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Medium-Term Culture of Primary Oral Squamous Cell Carcinoma in a Three-Dimensional Model: Effects on Cell Survival Following Topical 5-Fluororacile Delivery by Drug-Loaded Matrix Tablets

Giuseppina Campisi[1], Libero Italo Giannola[2,*], Alberto Fucarino[3], Antonella Marino Gammazza[3,5], Alessandro Pitruzzella[3,5], Vito Marcianò[3], Viviana De Caro[2], Maria Gabriella Siragusa[2], Giulia Giandalia[2], Domenico Compilato[1], Stephen T. Holgate[4], Donna E. Davies[4], Felicia Farina[3], Giovanni Zummo[3], Carlo Paderni[1] and Fabio Bucchieri[3,5]

[1]*Dipartimento di Discipline Chirurgiche ed Oncologiche, Università di Palermo, Palermo, Italy;* [2]*Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari, Università di Palermo, Palermo, Italy;* [3]*Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche, Università di Palermo, Palermo, Italy;* [4]*Infection, Inflammation and Immunity Division, School of Medicine, Southampton University, Southampton, UK;* [5]*Istituto Euro-Mediterraneo di Scienza e Tecnologia, IEMEST, Palermo, Italy*

Abstract: Since the activity of several conventional anticancer drugs is restricted by resistance mechanisms and dose-limiting side-effects, the design of formulations for local application on malignant lesions seems to be an efficient and promising drug delivery approach.

In this study, the effect of locally applied 5-FU on cell death was evaluated both in a SCC4/HEK001 model and in a newly proposed 3D outgrowth model of oral squamous cell carcinoma (OSCC). Initially, the optimal drug dose was established by delivery of solutions containing different amounts of 5-FU. The solution containing 1% (w/v) of 5-FU resulted effective in inducing cell death with complete eradication of cell colonies.

Buccal tablets were designed to deliver 5-FU locoregionally to the cancer lesions of the oral cavity. Tablets were prepared using a drug loaded matrix of acrylic/methacrylic acid copolymer containing 1% (w/w) of 5-FU and applied on 3D outgrowths. The drug release from tablets appeared to be sufficient to induce cell death as confirmed by transmission electron microscopy and enzymatic assay (TUNEL). After 120 h of treatment, when about 90% of the drug had been discharged from the tablets into the culture environment, 5-FU caused loss of cell-cell communications and apoptotic cell death. After 192 h, a complete disaggregation of the 3D oral outgrowths and the death of all the cells was observed.

Buccal matrix tablets could be considered a promising new approach to the locoregional treatment of OSCC. Risks of systemic toxicity are avoided since very low drug doses are delivered.

Keywords: 5-Fluorouracil, Locoregional drug delivery, Oral squamous cell carcinoma, Buccal tablets, Tissue engineering, 3D oral outgrowths.

1. INTRODUCTION

Although tumors of a variety of histologic types may arise within the oral cavity, more than 90% of these tumors are squamous cell carcinomas [1]. The risk factors most commonly associated with the progress of oral squamous cell carcinoma (OSCC) include cigarette smoking, exposure to second-hand smoke, the consumption of betel nuts, heavy alcohol consumption, infection with viruses and advanced age [2,3]. Tobacco and alcohol usage are the most important avoidable risks, although only a small fraction of people who are exposed to these agents develop OSCC. This cancer kills men at a much higher rate than women, and the mortality rate is highest among black men.

These considerations provide further confirmation to the concept of wide differences in genetic susceptibility to carcinogen exposure [4-6]. OSCC of the buccal mucosa can be especially aggressive. Cancer of the buccal mucosa arises more frequently from pre-existing leukoplakia than cancers of the oral cavity.

Molecular alterations causing oral carcinogenesis have been linked to genetic factors involving chromosomal aberrations, tumor suppressor genes, oncogenes, DNA mismatch repair genes, environmental and viral factors. It is likely that sequential and/or concomitant mutations or defects enable the transformed epithelial cells to proceed through precancerous progression and malignant conversion with a metastatic potential. In a few words, squamous cell carcinoma is characterized as exophytic or ulcerative lesions or a combination of both. Exophytic lesions are generally less common than ulcerative lesions; they grow slowly and are little infiltrative. In advanced cases, the exophytic lesions can become deeply infiltrative and invasive. On the other hand, ulcerative lesions are common and often appear as red or grey ulcers with heaped boundaries that bleed easily. These ulcerative lesions can be deeply infiltrative [7].

During the past decades, there has been an extensive evolution in the knowledge on oral cancer. Despite these developments, the 5-year overall patient survival has experienced little progression, while the quality of life of patients has notably improved [8].

Treatment of oral cancer has primarily relied on classical modalities encompassing surgery, radiation, and chemotherapy or a combination of these methods [9].

The surgical removal of the tumor may result in facial distortion and can adversely affect the patient's ability to speak and swallow. In addition, oral cancer surgery can lead to complications such as injury to neurovascular structures, reduced mouth opening associated with patient's perception of chewing deficit, wound infection, or salivary fistula. Post-operative sialoceles and fistulas may interfere with wound healing.

*Address correspondence to this author at the Dipartimento di Scienze e Tecnologie Molecolari e Biomolecolari (STEMBIO), Università degli Studi di Palermo, Via Archirafi 32, 90123 Palermo, Italy; Tel: +39 091 23891924; Fax: +39 091 23891962; E-mail: l.italo.giannola@unipa.it

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The most frequently observed complications resulting from radiation therapy include tooth decay and dental caries, tissue edema and erythema, decreased salivation or xerostomia, osteoradionecrosis of the mandible, periodontal disease and dysphagia [10]. The successful treatment of OSCC depends on the appropriate treatment of the primary site. Increased tumor size (T stage), the presence and number of nodal metastases (N stage), the spread of tumor outside of the lymph node capsule, and perineural invasion by the primary tumor are the most consistent adverse outcomes of delayed treatment. Cancer of the buccal mucosa has multiple potential routes of spread to the adjacent areas in the head and neck [11].

Despite the fact that some patients may be successfully treated with surgery and/or radiotherapy, the majority are treated with chemotherapy, in order to ameliorate the quality of life, convert inoperable tumors into operable as well as to improve the locoregional tumor control and long-term survival rate [12].

In addition to debulking surgery, the main treatment modality in cancer therapy is chemotherapy, nevertheless its role in treatment of patients with oral cancer is not well established yet.

