Clin Oral Invest (2012) 16:531–536
DOI 10.1007/s00784-011-0551-x

ORIGINAL ARTICLE

Addition of zinc methacrylate in dental polymers: MMP-2 inhibition and ultimate tensile strength evaluation

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Received: 23 July 2010 / Accepted: 16 March 2011 / Published online: 30 March 2011

Abstract This study evaluated the effect of zinc methacrylate (ZM) on the inhibition of matrix metalloproteinase 2 (MMP-2) and the ultimate tensile strength (UTS) of an experimental polymer. Enzymes secreted from mouse gingival tissues were analyzed by gelatin zymography in buffers containing 5 mM $CaCl_2$ (Tris–$CaCl_2$) in 50 mM Tris–HCl buffer with various concentrations of ZM (0.5, 1, 2, 4, 8, and 16 mM). The matrix metalloproteinases present in the conditioned media were characterized by immunoprecipitation. The polymer UTS evaluation was performed in eight groups with various concentrations of ZM (0, 0.5, 1, 2.5, 5, 10, 20, and 30 wt.%), in a mechanical testing machine. MMP-2 (62 kDa) was detected in the zymographic assays and inhibited by ZM in all tested concentrations. UTS data were submitted to one-way ANOVA and Tukey's test (α=0.05), and no significant differences were observed among groups, except in the polymer containing 30% ZM, presenting a significantly lower value when compared with the control group (p<0.05). The results suggest that ZM inhibits MMP-2 expression in all concentrations tested, while small concentrations did not affect the ultimate tensile strength of the polymer. Zinc methacrylate is a metalloproteinase inhibitor that can be copolymerized with other methacrylate monomers. Yet, the addition of ZM did not affect the resin bond strength. Thus, in vivo tests should be performed to evaluate the performance of this material.

Keywords Matrix metalloproteinase 2 · Gelatinase A · Polymers · Zymography · Ultimate tensile strength

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Introduction

Matrix metalloproteinases (MMPs) constitute a family of multidomain zinc endopeptidases involved in the degradation of extracellular matrix proteins and in a number of other important biological processes [1, 2]. At least 23 human MMPs have been characterized [3], with two of these MMPs [matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9)] attacking collagen and its by-products, such as gelatin [4]. MMPs can be divided into at least six subgroups according to the substratum: collagenases (MMP-1, MMP-8, and MMP-13), stromelysins (MMP-3, MMP-10, MMP-11, and MMP-

20), gelatinases or type IV collagenases (MMP-2 and MMP-9), matrilysin (MMP-7), metalloelastase (MMP-12), and membrane-type metalloproteinases (MMP-14, MMP-15, MMP-16, and MMP-17) [5].

Human odontoblasts are capable of synthesizing gelatinase A (MMP-2) during dentin formation [6]. MMP-2 is the metalloproteinase present in larger amounts in the human dentin [7]. Presumably after dentin mineralization, MMPs could be arrested in the mineralized phase and bound to matrix components or to hydroxyapatite crystals [8]. Eventually, MMPs could be released after exposure of the collagen network by acid-etching treatment in dentin and degrade the exposed collagen. Therefore, an important role has been attributed to MMPs in the durability of adhesive restorations [9], since MMPs degrade the hybrid layer collagen.

Contemporary dental adhesives show satisfactory performance in short-term investigations [10, 11]. However, longevity of the adhesive restorations remains a great challenge in contemporary adhesive dentistry [11]. The combined degradation of resin and collagen has been considered the major factor responsible for the degradation of dental adhesion. One of the proposed strategies used to increase the longevity of adhesive restorations has been the inhibition of MMPs present in the dentin collagen [9]. The application of chlorhexidine in the collagen network before the adhesive system is applied demonstrates the potential for possible MMP inhibition [12–15]. However, the chance of using copolymerizable MMP inhibitor materials is extremely promising, as chlorhexidine is not copolymerizable with the other methacrylate monomers being leached, ultimately leading to a decrease in its inhibitory effect over time.

