Enamel erosion and prevention efficacy characterized by Confocal Laser Scanning Microscope

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Running title: Eroded enamel characterized by CLSM

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Abstract

The aim of this study was to evaluate the erosion-inhibiting effect of two toothpastes on the development of erosion-like lesions, by a Confocal Laser Scanning Microscope (CLSM). Forty human enamel blocks were divided into five groups (n=8), in accordance to evaluate the GC MI Paste Plus and Oral B with stannous fluoride, applied as slurries and associated with toothbrush. Specimens were submitted to an erosion challenge from citric acid (0,5%, pH=2,8), for 5 minutes, 6 times a day, alternating in artificial saliva immersions. Reference group was not exposed to treatment. Part of specimens (Groups 02 and 03) was exposed twice daily just to slurries, for 2 minutes, therefore specimens from Groups 04 and 05 were also abraded, for 30 seconds. The enamel surfaces were morphological characterized using CLSM images, with mineral loss being measured using the resulting 3D images referenced to an un-challenged portion of the sample. Step values were compared using the One Way ANOVA test. CLSM was shown to be a viable, non-contact and simple technique to characterize eroded surfaces. The statistical difference in the step size was significant between the groups (p=0.001) and using multiple comparisons a statistically significant protective effect of toothpastes was shown when these were applied as slurries. Although groups submitted to tooth brush showed mineral loss similar to reference control group, due to the damages of abrasion associated.

Key Words: Fluoride, Tooth erosion, enamel surface

Introduction

There is evidence that the prevalence of dental erosion is steadily increasing (Jaeggi and Lussi, 2006) and its management has becoming an important aspect of the long-term health of dentition around the world. In the light of the difficulties involved in clinically detecting, monitoring and managing dental erosion, researchers are actively searching for new agents for the prevention, ~~or repair,~~ of dental erosion lesions and recently several strategies have been tested aiming to limit enamel erosion (Huysmans et al.; 2011; Moretto et al., 2010; Ranjitkar et al., 2009; Rios et al.; 2006).

It has been shown *in vitro* that fluoride treatments, such as sodium fluoride, amine fluoride or acidulated phosphate fluoride, form CaF_2-like layers on the tooth surface, which is unlikely to provide a preventive effect against erosion, as an acidic drink will rapidly dissolve the accessible CaF_2 and remove traces of any previous topical fluoride treatment (Larsen and Richards, 2002). In recent years several research groups (Ganss et al., 2011; Wiegand et al., 2010; Schlueter et al., 2009; Rees et al.; 2007; Magalhães et al., 2007) have investigated the preventive effects of different fluoride formulations on dental erosion in order to identify preparations or compounds that form precipitates other than CaF_2-like layers.

Agents based on milk products have been investigated for many years and currently, several different paste formulations are available as variations of MI Paste Plus (GC Corporation, Tokyo, Japan), which is based on a nano-complex of the milk protein casein phosphor-peptide (CPP) with amorphous calcium phosphate (ACP).

CPP binds to form nano-clusters of ACP preventing their growth to the critical size required for nucleation and phase transformation (Reynolds, 1998). The complex compound thus formed has demonstrated preventive and re-mineralization properties in the caries process (Reynolds et al., 1999). It has been claimed that CPP-ACP

promotes a supersaturated state close to dental hard tissues, making remineralisation of surface enamel possible (Rahiotis and Vougiouklakis, 2007).

One other agent that has shown promise under both mild and severe erosive conditions is the stannous ion (Ganss et al., 2004; Tinanoff, 1995). The application of tin-containing solutions leads to deposits on the tooth surface (Hove et al., 2008; Willumsen et al., 2004) and there are indications that these deposits are relatively resistant to acid dissolution (Hjortsjö et al., 2009). It is known that the stannous ion reacts with pure hydroxyapatite (Schlueter et al., 2007; Young et al., 2006) on the surface of the dental hard tissue (Willumsen et al., 2004), resulting in reduced solubility of hydroxyapatite or enamel (Tinanoff, 1995).

