The Pharma Innovation

ISSN: 2277- 7695
CODEN Code: PIHNBQ
ZDB-Number: 2663038-2
IC Journal No: 7725

Received: 05-03-2013
Accepted: 09-04-2013

Vol. 2 Issue 3 2013
Online Available at www.thepharmajournal.com

THE PHARMA INNOVATION - JOURNAL

Rifampicin and N-acteylcisteyne Inhibit Oral Bacterial Growth and Biofilm Formation

Hernandez-Romero Cindy[1], Hernandez-Delgadillo Rene[2], Galindo-Rodríguez Sergio[2], Reséndez-Pérez Diana[2], Flores-González María del Socorro[2] and Cabral-Romero Claudio[1*].

1. Facultad de Odontología, Universidad Autónoma de Nuevo León, UANL, Monterrey, Nuevo León, México.
   [E.mail: claudiohubble@hotmail.com, Tel: +52 (81) 83294000 Ext. 3153.]
2. Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, UANL, Monterrey, Nuevo León, México.

Dental plaque consists in a biofilm of pathogen microbes. Some oral bacteria have high homology with *M. tuberculosis* regarding growth, slow metabolism and cell division. Rifampicin is the first election to treat active tuberculosis; however there are no reports about the capability of rifampicin against oral bacteria. N-acetylcysteine (NAC) is a mucolytic agent with antibiofilm properties, but it is unknown their ability to disrupt oral bacteria biofilm. The aim of this study was determine the bactericidal and antibiofilm effectiveness of rifampicin/N-acetylcysteine against oral microbes. The bactericidal activity to interfere with oral bacteria growth was analyzed by cell viability MTT assay. Anti-biofilm activity explored by fluorescence microscopy. The result of MIC was 1µg/ml against periodontopathogen bacteria and 0.25µg/ml for *S. mutans*. Rifampicin was capable to inhibit the oral biofilm formation in inoculation time. NAC had antibiofilm activities at 200mg/ml and the mix rifampicin/NAC was capable to eradicate a 4-8 hrs. biofilm.

Keyword: Rifampicin, N-acetylcysteine, Bactericidal Agent, Antibiofilm, Oral Bacteria, *S. mutans*

1. Introduction

Bacteria in nature do not grow in nutrient-rich medium nor individual form. 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[1]. The most common biofilm is the dental plaque in oral cavity, being *Streptococcus mutans* the main etiological agent of dental caries worldwide[2, 3]. *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[4]. Periodontal disease is the second most common sickness in oral cavity,[5] being *Agregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* the main etiological agents[6].

The increasing prevalence of infections due multiresistant microorganisms has become one of the most important problems in modern medicine[7]. Dental area is not absent of this problem, being common the excessive use of antibiotics contributing to develop antimicrobial resistance. Significantly higher Minimal Inhibition Concentration (MIC) values were

noted in Spanish strains of *F. nucleatum* for penicillin, ciprofloxacin, of *P. intermedia* for penicillin, amoxicillin and tetracycline, and of *P. gingivalis* for tetracycline and ciprofloxacin. 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[8]. Periodontal microorganisms isolated from patients with chronic periodontitis can be resistant to the antimicrobial agents commonly used in anti-infective periodontal therapy[9]. 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.

