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CONCENTRATION OF COLLAGENASES (MMP-1, -8, -13) IN PATIENTS WITH CHRONICALLY INFLAMED DENTAL PULP TISSUE

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A b s t r a c t: Matrix metalloproteinases (MMPs) form an enzyme family capable of degrading almost all extracellular matrix (ECM) and basement membrane (BM) components. They play an important role in normal tissue remodelling and growth, as well as in many destructive pathological conditions such as inflammation, tumour growth and metastasis. The role of MMPs in the breakdown of pulp tissue of teeth with pulpitis has not yet been directly elucidated.

The purpose of this study was to evaluate the tissue levels of collagenases (MMP-1, -8, -13) and their distributions in the clinically healthy and chronically inflamed human dental pulps of 30 patients, aged 15–70 years. Twenty pulps were collected from subjects diagnosed with chronic pulpitis, and 10 control pulps were obtained from 10 subjects following molar extraction for orthodontic reasons. The levels of collagenases were determined with an enzyme-linked immunosorbent assay (ELISA). Results reveal that levels of collagenases were significantly higher in chronically inflamed vs. clinically normal pulps.

Overall, these results show that MMPs play an important role in ECM destruction during the inflammatory processes of pulpitis, as well as reflecting the special characteristics of them. This investigation opens a new opportunity for one contemporary

method for the diagnosis of pulp inflammations and monitoring of the inflammatory processes.

Key words: matrix metalloproteinases, collagenases, pulp tissue, pulpitis.

Introduction

MMPs comprise a family of at least 28 secreted or transmembrane enzymes collectively capable of processing and degrading various extracellular matrix (ECM) proteins and basement membrane (BM) components. Of these, at least 22 MMPs have so far been found to be expressed in human tissues. MMPs share a high protein sequence homology and have defined domain structures and thus, according to their structural properties, MMPs are classified into interstitial collagenases, gelatinases, membrane-type MMPs, stromelysins, matrilysins and other MMPs [7, 34].

Previous studies have indicated that matrix metalloproteinases (MMPs) may exist in the dentin-pulp complex [3, 9] and that some specific MMPs may participate in the regulation of dentin matrix mineralization [13]. In addition, some MMPs have been detected in dentinal caries lesions and chronic pulp tissue [30].

Collagenase-1 (MMP-1), collagenase-2 (MMP-8) and collagenase-3 (MMP-13) comprise the collagenase subfamily capable of initiating degradation of native fibrillar collagen types I, II, III, V and IX.

The story of MMPs began when Gross and Lapiére (1962) [12] detected collagenolytic activity in tissues of tadpoles, and called the respective enzyme collagenases. In oral tissues, this first characterized collagenase, MMP-1 (collagenase-1, interstitial collagenase, fibroblast collagenase), is detected in gingival fibroblasts capable of disrupting ECM collagen [36].

MMP-1 is the most often associated collagenase with normal tissue remodelling. MMP-1 is expressed by fibroblasts, endothelial cells, macrophages, hepatocytes, chondrocytes, osteoblasts, odontoclasts, tumour cells and migrating epidermal keratinocytes and its expression can be induced in certain inflammatory diseases and cancers [10]. It is constitutively expressed at low levels under normal physiological conditions; however, its expression may increase markedly in pathological conditions.

MMP-8 (neutrophil collagenase, collagenase-2) represents the second collagenase, and was originally thought to be synthesized and stored exclusively

in intracellular granules of human polymorphonuclear neutrophils (PMNs) in bone marrow [19]. MMP-8 has been purified from these granules, from which the PMNs secrete the enzyme, and it has been described as neutrophil type or polymorphonuclear type (PMN) MMP-8 [15]. Mesenchymal type MMP-8, differing from neutrophil MMP-8 in protein size, is expressed by human chondrocytes [5], rheumatoid synovial fibroblasts and endothelial cells [14]. It is also produced in keratinocytes, including oral squamous cell, carcinoma cells [21] and plasma cells [33]. Furthermore, MMP-8 has been found in human gingiva [32], saliva [16] (dental plaque [26] and demineralised dentinal caries lesions [30].