Other potential inhibitors, namely zinc oxide cements, metal salts, and zinc-based amalgam, have also been identified [16–18], although the possibility of a polymerizable inhibitor that is relatively stable in the polymeric matrix thus in permanent contact with the collagen of the hybrid layer can be considered an advantage. Based on previous studies [16, 18] demonstrating the MMP inhibitory effect of zinc-based dental materials, it is expected that zinc methacrylate (ZM) will show a feasible MMP inhibition effect due to the presence of zinc. Zinc methacrylate is a monomer containing a functional methacrylate group in its structure, also found in other monomers present in adhesive systems. Thus, the possibility of using a copolymerizable material with the other methacrylate monomers is highly promising. However, the addition of ZM should not affect the resin bond strength. Therefore, the purpose of this study was to determine the effect of a copolymerizable zinc derivative, zinc methacrylate, on the potential to inhibit MMP-2 activity and the ultimate tensile strength (UTS) of the polymers formed.

Materials and methods

Zymography assay

Chemicals

Cell culture medium and reagents were supplied by GIBCO (Grand Island, NY, USA). All the other reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise mentioned. Zinc methacrylate and methacrylic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

Collection and preparation of MMP-2

This study was approved by the local research ethics committee and was carried out in conformity with the Declaration of Helsinki. Gingival tissue specimens were dissected from the palatal, lingual, and buccal sites of two mice (aged 4 weeks). Immediately after excision, the tissue specimens were pooled and washed in Dulbecco's Modified Eagle Medium (DMEM) containing 80 mg/ml gentamicin at room temperature for 30 min. After washing, the specimens were incubated in DMEM containing 40 mg/ml gentamicin at 37°C for 24 h. Next, gingival fragments were discarded and the cell culture conditioned medium (DMEM) containing the secreted MMPs was centrifuged at 1,000 rpm for 5 min, and the supernatant frozen at −70°C until analysis for enzyme activity.

Zymography

Proteolytic activity was examined on 10% polyacrylamide gels containing 0.05% gelatin. Conditioned medium was mixed with an equal volume of nonreducing sample buffer (2% SDS; 125 mM Tris–HCl, pH 6.8, 10% glycerol and 0.001% bromophenol blue) followed by electrophoreses. After electrophoresis, gels were washed twice in 2% Triton X-100 for 60 min at room temperature and incubated at 37°C for 24 h in 50 mM Tris–HCl buffer, pH 7.4, containing 5 mM $CaCl_2$ (Tris–$CaCl_2$). After incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250 (Bio Rad, Richmond, CA). Gelatinolytic activity was detected as unstained bands.

Identification of MMP by means of specific chemical inhibition

To identify lytic bands present in conditioned media, parallel experiments of zymographic inhibitions were performed. Gelatin-containing gels were incubated in Tris–$CaCl_2$ buffer at 37°C for 24 h with the addition of 0.5 mM of ethylenediaminetetraacetic acid (EDTA; Reagen,

Brazil) to inhibit lytic activities due to MMP, while 0.5 mM *N*-ethyl-maleimide (NEM; Sigma-Aldrich, MO, USA) was used to inhibit activities caused by serine proteinases.

Characterization of MMP-2 by immunoprecipitation

Conditioned medium was subjected to an immunoprecipitation reaction with sheep anti-human MMP-2 IgG (The Binding Site, Birmingham, England). The immunocomplexes were precipitated with A-Sepharose protein beads (Pharmacia Biotech, Uppsala, Sweden) for 14 h at room temperature. After washing nonspecific Sepharose-adsorbed material with TBS, the immunoprecipitated material was eluted with nonreducing sample buffer for 10 min at 70°C and assayed by gelatin zymography.

