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The Inhibitory effect of Natural materials Black Tea Theaflavins on MMPs-2 and MMPs-9 activities in Dentine

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Abstract. The aim of the present study is to investigate the inhibitory effect of black tea theaflavins(TF) on gelatinases (MMP-2, MMP-9) in human dentine. Protein extracts from demineralized human dentine powder were treated with TFs concentration range from 0.1-1.6 mg/mL; Samples treated with EDTA was used as a positive control while untreated sample as a negative control; Zymography was utilized to assess MMP-2 and MMP-9 activities in dentin. The results demonstrated that untreated demineralized dentine showed the strongest band of MMPs activity on zymography, while EDTA treatment completely inhibited MMPs activity; MMPs activity was inhibited by TFs in a concentration dependent manner. We conclude that theaflavins inhibited MMP-2 and MMP-9 activities, suggesting that natural product theaflavins could be a potential supplementary therapy both for prevention and treatment of dentine caries.

1. Introduction

Caries is defined as a chronic and infectious disease caused by certain cariogenic bacteria. The bacteria aids dissolving the hard tissues of teeth like enamel, dentine and cementum. Being different from enamel, dentine contains less mineral and more organic material. Dentine carious process also differs from enamel caries for its construction. Dentine mineral dissolved and organic dentine extracellular matrix (ECM) exposed, followed by ECM degradation by proteases, cariogenic bacteria aggress toward pulp tissues. In the past, people thought proteases were secreted by bacteria. however, host-derived protease especially matrix metalloproteinases (MMPs) are recently found to play an important role in caries as well ^[1].

MMPs participate in normal cell functions and regulate many physiological processes. However, they also implicate in pathological conditions such as inflammation, tumor development, and arthritis. In the oral region, MMPs have implications in tooth development, pulp and periradicular pathoses, periodontal disease and dental caries. The dentine extracellular matrix contains type I collagen mainly,



which can be degraded by gelatinases (MMP-2 and MMP-9). Several studies have suggested that MMP-2 and MMP-9 can be found in caries lesions and have contribution to dentine matrix degradation^[2]. So people try to find out different ways to inhibit MMPs for decreasing caries. Recently nature MMPs inhibitors becomes popular for their nontoxicity and less side effects.

The black tea is one of the most popular drink in the world. It's fermented and contains four different theaflavins which are the main and active components in black tea. Some researches indicated that the extracts of black tea could inhibit *P. gingivalis* and inflammatory factors secreted by epithelial cells. Mughal et.al found that the MIC of methanolic extracts of black tea diluted in methanol was 0.6 mg/ml^[3]. Our previous study showed the MIC and MBC of theaflavins were 0.5 mg/ml and 1.0 mg/ml respectively.

The black tea exhibits its antibacterial character. However, to our knowledge there are few studies focus on the influence of theaflavins on gelatinases in dentine. Therefore, the aim of this study is to determine the capacity of theaflavins to inhibit MMP-2 and MMP-9 in dentine caries.^[1-3]

2. Methods and materials

2.1. Analysis of theaflavins

Commercial TFs (DeHe Bio-technology Company, Jiangsu, China) were analyzed on a high performance liquid chromatography (HPLC) system (Waters 1525 pump, 2487 dual γ absorbance detector, 717-plus injector). A Phenyl-Hexyl C18 reversed-phase column (250 mm $\times$ 4.6 mm inner diameter) was used for applications. The flow rate was 1mL/min.

2.2. Specimens preparation and demineralization

25 sound human molars were selected after informed consent had been obtained under a protocol approved by Zhejiang Chinese Medical University. Teeth were cleaned in distilled water and attached soft tissue was removed by a scalpel carefully. Enamel, roots and pulp tissue were removed with diamond burs under flowing water. Dentine powder was prepared by pulverizing liquid nitrogen-frozen coronal dentine with a hammer and pestle. The commercial 80% theaflavins (DeHe Bio-technology Company, Jiangsu, China) were diluted to concentrations of 1.6, 0.8, 0.4, 0.2 and 0.1 mg/mL using two-fold dilution method. Seven 500mg of aliquots of dentine powder were treated as follow. Group 1: dentine powder demineralized in 37% aqueous PA for 1 min and 10mM EDTA for 5 min; Group 2: dentine powder demineralized in 37% aqueous PA for 1 min; Group 3-7: dentine powder demineralized in 37% aqueous PA for 1 min and different concentrations of TFs solution for 5 min.

