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RESEARCH PAPER

Strategic Approaches for Enhancement of *In Vivo* Transbuccal Peptide Drug Delivery in Rabbits using Iontophoresis and Chemical Enhancers

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ABSTRACT

Purpose To evaluate the feasibility of iontophoresis and the combination effects with chemical enhancers on *in vivo* hypocalcemic effect of transbuccally delivered salmon calcitonin (sCT).

Methods *N*-acetyl-L-cysteine (NAC), sodium deoxyglycocholate (SDGC), and ethanol were used as chemical enhancers; and 0.5 mA/cm^2 fixed electric current was employed as a physical enhancer. sCT hydrogel was applied to rabbit buccal mucosa, and blood samples were obtained via the central auricular artery. Blood calcium level was measured by calcium kit and the conformational changes of buccal mucosa were investigated with FT-IR spectroscopy. Hematoxylin/eosin staining was used for the histological evaluation of buccal mucosa.

Results Iontophoresis groups except iontophoresis-NAC group showed significant hypocalcemic effect compared to negative control, in particular iontophoresis-SDGC combination group showed fast onset of action as well as sustained hypocalcemic effect ($p < 0.05$). FT-IR result demonstrated the reduction of buccal barrier function, and the histological study showed a decrease in buccal thickness as well as minor damage to the dermal-epidermal junctions in the enhancing method groups; however, the damaged tissues virtually recovered within 24 h after the removal of electrodes.

Conclusions Iontophoresis and combination with SDGC were found to be safe and potential strategies for transbuccal peptide delivery *in vivo*.

KEY WORDS buccal delivery · chemical enhancer · hypocalcemic effect · iontophoresis · peptide delivery

INTRODUCTION

Many endogenous peptides which regulate endocrine and other physiological conditions in the human body are highly efficient. For this reason, the use of bioactive peptides such as insulin, calcitonin, LHRH (Luteinizing hormone-releasing hormone) derivatives, and somatostatin have been extensively studied [1, 2]. Delivering therapeutically active biopharmaceuticals via the oral route, however, has been a challenge for decades because of the low bioavailability, resulting from poor intrinsic permeability of peptides and enzymatic degradation or extreme pH in the

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gastrointestinal tract. Therefore, they are unsuitable for oral delivery and have been mostly delivered via a parenteral route [1]. Although parenteral route has many benefits, there have been some serious problems with injection. As peptides have a short plasma elimination half-life, repeated injections are needed [1]. This is one of the most serious problems of injection, because it is fundamentally related to patient compliance. Another problem is side effects such as phlebitis or tissue necrosis due to repeated injection. Thus, potential alternative delivery methods via nasal, vaginal, rectal, oral, transdermal, and buccal routes have been considered in order to find solution [2–5].

Among the alternative routes, buccal tissue has been frequently studied because it offers a number of advantages. It can avoid the first-pass hepatic metabolism and gastrointestinal degradation. It also may provide easy administration with little irritation, thereby improving patient compliance [6]. The absorption of drug via the buccal route is not affected by the potential variations in the gastric-emptying rate or the presence of foods [7]. In addition, buccal mucosa shows a short recovery time after stress or damage compared to other mucosal sites. Moreover, it is more suitable for sustained drug delivery especially for peptide drugs and it is resistant to potential allergens due to the virtual lack of Langerhans cells [8].

Despite the non-keratinization of buccal mucosa, various permeation enhancing strategies are often required as the mucosal lining protects the body from external environments, and the intercellular lipid within buccal epithelial tissue acts as a physical barrier [6, 9]. Buccal mucosa is surrounded by the mucus layers of 40~300 μm thickness and involved in production and secretion of mucus consisting of mucin and suspended mineral salts in water [10]. In the buccal epithelia, mucus is particularly secreted by the major and minor salivary glands as part of saliva, unlike other stratified squamous epithelia in the body [8]. Thus, mucus is very important to maintain the barrier property of buccal tissue and saliva covering the mucus layers also acts as a barrier of buccal mucosa [10].

