UNIVERSITY OF MEDICINE AND PHARMACY ”IULIU HAȚIEGANU” CLUJ-NAPOCA

HABILITATION THESIS

SUMMARY

Innovative therapeutic approaches in periodontal disease in the context of local and systemic oxidative stress and metainflammation
Premises of the use of mesenchymal stem cells derived from human periodontium in tissue engineering

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The habilitation thesis „Innovative therapeutic approaches in periodontal disease in the context of local and systemic oxidative stress and metainflammation. Premises of the use of mesenchymal stem cells derived from human periodontium in tissue engineering" presents the main aspects of the author's scientific, didactic and professional activity, and the future perspectives regarding the academic career.

The thesis includes the following sections and chapters: the Introduction, the Scientific research, the Academic and Professional Career, the Main Directions for the Development of Scientific, Academic and Professional Career, the References and the Abbreviations.

The first chapter in the Scientific research focuses on the pathogenic mechanisms in periodontal disease involved in local and systemic inflammation, and the novel therapeutic approaches that modulate the response of the host to periodontal pathogens. The experimental and clinical studies demonstrated the role of various pathogenetic factors and the therapeutic efficacy of natural antioxidants. In an animal model, the intense expression of carboxymethyl lysine (CML) in the tissues of the oral cavity was associated with age, suggesting the involvement of advanced glycation end products (AGEs) in the aging process. In addition, in clinical trials, we used immunohistochemistry to demonstrate the high levels of CML accumulated in the gingival tissues in patients with chronic periodontitis. The increased expression of CML was associated with the stage of chronic periodontitis, diagnosed based on clinical criteria, and the degree of gingival inflammation, assessed by histological examination. These studies contribute to the understanding of the role of AGEs in periodontitis and age-related changes in the oral cavity tissues.

The implication of nitro-oxidative stress in experimentally induced periodontitis was demonstrated by the gradual increase in serum levels of specific markers: total oxidative status (TOS), total antioxidant capacity (TAC), total nitrites and nitrates (NOx) and the oxidative stress index (OSI), during the progression of periodontal inflammation. This was the first study assessing the dynamic changes in nitro-oxidative stress parameters during the evolution of periodontitis in an animal model. In experimental periodontitis induced in rats, curcumin administered orally, either as a single therapy or in combination with piperine as an adjuvant, significantly reduced the level of systemic nitro-oxidative stress. Local application of muco-adhesive curcumin gel did not influence serum levels of nitro-oxidative stress and did not show an additional antioxidant effect when combined with oral curcumin. This study was the first to assess the nitro-oxidative stress parameters as indicators for the therapeutic efficacy of curcumin in experimental periodontitis; moreover, we compared multiple routs for the administration of curcumin, and for the local application we used an innovative formulation: curcumin was incorporated into a muco-adhesive gel that exhibited adhesion to the oral mucosa and remained for an extended period, thus enabling the prolonged release of active ingredients into the gingival tissues.

The second chapter in the Scientific research section presents the *in vitro* studies performed on mesenchymal stem cells derived from the tissues in the oral cavity. In our

studies, we isolated four types of mesenchymal stem cells derived from the human periodontium: gingival chorion cells, gingival ligament cells, periodontal ligament cells, alveolar bone cells, as well as mesenchymal stem cells derived from the tuberosity bone, apical papilla and dental pulp. In order to characterize these cells, we demonstrated the expression of specific markers using immunocytochemistry and flow cytometry, as well as genetic methods - RT-PCR, and we determined the ability of these stem cells to differentiate into other mesodermal lineages: osteogenic, chondrogenic and neurogenic.

The *in vitro* studies allow the investigation of the pathogenetic mechanisms involved in various oral diseases and the testing of the cellular response to various substances, in order to develop innovative therapies. Based on this hypothesis, we studied the effect of *Porphyromonas gingivalis* lipopolysaccharide (LPS-Pg) and nicotine on the mesenchymal stem cells derived from the human periodontium, and the therapeutic effect of natural antioxidants: curcumin (CURC) and epigallocatechin-gallate (EGCG) and synthetic curcumin analogs. In cell cultures, LPS-Pg induced the production of intracellular free radicals, and nicotine exerted a dose- and time-dependent cytotoxic effect. Exposure to LPS-Pg and nicotine influenced, depending on the dose, the osteoblastic differentiation of mesenchymal stem cells derived from the human periodontium cultured in the osteogenic induction medium. These effects were demonstrated by assessing the presence of calcium deposits in the culture medium and by the expression of osteoblastic markers: osteopontin and osteocalcin. Depending on their origin, cells showed different responses to LPS-Pg and nicotine exposure.

