



Original Article

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



Soram Oh^a, Hyun-Sook Jung^a, Hyun-Jung Kim^a, Ji-Hyun Jang^a, Duck-Su Kim^a, Kyoung-Kyu Choi^a, Sun-Young Kim^{b*}

^a Department of Conservative Dentistry, School of Dentistry, Kyung Hee University, Seoul, South Korea

^b Department of Conservative Dentistry, Dental Research Institute, School of Dentistry, Seoul National University, Seoul, South Korea

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KEYWORDS

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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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* Corresponding author. Department of Conservative Dentistry and Dental Research Institute, School of Dentistry, Seoul National University, 101 Daehak-ro, Jongno-gu, Seoul, 03080, South Korea. Fax: +82 2 2072 5108.

E-mail address: denkim@snu.ac.kr (S.-Y. Kim).

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 Tolodano 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 μ g/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 μ l 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%

Table 1 Composition of incubation media.

Incubation medium	Composition	pH
Artificial saliva (AS)	50 mM HEPES, 5 mM CaCl ₂ ·2H ₂ O, 0.001 mM ZnCl ₂ , 150 mM NaCl, 100 U/ml penicillin	7.2
Doxycycline in AS (Doxy)	Artificial saliva + doxycycline (1:1)	7.4
Zinc chloride in AS (Zn)	Artificial saliva + 3.33 mg/ml Zinc chloride	7.0
	Artificial saliva + 6.82 mg/ml Zinc chloride	7.0
	Artificial saliva + 13.63 mg/ml Zinc chloride	7.0
	Artificial saliva + 27.263 mg/ml Zinc chloride	7.0

glycerol, 0.005% Brij 35) to be final concentration of 0.01 µg/µl, and 5 µl was added to each of corresponding samples. Three Eppendorf tubes were prepared for each subgroup. Each tube was stirred in a 37 °C incubator (Lab House, Pocheon, Korea) for 24 h, 1 week, or 2 weeks.

Enzyme-linked immunosorbent assay

Eppendorf tubes containing the specimens were centrifuged using a MicroCentrifuge (Gyrozen Co., Ltd., Seoul, Korea) at 13,000 rpm for 30 s and the supernatant was removed. The collagen degradation product, C-terminal telopeptide of type I collagens (ICTP) in the supernatant was measured using an ELISA kit (ICTP-EIA, Orion Diagnostica Oy, Espoo, Finland) according to the manufacturer's instructions. Briefly, medium separated from the sample and each standard solution was mixed and 50 µl of the mixture was placed into the microplate wells. Equal amount of enzyme conjugate and antiserum were added and the solutions shook at 600–1000 rpm for 2 h at room temperature. After 2 h, solutions in the microplate wells were removed, and the wells were washed 4 times with a wash buffer. Moisture was removed from the wells, then 100 µl of substrate was added to each well, and the plate was shaken at room temperature for 30 min. To every well, 100 µl of stop solution was added and mixed for 15–30 s. Absorbance at 450 nm was measured within 10 min using a microplate spectrophotometer (Bio-Rad, Hercules, CA, USA). Triplicate reactions per sample were performed.

Statistical analysis

ELISA results were statistically analyzed using repeated measure ANOVA for incubation media and incubation period and Duncan's *post hoc* analysis, with significance level of $\alpha = 0.05$.

Results

The average concentrations of ICTP according to the incubation media and period without exogenous MMP is shown

Table 2 Mean (SD) of ICTP concentration (µg/l) in conditioning media without exogenous MMP activity.

Mediums	Incubation time		
	24 h	1 week	2 weeks
AS ^a	29.44 (8.30)	37.78 (8.01)	64.79 (0.20)
Doxy ^d	6.61 (0.34)	9.01 (0.90)	9.60 (0.74)
3.33 mg/ml Zn ^b	15.88 (3.16)	20.32 (3.95)	24.86 (1.38)
6.82 mg/ml Zn ^{b,c}	11.72 (2.39)	15.01 (1.45)	25.34 (8.78)
13.63 mg/ml Zn ^{c,d}	10.08 (0.19)	12.41 (0.24)	12.25 (2.31)
27.26 mg/ml Zn ^{c,d}	11.63 (0.04)	13.26 (1.76)	16.53 (2.39)

