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ORIGINAL ARTICLE

# Influence of a mouthwash containing hydroxyapatite microclusters on bacterial adherence in situ

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**Abstract**
*Objective* The aim of the present study was to investigate the efficacy of a new preparation in dental prophylaxis containing zinc-carbonate hydroxyapatite microclusters (Biorepair) for oral biofilm management.
*Methods and materials* Initial biofilm formation was carried out in situ with bovine enamel slabs fixed to individual upper jaw splints worn by six subjects. Rinses with the customary preparation as well as with subfractions (hydroxyapatite microclusters in saline solution; liquid phase without particles) were adopted for 1 min in situ after 1 min of pellicle formation, and the bacterial colonization was recorded after 6 h and 12 h, respectively. Rinses with chlorhexidine served as a reference. The adherent microorganisms were quantified and visualized using DAPI staining and live–dead staining (BacLight). Furthermore, the effects on *Streptococcus mutans* bacteria were tested in vitro (BacLight).
*Results* Application of the customary preparation and of the separate components distinctly reduced the initial bacterial colonization of the enamel surface in situ as visualized and quantified with all techniques. After 12 h, $1.3\times10^7\pm2.0\times10^7$ bacteria/cm² were detected on unrinsed control samples with DAPI staining; $2.4\times10^6\pm3.3\times10^6$ after application of Biorepair (12 h after CHX-rinse; $1.3\times10^5\pm9.2\times10^4$). Also, pure hydroxyapatite microclusters in saline solution ($2.1\times10^6\pm3.0\times10^6$) as well as the liquid phase without particles ($5.1\times10^5\pm3.3\times10^5$) reduced the amount of adherent bacteria. Furthermore, antimicrobial effects on *S. mutans* were observed in vitro.
*Conclusion* The preparation is an effective compound for biofilm management in the oral cavity due to antiadherent and antibacterial effects.
*Clinical relevance* The tested mouthrinse seems to be a reasonable amendment for dental prophylaxis.

**Keywords** Nanomaterial · Mouthwash · Hydroxyapatite · Biofilm · Pellicle · Bacterial adherence

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## Introduction

Caries is the infectious disease with the highest prevalence worldwide. Tooth brushing, flossing, and application of fluoridated toothpastes have been the fundamentals in dental prophylaxis for biofilm management over years. However, there is still room for improvement though fluorides facilitate remineralisation and aggravate demineralisation [1–3]. The intraoral biofilm management cannot be rated as sufficient, and new biological and biomimetic approaches are required to optimize the prevention of caries [4–7]. In addition, from a theoretical point of view, it has been hypothesized that natural enamel wear due to abrasion, and attrition leads to a release of nano-sized hydroxyapatite crystallites in the oral cavity for potential physiological biofilm management and remineralisation of initial enamel lesions [7, 8]. It is noteworthy that prehistoric skulls as well as teeth of

omnivorous primates with considerable tooth-wear show little or no caries though carbohydrates are part of the diet [8]. Repair of demineralized enamel structures could be improved if supplied apatite particles fit to the size of the nano-sized defects and correspond to the scale of the smallest building units of the enamel, the hydroxyapatite crystallites. Besides effects on de- and remineralisation, also modulation of bacterial adhesion and effects on the bacteria are conceivable [7]. Due to modern diet, there is only little attrition, and such nano-sized hydroxyapatite-crystallites do not occur in the oral fluids [8, 9]. Accordingly, it is worthwhile to investigate if supplementation of hydroxyapatite nanoparticles or hydroxyapatite microclusters could contribute to oral biofilm management; examples are toothpastes and mouthrinses containing microclusters of zinc-carbonate hydroxyapatite crystallites [7, 9]. Biomimetic apatite nanocrystals and fluoride ions yield different effects with respect to interactions with the enamel surface [10]. The efficacy of such microclusters in a toothpaste preparation has been shown in vitro, especially for the purpose of remineralisation and prevention of demineralisation. A toothpaste based on hydroxyapatite microclusters has been tested with respect to remineralizing effects in vitro. It showed better effects with bovine dentine compared to amine fluoride toothpastes; comparable efficacy was recorded for enamel [11]. Furthermore, this type of toothpaste reduced enamel demineralization induced by a soft drink in vitro [12]. However, the potential impact on bacterial biofilm formation has not been investigated until now [9].

Therefore, the aim of the present study focussing on in situ experiments amended by in vitro tests was to investigate the potential capacity of an accredited compound containing clusters of zinc-carbonate hydroxyapatite nanoparticles as well as detergents and sugar alcohols for oral biofilm management (Biorepair; Dr. Kurt Wolff, Bielefeld, Germany). Thereby, antibacterial as well as antiadherent properties were to be considered. The following hypothesis was tested: The mouthrinse with hydroxyapatite microclusters reduces bacterial colonization of enamel surfaces.

## Materials and methods

### Specimens and subjects

Six subjects, members of the laboratory staff in Dresden, participated voluntarily in all the different in situ experiments. Visual oral examination was carried out by an experienced dentist (CH, Dresden). The subjects showed plaque and gingival indices close to zero and no untreated carious lesions. All in situ experiments were carried out in Dresden, in vitro experiments; fluorescence microscopic evaluation and electron microscopy were conducted in Dresden, Freiburg, and Homburg.

The study has been approved by the Medical Ethics Committee of the Medical Association of Saarland, Germany (# 18/10). Informed written consent was given by the subjects about participation in the study.