Furthermore, we investigated the influence of CURC and EGCG on the viability and proliferation of mesenchymal stem cells derived from human periodontium and the therapeutic effect after exposure to LPS-Pg and nicotine. The two substances had dose-dependent effects: in high concentration, curcumin had a cytotoxic effect, whereas EGCG was better tolerated. Low doses of CURC and EGCG had a discreet influence on cell proliferation and metabolic activity. Depending on the origin, the cells showed selective responses: the cells derived from the alveolar bone were the most sensitive to CURC and EGCG. Natural antioxidants had protective effect against LPS-Pg-induced oxidative stress, demonstrated by a decrease in levels of intracellular free radicals. Both substances increased the viability of cells exposed to nicotine, but CURC was more effective than EGCG. In general, treatments were more effective when higher concentrations of CURC and EGCG were used in combination with low doses of nicotine. In these *in vitro* studies, the novelty was the dynamic investigation of the production of intracellular free radicals under the influence of LPS-Pg and during the treatment with CURC and EGCG. These were the first studies focused on the cytotoxic effect of nicotine and the inhibition of bone differentiation exerted by LPS-Pg and nicotine, on four types of mesenchymal stem cells derived from human periodontal tissues.

In addition, we tested the effect of innovative formulations: liposomes with CURC and EGCG, and synthetic curcumin analogs in mesenchymal stem cell cultures derived from human periodontium. For optimizing the therapeutic effects of natural antioxidants, we prepared liposomes with CURC (L-CURC) and EGCG (L-EGCG), which were tested in cultures of mesenchymal stem cell derived from human periodontal ligament. Liposomes were studied for cytotoxicity and antioxidant activity after cells' exposure to LPS-Pg, and the effects were compared with free CURC and EGCG, respectively. The two substances had antioxidant effects in different doses: CURC and L-CURC were effective in lower doses, while all doses of EGCG and L-EGCG had antioxidant effect. The dynamic assessment showed that the therapeutic efficacy was maximum at different moments. For CURC and L-CURC, the therapeutic effect was delayed; in contrast, EGCG and L-EGCG were effective immediately after the addition to the culture medium, and higher doses had a prolonged effect. Both CURC and EGCG included in liposomes had a more intense antioxidant effect than the free substances. In addition, we determined the *in vitro* effect of five synthetic curcumin analogs on the synthesis of metalloproteinase-2 (MMP-2) and metalloproteinase-9 (MMP-9) in latent and active form, and Tissue Inhibitor of metalloproteinases (TIMP-1) in cultures of mesenchymal stem cells isolated from human periodontium. For the determination and dosing of MMP-2 and MMP-9 levels as pro-enzymes or activated enzymes, gelatin zymography was performed, and levels of TIMP-1 in culture medium were determined by ELISA. The synthetic curcumin analogs showed variable cytotoxicity and intracellular uptake depending on the chemical structure and concentrations used for treatments. Synthetic curcumines did not induce a detectable synthesis of pro-MMP-9, but influenced the synthesis of pro-MMP-2; however, MMP-2 and MMP-9 were not present in active forms. In addition, TIMP-1 levels increased in all cell types. Our results suggest that, due to increased intracellular uptake and the modulating effect on the balance between proteolytic enzymes and tissue inhibitors, some synthetic curcumins could be used as an alternative to natural curcumin, as an adjunct in periodontal therapy. Currently, this is the first *in vitro* study to investigate the effect of synthetic curcumin analogs on stem cells derived from oral tissues.

