

“VICTOR BABEȘ” UNIVERSITY OF MEDICINE AND PHARMACY
TIMIȘOARA
FACULTY OF MEDICINE
Department of Anatomy and Embriology

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DOCTORAL THESIS

Scientific Coordinator

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2020

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**IMMUNOHISTOCHEMICAL ANALYSIS OF GINGIVAL
PROLIFERATIVE PROCESSES ASSOCIATED WITH
FIXED ORTHODONTIC THERAPY**

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Introduction

The thesis includes an introduction, a general part and a special part, conclusions and personal contributions. The general part describes the general notions of anatomy, histology, physiology, orthodontics and periodontology, where all the information related to the dento-maxillary apparatus, oral cavity, oral hygiene, gingival inflammation, orthodontic appliances, restraints are explained in detail. In the special part, the studies that were done related to gingival hyperplasia and what we wanted to determine, starting from the methods of tissue collection to the results obtained, are described. Also in the special part are described the tests made in the laboratory on thermoplastic splints, these being a form of mobile containment, after the dental braces have been removed and their resistance to wear has been studied.

In the end, in the special part, we developed a questionnaire for patients, divided into 3 sectors, before treatment, during treatment and after its completion. The purpose of these questions was to see how informed patients are in general, if they follow the doctor's recommendations, how they feel during treatment and what financial possibilities they have. The aim of my work was to determine from an immuno-histo-chemical point of view, the cause of interdental gingival inflammations, which appeared in patients with fixed orthodontic appliances, shortly after they were applied, but also after a certain period of time, after they have been sectioned and given for analysis. Sections of gingival tissue were taken from the same place. We started from the premise that these gingival hyperplasias appeared as a result of mechanical stress and periodontal remodeling during orthodontic tooth movement. It should be mentioned that no patient who participated in the study had general diseases, and the oral and gingival status, as described in the periodontology is 0, without gingival inflammation. In addition to the fact that the patients had a very good oral hygiene, they also had professional hygiene in the dental office. After the completion of the orthodontic treatments, in order for the teeth not to return to the positions they were in before the treatments, restraints are resorted to, whether they are fixed or mobile. We chose to see the wear resistance of certain thermoplastic gutters, these being part of the mobile containment part. Also, the purpose of this study is to develop a questionnaire to properly assess the oral status of patients undergoing fixed orthodontic treatment.

Keywords: brackets, tissue, T cells, B cells, dendritic cells, VFR, ANOVA analysis

I. GENERAL PART

I.1. Anatomy and histology of the healthy periodontium

The dento-maxillary apparatus (DMA) consists of an internal support system (visceral skull with dento-alveolar arches), five muscle parts, salivary glands and two shells (one external - the skin and one internal - the oral mucosa). ADM includes the jaw and mandible in which the teeth are implanted, the temporo-mandibular joint, muscles, tongue, salivary glands, oral mucosa and skin.

The maxillary bone is a paired bone of the viscerocranium. It is part of the skeleton of the middle facial floor and consists of a body and four processes: frontal, zygomatic, alveolar and palatine. The body of the maxillary bone has four faces: anterior, posterior, orbital and nasal. The edges of the body of the maxillary bone are: anterior, posterior, superior, infraorbital and postero-superior.

The mandible is the middle and lower bone of the face, representing the only mobile bone of the skull. The mandible articulates with the temporal bone and forms the temporomandibular joint (TMJ).

The tooth ("dents" in Latin) is the hardest organ, colored in shades of white-yellow, located in the oral cavity. It is composed of the crown (larger in the molar area and smaller in the premolar, canine and incisor areas) and one or more roots (the molar area or the upper first premolars), these being implanted in the alveolar region of the maxillary bones (maxilla and mandible).

The tongue (Lingua in Latin) is an unpaired, muscular organ, covered by the mucosa, which has very good mobility, and a high sensitivity from a tactile point of view.

The salivary glands are exocrine glands in the oral cavity that produce saliva. The salivary glands are glands specific to vertebrates.

The periodontium was divided into 2 parts: the gum and the support device. Its main function is to protect the underlying tissues, and the supporting apparatus is composed of periodontal ligament, cementum and alveolar bone.

The gum is a soft tissue that lines the oral cavity and is of three types: marginal, adherent and interdental. It acts as a barrier against the penetration of microbes or other harmful agents into the underlying deep tissues.

The gingival epithelium has as its main cell type the keratinocyte. Other existing cells are nonkeratinocytes which include Langerhans cells, Merkel cells and melanocytes.

