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DOCTORAL THESIS

BIO-OXIDATIVE OZONE THERAPY IN DENTISTRY

- A B S T R A C T -

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SUMMARY

Ozone (O_3) is a naturally occurring compound that is the triatomic form of dioxygen (O_2). It is naturally present as a gas in the stratosphere to protect living organisms from UV (ultraviolet) radiation and is derived from oxygen through the action of UV light and electrical discharges; however, ozone is very unstable (half-life of 40 min at 20°C) and decays rapidly to its diatomic allotrope, which accounts for the very small amounts of ozone in the atmosphere compared to oxygen(1,2) . Ozone is recognized as a powerful oxidant and thus has been proposed as a disinfectant, deodorizer, cleaning and bleaching agent, food additive and air/water purifier.

Scientists are increasingly discovering that Bio-Oxidation is much more complex and important than previously recognized. There are many theories about the different functions of oxygen and hydrogen in the body. There is plenty of scientific evidence to support almost every one of these theories. When administered in gaseous form, it is a gaseous oxygen-ozone mixture, in which ozone accounts for no more than 5% of the total.

Bio-oxidative medicine, through its therapies, provides the body with more oxygen oxygenates it and improves in particular the uptake and use of oxygen at the cellular level. Adding extra oxygen has many benefits for both prevention and treatment of many types of conditions and diseases. By increasing the intracellular availability of oxygen, cells begin to oxidase-accumulated toxins, eliminate them, cells regenerate and inevitably tissues regenerate. On the other hand the chemical reaction involving oxygen is known as oxidation. Oxidation reactions can be slow or fast. The human body could not survive without oxidation reactions. In the biochemical context, oxidation refers to the loss or transfer of electrons from one atom or molecule to another, thus the natural by-product of physiological oxygen metabolism leads to the formation of reactive oxygen species, called free radicals (ROS). Reactive oxygen species play an important role in a huge number of biological processes. Many of them are necessary for life, such as the first line of defence against bacteria, viruses, yeast and parasites by antibodies and phagocytes such as granulocytes and macrophages. The main oxygen free radicals are peroxides, superoxides, hydroxyl radicals and singlet oxygen. These are derived from molecular oxygen under conditions of chemical reduction. Excessive growth of these ROS, can result in the destruction of cellular structures, a phenomenon known as oxidative stress (OS), which occurs in an imbalance favouring pro-oxidants over antioxidants.

The present study is an analytical and critical synthesis of the use of bio-oxidative ozone principles in dentistry. Many devices and products for the use of ozone in dentistry that can be found on the medical device market have little published scientific evidence in the field of dentistry.

The **main objective** of the thesis is to evaluate the biological effects of oxygenation by ozonation, on soft and hard tissues in the oral cavity. The thesis proposes to identify the protocols of ozone use in dentistry: the effectiveness of the time and type of ozonation on the pathogenic factors that cause the main oral pathologies: dental caries and periodontal disease.

The objective of the thesis is achieved by::

1. In vivo evaluation of the biological effects of gaseous ozone on the remineralization process of dental enamel, the potential of ozone for remineralization immediately and over time.
2. Evaluation of cytotoxicity, in vitro of ozone gas and changes that may occur on soft tissue specific cells Gingival fibroblasts (HGF) and gingival keratinocytes (PGK).
3. The analysis of the biooxidative effect and microstructural changes of dental enamel following ozone use at different time intervals.
4. Evaluation of the antibacterial effect of gaseous or potent ozone from *Mentha piperita* Essential Oil (MpEO)

The thesis is classically structured in 2 parts, the **General Part**, Literature review and the **Special Part**, Personal contributions.

The **General part** represents 1/3 of the thesis and includes the following chapters: properties and mechanisms of action of ozone (chapter 1), the action of ozone in dentistry (chapter 2), methods of use and ozone production devices used in dentistry (chapter 3).

The **Special part** represents 2/3 of the thesis and is structured in 4 studies, as follows:

1. Evaluation of the potential for remineralization of tooth enamel and arrest of initial enamel damage using gaseous ozone.
2. Evaluation of the bio-oxidative effect and cytotoxicity of gaseous ozone on gingival cells: gingival fibroblasts (HGF) and gingival keratinocytes (PGK)
3. Evaluation of the impact of ozone on the morphological and microstructural characteristics of dental enamel - *in vitro* study, SEM (Scanning Electron Microscope) and LTF (Laser Fluorescence Technology) analysis

4. The analysis of antimicrobial potential of ozone alone or in combination with *Mentha piperritaes* essential oil, against different bacteria: *Escherichia coli* (*E.Coli*), *Staphylococcus aureus* (*S.aureus*), *Pseudomonas aeruginosa* (*P.aeruginosa*), *Streptococcus mutans* (*S.mutans*), and *Candida albicans* (*C.albicans*) fungi, at different exposure times.

In the first and the second study, the ozone produced by HealOzone was applied in vivo to 68 M1s (first permanent molars), both maxillary and mandibular, on the occlusal surfaces at pit and fissure. The molars included in the study recorded values between 13 and 24 according to the DIAGNOdent Pen 2190 scale, this being the main inclusion/exclusion criterion for the investigated molars. Because the gas can make contact with primary gingival cells during the ozonation process, both human gingival fibroblasts and keratinocytes were exposed to different doses of ozone (20 s, 40 s, 60 s), and its effects were observed with the Olympus IX73 inverted microscope. The contact of ozone with the human primary gingival cells demonstrates cell sensitivity to the action of ozone, this being higher in fibroblasts compared to keratinocytes, but it is not considered toxic because all the changes are reversible at 48 h after exposure