In general, the mechanisms of chemical enhancers on mucosal absorption of macromolecules are mentioned; i) by changing mucus rheology, ii) by increasing the fluidity of lipid bilayer membrane, iii) by acting on the components at tight junctions, and so on [10]. In addition, physical enhancements such as iontophoresis and phonophoresis (or sonophoresis) have been considered to enhance the efficiency of transbuccal drug delivery. In particular, iontophoresis uses a low electric current and it is advantageous in trans-membrane delivery of charged, hydrophilic, and macromolecular drugs [11]. Some researchers have previously investigated the feasibility for transbuccal iontophoretic delivery of small molecule drugs [12, 13], but so far no studies have been reported regarding *in vivo* transbuccal iontophoretic delivery of macromolecular drugs, except for some *in vitro* studies [5, 14, 15].

In this study, we used sCT as a model peptide drug and evaluated *in vivo* transbuccal sCT delivery in rabbits. sCT is a polypeptidic hormone consisting of 32 amino acids, which influences calcium metabolism in the gut, kidney, and bone. It is secreted from C-cells in the thyroid glands, and plays a crucial role by inducing the inhibition of osteoclastic bone resorption. sCT has been used clinically for the treatment of Paget's disease, hypercalcemia, and postmenopausal osteoporosis [16]. The analgesic activity of sCT in fracture-induced bone pain has been well established both through clinical trial and specific experimental investigation [17, 18]. Although the mechanism of its analgesic effect is still unclear, it is thought to be a result of direct central action of calcitonin [17]. The pharmacological activity of sCT is similar to human calcitonin (hCT), but sCT has 40–50 times higher intrinsic potency than hCT [19].

Like many other bioactive peptides, the peroral administration of sCT has been considerably hindered by poor absorption and rapid proteolytic degradation. Therefore, most commercialized sCT products were developed to be injected or used as a nasal spray. These formulations need frequent administration for continuous action due to the short half-life of sCT and low bioavailability. Thus, buccal tissue may be an appropriate alternative delivery route for sCT because it is advantageous for the sustained release of drugs [8].

We have reported the first study for *in vitro* iontophoretic transbuccal delivery of peptide drug using porcine buccal tissues [5], and we report here first again *in vivo* transbuccal delivery of sCT, a model peptide, using iontophoresis. The proper selection of laboratory animal as well as the design of specific methodology is very important in order to evaluate drug permeation across the buccal membrane. Many researchers have used small animals such as rats or hamsters for *in vivo* buccal drug permeability studies [20]. However, such studies have a problem, because unlike humans, buccal epithelial tissues of most laboratory animals are keratinized. Thus, we consider rabbits as the most suitable animal for *in vivo* buccal drug delivery experiments as they are small with non-keratinized buccal mucosa similar to humans [21].

The purpose of the present study was to evaluate the effect of iontophoresis, chemical enhancers, and their combination on the *in vivo* hypocalcemic activity of sCT in rabbits. The conformational changes of rabbit buccal epithelial lipids were characterized with FT-IR spectroscopy, and the safety of enhancing methods on the buccal tissue and *in vivo* recovery after treatment were evaluated by hematoxylin-eosin staining.

MATERIALS AND METHODS

Materials

sCT was obtained from Bachem AG (Bubendorf, Switzerland). *N*-acetyl-L-cysteine (NAC), sodium

deoxyglycocholate (SDGC), and silver wires (diameter 1.0 mm) were purchased from Sigma Aldrich (St. Louis, MO, USA). Poloxamer 407 (Pluronic® F-127) was obtained from BASF Wyandotte (Parsippany, NJ, USA) and water-soluble chitosan (WSC) from crab shell was purchased from Jakwang Co., Ltd (Suwon, Republic of Korea). The deacetylation degree of WSC was c.a. 88% and it was used without further purification. The DC power supply was purchased by Unicorn Co., Ltd (Anyang, Republic of Korea), and a digital multimeter was provided by EZ Digital Co., Ltd (Bucheon, Republic of Korea). The Ca^{2+} kit (FUJI-dry-chem slide Ca-PIII), to analyze hypocalcemic effects of sCT, was purchased from FUJI film (Tokyo, Japan). All other reagents were of analytical grade and used as received without further purification.