The sulcular fluid is continuously secreted by the gingival connective tissue at the level of the gingival groove, through the epithelial wall. It contains components of connective and epithelial tissue, inflammatory cells, serum and microbial flora that colonize the gingival margin or sulcus.

I.2. Physiology of dental plaque

1. Dental film

Brushing your teeth removes the organic material from the tooth surface, but shortly after contact of the tooth surface with saliva, a fine acellular film with a thickness of 10µm, two layers and containing proteins from saliva, carbohydrates and glycoproteins with hexoses, hexosamine and fucose.

2. Formation of the plate matrix

In the absence of food, the extracellular matrix of the plaque is thin and contains insoluble calcium protein-phosphate complexes together with modified salivary glycoproteins.

3. Bacterial colonization

The already formed film is rapidly invaded by bacteria caused by: saliva, adjacent soft tissues, damaged surface of the enamel. The adhesive properties of microorganisms allow the colonization of the plaque.

I.3. Periodontal examination and immunology of the periodontium

SILNESS & LOE plate index

The index was introduced in 1964 by Loe and Silness. The lateral surfaces of each tooth are used in its determination. This system appreciates the thickness of plaque deposition at the gingival margin of the teeth.

The dental surfaces examined must be insulated and dry. With the rounded tip of the probe, scrape from the cervical to the occlusal on the investigated surface and determine the presence or absence of plaque deposits.

Gum health is the result of a balance between existing subgingival microorganisms and host resistance and is characterized by the absence of inflammatory gingival infiltrate. The gingival groove contains bacteria both in the case of healthy periodontium and in the case of the sick one.

In periodontal health, the microbial flora is represented by gram-positive bacteria, predominating species of Actinomyces and Streptococcus. Related to discrete gingival inflammation, a number of studies have shown the presence of a small number of inflammatory mediators PGE2, LTC4, and IL-1 alpha.

Orthodontic therapy can induce damage to the periodontium, with the appearance of secondary periodontal sequelae. Periodontal disease can affect the aesthetics and functionality of the teeth. Gingivitis occurs as a primary reaction to bacterial plaque. It consists of the vascular reaction, with increased accumulation of fluid and inflammatory cell infiltrate.

In early forms, T lymphocytes predominate, and in advanced forms, plasma cells appear.

With the intensification of the gingival inflammatory reaction, the body responds by increasing the hepatic synthesis of plasma proteins, with the role of healing the lesions.

I.4. Diagnostic. Prognosis. Treatment

The diagnosis is very important in establishing the treatment plan and in establishing the gingival diseases. Thus, the observation sheet will be completed, which will contain more relevant information.

I.5. Notions of orthodontics

Orthodontics is part of dentistry that has as its operating technique the movement of teeth, both coronary and root. Orthodontic appliances have three components: brackets, springs and accessories.

The brackets are attached to the dental crowns so that their springs and accessories will influence the position of the teeth. Brackets are devices used in orthodontics that align and straighten teeth and help position them in a person's bite.

They are often used to correct dental crowding, open occlusions, deep occlusions, reverse occlusions, teeth in malpositions and other pathologies of the maxillary and mandibular teeth and bones.

Orthodontic appliances often act as retentive factors for plate biofilm, so very good hygiene is essential throughout orthodontic treatment.

Regardless of the type of orthodontic appliance, it can cause gingival inflammation, which if left untreated leads to loss of attachment, alveolysis and the inevitable appearance of periodontal disease.

II. APPLIED RESEARCH

II.1. Introduction, objectives

The special part of this doctoral thesis is divided into three important points. The three studies are closely linked because they cannot be done separately.

II.2. Immunohistochemical analysis of gingival proliferative processes associated with fixed orthodontic therapy

Objective: Our study started from the hypothesis that the increase in gingival volume associated with fixed orthodontic treatment occurred during the use of orthodontic leveling and alignment arches, without inflammatory signs, due to mechanical stress and periodontal remodeling during orthodontic tooth movement.

In patients with gingival hypergrowth, a gingivectomy was performed and the tissues obtained were used for histological and immunohistochemical examination. Our goal is to observe the number of dendritic cells, the number of T cells and the number of B cells in the two stages, the early and the late.

Material and method: We selected and included in the study 70 patients (40 women and 30 men) aged between 18 and 45 years. The selected subjects showed a hypergrowth of the gums, covering one third or two thirds of the clinical crown of the teeth, diagnosed during active treatment with fixed orthodontic appliances (brackets). For each patient, two tissue samples were taken: one in the early stages of the lesion, coinciding with the use of leveling and alignment orthodontic arches, and another in the late stages of the lesion, coinciding with the use of finishing arches. Samples were taken from the same place in the oral cavity.