Abbreviations: AS, artificial saliva; Doxy, Doxycycline in AS; Zn, zinc chloride in AS. Same superscript letters mean no statistically significant difference ($P > 0.05$).

in [Table 2](#). The average concentration of ICTP was dependent on the incubation media and incubation period ($P < 0.05$). For the subgroups without exogenous MMP, ICTP for the AS group after 2-weeks incubation was set as 100% to denote relative amount of ICTP for each group ([Fig. 1](#)). Depending on the media used, amount of ICTP showed significant difference over time ($P < 0.001$). The AS group showed the highest amount of ICTP at all periods. The Doxy group showed the lowest amount of ICTP. For all concentrations, the Zn groups showed higher amount of ICTP compared to the Doxy group, and significantly less amount of ICTP compared to the AS group for all test periods ($P < 0.05$). With respect to zinc concentration, higher concentration of zinc showed stronger inhibition of collagen degradation ([Fig. 1](#) and [Table 2](#)). For all zinc concentrations, amount of ICTP slightly increased over time but collagen degradation was still inhibited compared to the AS group.

The average concentrations of ICTP according to the incubation media and period with exogenous MMP is shown in [Table 3](#). In the subgroups with exogenous MMP-2, ICTP value for the AS group after 2-weeks incubation was set as 100%, and relative amount of ICTP for the groups are shown in [Fig. 2](#). Comparing [Figs. 1](#) and [2](#), the AS group with exogenous MMP-2 showed higher amount of ICTP compared to the AS group without exogenous MMP-2 starting from 24 h of incubation. However, the amount of ICTP did not show significant change over incubation time for the AS group with exogenous MMP-2. The Doxy group with exogenous MMP exhibited strong inhibition of collagen degradation for all tested incubation periods as in the Doxy group without exogenous MMP. Inhibition of collagen degradation was also observed in all Zn groups. However, for Zn groups with zinc concentration of 6.82 mg/ml or higher, higher concentration did not result in increased inhibition of collagen degradation. For all zinc concentrations, amount of ICTP did not increase over time.

Discussion

This study first addressed the effect of zinc concentration on inhibition of MMP, thereby initiating research on the optimal zinc concentration. De Souza et al.²⁰ used

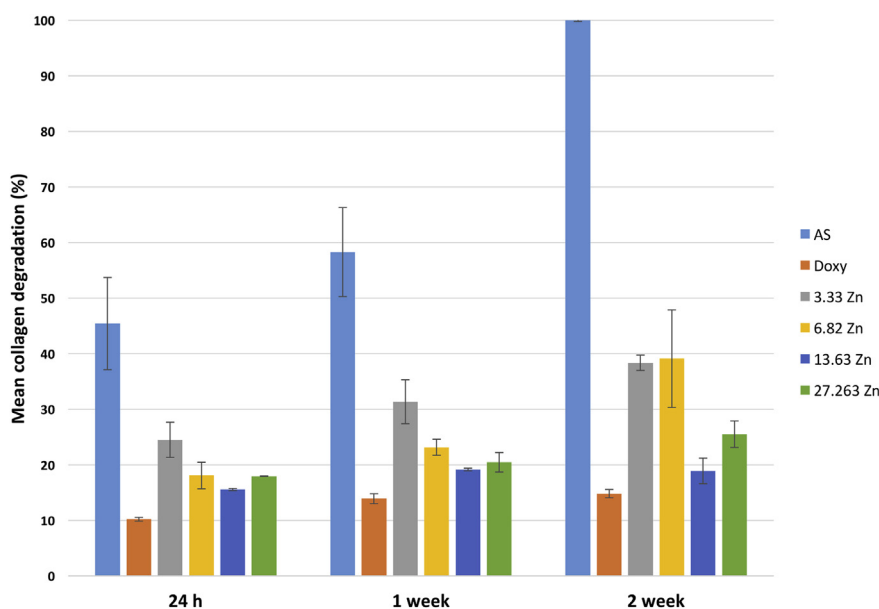


Fig. 1 Means of collagen degradation (%) of partially demineralized dentin in each experimental group without exogenous MMP activity.

