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Original Article

Cytotoxicity evaluation of a new ozonized olive oil

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ABSTRACT

Objective: The cytocompatibility of a new ozonized olive oil toward immortalized human gingival fibroblasts (HGFs) was evaluated and compared with two common antimicrobial agents based on chlorhexidine digluconate (CHX). **Materials and Methods:** The cytocompatibility of the samples was tested on immortalized HGF-1 cells by 3-(4, 5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were incubated for 2 or 24 h with increasing dilution of ozonized olive oil or CHX agents. The percentage of viable cells was calculated relative to control cells set to 100%. **Results:** The ozonized olive oil is cytocompatible, and the viability values of the cells treated for 2 or 24 h with increasing concentrations of ozonized olive oil were significantly higher ($P < 0.01$) compared with the values obtained using CHX. **Conclusions:** The present data demonstrate that due to its cytocompatibility, the new ozonized olive oil could be considered an alternative antibacterial agent.

Key words: 3-(4, 5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide test, cytotoxicity, gingival fibroblast, ozonized olive oil

INTRODUCTION

The use and approval of ozone therapy among dental and medical professionals have been increasing during recent years. It has been suggested for the cure of more than 250 different pathologies.[1] This is due to the characteristics of ozone including its high oxidative power, stimulation of blood circulation and immune response, its analgesic properties, and its strong antimicrobial activity against viruses, bacteria, fungi, and protozoa.[2]

Ozone is used in many dental therapies including tissue regeneration and postsurgery healing,[3] tooth surface remineralization and treatment of early dental caries,[4,5] root canal disinfection,[6] periodontal pocket therapy,[7] teeth whitening and management of tooth sensitivity,[8,9] and pain control and temporomandibular joint treatment.[10]

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Plaque biofilm is the main cause of both caries and periodontal disease. Ozone has been demonstrated to be useful to control oral infectious microorganisms in dental plaque.[11] In dental treatments, ozone can be applied in three different forms: ozone gas, ozonated water, and ozonated oil.[1] Ozonated water strongly inhibited the accumulation of experimental dental plaque.[12] Similarly, ozonated oils such as ozonated sunflower oil, olive oil, and groundnut oil were capable of inducing the reduction of many oral microorganisms.[13]

Ozone exerts its own antimicrobial action through the synergistic action of damaging the cytoplasmic membrane of cells and of inducing the modification of

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intracellular contents because of secondary oxidants effects.[14] This action is nonspecific and selective to microbial cells; however, the activity of ozone on human cells has been investigated considering the importance of complete biocompatibility of ozone in its use in dental practice.[15]

O-zone gel (Alnitec, Cremosano, CR, Italy) is a new ozonated olive oil with bactericidal and fungicidal properties whose use is suggested for the periodontal treatments of both gingivitis and periodontitis affections and for the root canal disinfection during endodontic therapies.

The aim of the present study is to evaluate the biocompatibility of this new ozonized olive oil on immortalized human gingival fibroblasts (HGFs) and to compare it with two common antimicrobial agents based on chlorhexidine digluconate (CHX). The null hypothesis of the study was that the ozonated oil did not demonstrate cytotoxic effects; therefore, there was no difference between the cytotoxic capability of the three different antimicrobial agents tested.

MATERIALS AND METHODS

A new ozonized olive oil was selected for this study: O-zone gel (Alnitec, Cremosano, CR, Italy). Cytocompatibility was compared with two common antimicrobial agents based on CHX : Corsodyl Dental Gel® (GlaxoSmithKline, Brentford, Middlesex, UK) and Plak Gel® (Polifarma, Rome, Italy).

Cell culture

Immortalized HGF-1 (ATCC CRL-2014) was obtained from the American Type Culture Collection and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 4 mM L-glutamine (Sigma-Aldrich), 1% penicillin, streptomycin (Sigma-Aldrich), and 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich). Cells were incubated at 37°C in 5% CO_2 in T75 flasks to approximately 95% confluence, liberated with trypsin-EDTA (0.05%; Sigma-Aldrich), and plated as reported below.

