Effects of Additional Use of Bioactive Glasses or a Hydroxyapatite Toothpaste on Remineralization of Artificial Lesions in vitro

Körner, Philipp; Schleich, Jana A; Wiedemeier, Daniel B; Attin, Thomas; Wegehaupt, Florian J

Abstract: OBJECTIVES This in vitro study aimed to evaluate and compare the effect of two different bioactive glasses, a hydroxyapatite-containing, fluoride-free toothpaste (HTP) and a fluoride toothpaste (FTP) on the remineralization behavior of initial caries lesions. MATERIALS AND METHODS A total of 100 bovine enamel samples were randomly allocated to five groups of 20 samples each: NC = negative control group (artificial saliva); HTP = HTP group (Karex); FTP = FTP group (Elmex caries protection, 1,400 ppm); FTP + BGnano = FTP followed by Actimins bioactive glass; FTP + BGamorph = FTP followed by Schott bioactive glass. Radiographic documentation (advanced transversal microradiography; aTMR) was applied before and after all samples were exposed to a demineralizing gel for 10 days. Over a period of 28 days, samples were covered twice a day (every 12 h) with a toothpaste slurry of the respective test group or with artificial saliva in NC for 60 s and brushed with 15 brushing strokes. Samples in FTP + BGnano and FTP + BGamorph were additionally treated with the respective bioactive glass slurry for 30 s after brushing with the FTP. In the meantime, all samples were stored in artificial saliva. After 28 days, the structure of all samples was assessed again using aTMR and compared to the values measured after demineralization. The statistical evaluation of the integrated mineral loss was performed using Kruskal-Wallis test followed by a post hoc Conover test. RESULTS The FTP revealed the significantly highest increase of mineral content while the HTP showed the significantly lowest remineralization. Compared to artificial saliva, the use of the HTP or the combined application of FTP followed by bioactive glasses (FTP + BGnano and FTP + BGamorph) showed no significant remineralization. CONCLUSION Under remineralizing in vitro conditions, brushing with 1,400 ppm FTP induced significantly more remineralization compared to storage in artificial saliva. The additional administration of both bioactive glasses as well as the substitutional brushing with an HTP resulted in significantly less remineralization compared to brushing with 1,400 ppm FTP.

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Effects of additional use of bioactive glasses or a hydroxyapatite toothpaste on remineralization of artificial lesions *in-vitro*

Philipp Körner1,*, Jana A. Schleich1, Daniel B. Wiedemeier2, Thomas Attin1, Florian J. Wegehaupt1

1 Clinic of Conservative and Preventive Dentistry, Center of Dental Medicine, University of Zurich, Zurich, Switzerland

2 Statistical Services, Center of Dental Medicine, University of Zurich, Zurich, Switzerland

Short Title: Remineralization with hydroxyapatite or bioactive glass

*Corresponding author at:
Clinic of Conservative and Preventive Dentistry, Center of Dental Medicine, University of Zurich, Plattenstrasse 11, CH-8032 Zurich, Switzerland,
Tel: +41 44 634 34 93, Fax: +41 44 634 33 54,
E-mail: philipp.koerner@zzm.uzh.ch

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ABSTRACT

Objectives: This *in-vitro*-study aimed to evaluate and compare the effect of two different bioactive glasses, a hydroxyapatite containing, fluoride-free toothpaste and a fluoride toothpaste on the remineralization behavior of initial caries lesions.

Materials and methods: A total of 100 bovine enamel samples were randomly allocated to five groups of 20 samples each: NC = negative control group (artificial saliva); HTP = hydroxyapatite containing, fluoride-free toothpaste (Karex); FTP = fluoride toothpaste (Elmex caries protection, 1400 ppm); FTP+BG$_{nano}$ = fluoride toothpaste followed by Actimins bioactive glass; FTP+BG$_{amorph}$ = fluoride toothpaste followed by Schott bioactive glass. Radiographic documentation (advanced transversal microradiography (aTMR)) was applied before and after all samples were exposed to a demineralizing gel for 10 days. During 28 days, samples were covered twice a day (every 12 h) with a toothpaste slurry of the respective test group or with artificial saliva in NC for 60 s and brushed with 15 brushing strokes. Samples in FTP+BG$_{nano}$ and FTP+BG$_{amorph}$ were additionally treated with the respective bioactive glass slurry for 30 s after brushing with the fluoride toothpaste. In the meantime, all samples were stored in artificial saliva. After 28 days, the structure of all samples was assessed again using aTMR and compared to the values measured after demineralization. The statistical evaluation of the integrated mineral loss was performed using Kruskal-Wallis test followed by a post-hoc Conover test.

