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Candesartan exhibits low intrinsic permeation capacity and affects buccal tissue viability and integrity: An *ex vivo* study in porcine buccal mucosa

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ABSTRACT

Candesartan is a nonpeptide angiotensin II receptor blocker that selectively binds to angiotensin II receptor subtype 1. It is administered orally in its ester form (candesartan cilexetil). However, its poor aqueous solubility results in its low bioavailability; therefore, other routes of administration must be explored. The buccal mucosa has been extensively studied as an alternative route for drug delivery as it improves the bioavailability of drugs administered via the peroral route. Porcine buccal mucosa has been widely used as an *ex vivo* model to study the permeability of various diffusants; however, studies on candesartan are limited. This study aimed to evaluate the *ex vivo* permeation profile of candesartan and its effects on the viability and integrity of porcine buccal mucosa. Initially, we evaluated the viability, integrity, and barrier function of the buccal tissue before performing permeability tests using freshly excised tissues or tissues after 12 h of resection. Here, three indicators were used: caffeine, β-estradiol, and FD-20 penetration; mucosal metabolic activity, as determined using MTT reduction assay; and haematoxylin and eosin staining. Our results indicated that the porcine buccal mucosa preserved its viability, integrity, and barrier function before the permeation assay, allowing the passage of molecules with a molecular mass of less than 20 kDa, such as caffeine, but not β-estradiol and FD-20. Furthermore, we analyzed the intrinsic capacity of candesartan to diffuse through the fresh porcine buccal mucosa under two pH conditions. The concentration of candesartan in the receptor chamber of Franz diffusion cell was quantified using ultra-high liquid chromatography. In the permeation assay, candesartan exhibited a low intrinsic permeation capacity that impacted the buccal tissue viability and integrity, suggesting that using the buccal mucosa as an alternative route of administration requires developing a pharmaceutical formulation that reduces the adverse effects on mucosa and increasing the buccal permeability of candesartan.

1. Introduction

Hypertension is currently the most prevalent noncommunicable disease. Globally, it affects 31.1% of the adult population (1.39 billion people) and is the leading cause of death worldwide (Kuehn, 2020). The therapeutic arsenal for treating hypertension includes multiple drugs that act through different mechanisms. However, only 37.6% of patients receiving medication have controlled blood pressure (Lamelas et al., 2019).

Most antihypertensive drugs are administered orally, but they exhibit poor bioavailability due to their poor aqueous solubility, low permeability, extensive hepatic first-pass metabolism, or instability in the gastrointestinal environment, thereby limiting their therapeutic effect and supporting the need to explore other routes of administration (Sharma et al., 2016).

Candesartan (CD) is a nonpeptide angiotensin II receptor blocker that selectively binds to angiotensin II receptor subtype 1 (AT1) and subsequently dissociates from it slowly (Mcclellan and Goa, 1998). The

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AT1 binding affinity of CD is 80 times greater than that of losartan. Moreover, the efficacy of CD is much higher or equivalent to many other commonly prescribed antihypertensive agents (Darwhekar et al., 2012). Among the eight clinically available angiotensin receptor blockers, CD has one of the lowest required daily doses to reduce blood pressure (16 and 32 mg/day) (Abraham et al., 2015), which could favor its absorption through an alternative route, such as the buccal mucosa. Chemically, it is a tetrazole derivative that is used as an ester prodrug (candesartan cilexetil [CC]) to treat hypertension (Husain et al., 2011); however, recent studies have also demonstrated its potential application as an antiviral agent (Loe et al., 2019), antibacterial agent (Xu et al., 2021), and a possible lead compound for the development of new anticancer drugs (Ni et al., 2020). During absorption from the gastrointestinal tract, CC is bioactivated via ester hydrolysis to liberate the free drug. However, CC is characterized by poor aqueous solubility within the physiological pH range, which results in its incomplete intestinal absorption and very low bioavailability (≤15%) after administration (Aly et al., 2020; Husain et al., 2011). Recent studies have focused on improving CD bioavailability using different strategies, such as solid dispersions (Gurunath et al., 2014; Sonawane et al., 2016); encapsulation (Anwar et al., 2020; Jena et al., 2019); nanoparticle formation (Nekkanti et al., 2009); and mucoadhesive buccal systems, including patches, tablets, and films (Mady et al., 2021; Malpure and Deore, 2016; Padmaja et al., 2018; Pansuriya et al., 2019; Reddy et al., 2018; Samanthula et al., 2021, 2019; Thulluru et al., 2011; Vinay and Ahmed, 2015).Nevertheless, none of these latter approaches assess the permeation of free drug through the buccal mucosa.

The buccal mucosa has been considered an attractive route for drug delivery as it offers many advantages over the peroral route (Madhav et al., 2009; Morantes et al., 2017). Drugs administered via the buccal route can reach the systemic circulation after overcoming mucosal barriers, thereby avoiding the first-pass effect and degradation in the gastrointestinal tract (Rossi et al., 2005; Şenel and Hincal, 2001). *Ex vivo* permeability measurements have been invaluable in predicting transbuccal drug absorption kinetics in vivo and exploring drug absorption mechanisms (Hoogstraate and Boddé, 1993; Lee et al., 2005). Franz diffusion cell (FDC) is one of the devices mostly used for *ex vivo* permeability studies; it is a system that allows determining the quantity and speed of drug diffusion through a barrier of synthetic or animal source (Kulkarni et al., 2010). Animal buccal mucosal tissues isolated from monkeys, dogs, and pigs are routinely used in *ex vivo* permeation assay due to their similarity to human buccal mucosa in terms of lack of keratinization (Hoogstraate and Boddé, 1993; Wang et al., 2022). However, porcine buccal mucosa is considered the most suitable *ex vivo* model because of its physiological and anatomical similarities with human buccal mucosa as well as its advantages in utility and cost (Wang et al., 2021).

