Comparison of the antibacterial activity of an ozonated oil with chlorhexidine digluconate and povidone-iodine. A disk diffusion test

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SUMMARY

Ozonated oils are antiseptics obtained from the chemical reaction between ozone and unsaturated fatty acids of vegetable oils. The aim of this study was to investigate the antimicrobial effectiveness of a commercially available ozonated oil (O_3-Oil), in comparison with 0.2% chlorhexidine digluconate (CHX) and 10% povidone-iodine (PVP-I) through a disk diffusion test. For each antiseptic a series of two-fold dilutions was made, obtaining seven dilutions: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128. The undiluted antiseptics and the seven dilutions were tested against two freeze-dried bacterial strains: *Staphylococcus aureus* (Sa) and *Porphyromonas gingivalis* (Pg). O_3-Oil showed significantly greater diameters of growth inhibition ($p<0.01$) than CHX and PVP-I in all dilutions for both tested strains. CHX lost any antibacterial efficacy when diluted more than 1:32. At the highest dilution, the diameters of growth inhibition against Sa were 20.67±0.58 mm and 15.33±0.58 mm, for O_3-Oil and PVP-I, respectively. At the same dilution, the diameters of growth inhibition against Pg were: 19.00 mm for O_3-Oil and 13.67±0.58 mm for PVP-I. The promising results obtained for the O_3-Oil, against the opportunistic Sa, and Pg, one of the main periodontal pathogens, suggest its potential applicability for periodontal treatment. Further preclinical and clinical investigations are warranted.

KEY WORDS: Ozonated oil, Antibacterial activity, *Staphylococcus aureus*, *Porphyromonas gingivalis*, Periodontal disease, Peri-implantitis.

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INTRODUCTION

Bacterial colonization of dental surfaces is considered to be the primary causative factor of periodontitis (Socransky *et al.*, 1998; Flemming, 1999).
The World Workshop of Periodontology defined *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia* as periodontal pathogens (Consensus Report., 1996). This designation is based on strong evidence supporting an etiologic role of periodontal diseases for these species. Other bacteria, classified in chromatic microbial complexes on the basis of their interaction, have also been related to periodontal diseases (Socransky *et al.*, 1998).
The periodontal impairment mostly results from an intricate sequence of host immune reactions to pathogens (Honda *et al.*, 2006). This process, conditioned by multiple factors, progressively involves the apical portions of the tooth support, inducing a gradual loss of periodontal attachment and alveolar bone, with tooth loss as the final outcome (Schwartz *et al.*, 1997). A similar pattern has been recognized for peri-implantitis (Lindhe *et al.*, 1992; Berglundh *et al.*, 2004).
The primary goal of periodontal therapy is to eradicate the periodontal pathogens within tooth and dental implants. Removal of subgingival biofilm

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and calculus deposits through ultrasonic and manual instruments is an effective and well-documented treatment (Checchi *et al.*, 1988; Checchi *et al.*, 1997). After this procedure, a decrease in the mean counts and number of sites colonized by *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia* has been observed for several weeks (Shiloah *et al.*, 1994; Darby *et al.*, 2005).

Unfavourable anatomy of roots, tissue invasive micro-organisms and bacterial invasion into dentinal tubules hamper the complete elimination all of pathogens from the periodontal pockets (Mombelli *et al.*, 2004). Moreover, a recently treated site may be re-colonized by pathogenic bacteria residing in other areas within the oral cavity (intra-oral niches, tonsils, dorsum of the tongue - this is called intra-oral translocation) (Beikler *et al.*, 2004). Therefore, there is currently considerable interest in the use of chemotherapeutic agents to assist root detoxification and periodontal pocket disinfection (Herrera *et al.*, 2002; Haffajee *et al.*, 2003; Mombelli *et al.*, 2004). However, the use of antibiotics can produce unpleasant side-effects including adverse host reactions and development of bacterial resistance (Mombelli *et al.*, 2004). Broad-spectrum antiseptic agents, like povidone-iodine (PVP-I) and chlorhexidine digluconate (CHX), seem to constitute a more desirable choice to avoid adverse reactions of antibiotics (Unsal *et al.*, 1994; Hoang *et al.*, 2003; Mombelli *et al.*, 2004; Krück *et al.*, 2012). Bacteria of the Staphylococcus genus are Gram-positive cocci responsible for a wide range of infections: bacteraemia, infective endocarditis, pneumonia, osteomyelitis, joint infections, diabetic foot ulcers and surgical site infections (Sheagren *et al.*, 1985; Le Thomas *et al.*, 2001; Dang *et al.*, 2003; Charles *et al.* 2004; Chambers, 2005a; Chambers, 2005b; Davis, 2005; Francis *et al.*, 2005; Mitchell *et al.*, 2005; Roberts *et al.*, 2005; Simon *et al.*, 2005; Galkowska *et al.*, 2009).

