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Remineralization effects of conventional and experimental ion-releasing materials in chemically or bacterially-induced dentin caries lesions

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ABSTRACT

Objectives. The aim of this study was to evaluate the remineralization effects of conventional and experimental ion-releasing materials on different artificial dentin carious lesions.

Methods. Forty human dentin discs were submitted to different demineralization protocols for simulated caries lesion: (D1) Shallow chemically-induced caries, (D2) deep chemically-induced caries, (D3) deep bacterially-induced caries. Each disc was divided in five parts; one of those served as baseline control. The remaining parts of each disc (n = 12–16/group) were treated using the following materials: EXP, an experimental resin-based bioactive material consisting of a self-etch primer and an adhesive containing a fluoride-doped bioglass; GIC, a glass ionomer cement (Riva LC); MTA, Mineral Trioxide Aggregate (ProRoot MTA); BIO, a calcium silicate cement (Biodentine). Specimens were mounted in a dual-chamber device to simulate the exposure to pulpal pressure and oral fluids. After 3 months, mineral and mechanical gains were assessed using transverse microradiography (vol% × μm) and microhardness measurements (VHN). Characterization using confocal microscopy and transmission electron microscopy (TEM) was also performed.

Results. All four restorative materials induced mineral gains regardless of the protocol for caries lesion, without significant differences between materials. Microhardness significantly increased in the groups BIO and MTA, but not GIC; EXP only provided hardness gains in D3-lesions. Fluorescence and confocal microscopy confirmed these results. There was a clear "top-down" remineralization in the groups BIO and MTA, and "bottom-up" intrafibrillar collagen remineralization in EXP.

Significance. Mineral gains did not always translate into hardness gains. Biodentine and MTA induced evident mineral precipitation, but intra/inter-fibrillar collagen mineral infiltration was only provided by biomimetic remineralisation via the use of the experimental adhesive. Complete remineralization of caries lesions remains a challenge.

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1. Introduction

Removal of carious tissue in deep dentin lesions comes with a significant risk of pulp exposure. Hence, selective carious tissue removal is recommended for deep lesions. During this selective removal, only hard dentin remains along the walls of the cavity, whereas demineralized, bacterially-contaminated dentin is left in pulpal areas and sealed beneath the restoration [1].

Conventional adhesives achieve low bond strengths when applied to such demineralized or contaminated dentin, as this layer of carious dentin can be only partially infiltrated by resin monomers. Such a "poorly" resin-infiltrated demineralized dentin is prone to hydrolytic and/or enzymatic degradation, which may eventually compromise the durability of composite restorations [2,3]. Contemporary caries management attempts to remineralise the sealed carious lesion through the use of ions-releasing dental materials (e.g. liners, bioactive adhesives and restoration materials), in addition to "natural" mineral gains mediated by odontoblasts and/or mineral-containing pulpal fluids [4–6].

While calcium hydroxide, the most common lining material, seems only limitedly able to the provide mineral gains, alternative materials like glass ionomer cements (GIC) [7–13], mineral trioxide aggregate (MTA) or novel calcium silicate cements have been advocated to increase the mineral content of lesions sealed beneath them [14]. More recently, experimental bioactive/biomimetic adhesives have been advocated to induce slow displacement of the residual water from water-filled voids within the incompletely infiltrated hybrid layer, via infiltration of collagen with nano-crystals of apatite, leading to recovery of mechanical properties and protection of collagen against proteolytic degradation [8,11,8–13].

The aim of this study was to evaluate the remineralization effects of conventional and experimental ion-releasing materials in chemically or bacterially-induced dentin caries lesions *in vitro*. This aim was accomplished by performing mineral content measurement (transverse microradiography), microhardness determination (Vickers Hardness, VHN) as well as confocal microscopy and transmission electron microscopy (TEM) ultra-morphology analysis. Our null-hypothesis was that the remineralisation assessed through transverse microradiography and microhardness would not differ significantly between the different materials tested in this study.

