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ORIGINAL ARTICLE

Effect of saliva substitutes in combination with fluorides on remineralization of subsurface dentin lesions

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Abstract
Purpose Saliva substitutes are prescribed to patients suffering from radiation-induced hyposalivation to alleviate oral complaints; however, some available products have shown to demineralize dentin. The purpose of this in vitro study was to evaluate the effects of two saliva substitutes in combination or not with fluoridation on remineralization of bovine dentin subsurface lesions.
Materials and methods Dentin specimens were demineralized, and stored in either mineral water (control; volvic, danone), Glandosane (cell pharm), or modified Saliva natura (SN, Medac) for 5 weeks (37°C). The following treatments were applied twice daily (n=12/group): (1) no treatment; (2) immersion in pure Elmex sensitive mouthrinse (250 ppm F^-; 10 min; Gaba); (3) brushing with Duraphat toothpaste (5,000 ppm F^-; Colgate)/storage solution slurry (5 s; ratio 1:3); (4) combination of treatments 2 and 3. Differences in mineral parameters before and after storage/treatment were microradiographically evaluated.
Results After 5 weeks, Glandosane-induced a significant demineralization of dentin specimens. The mineral loss of specimens stored in Glandosane was significantly higher compared to all other solutions ($p<0.05$), and this side effect was inhibited by the fluoride products ($p<0.05$; ANOVA, Tukey). Modified Saliva natura enabled considerable remineralization, and this was significantly increased by daily application of both fluoride products ($p<0.05$).
Conclusions Modified Saliva natura has remineralizing properties, while Glandosane is a demineralizing saliva substitute that should only be used with frequently applied fluorides in dentate patients.

Keywords Dentin · Fluoride · Microradiography · Remineralization · Saliva Substitute

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Introduction

Patients suffering from hyposalivation frequently experience a high caries activity. This relationship has been demonstrated in patients irradiated for tumors in the head and neck areas [1, 2]. The increased caries incidence in these patients is associated with side effects of hyposalivation such as the reduced antibacterial function, impaired remineralization of dental hard tissues, reduced buffering capacity, and compromised self-cleaning effects. In addition with consumption of soft high-carbohydrate foods and use of cariogenic saliva stimulants, these alterations stimulate rapid onset and progression of caries [2–4]. Since hyposalivation is generally restricted to palliative treatment, a rigorous supportive care is recommended to these patients, including daily application of

fluoride products in combination with meticulous oral hygiene and control of dietary intake [5, 6].

Saliva substitutes are most frequently applied for relieving the sensation of dry mouth conditions; however, with regard to supportive care in patients irradiated for head and neck cancer, these products should also protect dental hard tissues [7]. A few commercially available saliva substitutes demonstrated remineralizing effects, while others had a substantial demineralizing potential on enamel and dentin [7–9]. To increase the remineralizing capacities, fluorides, calcium, and phosphates have been added to saliva substitutes [10, 11]. Saliva substitutes supersaturated with respect to octacalcium phosphate (OCP) should preferably be used, since calcium and phosphates may form complexes with the polymer ingredients of artificial salivas [12, 13]. Recently, it has been shown that mucin and polysaccharide based saliva substitutes supersaturated with respect to OCP enabled remineralization of dentin [14, 15].

The topical use of fluoride products including mouthrinses, gels, and toothpastes has demonstrated a successful prevention of dental caries in irradiated patients [16–18]. Caries onset in these patients usually starts at the cervical dentin areas of the teeth [2]; while dentin seems to be directly affected by radiotherapy [19], no increased caries susceptibility could be found clinically, if adequate oral hygiene techniques are implemented [20]. Notwithstanding, only scanty information concerning the effects of saliva substitutes in combination with fluoride products on remineralization of initial dentin lesions is available in the literature [15]. Therefore, the aim of this in vitro study was to evaluate the effects of daily applications of fluoride products (including a mouthrinse and a high-fluoride toothpaste) in combination with two saliva substitutes on the mineralization of bovine dentin subsurface lesions. A commercially available and widely used saliva substitute with a known demineralizing potential (Glandosane (G); cell pharm, Hannover, Germany) was studied in comparison to a remineralizing product (modified Saliva natura solution (SN); Medac, Hamburg, Germany). It was hypothesized that the additional use of fluoride products (mouthrinse or toothpaste) would not result in less pronounced demineralization of G, and that fluoride products would not result in enhanced remineralization of SN (H_0), and these null hypotheses were tested against the alternative hypotheses of a difference.

