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ORIGINAL ARTICLE

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Effect of ozonized olive oil on oral levels of Candida spp. in patients with denture stomatitis

Efeito do óleo de oliva ozonizado sobre os níveis orais de *Candida* spp. em pacientes com estomatite protética

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ABSTRACT

Objective: The aim of this study was to evaluate the effect of ozonized olive oil (OZ) on the oral levels of *Candida* spp. in patients with denture stomatitis. **Material and Methods:** In vitro tests were performed to validate antifungal activity and to standardize OZ conditions. Antifungal activity was screened against *C. albicans* and five non-*albicans* species (*C. tropicalis, C. dubliniensis, C. krusei, C. guilliermondii, and C. parapsilosis*). Also, the effects on *C. albicans* planktonic and biofilm were evaluated. After validation, OZ was included in a therapeutic protocol of denture stomatitis in vivo. Thirty patients used OZ and 20 used sodium bicarbonate (SB) for 14 days. After 7 and 14 days, clinical evaluation, isolation and identification of yeasts were performed. Isolates were identified by phenotypic and genotypic tests. Ozonated oil showed in vitro antifungal activity against all species of *Candida*. Ozonated oil reduced the number of viable cells in *C. albicans* biofilms. Oral candidal levels were lower in relation to baseline both after after 14 days of treatment with SB and OZ. **Results:** A total of 493 *Candida* spp. isolates was obtained and 80% were identified as C. albicans. Remission of denture stomatitis was observed in all patients after 7 days of treatment in both groups. **Conclusion:** Within the limits of the study we can conclude that ozonized olive oil can be a new alternative for the control of biofilm in patients with denture stomatitis.

KEYWORDS

Ozone; Candida; Antifungal; Stomatitis; Denture.

RESUMO

Objetivo: O objetivo deste estudo foi avaliar o efeito do óleo ozonizado (OZ) sobre os níveis orais de *Candida* spp. em pacientes com estomatite protética. **Material e Métodos:** Testes *in vitro* foram realizados para validar a atividade antifúngica e padronizar as condições do OZ. A atividade antifúngica foi avaliada contra *C. albicans* e cinco espécies não-albicans (*C. tropicalis, C. dubliniensis, C. krusei, C. guilliermondii* e *C. parapsilosis*). Além disso, os efeitos sobre *C. albicans* planctônico e biofilme foram avaliados. Após validação, o OZ foi incluído em um protocolo terapêutico de estomatite protética *in vivo*. Trinta pacientes usaram OZ e 20 usaram bicarbonato de sódio (SB) por 14 dias. Após 7 e 14 dias, foram realizadas avaliações clínicas, isolamento e identificação de leveduras. Os isolados foram identificados por testes fenotípicos e genotípicos. O óleo ozonizado mostrou atividade antifúngica *in vitro* contra todas as espécies de *Candida*. O óleo ozonizado reduziu o número de células viáveis em biofilmes de C. albicans. Os níveis orais de candidíase foram menores em relação aos valores basais após 14 dias de tratamento com SB e OZ. **Resultados:** Um total de 493 *Candida* spp. isolados foram obtidos e 80% foram identificados como *C. albicans*. A remissão da estomatite protética foi observada em todos os pacientes após 7 dias de tratamento em ambos os grupos. **Conclusão:** Dentro dos limites do estudo podemos concluir que o óleo de oliva ozonizado pode ser uma nova alternativa para o controle do biofilme em pacientes com estomatite protética.

PALAVRAS-CHAVE

Ozônio; Candida; Antifúngicos; Estomatite; Prótese total.

INTRODUCTION

Denture stomatitis is a multifactorial condition associated to candidal infection, poor oral hygiene and denture hygiene, trauma, alterations in oral pH, and other systemic conditions [1,2]. Dentures predispose to *Candida* infection in the denture supporting area. Denture stomatitis may be related to materials presenting higher surface roughness [2].

Microbial biofilm accumulated on denture's surface may create a favorable environment to *Candida* spp. growth [3] and to the establishment of pathological condition. In addition, the structural organization and function of biofilm communities is shown to be less susceptible to antifungal agents [4]. *C. albicans* is the most frequently isolated species in cases of denture stomatitis (more than 80%) [5]. *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. stellatoidea*, *C. krusei* and *C. kefyr* are also found [6].

The most frequently recommended treatment for denture stomatitis includes topical polyene antifungals and azole agents [7]. Nevertheless, therapeutic protocol includes the control of predisposing factors, adequacy of dentures and biofilm control [8].