2. Materials and Methods

2.1. Study design

The study design is summarised in Fig. 1. The remineralization activity of four different ion-releasing materials applied onto artificial dentin carious lesions was tested in a dual-chamber device [14]. This device allowed simultaneous simulation of pulpal fluid flows onto the pulpal side of the carious dentin disc as well as possible remineralization from there, and restorative effects from the coronal side of the disc. Three types of artificial demineralized carious lesions were induced in human dentin discs: (D1) Shallow chemically induced lesions, (D2) deep chemically induced lesions, (D3) deep bacterially induced lesions. Baseline mineral loss was measured through transverse microradiography, and baseline surface microhardness determined. Dentin discs were restored (covered) using three conventional ion-releasing materials (GIC, MTA, Biodentine) or an experimental bioactive/biomimetic resin-based adhesive system. The specimens were subsequently exposed to cyclic artificial saliva and fluoride rinses for a period of 12 weeks. Afterwards, transverse microradiography and microhardness measurements were repeated. Moreover, three representative specimens from each group were selected and analyzed through TEM and confocal microscope.

2.2. Sample preparation

Forty extracted human permanent molars, devoid of any lesions and/or restorations, were obtained with an informed consent based on an ethical approved protocol (ethics committee of the Charité — Universitätsmedizin Berlin EA4/102/14). Both roots and coronal enamel were removed (Band Saw 300 cl; Exakt Apparatebau, Norderstedt, Germany) until the deep dentin was reached (the final disc consisted of 1.5 mm residual dentin measured using a C-calliper). Discs were plan-parallelised and polished using SiC abrasive papers 1200, 2400 and 4000 SiC (Mikroschleifsystem 400 CS,). The specimens were covered with acid-resistant nail varnish (Maybelline, New York, NY, USA) leaving a round window (5 mm diameter) on the occlusal surface; this latter was left uncovered.

Three types of artificial carious lesions were created in this unprotected window following different validated protocols [15–17]: (D1) Shallow chemically induced lesions (n = 12). These were created via storing the samples in 5 l of a demineralising solution (pH 5.3, 37 °C) containing 50 mM acetic acid, 3 mM $CaCl_2 \times H_2O$, 3 mM KH_2PO_2 and 6 mM methylhydroxydiphosphonate for 14 days. The pH of the solution was monitored daily (InLab micro, Mettler-Toledo, Giessen, Germany) and if necessary adjusted using HCl or 10 M KOH. Chemicals (except HCl) were purchased from Sigma Aldrich (St. Louis, USA), HCl was purchased from Roth (Karlsruhe, Germany). (D2) Deep chemically induced lesions (n = 12). These were created via storing the samples in the same acetic acid solution for 28 days. (D3) Bacterially induced deep lesions (n = 16). These were first demineralized in the solution of acetic acid for 7 d, followed by bacterial demineralization. For the latter, a computer-controlled continuous-culture biofilm model was used [18]. *Lactobacillus rhamnosus* (DSM 20247, DSMZ, Braunschweig, Germany) was cultured overnight in deMan-Rogosa-Sharpe (MRS) medium (Roth) at 37 °C. Cultures were then transferred to the biofilm model, where they were provided with MRS supplemented with 2% glucose via peristaltic multi-canal pumps (8152 Standard, MCP, Glattbrugg, Germany) for 10 min four times daily for a total of 2 days. Between simulated meals, biofilms were cultured at rest at 37 °C under 100% humidity. Finally, the biofilm was removed using a sterile swap, the specimens were well rinsed, the nail varnish completely removed from the coronal and pulpal surfaces [19], and from each disc, a perpendicular section was cut as baseline