Table 3 Mean (SD) of ICTP concentration ($\mu\text{g}/\text{l}$) in conditioning media with exogenous MMP activity.

Mediums	Incubation time		
	24 h	1 week	2 weeks
AS ^a	40.84 (7.62)	41.24 (1.34)	42.09 (0.62)
Doxy ^d	6.03 (0.63)	6.38 (0.48)	6.59 (0.20)
3.33 mg/ml Zn ^b	19.86 (1.82)	20.10 (2.41)	19.81 (0.58)
6.82 mg/ml Zn ^c	16.42 (0.69)	15.73 (1.15)	16.30 (0.31)
13.63 mg/ml Zn ^c	16.51 (0.51)	14.68 (0.76)	16.45 (0.48)
27.26 mg/ml Zn ^c	16.58 (0.51)	15.92 (0.68)	16.67 (1.71)

Abbreviations: AS, artificial saliva; Doxy, Doxycycline in AS; Zn, zinc chloride in AS.

Same superscript letters mean no statistically significant difference ($P > 0.05$).

zymography to demonstrate the inhibition of MMP activity for different zinc concentrations; however, this study used ground dentin, which are complex biological materials, and observed the physiological phenomenon of collagen degradation over time. In this study, inhibitory effect of zinc on MMP's collagen degradation was weaker than doxycycline. However, it was verified that zinc inhibited collagen degradation for at least 2 weeks. Also, higher concentration of zinc resulted in stronger inhibitive effect. In subgroups without exogenous MMP, Zn groups with higher concentration groups (13.63 mg/ml, 27.26 mg/ml) showed no significant differences with the Doxy group.

One of possible mechanisms for inhibition of collagen degradation by zinc is that zinc ion attaches to specific site on MMP to induce structural deformation, thereby deactivating the MMP.^{20,22} Larsen and Auld described the mechanism by which the carboxypeptidase A, a zinc metalloproteinase, is inhibited by zinc.²³ In their mechanism, catalytic zinc ion binds to the side chain of the active

site to forms zinc monohydroxide which inactivate the enzyme.²³ Similar mechanism may be responsible for inactivation of MMP. Another possible mechanism is that attachment of zinc ion to a specific site on collagen to cause change in three-dimensional structure of type I collagen.¹⁵ Alternatively, zinc may competitively attach to the site on collagen to which MMP also attaches to degrade collagen. If MMP is unable to attach to collagen for these reasons, MMP-mediated collagen degradation might be suppressed.

In this study, we conducted experiments on both samples with and without exogenous MMP. By adding exogenous MMP, effect of MMP was amplified to clarify the test results. Similar trends in the inhibition of collagen degradation for the samples with and without exogenous MMP may reflect that the endogenous MMP within the ground dentin is evidently effective in degradation of the collagen. Since experiments using exogenous MMP amplified the inhibitory effect of collagen degradation, it would be more reasonable to refer to experiments without exogenous MMP for inhibitory effect for different zinc concentrations. Based on measurement of degradation product of collagen by MMP inside the dentin, higher zinc concentration resulted in greater inhibition of collagen degradation in the current study. Further research is required to identify the optimal zinc concentration that is not biologically toxic but effective at inhibiting MMP at the same time.

Ground dentin was used as specimens in this study. Many studies on MMP inhibition used dentin blocks obtained from the center of crown of human or bovine tooth.^{12,15,22,24,25} There exist many types of MMP in the human dentin, and their distribution may differ depending on the location of dentin. Extent of mineralization or amount of MMP may also vary depending on the age of tooth. The more mineralized a tooth is, amount of collagen exposed by the same surface treatment may decrease, which will consequently result in different amount of active endogenous MMP. Considering

