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Mucoadhesive emulgel systems containing curcumin for oral squamous cell carcinoma treatment: from pre-formulation to cytotoxicity in tissue-engineering oral mucosa

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Abbreviations: ANVISA, Brazilian National Health Surveillance Agency; CUR, curcumin; C974P, Carbopol 974P; DMEM, Dulbecco's modified eagle's medium; HNC,

head and neck cancer; Ii, instability index; IM, isopropyl myristate; LVR, linear viscoelastic region; OSCC, oral squamous cell carcinoma; PAS, photoacoustic spectroscopy; PBS, phosphate-buffered saline; PC, polycarbophil; PEO, polyethylene oxide; PPO, polypropylene oxide; P407, poloxamer 407; PTFE, poly*tetrafluoroethylene*; SEM, scanning electron microscopy; SO, sesame oil

ABSTRACT

Current oral squamous cell carcinoma chemotherapies demonstrate off-target toxicity, which could be reduced by local delivery. Curcumin acts via many cellular targets to give anti-cancer properties; however the bioavailability is hindered by its physicochemical characteristics. The incorporation of curcumin into emulgel systems could be a promising approach for its solubilization and delivery. The aim of this work was to develop emulgel systems containing curcumin for the treatment of oral cancer. The emulgels containing curcumin were prepared with poloxamer 407, acrylic acid derivatives, oil phase (sesame oil or isopropyl myristate). The more stable system was evaluated for mechanical and rheological properties, as well as, the *in vitro* drug release profile, permeation and cytotoxic potential to oral mucosa models. The flow-throw system evidenced that the formulations could keep 5 min over porcine oral mucosa. Emulgel showed pseudoplastic behavior and a gelation temperature of 33 °C, which ensure their higher consistency. In addition, 70% of the incorporated curcumin was released within 24 hours in an *in vitro* drug release study and could permeate porcine oral mucosa. Monolayers cultures and tissue-engineered models showed the selectivity of the drug and systems for tumor cells. The physicochemical properties, subsequent release and permeation of curcumin to selectivity kill cancer cells could be improved by the incorporation into emulgel systems.

Keywords: emulgel, curcumin, tissue-engineering, oral squamous cell carcinoma, *ex vivo* permeation, mucoadhesion.

1. Introduction

Head and neck cancer (HNC), including cancers of the larynx, salivary glands, throat and lips, is the sixth most common malignancy worldwide (Colley et al., 2011; Hsu et al., 2014; Narihira et al., 2018). Approximately 90% of HNC are classified as squamous cell carcinoma. Oral squamous cell carcinoma (OSCC), originating in the mouth and lips (Chen et al., 2019; Narihira et al., 2018) is the eighth most common type of malignancy in developed countries, and the third most common form of cancer in developing countries (Shrivastava and Shakya, 2019). Although the oral cavity is easily accessible, late detection is common and contributes to a poor 54% 5 year survival rate and consequently over half million deaths worldwide each year (Brener et al., 2007; Goertzen et al., 2018; Nakagaki et al., 2018). OSCC is linked to a diverse range of risk factors including smoking, excessive alcohol consumption, human papillomavirus infection, the aging population, immunosuppression and UV radiation (Brener et al., 2007; Hsu et al., 2014; Narihira et al., 2018) which leads to the transformation of healthy oral keratinocytes (predominately in the floor of the mouth and on the lateral tongue), to pre-malignant dysplastic lesions and, in 10-20% of lesions, further progression to OSCC (Brener et al., 2007; Colley et al., 2011; Ettinger et al., 2019; Graciano et al., 2015; Hsu et al., 2010; Longo et al., 2011). Current treatment modalities consist of radiation, chemotherapy and surgery yet despite recent advances in chemo- and radiotherapy, the prognosis is still poor. Considering that treatment primarily happens in the outpatient setting, patients have shown many diverse effects and complications, including swallowing difficulties, xerostomia, malnutrition, oral mucositis, facial disfigurement that can be severe enough to lead to hospitalization (Hazelden et al., 2019; Narihira et al., 2018). Therapeutic strategies and effective

diagnosis are urgently needed to improve the prognosis of this deadly disease (Narihira et al., 2018).

Curcumin (CUR), a yellow polyphenol derived from turmeric roots (*Curcuma longa Linn*), is considered as a pleiotropic molecule. Among its diverse biological properties, anti-cancer activities have been reported in a wide range of cancer cells including liver, pancreatic, cervical, stomach, colon, breast and OSCC (Anand et al., 2008; de Souza Ferreira and Bruschi, 2019; Khan et al., 2018). Scientific studies have confirmed that CUR cellular effects are via multiple molecular paths including reduction of nuclear factor-κB (NF-κB) activated by carcinogens, inflammatory cytokines (interleukine-1 and tumoral necrosis factor) and extracellular stresses (UV light and smoke) (de Campos et al., 2017; Mazzarino et al., 2015; Sharma et al., 2006; Zlotogorski et al., 2013), increase in the expression and function of cytochrome P450 (CYP) 1A1 and/or CYP1B1 (Zlotogorski et al., 2013), increase in the expression of linking proteins of growth factor like insulin (IGFBP-5), decrease on the expression of PD-L1 and pSTAT3 by immunosuppressive pathway (Liao et al., 2018), down-regulates HIF-α (heterodimeric transcription factor) remarkably up-regulated in areca quid chewing associated-OSCC (Kunnumakkara et al., 2017; Lee et al., 2010), upregulating micro RNA-9 expression and inhibiting Wnt/β-catenin signaling pathway (Xiao et al., 2014). These molecular targets are involved in carcinogenesis and CUR acts via decreasing cell proliferation, migration and increasing cellular death and apoptosis rate (de Campos et al., 2017; Zlotogorski et al., 2013). Despite strong evidence for the pleiotropic activity of CUR in OSCC, the molecular displays challengeable physicochemical properties such as high lipophilicity (log P = 3.29) and photosensitivity, that limits the bioavailability, therapeutic effects and clinical applicability (Anande et al., 2008; Araiza-Calahorra et al., 2018; Santezi et al., 2018). There is a huge necessity to develop

drug delivery systems to improve the solubility and photosensitivity issues of CUR to enable clinical utilization (Akolade et al., 2018; Karavasili et al., 2019; Katas et al., 2017; Khan et al., 2018; Rodero et al., 2018).

Topical delivery of drugs directly to the oral mucosa is considered important in many epithelial diseases, including OSCC, to avoid unwanted side effects, systemic toxicity, as well as, first-pass metabolism (Bruschi and de Freitas, 2005; Hearnden et al., 2009). Moreover, the oral mucosa is an attractive site for the administration of drugs as it is less susceptible to damage or irritation with a fast recovery time (5 to 6 days) (Russo et al., 2016; Satheesh Madhav et al., 2012).

The ability to solubilize hydrophilic and hydrophobic molecules such as CUR, and also thicken and emulsionate the compounds of formulations are advantageous properties of poloxamer 407 (P407). These properties are due to its block structure containing hydrophilic regions of polyethylene oxide (PEO) and hydrophobic regions of polypropylene oxide (PPO), that assemble into polymeric micelles with temperature increase. These polymers help in the structuration of emulgels, which are formed by the combination of emulsions and gels. Emulgels enable the higher availability and solubilization of hydrophobic drugs, improving the stability and control of the delivery (Djekic et al., 2015; Ibrahim et al., 2013; Lopez-Martínez et al., 2015; Rocha et al., 2013; Shen et al., 2015). These systems are basically composed of aqueous phase, oil phase, emulsifying agent and gelling (Ajazuddin et al., 2013; Pant et al., 2015; Sultana et al., 2014). Sesame oil and isopropyl myristate were selected as the oil phase for the systems as they have been reported successfully for other emulgel formulations (Acharya et al., 2001; Singh et al., 2015; Subramanian et al., 2005). In addition, bioadhesive polymers, such as Carbopol 974P® (C974P) and polycarbophil (PC), were incorporated into the systems, as they increase contact, improve retention and clinical

efficacy; all important in the development of formulations for administration to the oral cavity (De Souza Ferreira et al., 2017; Sabrina Barbosa De Souza Ferreira et al., 2017).

