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Evaluation of Matrix Metalloproteinase-9 (MMP-9) in Dentine Pulp Complex of Normal and Carious Tooth by an Immunohistochemical Analysis- An Invitro Study

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KEYWORDS
MMP-9, MMP, Dental caries

ABSTRACT:

Introduction: Dental caries is a multifactorial disease that requires a susceptible host as well as cariogenic bacteria and diet in order to develop. MMP-9 is a gelatinases that have the ability to break down denatured collagen and type IV collagen (the main structure of extracellular matrix). The inhibition of MMPs may represent a key piece of the troubleshooting during dentin adhesion, and resulting in long-lasting hybrid layer.

Aim: To evaluate the possible role of Matrix Metalloproteinase-9 (MMP-9) in Dentine Pulp Complex during caries progression.

Objectives: To compare and evaluate the expression Matrix Metalloproteinase-9 (MMP-9) in Dentine Pulp Complex of normal teeth and carious teeth.

Methodology: 12 control (normal teeth) and 12 study (carious extracted teeth) Samples are collected and fixed in neutral buffered formalin. Decalcification done using EDTA. Tissue processing done. Evaluated for hematoxylin and eosin staining then immunohistochemical analysis is done.

Result: Brown coloured precipitate observed in the dentino-pulpal area and the cells of the pulp were considered as positive for MMP-9. The intensity of the staining reaction and the number cells expressing the MMP-9 stain were considered for the scoring of expression of MMP-9 for the particular tooth. Mann whitney U test revealed that MMP-9 staining score were significantly higher in test group compared to control group.

Conclusion: In our study we tested MMP-9 in dentine-pulp complex of two groups one with carious dentine and other with non-carious dentine. The non-carious teeth showed less positivity while comparing with carious teeth, which is one of the indicator and additive for carious progression.

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Introduction

The dentin-pulp complex consists of a tubular mineralized hard tissue compartment, dentin, and the vital soft tissue encompassed by and partly embedded into the dentin, i.e., odontoblasts and pulp tissue[1-3].

Dental pulp, the soft tissue compartment maintaining the tooth's vitality, is encased inside dentin. The term 'dentin-pulp complex' emphasizes the structural and functional integration of dentin and dental pulp, even though these tissues differ markedly with respect to their chemical composition and cellular components[4-8].
Dental caries is a multifactorial disease that requires a susceptible host as well as cariogenic bacteria and diet in order to develop. Caries results in hard tissue loss, which occurs in dentin by dissolution of dentinal minerals and, eventually, by degradation of the collagenous organic matrix[1-2].

Even though organic matrix breakdown is essential for lesion progression, the exact mechanisms and enzymes responsible for it are largely unknown. The members of the matrix metalloproteinase (MMP) enzyme family are in concert capable of degrading virtually all extracellular matrix components[9,10].

Several MMPs are found in normal dentin-pulp complex cells and tissues, and they are considered to be involved in many physiological processes during the formation and maintenance of the dentin-pulp complex[11-15]. However, their activity has also been suggested to contribute to the organic matrix degradation during dentin caries progression and to the repair and defense reactions elicited by caries in dentin-pulp complex cells[16-19].

MMP-9 is a gelatinases that have the ability to break down denatured collagen and type IV collagen (the main structure of extracellular matrix)[20]. MMP-9, has a molecular weight of 92 kDa, which is nearly absent or minimal in normal tissues and is secreted by dendritic and hematopoietic cells, neutrophils, macrophages, mast cells, fibroblasts and lymphocytes during teeth formation and pathological inflammation[21-24].

Feng et al. suggested that MMP-9 may play a role in controlling amelogenin processing and enamel formation. According to their study, mice that lacked the MMP-9 gene exhibit malformed teeth, loss of ameloblast polarization, immature ameloblast differentiation, and delayed tooth eruption[25].