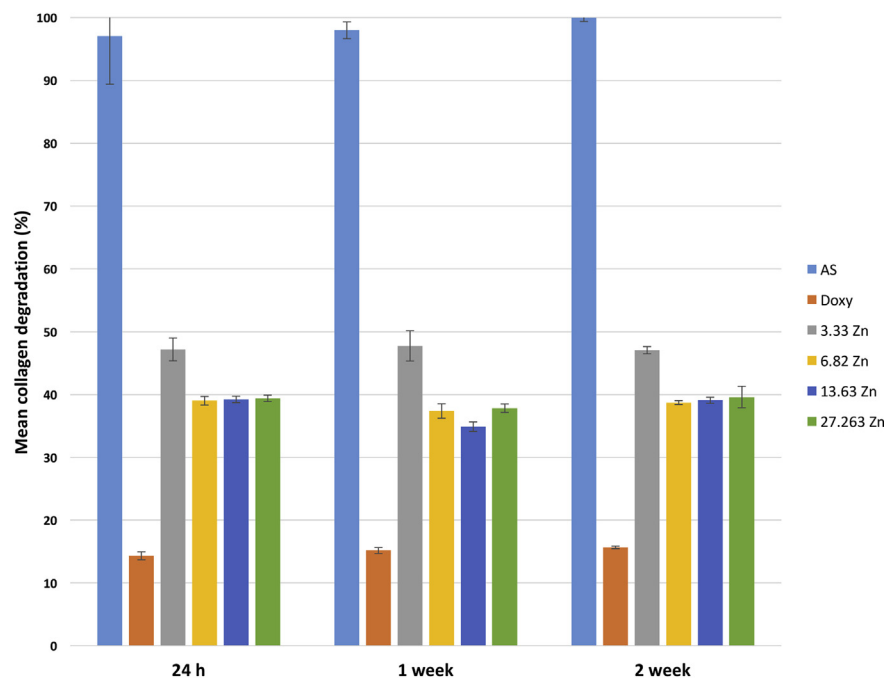


Fig. 2 Means of collagen degradation (%) of partially demineralized dentin in each experimental group with exogenous MMP activity.

these factors, we processed ground dentins from multiple teeth as a batch, and randomly distributed them into respective experimental groups. Ground dentin specimens have larger surface area than block-type dentin specimens, which would increase the amount of collagen matrix and available endogenous MMP.

To evaluate the effect of MMP-mediated collagen degradation, we used ELISA to measure ICTP that exist in the media after respective incubation period. ICTP is generated from degradation of type I collagen by active MMP.²⁶ Measurement of ICTP is one of the most reliable methods for quantifying activity of MMP toward type I collagen.¹⁷ Measurement of ICTP is known to have higher specificity and sensitivity compared to measurement of other collagen products such as hydroxyproline or C-terminal cross-linked telopeptide of type I collagen.¹⁷

Even though Doxy group exhibited significantly lower ICTP concentration (Figs. 1 and 2) compared to the AS group, it did not completely stop the MMP activity to degrade the collagen in dentin. This may be due to collagen degradation that occurred already during 6 h of tumbling in PBS. There has been a study that supports the claim that MMP can maintain its activity in PBS.²⁷ ICTP concentration slightly increased over time in the Doxy group (Fig. 1). Such result may be attributable to the fact that the specimens are ground dentin. Ground dentin has more collagen fibers and MMP exposed to the media compared to a dentin block. MMPs buried below collagen fibers may not be accessible to doxycycline due to small size of micropores produced by crosslinking of collagen fibers. Such MMPs below the crosslinked collagen fibers are thought to be responsible for the continued degradation of collagen.

In this study, we used ground dentin, which is a complex biological material, to observe the effectiveness of zinc on

inhibiting the physiological process of collagen degradation. The exact mechanism through which zinc inhibits collagen degradation is outside the scope of this study. We also attempted to identify the optimal zinc concentration for inhibiting collagen degradation, but the range of concentrations tested was limited. In further studies, range of zinc concentration should be put at smaller intervals to identify the optimal zinc concentration for inhibition of collagen degradation. In addition, incubation period should be set at a shorter interval in the future to confirm inhibitory effect of zinc over time.

In this study, we used ground human dentin to evaluate the effect of zinc at different concentrations (3.33, 6.82, 13.63, and 27.26 mg/ml) and known MMP inhibitor, doxycycline, on inhibiting MMP-mediated collagen degradation inside human dentin. By measuring ICTP, which is generated by degradation of type I collagen by active MMP, the following conclusions were drawn: all Zn groups for the four concentrations tested exhibited statistically significant inhibitory effect on collagen degradation and the inhibitory effect of zinc increased as the concentration of the zinc increased when exogenous MMP was not added.

Conflict of interest

All authors deny any conflicts of interest related to this study.

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