Confocal laser scanning reflection microscopy applied to the remineralization studies in incipient caries lesions

Microscopía confocal de reflexión de barrido láser aplicada a los estudios de remineralización en lesiones de caries incipientes

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ABSTRACT

To evaluate through roughness parameters measurement the penetration of calcium phosphate stabilized by casein phosphopeptides (CPP-ACP) in the subsurface zone of the incipient caries lesions known in dentistry as white spot lesion (WSL). To our knowledge, there is no data about roughness parameters to evaluate the demineralization-remineralization process in the body of the lesion. WSL was generated in vitro in 10 dental samples and sectioned in half of them (longitudinal section). CPP-ACP was applied on the WSL (3 minutes daily for 60 days). Roughness parameters (Rp and Ra) were measured on the longitudinal section with a laser scanning confocal microscope in the reflection acquisition mode (CLSRM), either in the WSL or sound enamel, before and after applying CPP-ACP. CLSRM is a non-contact method able to detect small irregularities more precisely due to the small spot of laser illumination (0.5 µm). Mixed linear models were carried out, using the treatment as a fixed factor and the tooth as a random factor (significance level 5%). Rp and Ra values in WSL area before applying the remineralization protocol (WSL pre) were significantly higher than in the sound enamel of the same samples. After applying CPP-ACP, Rp and Ra values decreased significantly with respect to the WSL pre and were similar to the values of these parameters in sound enamel. roughness parameters were used as an indirect way to measure the porosity of WSL at subsurface level. The decrease of these parameters could be interpreted as the remineralization of WSL by the ability of CPP-ACP to penetrate the body of the lesion. Clinical significance: WSL is characterized by the loss of minerals from the enamel and an increase in porosity at the subsurface level. The effectiveness of remineralizing substances would be determined by their ability to penetrate the body of the lesion and reduce porosity.

Keywords: porosity, casein phosphopeptide-amorphous calcium phosphate, enamel caries, tooth remineralization, confocal laser scanning reflection microscopy, human enamel.

RESUMEN

El objetivo de este trabajo fue evaluar mediante la medición de parámetros de rugosidad la penetración de fosfato cálcico estabilizado por fosfopéptidos de caseína (CPP-ACP) en la zona subsuperficial de las lesiones incipientes de caries, conocidas en odontología como lesión de mancha blanca (MB). Hasta donde sabemos, no existen datos sobre parámetros de rugosidad para evaluar el proceso de desmineralización-remineralización en el cuerpo de la lesión. La MB se generó in vitro en 10 muestras dentales, luego se seccionaron por la mitad de la MB (sección longitudinal). Se aplicó CPP-ACP en la MB (3 minutos diarios durante 60 días). Los parámetros de rugosidad (Rp y Ra) se midieron en la sección longitudinal con un microscopio confocal de barrido láser en el modo de adquisición de reflexión (CLSRM), tanto en la zona de MB como en el esmalte sano, antes y después de la aplicación de CPP-ACP. CLSRM es un método sin contacto capaz de detectar pequeñas irregularidades con mayor precisión debido al pequeño diámetro del haz del láser (0,5 µm). Se realizaron modelos lineales mixtos, utilizando el tratamiento como factor fijo y el diente como factor aleatorio (nivel de significancia 5%). Los valores de Rp y Ra en el área de MB antes de aplicar el protocolo de remineralización (MB pre) fueron significativamente más altos que en el esmalte sano de las mismas muestras. Después de aplicar CPP-ACP, los valores de Rp y Ra disminuyeron significativamente con respecto a MB pre y fueron similares a los valores de estos parámetros en el esmalte sano. Los parámetros de rugosidad se utilizaron como una forma indirecta de medir la porosidad de la MB a nivel del subsuperficial. La disminución de estos parámetros podría interpretarse como la remineralización de la MB por la capacidad de CPP-ACP de penetrar en el cuerpo de la lesión. Significancia clínica: La MB se caracteriza por la pérdida de minerales del esmalte y un aumento de la porosidad a nivel subsuperficial. La eficacia de las sustancias remineralizantes estaría determinada por su capacidad para penetrar en el cuerpo de la lesión y reducir la porosidad.

