Detection of Gelatinolytic Activity in Deciduous Sound Dentin

Sağlam Süt Dişi Dentininde Jelatinolitik Aktivite Tespiti

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ABSTRACT

INTRODUCTION: The purpose of present study was to demonstrate the intrinsic gelatinolytic activty in sound dentin of primary teeth by detecting and stimulating the activity of these enzymes.

METHODS: Sound dentin from 19 extracted primary teeth were collected and cryo-pulverized into a fine powder. Fluorescein conjugate-gelatin DQ Gelatin EnzCheck gelatinase/collagenase kit (Molecular Probes, Eugene, OR, USA) was used as the cleavage substrate. Intrinsic gelatinolytic enzymes were activated by treating sound dentin powder with 4-Aminophenylmercuric acetate (4-APMA, Sigma-Aldrich International GmbH). A full day (24 h) continuous record of fluorescence emission intensity (FEI) was targeted at 535 nm using 96-well plate reader spectrophotometer (Victor 5 Multilabel Plate Reader, PerkinElmer Life Sciences, Boston, MA, USA).

RESULTS: Gelatinolytic activity was detected in sound dentin of primary teeth (981,59±115) and 4-APMA treated dentin exhibited higher gelatinolytic activity (1961,78±204), p<0,05.

DISCUSSION AND CONCLUSION: Gelatinolytic enzymes are present in sound dentin of primary teeth and their activity is enhanced with 4-APMA. So, sound deciduous dentin possesses gelatinolytic enzymes which may lead to self-destruction if exposed to conditions that induce their activation.

Keywords: *deciduous tooth, dentin, enzyme activity, 4-APMA, primary tooth*

ÖZ

GİRİŞ ve AMAÇ: Bu çalışmanın amacı süt dişlerine ait dentin dokusundaki intrinsik jelatinolitik aktivitenin direkt olarak ve aktivitesinin uyarılarak artırılıp saptanmasıdır.

YÖNTEM ve GEREÇLER: On dokuz adet süt dişinden elde edilen sağlam dentin dondurulup pulverize edilerek ince bir toz haline getirilmiştir. Floresin bağlanmış jelatin DQ (Gelatin EnzCheck gelatinase/collagenase kit, Molecular Probes, Eugene, OR, ABD) jelatinolitik enzim substratı olarak kullanılmıştır. İntrinsik jelatinolitik enzim aktivitesi 4-Aminophenylmercuric acetate (4-APMA, Sigma-Aldrich International GmbH) kullanılarak aktive edilmiştir. 96'lı mikropetri içerisine koyulan örneklerin floresans emisyon miktarı (FEI), 535 nm eksitasyonda 24 saat boyunca petri okuyucu spektrofotometre ile kaydedilmiştir (Victor 5 Multilabel Plate Reader, PerkinElmer Life Sciences, Boston, MA, USA).

BULGULAR: Süt dişlerinde sağlam detine ait jelatinolitik aktivite tespit edilmiştir (981,59±115), ve 4-APMA maddesine maruz kalan dentin istatistiksel olarak daha fazla jelatinolitik aktivite göstermiştir (1961,78±204), (p<0,05).

TARTIŞMA ve SONUÇ: Jelatinolitik enzimler süt dişleri dentininde mevcuttur ve 4-APMA ile aktiviteleri artırılabilmektedir. Yani, jelatinolitik enzimlerin etkinliklerini artıracak bir durumda dentin dokusunda yıkım oluşması muhtemeldir.

Anahtar Kelimeler: *süt dişleri, dentin, enzim aktivitesi, 4-APMA*

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INTRODUCTION

As early as 1949s acid producing bacteria were reported to be responsible for the aggravation of caries.[1-3] Later, the mechanism of caries aggravation was understood better since the acid producing cariogenic bacteria was shown to degrade the inorganic portion of dentin[4] leading to the exposure of the organic collagen matrix[5]. Also, intrinsic proteolytic and gelatinolytic enzymes, mostly matrix metalloproteinases (MMPs) located in permanent dentin are activated in acidic environment, which in turn contribute to the breakdown of its organic matrix.[6-8]

It was shown that cariogenic bacteria are not capable to cleave dentin matrix sufficiently to result in a cavity formation.[6-9] Instead, intrinsic proteolytic and gelatinolytic activity of host enzymes are responsible for the degradation of the organic matrix during the caries progression. [10,11] Type of these enzymes and their site-specific and function-related properties are already well-documented for permanent dentin.[12-17] Tooth caries is the most prevalent chronic disease in children [18]; yet, given the fact that such destructive intrinsic enzyme activity has not been directly shown in sound deciduous dentin. The objective of this study was to investigate the gelatinolytic activity in primary teeth dentin. The null hypotheses tested were that there is no gelatinolytic activity in deciduous dentin and gelatinolytic activity is not enhanced when a MMP activator 4-Aminophenylmercuric acetate (4-APMA) is used.

