

Action of antimicrobial photodynamic therapy with red leds in microorganisms related to halitose

Controlled and randomized clinical trial

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Abstract

Introduction: Halitosis is the term used to describe any unpleasant odor relative to expired air regardless of its source. The prevalence of halitosis in the population is approximately 30%, of which 80 to 90% of the cases originate in the oral cavity resulting from proteolytic degradation by gram negative anaerobic bacteria. Antimicrobial photodynamic therapy (aPDT) has been widely used with very satisfactory results in the health sciences. It involves the use of a non-toxic dye, called photosensitizer (FS), and a light source of a specific wavelength in the presence of the environmental oxygen. This interaction is capable of creating toxic species that generate cell death. The objective of this controlled clinical study is to verify the effect of aPDT in the treatment of halitosis by evaluating the formation of volatile sulphur compounds with gas chromatography and microbiological analysis before and after treatment.

Materials and Methods: Young adults in the age group between 18 and 25 years with diagnosis of halitosis will be included in this research. The selected subjects will be divided into 3 groups: G1: aPDT; G2: scraper, and G3: aPDT and scraper. All subjects will be submitted to microbiological analysis and evaluation with Oral Chroma™ before, immediately after treatment, 7, 14, and 30 days after treatment. For the evaluation of the association of the categorical variables the Chi-square test and Fisher's Exact Test will be used. To compare the means the student *t* test and analysis of variance (ANOVA) will be used and to analyse the correlation between the continuous variables the correlation test by Pearson will be applied. In the analyses of the experimental differences in each group the Wilcoxon test will be used. For all analyses a level of significance of 95% ($P < .05$) will be considered.

Discussion: Halitosis treatment is a topic that still needs attention. The results of this trial could support decision-making by clinicians regarding aPDT using aPDT for treating halitosis.

Ethics committee: The Ethics Committee of University of Nove de Julho (UNINOVE) approved this project under process number 76402417.2.0000.5511, in accordance with the guidelines of the National Ethics Committee (CONEP). Any modifications to the protocol that may have an impact on the conduct of the study will be reported to the committee. An informed consent form (which was approved by the UNINOVE Ethics Committee) will be signed by the volunteers previous to their participation.

Number of clinical trials.gov NCT03656419

Data collection methods: The authors were previously trained to collect data and perform the evaluations. All authors are qualified in laser therapy. The same researchers will enroll patients and assign them to interventions. All data will be entered electronically. The participants' files will be stored in numerical order in a safe place and accessible only to the authors of this study.

Discontinuing intervention: If volunteers become ill or do not adapt to therapy, it will not be possible to continue laser therapy. Interventions will be made while the participants are at the university, to make it easier for them to come, avoiding absences. No adverse effects are expected.

Availability of data and materials: The datasets generated and analysed during the present study are available from the corresponding author at reasonable request. After the analysis of the data, volunteers will be invited to a meeting and the results will be shared and they will become public.

Funding: Fundings received: Fapesp – 2017/19983–5; CnpQ – 306324/2017–2

The authors have no conflicts of interest to disclose.

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Medicine (2019) 98:1(e13939)

Received: 6 December 2018 / Accepted: 10 December 2018

<http://dx.doi.org/10.1097/MD.0000000000013939>

Abbreviations: aPDT = antimicrobial photodynamic therapy, CH₃SCH₃ = dimethyl sulphide, CH₃SH = methylmercaptanes, FS = photosensitizer, H₂S = hydrogen sulfide, ppb = parts per billion, VSCs = volatile sulfur compounds.

Keywords: antimicrobial photodynamic therapy, halitosis, qPCR

1. Introduction

Halitosis is the term used to describe any unpleasant odor related to expired air regardless of its origin.^[1] The chemical components related to halitosis are volatile sulfur compounds (VSCs) such as hydrogen sulfide (H₂S), methylmercaptanes (CH₃SH), and dimethyl sulphide (CH₃SCH₃).^[2–5] The prevalence of halitosis in the population is approximately 30%, of which 80 to 90% of the cases originate in the oral cavity resulting from proteolytic degradation by gram negative anaerobic bacteria from sulfur-containing substrates in saliva, epithelial cells, blood, and food debris.^[6]