To date, treating *S. aureus* infections is a challenging task because of the continuing occurrence of resistance to antibiotics. Therefore the development of alternative agents to control multiresistant staphylococcal strains have been common themes in the staphylococcal literature over the last decade (Kurlenda *et al.*, 2012).

PVP-I is a water-soluble compound of iodine and the solubilizing agent polyvinylpyrrolidone. It is probably the most broad-spectrum antiseptic available to healthcare professionals. PVP-I has a broad antibacterial spectrum that covers Gram-negative and Gram-positive bacteria (Gocke *et al.*, 1985). This iodofor has bactericidal effects against anaerobic bacteria associated with periodontal disease: a 30 s application of 2 % PVP-I could effectively suppress *P. gingivalis, Aggregatibacter actinomycetemcomitans* and other periodontal pathogens *in vitro* (Caufield *et al.*, 1987; Hosaka *et al.*, 2012). PVP-I has a proven wide virucidal spectrum covering herpes viruses, influenza virus and HIV (Kawana *et al.*, 1997). It is also effective against *Candida Albicans* (Schreier *et al.*, 1997).

CHX is probably the best known and most widely used antiplaque agent in periodontal therapy to date. This antiseptic has a wide antimicrobial action, including a broad variety of Gram-positive and Gram-negative bacteria (Wade *et al.*, 1989). It is also effective against some yeasts like Candida and some viruses including herpes simplex and HIV (Kawana *et al.*, 1997; Fathilah *et al.*, 2012). The effectiveness of CHX as antiplaque agent depends on its ability to be absorbed on hard and soft oral tissues (substantivity) (Schiott *et al.*, 1970). Once absorbed, there is a slow release of the antiseptic, determining a prolonged persistence of antimicrobial action in the mouth (Bonesvoll *et al.*, 1974a; Bonesvoll *et al.*, 1974b; Gjermo *et al.*, 1974).

Over the last decade, many ozonated formulations have been introduced as alternative oral antiseptics. Ozonated oils (O_3-Oil), for instance, have a broad antibacterial spectrum that covers Gram-negative and Gram-positive (Siqueira *et al.*, 2000; Sechi *et al.*, 2001; Rodrigues *et al.*, 2004). O_3-Oil has also been proven to be effective against these eight periodontal putative pathogens *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*), *Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola*, and *Tannerella forsythia* (previously *T. forsythensis*), as assayed by multiplex PCR method, in a randomized controlled clinical trial (Patel *et al.*, 2012). O_3-Oil has also been proven to be effective against the following pathogenic fungal species: *Candida Albicans, Aspergillus fumigatus, Epidermophyton floccosum, Microsporum canis* and *Trichophyton rubrum* (Geweely, 2006).

The purpose of this preclinical study is to compare the antibacterial effectiveness of a commercially available O_3-Oil (Novox®) with CHX and PVP-I formulations against *Staphylococcus aureus* and *Porphyromonas gingivalis* through a disk diffusion method.

MATERIALS AND METHODS

Microbial species
In this study freeze-dried bacteria from the American Type Culture Collection® (ATCC®, USA) were used. The bacteria strains were: *Porphyromonas gingivalis* ATCC® 33277™ a Gram-negative anaerobic bacteria closely related to the periodontal disease and *Staphylococcus aureus* ATCC® 29213™, a Gram-positive bacteria.

Culture conditions
P. gingivalis frozen isolate was thawed and suspended in Brain-Heart Infusion (BHI) medium (BD Diagnostic Systems, Germany). This bacterial suspension was inoculated on Brucella agar plates supplemented with 5 µg/ml hemin, 1 µg/ml menadione and 5% sheep blood (BD Diagnostic Systems, Germany). Plates were incubated at 37°C under anaerobic conditions (5% CO_2, 10% H_2 and 85% N_2) for 3-5 days. *S. aureus* was first thawed in trypticase soy broth and later cultivated in trypticase soy agar plates at 37°C for 24 hours (BD Diagnostic Systems, Germany).
The identity of bacterial cultures was verified using standard methodology: each agar plate contained well-defined colonies of *P. gingivalis* and *S. aureus*. At this stage, microorganisms were harvested from the agar surface with sterile swabs and suspended in a sterile balanced saline solution. Samples were diluted so that the suspension turbidity was adjusted to 0.5 MacFarland Standard Units (1,5 x 10^8 CFU/ml).

Antiseptics
The following antiseptics have been tested: an ozonated extra virgin olive oil (Novox®, MOSS S.r.l., Lesa - Novara, Italy) with a peroxide value of 560/590 mmol-equiv/kg, chlorhexidine digluconate 0.2% (Dentosan®, Recordati S.p.A., Milan, Italy) and povidone-iodine 10% (Betadine®, MEDA Pharma S.p.A., Milan, Italy).
For each antiseptic a series of two-fold dilutions was made; obtaining seven dilutions: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128. The diluting agents were virgin olive oil for the ozonated product and saline solution for the others.