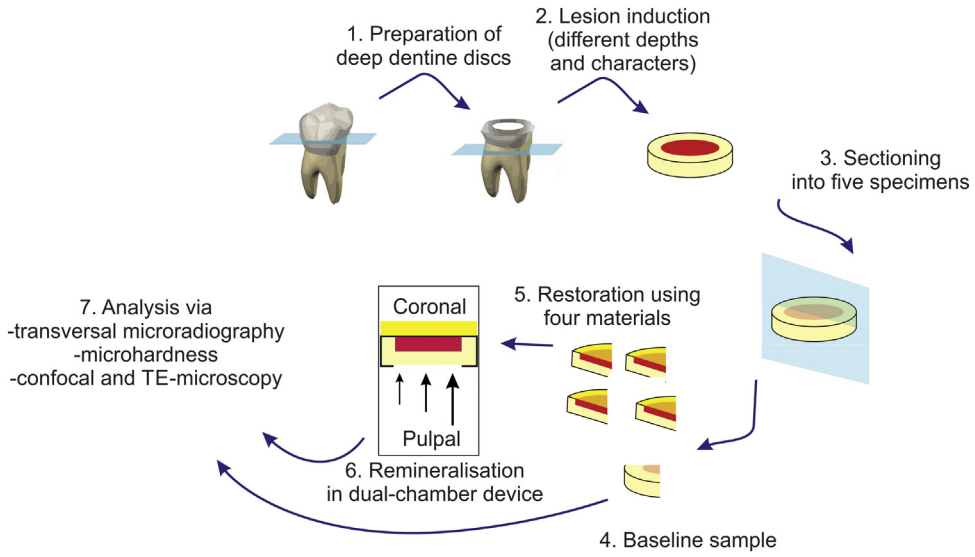

Fig. 1 – Schematic representation of the study design.

control. The remaining disc was cut into four experimental samples, each being restored with one different material.

2.3. *Restorative materials*

The following materials were tested in this study: (1) A glass ionomer cement (GIC), Riva LC (Riva, SDI, Bayswater, Australia). The GIC was not covered by any further material. (2) An experimental adhesive system (EXP) made of a self-etching primer (containing urethane dimethylacrylate (UDMA), hydroxyethyle methylacrylate (HEMA), triethylene gylcole-dimethacrylate (TEGDMA), glycerol dimethacrylate phosphate (GDMA-P), and ethoxylated bisphenol A dimethacrylate (Bis-EMA) and an adhesive containing UDMA, TEGDMA, HEMA, Bis-EMA and 50 w% fluoride-bioglass [20] (<50 μm particle size). All monomers used for the formulation of the experimental adhesive were purchased from Esstech (Essington, Pennsylvania, USA). The primer was applied onto dentin for 15 s, air-dried for 5 s to remove the solvent, and then covered with a layer of adhesive applied for 15 s. This latter was then air-dried to remove the excess deposited on the dentin surface and light-cured for 20 s with an LED-light-curing with an intensity of >1000 mW/cm^2 (Smartlite, Dentsply, Konstanz, Germany). Subsequently, a flowable composite (Tetric EvoFlow, Ivoclar Vivadent, Schaan, Liechtenstein) was placed in a 2 mm thick layer using the same curing protocol. (3) Biodentine (BIO, Septodont, Niederkassel, Germany), a tricalcium silicate cement that was mixed as per manufacturer's instructions and placed in a thin layer of 0.5–1.0 mm thickness. The cement was covered with a wet cotton pellet and left undisturbed for 15 min to achieve a proper setting reaction. Subsequently, these specimens were covered using a commercial adhesive (Clearfil SE Bond, Kuraray, Tokyo, Japan) applied according to manufacturer's instructions, followed by a flowable resin composite (Tetric EvoFlow), as previously described. (4) ProRoot MTA (MTA, Dentsply), mixed as per manufacturer's instructions and placed in a thin layer of 0.5–1.0 mm thickness. Also, this cement was covered with a wet cotton pellet and left undisturbed for 15 min. Finally, the specimens were restored as described above for group 3 (BIO).

2.4. *Remineralization phase*

Specimens were mounted on the lit of a custom-made dual-chamber system (Fig. 1), with pulpal and coronal surfaces being exposed two different chambers [14]. The use of this system allows to simultaneously provide remineralization from coronally (induced by the materials) and pulpal mineral delivery, as is the case clinically [21]. The coronal chamber was filled with a mucin-based medium to simulate the oral milieu [22]. Every 2 weeks, the coronal surfaces all the specimens were additionally rinsed with 200 ppm NaF solution for 5 min to allow possible fluoride recharge of GIC and to simulate a real scenario where toothpastes and/or mouthrinses are used. The pulpal chamber was immersed in simulated pulpal fluid (containing hepes buffer, calcium, magnesium, phosphate, as well as albumin) at a constant pulpal pressure of 2.94 kPa [14,23]. Chambers were stored at 37 °C and under gentle agitation (70 rpm) for three months, with coronal and pulpal fluids being replaced every two weeks.