In dental practice, the use of stem cells derived from various tissues in the oral cavity in combination with biomaterials opens new perspectives for developing cell therapies in regenerative medicine. Thus, the third chapter in the Scientific research section presents the results of the studies investigating the biocompatibility of porous titanium (Ti) matrices obtained by selective laser melting (SLM) technique. These matrices were tested *in vivo*, on animal model, and *in vitro*, in cultures of human mesenchymal stem cells isolated from oral cavity tissues. In order to evaluate osseointegration and to establish the importance of surface conditioning and pore size, we used Ti matrices with unmodified surface and Ti matrices coated with silicon-substituted nano-hydroxyapatite (Ti + nano-HapSi), with different pore size: 0.8 mm and 1 mm. The implants were inserted into the bone defects in rabbits, in the proximal third of the femur, and the bone fragments with the implants were harvested after 2, 4 and 6 months and processed for examination in light microscopy and scanning electron microscopy (SEM). Our results indicated different stages of healing process: after 2

months, endochondral ossification was characterized by the presence of hyaline cartilage adjacent to the implant, and after 4 months the healing was complete, with bone formation in the defect. The conditioning of the implant surface also influenced the new bone formation, that was more efficient for implants with Ti + nano-HapSi conditioned surface. SEM images of the matrices confirmed these observations and, in addition, indicated that the 0.8 mm pores provided better support for the cells developed on the surface of the implants and inside the pores. The *in vitro* study demonstrated that Ti matrices obtained by SLM, both unconditioned and with Hap-conditioned surface, were biocompatible in cultures of mesenchymal cell isolated from oral cavity tissues (dental pulp, apical papilla, inter-radicular alveolar bone and tuberosity bone) and promoted the formation of mineralized matrix. In particular, Hap-conditioned Ti matrices induced early mineralization. Mesenchymal cells derived from the inter-radicular alveolar bone seeded on these matrices showed high proliferative capacity and osteoblastic differentiation. Therefore, the experimental study demonstrated the importance of surface conditioning and pore size for osseointegration of Ti porous matrices, and the *in vitro* study was the first to compare the osteogenic potential of several types of human mesenchymal stem cells isolated from oral tissues, under the influence of Ti matrices unmodified or conditioned with Hap.

The forth chapter in the Scientific research section presents the microscopic aspects of gingival enlargement, depending on the etiology. The gingival enlargement induced by the treatment with Nifedipine was characterized by the following features: hyperplasia of the oral epithelium, with acanthosis and the formation of deep epithelial ridges, fibrosis in chorion, and ulcerations of the sulcular epithelium, associated with inflammatory infiltrate in the papillary chorion. The gingival squamous papillomas caused by the human papilloma virus (HPV), were characterized by koilocytosis in the spinous and superficial layers, dyskeratosis, hyperkeratosis and acanthosis. The peripheral giant cell granulomas of the oral cavity featured mononuclear cells and giant multinucleated cells, with various morphology in terms of size, amount and color of the cytoplasm, number and aspect of the nuclei, scattered in the connective-tissue stroma, among inflammatory cells.

The fifth chapter in the Scientific research section discusses the methods for assessing the metainflammation at the level of the oral cavity, taking into consideration the inter-relation between oral pathology and systemic diseases. This inter-relation makes possible the detection of certain morphological changes in the tissues of the oral cavity and the evaluation of salivary biomarkers, which could be interpreted for the diagnosis of local and systemic metainflammation in the metabolic syndrome. Oral exfoliative cytology, ultrasonography of the oral mucosa and the associated structures, and monitoring of saliva composition using intraoral sensors are new approaches to oral examination that we used in clinical trials. Oral exfoliative cytology facilitates the