All patients were treated orthodontically within the Orthodontic Discipline, in the Faculty of Dentistry, "Victor Babeș" University of Medicine and Pharmacy in Timișoara, Romania or in the private practice. All patients signed the informed consent and the samples were taken with the approval of the Ethics Committee of the University of Medicine and Pharmacy "Victor Babeș" from Timișoara

The selected patients were healthy, did not have systemic diseases and none of them underwent drug treatment, which could have affected their periodontal condition.

Tissue sampling: The instruments used in periodontal therapy have sharp edges, with the help of which fragments of soft and hard tissue, both healthy and pathological, are removed or shaped to achieve therapeutic goals. Even after first use, there is a degree of wear that results in the loss of the cutting character of these tools. Patients underwent dental prophylaxis and were instructed to maintain adequate oral hygiene.

One week later, patients were called in for tissue sampling. The gum was cleaned and disinfected with a sterile compress moistened with 70% ethanol. A local anesthetic with 10% lidocaine spray was applied.

Excision of interdental papillae or volume-modified tissues (hyperplastic or hypertrophic tissues) was performed using a soft tissue ceramic trimmer (Precicut, DFS DIAMON), similar to a high-speed FG cutter. Water cooling was not used.

The soft tissue trimmer was inserted into a dental turbine and used at an operating speed of 300000 rpm. At high speeds, due to friction between the tip of the instrument (made of Yttrium-stabilized zirconium nanostructure) and tissue, the heat generated produced rapid tissue coagulation and almost complete hemostasis without tissue burning. Recovery time was much shorter, and no healthy tissue was lost.

Histopathological examination: The gingival mucosa has a squamous multilayered epithelium located on a basement membrane, underlying a loose connective tissue, richly vascularized and innervated, which forms its own lamina. The epithelium forms extensive interdigitations, thus increasing the area of attachment, connecting with the underlying connective tissue. The epithelium of the gingival mucosa is keratinized on the external face and non-keratinized on the internal face. On the inner face of the gingival mucosa is found the presence of inflammatory infiltrate, whose cells have invaded their own lamina and penetrated the epithelium.

Immunohistochemical examination: The specimens were fixed in 10% buffered formalin for 48 hours and incorporated into paraffin. Tissues embedded in paraffin were cut into 5 μ m sections. All samples were stained using the H&E staining protocol. We performed an enzyme pretreatment (Leica Biosystems Binding Enzyme Pretreatment Kit, Newcastle upon Tyne, UK) for 10 minutes. Endogenous peroxidase blockade was performed with 3% hydrogen peroxide for 5 minutes. This step was followed by incubation for 15 minutes with CD8 and CD20 (Novocastra, Newcastle upon Tyne, UK, polyclonal rabbit antibodies, ready to use) as primary antibodies. We used a set of differentiation classes (CDs), antigen-specific monoclonal antibodies to detect different cell types in tissues. These included anti-CD20 (B cells), anti-CD8 (T suppressor cells and dendritic cells). The Bond Polymer Refine Detection System (Leica Biosystems, Newcastle upon Tyne, UK) was used for visualization. 3,3'-diaminobenzidine dihydrochloride was applied as a chromogen and we used hematoxylin for counterstaining.

The entire immunohistochemical procedure was performed with Leica Bond-Max autostainer (Leica Biosystems, Newcastle upon Tyne, UK). Image acquisition and analysis were performed using the Nikon Eclipse E600 microscope and Lucia G software for microscopic image analysis.

Results and discussions: Of the 70 gingival biopsies taken from patients, 12 cases did not show significant changes in the gums, while in the remaining 58 cases, significant changes were found after histopathological examination. Histopathological examination revealed a dense band of subepithelial collagen fibrous tissue. We also noticed an area strongly infiltrated with chronic inflammatory cells, with many plasma cells, lymphocytes and fewer dilated blood vessels.

Out of 58 gingival biopsies, 44 cases showed a significantly higher number of T cells ($p < 0.05$). In addition, a higher number of B cells was observed in the granulation tissues than in the gums ($p < 0.05$). In this type of progressive lesions induced by orthodontic treatment, the relative number of B cells (CD20), T cells and dendritic cells (CD8) was expressed, both in the early and late stages of gingival lesions.

