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Research paper

Enhanced transbuccal salmon calcitonin (sCT) delivery: Effect of chemical enhancers and electrical assistance on *in vitro* sCT buccal permeation

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ABSTRACT

This study investigates the combined effect of absorption enhancers and electrical assistance on transbuccal salmon calcitonin (sCT) delivery, using fresh swine buccal tissue. We placed 200 IU (40 μg/mL) of each sCT formulation—containing various concentrations of ethanol, *N*-acetyl-L-cysteine (NAC), and sodium deoxyglycocholate (SDGC)—onto the donor part of a Franz diffusion cell. Then, 0.5 mA/cm^2 of fixed anodal current was applied alone or combined with chemical enhancers. The amount of permeated sCT was analyzed using an ELISA kit, and biophysical changes of the buccal mucosa were investigated using FT-IR spectroscopy, and hematoxylin–eosin staining methods were used to evaluate histological alteration of the buccal tissues. The flux (J_s) of sCT increased with the addition of absorption enhancer groups, but it was significantly enhanced by the application of anodal iontophoresis (ITP). FT-IR study revealed that all groups caused an increase in lipid fluidity but only the groups containing SDGC showed statistically significant difference. Although the histological data of SDGC groups showed a possibility for tissue damage, the present enhancing methods appear to be safe. In conclusion, the combination of absorption enhancers and electrical assistance is a potential strategy for the enhancement of transbuccal sCT delivery.

1. Introduction

With the rapid development of biotechnology, some peptide drugs, such as insulin, calcitonin, luteinizing hormone-releasing hormone, and its derivatives have been launched on the market [1–4]. However, these peptide drugs have thus far been commercialized almost exclusively as the injection form. There have been requests for non-parenteral formulations of peptide drugs because the injection formulations bring about patient non-compliance issues, such as pain, needle phobia, phlebitis, and tissue necrosis induced by frequent injections [1].

To overcome the above-mentioned disadvantages of peptide injection formulations, potential alternative delivery strategies have been studied through nasal, vaginal, colorectal, oral, transdermal, and buccal routes [5]. Although the nasal route is attractive because of the ease of administration, side effects including rhinitis, rhinorrhea, and allergic rhinitis are induced by excipients such as absorption enhancers and surfactants [6,7]. Vaginal delivery of peptide drugs generally exhibits low and variable bioavailability [8]. The colorectal delivery route provides low enzymatic metabolic environments and longer residence times; however, this route also provides very limited bioavailability [9]. Systemic peptide drug delivery through an oral route is limited by the large molecular size, charges, and hydrophilicity of the peptide drug itself, as well as its susceptibility to proteolytic degradation within the GI tract and at biomembrane interfaces [10]. Transdermal or buccal routes have been recognized as desirable, offering better patient compliance than other delivery routes. Recently, transbuccal peptide delivery has been in the limelight because this route can avoid hepatic first-pass metabolism and gastrointestinal degradation. In addition, the buccal route is easy and safe for self-medication and has much better patient compliance than the parenteral route. Also, this route has fast onset of action, and little irritation is expected [11]. Moreover, buccal tissue has rapid recovery properties compared with other mucosal sites [1]. Hoogstraate and Wertz summarized well the potentials of buccal drug delivery compared with other administration routes [11].

To enhance the transbuccal drug delivery, absorption-enhancing methods have been introduced despite the non-keratinization of buccal tissue because the oral mucosa protects the body from external influences and the intercellular lipids within buccal tissue act as a physical barrier [11,12]. Among these enhancing methods, chemical enhancers are the general choice, but iontophoresis using a low

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electric current offers immense potential for the delivery of charged peptide drugs [13]. In transdermal drug delivery, a combination of two enhancing methods has been well established for the delivery of peptide drugs [13,14].

Recently, some researchers have reported iontophoretic delivery of small molecular chemical drugs through buccal tissues, in particular the enhanced delivery of atenolol·HCl and naltrexone [15–17]. However, there have been no reports regarding transbuccal delivery of peptide drugs using iontophoretic methods.

In the present study, we chose salmon calcitonin (sCT) as a model drug and evaluated *in vitro* sCT delivery through the porcine buccal route. sCT is a polypeptidic hormone composed of 32 amino acids and is secreted from C-cells of the thyroid gland. For the inhibition of bone resorption and excitation of bone formation by osteoclasts, sCT has been used clinically for the treatment of Paget's disease, hypercalcemia, and postmenopausal osteoporosis [7,18]. The general drawback of oral sCT delivery is low bioavailability [19], and the nasal formulations showed not only low bioavailability [18] but also side effects such as rhinitis, rhinorrhea, and allergic rhinitis [7]. Thus, we report here the first transbuccal delivery of sCT by the combination of chemical enhancers and electrical assistance (in the form of a fixed 0.5 mA/cm^2 current). To evaluate the irritation caused by the two enhancing methods, we used Fourier transform-infrared (FT-IR) spectroscopy to investigate conformational changes of the buccal epithelial lipids. In addition, histological evaluation of the buccal specimen was made to confirm any damage to tissue integrity after the application of the chemical and physical enhancing methods.

