In-vivo Anti-Fungal Potential of Ozonated Clove Oil Against *Microsporum Canis*

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ARTICLE INFO

Article history:
Received: 13 Jaunary 2023
Revised: 23 March 2023
Accepted: 2 April 2023

Keywords:
Antifungal
Clove oil
Dermatophyte
In-vivo
Ozonated oil

ABSTRACT

Dermatophytes are keratinophilic fungi that infect keratihized tissues causing diseases known as dermatophytoses. Dermatophytes are classified in three genera, *Epidermophyton*, *Microsporum*, and *Trichophyton*. The treatment of dermatophytoses is carried out using classical antifungals. The emergence of resistant strains has stimulated the development of natural medicines and the use of ozone gas. This investigation was performed to study the possibility of using ozonated clove oil in the recovery of guinea pigs inoculated artificially by one of major dermatophytes that cause tinea which is *Microsporum canis* (ATCC 8137). The percentage recovery of the dermatophyte infected guinea pigs when application of 4 µg/ml ozonated clove oil is equivalent to that obtained by using 2 µg/ml Itraconazole as antifungal reference drug. The count of demonstrated hematological values on using ozonated clove oil was better than inoculated control and was significantly close to the data obtained from guinea pigs treated with Itraconazole.

1. INTRODUCTION

A group of fungi that infect keratinized tissues (skin, hair, and nails) of humans and animals cause dermatophytoses (tinea or ringworm) are called dermatophytes. The major dermatophytes that cause ringworm are *Microsporum canis*, *M. gypseum*, *Trichophyton rubrum*, *T. mentagrophytes*, and *T. interdigitales* [1, 2].

Although the research regarding the synthesis of new antifungal drugs has intensified, however the treatment with systemic antifungal chemical agents such as azole derivatives has side effects, in particular, when these chemicals are used in long-term application [3]. Moreover, the global emergence of antifungal resistance is a growing threat to public health. These have open the way for finding out an alternative to chemical and conventional drugs. One of the possible approach is to use ozone therapy. Ozone is a gas that is naturally present in the stratosphere and has a high oxidative power. The latter characteristic has been positively considered and it has been used because of its bactericidal, fungicidal and virucidal activities. Ozone toxicity is due to oxidation reaction which occurs upon any collision between an ozone molecule and a molecule of an oxidizeable cellular components, particularly those containing double bonds, sulfhydryl groups, and phenolic rings [4]. Therefore, membrane phospholipids, intracellular enzymes, and genomic materials are targeted by ozone. These reactions result in cell damage and death of microorganisms. *Unlike the majority of fungi, our bodies are evolutionarily designed to protect themselves against single reactive oxygen. The protection is provided through the action of antioxidants such as vitamins A, C, E and others which are incorporated into protective enzymes of the cell membrane. Thus ozone does not harm healthy cells* [2, 5].

The reaction of ozone with clove oil occurs practically with the carbon–carbon double bonds present in unsaturated fatty acids producing different toxic products such as several oxygenated compounds, ozonides, aldehydes and peroxides. These compounds could be also responsible for the wide antimicrobial activity of ozonated clove oil [6, 7]. The safety of ozonated clove oil was reported by de Souza Pedrosa *et al.* [8]. The aim of this investigation was to investigate the possibility of using OCO (ozonated clove oil) in the recovery of guinea pigs inoculated by *M. canis.*

2. MATERIALS AND METHODS

2.1. Test organism

M. canis (ATCC 8137) was bought from the inquiry network for microbial strains of China. The molecular and morphology identification (DNA sequences, the spore morphology, and colony characters) were confirmed by Dr. Elhawary (Cairo University, Cairo, Egypt) using a molecular- based method reported previously [9]. *M. canis* was inoculated into fresh plates of sabouraud dextrose agar and incubated at 25°C or 30°C for 20 days (Figure 1).

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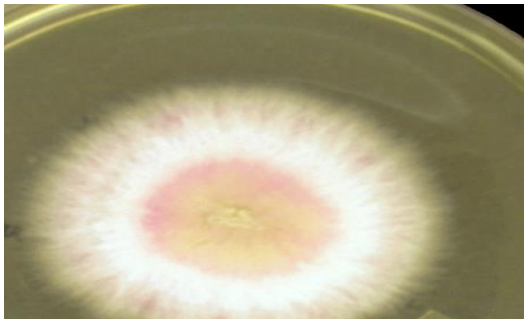

Fig. 1. Culture of *M. canis* ATTC 8137 on Sabouraud-Dextrose agar.