Our results showed that the proportion of T lymphocytes and dendritic cells was higher in the early stages than in the late stages ($p < 0.02$). B cells showed a higher number in the late stages. Dendritic cells migrated from the basal layer to the superficial epidermal layer.

Conclusions: In our study we observe that in the early stages of progressive lesions during orthodontic treatment, the number of dendritic cells and T increased, while in the late stages, the number of B cells was increased. Drug treatment will not be used to make the inflammation go away, and patients who will be treated with a fixed orthodontic appliance with brackets will be notified of the appearance and reappearance of inflammation in the gingival tissue.

II.3. Wear resistance under high load forces of four different polyethylene terephthalate glycol vacuum-formed orthodontic retainers, following orthodontic therapy

Introduction - Gutter: A gutter can have 2, 3, or 4 vertical walls, an edge adapted to the gingival contour, a single occlusal wall, an inner face and an outer face. In order to prevent fracture of the gutter during application, it is necessary that the walls have a uniform thickness. The vertical walls must be thick enough to have considerable mechanical strength.

Objectives: The aim of our study was to determine the wear resistance of vacuum-formed containment gutters (VFR) made of glycol-modified polyethylene terephthalate (PETG) from four different manufacturers (Essix, Leone, Erkodent, Bio-Art), under the action of high load forces (600 N), for 10000 cycles.

Forty thermoplastic sheets were divided into four groups, each group consisting of a set of ten sheets from each manufacturer, thermoformed on a pair of two study models with ideal occlusion. Instron 8874 equipment was used to simulate accelerated wear of VFR surfaces. VFR surfaces were visually inspected using a KEYENCE VHX-600 digital microscope, but this proved difficult due to the brightness of the surfaces.

Material and method: For this study we used forty thermoplastic foils (1.0 mm thick, square foils, measuring 125x125 mm) from four different manufacturers, made of polyethylene terephthalate glycol (PETG), recommended for vacuum-forming restraint devices. The samples were divided into four groups, each group consisting of a set of ten thermoplastic sheets from each manufacturer (five sheets for the upper dental arch and five for the lower dental arch). For a correct assessment of changes in the mass of the specimens, a handling protocol was established, before and after the test, which consisted of using a low-pressure air jet to remove material debris that occurred during the test. The specimens were handled with standard tweezers. After the wear test, the VFR surfaces were visually inspected and characterized using a KEYENCE VHX-600 digital microscope. A GOM industrial laser surface scanner was used to scan the VFR surfaces, and the 3D models were generated by GOM Inspect 3D software using the three overlapping marking method for scanning interior and exterior surfaces.

The scanner detected all positive or negative surface irregularities. The surface gloss was reduced using a standard white powder coating film.

Statistical analysis: Data were statistically analyzed using the specialized program (IBM SPSS, version 24, SPSS Inc., Chicago). The one-factor ANOVA analysis was used to test the differences in net mass loss after wear simulations between the four groups, for each arch ($\alpha = 0.05$).

The data did not contain aberrant values, as suggested by the evaluation of the box plot diagram, with one exception in the Bio-Art group for the upper arch. The aberrant value was included in the analysis because we concluded that it would not affect the final result, the aberrant value (0.018 mg) was not extreme and was close to the next highest value in the group (0.015 mg).

Results and discussions: The average total net mass loss in the upper arch groups (0.153 ± 0.048) was lower than the total average of the lower arch groups (0.549 ± 0.075), possibly due to the difference in force distribution between the two surfaces. (corresponding to the upper and lower arch), taking into account not only the vertical forces but also the rotational component. The one-factor ANOVA analysis did not show statistically significant differences in terms of net mass loss after wear simulations, between the four groups ($p = 0.183$ for the upper arch groups and $p = 0.300$ for the lower arch groups).

Conclusions: No statistically significant differences were found in the net weight loss after the wear simulations, between the four groups (Essix, Leone, Erkodent, Bio-Art) of the glycol-modified polyethylene terephthalate (PETG) containment gutters.

Visual inspection and characterization of surfaces under the digital microscope proved difficult for VFR materials due to surface gloss, however, several areas of wear were identified, with varying degrees of roughness, but generally similar between the four groups. , without obvious breaking points or perforations

II.4. Study on the development and validation of a questionnaire to assess the oral status of patients in different stages of treatment, pre-treatment or post-orthodontic treatment fixed with brackets

Introduction: In dentistry, it is not easy to make a correct diagnosis. In order for it to be the best, and for the patient to be satisfied with the final result of the treatment plan (treatment costs, waiting time until the end of orthodontic treatment, waiting time until implant insertion, waiting time until coverage dental implants), it is necessary to: thorough consultation; history; questionnaire on patients' wishes; medical imaging (radiographs, CBCT); the final discussion about the treatment possibilities (what is wanted and what is possible), the financial possibility of the patient and the choice of the type of treatment (cost of materials).