In the third study, the ozonation was performed with gaseous O₃ produced by HealOzone X4, the demineralization level was measured with the DiagnoDent Pen 2190 device, and the microstructure changes of enamel surface were observed using scanning electron microscopy (SEM) analysis. The results showed the exposure to O₃ for 40-50 seconds enhanced enamel micro-hardness and ensures a rate of remineralization between 96.82-97.38%. In search of new minimally invasive solutions in the treatment of caries and to offer antimicrobial support of the oral cavity, the use of O₃ as an alternative therapy to classical solutions may be a viable solution in dentistry

The fourth study aimed to obtain and analyse *Mentha piperita* essential oil (MpEO) for the prospect of being used as an enhancement agent for the antimicrobial potential of ozone against gram-positive and gram-negative bacteria and fungi. The research was done for different exposure times, and it gained time-dose relationships and time-effect correlations. *Mentha piperita* (Mp) essential oil (MpEO) was obtained via hydrodistillation and further analysed by using GC-MS. The broth microdilution assay was used to determine the strain inhibition/strain mass growth by using spectrophotometric optical density reading

(OD). The bacterial/mycelium growth rates (BGR/MGR) and the bacterial/mycelium inhibition rates (BIR/MIR) after ozone treatment in the presence and absence of MpEO on the ATTC strains were calculated; the minimum inhibition concentration (MIC) and statistical interpretations of the time–dose relationship and specific *t*-test correlations were determined. The effect of ozone on the following tested strains at maximum efficiency was observed after 55 s of single ozone exposure, in order of effect strength: *S. aureus* > *P. aeruginosa* > *E. coli* > *C. albicans* > *S. mutans*. For ozone with the addition of 2% MpEO (MIC), maximum efficacy was recorded at 5 s for these strains, in order of effect strength: *C. albicans* > *E. coli* > *P. aeruginosa* > *S. aureus* > *S. mutans*. The results suggest a new development and affinity regarding the cell membrane of the different microorganisms tested. In conclusion, the use of ozone, combined with MpEO, is sustained as an alternative therapy in plaque biofilm and suggested as helpful in controlling oral disease-causing microorganisms in medicine.

The present research was successful in archiving the aims as started at the beginning of the Special part.

The final concusions of this thesis are listed below:

1. Preservation of healthy tissues, minimally invasive interventions and stimulation of regenerative processes through tissue engineering are increasingly discussed in contemporary dentistry.
2. Bio-oxidative medicine, through its therapies, provides the body with more oxygen it and improves in particular the uptake and use of oxygen at the cellular level. Adding extra oxygen has many benefits for both prevention and treatment of many types of conditions and diseases.
3. One of the mayn objectives of the thesis is to describe and understand the effects of O₃ on dental enamel. The result was found to be statistically significant differences between initial and post-O₃ assessment ($p < 0.05$), and between post-O₃ and one month mineralization values ($p < 0.05$).
4. Between initial and post-O₃ situation, the difference between the degree of mineralization is statistically significant. (Mineralization was ($m = 1.77$ and standard error = 2.51, $p < 0.05$).

5. A 40 s exposure to O₃ (*HealOzone X4*) dental enamel is sufficient to remineralized a superficial occlusal enamel lesion (*DIAGNOdent Pen 2190*- value 15-24) to the value of healthy enamel (0-140)
6. The arch position of the tooth does not influence the O₃ mineralization process. After initial and one month following the ozone application, there were significant differences between the 4 molar on the arch tested teeth, but we found that the mean differences were not statistically significant, $p = 0.349$.
7. The remineralization process was initiated immediately, but continued over the next 30 days, and the greater the demineralization of the dental units, the more effective the remineralization process.
8. Enamel exposure to gaseous ozone (10, 20, 30, 40, 50 s) significantly improves the microstructural properties of dental enamel. The phenomena of malt quality improvement are even more pronounced after exposure of 50 sec to ozone treatment.
9. These changes depend on the exposure time and the opportunity to disrupt the proteins embedded in the enamel matrix by bio-oxidation.
10. The SEM images after 50s ozonized, show a leveling of the enamel surface, open inter prismatic canals and the prisms' ordering. At the higher the exposure, the better the appearance of the honeycomb with an orderly structure of the hydroxyapatite prisms and the cleaning of the interprismatic spaces was noticed.
11. Given the widespread use of ozone as a therapy for a multitude of dental diseases, it has become mandatory to assess the cytotoxic potential of ozone on soft tissue and oral cells (keratinocytes and fibroblasts) in gingival tissue that come into close contact with this agent.
12. The present study showed that exposure to 20, 40 and 60 s of gaseous ozone elicited a cell type-dependent response as follows: gingival keratinocytes- PGK (forming the outer layer of the gingiva) were affected by ozone after 60 s of exposure (changes in cell morphology and a decrease in confluence), while in the case of HGF-gingival fibroblasts, morphological changes were observed at the shortest exposure interval - 20 s.
13. Results of the study show an increased sensitivity of gingival fibroblasts to exposure to ozone gas compared to the Keratinocytes and could be used as a basis for further research on the harmful effects of ozone gas.
14. In the search for alternative antimicrobial solutions to synthetic antibiotics, the use of ozone in combination with essential oils can represent a viable solution with

applications in medicine and dentistry. The obtained results highlight that the association of ozone with MpEO leads to an increase in its efficiency and a decrease in the exposure time.

15. The use of O₃ in medicine requires the establishment of clear and precise protocols regarding the time and dose of used.

The use of O₃ in medicine requires the establishment of clear and precise protocols in terms of time and dose to be used. This thesis makes a valuable contribution to the implementation of regenerative, non-invasive technologies for the use of ozone bio-oxidation in dental medicine. This paper provides scientific evidence, which supports the use of gaseous ozone or ozone derivatives in non-invasive bio-oxidative therapies in modern, conservative dentistry.