Bacterial biofilms formed *in vitro* and *in vivo* on orthodontic appliances. Effect of antimicrobial agents

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RESUMEN. Los procesos cariogénicos y las infecciones gingivales y en la vecindad de los implantes pueden ocurrir como una consecuencia de la formación de biopelículas. A fin de prevenir estos procesos, se utilizan productos profilácticos tales como fluoruro de sodio (F), clorhexidina (C) y xilitol (X). El objetivo de este trabajo fue estudiar el efecto de las mezclas F + X y C + X sobre las biopelículas orales formadas en dispositivos para Ortodoncia. En los experimentos *in vitro* se utilizó un consorcio de estreptococos y como medio de cultivo Agar Mitis Salivarius o su composición modificada para cultivos líquidos líquido. Se sumergieron bandas de acero inoxidable en los medios inoculados durante 7 d y se siguió el crecimiento de la biopelícula a través de microscopia óptica realizada *in situ*. Las bacterias sésiles adheridas a las bandas fueron observadas después de teñirlas con naranja de acridina. Después de los períodos establecidos las bandas con las biopelículas fueron retiradas de los medios de cultivo y se transfirieron a tres frascos diferentes con: i) disolución reguladora de fosfatos, ii) un colutorio que contenía F (0,05 %) + X (10 %) y iii) un colutorio que contenía X (10 %) + C (0,12 %). Los resultados demostraron que es difícil predecir la eficacia de los agentes antimicrobianos (AA) contra las biopelículas orales basado en experiencias realizadas con células planctónicas. Los AA ensayados fueron capaces de difundir dentro de la biopelícula, modificar su microestructura, haciéndola más compacta, reducir el crecimiento de las bacterias sésiles y promover el desprendimiento de células. Sin embargo, el recrecimiento de la biopelícula podría ocurrir bajo mejores condiciones ambientales cuando finaliza el tratamiento.

ABSTRACT. Cariogenic processes and peri-implant and gingival infections can occur as a consequence of the biofilm formation. In order to prevent these processes, prophylactic products like sodium fluoride (F), clhorhexidine (C) and xylitol (X) are used. The aim of this work was to study the effect of commercially available mouth rinses containing F + X and C + X on oral biofilms formed on orthodontic appliances. A consortium of streptococci was used in the *in vitro* experiments and *Agar Mitis Salivarious* and its modified composition for liquid cultures. Stainless steel and elastic bands were immersed in the inoculated culture media for seven days and the growth of biofilms was followed in real time through optical microscopy made *in situ*. The sessile bacteria attached on the bands were also observed using epifluorescence microscopy after staining the sample with acridine orange. Subsequent to the preset periods the biofilmed bands were removed from the culture medium and were transferred to three different flasks with: i) a phosphate buffer, ii) a mouth rinse containing F (0.05 %) + X (10 %) iii) a mouth rinse containing X (10 %) + C (0.12 %). Results demonstrated that it is difficult to predict the efficacy of an AA against oral biofilms based on experiments performed with planktonic cells. The assayed AA were able to diffuse into the biofilm, change the microstructural characteristics of the biofilm which becomes more compact, slow down the growth of the sessile bacteria and promote the detachment of cells. However, re-growth of the biofilm could occur under better environmental conditions when the treatment finishes.

INTRODUCTION

Oral microorganisms can form biofilm on teeth and foreign materials such as orthodontic appliances and implants. In the oral environment insertion of orthodontic appliances tends to create new retentive areas which favour the local growth of streptococci increasing in turn the general infection level of these organisms.[1] Unfortunately, these regions are very difficult to disinfect and frequently microorganisms re-colinised the surface when the treatment finished.

Most of the tests, which were devoted to measure the caries tendency of a patient, are based on the enumeration of the microorganisms contained in saliva with and without the addition of the antimicrobial agents (AA). However, planktonic cells number may not be easily correlated with the number of sessile bacteria.

Chlorhexidine (C), sodium fluoride (F) and xylitol (X) are frequently used as AA in prophylactic treatments. The aim of this work was to evaluate the effectiveness of AA on biofilms formed on stainless steel and elastic bands *in vitro* and *in vivo*. The possible re-growth of the cells of the biofilm after treatment was also assayed.

MATERIALS AND METHODS

A consortium of streptococci isolated from the gingival area of bucal

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and lingual tooth surfaces of several patients was used in the *in vitro* experiments (Fig. 1). Agar Mitis Salivarius and its modified composition for liquid cultures (MLS) were employed as culture medium. Stainless steel Ponce bands and orthodontic arches were immersed in the inoculated culture media for seven days (Treatment **a**) and the growth of biofilms was followed in real time through optical microscopy (OM) made *in situ*, using an assemblage specially designed[2] and scanning electron microscopy (SEM) made *ex situ*. Subsequent to the preset period (7 d for *in vitro*) the biofilmed bands (Fig. 2) were removed from the culture medium and were transferred to three different flasks (Treatment **b**) containing: i) a phosphate buffer solution (BS: NaCl 8 g/L, KH_2PO_4 0.34 g/L, K_2HPO_4 1.21 g/L) (control), ii) a mouth rinse containing X (10%) + F (0.05 %), iii) a mouth rinse containing X (10 %) + C (0.12 %). 24 h later the samples were removed from these flasks. One of the samples was observed using OM and then by epifluorescence microscopy (EM) after staining it with acridine orange and another was scrapped, the removed cells were suspended in a BS and then they were enumerated in a Neubauer camera. A third sample was used to assess the re-growth of the biofilm in a fresh culture medium (Treatment **c**).