Systemic chemotherapy regimens appear to be efficient locally and in the subsumed prevention of metastatic spread, but they are also associated with severe toxicity and morbidity, potentially relevant to the survival rate. Many chemotherapeutic agents can simultaneously improve the survival rate of patients with cancer and result in adverse effects, owing to the lack of specificity. Anticancer drugs have toxic effects on normal tissues and have a very narrow therapeutic window. Recently, it has been reported that nerve cell function can also be disrupted and neurotoxicities (i.e. peripheral neuropathy, cognitive changes and humour alterations) could be developed [13]. Chemotherapy is beneficial for local control and survival improvement, although it does not always induce a substantially positive response. Many tumors present a satisfactory response when they are first exposed to chemotherapeutic agents. However, drug resistance occurs sooner or later in these tumors, and the majority of the patients develop progressive disease [14]. The lack of effective chemotherapy results in a high death rate in patients with OSCC. In particular, high doses of chemotherapeutic agents could cause the development of resistant cell lines that represent a major obstacle in cancer therapy [15].

5-Fluorouracil (5-FU) is currently used in systemic treatment of oral, gastrointestinal, breast, pancreas and skin neoplasms, either alone or associated with other drugs [9,12,15-17]. It is still one of the most widely used anticancer agents developed over the past 10 years, for chemotherapy regimens aimed at enhancing local tumor control, reducing distant metastases and preserving anatomical functions.

5-FU is an antimetabolite that induces apoptotic death of the cancer cells [16]. *In vitro* studies demonstrated that 5-FU, also in oral cancer, causes apoptosis that could be caspase-dependent and might vary in different tumor cell lines [9,12,15,16,18]. Nevertheless, to induce apoptosis, high 5-FU concentrations next to cancer cells are needed.

Since 5-FU is poorly absorbed from gastrointestinal tract, it is usually administered by slow intravenous continuous infusion or bolus. It has been demonstrated that protracted venous infusion is more effective, in terms of percentage of effectiveness, than intravenous bolus, although the biological mechanism responsible for this phenomenon is unclear [19].

After intravenous administration its plasmatic half-life is about 16 min and it is dose-dependent. Severe and unpredictable adverse reactions to 5-FU are mainly attributed to deficiency of dihydropyrimidine dehydrogenase (DPD), an enzyme encoded by the DPYD gene. This enzyme is the rate-limiting step in catabolism of 5-FU and deactivates more than 80% of its standard dose. The deficiency of DPD, resulting in poor enzymatic activity, increases the half-life of 5-FU and causes drug accumulation and severe toxicity [20]. The major toxic effects of 5-FU regimens result in myelosuppressive action and gastrointestinal toxic effect. Loss of the integrity of the gastrointestinal mucosal barrier associated with neutropenia is the most common cause for the risk of fatal toxicity associated with bolus 5-FU. Moreover, non-specific distribution of drug in tumors, as well as in normal tissues, can result in an undue toxicity [19].

The clinical use of 5-FU is strongly limited by the acquired or inherent resistance, which is thought to mediate therapeutic efficacy in cancer chemotherapy. To overcome 5-FU resistance in OSCC, a combination of anticancer agents is usually used to reduce these resistance determinants via alternative applications [21]. However, the inconveniences associated to the toxicity often prevent the use of the adequate dose and sufficient period for the desired results. These effects could be lowered by topical applications. Five percent 5-FU cream is approved by the FDA for the treatment of superficial basal cell carcinomas (BCC) but has so far been underutilized. The mechanism of action of topical 5-FU in treating superficial malignancies is thought to be similar to that of intravenous 5-FU [22-24].

It has been reported that low concentration formulations provide efficacy on skin diseases equal to that of currently available formulations containing high amounts of 5-FU [25].

Strategies able to increase the anticancer activity and to circumvent resistance are either discovery of new drugs or improving delivery of conventional chemotherapeutics. Established anticancer drugs have the advantage of well known pharmacological and toxicological profiles.

Our working hypothesis is that locoregional 5-FU delivery can reduce the risk of systemic toxicity while preserving the efficacy also in treatment of OSCC. To date, the most common local treatment is limited to chemoprevention. However, applications of a 2-3 min topical drug solution-painting onto premalignant lesions of oral mucosa has a controversial effectiveness. Usually, the oral saliva and mechanical activities will remove the medication painted on mucosal surface quickly. It is difficult to use such painting treatment to retain an agent solution on targeted tissue surface with adequate concentration and sufficient retention time [26]. Solid dosage forms applied on cancer lesions of buccal mucosa could be able to prolong the medication retention time on the targeted tissue, allowing high drug concentration in a local area, decreasing the risks of toxic and dose-dependent side-effects of systemic drug delivery, while they do not require expertise for administration.

Tissue-engineered oral mucosal equivalents have been developed for *in vitro* studies of biocompatibility, for mucosal irritation and for oral disease to better understand disease process and discover new treatments [27,28]. In the present work we have used a novel 3D *in vitro* model that enabled us to perform mid- to long-term cultures of both normal and malignant oral mucosa [29]. Using this model could lead to an increased understanding of the features of OSCC progression and to the discovery of new potential therapeutic strategies.

Following the development of a suitable formulation to be applied on culture of primary oral squamous cell carcinoma in a 3D model, in this study the effects on cell survival of topically applied 1% 5-FU tablets was evaluated as a potential therapeutic means against OSCC.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

5-Fluorouracil (5-FU), USP grade, 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide (MTT) and Proteinase K were purchased from Sigma-Aldrich (Milan, Italy). Acrylic and methacrylic acid copolymer (described in USP/NF as ammonio

methacrylate copolymer "type B") was kindly supplied by Rofarma (Milan, Italy). HCl was purchased from Titolchimica (Pontecchio, Italy) and isopropanol from Carlo Erba (Milan, Italy). Glutaraldehyde, osmium tetroxide (OsO_4), epoxy resin (Epon812) and epoxy accelerator (DMP30) were purchased from Electron Microscopy Sciences (Hatfield, PA, USA). 1,2-Propylene oxide was purchased from Merck (Darmstadt, Germany). 3,3'-Diaminobenzidine (DAB) was purchased from Millipore (Billerica, MA, USA). Buffer solution (pH 6.8) simulating saliva was prepared by dissolving NaCl (0.126 g), KCl (0.964 g), KSCN (0.189 g), KH_2PO_4 (0.655 g), Na_2SO_4 $10H_2O$ (0.763 g), NH_4Cl (0.178 g), $CaCl_2$ $2H_2O$ (0.228 g), $NaHCO_3$ (0.631 g), and urea (0.200 g) in 1 L of distilled water. Phosphate-buffered saline (PBS) Ca^{2+} and Mg^{2+}-free solution (pH 7.4) was prepared by dissolving KH_2PO_4 (0.144 g), anhydrous Na_2HPO_4 (0.795 g) and NaCl (9.0 g) in 1 L of distilled water.

All chemicals and solvents were of analytical grade and were used without further purification.

