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PHD THESIS

**EXPERIMENTAL AND STATISTICAL STUDIES ON
MALIGNANT ORAL PATHOLOGY AND THE
PROPHYLACTIC ROLE OF ORAL HYGIENE**

– R E S U M E –

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RESUME

Tumors in the oral cavity have an increased incidence from one year to another, according to data reported by the WHO. Chemotherapeutic drugs used to treat cancer have been shown to exert limited effectiveness based on the problems related to release, penetration and a moderate degree of selectivity for tumor cells, and in addition can cause severe damage to healthy tissues. Currently, the main prognostic variables of tumor pathology are the location, size of the tumor and the presence of cervical and distant lymph node metastases. The current classification of tumors is based on their anatomical and morphological distribution, aspects that lead to the application of a common therapy, homogeneous for a heterogeneous group of malignancies. Limited information at this time on the molecular and cellular aspects of oral mucosal carcinogenesis on the biological heterogeneity of these tumors requires the development of research strategies through *in vitro* cell culture techniques, with applicability for *in vivo* studies.

Dental and periodontal pathologies can be correlated with risk factors in tumor pathology. Overflowing fillings, maladaptive prosthetic restorations, incorrect, irritative dental debris associated with an unhealthy diet and poor oral hygiene are risk factors for the installation of tumor pathologies in the oral cavity. Dental caries has a multifactorial etiology in which there are involved three main factors: the host (saliva and teeth), the microflora (plaque) and the substrate (diet) with the mention that a fourth factor can be the time. The role of sugar (and other fermentable carbohydrates, such as refined flour) as a risk factor in the appearance and progression of tooth decay is overwhelming. Whether this initial demineralization reaches clinically detectable caries or whether the lesion is remineralized by plaque minerals depends on a number of factors, of which the amount and frequency of additional sugar intake are of the utmost importance. In the modern world, the products are very varied and nutrition, oral hygiene and periodic specialized professional controls play an important role. Awareness-raising actions undertaken by specialists need to be implemented and are currently very rare.

The aim of this research is to evaluate in detail through *in vitro* cellular techniques a series of compounds commonly used by both dentists and the general population. The research had four major objectives, which interfere and ensure a more detailed understanding of the processes involved in the studied oral pathologies. The first objective is related to *in vitro* studies focusing on cell cultures. The second objective involved *in vitro* testing on normal and tumor cells of compounds commonly found in contact with the oral mucosa. The third objective aimed at obtaining statistical data

related to the importance of oral hygiene, assessing the level of knowledge of the effects of these compounds frequently used by the population or by specialists (for example: (a) sugar substitutes, such as aspartame and xylitol found in “sugar-free” branded products; and (b) compounds commonly used in dental offices, such as chlorhexidine and baking soda, the latter being used frequently by the population in the food industry) in the installation and prevention of diseases in oral cavity. The last objective focused on the importance of using and investigating compounds of natural origin with beneficial action in oral tumor pathology. Therefore, the doctoral thesis is structured on two major chapters, according to the writing rules, namely the general part, respectively the special part.

The general part addresses in a first subchapter the latest aspects related to oral tumor pathology - risk factors, macroscopic and microscopic aspects, oral cancer management, and in the second subchapter are presented the experimental possibilities related to the evaluation of oral pathology and of prophylactic therapies with an emphasis on *in vitro* and *in vivo* evaluation techniques.

The special part comprises four main directions: (1) *In vitro* studies - cell cultures, (2) *In vitro* testing on normal and tumor cells of chemical / natural compounds frequently encountered on contact with the oral mucosa, (3) Statistical data related to the malignant oral pathology and the importance of oral hygiene and (4) Importance of the use and investigation of compounds of natural origin with beneficial action in oral tumor pathology. Each of the four chapters of the original research emphasizes the importance of the processes that take place in the oral cavity: (i) changes that take place at the level of keratinocytes and fibroblasts, at the level of tumor cells (squamous cell carcinoma), (ii) the action of various compounds frequently used or which are part of the therapeutic treatment of the most common dental diseases, which have a first contact with the oral mucosa - chlorhexidine (antiseptic used both by the dentists and in oral hygiene products such as mouthwash), aspartame and xylitol (artificial / natural sweeteners) and sodium bicarbonate (cleaning agent, used for alkalizing) on oral cells, (iii) statistical data related to the importance of oral hygiene, use of oral hygiene products, visit to the dental office and other factors that contribute to the prevention of dental diseases and the occurrence of malignancies associated with the oral cavity and (iv) the types of classes of compounds of natural origin that have a recognized pharmacological action in the preventive and curative approach to diseases of the oral cavity.

