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Research paper

Evaluation of galantamine transbuccal absorption by reconstituted human oral epithelium and porcine tissue as buccal mucosa models: Part I

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ABSTRACT

Over the last decade, interest in delivering drugs through buccal mucosa has increased. As a major limitation in buccal drug delivery could be the low permeability of the epithelium, the aim of this study was to evaluate the aptitude of galantamine, useful in Alzheimer's disease, to penetrate the buccal mucosa. The evaluation of the ability of galantamine to permeate through the buccal epithelium was investigated using two permeation models. Firstly, in vitro permeation experiments were carried out using reconstituted human oral non-keratinised epithelium and Transwell diffusion cells system. Results were validated by ex vivo experiments using porcine buccal mucosa as membrane and Franz type diffusion cells as permeation model. The entity of buccal permeation was expressed in terms of drug flux (J_s) and permeability coefficients (K_p). Data collected by in vitro and ex vivo experiments were in agreement and suggested that buccal mucosa does not block diffusion of galantamine. The effects of drug application on histology of tissue specimens used in every experiment were also studied: no sign of flogosis and no significant cytological or architectural changes were highlighted.

1. Introduction

Alzheimer's disease is characterized by progressive decline in memory with impairment of at least one other cognitive function; the median survival for affected patients is approximately 8 years from the onset of symptoms [1,2]. Degradation of cholinergic neurons in the cerebral cortex and other areas of the brain – resulting in cholinergic transmission and acetylcholine levels deficit – is considered the primary cause of cognitive decline. Consequently, research strategies have focused on cholinergic transmission enhancement [3]. To date, the most successful pharmacologic strategy has been the inhibition of acetylcholinesterase, the enzyme responsible of acetylcholine catabolism in the synaptic cleft [1]. Galantamine is a cholinesterase inhibitor that differs from other medications of the same class since it has also a modulating effect on nicotinic receptors which enhance the drug effects [4,5]. It has been suggested that this dual mode of action could produce a beneficial cascade of neurotransmitters, possibly affecting the serotoninergic and gamma-aminobutyric acid systems. Efficacy of galantamine on the cognitive, functional and behavioral symptoms of dementia in Alzheimer's, has been demonstrated in several large-scale clinical trials [6]. At present, galantamine is available in the market as either tablets or oral solutions, and two daily oral administrations are required [2]. Even if oral administration is convenient for most patients suffering from mental disorder, it is extremely difficult to follow scheduled dosage. So, an alternative way of galantamine administration could be helpful for the success of the therapy.

Due to its relative permeability, the buccal mucosa offers good opportunity for local/systemic pharmacological actions. Buccal delivery specifically refers to the drug administration through the mucosal membrane lining of the inner cheek [7–10]. This mucosa is richly vascularized, accessible for the administration and removal of a dosage form; moreover, drug delivery has a high patient acceptability compared to other non-oral routes. Drug administration on buccal mucosa could be helpful in adhering to a correct dosage regimen for Alzheimer's patients.

Following application of actives on buccal tissue, therapeutic efficacy mainly depends on the ability of drugs to permeate through the tissue fast enough, providing the required plasma concentrations. To establish the aptitude of drugs to permeate buccal mucosa, tissues from various animals have been used as models for human mucosa as well as cells grown in culture [11–14]. Porcine buccal mucosa is one of the most used in *ex vivo* studies; on the other hand, stratified

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cultured TR146 cell layers (the so-called reconstituted human oral epithelium) are analogous to normal human buccal epithelium and could be used in *in vitro* studies [15,16].

Aim of this study was to establish the aptitude of galantamine to penetrate the buccal mucosa using porcine buccal mucosa and reconstituted human oral non-keratinized epithelium as membrane models. Since topical application of drug could damage the structure of the biological tissue, we observed also the effects of galantamine local administration on the histology of buccal tissue.

2. Materials and methods

2.1. Materials

Galantamine hydrobromide, USP grade, was purchased from Biodar (Yavne, Israel), 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) and all components of buffer solutions were purchased from Sigma–Aldrich (Milano, Italy).

Simulated saliva was prepared using a buffer solution (pH 6.8) containing NaCl (0.126 g), KCl (0.964 g) KSCN (0.189 g), KH_2PO_4 (0.655 g), and urea (0.200 g) in 1 L of distilled water [17].