The aim to this study was to develop emulgel systems containing P407, C974P or PC, sesame oil or isopropyl myristate and CUR as a treatment for OSCC. We investigated the oil phase and bioadhesive polymers for improved physical. The best combination of P407, oil phase, bioadhesive polymer and CUR was evaluated for mechanical, mucoadhesive, rheological properties. Moreover, the *in vitro* drug release profile, permeation and cytotoxic potential to both OSCC monolayers and three-dimensional tissue engineering oral mucosa models of the emulgel systems were evaluated.

2. Materials and methods

2.1. Materials

Curcumin C3 complex® was kindly donated from Sabinsa® (West Windsor, USA). Poloxamer 407, mucin from porcine stomach type II, and phosphate buffered saline (PBS) were purchased from Sigma Aldrich (St. Louis, MO, USA). Carbopol 974P® and polycarbophil were received from Lubrizol (São Paulo, SP, Brazil). Isopropyl myristate and polysorbate 80 (Tween 80) were purchased from Synth® (Diadema, SP, Brazil), while sesame oil was received from Veris óleos vegetais® (Vinhedo, SP, Brazil). Triethanolamine, neutralizing agent, was purchased from Galena (Campinas, SP, Brazil).

2.2 Preparation of mucoadhesive formulations

Formulations were prepared containing 15% (w/w) P407, 0.1% (w/w) CUR and different mucoadhesive polymers and oil phases (Table 1). The amount of CUR was

dispersed in oil phase (isopropyl myristate or sesame oil) until complete homogeneity was achieved. Then, this dispersion was incorporated in P407 and purified water-polymeric dispersion, previously prepared. Subsequently, a C974P or PCB-dispersion was added to the mixture containing P407, oil phase, CUR and purified water, and agitated until the complete homogenization of the system. The pH of each formulation was adjusted to 7.0 with triethanolamine and the formulations were stored in a fridge for, at least, 24 hours before further analysis.

2.3. Preliminary stability studies

The studies were carried out in cycles of freeze thaw, where the nanostructured systems were submitted to -5± 2 °C for 24 hours and 40 ± 2°C for 24 hours, during 12 days and representing six cycles as described in Cosmetics Stability Guide from ANVISA (Brazil) (Agencia Nacional de Vigilância Sanitária, 2004). The systems were evaluated regarding the organoleptic characteristics (color, smell and aspect), morphological and size properties of oily droplets (mean diameter and polydispersity index), centrifugation sedimentation analysis (instability index) and drug content during time 0 and cycle 6 (Lima et al., 2008; Morais, 2006). Regarding the centrifugation sedimentation index, the samples were placed in a bucket in optical path of 2 mm and centrifuged at 4000 RPM for 1 hour in an analytical centrifuge (LUMiSizer, LUM GmbH, Berlin, Germany). The samples were exposed to the centrifuge force; an infra-red light illuminated the bucket allowing the measurement of the transmitted light according to time and position in all bucket length simultaneously. The transmittance profiles and instability index (Ii) were obtained (Hou et al., 2010; Xu et al., 2017). For the morphological analysis, images were taken from an optical microscope (Kozo Optics) at 400x, where Feret's diameter (oil droplet size by the measurement of two

tangents in opposite sides) was calculated from the measurement of approximately 800 droplets of oil phase for each formulation using Image Pro Plus 4® (Media Cybernetics, Inc., Rockville, USA) and droplet size distribution was estimated (Nova et al., 2019; Marcela B. Oliveira et al., 2018; Toledo Hijo et al., 2015; Tonon et al., 2011). For drug content of CUR in the emulgel, 1 g-formulations was dispersed in 5 ml of methanol, transferred to a 25 ml-volumetric flask and sonicated for 15 min in a sonicator water-bath (Unique ultra-cleaner 1400, Indaiatuba, Brazil). Subsequently, the volumetric flask was completed to 25.0 ml with methanol and the mixture was centrifuged at 4000 RPM for 5 min (Z36HK Hermle Labortechnik GmbH, Wehingen, Germany). The samples were filtered with a PTFE membrane (Sartorius, Goettingen, Germany) with 0.45 µm pore size and 20µl-sample was injected into a HPLC Shimadzu LC CBM 20[a] system (Tokyo, Japan) equipped with a manual injector (7725i) and a UV-Vis detector (SPD 20 A). The stationary phase was a C18 reversed phase column (5 µm X 4.6 mm X 250 mm) (Luna PFP, Phenomenex®, Torrance, USA) and a solution containing acetonitrile and acetic acid (1.5 %, v/v) is the mobile phase in a gradient elution. The peak area was detected at 425 nm and the flow was adjusted to 1.0 ml/min.

2.4. Mechanical analysis

A TA-XTplus Texture Analyzer (Stable Micro Systems, Surrey, England) was used for performing the textural profile analysis (TPA) and the measurement of the work required to expel the formulations from a syringe (syringeability).

TPA analysis was performed at 5 °C, 25 °C and 37 °C. 16 g-sample was carefully placed to bottles, avoiding the entrapment of air. In TPA mode, the samples were compressed two times by an analytical probe (10 mm diameter) at 2 mm/s with 15 s of delay period between the end of the first and the beginning of the second compression at

a defined depth (15 mm). The properties (hardness, compressibility, adhesiveness, elasticity and cohesiveness) were calculated from force-time and force-distance plots (de Francisco et al., 2019; Sabrina Barbosa De Souza Ferreira et al., 2017). At least, three replicates were performed for each analysis.

The work necessary to expel the formulations from a syringe (syringeability or resistance to compression of systems inside the syringe) was investigated using a texturometer TA-XTplus in compression mode (Jones et al., 1996). In 1ml-plastic syringes, the systems were carefully packed until 30 mm avoiding the entrapment of air. The determination was carried out at 25 °C, where each syringe was fixed vertically and pressed at 2.0 $mm.s^{-1}$ until reach the initial contact with the syringe plunger with at least three replicates (Borghi-Pangoni et al., 2015; Bruschi et al., 2007). A graph of force per distance was derived during the compression of the plunger.

2.5. In vitro evaluation of mucoadhesive force

In tension mode, the mucoadhesive properties of the systems with or without CUR were investigated using the same texture analyzer described previously. Initially, the compression of crude mucin provided mucin discs, which were hydrated in mucin solution 5% (w/w) for 30 s, placed in the bottom of the TPA probe and the excess of mucin solution was slightly removed with absorbent paper. Afterwards, a shallow container containing the sample was positioned behind the analytical probe, which was lowered until the mucin disc and sample were in contact and 0.1 N force was applied for 30 s to ensure intimate contact between the two surfaces. Then, 1.0 mm/s – speed was used to raise the probe and the force necessary to separate the mucin disc and the systems was determined, from the graph of force per time (Marcos Luciano Bruschi et al., 2007).

