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AFM study of the effects of collagenase and its inhibitors on dentine collagen fibrils

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ABSTRACT

Objective: This study evaluated the effects of exogenous collagenase and two collagenase inhibitors on the variation in microstructure of human collagen fibrils.
Methods: Dentine specimens which were sectioned from 6 freshly extracted human caries-free third molars were wet polished. Each specimen was divided into 4 parts which were treated as experimental groups (group 1, group 2, group 3) and the control group, respectively. All the specimens were etched and further treated with NaClOaq. Subsequently, the topography of each specimen was observed using atomic force microscopy (AFM) in tapping mode in air. Group 1 was then treated with a solution of collagenase II. Group 2 was treated with a solution of collagenase II and chlorhexidine (saturated solution). Group 3 was treated with a solution of collagenase II and captopril (0.3%). The control group was treated with a buffer solution. After 3 h and 6 h of treatment, the topography of the collagen fibrils was measured with AFM in air, respectively.
Results: AFM images of the dentine collagen fibrils were obtained after treatment with NaOClaq. Following further treatment with collagenase II, the topography of the collagen fibrils changed. Most reticular collagen fibrils disappeared after 6 h. After treatment with collagenase II in the presence of chlorhexidine or captopril for 3 h and 6 h, the morphology of the collagen fibres was not changed obviously.
Conclusions: Exogenous collagenase II effectively degraded human dentine collagen fibrils, and its collagenolytic activity was inhibited by the exogenous collagenase inhibitors, chlorhexidine and captopril.

1. Introduction

Dentine contains endogenous matrix metalloproteinases (MMPs),[1] which regulate the physiologic and pathologic metabolism of dentine collagen tissues.[2,3] As endogenous enzymes, MMPs may be activated during dental adhesive restoration. The activated endogenous collagenolytic and gelatinolytic activity may affect the integrity of the organic matrix (collagen fibres) at the bond interface, which has a negative impact on the durability of dentine bonding.[4–7]

Many experiments have been carried out to investigate the influence of endogenous enzyme activity on dentine bonding.[7–13] Recently, well-established short-term effects have been acquired by the application of collagenase inhibitor (chlorhexidine salts) onto the dentine bond interface which can improve the stability and durability of dentine bonding.[14–19] The special radicals of collagenase inhibitors can combine with collagenase molecules and deactivate its collagenolytic activity. The application of collagenase inhibitors onto the bond interface may retard degradation of the collagen matrix, maintaining the integrity of the organic phase of bonding,

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which contributes to bond stability and durability. As an agent with poor water solubility (0.08 g/100 ml), chlorhexidine can be used as a salt to improve water solubility, however, some of the inhibiting effect on collagenase is lost. Therefore, a feasible approach to acquiring optimal bonding performance is to find a specific collagenase inhibitor with high collagenase inhibition as well as good water solubility and then apply this inhibitor to dentine bonding by identifying the special radicals that can be combined with collagenase and make it lose effectiveness.

In this study, atomic force microscopy (AFM) was used to study the effects of collagenase and its inhibitors on dentine collagen fibrils. As a direct surface morphology observation technique, AFM has the advantage of continuous observation of the same specimen undergoing different treatments.[20] Dentine collagen fibrils can be observed by AFM[21] and we previously examined the real-time enzymatic degradation of dentine collagen fibrils in situ using AFM.[22] Therefore, the purpose of this study was to detect the inhibitory effects of selected collagenase inhibitors and observe the microscopic morphology changes on the dental surface (collagen fibrils) in the presence of collagenase as well as collagenase inhibitors using AFM. The collagenase inhibitors, chlorhexidine and captopril were used in this study and the collagenase used was collagenase II. The null hypotheses tested were (1) that collagenase II does not degrade dentine collagen fibrils and no dentine surface morphology changes were observed by AFM, and (2) that the use of collagenase inhibitors has no inhibitory effect on the enzymatic degradation of dentine collagen fibrils.

