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ORIGINAL ARTICLE

Nicole Birgit Arweiler · Thorsten Mathias Auschill
Nikolaos Donos · Anton Sculean

Antibacterial effect of an enamel matrix protein derivative on in vivo dental biofilm vitality

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Abstract The purpose of this observer-blind, randomised, five-cell crossover study was to examine the antibacterial efficacy of an enamel matrix protein derivative (EMD) on established supragingival plaque in vivo. Saline (NaCl) served as a negative control solution and chlorhexidine (CHX) as a positive one. Additionally, the propylene glycol alginate (PGA) vehicle and the 24% ethylenediaminetetra-acetate (EDTA) gel were tested. After professional oral prophylaxis, 14 volunteers refrained from all mechanical oral hygiene measures for the following 48 h to build up plaque. In randomised order, the following procedures were applied: (a) 10 ml of CHX (0.2%) or (b) 10 ml of NaCl were used as a mouthrinse for 1 min each. In the cases of (c) EMD (Emdogain), (d) PGA, or (e) 24% EDTA (PrefGel), 1 ml of each were applied with a syringe on the teeth. Two hours after application, plaque samples were taken from one upper and one lower molar, and the vitality of the biofilm microbiota was examined using the vital fluorescence technique. Biofilm vitality (VF%) was lower for EMD, PGA, and CHX by 19% ($P<0.0001$), 22% ($P=0.001$), and 35% ($P<0.0001$), respectively, than in negative controls. The EDTA showed similar vitality values to NaCl and was therefore not able to affect the biofilm flora significantly. The EMD and PGA displayed significantly reduced biofilm vitality compared to negative controls, which, however, could not reach the effect of the positive control (0.2% CHX). The present results demonstrate for the first time a direct influence of EMD on the vitality of supragingival dental plaque in vivo.

Keywords Antibacterial agents · Emdogain · Chlorhexidine · Dental plaque · Biofilm vitality

N.B. Arweiler (✉) · T.M. Auschill
Department of Operative Dentistry and Periodontology, Albert-Ludwigs University of Freiburg, Hugstetter Str. 55, 79106 Freiburg i. Brsg., Germany
e-mail: arweiler@zmk2.ukl.uni-freiburg.de
Tel.: +49-761-2704846, Fax: +49-761-2704762

N. Donos
Department of Periodontology, Eastman Dental Institute, London, UK

A. Sculean
Department of Periodontology and Conservative Dentistry, University of the Saarland, Homburg, Germany

Introduction

Application of an enamel matrix protein derivative (EMD) onto debrided and conditioned root surfaces promotes periodontal wound healing in animals and humans [14, 15, 23, 24, 25, 33]. The treatment of different types of periodontal defects with EMD has been shown to result in new formation of periodontal ligament (PDL) and alveolar bone [14, 15, 23, 24, 25, 33]. Recent findings from in vitro and in vivo experiments provide evidence that EMD modulates the behaviour of a variety of dental and nondental cell types in different ways. It was found to upregulate cyclic adenosine monophosphate (cAMP) levels and induce the synthesis and secretion of transforming growth factor-beta (TGF-β) and interleukin-6 (IL)-6 in cultured PDL cells and gingival fibroblasts [18, 30]. It inhibits epithelial growth and stimulates proliferation of preosteoblasts and differentiation of immature osteoblasts [17, 22]. Findings from recent in vitro studies indicate that EMD possesses an antimicrobial effect which may directly influence dental plaque vitality and/or inhibit further growth of bacteria [26, 28, 31]. However, these results were obtained from either in vitro (bacterial cultures) [28, 31] or ex vivo studies in which dental plaque was mechanically removed and then treated with EMD [26]. These experiments, in turn, may not accurately represent the clinical situation. Since substances proved antibacterial in simple in vitro systems may fail to show antibacterial effects in vivo [2, 11], EMD has still to prove its antibacterial efficacy after oral application.

Therefore, the aim of the present randomised crossover study was to investigate the in vivo effect of EMD on the vitality of supragingival dental plaque biofilm and

to compare it to those of the PGA vehicle (24% ethylenediaminetetra-acetate, or EDTA), 0.2% chlorhexidine (CHX), and NaCl solution.

