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The activity of ozonated olive oil against *Leishmania major* promastigotes

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ABSTRACT

Objective(s): Cutaneous Leishmaniasis is a common and endemic disease in Khorasan province in North-East of Iran. The pentavalant antimony (Sb V) is the mainstay of treatment that has many side effects and resistance to the drug has been reported. The microbicidal effect of ozone was proven in different microorganisms. Since there is no study in this respect and to achieve a low cost and effective treatment, we decided to evaluate the efficacy of ozone against promastigotes of *Leishmania major*, *in vitro*.

Materials and Methods: Ozonated olive oil was prepared after production of ozone by bubbling ozone-oxygen gas produced by ozone generator through olive oil until it solidified. Promastigotes of *L. major* were cultivated in two phasic media. After calculation of the number of promastigotes, they were incubated with ozonated olive oil (0, 0.626, 0.938, 1.25, 2.5, 5, 10 mcg/ml) at 28 ˚c for 24 hr. Parasites survival percentage was evaluated using MTS and microscopic assay, and then compared with Glucantime and non-ozonated olive oil.

Results: According to the results, there were significant differences in parasites survival percentage between ozonated olive oil and non-ozonated olive oil, at similar concentrations (P<0.001). Ozonated olive oil was more effective than Glucantime. According to MTS results, Glucantime and ozonated olive oil gel concentrations that are required to inhibit the growth of *L. major* promastigotes by 50% (IC_{50}), were 165 and 0.002 mg/ml, respectively.

Conclusion: Ozonated olive oil has *in vitro* activity against the promastigotes of *L. major* and this effect is dose dependent.

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Introduction

Leishmaniasis is a group of infectious diseases that are caused by different species of parasites belonging to the Leishmania genus (1). Although leishmaniasis is estimated to cause the ninth largest disease burden among individual infectious diseases, it is largely neglected in considerations of tropical disease priorities (2, 3). Leishmaniasis is endemic in 15 out of 30 provinces of Iran (4) and a recent study showed that Iran is among the top ten countries with the highest estimated case counts that together account for 70 to 75% of global estimated Cutaneous Leishmaniasis (CL) incidence (5).

It is a complex disease manifesting as cutaneous, muco-cutaneous and visceral forms. Amongst these forms, CL is the most common type of the disease and could result in severe skin infection (6). The choice of the treatment is dependent on size, number, and location of lesions, Leishmania species and also the accessibility of treatment modalities (7).

Systemic agents against Leishmaniasis are expensive and limited to a few drugs with inconsistent efficacy and unacceptable side effects (8-10). There is not any treatment for all forms of the disease, so far. The first line of the treatment of CL in Iran is pentavalent antimony (SbV). Resistance to SbV containing drugs is now well established and it was found to occur in some regions (11-13). Few alternatives to SbV are available and the resistant parasites are cross-resistant to some of them (14). So, an effective topical drug would be valuable if it offers a safe and unsupervised treatment at a reasonable cost. Various forms of topical treatment serve as alternative drugs in the control of leishmaniasis (15).

Ozone, an allotropic form of oxygen in nature, has a molecular weight of 48 and a density of one and a half times that of oxygen, and consists of a large

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overload of energy in its molecule. It has been used in human medical practice since long to kill bacteria, fungi, some protozoa, to inactivate viruses and to control hemorrhages (16-19). Related clinical trials have shown little side effects following topical ozone therapy (20-22).

Since no previous study had been done to evaluate the efficacy of ozone therapy on leishmaniasis, we decided to determine the effect of olive oil, ozonated olive oil and meglumine antimoniate (Glucantime) on promastigotes of *L. major*, *in vitro*, compare their anti-leishmanial activity and determine the most effective dose of ozonated olive oil on promastigotes of *L. major*.

Materials and Methods

This basic interventional study was done at Research Center for Cutaneous Leishmaniasis. Here, the efficacy of ozonated olive oil and Glucantime against promastigotes of *L. major* was evaluated and was compared with each other.