Palabras Claves: porosidad, fosfopéptido de caseína-fosfato de calcio amorfo, caries de esmalte, remineralización dental, microscopio confocal laser de reflexión, esmalte humano.

1 INTRODUCTION

The incipient caries lesion known in dentistry as white spot lesion (WSL) is the first clinical manifestation of tooth decay in enamel. It is described as a circumscribed loss of the translucency of the dental enamel, with a white opaque appearance, due to an optical phenomenon caused by the loss of minerals at the subsurface level. This situation leads to an increase in porosity at the ultrastructural level, which alters the refractive index and increases the scattering of light in the affected area (Sundararaj D et al., 2015).The study on the depth of dental enamel with WSL by polarized light microscopy was able to demonstrate a complex change in pore structure as the lesion develops (Darling AI, 1956; Darling AI, 1961).

The therapeutic approach to these lesions, from the philosophy of minimal intervention, is mainly based on the control of risk factors that are causing this disease and the application of remineralization substances with the aim of reversing the emerging carious lesions (Frencken JE et al., 2012).

The term 'remineralization' is defined as the process whereby calcium and phosphate ions are supplied from a source that is external to the tooth in order to promote ion deposition into crystal voids in demineralized enamel to produce net mineral gain (Cochrane NJ et al., 2010). Among the remineralization agents, CPP-ACP, is a bioactive material based on amorphous calcium phosphate (ACP) stabilized by casein phosphopeptide (CPP). In this formulation the casein phosphopeptides, through their cluster sequence –Ser(P)- Ser(P)-Ser(P)-Glu-Glu, are able to stabilize the bioactive calcium and phosphate ions in a metastable solution. Some studies have suggested that CPP-ACP is able to penetrate the subsurface level of the enamel promoting remineralization in the body of the caries lesion (Cross KJ et al., 2005; Reynolds EC et al., 2008; Zhou C et al., 2014). Nevertheless, there is not strict evidence supporting this penetration process within the body of the WSL (Pitts NB & Wefel JS, 2009; Zero DT, 2009; Borges BC et al., 2011).

The surface roughness methodology has been used by several researchers to evaluate the characteristics of dental structures surfaces and dental materials (Attia ML et al., 2015; Mullan F et al 2017). The surface roughness is conventionally defined by the set of real surface irregularities in a section of the material and is quantified by its vertical deviations (peaks and valleys). If these deviations are large, the surface is rough (Miller AZ et al., 2012).

There are several methods in the literature to measure the roughness of dental structures, such as contact profilometers (CP) or non-contact profilometers (Field J et al., 2010). Confocal laser scanning reflection microscopy (CLSRM) is a non-contact method, which has the advantage of reproducing the topography of the surface in a non-destructive way. In addition, and compared to CP, CLSRM is able to detect small irregularities more precisely due to the small laser focus spot (approximately 0.5 μm) (Field J et al., 2010; Cha JK et al., 2019).