The cylindrical enamel slabs (diameter, 5 mm; surface area, 19.63 $mm^2$; height, 1.5 mm) were gained from labial surfaces of bovine incisors of 2-year-old cattle with a trepan bur (Brasseler Komet, Lemgo, Germany). The animals were BSE negative. The surfaces of the enamel slabs were polished by wet grinding with abrasive paper (400–4,000 grit, FEPA-P, Struers-GmbH, Erkrath, Germany); the smear layer on the slabs was removed by ultrasonication in 2 %NaOCl (pharmacy of the Dresden university hospital) for 3 min. In the following, the samples were disinfected in ethanol (70 %, pharmacy of the Dresden university hospital) for another 3 min, washed in distilled water (pharmacy of the Dresden university hospital), and stored in fresh distilled water for 24 h before exposure in the oral cavity [13].

### Tested solutions

Biorepair (Biomimetisch wirkende Zahn- und Mundspüllösung mit künstlichem Zahnschmelz [biomimetic tooth and mouthrinse with artificial enamel]; Dr. Kurt Wolff, Bielefeld, Germany) based on zinc-carbonate hydroxyapatite microclusters (microrepair) was used in different dilutions (Table 1). Additional experiments were carried out with a preparation free of nanoparticles as well as with hydroxyapatite microclusters in saline solution (Table 1). The subfractions were provided by Dr. Kurt Wolff, Bielefeld, Germany. Furthermore, chlorhexidine (CHX) was used as a reference (chlorhexidine, 0.2 %, meridol perio chlorhexidine, GABA, Lörrach, Germany). The different rinses were adopted in a randomized manner by the subjects; the sequence of application was determined by drawing lots. The 6-h experiments were conducted first, then the 12-h experiments.

### BacLight viability assay, in vitro experiment

The LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Molecular probes, Darmstadt, Germany) adopts two nucleic acid stains—green-fluorescent SYTO 9 stain and red-fluorescent propidium iodide stain [14]. The assays were carried out according to manufacturer's instructions and the fluorescence intensity was measured in a 96-well plate reader. A suspension of *Streptococcus mutans* in saline solution was prepared after cultivation overnight; 50 % of the bacteria were inactivated with heat (1 h; 95 °C). Dilutions of Biorepair and chlorhexidine were prepared with saline solution (0.9 % NaCl, sterile). The vital bacteria were mixed 1:1 with the diluted preparations (CHX, Biorepair) and incubated for 10 min. Afterwards, these suspensions were mixed with heat-inactivated bacteria (0:100; 5:95; 25:75; 45:55; 50:50). A volume of 0.5 µl of BacLight-staining solution (component A and B 1:1) was added to

**Table 1** Principle composition of rinsing agents used in this study

| Mouthrinse | Main ingredients (principal composition) | Percentage |
|---|---|---|
| Biorepair | Water | >50 |
| Mouth and tooth rinsing solution (Dr. Wolff, Bielefeld, Germany) 8.348.51.037 | Sorbitol | <25 |
| | Alcohol denat | 4 |
| | Glycerin | <5 |
| | Xylitol | <5 |
| | Cellulose gum | <5 |
| | Zinc PCA | <1 |
| | Zinc hydroxyapatite | <1 |
| | Aroma | <1 |
| | Sodium lauryl sulfate | <1 |
| | Silica | <1 |
| | Ricinus communis seed oil | <1 |
| | Ammonium-acryloyldimethyltaurate/ VP-copolymer | <1 |
| | Sodium saccharin | <1 |
| | Sodium benzoate | <1 |
| | Sodium-myristol-sarcosinate | <0.1 |
| | Sodium-methyl-cocoyl-taurate | <0.1 |
| | Benzyl-alcohol | <0.1 |
| | Phenoxyethanol | <0.1 |
| | Limonene | <0.1 |
| Hydroxyapatite microcluster-solution (microrepair) | Hydroxyapatite microclusters in saline solution (Zinc hydroxyapatite ) | 0.75 |
| Biorepair free of hydroxyapatite microclusters | Water | >50 |
| | Sorbitol | >25 |
| | Alcohol denatured | 4 |
| | Glycerin | <5 |
| | Xylitol | <5 |
| | Cellulose gum | <5 |
| | Zinc PCA | <1 |
| | Aroma | <1 |
| | Sodium lauryl sulfate | <1 |
| | Silica | <1 |
| | Ricinus communis seed oil | <1 |
| | Ammonium-acryloyldimethyltaurate/ VP-copolymer | <1 |
| | Sodium saccharin | <1 |
| | Sodium benzoate | <1 |
| | Sodium-myristol-sarcosinate | <0.1 |
| | Sodium-methyl-cocoyl-taurate | <0.1 |
| | Benzyl-alcohol | <0.1 |
| | Phenoxyethanol | <0.1 |
| | Limonene | <0.1 |

250 μl of these mixtures. The staining was incubated for 10 min in a dark chamber. A volume of 100 μl from each sample was pipetted in a micro titer plate, and the fluorescence was measured. The excitation wavelength was 470 nm; emission was recorded at 530 nm for the vital and at 620 nm for the avital cells. The measurements were carried out in duplicates to equalize inhomogenities of the suspension. For evaluation of the recorded data, the ratio of

vital and dead/avital cells was calculated according to ratio= emission vital/emission dead bacteria. Experiments with saline solution served as a reference/negative control.