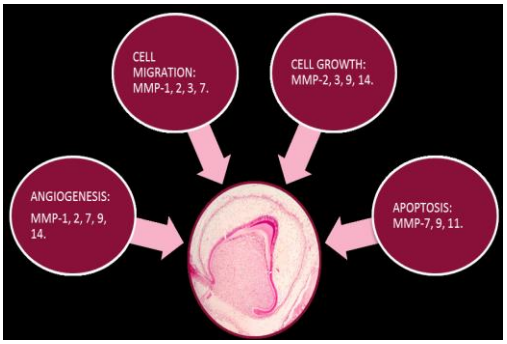

Fig 1. The connection between metalloproteinases and odontogenesis We can see the influence of MMPs on tooth development, and have found that MMP-2 or MMP-9 are involved in all stages.

When dentin is partially demineralized (whether by a caries process or by an acid attack caused by dental materials, lowering pH levels, creating a favorable environment), the latent forms MMP-9 is activated that lead to faster destruction of type IV collagen[26].
Since MMP-9 crucially contribute to all phases of tooth development, differentiation, growth, shaping, apoptosis and degradation of different dental and periodontal tissues[27-30]. It has become one of the important biomarkers of diseases including degradation of different dental tissues, reversible and irreversible pulpitis and apical periodontitis, as well as gingival and periodontal lesions.

Hybrid layer is the area of adhesion formed by the dentin collagen matrix and resin adhesive. Studies have shown that the bond between the adhesive systems and dentin weakens over time resulting in decrease in bond strength is related to the degradation of the hybrid layer[31]. After exposure to acid (etch-and-rinse adhesives) or acidic monomers (self-etch adhesives), the demineralized dentin collagen matrix is infiltrated with the applied adhesive resin.
The collagen matrix is vulnerable to enzymatic degradation by the endogenous collagenolytic enzymes, matrix metalloproteinases (MMPs) and cysteine cathepsins which play an important role in bond destruction[32,33]. The inhibition of MMPs may represent a key piece of the troubleshooting during dentin adhesion, and resulting in long-lasting hybrid layer.

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Immunohistochemistry (IHC) analysis is used for our study because it is one of the advanced and standard method for detecting the biomarkers and demonstrating the presence and location of proteins in tissue sections. Immunohistochemical staining is accomplished with antibodies that recognize the target protein. Since antibodies are highly specific, the antibody will bind only to the protein of interest in the tissue section. The antibody-antigen interaction is then visualized using either chromogenic detection, in which an enzyme conjugated to the antibody cleaves a substrate to produce a colored precipitate at the location of the protein, or fluorescent detection, in which a fluorophore is conjugated to the antibody and can be visualized using fluorescence microscopy.

This study is done based on the research question "Whether the MMP-9 localization increased in Dentine Pulp Complex of caries teeth while comparing with normal teeth?"

Preparation of the Samples and Immunostaining Samples

SAMPLE SIZE: Total 24

- Control group - 12 normal teeth extracted for prophylactic reason.
- Study group – 12 carious extracted teeth

Inclusion criteria :

- Only fully erupted extracted teeth were included for both groups.
- For control group teeth which are extracted for prophylactic reason is used.
- For study group teeth which are extracted due to caries are used.

Exclusion criteria :

- Grossly decayed tooth.
- Fractured tooth.
- Restored or RCT tooth

Methodology

Fixation of tooth samples

All the tooth samples were extracted and washed in saline immediately. The root of the teeth was cut 3 mms away from the cemento-enamel junction in order to improve the penetration of the fixative. Immediately all the tooth samples were immersed in 10% neutral buffered formalin for a minimum of 48 hours for better fixation of the pulpal tissue.