2. Materials and methods

2.1. Materials

Salmon calcitonin (sCT) was purchased from Bachem AG (Bubendorf, Switzerland). *N*-acetyl-L-cysteine (NAC), sodium deoxyglycocholate (SDGC), and silver wire (diameter 1.0 mm) were obtained from Sigma–Aldrich (St. Louis, USA). The DC power supply was obtained from UNICORN Co. (Anyang, Republic of Korea), and a digital multimeter was provided by EZ Digital Co. (Bucheon, Republic of Korea). An ELISA kit for the analysis of the permeated amount of sCT was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). All other chemicals and solvents were of reagent grade.

2.2. Experimental methods

2.2.1. Pretreatment of porcine buccal tissues

The porcine buccal tissues (cheek) were obtained from freshly sacrificed 6-month-old pigs weighing about 110 kg and were transferred within 1 h to a laboratory while maintaining a temperature of 4 °C to maintain cell viability. The slack connective tissues and adipose tissues were removed mechanically from the buccal mucosa using surgical scissors and scalpels. To obtain buccal epithelium, the tissues were soaked in pH 7.4 phosphate buffered saline (PBS) at 60 °C for 1 min following the protocol of Consuelo et al. [20]. Before and after the permeation experiments, the thickness of the buccal epithelial layer was measured using a vernier caliper (Mitutoyo, Japan). All measurement was taken three times.

2.2.2. Preparation of sCT formulations

We used pH 4.0 citrate buffer as the donor solution for several reasons. sCT maintains maximum stability near pH 3.3; thus, pH 4.0 was chosen to remain close to this yet minimize tissue irritation in actual application. Additionally, a pH below 4.0 may neutralize the negative charges of the cell membrane, which may reduce or reverse the direction of electroosmotic flow [18,21]. The isoelectric point (pI) of sCT is 10.4, and sCT will have a positive charge below this; thus, the positive charge of the peptide in pH 4.0 buffer will inevitably contribute to enhanced anodal delivery of sCT in anodal iontophoretic conditions.

The composition of control formulation was 50 mM citrate buffer, which contained 0.1% Tween 80® and 75 mM sodium chloride. The test formulations were prepared by adding enhancers to control formulation. All the formulation contained 200 IU (40 μg/ml) of sCT. The receptor solution was prepared with pH 7.4 phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} ions to mimic non-enzymatic plasma conditions of buccal tissue; this was made with Na_2HPO_4 (0.795 g), KH_2PO_4 (0.144 g), and NaCl (9.0 g) in 1 L of deionized water [15].

2.2.3. Preparation of Ag/AgCl electrode

The Ag/AgCl electrode was prepared by the method described by Jacobsen [16]. Briefly, 10 cm of Ag wire was soaked in distilled water, ethanol, and fuming nitric acid and rinsed thoroughly with distilled water. Each process was performed three times for 3 s. The wire was then dipped into 0.1 N HCl, and a regular 1.0 mA current was maintained for 24 h using another Ag wire as a cathode to coat AgCl to the surface of the first Ag wire.

2.2.4. In vitro sCT buccal permeation study

2.2.4.1. Effect of chemical enhancers on sCT permeation. The pretreated buccal tissues were fixed horizontally between the donor and receptor chambers of a Franz diffusion cell filled with pH 7.4 PBS. Before the permeation study, 1 mL of pH 7.4 PBS was preadded to the donor chamber for 30 min to maintain equilibrium between the donor and the receptor. The donor solution was removed completely with towels before the *in vitro* permeation study. A 1-mL sample of sCT formulation was loaded onto the donor chamber, and the bubbles within the receptor chamber were removed carefully to maintain an effective diffusion area. To investigate the effect of enhancers on sCT buccal permeation, we used 10% of ethanol, various concentrations (1%, 2% and 5%) of *N*-acetyl-L-cysteine (NAC), sodium deoxyglycocholate (SDGC), and a mixture of these as chemical enhancers. We withdrew 200 μL of sample from the receptor chamber at predetermined time intervals and replaced it with same volume of fresh PBS solution. Each experiment was performed on 37 °C for 8 h and repeated in triplicate.