Materials and methods

Preparation of the dentin specimens

Thirty-six recently extracted permanent bovine central incisors were used to prepare dentin specimens ($6\times4\times4$ mm^3). The specimens (n=144) were embedded in epoxy resin, while the natural surface was kept free from resin. The surfaces were ground flat and hand-polished (4,000 grit, silicium carbide; Struers, Copenhagen, Denmark). One quarter of each specimen's surface was covered with an acid-resistant nail varnish to serve as control of sound dentin. Subsurface lesions were created as described previously [15].

Treatment of specimens

Half of each demineralized surface was covered with nail varnish (internal demineralization control), and specimens were randomly allocated to three groups (n=48 each): Volvic mineral water ([W], control, pH 7.0; Danone, Frankfurt, Germany); G neutral (pH5.2; cell pharm, Hannover, Germany); and modified SN (pH5.98; Medac, Hamburg, Germany). The original product Saliva natura was experimentally modified with the addition of calcium, phosphates, and fluorides according to a previous study [11]. The degree of saturation (S) with respect to calcium-containing compounds (e.g., dicalcium phosphate dihydrate, DCPD; octacalcium phosphate, OCP; and hydroxyapatite, HA) was calculated, since the pH and the concentrations of certain ions were known (Table 1) [21]. Specimens were stored in the solutions for 5 weeks (25 ml, 37°C), and submitted to one of the following treatments twice daily (n=12/subgroup): no treatment (0); immersion in pure Elmex sensitive mouthrinse (250 ppm F^-; pH4.63; GABA, Lörrach, Germany) for 10 min (ES); forceless brushing with Duraphat toothpaste (5,000 ppm F^-; Colgate-Palmolive, Hamburg,

Table 1 Composition of the control solution and the saliva substitutes, and calculated saturations with respect to apatites

Solution	Calculated saturation of an aqueous solution with respect to:			pH value	Concentrations of the ions (mM)						
	DCPD	OCP	HA		Ca	PO_4	F	K	Cl	Na	Mg
Water	0.2	0.7	3.9	7.0	0.3	0.2	–	0.2	0.4	0.5	0.3
Glandosane	0.2	0.3	0.7	5.2	1.01	2.57	–	19.03	33.75	14.76	0.26
modified Saliva natura	1.2	2.0	7.4	5.98	3.2	5.0	0.1	6.7	6.3	–	–

DCPD dicalcium phosphate dihydrate, *OCP* octacalcium phosphate, *HA* hydroxyapatite

Table 2 Means with confidence intervals (CI 95%) of mineral losses (ΔZ; vol%×μm) after in vitro demineralization (ΔZ_{Demin}) and storage/treatment for 2 ($\Delta Z_{Effect\ 2}$) and 5 weeks ($\Delta Z_{Effect\ 5}$)

Solution	Treatment	Mineral loss (vol%×μm)							
		ΔZ_{Demin}		$\Delta Z_{Effect2}$		*p*	$\Delta Z_{Effect5}$		*p*
		Mean	CI 95%	Mean	CI 95%		Mean	CI 95%	
Water	0	3,068	2,943; 3,193	2,981	2,728; 3,234	1	3,134	2,838; 3,430	1
	ES	3,168	2,892; 3,445	3,052	2,725; 3,380	1	3,096	2,779; 3,414	1
	D	3,212	2,996; 3,428	3,209	2,912; 3,506	1	2,971	2,730; 3,211	0.492
	ES+D	3,069	2,687; 3,452	2,929	2,550; 3,309	1	2,870	2,490; 3,250	1
Glandosane	0	3,349	3,095; 3,602	4,980	4,651; 5,210	0.001*	7,144	6,496; 7,793	0.001*
	ES	3,169	2,865; 3,472	3,033	2,668; 3,399	0.612	3,096	2,856; 3,336	1
	D	3,177	2,950; 3,404	3,061	2,798; 3,324	1	3,172	2,879; 3,466	1
	ES+D	3,080	2,797; 3,362	3,065	2,738; 3,392	1	3,072	2,672; 3,473	1
modified Saliva natura	0	3,338	3,092; 3,585	2,978	2,638; 3,318	0.072	2,522	2,261; 2,782	0.001*
	ES	3,466	3,150; 3,781	2,963	2,592; 3,333	0.001*	2,881	2,604; 3,157	0.001*
	D	3,443	3,160; 3,725	2,901	2,660; 3,142	0.001*	2,858	2,531; 3,186	0.001*
	ES+D	3,245	2,979; 3,511	2,208	1,942; 2,474	0.001*	1,822	1,621; 2,023	0.001*

