IN VITRO STUDY OF DENTIN HYPERSENSITIVITY TREATED BY WHITLOCKITE GLASS-CERAMICS

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Abstract. *The aim of this study is to evaluate the potential of a bioactive glass based on the $3CaO.P_2O_5$-SiO_2-MgO-system and its glass-ceramics containing whitlockite on the remineralization of dentin as a possible treatment to dentin hypersensitivity. For that, 40 third molar human teeth were artificially demineralized and randomly distributed in 4 groups (n = 10): G1 - Negative Control (no treatment), G2 - Positive Control (treated by Bioglass® 45S5), G3 – BG (treated by bioactive glass based on $3CaO.P_2O_5$-SiO_2-MgO-system), and G4 – BGC (treated by bioactive whitlockite glass-ceramics). After treatment, the samples were emerged in artificial saliva and stored for 7 days in a controlled temperature of 37ºC. After that, ATR-FTIR was used to identify and quantitatively analyze the mineral variation of the dentin surface. The morphological aspects were evaluated by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The analysis confirmed the formation of hydroxyapatite on the surface of all the biomaterials studied, and it also showed that the results obtained with the glasss-ceramic are similar to those obtained with Bioglass®45S5 AFM and SEM examination showed that in the dentine specimens treated by bioactive glass and whitlockite glass-ceramic most of the dentinal tubules were completely occluded .*

Keywords: *Biomaterials, bioactive glass, glass ceramic, remineralization, dentin.*

1. INTRODUCTION

Dentin is a tubular, permeable, mineralized structure that composes the most part of the human teeth. This tissue is mainly formed by type I collagen fibrils, glycosaminoglycans, phosphoproteins, phospholipids and hydroxyapatite crystals.

The dentin demineralization can occur due to exposition of this tissue to acids that can be present in the oral environment, as well as a result of abrasion or attrition of dentinal exposed surface. This process is characterized by the loss of hydroxyapatite crystals and, as a consequence, the opening and exposition of dentinal tubules, which can result in hypersensitivity. (Wang *et al*. 2011)

Bioactive glasses and glass-ceramics are promising biomaterials in the field of bone regeneration and dentin remineralization due to their exceptional bioreactive properties that allows to easily form hydroxycarbonated apatite (HCA) when immersed in simulated body fluids. (Wang *et al*, 2011).

The clinical use of glass biomaterials became notorious with the creation of "*Bioglass® 45S5*", by L. Hench, that demonstrated to be able to reconstruct bone in a faster way than that achieved by the use of synthetic hydroxyapatite. Years later, T. Kokubo and coworkers created a bioactive glass-ceramic that, as "*Bioglass® 45S5*" exhibited bioactive properties , but with better mechanical properties, which increased its applications possibilities. (Gutierres *et al*, 2006).

Although it is already established that bioactive glasses and glass-ceramics from the $3CaO.P_2O_5$-SiO_2-MgO system have bioactive properties when exposed to simulated body fluids, and that the presence of whitlockite as crystalline phase in the bioactive glass-ceramic from that system contributes for increasing its biocompability and mechanical properties

(Daguano *et al*, 2013), there is still the need for a quantitative analysis that determines how successful was the connection between the biomaterial and the dentin tissue. In this context, this study performed a semi-quantitative analysis of the mineral variations on the dentin surface when treated with bioactive glass and glass-ceramic from the $3CaO.P_2O_5$-SiO_2-MgO system.

The concept behind this project is to study the potential of a bioactive glass based on the $3CaO.P_2O_5$-SiO_2-MgO system and its glass-ceramics containing whitlockite on the reconstruction of the mineral phase of the human dentin. A qualitative and semi-quantitative compositional analysis was performed using the Attenuated Total Reflection technique of Fourier Transform Infrared Spectroscopy (ATR-FTIR), a technique highly used on the characterization of hard tissues composition due to its non-destructive approach (Wang et all, 2011). Also, a qualitative analysis by Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) was performed to observe the occlusion of the dentin tubules by the formation of hydroxyapatite.