Decalcification of the samples

Slow decalcification using EDTA was preferred in order to preserve the architecture of the tissue to the maximum. The specimen were periodically examined by taking an X-ray for completion of decalcification procedure. Complete decalcification was achieved in 60 days. Section preparation for hematoxylin-eosin staining and immunohistochemistry. All specimens were processed routinely and embedded in paraffin wax. Two serial sections of 3-4 µm thickness were made from the tissue blocks. One section was prepared in egg albumin coated glass slide and stained with hematoxylin and eosin stain to confirm the quality and architecture of the dentino-pulpal complex. The second section was prepared in Poly-L-Lysine coated glass slides and used for immunohistochemistry.

Interpretation of MMP-9 staining and scoring criteria

Brown coloured precipitate observed in the dentino-pulpal area and the cells of the pulp were considered as positive for MMP-9. The intensity of the staining reaction and the number of cells expressing the MMP-9 stain were considered for the scoring of expression of MMP-9 for the particular tooth. The percentage of cells expressing MMP-9 was measured by counting the number of cells expressing brown colour in 100 cells and assigned a score in a scale of 0 to 4 as follows.

The final score of expression for each tooth sample was calculated by adding both intensity score and percentage of cells score and plotting it in a scale of 0 to 7.

Results

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Table 1: CONTROL SAMPLES (NON CARIES TEETH)

Sample no	Staining (Y/N)	Intensity 1 – Mild 2 – Moderate 3 - Intense	Scoring percentage 1 (< 25%) 2 (25-50%) 3 (50-75%) 4 (> 75%)	Score (Out of 7)
1	N	0	0	0
2	N	0	0	0
3	Y	1	1	2
4	N	0	0	0
5	N	0	0	0
6	Y	1	1	2
7	N	0	0	0
8	N	0	0	0
9	N	0	0	0
10	N	0	0	0
11	Y	1	1	2
12	N	0	0	0

Table 2: STUDY SAMPLES (CARIES TEETH)

Sample no	Staining (Y/N)	Intensity 1 – Mild 2 – Moderate 3 – Intense	Scoring percentage 1 (< 25%) 2 (25-50%) 3 (50-75%) 4 (> 75%)	Score (Out of 7)
1	Y	1	1	2
2	N	0	0	0
3	Y	1	1	2
4	Y	3	1	4
5	N	0	0	0
6	Y	2	1	3
7	Y	3	2	5
8	Y	3	3	6
9	Y	3	3	6
10	Y	3	2	5
11	Y	3	3	6
12	Y	2	1	3

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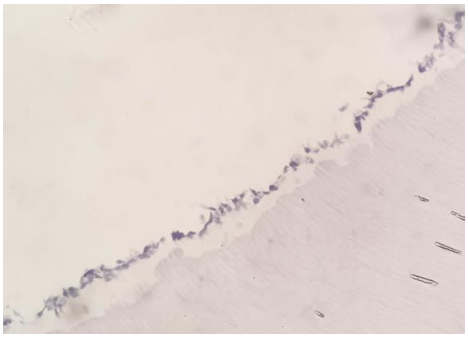

FIG 2. No Expression of MMP-9 in the Normal Tooth

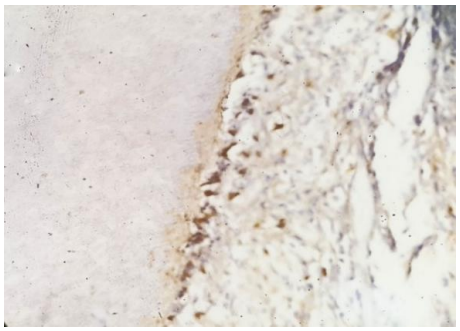

FIG 3. Mild Expression of MMP-9 in the Peripheral Cells of Pulp Tissue in Caries Tooth

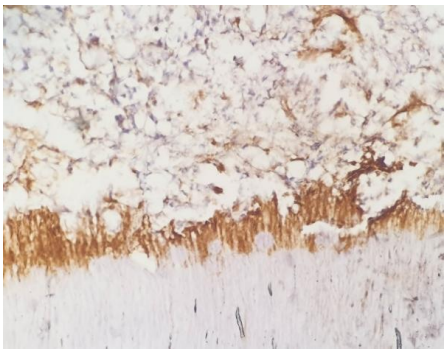

Fig 4. Moderate Expression of MMP-9 in the Cells of the Pulp and Dentinal Tubules Near the Pulp in Caries Tooth.