2.1.2. Cell Culture Reagents

Recombinant human EGF (hEGF) was purchased from Cell Signaling Technology (Beverly, MA, USA). L-Glutamine was obtained from Stem Cell Technologies (Tukwila, WA, USA). Hydrocortisone (H400) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Sigma-Aldrich (Milan, Italy). GIBCO™ Keratinocyte-Serum Free Medium (Keratinocyte-SFM) and Ham's F12 medium were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Fetal bovine Serum (FBS), Hanks BSS without phenol red (HBSS) and Phosphate Buffered Solution (PBS) were purchased from Lonza, (Cologne, Germany). Veloderm® (kindly supplied by the Bioskin Trade Company,Ancona, Italy) is a freely permeable, 0.1 mm thick membrane, commonly used as wound dressing. It is a natural micro-porous polymeric dressing, made from cellulose microfibrils called Crystalcell 77™; its fibers, as evidenced by scanning electron microscopy analysis, show regular structure with inter-fibrillar spaces large enough to allow transfer of large molecules from one to the other side of the membrane [30].

2.2. Methods

2.2.1. Cell Cultures

2.2.1.1. Cell Lines

Human squamous cell carcinoma cell line (SCC4, from LGC Standards, Milan, Italy) was grown in a 1: 1 mixture of DMEM and Ham's F12 medium supplemented with 2 mM L-glutamine, 400 ng/ml hydrocortisone and 10% FBS. These cells originated from a squamous cell carcinoma of the tongue of a 55 years old male patient. Human skin keratinocyte cell line (HEK001, from LGC Standards) was grown as a monolayer in Keratinocyte-SFM, supplemented with 5 ng/ml h-EGF and 2mM L-glutamine. These cells are derived from normal skin keratinocytes immortalized by human papillomavirus 16 (HPV-16) E6/E7, and were used as a control for the SCC4. Medium was changed every 2 days and both cell lines were cultured at 37°C, 5% CO_2 atmosphere in 25 cm^2 flasks (Corning Life Sciences, Amsterdam, The Netherlands). Subcultures before confluency were performed harvesting adherent cells by trypsin digestion in HBSS, washing with PBS and resuspending cells in fresh growth medium.

2.2.1.2. Three-dimensional Cancerous Oral Outgrowth Model

OSCC samples were obtained from patients referred to the Unit of Oral Medicine of the University of Palermo, Italy. The following adopted procedures, conforming to the relevant ethical guidelines for human research, were in agreement with the Helsinki Declaration of 1975, as revised in 1983, and approved by the Ethic Council of the Policlinico-Hospital of the University of Palermo. Each patient suffering from neoplastic oral lesion was subjected to topical antifungal therapy (miconazole 2% oral gel, Daktarin, Janssen-Cilag) 3 times daily before the biopsy, after written informed consent. After 1 min of treatment with commercial 0.2% chlorhexidine oral rinse (Broxo Din, Laboratorio Farmaceutico S.I.T), OSCC samples were obtained by using 6 mm diameter punch biopsy on the lesion margin with clinically healthy tissue. Each sample was divided into two parts. The outer part of the sample was fixed in formalin and sent for histo-cytopathological examination, while the inner part was immediately placed in fresh culture medium and processed for the 3D oral outgrowths.

The method employed to obtain 3D human oral outgrowths from normal human oral mucosa has already been described [29]. The 3D OSCC outgrowths were obtained similarly. Briefly, the biopsies were cut using a sterile scalpel into 0.5 mm^3 pieces and placed onto 6.5 mm Transwells (Becton Dickinson, Franklin Lakes, NJ, USA) embedded in 60 µl of Matrigel (Becton Dickinson). The Transwells were put on 24 wells culture plates (Corning Life Sciences) and 330 µl of growth medium mix was added to each well. This mix was constituted of Keratinocyte-SFM supplemented with 5 ng/ml of h-EGF and DMEM supplemented with 10% FBS (1: 1) which was placed underneath the nylon membrane of the Transwells. The outgrowths were cultured at 37°C in a 5% CO_2 atmosphere and the medium was changed every 48 h. An inverted light microscope equipped with phase contrast rings (LEICA DM-IRB, Leica Microsystems Srl, Milan, Italy) was used to monitor the outgrowths.

2.2.3. Cell Treatment

2.2.3.1. Cell Treatment with 5-FU Solutions

Cell lines, SCC4 and HEK001, were seeded at $1x10^4$ cells/ml in 24-well plates. After cells reached approximately 80% of confluence, they were rendered quiescent with 1% FBS medium for 24 h. 5-FU treatment of the outgrowths was performed when these had reached complete 3D differentiation (10-14 days). A stock solution was prepared by dissolving 5-FU in PBS, while the working solutions were obtained by diluting it in the growth medium. To identify in the cell lines the optimal 5-FU dilutions necessary to induce adequate levels of cell death (MTT test) 1 ml of 0.05, 0.1, 0.5, 1 and 5% (w/v) solutions respectively were added to the outgrowths in the culture wells. Treatment of the outgrowths was carried out for up to 10 days with 330 µl of the same solutions that were placed underneath the Transwell membrane; induction of cell death was evaluated morphologically. Untreated outgrowths were considered as control.

2.2.3.2. Cell Treatment with 5-FU Loaded Tablets

In order to evaluate the mid- to long-term effects of topical 5-FU treatment on cell survival, 3D OSCC outgrowths were treated with tablets containing 1% (w/w) of 5-FU (see section 2.2.7). Outgrowths were grown until complete 3D differentiation (10-14 days), and then aliquots (weighing 23 ± 1.0 mg) of tablets containing 5-FU were added on top of the matrigel layer of the outgrowths. To avoid possible adverse mechanical effects of the tablets on the cells, a Veloderm® membrane was positioned between the matrigel and the 5-FU loaded tablets. 200 µl of growth medium mix (Keratinocyte-SFM/DMEM 1: 1) was added on top of the tablets to facilitate drug delivery on the cell layers. Untreated controls, with drug unloaded matrix tablets positioned on top of the Veloderm® membrane, were used to verify the potential adverse effects of the Veloderm® and tablet system on cell survival. The treatment was carried out for up to 8 days with 48, 120 and 192 h time points chosen for the evaluation of the effects on cell survival. In experiments at 120 h and 48 h after the start of the experiment, the growth medium was collected from the bottom plate and replaced with fresh medium; in experiments at 192 h replacement was accomplished at respectively 48 and 120 h after the start. For all time points, outgrowths were processed for electron microscopy and TUNEL analysis as described in sections 2.2.6 and 2.2.7.