While good oral hygiene is of proven value in the prevention of periodontal disease and dental caries, frequent tooth brushing with abrasive oral hygiene products may enhance tooth damage (Lussi et al., 2011). Several studies have shown that softened enamel (such as that caused by acidic drinks) is very susceptible to scratching (Eisenburger et al., 2003; Jaeggi and Lussi, 1999; Lippert et al., 2004) and also highly unstable and can be easily removed by short and relatively gentle physical action (Eisenburger et al., 2003). Tooth brushing of eroded enamel thus leads to minor changes in its surface morphology and mechanical properties (Lippert et al., 2004).

The structural changes resulting from different challenges and anti-erosive treatments can be studied by qualitative methods, such as optical or electron microscopy, which can be used either alone or combined with quantitative measurements (Schlueter et al., 2011). Confocal laser scanning microscopy (CLSM) is a non-destructive 3D technique commonly used in biological imaging, capable of producing high-resolution images, by scanning the surface with a highly focused laser beam and using the principle of confocal imaging to reject light returned from out of focus layers, thus effectively optically sectioning the sample (Sheppard and Shotton,

1997). This has been recently applied to the analysis of eroded enamel surfaces to assess quantitative of tissue loss (Heurich et al., 2010). More recently, the 3D Focus Varying Microscope (FVM) also shows sensitive in detecting structural changes following erosive and abrasive processes, and it could also perform optical scans of the surface in three dimensions, and calculate surface roughness quantitatively without any surface damages (Lima et al., 2013, Passos et al., 2013, Ren et al., 2009).

The advantages of CLSM are the high resolution (sub-micron) images which are similar to low magnification Scanning Electron Microscopy (SEM) but without any of the problems of specimen preparation (Field et al., 2010). Systems are routinely capable of imaging at in excess of 10 frames per second and thus rapidly record the surface topography allowing quantification of the interface step between an eroded area and a sound enamel reference.

Therefore, the purpose of the present study was to perform an *in vitro* evaluation of enamel surfaces subjected to citric acid attack and to quantify the erosion-inhibiting and/or re-mineralising potential of specific anti-erosive agents applied with and without toothbrush abrasion. We sought (i) to verify if confocal laser scanning microscopy identify alterations on enamel structure and then (ii) to evaluate and compare the anti-erosive regime/paste efficacy.

Materials and Methods

Sample Preparation

Permission was granted by the Ethical Committee of the Federal University of Pernambuco - Recife PE, according to approval form (038/2010), and 20 third molar teeth were acquired from the tooth bank from the same Institution. All teeth were kept in 0.05% Chloramine T (Rio de Janeiro, RJ, Brazil) for 1 week for disinfection and

stored in a humid environment during the experimental stages. The selection criteria of included the absence of caries, cracks, fractures, grooves or surface decalcification under visual observation in natural light. Forty transverse-sectioned enamel specimens were prepared from the facial and/or lingual surface of the freshly extracted molars.

Each sample was embedded in acrylic resin (VIPI CRIL Plus, Pirassununga, SP, Brazil) and the natural surfaces were ground flat in a water-cooled mechanical grinder and carefully polished with sandpaper of decreasing grit (P600 and P1200 ground discs Buhler, Illinois, EUA) until the preparation resulted in an experimental surface area of at least 4 x 4mm^2. Final polishing was performed with a metallographic polishing cloth (SUPRA, Arotec, Sao Paulo, SP, Brazil) moistened with 5µm diamond polishing oil suspension (Buehler, Illinois, EUA).

Specimens were randomly divided into five groups initially [n=8]. Each sample was attached to a single holder and around one third of the experimental area of each specimen was covered by waterproof transparent adhesive tape (3M, St. Paul, Minnesota, EUA) to protect the reference area of un-etched enamel from the test regimens. To permit simultaneous immersion of all samples in the solution the teeth were attached by wire to the caps of the falcon tubes into which the test solutions were placed. The caps were then attached to a rod so that the samples could be inserted and removed from the solutions simultaneously.