Materials and methods

Sample size

A level of significance of α=0.05 and a power (1-β) of 0.99 were set. A reduction of 20% in biofilm vitality with a standard deviation of 10% was considered clinically relevant using recently published data [8]. For these input values, a minimum sample size of 12 was computed using statistical software (http://ebook.stat.ucla/calculators/powercalc) for two-sided null hypothesis (H_0).

Study population

Fourteen periodontally healthy dental students of the University of Saarland (nine females, five males) with a mean age of 26.4 years (range 24 to 31) volunteered for the study. Criteria for exclusion were the use of antibiotics during the previous 6 months or other antibacterial medicaments that could affect plaque growth, poor oral hygiene (papillary bleeding index, or PBI, according to Saxer and Mühlemann of >40% [21]), buccal restorations on upper and lower molars, fixed or removable orthodontic appliances, partial dentures, known allergy against ingredients of the test products, and pregnancy.

Products

The control solutions were: (1) NaCl solution (Ringer, Delta-Pharma, Pfullingen, Germany) and (2) chlorhexidine 0.2% (Corsodyl, SmithKline Beecham, Bühl, Germany). The test solutions were: (1) EMD (Emdogain, BIORA, Malmö, Sweden), consisting of enamel matrix derivative plus propylene glycol alginate (PGA) (pH 5.4 at 35°C), (2) PGA vehicle (BIORA) (pH 3.32 at 35°C), and (3) 24% EDTA (PrefGel, BIORA)

Study design and microbiological evaluation

After reviewing the aims of the trial and signing declarations of consent, all volunteers received professional tooth cleaning. For the next 2 days, all mechanical oral hygiene measures including flossing and the use of chewing gum were stopped. After 48 h of undisturbed plaque regrowth, one of the test solutions was applied – in randomised order – as follows: EMD (freshly mixed immediately before application), PGA, and EDTA (1 ml each) were applied on supragingival plaque on the buccal surfaces of different molars (teeth 26 and 36) for a period of 2 min. Excesses of the products were spit out and, in the case of EDTA, the volunteers were allowed to rinse with water.

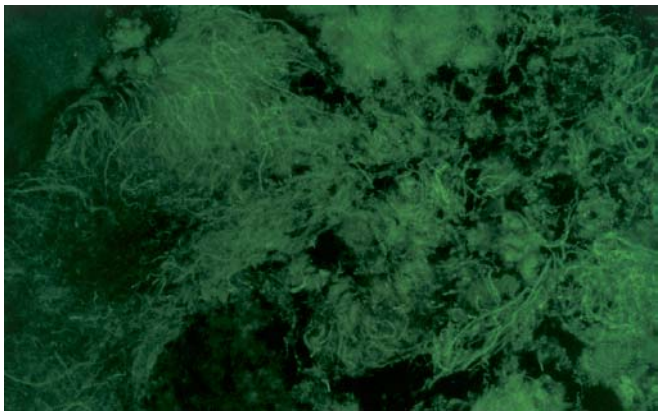

Fig. 1 Vital stained plaque sample containing mainly living cells (living cells are stained *green*)

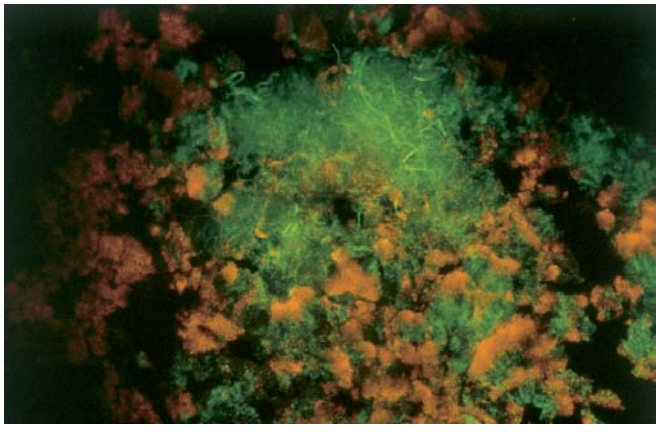

Fig. 2 Vital stained plaque sample containing mainly dead cells (dead cells are stained *red*)

Chlorhexidine or the NaCl solution (10 ml) were used as a mouthrinse by the volunteers for 1 min. The solutions were applied by a person not otherwise involved in the study. The volunteers were not allowed to eat, drink, or brush their teeth for the following 2 h.