Disk diffusion susceptibility testing
Microbial suspension with the Gram-negative bacteria was aseptically spread on petri dishes containing Brucella agar supplemented with 5 µg/ml hemin, 1 µg/ml menadione and 5% horse blood. Plates containing Müller-Hinton agar (Acumedia, USA) were seeded with *S. aureus*.
Nine cellulose disks (6 mm diameter sterile Whatman cellulose filters number 5, Germany) were impregnated with different concentrations of antiseptic and were placed onto agar surface. The disks were numbered starting from 0 to 8.
One hundred and fifty microliters of undiluted antiseptic were applied to disk number 0. From disk numbers 1 to 7, 150 microliters were applied of the seven antiseptic dilutions (1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128) previously prepared. Disk number 8 was impregnated with the diluting agent alone, as negative control for the experiment. *P. gingivalis*-containing plates were incubated at 37°C under anaerobic conditions for 48 h. Agar plates inoculated with S. aureus were incubated at 37°C for 24 h.
All disk diffusion tests were performed independently in triplicate. Antimicrobial activity was assessed by measuring the diameter (in millimeters) of the zone of growth inhibition surrounding cellulose disks. The diameter was measured using a caliper.

Statistical analysis
Data are presented as mean ± standard deviation (mean ± SD). Diameters of the zones of growth inhibition (O_3-Oil, CHX and PVP-I) were compared among groups using one-way analysis of variance (ANOVA). Tukey-Kramer method was performed as post-hoc test. A p value less than 0.01 was considered statistically significant.

RESULTS

Diameters of the zones of growth inhibition, in millimetres, produced by the three tested antiseptics are presented in Tables 1, 2, 3 and 4 as mean ± SD.

Results for *S. aureus* are reported in Tables 1 and 2 and Figure 1; results for *P. gingivalis* are reported in Tables 3 and 4 and Figure 2. All tested antiseptics revealed varying degrees of antibacterial activity against the two tested strains. Disk number 0, impregnated with undiluted antiseptics, showed the greatest diameters of growth inhibition. None of the tested strains were sensitive to the diluting agent alone (disk number 8 - negative control).

TABLE 1 - *Diameter in millimeters (mean ± SD) of the inhibition zones at different dilutions for ozonated oil (O_3-Oil), chlorhexidine (CHX) and povidone-iodine (PVP-I) on Staphylococcus aureus ATCC® 29213™.*

Disk	*Dilution*	*O3-Oil*	*CHX*	*PVP-I*	*F-ratio*
0	Undiluted	30,00	29,00	27,67±0,58	37*
1	1:2	28,33±0,58	25,33±0,58	26,00	33,5*
2	1:4	26,67±0,58	21,00	25,00	229*
3	1:8	25,00	19,67±0,58	24,00	217*
4	1:16	24,67±0,58	10,00	23,00	1741*
5	1:32	23,33±0,58	0	20,00±1,00	1075*
6	1:64	22,00	0	17,67±0,58	3667*
7	1:128	20,67±0,58	0	15,33±0,58	1554*
8	Neg. control	0	0	0	/

*Significant difference ($p<0.01$) among the groups using one-way ANOVA.
Critical value of $F(2,6) = 10,9247665009121$ for the 0.01 significance level.

TABLE 2 - *Differences between means with indication of their significance using Tukey-Kramer method as post-hoc test for Staphylococcus aureus ATCC® 29213™.*

Disk	*Dilution*	*O_3-Oil*	*CHX*	*MO_3 - MC*	*PVP-I*	*M_{O3} - MP*
0	Undiluted	30,00	29,00	1*	27,67±0,58	2,33**
1	1:2	28,33±0,58	25,33±0,58	3**	26,00	2,33**
2	1:4	26,67±0,58	21,00	5,66**	25,00	1,66**
3	1:8	25,00	19,67±0,58	5,33**	24,00	1*
4	1:16	24,67±0,58	10,00	14,66**	23,00	1,66**
5	1:32	23,33±0,58	0	23,33**	20,00±1,00	3,33**
6	1:64	22,00	0	22**	17,67±0,58	4,33**
7	1:128	20,67±0,58	0	20,66**	15,33±0,58	5,33**
8	Neg. control	0	0	0	0	0

*Significant difference at $p<0.05$; **Significant difference at $p<0.01$.
Mean of group O_3-Oil is denoted M_{O3}, mean of group CHX is denoted M_C and mean of group PVP-I is denoted M_P.