2. MATERIALS AND METHODS

2.1 Sample Preparation

Forty extracted sound third molar human teeth were obtained from the Human Teeth Bank of School of Dentistry of University of Sao Paulo. From each tooth it was obtained a 3 x 3 x 1mm dentin slab using a water cooled diamond saw (EXTEC Labcut 1010 Low Speed Diamond Saw). The cuts were made 3 mm below the occlusal surface of the teeth. A standard smear layer was created using a 600-grit silicon carbide for 60 s under constant water irrigation.

2.2 Bioactive glass and glass-ceramic treatment

Before receiving the biomaterials, the surface of samples was demineralized with a 0.5 M EDTA solution (pH 7.4) during 5 min and then washed with distilled water for 2 min to assure complete removal of the solution. After that, the samples were randomly distributed in four experimental groups (n = 10): G1 – Negative control (no treatment), G2 – Positive control (treated with *Bioglass® 45S5*), G3 – Treated with bioactive glass from the $3CaO.P_2O_5$-SiO_2-MgO system, and G4 – Treated with whitlockite bioactive glass-ceramic from the $3CaO.P_2O_5$-SiO_2-MgO system.

The treatment with *Bioglass® 45S5*, bioactive glass and whitlockite glass-ceramic from the $3CaO.P_2O_5$-SiO_2-MgO system was made by smearing 0.2 g of the biomaterial for 60 s, and then the samples were lightly washed with distilled water to remove the excess. After treatment, all samples were stored in 20 mL of artificial saliva for 7 days at controlled temperature of 37ºC to induce the remineralization process. The artificial saliva with the composition presented in Table 1, was replaced every day.

Table 1 – Composition of the artificial saliva used to induce dentin remineralization

Reagent	Manufacturer	Quantity
$Ca(NO_3)_2.4H_2O$	Sigma-Aldrich, MO, EUA	1.5 mM
$H_2NaPO_4.H_2O$	Sigma-Aldrich, MO, EUA	0.9 mM
Fluoride Standard	Orion, EUA	0.03 ppm
KCl	Sigma-Aldrich, MO, EUA	150 mM
TRIS	Sigma-Aldrich, MO, EUA	0.1 M

All quantities informed were used in the preparation of 1L of artificial saliva. To reach its functional pH (pH 5.0) solutions of NaOH 20% and HCl 20% were used.

2.3 Compositional analysis by ATR-FTIR spectroscopy

After the remineralization process the surface of the samples had their surface chemical composition analyzed by Attenuated Total Reflection technique of Fourier Transform Infrared Spectroscopy (ATR-FTIR), using a FTIR 610 (Varian Inc.). In each slab, it was collected a single spectrum in an area of 1.5mm^2 which corresponds to the size of Zn-Se crystal. Each spectrum had a background spectra subtracted during acquisition and was obtained with 80 scans, at a resolution of 4 cm^{-1} and in the range of 1800 to 650 cm^{-1}.

With the data obtained, a qualitative descriptive analysis was performed to identify the chemical changes that occurred after the remineralization process, identifying the specific functional groups of the dentin tissue, as well as a semi-quantitative comparison between the spectra obtained from the experimental groups, identifying the relative changes between different infrared absorption bands areas: from 800 cm^{-1} to 887 cm^{-1} for ν_2 carbonate, from 887 cm^{-1} to 1181 cm^{-1} for ν_3 phosphate, from 1181 cm^{-1} to 1296 cm^{-1}for amide III, from 1300 cm^{-1} to 1510 cm^{-1} for ν_3 e ν_4 carbonate, from 1510 cm^{-1} to 1580 cm^{-1} for amide II and from 1593 cm^{-1} to 1720 cm^{-1} for amide I. After selection of the bands, the background signal was subtracted and, for a semi-quantitative comparison between groups, the areas under the considered bands were calculated after normalization by the area of phosphate band (1300–900 cm^{-1}) using the OriginPro software (version 8.0 – OriginLab Corp.).

