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Antibacterial and Anti-Biofilm Activities of Ambroxol Against Oral Bacteria

Cabral-Romero Claudio[1*], Martínez-Sanmiguel Juan José[1], Reséndez-Pérez Diana[2], Flores-González María del Socorro[2], Hernandez-Delgadillo Rene[2]

1. Facultad de Odontologia, Universidad Autonoma de Nuevo Leon, UANL, Monterrey, Nuevo Leon, Mexico.
2. Instituto de Biotecnologia, Facultad de Ciencias Biológicas, Universidad Autonoma de Nuevo Leon, UANL, Monterrey, Nuevo Leon, Mexico.
[E.mail: claudiohubble@hotmail.com]

The first stage to develop periodontal disease and caries is to get a biofilm of oral bacteria. This oral biofilm presents similitude with bronchial mucus, therefore in theory we can use mucolytic agents to eradicate oral biofilm. Ambroxol is a mucolytic agent employed to eradicate this kind of mucus in patients with cystic fibrosis. However, there are no reports about the capability of ambroxol against oral bacteria. The aim of this study was determine the antimicrobial and antibiofilm effectiveness of ambroxol against oral bacteria. The bactericidal activity of ambroxol was determined by using the cell viability MTT assay. The Minimum Inhibitory Concentration (MIC) of ambroxol to interfere with oral bacteria growth was detected. At same time, the anti-biofilm activity of ambroxol was explored by fluorescence microscopy. Within our results, ambroxol showed an important bactericidal activity since 4 mg/ml as final concentration. The MIC estimated for ambroxol was 1mg/ml against oral bacteria. At this concentration, ambroxol was capable to inhibit the oral biofilm formation added in the inoculation time. Also, it was effective to remove a mature oral biofilm. As conclusion, ambroxol is a good therapeutic alternative for prevent or treatment of oral diseases.

Keyword: Mucolytic Agent, Antimicrobial Activity, Biofilm, *S. mutans*.

1. Introduction

Despite continuous efforts from medicine and pharmaceutical industry; the increasing antimicrobial resistance among microorganisms to common antibiotic has become the most important problem in current medicine[1]. Odontology practice is not absent of this problem, being frequent the excessive use of antibiotics contributing to develop antimicrobial resistance. Based on breakpoint concentrations, a higher number of resistant strains in Spain were found in *F. nucleatum* for penicillin, amoxicillin and metronidazole, in *Prevotella intermedia* for tetracycline and amoxicillin, and in *A. actinomycetemcomitans* for amoxicillin and azithromycin[2]. Periodontal microorganisms isolated from patients with chronic periodontitis can be resistant to the antimicrobial agents commonly used in anti-infective periodontal therapy[3]. The absence of new alternatives to treat efficiently the multiresistant pathogenic bacteria is a real problem and it is urgent to synthesize new broad spectrum drugs to fight antimicrobial resistance.

The microorganisms live in association into communities with other microorganisms in a

cooperative form, which is called biofilm. The biofilms can form on all kind of surfaces and interfaces, including the human body[4]. The most common biofilm is the dental plaque in oral cavity, being *Streptococcus mutans* the main etiological agent of dental caries worldwide[5, 6]. *S. mutans* has also been identified in endocarditis cases where they colonize endocardium and cardiac valves, probably due to for their ability to adhere to solid surfaces and form biofilms[7]. Periodontal disease is the second most common sickness in oral cavity [8], being *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* the main etiological agents[9].

The first stage to develop an oral disease is to get a biofilm of pathogen bacteria[9]. The cells inside a biofilm remain together through an exopolysaccharides matrix, that is very similar to bronchial mucus[4]. Interestingly, had not been used mucolytic agents to remove the oral biofilm to prevent oral diseases.