2.2.4.2. Effect of electrical assistance on sCT permeation. We evaluated the effects of electrical assistance alone or in combination with chemical enhancers. An Ag/AgCl electrode was used to prevent the production of protons in the anode, which would induce tissue irritation and decrease drug stability [22]. Electric resistance (Ω) of the tissues was measured before and after a permeation study. A constant 0.5 mA/cm^2 fixed electric current was applied using a DC power supply and maintained by a current controller. An Ag plate (diameter: 16 mm, thickness: 1 mm) was placed on the donor chamber as the anode, and an AgCl electrode was used as the cathode. The experimental conditions are depicted in Fig. 1. The rest experimental procedures were performed as mentioned earlier (Section 2.2.4.1), unless otherwise specified.

2.2.5. Analysis of permeated sCT

The amount of permeated sCT was analyzed with an ELISA kit, and the transbuccal parameters were measured and compared between formulations and experimental conditions. The steady state flux (J_s) was calculated from the linear part of the permeation curve using Equation (1), where Q_r is the total permeated sCT (ng), A is the cross-sectional diffusion area (cm^2), and t is the time of exposure (h).

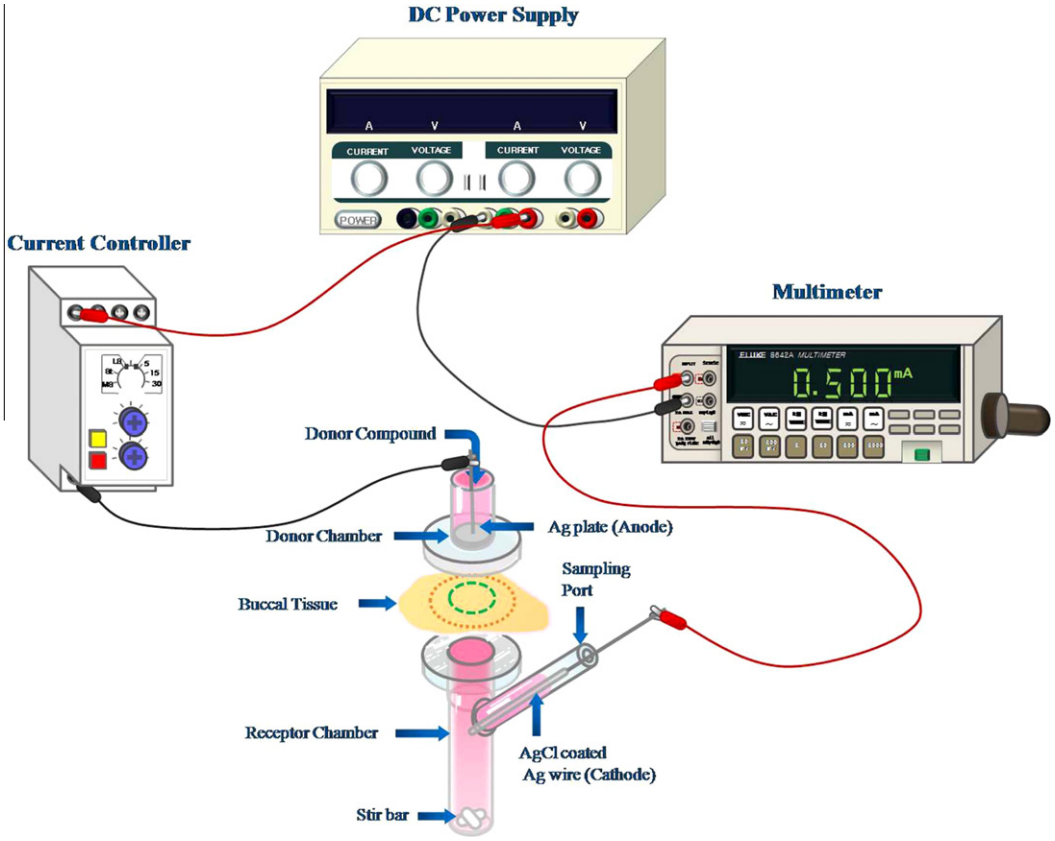

Fig. 1. Schematic drawing of the experimental design for iontophoresis. Effective diffusion area was 2.0 cm^2, and the receptor was maintained at 37 °C and stirred with a magnetic bar. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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

The permeability coefficient (K_p) was calculated using Eq. (2), where J_s is the flux from the steady state (ng cm^{-2} h^{-1}) and C_d represents the initial concentration in the donor chamber (ng cm^{-3}). The enhancement ratio (ER) was obtained by dividing the K_p value of each formulation with that of the control.

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

2.2.6. FT-IR spectroscopy study

A Fourier transform-infrared (FT-IR) spectrophotometer (Varian 1000 FT-IR, Varian, USA) was employed to evaluate the effects of chemical enhancers and electricity on the biophysical state of buccal tissues. The buccal samples were freeze-dried and ground in liquid nitrogen to prepare disks. The ground buccal tissues were mixed with KBr powder, and the pellets were prepared under conditions of 20 °C and 30% relative humidity. The pellet was prepared under 7.54 ton/cm^2 of pressure. The FT-IR spectra of porcine buccal samples were measured in the range of 4000–800 cm^{-1}, and spectra within 3000–2800 cm^{-1} were reviewed for the discussion of the results. The final spectrum was obtained from the averages of 32 scans.

