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Ascorbic Acid On Oral Microbial Growth and Biofilm Formation

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Multiresistance between pathogen microorganisms is one of main problems in modern medicine worldwide. The infectious diseases caused by forming biofilm microbes are the most difficult to eradicate in humans. In oral cavity, the first stage to develop dental caries or periodontal disease is to get an oral biofilm that is called “dental plaque”. Ascorbic acid is anantioxidant agent synthesized by plants and most animals, been employed as adjuvant in the treatment of cancer. However, there are not reports about antimicrobial activity of ascorbate against bacteria.Within our results, ascorbate showed an important bactericidal activity since 20 mg/ml as final concentration against *Streptococcus mutans*, *Staphylococcus aureus*, *Porphyromonas gingivalis*, *Candida albicans* and *Enterococcus faecalis*. The MIC estimated for ascorbate was 10 mg/ml against oral bacteria and fungus. At 20 mg/ml, ascorbate was capable to inhibit the oral biofilm formation added at the inoculation time. As conclusion, ascorbate is a natural therapeutic alternative for prevent oral diseases.

Keyword: Ascorbate, antimicrobial activity,biofilm, Oral bacteria.

1. Introduction

The continue increasing of multi-resistance among pathogen microorganisms to anti-biotic 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. Several reports have showed the detection of oral bacteria resistant to different kinds of antibiotics. In Spain were found *F. nucleatum* resistant for penicillin, amoxicillin and metronidazole, *Prevotella intermedia* for tetracycline and amoxicillin, and *A. actinomycetemcomitans* for amoxicillin and azithromycin[2]. 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 the microorganisms in a co-operative form, which is called biofilm. The biofilms can form on all kind of surfaces and interfaces, including the human body[3]. The most common bio film is the dental plaque in oral cavity, being *Streptococcus mutans* the main etiological agent of dental caries world wide[4,5]. *S. mutans* has also been identified in endocard it is cases where they colonize endocardium and cardiac valves, probably due to for the inability to

adhere to solid surfaces and form biofilms[6]. Periodontal disease is the second most common sickness in oral cavity[7], being *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* the main etiological agents[8]. Other microbes that colonize oral cavity are *Candida albicans*, associated with oral candidiasis[9], *Enterococcus faecalis* related with endodontic infections[10] and *Staphylococcus aureus* present in dental plaque[11].

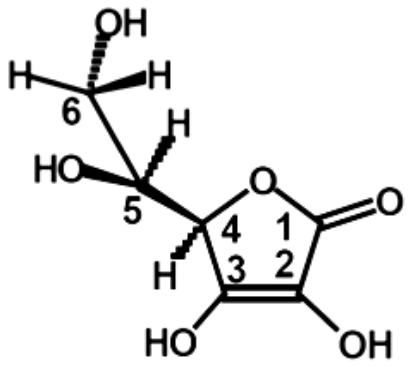
To prevent infectious diseases caused by microbes growing as biofilm, it is require non-antibiotics composites with antimicrobial and antibiofilm activities. Ascorbic acid (AS), also known as $AscH_2$, ascorbate or vitamin C is a water-soluble ketolactone with two ionizable hydroxyl groups (Figure 1)[12]. Plants and most animals synthesize ascorbate from glucose, taking place in liver in mammals. Humans, other primates, guinea-pigs and a few species of fruit-eating bats cannot synthesize AS because the gene encoding L-gulonolactone oxidase (GLO), the enzyme required for the last step in ascorbate synthesis, is not functional[13]. AS has been demonstrated to be an effective antioxidant, acting both directly, by reaction with aqueous peroxyl radicals, and indirectly, by restoring the antioxidant properties of fat-soluble vitamin E. The overall consequence of these antioxidant activities is the beneficial control of lipid peroxidation of cellular membranes including those surrounding as well as within intracellular organelles. AS is also used as adjuvant in the treatment of cancer. However, there are not previous reports about antimicrobial activity of ascorbate against bacteria, fungus or parasites.

The aim of this study was determine the bactericidal and antibiofilm effectiveness of ascorbate against oral bacteria. In this work, we present the first evidence of the antimicrobial and antibiofilm abilities of AS inhibiting the oral microbial growth and biofilm formation.