In pulpitis, the MMP-8 was expressed in various inflammatory cells, such as PMNs, macrophages, plasma cells and in endothelial cells. In pulpitis, MMP-8 was considered to originate from both PMN and non-PMN lineage cells. The main MMP-8 positivity was accumulated around the pulp abscess suggesting that it also contributes to the abscess formation [35].

MMP-13 (collagenase-3) was originally cloned from a human breast tumour [8]. It has the widest substrate selection among the interstitial collagenases and it is able to cleave various BM components. MMP-13 cleaves type II collagen more efficiently than types I and III, and among interstitial collagenases, it is most effective in cleaving gelatin [6]. The physiological expression of MMP-13 seems to be limited only to developing bone [17], wound healing and teeth [22]. It is widely expressed in pathological conditions including rheumatoid arthritis, osteoarthritis, periodontitis, chronic ulcerations, hypertrophic chondrocytes, osteoblasts as well as plasma cells and many carcinoma and melanoma cells [24]. MMP-13 is predicted to have an important role in tumour invasion and metastasis due to its wide substrate-specificity together with catalytic efficiency and its up regulated expression in cancer cells [29].

MMPs may have dual-roles; they can act in both healthy and disease conditions. Thus, the possible roles of MMPs in mature odontoblasts could be classified in the following subcategories: 1) Functions of MMPs in intact and healthy teeth in processes of physiological secondary dentine formation and mineralization, 2) MMPs participating in matrix degradation during dental injury, 3) Roles of MMPs in tertiary dentinogenesis, and 4) Roles of MMPs in pulpal inflammation [23].

The dual roles of MMPs were the main initiative moment, which gave us the idea of investigating pulp inflamation more elaborately, which set MMPs as the problem of this examination, and object of the investigation, i.e. their concentration in healthy and inflamated pulp tissue.

With all respect to the numerous contemporary aknowledgements aimed at underlining the MMP's role, upon which on this study is based, we have started from this genaral hipothesis:

MMPs are the proteolitic enzymes which have a crucial role during the degradation of structural macromolecules from the intersticial connective tissues in the extracellular matrix and basal membrane, affecting in that way against tissue destructive changes during inflamatory processes in pulp tissue.

The latest findings from different autors, as well as lack of personal data about the designate problem and subject of investigation, was a challenge to us to set up the folowing working hypothesis:

1. Degradation of the collagen of the organic matrix from the pulp tissue during chronic inflamation is an enzyme process. The hypothesis was that collagenses (MMP-1, -8, and -13) are invovlved in these destructive processes. Verification of the set hypothesis in the investigation method was carried out through following indicators:

– determantion of the concentration of MMP-1, -8 and -13 in inflamed pulp tissue with enzyme method.

2. Expression and activity of MMP in healthy tissue is quite low, but increases significantly during the inflammatory pathological process which leada to unwanted tissue dectruction. The hypothesis was that conccentration of the MMP-1, -8 and -13 increases with the spreading of the infection and after that pulp tissue is affectedresulting in chronic pulpitis in the patients. Verification of the set hypothesis in the examination method was performed through the following indicators:

– determination of the concentration of MMP-1, -8 and -13 in the healnthy pulp tissue of impacted third molars.

– comparison of the values of the concentration of MMP-1, -8 and -13 between healthy and pathologicali changed tissue.

Matherials and Methods

This study included 30 patients, both male and female, aged 15–70, investigated in the Dentistry Faculty (Endodontic and Oral Surgery Clinics) in Skopje. Laboratory analyses were done in the Faculty of Natural Sciences and Mathematics (Institute of Biology) in Skopje, with the approval of the Ethical Committee of the University St. Cyril and Methodius Faculty of Dentistry, and with the permission of the patients.

Examination materials were collected on the basis of clinical diagnosis after completely realized anamneses and clinical investigation with X-ray analysis.

Chronically inflamed pulps were obtained from 20 patients during endodontic treatment. Ten normal pulps that were obtained by extirpation of the pulp of impacted third molars after surgery were used as control specimens. Examination material was frozen at -80ºC as soon as possible and stored until the analysis, but not longer than 6 months.

For quantitative analysis of MMP-1, -8, -13 activities in all of the examined samples (chronic periapical tissues and healthy pulp tissue) Chemicon Collagenase Activity Assay Kit (ECM710) was used.