Natural human saliva (pH 6.8) was obtained from a healthy donor without any conditioning habits, i.e. smoking, alcohol, coffee, drinking or any other further habit able to alter its composition; it was collected from one of the Authors, after overnight fasting, first brushed his teeth and thoroughly rinsed the mouth using only deionized water, then sat in a relaxed position with the head in a slightly inclined forward position, allowing saliva to accumulate on the floor of the mouth. The first few millilitres of saliva were discarded. The accumulated saliva was then withdrawn using disposable sterile plastic pipettes until about 1.5 ml had been collected. The samples of saliva were not further handled to evaluate the drug behavior in environmental conditions similar to those of the administration site.

Phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free solution, pH 7.4, was prepared by dissolving KH_2PO_4 (0.144 g), anhydrous Na_2HPO_4 (0.795 g) and NaCl (9.0 g) in 1 L of distilled water. All chemicals and solvents were of analytical grade and were used without further purification. All other reagents for cell culture were obtained from Sigma and solutions for cell culture were prepared in endotoxin-free water.

2.2. Methods

2.2.1. Galantamine permeability studies

2.2.1.1. In vitro permeation of galantamine throughout reconstituted human oral epithelium. Galantamine permeation was investigated *in vitro* by measuring drug fluxes throughout TR146 cell layers, derived from a human neck metastasis originating from a buccal carcinoma, cultured on permeable polycarbonate inserts. Mucosal specimens were supplied by Skinethic Laboratories (Nice, France). Upon arrival, the bags containing the inserts with cultured cell layers were opened under sterile airflow. Each insert (area 0.5 cm^2) containing the epithelial tissue (100 μm thick) was taken out and any remaining agarose that adhered to the insert's walls was rapidly removed by gentle blotting on sterile filter paper and placed in culture dishes filled with maintenance medium (Skinethic). The culture dishes were placed overnight in the incubator at 37 °C, 5% CO_2 and saturated humidity. Before testing, the maintenance medium was changed by adding fresh medium. The permeability of galantamine was performed using filter-grown TR146 cell aged 12 days old. The experiments were maintained at a constant temperature of 37 °C [Polimix EH 2 bath equipped with a constant-rate adjustable stirrer RECO® S5 (Kinematica, Switzerland)], using the Transwell diffusion cells as a two-compartment open model.

Accurately measured amounts of drug solution (galantamine 1.5 mg in 0.5 ml of simulated saliva) were applied to the apical side of the cell layers. To avoid cell stress, PBS (pH 7.4, 25 ml) was used as acceptor fluid. The acceptor solution was stirred by means of a magnetic stirrer to avoid formation of stagnant boundary layers next to the membrane surface. An insert in which simulated saliva was applied to the apical side of the cell layer was used as control. At regular intervals, samples (0.5 ml) were withdrawn from the basolateral side of the acceptor compartment. To avoid saturation phenomena and maintain the "sink" conditions, the sample volume taken out was replaced by fresh fluid. The galantamine transferred from the donor to the acceptor compartment was monitored spectrophotometrically by measuring the drug that reached the acceptor fluid. The integrity of the TR146 cell tissue was monitored after each permeability study (see Sections 2.2.4 and 2.2.5). Results are reported as means ± SD of six different experiments in which inserts of a single time production batch were used ($P < 0.05$).

2.2.1.2. Ex vivo permeation of galantamine throughout porcine buccal mucosa. The permeation kinetic throughout the porcine buccal mucosa was evaluated using Franz type diffusion cells. Mucosal specimens (kindly supplying by Pig Farm, Pioppo, Palermo) were obtained from tissue removed from two freshly slaughtered domestic pigs. After sampling, all specimens were immediately placed in a refrigerated transport box and transferred to the laboratory within 1 h. Excesses of connective and adipose tissue were trimmed away until 0.8 ± 0.1 mm thick slides were obtained. Some specimens were used fresh; the remaining specimens were stored at −40 °C for periods up to six months. The frozen specimens were equilibrated in PBS, (pH 7.4) for 1 h at room temperature to thaw completely before starting experiments. To avoid damage of the epithelial surface, the mucosal samples were carefully cut to obtain suitable disks. These sections of mucosa were then mounted in the flow-through cell. Tissue disks were equilibrated for 1 h at 37 °C [Polimix EH 2 bath equipped with a constant-rate adjustable stirrer RECO® S5 (Kinematica, Switzerland)] adding PBS in both the donor and the acceptor compartment. This step was followed by the removal of PBS from the compartments.