Chemicals preparation

a) Olive Oil (food grade), was obtained from Tarom industrial company, north of Iran.

Olive oil (150 g) was warmed in water bath at 30 °C. Oxygen gas containing about 200 ppm ozone was then bubbled through the olive oil at a rate of 1.0 l/min for 50 hr to give ozonated olive oil as vaseline with the distinctive odor of ozone. The ozonated olive oil was stored in a refrigerator. Then 3-4 drops Tween 80 was added as an emulsifier. This product was diluted using Phenol red-free RPMI 1640 medium supplemented with 5% PBS and was tested at the concentrations of 0, 0.626, 0.938, 1.25, 2.5, 5, 10 mcg/ml.

b) Positive control: Glucantime (0, 45, 120, 150, 180 and 240 mg/ml) was prepared after dilution of Glucantime ampoule (1.5 g/5 ml, Aventis, France) as same as ozonated olive oil.

c) Olive oil as a negative control: Olive oil was mixed with 3-4 drops of Tween 80 as an emulsifier. Then, the mixture was diluted as mentioned above to prepare different concentrations.

Parasite culture

A number of 3×10^6 promastigotes of *L. major* (MRHO/IR/75/ER) were injected subcutaneously into the tails of BALB/c mice. Splenectomy was done and amastigotes in mice spleen were cultured in Novy-MacNeal-Nicolle (N.N.N) medium containing Agar (4 mg/100 ml) and defibrinated rabbit blood (10%). After transforming from amastigotes form, the promastigotes were incubated in RPMI 1640 (HIMED IA; AT 028) supplemented with 100 units/ml penicillin, streptomycin 100 µg/ml and 20% FBS at 28 ˚C (23).

Seven days later, the parasites were transformed to a culture flask containing 5-10 ml complete culture medium. The parasites were cultured for 2-3 days and used when they were at the stationary growth phase.

Promastigotes survival assay

At first, 200 µl of promastigotes suspension at the density of 1×10^7 parasite/ml was transferred to the wells of a 96-well plate and incubated at 28 ˚C for 24 hr. Then, incubation was continued in the presence of different concentrations of olive oil, ozonated olive oil or Glucantime for 3 hr. After 24 hr, promastigotes survival percentage was assessed using MTS solution ([4, 5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt). Optical density of the samples was read by an ELISA Micro plate Reader (USA, STAT FAX 2100) at 492 nm and parasite survival was calculated for all samples in comparison with the control. Also, under similar conditions, the number of alive promastigotes treated with olive oil, ozonated olive oil and Glucantime was also determined using a hemocytometer slide under a light microscope in a separate experiment seri and the promastigotes survival percentage was calculated relative to control group. Since Glucantime with MTS produces a color like the color of reduced MTS produced by alive parasites, we subtracted its optical absorption from all experimental groups treated with Glucantime.

Statistical analysis

Statistical analysis was performed using SPSS software version 16 by Kolmogorov-Smirnov, One way ANOVA and Tukey statistical tests and Independent sample t-test. The *P*-values of lower than 0.05 were considered significant. IC_{50} value (the concentration that is required to inhibit the growth of *L. major* promastigotes by 50%) was calculated using linear regression analysis or linear interpolation for Glucantime and ozonated olive oil gel.

Results

Figures 1 and 2 show that increasing concentrations of ozonated olive oil and Glucantime led to a decrease in parasite survival and had an increasing leishmanicidal activity against *L. major* promastigotes which was significantly different among different concentrations ($P<0.001$). Olive oil alone had no significant leishmanicidal activity. Comparison of data related to similar concentrations of olive oil and ozonated olive oil revealed significant differences between the two groups ($P<0.05$) (Figure 1). The anti-leishmanial activity at the highest concentration of ozonated olive oil was significantly more than that of Glucantime ($P<0.001$).

