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Antimicrobial activity of *Melaleuca alternifolia* nanoparticles in polymicrobial biofilm in situ

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ABSTRACT

Microbial biofilms represent a challenge in the treatment of infections, due to the low efficacy of the antimicrobials. This study evaluated the antimicrobial effect of nanoparticles of *Melaleuca alternifolia* (TTO) in dental biofilm. Thirty-eight volunteers used an oral device in situ in situ including four bovine enamel specimens for 07 days. From the fifth day four solutions were applied randomly for each specimen: Physiological Saline Solution (0.85% NaCl) (C+), Chlorhexidine 0.12% (CHX), *M. alternifolia* oil 0.3% (TTO), and a nanoparticle solution of 0.3% *M. alternifolia* oil (NPTTO). The nanoparticles of TTO were characterized for pH, IPD, medium size, zeta potential and Transmission Electron Microscopy. Antimicrobial activity was evaluated by viable microorganisms count and the structure of the biofilm by atomic force microscopy. The NPTTO presented pH 6.4, particle diameter of 197.9 ± 1 nm, polydispersion index of 0.242 ± 0.005, zeta potential of −7.12 mV and ± 0:27 spherical shape. The C+ resulted in 100% of bacterial vitality, while CHX, TTO and NPTTO showed 34.2%, 51.4% and 25.8%, respectively. The AFM images showed biofilms with an average roughness of 350 nm for C+, 275 nm for CHX, 500 nm for TTO and 100 nm for NPTTO. The NPTTO demonstrated excellent antimicrobial activity in the biofilm formed in situ and will possibly be used in future for the treatment/prevention of oral biofilms.

1. Introduction

Infections related to biofilms may occur on abiotic materials (catheters, prostheses, heart valves, endotracheal tubes and many others) and in host tissues (e.x. in epithelium, endothelium, mucosal surfaces and teeth) [1,2]. They were associated with significant morbidity and mortality [3,4].

The oral cavity has hundreds of bacterial species, incorporated in an extracellular matrix rich in polysaccharides, forming oral biofilms of multiple species. The formation of oral biofilm can be influenced by several environmental factors and hosts, as host immunity, pH, enzymes, saliva and antibiotics [5,6] When biofilms develop in the oral cavity they can cause diseases such as caries, periodontal diseases, root canal infections and peri implant diseases, affect bacterial virulence and in immunocompromised patients can lead to other potentially fatal systemic diseases [7–9].

Abbreviations: EPS, exopolysaccharides; TTO, *Melaleuca alternifolia*; NPTTO, *M. alternifolia* oil nanoparticles; CHX, chlorhexidine; PDI, polydispersity index; SLN, solid lipid nanoparticles; AFM, Atomic force microscopy

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The patients cooperation in the daily destructuring of dental biofilm is a challenge because many individuals are unable or unmotivated to perform this procedure with the necessary regularity and efficiency. Thus, the use of chemicals can be considered as part of oral hygiene to assist the mechanical control of dental biofilm [10].

Among the agents used to control dental biofilm, chlorhexidine (CHX) is considered the gold standard [11,12]. However, its action is diminished after 24 h in the more internal layers of biofilm (which limits its clinical efficacy in controlling gingival inflammation) [13], besides presenting adverse effects such as tooth staining and significant changes in taste [12,14]. In this context, *Melaleuca alternifolia* (TTO) has shown a broad spectrum of action and significant antimicrobial activity [15]. On the other hand, this essential oil has presented problems involving its physical properties including low miscibility in water and a high rate of volatilization, resulting in less bioavailability and low stability [16].

In the last decades, the nanostructuring of some products of natural origin has been one of the alternatives applied to solve the low solubility problems in aqueous media, thus avoiding the degradation of the drug when in contact with biological fluids. It also promotes the possibility of a sustained release of drug/product, reduces toxicity and increases antimicrobial activity [17–20].

Due to the composition of bacteria with a high degree of flexibility genomics (which have the capacity to express various phenotypes that increase intrinsic resistance to antibiotics), dental biofilm consists of an important model to evaluate the effect of new presentations of antimicrobial agents. In vitro dental biofilms contributed significantly to oral disease studies, but present limitations due to the diversity of their species and to the complex environment of the oral cavity. Therefore the in vitro results should be interpreted with caution [21,22]. Thus, it is of paramount importance that studies be performed on in situ models so that they can be analyzed *ex vivo* without distortion of the results. In this context, the objective of this study was to evaluate for the first time the antibiofilm effect of TTO oil and NPTTO in human dental biofilms using an in situ model.