2.2.7. Histological evaluation

After the permeation study, the buccal tissues were washed with saline solution. The washed tissues were fixed with 4% neutral buffered paraformaldehyde for 4 h, and the water was completely removed by soaking in 12%, 16%, and 20% sucrose solutions in turn. The fixed buccal samples were embedded within silver foil molds using Optimal Cutting Temperature compound. The mold was dipped into liquid nitrogen, and the samples were cryosectioned at 10 μm thickness using a cryotome (Microtome Cryostep HM 525, Germany) and stained with hematoxylin/eosin (H&E). The final samples were examined using a light microscope (Olympus BX 51, Japan) mounted with a digital camera (Olympus DP70, Japan). Three slides for every tissue specimen were prepared for microscopic examination.

2.2.8. Statistics

The results were represented as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA. The significance of differences in drug flux (J_s) and K_p was determined by one-way ANOVA using the Student–Newman–Keuls test and Tukey as the multiple comparison method. For all the data, a single, double, or triple asterisk was used if the p-value is less than the 0.05, 0.01, or 0.001 level of significance, respectively.

3. Results and discussion

Human buccal tissue is 500- to 800-μm-thick non-keratinized tissue. To simulate the *in vivo* environment of human cheek, we used about 500- to 800-μm-thick porcine buccal tissue, which is similar to the human tissue. In buccal delivery, the mucosa layer is considered a barrier to be overcome; thus, enhancing methods are required for transbuccal drug delivery. For the enhancement of sCT buccal delivery, we used three kinds of chemical enhancers (ethanol, NAC, and SDGC), the physical enhancing method of iontophoresis, and the combination of chemical and physical enhancement.

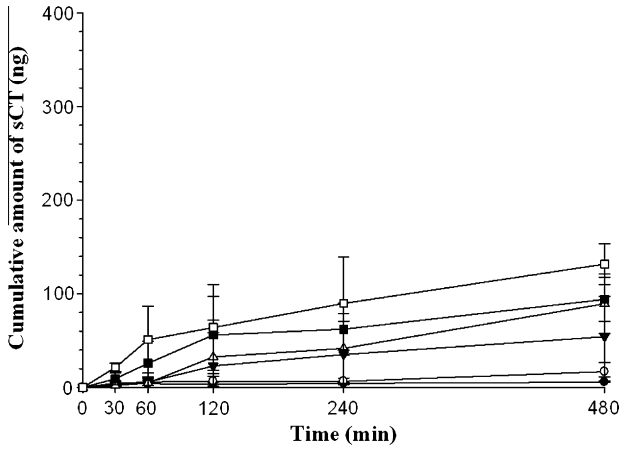

Fig. 2. Effect of NAC on the transbuccal delivery of sCT ($n = 3$). ●, control; ○, 10% ethanol; ▼, 1% NAC with no ethanol; △, 1% NAC with 10% ethanol; ■, 2% NAC with 10% ethanol; □, 5% NAC with 10% ethanol.

3.1. Effect of chemical enhancers on the transbuccal permeation of sCT

Ethanol is considered as a good chemical enhancer not only for transdermal delivery but for buccal delivery. The enhancement mechanism of ethanol is the alteration of the intercellular lipids of the buccal epithelium by perturbing the systematic arrangement of lipid molecules [23,24]. Although the J_s of sCT in the ethanol group increased compared with that of the control group, no statistically significant differences were observed (Fig. 2).

Mucolytic agents are useful for transmucosal delivery because these agents increase the permeation efficiency of drug molecules and the damage of mucosa can be minimized [25]. NAC, a mucolytic agent, has been used as a permeation enhancer for transmucosal delivery of hydrophilic macromolecules. Matsuyama et al. used the combination of NAC and non-ionic surfactant to enhance nasal delivery of sCT. NAC enhanced the accessibility of epithelial membrane to active pharmaceutical ingredient by decreasing the viscosity of mucus on the mucosa [25]. In our study, NAC enhanced the permeation of sCT, and the enhancing effect was insignificant but apparently NAC concentration dependent (Fig. 2).