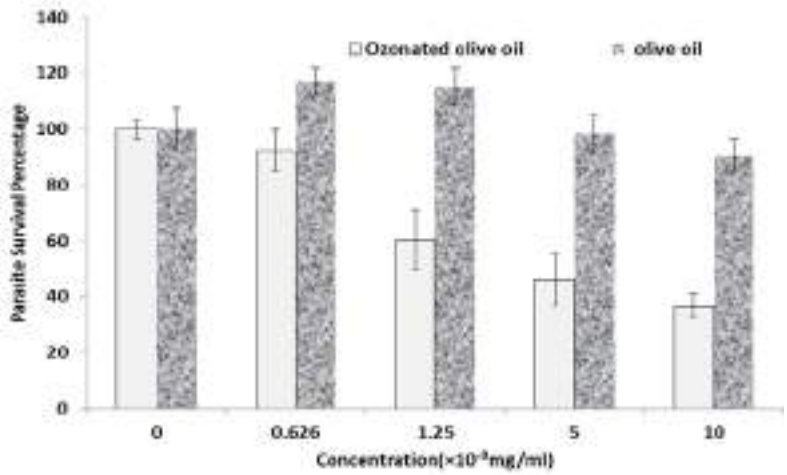

Figure 1. Comparison of mean parasite survival percentage in similar concentrations of ozonated olive oil and olive oil using MTS method. Data are shown as Mean±SD

Considering mean alive promastigotes determined by MTS method and light microscope, there were significant differences among different concentrations of Glucantime and some concentrations of ozonated olive oil ($P \leq 0.001$ and $P=0.01$, respectively). As it has been shown in Figures 2 and 3, on the basis of the microscopic observations and MTS assay data, IC_{50} were 120 mg/ml and 165 mg/ml for Glucantim and almost 2 and 3.5 mcg/ml for ozonated olive oil.

Discussion

Treatment of leishmaniasis is still dependant on pentavalent antimonial drugs, but the emergence of drug-resistant parasites shows treatment failures (11-13). So, seeking for a new inexpensive more effective safer drug which is preferably administered topically is a logical aim.

Ozone has been shown to be a powerful and reliable anti-microbial agent against bacteria, fungi, protozoa, and viruses. In a randomized study, ozonated sunflower oil was shown to be more effective than ketoconazole in the treatment of onychomycosis (22). Ozonated olive oil has germicidal action and was used in the treatment of tinea pedis (24). Inactivation of other pathogenic fungi like Candida albicans and Aspergillus nigerwas reported by Coronel *et al* (25). Also, ringworm infection caused by Trichophyton, Microsporum, and Epidermophyton species was studied by Gupta *et al* (26). Strong bactericidal activity of ozonated water against bacteria in plaque biofilm has been frequently reported in dentistry for improvement of periodontal diseases, chronic gingival disorders and oral hygiene (27). Moreover, Sechi *et al* noted an interesting antimicrobial activity for ozonated sunflower oil against Mycobacterium (28).

With respect to the toxic effects of ozone and its compounds, since there is no study on anti-

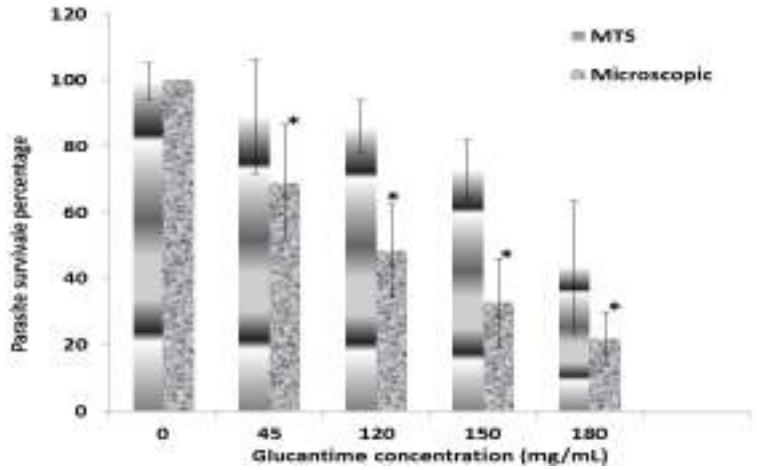

Figure 2. Promastigotes survival percentage after incubation with different concentrations of Glucantime. Data was obtained by microscopy and MTS assays