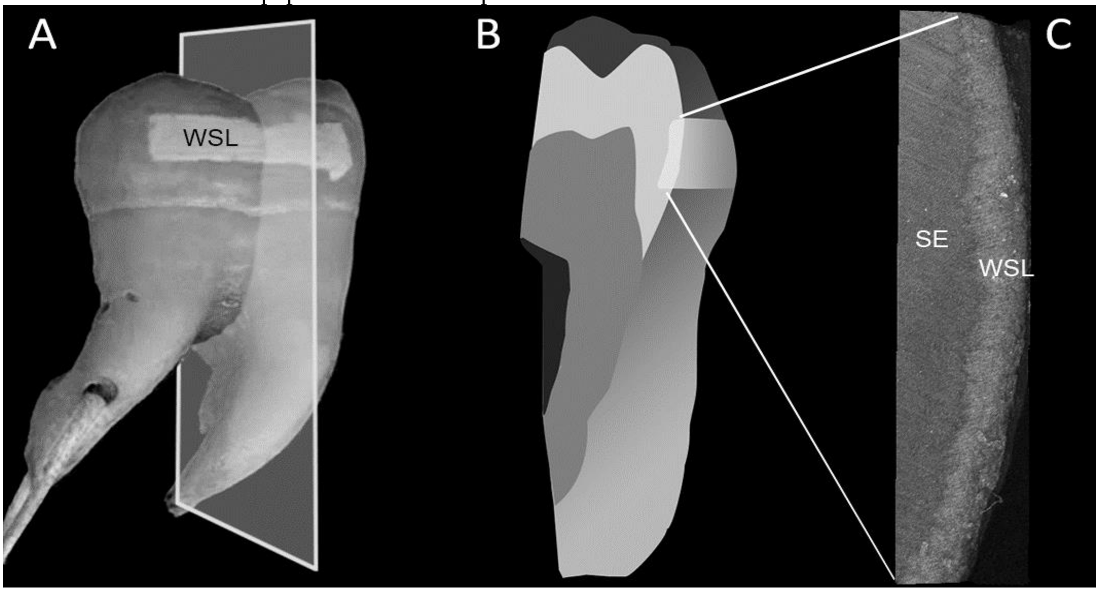
The surface roughness measurement could be a method used as an indirect way to evaluate porosity. Although there are publications that evaluate the surface roughness on the external surface of the enamel (Mukarromah A, 2018; Ulrich I et al., 2015), to our knowledge there is no data on the roughness in the subsurface zone of the WSL for evaluating the demineralization-remineralization process. The aim of this paper is to evaluate the penetration capacity of CPP-ACP in the subsurface zone of the WSL through roughness parameters measurement using CLSRM.

2 MATERIALS AND METHODS

2.1 SAMPLE PREPARATION AND WSL FORMATION

The Institutional Ethics Committee on Health Research, Project ODO-CIEIS N º 87, approved this study. Five retained third molars extracted according to the dentist's specific indications were used. The inclusion criterion involved these teeth being free of cavities, white spots or hypoplasia. Teeth were properly washed with distilled water and sectioned in mesial-distal direction, thereby obtaining a vestibular half and lingual or palatal half, making up a total sample of 10 segments (n=10). Each half was covered with acid-resistant nail polish (Colorama, Maybelline, New York, USA) exposing only an enamel window of 2x6 mm in the center. In order to generate the WSL, teeth were immersed in a demineralizing solution with pH 4.5 for 72h with a change of solution at 48h (de Rooij JF& Nancollas GH, 1984). After that time, samples were properly washed with distilled water, the nail polish was removed and the presence of the WSL was verified visually. Later, they were sectioned in the vestibule-palatal direction by half of the WSL; in this paper, this section will be called the **"longitudinal section"** of the sample (Fig. 1A). The cut was made with a metallographic cutter (Buehler ISOMET Low Speed Saw, Germany), using a 0.3 mm thick diamond disc (Buehler 15-LC, Germany), (Fig.1B). The longitudinal section was polished with Buehler disc of fine granulometry (120 µm) and felt disc with diamond paste of ¼ µm. In order to eliminate all types of impurities, the samples immersed in distilled water were washed with an ultrasonic frequency of 42 Hz (Denimed, Argentina) and stored in distilled water at 4ºC.

Figure 1. Schematic representation of sectional cut of the sample. A: white spot lesion (WSL) and the vestibule-palatal cut plane. B: Scheme of a longitudinal section after metallographic cut. C: Inset shows a typical reflection confocal image with the interest zones evaluated in this paper: WSL: white spot lesion. SE: sound enamel.



2.2 FLUORESCENCE CONFOCAL MICROSCOPY

The samples were immersed in a fresh solution of 0.1 mM rhodamine B (fluorophore) for one hour and then rinsed with distilled water. Images corresponding to the fluorescence of rhodamine B were registered using a confocal laser scanning fluorescence microscope (CLSFM) (LSM5 Pascal, Carl Zeiss, Germany) using a gaseous laser (543 nm) for excitation and collecting the fluorescence above 560 nm employing a long pass filter. The images were obtained using PlanApo objective lenses of 10X (NA 0.3) and / or 20X (NA 0.5) magnification (Fontana M et al., 1996; Berger SB et al., 2012).