SDGC is a bile salt that is widely used to improve the transbuccal delivery of drugs. At low concentration of SDGD below 10 mM, SDGC enhanced buccal permeation by interaction with polar intercellular lipids; thereby, the intercellular transport of hydrophilic compounds was dose dependently increased. At higher concentration of 100 mM SGDC, however, transcellular permeation was possible because SDGC solubilized the hydrophobic cell membrane lipids and diffused from the intercellular space into the cytoplasm [12]. Hoogstraate et al. [26] also reported that the 10 mM low-concentration SDGC enhanced the flux of FITC-dextran through paracellular routes, but high concentrations of 100 mM SDGC enhanced drug delivery through paracellular and transcellular routes. Our study tested SDGC concentrations of 1%, 2%, and 5%, and these concentrations are comparable with 21.2, 42.4, and 105.9 mM of SDGC, respectively. As shown in Fig. 3, the optimal concentration of SDGC for transbuccal sCT delivery was about 1% SDGC (21.2 mM). Because sCT has a strong positive charge in the present formulation, permeation enhancement by SDGC considered to be happened primarily via the paracellular pathway.

The combination of 10% ethanol with SDGC or NAC enhanced the delivery of sCT compared with the groups that contain single enhancers (Table 2). In the passive delivery groups, 1% SDGC with 10% ethanol and 5% NAC with 10% ethanol showed the maximum J_s.

Table 1
The changes of buccal epithelial thickness and resistance after permeation study ($n = 3$).

	Tissue thickness (mm)		Tissue resistance (%)
	Before	After	(After/before × 100)
Intact	0.867 ± 0.058	ND	98.4 ± 5.5
Control (no enhancer)	0.767 ± 0.029	1.117 ± 0.029	97.8 ± 8.1
10% EtOH	0.717 ± 0.029	0.900 ± 0.173	105.4 ± 4.7
1% NAC (no EtOH)	0.700 ± 0.000	1.200 ± 0.100	84.4 ± 14.5
1% NAC (10% EtOH)	0.767 ± 0.029	1.400 ± 0.100	52.4 ± 8.4
2% NAC (10% EtOH)	0.700 ± 0.050	0.967 ± 0.115	39.4 ± 18.3
5% NAC (10% EtOH)	0.783 ± 0.029	1.067 ± 0.058	17.3 ± 2.9
1% SDGC (no EtOH)	0.700 ± 0.000	1.283 ± 0.202	100.7 ± 7.7
1% SDGC (10% EtOH)	0.650 ± 0.050	1.317 ± 0.104	103.3 ± 5.9
2% SDGC (10% EtOH)	0.600 ± 0.050	1.317 ± 0.076	97.6 ± 10.1
5% SDGC (10% EtOH)	0.517 ± 0.029	1.017 ± 0.104	98.5 ± 9.0
ITP (no enhancer)	0.633 ± 0.029	0.700 ± 0.132	70.6 ± 22.0
ITP + 5% NAC (10% EtOH)	0.683 ± 0.029	0.967 ± 0.293	9.5 ± 1.8
ITP + 5% SDGC (10% EtOH)	0.717 ± 0.076	1.300 ± 0.100	78.8 ± 25.1

ND: Not Determined. NAC, *N*-acetyl-L-cysteine; SDGC, Sodium deoxyglycocholate; ITP, Iontophoresis.

3.2. Effect of iontophoresis on the transbuccal permeation of sCT

We measured the buccal thickness and electric resistance (Ω) of the buccal epithelium at initial and end points because this value would reflect the effect of electric current on the integrity of buccal

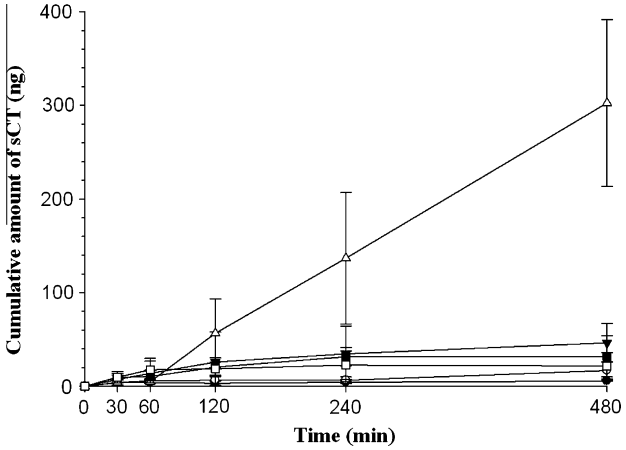

Fig. 3. Effect of SDGC on the transbuccal delivery of sCT ($n = 3$). ●, control; ○, 10% ethanol; ▼, 1% SDGC with no ethanol; △, 1% SDGC with 10% ethanol; ■, 2% SDGC with 10% ethanol; □, 5% SDGC with 10% ethanol.

Table 2
Permeation parameters calculated from sCT transbuccal delivery ($n = 3$).