### Exposure of the samples in the oral cavity

For in situ pellicle formation and bacterial colonization, individual upper jaw splints were vacuum-formed from 1.5-mm thick methacrylate foils. Cavities were prepared in the buccal aspects of the splints at the sites of the premolars and the first molar (six slabs per splint). The slabs were fixed on the splints using polyvinyl siloxane impression material (President Light Body, Coltene, Switzerland), exposing only the surfaces of the enamel slabs to the oral fluids. The splints were carried intraorally for 1 min, then rinses with the mouthwashes (1 min, 8 ml) were performed, and the samples were kept in the oral fluids for another 6 or 12 h. After intraoral exposure, the slabs were immediately dismounted from the splints and rinsed with running water for 5 s in order to remove non-adsorbed salivary remnants [13, 15].

### Fluorescence microscopic assays

The epifluorescence microscopic analyses were conducted at 1,000-fold magnification (Axioskop II, ZEISS, Oberkochen, Germany) as in several previous studies. The number of cells observed in 10 randomized microscopic ocular grid fields per sample was counted. The area of ocular grid fields (0.0156 mm²) allowed calculating the number of cells per cm² of evaluated enamel surface [13, 15–17].

### DAPI staining

DAPI (4′,6-diamidino-2-phenylindole, Merck, Darmstadt, Germany) forms fluorescent complexes with double-stranded DNA [13, 16, 18]. Thereby, DAPI shows fluorescence specificity for AT-clusters. DAPI is rapidly taken up into cellular DNA, yielding highly fluorescent nuclei without cytoplasmic fluorescence. The maximum of fluorescence is observed at a wavelength of 461 nm. The staining was conducted as described previously [13]. After the oral exposure, specimens were washed once in methanol. For staining, the slabs were covered with 1 ml DAPI–methanol (working solution, 1 μg/ml) and incubated in a dark chamber for 15 min at room temperature. Subsequently, the solution was poured off and the specimens were washed again with methanol, then air dried at room temperature. The slabs were sticked to a slide and analyzed by fluorescence microscopy.

### BacLight viability assay, in situ experiments

The BacLight kit was used for staining of enamel samples exposed to the oral fluids for visualization of vital and avital bacteria in the adherent state. Similar amounts of component A (Syto9 1.67 mM/propidium iodide, 1.67 mM, 300 μl DMSO) and B (Syto 9 dye, 1.67 mM/propidium iodide, 18.3 mM, 300 μl DMSO) were mixed; 2 μl were added to 1 ml of saline solution. The enamel samples were incubated with this solution in a dark chamber for 10 min. Afterwards, the samples were rinsed with saline solution and evaluated immediately with the fluorescence-microscope using the FDA-filter and the ethidium bromide filter.

### Statistics

Statistical evaluation was performed by Kruskal–Wallis test, additional pairwise comparison was carried out using the Mann–Whitney test; $p$ values were evaluated according to Bonferroni correction ($p<0.010$). The software used was SPSS 16.0 (IBM, Ehningen, Germany). A post hoc power analysis was performed for the significant effects calculated with Mann–Whitney test in order to determine the achieved power (G*Power 3.1.3, university of Düsseldorf, Germany) [19, 20].

## Results

### In vitro experiments with suspensions of bacteria

Considerable antibacterial effects were observed. In vitro experiments with suspensions of *S. mutans* indicated that Biorepair had pronounced impact on the viability of the bacteria even at high dilution. In contrast, the effect of chlorhexidine occurred in a dose-dependant manner (Fig. 1 a–c). Biorepair and the subfraction of Biorepair without hydroxyapatite microclusters had the same effect on *S. mutans* in vitro, whereas microclusters in saline solution had no effect as compared with the NaCl control and with the gold standard chlorhexidine in different dilutions (Fig. 1). The curve progression observed for undiluted Biorepair and for hydroxyapatite microclusters in NaCl is a result of the optical characteristics of these suspensions. The hydroxyapatite microcluster particles themselves were visualized with TEM; crystallites of 15- to 20-nm in length are aggregated as clusters; their diameter ranged between 100 and 300 nm (Fig. 2).

### In situ experiments

After 6–12 h, pronounced effects of Biorepair and its subfractions on the amount of adherent bacteria were observed with DAPI staining and BacLight, the effects are comparable to those observed after rinsing with chlorhexidine. Representative images visualizing the effects qualitatively are given in Fig. 3.

