Evaluation of indomethacin as matrix metalloproteinases inhibitor in human dentin

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Abstract

Objective: The objective was to determine a new experimental material, indomethacin’s inhibitory effect on the enzymatic activity of dentin collagen.

Materials and Methods: Fifteen freshly extracted teeth were collected and stored at 4°C until use. Enamel, roots, and remnant pulp tissue were removed, and dentin powder was obtained by pulverizing liquid nitrogen-frozen coronal dentin with a mortar pestle. The obtained protein extract from human dentin powder was treated with indomethacin and incubated. The inhibition of enzymatic activity was analyzed using plate assay method and zymographic analysis.

Results: Plate assay method and zymograms showed that indomethacin-treated samples inhibited dentin enzymatic activity.

Significance: Bond strength at the dentin adhesive interface decreases because of the hydrolytic degradation of dentin collagen. The inhibition of enzymes responsible for collagen degradation may improve the bond strength durability. This study demonstrates the efficacy of indomethacin in inhibiting enzymatic activity.

Keywords: Indomethacin; matrix metalloproteinase inhibitors; plate assay method

INTRODUCTION

Composite restorations are the most commonly used esthetic restorative materials in clinical practice. Despite the evolution of material sciences over the past decades, the longevity and clinical performance of composite restoration mainly depends on the bonding of composite restoration with tooth. Resin–dentin bonding relies on proper hybrid layer formation from resin infiltration in demineralized dentin collagen, which couples adhesives/resin composites to the underlying mineralized dentin. Enzymes present in dentin substrate can degrade the collagen fibrils by the activity of collagenolytic enzymes, leading to reduced bond strength with time.[1,2] Matrix metalloproteinases (MMPs) are the enzymes degrading extracellular matrix components.[3,4]

Till date, human dentin has shown to contain MMP-3 (stromelysin-1), MMP-8 (collagenase-2), MMP-2, MMP-9 (gelatinases), and MMP-20 (enamelysin).[5] The physiological roles of these enzymes in the dentin are still unknown, but they have been suggested in the formation of peritubular and tertiary dentin formation and the release of dentinal growth factors, which in turn, regulate the pulp defensive reactions.[3] Thus, with a change in different pH, the human dentin collagen matrix also exhibits various collagenolytic and gelatinolytic activities. Studies have investigated the preservation of the collagen matrix using various MMP inhibitors, which were effective, but for a short time, so the saga of quest for the best still continues.[6]

Indomethacin is a non-steroidal anti-inflammatory drug that inhibits cyclooxygenase (COX) and prostaglandin synthesis. Indomethacin is a nonselective COX inhibitor and exerts the inhibition of MMP-2 expression in human cancer cells.[7] Its role in dentistry as an enzyme inhibitor...
has not been verified yet, so a study was purposed to evaluate the inhibitory effect of indomethacin on MMPs.

**MATERIALS AND METHODS**

**Collection of teeth samples**
Fifteen freshly extracted teeth for orthodontic reasons were collected and stored at 4°C (Figure 1 shows the stored samples) in saline until use. After organic debris removal, teeth were sectioned at cementoenamel junction. The pulp tissue was removed using endodontic files. Cementum and enamel were removed from the radicular and coronal teeth fragments with diamond points operated in high-speed handpiece under water spray. Dentin extract was obtained by pulverizing liquid nitrogen-frozen coronal dentin with a steel mortar and pestle in the extraction buffer.

**Chemicals used in the study**
Acrylamide, bisacrylamide, trichloacetic acid, and Tris-HCl were purchased from Himedia Chemicals India Private Limited. All the reagents were of analytical grade.

**Preparation of indomethacin solution**
Indomethacin capsule powder (Inmecin – 25 mg, GM Pharmaceuticals) was dissolved in 20% ethanol solution.

**Dentin powder extraction**
To remove remaining organic debris from the pulverized dentin, it was rinsed twice with cold distilled water and extracted with 4 M guanidine-HCl and Tris-HCl, Ph 7.4 at 4°C. The guanidium chloride extract was clarified by centrifugation at 10,000 rpm for 20 min at 4°C. Protein content from the supernatant was quantified by spectrophotometry at 660 nm by Lowry’s method.[8]

Aliquots of 1 g each of dentin powder were obtained and treated as follows:
- GROUP 1 – untreated mineralized dentin powder
- GROUP 2 – dentin powder was treated with indomethacin solution (5 mg/ml) prepared in 20% ethanol for 30 min at room temperature.

**Gelatinase activity by plate assay method**
Agar gel 1.5% was prepared in distilled water with the inclusion of gelatin (2%) and autoclaved at 121 lbs for 30 min. The gel was allowed to solidify for 90 min and was divided into two parts and wells of 4 mm size diameter were made into the agar plate and filled with 0.5 ml of gelatinase enzyme source on the right-hand side part [A in Figure 2] and another with gelatinase and indomethacin on the left-hand side part [B in Figure 2] and incubated for 24 h. After 24 h, the enzymatic activity was assayed.

**Zymography analysis**
Aliquots of dentin were collected in two volumes: Volume 1 consisted of extracted supernatant samples and Volume 2 consisted of supernatant which was precipitated with 40% acetone, incubated for 2 h at -8°C, and then centrifuged at 6000 rpm for 10 min. Precipitate was resolubilized in the loading buffer. Samples were electrophorized under nonreducing conditions on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with polyacrylamide gel (15%) at 4°C.