2.6. Ex vivo flow liquid falling mucoadhesive evaluation

Porcine oral mucosa was taken from the cheek of young, white and freshly slaughtered pigs (from a slaughterhouse authorized from human consumption by Brazilian Agriculture Ministry). The cheek was gently washed with PBS buffer and the oral mucosa was excised with scissors and a surgical scalpel. The samples were stored at -18 °C in a PBS solutions and defrosted at room temperature on the experimental day after 48 hours of the oral mucosal preparation (Ferreira et al., 2019). These samples were used for the determination of mucoadhesive properties, as described by Oliveira and collaborators (Oliveira et al., 2018). In a chamber with temperature control (37 °C), the samples of oral mucosa were placed on the channel testing behind the syringe-pump system, where PBS flowed over the mucosal samples at a flow rate of 4 ml/min. Formulation (100 µl) was positioned and warmed over the mucosa to allow the proper gelation for 5 min. Subsequently, the buffer flowed over the set during 20 min and samples (elution liquid) were collected in 1, 2, 5, 10, 15, 20, with 1% (V/V) Tween 80 to let the complete solubilization of the drug. Samples were diluted with methanol (1:2) and the drug was quantified in the liquid chromatography method, as previously described. The retention data was determined by the deduction between the total of formulation and the amount of formulation flowed from the surface of the substrate. At least three replicates were carried out and fresh mucosa was used for each experimental assay (da Silva et al., 2017; Madsen et al., 2013;Oliveira et al., 2018).

2.7. Morphological analysis by scanning electron microscopy

An electron scanning microscope Quanta FEI (Thermo®, Oregon, USA) was used to investigate the morphological characteristics of the systems with and without CUR.

Initially, 2g-system was freeze-dried, the dried material was deposited over a double-sided tape, coated with colloidal gold under argon atmosphere. Then, micrographs were derived from the scanning electron microscopy (SEM).

2.8. *Rheometry*

Rheological analysis of systems was investigated at 5 °C, 25 °C and 37 °C ± 0.1 °C using a controlled stress rheometer (MARS II, Haake Thermo Fisher Scientific Inc., Newington, Germany) with parallel steel cone-plate geometry (35 mm diameter, separated by a fixed distance of 0.052 mm) for at least three replicates samples. Replicates were carefully positioned and allowed to equilibrate (1 min) before analysis to ensure minimal shearing of the sample. The continuous shear flow rheology was performed in flow mode, where downward and upward curves were obtained (shear rates from 0 to 2000 s^{-1}), increasing over 150s-period, retained at the high limit during 10 s, and, subsequently decreasing over 150s-time. The software RheoWin 4.10.0000 (Haake®) allowed the calculation of the hysteresis area. Moreover, Power Law (Oswald-de-Waele equation) rheological model was used to determine the flow properties (Equation 1) (Bruschi et al., 2007; De Souza Ferreira et al., 2017; Fabri et al., 2011):

$$\tau = K\dot{\gamma}^{n} \quad (1)$$

Where τ is shear stress (Pa), k is consistency index [$(Pa.s)^n$], $\dot{\gamma}$ is shear rate (s^{-1}), and n is the flow behavior index (dimensionless).

The yield value was evaluated by the rheological models Casson (Equation 2) and Herschel-Buckley (Equation 3) (Lee et al., 2009):

$$\tau = \sqrt[n]{(\tau_o^n + (\dot{\gamma} n_p))^n} \quad (2)$$

Where τ is shear stress (Pa), n is flow behavior index (dimensionless), τ_o is yield value (Pa), $\dot{\gamma}$ is shear rate (s^{-1}), and η_p is Casson plastic viscosity.

$$\tau=\tau_o+K\dot{\gamma}^n \quad (3)$$

Where τ is shear stress (Pa), τ_o is yield value (Pa), k is consistency index, $\dot{\gamma}$ is shear rate (s^{-1}) and n is flow behavior index (dimensionless).

The oscillatory rheology was performed in oscillatory mode. Initially, the linear viscoelastic region (LVR) was investigated for each system and the frequency sweep assay was carried out from 0.1 to 10.0 Hz at 5 °C, 25 °C and 37 °C. The viscoelastic characteristics of the emulgels, storage modulus (G'), loss modulus (G"), dynamic viscosity (η') and the loss modulus (tan δ) were calculated (de Francisco et al., 2019; De Souza Ferreira et al., 2017).

The gelation temperature ($T_{sol/gel}$) determination was performed in oscillatory mode with temperature ramp. Firstly, the LVR of each formulation was carried out at 5 °C and 60 °C. Subsequently, the temperature sweep analysis was performed over a range of 5-60 °C at 1.0 Hz-frequency and 10 °C /min-rate heating with controlled stress. RheoWin 4.10.0000 (Haake®) allowed the calculation of the viscoelastic properties (G', G", η' and tan δ) in each case. $T_{sol/gel}$ (temperature at which G' was halfway between solution and gel) was calculated for all systems where η' increased significantly by the rise of temperature (Gratieri et al., 2010; Lee et al., 2009; Tuğcu-Demiröz et al., 2013).

2.9. In vitro drug release profile investigation

The release of CUR from the emulgel was performed using double-wall glass beakers with temperature control (37 ± 0.5 °C). The emulgel (1.0 g) was positioned on the bottom of the recipient at 37 °C to allow complete gelation. Then, the release media containing aqueous solution of Tween 80 (1%, V/V) was carefully transferred to the vessel and kept under constant agitation to achieve sink condition (Borghi-Pangoni et al., 2015; Borghi-Pangoni et al., 2017). Aliquots of samples (500 µl) were collected in fixed time intervals (0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h and 24 h), replaced by the same volume of fresh medium. The aliquots were diluted in methanol (1:2), filtered with PTFE membranes and quantified by HPLC method, previously described (Oliveira et al., 2018). The plotting of the amount released versus time allowed the calculation of release profiles, which were fitted by Korsmeyer-Peppas equation (describes the drug release from polymeric matrix systems) (Zhang et al., 2010):

$$F = k.t^n \qquad (4)$$

Where F means the fraction the drug released, t represent time released, k is the incorporation kinetic constant of geometric and structural properties of the apparatus and n is the release exponent that tells mechanism of the drug release.

2.10. Ex vivo permeation assays

The permeation of CUR from emulgel systems was evaluated by Franz cell apparatus and photoacoustic spectroscopy (PAS) in porcine oral mucosa (taken from porcine cheek as previously described). Using Franz cell apparatus, the receptor media (1%, V/V Tween 80 in PBS) was placed in the receptor compartment of Franz cell,

maintained at 37 °C with constant stirring. The porcine oral mucosa was positioned between the receptor compartment and donor compartment, where 1 ml of formulation was homogeneously distributed. Medium (500 µl) was collected at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h and 24 h, diluted in methanol (1:2), filtered in PTFE and quantified in HPLC method and replaced by fresh medium. At least three replicates were carried out (Borghi-Pangoni et al., 2017; Junqueira et al., 2016). In order to quantify the retention of CUR in tissue, the porcine mucosa were taken from the recipient, cut in small pieces, placed in volumetric flask (5 ml) with methanol, sonicated for 15 min, completed, filtered in PTFE membranes and quantified in HPLC method, previously described, in at least three replicates.

For PAS, a set containing 30 µg of system over the surface of 1 cm³ of oral mucosa were allowed in contact during 30 min and, then the sample was investigated by photoacoustic spectroscopy. The homemade experimental equipment containing 1000 W Xenon arc lamp (Oriel, model 68820), as source light with 800W- nominal power was used for this essay. When passing through 3.16 mm input and output slits of a monochromator (Oriel, model 77250), the light was diffracted, and, subsequently, modulated at 13 Hz with a mechanical chopper (Stanford Research Systems, model SR 540) to focuses the sample. The photoacoustic signal obtained by pressure changes resulting in period heating of the sample, placed inside the photoacoustic cell and sealed with transparent quartz window (thickness of 2 mm and diameter of 8 mm), was collected by a capacitive microphone (Brüel and Klaer, model 2669). The influence of the depth of tissue which contributed to the photoacoustic signal used the thermal diffusion length (µs):

$$\mu_s = \sqrt{\frac{D}{\mu f}} \qquad (5)$$

Where D means the sample thermal diffusivity ($cm^{-2}s^{-1}$) and *f* represents the light modulation frequency (Hz). Considering the similarity between porcine and human oral mucosa, D= 4.1 x 10^{-4} cm^2 s^{-1} (Brown et al., 1993) and *f*=13 Hz. Consequently, μ_s was 31 µm for oral mucosa. Considering that the photoacoustic signal is proportional to the sample coefficient absorption, the photoacoustic signal was interpreted by band absorption spectra (Rosencwaig, 1980). All samples were normalized to the carbon black- sample to eliminate the source emission intensity in each wavelength (Ames et al., 2017). The spectra of at least three porcine oral mucosa was investigated from the illuminated side of tissue to be measured and, subsequently, the tissue was upside down to illuminate the opposite side (Borghi-Pangoni et al., 2016; Campanholi et al., 2018).

