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# **DOCTORATE THESIS**

**NONCONVENTIONAL THERAPIES FOR THE  
REGENERATION OF GINGIVAL TISSUES AFTER  
BISPHOSPHONATE TREATMENT**

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Bisphosphonates are the most used and effective anti-resorptive agents for the treatment of diseases in which bone resorption is caused by the increase of osteoclasts, such as osteoporosis, Paget's disease and tumours causing osteolysis.

Presently, oral bisphosphonates are widely used in the treatment of osteoporosis. Intravenous bisphosphonates are used in the treatment of bone metastatic diseases (consequences of malignant tumours of the breast, prostate, lung or kidney), of primary osteolytic pathology (multiple myeloma, Paget's disease of bone) or to ameliorate the effects of hypercalcemia in malignant diseases as well as the pain associated with osteolytic pathology. Their specific behaviour consists of the osteoclastic activity, a half-life over 10 years and the intervention in the bone turnover.

Bisphosphonates may have secondary effects in the oral-dental area by inhibition of the physiological dental mobility, reduction of the healing process of destroyed bones and the onset of maxillary and mandibular osteonecrosis.

Bisphosphonates are composed of two different radical groups which allow their classification. The first radical is responsible for chemical properties and pharmacokinetics, the second radical determines the potential and action mechanism. Basically, there are two types of bisphosphonates: non-amino bisphosphonates (non-N-BP) including Etidronat and Clodronate, and amino-bisphosphonates (N-BP), such as Zoledronate, Alendronate, Risedronate, Ibandronate and Pamidronate.

Even though bisphosphonate are frequently administered in clinical practice, their side effects are numerous. The side effects of bisphosphonates include oesophageal irritation, dysphagia, migraines, intestinal obstruction, articular and osseous pain, but the most important is maxillary osteonecrosis. The increasingly frequent prescription of this group of drugs led to an increase in the incidence of bisphosphonate-related maxillary osteonecrosis, with attempts to describe stages of this novel clinical entity, as well as to draft adequate prophylactic and therapeutic protocols but also to reduce their side effects.

In search of new methods to reduce the side effects of bisphosphonates, we identified a series of healing opportunities and restoration of cell viability by the use of ozone but also of the essential oil of *Salvia Officinales*. Ozone (O<sub>3</sub>) is a natural, highly reactive gaseous molecule formed of three oxygen atoms. The benefic power of ozone, either alone or in combination with other medicines is well established. Its most important characteristics are: (a) the antimicrobial activity against aerobic and anaerobic bacteria (especially against *Staphylococcus aureus*), fungi and viruses; (b) the stimulation of the circulatory system, increasing haemoglobin synthesis and the production of red blood cells, thus producing tisular oxygenation; (c) modulation of immune cells by acting as a cytokine and increasing their phagocytosis and diapedesis (d) stimulation of angiogenesis as well as the proliferation of fibroblasts; and (e) the capacity to reduce pain. The antimicrobial effect consists of the inactivation of bacteria, viruses and fungi. It destroys the integrity of the bacterial cell membrane by oxidation of phospholipids and lipoproteins.

The use of ozone to treat maxillary osteonecrosis after bisphosphonates therapy was first reported in 2006. The authors introduced ozone therapy together with surgical treatment and antibiotic therapy, reporting a reduction of symptoms caused by maxillary osteonecrosis after bisphosphonates therapy in 90% of cases. The action of ozone has various beneficial effects on oral tissues, including the remission of various mucosal changes, improvement of wound healing and the increase of oral cells turn-over rate.

Sage, scientifically known as *Salvia Officinalis*, is a medicinal plant used as a cosmetic, flavouring agent. It has antibacterial, antifungal, cariostatic, anti-plaque, antiviral,

astrigent properties. In dental medicine, it is used in the treatment of periodontal disease, in the prevention of halitosis and carious lesions.

The thesis is structured in four main parts 1. Introduction, 2. General Section with two chapters, 3. Specific Section with four chapters, and 4. Conclusions. The general section of the thesis will describe bisphosphonates, their classification, therapeutic recommendations, but also their side effects, especially over the maxillary bones. The general section also describes ozone and sage, the substances used in nonconventional therapies for the regeneration of gingival tissues after bisphosphonate therapy, but also their use in dental medicine.