Cytocompatibility test

Cells were plated at 1×10^4 into 96-well plates (Corning) and allowed to attach for 24 h at 37°C. The following day, the ozonized olive oil and the CHX agents namely Corsodyl Dental Gel® or Plak Gel® were diluted 1:10 for 4 times in DMEM. The cell medium was removed from the well, and 100 µL of each diluted test agent was applied to the cell monolayers. As negative control, fresh medium was used. After 2 or 24 h of incubation at 37°C, the cell medium was pipetted off from each well and HGFs viability determined using the 3-(4, 5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. 100 µL of MTT solution (Sigma-Aldrich) in RPMI-1640 without phenol red (Sigma-Aldrich) (5 mg/mL) was added to each well, and the monolayers were incubated for 4 h at 37°C. Then, the supernatant was removed, and the resulting formazan was dissolved by adding 100 µL dimethyl sulfoxide (Sigma-Aldrich) to each well. The optical density of formazan dye was read at 545 nm against 620 nm as background by ELISA reader (Bio-Rad, Hercules, CA, USA). The percentage of viable cells in each well was calculated relative to control cells set to 100%. Cytotoxicity responses were rated as severe (30%), moderate (30%–60%), mild (60%–90%), or noncytotoxic (>90%).[16]

Statistical analysis

Cell viability data were analyzed by one-way ANOVA, followed by Bonferroni's *post hoc* tests. Analyses were performed using Prism 4.0 (GraphPad Software Inc., La Jolla, CA, USA). Two-tailed $P = 0.05$ was considered statistically significant.

RESULTS

To evaluate cell viability in the presence of increasing dilution of the three gingival gels tested, an MTT assay was performed. The results are shown in Tables 1 and 2 and collectively represented in Figure 1.

No cytotoxic effect was measured by incubating the cell monolayers for 2 h with all the dilutions tested of the ozonized olive oil. The differences in cell viability were statistically significantly lower ($P < 0.01$) when the cell monolayers were incubated with increasing dilution of both CHX agents: Corsodyl Dental Gel® or Plak Gel®.

Table 1: Viability numerical values of the human gingival fibroblast treated for 2 h with increasing dilution of different gingival gels

Samples	2 h Dilutions			
	1:10	$1:10^2$	$1:10^3$	$1:10^4$
A	124.20±10.17 Noncytotoxic	124.02±13.25 Noncytotoxic	132.96±7.60 Noncytotoxic	133.09±7.57 Noncytotoxic
B	26.24±8.16 Severe	31.14±3.97 Moderate	30.29±4.47 Moderate	31.81±3.13 Moderate
C	28.87±5.19 Severe	30.35±3.65 Moderate	35.46±7.76 Moderate	44.08±15.92 Moderate

A: O-zone gel, B: Corsodyl Dental Gel®, C: Plak Gel®. Cytotoxicity responses were rated as severe (30%), moderate (30%–60%), mild (60%–90%) or noncytotoxic (>90%)