2.3. Enzyme extraction of dentine specimens

After treatment, all specimens were rinsed with distilled water for 2 times and centrifuged for 10 min (13000rpm) at 4°C. Supernatants were discarded and specimens were resuspended in 1mL extraction buffer (50mM Tris-HCl, pH 6.4, 50mM CaCl₂, 100mM NaCl, 0.1% Triton X-100, 0.1% NP-40, 0.1mM ZnCl₂, 0.02%NaN₃) and EDTA-free protease inhibitor cocktail (P8340, Sigma company) for 24h at 4°C. Supernatants were collected after centrifuging for 10 min at 4°C and protein was precipitated with cold acetone at -20°C for 2 h. The protein content was then centrifuged for 10 min and dissolved in 1X

loading buffer. Total protein concentration of the extracts of treated dentine powder was then quantified (Enhanced BCA Protein Assay Kit, Beyotime, Jiangsu, China).

2.4. Gelatin zymography

Gel preparation was performed according to the protocol described by Marta et al ^[4]. Proteins were electrophorized at 4°C under non-reducing conditions on 10% SDS-polyacrylamide gels copolymerized with 1g/L gelatin (porcine skin, Sigma company) at constant voltage (120V, 40mA, 120 min). Carefully removed the gel and placed it in renaturing buffer. Incubating the gel at room temperature for 30 min with gentle agitation and rinsed the gel with distilled water for 30 min. The gel was incubated in developing buffer at room temperature for 30 min and then replaced in another fresh developing buffer at 37°C for approximately 18h. Staining the gel with 0.5% Goomassie Brilliant Blue for 1h and destained in destaning buffer (50% methanol, 10% acetic acid, 40% water).

3. Results

The HPLC analysis demonstrated that TFs were mainly composed of TF, TF-3-G, TF-3'-G and TF-D-G (Fig.1). The concentration of the commercial theaflavins was 71.4% (Tab.1).

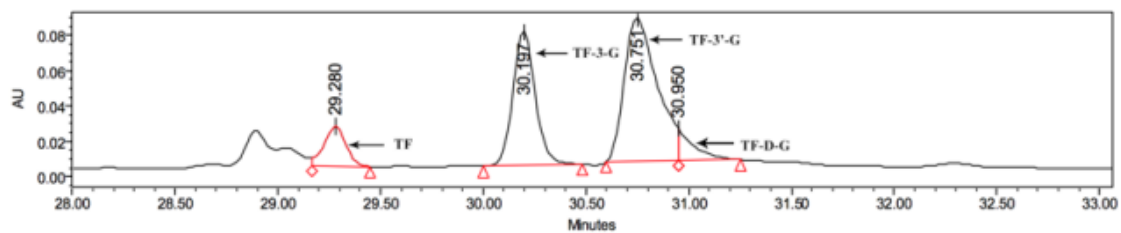


Fig.1-The retention times of theaflavins (TF), TF-3-G, TF-3'-G and TF-D-G were 29.3, 30.1, 30.7 and 30.9 min, respectively.

Name	Retention Time	Area	Area Percent	Concentration
TF	29.269	170075	9.31	12.925
TF-3-G	30.184	590574	32.34	44.174
TF-3'-G	30.736	949802	52.02	76.744
TF-D-G	30.950	115672	6.33	8.976
		1826123	100.00	71.410

Tab.1-The main components of theaflavins (TF) and their concentrations respectively. The total concentration of the commercial TFs was 71.4%.

Gelatin zymography showed four kinds of zymographic lytic bands ranged from 62 kDa to 130 kDa (Fig.2). The latent proforms (92 kDa) and activated enzyme forms (82 kDa) of MMP-9 as well as activated forms of MMP-2 (62 kDa) were detected. 130 kDa form was probably complexes of MMPs. Gelatinolytic activities varied amongst specimens, ranging from a strong band to trace amounts. With the increasing concentration of TFs, MMPs decreased activity. Demineralized dentin powder presented

the strongest band while the positive control showed nothing since they were inhibited by EDTA completely.

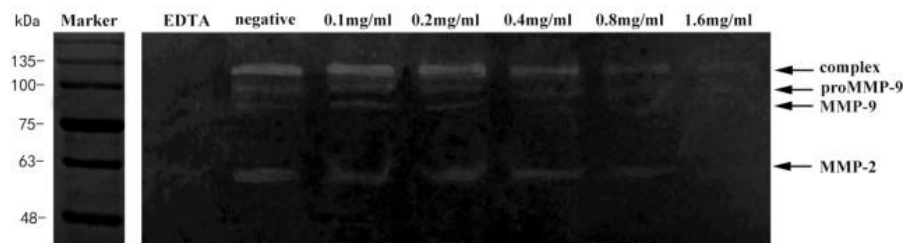


Fig.2- The zymography of MMP-2 and -9. Lane 1: protein extract of EDTA-treated demineralized dentine powder; Lane 2: protein extract of demineralized dentine powder without treatment; Lane 3-7: protein extracts of TFs-treated demineralized dentine powder.