DISCUSSION

H_0 hypothesis was rejected at 0.01 level of significance for both tested strains using one-way ANOVA. Tukey-Kramer method, as post-hoc test, revealed the following results. With regard to *S. aureus* (Figure 1), O_3-Oil showed a significantly better ($p<0.01$) antibacterial efficacy than 0.2% CHX and 10 % PVP-I in all dilutions except for undiluted CHX and 1:8 PVP-I. With regard to *P. gin-*

TABLE 3 - *Diameter in millimeters (mean ± SD) of the inhibition zones at different dilutions for ozonated oil (O_3-Oil), chlorhexidine (CHX) and povidone-iodine (PVP-I) on Porphyromonas gingivalis ATCC® 33277™.*

Disk	*Dilution*	*O3-Oil*	*CHX*	*PVP-I*	*F-ratio*
0	Undiluted	30,67±0,58	27,00	28,00	97*
1	1:2	29,00±1,00	24,00	25,00±1,00	31,5*
2	1:4	27,33±0,58	21,00±1,00	25,00	69,25*
3	1:8	25,00	18,33±0,58	23,33±0,58	162,50*
4	1:16	25,33±1,15	10,00±1,00	21,00	241*
5	1:32	24,00	0	19,00	65535*
6	1:64	20,33±0,58	0	17,00±1,00	802,75*
7	1:128	19,00	0	13,67±0,58	2593*
8	Neg. control	0	0	0	/

*Significant difference ($p<0.01$) among the groups using one-way ANOVA.
Critical value of $F(2,6) = 10,9247665009121$ for the 0.01 significance level.

TABLE 4 - *Differences between means with indication of their significance using Tukey-Kramer method as post-hoc test for Porphyromonas gingivalis ATCC® 33277™.*

Disk	*Dilution*	*O_3-Oil*	*CHX*	*M_{O3} - MC*	*PVP-I*	*M_{O3} - M_P*
0	Undiluted	30,67±0,58	27,00	3,66**	28,00	2,66**
1	1:2	29,00±1,00	24,00	5**	25,00±1,00	4**
2	1:4	27,33±0,58	21,00±1,00	6,33**	25,00	2,33*
3	1:8	25,00	18,33±0,58	6,66**	23,33±0,58	1,66*
4	1:16	25,33±1,15	10,00±1,00	15,33**	21,00	4,33**
5	1:32	24,00	0	24**	19,00	5**
6	1:64	20,33±0,58	0	20,33**	17,00±1,00	3,33**
7	1:128	19,00	0	19**	13,67±0,58	5,33**
8	Neg. control	0	0	0	0	0

*Significant difference at $p<0.05$. **Significant difference at $p<0.01$.
Mean of group O_3-Oil is denoted M_{O3}, mean of group CHX is denoted M_C and mean of group PVP-I is denoted M_P.

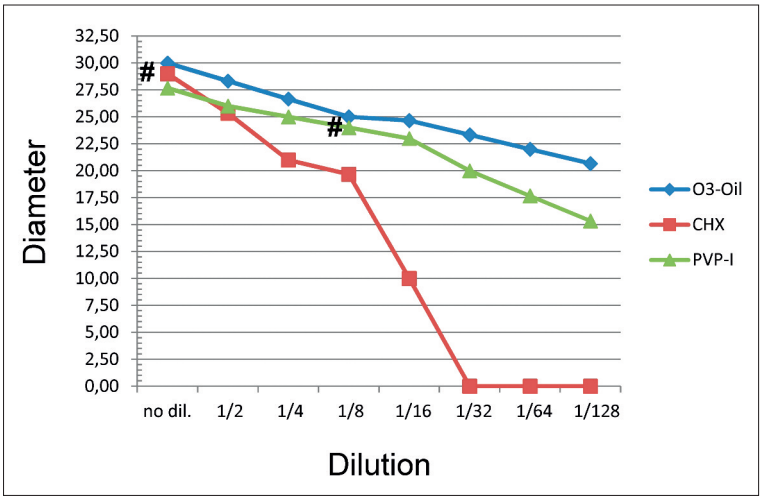

FIGURE 1 - *Mean diameters in millimeters of the inhibition zones at different dilutions for ozonated oil (O_3-Oil), chlorhexidine (CHX) and povidone-iodine (PVP-I) on Staphylococcus aureus ATCC® 29213™. #not significantly different from the O_3-Oil (test group) at the α = .01 level of significance using Tukey-Kramer method (see tables for details).*

givalis (Figure 2), O_3-Oil showed a significantly better (p<0.01) antibacterial efficacy than 0.2% CHX and 10% PVP-I in all dilutions except for 1:4 PVP-I and 1:8 PVP-I.

Although for both tested strains some diameter values of the two controls did not differ significantly (p<0.01) from the test group, we can argue that these results are equally interesting because they were statistically different at the 0.05 level of significance. As a whole, the results of the present study demonstrate that the tested O_3-Oil has a better antibacterial efficacy than 0.2% CHX and 10% PVP-I at all dilutions. In particular CHX lost any antibacterial efficacy when diluted more than 1:32 for both the bacterial strains tested.