The dorsum of the tongue and periodontal pockets are related as the major niches of bacteria that are responsible for the emission of VSCs. Some methods are used to diagnose halitosis: the organoleptic method, which is a subjective evaluation of the exhaled air of the mouth and nose, and a scale, is used to quantify this odor, depending greatly on the olfactory capacity of the evaluator. Sulphide monitors are devices that quantify the total value of VSC exhaled from the oral cavity. Gas chromatography is the most appropriate method to detect halitosis of different origins because it performs individual measurement of the three main gases (sulfhydryde, methylmercaptanes, and dimethyl sulphide), allowing to assess the intensity of the breath and its origin. However, the lack of standardization in the protocol for diagnosis and treatment of halitosis makes it difficult to compare the epidemiological data obtained in different countries.^[7,8]

Currently the treatment of halitosis is related to the masking of the odor or the decrease of the number of bacteria, through chemical and mechanical removal with the use of tongue scrapers and mouthwashes.^[5,9–13] Antimicrobial photodynamic therapy (aPDT) has been widely used with very satisfactory results in the health sciences. It involves the use of a non-toxic dye, called photosensitizer (FS), and a light source of a specific wavelength in the presence of the environmental oxygen. This interaction can create reactive oxygen species (ROC) that generate cell death.^[14–17] The advantages of this approach are to avoid resistance to target bacteria and damage to adjacent tissues as the antimicrobial effect is confined only to the areas covered by the photosensitizer and irradiated by light acting on the target organism rapidly, depending on the dose of light energy and power output. In addition, according to Wainwright, M.^[18] bacterial resistance to aPDT is unlikely, since the singlet oxygen and free radicals formed interact with various bacterial cell structures and different metabolic pathways.

Halitosis is considered one of the problems related to the impact of oral health on quality of life and is enough to affect the individual's perception about his life.^[19] Therefore, there is a need for new treatment alternatives for the patient, especially in the face of the technological advances in dentistry, so the patient's expectation regarding the final results can be met. The present study proposes to conduct a controlled clinical trial to evaluate the effectiveness of the application of aPDT in the tongue coating as a new way to control halitosis, mainly because it is a fast, non-invasive procedure and does not generate bacterial resistance.

2. Materials and methods

The study will follow the regulatory norms of research in humans with favourable opinion of the Research Ethics Committee of the University Nove de Julho number, and the participants will sign the informed consent form after clarifications for authorization of the participation in the research, according to Resolution 466/12 of the National Health Council. Type of study: controlled, quantitative, cross-sectional clinical study.

2.1. Hypothesis

Null hypothesis: There is no alteration of halitosis after the use of photodynamic therapy employing the use of blue dye and red LED. There is no microbiological alteration after antimicrobial photodynamic therapy. Experimental hypothesis: there is a decrease in halitosis after the use of photodynamic therapy using blue dye and red LED associated or not with the tongue scraper. There is microbiological alteration after antimicrobial photodynamic therapy.

2.2. Research subjects

For this study, young adults of both sexes, students and employees of the *Universidade Nove de Julho*, São Paulo will be evaluated.

2.3. Inclusion criteria

Young adults between the ages of 18 and 25, with an informed consent form and authorization for the diagnosis and treatment of halitosis. Young adults diagnosed with halitosis presenting Oralchroma S2H \geq 112 ppb and/or CH₃SH \geq 26.

2.4. Exclusion criteria

Individuals will be excluded from the study:

- With dentofacial anomalies, in orthodontic and/or orthopedic treatment,
- Using a removable device, implant and/or prosthesis,
- With periodontal disease,
- With carious lesions,
- In cancer treatment,
- On antibiotic treatment up to 1 month before the survey,
- Pregnant,
- With hypersensitivity to the photosensitizer to be used.

As the research is a randomized clinical study and seeking greater transparency and quality of this research, we will use the CONSORT (Consolidated Standards of Reporting Trials) recommendations (Fig. 1). The protocol is in accordance with the 2013 Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) Statement.