Cell cultures play an important role in the evaluation of chemical compounds, both of natural origin and synthesis, and also help to partially elucidate the mechanisms of

their biological activity. Mostly, based on the cell culture technique, chemical compounds are investigated to evaluate the beneficial potential (eg herbal extracts, volatile oils, infusions of various teas, polyphenols, etc.) or to evaluate toxicity (e.g. agents used for gingival retraction, chemical agents in oral hygiene products, etc.). The cell lines used in the present study were: primary gingival keratinocytes (PGK-ATCC® PCS-200-014™), primary gingival fibroblasts (HGF-ATCC® PCS-201-018™) and squamous cell carcinoma (SCC4 - ATCC® CRL-1624™) purchased from ATCC (American Type Cell Collection) as frozen samples. These were analyzed to assess the development in culture at different passages in order to make a correct characterization of their behavior (depending on the type and origin) for further testing. Gingival keratinocytes (figure 1) have been shown to have a low confluence after the first week of culture, subsequently forming colonies that have strong links between them, a feature of the epithelial phenotype. These changes decisively influence the selection of certain cell types for safety, toxicity, efficacy studies but also those aimed at creating models later correlated with *in vivo* experiments.

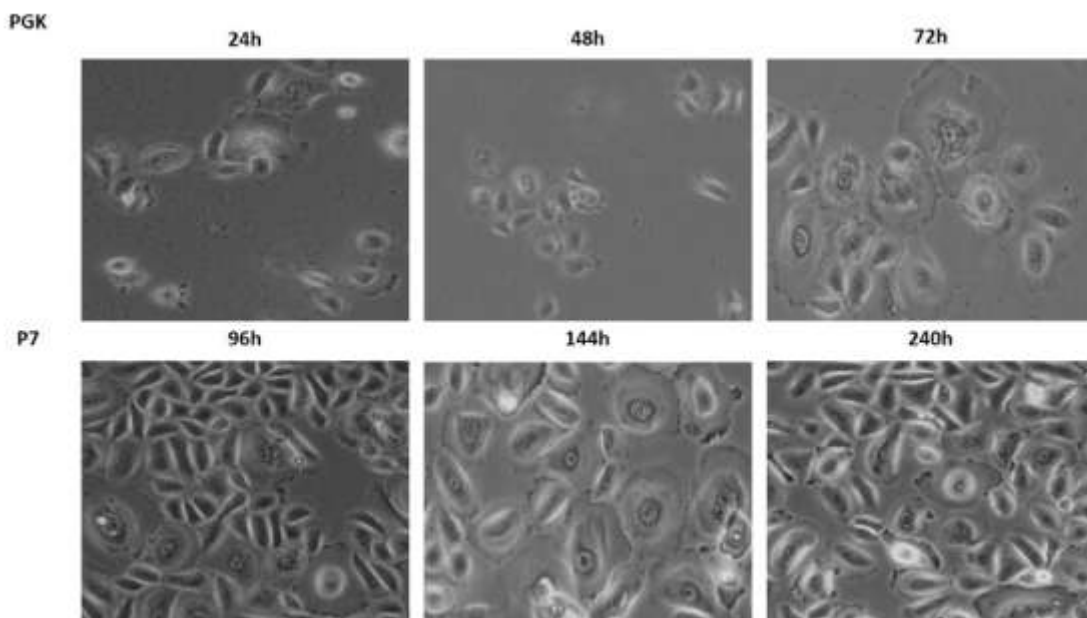


Figure 1. Appearance of primary gingival keratinocytes (passage 7) in culture at different time intervals (10x)

Primary human gingival fibroblasts (figure 2), as the number of passages increases, denote a confluence that does not suffer as significant differences as in the case of primary human gingival keratinocytes.

