The Study of Dentine Matrix Metalloproteinases (Mmps) Activity in Devitalized Teeth

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Abstract

The goal of this study was the need to update the chlorhexidine adhesive protocol in the treatment of devital teeth in the presence of TIMP1 and TIMP2 in fragments of removed teeth previously endodontically treated and removed according to indications using PCR.

The study was conducted on 38 human molars removed according to indications, which were divided into 2 groups: 1 group of the studied samples – 22 removed devital molars and 2 group – the control group - these are 16 third molars removed according to orthodontic indications. The removed teeth of each group were fragmented using a diamond-coated disk and air-water cooling.

According to the results it was found that MMP 2 and MMP 9 are contained not only in vital, but also in devital teeth. The use of a chlorhexidine adhesive Protocol is relevant in both clinical cases but depending on the type of MMP and the state of the pulp the concentration of chlorhexidine is selected.

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Introduction

Recently, the chlorhexidine adhesive protocol has become an integral part of the qualitative treatment of diseases of the hard tissues of teeth due to its unique ability to inhibit the activity of matrix metalloproteinases that cause disruption of the connection between dentin and adhesive. It has been clinically proven that the use of this adhesive protocol helps to improve the indicators of edge fit, as well as get rid of micro-flows, edge pigmentation and postoperative sensitivity[1].

The main goal of this protocol is to create a high-quality and stable hybrid layer, the main components of which are the primer and collagen fibers of dentin [2-5]. With the classical adhesive protocol, the degradation of the hybrid layer occurs as a result of the hydrolysis of collagen fibers by matrix metalloproteinases, which is why micro-flows are formed under the action of the fluid flow, the edge fit is violated [5,6].

Matrix metalloproteinases (MMPs) are Zn- and Ca-dependent endopeptidases that, after activation, destroy the components of the extracellular matrix. MMPs play an important role in many physiological processes, such as embryonic development, morphogenesis, dentinogenesis, reproduction and tissue remodeling, as well as in pathological processes of inflammation of the tooth pulp and the progression of the carious process. All MMPs are characterized by the presence of zinc ions Zn 2+ in the active center and the need for Ca 2+ ions to stabilize the molecule [7-9].

MMPs are classified into groups depending on their structural homology and substrate specificity: collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), transmembrane MMP or MT-MMP (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24 and MMP-24). 25), and 2009 Australian Dental Association 347 others (MMP-12, MMP-19, MMP-20, MMP-21, MMP-22, MMP-23, MMP-27 and MMP-28) [10-13].

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Researchers from different countries agree that the main cause of demineralized dentin degradation are MMP2 and MMP9. The dentin matrix contains mainly type I collagen, but also a small amount of type V collagen and non-collagenic proteins, such as protein I of the dentin matrix, phosphophorin and sialoprotein.

MMP-2 (gelatinase-A). This enzyme is activated by autolysis, which has a concentration-dependent character, the degree of which increases in the presence of heparin. Another activation mechanism is based on the interaction of pro-MMR-2 with two active MMR-14 and TIMR2 [14].

MMP-9 (gelatinase B) is a Zn—dependent endopeptidase synthesized and secreted as a monomer. Its structure is similar to MMP-2. Initially, MMP-9 is synthesized as an inactive enzyme.

Materials and methods

The tooth fragment was placed in 1 ml of RNA leiter ("QIAGEN", Germany), incubated for a day at +4C, samples were stored at -70 °C.

To isolate total RNA, the RNeasy Plus Mini Kit ("QIAGEN", Germany) was used. The tissue (about 30 mg) was homogenized in the attached Buffer Buffer RLT Plus with mercaptoethanol on a TissueLyser LT homogenizer ("QIAGEN", Germany) for 4 min at 40 Hz, the homogenate was centrifuged for 3 min at 9500g in an Eppendorf Mini Spin Plus centrifuge ("Eppendorf", Germany), the supernatant was transferred to a gDNA Eliminator for removal of genomic DNA. Centrifuged for 30 seconds at 8000g, an equal volume of 70% ethyl alcohol was added to the precipitate, thoroughly mixed. The sample was transferred to the RNeasy Spin Column, centrifuged for 30 sec at 8000g, Buffer RW1 was added, centrifuged for 30 sec at 8000g, Buffer RPE was added, centrifuged for 30 sec at 8000g. Buffer RPE was added, centrifuged for 2 min at 8000g, then at 9500g for drying, 40 µl of sterile water free of RNase was added, and centrifuged for 1 min at 8000g. The resulting RNA was stored at -70C.

