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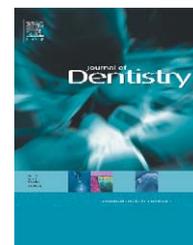
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## Enamel and dentine remineralization by nano-hydroxyapatite toothpastes

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### ABSTRACT

**Objectives:** This *in vitro* study evaluated the effects of nano-hydroxyapatite (n-HAp) toothpastes on remineralization of bovine enamel and dentine subsurface lesions.

**Methods:** Specimens were demineralized, randomly divided into five groups, and exposed to an aqueous remineralizing solution for two and five weeks (37 °C). Brushing procedures were performed with the respective toothpaste/storage solution slurry twice daily (2 × 5 s; total contact time of the slurries 2 × 120 s/d): storage in remineralizing solution only (0); additional brushing with B (20 wt% zinc carbonate nano-hydroxyapatite, ZnCO<sub>3</sub>/n-HAp); BS (24 wt% ZnCO<sub>3</sub>/n-HAp); E (0.14 wt% amine fluoride); or A (7 wt% pure n-HAp). Differences in mineral loss (ΔΔZ) before and after storage/treatment were microradiographically evaluated.

**Results:** Dentine groups 0, B, BS, and A showed significantly higher ΔΔZ values compared to E ( $p < 0.05$ ; ANOVA). Enamel ΔΔZ values of group A were significantly higher compared to group E ( $p < 0.05$ ), whilst no significant differences of these groups could be observed compared to 0, B, and BS ( $p > 0.05$ ).

**Conclusions:** With the *in vitro* conditions chosen, toothpastes containing n-HAp revealed higher remineralizing effects compared to amine fluoride toothpastes with bovine dentine, and comparable trends were obtained for enamel.

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## 1. Introduction

The process of de- and remineralization is governed by the degree of saturation of oral fluids (saliva and plaque) with respect to apatite minerals.<sup>1</sup> Given an appropriate change in conditions, remineralization may become the predominant process, thus leading to lesion repair.<sup>2,3</sup> To enhance lesion remineralization, increase of calcium or fluoride concentrations in the oral fluids would seem reasonable.<sup>4</sup>

For this purpose, fluorides have traditionally been used in various formulations, and the concomitant cariostatic mechanisms can be explained by an increased driving force for fluoridated apatites.<sup>5</sup> The decline in dental caries experienced in most industrialized countries can be attributed largely to the widespread use of fluorides,<sup>6</sup> and this preventive effect is mainly due to the formation of calcium fluoride-like precipitates hampering demineralization, whilst fluoride levels needed for remineralization are assumed to be higher than those to prevent lesion formation.<sup>7</sup>

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Nano-hydroxyapatite (n-HAp) is considered one of the most biocompatible and bioactive materials, and has gained wide acceptance in medicine and dentistry in recent years.<sup>8</sup> Whilst former attempts to use hydroxyapatites clinically did not succeed, synthesis of nano-scaled zinc carbonate hydroxyapatite ( $\text{ZnCO}_3/\text{n-HAp}$ ) yielded a significant progress, and showed considerable affinity to the enamel surface.<sup>9</sup> Nano-sized particles have similarity to the apatite crystals of tooth enamel in morphology and crystal structure.<sup>10</sup> Recently, a few reports have shown that n-HAp has some potential to repair dental enamel,<sup>11–15</sup> but no information is available for established dentine lesions. To date, it can be summarized that for remineralization of subsurface lesions by n-HAp containing products, different formulations have been developed, and early data have suggested remineralizing properties.<sup>8</sup> However, evidence is still incomplete to substantiate claims by manufacturers,<sup>16,17</sup> and, so far, none of these products has been shown to be more effective than fluorides.

Therefore, the aim of the present study was to evaluate the effects of daily treatment with different n-HAp toothpastes on the remineralization of bovine enamel and dentine subsurface lesions stored in a remineralizing solution. An amine fluoride toothpaste was used as a reference for comparative reasons. We hypothesized ( $H_0$ ) that additional brushing with n-HAp or fluoride toothpastes would result in equal remineralizing effects compared to a pure remineralizing solution (positive control). This null hypothesis was tested against the alternative hypothesis of a difference between products.