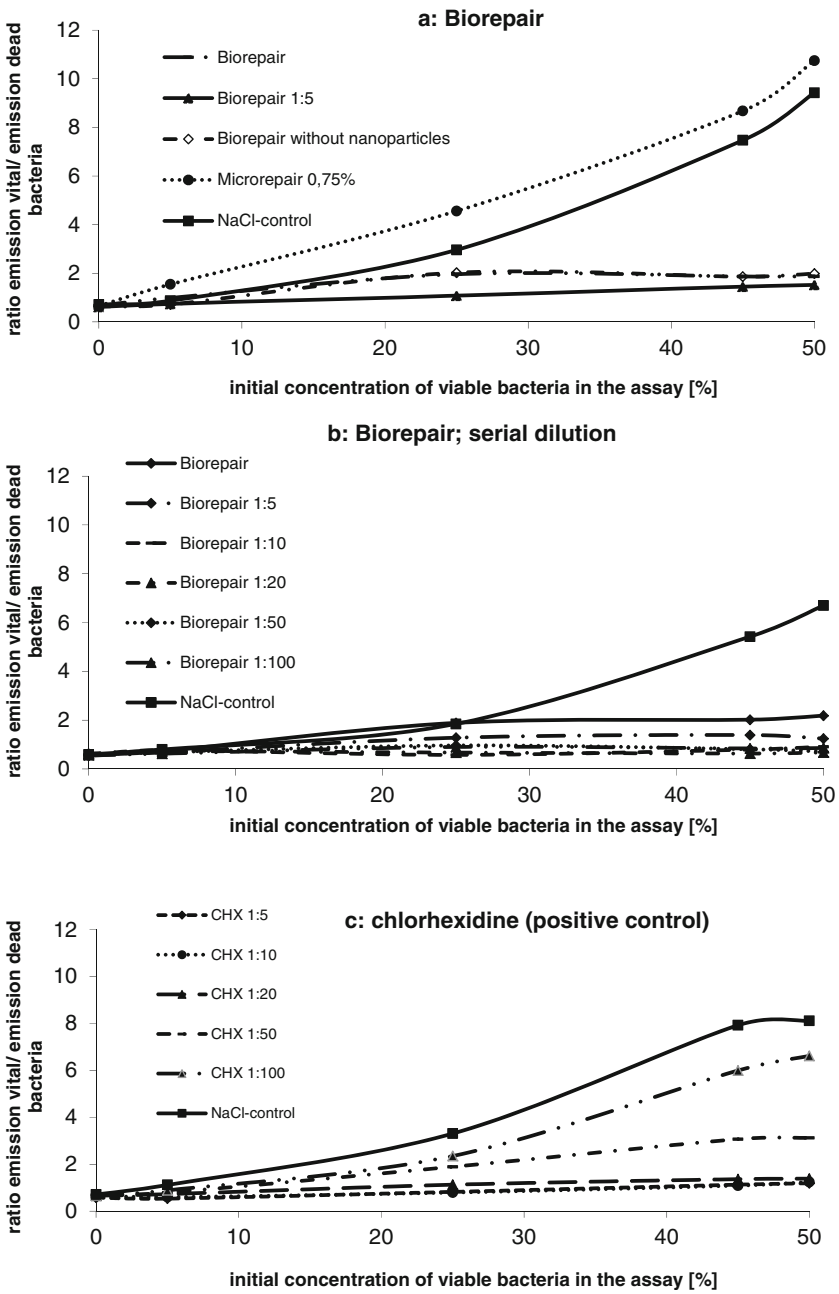



**Fig. 1** Effect of Biorepair with and without hydroxyapatite microclusters and of chlorhexidine (positive control) on the viability of *S. mutans* in vitro. A suspension of *S. mutans* was incubated with different dilutions of Biorepair and chlorhexidine, respectively. Saline solution served as a reference (negative control). After incubation, the samples were admixed to proportions of a suspension containing heat-inactivated bacteria, the amount of vital and dead bacteria was determined using the BacLight bacterial viability assay, the measurements were carried out in duplicates, and the ratio was calculated: emission 530 nm / emission 620 nm, representing emission vital / emission dead bacteria, respectively. **a** Effect of Biorepair and the respective subfractions; **b** Effect of Biorepair in a dose-dependent manner; **c** Effect of chlorhexidine in a dose-dependent manner. The curve progression observed for undiluted Biorepair and for hydroxyapatite microclusters in NaCl is a result of the optical characteristics of these suspensions.

Adherent bacteria on the enamel slabs were quantified after exposure to the oral fluids for 6 and 12 h following application of the different rinses by six subjects using DAPI and BacLight. There was a high interindividual and