2.4 Morphological evaluation by SEM and AFM

After the ATR-FTIR analysis, the morphological analysis of the samples was made by using a Scanning Electron Microscope, Phenon/FEI. An Atomic Force Microscope, AFM/SPM Series 5500 Agilent, was used to determine the topography of the samples. Each sample had six 70 x 70 µm images and six 40 x 40 µm images taken.

3. RESULTS

3.1 ATR-FTIR Spectroscopy

Figure 1A, 1B and 1C show the infrared spectra obtained for a sound dentin (fig. 1A), a comparison between a partly demineralized dentin and a sound dentin (fig. 1B), a comparison between the mean curves of all groups, respectively. In Figure 1A it is depicted the identification of the main components of sound dentin tissue, such as the intense bands of inorganic content (phosphate and carbonate), as well as the bands that correspond to the inorganic matrix of dentin (amides I, II and III). Also, a band of water is overlapped at the same infrared region of Amide I.

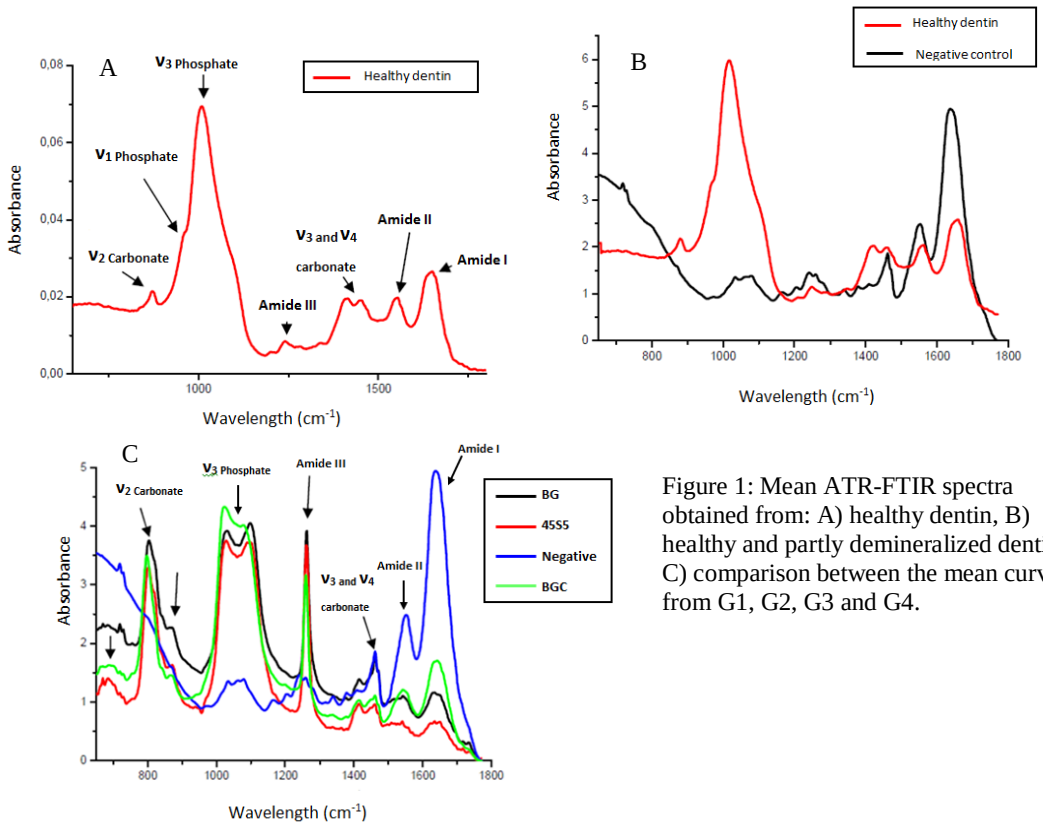

Figure 1: Mean ATR-FTIR spectra obtained from: A) healthy dentin, B) healthy and partly demineralized dentin, C) comparison between the mean curves from G1, G2, G3 and G4.