2.5. *Transverse microradiography (TMR)*

Microradiography was used to estimate the integrated mineral loss (ΔZ) of the baseline samples and of the remineralized samples. From each microradiograph, three standardized areas were used to determine mineral loss. Absolute mineral loss differences (ΔΔZ) were calculated for each area, with positive differences indicating mineral gains, and means for each sample used for statistical analysis.

A nickel-filtered copper X-ray source (PW 1730/10, Philips, Eindhoven, The Netherlands) operating at 20 kV and 10 mA with a vertical tube (PW 2213/20, Panalytical, Kassel) and a 280 mm radiation-to-film distance was used to obtain radiographs. Films (Fuji fine 71337, Fujifilm, Tokyo, Japan) were exposed for 5 s and developed under standardized

conditions according to the manufacturer's recommendations. The microradiographs were analyzed with a digital-image-analysing system (XC 77 CE, Sony, Tokyo, Japan) interfaced with a universal microscope (Axioskop2 60318, Zeiss, Oberkochen, Germany) and a personal computer (TMR for Windows 2.0.27.2, Inspector Research, Amsterdam, The Netherlands).

2.6. Microhardness assessment

Specimens were cut perpendicularly to the occlusal surface that had been in contact with the restorative materials. Two interfaces were obtained from each specimen and these were embedded in epoxy resin with the transverse cut face exposed. The surface was serially polished to a 1 μm finish. The Vickers hardness number (VHN) was calculated from ten diamond indents that were made at 50 μm below the anatomical occlusal surface of each specimen. A Vickers hardness tester (HMV, Shimadzu, Kyoto, Japan) was used with a load of 1 N (100 g) applied for 15 s.

2.7. Fluorescence and confocal microscopy

Three exemplary dentin specimens from the microhardness evaluation were employed for both optical fluorescence and confocal microscopy to ascertain the interfacial characteristics of the demineralized /remineralized dentin interfaces. The specimens were immersed in 1 wt% aqueous fluorescein dye solution for 24 h and subsequently rinsed in an ultrasonic bath for 2 min containing distilled water. The specimens were polished using 1200-grit SiC paper for 30 s followed by a further rinse in an ultrasonic bath (2 min). Baseline lesions were similarly prepared.

The specimens were first evaluated in a fluorescence optical microscope (DEM2500 LED) equipped with a LED light and a filter-pass (490–520 nm) and a 20 × NA 0.7 oil-immersion lens. Subsequently, the specimens were examined using a confocal laser scanning microscope (Leica SP5 CLSM; Leica, Heidelberg, Germany), equipped with a 63×/1.4 NA oil-immersion lens and a 514 nm argon/helium ion laser. Confocal laser scanning microscopy reflection and fluorescence images were obtained with a 1-μm z-step to optically section the specimens to a depth of up to 20 μm below the surface. The z-axis scan of the interface surface was compiled into a single projection using the Leica SP5 CLSM image-processing software (Leica). The configuration of the system was standardized and used at the same settings for the entire investigation. Each dentin interface was completely investigated and five micrographs representing the most common morphology features observed in the specimens were captured and recorded.

2.8. Transmission electron microscopy

Further three representative specimens from each group previously tested for microhardness were used for TEM. Specimens were fixed in Karnovsky's fixative, post-fixed with 1% osmium tetroxide, dehydrated in an ascending ethanol series (30–100%), transitioned through propylene oxide and embedded in epoxy resin. Baseline lesions were similarly prepared.

Thick sections (180–200 nm) of the caries-like lesions including the mineralized dentin base were prepared without additional demineralization and examined unstained for evaluating the overall effect of remineralization. Resin blocks were then trimmed for thin section preparation (90 nm). Examination was performed using a JEM-1230 TEM (JEOL, Tokyo, Japan) at 110 kV.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 20 (IBM, Chicago, IL, USA). Data distribution was assessed via Shapiro-Wilk-test. Generalized linear modelling (GLM) was performed to determine differences across groups. Level of significance was set at $p < 0.05$.