2. Material and Methods

2.1 Activation and Growing of Oral Bacteria

The oral microorganisms; *Streptococcus mutans, Porphyromonas gingivalis, Staphylococcus aureus, Enterococcus faecalis* and *Candida albicans* (ATCC number; 700611, BAA-308, 33592 and 11420 respectively) were grown in Tripticase Soy Broth (TSB, BD Biosciences, Franklin Lakes, NJ, USA) at 37 °C in aerobic conditions for 1-7 days. All cultures were prepared in the moment of their use and cell account was obtained employing a Neubauer chamber.



Ascorbic Acid
($AscH_2$)

Abbreviation: AS, ascorbic acid.

Fig 1: Chemical structure of acid ascorbic ($AscH_2$).

2.2 Preparation and Dilution of Ascorbic Acid

30 mg of AS were dissolved in 1 ml of TSB medium to get a stock solution of 30 mg/ml. Final concentrations of 1, 5, 10, 15 and 20 mg/ml were obtained through serial dilutions to explore the antimicrobial activity against oral microbes. Ascorbate (Fermont, Monterrey, N.L., Mexico) was mixed with TSB medium and vortex for two minutes. It was covered of light with aluminum foil. All solutions were prepared in the moment of their use.

2.3 Antimicrobial activity of Ascorbic Acid Against Oral Bacterial Growth

The antimicrobial effect of ascorbate on *S. mutans, P. gingivalis, S. aureus, E. faecalis* and *C. albicans* growth was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Biotium, Hayward, CA, USA)[14,15] according to the instructions of

manufacturer. Oral microorganisms were grown in TSB medium at 37°C, overnight in aerobic conditions. The microbes were counted using a Neubauer chamber and $1X10^4$ cells were in oculated in 100µl of TSB medium in 96 wells polystyrene plates. Three wells with only TSB medium were used as control of microorganism growth. 1.2 mg/ml of chlorhexidine (Ultra dent products, South Jordan, UT, USA) were used as an antimicrobial positive control. Several final concentrations of AS were employed to interfere with bacterial growth. The 96 wells plate was incubated at 37ºC in aerobic conditions for overnight. 10µl of MTT was added to each well, the plate was protected against light and incubated at 37°C for 2 hours. 200µl of Dimethylsulfoxide (DMSO) was added to dissolve the reduced MTT. The amount to five cells was determined by a Microplate Absorbance Reader (Bio-Rad Laboratories, Philadelphia, PA, USA) at 595nm. The experiment was repeated three times and the measured optical density were analyzed by descriptive statistics.