Figure. 1B shows the comparison of infrared spectra of a sound and a demineralized dentin (Group G1). It is possible to note a decrease in the intensity of phosphate and carbonate bands after demineralization, as well as an increased intensity of amides I and II bands. The same aspects are evidenced in the blue (mean curve for the negative control group) curve on fig. 1C,. The opposite can be observed in the red curve (mean curve for the positive control group), where the treatment with *Bioglass® 45S5* after the demineralization process promoted the expansions of the bands of phosphate and carbonate, as well as the suppression of the bands of amides.

The spectra obtained from the bioactive glass (fig. 1C) is very similar to that obtained from the positive control (fig.1C), where it can be noted an increase in the intensity of 801 cm^{-1}, 1097 cm^{-1}, 1016 cm^{-1} and 1261 cm^{-1} bands, which correspond to phosphate and carbonate bands. It is also noticed the expressive increase of the peak relative to phosphate in the spectra obtained from the samples of the bioactive glass-ceramic. Figure 1C shows a comparison between the mean infrared spectra of all experimental groups of this study, where it can be observed that all biomaterials used promoted the formation of the same infrared bands on demineralized dentin.

The semi-quantitative analysis of the material formed in all experimental groups is shown in Table 2. It can be noted a significant decrease ($p < 0.05$) on carbonate-to-phosphate ratio after all treatments, which indicates, according to the spectra obtained, a significant amount of phosphate formation due to treatments. In addition, no statistical differences were observed among the treated groups, which indicates that all treatments formed the same amount of hydroxyapatite on dentin.

Table 2: Area under the considered FTIR enamel bands, for each treatment, after normalization by phosphate band area. The statistical analysis was performed individually for each band (one-way ANOVA + Tukey's test), considering each treatment as a separate block (a.u. = arbitrary units).

Infrared bands	Normalized area by phosphate band (a.u.) ± standard deviation*			
	G1 - untreated	G2 – 45S5	G3 – bioactive glass	G4 – bioactive glass ceramic
ν_3 and ν_3 carbonate	1.44 ± 0.50[a]	0.064 ± 0.01[b]	0.13 ± 0.03[b]	0.21 ± 0.11[b]
amide I	9.32 ± 4.97[a]	0.036 ± 0.01[b]	0.20 ± 0.07[b,c]	0.28 ± 0.12[c]
amide II	1.46 ± 0.73[a,c]	0.004 ± 0.00[b]	0.04 ± 0.02[b,c]	0.03 ± 0.02[c]
amide III	1.06 ± 0.40[a]	0.131 ± 0.01[b]	0.11 ± 0.01[b]	0.11 ± 0.01[b]

*Distinct letters mean statistically significant differences according to Tukey's test.

The quantities of amides I, II and III in relation to phosphate indicates the proportion of collagen to hydroxyapatite. According to the Table 2, it was observed a significant decrease of this proportion after all treatments when compared to the untreated group. Also, it can be noted that the application of bioactive glass had a behavior similar to the application of *Bioglass® 45S5*, which is evidenced by the non-statistical difference between G2 and G3 groups concerning the quantities of amides I, II and III.

3.2 Morphological evaluation by SEM

Figure 2A and 2B show the SEM images obtained from G1 and G2 groups. In Figure 2A, it is possible to observe the dentin tissue with the exposition of dentinal tubules, which were fully or partially opened due to the demineralization process. After the application of the *Bioglass® 45S5*, it was observed the presence of agglomerated material on the dentin surface, with the presence of globules and dentin tubules totally covered. Figures 2C and 2D show the SEM images obtained for samples from G3 and G4.

