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Review

Scientific rational for the medical application of ozonized oils, an up-date

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Keywords

ozone,
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vegetable oils,
ozonated oils..

Abstract

The use of ozonized oils in medicine has recently gain importance, because it's recognized antimicrobial potential and the most frequent spread of microorganisms that are resistant to conventional antimicrobial agents. This paper reviews the general and main clinical applications of ozonated oils that have appeared in scientific literature between 1859-2020. The compound derived from the reaction of ozone with fatty acids and other substrates can acts as a germicide, immune stimulant and tissue restoration agent. The biological activities and stability of the ozonated oils allows the development of standard formulations that deliver the benefits of ozone, supported by pre-clinical and clinical studies. The quality of the ozonized oil should be of paramount importance for its use in clinic in order to guaranty its efficacy and avoid toxicity. Chemical and physical characterization and a precise peroxide values (as index of dosage) will be considering as a quality criterium of one medicinal ozonized oil preparation. Today the main applications of the ozonized oil are for external use essentially in dermatology, dental, ophthalmology and gynaecology, however there are evidences of immune-stimulating, germicide and repair effects when used orally. Additional clinical trials are need in order to support the clinical used of ozonized oils, but bases in standardized and stable formulations.

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Introduction

Early evidence on the clinical use of ozonized oils first appeared in scientific literature in 1859. This paper reviews the general and main clinical applications of ozonized oils that have appeared in scientific literature between 1859 - 2020. The oxidation products generated after the reaction of ozone with fatty acids and other substrates can act as a germicide, immune stimulant and tissue restoration agent. The biological activities and stability of the ozonized oils allow the development of standard formulations that deliver the benefits of ozone, supported by pre-clinical and clinical studies. The main clinical studies that support the use of ozonized oils apply ozonized sunflower oil or ozonized olive oil in different clinical conditions. The applications are essentially for external use, however there is evidence of immune-stimulating and repairing effects when used orally.[1]

In the world, there are millions of people affected by dirty traumatic lesions, infected wounds, chronic torpid ulcers, bed sores, oral infections, vaginitis, burns, herpetic lesions, fungal infections and insect stings, who suffer for a long time because the conventional topical treatments based on antibiotics and anti-inflammatory drugs are not sufficiently effective. Antibiotic-resistant strains of pathogenic bacteria are increasingly prevalent in hospitals and the community.[2] New antibiotics are needed to combat these bacterial pathogens, but progress in developing them is slow. Historically, most antibiotics have come from a small set of molecular scaffolds whose functional lifetimes have been extended by generations of synthetic tailoring. The emergence of multidrug resistance among the latest generation of pathogens suggests that the discovery of new scaffolds should be a priority.[3] Unfortunately, most physicians and nurses are not aware of the potency and efficacy of ozonized oil. [4]

Dr. Velio Bocci said "I would like to predict that the application of ozonized oil, a simple and inexpensive remedy, will become far more useful than expensive pharmaceutical creams and will herald a medical evolution for the topical treatment of topic ulcers and wounds. Under these terms, it is not exaggerated to proclaim ozone as the wonder drug of the XXI century.[4]

Interestingly, in spite of its instability, the O_3 molecule can be stabilized as an ozonide between the double bonds of a monounsaturated fatty acid such as oleic acid.[5] Ozonation of edible oil is performed by bubbling the gas mixture (O_2/O_3) for some hours. One gram of oil can bind up to 160 mg of ozone. As a consequence, ozonized oil remains stable for 2-3 years at 4 ºC depending on the method of stabilization. This preparation is proving to be ideal for the topical use of O_3 in the treatment of chronically infected cutaneous and mucosal areas of the body.[6] O_3 is widely recognized as one of the best bactericidal, antiviral and antifungal agent and therefore it is profitably and practically employed as ozonized oil with well-defined peroxide contents.

The ozonized oil is now used topically for the treatment of war wounds, anaerobic infections, herpetic infections (HHV I and II), trophic ulcers and burns, cellulitis, abscesses, anal fissures, decubitus ulcers (bed sores), fistulae, fungal diseases, furunculosis, gingivitis and vulvovaginitis.[4] Even radiodermatitis lesions in patients with cancer have been found to be beneficially influenced by exposure to a simple application of ozonized oil.[7]

The efficacy and safety of ozonized oil is closely linked to its quality control. One of the basic parameters to define the dosage and its clinical application is the peroxide value.[8] This indicator is critical to define the proper indication. It is estimated that low IP values (80 mEq O_2/kg to 120 mEq O_2/kg) are used mainly in cosmetics. Higher values of peroxide index between 200 mEq O_2/kg to 400 mEq O_2/kg, have a more important effect on the healing processes. On the other hand, the germicidal effects of the oils are more marked at IP values higher than 400 mEq O_2/kg. It is important that the IP measurement is carried out by a suitable method.[8] Regarding safety, it is important to rule out the presence of formaldehyde and to keep the acid number and malondialdehyde values within a controlled range. In this way, hypersensitivity reactions and irritation will be avoided.

Chemistry of the ozonized vegetable oils

To obtain ozonized oil, edible oil is bubbling with the gas mixture (O_2/O_3). During the reaction of O_3 with the fatty acid present in vegetable oils, lipoperoxides and ozonides (derived in: aldehydes, ketones, peroxides) are formed. In chemistry, especially biochemistry, a **fatty acid** is a carboxylic acid often with a long unbranched aliphatic tail (chain), which is either saturated or unsaturated. For example, **oleic acid** is a monounsaturated omega-9 fatty acid found in various animal and vegetable sources. It has the formula $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$. The term *Oleic* means related to, or derived from, *oil* or *olive* (Fig. 1).

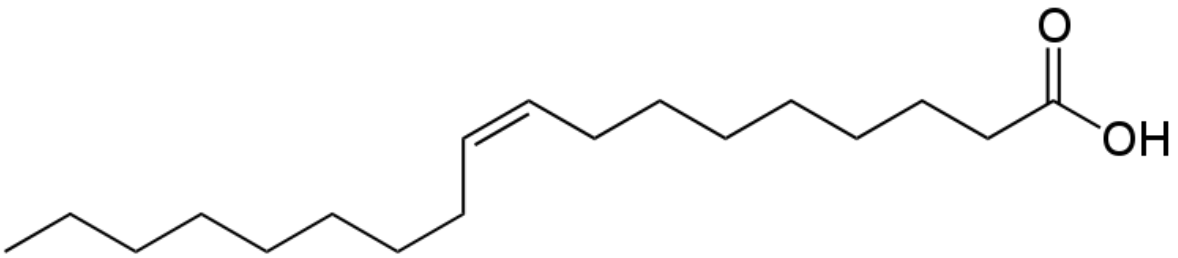

Figure 1. Representation of chemical structure of oleic acid.

The reaction of O_3 essentially with the unsaturated double bond of the fatty acid form different derivates, such as organic ozonides and lipoperoxides.

Organic ozonides are formed by the addition reactions of ozone and unsaturated compounds. They are intermediates in ozonolysis and have a **trioxolane** ring structure with a five-membered C-O-O-C-O ring (Fig. 2).[9,10] They usually appear in the form of foul-smelling oily liquids, and rapidly decompose in the presence of water to carbonyl compounds: aldehydes, ketones, peroxides.

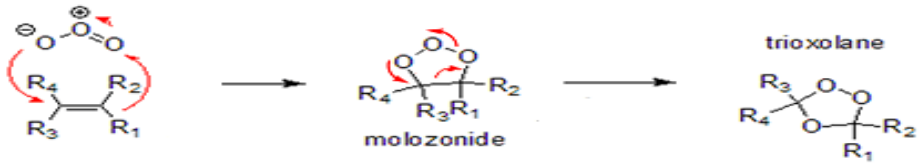

Figure 2. Representation of the mechanism of Criegee reaction.

In the generally accepted mechanism proposed by Rudolf Criegee in 1953, the alkene and ozone form an intermediate molozonide in a 1,3-dipolar cycloaddition. Next, the molozonide reverts to its corresponding **carbonyl oxide** (also called the *Criegee intermediate*) and aldehyde or ketone in a retro-1,3-dipolar cyclo-addition. The oxide and aldehyde or ketone react again in a 1,3-dipolar cyclo-addition or produce a stable ozonide intermediate (a trioxolane) (Fig. 3).

