Characterization of Healthy and Tumor Oral Cell Lines of Human Origin

The preliminary stage in the assessment of relevant chemical compounds with impact on dentistry

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The present study was aimed to evaluate the confluence percentage of three oral cell lines, namely primary gingival keratinocytes (PGK), primary gingival fibroblasts (HGF) and tongue squamous cell carcinoma (SCC-4). All cells have been monitored at different passages for 21 days. Evaluation of confluence percentage reveals the fact that primary gingival keratinocytes and tongue squamous cell carcinoma at small passages requires a period of about two weeks to reach a confluence of approximately 80% while for the gingival fibroblasts a period of about three times smaller is satisfactory.

Keywords: characterization, confluence, gingival keratinocytes, gingival fibroblasts, tumoral oral cells

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 biological activity exerted by them. Mostly, based on the cell culture technique, chemical compounds are investigated to evaluate the beneficial potential (*e.g.* medicinal herbal extracts, volatile oils, infusions of different teas, polyphenols, etc.) or to assess toxicity (*e.g.* chemical agents used for gingival retraction, chemical agents in products oral hygiene) (fig. 1).

In vitro assessment contributes to elucidating cellular mechanisms involved in the occurrence of toxic effects and is, most often, conducted on animal or human cell cultures. This type of test has a number of advantages, such as: (a) maintaining the cells in a viable state for as long as possible, allowing for the finalization of the ongoing experiments; (b) the production of live systems that serve to investigate toxicity, thereby avoiding direct contact with the living organism; (c) the possibility of cultivating human cell lines which play a very important role in experiments aimed at verifying the toxic effects of certain agents used in medical practice. In case of interaction between a toxic terminal and a host molecule, a series of reactions may occur at the cellular level involving: covalent or non-covalent bonds, electron transfer, enzymatic reactions, loss of hydrogen etc. depending on this being established the mechanism and the toxicity cell response [1].

Primary gingival keratinocytes (PGK) represent the first line of defense against bacterial attack by forming a barrier based on intercellular connections what makes them suitable models to assess the properties of the oral epithelium and the oral bacterial infections primary stages. It is important to know that these kind of cells present several key features like: a short life span and restricted availability *in vitro*. In addition, it was stated that the modalities of culturing primary gingival keratinocytes are limited since after 6-10 passages the cells become senescent and will entry into apoptosis [2,3]. These