Significant differences between values after demineralization (ΔZ_{Demin}) and those after storage/treatment for 2 ($\Delta Z_{Effect2}$) or 5 weeks ($\Delta Z_{Effect5}$) are indicated by asterisks ($p<0.05$; adjusted paired *t* test [factor ×12])

0 no treatment, *ES* Elmex Sensitive mouthrinse, *D* Duraphat toothpaste slurry, *ES+D* Elmex Sensitive mouthrinse+Duraphat toothpaste slurry

Germany)/storage solution slurry for 5 s with a total contact time of 2 min (D); immersion in Elmex-sensitive mouthrinse for 10 min and forceless brushing with Duraphat toothpaste slurry for 5 s with total contact time of 2 min (ES+D). The slurries were prepared with a ratio of 1:3 (Duraphat toothpaste-water/storage solutions), and the pH values were measured (8.28 for Duraphat/W; 7.52 for Duraphat/G, and 7.76 for Duraphat/SN). Specimens were forcelessly brushed with soft manual toothbrushes (Meridol; GABA, Lörrach, Germany). After each treatment, specimens were washed with deionized

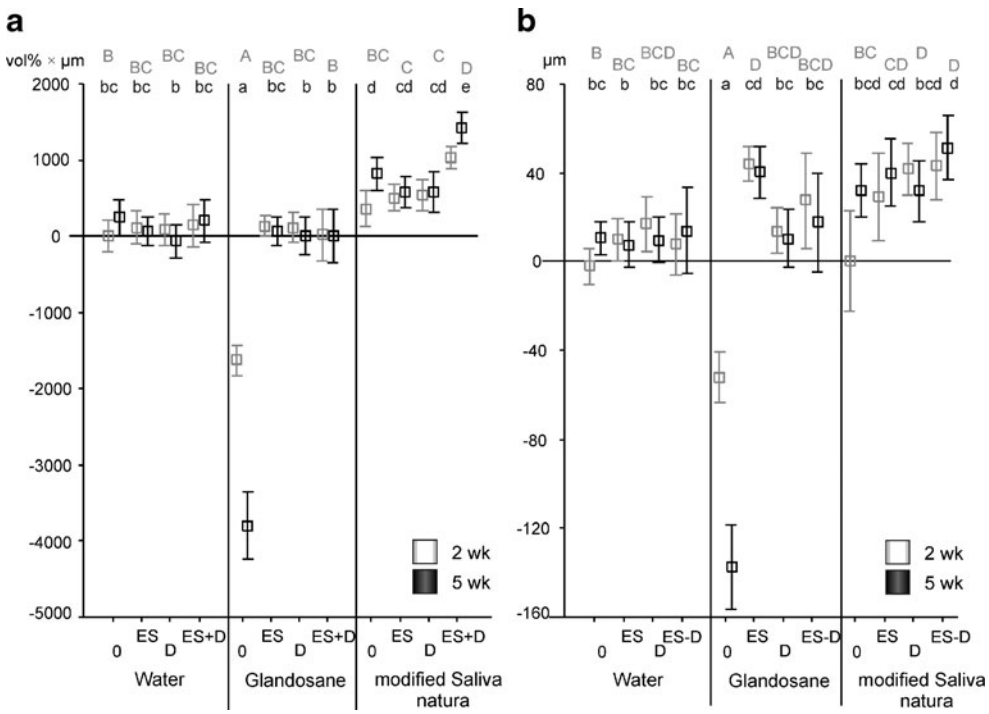

Fig. 1 Means with confidence intervals (CI 95%) of the changes in mineral loss (**a**; $\Delta\Delta Z_{Effect}$, vol%×μm) and lesion depths (**b**; ΔLD, μm) after storage [water (W, control group), Glandosane (G), modified Saliva natura (SN)] and treatment [no treatment (0), Elmex sensitive solution (ES), Duraphat toothpaste (D), Elmex sensitive solution+Duraphat toothpaste (ES+D)] of demineralized dentin specimens (subsurface lesions) for 2 weeks (*gray*) as well as after 5 weeks (*black*). *Different letters* indicate significant differences between groups (*gray capital letters*: 2 weeks; *black lower case* letters: 5 weeks; $p<0.05$; ANOVA, Tukey's post hoc test)

water (20 s). Storing solutions were replenished every 2 days. Half of each exposed specimens' surface (1.5×4 mm^2) was varnished after 2 weeks (effect area after 2 weeks).

Transversal microradiography analysis