In the donor compartment was then placed drug solution (3 mg of galantamine in 1.0 ml of simulated saliva or human saliva), in the acceptor compartment was placed PBS (26 ml). The acceptor solution was stirred by means of a magnetic stirrer. At regular time intervals, samples (0.5 ml) were withdrawn from the acceptor compartment. To avoid saturation phenomena and maintain the "sink" conditions, the sample volume taken out was replaced by fresh fluid. Each experiment was carried out for six hours. The integrity of the mucosal tissue was monitored after each permeability study (see Section 2.2.5). Results are reported as means ± SD of six different experiments in which inserts of a single time production batch were used ($P < 0.05$).

2.2.2. Drug assay

The cumulative amount permeated through reconstituted human oral epithelium and porcine buccal mucosa was calculated from the galantamine concentration in the acceptor medium and plotted as a function of time. Each experiment was performed six times using six different culture cell inserts of one single time production batch and six different samples of porcine buccal mucosa. Each data point on the plot represents the mean of the recorded values ($P < 0.05$).

In all experiments, the drug transferred from the donor to the acceptor compartment was monitored spectrophotometrically (UV/vis Shimadzu mod. 1700 Pharmaspec instrument) by measuring the amount that reached the acceptor fluid using the appropriate calibration curve and blank ($\lambda_{max} = 288.2$ nm, $E_{1\%} = 0.084$ in

PBS). The UV absorption peak was highly reproducible and linearly related to concentration over a range 0.001–0.4 mg/ml. At testing concentrations, acceptor media components used do not interfere significantly with the UV absorption of galantamine. The sensibility was less than 0.0001 mg/ml. Intraday and interday variations, observed during collection of experimental data, were lower than sensibility.

2.2.3. Data analysis

The flux values (J_s) across the membranes were calculated at the steady state per unit area by linear regression analysis of permeation data following the relationship:

$$J_s = \frac{Q_r}{A\,t}(\text{mg cm}^{-2}\,\text{h}^{-1}) \quad (1)$$

where Q_r is the quantity of galantamine which passes through the tissue into the receptor compartment (mg), A is the active cross-sectional area available for diffusion (cm^2) and t is the time of exposure (h).

The permeability coefficient (K_p) was then calculated by the relationship:

$$K_p = \frac{J_s}{C_d}(\text{cm h}^{-1}) \quad (2)$$

where J_s is the flux calculated at the steady state (mg cm^{-2} h^{-1}), C_d is the drug concentration in the donor compartment (mg cm^{-3}).

Flux and permeability coefficient values, obtained as average value of six replicated experiments, were reported with the standard deviations. All differences were statistically evaluated by the Student's t-test with the minimum levels of significance with $P \leqslant 0.05$.

2.2.4. Cell viability

At the end of the experiments, cell viability of cultured tissue was evaluated by measuring the mitochondrial dehydrogenase activities according to the MTT assay [18]. The mean optical density of the untreated control tissues was set to represent 100% of viability (MTT test, $n = 1$, OD = 0.999) and the results were quantified as percentage of the untreated controls.

2.2.5. Histological methods

Histomorphological analyses were performed on reconstituted human oral epithelium and porcine buccal tissues, to evaluate the pathological changes occurring in cell morphology and tissue organization, after permeation experiments. The reconstituted human oral epithelium was cut out from the insert together with the polycarbonate filter using a sharp scalpel. The tissues samples were fixed in 10% neutral-buffered formalin for 2 h, washed in water for 1 h, dehydrated in graded ethanol (60%, 80%, 90%, 95%, and 100%) and, after permeation in xylene, embedded in paraffin using the standard procedures. Formalin-fixed, paraffin embedded samples were cut into 4-μm-thick sections on a microtome with a disposable blade and conventionally stained with hematoxylin–eosin. Two different types of negative controls were included in the histomorphological analysis: blank controls and permeation controls.

Blank reconstituted human oral epithelium or porcine tissues controls were considered those samples not subjected to the experimental phase; permeation controls were those samples subjected to the experimental phase in the absence of drug. Slides were evaluated by optical microscopy by two of the authors in a blind and independent fashion. Microphotographs of relevant fields were taken at 40× and 100× magnification. Digital images were captured using a Leica DC 300F camera (Leica, Nidau, Switzerland) mounted on a Leitz DMRB microscope with the Leica IM50 Image Manager program version 1.20.

3. Results and discussion

The most important limitation in the development of a buccal drug delivery could be the low drug permeability of the epithelium, so the aptitude of galantamine to penetrate this barrier was assessed [19]. Firstly, the permeation kinetic was evaluated *in vitro* using reconstituted human oral epithelium as membrane and the Transwell system as a two-compartment linear open model.