Erosion Cycle

Before the erosion cycle, samples were soaked in commercially available artificial saliva (A.S Orthana Saliva, Andover, Hampshire, UK) for 24 hours. All specimens (Groups 1-5) were subjected to a cyclic demineralization and remineralisation procedure, with six demineralisation periods per day 5-min each; 0,5% citric acid, pH 2.8, (Anhydrous citric acid; Merck KGaA, Darmstadt, Germany), as an

adaptation of previously published method (Schlueter et al., 2009). There was a gap of around one and a half hours between each immersion and this cycle was repeated over three days.

The control reference samples were submitted only to cyclic demineralization and reinsertion in artificial saliva pH=7,0, for one and a half hours between acid attacks to simulate the oral environment. After each immersion, the specimens were taken out from the solution, and carefully washed using deionised water for 30 seconds to remove any residual acid or saliva.

Two preventive products were used: Casein Phosphor Peptide Amorphous Calcium Phosphate (CPP-ACP) plus Sodium Fluoride 900ppm available in GC MI Paste Plus (Mint flavour, GC Corporation, Tokyo, Japan); Stannous Fluoride 1100ppm and Sodium Fluoride 350ppm available in Oral B Pro Health (Proctor & Gamble, Weybridge, UK), tested by groups described on table 1.

Table 1: Specification of preventive products used.

Groups	Preventive Products	Brand	Toothbrush
G 01	---		
G 02	CPP-ACP NaF 900ppm	GC Corporation	
G 03	CPP-ACP NaF 900ppm	GC Corporation	Phillips Sonicare
G 04	SnF 1100ppm; NaF 350ppm	Proctor & Gamble	
G 05	SnF 1100ppm; NaF 350ppm	Proctor & Gamble	Phillips Sonicare

To compare the best treatment for the control or arrest of erosion based lesions, samples were subjected to the preventive solution cycling treatment. The toothpastes under test were combined into slurries in 1:3 ratio of deionized water and the slurries placed on the samples after the first and last erosion period each day, for 2

min on each occasion. Specimens of two groups were also submitted to abrasion by tooth brush. For the abrasion test, groups (03 and 05) were also brushed for 30 seconds within the slurry during the two minute immersion time, using an electrical toothbrush (Phillips Sonicare, HX6511/50, Andover, Massachusetts, USA) fixed in a mechanical set-up to control the brushing force to 2N.

Procedures were started in the morning, with the erosive solution renewed at the beginning of each day. The pH of all solutions was measured and controlled on each experimental day. All procedures were performed, avoiding agitation, at room temperature (20°C).

CLSM Measurements

Each sample was analysed by CLSM after the three days of erosive and preventive regime. Moving the microscope objective through the optical axis, it was possible to produce successive focal optical section at 1 micron step intervals and thus reconstruct a 3D image of the tooth. From the image stack it was then possible to quantify the height differences between the eroded and reference area.

The images were taken using a Nikon D-Eclipse C1 confocal microscope (NIKON Instruments Inc. Melville, New York, USA), with a 405 nm, 25 mW laser (Coherent, Santa Clara, California, USA) used to illuminate and excite the tooth samples with the power on the sample limited to around 100 μW. Fluorescence from the sample was detected in two different channels: blue (515-530 nm) and green (590-650 nm). An apochromatic 60x water dipping objective lens with an NA of 1.0 was used unless otherwise stated and optical sections were recorded at 1 micron depth intervals (accuracy of depth sections being +/- 50 nm). From the resulting image stack measurements could then be made on the height differences between the eroded and un-damaged areas along with a qualitative assessment of the surface finish of the samples.