Ambroxol [2-amino-3,5-dibromo-*N*-(trans-4-hydroxycyclohexyl) benzylamine] is a mucolytic agent that has been used for the treatment of chronic bronchitis[10]. The pharmacological effects of ambroxol (AMB) have been reported as mucoregulation on gland cells[11]. Furthermore, AMB exhibits antioxidant[12] and anti-inflammatory properties with reduction of the release of inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-2, IL-1, IL-4, IL-13 and interferon (IFN)-γ, from bronchoalveolar macrophages, monocytes and granulocytes[13,14]. Interestingly, the antimicrobial activity of AMB has not been extensively studied. There are not reports about the bactericidal activity of AMB against oral bacteria.

The aim of this study was determine the antimicrobial and antibiofilm effectiveness of AMB against oral bacteria. In this work, we present the first evidence of the antimicrobial abilities of AMB inhibiting the oral bacteria growth. The biocidal activity of AMB was very similar to the obtained with most common oral antiseptic, chlorhexidine. AMB constitutes an excellent option to interfere with oral bacteria biofilm formation.

2. Material and methods

2.1 Activation and Growing of Oral Bacteria

Oral bacteria were grown in Tripticase Soy Broth (TSB, BD DIFCO, Sparks MD, USA) at 37 °C in aerobic conditions for 7 days. The presence of *Aggregatibacter actinomycetemcomitans (A.a)* and *Streptococcus mutans* was determined by real time PCR using specific probe and oligonucleotides to amplify the 16S subunit ribosomal of each bacterium. As positive controls were employed ATCC strains of *S. mutans* and *A.a* (strain AU130, ATCC number; 700611 and ATCC number 29522, respecitvely).

2.2 Preparation and Dilution of Ambroxol

From an 30 mg/ml stock solution of AMB [2-amino-3,5-dibromo-*N*-(trans-4-hydroxycyclohexyl) benzylamine] were obtained final concentrations of 2, 1, 0.5, 0.1 and 0.01mg/ml to explore the antimicrobial activity against oral microbes. AMB (Bayer, Munich, Germany) was mixed with TSB medium and vortex for two minutes. It was covered of light with aluminum foil. All reacts were prepared in the moment of their use.

2.3 Antimicrobial Activity of Ambroxol Against Oral Bacterial Growth

The antimicrobial effect of AMB on *A.a* and *S. mutans* growth was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Biotium, Hayward, CA)[15, 16] according to the instructions of the manufacturer. Oral microorganisms were grown in TSB medium at 37°C, overnight in aerobic conditions. The bacteria were counted using a Neubauer chamber and $1X10^4$ cells were inoculated in 100 µl of TSB medium in 96 wells polystyrene plates. Three wells with only TSB medium were used as control of *A.a* and *S. mutans* growth. 0.12% clorhexidine (Ultradent products, South Jordan, UT) was used as an antimicrobial positive control. Several final concentrations of AMB were employed to interfere with bacterial growth. The 96 wells plate was incubated at 37ºC in aerobic conditions for overnight. 10 µl of MTT

was added to each well, the plate was protected against light and incubated at 37°C for 2 hours. 200 µl of Dimethylsulfoxide (DMSO) was added to dissolve the reduced MTT. The amount of live cells was determined by a Microplate Absorbance Reader (Biorad, Philadelphia, PA) at 595 nm. The experiment was repeated three times and the measured optical density were analyzed by descriptive statistics.

2.4 Anti-Biofilm Activity of Ambroxol Against Oral Biofilm

The anti-biofilm activity of AMB determined by microscopy of fluorescence following the methodology described above. To observe the biofilm, the SYTO 9 green dye (Invitrogen, Carlsbad, CA) was added at a final concentration of 20µM[16, 17]. The 96 wells plate was incubated for 30 minutes at room temperature and protected against light. The oral biofilm was visualized with a Carl Zeiss Z1 Axio Inverter microscope (Thornwood, NY) at 485nm.

