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Original Article

Effect of zinc on the collagen degradation in acid-etched dentin

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Abstract *Background/purpose:* Matrix metalloproteinases (MMPs) play a crucial role in the pathogenesis of dental caries, collapse of adhesive interface, and chemical erosion of teeth. The objective of this study was to investigate the inhibitory effect of zinc on collagen degradation.

Materials and methods: Human dentin was ground and demineralized by citric acid (pH 2.0). The demineralized ground dentin was incubated in six different media: artificial saliva (AS); 5 mg/ml doxycycline in AS; 3.33, 6.82, 13.63, and 27.26 mg/ml of zinc chloride (Zn) in AS. Each group was divided into two subgroups, and active MMP-2 was incorporated into one subgroup. Specimens were incubated for 24 h, 1 week, and 2 weeks. Collagen degradation product was assessed using ELISA. The results were analyzed using repeated measured ANOVA and Duncan's *post hoc* analysis ($\alpha = 0.05$).

Results: The amount of collagen degradation was the lowest in Doxy group. Zn groups showed a significant inhibitory effect in collagen degradation for all concentrations ($P < 0.05$). In subgroups without exogenous MMP-2, zinc-mediated inhibition increased in a concentration-dependent manner with increasing zinc concentration. The amount of collagen degradation product slightly increased with increased incubation time from 24 h to 2 weeks. However, in subgroups with exogenous MMP, the inhibitory effect of zinc on collagen degradation did not depend on zinc concentration.

Conclusion: All Zn groups for the four concentrations tested exhibited statistically significant inhibitory effect on collagen degradation.

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Introduction

The human dentin is composed of about 50 vol.% of mineral, 30 vol.% of organic matter, and 20 vol.% of water.[1] Of the organic matter, collagen fibers and non-collagenous proteins of dentin are buried among fine crystals of hydroxyapatite.[1] Collagen serves as an organic template for mineral deposition during tooth development, and thereafter supports the dentin structure and serves as substance for various enzymes to adsorb on.[2] Especially in the field of adhesive dentistry, collagen forms a hybrid layer with the adhesives for resin bonding to dentin and serves as the core component for bond strength.[3]

Many matrix metalloproteinases (MMPs) have been known to exist in pulp-dentin complex with various functions.[4] During tooth development, MMPs participate in the formation of organic matrix and reconstitute of normal tissue in the enamel and dentin prior to mineralization.[4,5] Such MMPs are later fossilized inside the dentin matrix as minerals deposit on the dentin.[5] Recently, various MMPS have been identified in the human dentin, including the gelatinases MMP-2 and MMP-9, as well as the collagenase, MMP-8 and stromelysin, MMP-3.[6–8] MMP-20 was also found in dentinal fluid.[9] MMPs are secreted by odontoblast or ameloblast and exist as proenzymes until activation through peptide cleavage by external stimuli such as acidic environment, bacterial enzyme, water, and heat.[10] Many studies have verified the involvement of activated MMP with dental caries, collapse of adhesive interface, and chemical erosion of teeth.[4,7,11,12]

A number of studies have been conducted to discover MMP inhibitors. Various agents such as chemically modified tetracycline, doxycycline, and minocycline have been researched as MMP inhibitors for treatment of periodontitis.[13,14] These are known to inhibit MMP-1, MMP-2, and MMP-12.[11,13,14] Zoledronate, a kind of bisphosphonate, inhibited protease activity of MMPs and reduced caries progression in dentin under fissure.[11] Epigallocatechin gallate, which is present in green tea, inhibited activation of MMP-2 and MMP-9.[11] In addition, EDTA inhibited activation of MMP by chelating zinc or calcium ions.[15,16] Treatment of acid-etched dentin with chlorhexidine inhibited collagen degradation by MMPs.[17,18] Galardin treatment of acid etched dentin also inhibited gelatin degradation and reduction of bond strength.[19]

Zinc has been widely used in clinical dentistry. It is an important component of dental materials such as dental cements and restorative materials, and it is also included in toothpaste and mouthwash as an active ingredient.[20] Some previous studies have focused on zinc as a MMP inhibitor. Zinc sulfate ($ZnSO_4$) was reported to strongly inhibit MMP-2 and MMP-9.[20] Zinc ion was also known to be the most effective among various divalent metal ions separated from dental amalgam in inhibiting protease activity of MMP-2 and MMP-9.[21] Moreover, according to Osorio et al.[22] and Toledano et al.,[15] excessive zinc ion inhibited collagen degradation by MMP. The amount of liberated collagen degradation product from acid-etched dentin in the zinc-containing solution was significantly lower than that of the artificial saliva, higher than that of doxycycline-added artificial saliva.[15,22] In those two previous studies, 3.33 mg/ml of zinc chloride solution was used.[15,22] Except these two studies, to our best knowledge, the effect of excessive amount of zinc on MMP inhibition has not been investigated elsewhere, so is still intriguing issue. In particular, there is no validation on adequate concentration of zinc for inhibition of collagen degradation in dentin.

Therefore, the objective of this study is to evaluate the effectiveness of zinc in inhibition of collagen degradation, by comparison with the known MMP inhibitor, doxycycline and to investigate the influence of various concentration of zinc's inhibitory effect of collagen degradation.

Materials and methods

Specimen preparation and demineralization

Extracted human third molars were collected at the department of oral and maxillofacial surgery with consent of patients. Teeth with caries or restoration were excluded. The third molars were washed with sterile phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA), then stored in PBS less than 6 h. The coronal dentin of third molars were collected using a high speed 104R, 103R diamond bur (Shofu Inc., Kyoto, Japan) and ground with mortar and pestle. The ground dentin sample was immediately stored in a −76 °C freezer (ULT Freeze, Thermo Fisher scientific Inc., USA & Canada). Specimens were partially demineralized by immersion in citric acid solution (pH 2.0) and tumbled for 12 h at room temperature. After the partial demineralization, specimens were washed three times with PBS. The specimens were tumbled again in PBS solution for 6 h to remove remaining citric acid. Subsequently, the ground dentin was freeze-dried for 24 h in a lyophilizer (IlshinBioBase, Dongducheon, South Korea). Dry weight of the ground dentin was measured using a micro balance (AX 200, Shimadzu Corporation, Kyoto, Japan). The ground dentin powder was divided into twelve groups and put into each tube. The dry mass was rehydrated in 0.9% NaCl containing 10 U/ml of penicillin G and 300 μg/ml of streptomycin was added to each tube to rehydrate the specimens for 24 h (pH 7.0).

Specimen incubation

The experimental groups were classified according to the medium in which a sample was submerged: (1) artificial saliva (AS), (2) AS with doxycycline (Doxy), (3–6) AS with zinc chloride, concentrations of 3.33, 6.82, 13.63, or 27.26 mg/ml (Zn) (Table 1). 500 μl of incubation medium was put into each Eppendorf tube which contains rehydrated ground dentin. Since prior studies used 3.33 mg/ml of zinc chloride, the identical concentration was applied in this study.[15,22] Additionally, 6.82, 13.63, and 27.26 mg/ml corresponding to 0.05M, 0.1M, and 0.2M respectively, were applied as an intention of finding out the optimal concentrations which exert comparable inhibition effect with doxycycline. Each group was divided into two subgroups according to either adding exogenous active MMP-2 (Calbiochem, Gibbstown, NJ, USA) or not. Active MMP-2 was diluted with buffer solution (50 mM borate, 5 mM $CaCl_2$, 20%

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