After in vitro storage/treatment, thin sections (100 μm) were prepared (Band Saw; Mikroschleifsystem). Subsequently, contact microradiographs (TMR) were obtained as described previously [8], and analyzed (TMR for Windows 2.0.27.2; Inspektor Research Systems, Amsterdam, The Netherlands) after blinding of the investigator to prevent any bias. During radiography procedures, dentin specimens were treated with ethylene glycol (99%; Sigma-Aldrich Chemie, Munich, Germany) to avoid shrinkage [22]. Mineral loss (ΔZ) and lesion depth (LD) of each specimen's surface area (sound; demineralized; effect after 2 and 5 weeks) were calculated. Subsequently, mineral losses and lesion depths of the demineralized (ΔZ_{Demin}, LD_{Demin}) and effect areas ($\Delta Z_{Effect\ 2}$/$LD_{Effect\ 2}$; $\Delta Z_{Effect\ 5}$/$LD_{Effect\ 5}$) were corrected by subtraction of the respective sound control values (ΔZ_{Sound}, LD_{Sound}). Changes in mineral losses ($\Delta\Delta Z_{Effect}=\Delta Z_{Demin}-\Delta Z_{Effect}$) and lesion depths ($\Delta LD_{Effect}=LD_{Demin}-LD_{Effect}$) were calculated for 2 and 5 weeks. Positive ΔΔZ or ΔLD values were considered as remineralization and negative results as demineralization (Fig. 1).

Statistical analysis

Statistical analyses were performed with SPSS 11.5 (SPSS, Munich, Germany) software. Data were tested for normal distribution (Kolmogorov-Smirnov test). ΔΔZ and ΔLD values were compared using one-way analysis of variance (ANOVA) and Tukey's post hoc tests. Differences in mineral loss and lesion depth before and after storage/treatment were compared using adjusted paired *t* test (Bonferroni correction with factor ×12). Level of significance was set at 5%.

Results

Morphological analysis of the specimens revealed surfaces with no signs of erosive or abrasive loss after demineralization or storage/treatment, respectively. Mineral loss (p=0.250) and lesion depth (p=0.848; ANOVA) values after demineralization (=baseline) did not differ significantly between the various groups.

In vitro storage in G resulted in significantly lower $\Delta\Delta Z_{Effect\ 2}$/$\Delta\Delta Z_{Effect\ 5}$ and $\Delta LD_{Effect\ 2}$/$\Delta LD_{Effect\ 5}$ values compared to SN and W (p<0.05; ANOVA, Tukey's post hoc test; Fig. 1). Although no typical erosion (loss of