O_3-Oil demonstrated a higher effectiveness than PVP-I even in greater dilutions. At higher dilutions (1:16, 1:32, 1:64 and 1:28) the O_3-Oil has greater inhibition haloes than PVP-I for both bacterial strains (the mean difference is 4±1 mm in diameter). These differences were statistically significant using the Tukey-Kramer method (p<0.01). The data of the present study are in accordance with the results of Sechi *et al.* (2001) who demonstrated, on a broth dilution test, the antibacterial activity of a sunflower O_3-Oil against *S. aureus* and other Gram-negative freeze-dried strains. Rodrigues *et al.* (2004) tested an ozonated sunflower oil against some yeasts and bacteria: they found a mean diameter of 42.4 mm for *S. Aureus* which is greater than the diameters obtained in the present paper. The results of the present study agree with the results of Siqueira *et al.* (2000) although they found lower mean di-

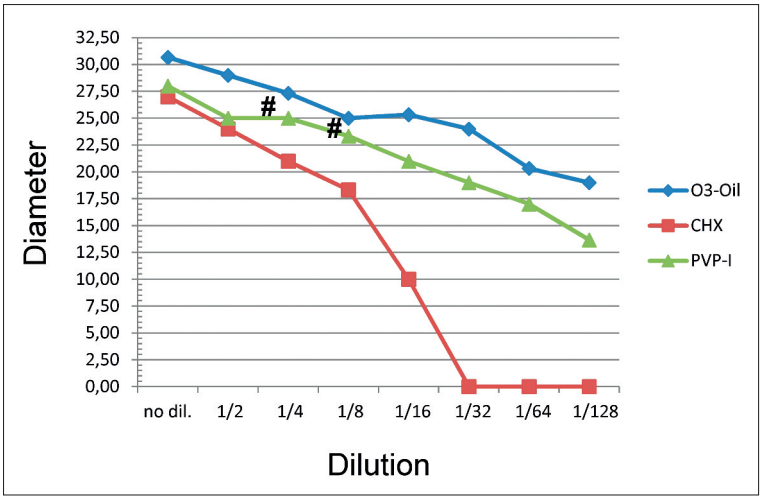

FIGURE 2 - *Mean diameters in millimeters of the inhibition zones at different dilutions for ozonated oil (O_3-Oil), chlorhexidine (CHX) and povidone-iodine (PVP-I) on Porphyromonas gingivalis ATCC® 33277™. #not significantly different from the O_3-Oil (test group) at the α = .01 level of significance using Tukey-Kramer method (see tables for details).*

ameter values 14.9±4.1 mm (against 11 bacterial strains) testing an undiluted sunflower O_3-Oil. However, instead of using cellulose filters, they made holes in the culture medium. They also did not mention how much O_3-Oil they applied in each hole. The results are different from our study because they tested different bacteria strains and different qualities of O_3-Oil.

In order to understand the different results observed in these studies, we have to explore the physicochemical properties of ozonated vegetable oils. O_3-Oil are antiseptics obtained from the chemical reaction between ozone and unsaturated fatty acids of vegetable oils. A new ozonated virgin olive oil with antiseptic indications (Novox®) has recently been introduced on the Italian market. Virgin olive oil contains mainly unsaturated fatty acids (mean value >80%) (Beltrán *et al.*, 2004; Dag *et al.*, 2011). Unsaturated fatty acids are fatty acids in which there is at least one carbon-to-carbon double bond (alkene) within the fatty acid chain.

The chemical reaction between ozone and carbon-to-carbon double bonds is called ozonolysis and is depicted in Figure 3. The mechanism of this reaction was first described by Criegee *et al.* in the 1960s and later confirmed by other authors. (Criegee, 1975; Kuczkowski, 1983; Geletneky *et al.*, 1998).

When ozone is combined with virgin olive oil, its primary targets are carbon to carbon double bonds of unsaturated fatty acids to form an initial unstable molozonide (1). The molozonide is very unstable and rapidly cleaves to a stable carbonyl compound (3) and a carbonyl oxide (2). In an anhydrous environment (II), carbonyl oxide quickly combines with carbonyl compound to produce a secondary ozonide (4). When the secondary ozonide comes into contact with tissues (1), the carbonyl oxide reacts with water to give hydroxyhydroperoxydes (5) and, ultimately, hydrogen peroxide (6) and a second mole of carbonyl compound (7). The possible mechanism by which O_3-Oil act as an antiseptic is the oxidation