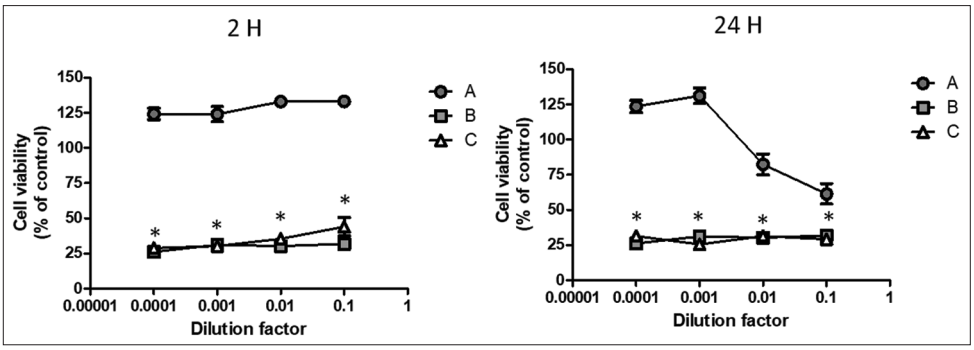

Figure 1: Time-dependent effects of different gingival gels on viability of human gingival fibroblast. Confluent human gingival fibroblast was treated for 2 or 24 h with 10-fold dilution of different gingival gels (A: O-zone gel; B: Corsodyl Dental Gel®; C: Plak Gel®). The cell viability was measured by the 3-(4, 5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide assay. Statistically significant ($P < 0.05$; Student's *t*-test) differences in values compared with the control value (untreated) are indicated by an asterisk. Bars and error bars represent the means and ± standard deviation from three independent determinations performed in triplicate

Table 2: Viability numerical values of the human gingival fibroblast treated for 24 h with increasing dilution of different gingival gels

Samples	24 h Dilutions			
	1:10	$1:10^2$	$1:10^3$	$1:10^4$
A	61.39±17.72 Moderate	82.38±18.14 Mild	131.16±13.48 Noncytotoxic	123.55±10.72 Noncytotoxic
B	28.12±8.16 Severe	32.16±3.79 Moderate	30.92±4.74 Moderate	31.18±3.31 Moderate
C	29.17±6.83 Severe	31.44±3.68 Moderate	30.57±7.75 Moderate	30.44±3.31 Moderate

A: O-zone gel, B: Corsodyl Dental Gel®, C: Plak Gel®. Cytotoxicity responses were rated as severe (30%), moderate (30%–60%), mild (60%–90%) or noncytotoxic (>90%)

Moreover, their cell viability (severe/moderate) data were also statistically significantly lower compared to the control ($P < 0.01$) (culture medium only).

No cytotoxic effect was measured by incubating 24 h the cell monolayers with the ozonized olive oil diluted 10^3 and 10^4 times. Instead, the cytotoxic effect was mild using the gel diluted 10^2 times. The cytotoxic effect was moderate using the gel diluted 10 times and the differences in cell viability were statistically significant compared to the control ($P < 0.05$). The differences in cell viability remained severe/moderate for all the gel dilutions tested and statistically significantly lower ($P < 0.01$) compared to both the cell monolayers treated with the ozonized olive oil and the control.

DISCUSSION

The production of ozone (O_3) is naturally obtained by the photodissociation of molecular oxygen (O_2) into activated oxygen atoms, which then react with further oxygen molecules.[1] This transient radical anion rapidly becomes protonated, generating hydrogen trioxide (HO_3), which, in turn, decomposes to an even more powerful oxidant, the hydroxyl radical (OH).[1]

For the treatment of infections, the use of ozone became an inherent element in such fields as surgery, dermatology, cosmetics, and dentistry during the last few years.[17]

Ozone therapy is based on various useful effects. Ozone is not only an antimicrobial agent, but it can also enhance blood circulation and immune response. Ozone can modulate cellular and humoral immune system, by the proliferation of immunocompetent cells and synthesis of immunoglobulins. It can stimulate the phagocytosis process, activate the macrophages, and increase the sensitivity of microorganisms to macrophages.[18]

The European Cooperation of Medical Ozone Societies forbids the direct intravenous injections of ozone/oxygen gas due to the risk of air embolism.[19] There are three basic forms of ozone application: ozone gas, ozonated water, and ozonated oil.[1] In this study, the new ozonized olive oil O-zone gel (Alnitec), suggested as a coadjutant in periodontal and endodontic treatments, was tested.