Results: The fluoride toothpaste (FTP) revealed the significantly highest increase of mineral content while the hydroxyapatite containing, fluoride-free toothpaste (HTP) showed the significantly lowest remineralization. Compared to artificial saliva, the use of the hydroxyapatite containing, fluoride-free toothpaste (HTP) or the combined application of fluoride toothpaste followed by bioactive glasses (FTP+BG$_{nano}$ and FTP+BG$_{amorph}$) showed no significant remineralization.
Conclusion: Under remineralizing in-vitro-conditions brushing with 1400 ppm fluoride toothpaste induced significantly more remineralization compared to storage in artificial saliva. The additional administration of both bioactive glasses as well as the substitutional brushing with a hydroxyapatite containing, fluoride-free toothpaste resulted in significantly less remineralization compared to brushing with 1400 ppm fluoride toothpaste.
INTRODUCTION

Although the prevalence of caries has drastically declined over the past decades, it still is one of the most common diseases worldwide [Lagerweij and van Loveren, 2015]. Dental enamel does not have a regenerative capacity after tooth eruption and caries lesions may occur at any time in the course of a lifetime [Selwitz et al., 2007]. Thus, the focus of modern dentistry should be the protection and preservation of dental hard tissues. In this context, a reduction of caries risk, the detection of tooth disease in an early stage and a preferably non-invasive approach are important factors in preventive dentistry [Wegehaupt et al., 2012]. A potentially non-invasive approach is the remineralization of initial caries lesion which can be supported by different substances. Beside established fluoride containing oral health care products, further approaches and products are discussed and evaluated in current literature. For example bioactive glasses composed from silicon dioxide, disodium oxide, calcium oxide and phosphorus pentoxide have been used in dentistry in the course of implantology, periodontal bone regeneration or treatment of dentin hypersensitivities [Hench, 2006]. They were shown to enable an in-vivo-response including osteoconductivity, adherence to bone by release of ions and the potential to form an apatite layer [Hench, 1991] and have been reported to induce mineralization of dentin surfaces [Forsback et al., 2004]. However, little is known and described in literature about the potential of bioactive glasses to promote remineralization of initial enamel caries lesions [Li et al., 2014]. Another approach is the remineralization of initial enamel lesion using artificial hydroxyapatite minerals which are seemingly analogous to natural enamel [Enax and Epple, 2018]. The artificial minerals aim to reinforce partially destroyed hydroxyapatite crystals in enamel through accumulation and incorporation of calcium and phosphate ions. While in some studies, hydroxyapatite containing toothpastes were shown to enable a remineralizing potential [Makeeva et al., 2016; Meyer et al., 2018], other studies couldn`t find a remineralizing effect for hydroxyapatite [Zhang et al., 2015; Esteves-Oliveira et al., 2017] or report missing evidence [Hellwig et al., 2018]. In this context, the microcrystalline hydroxyapatite containing toothpaste
Karex has recently been brought to market and promises caries protection without the use of fluoride. However, there are few studies investigating its remineralizing potential on initial enamel lesions [Schlagenhauf et al., 2019].

Therefore, aim of the present in-vitro-study was to determine the remineralizing potentials of either a fluoride toothpaste followed by two different kinds of bioactive glasses or a hydroxyapatite containing, fluoride-free toothpaste on initial enamel caries lesions and compare them to a fluoride toothpaste only. Hypothesis was that there is no significant difference in mineral content of samples with initial caries lesions after treating them with a fluoride toothpaste, a combination of a fluoride toothpaste followed by a bioactive glass (Actimins or Schott bioactive glass), a hydroxyapatite containing, fluoride-free toothpaste or solely with artificial saliva.

**MATERIAL AND METHODS**

The study design is illustrated in Figure 1.