Inhibition of metalloproteinase activity by zinc methacrylate

In order to examine the effect of different concentrations of zinc methacrylate on enzyme activity, conditioned medium containing MMPs was loaded on preparative gelatin-containing polyacrylamide gels. To show the specific effect of zinc present in the ZM, the effect of the same concentrations of methacrylic acid also was tested. After electrophoresis the gels were cut into strips of approximately 1 cm, and each strip was incubated at 37°C for 24 h in Tris–$CaCl_2$ buffer containing one of the following experimental conditions: Tris–$CaCl_2$ only (control), 0.5, 1, 2, 4, 8, and 16 mM of zinc methacrylate or methacrylic acid. After the addition of the monomers to the solution, pH was adjusted to 7.4. and the solution was then incubated at 37°C for 24 h in 50 mM Tris–HCl buffer containing 5 mM $CaCl_2$ (Tris–$CaCl_2$). After incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250. Each assay was performed in triplicate and was repeated at least twice.

Ultimate tensile strength assay

Chemicals

Triethylene glycol dimethacrylate (TEGDMA) and camphorquinone (CQ) were obtained from Esstech Inc. (Essington, PA, USA). Ethyl 4-dimethylamine benzoate (EDAB) was purchase from Aldrich Chemical Co. (Milwaukee).

Experimental resin formulation

The zinc methacrylate was added in concentrations of 0.5%, 1%, 2.5%, 5%, 10%, 20%, and 30% by mass to a resin model composed of TEGDMA, EDAB, and CQ, forming eight groups (Table 1). Polymer without zinc methacrylate was used as control. TEGDMA was chosen as a model to allow a better zinc methacrylate dissolution in this low-viscosity monomer.

Specimen preparation

Hourglass-shaped specimens (n=10) were made using an hourglass-shaped silicon head office 0.7 (±0.1)mm thick. The monomers were light activated for 20 s on each side with a light curing unit (Radii SDI, Victoria, Australia). The irradiance was measured with a digital power meter (Ophir Optronics, Danvers, USA) and resulted in 1,400 mW/cm^2. The specimens were polished with 600-grit silicon carbide paper and stored for 24 h at 37°C.

Ultimate tensile strength

After storage, each specimen was fixed in a device and loaded under tension using a universal testing machine (DL-500; Emic, São José dos Pinhais, Brazil) at a crosshead speed of 0.5 mm/min, and results were expressed in megapascal. Data analyses were performed using one-way ANOVA and Tukey's test at a 5% level of significance.

Results

Zymography

One major band was detected in the zymographic assays (Fig. 1). The band of larger intensity corresponded to the approximate molecular mass of 62 kDa (intermediate band). The band of 62 kDa is characterized as active form

Table 1 Composition of experimental polymer

Composition (%)	Group control	Group ZM 0.5	Group ZM 1	Group ZM 2.5	Group ZM 5	Group ZM 10	Group ZM 20	Group ZM 30
Zinc methacylate	0	0.5	1	2.5	5	10	20	30
TEGDMA	98.8	98.3	97.8	96.3	94.7	89.9	82.5	76.2
EDAB	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Camphoroquinone	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4

ZM zinc metacrylate,*TEGDMA* triethyleneglycol-dimethacrylate, *EDAB* ethyl dimethylamino-benzoate