2.11. Cell cultures

The cell lines, Cal27 (ATCC, Manassas, VA, USA, CRL-2095) isolated from a tongue squamous carcinoma (ECACC, Health Protection Agency Culture Collections, Salisbury, UK) and FaDu (LGC Promochem, Middlesex, UK), originally isolated from a hypopharyngeal tumor were used in this study. Cal27 were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose, while FaDu cells were cultivated in RPMI-1640. Both medium were supplemented with 10% (v/v) fetal calf serum (FCS), 100 UI/ml penicillin and 100 µg/ml streptomycin and 2 mM L-glutamine. Moreover, immortalized cell line FNB6 (a kind gift from Professor Keith Hunter) were originally isolated from normal oral keratinocytes. FNB6 were cultures in Green's medium containing DMEM high glucose and Ham's F12 medium 3:1 (v/v) enriched with 10% (V/V) FCS, 2 mM L-glutamine, 0.625 µg/ml amphotericin B, 100 UI/ml penicillin and 100 µg/ml streptomycin, 0.4 µg/ml hydrocortisone, 10 ng/ml epidermal growing factor,

0.18 mM adenine, 5 µg/ml transferrin and 0.2 µM triiodotironine. All cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere. When 80% confluence was reached, the cells were sub-cultivated using trypsin-EDTA.

2.12. *Cytotoxicity evaluation of CUR and formulations against buccal cell lines*

The viability of FNB6, Cal27, FaDu cell lines was investigated after 24 hours of treatment with CUR and the formulations with or without CUR. Initially, 2 x 10^5 cells were seed in each well of a 96-well plate. After overnight culture, increasing dilutions of CUR (previously solubilized in DMSO) or formulations with and without CUR in medium were added with final concentration of 2.5, 5, 10, 20, 40, 80, 120 and 240 µM and 10, 20, 300, 500, 1000, 1500 and 2000 µM, respectively, for 24 hours along with a media only negative control. Subsequently, the media with CUR was aspirated, cells were washed with PBS (three times) and 200 µl media added to each well. After a further 24 hours of incubation, 0.5 mg/ml MTT (Sigma, Poole Dorset, UK) solution was added to the cells, which were incubated for 1 hour at 37 °C. Finally, the solution was removed, and acidified isopropanol added to solubilize blue formazan crystals. The absorbance was measured in a plate reader at 570 nm, with 620 nm reference correction, at least three times for each cell line.

2.13. *Tissue-engineered oral mucosal equivalents*

Oral mucosal models were constructed as previously described (Colley et al., 2011; Jennings et al., 2016). Initially, freeze-dried rat-tail collagen previously dissolved in 0.1 M acetic acid to give a final concentration of 5 mg/ml, was mixed with L-glutamine, FBS, 10 x DMEM and reconstitution buffer (2.2% sodium bicarbonate, 4.8% HEPES, 0.248 % NaOH in purified water). The pH was adjusted to 7.4 with 2M NaOH.

Subsequently, NOF were added to the collagen gel at a concentration of 2.5 x 10^5 cells/ml. After gentle homogenization, 1 mL was transferred to 12-well hanging inserts (0.4 mm pore; Millipore) and allowed to set in a humidified atmosphere at 37 °C for 2 hours. The inserts were submerged in DMEM high glucose and incubated for 2 days, after which 5 x 10^5 normal oral keratinocytes (FNB6) or 2.5 x 10^5 Cal27 was seeded on top of each model. FNB6 models were cultured in Green's medium, while Cal27 models were cultured in DMEM high glucose supplemented as previously described. After a further 2 days, the models were raised to an air-to-liquid interface and cultured for 14 days.

2.14. Cytotoxicity using tissue-engineered oral mucosal

The cytotoxicity of the models was performed as recommended by the OECD standards (OECD 439) (OECD, 2015). Briefly, the CUR diluted in media (9 µM, 240 µM and 380 µM) and emulgel with and without CUR (pure and diluted in PBS, 1:4) were applied to the models, which were incubated for 24 h, washed three times with PBS and cultured for a further 42 hours in fresh medium. Subsequently, the models were washed with PBS and incubated in MTT solution (1 mg/ml) for 3 hours. After MTT solution was removed, acidified isopropanol (0.1 M HCl in 2-propanol) was added to each model with gentle agitation to remove the formazan crystals. The optical density was measured in a spectrophotometer (Tecan, Männdorf, Switzerland). Viability was expressed to the viability relative to the negative control (Colley et al., 2018). These analyses were performed in triplicate.

2.15. Histological analysis

In the histological analysis of tissue integrity post treatment, the models were removed from the medium, washed with PBS and fixed in 10% formalin overnight. Then, the models were subjected to routine histological processing and finally paraffin wax embedded. Using a Leica RM2235 microtome (Leica microsystems), sections (5 µM) were cut and stained with hematoxylin and eosin (Colley et al., 2018).

2.16. Statistical analysis

The CUR presence and temperature effect in consistency index, flow index, yield value, hysteresis area and textural parameters (hardness, compressibility, adhesiveness, elasticity and cohesiveness) were statistically compared using two-way variance analysis (ANOVA). In addition, one-way ANOVA was used to investigate the effect of CUR presence *in vitro* mucoadhesion and syringe-ability. Moreover, t-test student determined if the dynamic viscosity of formulations increased with temperature increase. Moreover, the effect of drug, formulation and time exposition was evaluated by two-way ANOVA. Tukey *post-hoc* test was used to compare individual groups of all cases of ANOVA.

3. Results and discussion

3.1. Preliminary stability studies

The preliminary stability studies, mainly by freeze-thaw cycles, displayed fundamental importance in the development of oil-water-based systems (Chhibber et al., 2019; Xu et al., 2016), including emulgels. These systems have shown great importance to solubilize hydrophobic drugs such as CUR in the hydrophobic core of P407 with the oil phase and the hydrophilic shell that would interact with mucoadhesive polymers including acrylic acid derivatives including C974P and PC. The oil phases chosen in

this study were sesame oil (SO) from *Sesamum indicum L.*, and isopropyl myristate (IM) (Acharya et al., 2001; Pant et al., 2015; Singh et al., 2015; Subramanian et al., 2005; Sultana et al., 2014). C974P and PC have different cross-linking rates, which impacts directly on the viscosity of solutions containing these polymers (De Souza Ferreira et al., 2017; De Souza Ferreira et al., 2017). In order to find the emulgels that would display the best interaction and absence of phase separation, the systems containing P407 and CUR had their physical stability tested in two different types of oil phase (OS and IM) and mucoadhesive polymers (C974P and PC) (Table 1).