While using the porcine buccal mucosa as an *ex vivo* permeability model, mucosal integrity, biological viability, and barrier function must be verified before and after the permeation assay (Wang et al., 2022). However, these properties have not been clearly documented in buccal permeation studies on CD. Furthermore, the time of experimentation is another crucial factor that directly affects the results of a permeation assay; however, this factor has not been thoroughly investigated in existing studies using CD. In the present study, we evaluated the *ex vivo* permeation profile of CD and its effects on the viability and integrity of porcine buccal mucosa before and after the permeation assay. The integrity of the buccal mucosa was verified by performing a permeation assay of the fluorescent marker FD20 and tissue barrier function using *β*-estradiol and caffeine, two compounds with different permeation profiles. *β*-estradiol is a low-permeability compound, whereas caffeine is a high-permeability compound (Nicolazzo et al., 2004). Tissue viability was assessed before and after the permeation assay using MTT reduction assay and histological examination, two techniques frequently used to assess buccal viability (Imbert and Cullander, 1999). Finally, under physiological (6.6–6.9) and acidic (5.5–5.7) pH conditions, the permeation assay showed that CD exhibited a low intrinsic permeation capacity, affecting buccal viability and integrity. Further studies are required to develop a pharmaceutical formulation capable of reducing adverse effects on the mucosa while increasing the buccal permeability of CD.

2. Materials and methods

2.1. Materials and reagents

CD was obtained from Alfa Aesar™GmbH (Germany). Caffeine, β-estradiol, MTT (3-(4,5-Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide), fluorescein isothiocyanate (FITC)-labeled dextran with a molecular weight of 20,000 Da (FD-20), Na_2CO_3, and other reagents were purchased from Sigma-Aldrich (USA). Dimethyl sulphoxide (DMSO) was purchased from AppliChem (USA). Krebs Ringer Bicarbonate (KBR) buffer consisted of 115.5 mM NaCl, 4.2 mM KCl, 21.9 mM $NaHCO_3$, 12.2 mM glucose, 4.0 mM HEPES, 1.2 mM $MgSO_4{\cdot}7H_2O$, 1.6 mM $NaH_2PO_4{\cdot}2H_2O$, and 2.5 mM $CaCl_2{\cdot}2H_2O$. $CaCl_2{\cdot}2H_2O$ was dissolved separately in half the final volume to avoid calcium precipitation. The final solution was filtered using a 0.22-μm filter and gassed with carbogen (95% O2:5% CO_2) for 40 min. Phosphate buffered saline (PBS) containing 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 136.7 mM NaCl, and 2.7 mM KCl was prepared in deionized water. For ultra-high performance liquid chromatography (UHPLC), milli-Q water, acetonitrile, methanol, and formic acid (Merk, High Performance Liquid Chromatographic (HPLC) grade) were used. All solvents were degassed by stirring at a reduced pressure before UHPLC.

2.2. Tissue preparation

The buccal tissues from freshly slaughtered domestic pigs (age: 4–6 months and weight: 80–110 kg) were collected from a local abattoir, Bogotá-Colombia. Samples without wounds or bruises were washed twice with saline solution (0.9% NaCl), transferred to a flask containing ice-cold KBR buffer (pH: 7.4), transported to the laboratory, and processed according to a previously described procedure (Kulkarni et al., 2010). In brief, excesses of connective tissues were trimmed using a scalpel blade, and the remaining buccal mucosa was immersed in a beaker containing saline solution (0.9% NaCl) at 60 °C for 1 min. Thereafter, the epithelium was mechanically peeled off using a fine spatula and placed in a Petri dish containing KBR buffer to prevent dehydration. At this point, the tissue was ready to be used in the experiments described below.

2.3. Assessment of buccal tissue viability using MTT reduction assay

MTT reduction assay was performed for the following four reasons: (i) to set a baseline or control TR index value to discriminate between viable and nonviable mucosa; (ii) to determine the impact of experimentation time on epithelial tissue viability; (iii) to establish the innocuousness of the vehicle in which CD and control molecules (caffeine and β-estradiol) were solubilized; and iv) to determine the effects of CD, caffeine, and β-estradiol on epithelium viability after permeation assays. The protocol used for the MTT reduction assay was modified from that of Imbert and Cullander (1999). In brief, 4-mm tissue samples were weighed and placed into a 24-well plate with 1 mL of MTT solution (2 mg/mL in PBS). The plate was then incubated for 2 h at 37 °C on a rotating platform at 100 rpm. Following incubation, unmetabolized MTT was removed, and the tissues were washed twice with 1 mL of PBS for 1 min and minced with surgical scissors to avoid tissue loss. Then, 1 mL of DMSO was added to each sample to extract formazan and stirred (100 rpm) overnight at room temperature on a rotating platform. The absorbance of formazan was measured at 560 nm using a plate reader (Tecan Genius, Infinite™ M200 PRO); wells containing DMSO were used as blank (Imbert and Cullander, 1999). The results have been reported as

the mitochondrial tetrazolium reductase activity index (TR index; absorbance per mg tissue). A sample of deactivated mucosa or that without any apparent metabolic activity was used as the damage control or nonviable mucosa (frozen buccal mucosa treated with 10% Triton-X100 in PBS for 1 h). All experiments (n = 10–24) were conducted using tissues from independent animals.

2.3.1. Impact of time on epithelial tissue viability

The epithelium was separated from the connective tissue using the method described in Section 2.2. The metabolic activity of samples isolated within 2 h postmortem (henceforth referred to as "fresh mucosa," $n = 24$) was compared with that of the tissue after 4 h of incubation ($n = 24$) under conditions simulating the permeation assay (first condition). Simultaneously, another group of buccal mucosa samples was mounted in the FDC to perform viability assay after 16 h of incubation. The buccal mucosa mounted in the FDC during 12 h was used as a control ($n = 24$) (second condition). The buccal tissue was mounted between FDC donor and receptor compartments. The KBR buffer was charged into the donor compartment, and the membrane was gently removed at the end of each condition and subjected to histological examination and viability studies using the MTT reduction assay.

2.4. Buccal tissue integrity study

Buccal tissue integrity was monitored using fluorescein isothiocyanate (FITC)-labeled dextran 20 kDa (FD20) absorption. Buccal mucosa samples were mounted in the FDC. After an equilibration period of 30 min, KBR buffer was replaced with an FD20 solution in KBR (37 °C; 200 μg/mL). FD20 appearance in the receptor chamber was determined based on the conditions described in Section 2.3.1. The fluorescence energy of the samples was detected using a fluorescence microplate reader (Tecan Genius, Infinite™ M200 PRO) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The results were expressed as relative units of fluorescence (RFU) and compared with those of fresh mucosa and deactivated mucosa (damaged) + FD20.