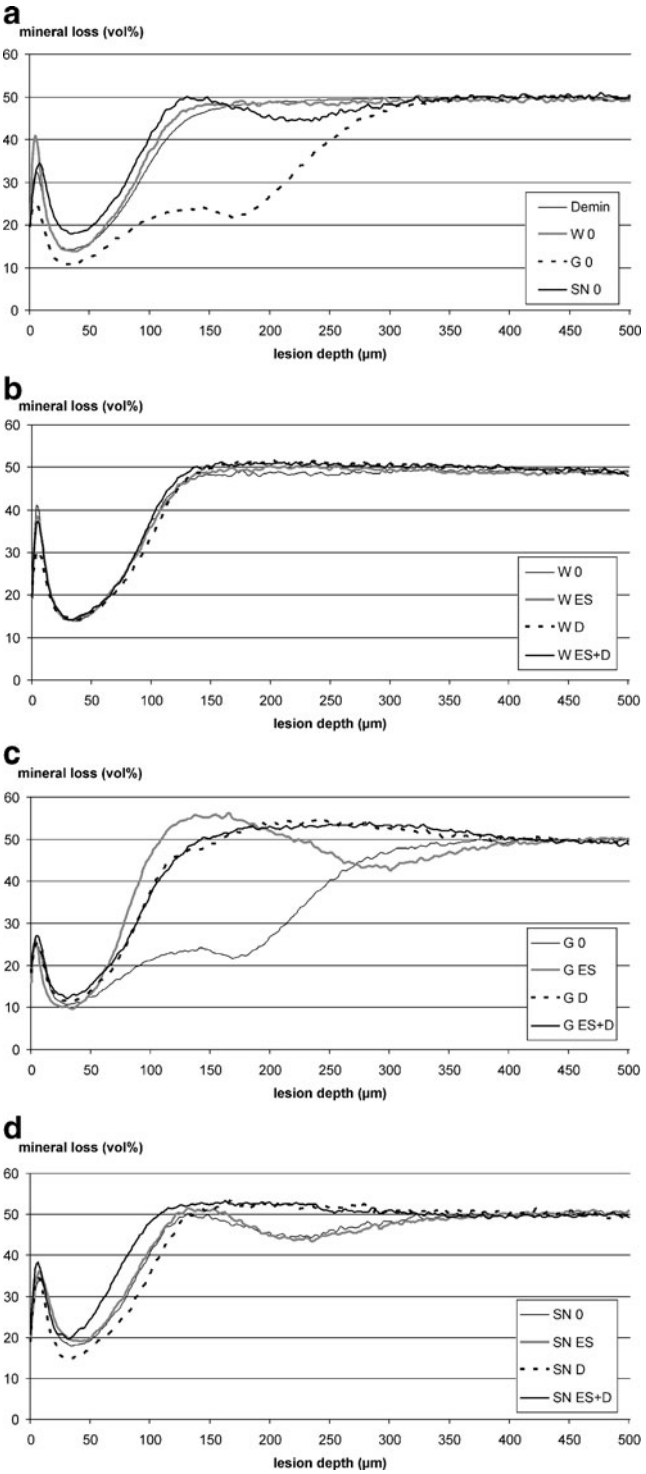

Fig. 2 Mean value graphs (ΔZ; vol%×μm) after 5 weeks storage/treatment of bovine dentin specimens. **a** Mineral loss after demineralization and only storage in the different solutions (baseline values); **b** mineral loss after storage/treatment in water (W): no treatment (0), Elmex sensitive solution (ES), Duraphat toothpaste (D), Elmex sensitive solution+Duraphat toothpaste (ES+D); **c** mineral loss after storage/treatment in Glandosane (G); **d** mineral loss after storage/treatment in modified Saliva natura (SN)

surface layer) could be detected, the lesions revealed a lower mineralized surface layer, along with a pronounced mineral loss in the body of the lesion (Fig. 2a). $\Delta\Delta Z_{Effect\ 5}$ values of specimens stored in SN were significantly higher compared to W ($p<0.05$).

Specimens stored in G and treated with fluoride products revealed significantly higher $\Delta\Delta Z_{Effect\ 2}/\Delta\Delta Z_{Effect\ 5}$ and $\Delta LD_{Effect\ 2}/\Delta LD_{Effect\ 5}$ compared to only storage in G ($p<0.05$; ANOVA, Tukey's post hoc test; Fig. 1). However, no significant differences were detected between specimens treated with either one or two fluoride products (G ES, G D, G ES+D). Application of both fluoride products in combination with storage in SN enabled significantly higher $\Delta\Delta Z_{Effect\ 2}/\Delta\Delta Z_{Effect\ 5}$ values compared to SN 0, SN ES, or SN D ($p<0.05$). With regard to mineral parameters, no significant differences could be observed between control subgroups, irrespective of fluoride use ($p>0.05$). The mean value graphs of mineral loss (ΔZ) after 5 weeks as a function of storage/treatment and depth of the lesion are illustrated in Fig. 2.

