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CONTRIBUTIONS TO THE HISTOLOGICAL STUDY OF NON-DEMINERALIZED COMPLEX BONE-METAL-SOFT TISSUE SAMPLES IN ORAL IMPLANTOLOGY

ABSTRACT

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Introduction:

The bone is considered the most important supporting tissue in the whole body. It is composed of cells, extracellular organic matrix and inorganic salts. Bone tissue is mineralized in layers that provide it with increased strength and high flexibility. It varies in formation, since it depends on the function performed in the organism to which it belongs. These functional and formation differences are also based on organic or inorganic processes incorporated or produced in the formation of a particular bone tissue. The most widespread mineral in the bone structure is hydroxyapatite which is made up of collagen, proteins and carbonated ions. The bone consists of about 70% minerals and 30% organic components, depending on the weight. Bone cells, unlike medullary cells, are rarer.

Bone physiology was better understood in the 1950s with the development of plastic packaging techniques for microscopic examination of non-demineralized sections of bone tissue. In the past, for examination, bone histology required the removal of the most important component, the mineral. New technologies have been considerably improved since then.

Treatment with dental implants has become in recent years of choice for edentulous patients, reporting success rates of over 95.4% after 5 years of function. Although these values are extraordinary, peri-implant inflammation is common and can cause bone resorption around implants and even their loss. The peri-implant bone is often observed, by imaging methods, in the initial phases after the insertion of the implants and/or after loading. These losses are usually attributed to the bone healing response, called physiological remodeling. As a result of this natural remodeling, the marginal bone can be reabsorbed due to the peri-implantation inflammation induced by the bacterial plaque and can progress towards the loss of osseointegration, which can negatively influence the long-term success of the treatment with dental implants. Peri-implant disease was defined in 2018 by the American Academy of Periodontology and the European Federation of Periodontology, within the Classification of Periodontal Diseases and Conditions and Peri-implantation, as a condition associated with microbial plaque. It begins in the soft tissues around dental implants, defined as peri-implantary mucositis, progressing to the hard support tissues, at which point peri-implantitis sets in.

The general part depicts the current state of knowledge in the field, histological technologies and protocols of preparation of complex non-demineralized sections.

The histological technique of obtaining sections of complex non-demineralized tissues, mainly aims to reveal the processes of bone regeneration or bone positioning that take place in the proximity of the inserted dental implants. These techniques are usually laborious and are indicated in the investigation of the biology of bone tissue. The use of non-decalcified tissues allows the differentiation of the mature bone from the immature one and the quantification of variables such as the

bone-implant interface, the density of the bone area and the speed of bone remodeling. The beginning of the processing of hard and soft tissue specimens containing metal alloys for metallographic analysis, respectively the technique of cutting-finishing histological samples, was first described by Donath and Breuner in 1982.

Several methods of inducing peri-implantitis in experimental models have been proposed, the human subjects being subject to ethical and moral regulations. It is well documented that the canine species has a natural susceptibility to periodontal disease, being therefore the most used experimental model. The cause-and-effect relationship between the colonization of the microbial plaque and the pathogenesis of the peri-disease the pathogenesis of peri-implantation disease was initially investigated in preclinical studies on animals by means of degradation of the hard and soft support of dental implants. In these models, the appearance of peri-implantation lesions was induced by submarginal application of cotton ligatures. To date, this experimental model of induction of peri-implantal defect is the gold standard, to investigate the pathogenesis and therapy of peri-implantation disease. With the increase in the number of investigations on these animal models, the need for ethical regulations has arisen, namely the ARRIVE guide, which recommends the use of as few animal models as possible (if the end of the experiment is *exitus*) and their use as their own controls ('within-subject experiments').

Although many sophisticated and expensive techniques have appeared with the development of technology in recent years, light field microscopy has remained the "gold standard", because it has proven to be an extremely valuable method of investigation, which allows the provision of important information on the processes of bone remodeling on non-demineralized specimens of oral hard tissue. However, the histological assessment of bone specimens, especially hybrid specimens, containing synthetic biomaterials, is technically challenging.

The special part includes three experimental studies, meant to propose a novel canine model of induced peri-implantitis and to simplify the classical processing protocol of non-demineralized soft tissues – hard tissues – dental implant complex specimens.

Thus, the conduct of personal research included a pilot study in which the degradation of soft and hard tissues around implants to which consecutive and alternative ligatures were applied to the same hemimandible on a canine model was analyzed histologically and radiologically. The influence of ligated implants on non-ligated implants was also investigated. When analyzing the histomorphometric parameters and on CBCT, it resulted in deep peri-implantation bags on the oral appearance and pronounced bone resorption on the vestibular aspect of the analyzed ligated implants and a less pronounced impairment of the unligatured implants, despite their proximity to the ligated ones. Histological analysis on thin non-demineralized sections under the light field microscope, confirmed intense inflammation of the soft tissue, a pronounced bone resorption and a greater amount of connective tissue infiltrated in ligated implants, compared to non-ligated ones. In the conclusion of the study, it was noted the successful evaluation of the unligabited experimental model of induction of peri-implantitis,

while also satisfying the requirements of designs using a small number of experimental animals.