2.5. Permeation studies for CD and control molecules

Before performing CD permeation assay, we investigated whether the mucosal barrier function was preserved during the processing conditions (sample collection, sample transport, and epithelium separation) described above. Here, two control molecules were used: caffeine and β-estradiol. β-estradiol diffusion through the membrane indicates decreased barrier function. Conversely, caffeine diffusion with a permeability coefficient between 4.0×10^{-6} cm/s and 10×10^{-6} cm/s indicates the functional state of the epithelial barrier (Hansen et al., 2018). For the permeation assay, a saturated CD solution (1 mg/mL) was prepared by adding 0.2 mM Na_2CO_3 in PBS (1:150). The solution was adjusted at two pH values: physiological pH in the oral cavity (6.6–6.9) and acidic pH (5.5–5.7). The acidic pH value was chosen considering that various extrinsic and intrinsic factors can modify the buffering capacity of the saliva, decreasing the intraoral pH and altering the permeability profiles of ionizable drugs. A mixture of PBS and ethylene glycol (10%) was used to solubilize β-estradiol (0.4 mg/mL). Caffeine solutions (1 mg/mL) were prepared in the same vehicle used to solubilize CD. *Ex vivo* permeation studies for CD and control molecules were performed using the FDC of the following two references: Perme Gear Inc., USA; 4G-01–00–05–03-S, for the experiments with CD and β-estradiol (donor volume: 350 μL, receptor volume: 3 mL, diffusion area: 0.2 cm^2) and Perme Gear Inc., USA; 4G-01–00–09–05, for the experiments with caffeine (donor volume: 1 mL, receptor volume: 5 mL, diffusion area: 0.64 cm^2). Porcine buccal mucosa samples were mounted between the donor and receptor compartments. The two compartments were held together with a clamp. The temperature of the receptor compartment was controlled via a water jacket connected to a circulating water system at 37 ºC. A small magnetic stirrer was placed in each cell to stir the receptor solution constantly. Before the permeation experiments, the tissues were equilibrated with the vehicle solutions for 30 min. Then, the vehicle solutions were replaced either with a saturated CD solution (1 mg/mL) prepared in acidic pH (5.5–5.7) and physiological pH (6.6–6.9) or with solutions containing control molecules. All openings, including the donor top and receptor arm, were occluded with parafilm® to prevent evaporation. Aliquots of 300 μL were collected from the receptor compartment every 30 min for 4 h and the cells were immediately refilled with the corresponding vehicle solution to keep the volume of the receptor chamber constant during the experiment. At the end of the experiment, the epithelium was gently removed from the receptor compartment. Then, the tissue was cut out in three sections with a circular punch (4 mm diameter). Two sections were placed in the MTT solution to assess viability and the third section was used for histological examination. The samples recollected from the receptor compartment were either directly analyzed via HPLC to quantify caffeine and β-estradiol or lyophilized and later analyzed via UHPLC (previous dissolution in 100 μL of methanol, sonicated, and centrifuged for 10 min at 6000 rpm) to quantify CD. All experiments were conducted using tissues from independent animals.

2.6. HPLC

2.6.1. Caffeine and β-estradiol

Shimadzu HPLC (Japan, Prominence-i model LC 2030), with a column of Gemini C18 ® C18 (150 × 4.6 mm, 5 μm) and flow rates of 1.0 mL/min (caffeine) and 1.8 mL/min (β-estradiol), was used. The mobile phase for caffeine was composed of water and methanol (55:45 v/v). The column oven temperature was set at 40 °C. The injection volume was 10 μL, and UV detection was performed at 273 nm. For β-estradiol, the mobile phase was composed of water and acetonitrile (50:50 v/v). The column oven temperature was set at 40 °C. The injection volume was 10 μL, and UV detection was performed at 225 nm.

2.6.2. CD

UHPLC was conducted on a Thermo Scientific Dionex Ultimate 3000 chromatograph equipped with a Dionex Ultimate 3000 diode array detector, Dionex Ultimate quaternary pump 3000 RS, in-line degasser, and autosampler. The column used was Gemini ® C18 (150 × 4.6 mm, 5 μm). The data were processed using Chromeleon Client software, version 6.80 SR15. Mobile phase A consisted of formic acid in water (0.51%) and phase B consisted of acetonitrile. The method consisted of a gradient with a constant flow of 1 mL/min, where phase B increased from 10% to 45% in 5 min and to 48% from 5 to 13 min and returned to the initial condition in 2 min. The column oven temperature was set at 25 ºC. The injection volume was 30 μL, and UV detection was performed at 260 nm.

2.6.3. Standard solution preparation

The standard stock solution of CD was prepared in methanol, and dilutions were made to obtain solutions of 120, 60, 24, 6, 1.5, and 0.12 μg/mL in triplicate. An additional calibration curve in a concentration range of 0.004–0.08 μg/mL was plotted to determine the limit of detection and quantitation. All standard solutions were filtered using a 0.22 μm membrane before UHPLC. A standard stock solution of caffeine (1 mg/mL) was prepared using 0.2 mM Na_2CO_3 in PBS (1:150). Then, the solution was serially diluted in the concentration range of 0.0610–500 μg/mL. All standard solutions were filtered through a 0.22 μm membrane before the analysis. A stock solution of β-estradiol was prepared by adding 2.3 mg of β-estradiol in 5.5 mL PBS + 10% ethylene glycol to obtain a final concentration of 418 μg/mL. Dilutions were made in the range of 0.02–418 μg/mL.

2.7. Histological examination

Histological examination was performed to identify potential

changes in tissue structure and cell morphology in response to experimental conditions and compounds used in this study. Buccal mucosa samples were fixed in 10% neutral formalin and embedded in paraffin blocks. The embedded samples were cut into 10 μm-thick sections and conventionally stained with hematoxylin and eosin. The tissue sections were examined using a ZEISS light microscope, and representative pictures were taken using an AxioCam ERc5 camera (Zeiss, Jena, Germany).

2.8. Statistical analysis

Statistical analyses were performed using the statistics package GraphPad Prism 9 (GraphPad Software Inc., La Jolla, CA, USA). Data were explored for normality using Shapiro–Wilk test. As appropriate, data were analyzed statistically using Student's *t*-test, Kruskal–Wallis test with Dunn's multiple comparisons test, and one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. A p-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Viability studies using MTT reduction assay and histological examination

Assessing tissue viability is fundamental for validating and interpreting results in *ex vivo* permeability assays. The mitochondrial TR index (absorbance per mg tissue) calculated using the MTT reduction assay has been extensively used to determine tissue viability. This index can be defined as the ability of the isolated tissue to retain biological functions, such as metabolism and enzyme activity (Wang et al., 2022). No consensus TR index values for viable and nonviable mucosa were found in the literature. Therefore, we determined control reference TR index values to discriminate between viable and nonviable mucosa.