The objectives of the present doctorate thesis aim to a) establish the epidemiological and clinical characteristics of maxillary bone osteonecrosis after bisphosphonates therapy in the population of Timi oara, b) assess the manner of conducting an anamnesis in dental medicine practices in the West of Romania, the interdisciplinary approach including the general practitioners and last but not least to evaluate their level of knowledge regarding the approach of patients treated with bisphosphonates, c) test the cytotoxicity of both oral and intravenous bisphosphonates on human gingival fibroblasts and to attempt counteracting these harmful effects by the benefic action of ozone d) use the essential oil of *Salvia Officinalis* as a nonconventional therapy for the regeneration of gingival tissues after bisphosphonates treatments by applying the oil on human gingival fibroblasts previously stimulated with bisphosphonates.

The specific section is structured in four chapters

1. Epidemiological study on the cytotoxic effects of bisphosphonate therapies in Timi oara
2. Epidemiological study regarding the information and knowledge of dentists on bisphosphonate therapy
3. The biological regeneration potential of ozone over gingival fibroblasts stimulated with bisphosphonates
4. Nonconventional therapy for the regeneration of gingival tissue after treatment with bisphosphonates using essential *Salvia Officinalis* oil

In the first chapter of the specific section entitled Epidemiological study on the cytotoxic effects of bisphosphonate therapies in Timi oara, an attempt was made to establish the epidemiological and clinical aspects of maxillary bones osteonecrosis after bisphosphonate treatments in the population of Timisoara. A retrospective study was conducted on a group of 122 patients admitted in the Clinic of Oral and Maxillo-Facial Surgery of the Timisoara Municipal Hospital with the diagnosis of maxillary osteonecrosis after bisphosphonate

treatment, during the period 2010- 2018. The cases resulted from the analysis of patients' hospital files where the following aspects were analysed: epidemiological (repartition of cases according to years of study, age groups and gender) and clinical data (presence of neoplasia, bone metastasis or other pathologies). The statistical analysis of the data revealed the highest incidence of cases in the year 2017, with osteonecrosis predominantly located in the mandible. The most cases of osteonecrosis secondary to bisphosphonate treatment were found in patients with mammary neoplasm, followed by prostate neoplasm and osteoporosis, thus the epidemiology of maxillary osteonecrosis secondary to bisphosphonate treatment is linked to the epidemiology of the main diagnosis. Most patients belonged to the 60-69 years age group, and the gender distribution was approximately even.

In the second chapter of the specific section entitled Epidemiological study regarding the information and knowledge of dentists on bisphosphonate therapy, an attempt was made to assess the manner of performing an anamnesis in dental medicine practices of the Western part of the country and the knowledge level of dentists regarding the approach of patients under bisphosphonates treatment. A questionnaire was applied to dentists who attended a conference in Western Romania. The questionnaire includes 21 questions and was structured in two main parts: the first part focuses on the manner the dentist performs the anamnesis and the connection he/she has with the general practitioner and the second part investigates the knowledge level and the approach of patients under bisphosphonate treatment. According to the interpretation of the results we may state that one of the most important elements in approaching a patient who addresses a dentist is a detailed and correct anamnesis in order to gather all the information regarding the medical history of the patient before initiating the treatment with bisphosphonates. Additionally, the anamnesis must be conducted throughout the entire treatment period. In this study, most dentists are aware of the medical indications of bisphosphonates but despite the fact that literature abounds in information on bisphosphonates and their adverse effects, there are dentists who do not know the effects of these drugs.

In the third chapter of the specific section entitled The biological regeneration potential of ozone over gingival fibroblasts stimulated with bisphosphonates the cytotoxicity of both oral and intravenous bisphosphonates on human gingival fibroblasts were tested and an attempt to counter these harmful effects through the beneficial action of ozone was made.