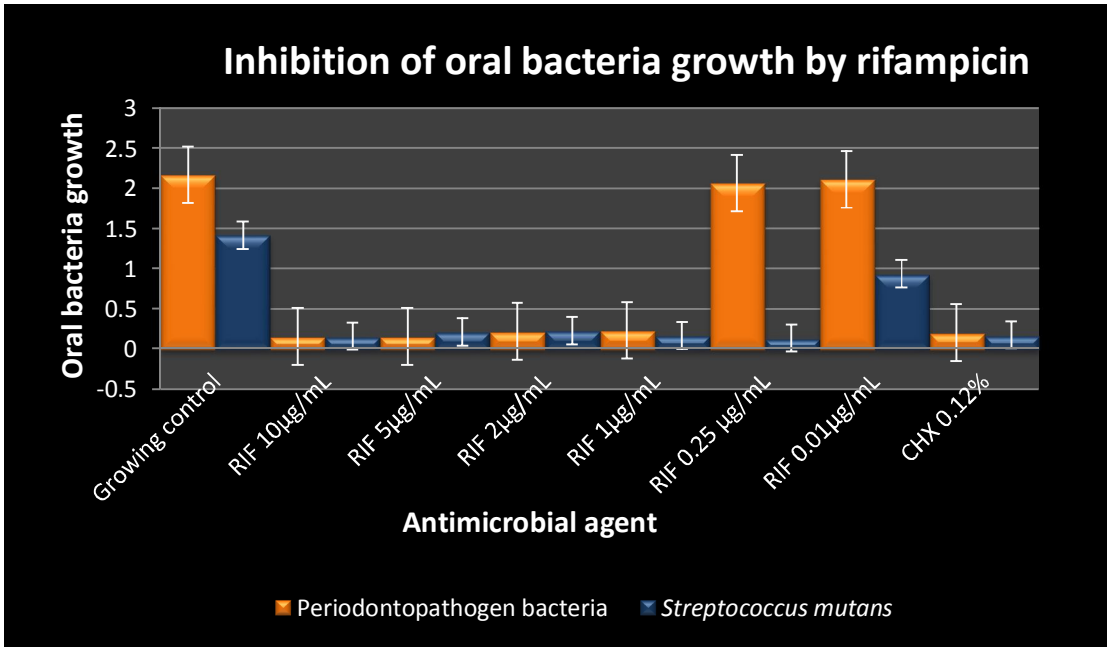

Figure 1. Bactericidal activity of rifampicin against oral bacteria. The Y axis shows the optical density units of *S. mutans* growth and *A.a* growth.. As growing control of bacteria was added culture media and 0.12% chlorhexidine was employed as positive inhibition control. Rifampicin was used at final concentrations of 10, 5, 2, 1, 0.25 and 0.01 µg/ml. All experiments were done by triplicate to assess the veracity of results.

The first step to develop periodontal disease is to get a biofilm of periodontopathogen bacteria[6]. These bacteria exhibit high homology with *Mycobacterium tuberculosis* regarding growth, slow metabolism and cell division. Rifampicin is one of few efficient antibiotics against multi-resistant bacteria, being the first election to treat active tuberculosis[10]. However, there are not reports about the capability of rifampicin to inhibit oral bacteria growth.

N-acetylcysteine (NAC) is a mucolytic agent that is considered a non-antibiotic drug with antibacterial properties. NAC decreases biofilm formation by a variety of bacteria[11-13] and reduces the production of an extracellular polysaccharide matrix, promoting the disruption of mature biofilms[14]. However, the possible effectiveness of NAC against oral bacteria biofilm has not been extensively explored. Recently, it was published that NAC inhibits growth and eradicates biofilm of *Enterococcus feacalis* ,[15] although there are no reports of NAC against the main oral bacteria, like *Streptococcus*

mutans, *Porphyromonas gingivalis* and *Agregatibacter actinomycetemcomitans*.

The aim of this study was determine the bactericidal and antibiofilm effectiveness of rifampicin and NAC against oral microorganisms. In this work, we present the first evidence of the antimicrobial abilities of rifampicin inhibiting the *S. mutans* and *A. actinomycetemcomitans* growth. The biocidal activity of rifampicin and NAC was very similar to the obtained with most common oral antiseptic, chlorhexidine. NAC eradicates the biofilm of *A.a* and *S. mutans* and in combination with rifampicin constitutes an excellent option to interfere with oral bacteria biofilm formation.