3. Results

The microradiographic analysis showed that the simulated carious lesion attained in the specimens with the D1 protocol had a median mineral loss ΔZ [$25^{th}/75^{th}$ percentiles] of 3978 [3622/5266] vol%×μm and microhardness of 37.4 (30.0/45.4) VHN. Those specimens processed with the D2 protocol had 5047 (4859/5569) vol%×μm and 31.9 (25.0/42.3) VHN, while the specimens in group D3 had 6649 (5991/8155) vol%×μm and 35.7 (25.9/43.6) VHN.

After the remineralization period, it was observed that all four restorative materials induced mineral gain without any significant difference between the groups ($p > 0.05$). However, differences in mineral gain were detected between lesion types; significantly higher values were observed in deep bacterially-induced lesions compared to shallow or deep chemically-induced lesions (Fig. 2A).

Microhardness was only selectively increased by the restorative materials. For instance, GIC failed to increase the hardness regardless of the lesion type (Fig. 2B). EXP provided significant hardness gain only in the specimens of group D3. BIO and MTA increased the hardness in all lesions types and these were significantly superior to GIC and EXP ($p < 0.05$).

The fluorescence and confocal microscopy confirmed the results obtained during microradiography and microhardness measurements. The demineralization process performed with bacteria (D3) created a deeply demineralized and denatured dentin, resembling natural residual lesions (Fig. 3A). The demineralization protocol D1 created only shallow lesions, which showed limited fluorescein uptake. The fluorescein uptake into the demineralized dentin created with D2 was deeper, while denaturation was not detected (Fig. 3B,C). None of the treatments had completely remineralized the lesions. However, D3-lesions showed detectable mineral precipitation after remineralization using BIO and MTA (Fig. 3D).

Such results were confirmed by TEM analysis, where it was possible to see clear mineral precipitation on the top of the dentin treated with BIO (Fig. 4A) and MTA (Fig. 4B). In these latter specimens, irregularly shaped (needle-like or globular) mineral crystals were often detected. In the case of dentin treated with GIC, there was no evidence of collagen remineralization (Fig. 4C). Specimens restored using EXP showed limited mineral precipitation on the top of the dentin, but localised