The selected subjects will be divided by block randomization into 3 groups (n per group = 13), according to the treatment to be performed. Opaque envelopes will be identified, and a sheet

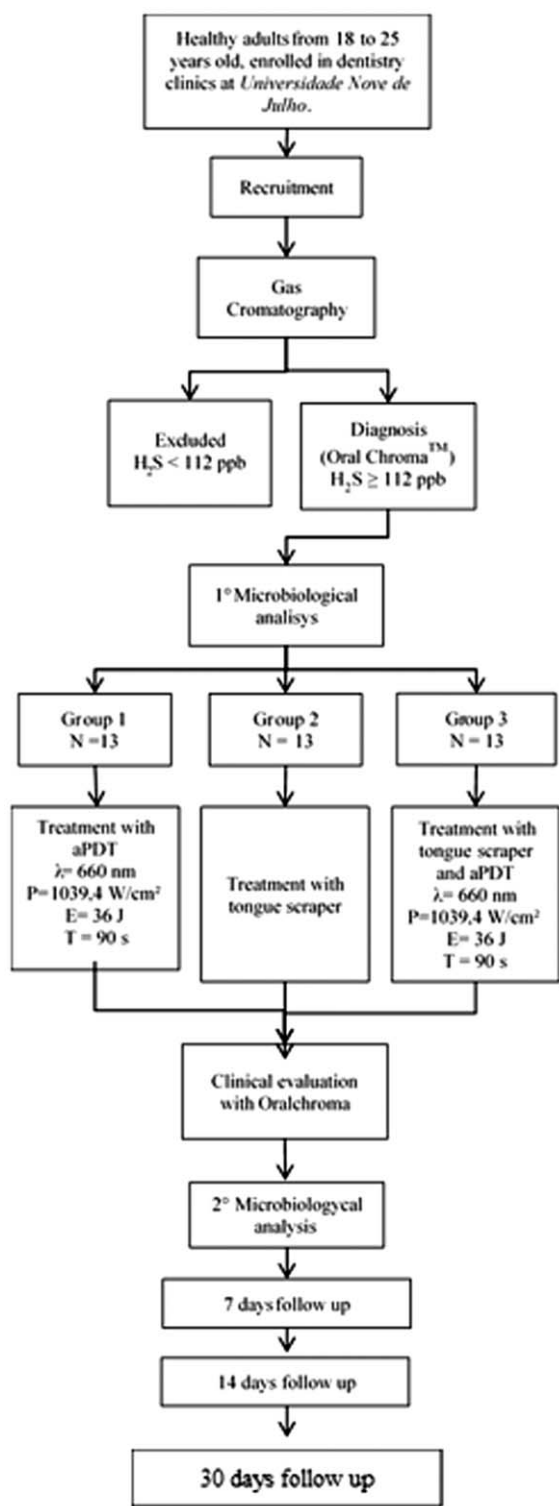


Figure 1. Flowchart.

containing the information of the corresponding experimental group will be inserted. There will be a 1 session for each group (Table 1). All samples from the subjects will be submitted to microbiological analysis and evaluation with Oral Chroma™ before and after treatment, followed by controls of 7, 14, and 30 days.

Table 1

Experimental condition.

Group	Halitosis	Treatment (1 section of 30 minutes)
1	SH2 ≥ 112 ppb CH ₃ SH ≥ 26 ppb	aPDT 4 points E = 36 J T = 90 s /point
2	SH2 ≥ 112 ppb CH ₃ SH ≥ 26 ppb	Tongue scraper - 10 scrapes in the tongue dorsum (Halitus)
3	SH2 ≥ 112 ppb CH ₃ SH ≥ 26 ppb	Tongue scraper - 10 scrapes in the tongue dorsum and aPDT 4 points E = 36 J T = 90 s /point

aPDT = antimicrobial photodynamic therapy.