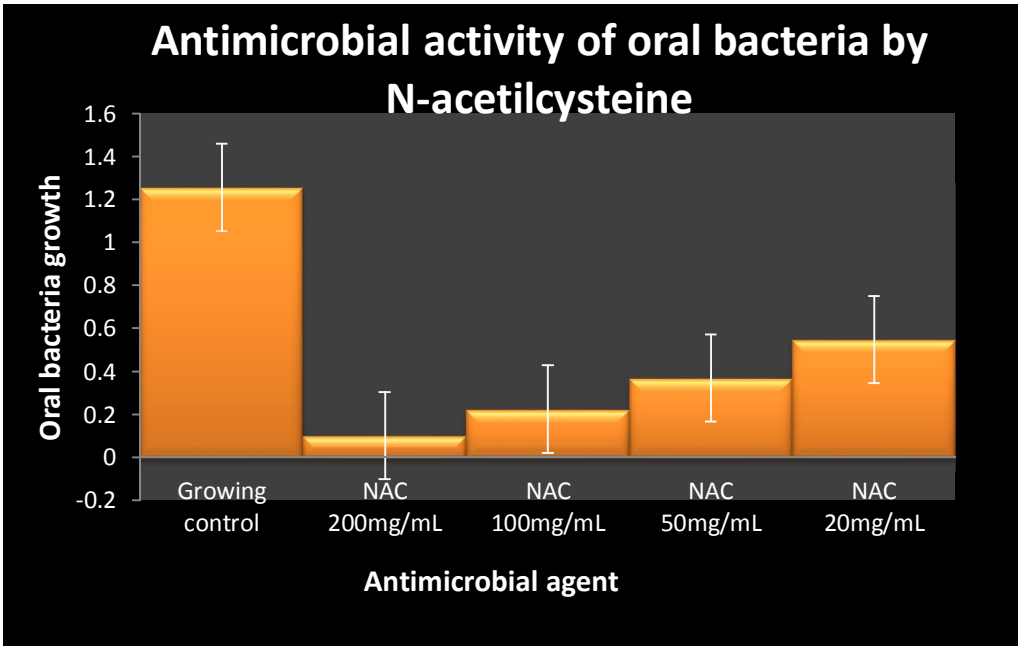

Figure 2. Bactericidal activity of NAC against oral bacteria. The Y axis shows the optical density units of oral bacteria growth. As growing control of microbes was added culture media and 0.12% chlorhexidine was employed as positive inhibition control. NAC was used at final concentrations of 200, 100, 50, and 20 mg/ml. All experiments were done by triplicate to assess the veracity of results.

2. Material and Methods

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

Samples from crevicular fluid were taken of patients with periodontal pockets around 5-10 mm of probing depth. 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 Rifampicin and NAC Preparation

A 100 µg/ml stock solution of rifampicin (Sigma-Aldrich, St. Louis, USA) was obtained dissolving 0.100 g in 100 µl of 96° ethanol and homogenized by vortex for five minutes. It was added 900 µl of Milli-Q water and vortex for two minutes. It was covered of light with aluminum foil. The stock solution of rifampicin was diluted to get final concentrations of 10, 5, 2, 1, 0.25 and 0.01µg/ml. In the case of NAC (Sigma-Aldrich, St. Louis, USA), it was prepared a 400mg/ml stock solution dissolving 0.4 g in 1 ml of TSB and vortex for two minutes. Final concentrations

of 200, 100, 50, and 20 mg/ml were obtained of NAC to explore the minimal inhibitory concentration. All reacts were prepared in the moment of their use.