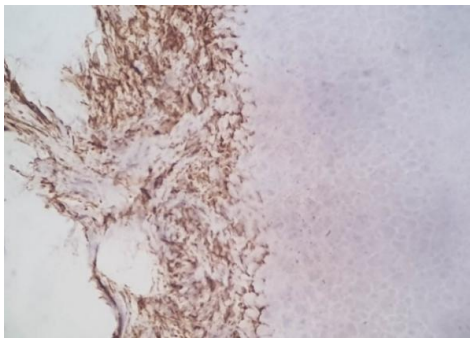

FIG 5. Photomicrograph of the Dentino-Pulpal Junction Showing Intense Expression of MMP-9 Antibody by the Pupal Cells in Caries Tooth.

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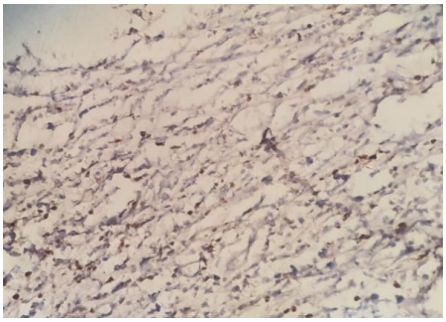

Fig 6. Intense Expression Of Mmp-9 In The Pulpal Cells Of Caries Tooth.

Statistical Analysis

Table 3: Comparison of Immunohistochemical staining score of MMP-9 in dentine-pulp complex between two groups

Groups	Median (50^{th} percentile)	Mean rank	Mann Whitney U value	p-value
Test group	3.5	17	18	0.001
Control group	0	8		

Mann Whitney test; p value <0.05 (significant)

Mann whitney U test revealed that MMP-9 staining score were significantly higher in test group (*Md*=3.5,n=12) compared to control group (*Md*=0,n=12) with a medium effect size r= 0.68

Discussion

The pulp is heterogeneous soft tissue with various cell types as well as neural and vessel components embedded in the extracellular matrix. Similarly, to other soft connective tissues, pulp involves ongoing ECM turnover, and pulpal MMPs have been suggested to contribute to this physiological process[10].

A caries attack causes many changes in the pulp, e.g. infiltration of inflammatory cells[34] and accumulation of pulpal cells into the cell-free zone.

In the early phase of dentinal caries, the metabolic activity of pulp cells increases[35]. Due to injury, interodontoblastic junctions may be impaired[36], whereupon pulpal proteins, even those originating from plasma[37], leak into dentin tubules and presumably also into carious dentin. In the case of severe injuries, PMN cells may also penetrate into dentinal tubules[38].

The findings on changes in MMP synthesis in advanced pulpal inflammation have been conflicting, as the levels of MMP-2 and -3, for example, have been reported to be both increased by Shin et al. 2002 and decreased by Gusman et al. 2002 in heavily inflamed pulp.

Immunohistochemistry (IHC) is considered to be an advanced form of histopathology. Immunohistochemistry is not usually used initially but is added when routine/regular histological testing is insufficient to form a diagnosis.

IHC uses primary antibodies to label a protein, then uses a secondary antibody which is bound to the primary one. In immunoperoxidase staining, an antibody is joined to an enzyme, peroxidase, that catalyses a reaction in which the protein is specifically stained brown. IHC can also involve fluorescently labelled antibody so that when viewed under a light microscope a certain pattern will be observed from the emitted fluorescence.

The IHC pattern is considered diagnostic, demonstrating nuclear, membranous or cytoplasmic patterns. IHC is often used in situations where a presence or absence of certain proteins,

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biomarkers can form a basis for a diagnosis. It can also be used to distinguish between two different disease processes that may otherwise appear similar to the pathologist.