2.3 REMINERALIZATION OF WSL *IN VITRO*

Mi Paste® (GC Corporation, Japan), which contains amorphous calcium phosphate stabilized by casein phosphopeptides (CPP-ACP) as an active ingredient, was used to promote remineralization. It was applied daily with a microbrush on the WSL for 3 minutes for 60 days. In order to ensure that Mi Paste only acted through the WSL, the surface of the longitudinal section of the sample was covered with acid-resistant nail polish (Colorama, Maybelline, New York, USA). During the experimental period, the samples were stored at 37°C in artificial saliva (Farmacia & Laboratorio Vip, Argentina) with complete renewal every 24h. After 60 days, the nail polish was removed and the samples were again immersed in distilled water, washed with an ultrasonic frequency of 42 Hz (Denimed, Argentina) and stored in distilled water at 4°C until processing.

2.4 CLSRM EVALUATION SURFACE ROUGHNESS MEASUREMENTS

The roughness parameters (Ra and Rp) were measured along a line on the longitudinal section of the samples (Fig. 1C), either in the WSL or SE area, before and after applying the remineralization protocol. Ra is defined as the arithmetical mean of the absolute values of the profile deviations (Zi) from the mean line of the roughness profile, while Rp is the average of peak height (Mitutoyo. Quick guide to surface roughness measurement, 2010). These parameters were obtained from the three-dimensional images obtained with a reflection laser scanning confocal microscope, OLS4000 LEXT (Olympus, Japan) and measured using the LEXT's software. To image the whole WSL area, which is approximately 2 mm in length, the microscope was configured to work with a "stitching" function. This function makes the microscope acquire a set of confocal image stacks sequentially that automatically join them -"stitch"- after acquisition delivering in one image the entire area of interest. Each stack of images covers an area of 220x220 μm, so approximately 5 to 10 stacks were needed to collect the full area of interest. The stack of images was acquired with a dedicated objective lens, 20x MPLAPON20X LEXT (NA 0.6), by adjusting the zoom of the microscope to get a lateral resolution of 0.2 μm/pixel. The jump (pitch) of the objective lens between each image of the stack was 0.4 μm and the lens was set to cover an axial depth of the sample of approximately 100 μm (Z direction). An evaluation length of 4 mm was used to carry out the determinations of the roughness measurements along each zone of interest (WSL or SE) in accordance with the international standard ISO 4288 (Mitutoyo. Quick guide to surface roughness measurement, 2010). This length is suggested for roughnesses that are in the range of $0.1\ \mu m < Ra \leq 2\ \mu m$ as is the case with this type of material. Since the WSL length was half the required evaluation length, the polygonal line tool of the LEXT software was used. This tool allows drawing a line along the longitudinal direction from the beginning of the zone of interest until its end and then returning in the opposite direction, but parallel to the first line. This procedure was repeated until the 4 mm length was achieved. The measurements of Ra and Rp were made in triplicate in each sample and then an average value of the measurement was calculated for both the SE and WSL areas before and after the treatment.