**Sample preparation and allocation**

A total of 100 samples were gained from bovine incisors and cut transversal to the buccal surface into parallel enamel samples with 500 µm thickness using a water-cooled saw microtome (SP1600, Leica Microsystems AG; Heerbrugg, Switzerland). Each sample was placed between two polycarbonate platelets (Makrolon, Bayer AG; Darmstadt, Germany), embedded in a light curing resin (LC Block-Out Resin, Ultradent Products, Inc.; South Jordan, USA) and light polymerized for 30 s (Bluephase Polywave, Ivoclar Vivadent AG; Schaan, Liechtenstein). Afterwards, enamel surfaces were standardized ground flat using a mill (BFW 40/E, Proxxon; Foehren, Germany) with 25 µm diamond burr (Finierer NR 840, Busch & Co. KG; Engelskirchen, Germany). In this course, the outer cementum and enamel layer was removed. An exemplary sample is illustrated in Figure 2. All 100 prepared samples were randomly assigned into five groups of 20 samples each, labeled and placed in one of five custom
made holding devices able to carry all 20 samples of the respective group (see Figure 3). During the process of preparation and until use, all samples were stored in non-fluoridated tap water.

**Demineralization**

Before demineralization the mineral content of all samples was assessed using aTMR (see mineral analysis). Artificial initial enamel lesions were created using both, a demineralizing gel and a demineralizing solution based on a thymol stock solution with lactic acid and calcium concentrate with defined pH value 4.4. The demineralization protocol is described by Amaechi et al. [Amaechi et al., 1998]. After ten days of demineralization, a mean lesion depth of 118.1 ± 20.2 µm was measured.

**Remineralization Procedure**

During 28 days, samples were covered twice a day (every 12 h) with a group specific toothpaste slurry (mix of toothpaste and artificial saliva at a weight ratio of 1:2) (HTP: Karex, Dr. Kurt Wolff GmbH; Bielefeld, Germany) (FTP, FTP+BG$_{\text{nano}}$, FTP+BG$_{\text{amorph}}$: Elmex caries protection, GABA; Therwil, Switzerland) or with artificial saliva (NC) for 60 s and brushed with 15 brushing strokes (1 stroke/second) (Paro S39, Esro AG; Kilchberg, Switzerland). A constant brushing force of about 1.0 N was applied by fixing a 100 g weight on the head of the toothbrush (see Figure 4). After brushing with the fluoride toothpaste slurry and rinsing with tap water for 30 s, samples of FTP+BG$_{\text{nano}}$ and FTP+BG$_{\text{amorph}}$ were additionally treated with slurries prepared from the respective bioactive glasses and tap water (weight ratio 2:1) for 30 s. The bioactive glass in FTP+BG$_{\text{nano}}$ (Actimins Dental Desensitizer, Datsing Bio-Tech Co.; Beijing, China) is a commercially available dental product and claims to contain bioactive nanocrystal powder mainly composed of calcium, phosphorus, sodium and silicon. The bioactive dental glass used in FTP+BG$_{\text{amorph}}$ (G018-144 = 45S5, Schott AG; Landshut,
Germany) is a typical amorphous bioactive glass (grain size SM 4.0 = $d_{50} = 4.0 \pm 1.0 \ \mu m$) composed of the four inorganic oxides SiO$_2$, CaO, Na$_2$O and P$_2$O$_5$.

Between treatments, all samples were stored in daily refreshed artificial saliva (pH 6.5) which was formulated according to Klimek et al. [Klimek et al., 1982] but without D-glucose ($C_6H_{12}O_6$) and ascorbic acid ($C_6H_8O_6$). The toothpaste- and bioactive glass slurries were renewed every 48 h. After 28 days, the mineral content of all samples was assessed again.

**Mineral analysis**

For mineral analysis the quantitative assessment of mineral content in calcified tissues was performed using advanced transversal microradiography (aTMR) as recently described by Becker et al. [Becker et al., 2020]. Mineral uptake/loss was assessed by calculating the difference ($\Delta Z$ in Vol.-% x $\mu m$) between the respective surface before and after remineralization (integrated mineral loss).

**Statistical analysis**

The dataset was analyzed using the software R [R Core Team, 2015] and the associated packages ggplot2 [Wickham, 2016] and PMCMR [Pohlert, 2014]. Due to deviations in homogeneity of variance for the measurement after 28 days, a non parametric test (Kruskal-Wallis), followed by a post-hoc Conover test was computed to check for differences between the five test groups. Additionally, corrections for multiple testing were made using the Bonferroni-Holm method. The level of significance was set at 5%.
RESULTS