G 0 induced significantly increased (demineralization) $\Delta Z_{Effect\ 2}/\Delta Z_{Effect\ 5}$ and $LD_{Effect\ 2}/LD_{Effect\ 5}$ values ($p<0.05$; adjusted paired t test; Tables 2 and 3), whereas SN 0 resulted in significantly decreased (remineralization) $\Delta Z_{Effect\ 5}/LD_{Effect\ 5}$ values ($p<0.05$) compared with baseline ($\Delta Z_{Demin/LDDemin}$). Compared to baseline (LD_{Demin}), specimens of G ES subgroup showed significantly decreased $LD_{Effect\ 2}/LD_{Effect\ 5}$ values ($p<0.05$). All specimens stored in SN and treated with fluoride products revealed significantly decreased $\Delta Z_{Effect\ 2}/\Delta Z_{Effect\ 5}$ and $LD_{Effect\ 2}$ (with exception of SN ES)/$LD_{Effect\ 5}$ values ($p<0.05$).

Discussion

The present in vitro study demonstrated that fluoride mouthrinse as well as high-fluoride toothpaste inhibited the previously observed detrimental effect of Glandosane [7–9], while modified Saliva natura in combination with both fluoride products revealed considerable remineralizing effects. Thus, the null hypotheses of this study were rejected.

The influence of saliva substitutes in combination with fluoride products was evaluated in the present study without the use of a pH-cycling model (alternating demineralizing and remineralizing protocols) to determine more efficiently the real effect of the artificial salivas with and without fluoride treatment. Since remineralizing effects of natural saliva cannot be expected in patients with extensive hyposalivation [1, 2], the 5-week storage period can be considered an extremely intensive contact that is not expected under clinical conditions. However, it should be considered that patients suffering from (radiation-induced) hyposalivation generally use saliva substitutes ad libitum, and similar effects might be conceivable, in particular after longer in vivo periods.

Table 3 Means with confidence intervals (CI 95%) of lesion depths (LD; µm) after in vitro demineralization (LD_{Demin}) and storage/treatment for 2 ($LD_{Effect\ 2}$) and 5 weeks ($LD_{Effect\ 5}$)

Solution	Treatment	Lesion depth (µm)							
		LD_{Demin}		$LD_{Effect2}$		p	$LD_{Effect5}$		p
		Mean	CI 95%	Mean	CI 95%		Mean	CI 95%	
Water	0	132	123; 141	115	108; 122	0.144	122	110; 134	0.804
	ES	136	127; 145	125	116; 135	0.480	128	117; 139	1
	D	133	123; 141	134	120; 147	1	121	114; 129	0.144
	ES+D	130	113; 146	122	104; 140	1	116	93; 138	1
Glandosane	0	137	121; 152	189	172; 205	0.001*	274	244; 304	0.001*
	ES	132	119; 146	88	80; 97	0.001*	92	87; 97	0.001*
	D	131	114; 148	117	108; 127	0.168	121	112; 130	1
	ES+D	136	116; 156	108	199; 117	0.204	118	109; 128	1
Modified Saliva natura	0	136	122; 149	135	117; 154	1	104	99; 109	0.001*
	ES	142	125; 159	113	100; 127	0.096	102	95; 109	0.001*
	D	146	135; 157	104	96; 112	0.001*	114	102; 125	0.001*
	ES+D	140	126; 155	97	92; 103	0.001*	8	8; 95	0.001*

Significant differences between values after demineralization (LD_{Demin}) and those after storage/treatment for 2 ($LD_{Effect2}$) or 5 weeks ($LD_{Effect5}$) are indicated by asterisks ($p<0.05$; adjusted paired t test [factor ×12])

0 no treatment, *ES* Elmex Sensitive mouthrinse, *D* Duraphat toothpaste slurry, *ES+D* Elmex Sensitive mouthrinse+Duraphat toothpaste slurry

In the present study, the control solution (non-carbonated mineral water) showed a negligible effect that was expected because of the neutral pH and the low saturation with respect to OCP and DCPD. In contrast, the commercially available saliva substitute Glandosane demonstrated a remarkably demineralizing effect. Similiar effects on dentin have been observed previously [8, 9]. Glandosane is a carboxymethylcellulose-based solution, with a pH value being substantially lower than the critical value assumed for dentin demineralization (6.0-6.5). The pH value, the unspecified amount of titrable acids (sorbic acid, hydrochloric acid), and, consequently, the low saturation with respect to OCP and DCPD (Table 1) could elucidate this progressive mineral loss.