2.5. Microbiological analysis

Samples of tongue coating will be collected using a sterile swab that will be passed on the surface of the tongue dorsum with a single movement and light pressure. Samples will be deposited in sterile tubes that will be identified and stored at -80°C until analysed. After thawing, the samples will be vortexed for 1 minute. For extraction of the bacterial DNA samples will be boiled for 10 minutes and then centrifuged at 10,000 rpm for 10 minutes. The supernatant will be placed in one microtube containing 100 µL phenol/chloroform/isoamyl alcohol (25: 24: 1), followed by ethanol precipitation. The purified DNA will be resuspended in TE buffers. The levels of *P. gingivalis*, *T. forsythia*, and *T. denticola* will be analysed by quantitative PCR. The quantitative analysis will be performed using real-time PCR using the Step One Plus Real-Time PCR System (Applied Biosystem, Foster City, CA) and fluorescently detected products using the Quantimix Easy SYG Kit (Biotools, Madrid, Spain), following the protocol recommended by the manufacturer. To the reaction 10l will be used SYBR Green 0.5 ul DNA template, 200 mM of each primer (*P. gingivalis* CATAGATATCAGGAACT CCGA TT and AAAGTGTAGCAACTACCGATGTGG; *T. forsythia* GGGTGAGTAACGCGTATGTAACCT and ACC-CAT CCGCAACCAATAAA, *T. denticola* CGTTCCTGGGCC TTGTACA and TAGCGACTTCAGGTACCCTCG; Universal bacteria CCATGAAGTCGGAATCGCTAG and GCTTGACGG GCGGTGT) in total volume of 20 µl. For the standard curve, reactions containing template DNA 2 to 2X105 copies of the analyzed gene (16S rRNA) will be performed using pTOPO plasmids in which the 16S genes of the 14 different organisms will be cloned. As a negative control sterile milliQ water will be added instead of DNA template. Reactions to 16S rRNA will be performed with initial denaturation at 95°C for 2 minutes followed by 36 cycles of 94°C for 30 seconds, 55°C for 1 minute and 72°C for 2 minutes and final extension at 72°C for 10 minutes 46. Fluorescence will be detected after each cycle and plotted using Step One Plus Real-Time PCR System software (Applied Biosystem, Foster City, CA). To ensure the specificity of the products detected by fluorescence and to avoid detection of primer dimers, the detection will be performed to a degree below the dissociation temperature of the amplicons. All samples will be analyzed in duplicate and each dilution of the plasmids to the standard curve in triplicate. The purpose of the microbiological evaluation will be to verify the effectiveness of the photodynamic



Figure 2. Oral Chroma.

therapy for the treatment of halitosis, complementing the clinical evaluation.^[20]

2.6. Assessment of halitosis level

The literature describes some methods for measuring halitosis, such as the organoleptic assessment of air emanated from the oral cavity,^[21,22] by sulfide monitor^[21,23,24] and by gas chromatography.^[24-26] However, it has already been demonstrated that the organoleptic test can be influenced by the olfactory ability, the emotional state of the examiner and by climatic conditions. Therefore, the Oral Chroma portable device (Abilit, Japan) (Fig. 2), using a highly sensitive semiconductor gas sensor, will be used for this study.

The air collection will follow the OralChroma™ Manual Instruction, in which the participant is instructed to make a mouthwash with cysteine (10mM) for 1 minute to differentiate the VSCs origin and remain mouth closed for 1 minute. A manufacturer’s own syringe for collection of mouth air will be introduced into the patient’s mouth with the plunger fully inserted. The patient closes his mouth, breathes through his nose and waits with his mouth closed for 1 minute. They will be asked not to touch the tip of the syringe with their tongue. The plunger will be pulled out, re-emptied into the patient’s mouth and pulled out again to fill the syringe with the breath sample. The tip of the syringe will be cleaned to remove moisture from the saliva, the gas injection needle will be placed in the syringe and the plunger will be adjusted to 0.5 ml. The air is injected into the door of the appliance in a single movement (Fig. 3).

OralChroma™ allows the capture of gas concentration values by measuring the VSC thresholds (from 0 to 1000 ppb), very accurately after 8 minutes. The results are stored on the device itself and can be retrieved and viewed at any time for comparison before and after treatment.

From the analysis of VSC captured by the system, we have:

- Sulfide: originates mainly from the bacteria present on the back of the tongue. Values above 112 ppb are indicators of halitosis.
- Methylmercaptanes: predominantly higher in the periodontal pockets. Values up to 26 ppb are considered normal. Periodontal disease typically results in a high ratio of methylmercaptanes/sulphydride (> 3:1)
- Dimethyl sulphide: may have periodontal or systemic (intestinal, hepatic, pulmonary) origin. It can also be caused, temporarily, by the ingestion of certain foods and beverages. The perception threshold of dimethylsulphide is the lowest, 8 ppb.