The results showed that the TR index of the viable mucosa (fresh mucosa) was 0.0353–0.0647, with a mean value of 0.0472 ± 0.0078 (n = 24), and that of the nonviable mucosa (deactivated) was 0.0010–0.0202, with a mean value of 0.0154 ± 0.00298 (n = 24); a significant difference in the TR index was observed between the viable and nonviable mucosa ($p < 0.0001$) (Fig. 1A). These results were consistent with the decrease in formazan production (Fig. 1B) and changes in tissue architecture observed in the histological examination (Fig. 1C and D). To set the baseline for nonviable mucosa, tissues with a severe injury (frozen buccal mucosa treated with 10% Triton-X100 in PBS for 1 h) were used because metabolic activity was still evident in frozen tissues (TR index, 0.0366 ± 0.00583, n = 10) or tissues boiled in water for 1 h (0.03598 ± 0.0076, n = 10), despite cell morphology alterations and tissue architecture changes in these specimens (data not shown).

The TR index has been extensively used to indicate the viability of various tissues, such as skin, cornea, ovarium, and porcine buccal mucosa; however, different studies report a wide range of values (between 0.00416 and 0.08) for viable tissues (Chuchuen et al., 2013; Imbert and Cullander, 1999; Loe et al., 2019; Nicolazzo et al., 2004; Roblegg et al., 2012). This variability in the TR index values could be explained by differences in the protocols used to process the samples, the time that elapses from slaughter, and the incubation time with the MTT reagent. Interestingly, the value reported in this study was comparable to that computed by Imbert and Cullander (1999) (TR index: 0.05), who adapted the MTT assay to assess buccal tissue viability for the first time.

On the other hand, we also noted a certain degree of inconsistency

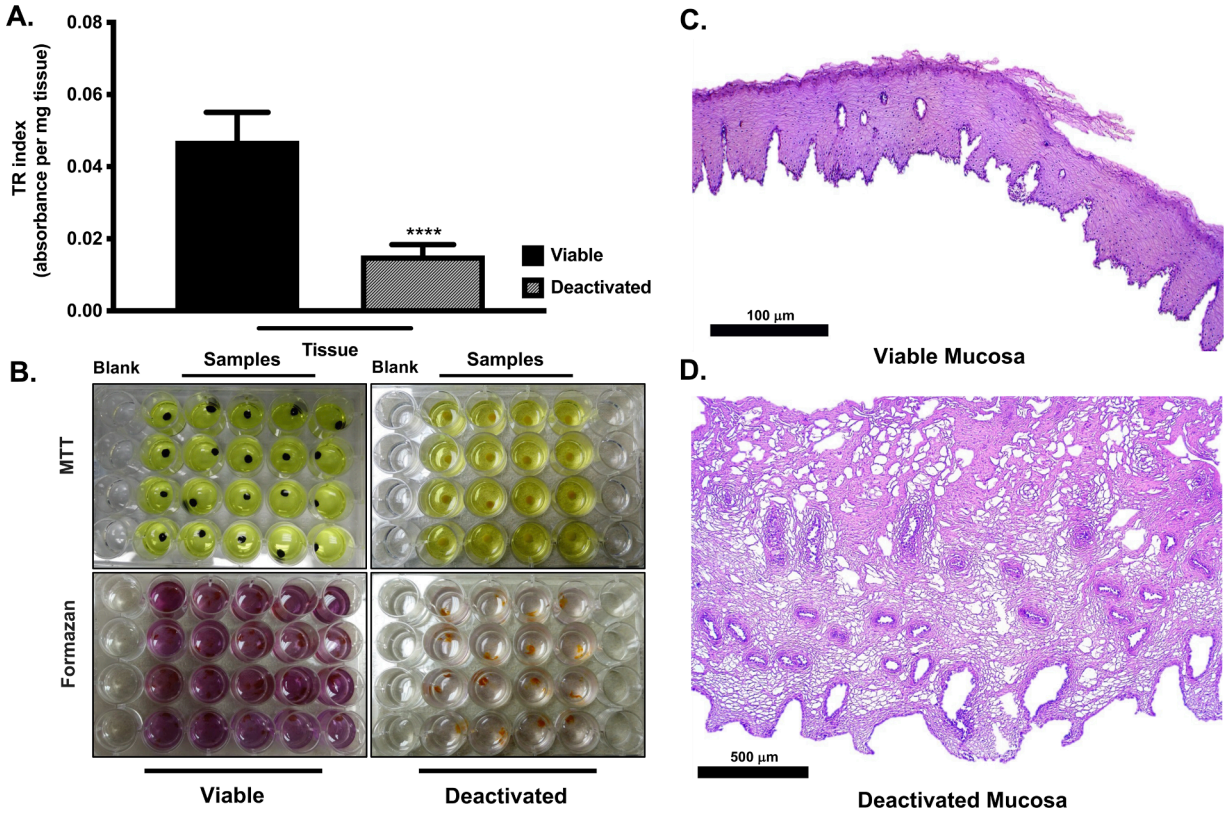

Fig. 1. MTT reduction assay and histological examination of porcine buccal mucosa before permeation assays. A. TR index values for viable and nonviable mucosa. Each bar represents the mean ± standard deviation (n = 24) for viable mucosa (TR index: 0.0472 ± 0.0078) and nonviable mucosa (TR index: 0.0154 ± 0.00298). Asterisk indicates statistical significance calculated using unpaired Student's *t*-test with Welch's correction (****) $P < 0.0001$. B. Production of formazan crystals from viable and nonviable mucosa. Blank (only DMSO). C. H&E staining of viable porcine buccal mucosa (tissue immediately after separation of the epithelium). D. H&E staining of nonviable or deactivated porcine buccal mucosa (frozen tissue treated with 10% Triton-X100 in PBS for 1 h).

between the results of MTT assay and histological evaluation of nonviable tissues, which has been previously published in the literature; while histological analysis showed significant damage to the frozen porcine buccal tissues, the MTT assay failed to show cell death (Nicolazzo et al., 2004). Although the explanations for this phenomenon are unclear, it can be suggested that the TR index is a good marker for significant damage to the metabolic activity, structure, and organization of the mucosa. Therefore, it is necessary to establish more sensitive methodologies for assessing buccal mucosa viability and obtaining results that can better correlate with the results of histological studies (Kulkarni et al., 2010).