## 2. Materials and methods

### 2.1. Specimen preparation and demineralization

From 35 bovine incisors 70 enamel specimens ( $6 \times 4 \times 4 \text{ mm}^3$ ) were prepared from the labial aspects. Dentine specimens ( $n = 85$ ) derived from the cervical regions ( $4 \times 3 \times 4 \text{ mm}^3$ ), and were prepared as described previously.<sup>18</sup> One quarter of each specimen's surface was covered with acid-resistant nail varnish (Jet-Set; Loreal, Karlsruhe, Germany) to serve as sound control. Following earlier studies, enamel lesions were prepared by immersion in a solution (5 l) containing  $6 \mu\text{M}$  methylhydroxydiphosphonate (MHDP), 3 mM calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), 3 mM potassium dihydrogen

phosphate ( $\text{KH}_2\text{PO}_4$ ), and 50 mM acetic acid ( $\text{CH}_3\text{COOH}$ ) (Merck, Darmstadt, Germany) at pH 4.95 in an incubator ( $37^\circ\text{C}$ ; BR 6000; Heraeus Kulzer) for 14 days.<sup>19</sup> Dentine lesions were prepared by immersion in a solution containing 0.0476 mM sodium fluoride (NaF), 2.2 mM calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), 2.2 mM potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), 50 mM acetic acid ( $\text{CH}_3\text{COOH}$ ), and 10 mM potassium hydroxide (KOH) (all chemicals from Merck) at pH 5.0 ( $37^\circ\text{C}$ ) for five days.<sup>20</sup> The solutions were not stirred or replaced during the demineralization period. The pH values of the demineralizing solutions were monitored daily (pH-electrode GE 100 BNC connected to pH-meter GMH 3510; Greisinger, Regenstauf, Germany), and slight elevations were corrected with small amounts of hydrochloric acid (HCl) to maintain a constant pH value between 4.94 and 4.96 for enamel as well as 4.99 and 5.01 for dentine during the demineralization period. Standard buffer solutions (Sigma-Aldrich, Steinheim, Germany) with nominal pH values of 4.0 and 7.0, respectively, and with an accuracy of 0.01 units were used to calibrate the pH metre.

### 2.2. Solution preparation and treatment of the specimens

Subsequently, half of each demineralized surface was covered with nail varnish (control of baseline demineralization) again. Specimens were randomly divided into five groups (enamel  $n = 14$ ; dentine  $n = 17$ ), and were separately stored in a remineralizing solution<sup>21,22</sup> for two and five weeks ( $37^\circ\text{C}$ ). In accordance with EN ISO 11609 (European standards for preparing artificial saliva/toothpaste slurries), the respective toothpaste (Table 1) was diluted in three parts (1:3) of the remineralizing solution to obtain a homogeneous slurry. Test products were commercially available toothpastes with either  $\text{ZnCO}_3/\text{n-HAp}$  or n-HAp (all without any fluorides) as active ingredients; a toothpaste containing amine fluorides served as reference group (Table 1). The pH values of the slurries were measured directly after preparation.

Subsequently, specimens were manually brushed by hand with a soft toothbrush (Meridol; GABA, Lörrach, Germany), and with minimum pressure; brushing procedures were carried out in each subgroup twice daily for 5 s each (with an additional contact time with the slurry of 115 s, thus resulting in a total contact time of 120 s). After each brushing treatment, specimens were washed with deionized water (10 s). Every two

**Table 1 – Treatment products, regimes and specimen grouping.**

Treatment products	Code	Active compound	Concentration	Treatment	pH
(Remineralizing solution)	0	Calcium and phosphate	1.5 mM	Only storage no further treatment	7.00
$\text{ZnCO}_3/\text{n-HAp}^a$	B	Zinc carbonate-	20 wt%	Slurry (ratio 1:3) from toothpaste	7.39
batch no. 928751019		nano-hydroxyapatite		with the remineralizing storage	
$\text{ZnCO}_3/\text{n-HAp}^a$	BS		24 wt%	solution and brushing for 5 s with a	7.34
batch no. 90001091_26-01-2010				total contact time of 120 s twice	
Fluoride <sup>b</sup>	E	Aminefluoride	0.14 wt%	daily in all groups	5.24
batch no. 435909					
n-HA <sup>c</sup>	A	Nano-hydroxyapatite	7 wt%		6.94
batch no. 20.10.11					

<sup>a</sup> BioRepair and BioRepair Sensitive; Dr. Kurt Wolff, Bielefeld, Germany.

<sup>b</sup> Elmex Kariesschutz; GABA, Lörrach, Germany.