2.2.4. Drug recovery in the Culture Medium after Cell Treatment with 5-FU Tablets

The withdrawals collected in experiments at 48, 120 and 192 h time points were analyzed to determine the amount of drug transferred onto the bottom plate. At the end of each experiment, the 5-FU recovery was evaluated as the cumulative amount of drug released from tablets and transferred into the culture medium through the outgrowths. Drug assays were carried out as described in section 2.2.9.2. As control, drug recovery assay was carried at the same time points in absence of 3D outgrowths to also evaluate drug release in the whole system.

2.2.5. Cell Viability Test

The mitochondrial activity of SCC4 and HEK001 cell lines, after exposure to 5-FU, was evaluated using the MTT assay. After 5 h and 24 h of incubation, the medium containing 5-FU was removed, MTT was dissolved in fresh growth medium mix and added to the cell lines at a final concentration of 0.5 mg/ml. Following a 2 h incubation period at 37°C, the converted dye was solubilized with 1 ml acidic isopropanol (0.04 M HCl in absolute isopropanol) and optical density (OD) was measured at 570 nm, with background subtraction at 690 nm, using a spectrophotometer (Ultrospec 1000, Amersham Pharmacia Biotech, Uppsala, Sweden). Cell viability was expressed as the percentage of the OD value of 5-FU-treated cells compared with untreated controls, according to the following equation: Viability = (OD sample/OD control) x 100. Each experiment was carried out in duplicate.

2.2.6. Electron Microscopy

Immediately after excision of the nylon membrane from the Transwells using a scalpel, the 3D OSCC outgrowths were cut in pieces and fixed in a solution of 2.5% glutaraldehyde in PBS for 20 minutes at room temperature. The pieces were post-fixed in 1% OsO_4 for 2 h, dehydrated in an ascending graded series of ethanol, infiltrated with Epon812, in propylene oxide (1: 3, 1: 2, 1: 1 for 30 min at room temperature respectively) and finally embedded in Epon812 with DMP30. The resin was then polymerized at 60°C for 48 h. Ultrathin and semithin sections were cut with an ultramicrotome (Ultracut E, Reichert-Jung, Depew, NY, USA) at different thicknesses and mounted on copper grids and on glass slides for further use.

2.2.7. In situ Apoptosis Detection

Semithin sections (about 2 μm thick) were cut with an ultramicrotome, collected on poly-L-lysine coated slides (Thermo Fisher Scientific, Illkirch Cedex, France), and air-dried at room temperature. A controlled corrosion of the section surface was obtained by etching the sections for at least 8 min in a solution composed of saturated sodium hydroxide in absolute ethanol diluted to 50% with absolute ethanol. After rehydration in descending graded series of ethanol to water, sections were treated with Proteinase K for 15 minutes at room temperature, and incubated with 3% hydrogen peroxide in PBS for 5 min. The ApopTag Peroxidase *In Situ* Apoptosis Detection kit (Millipore, Billerica, MS, USA) was used to label the free 3'-OH fragment DNA. Briefly, a working strength Terminal deoxynucleotidyl transferase solution was prepared according to the kit protocol and applied to the section for 1 h at 37°C. A stop/wash buffer solution was applied for 10 min at room temperature and slides were then treated with anti-digoxigenin-peroxidase for 30 min at room temperature. Samples were then washed four times in distilled water and DAB, as peroxidase substrate, was added for 10 min. Finally, the specimens were washed with distilled water and mounted under a coverslip in a permanent mounting media (Millipore). Three independent observers examined the specimens in a blind approach using a light microscope. Five optical fields for each section were selected and the total cell numbers and TUNEL-positive cell numbers were obtained from each. The percentage of TUNEL-positive cells is described as the percentage of TUNEL-positive cells relative to the total number of cells in each sample. All the observations were made at a magnification of 400 x and the means of triplicate counts were used for statistical analyses.

2.2.8. Preparation of 5-FU Loaded Matrix Tablets

Solid dispersion (matrix) containing 1% w/w of 5-FU was prepared as previously reported [31]. Briefly, the accurately weighed amount of 5-FU was solubilized in acetone. To the obtained clear solution, the appropriate amount of acrylic and methacrylic acid copolymer was added in order to obtain the final matrix. After solvent evaporation, the obtained solid mass was powdered and air-dried at room temperature for 24 h. The amount of 5-FU entrapped in the matrix was determined spectrophotometrically. Tablets (13 mm diameter, 1.33 cm^2 surface and 1.20 mm thickness, weighing 200 mg), were obtained by direct compression (10 tons) of drug loaded matrix using a hydraulic, single die, tabletting machine (IR Accessory PerkinElmer, Waltham, MS, USA), two flat-faced punches and a die. Before compression, the crushed matrix was passed through a standard mesh wire stainless steel sieve with an aperture size of 200 μm (Endecotts Ltd., London, UK).

2.2.8.1. Drug Release from Tablets

The drug release from tablets, in buffer solution simulating saliva, was assessed using the flow through system previously described [32]. Briefly, the system consists of a buffer solution simulating saliva container (100 ml) from which liquid is forced to a release chamber. The flow rate of saliva is controlled by a peristaltic pump (Bio-Rad Econo Pump, Hercules, CA, USA) and maintained constant (1.2 ml/h) during the experiments. In the chamber, the salivary film wetting the tablet is about 0.1 mm thick. During the flow through, simulating saliva wets the drug loaded tablet enriching it in 5-FU content. The temperature is controlled by submerging the chamber and the saliva container in a thermostatic bath (37 ± 0.1 C°). The drug amount in the saliva solution is then quantitatively determined spectrophotometrically (see section 2.2.9.2). Experiments were performed on six tablets of six different batches and mean results were reported (n=6). The residual drug content in the tablets after release studies was determined. The amount of drug released and the residual drug content in the tablet matched the original drug content within 2-6%. Release data were elaborated using Curve Expert version 1.3 and Kaleidagraph as software, and fitted to the equations usually applied in release studies [33]. Linear or non-linear least squares fitting methods were used to determine the optimum values for the parameters present in each equation. Fittings were validated by using chi-square test. A P value less than 0.05 was considered to be statistically significant.

2.2.9. Drug Assay

UV spectrophotometric quantitative determinations were accomplished using an UV/VIS Shimadzu mod. 1700, Pharmaspec instrument (Shimadzu, Kyoto, Japan). The absorption peaks were highly reproducible and linearly related to concentration over a range 0.001-0.4 mg/ml. At testing concentrations, PBS, artificial saliva and formulation components do not interfere significantly with the UV absorption of 5-FU. The sensibility was less than 0.0001 mg/ml. Intra- and inter-day variations, observed during collection of experimental data, were lower than sensibility.

HPLC analyses were performed with a Shimadzu LC-10AD_{VP} instrument equipped with a binary pump LC-10AD_{VP}, an UV SPD-M20A Diode Array detector, a 20 μl injector and a computer integrating apparatus (EZ Start 7.3 software).