2.2. Preparation of inoculum

From the culture, the spore suspensions prepared by flooding the slants with 0.85% saline containing 0.02% Tween 80 and then the fungal growth was gently scraped to dislodge the spores and hyphal fragments which were filtered through sterile gauze to remove hyphae. The resulting conidia suspension was counted with a haemocytometer then diluted in sterile distilled water to produce a working suspension of (8×10^4 conidia/ml).

2.3. Pathogenicity Test

Four- week- old guinea pigs (clinically free from ringworm lesions) were used in this study. The animal weight ranged from 200-350 gm and they were kept in stainless steel cages. The animals were kept for one week before start of the experiment. Twelve pigs were infected with tested dermatophyte by the general procedures of [10]. The guinea pigs were slightly sedated by injection of xylocaine ($2mg/kg^{-1}$). An area on the skin of the caudo-lateral thorax was clipped of hair and the surface of the skin was scarified with sterile sand paper. The dermatophyte suspension was applied to the scarified skin. The animal inoculation was started within 24 hours after fungal collection. Three pigs remained without inoculation and considered as normal control. After inoculation, the degree of infection was observed for 14 days.

2.4. Treatment

On day 15 post-inoculation, the experimental animals showed that more severe infection and divided into four groups (3 animals for each): (i) untreated group, (ii) 2 µg/ml of OCO group, (iii) 4 µg/ml OCO group, and (iv) positive control (2 µg/ml Itraconazole). The drugs were applied topically to the infected area (1mL) once a day for 14 days.

2.5. Clinical Evaluation

The guinea pigs were examined clinically through scoring of lesions on 7th, and 14th days post-treatment according to the method previously described [11]. The main criteria for the evaluation contained three points: scale, hair loss and inflammation. The scale was scored from 0 to 3 as follows: 0: no infection; 1: slightly scale areas; 2: medium scale areas; 3: large areas of scale. The hair loss was scored from 0 to 3 as indicated: 0: no loss of hair; 1: loss of hair < 25%; 2: loss of hair < 50%; 3: loss of hair > 50%. The inflammation was scored from 0 to 3 as follows: 0: no infection; 1: slightly erythematous of skin; 2: erythematous with papulo vesicular areas; 3: marked erosion and crust in places. The total score of each animal was obtained by adding the scores of scale, hair loss and inflammation together, and the maximum score per animal was 9.

2.6. Mycological Evaluation

To evaluate the rate of mycological cure, hair and skin at the infected area of each animal was scraped, and examined under a light microscope after adding of 10% KOH. The microscopic result is generally considered positive when finding arthroconidia, conidia, or hyphae. The samples were incubated at 30°C for 7 days. Species identification was done on the basis of the morphology of colonies as previously described [12]. A negative result of microscopy and culture were considered complete mycological cure. The effectiveness of OCO was assessed with scores ranging from 0 to 5 based on the number of fungal burden of skin samples of each animal. Percent efficacy rates were calculated as follows: Percent efficacy rates = $100 - T \times 100/C$, where T is the number of fungal burdens of treated group; C is the number of fungal burdens of untreated group.

2.7. Blood Evaluation

Different physiological aspects of blood samples of the normal non-inoculated, inoculated non-treated and inoculated treated guinea pigs were estimated. The item aspects included haemoglobin, RBCs, leucocytes, neutrophills, eosinophills, basophiles, monocytes, and lymphocytes.

2.8. Statistical Analyses

All results were calculated as the means and standard (M ± SD). The results were analyzed for statistical significance by one-way analysis of variance (ANOVA) followed by Student's t-test; $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Clinical Evaluation

All animals were monitored for clinical signs twice a day throughout the study. High clinical scores indicated severe skin lesions caused by fungal infection. Marked lesions, such as hair loss, scaly skin, and visible papulo vesicle of the affected area, appeared on day 7 post-infection and reached highest degree on day 14. Although the damage of control group has been reduced after treatment for 7 days, the lesion was still obvious through 14-day treatment. The results showed that ozonated clove oil could improve skin lesions in a dose-dependent manner in the fungi-infected pigs.