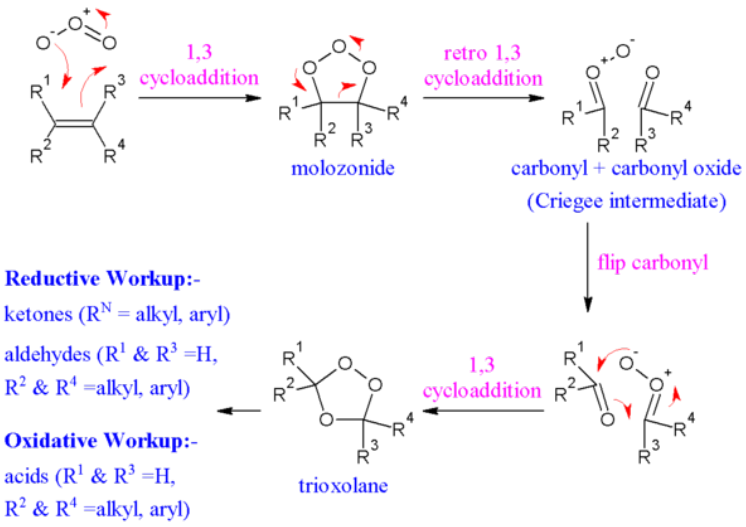

Figure 3. Representation of the steps of Criegee reaction to form trioxolane.

Evidence for this mechanism is found in isotopic labelling. When ^{17}O-labelled benzaldehyde reacts with carbonyl oxides, the label ends up exclusively in the ether linkage of the ozonide. There is still dispute over whether the molozonide collapses via a concerted or radical process; this may also exhibit a substrate dependence.

Lipid peroxidation refers to the oxidative degradation of lipids. This process proceeds and most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylene -CH2- groups that possess especially reactive hydrogen (Fig. 4).

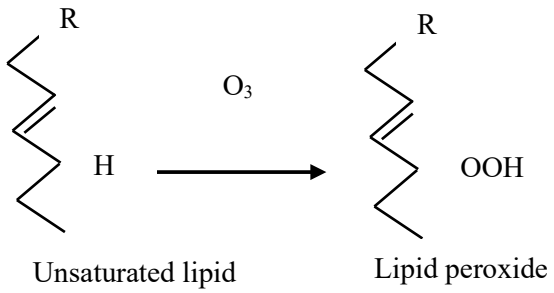

Figure 4. Formation of lipid peroxide during reaction of unsaturated lipid with ozone.

The reaction of ozone with vegetable oils occurs almost exclusively with the carbon-carbon double bonds present in unsaturated fatty acids producing, in addition to lipid peroxides and ozonides, several oxygenated compounds: aldehydes, diperoxides and polyperoxides; and these compounds could be also responsible for the wide antimicrobial activity of ozonized oils.[11] Unsaturated lipid substrates react with insufflate gaseous O_2/O_3 mixture leading to therapeutically active ozonized derivatives (Fig. 5).

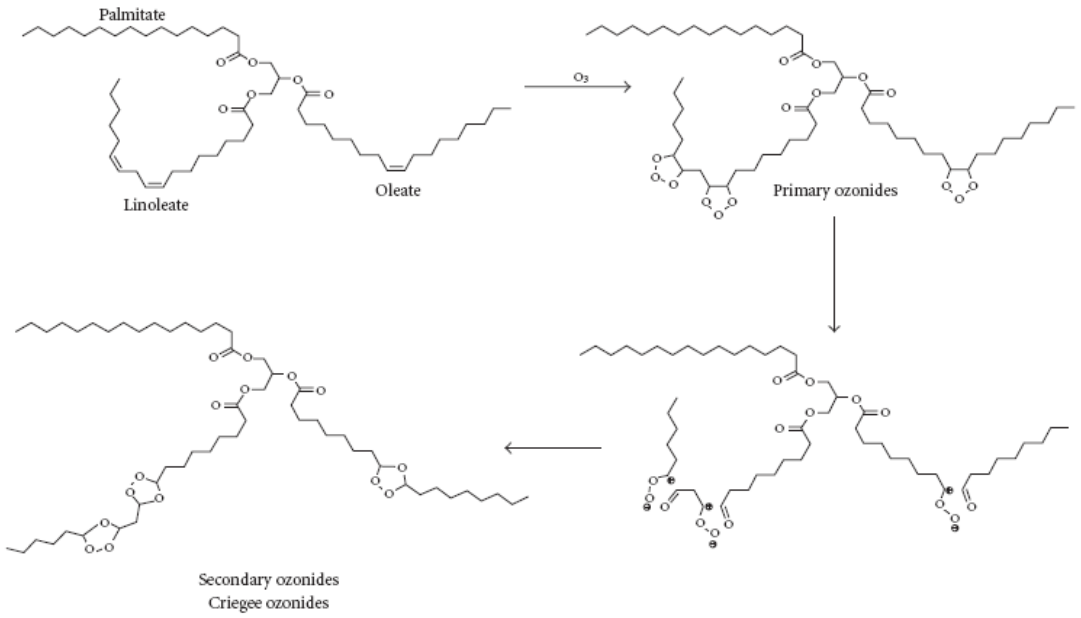

Figure 5. Representative chemical structures of ozonized derivatives which are formed by chemical reaction of ozone with unsaturated triglycerides. The primary ozonides are transient, unstable species which rearrange in the normal, secondary ozonides also known as Criegee ozonides.[12]

In summary, main oxygenated compounds that could possibly be obtained in the reaction of a fatty acid with ozone are: peroxides and aldehydes (Fig.6).[13] The peroxides are the most important products formed. This group includes ozonides, hydroperoxides, polymeric peroxides and other organic peroxides and, probably, is responsible for the wide biological activity of described ozonized vegetable oils.[14]

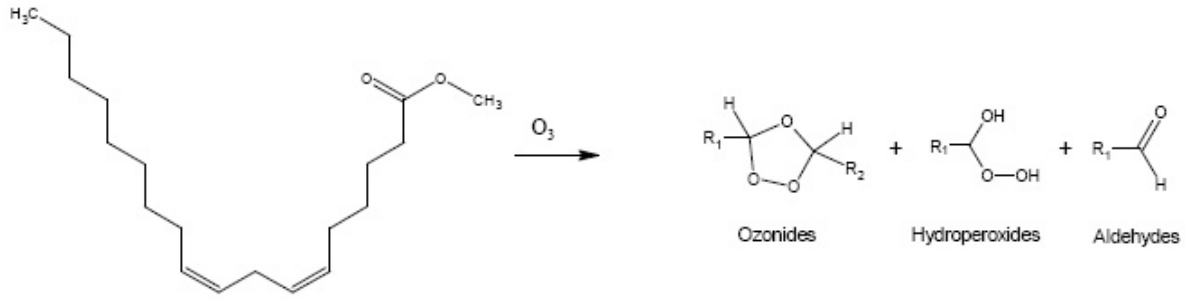

Figure 6. Oxygenated compounds obtained during the reaction of ozone with methyl linoleate.[15,16]

Quality of ozonized vegetable oils

From an industrial applicative viewpoint, the overall quality of ozonized derivatives depends upon several parameters, such as: 1) the type and the quality of ozone generators; 2) the ozonation conditions, in terms of reactors and time, material type and amount, presence of water and/or catalysers; 3) the efficacy of the ozone generator, in terms of O_3 concentration output, gas flow, gas carrier. As for the latter, the use of hight quality O_2 grade as a precursor, instead of air, is an important point to be considered; in fact, air feedstock (containing about 78 % of nitrogen) used for the ozonation of unsaturated substrates could lead to the production of potentially toxic nitrated by-products, and to a significant decrease of the ozonation efficiency. Another important feature is that ozonized oil has to be unequivocally characterized in terms of the species contents as well as the reaction kinetics. For these purposes, the knowledge of the physicochemical properties of ozonized vegetable oils during production has a great importance for their characterization and identification.[12]

For determining the quality of ozonized products, analytical methods such as peroxide, acid and iodine values, relative density, viscosity are usually carried out. The peroxide value (PV) represents the quantity of peroxide in the sample; acid value (AV) represents the present free acids; and iodine value (IV) is a measure of total number of double bonds in the sample. All values are well described according to the European pharmacopoeia[17] and official methods of analysis of the Association of Official Analytical Chemists (AOAC, 2019 and OACS, 1995).[18]

The PV represents the quantity of peroxide expressed in milliequivalents of active oxygen contained in a 1000 g sample (mEq O_2/kg). In the case of materials characterized by a high peroxide content, some authors determined the PV introducing changes into the method described in the official monograph due to the slow iodide reactivity with dialkyl peroxides.[19,20] In accordance with the official methods of analysis, after addition of potassium iodide, the sample is allowed to stand for 1 min so that the peroxide oxidizes iodide to iodine. During the ozonolysis of sunflower oil, polymeric peroxides and other organic peroxides are formed, and due to the high concentration of peroxides a long reaction time is required for these compounds to oxidize iodide to iodine.[21,22] Some methods include increased reaction time and reflux until from 30 ºC to 60 ºC. Peroxide content of ozonized sunflower oil using iodometric assay achieves the maximum values at 24 h of reaction time. Other difficulties found in the iodometric assay are the susceptibility to interference by molecular oxygen as well as the reaction of liberated iodine with other components in the system.[23]

The assay of peroxides concentration is essential in order to establish the therapeutic dose of ozonized oil. The lack of a standardized method adapted to high levels of peroxide is one of the challengers in the quality control of ozonized oil. Table 1 shows an example of a quality control report of the sunflower ozonized oil Ozonia 3000® by Innovares (Italy). In order to avoid variabilities is recommended to use standardized methods to assay the PV, as the method recommended by the ISCO3.[8]

Table 1. Example of quality report of a **c**hemical and physical characteristics of an ozonized oil. (Ozonia 3000® (Innovares, Italy)).