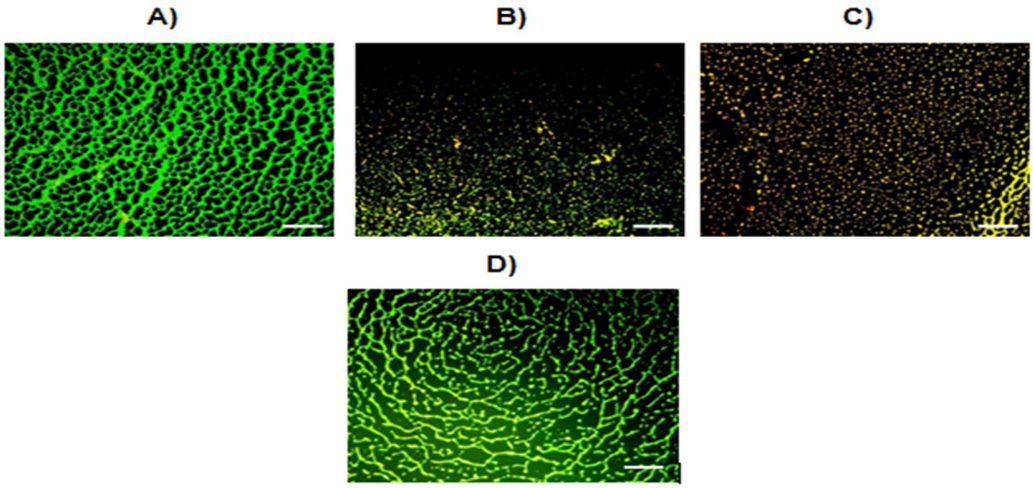

Figure 3. Inhibition of *S. mutans* biofilm formation by rifampicin using fluorescence microscopy. A) As growing control of *S. mutans* was added culture media and B) 0.12% chlorhexidine was employed as positive inhibition control. C) and D) Rifampicin was used at a final concentrations of 10 and 0.01 µg/ml, respectively. All experiments were done by triplicate to assess the veracity of results. The presence of yellow/orange color is indicative of dead cells, while green color represents live cells. Scale bars, 10 µm.

2.3 Determination of Minimal Inhibitory Concentration of Rifampicin

The minimal inhibitory concentration (MIC) was determined as previously described[16]. 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

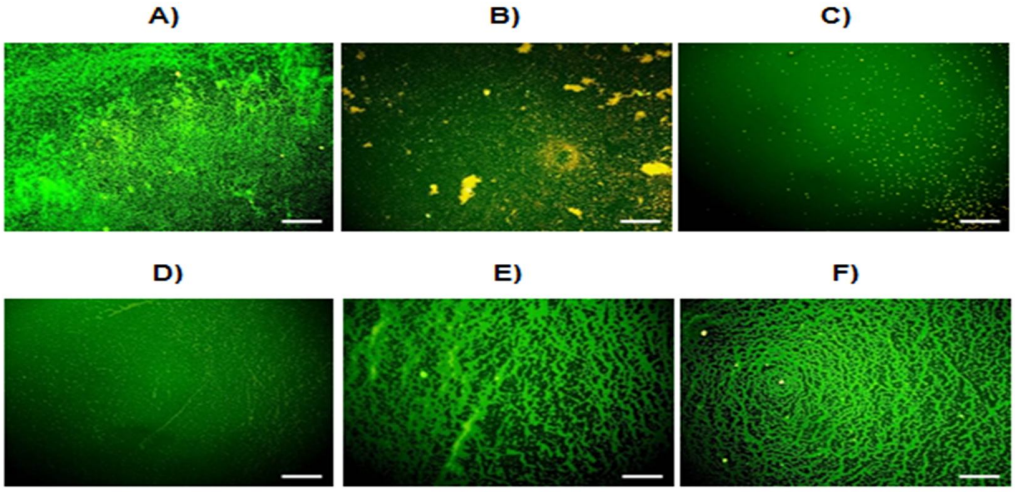

Figure 4. Inhibition of *S. mutans* biofilm formation by NAC using fluorescence microscopy. A) As growing control of *S. mutans* was added culture media and B) 0.12% chlorhexidine was employed as positive inhibition control. C), D), E) and F) NAC was used at a final concentrations of 200, 100, 50 and 20 mg/ml, respectively. All experiments were done by triplicate to assess the veracity of results. The presence of yellow/orange color is indicative of dead cells, while green color represents live cells. Scale bars, 10 µm.

$1x10^6$ CFU were obtained by dilution of the 5 tube in the McFarland scale. Rifampicin was diluted to final concentrations of 10, 5, 2, 1, 0.25 and 0.01 µg/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 antibiotic. 0.12% chlorhexidine was used as a positive control of inhibition.