Interestingly, specimens stored in modified Saliva natura, a polysaccharide-based solution, revealed a considerable mineral gain and lesion depth reduction. After 5 weeks, the mineral gain induced by modified Saliva natura was higher in comparison to the other solutions and to the baseline values after demineralization. These results could be explained by the supersaturation of this saliva substitute (OCP and DCPD). A recent study revealed that the original (not experimentally modified) product Saliva natura (OCP=0) demonstrated demineralizing effects on dentin [23].

It may be argued that an acquired pellicle (in particular a mature one with a formation time of several days) might protect the dentin surfaces from any acid challenge [24]. On the other hand, a matured pellicle also would impede the remineralization effects driven by the modified Saliva natura solution. Thus, the present setup simulated a best-case scenario [25], and was comparable to a severe hyposalivation, including lack of pellicle formation.

Products with higher fluoride concentration should be more effective than those containing lower fluoride levels [26, 27]. Thus, two different fluoride containing products were used in this study: a mouthrinse solution with low fluoride amounts, and a high-concentrated toothpaste. All fluoride treatments were capable to prevent further demineralization of specimens stored in Glandosane. The fluoride treatments with mouthrinse and/or toothpaste should have resulted in a distinct calcium fluoride-like layer on the specimens' surfaces [28, 29], which should have been dissolved over time by the potentially demineralizing effects of Glandosane. These precipitates on the specimens' surface might have acted as a fluoride reservoir, thus hampering the demineralization caused by Glandosane [28]. However, it should be kept in mind that calcium fluoride obviously dissolves rapidly even at almost neutral pH values [30]; in a recent study, more than 90% of the precipitated calcium fluoride was dissolved after application of aminefluoride or sodium fluoride within a few days. Therefore, the present study used a protocol that emphasized a protocol of daily fluoridation.

Modified Saliva natura revealed a remineralizing effect, and the additional use of only one fluoride product did not enhance remineralization. However, mineral deposition of both the lesion body and the surface layer was significantly increased after treatment with modified Saliva natura in combination with both fluoride products. Recently, a more pronounced remineralization of subsurface lesions was observed with an acidic solution compared to a neutral remineralizing one [31]. This could explain the absence of mineral gain with the use of the neutral high-concentrated toothpaste compared to the combination of toothpaste/acidic mouthrinse (see Fig. 2d), and this would be in accordance with a previous paper reporting a significant reversal of primary root caries lesions after use of a toothpaste/fluoride mouthrinse [32]

One well-documented problem of any multiple-step protocol could be the patient compliance, and, indeed, the long-term compliance of patients irradiated for head and neck tumors with a two-step regimen was very poor [33]. Notwithstanding, the use of a remineralizing saliva substitute containing fluorides, calcium, and phosphates is strongly recommended for dentate patients suffering from hyposalivation since lack of calcium and phosphate ions which are present in natural saliva is considered the limiting factor of remineralization of carious lesions, even in the presence of high fluoride concentrations. The results of the present study seem to support this.

In the control group, the mineral profile of the specimens was very similar, regardless of the application of fluoride products. Moreover, the small amount of calcium and phosphate ions in the water-based control, together with the neutral pH, could explain the absence of any remineralizing effect [34].

Within the limited protocol of the present study, it can be concluded that the use of fluoride products (mouthrinse and/or high-concentrated toothpaste) inhibited the detrimental effects of Glandosane. Modified Saliva natura revealed remineralizing effects, and the combination with both fluoride products' application yielded the most pronounced remineralization under the in vitro conditions chosen. Thus, within the concept of supportive care in head and neck cancer patients, the use of frequently applied fluoride products in addition to remineralizing saliva substitute should be preferred.

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