Animal Treatment

All experimental procedures were designed according to the 3R (Replacement, Refinement and Reduction) principle, and approved by the Institutional Animal Care and Use Committee (Protocol No. IJ-2011-4). The rabbits were kept under pathogen-free conditions in the Animal Center of Inje University, Republic of Korea. Male New Zealand white rabbits of 2.8±0.2 kg were used in this study. Rabbits were anesthetized with a solution of equal amounts of Zoletil 50® (Virbac Lab, France) and Xylazine HCl (Rompun®, Bayer, Korea). While the experiments were performed, the anesthesia was maintained by continuous infusion of the mixed anesthetic solution (12 $mg \cdot kg^{-1} \cdot h^{-1}$) using portable electronic infusion pump (Accumate® 1000, Wooyoung Medical, Korea).

Effects of Enhancing Methods on *In Vivo* Hypocalcemic Effects

Formulation of Hydrogel

We used pH 4.0 citrate buffer to prepare sCT hydrogel for several reasons. pH 4.0 was chosen to stabilize sCT because its maximum stability is maintained near pH 3.3 [22]. A pH lower than 4.0 was not selected to avoid buccal tissue irritation and to maintain the pH of the isoelectric point (pI) of the mucosa because the pI of rabbit buccal mucosa is known to be 2.7 [19, 23]. In addition, a lower pH than 4.0 can neutralize the negative electric charge of the tissue membrane which will reduce (or even reverse) the direction of electroosmotic flow, which would oppose anodal delivery. We used pH below 10.4, pI of sCT, because pH below 10.4 generates a positive charge on sCT for anodal iontophoretic delivery [5, 19]. Furthermore, chitosan is poorly water soluble above pH 6.0, where it loses its positive charge and finally precipitates from the solution [24].

In pH 4.0 citrate buffer, 150 mM sodium chloride and 15% Poloxamer 407 were added. In addition, the mucoadhesive property of the hydrogel and the stability of sCT were obtained by adding 2% (*w/v*) chitosan and 0.1% bovine serum albumin (BSA) to the formulation, respectively. The control formulation was prepared by these steps and the test hydrogels were prepared by the addition of enhancers into control formulation. All prepared formulations, free-sCT formulations, were stored in the refrigerator below 8°C overnight to degas and to stabilize the gel matrix. Before adding the active pharmaceutical ingredient, 0.5 mL of each hydrogel was loaded in individually prepared insulated-rubber mold embedded on the round electrode, and dry procedure was proceeded for 1 h at 40°C. These dry step was for mucoadhesiveness of hydrogel because dry or partially hydrated form is advantageous than fully hydrate form [25]. And then sCT stock solution was loaded into the each hydrogel. Final hydrogel formulations contained 200 IU of sCT (administration dose: 66 IU/kg) and are depicted in Fig. 1.

Effects of Chemical Enhancers on In Vivo Hypocalcemic Effects

In our previous study, we investigated the effect of different concentrations of chemical enhancers and a mixture of them on buccal permeation of sCT using porcine buccal mucosa *in vitro* [5]. Based on the results from that study, two chemical enhancer groups were used: i) a mixture of 1% sodium deoxyglycocholate (SDGC) with 10% ethanol, and ii) a mixture of 5%*N*-acetyl-L-cysteine (NAC) with 10% ethanol. Before applying the sCT formulations to the rabbits, their central auricular arteries were catheterized with a 22-gauge catheter needle, and blood was sampled at predetermined times. The rabbit was anesthetized with Zoletil 50® and Xylazine HCl (Rompun®), and then sCT formulations were placed onto the rabbit buccal mucosa. Experimental design to evaluate the effect of chemical enhancers was similar with Fig. 2, excluding electrical instruments (power supply, multimeter, current controller). After blood sampling, the same volume of 1.0% heparinized fresh saline was injected via an ear vein. The blood samples were allowed to clot for 30 min at room temperature and the serum was separated by