In our study MMP-9 was evaluated using immunohistochemical analysis because immunohistochemical analysis is one of the standard tests for identifying biomarkers present in teeth.

Odontoblastic MMPs in sound and carious human teeth

Odontoblasts are the first cells to encounter the caries stimulus, and they are thought to have an important role in the dentin-pulp complex repair and defence reactions by forming intratubular and tertiary dentin[39].

Steinman et al. 1980 concluded the factors favouring caries progression in teeth with dentin-pulp complex soft tissues left may include nutritive factors for cariogenic bacteria, but also undefined disturbed pulpal function. This pulpal dysfunction might also include increased secretion and/or activation of odontoblastic, and possibly also pulpal, MMPs.

MMPs in dentin

Even though mineralized dentin is not remodelled in the same manner as bone, it cannot be considered an inert tissue. Mineralized dentin contains various growth factors reviewed by Goldberg & Smith 2004 and proteinases, including a latent collagenolytic enzyme and gelatinolytic enzymes[40], specifically MMP-2.

In our study sound dentin exhibited very weak immunoreactivity comparatively with carious group.

Dentinal proteinases have been suggested to contribute to dentin caries progression. They may also degrade the collagen fibrils involved in the adhesion of dental composite restorations, leading to a failure of the restoration. However, dentinal proteinases may also have a defensive role during a caries attack, e.g., by releasing and activating growth factors needed in signalling for tissue repair in the dentin-pulp complex[41,42].

MMPs are suggested to be involved in dentin formation[10,15,43], and at least some of these MMPs become embedded into mineralized dentin.

Tjäderhane et al., 1998[41] suggested that host-derived MMPs participate in dentin destruction following demineralization by bacterial acids and therefore in the control or progression of carious decay, MMPS play an important role.

Matrix metalloproteinases in dental caries

Dental caries is characterized by demineralization of the inorganic portion of the tooth, and disintegration of the organic tissues of the tooth. The major organic component of dentin is Type I collagen. The dentin extracellular matrix can be compromised by proteolytic degradation, which happens in the presence of collagenases (MMP-1, MMP-8, MMP-13) and gelatinases (MMP-2, MMP-9)[44,45].

Dentin bound MMPs also seem to have a defensive role in dentin caries progression by lieu of releasing growth factors. These dentin bound growth factors help in the upregulation of dentin-pulp complex defensive reactions under caries lesions[46].

MMP-1, MMP-8 and MMP-13 are the most important human collagenases. While MMP-8 targets Type I collagen, MMP-1 targets Type III and MMP-13 prefers Type II collagen. The presence of these MMPs demonstrate the role that host derived MMPs are involved in the degradation of dentin organic matrix during caries progression[47,48].

The major source for MMPs in carious dentin has been claimed to be the gingival crevicular fluid from where they are distributed to the whole saliva[46]. The dentin-pulp complex has also been stated as a possible source of MMP-2 and MMP-9. The role of MMPs in physiological conditions is further supported by the presence of a latent collagenase in demineralized human dentin matrix.

MMPs in pulpal pathologies

Pulpal and periradicular diseases are responses of the immune system to microbiota, resulting in production of inflammatory and resorptive factors. The production of tissue type plasminogen activated factors, pro-inflammatory cytokines, substance P and vascular endothelial growth factor expression[49].

In the case of pulpal inflammation. there is degradation of matrix protein which is mediated by endopeptidases. MMPs are able to degrade all extracellular matrix proteins. The denatured gelatins namely gelatin,

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laminin, elastin, fibronectin and the basement membrane zone associated collagen are degraded by MMP-2 and MMP-9 which are characterized as gelatinases. The proteolysis of extra cellular matrix seems to be a key initiating event for progression of inflammatory processes.