2. Materials and methods

2.1. Preparation and characterization of M. alternifolia nanoparticles

The *M. alternifolia* oil was commercially obtained from Importadora Chemical Delaware® Ltda, Brazil and the solid lipid nanoparticles (SLN) containing the *M. alternifolia* oil were donated by the INVENTIVA® company. The NLS were prepared with 7.5% of oil using a method based on high pressure homogenization. Cetyl palmitate was used as a solid lipid and Polysorbate 80 as surfactant. The total solids concentration was 18.6%.

After the preparation, the nanoparticles were characterized as to particle size, polydispersity index (PDI) and zeta potential using Zetasizer Nano-ZS ZEN 3600 (Malvern, England) and the pH was determined using a potentiometer (Digimed®) calibrated (with standard solutions of pH 4.0 and 7.0). Each measurement was performed in three replicates. The morphology of the nanoparticles was analyzed by transmission electron microscopy operating at 80 kV (TEM; Jeol, JEM 1200 Exll, Japan). Diluted suspensions (1:10 v/v in water) were deposited on a specimen grid (Formvar-Carbon support films) negatively stained with uranyl acetate solution (2% w/v) and observed at different magnifications.

2.2. Preparation and use of the device in situ

Intraoral devices with niches for the insertion of test specimens (Fig. 1A) were made in plaster models of the upper arch of each participant. A numbered identification (1–4) was placed beside each niche for the subsequent implementation of solutions. This identification was visible throughout the experimental period. Specimens measuring 5 mm × 5 mm x 1 mm thick were prepared from bovine incisor teeth. The measurements were checked with a digital caliper (Jomarca®) and later the specimens were autoclaved. Four specimens were fixed with cyanoacrylate glue (Super Bonder®) in device niches, and freely released into the oral environment (Fig. 1A) After fixation, randomization was performed to choose the solution to be applied to each specimen. According to randomization, a sticker was glued to the solution indicating the niche number to which this solution should be applied.

2.3. Test products

The following solutions were tested: 0.12% chlorhexidine (Periogard® Colgate, Brazil), *M. alternifolia* oil 0.3%, nanoparticles containing *M. alternifolia* oil and 0.3% saline (NaCl solution 0.85%) as a positive control for biofilm growth. The four solutions were delivered to participants in different numbered glass bottles (1–4), with a screw cap with eyedropper for application. Further, a toothpaste without active ingredient and fluorine (carbopol gel 6%) and a container with saline were given to the participants.

2.4. Sample calculation

The number of test specimens for the application of each agent was determined from a pilot, study where there was an average difference of 2500 CFUs with a standard deviation of 1700 CFUs (between control and CHX) for each agent. Considering this mean difference, β 90%, α 5% and a paired design were estimated for each agent 34 specimens. Given some dropouts, 38 volunteers were included.

2.5. Design and study

We used an in situ delineation with subsequent in vitro microbiological analysis intraoral biofilm. The sample consisted of 38 individuals of both genders (21.94 ± 2.17 years), students from the Centro Universitário Franciscano, Santa Maria, Brazil. The participants were in good general health, had not used any antimicrobial/anti-inflammatory for at least three months were not oral breathers, did not have periodontitis (17), did not smoke and did not have any kind of allergy to 2 components of the substances. The research project was approved by the Ethics Committee (CEP) of the Centro Universitário Franciscano under number 23379414.0.0000.5306.

2.6. Experimental procedures

Two days before the beginning of the experimental period, volunteers began using a toothpaste active compound without fluorine. At baseline, the devices were inserted and maintained by the volunteers for 07 days. It was recommended that during these seven days the device be removed only for eating, oral hygiene and application of solutions, at such times being kept immersed in saline. No solution was applied to the specimens for the first four days, so that biofilm would form and mature. From the fifth day on it was requested that 01 drop of each solution (1–4 bottles) be applied to the respective specimens every 12 h. The experimental design is summarized in the experimental flowchart shown in Fig. 1B. After 07 days of use, the devices were removed from the oral cavity and analyzed microbiologically.

In order to analyze bacterial viability, the specimens were removed from the devices in situ and immediately transferred to eppendorf microvials containing 1 mL of saline and kept in a sonicator for 15 min (time previously determined in a pilot test, data not shown) to release the bacteria attached to the enamel surface. The samples were homogenized by vortexing (3000 rpm) for 30 s and diluted to 1: 100 and 1:1.000 in sterile saline. The method used to plate 10 μL aliquots was to spread them on Mueller Hinton agar (Merck®). The Petri plates were incubated aerobically for 24 h at 37 °C and the colonies were counted after 24-h incubation. The results were expressed in percentage