Reconstituted human oral epithelium is constituted by 4–6 layers of TR146 cells, derived from a human neck metastasis originating from a buccal carcinoma. The cells have been shown to be able to grow on polycarbonate permeable inserts and form cell layers resembling the stratified human buccal tissue; in particular, stratified cultured TR146 cell layers at 12th day of culture (100 μm thick) are analogous to normal human buccal epithelium, especially for morphology and ultrastructure [14,15].

All experiments were carried out for 3 h to avoid changes in permeability characteristics; in fact we experienced that cultured tissues, out of the maintenance medium, live and remain intact for only 3–4 h [20]. Prolonging the experiments over this time the cells, out of the culture or maintenance medium, could die and the collected data might lose accuracy.

Results are reported as the cumulative amount of galantamine, which enters the cell layers and arrives to receiver fluid, vs time (Fig. 1a). At the end of each experiment, the sum of residual drug content in the donor compartment and the amount of transferred drug matched the original administered dose, confirming that no drug was entrapped in the cell layers.

Permeation profile through reconstituted human oral epithelium shows a linear trend and absence of lag time. Fluxes (J_s) and permeability coefficients (K_p) were estimated at the steady state from the linear portion of the plot (Fig. 1b), when the sink conditions were retained. The J_s and K_p values were calculated by equations (1) and (2), and resulted 0.216 ± 0.012 mg/cm^2 h and 0.072. ± 0.003 cm/h, respectively. However, the TR146 cell cultured on inserts might have some limitations as permeability barriers. In this model, indeed, membrane-coating granules, lipoidal content between the epithelial layers, and intercellular connections are different than in *ex vivo* tissues, and a basal lamina is absent [12]. Moreover, the cultured tissue is about 100 μm thick while the epithelium of the human buccal mucosa is 500–800 μm thick (40–50 cell layers with about 12.5–20.0 μm for each layer) [15,21], our data on galantamine permeation through reconstituted human oral epithelium need further validation on a more thick tissue. To assess drug permeability, buccal mucosa from various animals (rabbits, dogs, monkeys, hamsters and pigs) has been used as models for human mucosa. However, when compared to the other animal models, porcine buccal mucosa has been considered the most representative model for human tissue as it is non-keratinised like human buccal mucosa [11]. Moreover, studies on bidirectional permeability through reconstituted human oral epithelium have shown close correlation to the data obtained using porcine buccal mucosa [16]. So, we measured drug fluxes and permeability coefficients throughout domestic pig mucosa 800 μm thick. In order to avoid possible uncertainties attributable to the saliva composition, comparative experiments were carried out using both buffer solution simulating saliva and natural human saliva. A limitation, however, in this work could be the use of saliva collected from a single person.

Fig. 2 shows drug movement, expressed as cumulative galantamine amount from the mucosal to the serosal side of the porcine epithelium vs time. Permeation profile through porcine buccal mucosa shows a linear trend. No significant differences were observed using artificial saliva or natural human saliva as donor medium.

As above, flux values (J_s) across the membrane were calculated at the steady state per unit area from the slope of the linear portion of the plot and the mean value resulted in 0.127 ± 0.010 mg/cm^2 h.