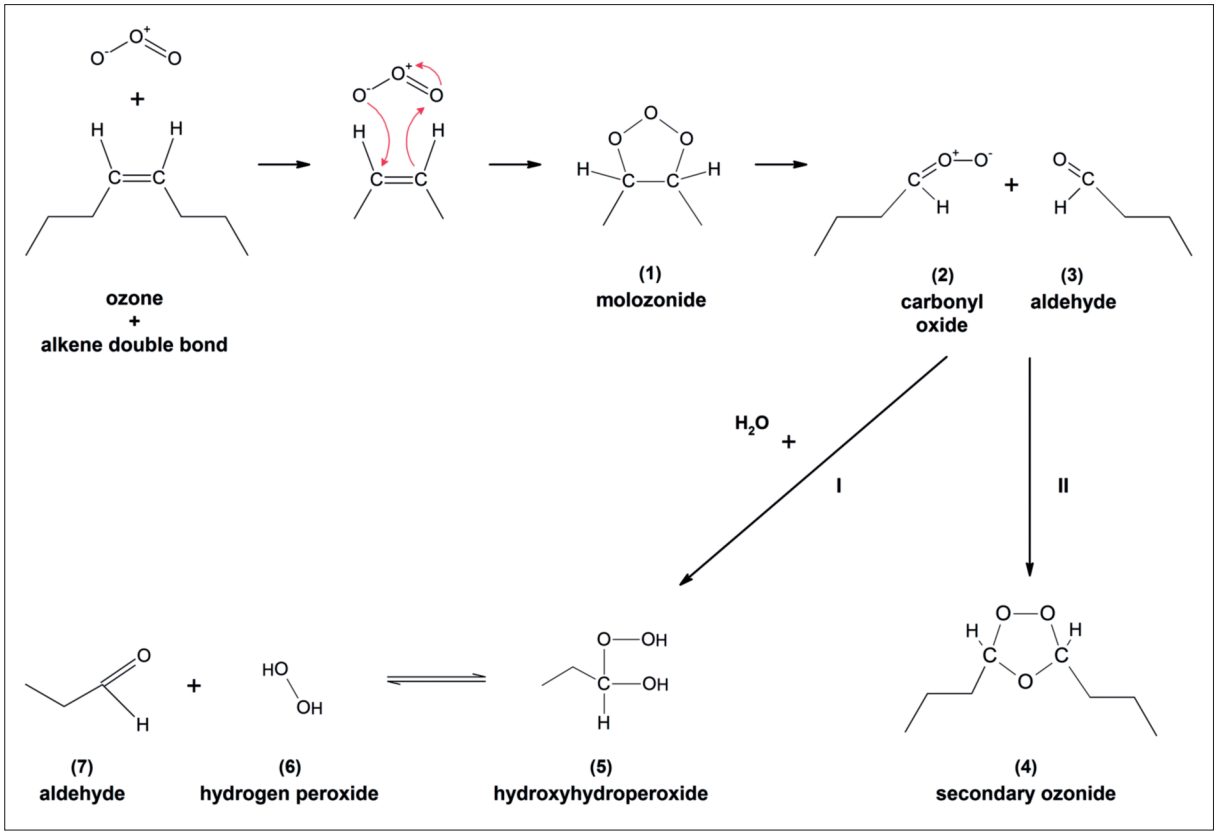

FIGURE 3 - *Criegee Mechanism for carbon-to-carbon double bonds of unsaturated fatty acids*

of microorganisms through a slow release of peroxides (Travagli *et al.*, 2010; Valacchi *et al.*, 2011). However, the ozonolysis reaction is meaningless if we are not able to quantify how much peroxide could be released by O_3-Oil. The main quantitative methods developed for determining the quality of O_3-Oil are: peroxide value, acid value and iodine value. The peroxide value (I_P) is the number that expresses in milliequivalents of active oxygen the quantity of peroxide contained in 1000 g of the substance (British Pharmacopoeia, 2012). I_P is an indicator of how much active oxygen could be released: the higher I_P, the stronger antimicrobial effectiveness of O_3-Oil (Díaz *et al.*, 2006; Travagli *et al.*, 2010). The acid value (I_A) is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralise the free acids present in 1 g of the substance (British Pharmacopoeia, 2012). I_A is a measure of the products of oxidation such as carbonyl oxides and carbonyl compounds, as a result of the ozonolysis reaction (Díaz *et al.*, 2006; Travagli *et al.*, 2010). The iodine value (I_I) is the number that expresses in grams the quantity of halogen, calculated as iodine, that can be fixed in the prescribed conditions by 100 g of the substance (British Pharmacopoeia, 2012). I_I is a marker of double bond content in O_3-Oil; ozonolysis reaction leads to the rapid decrease of iodine value: if I_I is zero, all unsaturated groups have reacted with ozone (Díaz *et al.*, 2006; Travagli *et al.*, 2010).