2.2.9.1. Drug Content Into the Matrix

Aliquots of randomly selected matrix of each batch were accurately weighed (25.0 ± 2.0 mg), transferred into a 25 ml flask, sonicated (Branson B 1200 cleaner, Branson Ultrasonic Corporation, Danbury, CT, USA), and brought to volume with acetonitrile to solubilize all components of formulation. The amount of 5-FU

loaded in the matrix was measured spectrophotometrically (λ_{max} = 263.3; $E_{1\%}$= 0.546 in acetonitrile).

2.2.9.2. Drug Assay in Release Studies

5-FU released from tablets in buffer solution simulating saliva was evaluated spectrophotometrically at λ_{max} 266 nm ($E_{1\%}$= 0.490 in simulating saliva).

5-FU release from tablets in the culture medium was assessed by HPLC analysis. Chromatographic separation was achieved on a reversed-phase C18-column (Gemini-NX, Phenomenex, 5 µm, 15 cm x 4.6 mm), a mobile phase consisted of 100% 25 mM buffer phosphate (pH 6.8). The flow rate was set at 1 ml/min and the UV wavelength at 266 nm. In these conditions, the retention time for 5-FU was 3.8 min. Standard curves were used for quantification of integrated areas under peaks. The calibration curves were performed at the concentration range of 0.05 to 0.54 mg/ml. In these conditions LOQ was 0.0015 mg/ml and LOD was 0.0010 mg/ml.

The same analytical method was used to evaluate the cumulative 5-FU recovery following drug release from tablets and permeation throughout the outgrowths.

2.2.10. Stability of Free 5-FU in Culture Medium

Due to stability and/or possible drug interaction with the components of the culture medium, the active availability could be reduced. Concentration of 5-FU was observed after pre-incubation in culture medium for several days: no chemical alterations or decrease of concentration were observed in the drug after 10 days of pre-incubation.

2.2.11. Data Analysis

All data, presented as average values of six experiments in duplicate, are reported with the standard deviations. Release data analyses were elaborated with Curve Expert version 1.3 and Kaleidagraph. Linear or non-linear least squares fitting methods were used to determine the optimum values for the parameters present in each equation. Fittings were validated by using chi-square test. Differences were statistically evaluated by the Student's t-test; a p value of ≤ 0.05 was considered significant.

3. RESULTS

3.1. Effects of Different 5-FU Solutions on Cell Viability in SCC and HEK Cells: MTT Test

MTT assays were carried out to evaluate the cell viability upon treatment with five different 5-FU solution concentrations and to select the optimal conditions for the death of squamous carcinoma cells. HEK cells were used as a control cell line to verify eventual differences in the response to 5-FU. Fig. (**1**) shows that both the normal (HEK001) and neoplastic (SCC-4) cell lines were sensitive to 5-FU treatment only after 24 h. A pronounced increase in cell death was observed with the increase of drug concentration. Results were similar amongst the two cell lines. The 5-FU concentration that was exerting the optimal effect in terms of cell death was 1% (64.488 ± 5.2 in HEK001, 52.466 ± 6.3 in SCC-4), as expressed by a marked and significant reduction of overall viability. Above this concentration, cell death levels were too high [Fig. (**1**).] and the majority of the cells were detached from their plastic substratum. The 1% 5-FU solution was therefore deemed to be the best concentration to use in the subsequent experiments.

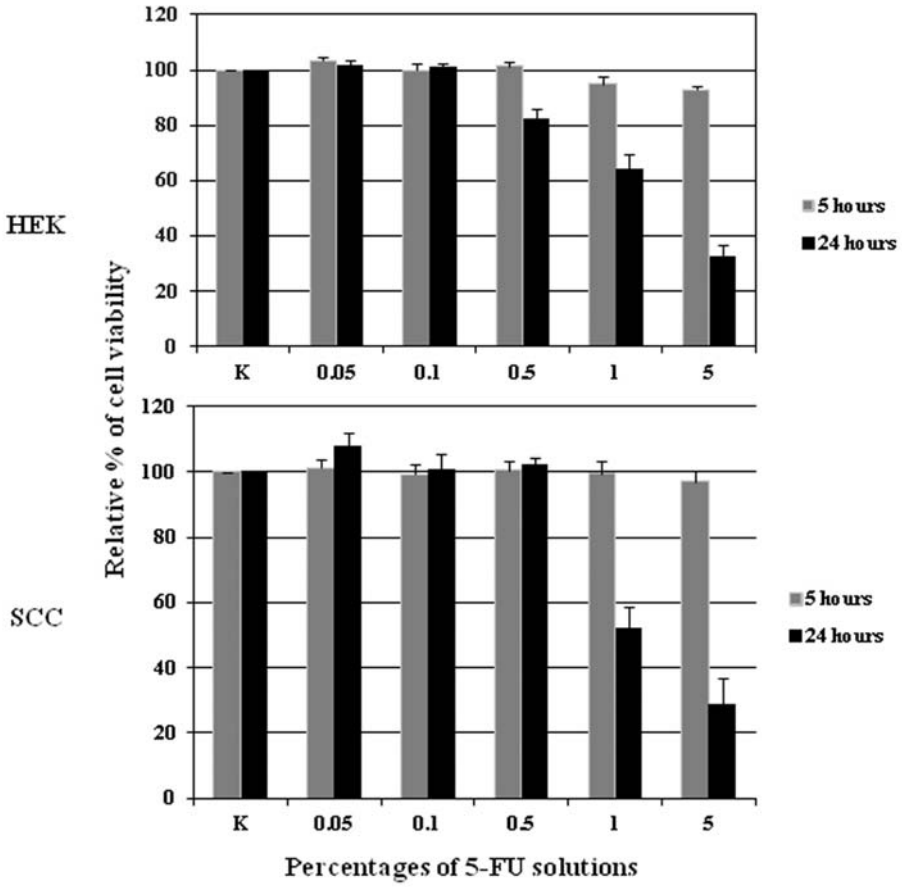

Fig. (1). Effects of 5-FU solution on cell viability in HEK001 and SCC-4 cell lines: MTT test. Results after 5h and 24h of treatment with increasing concentrations of 5-FU. Cell viability is expressed as the percentage of the OD value of 5-FU-treated cells compared with untreated controls. Histograms represent means ± SD (n = 6).