For analyzing non-demineralized sections of greater thicknesses, the confocal laser scanning microscope can be used because it penetrates tissues to a depth of 300-500 microns and reflects images that are not only at the surface of the specimen. The 2D images obtained can be the basis of three-dimensional reconstructions, where you can observe the non-demineralized bone matrix, the bone-implant interface and the mineralized bone.

The second part of the personal research included a "proof of concept" study to evaluate tissue degradation under the confocal microscope with laser scanning, using the autofluorescence of the tissues around the ligated and non-ligated implants. The influence of thickness was also analyzed sections on the accuracy of histological observations in comparison with "thin" ones used under the light-field microscope. The results of the study included higher tissue resorption values in histological analysis of "thick" (250-300 microns) sections than "thin" (30 microns). In the conclusion of the study it was noted the significant contribution of the analysis to the confocal microscope with laser scanning of the "thick" blades by the three-dimensional reconstructions obtained, the preparation of which is easier than the classic "thin" ones, . Also, although this type of investigation may require more advanced technology, the quantity and quality of the data obtained, I can justify this approach.

For the supplementation of the data set, regarding the processes that occur during tissue degradation in experimental peri-implantitis, fluorochromic substances can be administered intravitally. Sections prepared for the analysis of fluorochromic markers are usually thin, in order to highlight the markers, do not require additional staining, as in the case of light field microscopy, and are analyzed under a fluorescent microscopic equipment, to which various filters such as UV, green, blue or red ones are added. This method is simple, stable and is based on the detection of newly formed or resorbed bone and can be used as a mark of when these bone processes occur.

The third part of personal research includes the investigation of tissue degradation obtained by placing ligatures around implants to a canine pattern, using two intravital fluorochromic markings, under three filters under the fluorescence microscope, as an alternative to evaluation under the microscope in the luminous field. The results of the study confirmed the bone remodeling activity around the implants, during the healing period, at observation under the fluorescence microscope and intense tissue inflammation, observed under the light field microscope. The findings of the study included the complementary usefulness of fluorescence to the "gold standard" represented by the investigations under the light field microscope, providing sufficient and solid information on bone aposition processes.

To date, there is no consensus in the literature on the accuracy of the method of experimental induction of peri-implantitis in animal models. Also, no consensus was observed in choosing the thickness of the sections of non-demineralized specimens of soft tissue – bone tissue – dental implant, which makes it difficult to choose a technique that simultaneously provides histological relevance, with low costs and minimal processing time.

Conclusions:

1. Currently, the lack of a consensus regarding the accuracy of the way of inducing experimental peri-implantitis in the canine model, as well as the uncertainty of the influence that ligatures have on the soft and hard tissues around implants, make the research of this direction to be of utmost importance in the peri-implant literature.

2. From the research of the specialized literature it appears that some authors consider ligatures as a factor of inducing the appearance of peri-implantitis, while other authors state that ligatures act as a foreign body, to which the body reacts naturally. This is an insufficiently proven aspect and further studies are needed in this direction.

3. Currently, after decades of research, the study of peri-implantation therapies is based on models of induction of experimental peri-implantitis in animal models that are not completely in line with the physiopathogenicity of human peri-implantitis.

4. The present research proposed the introduction of the unligabited experimental model of spontaneous occurrence of peri-implantation inflammation, while also meeting the requirements for the design of experimental studies with a small number of used animal models.

5. The influence of implants affected by severe peri-implantitis on neighboring implants was evaluated and highlighted the existence of tissue loss.

6. These results can influence the choice of therapeutic approach at the time of confrontation with varying degrees of severity of the disease in neighboring implants.

7. To date, there is no consensus in the literature on the thickness of the sections analyzed, which seems to be dictated more by the personal preferences of the authors, based on certain purposes of the research and less on a clear correlation between the thickness of the sections and the histological results pursued.

8. In this regard, the present research proposes a simplification of the traditional histological protocol, through the use of "thick" sections (250-300 microns), of complex non-demineralized tissues of peri-implantitis experimentally induced to a canine model, which, properly embedded and manipulated, can ensure the easy obtaining of relevant information, examined under CLSM.

9. The information thus obtained may lack the precision of that obtained by viewing 'thin' sections below the LM, but may provide complementary data, such as 3D reconstructions. Although investigating these specimens at CLSM may seem complicated and technologically demanding, the information obtained is numerous and can justify this approach.

10. Currently, it is known that sections of complex non-demineralized preparations of experimentally induced peri-implantitis to a canine pattern provide information in a quick and easy way on the mechanism of bone resorption.

11. In the present research, the "thin" sections under a fluorescence microscope have been

successfully compared with the same sections visualized under the light field microscope to provide information on the processes of bone aposition.

12. By providing sufficient and relevant information, fluorescence microscopy has shown that it is a complementary approach, which allows the evaluation of the bone activity around the dental implants from the moment of insertion to the moment of complete integration, and subsequently, until the complete resorption during the progression of the peri-implantation disease.

13. Although in the researches in this thesis it has been observed that the simplification of histological protocols is possible through the use of modern technologies, the limitations of the studies call for the development of new research in the field; future studies will focus on refining the methods of inducing experimental peri-implantitis, on simplifying histological protocols and on the risk-free manipulation of complex specimens of bone tissue – soft tissue – dental implant.