Regarding biofilm control, commercial denture cleansing agents have been extensively studied [9,10]. Otherwise, some alternative solutions for prosthesis disinfection have been proposed, such as 10% vinegar [11]. The immersion of the denture in 2.5% sodium hypochlorite solution and the use of topical antifungals have been reported as valid methods treatment protocols for denture stomatitis [12].

The oxidative capacity of ozone is the basis of its biocide activity against fungi [13], bacteria [14], and viruses [15]. It has been reported that the ozonization of oil does not alter the therapeutic properties of ozone and keep it in stable form [16]. Reports on the effectiveness for the treatment of cutaneous infections can also be found [17]. The effect of ozone treatment on cell growth and structural changes in bacteria such as *E. coli*, *Salmonella* sp., *Staphylococcus aureus* and *Bacillus subtilis* has been demonstrated [18]. The antifungal effect of ozonized sunflower oil against yeasts related to onychomycosis by disk diffusion method was studied [19].

The use of ozonated water has been proposed in Dentistry due to the antimicrobial potential against a variety of oral pathogens [20,21]. Silveira et al. [22], in a study with dogs, reported 77% of treatment success rate with the use of ozonized oil as an intracanal medication. This percentage was comparable to the observed for calcium hydroxide and camphorated paramonochlorophenol (74%). Ozonized oil associated to zinc oxide showed to be an alternative filling material for root canal obturation in infected primary teeth [23]. Kollmuss et al. [24] reported the effect of gas ozone and ozonated water on cariogenic biofilm.

Ozone has been cited as a promising alternative for the treatment of several superficial infections, including those caused by *Candida* spp. However, the potential use of the ozonized oil for the control of denture stomatitis is still unexplored. For this reason, this study aimed to evaluate the effect of ozonized olive oil on the oral levels of *Candida* spp. in patients with denture stomatitis.

MATERIAL AND METHODS

Ozonized olive oil

An aliquot of 1000 mL of extra virgin olive oil was maintained for 16 h into a bubbling reactor in water bath at 25ºC with oxygen flow. Ozone was generated by an ozone generator (Ozoxi, model 5C) at a constant flow rate. This equipment produces 5.0 g/h of ozone. The flow rate was adjusted and the temperature of the oil did not exceed 35ºC. Olive oil was saturated with ozone until a thick gel was obtained. The end point of the reaction was obtained when the

peroxide values remained stable. The peroxide value was 900 milliequivalents per liter (mEq/L).

In vitro antimicrobial activity of ozonized olive oil

Agar diffusion assay and determination of minimum fungicide concentration

This study was previously submitted and approved by Local Ethical Committee (Protocol number 058/00 – PH/CEP). Reference strains of *C. albicans* (ATCC18804 and ATCC36802), *C. tropicalis* (ATCC13803), *C. dubliniensis* (NCPF3108), *C. krusei* (ATCC6258), C. guilliermondii (FCF205) and *C. parapsilosis* (ATCC22019) were evaluated. Twenty *C. albicans* and twenty *C. tropicalis* samples, previously isolated from the oral cavity of control individuals and stored at -80°C, were included in the study. The control individuals were healthy volunteers, without any local or systemic diseases. Inclusion criteria was the absence of antimicrobial therapy 60 days before the sampling.

Values of minimum fungicide concentration (MFC) were determined by agar dilution method. Initially, isolates were inoculated on Sabouraud dextrose agar (Difco, Detroit, USA) and incubated at 37° C for 24 h aerobically. A standardized suspension of cells (0.5 McFarland scale) was obtained in sterile saline solution (NaCl 0.85%). Plates containing serial dilutions (50% - 0.37%) of ozonized oil, solubilized in Tween 80, in RPMI medium buffered with MOPS were obtained. The isolates were inoculated with the aid of a Steers replicator. Then, the plates were incubated at 37°C for 48 h. The experiment was performed in duplicate. After the incubation period, reading was based on growth of the isolates tested in various dilutions. The MFC was defined as the lowest concentration that inhibited the growth of the samples.

In vitro C. albicans biofilm eradication by ozonized oil

Specimens of self-cured resin (1 cm^2) (Clássico, São Paulo, Brazil) were obtained, immersed into tubes containing water and autoclaved for 15 min at 121oC. They were randomly distributed into 24-wells culture cell plates. The study groups were ozonized oil (OZ) and sodium bicarbonate (SB, control) (n=8).