<sup>c</sup> ApaCare; Cumdente, Tübingen, Germany.

days the remineralizing solutions were replenished (250 ml per group each time), and pH values were checked. After two weeks, half of the exposed surfaces were nail-varnished to evaluate interim effects (effect after two weeks).

### 2.3. Transverse microradiography

After *in vitro* exposure, thin sections (100  $\mu\text{m}$ ) were prepared. Following, contact microradiographs of the specimens were obtained by transverse microradiography, and these were evaluated using a dedicated software (TMR for Windows 2.0.27.2; Inspektor Research System, Amsterdam, The Netherlands) as described previously,<sup>23,24</sup> ethylene glycol ( $\text{C}_2\text{H}_4(\text{OH})_2$ ) (99%; Sigma-Aldrich, Munich, Germany) was used to avoid shrinkage of dentine lesions.<sup>25</sup> The investigator was blinded with respect to group allocation.

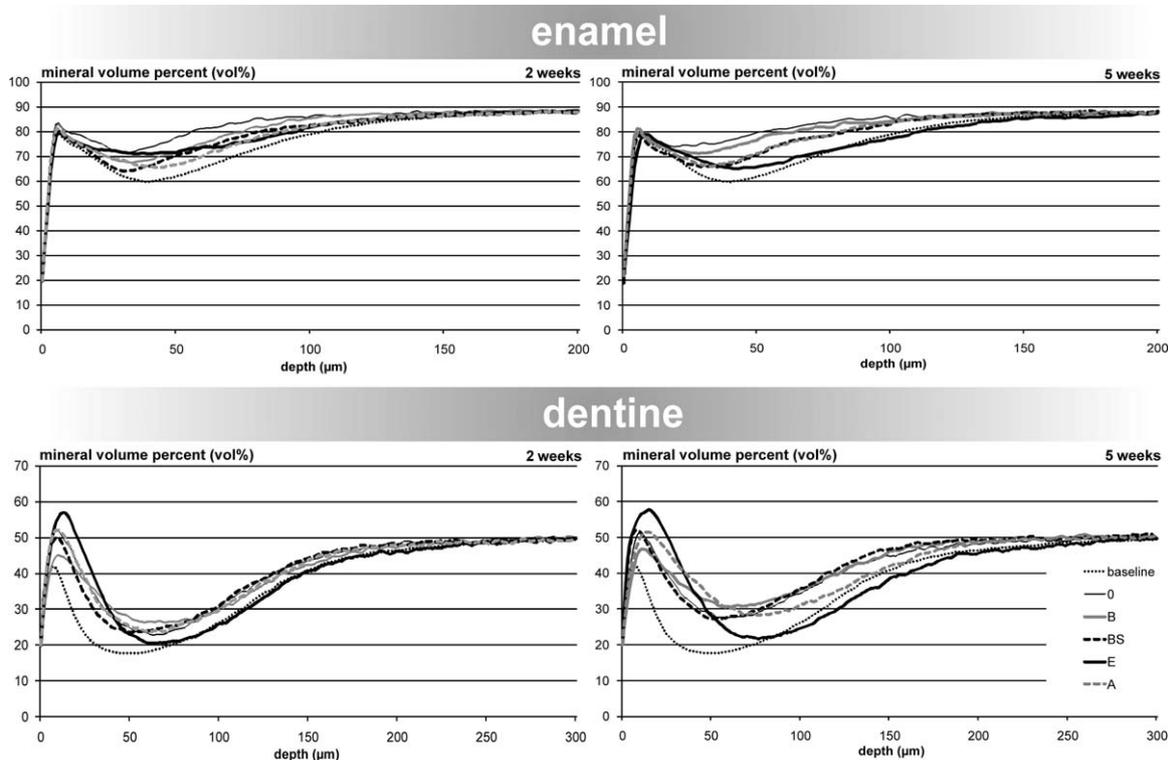
Mineral density profiles were evaluated from which integrated mineral loss ( $\Delta Z$ ) and lesion depth (LD) values were calculated following initial demineralization ( $\Delta Z_{\text{Demin}}$ ,  $\text{LD}_{\text{Demin}}$ ) and after treatment for either two ( $\Delta Z_{\text{Effect } 2}$ ,  $\text{LD}_{\text{Effect } 2}$ ) or five weeks ( $\Delta Z_{\text{Effect } 5}$ ,  $\text{LD}_{\text{Effect } 5}$ ). Each pair of values was corrected by subtracting the respective values for sound enamel ( $\Delta Z_{\text{Sound}}$  and  $\text{LD}_{\text{Sound}}$ ) before data analysis. Changes in mineral loss ( $\Delta\Delta Z_2 = \Delta Z_{\text{Demin}} - \Delta Z_{\text{Effect } 2}$ ,  $\Delta\Delta Z_5 = \Delta Z_{\text{Demin}} - \Delta Z_{\text{Effect } 5}$ ) and lesion depth ( $\Delta\text{LD}_2 = \text{LD}_{\text{Demin}} - \text{LD}_{\text{Effect } 2}$ ,  $\Delta\text{LD}_5 = \text{LD}_{\text{Demin}} - \text{LD}_{\text{Effect } 5}$ ) were analyzed for treatment differences. Positive and negative values of  $\Delta\Delta Z$  or  $\Delta\text{LD}$  indicated net remineralization and net demineralization, respectively.