Two hours after application of the respective products, plaque samples were taken from the buccal sites of molars 26 and 36 with a sterile curette, smeared on a sterile slide, and vital-stained with fluorescein diacetate (FDA) and ethidium bromide (EB) to visualise the percentage of living (green) and dead (red) cells [19]. Fluorescein diacetate is not fluorescent but membrane-soluble. In vital cells, it is metabolised to fluorescein, which fluoresces green, and is no longer able to leave the cell. Therefore, living cells are stained green. Dead cells are not able to metabolise the FDA. The contrastain with EB binds to the nucleic acids of dead cells and stains them red. This method allows living and dead cells to be stained simultaneously (Fig. 1, Fig. 2). A dichotomous decision (living/dead) can be made for each single cell.

Table 1 Vitality values (VF) and significance levels compared to NaCl (by paired *t*-test). The level of significance was set at $P \leq 0.05$. *Red.* reductions compared to NaCl, *Ns* not significant

	VF (%)	Red.	*P* value
NaCl	86.7±5.7		
EDTA	82.0±7.4	5%	0.15, ns
EMD	70.4±9.6	19%	<0.0001
PGA	67.5±14.9	22%	0.001
CHX	56.2±13.7	35%	<0.0001

After a staining reaction time of 2 min, a cover glass was pressed onto the plaque samples for immediate evaluation with a Leitz DMR B fluorescence microscope (Leica, Wetzlar, Germany). The vitality of the sample (VF) was assessed by a modified counting procedure and the percentage of vital bacteria in the sampled biofilm calculated.

After a 4-day washout period in which the subjects could follow their normal personal oral hygiene measures with a standard toothpaste (Aronal Forte, GABA, Switzerland), a new test cycle was started, so that each subject received each treatment. During this period, the use of antibiotics or other antibacterial medicaments would have led to exclusion from the study.

Statistical evaluation

After completion of the study, statistical analysis was performed using Statistical Package of Social Science/SPSS software, version 7.5.2G. Mean values and standard deviations of the biofilm vitality (VF%) were calculated. Since the data were normally distributed (tested by Kolmogorov-Smirnov), groups were compared using Student's paired *t*-test. Bonferrroni adjustments were not conducted, according to Perneger [20].

Results

All fourteen volunteers completed the study. No severe adverse effects were observed. Vitality values together with the results of statistical analysis are shown in Table 1. Compared to NaCl, the application of EMD, PGA, and CHX on supragingival plaque led to significant reductions in vitality values: 19% ($P<0.0001$), 22% ($P=0.001$), and 35% ($P<0.0001$), respectively. The EDTA showed vitality values similar to those of NaCl (VF_{EDTA} 82.0%, VF_{NaCl} 86.7%) and did not affect the biofilm flora significantly.

Discussion

This investigation evaluated the antibacterial properties of EMD. The study design was comparable to that of a very recent in vivo study testing the influence of active agents on dental biofilm [8]. These effects of antibacterial substances are normally investigated on healthy subjects [1]. Moreover, it is widely accepted that the efficacy of antibacterial or antiplaque agents (e.g. essential oils, amine fluoride, stannous fluoride, metal salts) should be generally tested against negative and positive controls [3]. Chlorhexidine is generally accepted as a positive control for testing the efficacy of possible antibacterial agents such as EMD [16]. Therefore, in the present study, water and CHX were applied according to a well-established protocol (i.e. 1-min rinses) for plaque removal and plaque regrowth trials [1]. Due to their high viscosity, the test products (EMD, PGA, and EDTA) could not be applied in the same way as a conventional mouthrinse. However, the aim was to test them in the amounts usually employed during surgical procedures and compare the effects against those of the gold standard, CHX. Since the primary aim of routinely prescribed CHX rinses following periodontal surgery is to prevent bacterial infection, it is important to point out that this study was designed to test the effect of EMD on supragingival plaque flora. Furthermore, since there are absolutely no data on the effect of EMD on in vivo dental plaque, it was necessary to compare its effect to that of a proven antibacterial substance. In this context, it should be kept in mind that, based on the present findings, no conclusion can be drawn regarding the possible effect of EMD on the subgingival biofilm.