Images Analysis

Images were plotted using Image J public domain software [http://rsbweb.nih.gov/ij]. The individual image slices were initially combined into an image stack for each series of confocal sections and the resulting stacks then used for the subsequent image qualitative description by observers. Using the 3D reconstructed images the XZ (Z being defined as into the tooth) profile was examined and the average height difference between the eroded and un-eroded sections measured, considering a distance of 40 um from interface, as the point/level of eroded area. No other image processing was undertaken on the images and the standard “grayscale” look-up table is used in all images presented.

Statistics

Data were organized into an Excel spreadsheet (Microsoft Office 2007) and analyzed using SPSS 13.0 (Statistical Package for the Social Sciences, Chicago, USA) for Windows. Statistical tests were guided after a Komogorov-Smirnov test was used to evaluate the normality of the data. The One Way ANOVA test was performed for comparison among groups. All tests were applied with 95% confidence.

Results

Around 350 images were analyzed and typical images are shown below in Figure 1. Figure 1a shows a representative sample of a polished area of ~~sound~~ enamel (areas under the protective tape) with the surface appearing quite smooth, the prisms and organic matrix not well defined. Both the returned fluorescence and reflected light shows little scattering even to a depth of around 25 microns below the surface. The absence of clear enamel structure seems to correspond to the aprismatic layer of enamel produced during the polishing procedure.

The typical appearance of eroded enamel submitted only to citric acid attack is shown in Figure 1b and 1c. Samples subjected to the preventive treatments of toothpaste slurries showed differences in the resulting enamel morphology. As shown in Figure 2a, the sample treated with CPP-ACP NaF (G 02) demonstrated a similar appearance to the control-eroded group (Figure 1c), with areas of mineral loss, though the XZ section is perhaps not as rough. Samples subjected to toothpaste slurries containing SnF_2 (G 04) present a lower level of fluorescence (compared to the control eroded sample) and it appears as though a thin layer of stannous fluoride is covering the enamel surface. The layer is not uniform and appears as a series of swirls, as observed on Figure 2b.

In the groups where samples were abraded for 30 seconds during the toothpaste slurry treatment, it was possible to distinguish toothbrush effects on the eroded enamel. In the CPP-ACP NaF group (G 03) lines of brushing in specific directions can be observed, with greater mineral loss at the top of enamel rods, leaving the rod boundary well defined (Figure 2c). Samples submitted to tooth brushing during the Stannous Fluoride treatment (G 05) showed brushing effects, as a mixed appearance with areas of etched prisms combined with areas where a surface layer appears to cover enamel (Figure 2d).

As can be seen in Figure 3 at the interface between the exposed and protected enamel during the acid attack a significant step develops. Visually it is clear that the stannous containing compound alone (G 04) shows less damage than the samples protected by tooth mouse and no protection.

In order to quantify these visual differences the average height change was measured using the XZ projection. Table 2 shows the average height change for each group, together with the standard deviation and percentage mineral loss. The statistical difference in the step size was significant between the groups, as demonstrated by

One Way ANOVA test (p=0.001). The Mann-Whitney test analyzing by multiple comparisons showed statistically significant protective effect of SnF_2 (G 04, p=0.001) and CPP-ACP NaF (G 02, p=0.041) when applied as toothpaste slurries.

Discussion

This study aimed to elucidate the effects of two different fluoride toothpastes on eroded dental hard tissue using confocal laser scanning microscope and quantifiable differences were recorded in line with the expected findings. At present there is no generally accepted standard protocol used in erosion studies *in vitro*, nor a previously reported reliable method of quantifying mineral loss non-destructively. A representative acidic challenge was necessary to promote alterations and facilitate demonstration of the effects of preventive agents. Therefore, an immersion time of 5 min cycles was selected to simulate clinical conditions, though the precise timings may require further optimisation. The erosive cycling model can be considered to be of medium severity, with for a daily exposure of 30 minutes - this was repeated for three days.