2.5 Determination of Minimal Inhibitory Concentration of Ambroxol

The minimal inhibitory concentration (MIC) was determined as previously described[18]. Briefly, it was obtained a 5 tube in the McFarland scale with $1x10^9$ CFU. *A. a* and *S. mutans* were grown in TSB agar and incubated at 37°C for 24 hours. One colony was inoculated in 5 ml of TSB medium and incubated at 37°C for 24 hours. The bacteria count was determined with a Neubauer chamber. Tubes with a final concentration of $1x10^6$ CFU were obtained by dilution of the 5 tube in the McFarland scale. AMB was used to final concentrations of 2, 1, 0.5, 0.1 and 0.01 mg/ml and mixed with bacterial suspension. They were incubated at 37°C in aerobic conditions for 24 hours. The MIC was determined from the presence or absence of turbidity in the different tubes containing the mucolytic agent. 0.12% clorhexidine was used as a positive control of inhibition.

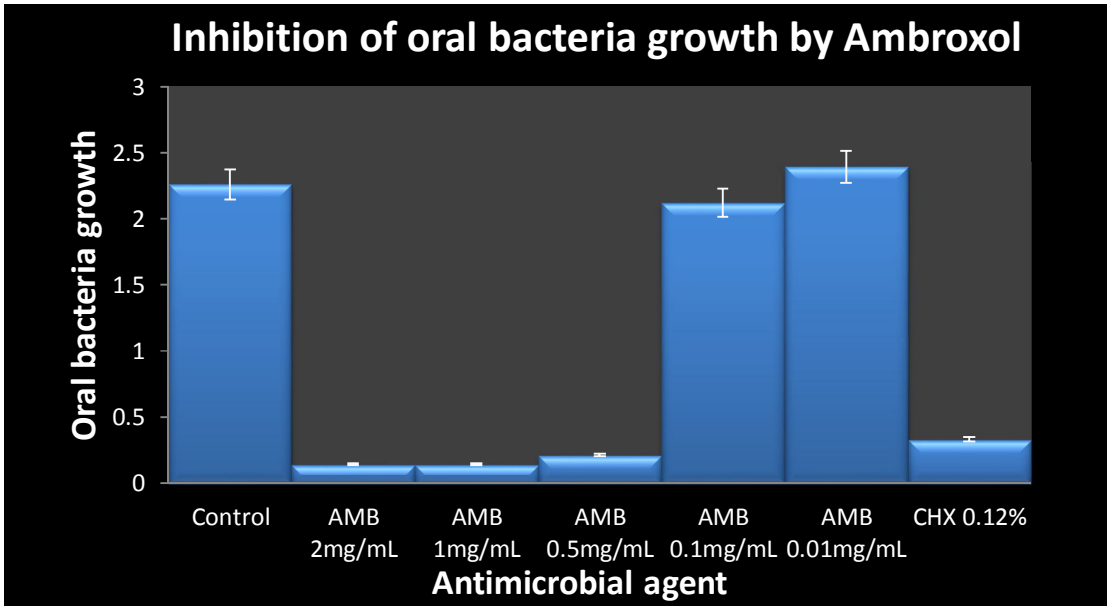

Figure 1. Bactericidal activity of ambroxol against A.a and *S. mutans* growth. The Y axis shows the optical density units of microbial growth. As growing control of bacteria was added culture media and 0.12% clorhexidine was employed as positive inhibition control. AMB was used at final concentrations of 2, 1, 0.5, 0.1 and 0.01 mg/ml. All experiments were done by triplicate to assess the veracity of results.

3. Results

3.1 Detection of *A. actinomycetemcomitans* and *S. mutans* by Real Time PCR

The presence of *A.a* and *S. mutans* was confirmed by real time PCR. It was detected *A.a* and *S. mutans* from crevicular fluid in patient samples with periodontal disease. These data supports the association of *A.a* with development and aggressive of periodontal disease. The susceptibility of these oral bacteria against ambroxol was determined right after.

3.2 Antimicrobial Activity of Ambroxol Against Oral Microbes

To explore the antimicrobial activity of AMB against oral bacteria, their effect under *A.a* and *S. mutans* growth was determined. The results showed that AMB was capable to inhibit the bacterial growth since 0.5 mg/ml. 2 mg/ml of ambroxol reduced the number of microorganisms by 90%, in comparison to microbes grown in medium alone (Figure 1). Similarly, the treatment with 0.12% clorhexidine (inhibition control) showed an 80% of reduction in the number of bacteria, when compared with non-treated cells (Figure 1). These data suggest that AMB was as an effective as chlorhexidine to inhibit the oral bacterial growth.

