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SHORT COMMUNICATION

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Association of chlorhexidine and high fluoride dentifrice on *Streptococcus mutans* viability using an in vitro biofilm model

Associação de clorexidina e dentifricio com alta concentração de Flúor na viabilidade de *Streptococcus mutans* em um modelo de biofilmes in vitro

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ABSTRACT

Objective: this *in vitro* study investigated the effect of high-fluoride dentifrice, Chlorhexidine (CHX), and their association on the viability of *Streptococcus mutans* using a biofilm model. **Material and Method:** biofilms were anaerobically grown on glass slides that were vertically suspended in 24-well plates for 5 days. After 48 h of initial growth, biofilms were treated for the next 72 h, 2x/day with 0.12% CHX and 2%, F as 0.08% and 0.4% NaF and their association. **Results:** CHX treatment decreased the bacteria counts either alone or in association with both F concentrations, when compared with control group and the F treatments alone ($p < 0.05$). **Conclusion:** no additional effect was observed when CHX and F were used in combination, when compared with CHX used alone.

KEYWORDS

Fluoride; Chlorhexidine; *Streptococcus mutans*.

RESUMO

Objetivo: este estudo avaliou, o efeito do dentifrício com alta concentração de fluor (F), da clorexidina (CHX) e da associação destes na viabilidade de Streptococcus Mutans (*SM*) utilizando um modelo de biofilme *in vitro*. **Materiais e Métodos:** biofilme cresceram anaerobicamente em lamínulas de vidro suspensas, verticalmente, em placas de 24 poços por 5 dias. Após 48h do crescimento inicial, o biofilme formado foi submetido a um tratamento por 72h, 2x/dia com CHX 0.12%, F na forma de NaF a 0.08% e 0.4%, suas associações, CHX 2% (controle positivo) e solução salina (controle negativo). Os dados obtidos foram transformados e submetidos ao ANOVA e teste de Tukey e em seguida analisados por meio do SAS, com significância fixada em 5%. **Resultados:** isolada ou em associação com as diferentes concentrações de F, a CHX demonstrou maior potencial em reduzir os níveis de SM quando comparada ao uso isolado de F em ambas concentrações ou com o controle negativo ($p < 0,05$). **Conclusão:** o uso da combinação de F e CHX não apresentou efeito adicional na redução dos níveis de *SM* quando comparado ao uso isolado de CHX.

PALAVRAS-CHAVE

Fluoreto; Clorexidina; *Streptococcus mutans*.

INTRODUCTION

S. *mutans* are closely related with the development of dental caries due their acidogenic and aciduric properties and their capacity to use dietary carbohydrates to synthesize extracellular polysaccharides. They are considered the most cariogenic microorganisms in dental biofilm [1]. Therefore, S. *mutans* biofilms models have been used to assess the efficacy of antimicrobial agents on biofilm cells, due to the difficulties of developing *in vivo* studies in controlled cariogenic situations [2].

Regarding caries prevention, many agents have been developed to act on de-remineralization process or interfere on the metabolism/growth of dental biofilms [3]. Fluoride (F) play an important role on caries prevention, mainly because its effect on dental hard tissues [4]. Nevertheless, an additional preventive effect of F is the reduction of acid production by inhibiting bacterial glycolysis. Also, in high concentrations, F alters the bacterial metabolism by a direct inhibitory effect on the pumping protons associated with membrane H + ATPase [5]. The intraoral F levels substantially increase after use of high-F concentrations [6,7] and thus should be indicated for the prevention of caries processes and high risk patients treatment [8]. However, little is known about the effect of high-F dentifrice on metabolism of oral bacteria.

On the other hand, Chlorhexidine (CHX) has been widely used for the control of caries and cariogenic biofilms, and it is acknowledged as the gold standard of antimicrobial agents for antiplaque treatments. CHX specifically reduces S. *mutans* on biofilm and saliva [9] and their use can enhance the F-remineralazing effect, since it suppresses bacterial growth. In fact, chemical control of biofilm is an important additional resource to the mechanical control achieved with brushing [10].

A greater F cariostatic effect has been suggested when it is combined with antimicrobial agents [11]. The use of antimicrobials associated with F may be necessary, particularly for the treatment of patients under high risk/activity of caries. However, there is little information available about the effect of association of CHX and F on different concentrations on viability of oral bacteria. Therefore, this study aimed to investigate the association of CHX and high concentration F-dentifrice on the viability of S. *mutans* using an *in vitro* biofilm model.