Each sample was macerated in Phosphate-Buffered Saline – PBS (1.5 ml). Homogenized samples were centrifuged with Eppendorf-Centrifuge at 10,000 g for 10 min. The supernatant was detected in new test tubes and used for following analysis. In a homogenized mixture with the Bradford micro method using a series of 5 standards of bovine-serum albumin, and then measuring the absorbance on 450 nm with a spectrophotometer, the concentration of total proteins was determined. With interpolation from the standard curve, concentration of the proteins in samples was measured. A micro-plate reader (Anthos ht III) was used to measure the absorbance at 450 nm. Values of the absorbance of each standard were corrected according to protein concentration. By adding 100 μL of Stop Solution to each well, the bright yellow converted to a bright blue coloured product and the enzyme reaction was stopped.

The MMP concentration of each sample was normalized vis-a-vis concentration of proteins in each sample. A standard curve was designed with software programme Curve Expert 1.3. With interpolation of the values, MMP-1, -8, -13 the concentration was calculated.

Statistical analysis for comparison of values to define the significance between specimens of the examined material used Kruskall-Wallis 1-way ANOVA and Mann-Whitney U-test descriptive and analytical statistical methods. A p-value less than 0.05 were considered significant.

Results

According to their age patients were divided in to five groups. Table 1 shows the distribution of the examined groups according to gender and age. Of the total 30 patients, most were from 30 to 39 years old. Analysis of the data for the examined groups according to age made with the Chi-Square test shows that there is no significant differences between them (X^2 = 8,460; df = 8; p = 0,390).

Table 1

Distribution of gender and age in examination and control group

Age	*Gender*		*Examination group*	*Control group*
			Pulpitis chronica	**Dens impacta**
< 20 years	male	Number	/	1
		%	/	100
	female	Number	2	1
		%	66.7	33.3
20–29 years	male	Number	5	2
		%	71.4	28.6
	female	Number	4	1
		%	80	20
30–39 years	male	Number	2	1
		%	66.7	33.3
	female	Number	3	3
		%	50	50
40–49 years	male	Number	1	1
		%	50	50
	female	Number	2	/
		%	100	/
> 50 years	male	Number	/	/
		%	/	/
	female	Number	1	/

Table 2 shows the distribution of the teeth in the examined groups according to representation in the jaw. In the patients with chronic pulpitis and also from the control group most of the teeth were in the maxilla. Analysis with Chi-Square test shows that there is no significant difference between the examined groups and representation in the jaw (X^2 = 3,112; df = 4; p = 0,539).

Table 3 shows minimal and maximal values of absorbance Abs, and also values of Mediana, standard deviation and median arithmetical values. Minimal values of absorbance in the patients with Pulpitis chornica were 0.125, and maximal 0.81. In the control group values of absorbance were lower, from 0.011 to 0.187.

Table 2

Distribution of the teeth in examination and control group according to representation in the jaw

Jaw		Examination group	Control group	Total
		Pulpitis chronica	Dens impacta	
maxilla	no.	15	6	21
	%	71.4	28.6	70
mandible	no.	5	4	7
	%	71.4	57.1	23.3

Table 3

Absorbance Abs (450 nm) in the examination samples

Clinical diagnosis	Number (n)	min	max	median	$\overline{X}$	σ
Pulpitis chronica	20	0.125	0.81	0.519	0.499	± 0.189
Dens impacta	10	0.011	0.187	0.069	0.076	± 0.056

Table 4 shows the concentration of collagenases in specimens of examination materials from patients with chronic pulpitis and from the control group.

Obtained results from the analysis of the mean values of the concentrations of collagenases in both groups showed a high statistical connection (ANOVA F = 67475; df = 4; p = 0000; p < 0.01). Because the value of F was smaller according to international standards for bio-medical sciences, zero hypotheses were declined. A high statistical significance was also obtained with the Kruskal-Wallis test (X^2 = 59.363; df = 4; p = 0.000; p < 0.01) and the Mediana test (X^2 = 37.400; df = 4; p = 0.000; p < 0.01).