3.3 Biofilm Inhibitory Activity of Ambroxol

Once determined the antimicrobial effectiveness of AMB, we analyze their possible anti-biofilm activity against oral bacteria biofilm. This ability was evaluated by fluorescence microscopy. The results showed a complete inhibition of biofilm formation by clorhexidine (Fig. 2B) and AMB (Figure 2C), compared to control (Fig. 2 A). 0.01 mg/ml AMB was incapable to interfere with *A.a* and *S. mutans* biofilm formation. These results correlate with previous data obtained by MTT assay, indicating that at 0.01 mg/ml AMB has not antimicrobial nor anti-biofilm activities. AMB was able to inhibit the oral biofilm formation if was added at 0-4 hrs. post-inoculation times (Fig. 3C and 3D). AMB was incapable to remove an 8-16 hrs. mature biofilm (Fig. 3E and 3F). All together, these results suggest that AMB is effective disrupting the oral bacteria biofilm.

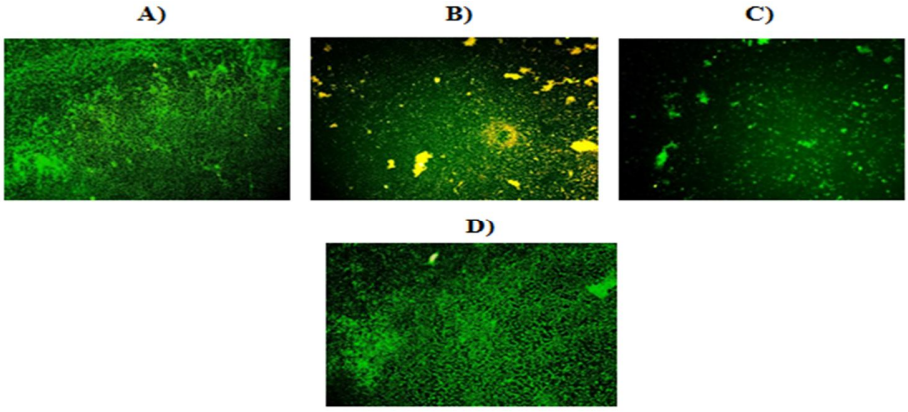

Figure 2. Inhibition of oral biofilm formation by ambroxol using fluorescence microscopy. A) As growing control of microorganisms was added culture media and B) 0.12% clorhexidine was employed as positive inhibition control. C) and D) AMB was used at a final concentrations of 2 and 0.01 mg/ml, respectively. Both chlorhexidine and ambroxol were added at inoculation time. All experiments were done by triplicate to assess the veracity of results.