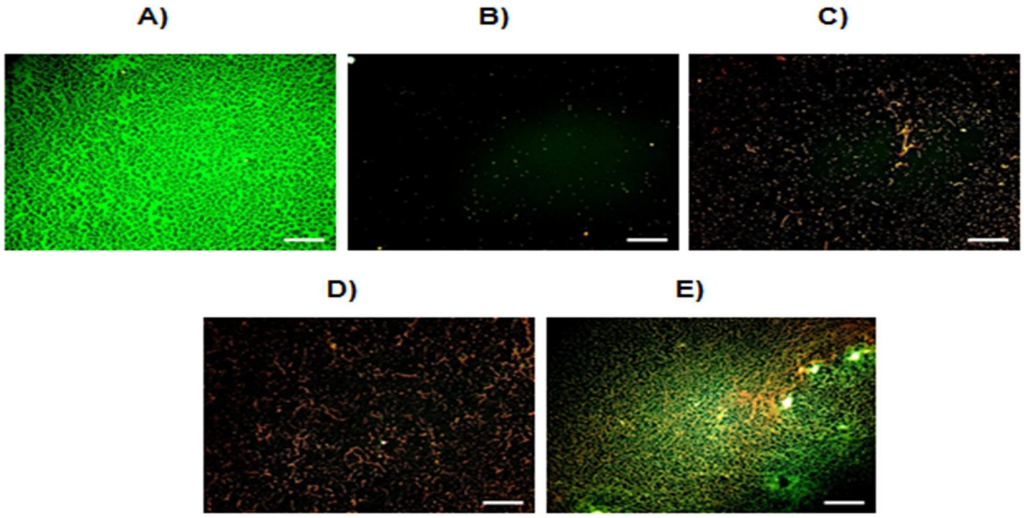

Figure 5. Inhibition of oral biofilm formation by rifampicin/NAC using fluorescence microscopy. A) As growing control of oral bacteria was added culture media. Rifampicin/NAC were used at final concentrations of 1 µg/ml and 200 mg/ml, respectively. B), C), D) and E) Rifampicin/NAC were added at 0, 4, 8 and 16 hrs. post-inoculation times, respectively. All experiments were done by triplicate to assess the veracity of results. The presence of yellow/orange color is indicative of dead cells, while green color represents live cells. Scale bars, 10 µm.

2.4 Biofilm Inhibitory Activity of Rifampicin

The anti-biofilm activity of rifampicin was 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 [17], [18]. The 96 wells plate was incubated for 30 minutes at room temperature and protected against light. The *A.a* and *S. mutans* biofilms were visualized with a Carl Zeiss Z1 Axio Inverter microscope (Thornwood, NY) at 485nm.

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 rifampicin and NAC was determined right after.

3.2 Antimicrobial Activity of Rifampicin/NAC against *A. actinomycetemcomitans* and *S. mutans* Growth

To explore the antimicrobial activity of rifampicin and NAC against oral bacteria, their effect under *A.a* and *S. mutans* growth was determined. The results showed rifampicin and NAC were effectiveness to inhibit the bacterial growth (Figure 1 and 2). Since 10 µg/ml, rifampicin reduced the number of bacteria (A.a and *S. mutans*, respectively) by 100%, in comparison to bacteria grown in medium alone (Fig 1). Similarly, the treatment with 0.12%

chlorhexidine (inhibition control) showed a 100% of reduction in the number of bacteria, when compared with non-treated cells (Fig 1). In the case of NAC, the most important effect was observed at 200 mg/ml, detecting a phenomenal dose dependent until 20 mg/ml (Fig 2). These data suggest the capacity of rifampicin and NAC as antimicrobial agents against oral bacteria.

3.3 Biofilm Inhibitory Activity of Rifampicin and NAC

Once determined the bactericidal effectiveness of rifampicin and NAC against oral bacteria, we analyze the possible biofilm inhibition of *A.a*. and *S. mutans* by rifampicin/NAC. The anti-biofilm activity was determined by fluorescence microscopy. The results showed a complete inhibition of biofilm formation by 0.12% of chlorhexidine (Fig. 3B) and 10 µg/ml of rifampicin (Figure 2C), compared to control (Fig. 3 A). The oral biofilm was not disrupted when rifampicin was added at 0.01 µg/ml as final concentration (Fig. 3D). In the case of NAC, It inhibited the biofilm formation only at 200 and 100 mg/ml (Fig. 4C and D). The biofilm was unaltered when NAC was added at 50 or 20 mg/ml (Fig. 4E and F). When rifampicin and NAC were mix (at final concentrations of 1µg/ml and 200 mg/ml, respectively) their antibiofilm potential was better. The mix was capable not just to inhibit the biofilm formation, even eradicated it after 4-8 hours post-inoculation (Fig. 5C and D). All together, these results are agreed with above describe in antimicrobial assays, suggesting antibiofilm activity of rifampicin/NAC is depend on antimicrobial activity.