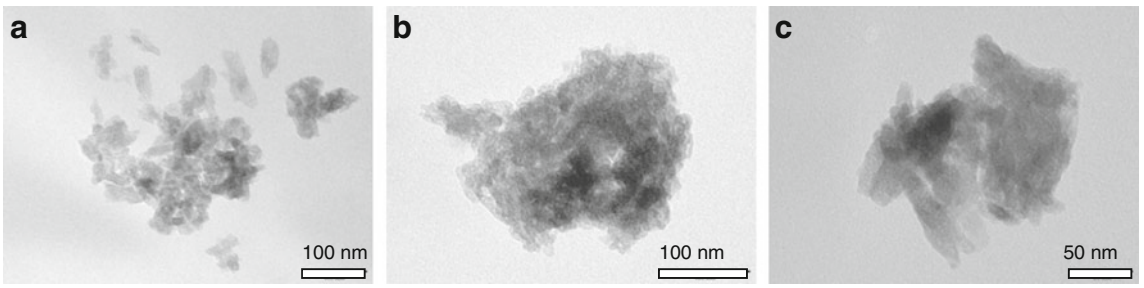
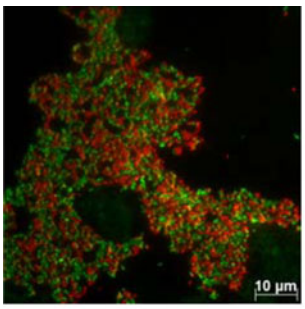
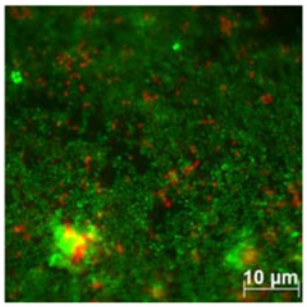
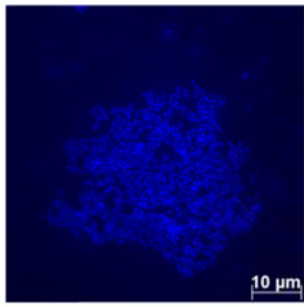
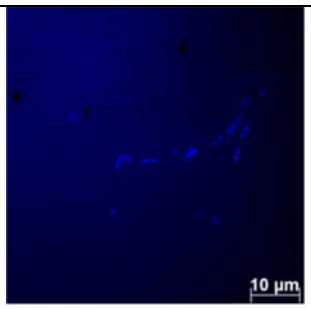
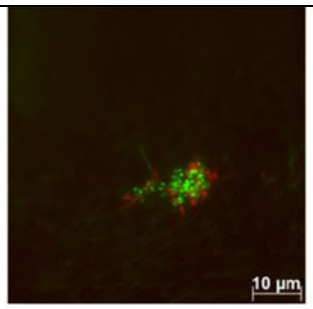
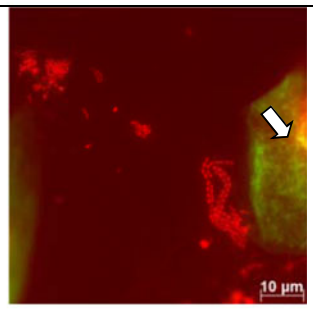
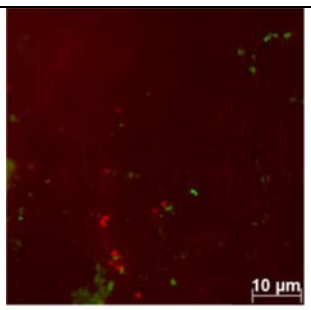
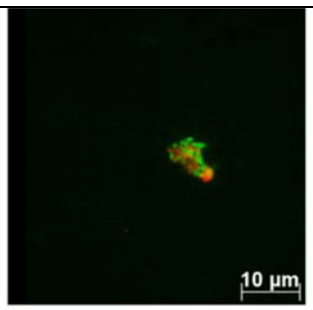
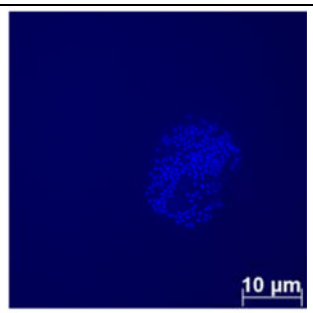
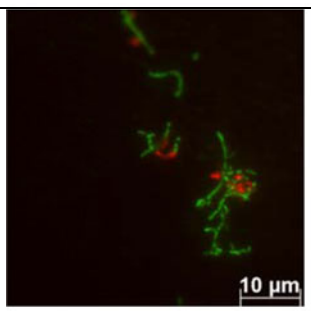
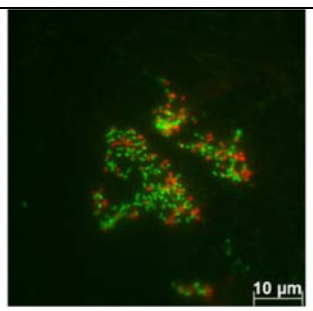
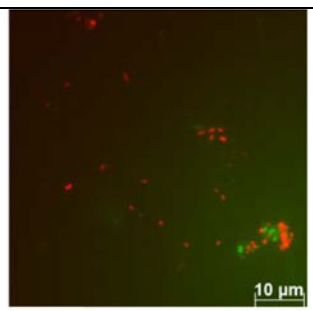
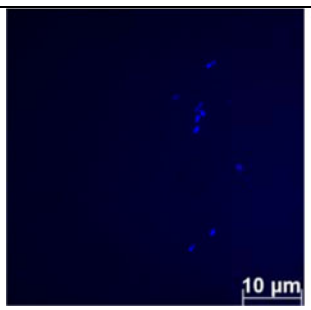
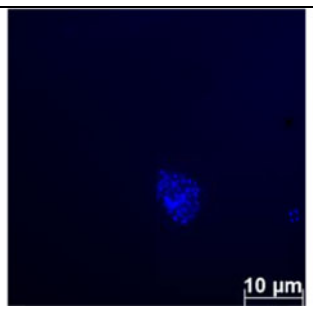
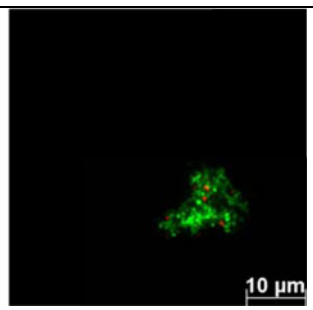



**Fig. 2** TEM-analysis: hydroxyapatite microcluster-particles; the particles were placed on grids for visualization. Crystallites of 15–20 nm in length are aggregated as unorderd clusters. Original magnification: **a**: 180,000-fold, **b**: 250,000-fold, **c**: 340,000-fold

| | | | |
|---|---|---|---|
| Control samples |   6 h |   12 h |   12 h |
| Biorepair |   6 h |   6 h |   12 h |
| Biorepair free of hydroxyapatite microclusters |   6 h |   6 h |   12 h |
| microclusters in saline solution |   6 h |   6 h |   12 h |
| chlorhexidine |   6 h |   12 h |   12 h |