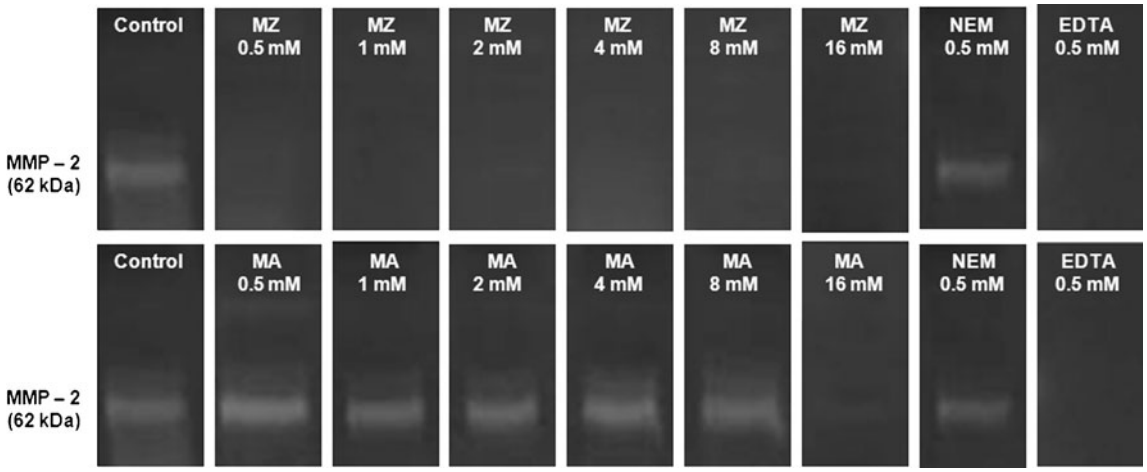

Fig. 1 Gelatin zymography showing the inhibition of MMP-2 expression by zinc methacrylate and the lower inhibitory effect of methacrylic acid in conditioned Tris–$CaCl_2$ buffer after 24 h of incubation. Characterization of the enzymes as metalloproteinases since their expressions were inhibited by EDTA, and NEM had no effect on enzyme activity

of MMP-2. This enzyme was characterized as matrix metalloproteinase since its expression was inhibited by EDTA (Fig. 1), while NEM (a thiol proteinase inhibitor) had no effect on the enzyme activity (Fig. 1). Even with low concentrations of zinc methacrylate (0.5, 1, 2, 4, 8, and 16 mM), the bands were completely inhibited after incubation for 24 h in a solution containing 5 mM $CaCl_2$ (Tris–$CaCl_2$) in 50 mM Tris–HCl buffer with addition of zinc methacrylate (Fig. 1). On the other hand, methacrylic acid showed a lower inhibitory effect, since the inhibitory effect was observed only with 16 mM (Fig. 1). This suggests that the zinc present in the methacrylate monomer plays an important role in MMP-2 inhibition by zinc methacrylate.

Ultimate tensile strength

The UTS mean and standard deviations are shown in Table 2. ANOVA followed by Tukey's test showed that the group containing 30% zinc methacrylate showed significantly lower UTS when compared with the control ($p<0.02$).

Table 2 Mean (±SD) of ultimate tensile strength in megapascal

Group	Ultimate tensile strength
Control	41.5 (±8.1)[a]
ZM 0.5	34.4 (±10.5)[ab]
ZM 1	36.1 (±7.8)[ab]
ZM 2.5	35.7 (±10.1)[ab]
ZM 5	32.1 (±10.9)[ab]
ZM 10	38.9 (±6.9)[a]
ZM 20	30.0 (±4.0)[ab]
ZM 30	25.6 (±4.9)[b]

Groups identified by different superscript letters are statistically different ([a] $p<0.002$ and [b] $p<0.001$)

Discussion

MMP-2 is the major gelatinase constituent of dentin [7] and is known as the enzyme active in the degradation of denatured type I collagen, type IV collagen, elastase, and several other components of the extracellular matrix [4, 7]. Therefore, evidences indicate that MMP-2 plays an important role in the failure of adhesive restorations in the course of time [9]. The inhibition of these enzymes has been discussed as an important factor in maintaining the hybrid layer [13, 15]. As a result, the possibility of adding a MMP inhibitor to adhesive systems could be promising with regard to improving the longevity of adhesive restorations.