High levels of these neutrophil enzymes indicate a level of tissue breakdown that is beyond repair- a condition that is termed irreversible pulpitis. Matrix metallopeptidase 9 (MMP-9) or neutrophil gelatinase is a proteolytic enzyme (endopeptidase) produced by neutrophil granulocytes.

Zehnder at al reported that these neutrophil enzymes could be considered as markers of pulpal disease. In a classic report, they demonstrated that dentinal fluid samples from symptomatic teeth had significantly higher MMP-9 levels than those from clinically healthy counterparts. This could possibly evolve into a non-invasive, chairside diagnostic marker of pulpal diseases and prognostic markers of procedures like deep caries management[50].

MMP family proteins elicit dual roles in the pathogenesis of inflammation, stimulating protective innate and/or adaptive immune functions, as well as tissue destruction[51]. MMP-3 elicits stimulatory effects on the proliferation and the migration of endothelial cells as well as anti-apoptotic effects on these cells in vitro[52,53].

In our study we tested MMP-9 in dentine-pulp complex of two groups one with carious dentine and other with non-carious dentine. The non-carious teeth showed less positivity while comparing with carious teeth, which is one of the indicator and additive for carious progression.

The MMP-9 immunoexpression pattern found in our study, with greater expression being found close to caries lesions. This study shows MMP-9 upregulation in dentin of human caries teeth. In particular, its immunoexpression was weak and confined to the peritubular area in sound dentin and strong at the dentin-pulp complex junction and in pulp tissues of caries teeth, where it decreased as the distance from the decay process increased.

Nonetheless, an important role of MMPs in progression and maintenance of the caries process is proved by several recent studies of the effect of synthetic MMP inhibitors, such as doxycycline and chlorhexidine, in reducing collagen degradation in demineralized dentin. MMPs are exposed and activated by acidic agents during adhesive bonding procedures. If these matrix-bound, activated MMPs are not fully infiltrated with adhesive resin, they can slowly degrade the collagen fibrils at the resin–dentin-bonded interface[40].

When dentin MMPs are exposed and activated by self-etch or total-etch adhesives[54,55], these enzymes degrade type I collagen. As collagen fibrils are incompletely infiltrated with resin monomers[56,57], MMPs may degrade the collagen within incompletely resin-infiltrated hybrid layers, decreasing the longevity of bonded restorations[58,59]. Intense MMP-2 and MMP-9 activities were detected at the basal part of the hybrid layer[59].

New strategies to prevent the degradation of dentin bonds may be crucial to increase the longevity of bonded restorations. Therefore, the use of exogenous MMP inhibitors, such as chlorhexidine, gallardin, and flavonols may be an effective strategy to improve the longevity of adhesive restorations . Synthetic MMP inhibitors must contain a functional group such as a carboxylic acid that can interact ionically with the Zn2+ ion in the MMP molecule. For longlasting adhesion of restorative MMPs inhibitors can be used.

Our study also proves the presence of MMP-9 in the dentine pulp complex. So using selective MMPs inhibitors helps in the longitivity of the restoration. Further research are required for identifying the selective MMP Inhibitors which will be useful for restorative dentistry.

Conclusion

The degradation of the collagenous organic matrix of dentin during caries progression is an enzymatic process. Some of the enzymes involved in this process may be of host origin, and specifically host MMP-9 were here hypothesized to take part in the breakdown of the dentin organic matrix.

Taking into consideration the enhanced presence of MMPs at the site of carious lesion, inflamed pulp, together with the fact that when this inflammatory response subsides, the level of MMPs diminishes, it can be concluded that MMPs do play an important role in the degradation of the collagenous structure and spread of the pathology. At the same time, they are an essential

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component of the tissue in the physiologic process of tissue remodelling.

Further research is warranted to elucidate the role of MMPs in dentin-pulp complex organisation, pulp pathology, and caries pathogenesis and evolution. Since MMP inhibitors have been shown to slow down the progression of dental caries their utilisation in caries prevention should be further investigated.

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