Seven *C. albicans* isolates obtained from denture-related stomatitis and *C. albicans* ATCC 18804 were plated onto Sabouraud dextrose agar and incubated for 24h at 37oC. After this period, standardized suspensions containing 10^6 cells/ml in phosphate buffered saline (PBS 0.1 M e pH 7.2) were obtained spectrophotometrically (Micronal® B582). Then, 3 ml of RPMI 1640 without sodium bicarbonate with L-alanine (Himedia, Mumbai, India) supplemented with 2% glucose and buffered to pH 6.5 with MOPS (Sigma, St. Louis, USA) were added to each well containing the specimen. An aliquot of 300 μL of each fungal suspension was added to each well and after gently agitation, plates were incubated at 37oC for 48h. After 24h of incubation the culture medium was refreshed.

After the period of biofilm formation, the specimens were carefully removed from the culture medium and transferred to new 24-well plates containing 3 mL per well of sterile phosphate buffered saline (PBS 0.1M e pH 7.2) for removing non- adherent cells. Specimens from the SB group were immersed in 3 mL of a sterile 3% sodium bicarbonate solution (Sigma, Detroit, USA) during 5 min. Specimens from the OZ group were maintained in contact (immersed) in 1.5 g of ozonized oil during 5 min. After this period, the specimens were completely covered with the ozonized oil.

After this period, coupons were transferred to tubes containing 5 mL of PBS and vortexed for 1 min. Suspension was diluted to 10^{-2} and 10^{-4} in sterile physiologic solution and plated on

Sabouraud dextrose agar (Himedia, Mumbai, India) in duplicate. Plates were incubated for 48 h at 37°C. The value of colony- forming units per milliliter (CFU/mL) was calculated for each group after.

In vivo evaluation

The protocol of *in vivo* evaluation was previously evaluated and approved by Local Ethical Committee of Research involving human participants (070/06-PH/CEP). The inclusion criteria for denture stomatitis group were: denture wearers with clinical diagnosis of stomatitis, according to Newton [25]. They were diagnosed in the Oral Medicine Clinic from São Paulo State University (Unesp), Institute of Science and Technology and among institutionalized elderly. The non-inclusion criteria were use of antimicrobials for 60 days before the sampling, decompensated diabetes mellitus and the presence of other oral lesions (ie. cancer).

The patients were informed about the aims of the study and were invited to participate. Those who agreed to participate signed an informed consent form. The patients were divided randomly into 2 groups according to the adopted treatment protocol: i) sodium bicarbonate (SB) (n=20; mean age 65.7 yrs), added as control group; ii) ozonized oil (OZ) (n=30; mean age 66.8 yrs).

Anamnesis was performed and all patients were intra-orally examined. After, all patients were asked to adopt the following general procedures: brushing the denture after meals and remove the denture every night.

Besides of these cleansing general procedures, patients were randomly divided according to the testing group. For ozonized group (OZ), patients were oriented to follow the protocol: after cleaning the denture, rinse with water, dry the surface and apply the ozonized oil. Application was made by cotton sticks, 3 times a day (after meals) for 14 days. Standardized ozonized olive oil was given to the patients. They were instructed to maintain the product at 4oC. Sodium bicarbonate group (SB) patients were oriented to rinse the mouth out with a sodium bicarbonate (Masterfoods, Campinas, Brazil) solution containing one coffee spoon (around 3 grams) in 100 ml of filtered water for 3 min, 3 times a day (after meals) for 14 days. SB group (currently adopted clinical protocol) was added as a control for OZ group.

Before the treatment, 7 and 14 days after, oral rinse samples were obtained in 10 mL of sterile phosphate buffered saline (PBS 0,1M e pH 7.2) during 1 min. A swab sample from the denture base was also collected for microbiological analyses. The swab was immediately transferred to tubes containing 2 mL of sterile saline solution (NaCl 0.9%). Samples were maintained in ice and transported immediately to the laboratory. All the samples were plated in a maximum period of 3 h from sampling.

Oral rinses samples were centrifuged for 10 min at 8.000 g and the supernatant was discarded. Then, 2.5 mL of PBS was added to the pellet. PBS dilutions of 10^{-1} and 10^{-2} were obtained and a 0.1 mL aliquot of each suspension was plated on Sabouraud dextrose agar (Difco, Detroit, USA) chloramphenicol supplemented (Sigma, St. Louis, USA) (0.1 mg/mL of the culture medium). Plates were incubated aerobically at 37°C for 48 h. After this period, characteristic colonies were counted and the number of colony-forming units per milliliter (CFU/ml) was obtained. Five colonies representative of each morphology observed in the plate were submitted to microscopic confirmation and were transferred to tubes containing Sabouraud dextrose agar (Difco, Detroit, USA). Tubes were incubated for 48h at 37°C and after this period they were maintained at 4°C until identification. Phenotypic identification included germ tube formation in bovine serum (Sigma, St. Louis,

USA), growth in corn meal (Oxoid, Hampshire, England) - Tween 80 (Sigma, St. Louis, USA) agar, fermentation and assimilation of carbohydrates [26].