During the development of new pharmaceutical buccal dosage forms, the choice of dose for local administration is very important (Hao and Heng, 2003). Considering that these systems aim the application in oral squamous-cell carcinoma, the drug content should be in the therapeutic levels not leading to toxic effects to normal cells and, at the same time, presenting effects in cancer cells. Previous studies in oral cells (Jelezova et al., 2015; Kim et al., 2012; Mazzarino et al., 2015) have demonstrated the CUR concentrations which show cytotoxic effects and lead to cellular apoptosis are around 40 and 100 µM. Cheng and collaborators (Cheng et al., 2001) performed phase I clinical studies in humans and found the absence of toxic effects, after the oral administration of up to 8000 mg CUR per day. Besides the therapeutic effects, it is important to use a CUR concentration which P407 can carry. Studies have demonstrated that P407 shows high carry capacity of CUR by partitioning inside the hydrophobic core by the self-assembly of triblock polymer with a temperature increase (Sahu et al., 2011). Considering the concentrations which present toxic effect versus oral squamous cells and the necessity to use an excess of CUR due to release and permeation processes, the concentration of CUR used 0.1% (w/w). This CUR content is suitable to administer the

desirable amount of formulation in each dose, considering the defined therapeutic regimen.

The formulations (Table 1) were exposed to thermal stress and gravity acceleration to investigate their stability and changes in the physico-chemical properties. Figure S1 (Supplementary data) shows the formulations before (after 24 hours at 40°C) and after the end of the freeze-thaw cycles (after 24 hours at -5 °C). I it was possible to verify that the formulations F2, F3 and F4 showed a brownish coloring with CUR precipitate after the end of the freeze-thaw cycles (Figure S1B, supplementary data). Formulation F1 displayed a yellowish color with the absence of CUR precipitates. This suggests that the interaction between P407, C974P and SO provided better incorporation of CUR in the hydrophobic P407-core which hindered the formation of CUR-precipitates. Moreover, C974P and SO display higher viscosity than PC and IM, which provides a stronger structuration.

Formulations had their stability evaluated in the analytical dispersion analyzer (LUMsizer®) at time 0 (Caddeo et al., 2013). The graphs of the position of the formulations inside the bucket (mm) versus the transmittance profile during the analyzed period showed (Figure S1A, supplementary data) slight changes in transmittance profiles for the formulations containing IM were observed. Alterations in a transmittance profile mean a physical destabilization of the formulation, whereas an overlapping profile means that these formulations are stable with gravity acceleration. Moreover, this equipment provides the instability index (Ii) for each formulation ranging from 0 to 1. The instability index equal to 1 means an unstable system, while Ii equal to 0 means a stable system. The results reveal that the emulgels containing SO (F1 and F3) displayed the lowest instability index values, while, systems containing IM (F2 and F4) demonstrated the highest instability index (Table 1). These results are due to the

structural characteristics of oil phases, SO is composed of a mixture of fatty acids including mainly oleic acid and linoleic acid, with one or two insaturations of carbonic chains composed of eighteen carbons. On the other hand, IM is a synthetic compound composed of propano-2-ol and fatty acids including myristic acid with fourteen carbon chains, providing lower hydrophobicity, density and viscosity (Rowe et al., 2009). Moreover, F1 and F3 differ by the presence of a mucoadhesive polymer, as F1 also contains C974P. This carbomer creates higher cross-linking, which renders a higher viscosity and better structuration to the preparations, as previously reported (De Souza Ferreira et al., 2017). A lower instability index was found for systems containing C974P and SO, which confirms the result reported for the freeze-thaw cycle experiments.

[Insert Table 1]

In order to evaluate possible alterations in the morphology and mean diameter of oily droplets of the preparations optical microscopy was employed during the initial phase and after the 6th cycle. All preparations displayed similar morphologies with rounded and dispersed droplets, as shown in Figure S1C (supplementary data).

The mean diameter of oily droplets and polydispersity index of oily droplets of the preparations are displayed in Table 1. Systems composed of SO demonstrated the highest polydispersity index at time 0 due to its composition of a mixture of fatty acids (Rowe et al., 2009). This characteristic is not desirable since it represents low uniformity in droplet size (Nova et al., 2019; Oliveira et al., 2018). At the end of these studies, the values decreased, which represents higher homogeneity of the droplet size.

The type of bioadhesive polymer incorporated influenced the mean diameter of oily droplets of the preparations. Emulgels containing Carbopol 974P® exhibited slightly

lower mean diameter of oily droplets in comparison to PC. The higher cross-linking in C974P, which has been reported to provide higher viscosity (De Souza Ferreira et al., 2017), probably hinder the coalescence of oil phase droplets and probably a better incorporation of CUR. Comparing the mean diameter of oily droplets at the beginning and end of the preliminary stability studies, F1 displayed the lower mean diameter of oily droplets that is a favorable characteristic, as well as, better stabilization. The determination of mean diameter of oily droplets and polydispersity index was only performed for F1, due to the absence of CUR-precipitates.

The CUR content in the emulgels was determined at the beginning and end of the preliminary stability studies (Table 1). All preparations displayed CUR content close to the theoretical value (0.1%), at time 0 and without significant statistical differences ($p > 0.05$). After thermal stress, F1 displayed similar drug content ($p > 0.05$).

The emulgel F1 showed favorable characteristics, including the absence of CUR-precipitates and lower instability indexes after the centrifugation sedimentation analysis. Furthermore, a decrease in the mean diameter of oily droplets and a good polydispersity index made F1 the best preparation for further characterization.

3.2. Mechanical characterization

TPA constitutes an important investigation, since the systems can display resistance to saliva (Baloglu et al., 2011; Carvalho et al., 2013) and the results are displayed in Fig. 1.

[Insert Figure 1]

Hardness and compressibility represent the force necessary to remove the formulation from the packaging material, its spreadability over the mucosa (uniform layer), avoiding the discomfort to the patient and the applications of the emulgel in the buccal cavity, respectively. Adhesiveness is a desirable parameter for such systems aimed at the wet environment of the oral mucosa expresses the work required to overcome the polycarbonate probe surface (Baloglu et al., 2011; Tuğcu-Demiröz et al., 2013). The presence of CUR in the formulations decreased the compressibility significantly ($p < 0.05$), which represents a promising finding to facilitate the administration of the emulgels over the oral mucosa. The presence of CUR did not show a significant influence on the other parameters (hardness, adhesiveness, elasticity and cohesiveness). Hardness, compressibility and adhesiveness were all significantly increased ($p < 0.05$) by the increase in temperature, due to the thermoresponsiveness of P407. At low temperatures, these system tend to be less viscous in a monomeric state and the increase in temperature promotes the dehydration of PPO- chains in the core and establishment of micelles where the oil phase is entrapped with CUR, increasing the viscosity (Alexandridis et al., 1994; De Souza Ferreira et al., 2015).

Elasticity and cohesiveness are properties related to the semi-solid ability to flow and return to its initial state and restructuration and molecular interactions after subsequent shear stresses, respectively (De Souza Ferreira et al., 2015; Pereira et al., 2013; Tuğcu-Demiröz et al., 2013). Both these parameters were not affected by either the incorporation of CUR or increase of temperature. Moreover, the adhesiveness, preliminary evidence of adhesion, did not show a significant increase, which is possibly due to the lower specificity interaction evaluated by the adhesion between formulation and polycarbonate probe (De Souza Ferreira et al., 2017).

In order to simulate the administration of the systems over the oral mucosa, the work required to expel the emulgel from a syringe was also evaluated. The syringeability of CUR-emulgel (47.5910 ± 1.0517 N.mm) was slightly lower than emulgel without CUR (49.9990 ± 2.3954 N.mm); however the effect of the incorporation of CUR was not significant ($p > 0.05$).