Significant ($P < 0.05$) reduction of the average lesion that treated with ozonated clove oil (4 µg/ml) when compared with control group and Itraconazole treated group, and all the animals had a complete reduction of clinical signs after treatment for 14 days.

Most of the skin lesions have been restored, but the hair loss still did not recover completely in case of 4 µg/ml dose of OCO. The positive drug (2 µg/ml Itraconazole) significantly ($P > 0.5$) reduced clinical scores (Figure 2 & Figure 3).

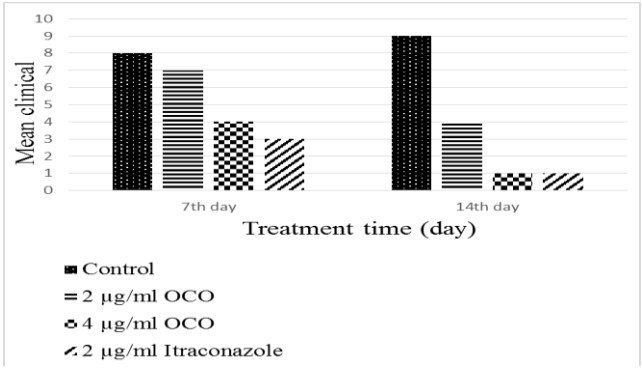

Fig. 2. Clinical scores of skin lesions in guinea pigs infected with *M. canis*. Values represented are the mean ± SD (n = 10). Significant differences are mentioned with different alphabets at the level of $P < 0.05$.

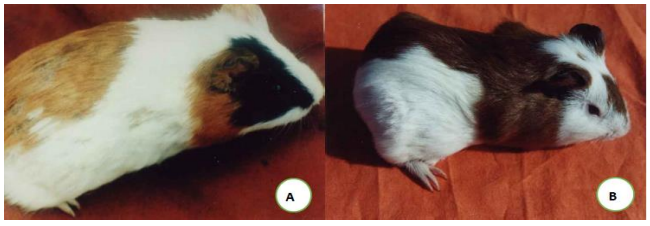

Fig. 3. Effect of 4 µg/ml of OCO on recovery of the guinea pigs infected with *M. canis* (A) as compared with 4 µg/ml of Itraconazole as a reference drug (B).

3.2. Mycological Evaluation

The rate of mycological cure was evaluated after treatment for 7 days and 14 days, respectively (Table 1). In infected animals, compared with the untreated control, the mycological efficacy rates of (2, 4 µg/ml) OCO-treated groups and 2 µg/ml Itraconazole treated group were 72.0%, 89.0%, and 91.0% respectively, after treatment for 7 days. That treated with OCO (4 µg/ml) possessed mycological cure efficacy (97.5%) which is equal to Itraconazole on day 14 post-treatment.

Table 1. Efficacy rates of OCO and Itraconazole on clearance of the fungi in experimental guinea pigs

Experimental groups	Mycological cure efficacy (%)	
	7^{th} day	14^{th} day
2µg/ml OCO-treated groups	72	80.5
4µg/ml OCO-treated groups	89	97.5
2 µg/ml Itraconazole treated group	91	97.5

The effect of ozonated oil and Itraconazole treatment of guinea pigs inoculated with *M. canis*, as a representative example, on different hematological values of blood pictures after five weeks post treatment is shown in Table 2. The demonstrated hematological values included hemoglobin, RBCs, leucocytes, neutrophils, basophils, monocytes and lymphocytes. Mostly the count of these item aspects on using ozonated oil was better than inoculated control and was significantly close to the data obtained from guinea pigs treated with Itraconazole. However, most items of treated pigs were deviated as compared with the normal control.