After 10 days of demineralization each of the 100 samples showed caries-like lesions with relatively high mineralized outer surfaces compared to the demineralized subsurface lesions. The mean lesion depth was $118.1 \pm 20.2 \, \mu m$ and the mean integrated mineral loss $3055.6 \pm 632.7 \, \text{Vol.-}\% \times \mu m$. There were no significant differences between the groups. Within the groups, mineral loss ranged from $2915.5 \pm 716.7 - 3354.1 \pm 409.1 \, \text{Vol.-}\% \times \mu m$. The difference of integrated mineral loss ($\Delta Z$ in Vol.-\% x \mu m) between baseline measurement ($\Delta Z_{\text{Baseline}}$ in Vol.-\% x \mu m) and measurement after 28 days ($\Delta Z_{\text{Remin}}$ in Vol.-\% x \mu m) for all five test groups is illustrated in Figure 5.

The fluoride toothpaste (FTP) revealed the significantly highest increase of mineral content ($p < 0.05$) overall and also showed significantly higher remineralization than artificial saliva (NC). The hydroxyapatite-containing toothpaste (HTP) showed the significantly lowest remineralization ($p < 0.05$). Compared to NC, no significant remineralization could be achieved using the hydroxyapatite containing, fluoride-free toothpaste (HTP) or the combined application of fluoride toothpaste followed by bioactive glasses (FTP+BG$_{\text{nano}}$ and FTP+BG$_{\text{amorph}}$).
DISCUSSION

The present *in-vitro*-study revealed significant differences between the test groups in terms of remineralization potential of initial caries lesions leading to the denial of the hypothesis. The fluoride toothpaste showed the significantly highest increase of mineral content after 28 days, the hydroxyapatite containing, fluoride-free toothpaste the lowest. The combined application of fluoride toothpaste followed by the respective bioactive glasses revealed no significantly higher remineralization compared to the control group.

Enamel samples in this study were prepared from bovine incisors which have been used and discussed in multiple studies investigating demineralized dental hard tissues and can be regarded as suitable substitute for human enamel [Kielbassa et al., 2001]. Artificial caries lesions were created according to an established demineralization protocol by Amaechi et al. [Amaechi et al., 1998] with additional thymol which was added for antibacterial reasons. The mean lesion depth in this study (118.1 ± 20.2 µm) was in the range of most *in-vitro*- and *in-situ*-studies investigating de- and remineralization of teeth (50 – 150 µm) [White, 1995]. The quantity (2 times/day) and duration (60 s) of toothbrushing, as well as the amount (n = 15), frequency (1 stroke/second) and application force (1 N) of the performed brushing strokes aimed to simulate realistic *in-situ*-conditions. However, it should be mentioned that no biofilm had to be removed in this model, thus brushing was only performed to apply the tested agents. Furthermore, it has to be considered that the storage of samples in artificial saliva between the brushing periods is not able to adequately imitate the intraoral mineralizing processes [White, 1995]. Remineralization *in-vitro* is likely to be greater than *in-vivo* as proteins (e.g. statherine) in natural human saliva bind calcium and thus inhibit calcium phosphate precipitation [Shellis et al., 2011]. Other modifying factors such as pellicle formation, bacteria and fluoride in saliva and plaque fluid were not regarded in this study. Furthermore, it has to be considered that compared to a pH-cycling-model, the study design does also not adequately reflect the dynamic process of caries progression with alternating phases of de- and remineralization. Thus, this
study can provide information about the remineralizing potential of the used products, but not about caries inhibiting properties. As products claiming to enable remineralization should be capable to induce remineralization in a net-remineralizing \textit{in-vitro}-model, this study has to be regarded as initial investigation. Agents enabling net-remineralization can then be investigated in a model with periodic pH-changes (simulating net-re- and net-demineralizing conditions) as a next step.

Transversal microradiography (TMR) is commonly used for mineral analysis of calcified tissues and assessment of change in mineral content of enamel samples [Gmür et al., 2006]. The advanced technique as recently described by Becker et al. [Becker et al., 2020] was used in this study as it enables even more precise and reliable analyses going along with facilitated sample preparation and test procedures.