3.2. Effects of 5-FU Solution on Cell Viability in Oral Outgrowths: EM Observations

To verify the sensitivity of our newly developed 3D OSCC outgrowths to 5-FU, these were first grown until complete 3D differentiation (this usually required 10 to 14 days) and then treated with 1% 5-FU solution for an additional 10 days. One of the advantages of our outgrowth model is in fact the possibility to follow mid- to long-term exposures. As shown in Fig. (**2**), that shows a typical phase contrast micrograph obtained from an outgrowth that was differentiated for 10 days, after 10 additional days of treatment with 1% 5-FU solution, the outgrowth margins had considerably shrunk towards the initial biopsy fragment [Fig. (**2**). **B**] when compared to the untreated control [Fig. (**2**). **A**] that instead covered the surface of the nylon membrane of the Transwell completely. These experiments were repeated with outgrowths obtained from 6 different OSCC patients and the results obtained were consistent throughout, suggesting a definite effect of the 5-FU treatment on cell death induction.

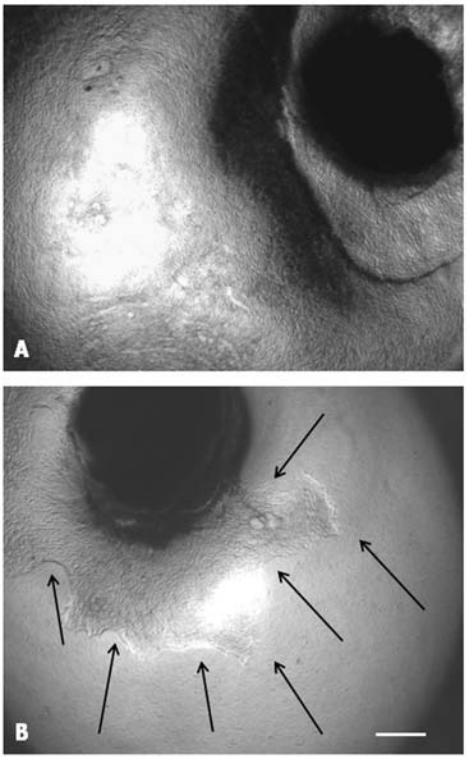

Fig. (2). Morphological appearance of 3D outgrowths treated for 10 days with 1% 5-FU solution. 3D outgrowths were cultured for 10 days until complete differentiation, and then treated for additional 10 days with (B) or without (A, untreated control) 1% (w/v) 5-FU solution. Arrows show the margins of the outgrowths as they recede towards the initial biopsy fragment as a consequence of the treatment. Bar = 1mm.

3.3. 5-FU Release from Tablets

Drug release tests, in buffer solution simulating saliva, were performed using a flow through cell system able to simulate the buccal conditions, including the saliva turnover of the buccal environment [32]. The cumulative amount of 5-FU released was plotted as a function of time as shown in Fig. (**3**). Each data point on the plot represents the mean of the recorded values. The drug discharge pattern showed a slow and regular drug release; it was elaborated according to equations usually applied in release studies [33]. The collected data showed that 5-FU release from tablets follows an Higuchian trend, thus suggesting that diffusion through the inert polymer matrix is the prevailing mechanism of dissolution.

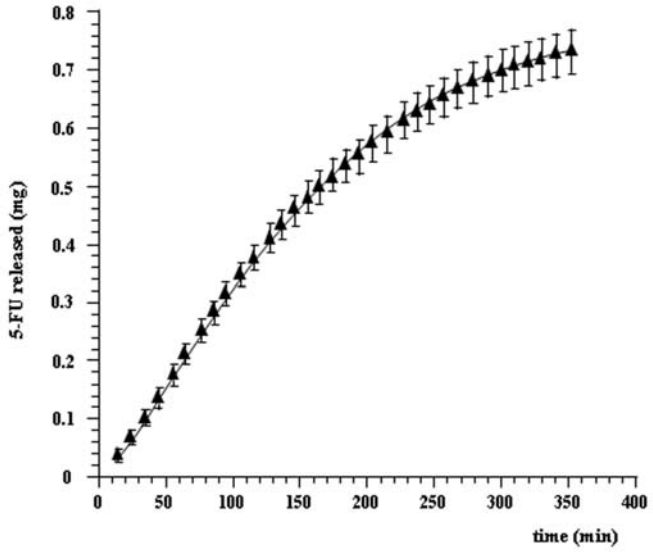

Fig. (3). *In vitro* profile of 5-FU released from tablets containing 1% w/w of 5-FU in buffer solution simulating saliva. Values are presented as means ± SD (n = 6).

3.4. 5-FU Released from Tablets and Transferred Throughout 3D Oral Outgrowths Into the Acceptor Medium

In order to evaluate levels of 5-FU released from tablets and transferred in the acceptor system across 3D outgrowths, at 48, 120 and 192 h time points, the culture medium was withdrawn from the bottom plate (see sections 2.2.3.2 and 2.2.4) and analyzed by HPLC. Drug recovery was calculated both as the amount transferred into the bottom plate and the total amount released in the whole system. Results are summarized in the Table.

Table. Amounts of 5-FU recovered in the bottom plate, in the whole system, and percentage of the dose released from tablets after administration of 1% (w/w) drug loaded tablets on 3D outgrowths grown until complete 3D differentiation (10-14 days)

Time (h)	5-FU transferred into the bottom plate (mg) ± SD	5-FU recovered in the system (mg) ± SD	Dose fraction (%) recovered in the system ± SD
48	0.106 ± 0.003	0.191 ± 0.006	83.0 ± 2.5
120	0.153 ± 0.007	0.204 ± 0.009	91.0 ± 1.7
192	0.198 ± 0.008	0.225 ± 0.001	97.8 ± 0.4

SD = Standard Deviation (n = 6)

3.5. Effects of 5-FU Topically Delivered by Drug-loaded Tablets on Cell Viability in Oral Outgrowths: EM Observations

To evaluate the long-term effects of 5-FU on 3D oral outgrowths' cell viability, these were treated for 48, 120 and 192 h with 1% 5-FU loaded tablets and prepared for transmission electron microscopy analysis. Fig. (**4**) shows that after 120 h of treatment, the squamous epithelial component of the cancerous oral outgrowths showed a marked reduction of its layers [Fig. (**4**). **B**] and some morphological features typical of apoptotic cell death [Fig. (**4**). **D**, **E** and **F**] when compared to the untreated controls [Fig. (**4**). **A** and **C**]. In particular, the treatment determined a complete loss of