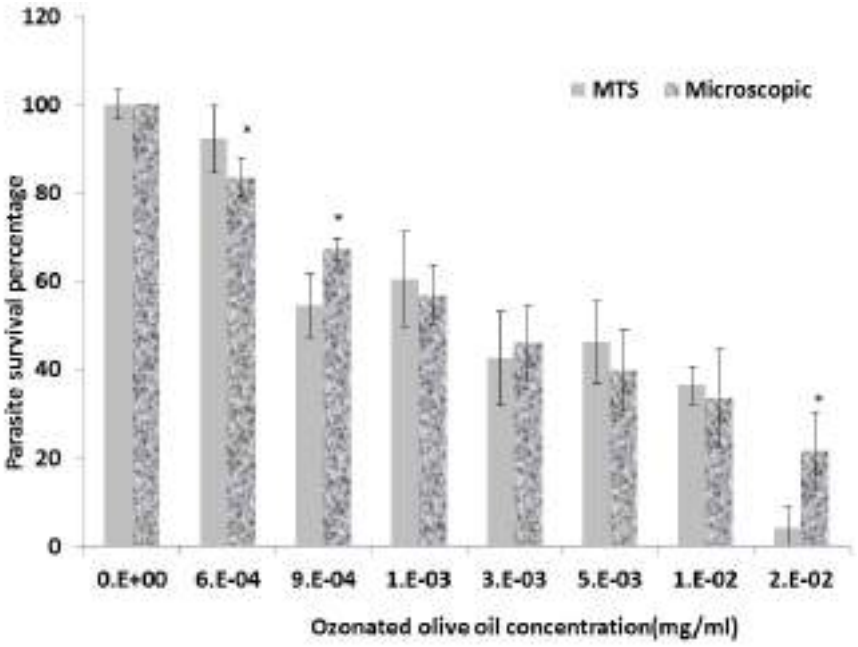

Figure 3. Promastigotes survival after incubation with different concentrations of ozonated olive oil as evaluated by microscopy and MTS assays. Data are expressed as Mean±SD

leishmanial activity, we decided to evaluate the efficacy of this easy and low cost drug on promastigotes of *L. major*. In this study, ozonated olive oil was used against *L. major*, *in-vitro*. Ozonated water and ozonated olive oil are ideal delivery systems as they have the capacity to entrap and then release oxygen/ozone (27).

According to our results, ozonated olive oil was effective against promastigotes of *L. major* at different concentrations. Conspicuously, in comparison with Glucantime as positive control, mean alive promastigotes percentage following treatment with the highest concentration of ozonated olive oil was significantly less.

The mechanism that explains how ozone decreases the number of leishmania parasite has not been studied yet. But it is generally accepted that the oxidant potential of ozone induces the destruction of cell walls and cytoplasmic membranes of bacteria and fungi. The reaction of ozone with olive oil occurs almost exclusively with c-c double bonds present in unsaturated fatty acids and produces different toxic products such as several oxygenated compounds, hydroperoxide, polyperoxides, aldehydes, ozonide and diperoxides that might be responsible for wide antimicrobial activity of ozonated olive oil (29-31).

During this process, ozone attacks glycoproteins, glycolipids, and other amino acids and inhibits and blocks the enzymatic control system of the cell. The degradation of nucleic acid was parallel to what happened to enzymatic activities. This leads to an increasing in membrane permeability that plays a key role in cell viability, leading to prompt functional cessation. It lets ozone molecules penetrating to the cell resulting in the microorganism death (32).

The wide availability of olive oil makes ozonated olive oil an inexpensive accessible anti-microbial agent and hopefully an alternative (or adjunctive) treatment of cutaneous leishmaniasis.

Conclusion

The results showed anti-leishmanial effect of ozonated olive oil. We recommend more studies on intracellular amastigotes and in infected animal models.

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