Analysis	**Method**	**Limits**
Density	European Pharmacopoeia	0.98 ± 0.02 g/mL
Acid Index	European Pharmacopoeia	26.50 ± 5.50 mg KOH/1 g
Index of peroxides	European Pharmacopoeia Modified	3300 ± 300 mEq O_2/kg
Iodine value	European Pharmacopoeia	13 - 53 g/100 g
Viscosity	European Pharmacopoeia	625.00 ± 40.00 mPa·s
Formaldehyde	EPA 8315 A:1996	< 0.0005
Arsenic		<100 µg/kg
Cadmium	C.E. 629/2008	<1000 µg/kg
Chrome		<50 µg/kg
Mercury	U.E.420/2011	<1000 µg/kg
Nickel		<200 µg/kg
Lead	C.E. 1881/06	<100 µg/kg
Benzopyrenes	C.E. 1881/06	<2 µg/kg
Benzopyrenes + benzo anthracene + benzo fluoranthene + chrysene		<10 µg/kg*
Total dioxin	OMSPCDD/ F-TEQ C.E. 1881/06	<0.75 pg/g

Legend: For the coconut oil the limit is 20 µg/kg. The analysis of physical chemical indicators should be included for each batch. While the presence of heavy metals, formaldehyde, malonyldialdehyde or pesticides and the structural characterization of the oil using methods such as U.V., infrared and mass spectroscopy; could be carried out when the production parameters or the origin of the raw material are modified.

How does ozonized oil act?

Ozonized oil acts by different mechanism, depending of the dose in terms of PV. Probably, when the stable triozonide comes into contact with the warm exudates of the wound, it slowly decomposes to reactive ozone, which readily dissolves in water, generating hydrogen peroxide and lipoperoxides that can explain the prolonged disinfectant and stimulatory activity. If it is correct, this reasoning implies that we should have titrated preparations with high, medium or low triozonide concentrations to be used during the inflammatory septic phase I, regenerating phase II or remodeling phase III, respectively. These phases have been related to the rapidly changing cell types and to the release of cytokines and growth factors that modulate the complex healing process.[4]

On the other hand, it has recently been observed that olive oil, which during ozonation traps O_3 in the form of a stable ozonide, when applied to all sorts of acute and chronic cutaneous infections, slowly release O_3 which, in comparison with conventional creams, displays effective disinfectant and stimulatory activities that lead to rapid healing.[6] In addition. it has been demonstrated that antimicrobial effect is not only attributable to the ozonides present in the ozonized oil, but to the all complex mixture of compounds derived from the ozonization process,[24] like formaldehyde.[25] However, for toxicological reason is preferable to avoid the formation of formaldehyde during the production of an medical ozonized oil. After the contact ozonized oil - microorganism it has been observed severe alteration of the cytoplasm.[26] In addition, application of ozonized oil leads to a significant reduction in amylase, lipase, keratinase and urease enzymes activities in the microorganism in line with a reduction in nucleic acid content.[27]

A recent study was undertaken to evaluate the therapeutic effects of topical ozonized olive oil on acute cutaneous wound healing in a guinea pig model and also to elucidate its therapeutic mechanism.[28] After creating full-thickness skin wounds on the backs of guinea pigs by using a 6 mm punch biopsy, authors examined the wound healing effect of topically applied ozonized olive oil (ozone group), as compared to the pure olive oil (oil group) and non-treatment (control group). The ozone group of guinea pigs had a significantly smaller wound size and a residual wound area than the oil group, on days 5 ($p < 0.05$) and 7 ($p < 0.01$ and $p < 0.05$) after wound surgery, respectively. Both hematoxylin-eosin staining and Masson-trichrome staining revealed an increased intensity of collagen fibres and a greater number of fibroblasts in the ozone group than that in the oil group on day 7. Immunohistochemical staining demonstrated upregulation of platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) expressions, but not fibroblast growth factor expression in the ozone group on day 7, as compared with the oil group. In conclusion, these results demonstrate that topical application of ozonized olive oil can accelerate acute cutaneous wound repair in guinea pigs in association with the increased expression of PDGF, TGF-β, and VEGF.

Even when the exact action mechanism of the ozonized oil is not described there are much pre-clinical and clinical evidence of its antimicrobial and wound healing beneficial efficacy. As antimicrobial the most sensible bacterium is *Staphylococus aureus* and the main resistant is *Pseudomonas aeruginosa*. [24] A recent *in vitro* study confirms the microorganism sensibility to ozonized oil in that way (from more to less sensibility): *Staphylococus aureus* > *Candida albicans* > *Escherichia coli* > *Pseudomonas aeruginosa* > *Enterococcus faecalis*. [29] In general, a lethal effect of ozonized oil is evident when it is applied to multi-resistant strain of *Staphylococcus epidermis*, *Stafilococcus aureus*, also when it is applied to fungi from the genus *Trichophyton*, *Epidermophyton* and *Microsporum*, yeast as *Candida albicans* and protozoan as *Giardia lamblia.*[27,30,31]

A comparison regarded to the antimicrobial effectiveness of ozonized extra virgin olive oil (peroxide value of 560/590 mEq/kg) with 0.2 % chlorhexidine digluconate and 10 % povidone-iodine through a disk diffusion test was done recently.[32] Ozonized oil showed a significant better behaviour that the references. This effect on one of the main periodontal pathogens, suggests its potential applicability for periodontal treatment.[32] The wound healing action mechanism of ozonized oil may be connected in part to its antimicrobial effect, but also with its ability to promote the liberation of gown factors,[33] activate local antioxidant mechanism[34,35] and promote tissue reparation.[36]

The theoretical sequence of wound healing has been schematically represented to happen in three successive stages. The scheme presented in Fig. 7 shows three phases: Phase I indicates the inflammation stage, normally lasting 2-3 days. The bacterial infection successive to a trauma, diabetes, local ischaemia and possibly antibiotic resistance, can become chronic unless it is possible to intervene with ozonized oils. Phase I corresponds to the intermediate stage and normally lasts two weeks. The synthesis of extracellular matrix (fibronectin, collagen III/I, hyaluronic acid and chondroitin sulphate) is accompanied by an active proliferation of fibroblasts and keratinocytes. The use of ozonized oil not only prevents a superinfection, but stimulates the initial tissue reconstruction. Phase III, includes the final healing and scar tissue remodelling and may take a long time in elderly and/or diabetic patients. In some cases, excessive release of Transforming Growth Factor (TGF-β 1) may stimulate excessive fibrogenesis with cheloid formation.[4]

The effect of sunflower ozonized oil (Peroxide value 75 mEq/kg – 100 mEq/kg,[37]) topical applications for 12 weeks in a total of 30 patients suffering from second-degree skin burns in the phase of reepithelization was studied. Skin burn was subdivided in two symmetrical parts. One part was treated with occlusive application of ozonized oil; the contralateral part of the lesion was treated with topical application of hyaluronic acid gel, once a day for 12 weeks. Ozonized oil was

more effective than hyaluronic acid in reducing symptoms related to skin burns, but it could be more effective in preventing the post-lesional hyperpigmentation.[37]

However, the word *ozonized* is itself without scientific meaning if it is not associated with *how much* peroxides are present in the oil. In fact, from a therapeutic point of view, the ozonide compositions have the capacity to deliver active O_2 and/or other useful species deep within the lesion without causing primary skin irritation. The few studies concerned with the therapeutic effects of ozonized oils on acute cutaneous wound healing in animal models did not investigate the dose/effect response, expressed as the amount of peroxides existing in the ozonized derivative used.[28] Recently, a quantitative evaluation of the therapeutic effect of topically applied ozonized sesame oil on acute cutaneous wound healing in mice as animal model has been developed.[38] The results indicate that both low (< 1000) and high doses (> 3000), as expressed in terms of peroxide value, delay cutaneous wound healing. Such evidence is reinforced by a number of results between groups where the «middle» concentration (about 1500) has the most beneficial effect in accelerating the wound closure ratio.