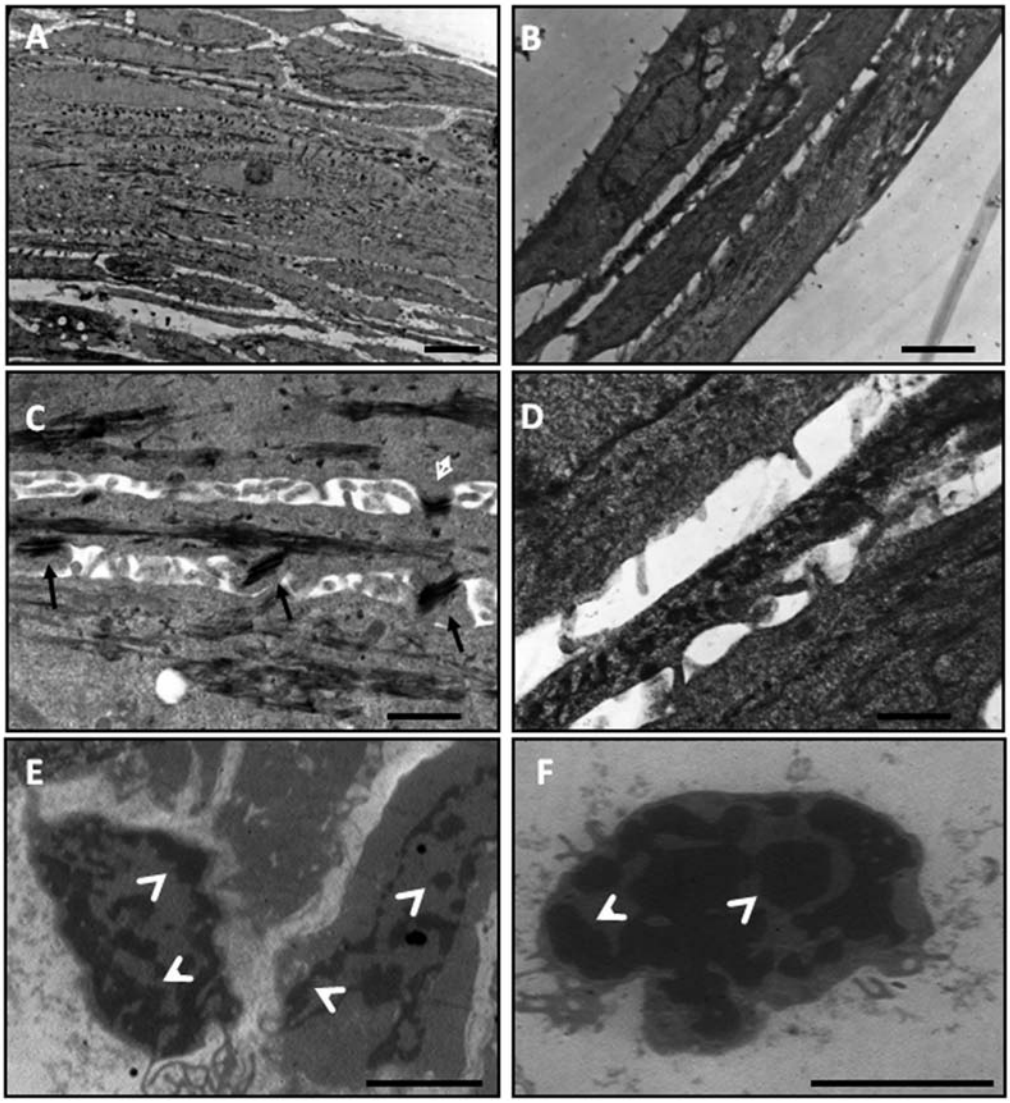

Fig. (4). Morphological appearance of 3D outgrowths treated with 1% 5-FU tablets for 120 hours. 3D outgrowths were grown until complete 3D differentiation (10-14 days), and then treated with 1% (w/w) 5-FU tablets for 120 h (B, D, E and F). A and C are untreated controls (drug unloaded matrix tablets). In B it is possible to observe a marked reduction of the epithelial layers. In D loss of desmosomal junctions between the epithelial cells is shown. Arrows in C show desmosomes. E and F show typical features of apoptosis such as chromatic condensation and apoptotic bodies respectively. Bars in A, B, E and F = 5μm, bars in C and D = 500nm.

the desmosomal junctions between the epithelial cells [Fig. (**4**). **D**] when compared to the untreated outgrowths [Fig. (**4**). **C**], chromatin and cytoplasm condensation [Fig. (**4**). **E**] and presence of apoptotic bodies [Fig. (**4**). **F**]. After 192 h of treatment with the 1% 5-FU loaded tablets all cells were detached and therefore it was not possible to perform any kind of morphological evaluation. These experiments were repeated with outgrowths obtained from 6 different OSCC patients and the results obtained were very consistent, suggesting that 5-FU treatment determined induction of apoptosis.

3.6. Effects of 5-FU Topically Delivered by Drug-loaded Tablets on Cell Viability in Oral Outgrowths: TUNEL Staining

In order to confirm that long-term 5-FU treatment did indeed induce apoptotic cell death in our 3D OSCC model, as already suggested by the EM observations, TUNEL was employed. As shown in Fig. (**5**), after 120 h of treatment with 1% 5-FU loaded tablets, the vast majority of nuclei were stained positively for TUNEL [Fig. (**5**). **B**] compared to the untreated control [Fig. (**5**). **A**] where only a few nuclei were positive. The graph in Fig. (**5**). **C** shows that at 48 h TUNEL positive cells were few (around 10%) in both treated outgrowths and untreated controls; after 120 h there was a significant increase of TUNEL positive cells in treated outgrowths when compared to untreated controls (80.43 ± 10.87 *vs* 12.54 ± 5.6, $p<0.001$); finally, after 192 h the nuclei of the few cells that were still attached to their substrate in the treated outgrowths were all positive for the TUNEL assay whereas the untreated controls were still showing, even after such a long time in culture, very low levels of apoptosis.

4. DISCUSSION

The current treatment of OSCC is not still satisfactory in terms of patient survival and the role of chemotherapy in the treatment of patients with oral cancer is not well established yet. Consequently, there is an urgent need for new treatment paradigms to achieve better efficacy, and avoid the side effects and long term morbidity of existing therapeutic protocols. The ideal situation would be to find drug formulations that will allow lower individual doses but yet result in enhanced cell killing with less side effects. To achieve