MATERIALS AND METHODS

Primary teeth (N=19) were extracted due to mobility associated with root resorption in the surgery and pediatric dentistry departments at UIC. The teeth were placed in a container with CO_2 ice and kept frozen until use. Present research proposal was approved by the institutional review board of the University of Illinois at Chicago, which is complied with the principles of Declaration of Helsinki with a year and an approval number of "#2015-0453". Consent forms were signed by the parents or the parents with Child Foster care or legal guardianship. Collected teeth were inspected in terms of the presence of dentin caries, and teeth with caries were excluded before cutting off dentin. The sound dentin was obtained using the primary tooth crowns after removing enamel with diamond burs connected to high-speed handpiece under copious water irrigation, and cryo-pulverized further using the device CryoMill (Retsch GmbH, Retsch-Allee 1-5, 42781 Haan, Germany) into a fine powder. Next, dentin powder was demineralized using 10% phosphoric acid for five hours, followed by five times rinse in water using a centrifuge.[17] After accomplishing lyophilization dry weigth was measured to be around 250 mg, and dentin powder was stored at -20°C.

The experimental groups and workflow were explained in Figure 1, included assessment of gelatinolytic activity of sound dentin as well as its exposure to 4-APMA. Fluorescein conjugated gelatin (DQ gelatin, EnzCheck gelatinase/collagenase kit) was used as the cleavage substrate. A reaction buffer was created for the optimal enzyme activity: 50 mL of 0,5 M Tris-HCl, 1,5 M NaCl, 50 mM $CaCl_2$, 2 mM Sodium Azide, pH 7,4. First an addition of 80 µL reaction buffer to the wells were performed using black 96-well plates (generic 8x12 size). Next, 20 µL DQ solution (1,0 mg/mL) was added to the wells; since, DQ gelatin concentrations fall between 12,5-100 µg/mL were resulted in tractable enzyme-substrate interactions in our pilot trials and in previously published studies.[16] As an external control, a stock solution of Clostridium collagenase (Collagenase, Type IV from *Clostridium histolyticum,* Sigma-Aldrich International GmbH) prepared to be 1000 U/mL. 100 µL of the Clostridium collagenase enzyme solution (positive control), or 100 µL of reaction buffer was dispersed into the wells (negative control).

Final volume in wells is 200 µL, and the groups were designed as follows:

Negative control: 80 µL buffer, 20 DQ µL gelatin, 100 µL buffer. *Positive control:* 80 µL buffer, 20 DQ µL gelatin, 100 µL enzyme solution of Clostridium collagenase dissolved in same reaction buffer. *Intrinsic gelatinolytic activity of sound dentin:*80 µL buffer, 20 µL DQ gelatin, 90 µL buffer, 10 µL suspension of sound dentin powder treated with pH 7,4 distilled water only. *4-APMA stimulated intrinsic gelatinolytic activity of sound dentin:* Based on the previous studies' methodology to stimulate gelatinolytic enzymes [12,19] 2 mM final concentration of 4-APMA (Sigma-Aldrich International GmbH) in wells (0,4 µL 4-APMA from the stock solution of 1 M was placed into each well). 80 µL buffer, 20 µL DQ gelatin, 90 µL buffer, 10 µL suspension of sound dentin powder.