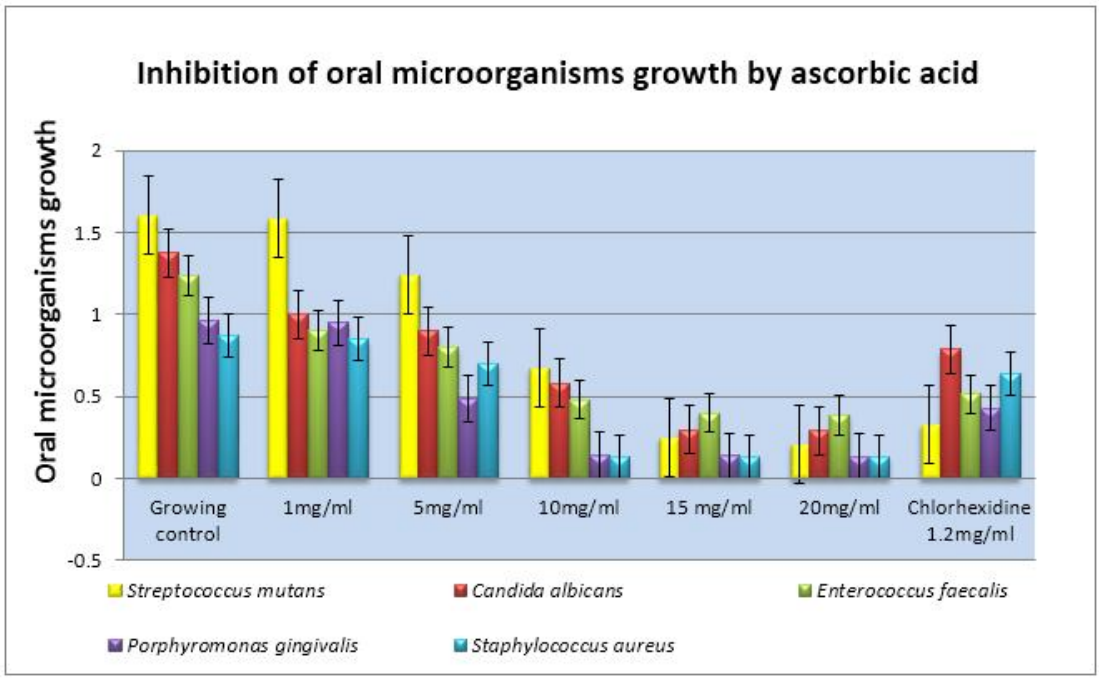


Fig 2: Antimicrobial activity of ascorbate against oral microbes. The Y axis shows the optical density units of microbial growth. As growing control of bacteria/fungus was added culture media and 1.2 mg/ml chlorhexidine was employed as positive inhibition control. Ascorbate was used at final concentrations of 1, 5, 10, 15 and 20 mg/ml. All experiments were done by triplicate to assess the veracity of results.

2.4 Antibiofilm activity of Ascorbic acid against oral biofilm

The antibiofilm activity of AS was determined by microscopy of fluorescence following the methodology described above. To observe the biofilm, the SYTO® 9 green fluorescent nucleic acid stain (Life Technologies, Carlsbad, CA, USA) was added at a final concentration of 20µM[15,16]. The 96 wells plate was incubated for 30 minutes at room temperature and protected against light. The oral biofilm was visualized at 485 nm with an inverter Carl Zeiss microscope (Carl Zeiss Meditec, Jena, Germany).

2.5 Determination of Minimal Inhibitory Concentration of Ascorbic Acid

The minimal inhibitory concentration (MIC) of AS was determined as previously described[17]. 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 5ml 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. Ascorbate was used to final concentrations of 1, 5, 10, 15 and 20mg/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 at 600 nm using a microplate absorbance reader (Biorad, Philadelphia, PA). The assay was done in triplicate and the measured optical density was further analyzed by descriptive statistics.