	J_s (ng cm^{-2} h^{-1})	K_p (cm/h) × 10^{-3}	ER
Control (no enhancer)	0.356 ± 0.015	0.009 ± 0.000	1.0
10% EtOH	1.056 ± 0.614	0.027 ± 0.016	3.0
1% NAC (no EtOH)	3.382 ± 2.691	0.086 ± 0.068	9.5
1% NAC (10% EtOH)	5.585 ± 1.976*	0.142 ± 0.050*	15.7
2% NAC (10% EtOH)	5.874 ± 1.480*	0.149 ± 0.038*	16.5
5% NAC (10% EtOH)	8.233 ± 1.381*	0.209 ± 0.035*	23.1
1% SDGC (no EtOH)	2.906 ± 1.296	0.074 ± 0.033	8.2
1% SDGC (10% EtOH)	18.905 ± 5.571**	0.481 ± 0.142**	53.1
2% SDGC (10% EtOH)	2.000 ± 1.376	0.051 ± 0.035	5.6
5% SDGC (10% EtOH)	1.360 ± 0.880	0.035 ± 0.022	3.8
ITP (no enhancer)	23.554 ± 10.534***	0.599 ± 0.268***	66.1
ITP + 5% NAC (10% EtOH)	28.247 ± 9.322***	0.718 ± 0.237***	79.3
ITP + 5% SDGC (10% EtOH)	58.703 ± 7.223***	1.493 ± 0.184***	164.8

ER: Enhancement ratio; NAC, *N*-acetyl-L-cysteine; SDGC, Sodium deoxyglycocholate; ITP, Iontophoresis.
* $p < 0.05$,
** $p < 0.01$,
*** $p < 0.001$.

tissues. After the permeation study, the thickness of buccal tissues was increased in all groups but the electric resistance (Ω) of the buccal epithelium was decreased in NAC and iontophoresis applied groups (Table 1).

Iontophoresis reduces electric resistance of tissue membrane [27] and NAC affects the electrical properties of epithelial cells by reducing trans-epithelial voltage (V_m) and increasing whole-cell conductance (G_m) [28]. As shown in Table 1, the electric resistances of NAC and iontophoresis applied groups were decreased compared with those of the control group. On combining iontophoresis with 5% NAC with 10% ethanol, the electric resistance of buccal epithelium was decreased dramatically because NAC solubilizes the mucus layer, which is the main barrier for buccal permeation of sCT [25].

Iontophoresis, a physical enhancing method using electricity, has been used to increase local or systemic delivery of hydrophilic and charged compounds. The mechanism of iontophoresis enhancement is well known to be a combination of electro-repulsion and electro-osmosis, and this method enhances drug delivery mainly through the paracellular pathway of the biological membrane [15,16,29,30]. We used an Ag/AgCl electrode as the active electrode for the iontophoretic delivery because inactive electrodes such as carbon or platinum induce proton production that causes tissue irritation and reduces stability or delivery effect of drugs [22]. When 0.5 mA/cm^2 of fixed anodal current was applied for the transbuccal sCT delivery, the J_s was significantly enhanced compared with that of the absorption enhancer groups. The enhancement ratio (ER) of the iontophoresis groups was at least 66-fold higher than that of the control group. The combination of iontophoresis and chemical enhancers further enhanced transbuccal sCT delivery, providing an approximately 165-fold increase in the ER value (Fig. 4, Table 2).

3.3. FT-IR spectroscopy study

The physical attributes of buccal tissue affect the drug flux and are closely related to the understanding of permeation mechanisms [31]. The lipid bilayers exist in the gel state (ordered) or the sol state (fluidized), and the status of a lipid bilayer can be seen by the FT-IR study of its components [32]. Thus, FT-IR spectroscopy was employed to evaluate the effect of permeation-enhancing methods on the biophysical properties of buccal epithelial lipids. The peaks near 2850 and 2920 cm^{-1} are consider important because they have

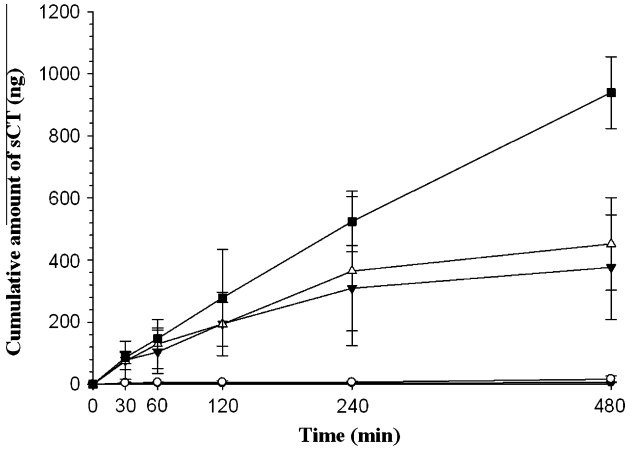

Fig. 4. Effect of electrical assistance on the transbuccal delivery of sCT ($n = 3$). ●, control; ○, 10% ethanol; ▼, iontophoresis with no ethanol; △, iontophoresis and 5% NAC with 10% ethanol; ■, iontophoresis and 5% SDGC with 10% ethanol.