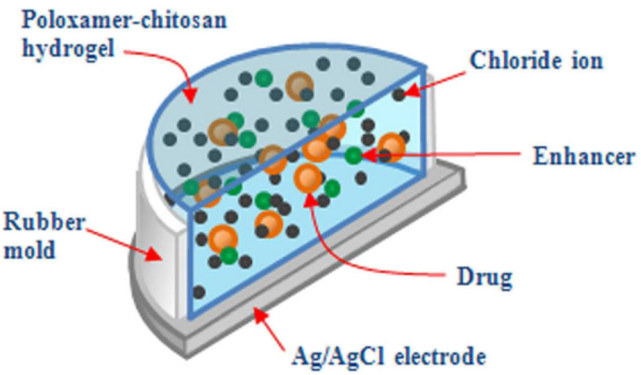


Fig. 1 Scheme of sCT buccal formulation. Chitosan provides mucoadhesiveness to the hydrogel and the Ag/AgCl electrode-rubber mold supports the shape and integrity of hydrogel.

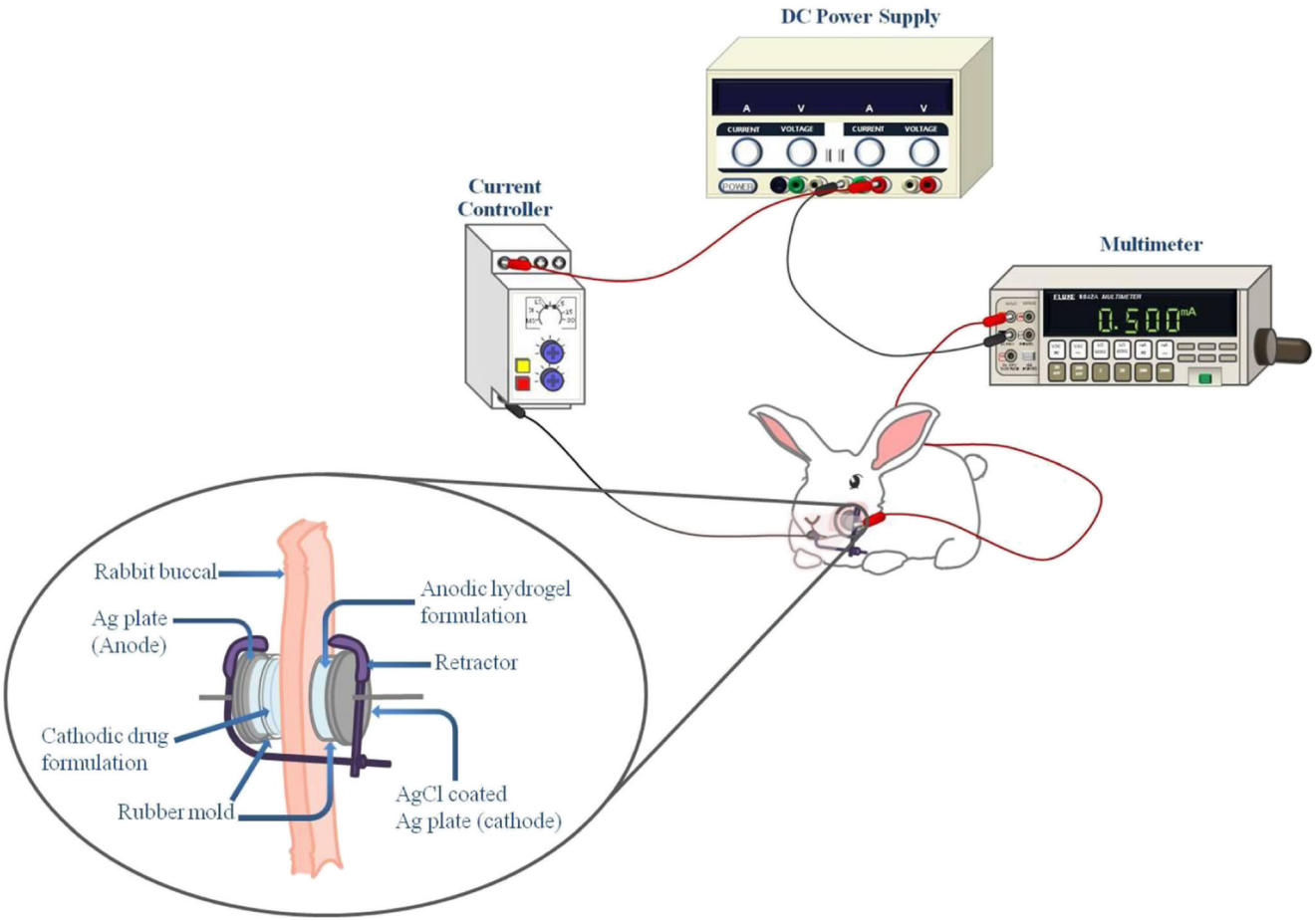


Fig. 2 Schematic drawing of experimental design for *in vivo* iontophoretic sCT buccal delivery. sCT containing hydrogel formulation was applied with insulated rubber mold onto the viable buccal mucosa of rabbits *in vivo*. The rabbits were anesthetized during permeation study. A continuous fixed 0.5 mA/cm^2 electric current was controlled by current controller.

centrifugation at 1,000×g, 4°C for 20 min. The rabbits were sacrificed after the last blood sampling and the sCT formulation applied to buccal tissues were carefully removed by surgical scalpel to evaluate tissue viability. Each experiment was performed for 4 h and repeated in triplicate.

Preparation of Ag/AgCl Electrode

The Ag/AgCl electrodes were prepared by Jacobsen's method [13]. Briefly, circular Ag plates (two sizes; 1.13 cm^2 for the buccal mucosa and 1.77 cm^2 for the skin) were attached to 1 cm of Ag wire, and the larger one for application onto the skin was dipped serially into distilled water, ethanol, and fuming nitric acid, and rinsed thoroughly with distilled water. Then it was immersed into 0.1 N HCl, and 1.0 mA of fixed current was maintained for 24 h using another Ag wire as a cathode to coat AgCl to the surface of the Ag plate.

Effects of Iontophoresis on In Vivo Hypocalcemic Effects

