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Microrganism Collected in Periodontal Pocket Inactivation through Photodynamic Therapy: Pilot Project of an *In vitro* Study

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Abstract

This pilot study evaluated *in vitro* whether photodynamic therapy and the application of methylene blue are effective methods for eliminating microorganisms collected in periodontal pockets deeper than 5mm and cultivated in a petri dish. The study design consisted in the division of control group (G1) and two test groups (G2 and G3). Material resulting from subgingival scraping was collected in a region were periodontal pocket range from 5 to 7 mm in 1 patient, and this material was used to make a smear in a previously prepared culture medium, using a flamed bacteriological loop, inside a laminar flow hood. The growth of the cultures was monitored in a 36°C incubator during 72 hours and after that period, tests of application of methylene blue 0.005% were performed in G2 and methylene blue 0.005% associated with photodynamic therapy in G3. 48 hours after this intervention, the cell colonies were removed from the culture medium using a bacteriological loop and applied to a glass plate, to perform the Gram staining method. The results indicated that there was no visible reduction in the size of cell cultures in G2 in relation to the control (G1), but there was a visible reduction in G3 in relation to the other groups. Microscopic analysis of gram staining indicated a significant reduction in the count of gram positive and negative bacteria in G3, but the same did not occur in G2 compared to G1. This pilot study demonstrated that photodynamic therapy is effective in inactivating microorganisms cultivated *in vitro* and that methylene blue at 0.005% concentration do not have enough antimicrobial effect to represent visible change in electron microscopy at 10X magnification.

Key words: Pilot study • Methylene blue • Photodynamic therapy

Introduction

According to the May 2014 publication of the World Health Organization (WHO), bacterial resistance to antibiotics is a current public health problem. Given this context, it is necessary to search for new adjuvant therapies that aim to complement conventional pharmacological treatments, without compromising the effectiveness in eliminating microorganisms and collaborating to mitigate the negative impacts on the patient undergoing treatment. Photodynamic Therapy (PDT) is presented. Therapy (PDT), which has as its mechanism the association of various Photosensitizers (PS) and low power laser with specific wavelengths in the presence of oxygen, which culminates in the formation of reactive oxygen species that are capable of causing damage to the cellular structures and promote microbial death. Studies conclude that PDT is a non-invasive, safe and agile treatment alternative, being conducive to fighting bacterial infections. Furthermore, it is applied topically at the site of infection and does not cause bacterial resistance [1].

Studies show that bacterial resistance consists of the multiplication of microorganisms (OM) that, in the presence of antimicrobials in higher concentrations than in therapeutic administrations, are resistant. This fact characterizes a "selective pressure", which, due to natural selection, survives the fittest microorganisms or those that present resistance genes. These genes can be propagated to other MOs of the same species, or even to different species, promoting the increase and development of pathogens in the presence of substances, which would commonly be sensitive.

As a result of this event, microbial resistance is currently a global public health problem and numerous factors are contributing to its spread, such as: the indiscriminate use of antibiotics by the population, improper prescriptions, incorrect dosage and posology, as well as treatments based on in the initial use of antimicrobials that fight the most likely agents of infection. Faced with this circumstance, it is necessary to disseminate the use of supplementary therapies to antibiotic therapy in order to combat microbial resistance, among which PDT stands out. The treatment of diseases through light, known as phototherapy, was the object of study by Niels Finsen, who approached the therapeutic resource in different ways and for this reason, the scholar won the Nobel Prize in 1901 [2]. In the early days, PDT was used as a form of treatment for human tumors through the topical application of a dye and the lighting of a lamp. Subsequently, the photosensitizer began to be administered systemically associated with the laser, as it is a more punctual light source, and possible to be used with the ideal wavelength to stimulate the photosensitizer [3].

PDT consists of the topical application of a photosensitizer, which will be subjected to a light source with a certain wavelength in the presence of oxygen, culminating in the production of reactive oxygen species leading to oxidative stress and cytotoxicity of microorganisms [4]. The photosensitizer enters the cell when excited by the specific laser wavelength compatible with its excitatory spectrum and absorbs the radiant energy to reach an excited singlet state, starting from its fundamental singlet condition of lowest energy. Furthermore, it obtains an extended triplet excited state, which is the active form of the photosensitizer. In the presence of oxygen, the photosensitizer in a lower-energy fundamental singlet state relates to the surrounding biomolecules through two types of photo-oxidative mechanisms: type I and type II. The type I mechanism involves the transfer of an electron or a hydrogen atom, generating intermediate radicals that interact with oxygen to produce hydroxyl radicals, peroxides and superoxides, initiating chain reactions of free radicals. Hence, the type II mechanism where the photosensitizer in the lowest energy ground state singlet oxygen transfers energy to the ground state (triplet state) oxygen molecules to give the higher energy level singlet oxygen with strong oxidizing properties [5]. Complementary techniques such as PDT, in addition to tests that assess the bacteriostatic or

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bactericidal potential of methylene blue, become evident. Thus, this pilot study was developed, which aims to evaluate *in vitro* whether photodynamic therapy and the application of methylene blue are effective methods for eliminating microorganisms collected in periodontal pockets deeper than 5mm and cultivated in Petri dish.