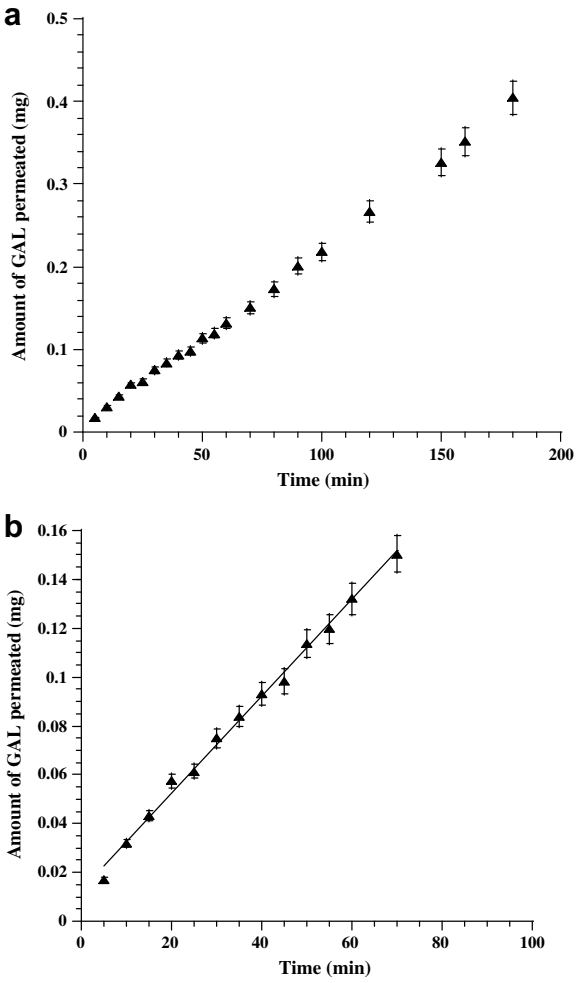

Fig. 1. (a) Plot of cumulative amount-time of galantamine permeated across reconstituted human epithelium into receiver chamber, using simulated saliva as donor medium and PBS as receptor medium. Values are reported as means ± SD ($n = 6$). (b) Linear portion of the permeation profile of galantamine through reconstituted human epithelium.

The permeability coefficients (K_p) were processed by diving the flux values by the drug concentration in the donor compartment: the mean value was 0.042 ± 0.003 cm/h. As seen for reconstituted human oral epithelium experiments, the sum of residual drug content in the donor compartment and the amount of transferred drug matched the original administered dose, confirming that no drug was entrapped in the membrane.

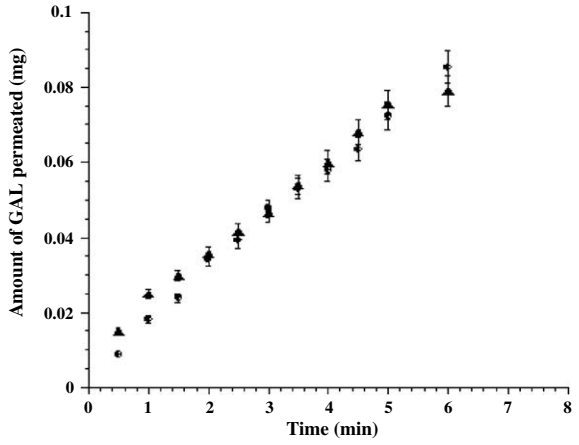

Fig. 2. Plot of cumulative amount-time of galantamine permeated across porcine buccal mucosa into receiver chamber, using (▲) natural human saliva or (•) simulated saliva as donor medium, and PBS as receptor phase. Values are presented as the means ± SD ($n = 6$).

Taking into account the different thickness of the two membrane models used in permeation studies, the obtained data for porcine buccal mucosa were in agreement with those observed for reconstituted human oral epithelium.

Considering that the drug itself could cause some changes in the structure of buccal mucosa (e.g. cellular swelling, spongiosis, epithelial thinning or atrophy, vesiculation as well as cell death [19,22–24]) we investigated the histomorphological effects of drug on the tissue specimens used.

In the *in vitro* permeation experiments on reconstituted human oral epithelium, the two types of negative controls, blank controls and permeation controls, showed not-keratinized squamous-cell epithelia composed by an average of five cellular layers; reconstituted human oral epithelium samples, submitted to galantamine passive diffusion, showed the absence of significant changes in cell morphology or tissue structure (Fig. 3).

Also in samples of porcine tissue used in *ex vivo* permeation experiments, no significant cytological or architectural changes