4. Discussion

As we all know, the cariogenic biofilm adheres to teeth surface resulting in caries. The bacteria especially streptococcus mutans lead to localized demineralization and destruction of dentine hard tissue by producing acids. Except for the influence of bacteria, MMPs are found to play a role in caries as well. This proteinase family contribute to homeostasis of tissues and participate in physiological processes like angiogenesis and bone remodeling. However, they also lead to a series of pathological processes(2). MMP-2 and MMP-9, known as gelatinases, can degrade type IV collagen which is the main component of ECM. Therefore, they can always be detected in inflammatory periodontal tissues. Type I collagen constitutes nearly 90% of the dentine organic matrix. In general, collagens are degraded by collagenases and then lose the triple-helical conformation. After that, they can be further degraded by MMP-2 and MMP-9. So gelatinases activities can be observed in caries lesions. Different with MMP-9, MMP-2 also has the ability to degrade collagen^[4].

Early research has found that 0.03% chlorhexidine can completely inhibit MMP-2 and MMP-9. The inhibition of gelatinases by sodium fluoride is reversible at low concentrations (250-1500 ppm), but irreversible at 5000 ppm. However, CHX and fluoride have limitations for their side effects. For instance, desquamations in the oral mucosa and discolored tongues and tooth surfaces are observed when using CHX mouth washes. Excess fluoride ingestion during tooth formation can result in dental fluorosis which leading to a loss of enamel surface^[5]. Because of these side effects, many researchers begin to focus on natural plants. Tea is a popular drink in the world. Lots of studies indicate that green tea has a significant effect on cariogenic bacteria and MMPs^[6]. EGCG, the main component of green tea, can inhibit MMP-2 and MMP-9 directly. The mechanism may be that EGCG has metal- chelating property or it can directly bind to gelatinases. Black tea accounts for 78% of the tea production worldwide. It has more active components than green tea for its oxidation during fermentation procedure. Theaflavins are the main polyphenols which are the standard to evaluate black tea quality and the important bioactive substance in black tea. Studies of TFs range from antioxidant to anticancer^[6], while small amounts of them focus on the potential effects in oral diseases. Among these

studies, antibacteria effect is observed the most. Black tea extract can inhibit *S. mutans* and the inhibitory effect of black tea is even in lower concentration than green tea^[7]. However, few studies observe the inhibitory effect of TFs on MMPs, which are also the important facts in dentine caries. Therefore, it's necessary to evaluate if TFs could suppress MMP-2 and MMP-9 efficiently.

This study revealed four different kinds of zymographic lytic bands. MMP-9 was detected at two molecular weight. 92 kDa indicated the latent proforms and 82 kDa showed the activated enzyme forms. The activated forms of MMP-2 were detected as well while the latent proforms of it were not observed. There were 130 kDa forms which might probably be complexes of MMPs. But in some studies, they were thought to be homodimers of MMP-9^[8]. The relation between gelatinolytic activities and concentrations of TFs was obvious. Demineralized dentin powder without TFs treatment presented the strongest band. With the increasing of concentrations, the activities of MMPs decreased. The band could nearly not be seen when the dentine powder was treated with 1.6mg/mL of TFs. The positive control showed nothing since they were inhibited by EDTA completely. So we could conclude that TFs could inhibit the activities of MMP-2 and MMP-9 especially in a high concentration. The reason why TFs can inhibit gelatinases may because they are flavonoids. Flavonoid is proved to chelate metal efficiently. So the inhibition of MMP-2 and MMP-9 activities by TFs may be related to the ability to chelate zinc metal which is essential for enzymatic activity. Otherwise, the 130 kDa forms and MMP-2 were easy to observe indicating their high concentrations in enzyme extract. Compared with MMP-9, MMP-2 is suggested to be the major gelatinase in human dentin acting in the organization of predentine, initiation of dentine formation and lateral extension of caries along DEJ^[9]. Charadram also found the activity of MMP-2 in dentine caries was higher than those in healthy dentine^[10]. This suggest MMP-2 plays a very active role in the dentine caries procedure.

In summary, the present study shows that TFs can efficiently inhibit the activity of gelatinases. This inhibition suggests that TFs may be a promising method both in the prevention and treatment in dentine caries.

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