Materials and Methods

This study was submited and approved on clinical ethics committee of Instituto Cultural Newton Paiva Ferreira LTDA under the registration number CAAE: 68617423.2.0000.5097. *In vitro* observational study was performed in 3 groups. Control group (G1), cell culture did not receive any intervention. In test group 1 (G2) the cell culture received 10 IU (international units) of Methylene Blue 0.005% (Quimiolux, DMC, Brazil). The cell culture in test group 3 (G3) received 10 IU (international units) of Methylene Blue 0.005% (Quimiolux, DMC, Brazil) and after 3 minutes photodynamic therapy was applied with an MMOptics device (LASER Duo, MMO, São Carlos, São Paulo, Brazil), following the decontamination protocol recommended by the manufacturer in the L1 function for 180 seconds (18J of energy).

The sample size for this pilot study consisted of 1 patient, who agreed to the terms of the free and informed consent form. Material resulting from subgingival scraping was collected in a region with a periodontal pocket ranging from 5 to 7mm, using Gracey curettes 11-12, 13-14 and 7-8. Upon macroscopic examination, the material obtained after scraping consisted of a mixture of softened biofilm, calcified biofilm and blood, which was immediately conditioned inside a previously sterilized Eppendorf -type tube. The Eppendorf containing the material collected from the periodontal pocket was immediately placed in a refrigerated thermal container and taken to the microbiology laboratory of the Instituto Cultural Newton Paiva Ferreira, where it was used to make a smear in a previously prepared culture medium, using a flamed bacteriological loop, inside the flow chapel. For the development of culture media, 2 Petri dishes containing BHI broth were used. One of the plates was divided into two halves, one half being used for G1 and the other half for G2. Test group 2 (G3) was cultivated in a separate Petri dish to prevent possible light source dispersion from interfering with G1 and G2. The growth of the cultures in the greenhouse was observed for 72 hours before the beginning of the tests. After this period, the proposed therapies were applied and 48 hours after the intervention, the cell colonies were removed from the culture medium with the aid of a bacteriological loop and applied to a glass plate, to perform the Gram staining method.

Results

After 48 hours of carrying out the tests, there was no visible reduction in the size of cell cultures in G2 in relation to the control (G1), but there was a visible reduction in G3 in relation to the other groups. The result of the gram staining test was evaluated in an electronic microscope at 10X magnification and also did not indicate differences between G1 and G2, both of which presented gram- positive cocci and bacilli and gram- negative bacilli, surrounded by a film of extracellular biofilm matrix. As for G3, few cells remained adhered to the glass plate during the Gram staining method, indicating that the material removed from the culture medium probably did not have viable cells to the point of maintaining active primary adhesion, co-adhesion and co-aggregation stages of the biofilm and consequently, the extracellular matrix.

Discussion

Methylene blue is a phenothiazine, which is a heterocyclic aromatic chemical complex, appearing as a solid green powder, darkened and odorless at room temperature, however, when dissolved in water, it becomes a blue solution. It is one of the most used photosensitizers and has beneficial characteristics for use in PDT, such as, for example, too much absorption in the 660mm extension, little toxicity, high solubility in water and for these reasons it is the photosensitizer of choice in several studies, as well as in this pilot project.

According to the study presented by Júnior ECF, et al. [6], which states that this dye has bactericidal properties (promotes bacterial death) on gram-negative and gram-positive bacteria, as a result of its ability to penetrate the outer membrane of the bacteria through interaction with proteins surface exerting its function of bacterial inactivation. Furthermore, it also mentions that factors related to methylene blue, such as concentration and diluent, are of great value for its bactericidal action, a fact that may explain the lack of efficiency of the photosensitizer in question used alone in the present study in relation to the group control, both in the macroscopic aspect and in the microscopic analysis.

Conclusion

In the present study, PDT is considered a promissory method to eliminate periodontal pockets microorganisms. In addition, it is a potential adjuvant in dental treatments, with the aim of mitigating the factors that contribute to the increase in bacterial resistance and indiscriminate use of antibiotics. This pilot study demonstrated that photodynamic therapy is effective in inactivating microorganisms cultivated *in vitro* and that methylene blue at 0.005% concentration do not have enough antimicrobial effect to represent visible change in electron microscopy at 10X magnification.

Acknowledgement

None.

Conflict of Interest

None.

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