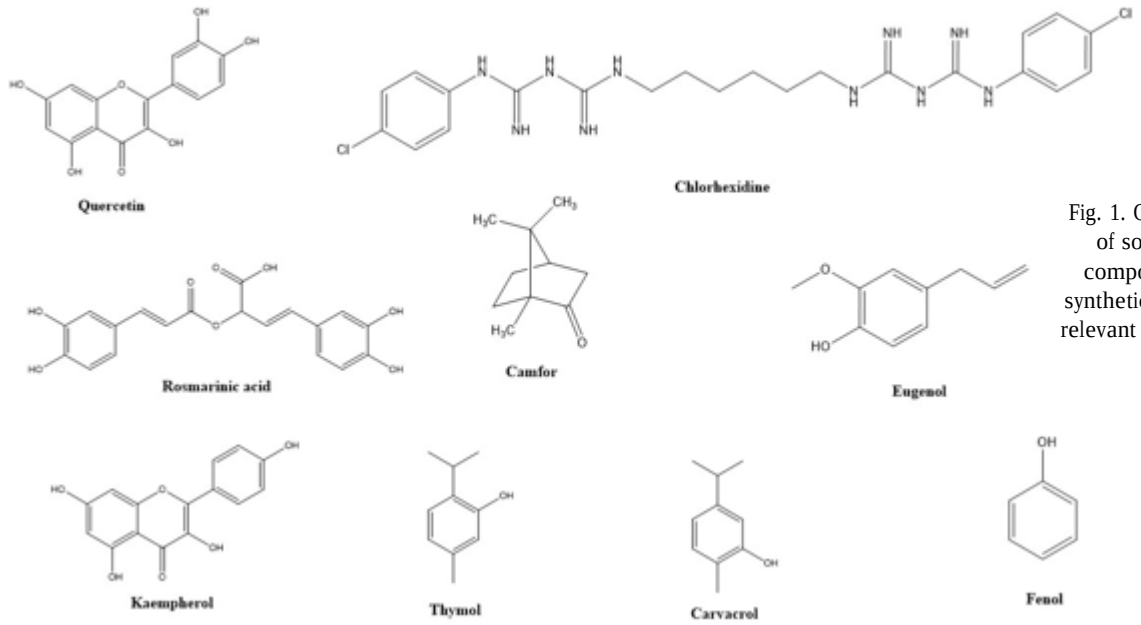

Fig. 1. Chemical structures of some both type of compounds natural and synthetic, respectively with relevant impact on dentistry

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drawbacks restricted the use of primary gingival keratinocytes in research and forced the researchers to find novel approaches, one of the most frequently applied resolution being the development of an immortalized gingival keratinocyte cell line that resembles the histology of the primary cell counterpart [4,5].

Gingival fibroblasts (HGF) are situated in lamina propria and play key roles in maintaining the integrity of the extracellular matrix and the interaction with the primary keratinocytes and the immune cells via soluble mediators in order to coordinate tissue repair process and immune reactions. These cells are considered to be a heterogeneous population due to their multiple functions, their different response to growth factors and their capacity to produce specific extracellular matrix proteins during the healing process [6]. It is known that primary gingival keratinocytes are responsible for the wound healing process (proliferation and migration from the wound edge, differentiation, and reepithelialization) whereas the fibroblasts must restore tissue integrity [5]. Primary cells of human oral tissue are used for multiple applications like: oral biology research, study of differentiation processes, drug efficacy/toxicity ratio, chromosomal analysis [7], evaluation of biomaterials used for dental implants [8,9,11], study of antifungal agents from denture adhesives [10,12], etc.

This study was aimed to monitor healthy - primary gingival keratinocytes and fibroblasts and tumor - tongue squamous cell carcinoma in culture at different passages for 21 days.

Experimental part

Materials and methods

Cell lines and reagents

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 tongue squamous cell carcinoma (SCC-4 - ATCC® CRL-1624™) acquired from ATCC (American Type cell Collection) as frozen vials. The cell lines data are presented in table I.

The specific reagents for cell culture (Dermal Cell Basal Medium - ATCC® PCS-200-030™, Keratinocyte Growth Kit - ATCC® PCS-200-040™; Fibroblast Basal Medium-ATCC PCS-201-030 and the specific growth kit - ATCC PCS-201-041, DMEM:F12 Medium -ATCC 30-2006), hydrocortisone, fetal bovine serum - FBS, antibiotic mixture of penicillin/streptomycin, phosphate saline buffer - PBS, Trypsin/EDTA, and Trypan Blue were acquired from Sigma Aldrich (Germany) and ATCC.

Cell type	*Age/gender*	*Tissue*	*Disease*
PGK	60 years/female	jaw	normal
HGF	60 years/female	gingival	normal
SCC-4	55 years/male	tongue	squamous cell carcinoma

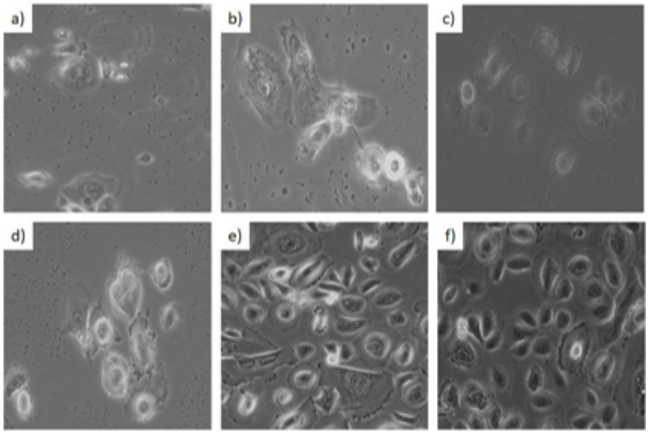

Fig. 2. The appearance of primary gingival keratinocytes (passage 1) in culture: a-day 1; b-day 2; c -day 5; d-day 7; e-day 15; and f-day 21