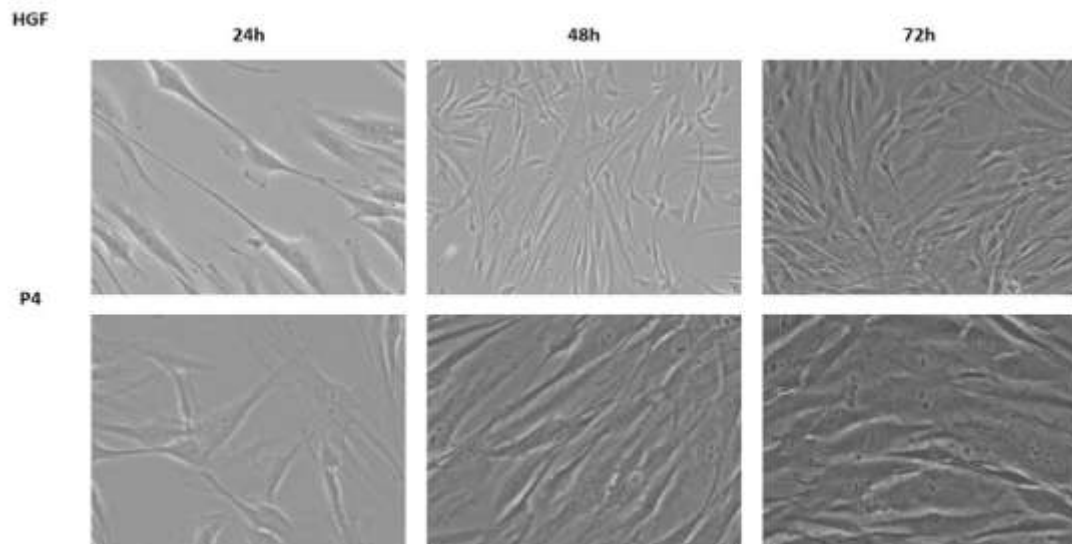


Figure 2. Appearance of primary gingival fibroblasts (passage 4) in culture at different time intervals (10x top row, 20x bottom row)

At small passages, cell growth and morphology are not influenced by time, the confluence being reached even after a few days, and in larger passages they reach confluence quickly compared to keratinocytes, viability not being decisively influenced. Tumor cells (figure 3) show an obvious difference in development in culture. They need a very long time to reach a sufficient confluence for experiments.

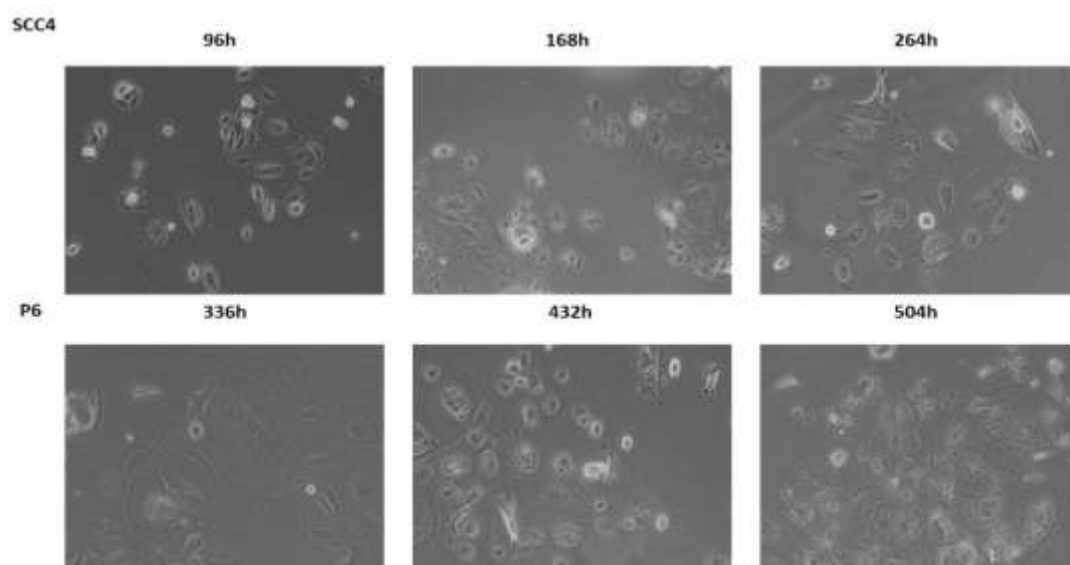


Figure 3. Appearance of squamous cell carcinoma cells of the tongue (passage 6) in culture at different time intervals (10x)

During the experiments conducted also in order to monitor the confluence of the three cell types, the following issues were observed: at small passages a confluence of over 80% is obtained after a period of about two weeks in the case of human gingival keratinocytes and squamous cell carcinoma whereas, for gingival fibroblasts, a period of about 3 times shorter is required to reach the same confluence; at intermediate passages, tumor cells surprisingly, need a longer time to reach optimal confluence to begin the development of specific experiments, while normal cells show faster growth directly proportional to obtaining optimal confluence in a shorter time. Extensive studies on the percentage of confluence based on comparative passages between normal cell lines and oral tumors have not been performed or are not presented in the literature.