◀ **Fig. 3** DAPI staining (*blue*) and live–dead staining (*red*/*green*): representative images of control samples and of specimens after application of the mouthrinses in situ. On control samples, broad monolayers of bacteria were visible covering large areas of the enamel surfaces after 6–12 h of oral exposure. The different rinses were adopted for 1 min following 3 min of pellicle formation. Application of customary Biorepair, Biorepair free of hydroxyapatite microclusters, of microclusters in saline solution, and of chlorhexidine reduced bacterial adherence over 6–12 h considerably. The structures in one image are extensions of an epithelial cell which is sometimes detected on the samples (*arrow*)

intraindividual variability. However, a significant reduction of the adherent bacteria was achieved with all rinses except of nanoparticles in saline solution after 6 h as shown with DAPI (Fig. 4; Kruskal–Wallis test, $p<0.001$; Mann–Whitney test; Table 2). In general, pure hydroxyapatite microclusters in saline solution were slightly less effective than Biorepair, Biorepair without micro repair or CHX, respectively. Also, after 12 h, an effect of the rinses was observed with DAPI though not statistically significant (Kruskal–Wallis test, n.s., Table 2). Additional pairwise comparison indicated that chlorhexidine reduced the amount of adherent bacteria significantly (Table 2).

The BacLight assay confirmed the general reduction of adherent bacteria induced by rinsing with the different solutions (Fig. 5). Not only after 6-h but also after 12-h effects of the rinses were observed for vital as well as for avital bacteria. After 6 h, the amount of vital bacteria was reduced significantly by all rinses except of pure hydroxyapatite microclusters in saline solution, whereas the amount of adherent avital

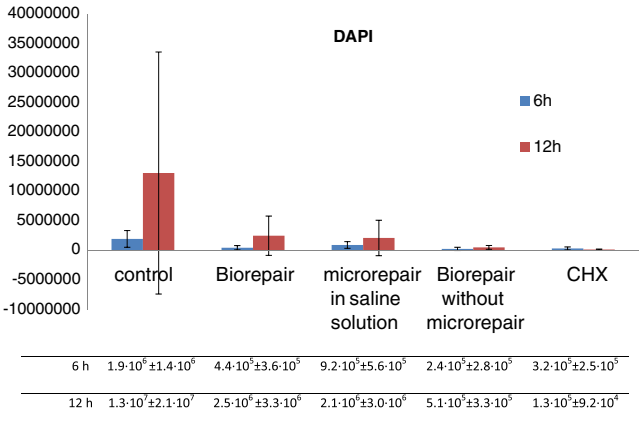



**Fig. 4** Impact of Biorepair and of chlorhexidine on the initial bacterial colonization of enamel in situ, DAPI staining. After 1-min pellicle formation, subjects were rinsed with 8 ml Biorepair, hydroxyapatite microcluster-particles in solution, and Biorepair subfraction without hydroxyapatite microcluster-particles or chlorhexidine, respectively, for 1 min. In the following, enamel slabs were exposed to the oral fluids for another 6 or 12 h. Samples carried in the oral cavity for 6 or 12 h without rinsing served as controls, MV±SD, $n=6$ subjects, two samples/subject and subgroup. The Kruskal–Wallis test indicated a significant impact of the rinses after 6 h ($p<0.001$)(12 h: n.s.). Additional pairwise comparison was carried out with the Mann–Whitney test (Table 2)

bacteria was not affected significantly, even not by CHX (Table 2). After 12 h, all rinses reduced the amount of viable bacteria significantly; the amount of avital bacteria was diminished significantly by all rises except of nanoparticles in saline solution which failed significance (Table 2).

Thereby, the proportion of avital bacteria increased as indicated by the ratio of avital to vital cells. It ranged between 0.1 (6 h) and 0.39 in controls (12 h). After 6 h, the ratio amounted to 13 in the Biorepair -group (12 h, 1.8). Also after application of Biorepair without nanoparticles (6 h, 29.0; 12 h, 15.6) and after rinsing with pure particles in saline solution (6 h, 2.0; 12 h, 18.0), a shift of the ratio was observed indicating antibacterial mechanisms besides the antiadherent effects. After rinsing with chlorhexidine, the ratio amounted to 190 (6 h) or 10.2 (12 h) which confirms the antibacterial effect of this agent.

## Discussion

In the present pilot study, the antibacterial and antiadherent efficacy of an accredited mouthrinse containing clustered hydroxyapatite microclusters has been demonstrated for the first time, the hypothesis tested was confirmed. Thereby, the effects of the preparation observed in situ were comparable to those recorded for the gold standard chlorhexidine; the in vitro experiments yielded even better results for Biorepair.