Bisphosphonates are compounds with an important activity on bone recovery due to their involvement in the osseous metabolism and inherent osseous diseases. The main side effect of their administration is represented by maxillary osteonecrosis in the oral-dental area. For this study we used 4 commercially available products, very often prescribed as specific therapy, belonging to two different bisphosphonates classes (oral and injectable) and not only the active substances, in order to monitor their cytotoxic effect on gingival fibroblasts

and further observe the protective proliferation and regeneration effect of ozone on gingival fibroblasts. In order to verify the potential toxicity of bisphosphonates, the Alamar blue test was applied. Briefly, gingival fibroblastic cells were inoculated in plates with 96 microwells ( $1 \times 10^4$  cells/microwell/ 200  $\mu$ L) and left to grow until the adequate confluence is reached (24-48 hours). Various concentrations (1,5; 2,5; 5 and 10  $\mu$ M) of the tested compounds (Actonel-A; Fosamax-F; Ossica-O; Zolendronic-Z acid) were added to the fresh culture medium. After 24 hours, 20  $\mu$ l of Alamar blue was added with further incubation for 3 hours at 37 ° C and then the absorption values at 570 and 600 nm were measured with the xMark™ microplate spectrometer (Biorad). In order to determine the effect of ozone (saline O<sub>3</sub> - 80  $\mu$ g / ml) on the viability of cells and its impact on the cell recovery capacity after the toxic effect of the tested compounds, the gingival fibroblasts were stimulated with Actonel and Fosamax (compounds which proved to be cytotoxic, 2.5, 5 and 10  $\mu$ M) for 24 hours. The old medium was removed and 100  $\mu$ L of fresh medium and 100  $\mu$ L of saline ozone solution/microwell were added for 24 hours. We may state that not all bisphosphonates (Zolendronic acid, Ossica, Fosamax, Actonel) have cytotoxic effects on primary gingival fibroblasts, only those with oral administration (Actonel and Fosamax), but the stimulation with ozone solution of destroyed fibroblasts led to a significant increase of the per cent of viable cells and improved the capacity of cells to recover by stimulating their growth and proliferation. We may state that ozone has a protective effect by regeneration of gingival fibroblasts after prior degeneration induced by exposure to bisphosphonates in Fosamax and Actonel.

In the last chapter of the specific section entitled Nonconventional therapy for the regeneration of gingival tissue after treatment with bisphosphonates using essential *Salvia Officinalis* oil the essential oil was applied in vitro on human gingival fibroblasts previously stimulated with bisphosphonates in order to demonstrate its efficacy in cellular regeneration.

The gingival fibroblasts were inoculated in 96 microwell plates ( $1 \times 10^4$  cells/microwell/ 200  $\mu$ L) and left to grow until the adequate confluence was reached (24-48 hours). Various concentrations (2.5; 5 and 10  $\mu$ M) of the tested compounds (Actonel; Fosamax) were added in fresh culture medium. The effects of the tested compounds solutions on the morphology of cells were evaluated by image acquisition before the addition of compounds and after the stimulation period (24 and 48 hours, respectively). The images were obtained using the Olympus IX73 inverted microscope with a DP74 photo camera and equipped with the CellSens V1.15 software (Olympus, Tokyo, Japonia). In order to determine the effect of the *Salvia Officinalis* essential oil on the viability of cells and its impact on the capacity of cells to recover after the toxicity of the tested compounds, the gingival fibroblasts were initially stimulated with Actonel and Fosamax for 24 hours. The old medium was removed and 100  $\mu$ L of fresh medium together with 20  $\mu$ L of *Salvia Officinalis* essential oil/microwell were added for 24 hours. Following bisphosphonates stimulation, a morphological degradation of



the cells stimulated only with Actonel or Fosamax in various concentrations occurs, while in gingival fibroblasts initially stimulated with bisphosphonates and further exposed to addition of *Salvia Officinalis* essential oil the morphology is almost similar to normal indicating that the essential oil had a protective effect against the toxicity of bisphosphonates.

The general conclusions of the doctorate thesis, described in detail in the last chapter were

- The incidence of maxillary osteonecrosis secondary to bisphosphonates treatment is increasing which requires prevention and novel treatment methods for this disease
- The correct anamnesis and informing the dentists on the effects of bisphosphonates on maxillary bones are important factors for the prevention of osteonecrosis.
- Ozone and *Salvia Officinalis* essential oil present beneficial effects in vitro as well as the capacity to reduce the cytotoxicity on human gingival fibroblasts previously stimulated with bisphosphonates