The confocal images provide some evidence of the processes taking place during the etching cycles. The initial polishing of the samples, to produce a uniform starting point left, as anticipated, an aprismatic area, as observed on sound reference region. However, the results of eroded surfaces show variations within the group, in which, even allowing for the same etch time, the emerging enamel rods have different shapes. This variation can be partly explained by the nature of the enamel rod following an S-shape course on the horizontal plane from DEJ to the surface. When the enamel specimens are prepared, the grinding should ideally occur at 90° to the enamel rods in order to achieve an evenly etchable surface (Hjortsjö et al., 2010). Due to the nature curvature of the teeth this may not always occur when an area of around 3 x 3 mm is required, hence the alignment of the exposed enamel rods in the various samples may

be different, thus affecting their etch susceptibility and their response to fluoride treatment.

The eroded surface showed a clearly visible increase in fluorescence, with areas of mild alteration of the enamel organic matrix, evidenced by the more apparent 'honeycomb' morphology. In areas where the eroded enamel prisms were clearly exposed, the 'honeycomb' morphology was better defined, with an apparently greater loss of organic matrix. Erosion of the rod boundary appears as a lower level of detected fluorescence from around each prism. The interaction of the light with such microscopic surfaces with large changes in the refractive indices of materials is complex and the exact reasons for the appearance of features is open to debate but it is clear that the method of fluorescence confocal microscopy clearly shows the changes in surface morphology. Surface roughness can also be confirmed through the transverse section image, and this can again be quantified using more advanced image processing methods than used in these preliminary measurements.

Previously, studies have assumed that tissue loss values of the order of 10-15 μm compared to the negative control group are sufficient for demonstration of differentiation of agent effects (Ganss et al., 2012). In this study, the erosive procedure was more intense, due to an acid etch time of five rather than two minutes, and the cycles completed in 3 days rather than 10. However, this resulted in a step height of 15.3μm (+/-4.8) of similar magnitude to the previous slower etch and perhaps more suited to a high throughput initial screen.

In this study, casein phospho-peptide as a component of a tooth cream in combination with ACP, although not indicated for daily use, was investigated for anti-erosive effects and abrasion prevention. It is known that CPP-ACP limits the free calcium and phosphate ion activities, thus helping to maintain a state of super-saturation, which decreases demineralization (Reynolds, 2008), although there are

conflicting results about its effectiveness (Rees et al., 2007; Wang et al., 2011; Wegehaupt and Attin, 2010). We observed a weaker protective effect, of 39% mineral loss reduction, against acid challenge, although still statistically significant (p=0.041).

There is preliminary evidence that toothpastes containing the Sn^{2+} ion could be promising agents in the prevention of acid based erosion (Ganss et al., 2008; Huysmans et al., 2011). In this study a toothpaste containing stannous fluoride was tested and a continuous surface coating appeared on the treated samples. One of the suggested mechanisms of erosion prevention of SnF_2 is the promotion of a protective layer on the tooth surface (Huysmans et al., 2011) and stannous fluoride has been demonstrated to be capable of depositing appreciable levels of tin on enamel. Using CLSM for surface analysis, we observed an area of minimum fluorescence, similar to a dehydrated surface, and as previously described, even when analysed using the water dipping objective lens through distilled water the tin protective layer was stable.

As noted, surfaces treated additionally with tooth-brushing showed decreased protection, probably due to the abrasion effects of physical forces. The stannous layer was abraded and totally removed in some areas. CLSM XZ sections supported the evidence that the SnF_2 does form a thin, but not strong, protective layer on the tooth. Comparing interface values of the step no statistical difference was found between the negative control and the tooth-brushed groups.

CLSM has high resolution that was sufficient to evaluate erosion effects on samples with minimum sample preparation. CLSM was shown to be an alternative to scanning electron microscope (in environmental mode) that facilitated evaluation without damage. The non-contact method is a significant advance as there is no risk of damaging the delicate protein matrix left exposed after erosive attack, which may well play a role in supporting the re-mineralisation process. In this limited study toothpaste containing SnF_2 showed a significant (p=0.001) protective ability of 70% against acid

based erosion, though the use of a toothbrush reduced 95% of its effectiveness. Abrasion promoted by toothbrush procedures also reduced 80% of the protective effects of CPP-ACP NaF.