2. Materials and methods

2.1. *Specimen preparation*

Six fresh caries-free third molars (from patients 20 to 25 years of age, both genders) were extracted during orthodontics treatment, following informed consent approved by the ethics committee of the Affiliated Stomatologic Hospital of the College of Medicine, Zhejiang University. The periodontal membrane of the extracted teeth was removed and the teeth were stored in normal saline at 4 °C before preparation. A specimen of midcoronal dentine of uniform thickness (approximately 1 mm) from each tooth was sectioned perpendicular to the long axis of the tooth. The surface of each specimen was wet polished with 600- to 5000-grit SiC polishing paper, and each specimen was then divided into 4 parts along the middle point and these were treated as experimental groups (E1, E2, E3) and the control group (C), respectively.

2.2. *Collagenase and collagenase inhibitor solution preparation*

Collagenase II (C6885, Sigma) was used in this study, and the collagenase inhibitors used were pure chlorhexidine (98%, Sigma) and captopril (98%, Sigma). Tricine buffer solution was selected and the compounding was 50 mmol/L Tricine, 12 mmol/L $CaCl_2$ and 400 mmol/L NaCl (pH 7.5).[22] Preparation of the chlorhexidine solution was as follows: chlorhexidine powder (0.1 g) was dissolved in deionized water (40 ml), ultrasonically mixed for 3 min and then placed in 25 °C environment in order to allow complete deposition. A filtered saturated supernate was prepared for use. Preparation of the Tricine buffer solution containing chlorhexidine was as follows: chlorhexidine powder (0.1 g) was dissolved in Tricine buffer solution (40 ml). The preparation method was as above and a filtered saturated supernate was prepared for later use. Preparation of the captopril solution was as follows: captopril powder (0.15 g) was dissolved in deionized water (50 ml) at a concentration of 0.3%. Preparation of the Tricine buffer containing captopril was as follows: captopril powder (0.15 g) was dissolved in Tricine buffer solution (50 ml) at a concentration of 0.3%.

Collagenase solution and collagenase inhibitor solution were prepared as follows:

- Collagenase II solution: 2 ml Tricine buffer solution, 1 ml deionized water, and 100 mg collagenase II.
- Collagenase II solution containing chlorhexidine: 2 ml Tricine buffer solution containing chlorhexidine, 1 ml chlorhexidine solution, and 100 mg collagenase II.
- Collagenase II solution containing captopril: 2 ml Tricine buffer solution containing captopril, 1 ml captopril solution, and 100 mg collagenase II.
- Control solution: 2 ml Tricine buffer solution, and 1 ml deionized water.

2.3. *Treatment of dentine specimens*

The prepared dentine specimens (E1, E2, E3, and C) were ultrasonically cleaned in deionized water for 15 min before use. Firstly, the dentine specimens were etched with 10% (w/w) citric acid for 15 s and rinsed thoroughly with deionized water, the specimens were then treated with an aqueous solution of 10% by volume NaOCl for 120 s. After thoroughly rinsing with deionized water, the specimens were gently blow-dried and then immediately examined by AFM in tapping mode in air. When the examination of each specimen was complete, the specimen was immediately immersed in deionized water in order to minimize structural destruction of the specimen surface. Later, the specimens were treatment as follows: Experimental groups: E1 was placed in collagenase II solution, E2 was placed in collagenase II solution containing chlorhexidine; E3 was placed in collagenase II solution containing captopril. The dentine specimen in the control group (C) was placed in control buffer solution. The treatment solution containing the specimen was placed in a sealed bottle and gentle shaking (for 10 s) of the bottle was carried out every half hour. After treatment for 3 h and 6 h in these solutions, respectively, the specimens were rinsed with deionized water and gently blow-dried. Subsequently, AFM in tapping mode was immediately used to examine the microscopic surface morphology of the specimens. Treatment of the specimens in these solutions was carried out at a constant temperature of 32 °C.

2.4. *AFM observation*

An AFM (Veeco/DI, NanoScope IVa, USA) was used in this study. After treatment, the specimens were gently blow-dried