3.3. Mucoadhesive properties

A mucin disc partially hydrated was employed to determine the mucoadhesive properties by detachment force between the two surfaces (mucin disc and formulation) over time (Andrews et al., 2005; da Silva et al., 2017). The mucoadhesion strengths of the formulations with and without CUR were 0.1859 ±0.0086 N and 0.1644 ±0.0120 N, respectively. CUR presence significantly increased ($p = 0.000921$) the mucoadhesive force of the formulations.

The mucoadhesive properties of systems were also evaluated by the falling liquid method, which evaluates the ability of a system to maintain adhered to the surface of oral mucosa with falling liquid (PBS) dropped over the sample at 4 ml/min for 20 minutes (da Silva et al., 2017; Madsen et al., 2013; Oliveira et al., 2018). Despite the wide variability in the salivary flow rate in physiological conditions, this flow rate was chosen to investigate how the formulation would behave in harsh conditions simulating stimulated salivary flow (Humphrey and Williamson, 2001; Iorgulescu, 2009; Navazesh and Kumar, 2008). The detection of the amount of gel adhered is calculated indirectly by determining the amount of CUR eluted with buffer from the beaker by HPLC. This analysis was performed for just the CUR-emulgel as shown in Fig. 2. There was a significant decrease ($p < 0.05$) in the cumulative retention of the emulgel for all time intervals investigated (1, 2, 5, 10 and 20 min). Thus, the amount of gel over the oral

mucosa decreases significantly. After 10 and 20 min, the emulgel was completely removed from the oral mucosa and it was observed the presence of formulation only until 5 min.

[Insert Figure 2]

3.4. Morphological analysis by SEM

The morphological properties of the possible network formed by the emulgel with and without CUR were investigated by SEM (Fig. 3). Fragments and heterogeneous structures were observed in the micrographs of the emulgel, between a well-defined polymeric network without the presence of diverse channels commonly found in the micrographs of gels (Campanholi et al., 2018). Moreover, it was possible to observe some sparse holes were probably the oil phase would be located and rough surface in an oriented direction. Conversely, the formulations containing CUR (Fig. 3C and 3D) displayed a smoother surface with less holes and some possible droplets of oil phase sparsely distributed.

[Insert Fig. 3]

3.5. Rheological characterization

The continuous shear rheology of emulgels in the presence and absence of CUR were investigated at 5 °C, 25 °C and 37 °C (Figure S2, supplementary data). All formulations displayed non-Newtonian behavior. The increase in temperature increased the shear stress required to the formulations flow as a result of the increase in viscosity,

mainly at 5 and 25 °C, due to the thermoresponsive polymer, P407 (De Souza Ferreira et al., 2015; Dumortier et al., 2006). The emulgels with and in the absence of CUR showed similar rheological characteristics at 5 °C, because of the P407 conformation.

Both formulations were investigated until 1000 s^{-1} at 37 °C, but the preparations containing CUR showed higher thixotropy area. This is considered a favorable property for emulgels which has been reported to promote higher stability to the systems and bioavailability of the drug (Ajazuddin et al., 2013).

The consistency index (k) and flow behavior index (n) were calculated by Ostwald de Waele equation (power law), which are expressed in Table 2. The effects of temperature increase and CUR presence were statistically evaluated by k and n (Bruschi et al., 2007; De Souza Ferreira et al., 2017; Jones et al., 2009).

There was a significant increase ($p < 0.05$) of consistency index (K) and significant decrease ($p < 0.05$) flow behavior index (n) due to the increase of temperature to 37 °C and incorporation of CUR. The structural organization of P407 containing SO and CUR in the hydrophobic core and the hydrophilic shell interacting with C974P, enabled the formation of viscous systems (Dumortier et al., 2006). During the analyses, the oil droplets got probably aligned in the shear direction, as previously demonstrated in the scanning electron microscopy of other emulgel systems (Santos et al., 2019), with the CUR incorporation the droplets movement was probably hampered due to the CUR interactions with PPO-portions and SO-fatty acids, increasing the system viscosity. The n values were lower than 1, demonstrating a non-Newtonian characteristic, which is typical of semisolid systems (Hemphill et al., 1993).

[Insert Table 2]

It was found a significant increase ($p < 0.05$) of the hysteresis area due to the incorporation of CUR in the emulgels. Hence, there is an improvement of the thixotropy indicating the higher malleability during the preparation of these systems with the application of high shear rates including agitation and pumping, as well as, during the application of the emulgels in the buccal cavity (De Souza Ferreira et al., 2015).

The viscoelastic properties of the preparations (Figure S3, supplementary data) were dependent of the oscillatory frequency, temperature, and CUR presence. Formulations showed higher G' and G" values at 37°C and at the highest oscillatory frequencies, due to the structuration of P407. The preparations containing CUR displayed increase G' and G" when compared to preparations without CUR, mainly at 37°C.

The η' of preparations decreased due to the increase of the oscillatory frequency. On the other hand, the η' increased as the temperature increase and in the CUR presence. All preparations displayed viscoelastic behavior ($\tan \delta < 1$), which is typical and desirable for pharmaceutical semisolid preparations, with consequent, higher retention in the buccal cavity and higher drug delivery control (Andrews et al., 2005; Baloglu et al., 2011; De Souza Ferreira et al., 2015; Jones et al., 2009). In general, the CUR presence did not change the viscoelastic properties of preparation ($p > 0.05$), indicating the oscillatory characteristics are due to the secondary interactions between P407, C974P and SO.

The influence of CUR on $T_{sol/gel}$ of the system was also determined. The drug presence significantly decreased the gelation temperature ($p < 0.05$). CUR-emulgel (F1) displayed $T_{sol/gel}$ of 33.2750 ± 0.0614 °C, while the emulgel without CUR displayed 35.6230 ± 0.0462 °C. These values were found in the acceptable temperature range necessary to provide the structuration of the formulations, as previously described.

Therefore, these preparations have shown favorable characteristics for administration in the oral cavity, without the risk of seepage and loss at the application site (Bruschi et al., 2007).

3.6. In vitro drug release profile

In order to simulate the behavior of the formulation inside the buccal cavity, *in vitro* release profiles of CUR from emulgel were performed (Fig. 4). The release media was Tween 80 (1%, w/v) to enable the solubilization of CUR, due to its hydrophobic characteristic (Borghi-Pangoni et al., 2017; de Souza Ferreira and Bruschi, 2019).

[Insert Figure 4]

The *in vitro* drug release of CUR from the emulgels was slow; after 8 hours only 30% of drug was released. At the end of the test (24 hours), the formulations had released around 70% CUR, which was considered satisfactory for buccal application. In addition, it was possible to observe the presence of the formulation after 24 hours, which provides a prediction of the unstimulated salivary flow. Even though the *ex vivo* falling liquid test demonstrated that the formulation was washed away in 10 minutes (4 mL/min flow rate). Therefore, it is not possible to ensure if the formulation would keep in the application during 24 hours for CUR release. Future investigations using more biorelevant conditions are necessary. The formulations showed first order profile, due to its composition (Korsmeyer et al., 1983), the preparations had their mechanism release evaluated regarding the general equation (equation 4). The *in vitro* release profile of CUR was fitted by Korsmeyer-Peppas (Power Law) and the release exponent value (n) determined to be 0.5415. The n was close to 0.5, which is considered as Fickian

diffusion model, but also, intermediate between 0.5 and 1.0, well known as anomalous transport. Considering the rheological and texture characteristics of this preparation and the high viscosity, as well as the presence of oil phase, the CUR release mechanism was mainly related by diffusion and, the relaxation of the polymer chains. The high speed of solvent diffusion to inside the matrix and the low velocity of polymer chains relaxing induced the formation of a solvent penetration gradient. The concentration of solvent decreases exponentially from the totally swollen region to the center of the matrix (Bruschi, 2015).