Isolates phenotypically identified as *C. albicans* or *C. dubliniensis* were submitted to molecular identification. *Candida* genomic DNA was prepared as described previously [27]. Multiplex PCR was performed in a final volume of 10 μL using PCR Master Mix (Promega Corporation, Wiscosin, USA) under the standard conditions for Master Mix and 1 μL of DNA template. The reaction also contained 5 μM of each universal fungal primers and *C. dubliniensis* specific primers (Integrated DNA Technologies, California, USA) [28]. Cycling conditions consisted of 3 min at 95°C followed by 30 cycles of 30 s at 95°C, 30s at 58°C, 60 s at 72°C, followed by 72°C for 10min. In all reactions, *C. albicans* (ATCC 18804) and *C. dubliniensis* (NCPF 3108) were included as positive control. A negative control run was performed with sterilized water in the PCR mixture. Amplification products were separated by electrophoresis through 2% (w/v) agarose gel (Bio America, Florida, USA) containing 25 μM ethidium bromide (Calbiochem, California, USA) and visualized on a UV transilluminator (Foto/UV 26, Fotodyne Inc.). A DNA ladder of 1000 pb (Fermentas Lifescience, Maryland, USA) was used as molecular size standard.

After 7 and 14 days, patients were followed-up by the same professional. Extra and intra-oral examination was performed. The absence of erythematous areas was considered as denture stomatitis remission.

Data Analyses

The results were analyzed by descriptive and inferential statistical analysis, with the alpha level set at 0.05, using statistical software (MINITAB for Windows program, version 2000, 13.1; Minitab Inc, State College, Pa). Values of CFU/mL were compared between SB and OZ groups at each period of evaluation by Mann-Whitney test.

RESULTS

In vitro evaluation

The antimicrobial activity of ozonized oil against *Candida* species was expressed by values of minimum fungicide concentration (MFC) and represented in Table 1. Ozonized oil showed effective antimicrobial activity against all species of *Candida*. Thus, for *C. krusei* and other clinical isolates of C. albicans the MFC was 0.75%, and for isolates of *C. tropicalis* and other standard samples tested MFC was 1.5%.

Ozonized oil was more effective in the eradication of *C. albicans* biofilm in vitro when compared to sodium bicarbonate. Mean values of fungal counts after *C. albicans* biofilm treatment with ozonized oil (0.79 x 10^6 cells/mL) were significantly lower than when treated with sodium bicarbonate (1.95 x 10^6 cells/mL) ($p = 0.040$) (Figure 1).

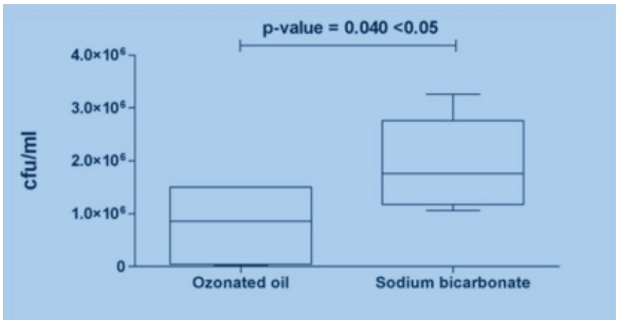


Figure 1 - Fungal counts, expressed in CFU/mL (mean and standard deviation), after *C. albicans* biofilm treatment with ozonated oil and sodium bicarbonate.

In vivo evaluation

Yeasts counts were reduced both in SB and OZ groups after 14 days of treatment in relation to baseline.

Statistically significant differences were observed between SB and OZ groups after 7

days of treatment both when oral rinse and swab samplings were analyzed (Tables 2 and 3). Unexpected arise in yeasts counts after 7 days of treatment with ozonized oil was observed when oral rinses samples were analyzed. However, after 14 days of treatment, the median values were similar to SB group.

A total of 493 *Candida* spp. isolates was obtained, 250 from the oral rinses and 243 from the dentures. *Candida* species identified are shown in Table 4. Regarding the isolated species, *C. albicans* was found in 96% (n=48) of the studied patients in both groups.