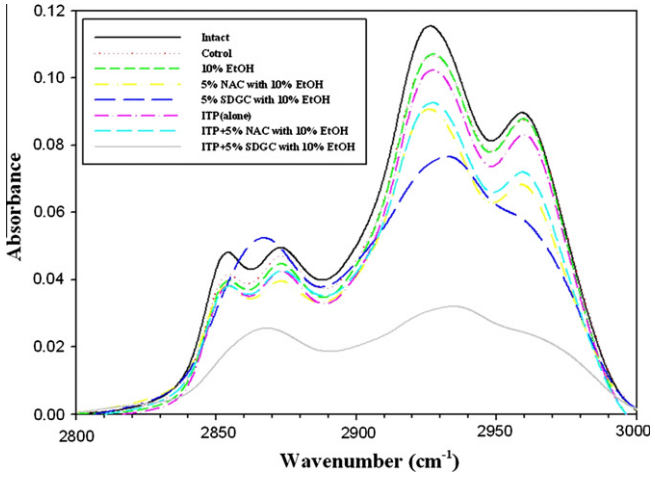

Fig. 5. FT-IR spectra of the excised porcine buccal mucosa. Symmetric and asymmetric C—H bond stretching absorbances were observed after the application of enhancing methods. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

been shown to represent the symmetric and asymmetric carbon–hydrogen (C—H) stretching of the lipids, respectively [33].

The increase in lipid bilayer fluidity that is intimately connected with the transbuccal drug flux can be explained by two mechanisms. The first mechanism is the numerical increase in gauche conformers into the lipid hydrocarbon chains, which results in increasing molecular motion as the gel to liquid crystalline transition occurs, and the status can be shown as the higher wave number shift of the symmetric and asymmetric C—H stretching absorbance peaks [34–37]. The second mechanism is the growth in translational movement or mobility of lipid acyl chains, which causes a broadening of the C—H stretching absorbance peaks at 2850 and 2920 cm^{-1} [38,39]. Moreover, the reduction in the C—H stretching frequency is also related to the increase in transbuccal drug flux, which can be seen when the epithelial lipids are extracted by the permeation-enhancing methods [31,40].

Fig. 5 depicts FT-IR spectra from 3000–2800 cm^{-1} of the buccal epithelia treated with chemical or physical enhancing methods. The test groups containing NAC exhibit a reduction in the C—H stretching frequency as a result of epithelial lipid extraction by NAC. The FT-IR spectra of SDGC alone and in combination with iontophoresis show not only the broadening of the peak but also a decrease in peak intensity, and the peak shifts towards higher wave numbers (Table 3). In other words, the treatment of buccal epithelium with SDGC alone or in combination with iontophoresis caused an increase in both the lipid bilayer fluidity and the epithelial lipid extraction, ultimately increasing transbuccal permeation of the sCT. Compared with the control, the C—H stretching absorbance peak of the sample experiencing solely iontophoresis shows no difference except for a slight reduction in peak intensity. Such a feature might result from the fact that iontophoresis is solely a physical enhancing method that uses electric current [15]. The combination of SDGC and iontophoresis greatly increased the lipid fluidity, but no significant differences were seen in the C—H stretching absorbance peaks between NAC alone and combination of NAC with iontophoresis (Fig. 5).

3.4. Histological study

Because permeation-enhancing methods may cause some structural change in the buccal tissue, we carried out a histological study to investigate such effects. Cryosections of the buccal epithelia stained with hematoxylin/eosin (H&E) are shown in Fig. 6. Com-

Table 3
Effect of enhancing methods on the asymmetric and symmetric C—H bond stretching absorbance peaks of buccal epithelium as measured by FT-IR ($n = 3$).

	Intact	Control (no enhancer)	10% EtOH	5% SDGC with 10% EtOH	5% NAC with 10% EtOH	ITP (no enhancer)	ITP + 5% SDGC with 10% EtOH	ITP + 5% NAC with 10% EtOH
Symmetric 2850 cm^{-1}	2854.9 ±0.35	2855.03 ±0.15	2854.9 ±0.42	2866.63 ±0.31	2854.3 ±0.17	2854.9 ±0.28	2866.73 ±1.07	2855.0 ±0.26
Asymmetric 2920 cm^{-1}	2926.93 ±0.55	2927.43 ±0.35	2927.05 ±0.78	2931.67 ±0.57	2926.53 ±1.69	2927.2 ±0.42	2933.03 ±1.27	2926.77 ±0.46

NAC, *N*-acetyl-L-cysteine; SDGC, Sodium deoxyglycocholate; ITP, Iontophoresis.