3.4 Determination of Minimal Inhibitory Concentration of Rifampicin and NAC

In order to characterize the antimicrobial activity of rifampicin and NAC against oral bacteria, we determined that minimal inhibitory concentration of rifampicin and NAC. The results obtained for rifampicin were 1 and 0.25 µg/ml for inhibit *A.a* and *S. mutans* growth, respectively. In the case of NAC, 100 mg/ml were the minimal concentration required to interfere with *A.a* and *S. mutans* growth. These results are important data to know the minimal effective quantity of rifampicin and NAC that is required to inhibit oral bacterial growth.

4. Discussion

Rifampicin is a semisynthetic derivative from rifamycin B, produced by *Streptomyces mediterranei*[19] and constitutes the first election to treat active tuberculosis, leprosy, and other infectious diseases[20]. Their action mechanism consist in interfere with RNA synthesis of bacteria through their binding to β subunit of RNA polymerase dependent of DNA, causing the interruption of RNA translation. Actually, rifampicin is not employed to treat oral diseases, but due similarity between *M. tuberculosis* and periodontopathogen bacteria, it will be an interesting option.

NAC is considered a non-antibiotic drug that has antibacterial and antibiofilm properties. Since NAC was founded in 1977, [21] it has been reported that NAC has the ability to inhibit the growth of both gram-positive and gram-negative bacteria, including *S. aureus*, *P aeruginosa*, *K. pneumoniae* and *Enterobacter clocae*. NAC seems to be more effective against gram-negative bacteria, like *P. aeruginosa* requiring a MIC 2-20 µg/ml. The mechanism for the antibacterial effect of NAC may be acting as a competitive inhibitor against cysteine or, reacting with bacterial cell proteins [22].

Here we present evidence of bactericidal activity of rifampicin and NAC against oral microbes. Their efficacy in inhibiting the *A.a* and *S. mutans* growth was better than chlorhexidine. There are no previous reports of rifampicin inhibiting the oral microorganisms' growth. It has been reported that NAC inhibited the growth and biofilm formation of several microbes including the oral bacteria *Enterococcus faecalis*[15], associated with endodontic biofilms. The minimal concentrations of rifampicin to inhibit the bacterial growth were 1 and 0.25 µg/ml (against *A.a* and *S. mutans*, correspondingly), while the MIC of NAC was 100 mg/ml versus oral bacteria. These data are relevant to take it account if

rifampicin/NAC will be incorporated into a topic gel. These results indicate that rifampicin and NAC are antimicrobial agents as good as the most commonly used oral antiseptic. In this work it was obtained a MIC value of NAC 5 times higher to inactive the oral microorganisms growth in comparison with *Pseudomonas aeruginosa*[22]. May be oral microbes require more quantity of NAC to be inactivated due their bacterial wall rich in exopolysaccharides. Previously, it was reported that NAC has synergy with different antibiotics, like carbencillin and ciprofloxacin against *P. aeruginosa* growth[22, 23]. Here, we detected an interesting phenomenon including rifampicin plus NAC to inhibit and eradicate the oral biofilm.

In order to assess if rifampicin and NAC 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 rifampicin/NAC at the inoculation time. In presence of chlorexidine and rifampicin/NAC we just observed cellular debris identified by yellow-orange color 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. Rifampicin was incapable to detached biofilm of 4-16 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 rifampicin alone. Earlier studies have shown that NAC decrease biofilm formation and promote the disruption of mature biofilms. [11, 14] It was reported that NAC at 0.5mg/ml detached mature *Pseudomonas aeruginosa* biofilms[22] and at 50mg/ml effectively eradicated *Enterococcus faecalis* biofilm[15]. Our data are agreed with these previous reports, just differing in NAC concentration and supporting the bactericidal activity found by MTT assays. We detected an interesting phenomenon including rifampicin plus NAC to inhibit and eradicate the oral biofilm. This is important because a person could eliminate their dental plaque without use a gel containing rifampicin/NAC.