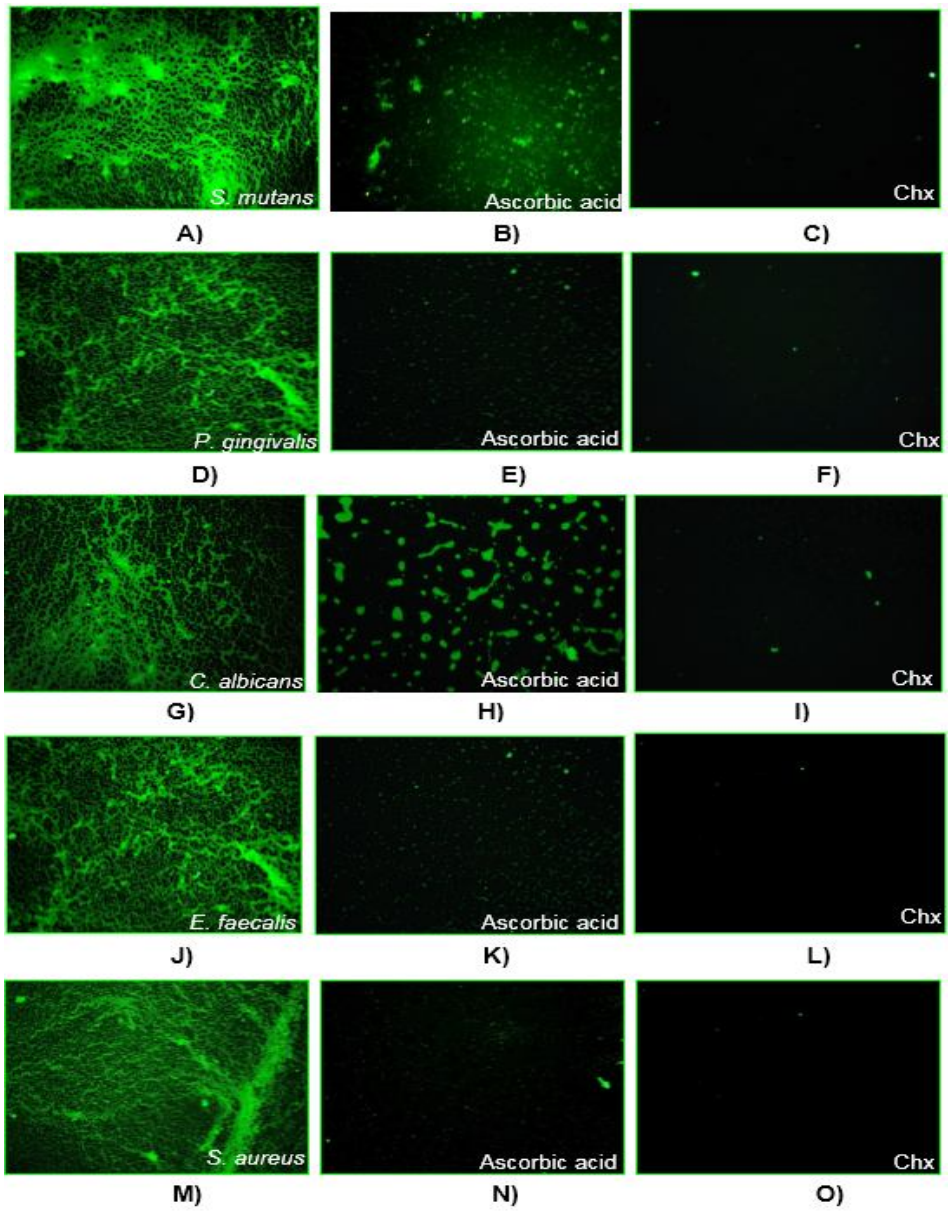


Fig 3: Inhibition of oral biofilm formation by ascorbate using fluorescence microscopy. As growing control of microorganisms was added culture media (A, D, G, J and M) and 1.2 mg/ml chlorhexidine was employed as positive inhibition control (C, F, I, L and O). Ascorbate was used at a final concentration of 15 mg/ml (B, E, H, K and N). Both chlorhexidine and ascorbate were added at inoculation time. All experiments were done by triplicate to assess the veracity of results.

3. Results

3.1 Antimicrobial activity of Ascorbic acid against oral microbes

To explore the antimicrobial activity of ascorbate against oral microorganisms, their effect under *S. mutans, P. gingivalis, S. aureus, E. faecalis* and *C. albicans* growth was determined. The results showed that ascorbate was capable to inhibit the microbe's growth since 10 mg/ml. 20 mg/ml of AS reduced the number of microorganisms by 90%, in comparison to microbes grown in medium alone (Figure 2). Similarly, the treatment with 1.2 mg/ml chlorhexidine (inhibition control) showed an 80% of reduction in the number of bacteria, when compared with non-treated cells (Figure 2). There was not big difference in the inhibitory effect of AS against *C. albicans*, suggesting their antimycotical activity was good as bactericidal one. Interestingly, the antibacterial activity of ascorbate was higher against *S. aureus* and *P. gingivalis*, one of multiresistant bacteria worldwide and main etiological agent of periodontitis, respectively. These data suggest that AS was as an effective as chlorhexidine to inhibit the oral microbial growth.