The study shows that EMD, PGA, and CHX possess significantly high antimicrobial effects when compared to a standard NaCl solution or a 24% EDTA gel. These findings are in agreement with those from a recent in vitro experiment [26] in which the same control solutions and vital fluorescence technique were used, but not in the oral environment.

The vital fluorescence technique used has become an established method of investigating the influence of antimicrobial agents and dental restorative materials on dental plaque [6, 7, 8, 9, 34]. It was previously demonstrated that the vital fluorescence technique yields comparable results to those obtained in bacteriological cultures [19]. Although several authors point out that the biofilmic nature of dental plaque should be considered when evaluating the antimicrobial properties of agents which might be useful in preventing biofilm formation [5, 12], the usual methodologies are inappropriate for use against biofilm-related oral infections [32]. In contrast to several studies dealing with the antibacterial effects of bioactive glass and bacteria suspensions [4, 10, 29], the vital fluorescence technique allows evaluation of the direct effects of substances on biofilms in oral conditions [13]. However, this special staining technique differentiates only between dead and vital bacteria, and only a rough differentiation between rods and cocci is possible under light microscopy. Even though the sense of the vital fluorescence technique is not to identify bacterial species, it was seen that rods and cocci were equally affected by the active substances. A differentiation between superficial and deeper plaque layers is not possible, either, since the entire amount of plaque was scraped off the teeth. To ex-

amine undisturbed biofilms in their natural hydrated structure, other methods will be needed. Confocal laser scanning microscopy in combination with the vital fluorescence technique offers the opportunity to visualise biofilms in situ (grown on removable substrates) without destroying their delicate three-dimensional structure [9].

It should be emphasised that, in the previous in vitro study, the reductions in bacterial vitality for EMD, PGA, and CHX were much more pronounced (72%, 74%, and 58%, respectively) [26] than in the present in vivo study (19%, 22%, and 35%, respectively), in which CHX exhibited the highest reductions. This might be due to multiple influences in the oral environment and the known excellent substantivity of CHX [16]. Furthermore, these results are consistent with previous reports which indicate that values derived from in vitro studies are not exact predictors of in vivo action [2, 11]. The reduced biofilm vitality values in the present study, however, are in agreement with previous findings in which reductions of 44% were found 2 h after rinsing with CHX on established plaque [8]. The vitality values obtained with EMD and PGA could be viewed as clinically relevant, since they are in a similar range as known antibacterial agents such as amine fluoride, stannous fluoride, and triclosan/copolymer [6, 7, 8, 13].

As already suggested in the former in vitro study [26], the present data seem to indicate that the antibacterial effect of EMD is strongly influenced by the PGA vehicle, which possesses a low pH and may perturb bacterial cell metabolism. On the other hand, very recent data have shown that EMD itself may also influence the adherence of certain oral bacteria and also markedly inhibit the growth of gram-negative periodontal pathogens [28, 31].

Since EMD is usually applied to cleaned root surfaces in conjunction with periodontal surgery, it may be anticipated that the elicited antibacterial effect might also affect the subgingival plaque biofilm, which consists mainly of gram-negative microbiota. Furthermore, recent data provide evidence that, once precipitated, EMD can be detected on treated root surfaces for at least 4 weeks [27]. Moreover, in vitro data have shown that, once precipitated onto a hard surface, EMD may inhibit the growth of certain gram-negative bacteria [31]. Thus, all the available data taken together seem to indicate that EMD may influence the oral microbiota. However, to gain a better understanding of its antibacterial effects, further studies are needed to elucidate to what extent EMD affects the in vivo subgingival biofilm.

Conclusion

The present results demonstrate for the first time that EMD has a direct influence on the vitality of supragingival dental plaque in vivo.

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