To avoid changes in halimetry, the examination will be conducted in the morning^[26] and participants will be instructed

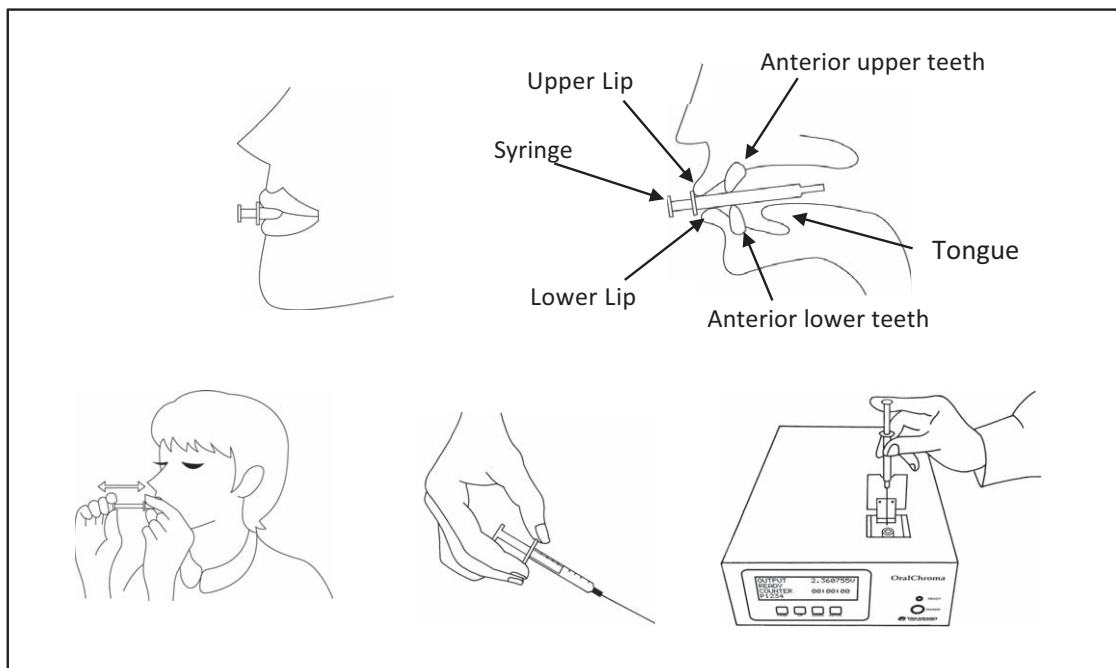


Figure 3. Halimetry procedure.

to follow the following guidelines: 48 hours before the evaluation, avoid ingesting food with garlic, onion and strong seasoning, alcohol consumption and use of oral antiseptic. On the day of the evaluation, in the morning, you can eat up to 2 hours before the examination, abstain from coffee, candies, chewing gum, oral hygiene products, and perfumed personnel (aftershave, deodorant, perfume, creams and/or tonic) and the tooth brushing should be done only with water.^[27]

2.7. Application of aPDT

For the photodynamic therapy, an equipment developed for this project will be used, with emission of red LED (660 nm) and tip of 2.84 cm² in diameter. At the moment of application of the aPDT, only the volunteer to be treated and the professional responsible will be present, both using specific eye protection glasses. The active tip of the laser will be coated with clear disposable plastic (PVC) (avoiding cross contamination) and the professional will be properly dressed (Fig. 4). Methylene blue will be used as the photosensitizing agent, at a concentration of 0.005% (165 μm), to be applied in enough quantity to cover the middle third and back of the tongue for 2 minutes for incubation. The excess will be removed with a sucker in order to maintain the surface wet with the photosensitizer itself, without the use of water. Four points will be irradiated with 1 cm between them, considering the scattering halo and the effectiveness of aPDT (Fig. 5). Based on previous studies carried out with aPDT for the treatment of halitosis^[21-24] the apparatus will be previously calibrated with