During the ozonolysis reaction there is typically an increase in peroxide and acid values and a decrease of iodine value (Díaz *et al.*, 2006; Skalska *et al.*, 2009; Sega *et al.*, 2010). Díaz *et al.* (2006) demonstrated that an ozonated olive oil at the peroxide value of 2506 mmol-equiv/kg has a MIC and BIC for the bacterial strain *Staphylococcus aureus* ATCC® 6538™ of 0.95 mg/ml and 11.12 mg/ml respectively. Similarly, Lezcano *et al.* (2000), studied the activity of an ozonated sunflower oil with a peroxide value ranging from 500 to 800 mmol-equiv/kg on *Staphylococcus aureus* ATCC® 25923™. The value of MIC was 9.5 mg/ml and the value of BIC was 356 mg/ml. These results were later confirmed by Sechi *et al.* (2001) testing the same ozonated oil against *Staphylococcus aureus* ATCC® 29213™.

The discrepancy among these results could be attributed to different ATCC strains, different peroxide values, different acid values and different iodine values. The peroxide values diversity of the O_3-Oil tested could also explain the differences in diameters between this paper and the repots by Rodrigues *et al.* (2004) and Siqueira *et al.* (2000). These considerations bring us to two important conclusions. First of all, O_3-Oil manufacturers should describe the peroxide, acid and iodine values on the product label. Second, further studies considering the standardization of ozonolysis procedures for vegetable oils are warranted.

The use of O_3-Oil could raise some toxicological questions. However, Azarpazhooh *et al.* (2008), in their systematic review of literature, stated that there is good evidence of ozone biocompatibility with human oral epithelial cells, gingival fibroblast, and periodontal cells.

Staphylococcal infections are particularly difficult to treat because *S. aureus* is able to develop resistance to antimicrobial drugs. Since new antibiotics have become available, staphylococci have developed efficient mechanisms to inactivate them. In the early 1940s penicillin became available for civilian use. However, in 1942, penicillin-resistant staphylococci were isolated. In order to resolve this problem, in 1959, a new antibiotic called methicillin was introduced into clinical practice. Nevertheless, just one year later, methicillin-resistant *S. aureus* isolates (MRSA) were recognized (Jevons *et al.*, 1963). Since this event, MRSA has spread globally in many hospitals and communities as causative agent of infections (Chambers, 1997; Chambers, 2001; Vandenesch *et al.*, 2003). To date, clear evidence suggests that some *S. aureus* strains manifest reduced susceptibility or resistance to vancomycin and other glycopeptides (CDC, 2002; Chang *et al.* 2003; Howden *et al.*, 2010). This is a serious clinical problem because glycopeptides are considered the gold standard for treating MRSA infections (Liu *et al.*, 2011a; Liu *et al.*, 2011b). Staphylococcal strains are the most common multidrug-resistant organisms causing health care-associated infections. Klein *et al.* (2007) conducted an epidemiological study in the USA from 1999 to 2005 and estimated that the number of *S. aureus*-related hospitalizations increased 62% (from 294,570 to 477,927) and MRSA-related hospitalizations more than doubled, from 127,036 to 278,203. Skin and soft tissues are the most common sites of infection (42.9%) (Jarvis *et al.*, 2012).

S. aureus is also significantly higher in number and frequency in subgingival biofilm of peri-implantitis than in healthy dental implants (Rams *et al.*, 1990; Leonhardt *et al.*, 1999; Persson *et al.*, 2013).

This clear evidence indicates that multiresistant staphylococcal strains are a serious clinical problem because of increasing prevalence and the continuing occurrence of resistance to antibiotics. Therefore, a topic of great interest in the literature is the development of new and effective therapy alternatives to antibiotics.

Kurlenda *et al.* (2012) recently reviewed the main alternatives proposed by the literature: antimicrobial peptides (e.g. bacteriocins and lysostaphin), plant-derived compounds (e.g. stilbenoids and flavonoids), animal-derived compounds (e.g. propolis), photodynamic therapy and vaccines. The encouraging results of the present paper suggest that ozonated oils could be taken into account as an innovative therapeutic option for treating multi-resistant staphylococcal infections and especially skin and soft-tissue infections such as: surgical sit infections, burns infections and diabetic foot ulcer infections.

The other tested strains in this study was *P. gingivalis*, one of the main periodontal pathogens (Consensus Report., 1996). This bacterium is a Gram-negative, anaerobic, non-motile, asaccharolytic rod that forms round, convex black-pigmented colonies. There is clear evidence that *P. gingivalis* is more common in number and frequency in deteriorating periodontal sites, whereas it is absent or in low numbers in healthy sites (Dzink *et al.*, 1988; Kamma *et al.*, 2001). Furthermore, *P. gingivalis* is significantly decreased in successfully treated sites (Haffajee *et al.*, 1997). This species has been shown to produce a large range of virulence factors: collagenase, endotoxin, fibrinolysin, haemolysin, fibroblast inhibitory factors, factors that inhibit migration of PMNs across epithelial barriers, bone resorption-inducing factor, and so forth (Haffajee *et al.*, 1994). *P. gingivalis* is not solely responsible for periodontal disease: clear evidence suggests that both periodontitis and peri-implantitis harbor the same type of bacteria species (Cortelli *et al.*, 2013).