### 2.4. Statistical analyses

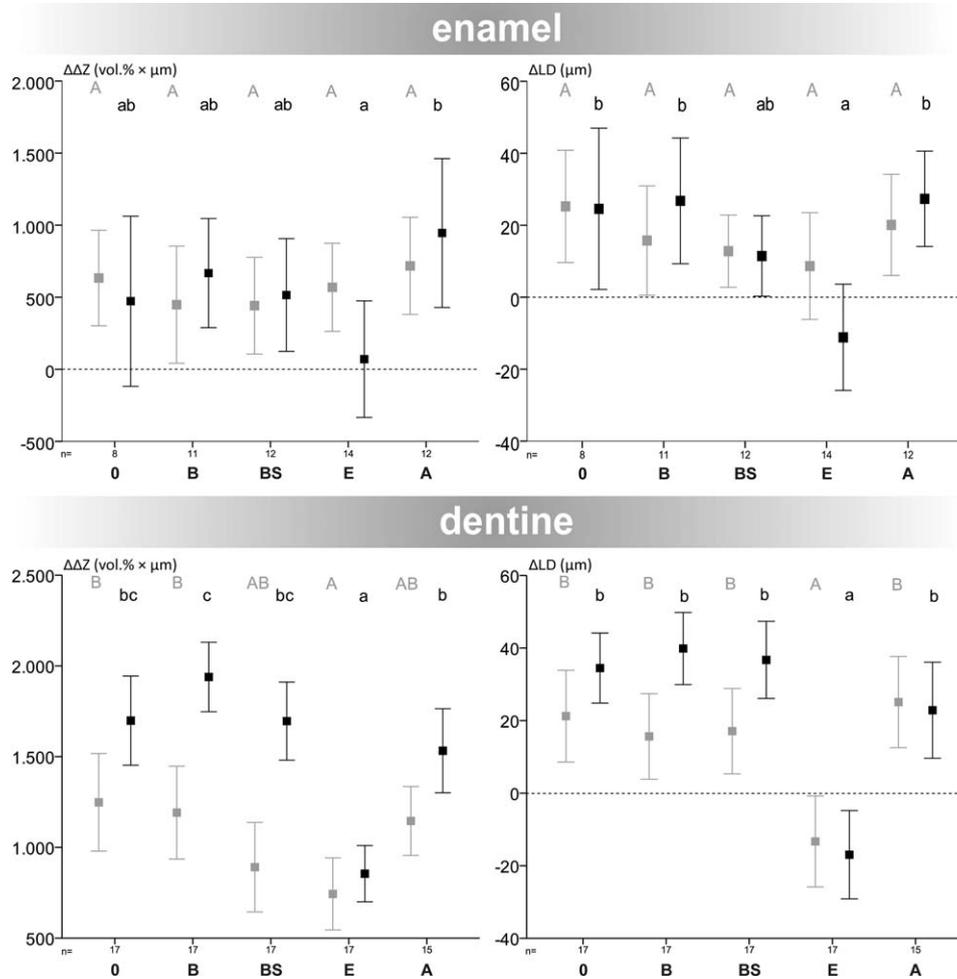
Normal distribution of  $\Delta\Delta Z$  and  $\Delta\text{LD}$  was tested (Kolmogorov-Smirnov). For overall comparison of solutions one-way ANOVA was applied; pairwise comparisons used Tukey's *post hoc* tests. Comparisons of changes in mineral loss and lesion depth before and after storage/treatment were performed by adjusted paired t-test (Bonferroni; correction factor  $\times 5$ ). Level of significance was set at  $\alpha = 0.05$  (two-sided). Statistical analyses were performed using PASW for Windows (version 18.0; SPSS, Chicago, IL).