Previous studies have demonstrated that the viability of the buccal tissue decreases as soon as the animal is sacrificed (Nicolazzo et al., 2004; Roblegg et al., 2012). As viability is reduced, the ability of the epithelium to maintain its barrier function is also compromised (Erickson-Direnzo et al., 2015). In this sense, the impact of time on epithelial tissue viability under conditions that mimicked the permeation assay was explored. For this reason, the TR index was calculated under two different conditions: (i) 4 h after and (ii) 16 h after mounting the buccal mucosa in the FDC, using fresh mucosa and buccal mucosa mounted for 12 h as controls, respectively. In both cases, buccal tissues were mounted simultaneously and only exposed to the KBR buffer. The time for the second condition was determined by considering the following points: (1) previous studies suggest that porcine buccal mucosa remains viable for up to 12 h (Nicolazzo et al., 2003) and (2) to solve a logistical problem and define whether the permeation assay should be performed the same or the next day.

The histological examination and mitochondrial tetrazolium reductase activity analysis revealed that the samples preserved their metabolic activity, cell morphology, and tissue architecture 4 h after mounting in the FDC. These results were consistent with those observed for the control (fresh mucosa) and those reported in previous studies (Figueiras et al., 2009; Imbert and Cullander, 1999). However, after 16 h, pathological manifestations, such as nuclei loss, cytoplasmic pallor (vacuolization), nuclear pyknosis, and cellular alignment loss, were observed (Fig. 2A), similar to the control after 12 h of incubation. In addition, the metabolic activity analysis showed that the mean TR index values estimated after 12 and 16 h of incubation were significantly lower than those of the fresh mucosa (0.0314 ± 0.0053 and 0.0289 ± 0.0049, respectively; $p < 0.0001$). In contrast, the TR index at 4 h was similar to that of the control group (0.0478 ± 0.0076 and 0.0472 ± 0.0078, respectively; $p > 0.05$), suggesting that it is an appropriate time to perform permeability studies. Neither histomorphological changes nor cytopathic effects were observed in these specimens (Fig. 2A). Recently, it has been reported that the buccal mucosa remains viable for up to 4 h. Wang et al. (2022) demonstrated that mucosal viability can be prolonged for up to 24 h when KBR is supplemented with 1% FBS and the FDC is placed in 5% CO_2, but their experimental conditions were different from ours. In the future, it would be interesting to determine whether these conditions can prolong mucosal viability in permeation assays using CD and other potential candidates administered via oral routes.

To determine the innocuousness of the vehicles employed, i.e., CD and control molecules (caffeine and β-estradiol), on tissue viability at the optimal time (4 h), TR index values were calculated, and the results are shown in Fig. 3. As expected, the TR index of the mucosa exposed to CD and caffeine was comparable to that of the fresh mucosa. However, a slight decrease in the TR index of the tissue exposed to β-estradiol was observed (Fig. 3A), which can be attributed to the usage of ethylene glycol. This alcohol can be used as a solvent in cutaneous permeability studies (Møllgaard and Hoelgaard, 1983); however, it is an osmotically active compound that causes metabolic disturbances and cell damage (Jammalamadaka and Raissi, 2010). Ethylene glycol is mainly metabolized in the liver to organic acid metabolites capable of promoting cell damage. Alcohol dehydrogenase is one of the enzymes responsible for the metabolism of ethylene glycol. This enzyme is expressed in the oral epithelium, and its activity may impair the viability of the buccal mucosa (Hedberg et al., 2000).

Interestingly, histopathological examination revealed that the tissue sections from fresh mucosa and mucosa exposed to the vehicles exhibited a normal histological structure, with few vacuoles (Fig. 3B). In contrast, CD and β-estradiol affected the buccal tissue and metabolic activity, significantly reducing TR index values (Fig. 3A). This reduction in TR index values could be associated with a drug entrapping within lipophilic domains of the buccal mucosa, resulting in moderate buccal epithelium irritation. These irritation signs observed in the tissue were consistent with those reported in other studies on irritant-inducing substances (Miles et al., 2014; Nakane et al., 1996; Şenel and Hincal,

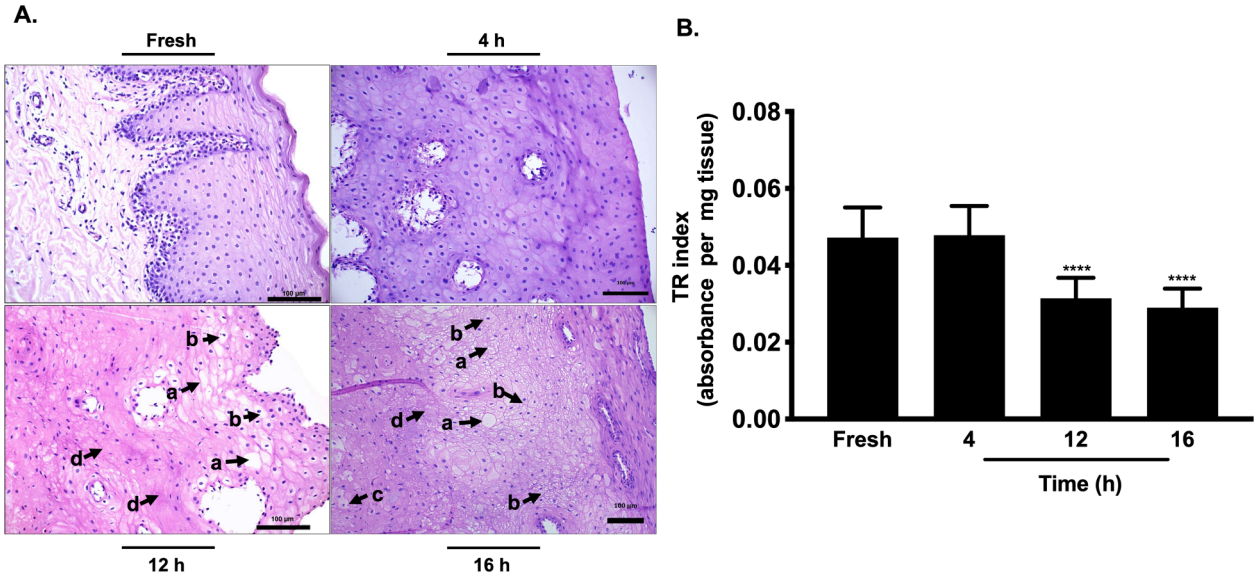

Fig. 2. Impact of experimental time on porcine buccal mucosa viability in permeation assays. A. H&E staining of fresh mucosa and mucosa after 4 h, 12 h, and 16 h of experimentation. The arrows indicate the most frequent damage observed in the tissue after 12 h and 16 h of experimentation. a. Vacuolization and cytoplasmic pallor with loss of nuclei; b. Vacuolization and cytoplasmic pallor without loss of nuclei; c. Changes in cell morphology; d. Loss of alignment of cell layers. B. TR index at different experimental times. Each bar represents the mean ± standard deviation ($n = 24$). Asterisk indicates statistical significance with respect to fresh mucosa calculated using nonparametric Kruskal–Wallis test with Dunn's multiple comparisons tests (****) $P < 0.0001$.