3.7. Ex vivo permeation assays

Permeation assay using Franz cell enable the measurement of the CUR amount in the receptor medium, the drug retained in the mucosa and the permeation kinetic profile may be determined. The slow permeation of the drug through the mucosa without reaching the systemic circulation is desirable for the CUR emulgels which are aimed at local application (Junqueira et al., 2016). The drug did not reach the receptor compartment and 55.0885 ±12.1636 µg/cm³ (6.5467 ± 0.1187 %) of CUR was retained in the porcine oral mucosa, which represents an advantageous characteristic, as previously discussed.

The investigation of the optical spectral absorption by a photoacoustic signal created by the interaction of matter with radiation of known wavelength is known as the basis of photoacoustic spectroscopy (PAS). This is a qualitative method, as it determines if the drug can permeate or not and the depth of tissue permeated. Porcine oral mucosa was used to evaluate the permeation ability of CUR from emulgels by PAS (Fig. 5).

[Insert Figure 5]

The spectra of optical absorption of CUR and the emulgel systems with and without CUR (Fig. 5A) were performed to allow visualization of the constituents' bands by the decomposition of the spectra. Thus, an intense band from 300 to 700 nm, with centers around 315, 427, 515 and 659 nm could be observed. The main absorption band is 427 nm, due to ether and the aromatic rings of hydroxyl (Crivello and Bulut, 2005). The emulgel 15/0.50/0.75 showed a large band between 300 and 350 nm, whereas CUR emulgel 15/0.50/0.75/0.10 displayed from 300 to 500 nm.

In the optical spectra of mucosa (Fig. 5B), it was possible to observe that all samples exhibited a band at 415 nm, that belong to blood vessel. Moreover, a band that varies from 500 to 615 nm could be observed in all samples. Additionally, the area of permeation behavior at 427 nm (Fig. 5C) demonstrates that free CUR permeated more intensely than CUR incorporated in the emulgel. This finding is explained by the release and permeation through the mucosal layers for CUR-emulgel. CUR could permeate the whole mucosa (818 μm) since the thermal diffusivity wavelength (μ_s) was 31 μm for both sides of the tissue.

3.8. Cytotoxicity of monolayers cell cultures

Despite the oral cavity being considered an excellent route for drug delivery systems, local tolerance to the oral mucosa and *in vitro* activity are fundamental aspects that should be investigated in the development of drug delivery systems for OSCC applications containing CUR. This is especially true for formulations with mucoadhesive properties (Kulkarni et al., 2016; Teubl et al., 2013; Zeng et al., 2015). Cytotoxicity was assessed by MTT assay, which evaluates the cell metabolism by

mitochondrial activity. Viable cells are able to convert tetrazolium salt to insoluble purple formazan by mitochondrial succinic dehydrogenase activity (Fotakis and Timbrell, 2006; Zeng et al., 2015). The toxicity test after 24 hours of treatment with CUR and formulations with and without CUR against cancerous cell lines, Cal27 and FaDu and healthy cell line including FNB6 were performed to evaluate the selectivity of CUR activity by cancerous cell lines. This incubation period was used since during the *in vitro* release investigation 70% CUR could be released and the formulation was retained after 24 hours, predicting the unstimulated salivary flow. The application of the formulation in the oral cavity is subjected to the removal physiological mechanisms by salivary flow, speech and swallowing (Bruschi and de Freitas, 2005).

Cal27 were the most susceptible cell line to free CUR and formulations with and without CUR. Although FNB6 are considered as normal, they were more susceptible to free CUR than FaDu cells. This is explained by their basal origin; hence, they do not represent the mature cells from the superior layer of oral epithelia but represent cells from just the basal layer which are actively dividing (Jennings et al., 2016). As a consequence, it is necessary to perform cytotoxicity tests in tissue-engineered models, which confirm the toxicity of the preparations. Comparative graphs and IC_{50} values are displayed in Fig. 6.

The viability of Cal27 was significantly decreased ($p < 0.05$) when treated with the formulation with and without CUR. This can be explained by the surfactant properties of P407, which could influence the test.

Despite FaDu cells displayed smaller changes in the viability compared to Cal27, the IC_{50} values were lower when compared to healthy immortalized FNB6 cell lines and the effect of CUR in the formulations influenced in the viability of the preparations. Thus, the viability of FaDu cell lines was significantly higher ($p < 0.05$) when the cells

were treated with CUR emulgel in comparison to free CUR, which is can be explained by the release and permeation processes. In addition, the presence of free water in RPMI media is enough to solubilize CUR and facilitate its biological activity. Furthermore, the incorporation of the drug in the emulgel systems caused the IC_{50} values to significantly decrease ($p < 0.05$).

It was observed that for the FNB6 cell line, incorporation of the drug in the formulations increased the cellular viability due to the activity of free water, since Green's medium is a rich media supplemented with a number of organic molecules and growth factors essential for the maintenance and proliferation of these cell lines (Jennings et al., 2016). Furthermore, the formulations without CUR showed IC_{50} values significantly lower than emulgel systems containing CUR (Fig. 6).

[Insert Figure 6]

Cal 27 again had the lowest IC_{50} which suggests that the composition did not influence the susceptibility of this cell line to the drug. However, the IC_{50} value could be influenced by the concentration of P407 which can be found in the monomeric conformation and, interfere with the viability of these cells.

3.9. Evaluation of cytotoxicity using tissue-engineering oral mucosa models

The use of relevant three-dimensional models are considered as a more physiologically method for the *in vitro* evaluation of cytotoxicity, since monolayer cultures does not reproduce the complexity of the microenvironment and a number of reports have demonstrated that they do not properly simulate the cellular toxicity of drug and drug delivery systems (Jennings et al., 2016). Tissue-engineered 3D models

involve the culture of keratinocytes at an air to liquid interface on top of a fibroblast populated collagen type I matrix. The models simulate the interactions between mesenchyme and epithelium, which is essential for the development and maintenance of a normal epithelium. Furthermore, these interactions influence diverse processes including the keratinocytes proliferation, migration, differentiation and formation of basal membrane in the epithelial-dermis junction by the synthesis of extracellular matrix components (Colley et al., 2011).

In order to evaluate the cytotoxicity of CUR, three-dimensional models of oral mucosa were generated using tissue-engineering, in order to simulate normal oral mucosa using FNB6 (immortalized keratinocytes) (Jennings et al., 2016) and tongue squamous carcinoma (Cal 27) (Colley et al., 2011). Despite, the cell line most commonly used to simulate normal oral mucosa is TR-146 (metastatic buccal carcinoma), they are not considered appropriate due to the lack of fully differentiation and, thus, they cannot accurately represent normal epithelium (Jacobsen et al., 1995; Said, 2018). Other alternative to TR-146 would be the use of keratinocytes from primary cells, but their short lifespan and donor to donor variability limits their use. Thus, the use of immortalized cells which over express telomerase reverse transcriptase-2 (TERT-2) for unlimited proliferation, are considered a suitable cell source for generation of the models (Jennings et al., 2016).

When comparing the IC_{50} values of Cal27 and FaDu, Cal 27 showed a much lower IC_{50} due to its susceptibility for both free drug and formulations. Thus, FaDu models were not prepared because of its higher IC_{50}, as well as, its complexity, hence, a much higher CUR concentration would be required to kill the models and consequently, much more formulation.

The use of tissue-engineering oral mucosa for cytotoxicity permits the investigation of drug delivery systems for intra-epithelial activity and presents pre-clinical toxicity testing before *in vivo* trials (Moharamzadeh et al., 2017). Cytotoxicity assays were performed following the OECD guide (OECD, 2015), which describes the cytotoxicity tests in skin and mucosal equivalent models. Three curcumin concentrations were tested 9 µM, 240 µM and 380 µM, which represents the IC_{50} in monolayer cultures, maximum concentration tested in monolayer and the maximum concentration able to display activity without being influenced by DMSO, respectively. The percentage viability was calculated by normalizing to a negative control (DMSO concentration equivalent to the higher drug tested diluted in medium) (Fig. 7).