## 3. Results

Thirteen enamel and two dentine specimens were lost with preparation procedures. All de- and remineralized specimens developed subsurface lesions consistently revealing a surface layer that was more mineralized than the body of the lesion, and none of the treatment regimens yielded surface erosions. Baseline  $\Delta Z_{\text{Demin}}$  and  $\text{LD}_{\text{Demin}}$  values (after demineralization) did not differ significantly between the various groups ( $p > 0.161$ ; ANOVA). With dentine, specimens of group E revealed a hypermineralized surface layer (with an increased thickness), and subsurface lesions could be found with all groups (Fig. 1). The pH values of the remineralizing solutions remained stable for the experimental period.



**Fig. 1** – Mean mineral density profiles (enamel and dentine) after two and five weeks with or without additional toothpaste treatment (0 = no further treatment; B =  $\text{ZnCO}_3/\text{n-HAp}$  20 wt%; BS =  $\text{ZnCO}_3/\text{n-HAp}$  24 wt%; E = amine fluoride 0.14 wt%; A = n-HA 7 wt%) compared to baseline. Lesion bodies and surface layers of baseline lesions consistently remineralized; hypermineralization of dentine surface layer occurred with group E, but without any decrease of lesion depths.



**Fig. 2 – Means and confidence intervals (95%; enamel and dentine) of differences in mineral change ( $\Delta\Delta Z$ ; vol%  $\times$   $\mu\text{m}$ ) and lesion depth ( $\Delta LD$ ;  $\mu\text{m}$ ) after two (grey) and five weeks (black) storage/treatment (0 = only storage and no further treatment; B = ZnCO<sub>3</sub>/n-HAp 20 wt%; BS = ZnCO<sub>3</sub>/n-HAp 24 wt%; E = amine fluoride 0.14 wt%; A = n-HA 7 wt%). Different letters indicate significant differences between groups within each storage/treatment period ( $p < 0.05$ ; ANOVA, Tukey post hoc test).**

Enamel  $\Delta\Delta Z_{\text{Effect } 2}$  and  $\Delta LD_{\text{Effect } 2}$  values did not differ significantly between groups ( $p > 0.705$ ; ANOVA, Tukey; Fig. 2).  $\Delta\Delta Z_{\text{Effect } 5}$  values of group A were significantly higher compared to group E ( $p = 0.017$ ), whilst no significant differences of both groups could be observed compared to 0, B, and BS ( $p > 0.221$ ). Comparable results were evaluated for lesion depths after both periods. With dentine, significantly higher  $\Delta\Delta Z_{\text{Effect } 2}$  values could be observed for groups 0 and B compared to E ( $p < 0.05$ ), whilst no differences could be seen compared to BS and A ( $p > 0.101$ ). Groups 0, B, BS, and A showed significantly higher  $\Delta\Delta Z_{\text{Effect } 5}$  and  $\Delta LD_{\text{Effect } 2}/\Delta LD_{\text{Effect } 5}$  values compared to E ( $p < 0.05$ ).

Enamel groups 0, E, and A showed significantly decreased  $\Delta Z_{\text{Effect } 2}$  values compared with baseline demineralization ( $p < 0.05$ ; adjusted t-test, Table 2); B and A significantly decreased  $\Delta Z_{\text{Effect } 5}$  values ( $p < 0.05$ ). Comparable LD<sub>Effect 2</sub> / LD<sub>Effect 5</sub> values were observed. All dentine specimens revealed significantly decreased  $\Delta Z_{\text{Effect } 2}/\Delta Z_{\text{Effect } 5}$  values if compared with baseline ( $p < 0.05$ ). LD<sub>Effect 5</sub> values of groups 0, B, BS, and A decreased significantly compared with baseline ( $p < 0.05$ ), whereas values increased for E ( $p < 0.05$ ).

#### 4. Discussion

The present *in vitro* study mainly showed that the different nano-hydroxyapatite toothpastes exert similar capacities to remineralize enamel and dentine subsurface lesions. Furthermore, the fluoride toothpaste displayed the lowest remineralizing effects on both hard tissues, along with an increase in lesion depths. Thus, the null hypothesis (stating that additional brushing with n-HAp or fluoride toothpastes would not result in significantly different remineralizing effects compared to control) was partially rejected.