METHODS

Experimental Design

S. *mutans* ATCC 25175 biofilms were grown on circular glass slides (13mm-diameter) placed in a vertical position in 24-well plates held by an orthodontic wire. Biofilms were anaerobically grown in Brain Heart Infusion (BHI) Broth (HIMEDIA Laboratories, Vadhani Industrial State, LBS MARG, India) at 37oC, for 5 days with the media supplemented with 1% sucrose during 8 hours and the remaining 16h without sucrose. After 48 h, the biofilms (n=6 for each treatment) were imersed 2x/day for 1 min with one of the following solutions: 1) 0.9% NaCl (Control); 2) 0.08% NaF; 3) 0.4% NaF; 4) 0.12% chlorhexidine digluconate (0.12% CHX); 5) 0.08% NaF + 0.12% CHX; 6) 0.4% NaF + 0.12% CHX and 7) 2% chlorhexidine digluconate (2% CHX). At the end of experimental phase, viable bacteria of all biofilm samples were determined. The experimental protocol was done in duplicate. For statistical evaluation, each biofilm was considered an experimental block.

Biofilm Growth

Frozen stocks of S. *mutans* ATCC 25175 were inoculated into 5 mL in BHI broth and incubated at 37 °C for 24 h under anaerobic atmosphere. Afterwards, microorganisms were inoculated in BHI Agar (HIMEDIA Laboratories, Vadhani Industrial State, LBS MARG, India). After 48 h under anaerobic conditions, one

colony of S. *mutans* was transferred to individual tubes containing 100 mL of BHI Broth. After incubation under anaerobic atmosphere at 37 °C for an additional 18 h, 2 ml of the suspension was transferred to 24-well plates containing the glass slides. Biofilms were grown for 48 h without any treatment and after this time, treatments were carried out for the next 72 h. The biofilms were exposed for 8 h to a BHI broth containing 1% sucrose and the remaining 16 h they remained in a 0.1 mM glucose (salivary basal concentration). The media were changed daily.

Treatments

The treatment solutions were prepared in a manipulation pharmacy. The chosen F concentrations was based on the use of conventional F dentifrice (1100 ppm F) and high fluoride dentifrice (5000 ppm F) after dissolution by saliva, leading to a final concentration of 350 ppm F (or 0.08% NaF) and 1650 ppm F (or 0.4% NaF) for conventional and high concentration dentifrice, respectively. The treatments were performed 2x/day (at 9:00 am and 5:00 pm) with the following solutions: 1) 0.9% NaCl (Control), 2), 3) 0.4% NaF, 4) 0.12% CHX, 5) 0.08% NaF + 0.12% CHX, 6) 0.4% NaF + 0.12% CHX and 7) 2% CHX. After each treatment, the biofilms on glass discs were rinsed in 0.9% NaCl and returned to the 24-well plates containing the media.

Biofilm Collection and Bacterial Viability

The glass discs with the biofilms were removed from the orthodontic wire and transferred into 2 ml of saline solution (0.9% NaCl). Then, biofilms were sonicated for 1 min using an ultrasonic water bath and then they were vortexed. An aliquot of 100 μl of the suspension was diluted in 0.9% NaCl in series up to 10-7 and 3 drops of 20 μl of each dilution were inoculated on BHI agar to determine the number of viable microorganisms. The plates were incubated for 48 h at 37 °C under anaerobic conditions. CFU were counted and the results were expressed as CFU/ml.

Statistical Analysis

The assumptions of equality of variances and normal distribution of errors were checked and the response variable was transformed to log10. The transformed variable was submitted to ANOVA followed by Tukey test with the significance level set at 5%. The statistical analysis was performed with SAS software (version 9.0).

RESULTS

The effects of the treatments on the viability of the S. *mutans* are shown in Figure 1. Among all the test groups, significantly lower counts was observed for the treatments with 2% CHX ($p < 0.05$). Similarly, the 0.12% CHX treatment decreased the bacteria counts either alone or in association with both F concentrations, when compared with control group and the F treatments alone ($p < 0.05$). Viable bacteria counts of biofilm treated with 0.08% NaF did not differ from those of the control. However, 0.4% NaF group showed statistically lower counts than control group ($p < 0.05$).

DISCUSSION

Biofilm models are important tools to evaluate the biochemical and microbiological composition of biofilm formed under different conditions or the changes caused on the substratum surface to which the biofilm is attached [12]. This study evaluated the reduction levels of S. *mutans* colonies of biofilm formed *in vitro*, after treatment using CHX and F in different concentrations and in combination. The experimental model used simulates some oral cavity conditions, but has limitations that are inherent to any *in vitro* experimental design.

Fluoride at conventional concentration (0.08% NaF) showed no effect on viable bacteria counts (Figure 1). This finding is supported by the evidence that at least a 10 ppm of F constant concentration in the media is necessary to have some bacterial effect [13]. One possible explanation is that the time of treatment (1 min)

was not sufficient to reach the F concentration necessary to exert any antibacterial effect. On the other hand, high F concentrations inhibits the colonization, metabolism and growth of bacteria, and prevent biofilm maturation [14], which can explain the slight reduction of these bacteria after treatment with high concentration solution (Figure 1).