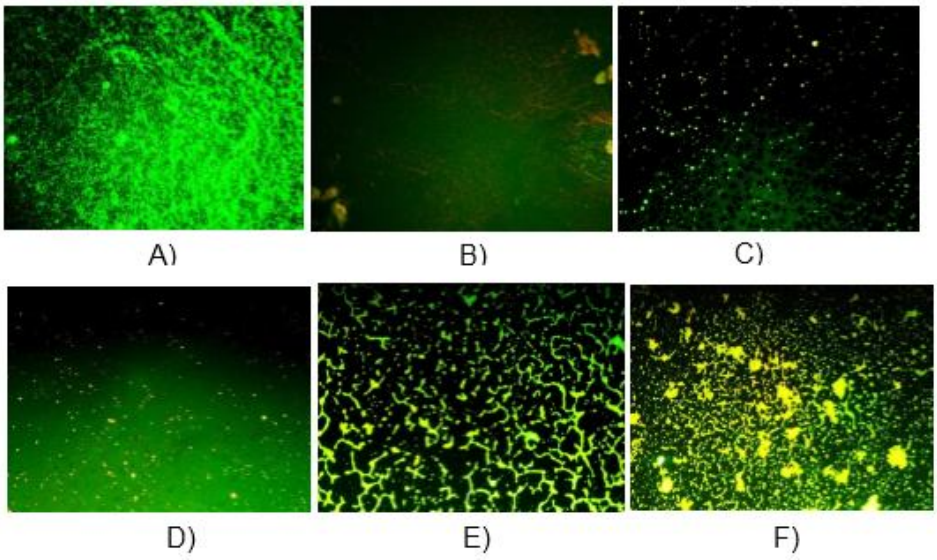


Fig 4: Inhibition of oral biofilm formation by ascorbate at different post-inoculation times, using fluorescence microscopy. A) As growing control of microbes was added culture media and B) 1.2 mg/ml chlorhexidine was employed as positive inhibition control. C), D), E) and F) correspond to AMB that it 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.

3.2 Biofilm inhibitory activity of Ascorbic acid

Once determined the antimicrobial effectiveness of AS, we analyze their possible antibiofilm activity against oral microbes. This ability was evaluated by fluorescence microscopy. The results showed a complete inhibition of biofilm formation by chlorhexidine (Fig.3 C, F, I, L and O) and ascorbate (Figure 3B, E, H, K and N), compared to control (Fig. 3A, D, G, J and M). These results correlate with previous data obtained by MTT assay, indicating that at 15 mg/ml AS inhibit the oral biofilm formation.

3.3 Determination of minimal inhibitory concentration of Ascorbic acid

In order to get a more detail characterization of the antimicrobial activity of AS against oral microorganisms, we determined their minimal inhibitory concentration. The result obtained for ascorbate was 10mg/ml for inhibit oral microbial growth. This result is a relevant datum to know

the minimal effective quantity of AS that is required to interfere with oral microbial growth.

4. Discussion

Ascorbic acid (ascorbate or vitamin C) is a natural antioxidant agent that is synthetized by plants and most animals except humans, primates and guinea pigs[13]. The typical concentration of AS in plasma of healthy humans is 40-80 µM, acting with vitamin E as co-antioxidant to protect Low Density Lipoproteins (LDL) from damage induced by aqueous peroxyl radicals[18]. In humans and animal tissues, the highest concentrations of AS are in the adrenal and pituitary glands[19,20]. The use of high-dose AS in treating cancer patients began in the 1970s. These early studies demonstrated beneficial effects of high-dose AS[21,22], however other reports suggest that ascorbate did not show any benefit[23,24]. The controversy was due to way of administration, only intravenous administration of ascorbate can yield high plasma levels, i.e. pharmacological levels.

Here we present evidence of biocidal activity of ascorbate against oral microorganisms. Their efficacy in inhibiting *S. mutans, S. aureus, P. gingivalis, C. albicans and E. faecalis* growth was better than chlorhexidine. Interestingly, the antibacterial activity of AS was higher against *S. aureus* and *P. gingivalis*, one of multiresistant bacteria worldwide and main etiological agent of periodontitis, respectively. The antimycotical activity of AS against *C. albicans* was very similar to bactericidal activity one, suggesting their action mechanism could be the same to kill bacteria and fungus. The minimal concentration of AS to inhibit the microbial growth was 10 mg/ml. This datum is relevant to take it account if AS will be incorporated into a mouthwash or tooth paste. These results indicate that AS is antimicrobial agent as good as the most commonly used oral antiseptic. We did not found any previous reports about bactericidal activity of AS to compare our results.

In order to assess if ascorbate had the potential to interfere or remove the oral biofilm formation, their antibiofilm activity was studied. Surprisingly, the effect antibiofilm formation was total against all microbes analyzed using AS at the inoculation time. In presence of chlorexidine and ascorbate we just observed cellular debris on a dark background; mainly DNA of dead microorganism with accumulates of dye, supporting the data previously obtained by MTT assays. Morphologically, these dye accumulates clearly differ from bacterial biofilm. Ascorbate was incapable to detached biofilm of 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 antibiofilm activity of AS against any bacteria. We do not know how exactly AS kill the microorganisms, but we hypothesize ascorbate penetrate in cells and alter their oxido-reduction reactions through their antioxidant properties.

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

5. Conclusion

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

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