Rationales for using bovine enamel and dentine specimens have been discussed previously,<sup>26</sup> and this source represents an accepted substitute for human dental hard substances.<sup>27-29</sup> Furthermore, several individual factors could have potential impact on remineralization (*e.g.*, behavioural changes, activity of the lesion, depth of the lesion, diet, stimulation of salivary flow, antibacterial and plaque removal strategies, brushing with fluoride toothpaste),<sup>3,30,31</sup> and these factors may modulate the natural process of lesion arrest (or repair).

**Table 2 – Means with confidence intervals (CI 95%) of mineral losses ( $\Delta Z$ ; vol%  $\times$   $\mu\text{m}$ ) and lesion depths (LD;  $\mu\text{m}$ ) of enamel and dentine specimens after *in vitro* demineralization ( $\Delta Z_{\text{Demin}}$ ,  $\text{LD}_{\text{Demin}}$ ) and storage/treatment for two ( $\Delta Z_{\text{Effect 2}}$ ,  $\text{LD}_{\text{Effect 2}}$ ) and five weeks ( $\Delta Z_{\text{Effect 5}}$ ,  $\text{LD}_{\text{Effect 5}}$ ).**

Enamel								
Code	Mineral loss (vol% $\times$ $\mu\text{m}$ )							
	$\Delta Z_{\text{Demin}}$		$\Delta Z_{\text{Effect 2}}$			$\Delta Z_{\text{Effect 5}}$		
	Mean	CI 95%	Mean	CI 95%	<i>p</i>	Mean	CI 95%	<i>p</i>
O	1288	942;1633	655	420;889	0.015	816	410;1221	1.000
B	1572	1014;2131	1124	705;1543	0.170	905	309;1502	0.015
BS	1848	1236;2460	1407	623;2191	0.075	1333	838;1828	0.070
E	1633	1317;1948	1064	625;1503	0.005	1563	994;2131	1.000
A	2147	1547;2746	1429	817;2042	0.005	1202	737;1666	0.010
Code	Lesion depth ( $\mu\text{m}$ )							
	$\text{LD}_{\text{Demin}}$		$\text{LD}_{\text{Effect 2}}$			$\text{LD}_{\text{Effect 5}}$		
	Mean	CI 95%	Mean	CI 95%	<i>p</i>	Mean	CI 95%	<i>p</i>
O	83	67;97	58	45;71	0.030	58	39;78	0.180
B	86	70;102	70	52;88	0.220	59	40;78	0.035
BS	90	75;104	77	57;97	0.085	78	61;95	0.230
E	87	77;97	79	61;96	1.000	99	79;118	0.635
A	102	91;114	82	62;102	0.045	75	61;89	0.005
Dentine								
Code	Mineral loss (vol% $\times$ $\mu\text{m}$ )							
	$\Delta Z_{\text{Demin}}$		$\Delta Z_{\text{Effect 2}}$			$\Delta Z_{\text{Effect 5}}$		
	Mean	CI 95%	Mean	CI 95%	<i>p</i>	Mean	CI 95%	<i>p</i>
O	3916	3540;4291	2667	2400;2935	0.0005	2217	1923;2511	0.0005
B	3919	3632;4205	2727	2448;3007	0.0005	1980	1739;2220	0.0005
BS	3708	3539;3878	2818	2579;3056	0.0005	2013	1802;2224	0.0005
E	3888	3605;4172	3145	2876;3413	0.0005	3033	2751;3316	0.0005
A	3870	3533;4207	2724	2465;2983	0.0005	2337	1961;2713	0.0005
Code	Lesion depth ( $\mu\text{m}$ )							
	$\text{LD}_{\text{Demin}}$		$\text{LD}_{\text{Effect 2}}$			$\text{LD}_{\text{Effect 5}}$		
	Mean	CI 95%	Mean	CI 95%	<i>p</i>	Mean	CI 95%	<i>p</i>
O	197	180;214	176	162;189	0.015	162	144;180	0.0005
B	191	181;201	176	162;189	0.065	151	140;163	0.0005
BS	185	172;198	168	152;184	0.035	149	140;157	0.0005
E	193	179;206	206	193;219	0.195	210	195;224	0.045
A	199	182;215	173	160;187	0.005	176	153;198	0.010

*p*-Values of differences between the values after demineralization and storage/treatment for either two or five weeks within each group as analyzed by adjusted paired *t*-tests (Bonferroni correction factor  $\times 5$ ) are given. Pairs differing significantly are highlighted (grey: demineralization; black: remineralization). Treatment code: O = only storage and no further treatment; B = ZnCO<sub>3</sub>/n-HAp 20 wt%; BS = ZnCO<sub>3</sub>/n-HAp 24 wt%; E = amine fluoride 0.14 wt%; A = n-HA 7 wt%.