We investigated the single effect of electrical assistance and combination effect with chemical enhancers on the hypocalcemic effect of sCT. An Ag/AgCl electrode was selected to prevent the production of protons in the anode, which could irritate the buccal tissue, and decline of drug stability [26]. 0.5 mA/cm^2 of fixed electric current was constantly applied by a DC power supply and controlled using a current controller. An Ag plate (diameter: 12 mm, thickness: 1 mm, area: 1.13 cm^2) was placed on rabbit buccal mucosa as an anode, and an AgCl cathode (diameter: 15 mm, thickness: 1 mm, area: 1.77 cm^2) was applied to the shaven cheek skin. The experimental situations are depicted in Fig. 2. Unless otherwise mentioned, the rest procedures were performed as described in the previous section. Detailed informations for chemical and physical enhancement methods were summarized in Table I.

Intravenous Bolus Injection of sCT

The rabbits were anesthetized as described in the previous section. 100 IU of sCT (administration dose: 33 IU/kg) dissolved in the citrate buffer was injected via an ear vein using a 1 mL disposable syringe with 26-gauge needle. Blood samples were collected at the desired time points and the same volume

Table I Informations for Chemical and Physical Enhancement Methods Included in Each Group

Groups	NC	No enhancer	NAC	SDGC	Iontophoresis	Iontophoresis-NAC	Iontophoresis-SDGC	
API	sCT	–	+	+	+	+	+	+
Chemical Enhancement	10% ethanol	–	–	+	+	–	+	+
	5% NAC	–	–	+	–	–	+	–
	1% SDGC	–	–	–	+	–	–	+
Physical Enhancement	iontophoresis (0.5 mA/cm^2)	–	–	–	–	+	+	+

NC Negative Control (no sCT formulation); *NAC N*-acetyl-L-cysteine; *SDGC* Sodium deoxyglycocholate

of 1% heparinized fresh saline was injected via an ear vein. Blood sampling and pretreatments were performed as described in the previous section.