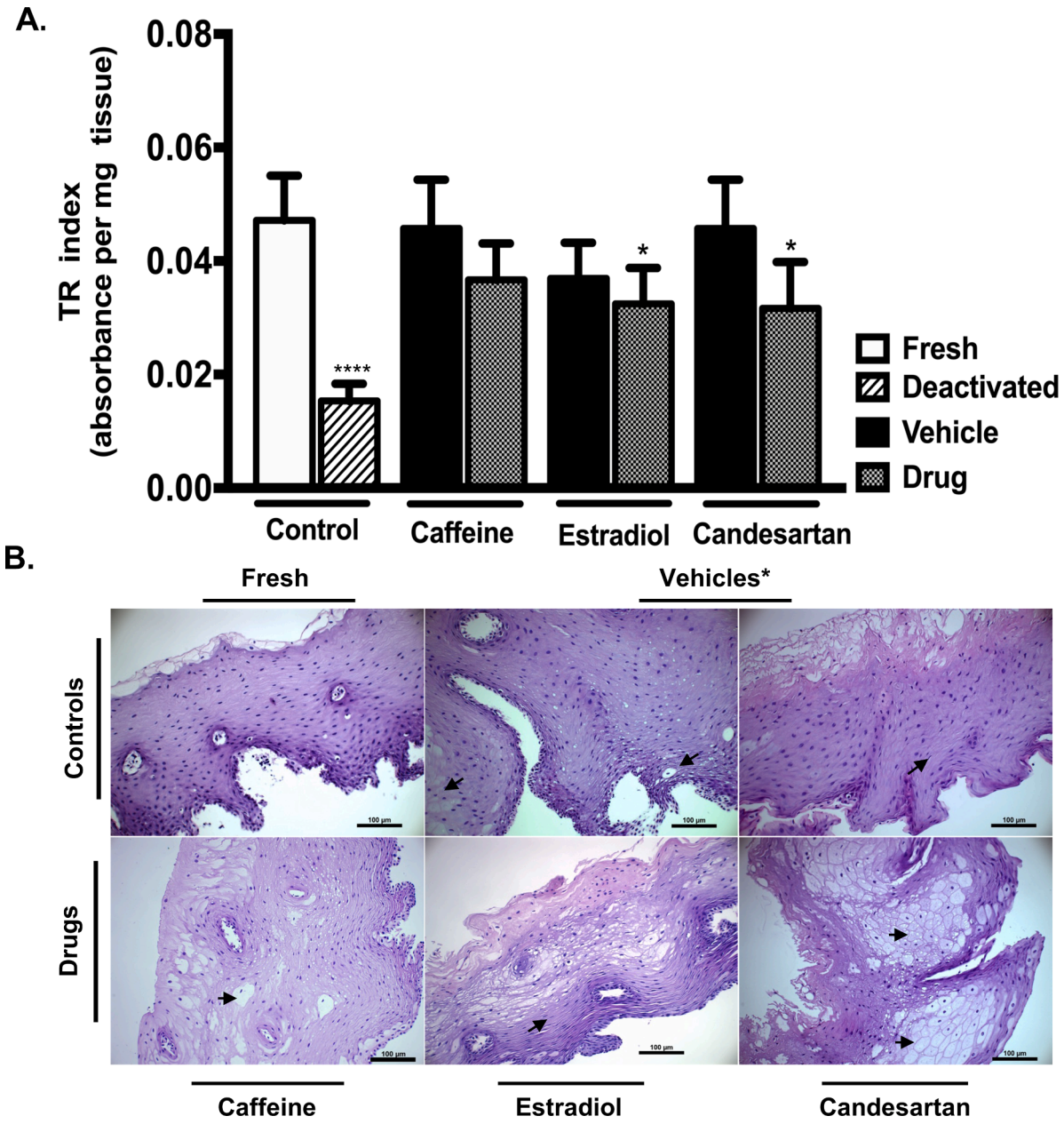

Fig. 3. MTT reduction assay and histological examination of porcine buccal mucosa after permeation assays. A. Effect of vehicles and drugs: CD, caffeine, and β-estradiol on the viability of porcine oral mucosa. Each bar represents the mean ± standard deviation of TR index ($n = 12$). Asterisk indicates statistical significance with respect to the control (fresh mucosa) calculated using one-way ANOVA with Tukey's multiple comparisons tests (*) ($P<0.05$), (****) ($P<0.0001$). B. H&E staining of fresh buccal mucosa and mucosa exposed to vehicles and drugs. The arrows indicate architecture changes, edematous cells, shrunk cells, and spherical vacuoles. * The vehicle used for CD is the same as that used for caffeine.

2001; Yoshimoto et al., 2019). Frequently observed alterations in the buccal tissue, including changes in tissue architecture and presence of edematous cells with extensive cytoplasmic vacuolation (which appear larger and weakly eosin-stained under the microscope), have been shown in Fig. 3B.

Cytoplasmic vacuolization is frequently observed after exposure to pharmaceutical agents and it results from the physicochemical interaction between drug and tissue (Aki et al., 2012). In our study, all drugs induced cytoplasmic vacuolization; however, this phenomenon was more evident in tissues exposed to hydrophobic agents (i.e., CD and β-estradiol). We suggest that cytoplasmic vacuolization and other alterations observed in the buccal tissue could be reversed if basal lamina cells were not affected by the treatment. Moreover, the buccal epithelium has the ability to promote damaged tissue repair (Groeger and Meyle, 2015). The molecular mechanism by which CD and β-estradiol cause tissue injury remains unclear. However, it has been demonstrated that some drugs or chemicals that induce irritation affect cells by inhibiting or inducing enzymes, altering metabolic pathways, producing free radicals, increasing membrane permeability, or damaging chromosomes or structural components of the cell via osmotic stress or drug accumulation in organelles (Miller and Zachary, 2017).