The present set-up used abraded and polished specimens; a recent study reported that the *in vitro* demineralization pattern of unabraded samples more closely resembles the pattern of a natural white spot lesions. However, the inter-sample variation was greater than with abraded specimens,<sup>31</sup> and, therefore, we used abraded specimens for standardization reasons.<sup>27</sup> The current brushing procedure was accomplished by brushing the specimens with toothpaste/remineralizing solution slurry for 5 s (with 120 s total contact time of the slurry) twice daily. The specimens were manually brushed without any considerable force by the same operator. Indeed, this should not be considered as a completely standardized procedure (i.e., using a brushing machine), even if slightly differing forces should have averaged during the study period.

Some specimens were lost during preparation for TMR. Main problems were surface losses due to sawing or polishing, and these brittle specimens were not suitable for further investigation. In some cases, thin section preparation could be repeated, but this procedure was limited, due to the small dimensions of the specimens. This problem can be only avoided by using non-destructive techniques (like T-WIM).<sup>32</sup> However, due to the surface misalignment in the outer  $\sim 15 \mu\text{m}$ , this method was not considered useful for the current experimental set-up.

In a clinical setting, toothpaste will be diluted, and this strongly depends on individual salivary secretion,<sup>33</sup> with the present experimental set-up, one part of toothpaste was dissolved in three parts (1:3) of remineralizing solution to obtain a homogeneous slurry. A major factor of the de- and reminer-

alization equilibrium of enamel is the ambient pH. For slightly acidic fluoride toothpaste slurries with a pH between 4.5 and 5.1, increased remineralization could be observed compared to higher pH values;<sup>34</sup> a pH of 5.24 was evaluated with the fluoride toothpaste (according to the manufacturer, the pH is 4.6 for 10% in water). However, only pH values higher than 5.5 have been assumed to promote lesion arrest and to facilitate remineralization.<sup>30</sup> In contrast, pH values of n-HAp toothpastes slurries have not been studied up to now, and it might be speculated that the higher pH values of the n-HAp slurries increased remineralization. Recently, for calcium phosphate based solutions a higher mineral gain could be observed with a pH of 6.5 compared to 5.5.<sup>19</sup> Moreover, a (constant) remineralization model was used to evaluate the effects of the different toothpastes (n-HAp or ZnCO<sub>3</sub>/n-HAp versus fluoride). Whilst pH-cycling experiments (including demineralizing periods) might mimic the clinical dynamics more adequately, remineralization-only models offer the opportunity to effectively monitor caries-preventive regimens on dental hard tissues on a short-time basis, thus simulating a best-case scenario.<sup>35</sup> With the present approach, initial screening of the effects of hydroxyapatite was accomplished, thus highlighting the advantages of experimental control, even if the breadth of relevant biological aspects was limited.<sup>36</sup> Notwithstanding, the current results provide valuable information on n-HAp containing toothpastes, and are considered a sound basis for further experiments.

Dental enamel comprises by 85–90 vol% of a calcium-deficient carbonate hydroxyapatite, whilst dentine contains considerably lower amounts (~50 vol%).<sup>37</sup> With this in mind, in environments supersaturated with respect to apatites, quantity of dentine remineralization should be higher compared to enamel,<sup>38</sup> and this was corroborated by the present results. Thus, the current findings with dentine as substrate seem to indicate a meaningful direction, whilst the observations with enamel lesions are of predominantly confirmative value, but not less momentous.

Treatment of specimens with n-HAp or ZnCO<sub>3</sub>/n-HAp toothpastes did not show any superior effects, but results were comparable to the pure remineralizing solution. From this outcome, one might speculate that n-HAp had no influence at all. However, it should be considered that the used remineralizing control (Buskes' solution) represents a solution with a substantial remineralizing potential,<sup>21</sup> and, therefore, allegorized a positive control. As a consequence, treatment with n-HAp toothpastes revealed no additionally beneficial effect on remineralization. Therefore, usage of a solution with a lower remineralizing potential<sup>24</sup> in combination with n-HAp toothpastes might have resulted in superior effects on mineralization compared to only storage under remineralizing conditions. Future pH cycling studies should elucidate these assumptions and are indicated to verify the observed results.