In conclusion, eroded samples showed loss of the organic matrix and exposure of enamel rods, increasing the irregularity of the enamel surface. The stannous fluoride within the Oral B toothpaste and the CPP-ACP NaF in the Tooth Mousse Plus demonstrate mineral loss reduction of 70%, and 39%, respectively. However, abrasion damage decreased those protective effects. Confocal microscopy is an excellent non-contact method for the monitoring of acid erosion and re-mineralisation on enamel. This study also shows the potential of this method to quantify the effect of acid based erosion *in vitro*, which may be suitable for high throughput screening of new toothpaste formulations in relation to protection against acid attack

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Declaration of Interests

The authors certify that there are no conflicts of interest in this study. The study was funded by public Brazilian agencies, and they had no input into the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

On behalf of the authors.

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Table 2. Tissue loss (µm) in all groups (mean ± SD) after three days of in vitro demineralization and relative mineral loss (percentage of control group).

	Control Group	**CPP-ACP NaF (S)**	**CPP-ACP NaF (TB)**	**SnF_2 + NaF (S)**	**SnF_2 + NaF (TB)**
Average Height Loss	15.3 ±4.8^{A}	9.3 ±4.9^{B}	14.2 ±6.8^{A}	4.6 ±1.3^{B}	10.3 ±3.1^{A}
% Reduction of enamel Loss	-	39.2%	7.2%	70%	32.7%

One Way ANOVA test p=0.001.Data sharing the same superscript letter are not significantly different.

Figure Legends

Figure 1: CLSM typical images of sound enamel surface (a); soft eroded surface (b); and areas of aggressive eroded surface (c). Below each Figures (a), (b) and (c), there is a XZ (transversal) section taken from each reconstructed images surface.

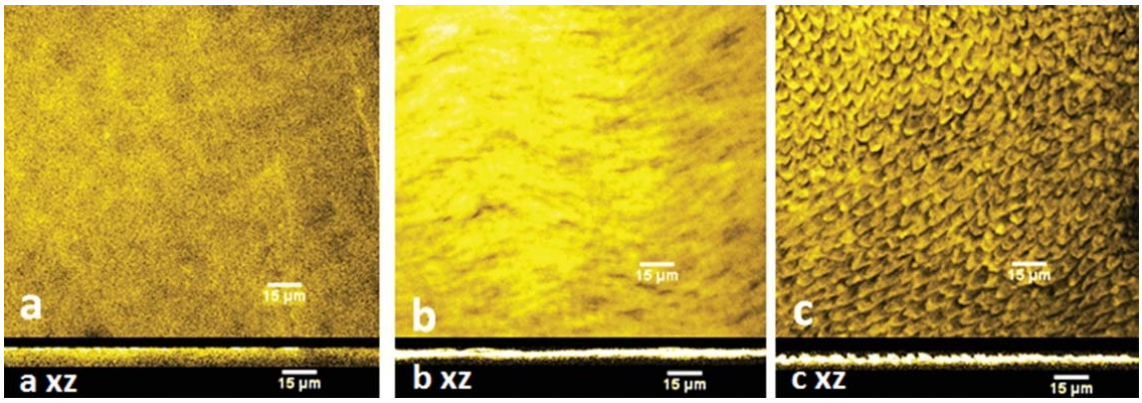

Figure 2: CLSM of typical enamel surfaces treated with prevention products: (a) G 02: CPP-ACP NaF Solution; (b) G 04: Oral B SnF_2 Solution; (c) G 03: CPP-ACP NaF Tooth-brushed; and (d) G 05: Oral B SnF_2 tooth-brushed effects. Below each image, there is a transversal XZ section image taken from the reconstructed images.