Culture method

The cells were culture in the specific culture medium as follows: primary gingival keratinocytes (PGK) were cultured in Dermal Cell Basal Medium supplemented with the keratinocyte Growth Kit, primary gingival fibroblasts (HGF) in Fibroblast Basal Medium- supplemented with the specific growth kit and SCC-4 cells in DMEM:F12 Medium supplemented with fetal bovine serum – 10 %. To all culture media it was added an antibiotic mixture (100 U/mL penicillin and 100 ìg/mL streptomycin). The cells were kept in standard conditions: humidified atmosphere with 5% CO_2 at a temperature of 37°C and were passaged every 2-3 days.

Counting the cells was done using the Countess II FL instrument (Thermo Fisher Scientific, USA) in the presence of the Trypan Blue reagent. Trypan blue is the reagent used to check the viability of the cells, and the principle of action is the ability to penetrate inside dead cells by coloring them in dark blue, while the living ones are not affected, being bright when viewed under the microscope.

Results and discussions

After selecting a cell line, a cell type, the non-homogeneous growth and cell selection occur at small passages and obtaining an identical confluence in experiments is very difficult to achieve.

In the first stage after receiving the frozen vials, each cell line was cultured in the specific culture medium. It is important to mention that at the first culture the confluence of 80-90% can be attained after a longer period of time (one week or more) which is cell-type dependent as compared to the cells that are after several passages when the confluence is reached in most cases at 2-3 days.

The shape and the morphology of the cells used in this study are different and these differences can be observed in the following images.

The primary gingival keratinocytes (PGK - fig.1) present an epithelial morphology, with round shape and cobblestone appearance and strong adhesion properties. According to the manufacturer (ATCC), these cells were isolated from the jaw of a female and present adherent properties. PGK suffered two passages and were cryopreserved at this passage (procedure conducted by the manufacturer), and by this information it is highlighted that this cell line is not immortalized. As can be seen in figure 2f at a confluence around 80%, the cells are associated with each other in colonies.

It can be observed in figure 2 that the confluence of the gingival keratinocytes was low (around 15%) after 7 days

Table I

CHARACTERIZATION OF THE NORMAL AND TUMORAL CELLS UTILIZED IN THE PRESENT STUDY

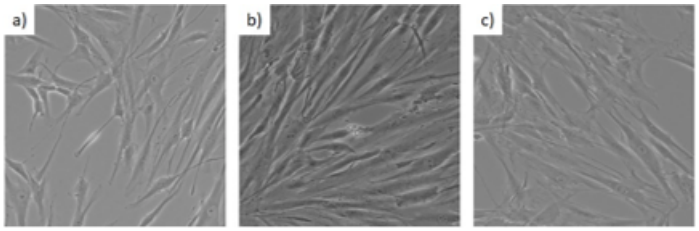

Fig. 3. The appearance of human gingival fibroblasts (passage 3) in culture: a -day 2; b-day 3 and c-day 5

in culture (the reason is that the cells were at the first passage after were received from ATCC), but after two more days the cells reached a 50-60% confluence. In the day 11 the cells formed colonies presenting strong bonds between them, a characteristic of the epithelial phenotype.

In the case of human gingival fibroblasts (HGF- fig. 3) which were at the third passage after the first defreeze and culture process, the confluence of over 90% was reached faster (after 4 days), at 48 h in culture the flask being already 50-60% full (fig. 3). Fibroblasts present a spindle shape with prolongations, the bonds between the cells being loose as compared to keratinocytes, this feature offering them the ability to migrate and invade the surrounding tissues. According to the product sheet provided by the manufacturer (ATCC), HGF were obtained in the same manner as keratinocytes: isolation from a female gingival tissue and cryopreserved at passage 2 in cryopreservation solution and are characterized as adherent, bipolar, refractile and spindle-shaped cells.