There is currently no doubt about the beneficial effects of scaling and root planing (SRP) for treating periodontal diseases (Haffajee *et al.*, 1997; Checchi *et al.*, 2002). The effects of combining SRP with locally administered antimicrobial agents has been evaluated in a number of studies (Unsal *et al.*, 1994; Hoang *et al.*, 2003; Perinetti *et al.*, 2004; Tomasi *et al.*, 2004; Cosyn *et al.*, 2005; Quirynen *et al.*, 2006; Renvert *et al.*, 2006; Cosyn *et al.*, 2006; Cosyn *et al.*, 2007a; Cosyn *et al.*, 2007b; Krück *et al.* 2012). As a whole, these studies suggest that adjunctive locally administered antimicrobial agents produce an additional improvement in clinical and microbiological parameters in comparison with SRP alone.

Although there is strong evidence supporting the use of several local antimicrobial agents with SRP, there is only one clinical study which evaluated the effectiveness of a locally administered O_3-Oil as an adjunct to scaling and root planing (Patel *et al.*, 2012). In their randomized controlled clinical trial, Patel *et al.* (2012) demonstrated that the subgingival administration of ozonated olive oil as an adjunct to SRP resulted in a significant improvement of clinical and microbiological parameters in comparison with SRP alone. Given the evidence provided by this paper, further research on the effectiveness of local administration of ozonated olive oil for treatment of periodontitis and peri-implantitis is warranted.

During the colonization of oral hard surfaces, bacteria tend to adhere to each other on the tooth surface leading to the formation of a biofilm. This community of microorganisms is embedded in a self-produced matrix and is firmly stuck to tooth surfaces and dental implants. Bacterial species growing within the biofilm enjoy many advantages over single cell bacteria. The most direct advantages are protection from host immune defence mechanisms, protection from competing microorganisms and protection from antibacterial agents such as antibiotics or antiseptics (Socransky *et al.*, 2002; Marsh, 2005). Biofilm formation is also an important problem in the antibiotic therapy of staphylococcal infections. If we accept this premise, this study has one important limitation. Its results are not valid for clinical purposes, because organisms growing in biofilm are more resistant to antimicrobial agents than the same species growing in vitro. More clinical trials on the effectiveness of ozonated oils against biofilm on tooth surfaces and dental implants are warranted.

Another shortcoming of our study is that we test-

ed the antimicrobial efficacy against freeze-dried microorganisms instead of bacterial strains coming from microbial samples collected from patients. If we had collected microbial samples from patients to test the antiseptics against a larger number of strains, the results would have been of greater scientific value.

To date, the evidence evaluating the effect of O_3-Oil in dentistry is poor. It would be interesting to further evaluate the potential beneficial effects of this new antiseptic for treating widely diffused infectious diseases of the oral cavity such as periodontitis and peri-implantitis. Cross-sectional studies in Europe show that moderate periodontitis occurs in 38-27% of the population and severe periodontits occurs in 13-11% of the population (Hugoson *et al.*, 1992; Hugoson *et al.*, 1998; Hugoson *et al.*, 2005). Cross-sectional studies on implant-treated patients show that peri-implantitis in present in 28-56% of patients and in 12-43% of implant sites (Fransson *et al.*, 2005; Zitzmann *et al.*, 2008). This means that treating periodontitis and peri-implantitis is important for improving the oral health of a large portion of the population.

Nowadays, dental implants are one of the most common treatment options used in the replacement of missing teeth. The increasing number of inserted dental implants has resulted in an increased frequency of peri-implantitis and subsequent implant loss. The screw-shaped design of the implants, combined with various surface modifications of titanium, may facilitate biofilm accumulation. Periodontal treatment regimes were assigned to peri-implantitis, but they are mainly based on individual experiences and preferences, and not standardized or scientifically justified. It would be of great scientific value to investigate the role of O_3-Oil as antiseptics in the treatment of periodontitis and peri-implantitis. If O_3-Oil is demonstrated to be an effective antiseptic, it will be possible to offer easier and more socially affordable treatment protocols for periodontitis and peri-implantitis. Developing easier treatment protocols means that thorough and safe treatment could also be provided for medically compromised patients who cannot undergo conventional invasive procedures. The effectiveness of the O_3-Oil would be economically relevant since its industrial production is cheaper than other widely used antiseptics. Having an effective and low-cost antiseptic could provide dental treatments for patients who usually cannot afford dental therapy.

Within the limitations of this *in vitro* study, the data presented in this work suggest that Novox®, an ozonated extravirgin olive oil, is a more effective antiseptic than chlorhexidine digluconate and povidone-iodine against *S. aureus* and the periodontal pathogen *P. gingivalis*.

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