A direct incorporation of n-HAp or ZnCO<sub>3</sub>/n-HAp particles into the lesions cannot be deduced from the present microradiographic data; however, from previous studies it is known, that crystal growth can be generated with CO<sub>3</sub>/n-HAp particles.<sup>9,39</sup> Nonetheless, the present results are hardly comparable, since a control group (i.e., only storage in artificial saliva) was missing in the mentioned papers. With the present set-up, dentine specimens of group B revealed the highest mineral gain of all groups after five weeks. Since the tested n-HAp toothpastes contained

various active compounds (zinc carbonate nano-hydroxyapatite versus nano-hydroxyapatite), no direct inference seems derivable from the present data; additionally, from a recent paper, it is known that different n-HAp concentrations (>5%) seem to be of minor importance.<sup>12</sup> Consequently, it seems reasonable to assume that the higher pH value of group B slurry favoured remineralization by incorporation of n-HAp particles into the dentine lesions. Moreover, with other products (i.e., CPP-ACP) a reduced fall in plaque pH following an immediate carbohydrate challenge has been reported<sup>17</sup> and this should be an interesting focus even for n-HAp toothpastes.

It should be emphasized that the used fluoride toothpaste (containing amine fluorides) is one of the well-known and widely used cariostatic products on the market (with documented remineralizing effects being higher than those of toothpastes containing sodium fluorides or monofluorophosphates),<sup>35</sup> and, therefore, has been included for comparative reasons. However, enamel and dentine specimens of group E revealed lower mineral gains compared to all other groups (including the controls). Additionally, dentine specimens treated with E revealed a hypermineralization of the surface layer (with an increased thickness), and it might be surmised that a distinct calcium fluoride-like layer on the specimens' surfaces should have been established by this regimen.<sup>40</sup> Moreover, when preparing the slurry, the degree of saturation with respect to calcium fluoride should have increased, and calcium-fluoride-like precipitates should have been favoured.<sup>41</sup> Such precipitates may have blocked any further ion transport into deeper lesion parts by decreasing the pore volume of the surface layer and obstructing the diffusion pathways,<sup>30</sup> and this could have inhibited further remineralization.<sup>42</sup> Furthermore, the observed hypermineralization of the surface layer was accompanied by an increase in lesion depth. Most likely, the low pH (prevailing during brushing with the slurry of group E) caused a redistribution of calcium and phosphates, and minerals situated at the bottom of the lesion should have diffused outwards and re-precipitated at the surface layer. This would be in accordance with the observation that fluorides can drive demineralization further into enamel by making the surface less soluble.<sup>43</sup>

Because of the different active toothpaste compounds, the pH of the amine fluoride toothpaste slurry was nearly two units lower compared to the hydroxyapatite toothpastes (5.24 and 6.94–7.34, respectively). Due to the lower pH, surface layer mineralization should have increased compared to a higher pH.<sup>44</sup> Groups treated with hydroxyapatite toothpaste revealed remineralized subsurface lesions compared to baseline, but without any hypermineralization. The used nano-sized particles (20 nm in size, with granular dimensions up to 100–150 nm)<sup>9</sup> as well as the calcium arising from storage solution should have followed a concentration gradient (with the solution higher than the subsurface lesion), thus leading to a remineralizing effect in deeper lesion parts.<sup>38</sup>

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## 5. Conclusions

The prevention of tooth decay and the treatment of lesions are ongoing challenges in dentistry, and nanotechnology has been claimed as one of the most revolutionary approaches in this

field. Notwithstanding, at the moment, the applied and marketable dental products have been studied rarely.<sup>8</sup> Interestingly enough, within the limitations of the present *in vitro* set-up, the different nano-hydroxyapatite toothpastes revealed similar remineralizing capacities with enamel and dentine lesions. For dentine, even higher remineralization effects could be achieved with n-HAp or ZnCO<sub>3</sub>/n-HAp toothpastes compared to the amine fluoride dentifrice. From the present outcome, we therefore speculate that nano-hydroxyapatite in dental products might help to promote remineralization. However, it is pertinent to note, that this experimental study did not take into account all oral factors; in particular, the complexity of any tooth–pellicle–plaque–saliva interface was not simulated. Hence, the current findings should be confirmed in future *in vitro* pH-cycling studies and clinical settings.

### Declaration of interests

The authors declare that they have no conflict of interest.

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