To obtain cDNA from the RNA matrix, a ready-made set of reagents MMLV RT Kit ("Eurogen", Russia) was used, the reaction was carried out according to the attached instructions. 2 µl of random decanucleotide primer (Random dN) and 1 µl of sterile RNase-free water were added to 6 µl of RNA, heated at +70C in a Termit thermostat (DNA Technology, Russia) for 2 min to melt secondary RNA structures, then stored on ice (+4C). 4 ml of 5X buffer was added to the reaction mixture for the synthesis of the first chain (280mM Tris-HCl, 375 mM KCl, 30 mM MgCl2, pH 8,7), 2 ml of dNTP mixture, 2 ml of DTT and 2 ml of sterile water free of RNase. Immediately before the reaction, 1 ml of MMLV revertase (reverse transcriptase of mouse leukemia virus) was added to the mixture and added to the RNA. The test tubes were heated in the Gnome thermostat (DNA Technology, Russia) at +39C for 60 minutes, then at +70C for 10 minutes in the Termit thermostat (DNA Technology, Russia). The synthesized cDNA was diluted with sterile water free of RNase. The resulting cDNA libraries were frozen and stored at -70C.

Results

Isolation of RNA

The tooth fragment was placed in 1 ml of RNA leiter ("QIAGEN", Germany), incubated for a day at +4C, samples were stored at -70 °C.

To isolate total RNA, the RNeasy Plus Mini Kit ("QIAGEN", Germany) was used. The tissue (about 30 mg) was homogenized in the attached Buffer Buffer RLT Plus with mercaptoethanol on a TissueLyser LT homogenizer ("QIAGEN", Germany) for 4 min at 40 Hz, the homogenate was centrifuged for 3 min at 9500g in an Eppendorf Mini Spin Plus centrifuge ("Eppendorf", Germany), the supernatant was transferred to a gDNA Eliminator for removal of genomic DNA. Centrifuged for 30 seconds at 8000g, an equal volume of 70% ethyl alcohol was added to the precipitate, thoroughly mixed. The sample was transferred to the RNeasy Spin Column, centrifuged for 30 sec at 8000g, Buffer RW1 was added, centrifuged for 30 sec at 8000g, Buffer RPE was added, centrifuged for 30 sec at 8000g. Buffer RPE was added, centrifuged for 2 min at 8000g, then at 9500g for drying, 40 µl of sterile water free of RNase was added, and centrifuged for 1 min at 8000g. The resulting RNA was stored at -70C.

Reverse transcription

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Real-time polymerase chain reaction

Using a mixture for PCR qPCRmix-HS SYBR (Eurogen, Russia) containing the fluorescent intercalating dye Sybr Green I, according to the attached instructions, the expression levels of MMP9, TIMP1 and TIMP2 mRNA were determined relative to the expression level of GAPDH mRNA on a Real-Time DTprime amplifier (DNA Technology, Russia). 1 ml of cDNA solution and 1 ml of primer were added to the sample. Primers for PCR were selected using the on-line Primer-BLAST program in accordance with generally accepted requirements. Primers synthesized by Eurogen (Russia) were used. The volume of the mixture was adjusted to 25 µl. To analyze gene expression, the method of determining the threshold cycle (Ct) and calculating the relative gene expression according to M.W. Pfaffl (2001) was used. The relative mRNA concentration of these genes was calculated by direct comparison of the data according to the formula: [A]0/[B]0 = EDC(T), where [A]0 is the initial concentration of the gene mRNA in the PCR mixture, [B]0 is the initial concentration of GAPDH mRNA in the PCR mixture, E is the reaction efficiency (assumed to be equal to 1.98), DS(T) is the difference between the threshold cycles of GAPDH and the desired gene. List of primers used shown in table 1.

Gene	Primer	5' к 3'
GAPDH	Straight	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGA
MMP9	Straight	AGGTTCGACGTGAAGGCG
	Reverse	GCCGTCCTGTACTGAAGGAG
TIMP1	Straight	CCTTCCAGGTGTTTCCCTGTT
	Reverse	TCCGGAAGAAAGATGGGAGTG
TIMP2	Straight	GACCCACAAGGAGATTGGGG
	Reverse	CGGAGACGACTGGTCTATGC

Table 1. List of primers used.

Quantitative PCR results for TIMP1 and TIMP2. A multiple change in relation to the GAPDH used as a reference is given. The data is collected from 5 independent repeats (Table 2).

Group	TIMP1		TIMP2	
	Mean value	Standard deviation	Mean value	Standard deviation
Control	0,541	0,015	0,735	0,015
Experience	0,579	0,009	0,711	0,009

Table 2. Quantitative PCR results for TIMP1 and TIMP2.