The significantly highest increase of mineral content was observed for the fluoride toothpaste (1400 ppm). In a review of literature [Li et al., 2014] as well as in other studies [Lippert and Juthani, 2015; Wierichs et al., 2017] investigating remineralization of enamel, likewise pronounced remineralizing effects of fluoride are described which can mainly be attributed to a precipitation of apatite crystals in enamel. The effects of fluoride on the oral cavity and the entire human organism are well known and investigated [Buzalaf and Whitford, 2011; Buzalaf et al., 2011]. Therefore, current guidelines consistently recommend fluoridation of initially demineralized enamel to enhance remineralization [Geurtsen et al., 2016]. Several studies demonstrated that the effect of fluoride agents might be increased by reducing their pH [Brighenti et al., 2006; Alves et al., 2007; Yamazaki and Margolis, 2008]. The adsorption of mineral ions into the lesion increases with decreasing pH. Thus, the remineralizing effect of acidic agents is supposed to be significantly higher than the effect observed for neutral agents [Yamazaki and Margolis, 2008]. The fluoride toothpaste used in this study is acidic, whereas the hydroxyapatite containing, fluoride-free toothpaste (Karex) seems to be neutral. Potentially, this fact might have influenced the results and contributed to the significantly lowest
remineralizing potential of the investigated micro-crystalline hydroxyapatite containing, fluoride-free toothpaste. Still, it has to be considered, that *in-vivo* saliva might buffer the low pH of an acidic toothpaste potentially alleviating this effect.

Different studies describe ambivalent effects of micro-hydroxyapatite on the remineralizing potential of demineralized enamel [Li et al., 2008; Huang et al., 2011; Schlagenhauf et al., 2019]. At the same time, other studies report a significant remineralizing effect for nano-hydroxyapatite [Huang et al., 2010; Makeeva et al., 2016; Manchery et al., 2019]. In general, it has to be taken into consideration that little is known about the interaction of applied hydroxyapatite minerals with dental hard tissues and to this day, it is not conclusively clarified whether a desired remineralizing or inhibiting effect on caries, erosion or plaque can be achieved using this active substance [Hellwig et al., 2018]. Possibly, there might be differences in the effect mechanism related to the size of hydroxyapatite particles [Li et al., 2008]. An *in-vitro*-study showed that nano-hydroxyapatite enables remineralization of demineralized enamel while no difference could be observed between the negative control (water) and micro-hydroxyapatite [Huang et al., 2011]. The investigated toothpaste in this study contains micro-hydroxyapatite. It might be suspected that the micro-hydroxyapatite particles are less able to penetrate the lesion body of demineralized enamel but at the same time might form a sealing deposition on the porous surface limiting the diffusion and accumulation of salivary minerals in the lesion and thus hampering remineralization. This would explain the lowest measured remineralizing potential in this study which was even lower than the one in the control group where only artificial saliva was applied.

The Schott bioactive glass is a typical amorphous bioactive glass and claims to have a remineralizing and strengthening effect on human hard tissue and to be beneficial for the treatment of acid-caused enamel erosion by releasing ions able to form a mineral matrix equivalent to that of hydroxyapatite. The Actimins bioactive glass seems to exhibit SiO$_2$ as crystalline phase and contains bioactive nanocrystal powder. Beside functioning as desensitizer,
Actimins claims to promote dental remineralization by raising the intraoral pH value to facilitate the formation of hydroxyapatite, by releasing minerals (silica, calcium, phosphorous) and by crystalizing an emerged calcium and phosphate layer into carbonated hydroxyapatite. A significant increase of enamel surface hardness (KHN) could be observed *in-vitro* for specimens treated with bioactive glasses [Milly et al., 2014]. However, the glasses seem not to be able to reduce lesion depth. It is rather assumed that the precipitation of calcium und phosphate is confined to outer surface layers [Milly et al., 2014]. Furthermore, it has to be considered that the interaction of calcium, phosphate and fluoride might result in precipitation of ions leading to a reduced amount of minerals for remineralization on the one hand and a limited diffusion of minerals into the lesion depth caused by precipitate accumulation on the lesion surface on the other [Wefel, 2009]. The results of this study seem to confirm this assumption as in the bioactive glass containing groups significantly less remineralization was observed compared to the group in which solely the fluoride toothpaste was applied. Still, it should be kept in mind that the additional application procedure of the bioactive glass slurry in FTP+BG$_{\text{nano}}$ and FTP+BG$_{\text{amorph}}$ compared to no further intervention in NC, HTP and FTP might be a potential bias.

Clearly, further *in-vitro*- and *in-vivo*-studies, especially investigating bioactive glasses, are needed in order to gain better knowledge and understanding about the different influence factors and their effect on initial caries lesions. Especially pH-cycling protocols should be included and to further understand the mode of action of bioglasses, the release of relevant active ions should be determined.