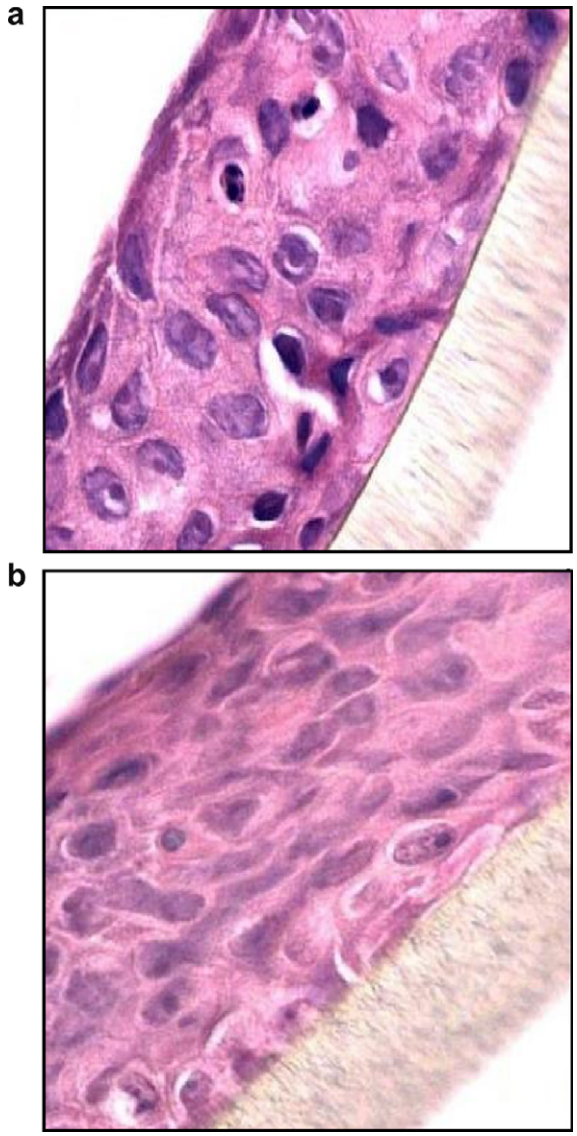

Fig. 3. Microphotographs of formalin-fixed paraffin embedded cross-sections of reconstituted human epithelium: (a) control untreated, (b) sample subjected to simple diffusion of galantamine in simulated saliva (magnification 100×).

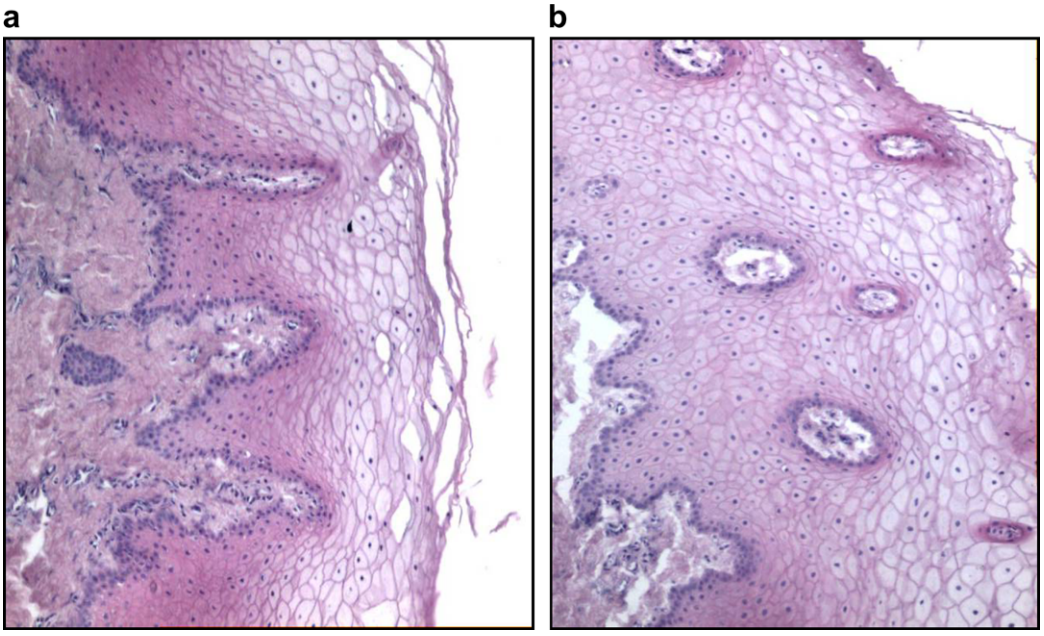

Fig. 4. Microphotographs of formalin-fixed paraffin embedded cross-sections of porcine buccal mucosa: (a) control untreated, (b) sample subjected to simple diffusion of galantamine in natural human saliva (magnification 40×).

were highlighted. In Fig. 4a, it is shown a microphotograph of untreated sample which reveals the appearance of the superficial cells of the epithelium. The samples submitted to galantamine diffusion (Fig. 4b) did not show significant alterations in cell morphology or epithelium structure. No sign of flogosis was found in any of mucosal specimens treated.

The collected data suggested that buccal mucosa does not block galantamine diffusion and the drug passively crosses the membrane, as shown by kinetic behavior; moreover, the drug causes no damage on the buccal tissue.

In conclusion, on the basis of our results, the buccal mucosa could represent an alternative way for galantamine administration. This study is part of the European Project "*Intellidrug: intraoral medicine delivery microsystem to treat addiction and chronic disease*" (Sixth Framework, Project n° 002243 IST; www.intellidrug.org).

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