2.5 STATISTICAL ANALYSIS

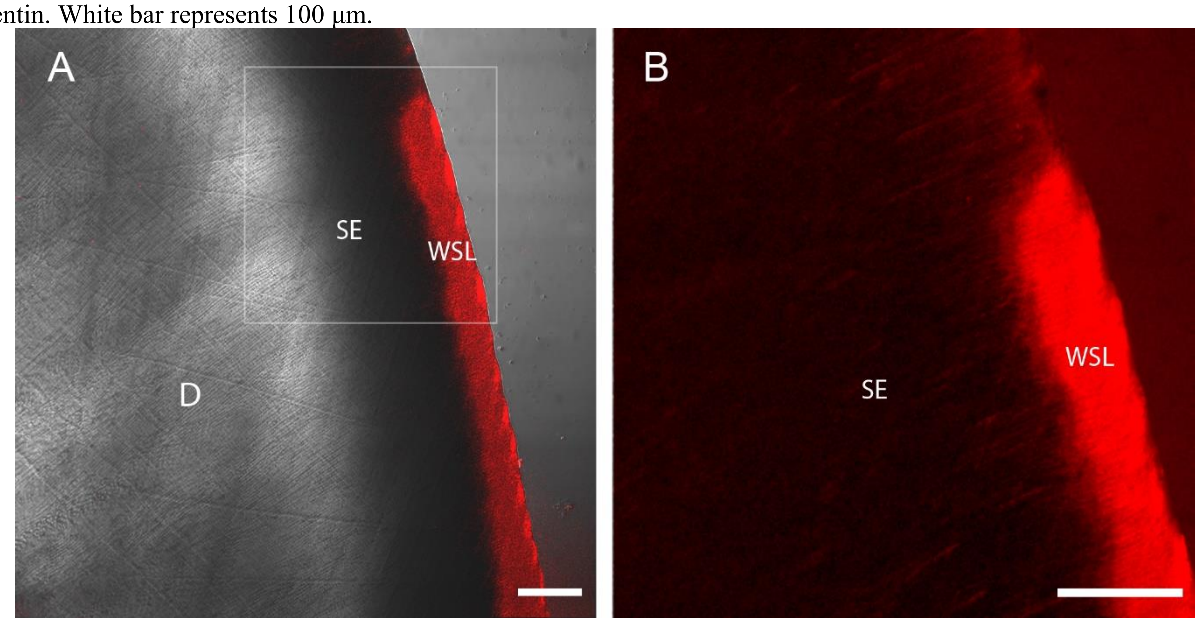
The results are expressed as the mean ± standard error. In order to find significant differences, mixed linear models were implemented, using the treatment as a fixed factor and the tooth as a random factor. The InfoStat software was used and the level of significance was 5%.

3 RESULTS

3.1 FLUORESCENCE CONFOCAL MICROSCOPY OF THE WSL

Three samples were chosen at random to verify the typical characteristics of the WSL by rhodamine B penetration evaluated by fluorescence confocal microscopy. Images showed the fluorophore penetration in the WSL area which corresponds with the visual inspection of the lesion. The WSL surface shows interspersed areas with higher and lower rhodamine B concentration while its penetration in the subsurface area of the lesion was more homogeneous corresponding with the area of greatest mineral loss. Inversely the areas of sound enamel appear black (absence of fluorophore) (Fig. 2). The depth of the WSL generated *in vitro* was around 50 µm.

Figure 2. Fluorescence confocal microscopy of WSL. A: Overlay of rhodamine B fluorescence and transmission confocal image. B: Magnified fluorescence confocal image corresponding to the box in A. SE: sound enamel, WSL: white spot lesion, D: dentin. White bar represents 100 µm.



3.2 ROUGHNESS PARAMETERS TO TEST THE REMINERALIZATION PROCESS: THE CUT-OFF WAVELENGTH CHOICE

A critical threshold that discriminates the roughness irregularities from the waviness of a surface is the cut-off wavelength value (Fig. 3). Since no specific cut-off wavelength values have been described to evaluate roughness parameters on dental surfaces, different cut-off

wavelength values were explored (800 to 5 µm) in order to find the maximum differences in roughness between SE and the WSL before starting the treatment with the remineralizing agent. Figure 4 shows the Ra and Rp parameter values for SE and WSL surfaces at different cut-off values. Based on these results, the cut-off wavelength of 400 µm was chosen to perform all of the roughness measurements in this study, since it represents the maximum difference between both surfaces and enhances sensitivity of the method.

Figure 3. Roughness profiles variation at different cut-off wavelengths from a representative sample.