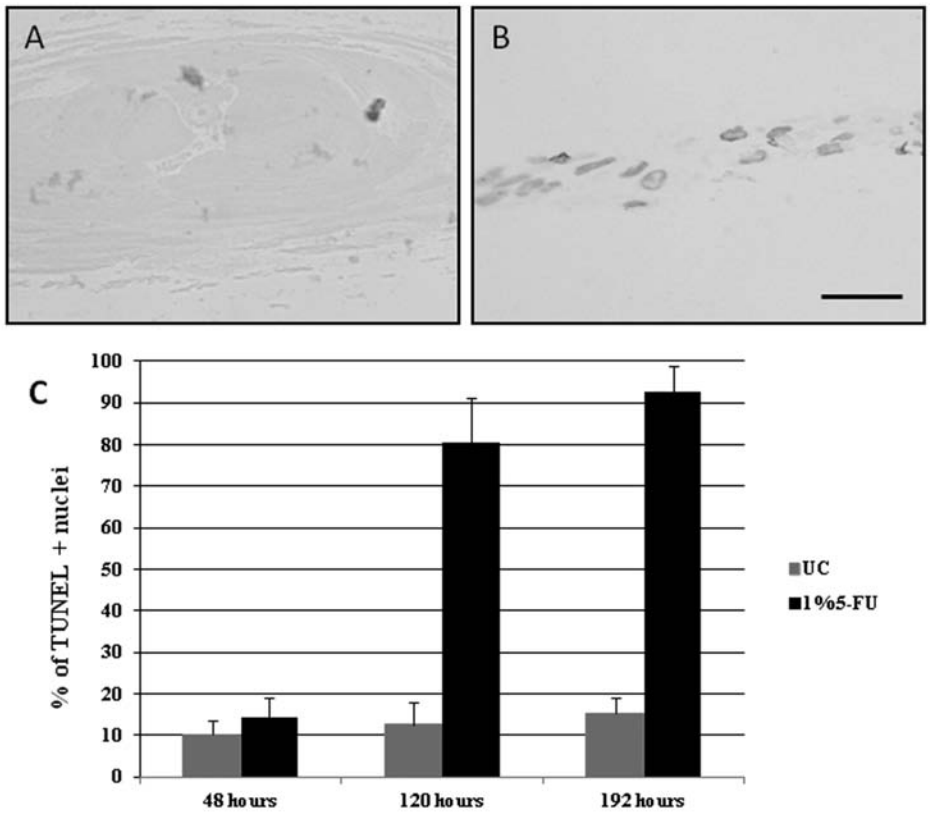

Fig. (5). Effects of 1% 5-FU loaded tablets on apoptosis in 3D oral outgrowths: TUNEL staining. 3D outgrowths were grown until complete 3D differentiation (10-14 days), and then treated with 1% (w/w) 5-FU tablets for 48, 120 and 192 h, and prepared for TUNEL assay. A and B are typical light microscope fields (400x magnification) obtained from outgrowths treated with drug loaded (B) or unloaded (untreated control, A) tablets for 120 h . Bar = 40 μm. C is the graph representing the means ± SD of quantifications obtained from 6 different experiments in duplicate after 48, 120 and 192 h in untreated controls (UC) and treated outgrowths (1% 5-FU).

this objective, the chemotherapeutic agent 5-FU, loaded into matrix tablets, locally applied on mucosal neoplastic lesions could increase the drug levels in the tumor area, and allow treating OSCC by delivering small drug amounts, just sufficient to give the desired effect on the neoplastic lesion without the severe systemic adverse effects. Cytotoxic chemotherapy is routinely used in the treatment of cancer, and has been an important factor in increasing 5-year survival rates for some types of cancer.

In this study, the effect of locally applied 5-FU on cell death was evaluated both on classic cell line models (HEK001 and SCC-4) and on a three-dimensional OSCC outgrowth model.

Initially, the optimal drug dose was established by delivery of solutions containing different amounts of 5-FU. The solution containing 1% of 5-FU resulted effective in inducing cell death with complete eradication of cell colonies. In a previous paper [31] we observed that solution containing 1% of 5-FU induced high degrees of apoptosis and foci of necrosis also on both a multilayer culture of the human TR146 keratinocyte cell line and healthy porcine buccal mucosa.

The drug solution was then tested on 3D OSCC outgrowths where it determined a reduction of outgrowth proliferation as shown by the marked shrinking of the margins of the outgrowth during the treatment.

The observed reduction in cell viability prompted us to investigate the effects of local application of solid dosage forms on the 3D OSCC outgrowth model.

Drug loaded matrices containing 1% of 5-FU were designed using the acrylic/methacrylic acid copolymer (described in USP/NF as ammonio methacrylate copolymer "type B") as this is a biocompatible, non-swelling and insoluble copolymer, sparingly permeable to water, unaffected by physiological pH values, that has been widely used in the preparation of various drug delivery systems. Buccal tablets were then prepared by direct compression of drug loaded matrices. A precondition for locoregional delivery of drugs is, in addition to the biocompatibility of the putative formulation, an appropriate drug release at the target site. To verify the ability of tablets to discharge drug amounts adequate to produce effective concentrations on tumoral tissue, the cumulative release of 5-FU into the saliva was determined in a time-dependent manner. To act as a reservoir, which provides a drug depot adjacent to the cancer lesion, a rapid release is not desired. Our results showed a slow and regular drug release that follows an Higuchian trend, thus suggesting that diffusion through the inert polymer matrix is the prevailing mechanism of dissolution.

The release of 5-FU from tablets and the drug transfer in the bottom well of the Transwell system across outgrowths, was evaluated at 48, 120 and 192 h time points. From the data reported in the Table it is possible to observe that after 48 h, about 83% of the 5-FU was discharged from tablets, whereas about 91 % was released after 120 h. Of course, after 192 h, the drug dose released was near the loaded (98%).

After showing that the drug was released from the tablets into culture medium, its activity was determined in 3D OSCC outgrowths by analysing cell death induction both morphologically by transmission electron microscopy and enzymatically by TUNEL assay. Our results show that after 120 h of treatment, when, as already stated, around 90% of the drug had been released from the

tablets through the Matrigel and into the cell layers, 5-FU caused loss of cell-cell communications and apoptotic cell death. This initially caused a dramatic reduction in the number of epithelial cell layers and eventually, after 192 h, when almost all the drug had been released from the tablet, determined a complete disaggregation of the 3D OSCC outgrowths and the death of all the cells contained within by apoptosis.

Since, beyond the drug used, other formulation components could cause some consequence on cell viability, analogous analyses were performed after application of drug unloaded matrix tablets. No significant effects were observed and no signs of cell death was found. The acrylic/methacrylic acid copolymer should not constitute a key obstacle to local drug delivery on tumor lesions.

5. CONCLUSIONS

Here we report for the first time the use of a newly developed 3D OSCC outgrowth as an *ex vivo* model to test the effects of drugs administration on neoplastic human oral mucosa.

Matrix tablet formulations containing 1% of 5-FU appear to be suitable for the release of adequate drug amounts and could be considered as a promising new approach to locoregional treatment of OSCC. The results herein reported represent a valuable basis for detailed animal studies addressed to the *in vivo* application.

We believe that by locoregional drug delivery the risks of toxicity associated with the anticancer chemoterapy must be very low as a consequence of a summary of different factors, such as the avoidance of systemic delivery, the very low drug doses administered, the high drug concentration next to the target tissue and the chemotherapic agent chosen. Moreover, locoregional OSCC therapy could improve patient compliance.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

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