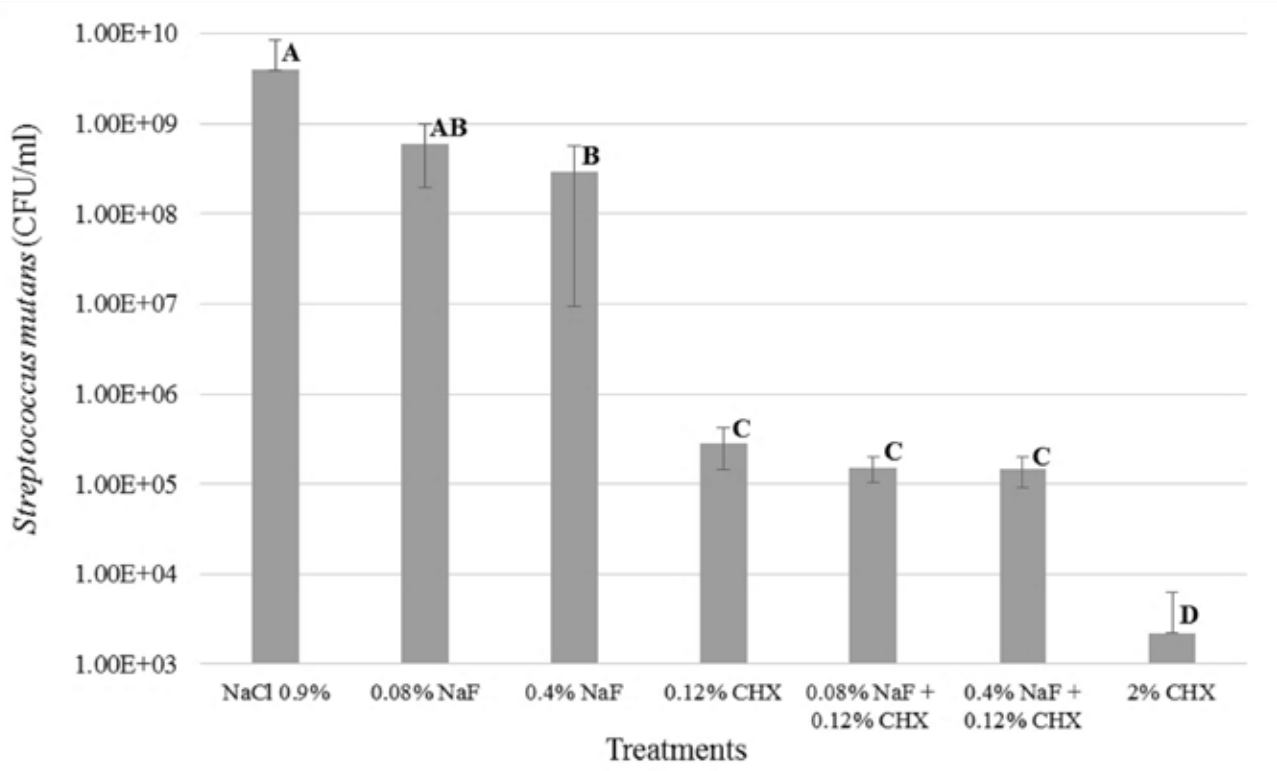

Figure 1 - Means of viable bacteria (CFU/mL) in the biofilms according to the treatments (n=6). Data were log-transformed. Vertical bars represent the standard deviations. Different letters indicate significant differences among the groups (p « 0.05).

The use of dentifrice containing 5000 ppm F, for example, significantly reduces the amount of accumulated biofilm possibly due to a reduction in the bacterial tolerance to acid, which does not occur when low doses of F are used [15]. In fact, the effectiveness of any strategy using F depends on its bioavailability in the oral cavity. This can be easily achieved by the use of high F concentrations, such as when a 5000 ppm F dentifrice is used [16]. After using 5000 ppm F dentifrice, F concentration in biofilm would reach around 14 ppm F [17].

With regard to CHX, the treatment 2x/day irrespective of concentration used (0.12% or 2%) had a strong bactericidal effect on the viable S. *mutans* of the biofilm. This is a well-established effect of CHX [18]. Although only two concentrations of CHX were evaluated in this study, the results corroborate others that showed a dose-response effect of CHX [12,19]. On the other hand, the combination of 0.12 % CHX and F in both concentrations showed no additional effect, when compared with 0.12% CHX alone. This result confirms the strong suppressive effect of CHX on S. *mutans* over the weak effect of F [4] even in high concentration.

CONCLUSION

In conclusion, analysis of the data suggests that the combination of CHX and F have no additional effect on SM reduction in comparison with the use of CHX alone, however further studies evaluating the shift caused by this association on the dental substrate may be necessary.

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