The impact on bacterial adhesion was investigated in situ, as bioadhesion processes under in vivo and in vitro conditions differ considerably [21]. Only in situ or in vivo experiments give optimal insight into the interactions of oral health care devices with the dental hard tissues and the oral biofilm. As in many previous publications, bovine enamel slabs fixed to individual upper jaw splints were adopted, the different fluorescence microscopic assays have also been used in former studies investigating initial oral bioadhesion [13, 15–17, 22–24]. This methodical approach has certain drawbacks, sometimes the bacteria are difficult to count, and typically a considerable variability of the results is observed. Hence, the power of the significant effects calculated with Mann–Whitney test ranged between 0.110 and 1.000 depending on the respective standard deviation of the compared subgroups as indicated by the post hoc power analysis (Table 2). This has to be considered when interpreting the data. However, this corresponds to previous studies and seems to be characteristic for bioadhesion in the oral cavity [13, 15–17, 22–24]. Despite this fact, the experimental setup offers direct insight into the process of bioadhesion to enamel in the oral cavity which cannot be achieved by other methods, e.g., determination of the colony forming units after desorption. This fundamental difference of the standard culture plate method on the one hand and the visualization and quantification of the adherent bacteria on the other has to be kept in mind when interpreting the present findings

**Table 2** Mann–Whitney test ($p<0.010$)

| | Biorepair | Microrepair in saline solution | Biorepair without microrepair | CHX |
|---|---|---|---|---|
| DAPI 6 h | | | | |
| control | 0.000 (0.807) | 0.050 | 0.000 (0.911) | 0.000 (0.882) |
| Biorepair | | 0.015 | 0.180 | 0.722 |
| Microrepair in saline solution | | | 0.001 (0.865) | 0.003 (0.778) |
| Biorepair without microrepair | | | | 0.143 |
| DAPI 12 h | | | | |
| control | 0.329 | 0.132 | 0.025 | 0.000 (0.350) |
| Biorepair | | 0.956 | 0.240 | 0.027 |
| Microrepair in saline solution | | | 0.377 | 0.003 (0.402) |
| Biorepair without microrepair | | | | 0.007 (0.882) |
| Viable BacLight 6 h | | | | |
| control | 0.000 (1.000) | 0.150 | 0.000 (1.000) | 0.000 (1.000) |
| Biorepair | | 0.000 (0.242) | 0.722 | 0.059 |
| Microrepair in saline solution | | | 0.001 (0.261) | 0.001 (0.277) |
| Biorepair without microrepair | | | | 0.579 |
| Viable BacLight 12 h | | | | |
| Control | 0.000 (0.177) | 0.000 (0.182) | 0.000 (0.182) | 0.000 (0.183) |
| Biorepair | | 0.094 | 0.608 | 0.391 |
| Microrepair in saline solution | | | 0.257 | 0.165 |
| Biorepair without microrepair | | | | 1.000 |
| Dead BacLight 6 h | | | | |
| Control | 0.048 (0.460) | 0.662 | 0.165 | 0.028 |
| Biorepair | | 0.020 | 0.923 | 0.872 |
| Microrepair in saline solution | | | 0.005 (0.408) | 0.006 (0.437) |
| Biorepair without microrepair | | | | 0.912 |
| Dead BacLight 12 h | | | | |
| Control | 0.003 (0.110) | 0.058 | 0.001 (0.114) | 0.000 (0.126) |
| Biorepair | | 0.080 | 0.984 | 0.007 (0.365) |
| Microrepair in saline solution | | | 0.022 | 0.000 (0.948) |
| Biorepair without microrepair | | | | 0.000 (0.506) |

The results of the post hoc power analysis for the significant differences are given in parentheses

[13, 16, 17, 22–24]. Another limitation of the present study certainly is the small number of subjects. The number of different experimental groups and the complexity of the methods was the reason to enroll a small number of participants as in many previous comparable in situ studies [16, 17, 23]. The aim of the study was to show up the assumed principal effects of the preparation on bacterial adhesion. Anyway, the quantification of the adherent bacteria based on fluorescence microscopy indicated a clear reduction of the bacterial colonization. After 6 h as well as after 12 h, distinctly less bacteria were detectable than on the respective controls. DAPI staining offers no information on the viability of the adhering bacteria. Thus, the BacLight assay was used additionally, confirming the results gained with DAPI. Furthermore, a shift of the ratio avital/vital bacteria was recorded, indicating not only antiadherent but also antibacterial properties of the tested preparations. This antimicrobial effect was confirmed in additional in vitro experiments carried out with suspensions of *S. mutans*. For this purpose, a modification of the BacLight viability assay (live/dead staining) was adopted. As shown with this assay, the tested mouthrinse yielded high antibacterial efficacy comparable to chlorhexidine. In higher dilutions, Biorepair still revealed considerable effects, whereas the antibacterial