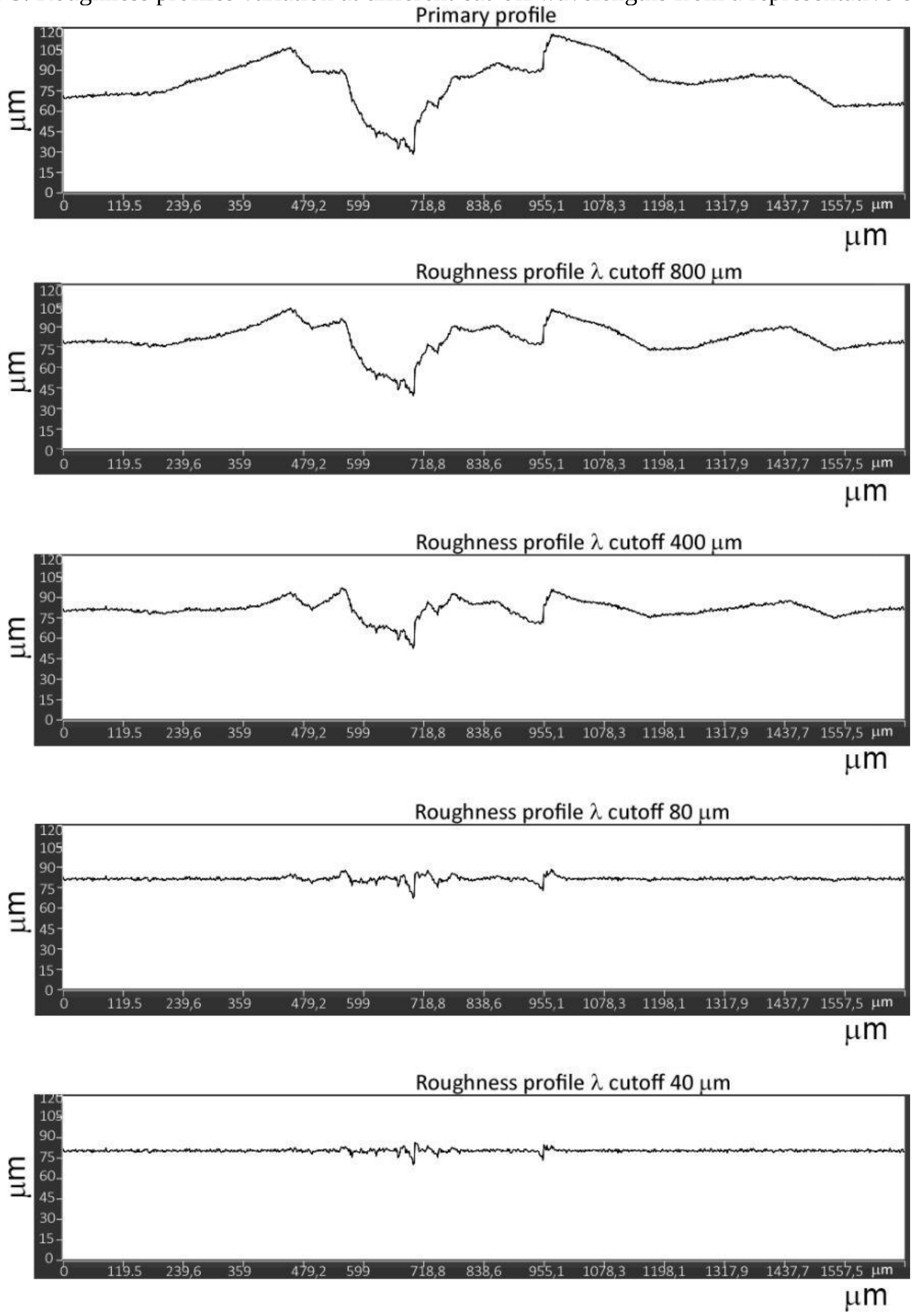