observation of cytological changes in the apparently normal mucosa, and is especially useful in patients who use tobacco and alcohol, and thus, have a significant risk of developing premalignant and malignant lesions. Microscopic evaluation of cells exfoliated from the oral mucosa and included in cytoblocks revealed various morphological changes in the epithelial cells from the superficial and intermediate layers, as well as the presence of microbial colonies. Various chronic conditions, such as diabetes and hypertension, tobacco and alcohol use, and malignant oral lesions may be associated with characteristic changes in oral epithelial cells. In addition, the intracellular accumulation of biomarkers, such as AGEs associated with metainflammation, could allow the assessment of health status in patients with metabolic syndrome. Thus, oral exfoliative cytology could be considered as a non-invasive screening method for early diagnosis or monitoring of these conditions. High frequency ultrasonography (HFU) is a modern imaging method, useful for determining morphological changes in the oral mucosa and facial skin. HFU of the oral mucosa and facial skin showed changes in the ultrasound parameters such as: tissue density, thickness of the epidermis and dermis of the skin, the epithelium and chorion of the oral mucosa, depending on age, gender, general pathology, tobacco use and exposure to ultraviolet (UV) radiation. Establishing the association between general pathology and ultrasound aspects of oral mucosa and facial skin would allow the implementation of HFU as a paraclinical diagnostic method in metabolic syndrome and age-related diseases. Saliva is the most available biofluid that can be easily obtained in the dental office, suitable for the measurement of specific biomarkers for the screening of various conditions. Moreover, the use of intra-oral sensors in order to monitor the salivary biomarkers could indicate the presence of a pathological condition and the efficacy of therapeutic interventions. The innovative optical sensor based on spectral detection using an optical fiber has the advantage of being integrated in an intraoral mouthguard, for real-time monitoring of salivary biomarkers.

The chapter Academic and Professional Career describes the didactic activity, the scientific and research activity and the medical activity after graduating the Faculty of Dental Medicine at the University of Medicine and Pharmacy “Iuliu Haţieganu”, Cluj-Napoca.

The didactic activity in the Histology Department, Faculty of Medicine, carried out since 2003 and until now, consisted in practical works and lectures presented to the Romanian, English and French students at the Faculties of Medicine and Dental Medicine. The author started her academic career in 2003 as an university assistant; since 2009, she filled the position of lecturer until 2019, and now she works as an associate professor. During the 18 years of activity in the field of Histology, the author participated as collaborator, author or editor of 18 university books, in Romanian, English and French: 16 Histology courses and exercises for students (two of which as coordinating author and 14 collaborator or author), and two practical workbooks. She participated as a collaborator in the postgraduate course for residents in the specialty of Pathological Anatomy and presented the course entitled "Histology and pathology of the thyroid gland and adrenal glands" in 2014, 2016 and 2018. She also participated in organizing

workshops for students: "Basic techniques in Histology Laboratory - hands-on" within Medicalis in the Discipline of Histology, and workshops for young doctors. She prepared didactic materials: courses and practical works in power point format, schemes and drawings, taking images of histological preparations for making didactic atlases. She was also actively involved in assessing students' knowledge by preparing questions for the tests during the semester and for the exams, by correcting tests and written assignments, and by organizing oral seminars in practical exams. She coordinated the elaboration of the license theses of students from the Faculties of Medicine and Dental Medicine. She is also the tutor of Series 2, the Faculty of Medicine in English, 3rd year. She took part in the exam committees organized within the University for simulating admission, admission, license and residency and the competitions for teaching positions within the Histology Department.

The scientific and research activity encompasses the PhD studies, the clinical, experimental and the *in vitro* studies within the research grants, the active participation at scientific manifestations, national and international congresses, workshops and round tables with oral and poster presentations. In 2012, she completed the PhD thesis entitled "Studies regarding the role of immunological factors in etiology of periodontal disease and therapeutic approaches". She was member in several research grants: two national grants, one international ERANET-HORIZON 2020 grant, an internal grant financed by the University of Medicine and Pharmacy Cluj-Napoca, and target member in the TRANSCENT project, in the European Social Found Human Resources Development Operational Programme 2007-2013. As result of the scientific activity, she published, as an author, 2 specialty books in national editing houses, 18 chapters in national books, 91 papers published in abstract in the volumes of national and international congresses, 77 papers published *in extenso:* 38 in ISI Journals (19 as main author and 19 as co-autor) and 39 papers in BDI indexed Journals (23 as main author and 16 as co-autor). Of these publications, 7 articles received the UEFISCDI award - National Plan for Research, Development and Innovation for 2015-2020, PNCDI III, P1 - Development of the national research and development system, Subprogramme 1.1 - HUMAN RESOURCES, "Awarding research results - articles ", PN-III-P1-1.1-PRECISI. The author also participated in the development of 3 pending inventions: (i) Ilea Aranka, Boşca Adina Bianca, Soriţău Olga, Guţiu Eugen, Câmpian Radu Septimiu. "Artificial dental root and process for obtaining it from polylactic acid grafted with mesenchymal stem cells" - no. OSIM - A 00135 / 27.02.2019; (ii) Farago Paul, Gălătuş Ramona-Voichiţa, Groza Robert-Gheorghe, Băbţan Anida Maria, Feurdean Nicoleta Claudia, Petrescu Bianca Nausica, Boşca Adina Bianca, Ilea Aranka. "Salivary optical sensor made by lateral coupling of an optical fiber with surface emission and a fluorescent optical fiber integrated in an intra-oral device" - no. OSIM A 00136 / 27.02.2019; (3) Tertiş Mihaela Claudia, Cristea Victoria Cecilia, Băbţan Anida-Maria, Feurdean Nicoleta Claudia, Uriciuc Willi-Andrei, Boşca Adina Bianca, Ilea