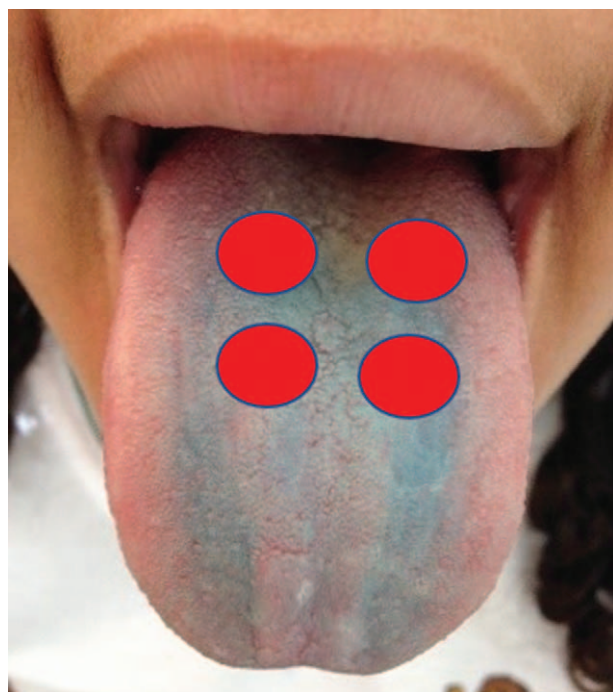


Figure 4. Implementation points for the aPDT. aPDT = antimicrobial photodynamic therapy.

Grupo	Tamanho amostral
1	13
2	13
3	13

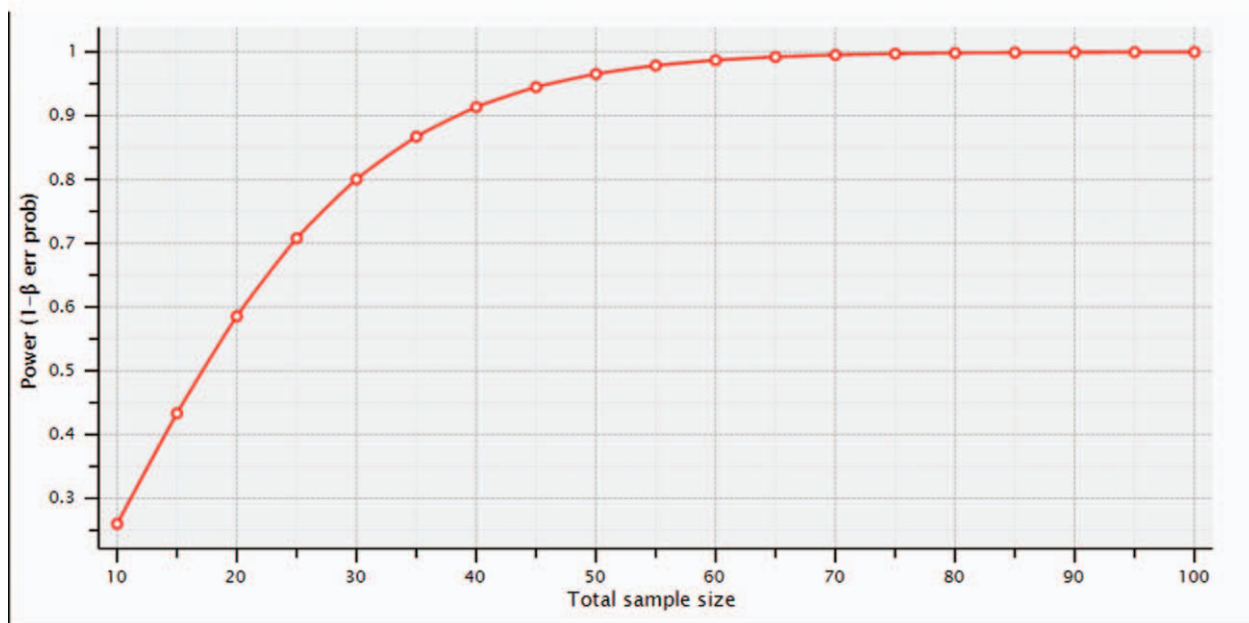


Figure 5. Shows an adjustment of the power of the test as a function of the total sample size.

Table 2

Shows the radiometric and spectroscopic parameters of the equipment developed for this project.

Paramêtros	
Central wavelength [nm]	658.17
Spectral bandwidth (FWHM) [nm]	20.21
Operating mode	Continuous wave
Average radiant power [mW]	400
Polarization	Randomizado
Aperture radius [cm]	0.7
Power Density [mW/cm ²]	1039.4
Beam profile	Multimode
Beam spot size at target [cm ²]	0.38
Irradiance at target [mW/cm ²]	1039.4
Exposure duration [s]	90
Energy density [J/cm ²]	93.5
Radiant energy [J]	36
Number of points irradiated	4
Total area irradiated [cm ²]	1.54
Application technique	Contato
Number of treatment sessions	1
Frequency of treatment sessions	Single irradiation
Total radiant energy [J]	144

wavelength 660 nm, with energy of 36 J, power of 400 mW for Groups 1 and 3 that will be irradiated for 90 seconds per point, creep of 1039.4 mW/cm² (Table 2).^[28]

The method of point application will be used in direct contact with the tongue.