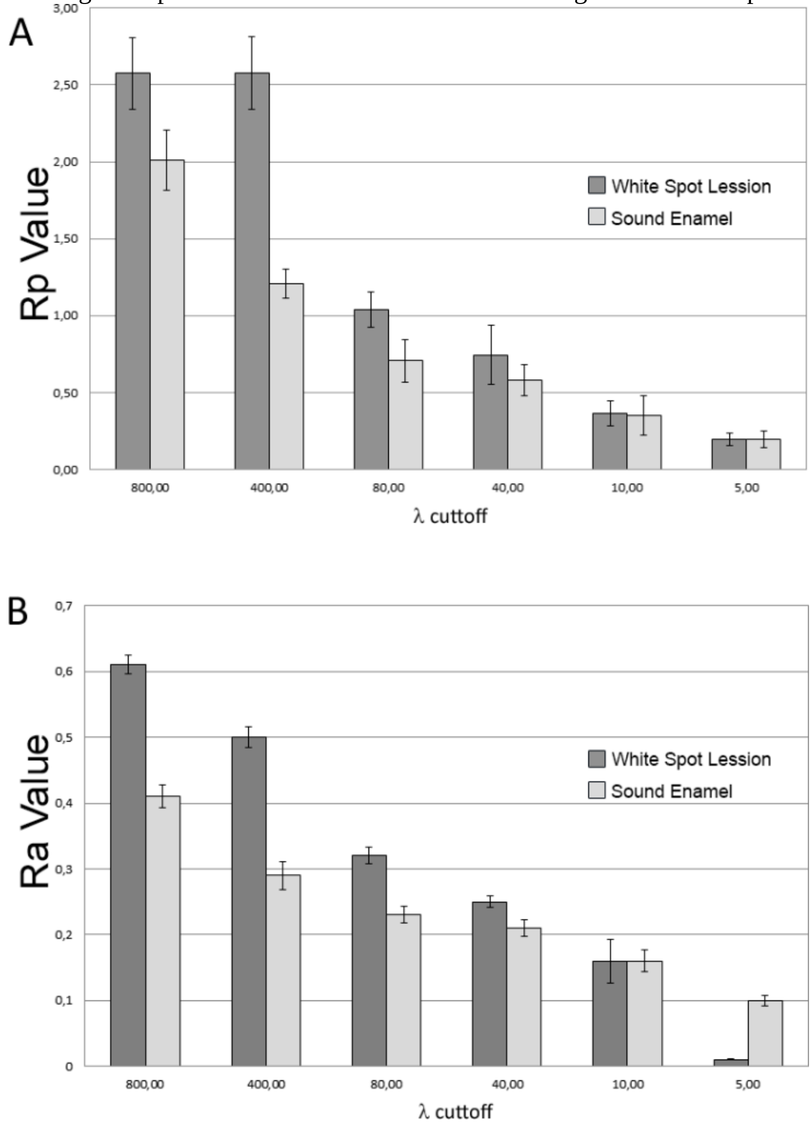