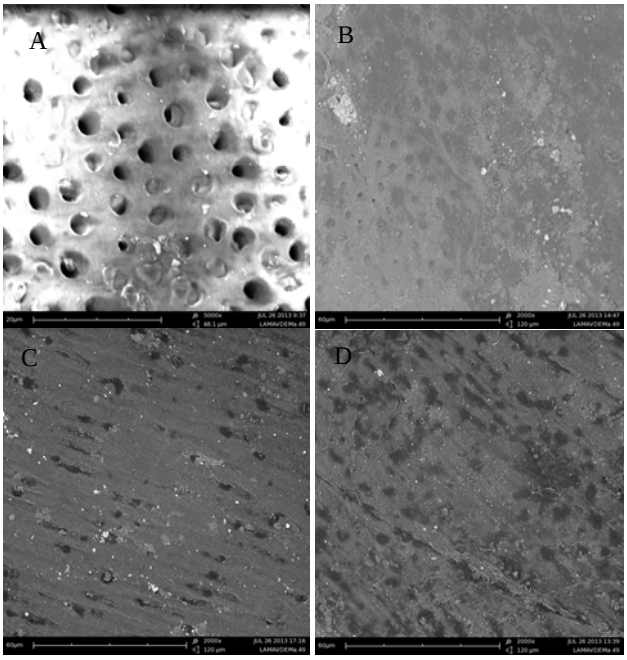

Figure 2: SEM images of samples from: A) G1 - negative control group, B) G2 - positive control group, C) G3 – treated with bioactive glass and D) G4 – treated with bioactive whitlockite glass-ceramic. After the process of remineralization. Original magnification: 5000x. TROCAR AS IMAGENS.

The samples that received the treatment with the bioactive whitlockite glass-ceramic (group 4) (fig. 2D) presented a thick layer of hydroxyapatite on the surface, covering completely the tubules that were opened by demineralization. The samples from G3, treated with bioactive glass, also presented their tubules coated by hydroxyapatite.

Comparing figures 2C and 2D it is perceptible that the formation of hydroxyapatite on the samples from G4 was more uniform that on the samples from G3. In fig. 2D it is possible to notice a nearly continuous precipitate throughout the entire surface of the samples treated with the bioactive whitlockite glass-ceramic , while fig. 2C shows that, although there is satisfactory layer of hydroxyapatite formed, it does not extend as uniformly trough the sample surface . Even so, it is clear that the dentin tubules in the samples treated with the bioactive glass and the bioactive glass-ceramic from the $3CaO.P_2O_5$-SiO_2-MgO system are completely covered.

3.3 AFM inspection

The topographic AFM analysis resulted in several 40 x 40 µm images of dentin samples with different treatments. Figure 3A to 3D show the AFM images from G1, G2, G3 and G4, respectively.