Table 4

Concentration of collagenases (ng/mL) in examination samples

Clinical diagnosis	Number (n)	min	max	median	$\overline{X}$	σ
Pulpitis chronica	20	0.1	1.28	0.35	0.47	± 0.38
Dens impacta	10	0.00	0.02	0.01	0.01	± 0.009

Obtained results for the concentration of collagenases according to clinical diagnosis in the specimens of the examination material are shown in Table 5. In most of the patients with a diagnosis of Pulpitis chronica (17 specimens; 56.6%) concentration was within borders from 0.1–0,99 ng/mL and only in 3 specimens (10%) was concentration within a border from 1–2.99 ng/mL. In the control group the concentration of collagenases was smaller than 0.09 ng/mL, and here the smallest values of concentration of MMP were registered.

Table 5

Concentration of collagenases (ng/mL) in examination samples

Collagenases (ng/mL)	**Clinical diagnosis**		
	Pulpitis chronica	**Dens impacta**	***Total***
	Number (%)	Number (%)	Number (%)
< 0.09	/	10 (100)	10 (33.3)
0.1–0.9	17 (100)	/	17 (56.6)
1–2.99	3 (100)	/	3 (10)
3–4.99	/	/	/
> 5	/	/	/

Discussion

After analysis and worked data, our results were compared with results from other authors. In this way we were able to interpret the problem set up in this study.

Through the investigations in this research we made an effort to give an answer to the questions that relate to the activity and role of collagenases in patients with pulpitis chronica.

With respect to the complexity of the aims and conventional methods that are present in everyday dental practice, we thought that our results might provide an answer concerning the etiology and pathogenesis of pulpitis chronica and also enzyme reactions in which MMPs have a crucial role.

Human dentine-pulp complex cells express several matrix metalloproteases (MMPs), including MMP-1, -8 and -13. [18, 27, 31]

With this study we confirm that degradation of the collagen of the organic matrix from pulp tissue during chronic inflammation is an enzyme process and that collagenses (MMP-1, -8, and -13) are invovlved in these destructive process.

The hypothesis was that the cocnentration of MMP-1, -8 and -13 increases with the spread of the infection and after that pulp tissue was affected, reflectedin chronic pulpitis in the patients. Confirmation was achieved with the detection of high levels of collagenases concentration in inflamed pulp tissue. The confirmation of the hypothesis was set up due to higher values of collagenases concentrations form 0.1–0.99 ng/mL in 17 specimens, and from 1–2.99 ng/mL in 3 specimens from the patients with a diagnosis of Pulpitis chro-nica.

The hypothesis concerning the increasing of collagenases cocnentration with the spread of the infection was accepted. The confirmation was set up due to the low values of collagenases concentration less than 0.09 ng/mL in the patients from the control group formed from impacted third molars, which means that the expression and activity of collagenases in physiological healthy tissue was very low, and increased in inflamed pulp tissue.

These results pprove the set hypothesis and suggest that MMPs play an important role in pulp tissue destruction of chronic inflamed pulp.

In the study by Palosaari H. [23] the expression level of MMP-8 was quite low in the pulp tissue compared to MMP-13, which leads to the possible conclusion that MMP-13 is the main collagenase in pulp tissue together with MMP-1.

This is supported by the recent study utilizing cDNA microarray, in which MMP-13 expression was extremely high in the pulp tissue compared to all the other MMPs studied [28].

Since pulp tissue inflammation is an essential defensive reaction in the protection of the dentine-pulp complex, it is possible that MMPs may have a role during pulpal inflammation by mediating the breakdown of the pulpal connective tissue, enabling migration of odontoblast-like cells [11].

According to Bergenholtz G. [2], MMP-8 in pulpal inflammatory lesions is mainly of PMN origin. PMNs are cells forming pulp abscesses, and thus activated MMP-8 may well participate in the tissue destruction of pulp necrosis and abscesses. PMNs are migrant and recruiting cells able to penetrate dentinal tubules.

The down regulation of MMP-8 may be a factor leading to reparative dentine formation because it is regarded as essential for modulating tissues during normal dentine formation. It is evident that MMPs expressed by odontoblasts may have a role in dentine and reparative dentine formation and the growth factors can act as MMP regulators, as well as regulate collagen synthesis. In the study by Wahlgren J [35].