Research has continued to evaluate the cytotoxic activity of aspartame (Asp), chlorhexidine (Chx), xylitol (Xyl) and sodium bicarbonate (NaB) on primary human gingival keratinocytes and fibroblasts but also on squamous cell carcinoma. Thus, the morphology of the cells and their viability in the presence of different concentrations of test compounds were studied. Primary human gingival keratinocytes have an intact morphology and a similar size and shape as untreated controls up to a chemical compound concentration of approximately 25 μM ; clear morphological changes observed under the phase contrast microscope occur after exposure to compounds with high concentrations (50 μM) after 24 h post-stimulation. Primary human gingival fibroblasts, in the presence of aspartame and xylitol at the highest concentrations, 50 μM , underwent significant changes in cell shape, detachment from the culture plate and impaired viability. Tumor cells in the presence of chlorhexidine changed their form relatively in the first period after exposure to the compound, after which, at 24 hours, cells in the presence of 50 μM corhexidine underwent changes in shape and development but not significant; the same observations can be made when using aspartame, xylitol and sodium bicarbonate. The changes are noticeable at the high concentrations used, 50 μM , and while at low concentrations the cells do not appear to be affected. All four compounds tested caused a decrease in tumor cell viability in a concentration-time manner. After 24 h of incubation, no statistically significant differences could be detected between the following compounds, chlorhexidine, aspartame and xylitol at 5 and 10 μM , but the concentrations of the compounds tested above 10 μM resulted in a decrease in cell viability in a dose-dependent manner especially in squamous cell carcinoma cells.

By preventing the occurrence of medical problems related to the oral cavity, a major contribution is made to improving the health of a population as well as reducing costs related to addressing certain socio-economic problems in the medical field. On the other hand, oral hygiene is more developed in urban areas compared to rural

areas, this being more and more accentuated in Romania. Also, depending on the oral hygiene products used and the existence of various pathologies, there may be side effects, especially in cases where medication is administered without a medical recommendation. The main purpose of this paper was to identify the role of the link between the patient and the dentist and the importance of knowledge and proper use of certain products to prevent oral problems and is expected to begin appropriate programs to raise public awareness of hygiene and the prevention of diseases related to this area.

The recorded data highlight important aspects in addressing the issue of public awareness in maintaining oral health. Most of the participants do not show up at the dental office in time, do not know very well or do not pay attention to the composition of oral hygiene products, do not differentiate between prevention and treatment, are not sufficiently informed about the possible occurrence of serious diseases. These issues need to be deepened in order to conduct certain awareness-raising campaigns for all segments of the population and to prevent serious diseases and also to take into account the socio-economic aspects related to this area.

The plant sources are multiple and still represent an incomplete field explored in terms of their use in the therapeutic approach to diseases of the oral cavity. Plants and compounds of natural origin are an important alternative due to their high efficacy and low toxicity. One of the most important biological activities presented by phytocompounds is the antibacterial action, useful in the treatment of most oral health problems. An advantage of medicinal plants is that they have a complex mechanism of antibacterial action, which is why the resistance of bacteria is diminished. The advantage of using natural extracts stems from the synergistic effect of existing compounds in plants that enhance their activity leading to an improved therapeutic effect. Natural compounds should be analyzed in combination with other phytocompounds, as well as with conventional drugs currently used in therapy to observe in more detail the synergistic effect between them and to act on a tumor pathology located in the oral cavity.

Original contributions are related to: (i) clarification of certain issues related to the use of healthy, gingival and tumor human cells in order to select a complex method in future *in vitro* studies; (ii) the importance of *in vitro* testing of certain compounds that are frequently in contact with the oral mucosa - chlorhexidine, aspartame, xylitol and sodium bicarbonate - in order to determine the behavior of cells involved in the body's defense processes; (iii) the analysis of the dentist-patient relationship and the importance of oral hygiene through the elaboration of a questionnaire distributed in

Timiș and Arad counties that contributed to the collection of important data and (iv) detailed analysis of compounds of natural origin used in dental practice either with a preventive role or with a preventive role.

Future research directions should include: the search for specific biomarkers for a particular oral disease that begins with keratinocytes and genetic instability; complex *in vivo* or *in vitro* testing on reconstructed human tissue of the compounds used in the present study but also other compounds of plant origin with increased efficacy and, last but not least, the development of programs to raise public awareness of the importance of oral hygiene.