The obtained gels were stained in 0.2% Coomassie Brilliant Blue R-250 and destained in the destaining buffer (50% methanol, 10% acetic acid, and 40% water).

**RESULTS**
Protein concentration estimated using Lowry’s method in the specimen was 1260 µg/ml. In the plate assay method, a zone of hydrolysis was seen around the well with gelatinase source [B in Figure 3] which indicates the enzymatic activity of the specimen, and on the counterpart, there was no zone.
of hydrolysis in the specimen mixed with indomethacin [A in Figure 3].

**Gelatin zymographic analysis**
Gelatin zymography analysis showed the molecular mass of the gelatinase enzyme was 80 kDa. The zone of hydrolysis was seen with gelatinase enzyme, whereas in specimens with indomethacin inhibited dentin gelatinolytic activity, and no zone of hydrolysis was observed. Figure 4 shows dye in zone 1 and zone 3; zone 4 shows hydrolysis (half-moon shaped); and zone 5 and 6 show no hydrolysis.

**DISCUSSION**
The dentin–resin interface lose their bond to dentin with time, and hybrid layer degradation is related to that loss of bond strength.\(^9\) MMP is endogenous Zn- and Ca-dependent enzymes capable of degrading essentially all extracellular components. The MMP family contains 23 members in humans. Substrate specific and homological classification of MMPs divides them into six groups, which include collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs. The biological activity of MMPs can be maintained by gene transcription, compartmentalization, enzyme activation, and inactivation. Some MMPs have been identified for the damage of collagen degradation in dentin.\(^3\) Studies have shown that the preservation of the collagen matrix integrity is important to improve the dentin bond durability.\(^5\)

Previous studies have demonstrated chlorhexidine as a mostly used inhibitor and related improvement in the stability of the hybrid layer.\(^10\) Chlorhexidine digluconate has the ability to inactivate MMP-2, 8, and 9.\(^11\) Recent studies have shown that chlorhexidine molecule is large and water-soluble, it may leach out of the hybrid layer.\(^12\) It can preserve collagen activity for at least 6 months, but this integrity was degraded after 1 year.\(^6\) Recent studies have shown that chlorhexidine has a toxic effect on odontoblast-like cells and stem cells from human exfoliated deciduous teeth.\(^13,14\) Therefore, it is relevant to find an alternative MMP inhibitor to improve bonding efficacy.

The result of this study has shown MMP-2 and MMP-9 (gelatinase enzyme) activity in the dentin. In addition, the present data are the first time evidence of the application of indomethacin to human dentin specimens inhibiting dentin MMPs (gelatinolytic) activity by the plate assay method and gelatin zymographic analysis as compared to control specimens. In the plate assay method, the gelatinase enzyme source hydrolyzed the gelatine substrate that was present in agar medium, and after 24 h of incubation, it showed a zone of hydrolysis around the enzyme source, which is indicative of active enzymatic activity. Whereas in the counterpart, the gelatinase enzyme source mixed with indomethacin showed no zone of hydrolysis and showed the complete inhibition of the activity of the enzyme source, which was indicative of indomethacin inhibited the enzymatic activity of the gelatine source.

In gelatin zymography analysis, the gelatinases activity was assessed. Gelatinases (MMP-2 and MMP-9) demonstrate a unique sort of structural similarity that makes them different from other MMPs like catalytic domains fibronectin like type II modules. These modules form collagen-binding domains and interact specifically with gelatine and provide positions to substrates for cleavage.\(^15,16\) Gelatin zymography is a sensitive quantifiable polyacrylamide gel-based electrophoretic approach to degrade one of their substrates. In the present study, gelatinases enzyme source from dentin hydrolyzed SDS-polyacrylamide gel impregnated with gelatin substrate under nonreducing conditions. After the staining of the obtained gel, both the latent and active forms of gelatinases exhibited gelatinolytic activity which was observed by the formation of a zone of hydrolysis.
of the hydrolytic zone. In the counterpart when the gelatinase enzyme source was treated with indomethacin solution, and a clear zone of inhibition was appreciated.

Indomethacin is a nonselective COX inhibitor and commonly used medication to reduce pain, fever, stiffness, and swelling from inflammation. It inhibits inflammation by inhibiting COX pathway. The MMPs have been observed in almost every human disease, in which inflammation is present. The acid etching during the bonding procedure leads to the activation of MMPs. The suppression of MMPs by indomethacin may be attributed to the presence of an indole group through repression of gene transcription, thereby inhibiting the gelatinolytic degradation.

Indomethacin is an ethanol-based substrate, and indomethacin has a low molecular weight in comparison with the chlorhexidine, and it was also able to inhibit MMPs, therefore indomethacin may be a better alternative than chlorhexidine in penetration into the dentinal tubules, inhibiting the MMPs and increasing the bond strength of dentin resin interface, however, further studies in this regard are indicated.

**CONCLUSION**

Within the limitations of the study, it can be concluded that indomethacin can inhibit MMPs activity, which may aid in increasing the bond strength. Further studies with regard to penetration of indomethacin in dentinal tubules solubility in bonding agents are indicated.

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**Conflicts of interest**

There are no conflicts of interest.

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