Aranka. "Electrochemical sensor printed on planar support integrated on an intra-oral device for direct and simultaneous electrochemical detection of advanced glycation agents in saliva" - no. OSIM - A 2020 00171 / 01.04.2020A. Several works presented at national and international congresses and conferences were awarded with 45 medals and distinctions. She also collaborated as reviewer with ISI and BDI national and international journals, and she is member of the Editorial Board of Inflammation as Review Editor of the journal Frontiers in Immunology.

The professional career covers the medical activity as training medical doctor, (2000-2001), medical doctor –residency in General Dentistry (2001-2003), and medical doctor specialized in General Dentistry (2003 - present). During her professional training, she obtained the Diplôme Approfondi de Langue Française (DALF) awarded by the Ministère de l'Éducation Nationale de la République Française in 2000, and the First Certificate in English - Cambridge ESOL Level 2 Certificate in ESOL International, University of Cambridge in 2013. She also attended courses and workshops organized by the University of Medicine and Pharmacy "Iuliu Haţieganu", Cluj-Napoca: Problem Based Learning - 2013, Strategies for Using IEEE Xplore, Open Access, How to Publish - 2014, Scientific Writing - 2014, Writing in the Sciences - 2014, PRIME Partnerships in International Medical Education - 2015, “Personal Digital Library” - 2015, Writing MCQ (Multiple Choice Questions) - 2017, Team Based Learning - 2017, Psychopedagogy - 2017, CADMED Moodle Platform - 2019. She is a member of the following scientific and professional associations: the Romanian College of Dentists, the Romanian Society of Morphology and the Romanian Society of Cell Biology.

The chapter Main Directions for the Development of Scientific, Academic and Professional Career presents the future perspectives regarding the research, didactic and medical activities. The future studies on cell cultures aim at assessing the applicability of mesenchymal stem cells in regenerative therapy of oral cavity tissues, to evaluate the cytotoxic effect of new dental materials and to test the efficacy of natural compounds or plant extracts for local therapies in various pathologies of the oral mucosa and periodontium. The experimental studies will be carried out within the project PN-III-P2-2.1-PED-2019- 3664, “Customized intelligent matrices for tissue regeneration and control of metainflammation” (2020-2022). The intelligent matrices based on Ag-HAP + Doxy / PLA and Ag-HAP-Si + Doxy / PLA nanofibers obtained by electrospinning will be implanted at the level of experimentally induced periodontal lesions on animal model. The clinical studies will be the continuation and the interpretation of the results of the COFUND-ERA-HDHL ERANET project, European and International Cooperation - Subprogram 3.2 - Horizon 2020, PNCDI III Program - Biomarkers for Nutrition and Health. ”Innovative Technological Approaches for validation of Salivary AGEs as novel biomarkers in evaluation of risk factors in diet-related diseases” - SALIVAGES. The author will participate in national and international scientific events for the dissemination of research results, for her professional development and for establishing contacts with researchers from other universities in the country and abroad, in order to collaborate in future research projects.

The teaching activity at the Histology Department will be focused on courses and practical works for the students of the Faculties of Medicine and Dental Medicine in accordance with the educational and curricular objectives at the highest standards in terms of content and teaching method, with the permanent updating of the courses, and by an interactive approach in delivering the information.

Furthermore, the author will develop her professional skills, by actively participating in congresses, postgraduate courses and workshops, will constantly improve her abilities to coordinate research teams, to organize didactic activities and to facilitate learning and research.