**CONCLUSION**

Within the limitations of this study the following conclusions can be drawn after 28 days testing under remineralizing *in-vitro*-conditions:
1. Brushing with 1400 ppm fluoride toothpaste enabled significantly more remineralization compared to storage in artificial saliva.

2. The application of bioactive glasses in addition to 1400 ppm fluoride toothpaste resulted in significantly less remineralization compared to brushing only with the 1400 ppm fluoride toothpaste.

3. Brushing with the micro-hydroxyapatite containing, fluoride-free toothpaste showed significantly less remineralization than brushing with 1400 ppm fluoride toothpaste.

Therefore, affected patients with initial caries lesions should rather choose a fluoride toothpaste instead of the other tested toothpaste systems.

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STATEMENT OF ETHICS

The study required no ethical approval. Bovine teeth were received as byproduct from a local slaughterhouse in Zurich.

DISCLOSURE STATEMENT

The authors declare no potential conflicts of interest with respect to the authorship and publication of this article.
AUTHOR CONTRIBUTIONS

Philipp Körner: Resident, Clinic of Conservative and Preventive Dentistry, Center of Dental Medicine, University of Zurich, Plattenstrasse 11, CH-8032 Zurich, Switzerland.
Wrote the manuscript.

Jana A. Schleich: Postgraduate, Clinic of Conservative and Preventive Dentistry, Center of Dental Medicine, University of Zurich, Plattenstrasse 11, CH-8032 Zurich, Switzerland. Performed the clinical experiment, wrote the doctor’s thesis, proofread the manuscript.

Daniel B. Wiedemeier: Research Associate in Data Analysis and Statistics, Center of Dental Medicine, University of Zurich, Plattenstrasse 11, CH-8032 Zurich, Switzerland. Performed statistical evaluation, proofread the manuscript.

Thomas Attin: Professor and Director, Clinic of Conservative and Preventive Dentistry, Center of Dental Medicine, University of Zurich, Plattenstrasse 11, CH-8032 Zurich, Switzerland. Research idea, contributed substantially to discussion and writing the paper, proofread the manuscript.

Florian J. Wegehaupt: Head of Division of Preventive Dentistry and Oral Epidemiology, Clinic of Conservative and Preventive Dentistry, Center of Dental Medicine, University of Zurich, Plattenstrasse 11, CH-8032 Zurich, Switzerland. Research idea, hypothesis, experimental design, contributed substantially to discussion and writing the paper, proofread the manuscript.
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FIGURE LEGENDS

Fig. 1. Study design.

Fig. 2. Enamel sample placed between two polycarbonate platelets and embedded in a light curing resin.

Fig. 3. Custom made holding devices able to carry all 20 samples of the respective group.

Fig. 4. 100 g weight placed on the head of a toothbrush to enable a constant brushing force.

Fig. 5. Boxplot of the difference of integrated mineral loss [Vol.-% x µm] in the five test groups (NC, HTP, FTP, FTP+BG_{nano}, FTP+BG_{amorph}). The higher the difference in integrated mineral loss, the more remineralization took place. The horizontal line in the box represents the median value, the box represents the 25th and 75th percentile and whiskers represent the 5th and 95th percentile. Significantly different values are marked with different capital letters.
Bovine enamel samples (n = 100)

Random allocation to five groups (n = 20 per group)

1st assessment of mineral content (aTMR)

Demineralization for 10 d

2nd assessment of mineral content (aTMR)

Remineralization-Cycle for 28 d (every 12 h)

|                  | NC | HTP | FTP | FTP+BG_{nano} | FTP+BG_{amorph} |
|------------------|----|-----|-----|--------------|-----------------|
| Artificial saliva|    |     |     |              |                 |
| Karex            |    |     |     |              |                 |
| Elmex caries     |    |     |     |              |                 |
| protection       |    |     |     |              |                 |
| Elmex caries     |    |     |     |              |                 |
| protection       |    |     |     |              |                 |
| Elmex caries     |    |     |     |              |                 |
| protection       |    |     |     |              |                 |

Rinsing with tap water for 30 s

- Application (30 s) of slurries prepared from bioactive glasses and water

- Actimins

- Schott

- Rinsing with tap water for 30 s

Storage in artificial saliva between the cycles

3rd assessment of mineral content (aTMR)

Calculation of mineral recovery/loss ($\Delta Z$ in Vol.-% x $\mu$m) = integrated mineral loss