Studies have shown that divalent metal ions such as zinc can effectively inhibit MMP proteolytic activity [16–18]. Dental materials with zinc in their composition, e.g., zinc oxide–eugenol cements, zinc-containing amalgams, zinc phosphate, have also been shown to be metalloproteinase 2 and 9 inhibitors [16–18]. The mechanism of enzyme inactivation by metals is not completely understood. It is assumed that metal ions bind with amino acid residues, causing conformational changes that inactivate the catalytic function of enzymes. Previous work has shown that the mechanism of zinc inhibition of carboxypeptidase A, a zinc metalloproteinase, occurs due to the formation of zinc monohydroxide that bridges the catalytic zinc ion to a side chain in the active site of the enzyme [19]. The noncompetitive inhibition by other heavy metal ions is attributed to binding of the ion to a site distinct from the active site.

The results of the present study suggest that ZM is a strong inhibitor of MMP-2 in all concentrations evaluated. On the other hand, methacrylic acid showed a lower inhibitory effect. Recently, the authors' research group reported that coordination of the oxygen from a hydroxyl group present in the 2-hydroxyethyl methacrylate (HEMA) with the cation (Zn^{2+}) present in the catalytic domain of the MMP-2 could create a reversible inhibition of metalloproteinases [20]. One could propose that this hypothetical mechanism may also explain the inhibitory effect of methacrylic acid, since methacrylic acid also has a hydroxyl group and a molecular formula similar to HEMA. Moreover, the inhibitory effect can be explained by a Lewis-type acid–base interaction between the carbonyl and Zn^{2+} present in the catalytic domain of the MMP-2 [21]. Thus, the presence of zinc in the methacrylate monomer seems to play an important role in the ability to inhibit MMPs.

In addition, it also demonstrates the possibility of adding the ZM and preserving the ultimate cohesive strength of the polymer, because at a ZM concentration of only 30%, the polymer showed a decrease in UTS, probably due to the large amount of ZM in the system. UTS is an essential test to evaluate material properties, as the restorative material strength may be related to maintaining the restoration longevity. It is important to point out that despite mechanical properties studied, it is necessary to evaluate the biocompatibility of ZM and its MMPs inhibition potential in dentin in vivo.

The addition of an MMPs inhibitor and its copolymerization with other components of the adhesive system could be an advance in the development of an adhesive system. Other MMPs inhibitors such as chlorhexidine (CHX) have been proposed to increase the longevity of adhesive restorations [12, 14, 15]. The application of CHX after collagen network exposure by acid-etching treatment may inhibit MMP-2 by denaturizing proteins or a cation-chelating mechanism [10]. The type of inhibition occurs according to the CHX concentration [22]. However, chlorhexidine is not copolymerizable with the other methacrylate monomers and is leached, which could challenge its inhibitory effect over time.

The addition of MMP inhibitors in adhesive systems is relevant, because previous studies have considered them to be dentin MMPs activators [23, 24]. However, it is important to point out that the inhibitory effect of the existent monomers in the adhesive systems has not been studied. Two recent studies conducted by the authors' research group demonstrated that 2-hydroxyethyl methacrylate and triethylene glycol dimethacrylate showed some MMP-2 inhibitory effect [20, 21]. However, it seems that these monomers do not inhibit MMP-2 in vivo. Therefore, studies proposing new or existent monomers that inhibit MMPs could be an important contribution to increasing the longevity of adhesive restorations. Conversely, in spite of the promising results of the present study, the inhibitory effect of ZM cannot be extrapolated to in vivo situations. Thus, studies must be conducted to evaluate the effect of ZM as an inhibitor of MMPs in vivo.

Conclusion

In summary, the results of the present study demonstrated that zinc methacrylate was a strong inhibitor of MMP-2 and when added in small concentrations, it did not affect the ultimate tensile strength of the polymer. Clearly, studies evaluating the longevity of adhesion must be conducted.

Acknowledgments The authors were grateful to CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the scholarship, Esstech Inc. for the donation of the reagents, Angelus Science and Technology for the partial financial support to research project and professor Tatiana Pereira Cenci for the valuable suggestions.

Conflict of interest The authors declare that they have no conflict of interest.

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