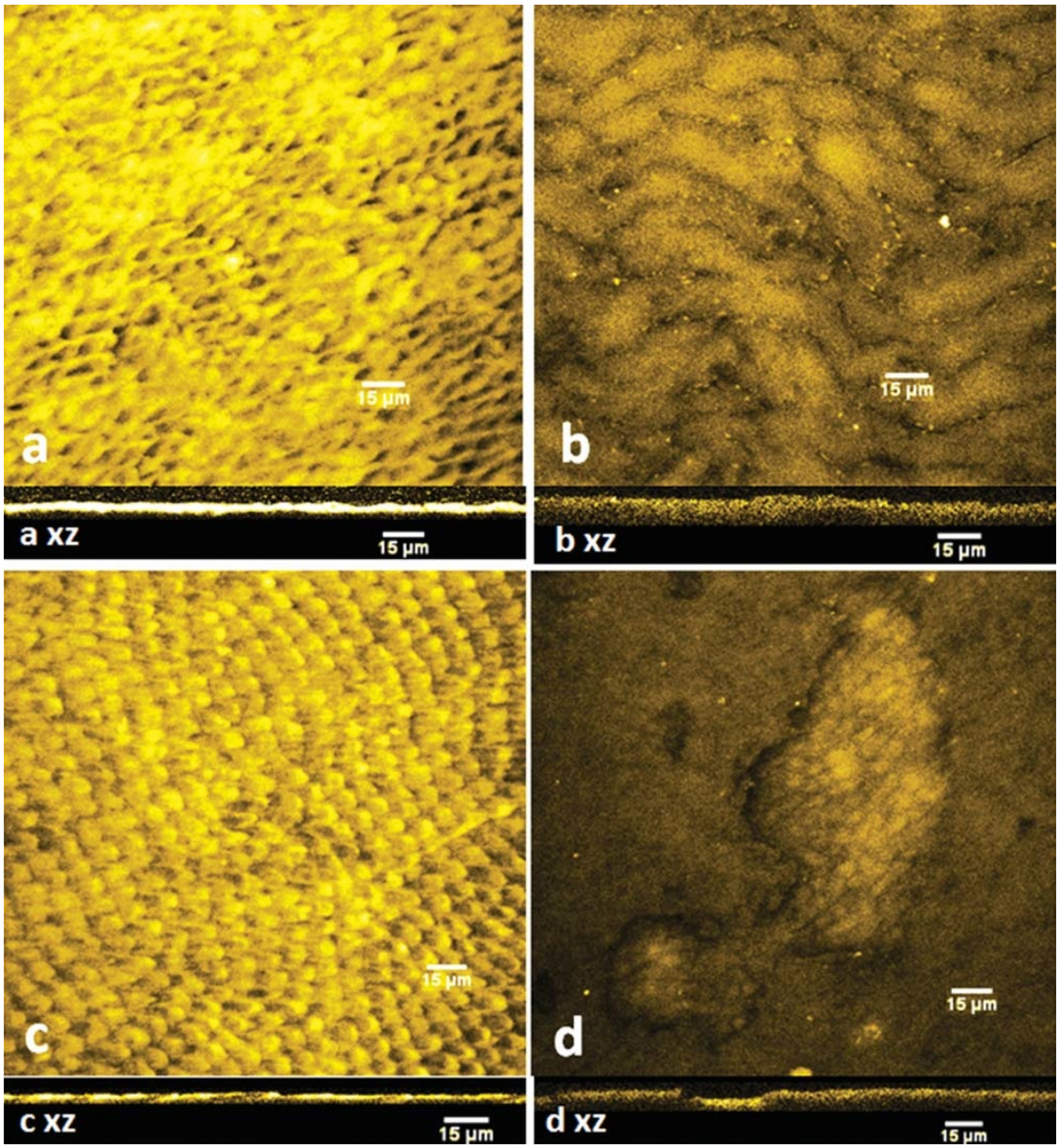

Figure 3: CLSM image on XZ sections of representative interface between sound and eroded area of each tested group at the end of cycle regime. (An interface reference between change of level is indicated by narrows).