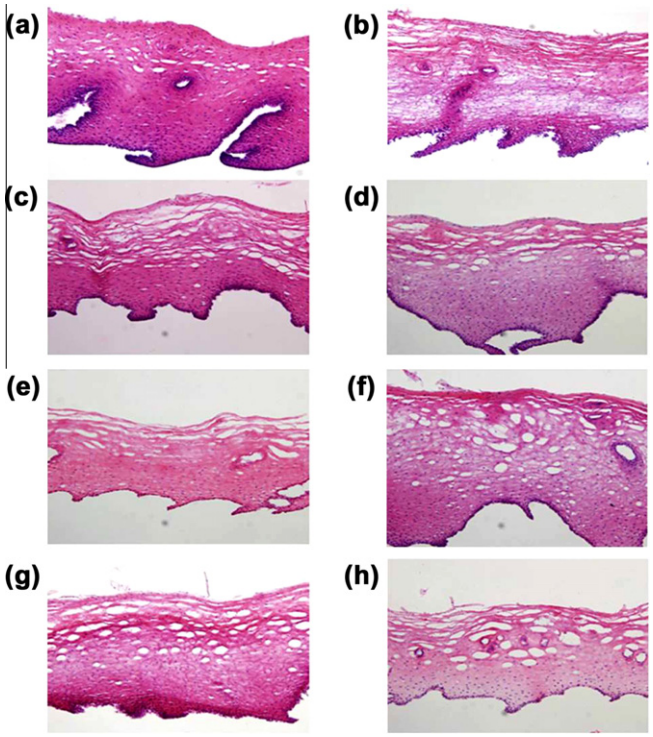

Fig. 6. Microphotographs of formalin-fixed, OCT-embedded cross-sections of porcine buccal mucosa: (a) Intact, untreated. (b) Control, treated with pH 7.4 PBS for 8 h. (c) EtOH, 10% ethanol treatment for 8 h. (d) NAC, 5% NAC with 10% ethanol treatment for 8 h. (e) SDGC, 5% SDGC with 10% ethanol treatment for 8 h. (f) ITP, 0.5 mA/cm^2 of fixed anodal current treatment for 8 h. (g) ITP + NAC, 0.5 mA/cm^2 of fixed anodal current with 5% NAC (10% ethanol) treatment for 8 h. (h) ITP + SDGC, 0.5 mA/cm^2 of fixed anodal current with 5% SDGC (10% ethanol) treatment for 8 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pared with the untreated no enhancer group, the control group treated with pH 7.4 PBS was slightly swelled (Fig. 6b), and the group treated with 10% ethanol exhibited slight enlargement of some cell layers (Fig. 6c). Photographs of tissue treated with NAC, iontophoresis, and a combination of these (Fig. 6d, f and g) were similar to those of the 10% ethanol group. However, Veuillez et al. [41] have reported that 30% ethanol caused little or no damage to the structure of porcine buccal epithelium. They also observed that the desquamation of the surface layer and the swelling of the epithelial cells in their study samples were no different from what is observed due to the passage of time. Similarly, desquamation of the surface layer and swelling of the epithelial cells were seen in the images of our present results, but we could regard them as natural phenomena.

Both SDGC alone and SDGC in combination with iontophoresis (Fig. 6e and h) exhibited a noticeable reduction in the thickness of the surface layer of the epithelium, whereas such changes were not apparent in other groups. Squier et al. [42,43] carried out an *in vivo* study using rabbit and porcine non-keratinized oral mucosa and discussed that the permeation barrier was at the surface layer of the buccal epithelium. According to Squier et al. [42,43], the dramatic increase in drug flux caused by SDGC alone and SDGC in combination with iontophoresis was due to the loss of the permeation barrier. Likewise, we found a decrease in the number of cell nuclei, as well as a mild loss of nuclei polarity in tissue treated with SDGC alone and with a combination of SDGC and iontophoresis (Fig. 6e and h). Consequently, there were no significant cytological or structural alterations in the test groups, with the exception of the groups treated with SDGC.

4. Conclusion

The present study shows that the combinations of NAC/ethanol and SDGC/ethanol have potential for the buccal delivery of sCT and that application together with anodal iontophoresis was a more helpful strategy. The FT-IR study validated the effect of the enhancing methods by measuring the changes in lipid fluidity of the test groups, and the J_s of sCT was proportional to the augmentation of lipid fluidity. Histological evaluation showed that SDGC caused cytological or structural changes in the buccal epithelium. Although the histological data of tissue treated with SDGC alone and in combination with iontophoresis show some possibility for tissue damage, the present enhancing methods are considered safe. From the present study, we conclude that the combination of absorption enhancers and electrical assistance is a potential strategy for the enhancement of transbuccal sCT delivery.

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