Objectives: The main objective of this study is to develop a questionnaire to properly assess the oral status of patients undergoing fixed orthodontic treatment.

Material and method: To start the research, 100 patients were selected, aged between 18 - 45 years, living in both urban and rural areas, of both sexes. The questionnaires were divided to be completed at the beginning with data related to age, sex and environment in which they live, they being anonymous. They were kept in the patient's file, to know whose it is until the end of the treatment. After completing 100 questionnaires, they were removed from the files and given for statistical interpretation.

Discussions: Through the answers we received, we notice that there are some differences between those who live in urban areas and those who live in rural areas. 46 patients live in urban areas, among those who decided, through self-assessment, that

they have dento-maxillary anomalies, unlike 19 of them, who live in rural areas. The results of the questionnaire show us, surprisingly, but also pleasantly, that all 100 patients are satisfied with oral health, and regarding brushing their teeth, except for 6 of them, they wash between 2 and 4 times a day. In hygienic, complementary procedures, dental floss, mouthwash or mouthwash, the most conscientious and educated in this regard are those in urban areas, men being in almost double the number of women. The subject of our immunohistochemical analysis study shows that 98 of the subjects suffered during orthodontic treatment from gingival bleeding, pain when activating the orthodontic appliance and changes in the volume of the gums.

Conclusions: The conclusions of these questionnaires show us, in turn, that most patients are already informed, from various sources, be they media, internet or from friends who are already or have been in orthodontic treatments. The differences in financial level are observed, most of them being those from the urban area, those who can afford more expensive treatments, but they are also more willing than those from the rural area, to accept some treatments for a longer period of time, here we are talking about 1 and 4 years of fixed orthodontic treatment, but also what follows by wearing restraint.

II.5. Conclusions and personal contributions

a. In the first part of the thesis, the study on the increase in gingival volume associated with fixed orthodontic treatment tells us that gingival inflammations occurred during the use of leveling and alignment orthodontic arches. These come as a result of mechanical stress and periodontal reshaping during orthodontic tooth movement. We had as a number of participants in the study 70 patients, we demonstrated and analyzed that in the early stages of progressive lesions during orthodontic treatment, the number of dendritic cells and T cells increased, while in the late stages, the number of B cells was raised. The personal contribution is related to the fact that we have shown that the gingival inflammations will return to the same place, after they have been excised. . Drug treatment will not be used to make the inflammation go away, and patients who will be treated with a fixed orthodontic appliance with brackets will be notified about the appearance and reappearance of inflammation in the gingival tissue. They will also be notified of any pain in the dental units where the gingival tissue will become inflamed.

b. In the second part of the thesis, we studied restraint, which is used after the orthodontic treatment has ended. The need for this mobilizable restraint comes to the aid of orthodontic treatment. The whole human body has memory . That is why restraint is mandatory for orthodontic treatment to have the best results. We studied the resistance of these VFRs to see what major differences there are between various types and manufacturing companies. In this case, no statistically significant differences were found in the net weight loss after wear simulations, between the four groups (Essix, Leone, Erkodent, Bio-Art) of the glycol-modified polyethylene terephthalate (PETG) containment gutters. As a personal contribution, we noticed that at the 10,000 mastication cycles under the action of high loading forces, the wear was not considerable, which will lead us to inform the patient from the beginning about the costs related to restraint. A patient will be

able to wear such a splint for a longer period of time and thus his costs related to future containment splints will be lower.

c. In the last study, we developed a questionnaire, in which we wanted to establish from the beginning the type of treatment and the costs related to it. Of course, we will never be able to establish the period of a treatment from the beginning, because difficulties related to bone density, dental structure, personal hygiene, etc. can occur. After the specialist consultation, completing the observation sheet with personal data and interpreting radiographs (rx), teleradiographs (trx) and / or CBCT, we handed patients this questionnaire in which we wanted to know in stages the financial possibilities and their wishes. related to orthodontic and post-orthodontic treatments. We were also interested in finding out and keeping an eye on each patient, in connection with the gingival inflammations we studied. The development of this questionnaire will allow us in the future to save time in collecting data and to approach a patient correctly from the beginning by establishing the treatment and we will thus avoid putting some of them in unpleasant situations due to the financial situation.

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