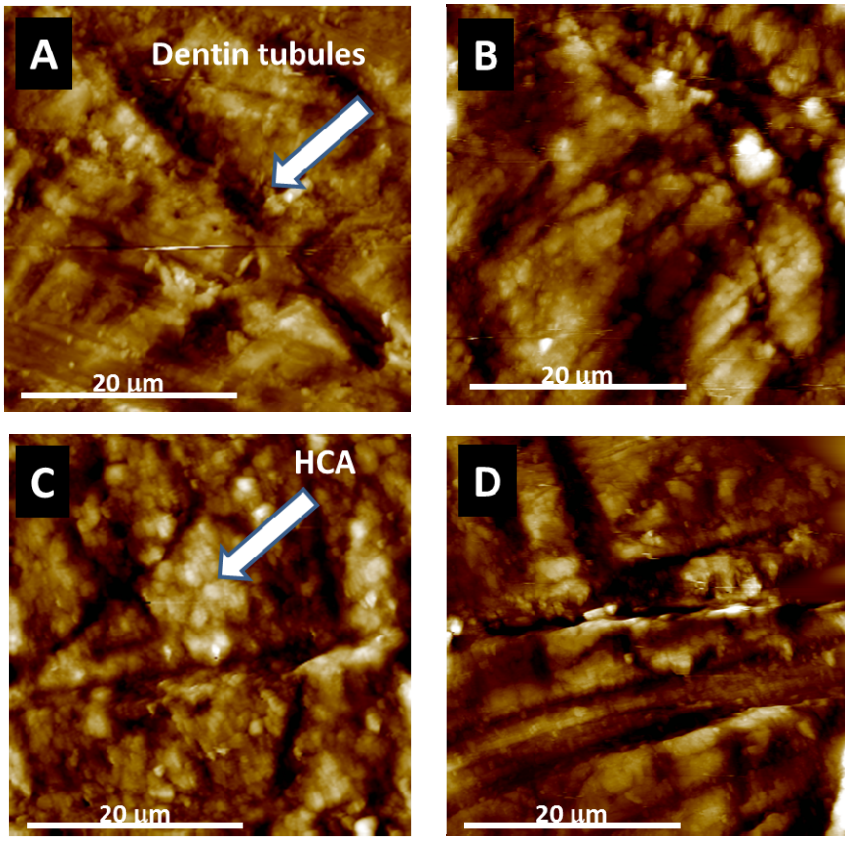

Figure 4: AFM images: A) G1 - negative control group, B) G2 - positive control group, C) G3 – treated with bioactive glass and D) G4 – treated with bioactive whitlockite glass-ceramic.

In fig. 3A is possible to see opened dentin tubules after the seven days immersion in artificial saliva. In the following images (fig. 3B to 3D) a different type of topology is observed, with the dentin tubules partially covered by the deposition of hydroxyapatite. The images also suggest a smoother surface for the samples that received the some sort of treatment.

4. DISCUSSION

The dentin hypersensitivity is a common clinical condition that results from the exposition and opening of dentin tubules. Although there are several alternatives for clinical management of this problem, there is still the need of treatments that can last for a long period of time. In this way, the use of bioactive glasses has been suggested as a promising alternative, since these materials can propitiate a regeneration of hard tissues due to the formation of hydroxycarbonated apatite. It this way, it is necessary to evaluate the efficacy of some bioactive glasses and bioactive glass-ceramic on demineralized dentin.

In this study, the compositional analysis by ATR-FTIR showed that all groups that received treatment present a higher quantity of phosphate and carbonate when compared to the spectra of the negative group. It's also possible to see the formation of new peaks in all the treatment groups, indicating the formation of phosphate and carbonate, covering the previously exposed dentin. This is also represented by the suppression of the amide peaks, which evidences that it was formed a crystalline material that covered the entire organic matrix exposed by the demineralization process.

Daguano *et al*. (Daguano *et al*, 2013) showed that the bioactive glass-ceramic used in this study, V775-4, has significant similarities with *Bioglass® 45S5* in terms of surface reactivity. In the present study, the similarity XXXX was confirmed by the proximity of the values encountered on the area of the bands of phosphate of the groups treated with bioactive glass-ceramic from the $3CaO.P_2O_5$-SiO_2-MgO system and treated with *Bioglass® 45S5*. The bioactive glass from the $3CaO.P_2O_5$-SiO_2-MgO system also showed a considerable band of phosphate, which confirms the presence of hydroxyapatite. It is important to point out that the time needed by the bioactive glass to form the hydroxyapatite is five times higher than the time needed by the bioactive glass-ceramic, as shown by in vitro bioactivity tests conducted by Daguano *et al* (Daguano *et al*, 2013). The importance of the presence of the phosphate is due to its similarity with the bone and dentin mineral matrix, which favors the biocompability of the material. (Silva *et al*, 2012).

The carbonate band is less expressive than the phosphate band, which brought difficulties to the area calculation. Nevertheless, again, it is visible the high similarity between the regions of the carbonate band of the bioactive glass-ceramic from the $3CaO.P_2O_5$-SiO_2-MgO system and the *Bioglass® 45S5*.