Figure 4. Rp and Ra roughness parameters at different cut-off wavelength values. A: Rp values, B: Ra Values.



3.3 WSL REMINERALIZATION WITH CPP-ACP

Table 1 shows the results of the roughness parameters measurements. Rp and Ra parameters in the WSL area before applying the remineralization protocol (WSL Pre) were significantly higher than in the SE of the same samples. When we compared roughness parameters in the WSL area before and after applying CPP-ACP (WSL Post), Rp and Ra parameters decreased significantly with respect to the WSL Pre and were similar to the values of these parameters in the SE, which received no CPP-ACP.

Table 1. Roughness parameters Rp and Ra in µm. WSL Pre versus SE: ζ $p<0.01$; WSL Pre versus WSL Post: ▲$p<0.05$; Sample size: n=10.

	WSL Pre	WSL Post	SE
Rp	2,02±0,15 ζ▲	1,22±0,07	1,14±0,06
Ra	0,59±0,06 ζ€	0,31±0,01	0,30±0,01

4 DISCUSSION

In this study the white spot lesion was generated *in vitro* in human third molars using a demineralizing solution (de Rooij JF& Nancollas GH, 1984). In a previous research, we established that the minimum demineralization time required for all samples to have the clinical appearance of WSL is 72 h (Tolcachir BR et al., 2015). The loss of the typical translucence of the enamel in WSL is due to the water that lodges in the pores and in the intercrystalline space modifying the refractive index of the hydroxyapatite (Fejerskov O, 2004; Braly A et al. 2007). This *in vitro* study has shown that water soluble, low-molecular weight rhodamine B readily penetrated into the demineralized enamel. This made it possible to determine the structural characteristics of the WSL by means of fluorescence confocal microscopy. Other authors have also used this methodology for the same purpose (González-Cabezas C et al., 1998; Berger SB et al., 2012; Kwon SR et al., 2012). In our observations, the surface enamel of the WSL showed interspersed areas of higher and lower mineralization (Fig. 2). The latter could be considered as the entryways of the CPP-ACP to the subsurface lesion area, where most mineral loss occurs. The increase in porosity in the subsurface zone of the WSL was demonstrated by polarized light microscopy (Karlsson L, 2010). It bears mentioning that porosity was related to surface roughness (Ra) in a study of different types of limestones used in construction. The finding of a positive logarithmic correlation between these two variables indicates that small changes in the porosity of low porous stones will induce small changes in their surface roughness; while for more porous stones small changes in this parameter have a greater impact on the roughness (Miller AZ et al., 2012).

In recent years, studies have been published that measure roughness parameters in different dental structures, in tooth whitening treatments, and to evaluate the remineralization process. However, these studies do not evaluate the roughness at the subsurface level of the WSL. Wang et al., 2016, used scanning electron microscope and atomic force microscope to measure surface roughness as a method to evaluate the remineralization effectiveness of artificial enamel lesions of different bioactive elements contained in toothpastes. They found a decrease in the surface roughness of the WSL after the application of remineralization substances; however, this decrease was greater when they applied arginine 8% calcium carbonate (ACC, Colgate Sensitive Pro-Relief) or calcium phosphosilicate sodium (NovaMin ®) than when using CPP-ACP. Elkassas & Arafa, 2014, also evaluated the enamel remineralization with different calcium and fluoride phosphate release systems using surface roughness. They observed an increase in Ra in the demineralized enamel with respect to sound enamel and a decrease in this parameter with the application of remineralizing substances in a variable way depending on the product applied. On the other hand, Zhao & Ren, 2016 and Ulrich et al., 2015 also measured surface roughness to evaluate the behavior of resins infiltration in WSL.

All of these authors, as we have already mentioned, measured the roughness parameters on the surface of the affected enamel and not at the subsurface level of the WSL. Yet, it is possible to show the presence of pores through irregularities (depressions and peaks) in the longitudinal section of our samples. (Fig. 1 B and C). Thus, in this study, roughness parameters were measured in the subsurface area of the WSL and sound enamel, before and after applying CPP-ACP by CLSRM. The versatility of the LEXT confocal software (Olympus. LEXT OLS4100 Industrial Laser Confocal Microscopes - Olympus 3D Laser Measuring Solution. 2017) enabled us to reach the sampling length established in the standard, choosing the value of the cut-off where we could detect the maximum differences between the parameters (400 μm) increasing the sensitivity of the method (Fig. 3).

Table 1 showed that Rp and Ra values were significantly higher in the subsurface area of the WSL than in the sound enamel of the same samples. When the measurements were made in the same samples after the application of the remineralization substance, Rp and Ra values decreased significantly, showing sound enamel values. This change indicates that CPP-ACP has penetrated at the subsurface zone of the WSL filling the pores with a recrystallization process or with the formation of other mineral phases. Consistent with this, in a previous study we found that treatment with CPP-ACP improves the clinical appearance of WSL by mimicking the appearance of sound enamel (Tolcachir BR et al., 2015). In this investigation, the study of the roughness in longitudinal sections at different depths of the enamel was considered an indirect indicator of porosity at the subsurface of WSL. Therefore, the decrease in roughness parameters are interpreted as the remineralization of the subsurface zone by the ability of CPP-ACP to penetrate the body of the lesion. The characteristics of the mineral phase formed by the application of CPP-ACP in the subsurface zone of the WSL are not known and are the subject of further investigation.

5 CONCLUSION

In this study, roughness parameters were used as an indirect way to measure the porosity of WSL at subsurface level. The decrease of these parameters could be interpreted as the remineralization of WSL by the ability of CPP-ACP to penetrate the body of the lesion. This was carried out by analyzing the changes in the roughness values at the subsurface level both in the WSL area and in the sound enamel (SE) in the same sample before and after the treatment.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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