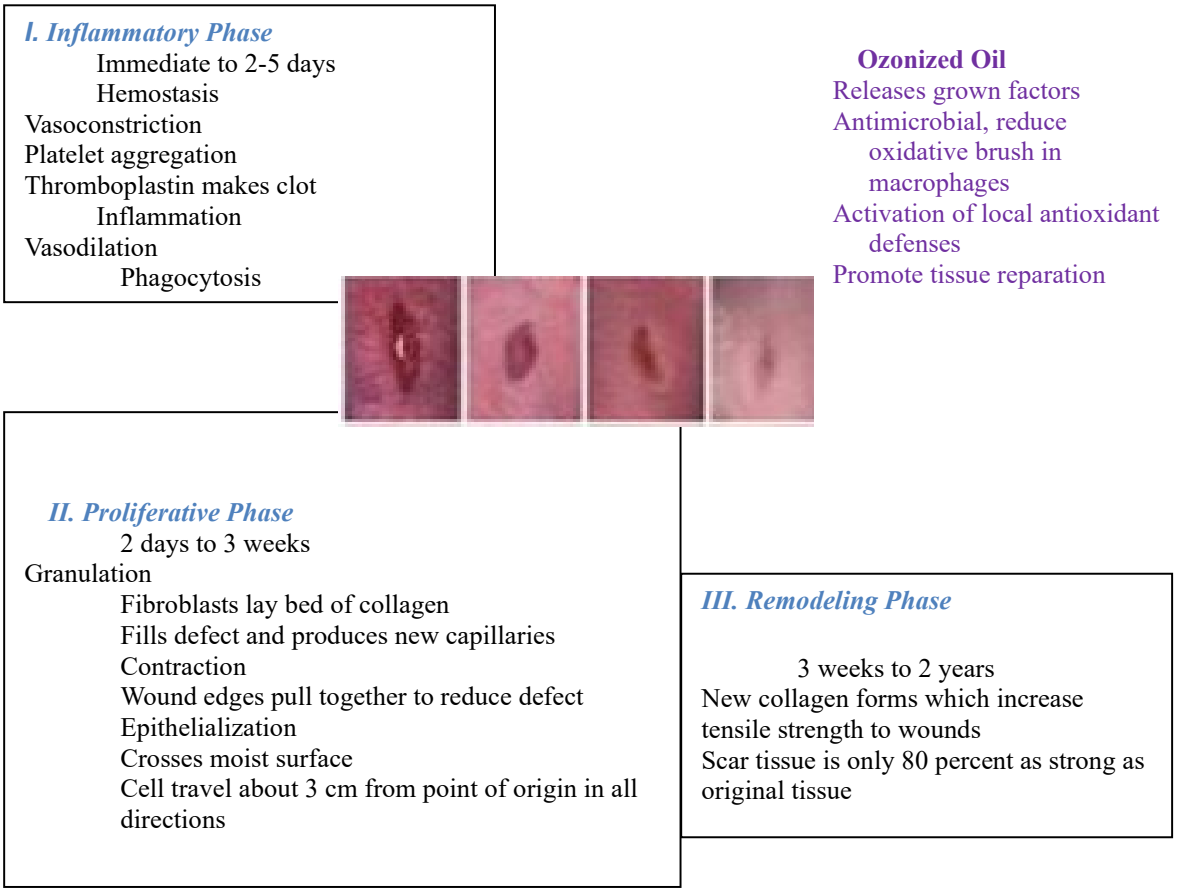

Figure 7. Possible action mechanism of ozonized oil during wound healing. The entire wound healing process is a complex series of events that begins at the moment of injury and can continue for months to years. Ozonized oil components may act in different steps of the wound healing process by different mechanisms of action.[1]

In summary, the possible action mechanism of ozonized oil should be due to: 1) Direct oxidation (germicide): slow release of O_3, trioxolane and lipoperoxides can destroy by oxidation the infective germs.[6,25,39] It has been hypothesized that the antimicrobial mechanism of ozonized oil involves 1,2,4-trioxolane present in the ozonized oil. When it is added to the warm exudates film of the ulcer, it slowly decomposes generating local oxygen, H_2O_2 as reactive oxygen species (ROS), and a trace of lipid oxidation products (4-HNE).[29] Such a cascade can explain the prolonged disinfectant action and stimulation of proliferative activity of fibroblasts and keratinoblasts.[39] 2) Cytotoxicity: Trioxolane, lipoperoxides and aldehydes are cytotoxic to microorganism; they can inactivate enzymatic pathways by mechanisms involving disruption of nuclear mediators.[27] 3) Grow factors Release: O_3 and other oxidized oil components can release grow factor from platelets[4] or from the local tissues (increased expression of PDGF, TGF-β, and VEGF) that act as tissue remodeling factors.[28] In addition, Ozonized oil has been observed to facilitate the wound healing via increasing fibroblast migration and epithelial–mesenchymal transition (EMT) process via PI3K/Akt/mTOR signaling pathway *in vivo* and *in vitro*.[40] 4) Oxidative pre-conditioning, local oxidation of tissue by oxidized oil components can stimulate the expression of endogenous antioxidant mechanism[34,35] and promote tissue reparation. [36]

It is very likely that all the mechanisms do not take place simultaneously. For example, the antioxidant effect (oxidative pre-conditioning) takes place at low peroxide vales and is exploited more in cosmetics with ozonized oils. On the other hand, the germicidal effect takes place at higher peroxide values. This is why it is essential to know the peroxide value of the formulation.

Current use of ozonized oils in dermatology

Velio Bocci*: "As soon as the medical community will appreciate their efficacy, ozonized oil will become indispensable tools in chronic wound healing units".*[4]

The germicidal properties of the ozonized vegetable oil have been already established. The ozonized vegetable oils have been used in the treatment of microbial infections of the skin (dermatitis, sores, infected wounds, fistulas, acne, infected burns and ulcers), in the treatment of nasal, ear and vaginal infections (U.S. Pat. No. 984,722,[41] U.S. Pat 5,270,344,[42] U.S. Pat 5,364,879,[43] U.S. Pat 2,356,062;[44] U.S. Pat 3,504,038)[45] and in post-operatory disorders. They have been also used in the treatment of gastroduodenal ulcers (Pat. WO 01/37829 A1),[46] against intestinal infections (U.S. Pat 5,364,879)[43] or erysipelas (Pat RU 2040235 A)[47] they have been recently used in the treatment of the *Giardia lamblia* (Pat. WO 01/37829 A1)[46] *Tynea Pedis*, recidivating genital *Herpes simplex*, *Helicobacter pylori* infection and in external hemorrhoids and bedsores.[24] Treatment of asthma, gastroduodenal ulcers (US 925590 A),[48] treatment of infections caused by pinworms, human papilloma virus (HPV), and fungi, such as microorganisms of the genus *Candida* (WO 03/085072 A1).[49]

The ozonized vegetable oils and fats have been also used in cosmetics. Since the 1950s, in France, the ozonized solutions have been used as cosmetics, directly on the skin or in baths, as stimulants, purifiers, as decongestant, tranquilizers and regenerating substances of the epidermal tissue. The properties for stimulating the tissue regeneration, the oxygenation of the cells and tissues and the moderated whitening properties are added to the acknowledged germicidal activity of the products from the ozonation of unsaturated compounds, such as terpenes, fatty acids, triglycerides and vegetable oils in the cosmetic applications. The highly oxygenated compounds, such as the ozonized vegetable oils, favour the flexibility and the softening of the skin and is used also to prepare creams for repairing the epithelial tissue (Pat. WO 01/37829 A1).[46]

How and when ozonized oils are used? Chronic wounds range from diabetic food to putrid and deep ulcers due to limb atherosclerosis, or trauma and burns. Moreover, both immunosuppressive chemotherapy and/or malnutrition cause abscesses, anal fissures and fistulae, bed sores, furunculosis, and osteomyelitis which are difficult to treat and often fail after prolonged treatments. Treatment of wounds of different aetiologies constitutes a major part of the total health care budget. It is estimated that 1,5-2 million people in Europe suffer from acute or chronic wounds.[50] Various types of disinfectants, antibiotics, antifungal, antiprotozoal, and growth factors are scarcely effective because the deranged metabolism and local hypoxia are not modified. Several other approaches such as vacuum therapy, maggot therapy and devices for providing topical oxygen therapy in a clinical setting have been proposed and variably used. This last approach has a rationale in the sense that enhanced oxygenation is useful for activating the metabolism and cell proliferation of ischemic tissues. However, it has also considerable limitations because it is a cumbersome therapy, with minimal disinfectant activity and modifications of the fundamental pathogenetic mechanisms.[12]

Ozonized oil preparation is proving to be ideal for the topical use in the treatment of chronically infected cutaneus and mucosal areas of the body. In addition, it was used to reduce the muscular fatigue.[51] Ozonized oil has been applied in human pathologies involving germs. Regularly ozonized oil formulations are topical apply lightly to the affected area twice daily or as prescribed by the physician. Before the application, the damaged skin surface must be cleaned by removing necrotic tissue, pus, loose fibrin deposition, and excess of fluid exudates. Ozonized oil has a wide range of antimicrobial effect, are useful to treat topical fungi, bacterial and virus infections. In addition, ozonized oil are used in the treatment of bedsores and in prophylaxis of diabetic food. Interactions are not well documented, but is preferable to do not mix ozonized oil with any other drugs or cosmetic. Adverse effect in dermatologic will occur with low frequency: Skin rashes (rarely), skin-burning sensation, pruritus and erythema (0.3 %) and contact dermatitis.[52]

5.1. Ozonized oils and regenerative medicine in skin ulcer/wounds

The rationale for using ozonized oil combined with regenerative medicine in the treatment of wounds and ulcers is basically based on the following criteria:

1) The pre-treatment of the wound/ulcer with ozonized oils allows the preparation of a micro environment suitable for receiving a (Plasma Rich in Platelets) PRP graft, and therefore a greater probability of therapeutic success.
2) The frequency of PRP treatment for physiological reasons is limited to a number of interventions with a frequency of 14 to 21 days. In the meantime, continuous treatment, even at home, with ozonized oil could contribute as an adjuvant therapy to: a) keep the wound area under microbiological control. b) further simulate the release of growth factors through the interaction of the oil components with the platelets present in the wound.