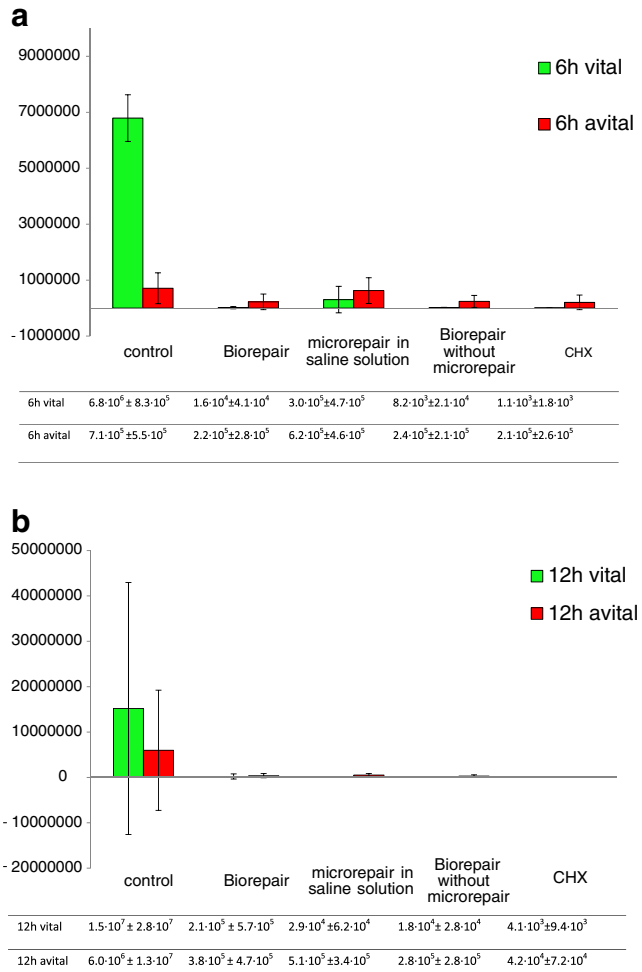



**Fig. 5** Impact of Biorepair and of chlorhexidine on the initial bacterial colonization of enamel in situ, Baclight staining. After 1-min pellicle formation, subjects were rinsed with 8 ml Biorepair, hydroxyapatite microcluster-particles in solution, and Biorepair subfraction without hydroxyapatite microcluster-particles or chlorhexidine, respectively, for 1 min. In the following, enamel slabs were exposed to the oral fluids for another 6 or 12 h. Samples carried in the oral cavity for 6 or 12 h without rinsing served as controls, MV±SD, $n=6$ subjects, two samples/subject and subgroup. **a** 6-h data: The Kruskal–Wallis test indicated a significant impact of the rinses for vital and avital bacteria ($p \leq 0.001$). **b** 12-h data: The Kruskal–Wallis test indicated a significant impact of the rinses for vital and avital bacteria ($p \leq 0.002$). Additional pairwise comparison was carried out with the Mann–Whitney test (Table 2)

impact of chlorhexidine decreased in a dose-dependent manner. However, pure nanoparticles in saline solution had no effect in vitro. Despite these observations, it has to be kept in mind that other compounds besides viable and dead bacterial cells could be stained. This is a general problem when fluorescent dyes are used and applies for all subfractions or preparations tested. Accordingly, the BacLight in vitro assay is rather a semi-quantitative approach.

Several components of the mouthrinse may account for the observed antiadherent as well as antibacterial effects. One mechanism contributing to these remarkable observations is probably the antimicrobial effect of the zinc contained in the solution [25, 26]. The antibacterial efficacy of ZnO nanoparticles has been demonstrated recently in an in vitro study [27]. Furthermore, sugar alcohols such as sorbitol and especially xylitol are known to have antimicrobial properties [28]. Also, the detergent sodium lauryl sulfate has surface active properties [29]. In addition to these well-accepted effects, the antiadherent but not the antimicrobial effects can be attributed to the size of the adopted biomimetic hydroxyapatite nano particles mimicking the smallest building units of the dental enamel, the enamel crystallites; the respective microclusters of Biorepair are composed of nano sized crystallites [30]. These particles could fit well to the initial microscopic defects occurring due to dental erosions or caries [8, 9, 30]. Moreover, the interactions with the bacteria might be facilitated. Due to size effects, hydroxyapatite nanoparticles can interact directly with the bacterial membrane. Other in vitro studies indicated that non-aggregated and clustered hydroxyl-apatite nanocrystallite particles (average size 100×10×5 nm) can bind to the bacterial membrane, thereby interacting with the adhesins to reduce bacterial adherence [31].

Pyrrolidone carboxylic acid complexed with zinc is contained in the preparation (Zn PCA). PCA is a physiological component of the natural moisturizing factor in human skin [32]. It may promote the formation of fibrillar networks in interaction with the pellicle and the accumulation of nano sized hydroxyapatite crystallites originating from hydroxyapatite microcluster clusters.

A different nanomaterial based on casein phosphopeptide stabilized amorphous calcium phosphate complexes with a diameter of 2.12 nm has been shown to have a high affinity to the bacterial surface as well as to the bacterial biofilm in general [6, 33–35]. Bacterial receptors are blocked; co-aggregation and bacterial adhesion to the pellicle are reduced [33–35]. Comparable mechanisms might occur with the tested microclusters and their nano sized components, the hydroxyapatite crystallites.

All in all, the preparation investigated in the present study seems to be an elaborate cocktail of efficacious components with impact on oral biofilm formation. This effect will also be a benefit for the prevention of periodontal diseases, but the impact on the soft tissues was not part of the investigation. Despite the promising results of the present study, further clinical studies are necessary to prove the long term effects of Biorepair in comparison or fluoride-based preparations.

Nanomaterials and their potential toxicological effects are albeit discussed extensively [36–39]. However, if biological or biomimetic nanomaterials are adopted, mimicking the smallest building units of dental enamel, side effects seem to be unlikely since it is assumed that hydroxyapatite particles dissolve in the gastric fluid in case of ingestion. Furthermore, the tested particles seem to reduce bacterial adhesion without killing the bacteria; this means biofilm management without effects on the ecology of the oral cavity.

## Conclusion

The tested preparation containing hydroxyapatite microclusters, sorbitol, and xylitol is of considerable interest for biomimetic strategies in dental prophylaxis. This applies in particular for the biofilm management. The antiadhesive effects can be attributed to components of the liquid phase, such as xylitol, but also to the hydroxyapatite microclusters, whereas antibacterial characteristics seem to be promoted mainly by components of the liquid phase, and less by the hydroxyapatite microclusters.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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