Primary dentin powder was treated with deionized water (pH 7,4) for 3 minutes, followed by rinsing,

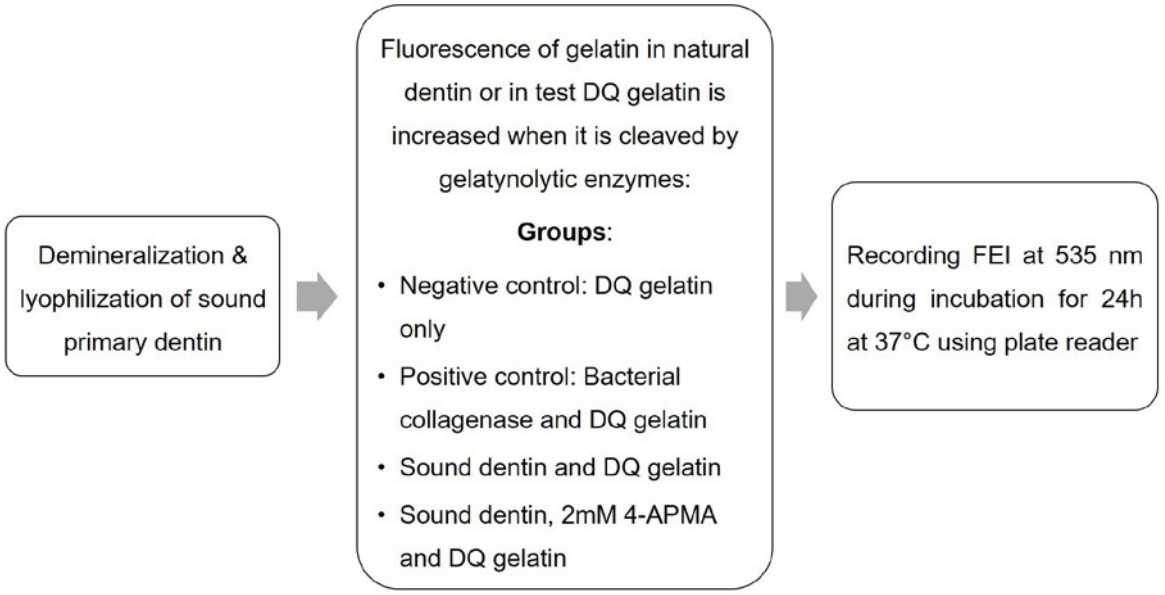

Figure-1 The experimental groups and workflow were explained.

centrifuging and re-suspending in the reaction buffer. A total of 10 µL of the suspended powder was used in triplicate in reaction buffer either with or without a 2 mM 4-APMA. The samples were incubated at 37°C in a 96 well plate and fluorescence emission intensity (FEI) was recorded continuously at 535 nm every hour for 24 hours using a spectrophotometer (Victor 5 Multilabel Plate Reader, PerkinElmer Life Sciences, Boston, MA, USA). For each time point, corrections were done automatically for the background fluorescence by subtracting the value derived from the readings of non-enzyme control.

Gelatinolytic enzymatic activity, expressed as FEI, was determined in three separate experiments using different batches, measured in triplicates (n = 9 per time point). Data were analyzed using SPSS (IBM Corp, version 22,0, Armonk, NY, USA). Activity of enzymes based on the digestion of the fluorescin tagged gelatin was recorded as fluorescence increase. Kruskal-Wallis with Mann-Whitney U-test was performed using data for all time points (SPSS) (α=0,05).

RESULTS

The null hypotheses was rejected since gelatinolytic activity in sound deciduous dentin was detected and gelatinolytic activity of its intrinsic enzymes was enhanced when a MMP activator (4-APMA) is used. Sound dentin with 4-APMA demonstrated statistically greater florescence compared to untreated sound dentin ($p<0,05$). Gelatinolytic activity of sound dentin over 24-hour incubation was depicted in Figure 2. The continuous gelatinolytic activity of sound dentin was shown in Figure 3.

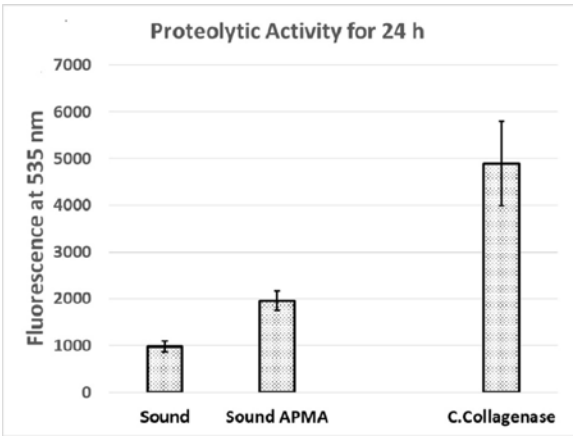

Figure-2 The mean gelatinolytic activity with standard deviation.

