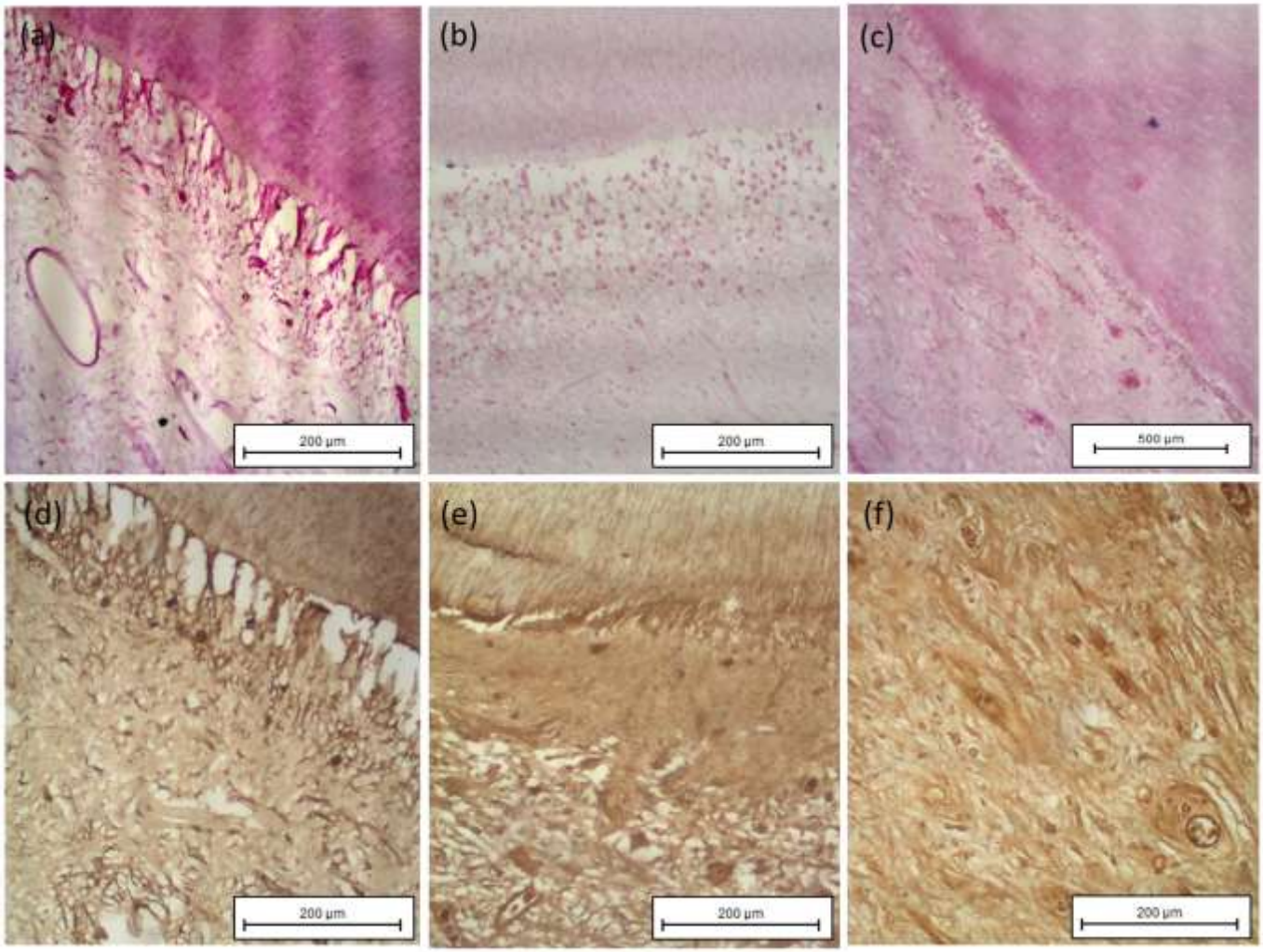
Figure 1: Histological features for XP Bond bonding agent group. (a) After 30 days: moderate inflammatory infiltrate, with a disorganized pulp tissue morphology and odontoblast layer (HE - Bars indicate 200µm). (b) After 90 days: slight inflammatory infiltrate with a disorganized pulp tissue morphology and odontoblast layer (HE - Bars indicate 200µm). (c) After 120 days: slight inflammatory infiltrate with a disorganized pulp tissue morphology and odontoblast layer (HE - Bars indicate 200µm). Immunohistochemical staining for XP Bond bonding agent group. (d) After 30 days: moderate and focal expression (IHC - Bars indicate 200µm). (e) After 90 days: moderate and focal expression (IHC - Bars indicate 200µm). (f) After 120 days: moderate and focal expression (IHC - Bars indicate 200µm).