There is a lot of scientific evidence on the positive role of PRP and ozonized oils in tissue regeneration and wound healing. Furthermore, an important advantage of autologous PRP and ozonized oils, in the clinical setting, is that they have no negative effects. Most likely, based on the mechanism of action of both, there is a synergy when they are used simultaneously.

Current use of ozonized oils in gynaecology

6.1. Introduction

Vaginitis is the general term for disorders of the vagina caused by infection, inflammation, or changes in the normal vaginal microbial environment. Microbiota, signs and symptoms are similar, irrespective of the underlying aetiology. Vaginitis is often the result of infectious agents. Recurrent vulvovaginal candidiasis, multiple episodes of vulvovaginal candidiasis within a 12-month period, adversely affects quality of life, mental health, and sexual activity.[53] The most common infections, bacterial vaginosis (BV), *Candida* vulvovaginitis, and trichomoniasis, account for over 90 % of infections. Cervicitis, typically from sexually transmitted infections, such as gonorrhoea, chlamydiosis and mycoplasmosis, can also present as nonspecific vaginal symptoms. Women with vaginitis typically present with one or more of the following: change in vaginal discharge, pruritus, burning, irritation, erythema, dyspareunia, spotting, and dysuria. Given the nonspecific nature of vaginitis symptoms, laboratory documentation of the etiology of vaginitis is mandatory before initiating therapy. The main steps in the initial evaluation of women with symptoms of vaginitis are to obtain a history and perform a physical examination, measure vaginal pH, test for vaginal and cervical infections, treat according to abnormal results, and reassess after targeted treatment.[54]

6.2. Pre-clinical studies

A recent study investigated the antimicrobial effectiveness of a commercially available olive ozonized oil (O_3-Oil, 560-590 mEq/kg), in comparison with 0.2 % chlorhexidine digluconate (CHX) and 10 % povidone-iodine (PVP-I) through a disk diffusion test. 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 O3-Oil and 13.67 ± 0.58 mm for PVP-I.[32]

Ozonized oil is very effective against *Candida albicans*. For ozonized Theobroma oil, minimum inhibitory concentration and minimum fungicide concentration values are between 5 and 3.75 mg/mL and 11.58 and 5.78 mg/mL for peroxides indexes of 1002 and 1200 mEq O_2/kg of sample, respectively.[55] Ozonized olive oil was investigated for its capacity to inhibit growth of 38 yeast strains of *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, and *Saprochaete capitata*. Ozonized olive oil 1352 mEq O_2/kg was the main effective. Fluconazole was chosen as control antifungal agent. This study demonstrated that ozonized olive oil may help to control some fluconazole-resistant and dose-dependent sensitive fungal strains.[56] Sunflower seed ozonized oil with a peroxide value equivalent to 356 mEq O_2/kg was efficacious against several clinical fungal strains: *Candida parapsilosis*, *Candida albicans*, *Trichosporon asahii*, *Candida tropicalis* and *Candida guilliermondii*.[57] *In vivo* studies also demonstrated that Sunflower seed ozonized oil has significant antimicrobial activity, anti-inflammatory and wound-healing properties, as compared to other commercially available antimicrobial agents.[58]

Animals intra vaginally infected with *Candida albicans* treated with ovules of 20 % Theobroma ozonized oil 220 mEq O_2/kg resulted in a decrease of 0.7 log of the number of microorganisms after 5 days of treatment; moreover, there have been not found signs of infection in rats after 10 days. This result was very similar to the one obtained with ketoconazole.[59] This is in line with antifungal *in vitro* effect of ozonized oil against *Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis.*[60]

6.3. Clinical studies

One hundred patients with confirmed vulvovaginal candidiasis were randomly classified into two groups and treated by ozonized olive oil or clotrimazole for 7 days. The study outcomes were changes in itching, burning, leucorrhea and cell culture before and after the treatment, which were evaluated by an interview and paraclinical examination. Ozonized oil and clotrimazole both reduced the symptoms significantly and led to negative specimen cultures ($p < 0.05$). There was no significant difference between the two groups in their effect on itching, leucorrhea and culture ($p > 0.05$). However, ozonized oil decreased burning sensation significantly better than clotrimazole ($p < 0.05$).[61-63]

Ozonized oil has been used to cure wounds of the cervix uteri after destructive treatments in 50 patients. A control group consisted of 57 women who received no topical treatment after applying the destructive technique. In the study group, the use of ozonized olive oil was found to significantly shorten the alteration and epithelization phases and there was a significant reduction in the time of complete wound epithelization after LASER (to 14 ± 0.4 days on average) and radiosurgical (to 16.1 ± 0.6 days) treatments as compared to the control group (22.3 ± 1.7 days). Ozonized olive oil used after destructive interventions for cervical diseases reduces the time of wound healing by an average of 6.2 ± 2.15 days, which can enhance the clinical efficiency of treatment.[64]

Patients (150) were selected with ages ranging from 30 to 50 years, with vulvovaginitis for at least 6 months of evolution, refractory to usual drug treatment and positive cultures for candidiasis. It was performed one hydrocolontherapy with ozonized water. It was given: diet low in carbohydrates of high glycaemic index, daily intravaginal instillations with ozonized water (10 sessions), daily intravaginal ozone insufflations at concentration of 20 µg/mL at a flow rate of 0.2 L/min during 10 min (10 sessions), application of ozonized oil (sunflower ozonized oil) with PV of 600 and 400, during 10 days, 4 major autohemotherapy at 2.0 µg once a week. At the end of treatment were repopulated the vaginal microbiota with *Lactobacillus vaginal* tablets for 7 days and repopulated the intestinal microbiota with Lactobacillus using oral route, during 1 month. Results: 85 % of patients favourably responded to treatment, 10 % remained asymptomatic for a period of less than one year and 5 % of patients did not respond to treatment. Intravaginal ozone therapy offered an effective alternative to conventional treatment with usual fungicides, not only achieved a remission of symptoms and negative cultures of vaginal exudates in patients with vulvovaginitis, but also increased IgA and Lactobacillus in the vaginal epithelium. It also served to restore the body´s own defence abilities by stimulating the normalization of vaginal mucosa local immunity, without producing a disturbing effect on the saprophytes.[65]

Ozonized Sunflower Oil in the Treatment of the Infection Caused by the Human Papilloma Virus: Sixteen women with the human papilloma virus (HPV) in the vagina or in the cervix were studied and treated with embrocations of ozonized sunflower oil on the affected areas, using the speculum for the curing. The treatment was daily performed for 15 days. The results, by colposcopy and cytology, showed an effectiveness of 94 %. [24,66] (CU patent 22749)

6.4. Remarks

The application of ozonized oil ranging dose (220-1200) mEq O_2/kg in case of vaginitis significantly reduces the symptoms and led to negative specimen cultures. Moreover, it may help to control antibiotic-resistance. It also serves to restore the body´s own defence abilities by stimulating the normalization of vaginal mucosa local immunity without producing a disturbing effect on the saprophytes. In addition, it shortens the alteration and epithelization phases and significantly reduces the time of complete wound epithelization.