In the case of *in vivo* experiments elastic and metallic orthodontic bands were removed from the patients after different periods between 3 and 15 d . A set of patients used a mouth rinse containing C once a day and the other set did not employ it. Subsequent to each treatment, the bands were successively sonicated for 10 s, agitated three times to removed the cells and afterwards the suspended bacteria were enumerated in a Neubauer camera.

Experiments were made in triplicate.

RESULTS AND DISCUSSION

Table 1 shows the results when the biofilmed samples (Treatment a) were immersed for seven days in the BS (i), F+X medium (ii) and C+X medium (iii) (treatment b).

Bacteria surface density was significantly reduced ($p < 0.05$) in the presence of AA. However, the mixture C + X (iii) seems to be more effective than F + X (ii). OM observations showed that after treatment b the colonies became more compact, and changed their microstructural characteristics. According to EM observations the cells were not only killed but were also detached from the surface because those that continue alive (orange cells) plus those that were killed or remained with reduced metabolic activity (green) were lower than those enumerated before Treatment b. Enumeration of planktonic cells showed a decrease (higher than three orders with respect to the BS) in the number of surviving cells after Treatment b (ii) and (iii).

The bands treated according c were immersed in flasks containing MSL medium to assess the possibility of the biofilm re-growth. A significant increase in the number of the cells was only observed in the buffer without AA. However, the survival treated cells could grow in Agar Mitis Salivarius medium after seven days.

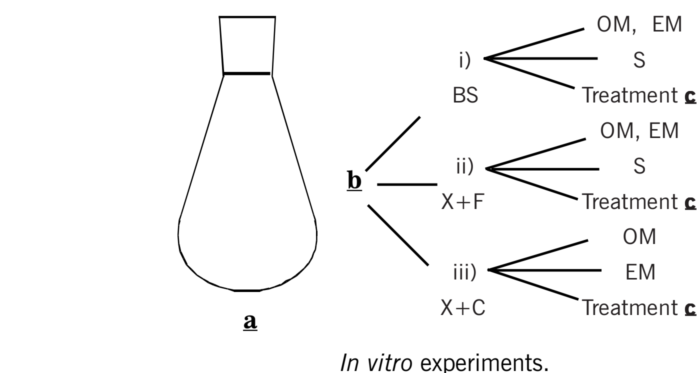

Fig. 1. *Nine biofilmed samples (Treatment* ***a****) were transferred to three different cells containing medium (i), (ii) or (iii) (Treatment* ***b****) and after the preset periods were observed through in situ optical microscopy (OM) and epifluorescence microscopy (EM); scrapped (S) for subsequent enumeration or transferred to another cell to performed treatment* ***c****.*

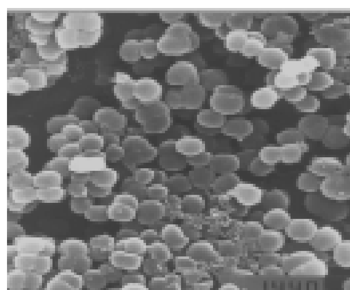

Fig. 2. *Scanning electron microscopy of a stainless steel Ponce band with a biofilm of streptococci.*

Table 1. Effect of antimicrobial agents on biofilms formed *in vitro*.

Solution--AA	Number of bacteria (cells $\cdot 10^{-7}/cm^2$)	Number of bacteria (% of the BS number)
BS (i)	18.2 ± 0.24	100 ± 1.3
F + X (ii)	3.9 ± 0.01	21 ± 0.05
C + X (iii)	0.8 ± 0.05	4.4 ± 0.25

In vivo experiments showed that the number of cells attached on the elastic bands was higher than that of metallic bands. Additionally, the bands removed from patients, which used the C-containing mouth rinse, had less than 60 % of the attached bacteria than those that did not used the prophylactic product.

CONCLUSIONS

Results showed that the efficacy of AA against planktonic cells was higher than those corresponding to sessile cells. Thus, it is dangerous to predict the efficacy of an AA against oral biofilms based on experiments performed with planktonic cells. Microscopic observations revealed that the AA assayed were able to diffuse into the biofilm, modify the morphology of bacterial colonies, inhibit the growth of the sessile bacteria and favour the detachment of biofilm patches. However, a significant number of sessile cells with a more resistant physiological state could remain alive after AA treatments and re-grow slowly under better environmental conditions when the treatment finishes.

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