In this work we focused on the effectiveness of rifampicin/NAC in inhibiting the oral bacteria growth. All together, the experimental data suggest that rifampicin/NAC could be an interesting alternative to combat the bacterial infections at the origin of biofilms. The property of rifampicin/NAC could be used in oral health, supporting the antimicrobial and anti-biofilm activities of oral antiseptics.

5. Conclusion

The rifampicin in combination with NAC is an excellent therapeutic alternative for remove dental plaque and treatment of periodontal disease which promotes more favorable results in the clinical evaluation of patients.

6. Acknowledgements

The authors wish to thank Vilma Suarez from FO UANL, for activation and growing oral bacteria. Also, the authors appreciate the support of Salomé Elizondo-Rodríguez from FCB UANL for their technical assistance in fluorescence microscopy. René Hernandez-Delgadillo wishes to express their grateful to CONACyt for their scholarship. Finally, Claudio Cabral-Romero wants to thank to CONACyT for financing the project CB-2012/183825 and to UANL for the grant PAICYT-UANL-2012.

7. References

1. Costerton, J.W., Overview of microbial biofilms. J Ind Microbiol, 1995. 15(3): p. 137-40.
2. Costerton, J.W., Introduction to biofilm. Int J Antimicrob Agents, 1999. 11(3-4): p. 217-21; discussion 237-9.
3. Jenkinson, H.F., Adherence and accumulation of oral streptococci. Trends Microbiol, 1994. 2(6): p. 209-12.
4. Guntheroth, W.G., How important are dental procedures as a cause of infective endocarditis? Am J Cardiol, 1984. 54(7): p. 797-801.
5. 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.

6. Benakanakere, M. and D.F. Kinane, Innate cellular responses to the periodontal biofilm. Front Oral Biol, 2012. 15: p. 41-55.
7. 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.
8. 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.
9. 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.
10. Lucchesi, M., et al., [The therapeutic action of Rifampicin, a derivative of 3-(4-methyl-1-piperazinyl-iminomethyl)-rifamycin SV, in pulmonary tuberculosis]. Ann Ist Carlo Forlanini, 1967. 27(3): p. 199-227.
11. Marchese, A., et al., Effect of fosfomycin alone and in combination with N-acetylcysteine on E. coli biofilms. Int J Antimicrob Agents, 2003. 22 Suppl 2: p. 95-100.
12. Perez-Giraldo, C., et al., Influence of N-acetylcysteine on the formation of biofilm by Staphylococcus epidermidis. J Antimicrob Chemother, 1997. 39(5): p. 643-6.
13. Schwandt, L.Q., et al., Prevention of biofilm formation by dairy products and N-acetylcysteine on voice prostheses in an artificial throat. Acta Otolaryngol, 2004. 124(6): p. 726-31.
14. Olofsson, A.C., M. Hermansson, and H. Elwing, N-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces. Appl Environ Microbiol, 2003. 69(8): p. 4814-22.
15. Quah, S.Y., et al., N-acetylcysteine inhibits growth and eradicates biofilm of Enterococcus faecalis. J Endod, 2012. 38(1): p. 81-5.
16. Andrews, J.M., Determination of minimum inhibitory concentrations. J Antimicrob Chemother, 2001. 48 Suppl 1: p. 5-16.
17. 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.
18. Frey, T., Nucleic acid dyes for detection of apoptosis in live cells. Cytometry, 1995. 21(3): p. 265-74.
19. Stowe, C.D. and R.F. Jacobs, Treatment of tuberculous infection and disease in children: the North American perspective. Paediatr Drugs, 1999. 1(4): p. 299-312.
20. Pahkla, R., et al., Comparative bioavailability of three different preparations of rifampicin. J Clin Pharm Ther, 1999. 24(3): p. 219-25.
21. Parry, M.F. and H.C. Neu, Effect of N-acetylcysteine on antibiotic activity and bacterial growth in vitro. J Clin Microbiol, 1977. 5(1): p. 58-61.
22. Zhao, T. and Y. Liu, N-acetylcysteine inhibit biofilms produced by Pseudomonas aeruginosa. BMC Microbiol, 2010. 10: p. 140.
23. Roberts, D. and P. Cole, N-acetylcysteine potentiates the anti-pseudomonas activity of carbenicillin in vitro. J Infect, 1981. 3(4): p. 353-9.