2.8. Sample calculation

For the calculation of the sample size, the data of the work by A. Costa Costa and Mota et al was used. Effect of photodynamic therapy for the treatment of halitosis in adolescents—a controlled, microbiological, clinical trial.^[29]

Initially we established an error $err = |(x_1)^- - (x_2)^-|$, where $(x_1)^-$ and $(x_2)^-$ are the baseline values for periodontal treatment with PDT. From this error, the effect size, given by

$$err / \sqrt{(\sigma_1^2 + \sigma_2^2)}$$

where σ_1^2 and σ_2^2 are the variances of groups 1 and 2, respectively.

Assuming that the studied groups have a normal or approximately normal distribution, that the sample size will be sufficiently large and that a 2-tailed test will be used, for a significance level $\alpha = 0.05$ and maintaining the power of the test $1 - \beta = 0.90$ we have: In Figure 5 it is observed that with a total sample size of 39 subjects, that is, 3 groups with 13 samples each, the statistical difference should be demonstrated, keeping the power of the test greater than or equal to 0.90. If the normal distribution hypothesis is rejected, the sample size should be corrected by approximately 5%.

2.9. Organization and statistical processing of data

The data will be tabulated and treated in the program Bioestat 5.0. The descriptive statistics of the data will be performed. For the evaluation of the association of categorical variables, the Chi-square test and Fisher's Exact Test will be used. To compare the means, the Student's *t* test and Analysis of variance (ANOVA) will be used and to analyze the correlation between the continuous variables the test of Pearson correlation will be

applied. In the analyzes of the experimental differences in each group the Wilcoxon test will be used. For all analyzes a level of significance of 95% ($P < .05$) will be considered.

3. Discussion

Halitosis plays an important role in communication and is related to quality of life and social interaction. Quality of life refers to a person's perception of their position in the system they live in relation to their goals, standard expectations and concerns. It is a comprehensive concept, which is affected by the physical, psychological and social relationship, and relationship with the environment in which it lives. And halitosis is considered one of the problems related to the impact of oral health on quality of life and is enough to affect the individual's perception about his life.^[19]

Therefore, the need for new treatment alternatives for the patient, especially in the face of the technological advances in dentistry, can work better with the patient's expectation regarding the results.

Because it is a condition that involves mainly anaerobic bacteria, which release the SVC in the exhaled air and are responsible for the bad smell. Its main cause is related to the presence of these microorganisms in the lingual flap. And so, it must work to reduce the number of microorganisms responsible for this problem.

There is still a great deal of divergence in the literature regarding the epidemiological data of this condition due to the lack of standardization in the diagnosis, and the treatment proposed in the literature is broad that consists of the chemical and mechanical reduction of the tongue coating.^[9–13]

In the initial studies in this area it was possible to observe an immediate result, with the elimination of bad odor by the reduction of CSV concentration specifically of the levels of SH2 when the treatment with photodynamic therapy was used in the dorsum of adolescents with halitosis.^[29–33]

The present study proposes to conduct a controlled clinical trial to evaluate the effectiveness of the application of aPDT in the tongue coating as a new proposal for treatment and control of halitosis, mainly because it is a fast, non-invasive procedure and does not generate bacterial resistance, as evidenced in the studies cited below and published.

Not generating bacterial resistance can be considered one of the great advantages of aPDT, due to the great problem that we are currently with the development of superbugs being these resistant to the broader antibiotics.

Author contributions

Conceive and design the study: ACCM, MLLG, SKB, MPAM; will perform the experiment: ACCM, MLLG, LA, ACRTH, AL, ESAS; will analyse the data: AMD, RAMF, KPSE, SKB; will perform the statistical analysis: LJM, AMD; write the paper: ACCM, MLLG, SKB.

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Writing – review & editing: Anna Carolina Ratto Tempestine Horliana, Lara Jansinsk Motta, Sandra Kalil Bussadori.

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