The tongue squamous cell carcinoma cells (SCC-4) displayed in figure 4, are at the first passage after acquiring procedure and it can be seen that the percentage of cells in the culture flasks is rather low even at 96 h, what means that it requires longer time periods to reach a 90% confluence. These cells present an epithelial-like morphology, the shape of the cells being somehow round. According to the provider (ATCC), SCC-4 cells were isolated from a tongue carcinoma biopsy collected from a 55-years old male and present adherent properties and epidermal keratins.

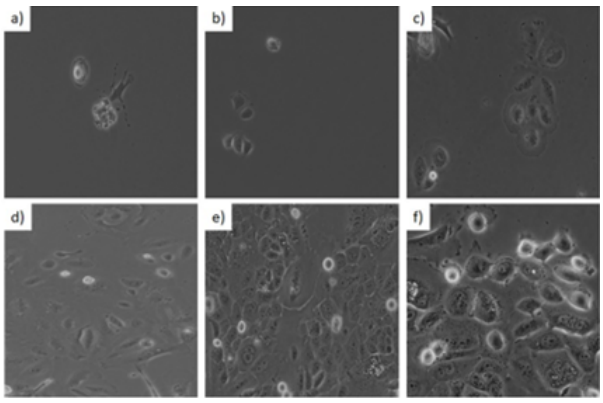

Fig. 4. The appearance of SCC-4 - human tongue squamous cell carcinoma (passage 1) in culture: a -day 1; b - day 2; c- day 5; d-day 7; e- day 15; and f - day 21

During the experiments in order to monitor the confluence of the three cell types, a number of changes were observed: at small passages a confluence of over 80% is obtained after a period of about two weeks in the case of gingival keratinocytes and squamous cell carcinoma while for gingival fibroblast whereas for gingival fibroblasts a period of about 3 times less is needed to reach same confluence (fig. 5). In the case of intermediate passages, surprising tumor cells require a longer time to

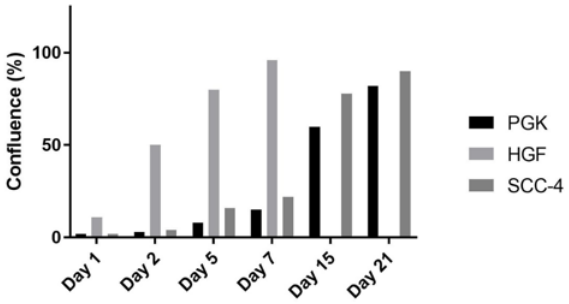

Fig. 5. The evolution of confluence percentage of normal and tumoral cells during the experiment at small passages [1-3]

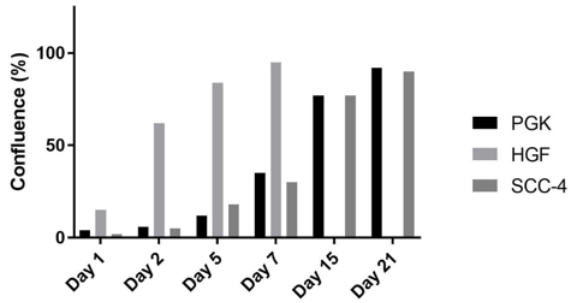

Fig. 6. The evolution of confluence percentage of normal and tumoral cells during the experiment at intermediate passages [10-13]

reach the optimum confluence to start the development of specific experiments while normal cells exhibit faster growth directly proportional to achieving optimal confluence in a shorter time (fig. 6).

Some studies points out that confluence percentage as well as number of passages meaningfully influences global gene expression patterns. Small passages of cells in culture are often taken into account as heterogeneous due to the fact that the majority of changes occur in the first few passages [11]. However, comprehensive studies on the percentage of confluence based on comparative passages between normal cell lines and oral tumors have not been performed.

Conclusions

Cell-based tests are frequently used for screening collections of chemical compounds to evaluate if the structures investigated exert effects regarding proliferation or cytotoxicity. The confluence percentage is determinant in conducting experiments and plays a key role in accurate assessment of test compounds. Regarding human gingival keratinocytes and fibroblasts at small passages, the optimal confluence to start the experiments is reached after about two weeks, 4-5 days respectively while for tumor cells, squamous cell carcinoma, also a period of two weeks it's necessary.

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