The results found in this study met the initial expectations based on the literature (Wang *et al*, 2011) (Daguano *et al*, 2013) (Mundstock *et al*, 2012). Daguano *et al* showed that the bioactive glass-ceramic used on this project, V775-4 from the $3CaO.P_2O_5$-SiO_2-MgO system, possesses whitlockite as its main crystalline phase, which favors the biocompability of the material, and is consistent with the results achieved in that study (Daguano *et al*, 2013). This same work shows that the bioactive glass-ceramic V775-4 takes very little time to form a layer of hydroxyapatite, approximately of one day, which is also due to the presence of whitlockite. According to the authors, this relation can be explained as follows: the whitlockite is a more soluble phase, and consequently, causes a super-saturation of the environment where the sample is located, which creates favorable local conditions for a fast

crystallization of hydroxyapatite, increasing the biocompability of the material. (Daguano *et al*, 2013)

This same presence of whitlockite explains the difference found between the bioactive glass and the glass-ceramic with respect to the formation of hydroxyapatite Being whitlockite a crystalline phase, and the bioactive glass completely amorphous it should be expected thatthe bioactive glass exhibited a higher bioactivity than the bioactive glass-ceramics. As discussed in this study the presence of whitlockite in the bioactive glass-ceramic inverts this expectation providing a bioactive response of the glass-ceramic much faster than the one of the parent bioactive glass. .

The interpretation of the ATR- FTIR spectra was not straightforward in particular due to the presence of two new bands that were not completely identified. The heterogeneity of the hydroxyapatite is also visible through the SEM images presented on this article. The typical morphological "cauliflower" pattern of hydroxyapatite forming on the surface of bioactive samples, as reported in the literature (Mundstock *et al*, 2012)was also obtained in all bioactive glasses studied in this project.

Furthermore, the same similarities between the bioactive glass-ceramic from the $3CaO.P_2O_5$-SiO_2-MgO system and *Bioglass® 45S5* found on Daguano *et al* article were also found on the spectra obtained and on the quantitative results presented. (Daguano *et al*, 2013)

Another work by Daguano *et al* (Daguano *et al*, 2012) discussed the mechanical properties of bioactive glass-ceramic with whitlockite as their crystalline phase. In this work (Daguano *et al*, 2012) it was related that the presence of whitlockite increases mechanical properties such as bending strength and hardness, probably due to the fact that the crystallization process can act as an toughening mechanism, as the acicular whitlockite phase causes crack-deflection. However, the same study (Daguano *et al*, 2012) indicates that this increase on mechanical properties can be lost with heat-treatments over 1100ºC, due to the increased porosity. Similar results were indicated in studies conducted with bioactive glass-ceramic from the P_2O_5-Na_2O-CaO-SiO_2 system. (Peitl *et al*, 2012)

With the SEM and AFM images it was possible to observe the morphological and topographic changes that occurred after the remineralization process. The SEM micrographs show that the hydroxyapatite formed on the G4 samples has a more crystalline aspect, due to the presence of whitlockite in the bioactive glass-ceramic (Daguano *et al*, 2013). The AFM images also indicate the occlusion of the dentin tubules by the formation of hydroxyapatite on the surface of the samples. The images can also suggest that the samples that received treatment with a bioactive glass or glass-ceramic have a smoother surface than the sample from the negative control group. This initial qualitative topographic analysis indicates that dentin surfaces treated with bioactive glasses and glass-ceramic are less rough than a demineralized dentin, and therefore, less likely to be affected by the adhesion of bacteria and plaques (Wang *et all*, 2011). A quantitative analysis of the roughness will be necessary to determine the differences at the submicron scale in the topographic features of the surface samples in the several experimental groups.

5. CONCLUSION

It was possible to conclude that both the bioactive glass and the bioactive glass-ceramic from the $3CaO.P_2O_5$-SiO_2-MgO system promoted the formation of hydroxycarbonated apatite on the demineralized dentin, in a way similar to that of *Bioglass® 45S5*. In this way, both biomaterials have a high potential for a future clinical application for dentin remineralization.

Another factor that allows to conclude that bioactive glasses studied here have a high potential for remineralization is their similarity with each other and with the gold standard

bioactive glass, *Bioglass® 45S5*, where, again, the bioactive glass-ceramic stands out in its similarity with the positive control of this project and for its mechanical properties.

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