Three patients from OZ group and 3 from SB group showed more than one species of *Candida* spp. in the oral cavity. Among patients treated with OZ, one patient (3.33%) was negative to *Candida* spp. in second sampling and 46.6% were negative in the third sampling. In SB patients, three patients (15%) were negative to *Candida* spp. in the second sampling and 10% in the third sampling.

Remission of denture stomatitis were observed in all patients after 7 days of treatment both in SB and OZ groups.

DISCUSSION

Despite of the multifactorial etiology of denture stomatitis, this can be mainly related to local factors such as biofilm accumulation [26]. This study focused in *Candida* spp. infection as dentures are a predisposing factor to denture stomatitis. For this reason, *C. albicans* was selected as the microbial indicator. This study was designed to evaluate the *in vitro* and *in vivo* effectiveness of ozonized oil on *Candida* spp.. Besides, sodium bicarbonate was selected for comparative purposes, due to its reported anti-candidal effects [29] and currently use in clinical protocols.

C. albicans samples were susceptible to ozonized olive oil with MIC values ranging from 1.50 to 0.75. These values were higher than those observed by Geweely [30] where MIC values ranged from 0.78 to 0.53. This difference can be related to different clinical strains origins. Another factor to be considered is the oil ozone concentration. In the present study the value of ozone concentration was 900 mg/L, while in Geweely [31] this value was 650 mmol kg^{-1}.

The incorporation of ozone to olive oil was performed due to previous reports on high instability and advantageous longer-term action when compared to water and gas vehicles [29]. Furthermore, the toxicological studies of ozonized oil (Oleozon) did not show toxic effects [32]. Cytoprotective effects of ozonized sunflower oil on rat gastric mucosa are reported [33]. These evidences motivated the study of ozonized oil in denture stomatitis.

Despite several previous reports on ozonized water, the oil was used in this study. Ozone diluted in oil maintains its therapeutic properties and stability [34], besides of maintaining a substance called ozonide in the chemical structure of the oil. As ozonide is stable under low temperatures, patients were asked to maintain the oil in refrigerator. Ozonized water is not stable and its half-life in pH 7.0 is approximately 12 minutes [35]. Oizumi et al. [33] reported that gaseous ozone seems to be an effective method for denture cleaning. However, the gaseous form is very difficult to generate and difficult to be applied by the patients. Considering these evidences, ozonized oil may be a good therapeutic alternative for its usage and storage facilities. Moreover, ozonized oil allowed more time of with mucosa and this can be considered as an advantage over sodium bicarbonate solution.

Ozonized oil was more effective in the reduction of candidal counts, however sodium bicarbonate solution also reduced candidal counts and can be considered a good therapeutic alternative. This result corroborates previous studies that reported good activity of this

substance against *C. albicans*. According to De Bernardis et al. [36], sodium bicarbonate is an alkali that can reduce the pathogenic potential of *C. albicans*, interfering in the expression of virulence genes.

In the OZ group, a reduction in fungal counts was observed after 7 days and arrived to similar levels when compared to SB group after 14 days. This increase might be correlated to the activity of ozonized oil on *Candida* adherence, increasing the detection of fungal cells in microbiologic evaluation of oral rinses.

Besides of the use of ozonized oil or sodium bicarbonate, our study corroborates the importance of a correct denture cleaning procedure for the reduction of *Candida* spp. oral counts [6]. The adoption of immersion in a solution of vinegar possibly complemented the use of ozonized oil [10]. An effective therapeutic treatment associated to a detailed anamnesis is of utmost importance for a correct diagnosis and an effective treatment aiming a better life quality to denture wearers.

Regarding the identification of *Candida* species, *C. albicans* was the most frequently found, corroborating to previous reports [37]. *C. glabrata* and *C. tropicalis* were also identified and some patients showed more than one species. Previous studies also reported non-*albicans* species isolation from stomatitis lesions and also combination of different species [38].

Clinical remission of denture stomatitis was observed when the treatment protocol included oral hygiene of prostheses and use of ozonized oil or sodium bicarbonate solution. After 14 days, the environmental alkalization promoted by sodium bicarbonate and the clinical remission of the erythematous areas were not always associated with reduction in candida counts. This observation is in accordance to previous study [39]. The alkalization capacity of sodium bicarbonate has been previously correlated to a probable reduction in the fungal pathogenicity [40].

Within the limits of the study we can conclude that ozonized oil may be a new alternative for the control of denture stomatitis, showing similar clinical performance when compared to sodium bicarbonate.

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STATEMENT CONFLICT OF INTEREST:

There is no conflict of interest involving the authors of this paper.

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