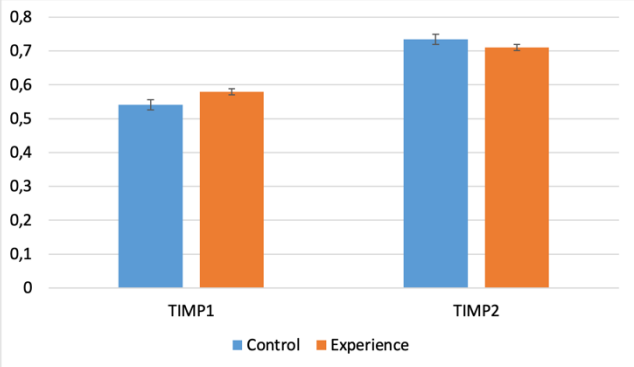

Figure 1. Quantitative PCR results for TIMP1 and TIMP2.

Quantitative PCR results for TIMP1 and TIMP2. A multiple change in relation to the GAPDH used as a reference is given. The data is collected from 5 independent repeats (Figure 1).

Table and Figure present aggregated quantitative PCR results for TIMP1 and TIMP2.

Discussion

The inhibitory effect of chlorhexidine depends on the concentration. The minimum concentration of chlorhexidine, which leads to complete inhibition of the activity of MMP-9, was 0.002%, whereas the activity of MMP-2 is much more sensitive, since it is inhibited at a concentration of chlorhexidine of 0.0001%. At a

concentration of 0.03% chlorhexidine, the activity of gelatinases MMP-2 and -9 is completely inhibited. It is likely that at high concentrations of chlorhexidine, MMP-2 is inactivated by protein denaturation rather than chelation of cations [16,17]. The chlorhexidine adhesive protocol consists of the following steps:

1. Etching of enamel (15-30 seconds) and dentin (up to 12 seconds) with 35-37% orthophosphoric acid;
2. Washing the formed carious cavity with distilled water (30 seconds) and drying;
3. Treatment with 2% chlorhexidine bigluconate solution (60 seconds). The solution is not washed off, but slightly dried.
4. Application of the adhesive system.
5. Polymerization of the adhesive.

When etching dentin with 37% orthophosphoric acid, the smear layer is removed and the collagen fibers are exposed, it is important to understand that only the mineral component is removed (dentin composition before etching: apatites 50%, collagen 30%, water 20%; dentin composition after etching: apatites 0%, collagen 30%, water 70%), consequently, the proteins of the intratubular space also remain. At this stage, it is necessary to understand that if the exposure time of the etching agent is violated, the recommended depth of dissolution of dentin, which is 5-7 microns, will also be violated, and the length of the reaction group of the primer monomer will not allow it to react with the OH group of apatites and the amino group of collagen for the entire length specified by the error, as a result of which it is not possible to achieve high-quality impregnation of collagen fibrils are monomerized, as a result, the MMP will have an additional habitat due to nanofluxes [7].

An important criterion for the next stage of treatment is the complete inactivation of dentin gelatinases, for this they are treated with chelating agents. Chlorhexidine is used in practice, it is clinically proven that 0.001% is enough for inactivation of MMP-2, 0.02% for MMP-8 and 0.002% for MMP-9, however, in most studies presented earlier, a 2% solution of chlorhexidine was used, since it interacts with dentin hydroxyapatites and forms a complex, this phenomenon it is called "biosubstance", due to this it is possible to achieve a prolonged action. However, as stated in [31], the complete inactivation of MMP is sufficient for a concentration exceeding the threshold of 0.04%[5]. When chlorhexidine and MP interact, the latter are inactivated due to binding to the sulfhydryl groups of the active site of the MMP, as well as due to competition for Ca2+ and Zn2+, which are necessary for MMP activity [18,19].

Based on the data obtained, we came to the conclusion that the chlorhexidine protocol is an important step for creating a high-quality, durable hybrid layer. In both devital and vital teeth, chlorhexidine will improve the indicators of marginal fit and delay clinically significant degeneration of the hybrid layer. It has been experimentally proved that at a concentration of chlorhexidine of more than 0.04%, complete inactivation of MMP occurs, at a concentration of 2%, prolonged action can be achieved. However, the question remains which concentration is optimal for practical use, depending on the vitality of the pulp.The data of immunohistochemical studies and the detection of MMP-2, MMP-9, TIMP-1, TIMP-2 in the processes of odontoblasts, periodontoblastic space, enamel-dentine junction, predentine, periculpar dentine with any acid activation at the stage of removal of the smear layer confirm the extreme importance of inhibition of the formed gelatinases using the chlorhexidine protocol in exposure up to 60 seconds at the stage of adhesive preparation in both vital and non-vital teeth.

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Declaration of Interest

The authors report no conflict of interest.

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