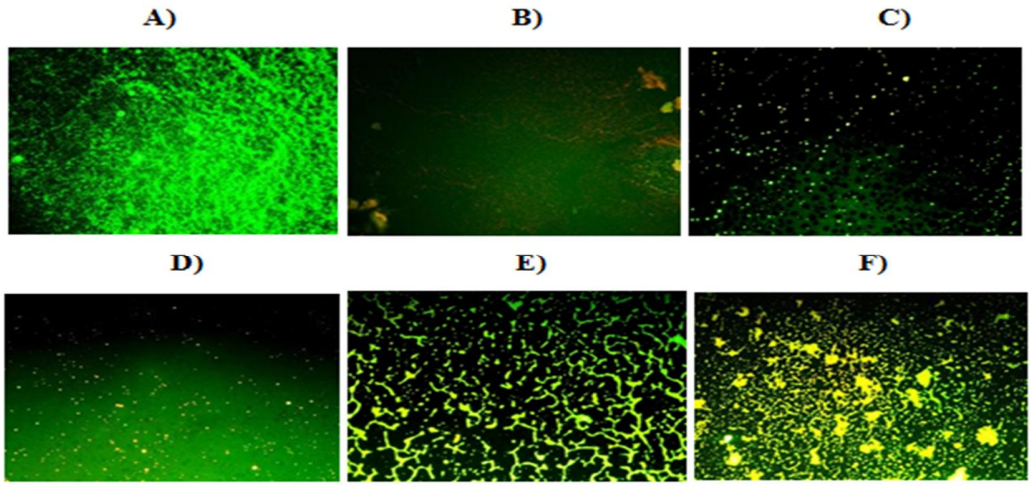

Figure 3. Inhibition of oral biofilm formation by ambroxol at different post-inoculation times, using fluorescence microscopy. A) As growing control of microbes was added culture media and B) 0.12% clorhexidine was employed as positive inhibition control. C), D), E) and F) correspond to AMB that it was used at a final concentration of 2 mg/ml inoculated at 0, 4, 8 and 16 hrs. post-inoculation times. All experiments were done by triplicate to assess the veracity of results.

3.4 Determination of Minimal Inhibitory Concentration of Ambroxol

In order to get a more detail characterization of the antimicrobial activity of AMB against oral bacteria, we determined their minimal inhibitory concentration. The result obtained for AMB was 0.5 mg/ml for inhibit oral microbial growth. This result is a relevant datum to know the minimal effective quantity of ambroxol that is required to interfere with oral bacterial growth.

4. Discussion

Mucolytics are drugs that change the biophysical properties of secretions by degrading the mucin polymers, DNA, fibrin, or F-actin in airway secretions, generally decreasing viscosity[19]. Among classic mucolytic agents are AMB and N-acetylcysteine (NAC), this last is considered a non-antibiotic drug that has antibacterial and antibiofilm properties. Interestingly, although oral biofilm is very similar to bronchial mucus, the used of mucolytic agents to eradicate the oral biofilm have not been extensively explored.

Here we present the first evidence of antimicrobial activity of AMB against oral bacteria. Their efficacy in inhibiting the *A. a* and *S. mutans* growth was better than clorhexidine. The minimal concentration of ambroxol to inhibit the microbial growth was 0. 5 mg/ml. This datum is relevant to take it account if AMB will be incorporated into a mouthwash or tooth paste. These results indicate that AMB is antimicrobial agent as good as the most commonly used oral antiseptic. Previously, it has been reported that AMB kill to *Pseudomonas aeruginosa,* decreasing the bacterial load into a biofilm[20]. However, in this work we employed the half of quantity of AMB to determine their bactericidal activity in comparison with this report. We did not found another report about bactericidal activity of AMB.

In order to assess if AMB had the potential to interfere or remove the oral biofilm formation, their anti-biofilm activity was studied. Surprisingly, the effect anti-biofilm formation was total using ambroxol at the inoculation time. In presence of chlorexidine and ambroxol we just observed cellular debris on a dark background; mainly DNA of dead bacteria with accumulates of dye, supporting the data previously obtained by MTT assays. Morphologically, these dye accumulates clearly differ from bacterial biofilm. Ambroxol was incapable to detached biofilm of 8-16 hrs. post inoculation, suggesting that is the

antimicrobial activity who kill the cells to interfere with biofilm formation. There are not previous reports about anti-biofilm activity of AMB against oral bacteria. Recent studies with *Pseudomonas aeruginosa* have shown that AMB decrease biofilm formation[20]. Similarity, it has been published that AMB prevent bacterial adherence to mammalian cells[21]. AMB increased the susceptibility of biofilm forming of *Candida parapsilosis* to voriconazole[22]. Our data are agreed with these previous reports, supporting the bactericidal activity of AMB found by MTT assays.

In this work we focused on the effectiveness of ambroxol in inhibiting the growth of oral bacteria. All together, the experimental data suggest that ambroxol could be an interesting alternative to combat the oral diseases. The property of ambroxol could be used in odontological practice, supporting the antimicrobial and anti-biofilm activities of oral antiseptics or tooth paste.

