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

# *Abstract*

*Objective:* The aim was to evaluate the effect of Ozonized oil (OZ) on oral candidal levels in denture stomatitis patients. 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. 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. Patients used OZ or sodium bicarbonate (SB) for 14 days. After 7 and 14 days, clinical evaluation, isolation and identification of yeasts were performed. OZ showed activity against all species of *Candida*. OZ reduced the number of viable cells in *C. albicans* biofilms. Oral candidal levels were lower in relation to baseline both after treatment with SB and OZ after 14 days of treatment. Results: A total of 493 *Candida* spp. isolates was obtained and 80% were identified as *C. albicans*. Remission of erythematous stomatitis lesions was observed in all patients after 7 days of treatment both groups. Conclusions: Within the limits of the study we can conclude that ozonized oil can be a new alternative for the control of biofilm in patients with denture stomatitis.

*Key-words:* Ozone; *Candida*; antifungal; stomatitis; denture.

# *Introduction*

Denture stomatitis is a multifactorial condition associated to candidal infection, poor oral hygiene, trauma, alterations in oral pH, and other systemic conditions [1,2].

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

The most frequently recommended treatment for erythematous stomatitis includes topical polyene antifungals and azole agents [7]. Also, the control of predisposing factors, adequacy of dentures and biofilm control are highly recommended as part of the therapeutic protocol [8].

Regarding biofilm control, commercial denture cleansing agents have been extensively studied [9,10]. Also, some alternative solutions for prosthesis disinfection have been proposed, such as 10% vinegar [11]. The immersion in 2.5% sodium hypochlorite and use of topical antifungals has been reported as valid 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]. Also, reports on the effectiveness for the treatment of cutaneous infections can 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 also reported [19].

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 that the treatment success rate with use of ozonized oil as intracanal medication was 77%. This percentage was comparable to the observed for calcium hydroxide/camphorated paramonochlorophenol (74%). Ozonized oil associated to zinc oxide showed to be a valid alternative obturing material in infected primary teeth [23]. Kollmuss et al. [24] reported the effect of gas ozone and ozonated water on cariogenic biofilm. The application of ozonized oil on oral levels of *Candida* spp. and possible application in the control of denture stomatitis is still unknown.

This study aimed to evaluate the effect of ozonated olive oil on the oral levels of *Candida* spp. in patients with denture stomatitis.

# *Material and methods*

*Ozonated olive oil*

An aliquot of 100 ml of extra virgin olive oil was maintained into a bubbling reactor in water bath at 25ºC. Ozone was generated by an ozone generator (Ozoxi, model RADAST 10C) at a constant flow rate. 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 remain stable. The peroxide value was 900 meq/l.

# *In vitro* antimicrobial activity of ozonized 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. Also, 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.

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 hours 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 hours. 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 ozonated oil*

Specimens of self-cured resin (1 cm2) (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 Ozonated 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 106 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 were 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 immersed in 1.5 g of ozonated oil during 5 min.

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 37oC and after the value of colony- forming units per milliliter (cfu/ml) was calculated for each group.

*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). Patients were denture users with clinical diagnosis of stomatitis, according to Newton [25]. They were diagnosed in the Dental School and among institutionalized elderly.

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

Anamnesis was performed and all patients were intra-orally examined. After, all the 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. Ozonated group (OZ) patients were asked to dry the palatal surface of the dentures after brushing and topically apply ozonated oil. The application was done by using cotton sticks, 3 times a day (after meals) for 14 days. Standardized ozonated oil was given to the patients and they were asked to maintain the product at 4oC. Sodium bicarbonate group (SB) patients were asked to rinse the mouth using a solution containing one coffee spoon (around 3 grams) of sodium bicarbonate (Masterfoods, Campinas, Brazil) in 100 ml of filtered water for 3 min, 3 times a day (after meals) for 14 days. Sodium bicarbonate group was added as a control for ozonized group (currently adopted clinical protocol).

Before the treatment, 7 and 14 days after, oral rinses samples were obtained in 10 ml of sterile phosphate buffered saline (PBS 0,1M e pH 7.2) during 1 min. Also, a sample from the denture base was collected for microbiological analyses by the aid of a swab. The swab was immediately transferred to tubes containing 2 ml of sterile saline solution (NaCl 0.9%). The 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.

Samples of oral rinses 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. Dilutions of 10-1 and 10-2 in PBS were obtained and a 0.1 ml aliquot of each suspension was plated on Sabouraud dextrose agar (Difco, Detroit, USA) supplemented with chloramphenicol (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 l 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 30s at 95°C, 30s at 58°C, 60s 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. Oral lesions were clinically evaluated. The absence of erythematous areas was considered as lesions’ 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 BS 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. Ozonated 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%.

Ozonated 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 ozonated oil (0.79 x 106 cells/ml) were significantly lower than when treated with sodium bicarbonate (1.95 x 106 cells/ml) (p = 0.040) (Figure 1).

*In vivo* evaluation

Yeasts counts were reduced both in sodium bicarbonate and ozonized oil 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 ozonated oil was observed when oral rinses samples were analyzed. However, after 14 days of treatment, the median values were similar to sodium bicarbonate 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 erythematous stomatitis lesions 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, considering that it is an important predisposing factor in denture-related stomatitis. For this reason, *C. albicans* was selected as the microbial indicator. This study was designed to evaluate the effectiveness of ozonized oil on *Candida* spp. by *in vitro* and *in vivo* approaches. Besides, sodium bicarbonate was selected for comparative purposes, due to its reported anti-candidal effects [29] and currently used 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 maybe can be related to the different origins of the clinical strains. Another factor to be considered is the ozone concentration in the oils. 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 time of 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 were also reported [33]. These evidences motivated the study of ozonized oil in oral stomatitis.

Although there are several previous reports on ozonized water, in this study the oil was used. 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 that can facilitate application and storage. Moreover, ozonized oil promoted a higher contact time between mucosa and denture base and this can be considered an advantage in relation to sodium bicarbonate solution.

Although ozonized oil was more effective in the reduction of candidal counts, 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 and could reduce the pathogenic potential of *C. albicans*, interfering in the expression of virulence genes.

In the ozonized oil group, a reduction in fungal counts was observed after 7 days and arrived to similar levels when compared to sodium bicarbonate 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 by 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 users [26].

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 erythematous candidosis lesions was observed when the treatment protocol included oral hygiene of prostheses and use of both ozonized oil and sodium bicarbonate solution. After 14 days, the environmental alkalization promoted by sodium bicarbonate and the clinical remission of the lesions 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.

*Acknowledgements and conflict of interests*

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*Statement conflict of Interest:*

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

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

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# Disclosure of interest:

The authors declare that they have no conflict of interest.

# Ethical Approval:

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The protocol of *in vivo* evaluation was previously evaluated and approved by Local Ethical Committee of Research involving human participants (070/06-PH/CEP).

# Informed consent:

Informed consent was obtained from all individual participants included in the study.