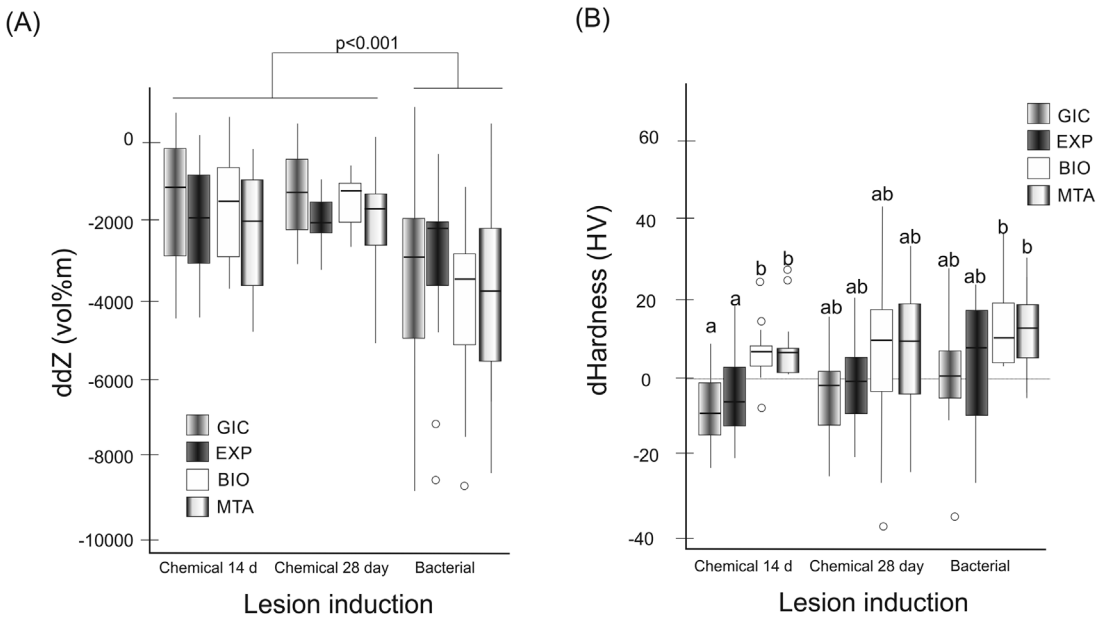

Fig. 2 – Mineral (A) and hardness (B) gains induced by the different materials. Absolute mineral differences (ddZ) and hardness differences (dHV) provided by the glass ionomer cement (GIC), the experimental adhesive system (EXP), Biodentine (BIO) and Mineral Trioxide Aggregate (MTA) compared with baseline are shown. Different lesion types were remineralized (D1: chemical 14 days, D2: chemical 28 days, D3: bacterial). Note that mineral gains are indicated by negative values (the mineral loss was reduced), while hardness gains are indicated by positive values (hardness was increased). Mineral differences were only detected between lesion types; being significantly higher in bacterially induced lesions than chemically induced ones. In contrast, hardness gains differed between materials. Shared superscript letters indicate no significant difference between the groups. Line and box: Median and 25th/75th percentiles, whiskers: min./max. after exclusion of outliers, circles: outliers.

"bottom-up" intrafibrillar collagen remineralization in D1- and D2-lesions, with the collagen morphology resembling that of sound dentin (Fig. 4D).

4. Discussion

Our results warrant partial rejection of the null hypothesis that the remineralization (mineral and hardness gain, assessed through transverse microradiography and microhardness) would not differ significantly between the tested materials.

Different increases in mineral content were detected between the three simulated lesion types (D1, D2 and D3). Indeed, higher relative mineral gains were observed in deep bacterially-induced lesions (D3) compared to shallow (D1) or deep chemically-induced ones (D2). Conversely, the increase in microhardness was material-dependent; only BIO and MTA induced a significant increase of hardness in all lesions types of demineralized dentin lesions. However, complete remineralization of the simulated lesions was not achieved with any material.

In details, it was found that both BIO and MTA formed a layer of intermediate, structurally altered remineralized tissue between the cement and the dentin. This is in agreement with the findings reported by Atmeh et al. [24], who showed that Biodentine was able to form a mineral infiltration zone in dentin, just beneath the cement [24]. Formation of such layer was suggested to be the result of carbonate and/or apatite formation within the superficial dentin tubules [25,26]. However, our TEM results revealed that such a mineral precipitation achieved both with BIO and MTA was characterized by the absence of collagen fibrils. It is well known that calcium silicate cements form calcium hydroxide, which is a highly alkaline material (pH = 13), and hence may cause caustic degradation of exposed demineralized collagen (as is found in carious dentin). This effect is mediated by the breakdown of intermolecular bonds in the collagen fibrils, increasing their water absorption and leading to swelling [27] as well as changing collagen conformation [24]. Thus, calcium silicate cements may deliver a great deal of mineral to the demineralized dentin, thereby increasing its hardness, but with no possibility to achieve intrafibrillar remineralization and recovery of a sound module of elasticity [28].

In our study, GIC provided mineral, but not hardness gains to demineralized dentin. Hence, while artificial carious dentin can be "remineralized" using GIC [29], even to significant depths [14], the formation of carbonated apatite inside and around collagen fibrils in demineralized dentin cannot be expected.

Our results demonstrate that the use of the experimental adhesive containing fluoride-doped bioglass (EXP) provided mineral and microhardness gains, especially in the lesion attained using the bacteria-mediated demineralization protocol. The bioglass used in EXP had been utilized in a previous study, where it was able to alkalinize the incubation media,