Ozonized oil and its application in dentistry

7.1. Periodontitis

Application of the Ozonized Sunflower Oil in Periodontitis: A clinical, phase III, randomized, controlled and simple blind trial was done, using only ozonized oil and combination therapy. The sample belonged to 50 patients, divided into 5 groups of 10 patients each one: Group A – Treaties with ozone gas. Group B – sunflower ozonized oil (Oleozon®). Group C – ozonized water. Group D – treatment of ozone combined with the three modalities (gas, ozonized water and Oleozon®. Group Z (control) – conventional treatment. The groups A, B, C and D were the experimental groups. Clinical and microbiolgical evaluation were performed. Effectiveness of the treatment and adverse events were evaluated. Clinical evaluation went satisfactory to the month of the treatment in 84.6 % of the studied places, with better results in the group D (96 %), with significant differences between the experimental groups and the control one. The microbiological evaluation was satisfactory and increased to 85.4 % to the six months of the study. The experimental group D prevailed (96.6 %). The effectiveness was good in 85.4 % of the sample, prevailing in the experimental group D with 96.6 %, followed by the group A. The percentage of adverse events was low, 1.5 %. This study concluded that: the clinical and microbiological evaluation showed satisfactory results, associated to a low percentage of adverse events (with gas ozone only). The combined ozone therapy was the most effective treatment for this type of periodontitis.[67]

A study evaluated the effect of subgingival application of ozonized olive oil gel as an adjunct to scaling and root planning (SRP) in aggressive periodontitis. Thirty patients were randomly selected and equally divided into: Group I received SRP only, group II received SRP and ozonized olive oil gel. Subgingival application of ozone gel was performed following initial SRP and at 7, 14 and 21 days. Clinical measurements included pocket depth (PD), plaque index (PI), gingival index (GI), bleeding on probing (BOP) and clinical attachment level (CAL). Real time polymerase chain reaction (PCR) was carried out to determine the effect of the treatment on both *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Porphyromonas gingivalis* (*Pg*). Clinical measurements and plaque samples for PCR were recorded at baseline, one, three and six months after treatment. The results showed improvement in all clinical parameters in group II, which was maintained up to six months ($p < 0.05$). However, this improvement was best following one month but gradually decreased at 3 and 6 months. Whereas SRP alone resulted in a significant improvement only up to one month for BOP, PPD and CAL parameters and up to three months for the PI and GI scores as compared to baseline values. It revealed significant reduction of the mean *Pg* and *Aa* DNA copies at 1 and 3 months for group II, whereas group I resulted in slight reduction up to 1 month only followed by gradual increase reaching baseline values. There was no significant difference between groups at three and six months regarding *Pg* DNA copies. There was a significant difference between groups at the one- and three-months' periods in term of number of *Aa* copies ($p < 0.001$, $p < 0.05$ respectively). The study concluded that ozonized oil gel could be a promising adjunct to SRP in the treatment of aggressive periodontitis.[68]

Similar results were found when the efficacy of ozonized olive oil was evaluated as a monotherapy and an adjunct to scaling and root planning in the treatment of chronic periodontitis. A split mouth, double-blinded, randomized controlled clinical trial was conducted on 20 subjects diagnosed with chronic periodontitis. Quadrants of each subject were randomly assigned to four groups and treated accordingly: Group A, scaling and SRP; Group B, topical ozonized olive oil as an adjunct to scaling and root planning; Group C, topical ozonized olive oil as a monotherapy and Group D, topical chlorhexidine gel as a monotherapy. The quadrants were analysed clinically by plaque index, gingival index, sulcus bleeding index, probing pocket depth, and clinical attachment level at baseline, 2, 4, 6 and 8 weeks of time intervals. The subjects were also analysed for perceived pain, discomfort or tooth hypersensitivity (quadrant wise) on a Visual Analogue Scale (VAS). Additionally, subgingival plaque samples were collected from the two predetermined sites of each quadrant at baseline, 4 and 8 weeks for the analysis of total bacterial counts (TBCs) and the detection of frequency of eight putative periodontopathogens by PCR method. The adjunctive use of the ozonized oil with SRP resulted in a significant improvement ($p < 0.001$) of clinical parameters as well as microbiological parameters over the time and in comparison, to the control groups. The ozonized oil as monotherapy also showed a significant improvement ($p < 0.001$) in clinical parameters as well as microbiological parameters over the

time without any documented side effects. However, there was a significant increase ($p < 0.05$) in dentinal hypersensitivity following ozonized oil as an adjunct to scaling and root planning therapy. The ozonized oil, as an adjunctive therapy as well as a monotherapy is efficient in improving periodontal conditions. [69]

Application of the Ozonized Sunflower Oil in Periodontitis: In this study, ozonized sunflower oil was used in order to evaluate its effect on the treatment of moderate simple periodontitis and for preventing its recidivation. A randomized, controlled and single-blind phase III clinical trial was performed in 84 patients, older than 35 years, from both sexes. Ozonized sunflower oil was topically applied to 42 patients on the operated area and on the 7th, 14th and 21th days after operation on the adjacent periodontal tissues. The control group was formed by 42 patients that received the conventional treatment with chlorhexidine (aqueous solution 0.2 %). An analytic index of hygiene, clinical and radiographical tests and microbiological controls were applied to the patients, at the beginning, on the 21th, 90th and 180th days and with intervals of 1 month until 9 months after operation. The effectiveness of the treatment after 180 d was considered as: good (satisfactory clinical and microbiological assessments) in 98 % of the patients of the group treated with ozonized sunflower oil and in 78 % of the control group; fairly good (some of the assessments were not satisfactory) for 2 and 17 %, respectively, and in the category of bad (both assessments were not satisfactory) only 5 % appeared in the control group. Recidivation was more frequent in the control group (15 %) than in the group treated with ozonized sunflower oil (5 %). In general, the best clinical results (best evolution and healing during the study) and microbiological results were obtained in the group treated with ozonized sunflower oil, and also a lower percentage of recidivation was found in that group. No side effects were observed.[24] (CU patent 22749)

7.2. Alveolitis

Application of the Ozonized Sunflower Oil in the Treatment of Alveolitis: The ozonized sunflower oil was used as the only drug in the treatment of alveolitis. The results were compared to those corresponding to a control group, where Alvogil® (iodine) was used as local treatment, besides applying an oral antibiotic.

The sample was formed by 100 adult patients, randomly distributed into two groups, with 50 patients each. Healings were performed every 72 h and visits to the doctor were carried out as required. The healing criterion considered was the formation of a healing tissue and the decrease or elimination of the pain. The healing was reached in 43 % of the patients treated with ozonized sunflower oil and in 41 % of the patients treated with Alvogil®, without any significant difference between both groups. However, patients treated with ozonized sunflower oil healed most rapidly

and they only required two or three visits to the doctor, regarding the patients healed with Alvogil® that required four to six visits to the doctor.[24,70] In alveolar osteitis, and the treatment of the pain derived for this pathology, the most potent effect was observed for the ozone gas, then ozonized water and finally ozonized oils.[71]

7.3. Gingivitis

Treatment of the Acute Ulcer-Necrotizing Gingivitis with Ozonized Sunflower Oil: A random phase III clinical assay was performed in a group of 48 patients suffering from acute ulcer-necrotizing gingivitis. From those patients, 24 formed the group with ozonized sunflower oil, by topical applications on the lesions, three times a day, for 7 days.

The control group (24 patients) was treated with local applications of aqueous solution of sodium perborate, as risings, with similar periodicity to that of the group treated with ozonized sunflower oil. The tests were performed 3 or 7 days after the beginning of the treatments. In the group treated with ozonized sunflower oil, 75 % of the patients were healed compared to the control group that reached 29.2 %, with a significant difference ($p < 0.01$). Regarding the signs and symptoms assessed: gingival bleeding, signs of local acute swelling and gingival pain, they disappeared more rapidly in the group treated with ozonized sunflower oil.[24]

In a case report study on the efficacy of a formulation based on ozonized sunflower oil [450 PV] (Ozoral®, Innovares, Italy), a once-a-day application of the formula for three weeks reduced by 55 % the total bacterial load. Results also showed a reduction of 58 % in the pathogenic microbial population.[72]

7.4. Radicular ducts

Application of the Ozonized Sunflower Oil in the Treatment of Infected Radicular Ducts: The sample was constituted by 200 adult patients presenting radiolucid rarefaction areas, with or without fistulas in monoradicular teeth. The patients for the study were randomly allocated into two groups of 100 patients each. The test group received healings with ozonized sunflower oil, by sterile cotton balls impregnated with the oil and put into the cavity, at the entrance of the ducts.