3.2. Mucosal integrity and barrier function

The altered integrity and impaired barrier function of the buccal mucosa can lead to increased permeability and misinterpretation of results in drug absorption studies. The technique to separate the buccal epithelium from connective tissue, storage conditions, and experimental

time are variables that may affect integrity and barrier function (Bhati and Nagrajan, 2012). We performed the FD20 permeation assay to assess the effects of experimental time on mucosal integrity. Here, fresh mucosa and damaged or nonviable mucosa, i.e., frozen buccal mucosa treated with 10% Triton-X100 in PBS for 1 h, were used as the control. Our results showed that samples collected from the receptor compartment of the Franz cell with damaged mucosa had the highest RFU values, reaching 3000 times higher than those observed for fresh mucosa at 4, 12 and 16 h after treatment, indicating that treatment with 10% Triton-X100 causes loss of integrity (Fig. 4). On the other hand, RFU values at 4 h showed no statistical differences compared with fresh mucosa ($p > 0.05$), suggesting that the mucosa remains intact for up to 4 h post treatment. A moderate but statistically significant increase in mean RFU values was observed at 12 and 16 h, indicating that experimental times may alter the integrity and barrier function of the mucosa. These results are compatible with viability loss at 16 h after treatment in metabolic activity assays.

FD20 has been identified as a fluorescent marker for evaluating the integrity of porcine buccal mucosa (Wang et al., 2022). The use of this compound as an integrity marker was initially reported by Janet Hoogstraate and Boddé (1993), who evaluated the permeability of the porcine oral mucosa against dextran of different molecular weights. They showed that only dextrans with a molecular weight of <20 kDa permeated the epithelium of the oral mucosa. More recent research reveals that the detection of >0.6% of FD20 in the receptor compartment of Franz cell after 4 h of experimentation indicates the loss of tissue integrity (Berka et al., 2020). In 2010, Kulkarni et al. compared the integrity of fresh and frozen tissues to ensure that the permeability profiles obtained were not a consequence of a compromised mucosa and demonstrated that the compound permeability was higher in frozen tissues than in fresh tissues, suggesting that fresh membranes should be used in drug permeation assays (Kulkarni et al., 2010). In our study, histological evaluation after FD20 passage at different experimental times showed that the mucous membranes exposed for 4 h presented an epithelium with cells with a conserved structure, similar to fresh mucosa. Whereas buccal tissues exposed for 12 h and 16 h of experimentation showed cytoplasmic vacuolization in most cells and loss of some nuclei. In mucous membrane micrographs at 16 h of experimentation, structural loss was observed for some cells (data not shown).

The barrier function of the buccal mucosa was also explored, and caffeine and β-estradiol, two compounds with different physicochemical properties and transport routes, were used as model permeants. Caffeine is a hydrophilic compound that preferentially permeates via the paracellular route and has been recommended as a model compound for in vivo, *ex vivo*, and in vitro buccal penetration testing because of its well-known penetration (Castro et al., 2016; Kulkarni et al., 2009, 2010, 2011; Nicolazzo et al., 2004). In contrast, β-estradiol preferentially permeates through the transcellular route. However, due to its lipid nature, it can be retained in the mucosa, leading to a low permeation rate and lag times of more than 24 h (Møllgaard and Hoelgaard, 1983). The diffusion of β-estradiol through the buccal epithelium at 4 h after the permeation assay could be interpreted as the loss of barrier function.

As expected, caffeine permeated the buccal mucosa with a passing profile linear after 60 min of assay and a mean permeability coefficient of $6.16 \pm 1.40 \times 10^{-6}$ cm/s, a value that indicates the functional state of the epithelial barrier (Hansen et al., 2018) and was comparable to that calculated by Kulkarni et al. (2009) ($6.03 \pm 0.36 \times 10^{-6}$ cm/s) and Kulkarni et al. (2010) ($6.13 \pm 0.75 \times 10^{-6}$ cm/s). In the present study, permeability coefficients of the mucosal tissue for caffeine did not exceed 1.38×10^{-5} cm/s, indicating that the collection, transport, and processing protocols employed here preserved mucosal viability and integrity for up to 4 h. This value was used as a reference to indicate a decrease in mucosal barrier function, as a previous study showed that the order of magnitude of the permeability coefficient for caffeine increased from 10^{-6} to 10^{-5} in tissues with integrity compromised (Kulkarni et al., 2010). On the other hand, permeability assay for β-estradiol was performed as described in the methodology, and samples from the receptor compartment were quantified according to the chromatographic conditions established for β-estradiol. However, the permeability kinetics profile was not achieved because the passage of β-estradiol through the porcine buccal mucosa was below the limit of quantification, indicating that the integrity of the buccal mucosa was preserved before and after the permeation assay.

Despite a decrease in buccal mucosa viability after the permeation assay (Fig. 3A), we observed that this experiment did not significantly alter β-estradiol permeability since the drug was not detected in the donor compartment. Some studies have investigated the relationship between tissue viability and barrier function. Nicolazzo et al. (2003) found that the barrier properties of buccal tissue remain intact even when tissue viability is reduced, which is consistent with our findings. In contrast, Erickson-Direnzo et al. (2015) reported findings that are inconsistent with both (Nicolazzo et al., 2003) and our study.

Other studies have already reported β-estradiol permeability through the buccal mucosa (Nicolazzo et al., 2005), vaginal mucosa (Van Eyk and Van Der Bijl, 2004), and skin (Møllgaard and Hoelgaard, 1983). However, these results differ from ours, likely because tritium-labeled β-estradiol was used in permeation assays, and their experiments were performed after more than 4 h.

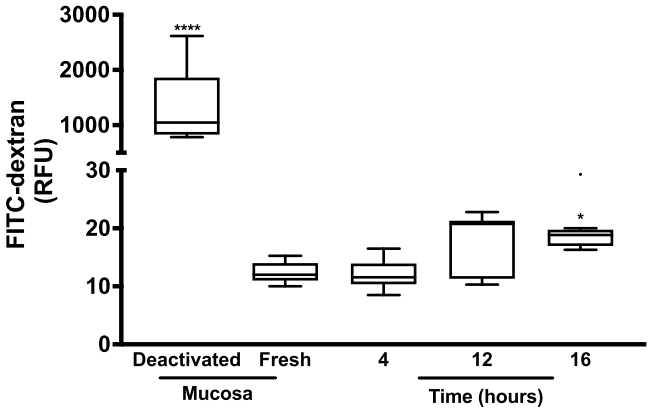

Fig. 4. Impact of experimental time on porcine buccal mucosa integrity in permeation assays with FD20. Asterisk indicates statistical significance with respect to control (fresh mucosa) calculated using nonparametric Kruskal–Wallis test with Dunn's multiple comparisons test (*) ($P < 0.05$), (****) ($P < 0.0001$).