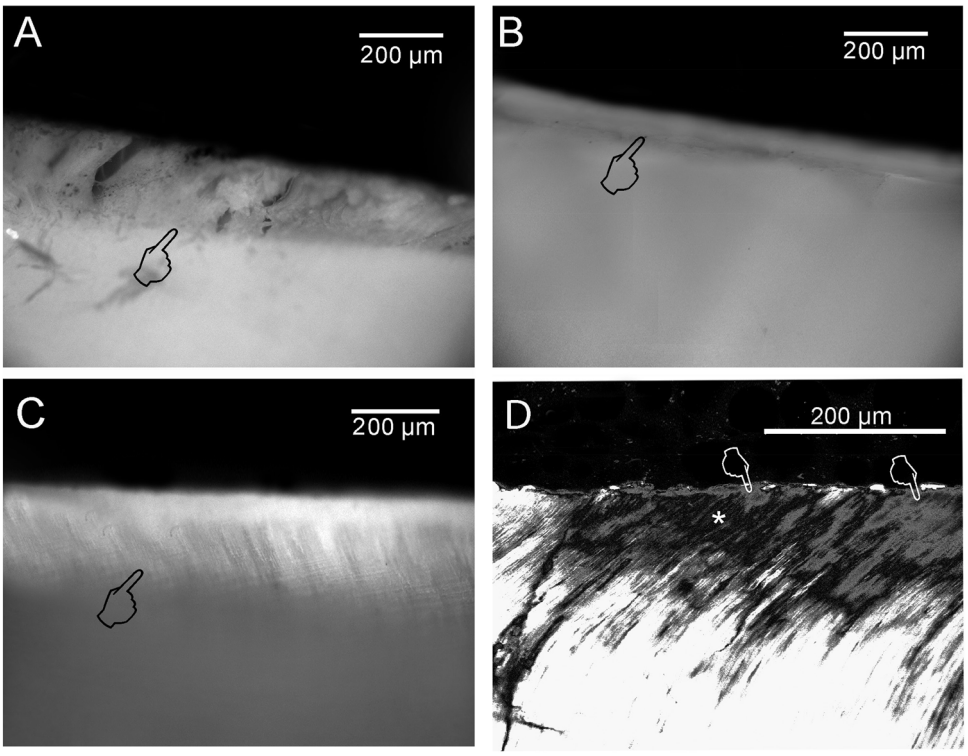

Fig. 3 – Fluorescence and confocal microscopic images of remineralized dentin specimens. (A) The fluorescence microscopy showed that the demineralization process performed in dentin with bacteria (D3) was able to create a layer of deeply demineralized and denatured dentin (Pointer). (B) The chemical demineralization process D1 was able to demineralise the dentin without creating any denaturation (Fig. 2b). However, this demineralized layer was not as deep (pointer) as the one observed in the specimens chemically demineralized using the process D2. (C) In this confocal image it is possible to see that the demineralization area (pointer: fluorescein uptake from the demineralized dentin collagen) is deeper than 200 µm. D) The only materials to show evident mineral precipitation in demineralized dentin (pointers) during confocal microscopy evaluation were MTA (shown here in a D3-lesion) and BIO. Nevertheless, large areas of the dentin remain demineralized (*).

reduce the solubilization of dentin collagen telopeptides, and significantly increase the stiffness of the demineralized dentin matrices [20]. It is has been advocated that bioactive glasses can release $Si(OH)_4$, which then binds to demineralized collagen and polymerizes into a absorbent SiO_2-rich layer. This layer might assist in the precipitation of amorphous calcium phosphate [30], and subsequent conversion into nonstoichiometric apatite [31]. Moreover, an alkaline environment rich in minerals may allow $Si(OH)_4$ condensation and Ca^{2+} and PO_4^{3-} precipitation; this may help to fossilize metalloproteinases and cathepsins and reduce their proteolytic activity [32]. This may explain why collagen was preserved in the specimens tested in this study (Fig. 4D). In contrast, the two calcium silicate cements (BIO and MTA) may have replaced collagen by minerals via caustic degradation, as previously described.

Our TEM results confirmed that it was possible to obtain an intrafibrillar "bottom-up" remineralization of the collagen fibrils via EXP in some, but not all zones of the demineralized dentin. We believe that this was possible due to the presence of two biomimetic analogues of remineralization in the experimental primer. The application of such biomimetic analogues has been found to promote hierarchical intrafibrillar deposition of needle-like crystallites, hence stabilizing bond strengths and reducing nanoleakage [33]. Furthermore, such biomimetic remineralization allows to slowly "back-fill" residual water trees in the hybrid layer with apatite crystallites via pre-nucleation clusters to produce fluidic, polymer-stabilised amorphous calcium phosphate nano-precursors [30,34].