The change of the cure was performed every 48 h. In the control group, the healing was made at the same place and similarly, using a liquid bactericide (Cresophen®). In this group, a similar application was performed seven days later. Radiological and clinical tests were carried out to the patients at the start and at the end of the treatment. In the group treated with ozonized sunflower oil, the results were better, with 91 % of improvement compared to the control group

(55 %) with significant differences ($p < 0.01$). 88 % and 5% of the patients healed with ozonized sunflower oil and Cresophen®, respectively, showing significant differences between both groups. Patients treated with ozonized sunflower oil needed two or three visits to the doctor's office, while most of the healed patients of the control group required four to six visits to the doctor's office.[24]

A study evaluated in dogs the response of the periradicular tissues to the endodontic treatment of infected root canals performed in a single visit or in two visits, using different interappointment dressings. Periradicular lesions were induced by inoculating Enterococcus faecalis in the root canals. After confirming that a periradicular lesion developed, the root canals were treated within one or two visits, using either ozonized oil or calcium hydroxide in camphorated paramonochlorophenol (CMCP) as an intracanal medication. After 6 months, the animals were sacrificed and the specimens were processed for histological and histobacteriological analysis. The root canals treated in a single visit showed a success rate of 46 %. When a calcium hydroxide/CMCP-based interappointment intracanal medication was used, 74 % of the cases were categorized as success. In cases where ozonized oil was used as the intracanal medication, a success rate of 77 % was observed. These results demonstrated that the two-visit treatment offered a higher success rate compared to one-visit therapy. In addition, ozonized oil may potentially be used as an intracanal medication.[36] Ozonized oil is effective against microorganisms infecting the root canal (eg. *Enterococcus faecalis*, *Streptococcus mutans* and *Candida albicans*) with an appropriate safety pattern.[73]

7.5. Gingivostomatitis

Comparative Study of the Effect of the Ozonized Sunflower Oil in Gingivostomatitis in Relation with Conventional Treatments: One hundred sixty children suffering from aphthous gingivostomatitis, between 0 and 15 years old were treated. The clinical symptoms of the children were fever, marked anorexia, salivation, gingival pain, asthenia and uneasiness of several days of evolution. The experimental group (60 children) were daily treated with touches of ozonized sunflower oil and the control groups with three different products (by following a procedure similar to that used for the experimental group): iodoxuridine (60 children), hibitane (20 children), boroglycerine (20 children). Between the third and the seventh days of treatment, the complete healing of the lesions was reached in 75 % of the patients treated with ozonized sunflower oil and in 6 % of the patients from the control group, with statistically significant differences ($p < 0.001$), regarding the other control treatments applied in a similar period of time.[24]

7.6. Viral infections

Application of the Ozonized Sunflower Oil in the Treatment of the Acute Herpetic Gingivostomatitis: This study covered the treatment of 113 patients with antecedents of acute herpetic gingivostomatitis, and they were daily treated with ozonized sunflower oil. In 76.9 % of those patients, the symptoms disappeared after a three-day treatment; in 20.4 % they disappeared on the seventh day of treatment and in 2.7 % the symptoms disappeared on the tenth day. The microorganism most frequently isolated in the lesions was the *Staphylococcus aureus.*[24]. In an additional study involving 2596 patients, the efficacy was 92.7 % (2007 patients cured) with 0.3 % of adverse reactions.[74]

7.7. Other applications

It has been recently demonstrated, in animal models, that topically-applied ozonized oil, may have a positive influence in **bone density** and in the quality of **osseointegration** around dental implants.[75] In addition, the combination of treatments using local ozone therapy and the application of a formulation based in ozonized sunflower oil [450 PV] (Ozoral®, Innovares, Italy) for 60 days, in a case of **peri-implantitis** showed the restoration of the pathological condition. It was shown that symptoms as: edema, bleeding and purulence, disappeared during the treatment. Moreover, tissues re-epithelization was observed. In addition, It was note, using evolutive radiographic image, new formation of bone after treatment.[76] In addition, it has been recently demonstrated, in animal models, that topically-applied ozonized oil, may have a positive influence in bone density and in the quality of osseointegration around dental implants.[77]

Ozonized oil also showed beneficial effect in oral Lichen planus and lichenoid lesions, that comprise a group of disorders of the oral mucosa that likely represent a common reaction pattern to 1 or more unknown antigens.[78] A formulation based on ozonized sunflower oil [450 PV] (Ozoral®, Innovares, Italy) applied for 4 week in 20 patients showed reduction in pain after the first application and a reduction in the local inflammation.[79] In addition, Lichen planus was also treated with olive[80] o sesame[81] ozonized oils with excellent improvement, unfortunately in this cases the IP of the oils was no declared.

A study evaluated the efficacy of ozonized olive oil with or without adjunctive application of mineral wash containing calcium sodium phosphosilicate on the reversal of post-surgical root **dentin hypersensitivity**. A double-blinded, randomized controlled clinical trial was conducted on 51 participants with root dentin hypersensitivity (RDH). Participants were randomly assigned to 4 groups: Group A, ozonized olive oil: Group B, ozonized olive oil and mineral wash: Group C, placebo olive oil (PPO) and mineral wash: Group D, placebo olive oil only. Active treatment was

carried out in-clinic and followed by at-home care with a remineralising paste. The response to various pain stimuli was periodically assessed with a visual analogue scale. Additionally, scanning electron microscopic study assessed the dentinal tubule occlusion and change in tubular surface area after treatment. The group B participants showed a significant decrease in tooth level and global sensitivity over the period ($p < 0.001$). Moreover, the intergroup comparison also revealed a significant result ($p < 0.001$). Similarly, participants of group C also showed a significant reduction in sensitivity over the period ($p < 0.001$). Whereas, no significant ($p > 0.05$) difference was detected between group A and group D for tooth level and global sensitivity analysis. The study result showed a significantly ($p < 0.001$) enhanced tubule occlusion and decreased tubular surface area in group B specimens compared to other group specimens. Ozonized oil, as a monotherapy is not efficient in reducing post-surgical RDH. However, the adjunctive application of mineral wash containing calcium sodium phosphosilicate has positive impact on the reversal of post-surgical root dentin hypersensitivity.[82]

A very new trend is the use of ozonating oil to perform the ***oil pulling***[83] but clinical trials should be done. In Ninety-six subjects with a diagnosis of periodontitis, scaling and root plaining with the aid of ozonized olive oil mouthwash were found to be more effective on salivary Extracellular Matrix Metalloproteinases (MMPs) reduction than scaling and root plaining alone. MMP-8 play a pivotal role in the damage to the periodontal tissue in patients with periodontitis.[84]

A study was focused to evaluate the efficacy of ozonized olive oil in the treatment of **oral lesions and conditions**. A longitudinal study was carried out on 50 patients (aphthous ulcerations, herpes labialis, oral candidiasis, oral lichen planus, and angular cheilitis). The ozonized olive oil was applied twice daily until the lesion regressed for a maximum of 6 months. All the lesions regressed in patients with aphthous ulcerations, herpes labialis, oral candidiasis and angular cheilitis or showed improvement in the signs and symptoms in oral lichen planus patients. No toxicity or side effect was observed in any of the patients. Ozone therapy required a gaseous form to be more effective, but topical form could also bring out the positive results without any toxicity or side effect. Hence, it can be considered as a minimally invasive therapy for the oral infective and immunological conditions.[85]

A case report showed the effect of ozonized oil in an exophytic fibrous gingival lesion. A 42-years female patient was selected, who presented a mild to moderately painful, exophytic, fibrous lesion on the upper anterior gingiva. This gingival lesion was treated with 2 mL of ozonized oil, thrice daily for one week. After the therapy, the postoperative outcomes were measured and analysed. Finally, the lesion was subjected to an excisional biopsy and a histopathological evaluation. After the therapy, the patient revealed that there was less pain. On examination of the lesion, an improvement was observed in the clinical sign of the inflammation and also a reduction in the

surface ulceration. During the final biopsy, less bleeding was observed. The morphometrical analysis showed a reduction in the size of the lesion. The histopathological analysis showed a reduction in the collagen fibres and in the inflammatory cells in the connective tissue stroma. Topical ozonized oil therapy provided potential benefits for the treatment of exophytic gingival lesions. The observed benefits in present case report needs to be verified in future with well-controlled clinical trials.[86]

Miscellaneous application

Use of ozonized oil in Ophthalmology: In a prospective, single-blind, randomized, parallel-groups trial. Eighty patients with a clinical diagnosis of presumed viral conjunctivitis were randomly divided into two treatment groups: a study group and a control group, 40 for each group. Patients in the study group received topical tobramycin 0.3 % / dexamethasone 0.1 % eye drops, plus ozonized oil eye drops, both four times daily; patients in the control group received only topical tobramycin 0.3 %/dexamethasone eye drops four times daily. The treatment was for seven days in both groups. The use of ozonized-oil containing eye drops in combination with topical tobramycin 0.3 %/dexamethasone 0.1 % eye drops four times daily seemed to reduce the signs of conjunctivitis, and the duration of viral infection, although it did not affect the subepithelial corneal infiltrates appearance.[87] The successful treatment of conjunctivitis, keratoconjunctivitis and corneal ulcers in veterinary medicine has also been reported.[88]

Ozonized Sunflower Oil in the Treatment of Epidemic Hemorrhagic Conjunctivitis (EHC). EHC is a self-limited, conjunctiva inflammation of viral etiology which affects all ages and takes place in epidemic form. Its main symptoms are sensation of foreign bodies, lacrimation, photosensitivity, general discomfort and pain. Its critical signs are subconjunctival haemorrhages, follicular reaction and pre-auricular adenopathy. Also, serous secretion, chemosis, superficial punctate keratitis and palpebral ptosis are observed. Taking into account the broad-spectrum germicide of ozonized sunflower oil, as well as its degree of anti-inflammatory character, a study evaluated the effectiveness of this medication in its collyrium form for the treatment of EHC. In “Dr. Salvador Allende” Clinical Hospital, 20 patients were treated with EHC in October, 2009. Twelve of them received treatment with ozonized sunflower oil collyrium (one drop twice per day), 8 were used as control group and received conventional treatment (cold compresses, non-steroidal antiinflammatory drugs, yodoxuridine in collyrium or recombinant alfa-2b interferon). All patients treated with ozonized sunflower oil underwent a fast evolution toward recovery. In 72 h, they showed signs of great improvement and in 1 week they were totally cured. No patients presented any complication. In the control group the evolution was more prolonged, mainly in patients showing complications (3 with keratitis). Treatment of EHC with ozonized sunflower oil collyrium provides very positive results in this disease.[89]

Application of Ozonized Sunflower Oil in Acute Tonsillitis: Fifteen patients suffering from acute tonsillitis were studied and daily treated with ozonized sunflower oil in the oropharyngeal area for a week. Microbiological controls (pharyngeal exudate) and physical tests of the oropharyngeal area were performed to those patients at the beginning and at the end of the treatment.