The oxidative power of ozone is 1.5 times greater than that of chloride when used as an antimicrobial agent.[1] This effect of oxidation gives to ozone its most important property: its bactericidal, virucidal, and fungicidal activity.[12] According to microbiological studies, ozone is capable of killing all the known types of Gram-positive and Gram-negative bacteria, including the *Pseudomonas aeruginosa* and *Escherichia coli*, both of which are extremely resistant to antibiotics.[1] This antimicrobial capacity is the result of ozone effects on cells such as damaging the cytoplasmic membrane due

to ozonolysis of dual bonds and inducing changes of cytoplasmic contents. This action seems not to damage human body cells; the reason attributed to this is antioxidant ability of mammalian cells.[20]

In our study, the cytocompatibility of ozone (and in this case of an ozonated oil) toward human cells was tested. The cytotoxicity of the different antimicrobial agents has been investigated, using the MTT assay. The MTT test is a standard colorimetric assay for measuring the activity of enzymes that reduce the MTT to formazan (a salt blue) in the mitochondria, giving the substance a blue/purple color. This technique has been widely used to characterize the cytocompatibility of various dental materials.[21]

The null hypothesis of this study was partially rejected. In fact, the new ozonated oil did not show any cytotoxic effects; however, differences between the cytotoxic capabilities of the three different antimicrobial agents tested were demonstrated.

No cytotoxic activity of the ozonated olive oil was revealed by incubating the cell monolayers for 2 h with all the dilutions tested. However, all the increasing dilutions of both CHX agents demonstrated moderate or severe cytotoxic responses after 2 h of testing. After 24 h of incubation, no cytotoxic effect was measured for the ozonized olive oil diluted 10^3 and 10^4 times. However, a mild cytotoxicity was registered using the ozone oil diluted 10^2 and 10 times. The cell viability of both the CHX agents tested remained severe/moderate for all the dilutions even after 24 h of experimentation.

Recent studies have assessed the antimicrobial capability of ozonated oils against different microbiological strains. Gram-negative bacteria, such as *Porphyromonas endodontalis* and *Porphyromonas gingivalis*, proved to be more sensitive to ozone oil than Gram-positive streptococci.[22] Huth *et al*. compared the bactericidal effects of ozone oil against periodontal microorganisms; ozone gel showed more effectiveness than 0.2% CHX.[23] In another study, the effect of oral irrigations with ozonated oil, 0.2% CHX, or 10% povidone–iodine in patients with chronic periodontal disease was evaluated, stating that the use of ozone can serve as potent atraumatic alternative to treat periodontal pockets nonsurgically.[24] In root canal therapy, ozonated oils proved to be more efficient than the conventional irrigation by sodium hypochlorite and sodium peroxide combination.[13]

However, few studies have evaluated the cytocompatibility of ozonated oils, comparing it with that of other antimicrobial agents used in periodontal or endodontic therapies. In this study, the ozone oil tested prove to be a biocompatible agent; contrariwise, the CHX-based gels showed higher degrees of cytotoxicity both after 2 and 24 h of incubation. Only after 24 h, a fair cytotoxic effect of O-zone gel diluted 10^2 and 10 times was registered. Anyhow, we suppose that this value of cytotoxicity of ozone gel after 24 h may be considered irrelevant because the product applied to the gingiva tends to dilute progressively over time, and after 24 h, it has abundantly diluted with saliva.

The findings of our study are in accordance with recent studies. Nagayoshi *et al*. compared the biological properties of an ozone oil with that of sodium hypochlorite, thus demonstrating that the metabolic activity of human fibroblasts was high when treated with ozonated oil.[25] Other authors showed less cytotoxic effect of ozone, as a potential antiseptic agent, if compared to other antimicrobials such as CHX, sodium hypochlorite, or hydrogen peroxide.[26] Therefore, ozone gel fulfills optimal cell biological characteristics in terms of biocompatibility for oral application.[15]

CONCLUSIONS

Within the limitations of this *in vitro* study, the ozonated olive oil tested proved to be a biocompatible agent, while the chlorhexidine-based gels demonstrated higher cytotoxic effects. Due to its cytocompatibility, ozonated olive oil may be considered an alternative to conventional antimicrobials agents in periodontal or endodontic treatments.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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