3.3. CD permeability

Once we confirmed that the viability and barrier function of the buccal mucosa remained intact under our experimental conditions, the permeation profile of CD was analyzed at 4 h (240 min) in two pH ranges: acidic (5.5–5.7) and physiological (6.6–6.9) pH. The pH of human saliva is variable, ranging from 5.3 (low flow) to 7.8 (peak flow). The flow and buffering capacity of the saliva can vary in patients with oral and systemic pathologies (e.g., hypertension), leading to a decreased pH close to the critical value of 5.5 (Kagawa et al., 2013; Pedersen et al., 2018; Persson, 1998). These variations in the oral pH may play a crucial role in the ionization status and permeability of drugs, such as CD (Humphrey and Williamson, 2001). Therefore, it is essential to assess the pH in the oral cavity. Here, apparent (P_{app}) or effective (P_{eff}) permeability parameters cannot be used because CD kinetics in the donor compartment was nonlinear at the time being evaluated (4 h). Instead, the CD concentration in the donor compartment was analyzed due to the direct relationship between permeability and passage concentration (Narula et al., 2022; Volpe, 2010).

In the permeation assay under acidic conditions, the passage of CD through the buccal mucosa showed an upward trend over time, with a

concentration peak of 2 μg/mL after 240 min (Fig. 5); this concentration was lower than that observed in a previous study (Gao et al., 2011). This discrepancy can be explained by pH effects, including ionized and molecular species determined by the acidity constant (pK_{a1} = 4.0), on the passage of CD (Cagigal et al., 2001; Kokate et al., 2008). Conversely, the passage at physiological pH had a maximum concentration of 4 μg/mL at 120 min; however, after this time, the concentration of CD significantly decreased in the receptor chamber, likely due to drug accumulation of the drug in the bucal mucosa. The mechanism of drug retention has already been reported for other molecules, including miconazole (De Caro et al., 2021), tertatolol, alprenolol, propranolol, and labetalol (Nielsen and Rassing, 2000). To the best of our knowledge, this is the first study to analyze the permeability of CD in the oral mucosa, and therefore, there is not enough available information to fully understand our results. Further studies are required to identify all variables involved in the permeation profile of CD in the buccal mucosa.

3.4. Limitations and future perspectives

CD is orally administered only in the form of tablets. Despite the proven potency and pharmacological properties of CD, its limited solubility and low bioavailability support the need to identify alternative routes of administration. Our study explores the potential of CD to be absorbed through the oral mucosa to determine the permeation kinetics of CD through the buccal mucosa. The tissue viability assays supported the usage of fresh mucosa and the realization of performance permeation assays at 4 h; however, due to the lipophilic and ionizable nature of CD, it may be necessary to increase the permeation time to establish whether the observed profile is maintained or changed. We believe that the viability of the tissue could be preserved for up to 24 h using KBR buffer supplemented with 1% FBS and maintaining FDCs at 5% CO_2, as already described in a previous study (Wang et al., 2022). The main limitation of the present study was that the amount of CD trapped in the buccal tissue was not quantified. A methodology to extract and quantify drugs trapped in the buccal epithelium should be developed in the future. This approach might help understand the CD permeation profile in the buccal mucosa. Here, CD showed a low intrinsic permeation capacity and affected the viability and integrity of the buccal tissue. Nonetheless, in the future, it would be interesting to develop a formulation including permeability enhancers that facilitate the passage of CD through the buccal mucosa to overcome the difficulties observed in this study.

4. Conclusion

This study demonstrated that porcine buccal mucosa used as an *ex vivo* model in permeation studies retains its viability, integrity, and barrier function for up to 4 h. After 12 h and 16 h of experimentation, the buccal mucosa became metabolically and functionally compromised, suggesting that the use of buccal mucosa from freshly isolated tissue is best for performing permeation studies. The permeation profiles of caffeine and β-estradiol were as expected under our assay conditions, validating the barrier function of our *ex vivo* model. This study also showed that CD had a low intrinsic permeation capacity through the buccal mucosa and induced changes in the metabolic activity, cell morphology, and tissue architecture, indicating that CD alters tissue viability and integrity. These findings suggest that a formulation that reduces adverse effects and increases the buccal permeability of CD should be developed.

CRediT authorship contribution statement

Yenny M. Garcia-Tarazona: Methodology, Investigation, Writing – original draft. **Sandra Johanna Morantes:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **José Francisco Ibla Gordillo:** Investigation, Formal analysis, Writing – review & editing. **Paula Sepúlveda:** Writing – review & editing, Formal analysis. **Freddy A. Ramos:** Writing – review & editing, Formal analysis. **Gloria Inés Lafaurie:** Conceptualization, Supervision, Writing – review & editing.

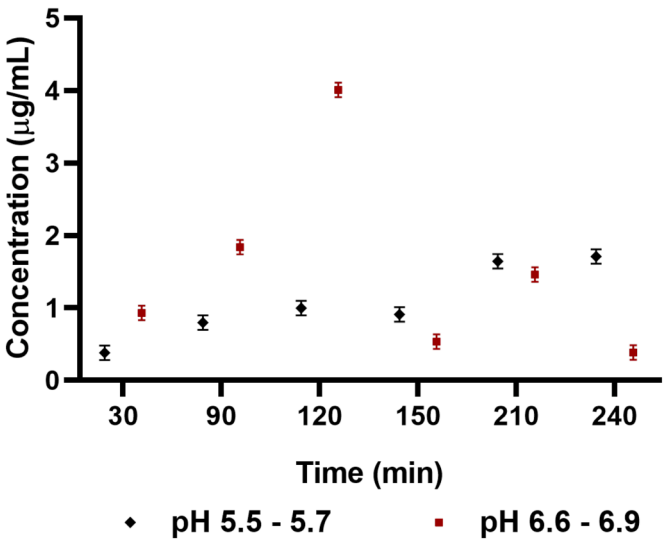

Fig. 5. Comparison of the *ex vivo* permeation profile of CD through the porcine buccal mucosa as a function of time (min) at two pH ranges after 4 h of experimentation using Franz diffusion cells. Values represent the mean concentration (μg/mL) of three trials ± standard deviation. pH = 5.5–5.7(black square) and pH = 6.6–6.9 (red square).

Data availability

Data will be made available on request.

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