Among the microorganisms in the first exudate, we found *Streptococcus pyogenes*, *Haemophylus influenzae*, *Bordetella pertussis*, and others. At the end of the treatment, all patients were cured, taking into account the microbiological and clinical tests performed. [24] (CU patent 22749)

Application of Ozonized Theobroma Oil in the Treatment of Tynea Pedis: Fifty patients with a diagnosis of *tynea pedis*, randomly distributed into two groups of study, 25 patients in each group, were studied. The experimental group was treated with an ointment containing 20 % ozonized Theobroma oil, for 6 weeks, twice a day and the control group was treated with Whitfield ointment with no sulphur with a similar plan of treatment. The healing criterion was the presence of negative microbiological exudate. A healing of 85 % and 20 % in the experimental and control groups, respectively, with significant difference between both groups was obtained. [24] (CU patent 22749)

Sunflower and Olive ozonized oils are fungicide, active against fungi that originate superficial mycosis in humans, such as *Candida albicans*, *Trichophyton mentagraphytes*, *Microsporum canis*, *Thichophyton rubrum.* [24,66] (CU patent 22749). Topical Sunflower ozonized oil was evaluated in a controlled randomized phase III assay, using ketoconazole (Nizoral®) as the comparing group. The results showed no significant differences between the two medications, nor side-effect or bacterial superinfection were observed in the study.[24]

Application of Ozonized Sunflower Oil in the Treatment of Lower Limb Ulcers Caused by Chronic Venous Insufficiency: A study was performed with 20 patients with lower limb ulcers caused by chronic venous insufficiency with less than five years of evolution. Both groups were treated with venous rest, hyposodic diet and analgesic drugs. Besides, a mechanical disinfection with benzalkonium chloride 1/5000 was performed twice a day. After disinfection, ozonized sunflower oil was applied to the experimental group and antibiotic ointments, according to the isolated germ, were applied to the control group. An amelioration of the inflammatory signs after 72 h and the appearance of granulation tissue after the fifth day were observed in the experimental group, whereas in the control group, both the evolution and the disappearance of signs and symptoms lasted more. [24] (CU patent 22749)

Application of the Ozonized Sunflower Oil in the Treatment of Bedsores: Twenty patients suffering from bedsores in the sacral region were studied and randomly distributed into two groups of 10 patients each. The experimental group was treated with ozonized sunflower oil, twice a day, and the control group was treated with ointments, according to the germ present, with a similar plan of treatment. All the patients succeeded in the healing of their wounds. In the group treated with ozonized sunflower oil, the time of healing was shorter and it was not necessary to perform any antibiogram, because of the wide germicidal power of ozonized oil. [24] (CU patent 22749)

Application of the Ozonized Oil in the Treatment of Fistulae and Chronic Surgical Wounds: In a study involving 28 patients suffering from fistulae and chronic surgical wounds a fully effectiveness in 27 cases without side effect was found.[7] Ozonized oil was also used in the treatment of osteonecrosis of the jaw in patients with bone metastases.[90] A clinical trial was developed in eight patients who underwent radio- therapy for the treatment of cervical or neck cancer and diagnosed with Osteoradionecrosis of the jaws. The exposed bone lesion and osteomucosal margin were cleaned with manual debridement and treated with topical applications of ozonized oil for 10 minutes with successful results. [91]

Ozonized oil has also proved to be very effective in burns.[4] In addition, ozonized oils are used for the long-term treatment of injuries, burns and local infections such as skin and nail mycosis, as well as in the follow-up treatment of ulcus cruris and decubitus ulcers.[92]

Ozonized sunflower oil shows inhibition and lethal activity in case of *Giardia lamblia* infections.[49] In addition, ozonated olive oil can have synergistic effects with glucantime in the treatment of cutaneous leishmaniasis, a clinical trial reported the reduction lesion size after the simultaneous application of glucantime and ozonized oil, in patient affected of cutaneous leishmaniasis.[93]

Safety of ozonized oils

The first report of use of ozonized oil was done in 1859,[94] the ozonized oil was used by ingestion in patients with pneumonia, and was observed an improvement in health conditions. The diverse tests performed with ozonized sunflower oil showed the safety of this kind of products: toxicological tests, histological tests, mutagenic tests,[95,96] genotoxic tests[97,98] and teratogenic tests[24] put in evidence the safety of this type of products. Ophthalmic irritability and dermic irritability test,[99] classified ozonized sunflower oil as non-irritant.[100] The sensitizing effect of sunflower ozonized oil was also tested resulting in no sensitizing effect.[101] In clinical assays using ozonized oil in the treatment of infective lesion, side-effect was not reported.[102] The same trends is observe for other ozonized oil, for example: cytotoxicity test for ozonized hazelnut oil concluded that hazelnut oil is safe.[103] Ozonized olive oil with a PV of 2700–2900 mEq O_2/kg oil for short-

term incubation was non-cytotoxic in L929 fibroblast cell line.[104] Vaginal irritability test for ozonized Theobroma oil showed that the oil do not cause irritability.[105]

Studies about the metabolism of ozonized sunflower oil shown that oral administration to Wistar rats has produced changes in the urinary content of dicarboxylic organic acids. Among others heptanedioic (pimelic acid) and nonanedioic acids (azelaic acid) were the major increased dicarboxylic acids found.[106]

Ozonized olive oil at dose of 3 and 6 mg/kg administered by oral way, showed a macroscopic and microscopic significant reduction in the damage scores in an experimental model of ulcerative colitis. In addition, CAT, GSH-Px, and SOD activities were significantly increased in the distal colon of inflamed animals pretreated with ozonized oil with respect to control group dose dependently. This experiment provide evidence that the protective effects of ozonized oil are mediated by stimulation of some antioxidant enzymes.[102] The same effect was observed in an ethanol-induced ulcers experimental model in rats.[34]

Rectal administration of ozonized oil to Wister rats, performed every other day for a total of 10 showed no pathologic morphological finding in the tissues examined. It was observed not any difference between the mucous membranes of the control group and the rest of the treated animals.[107]

Quality

A quality ozonized oil to be used with medical purpose should be prepared following the good manufacture practice. That means a strictly quality control during its production in a high-quality reactor by fixing the quality of the raw materials and important reaction variables as: time of reaction, ozone concentration, ozone sources, burbling size, reaction temperature and others. A quality control of the active component (ozonized oil) should involve chemico-physical analysis, microbiological analysis and biological analysis. Biological analysis should demonstrate the pharmacological effect attributed to the oil and the absence of toxicity. Microbiology should demonstrate the microbiological quality of the preparation. Chemical / physical analysis will be done to guaranty the homogeneous chemical content of active component and the stability. Chemical analysis will involve the measurement of the content of lipoperoxides and aldehydes, iodine and saponification indices. Physical analysis will take into consideration the acid values, density and viscosity of the active component. Test will be done according to the pharmacopeia methods and should be also used to demonstrate the stability of the preparation.[108]

Conclusions

The medical use of ozonated oils in infectious diseases has numerous advantages over conventional therapies. This type of therapies reduces the cost, has similar or superior effects to traditional antibiotics, result in a broad antimicrobial spectrum and a low rate of adverse events. The products of lipid oxidation produced after the reaction of ozone with fatty acids and other substrates, generate compounds with germicidal, immunostimulant and tissue repair activity. The stability of ozonated preparations allows the development of conventional formulations for clinical use. The hight standard quality control are required for he ozonized oil as raw materials of pharmaceutical in order to guaranty its efficacy and avoid toxicity. Chemical and physical characterization and a precise PV (as index of dosage) will be considering as a quality criterium. Today the main applications of the ozonized oil are for external use essentially in dermatology, dental, ophthalmology and gynaecology, however there are evidences of immune-stimulating, germicide and repair effects when used orally. Additional clinical trials are need in order to support the clinical used of ozonized oils, but bases in standardized and stable formulations.

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