[Insert Figure 7]

Tissue-engineered normal oral mucosa (FNB6) was not significantly affected ($p < 0.05$) by the increase of CUR concentration with a relative viability approximately 90%. Considering the OECD recommendation which compounds are considered as safe when the relative viability is higher than 50%, the CUR was considered safe for normal oral mucosa until above 380 µM. In higher CUR concentrations, the relative viability would be influenced by the DMSO concentration. On the other hand, the relative viability of tissue-engineered cancerous oral mucosa model (Cal 27) displayed a significantly decrease ($p < 0.05$) in the relative viability (%), with the higher concentration displaying a relative viability lower than 50%.

Histological analysis was performed on tissue-engineered oral mucosa for normal mucosa (FNB6) and tongue squamous cell carcinoma (Cal 27) to evaluate the basic structure of the models using hematoxylin and eosin staining of the negative control and

models treated with 380 µM CUR. Three layers were observed: epithelium, basement membrane and fibroblast-populated connective tissue.

These results allow the elucidation of activity mechanism and explain the reason of different relative viability between the models, which is probably related to the anti-proliferative activity. Since, the tissue-engineered of normal oral mucosa have keratinocytes with high proliferative capacity in the basal layer of epithelia, which are able to differentiate and stratify (Colley et al., 2011; Hearnden et al., 2009). On the other hand, the tongue squamous carcinoma oral mucosa model presents tumor cells with high proliferative capacity, which could have their growth inhibited by CUR. The relative viability differences between Cal27 and FNB6 were not observed in monolayer cultures, as both Cal27 and FNB6 have high proliferative capacity in 2D. Thus, the 3D models represent more physiological characteristics of mucosa and are able to show more accurate results to reflect exposure to the 3D oral mucosa. Moreover, the anti-proliferative activity of CUR could be confirmed by the immunohistochemistry analysis (Figure S8, supplementary data).

The evaluation of cytotoxicity of emulgel systems was performed using the formulations without dilution, since the medium is placed underneath the inserts with the models (basolateral compartment) and the cells would not die due to being smothered (common issue in monolayer cultures), and also diluted in PBS to simulate the probable dilution of the systems when there is contact with the salivary flow. The same concentrations were tested for emulgel in the absence of CUR; the negative control was PBS buffer. The cytotoxicity graph and the relative viability for FNB6 and Cal 27 are shown in Fig. 7.

Thus, the tissue-engineered normal oral mucosa (FNB6) displayed relative viability around 80%, thus, it was higher than 50%, in the formulations with and without CUR.

Thus, they are considered as safe, due to absence of strong influence in the viability of normal oral mucosa at an air-liquid interface.

Regarding Cal27, the viability was unchanged by incubation with preparations without CUR; around 80%. On the other hand, preparations containing CUR displayed a dose-response activity and the relative viability was 50% for CUR emulgel and 80% for emulgel diluted in PBS (1:4).

When comparing the cytotoxicity results of formulations incubated with monolayer cultures and tissue-engineered models, it is evident that the complexity of the models reduces the efficacy of the formulation with and without CUR. It has been reported in literature (Moharamzadeh et al., 2012; Sun et al., 2006) that when experiments are moved from monolayer cultures to three-dimensional cultures, a cytoprotective effect is observed with higher IC_{50} for tissue-engineered models. Furthermore, cytokines and growth factors released in 3D models presented in both non-viable and viable cells are different of those from monolayer culture (Xu et al., 2009). Moreover, some authors (Moharamzadeh et al., 2012) have carried out experiments comparing the response of freshly excised human oral mucosa and 3D tissue-engineered mucosa to ethanol and sodium lauryl sulfate and observed similar responses.

4. Conclusion

Emulgel systems containing CUR were developed for oral squamous cell carcinoma treatment. The systems preliminary stability, mechanical, mucoadhesive, rheological properties were investigated, as well as, *in vitro* drug release, *ex vivo* permeation, retention characteristics determined. Moreover, biological studies were performed in order to investigate the influence of CUR free or when incorporated in the formulations on the viability of healthy and tumor cells using both monolayer cultures and tissue-

engineered models. Preliminary stability studies evidenced that the emulgels containing SO and C974P could display lower instability index, smaller mean diameter of oily droplets, providing higher stability. Mechanical, softness and syringe-ability parameters were not influenced by the incorporation of CUR in the emulgel systems. CUR-emulgel remained adhered to the porcine oral mucosa for 5 min. Besides, the incorporation of CUR in the emulgel systems did not interfere with the flow and oscillatory rheology but a decrease in the gelation temperature which ensure the ability to not move. Despite, 70% CUR being released in the *in vitro* release profile assays, the drug could not permeate the porcine oral mucosa and was retained in the oral mucosa. Biological assays evidenced that CUR affected Cal 27 and FNB6 monolayer viability. When the drug was incorporated in the formulations the IC_{50} for Cal 27 cells decreased, whereas the IC_{50} of FNB6 cells increased. Moreover, the tissue-engineered models have shown to be much more realistic tool in the cytotoxicity evaluation of these formulations, once the drug and formulations had a relative viability higher in FNB6 models than Cal27 models, demonstrating the selectivity of the drug and formulations for cancer models. Thus, these nanostructured emulgel systems demonstrated interesting results and selectivity to cancer cells in tissue-engineered models; which have been shown to simulate the human oral mucosa more closely.

Declaration of Competing Interest

The authors of this study declare no conflicts of interest.

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Figure captions

Fig. 1. Textural properties evaluated at 5, 25 and 37 °C for emulgels containing P407, C974P, SO and/or CUR: (A) Hardness, (B) Compressibility, (C) Adhesiveness, (D) Elasticity, (E) Cohesiveness. At least, three replicates were evaluated for each value.

Fig. 2. (A) Cumulative retention profile of emulgel containing P407, C974P, SO and CUR retention on porcine oral mucosa over time. Each dot represents the average (± standard deviation), of at least, three replicates. (B) Porcine oral mucosa after the falling liquid test (20 min).

Fig. 3. Scanning electron micrographs of emulgels containing 15% (w/w) P407, 0.50% (w/w) C974P, 0.75% SO, without CUR original magnification x1500 (A) and x 5000 (B) and containing CUR original magnification x1200 (C) and x4600 (D).

Fig. 4. Curcumin (CUR) release profile from emulgel systems containing 15% (w/w) P407, 0.50% (w/w) C974P, 0.75% (w/w) SO and 0.1% (w/w) CUR.

Fig. 5. Optical absorption spectra from PAS: (A) CUR and emulgel system with and without CUR, (B) permeation of CUR through the mucosa, (C) permeation behavior area of absorption band in 427 nm in porcine oral mucosa.

Fig. 6. Cytotoxicity of formulations: (A) cell survival curves of drug and emulgel, (B) IC_{50} comparisons of CUR and emulgel for two SCC (FaDu and Cal27) and one normal

oral (FNB6) cell line. Each IC_{50} value and percent cell survival represent average ± SEM (n=3).

Fig. 7. Relative percentage viability (%) and representative images of (A) FNB6 oral mucosa and (B) cancerous oral mucosa (Cal27) treated with negative control and CUR in the concentrations 9, 240 and 380 µM (C) FNB6 oral mucosa and (D) Cal27 oral mucosa after the incubation with emulgel and CUR emulgel with and without CUR without dilution and diluted 1:4. The positive control represents the models treated with 5% sodium dodecyl sulfate and negative control represents models treated with PBS buffer and the higher concentration of DMSO used to dissolve CUR. Scale bar= 300 µm.