Source: Authors.

Histological features Futurabond DC bonding agent in each interval group can be exemplified in Figure 2. For the 30- and 90-day intervals in the Futurabond DC dentin bonding agent group, all of the specimens exhibited a slight inflammatory infiltrate close to the odontoblastic layer and a disorganized odontoblastic layer, although the central portion of the dental pulp was normal (Fig. 2(a)/2(b)). After 120 days, normal tissue morphology was observed, with a slight (or absent) inflammatory infiltrate (Fig. 2(c)). Regarding the MMP-9 immunoexpression, all specimens within the 30 and 90-day intervals and one 120-day interval specimen exhibited moderate and focal expression with predominant localization in the odontoblastic layer and inflammatory infiltrate (Fig. 2(d)/2(e)). In the other two specimens of the 120-day interval, no MMP-9 expression was observed (Fig. 2(f)).

Figure 2: Histological features for Futurabond DC bonding agent group. (a) After 30 days: slight inflammatory infiltrate closes the odontoblast layer, with normal pulp tissue morphology (HE - Bars indicate 200µm). (b) After 90 days: slight inflammatory infiltrate near the odontoblast layer (HE - Bars indicate 200µm). (c) After 120 days: normal tissue morphology with a slight inflammatory infiltrate. Immunohistochemical staining for Futurabond DC (HE - Bars indicate 500µm). (d) After 30 days: moderate and focal expression with predominant localization in the odontoblastic layer and inflammatory infiltrate (IHC - Bars indicate 200µm). (e) After 90 days: moderate and focal expression (IHC - Bars indicate 200µm). (f) After 120 days: no MMP-9 expression (IHC - Bars indicate 200µm).



Source: Authors.

4. Discussion

This study observed that previous acid etching led to a greater inflammatory cellular response and expression of MMP-9. The hybridization technique may have a cytotoxic effect on pulp tissue after acid etching (de Souza Costa *et al*. 2014). The toxic effects of materials may risk cell function and at long last cause cell death (Ferracane *et al*. 2010). The response of the dentin-pulp complex to the application of a dentin bonding agent in deep cavities in human teeth has been previously analyzed, however, the *in vivo* biological effect comparing adhesive protocols has not been widely studied and deserves further attention. Büyükgüral and Cehreli (2008) reported that restorative procedures in deep cavities may cause irreversible pulp tissue reactions, particularly when the RDT is less than 0.5 mm. Ballal *et al*. (2017) found more MMP-9 in

deep carious lesions than from in the shallow one. The intensity of the pulp reaction increased as the remaining dentin thickness decreased (Hebling *et al.* 1999). In the present study, the remaining dentin was approximately 2 mm. This quantity of remaining dentin can be considered a protective barrier for the pulp against injuries from the adhesives irritant agents (Alves & Sobral 2015).

The XP Bond group exhibited a slight inflammatory cellular response in most specimens. However, a moderate response was also observed. This increase in the inflammatory cell response may have occurred due to the pre-treatment with 37% phosphoric acid. Etching exposes collagen fibers that will be filled by the adhesive system at a later stage. It is known that the chemical diffusion gradient of the bonding agent prevents the complete infiltration of adhesives (Strobel & Hellwig 2015). The insufficient penetration of the etch-and-rinse DBA monomers along the entire length of the demineralized dentin leaves unfilled gaps (da Silva *et al*. 2014). The presence of exposed collagen fibrils leads to proteolytic degradation and an increased pulp inflammatory response (Elias *et al*. 2015, Bianchi *et al*. 2013).

In addition to the increased proteolytic activity, acid etching increases the permeability of dentinal tubules, furthering the contact between the cytotoxic substances in dentin bonding agents and the pulp tissue (Shafiei *et al.* 2014), even in deep cavities where dentinal tubules are naturally larger. Monomers' leach may occur in the first 24 hours until a month after the restorative procedure and trigger pulp inflammation and MMP induction (Lovász *et al*. 2021).

Concerning the organization criteria of pulp tissue, the odontoblastic layer was found to be disorganized. Assuming that odontoblasts are the most peripheral pulp cells, they are more susceptible to aggressions arising from the application of dental materials (Elias *et al*. 2015, de Souza Costa *et al.* 2014). Therefore, it is believed that the XP Bond, through its components and the associated tooth surface treatment, caused *stimuli* that led to reversible cellular damage in the odontoblastic layer. This is a transitory event which may lead to some form of defense response (Murray *et al.* 2002).