This study has a number of limitations. First, we used – albeit different – artificial lesions in this study, for reasons of availability and standardization. Future studies should assess if our findings are applicable to genuine dentin carious lesions. These lesions should be more difficult to remineralize due to their variability in lesion depths and degrees of tubular occlusion [15,35]. Moreover, unlike artificial caries-like lesions, demineralization in real human caries-affected dentin is often manifested as sporadic islands of demineralization instead of a continuous demineralization gradient from the lesion surface to the lesion base [36]. Second, only representative commercial and experimental materials have been used; a wide range of calcium silicates is available and could have been tested [37]. Third, we only assessed mineral and microhardness gains, and further studied ultrastructural effects. Material effects on the pulp or on bacteria within contaminated carious lesions have not been tested here [19]. Finally, the clinical relevance of our findings remains unclear; for instance, the impact of remineralization on restoration longevity cannot be directly inferred.

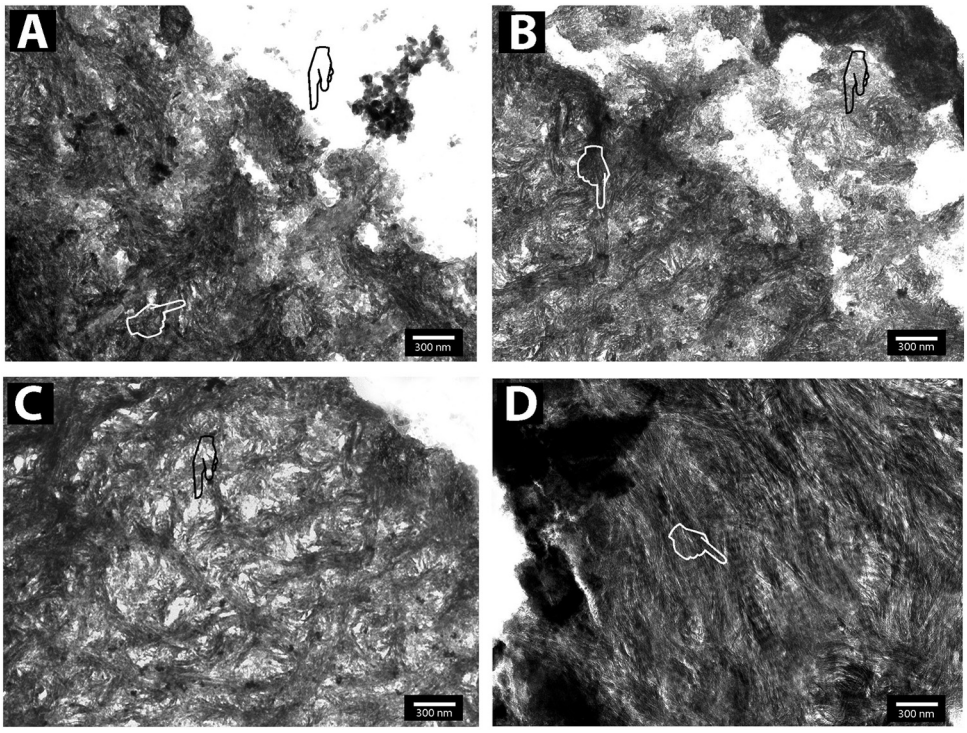

Fig. 4 – TEM assessment of remineralized lesions. (A) Representative TEM image of a dentin mineralized using BIO. A clear mineral precipitation is detectable on the top of the dentin (black pointer), and minerals with different shapes (needle-like or globular) and dimension (white pointer) are present within the demineralized dentin. (B) The same situation was observed with dentin mineralized using MTA. Evident mineral precipitation is detectable on the top of the dentin (black pointer), and minerals with different shapes and dimension (white pointer) can be seen within the demineralized dentin. (C) Dentin specimen treated with GIC. In this case there was no evidence of collagen remineralization (black pointer). (D) Dentin treated using EXP showed in a several cases intrafibrillar remineralization (white pointer) in all three types of lesions, with the collagen morphology resembling that of sound dentin.

5. Conclusions

Within the limitation of this study, we affirmed that complete remineralization of carious lesions remain a challenge. However, Biodentine and MTA may provide effective mineral precipitation and microhardness recovery in different artificial carious lesions. Nevertheless, experimental adhesives, containing ions-releasing fluoride-doped bioglass and biomimetic analogues of remineralization, may be able to allow intrafibrillar collagen mineral infiltration.

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