5. Conclusion

Ambroxol is an excellent therapeutic alternative for the treatment of oral diseases, which promotes more favorable results in the clinical evaluation of patients.

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7. References

1. Falagas, M.E., K.N. Fragoulis, and I. Karydis, A comparative study on the cost of new antibiotics and drugs of other therapeutic categories. PLoS One, 2006. 1: p. e11.
2. van Winkelhoff, A.J., et al., Antimicrobial profiles of periodontal pathogens isolated from periodontitis patients in The Netherlands and Spain. J Clin Periodontol, 2005. 32(8): p. 893-8.
3. Ardila, C.M., M.I. Granada, and I.C. Guzman, Antibiotic resistance of subgingival species in chronic periodontitis patients. J Periodontal Res, 2010. 45(4): p. 557-63.
4. Costerton, J.W., Overview of microbial biofilms. J Ind Microbiol, 1995. 15(3): p. 137-40.
5. Costerton, J.W., Introduction to biofilm. Int J Antimicrob Agents, 1999. 11(3-4): p. 217-21; discussion 237-9.
6. Jenkinson, H.F., Adherence and accumulation of oral streptococci. Trends Microbiol, 1994. 2(6): p. 209-12.
7. Guntheroth, W.G., How important are dental procedures as a cause of infective endocarditis? Am J Cardiol, 1984. 54(7): p. 797-801.
8. Beikler, T. and T.F. Flemmig, Oral biofilm-associated diseases: trends and implications for quality of life, systemic health and expenditures. Periodontol 2000, 2011. 55(1): p. 87-103.
9. Benakanakere, M. and D.F. Kinane, Innate cellular responses to the periodontal biofilm. Front Oral Biol, 2012. 15: p. 41-55.
10. Germouty, J. and J.L. Jirou-Najou, Clinical efficacy of ambroxol in the treatment of bronchial stasis. Clinical trial in 120 patients at two different doses. Respiration, 1987. 51 Suppl 1: p. 37-41.
11. Heath, M.F. and W. Jacobson, The inhibition of lysosomal phospholipase A from rabbit lung by ambroxol and its consequences for pulmonary surfactant. Lung, 1985. 163(6): p. 337-44.
12. Gillissen, A., et al., Oxidant scavenger function of ambroxol in vitro: a comparison with N-acetylcysteine. Res Exp Med (Berl), 1997. 196(6): p. 389-98.
13. Pfeifer, S., et al., Reduction of cytokine release of blood and bronchoalveolar mononuclear cells by ambroxol. Eur J Med Res, 1997. 2(3): p. 129-32.
14. Gibbs, B.F., et al., Ambroxol inhibits the release of histamine, leukotrienes and cytokines from human leukocytes and mast cells. Inflamm Res, 1999. 48(2): p. 86-93.
15. Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983. 65(1-2): p. 55-63.
16. Yue, H., et al., An evaluation of the performance of cDNA microarrays for detecting changes in global mRNA expression. Nucleic Acids Res, 2001. 29(8): p. E41-1.

17. Frey, T., Nucleic acid dyes for detection of apoptosis in live cells. Cytometry, 1995. 21(3): p. 265-74.
18. Andrews, J.M., Determination of minimum inhibitory concentrations. J Antimicrob Chemother, 2001. 48 Suppl 1: p. 5-16.
19. Rubin, B.K., Mucolytics, expectorants, and mucokinetic medications. Respir Care, 2007. 52(7): p. 859-65.
20. Li, F., et al., Effect of ambroxol on pneumonia caused by Pseudomonas aeruginosa with biofilm formation in an endotracheal intubation rat model. Chemotherapy, 2011. 57(2): p. 173-80.
21. Hafez, M.M., et al., Activity of some mucolytics against bacterial adherence to mammalian cells. Appl Biochem Biotechnol, 2009. 158(1): p. 97-112.
22. Pulcrano, G., et al., Ambroxol influences voriconazole resistance of Candida parapsilosis biofilm. FEMS Yeast Res, 2012. 12(4): p. 430-8.