In the study by Andonovska B et al. [1] concentration of collagenases (MMP-1, -8, -13) in chronically inflamed pulp tissue was within very low margins, which means that the inflammation process is still localized only in the pulp tissue and had not affected periapical connective tissue.

In the study of Shin et al. [25] concentrations of MMP-1 in all experimental groups were significantly higher than in the control ($p < 0.05$). The acute pulpitis and control groups were significantly different in terms of their MMP-2 levels ($p < 0.05$). The concentration of MMP-3 in acute pulpitis was significantly higher than in the control and chronic pulpitis groups ($p < 0.05$). Immunohistochemically, MMP-1 and MMP-3 were localized in the infiltrating neutrophils, macrophages, and extracellular matrix of the acute pulpitis group.

Conclusions

On the basis of our findings we came to these conclusions about the role and relevance of the collagenases (MMP-1, -8, -13) in the inflammation of pulp tissue:

1. Degradation and synthesis of the components of ECM in healthy tissue are in constant balance. Expression and activity of the MMPs in healthy tissue are very low. The measured concentration of collagenases in not inflamed tissue of impacted third molars varies from 0.00 ng/mL to 0.02 ng/mL.

2. Collagenases were involved in the degradation of collagen from the organic matrix of dental pulp tissue during chronic inflammations.

3. The concentrations of collagenases in chronic inflammation tissue in patients with chronic pulpitis were within borders from 0.1 ng/mL to 1.28 ng/mL, which means that the inflammation process was still localization only in pulp tissue and had not affected periapical connective tissue.

4. Values of the concentration of collagenase grow proportionally with the clinical picture of the infection and spread of the infection.

5. This investigation opens a new opportunity to one contemporary method for diagnosis of pulp inflammations and monitoring of the inflamematory processes.

6. The first step in future studies should be to confirm the location and activity of different MMPs and TIMPs in healthy and diseased dentine-pulp complex tissue. Also, *in vivo* studies aiming to selectively regulate MMP-TIMP–expression and activity in the dentine-pulp complex may open a pathway for improved means to master pulpal diseases in clinical work.

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Резиме

КОНЦЕНТРАЦИЈА НА КОЛАГЕНАЗИТЕ (ММП-1, -8, -13) КАЈ ПАЦИЕНТИ СО ХРОНИЧНО ВОСПАЛЕНО ТКИВО НА ЗАБНАТА ПУЛПА

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Матрик металопротеиназите (ММП) формираат ензимска фамилија способна за деградација на речиси сите компоненти на екстрацелуларниот матрикс (ЕЦМ) и базалната мембрана (БМ). Тие играат мошне значителна улога во нормалното ткивно ремоделирање и растот, како и во многу деструктивни физиолошки состојби, како на пример, воспалението, растот на туморите и појавата на метастази. Улогата на ММП во уништувањето на ткивото на забната пулпа со пулпитис сè уште не е доволно разјаснета.

Целта на оваа студија е да се определат ткивните нивоа на колагеназите (ММП-1, -8, -13) и нивната дистрибуција кај клинички здравото и хронично воспаленото пулпино ткиво кај 30 пациенти на возраст од 15 до 70 години. Дваесет забни пулпи беа собрани од пациенти со дијагностициран хроничен пулпитис и 10 контролни пулпи беа земени од 10 пациенти кај кои беше спроведена оралнохируршка интервенција поради ортодонтски причини. Нивоата на колагеназите беа детерминирани со ензимска метода (ЕЛИСА). Резултатите потврдија дека нивоата на колагеназите беа многу повисоки кај пациентите со хронично воспаленото ткиво на пулпата во однос на нормалното, здраво ткиво.

Овие резултати покажуваат дека ММП имаат значителна улога во деструкцијата на ЕЦМ за време на воспалителниот процес кај пациентите со пулпитис, но и ги рефлектираат нивните специјални карактеристики. Овие испитувања отвораат нови можности кон оние секојдневни дијагностички методи за дијагностицирање на воспаленијата на пулпиното ткиво како и мониторинг на воспалителните процеси.

Клучни зборови: матрикс металопротеинази, колагенази, пулпино ткиво, пулпитис.

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