Table 2. Effect of ozonated clove oil and Itraconazole treatment of guinea pigs inoculated with M. canis on different hematological values of blood pictures after five weeks post treatment

Item aspect	Control (normal)	Inoculated Non-treated	Inoculated and treated	
			Ozonated clove oil (4 µg/ml)	Itraconazol (2 µg/ml)
Hemoglobin (g/dL))	17.30±3.87	12.50± 3.03	15.71±3.11	15.50±2.57
RBCS (X 10^6/ µL)	4.60±1.06	3.70±0.65	4.83±0.87	5.42±0.91
Leucocytes (X 10^3/ µL)	5.20±1.15	12.40±2.93	8.93±1.93	9.64±1.43
Neutrophils (X 10^3/ µL)	0.59±0.19	0.74±0.18	0.71±0.21	0.68±0.16
Eosinophils (X 10^3/ µL)	0.41±0.09	0.72±0.21	0.77±0.25	0.83±0.31
Basophils (X 10^3/ µL)	0.00	0.00	0.00	0.00
Monocytes (X 10^3/ µL)	5.70±1.31	4.00±1.05	4.45±1.06	4.48±0.94
Lymphocytes (X 10^3/ µL)	4.20±0.79	1.40±0.09	3.1±0.72	3.00±0.68

4. DISCUSSION

Most of synthetic antifungal drugs have side effects especially when used in long term application. An alternative to chemical drugs is the use ozone therapy. Ouf *et al.* [2] reported the evaluation of efficacy of ozonated olive oil as antifungal agent on growth and spore germination of some dermatophytes, and he found that the application of ozone in the form of ozonated oil appears to be more efficacious than gaseous ozone. Ozonated vegetable oils have been attributed fungicidal effects. The higher toxicity of ozonated oil is may be related to the decrease of fatty chain unsaturation as result of long time ozonation, formation of ozonide, increase in peroxide and acid values [2, 13]**.** Ozonated oil has shown to be effective against some bacteria like *staphylococci*, *streptococci*, *Pseudomonas*, *Escherichia coli* and especially *Mycobacteria* and has been applied for the treatment of fungal infections [14].

Ouf *et al.* reported an increase in leakage of electrolytes and sugar after treatment with ozonated oil in the case of *M. gypseum*, *M. canis*, *T. interdigitale*, *T. mentagrophytes* and *T. rubrum*, and the high leakage may be attributed to the weakening of the membrane permeability which influences the normal physiological functioning of the cells, change in fractionating the cell into outer protein and plasma membrane protein and DNA damage mediated by singlet oxygen [2].

The application of ozonated oil was effective in producing high loss in enzyme production (keratinase, urease, alkaline phosphatase, amylase and lipase) which play an important role in the pathogenesis [15]. Cataldo, suggested that the changes in protein enzymes may be connected with the partial oxidation of the aromatic monomeric units of the proteins and/or cysteine units [16].

Based on the *in vitro* studies, a treatment regime was developed for *in vivo* using experimentally infected animals. In a trail to apply a control measure against dermatophytosis using ozonated clove oil, the results revealed a successful recovering of guinea pigs, artificially infected with *M. canis.* Application of ozonated oil achieved different degree of curing of guinea pigs infected with the tested dermatophyte depending on the percentage recovery reached 97.5% in *M. canis* on application of 4 µg/ml ozonated oil which is equivalent to 2 µg/ml Itraconazole as antifungal reference drug. The mycotic infection of guinea pigs was associated with a decrease in the count of haemoglobin, red blood cells and lymphocytes while it caused an increase in leucocytes, neutrophills and eosinophills. The count of these physiological aspects approach the normal levels two months after treatment with ozonated oil. Svirid *et al.* examined blood samples from 194 patients with athlete's foot and found that the phagocytes showed depression of their antifungal activity [17, 18].

They also observed that mycotic infection was associated with depressed acid phosphatase and increased alkaline phosphatase activity. Also there was a depression of neutrophills and an increase of macrophage activity. The obtained results reveal the possibility of applying ozone therapy to tissues infected with dermatophytes as an efficient approach for treatment of human mycosis.

5. CONCLUSION

In this study, we found ozonated clove oil has antifungal activity against *M. canis* ATCC 8137 *in-vitvo* and it exerted a significant clinical efficacy against dermatophytosis in guinea pig model if applied topically and the percentage recovery reached 97.5% on application of 4 µg/ml OCO which is equivalent to that obtained by using 2 µg/ml Itraconazole. The count of demonstrated hematological values obtained from guinea pigs treated with 4 µg/ml OCO was significantly close to that obtained from guinea pigs treated with 2 µg/ml Itraconazole.

ETHICAL APPROVAL

The experimental protocol was approved by the Institutional Animal Care and Use Committee at Cairo University (approval number CUFS-0109).

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