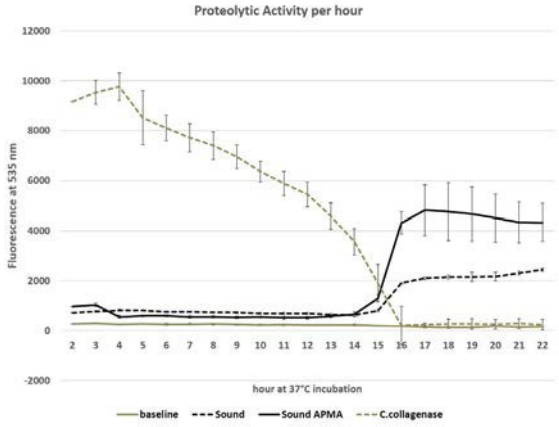

Figure-3 Continuous gelatinolytic activity in 24 h.

DISCUSSION

The present study investigated the gelatinolytic activity of deciduous sound dentin. A well-known MMP activator 4-APMA was also tested on deciduous sound dentin to see if the activation is possible on intrinsic gelatinolytic enzymes. The null hypotheses was rejected since gelatinolytic activity in sound deciduous dentin was detected and gelatinolytic activity of its intrinsic enzymes was enhanced when a MMP activator (4-APMA) is used. Thus, findings of this study demonstrated deciduous dentin possess intrinsic enzymatic activity, thus rejecting the null hypothesis.

Within the limitations of the present study, 5 h exposure to 10% PA did not seem to denature the gelatynolytic enzymes in deciduous dentin since their activity was detected and further activated. In permanent dentin, it was shown that 37% PA short exposure also does not denature the endogenous proteases.[19] Matrix-bound gelatynolitic enzymes for permanent dentin are far more stable to thermal or chemical denaturation than are same enzymes in soluble form. [20, 21] The closeness of the active site of MMPs and cathepsins to their collagen-binding sites [22, 23] explains how stable collagen-bound MMPs are compared to soluble counterparts.

Differences of deciduous dentin structurally and histologically from permanent dentin makes deciduous dentin weaker and thus makes it prone to aggressive caries progression.[24] Sound permanent dentin harbors latent proteolytic and gelatinolytic enzymes (MMPs) which activate while carious progression begins and MMPs contribute to the destruction in the organic matrix of dentin.[6,7,14] When the results of the present study is compared to the data exist in the literature for permanent teeth, gelatinolytic enzymes of sound permanent dentin was reported to be present in both (active and inactive) state[7] while some level of activity in gelatinolytic enzymes were demonstrated in deciduous dentin. However, gelatinolytic enzymes in deciduous sound dentin might be activated during the demineralization process of the dentin powders.[19] Increase in collagenolytic and gelatinolytic activities of permanent dentin is well-reported for both etch-and-rinse and self-etching bond agents,[25] and the clinical relevance of the obtained data is having enzymes in active state after acid treatment that may predispose deciduous dentin to the caries initiation. Herein, deciduous sound dentin treated with a stimulator (4-APMA) showed statistically greater gelatinolytic activity compared to untreated sound deciduous dentin, demonstrating the destructive potential of these enzymes under stimulant conditions.

For the permanent teeth dentin, the role of the intrinsic gelatinolytic/proteolytic enzymes are well-known in caries progression.[26] However, our knowledge about intrinsic enzymes in deciduous dentin is limited even though there are some recent attempts to locate them in situ.[27] The methodology used in present study permitted to evaluate enzymatic activity continuously and revealed the presence of gelatinolytic activity first time in sound primary dentin. However, present study lacks to locate and characterize gelatinolytic enzymes in primary teeth dentin. Based on the limitations in our study, characterization of gelatinolytic enzymes and distribution in human primary dentin using immunohistochemical and biochemical assays should be the next step to elucidate their function and identification.

CONCLUSIONS

Intrinsic enzyme activity and stimulation in deciduous dentin was shown in present study. The potent gelatinolytic activity of sound deciduous dentin and its direct stimulation may aggravate the process in case of a caries initiation. Enzyme inhibitory approach may be the choice to protect deciduous teeth dentin against caries progression.

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