The favorable results observed in the Futurabond DC group may be explained by the dentin pre-treatment method involved and the composition of the bond agent. The weak acid in the Futurabond DC bonding agent (Arrais & Giannini 2002) and the interposition of a (resin-impregnated) smear along the dentin surface results in what may be considered as a positive biological response, despite the fact that the DBA contains components considered to be irritants (Murray *et al.* 2002). The smear layer obliterates the dentinal tubules, reducing dentin permeability and limiting the toxicity of self-etching bonding agents on the pulp (de Souza Costa *et al*. 2014). De Munck *et al.* (2009) analyzed *in vitro* the MMP-2 and MMP-9 release after using DBA through zymography and observed endogenous enzymatic activity only for etch-and-rinse adhesive against mild self-etching adhesive.

In the first stage of the analysis, tissue disorganization was observed along almost the entire length of the odontoblastic layer, with normal central pulp tissue morphology. Interestingly, after 120 days, the odontoblastic layer had been reorganized ("*in palisade*") and involved all of the dentine, with no inflammatory cells. The dental pulp has an elevated inherent regenerative potential (Tran-Hung *et al.* 2012). This could be explained by the reparative capacity of ectomesenchymal cells, which reactivate the condition of the affected pulp, thereby favoring the constant remodeling of the odontoblastic layer. This pulp response proves the reparative capacity of the pulp tissue (de Souza Costa *et al*. 2014, Hebling *et al.* 2010), mainly due to the decrease in dentin permeability owing to the deposition of reactionary dentin over time (Büyükgüral & Cehreli 2008).

O'Boskey Jr and Panagakos (1998) proposed that inflammatory cytokines stimulate gelatinase levels in human pulp connective tissue cells, and MMPs were stimulated during the intense pulpal inflammatory process as in any other inflammatory process. MMP-9 has already been detected in both mineralized and demineralized dentin (Van Strijp *et al.* 2003). MMPs-9 are synthesized by the odontoblasts (Sulkala *et al*. 2002) and data present in the literature have showed the

presence of MMP-9 in healthy and inflamed pulps. According to the results of the present study, we suggest the relation of the expression of the MMP-9 enzyme with the regions of the pulp tissue where there was disorganization of the odontoblasts layer and the inflammatory cells near the congestive vessels.

In the study by Gusman *et al.* (2002), the levels of MMPs (-1, -2, -3, and -9) were evaluated in human dental pulps under conditions of normality and inflammation, through enzyme linked immunosorbent assay (ELISA) and zymography. According to the results, the high absolute quantification of MMP-9 in inflamed pulp and the positive correlation with gelatinolytic activity suggest that MMP-9 is important in the degradation of pulp tissue in inflammatory processes. Costa *et al*. (2021) evaluated the expression of MMP-9 after using a etch-and-rinse adhesive on human teeth and observed that the immunoreactivity of MMP-9 was considered intense in the most of specimens in the first storage intervals, before decreasing over time.

The expression of MMP-9 at the 30- and 90-day intervals for both Futurabond DC and XP Bond was considered moderate, located in the odontoblasts layer, and in inflammatory cells, thus the considerations are similar for both adhesive systems, denoting the influence of factors such as: surgical technique of cavity preparation, depth of preparation and restorative technique. For the 120-day interval, in two specimens of the Futurabond DC group, expression of MMP-9 was not visualized. We believe that this result is related to the tissue normality found and for not having presented any inflammatory conditions.

In the groups of the Futurabond DC adhesive system, the capacity of pulp tissue to be repaired in the face of low intensity stimuli in medium depth cavities was clearly observed. In addition to the reaction caused by dentin adhesives, the influence of phosphoric acid on process is extremely important because the acids, when applied directly on the dentin, facilitates fluid movement (Hashimoto *et al*. 2004), as well as the transport of substances that are part of the composition of the adhesive systems up to the pulp. However, in self-etching adhesive systems, such as Futurabond DC, the acids are weaker than the phosphoric acid (Perdigão 2007), which may lead to a lower inflammatory response and, consequently, lower proteolytic activity, as observed in the present study.

5. Conclusion

In the present study, the two methods of dentin pre-treatment ("etch-and-rinse" and "self-etch") generated different inflammatory cellular responses and could provide practical parameters for clinical decision making. Considering the limitations and the results of the present study, as well as the findings described in the literature and seeing the need to simplify the dental clinical practice, we believe that the self-etching adhesive systems are the best choice in thin RDT cavities. Further longitudinal *in vivo* studies are needed to investigate the expression of proteolytic molecules capable of degrading the hybrid layer and contribute to the decrease of the adhesive bond stability and the substances capable of inhibiting this activity.

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