Intranasal Delivery of sCT

In spite of same dosage, intranasal bioavailability of liquid formulation can vary by total dosing volume and the number of administration because the administration volume of the nasal cavity is limited [27]. Based on that study, an optimal dosage in this study was achieved by dosing twice, once into each nostril.

The nasal formulation was administered by Schipper's method [28]. The rabbits were anesthetized as described above, 200 IU of sCT (administration dose: 66 IU/kg) dissolved in the citrate buffer was administered with a Hamilton microliter syringe attached with 5 cm polyethylene tubing (medical grade, 0.58 mm id) on the nasal mucosa of rabbits laid straightly on their side. The tubing was inserted 1.0–1.5 cm into a naris, and a total volume of 100 μL of fresh sCT formulations were instilled into both nostrils (50 μL each) of the rabbit. The blood samples were collected at the desired time points and the same volume of 1% heparinized fresh saline was injected via an ear vein. Blood sampling and pretreatments were performed as described in the previous section.

The Measurement of Blood Calcium Level

Normal calcium concentration of New Zealand white rabbits is 10.1–15.8 mg/dl (mean 12.8±1.04 mg/dl). We checked blood calcium levels of rabbits before the experiment and excluded the rabbits showing abnormal calcium levels. The calcium levels of serum samples were measured using Ca^{2+} kit (Fuji Dry-Chem Slide Ca-PIII, Fujifilm, Japan).

Fourier Transform-Infrared (FT-IR) Spectroscopy

A FT-IR spectrophotometer (Varian 1000 FT-IR, Varian, USA) was used to investigate the effects of chemical and physical enhancing methods on the biophysical changes of the buccal mucosal lipids which are closely related to the permeability of buccal mucosa [29].

The rabbits were sacrificed after the final blood sampling and the buccal tissues applied with sCT formulation were incised with a surgical scalpel. The connective and adipose tissues were trimmed using scissors to obtain the buccal epithelium. The pretreated buccal samples were freeze-dried and ground in a pestle and mortar under liquid nitrogen to prepare disks. The ground buccal samples were mixed with KBr powder and the disks were prepared under 7.54 kgf/cm^2 of pressure. The conditions for the disk preparation were 20°C and 30% relative humidity. The FT-IR spectra of rabbit buccal samples were obtained in the frequency range of 800–4,000 cm^{-1} and the final spectrum was shown as the average of 32 scans with 4 cm^{-1} resolution. We focused on two characteristic absorption bands at around 2,850 and 2,920 cm^{-1} which denote the symmetric and asymmetric C-H stretching vibrations of the buccal mucosal lipids, respectively.

Histological Study

The effects of chemical enhancers and electricity on tissue integrity were evaluated with the buccal tissues obtained immediately after the last blood sampling. We also evaluated *in situ* recovery abilities of damaged buccal tissue by keeping the rabbits for 24 h from the end of *in vivo* transbuccal permeation study. For histological analysis, the buccal epithelia were cut off from the sacrificed rabbits using surgical scalpel and scissors. The tissues were washed with saline solution and fixed in 4% neutral-buffered paraformaldehyde for 4 h, and the inner water of the tissue was completely eliminated by immersing into sucrose solutions in the order of 12, 16, and 20%. The fixed buccal samples were embedded within silver foil mold filled with optimal cutting temperature (OCT) compound, and then the mold was carefully dipped into liquid nitrogen. The embedded sample was cut into 10 μm-thick sections on a cryotome (Microtome Cryostep HM 525, Germany) with disposable blade, and conventionally stained with hematoxylin-eosin (H&E). Photomicrographs of the stained samples were taken at 200-fold magnification. Digital images were captured using a digital camera (Olympus DP70, Japan) mounted on a light microscope (Olympus BX 51, Japan). The

slide preparation of every tissue specimen for microscopic examination was performed in triplicate.

Statistical Analysis

All experiments for *in vivo* hypocalcemic effect were repeated in triplicate and the data were expressed as mean ± standard deviation (S.D.). Statistical analysis at each sampling time was performed by a one-way analysis of variance (ANOVA). The significance of differences in % of blood calcium level was determined by one-way ANOVA with post-hoc Tukey HSD (Honestly Significant Differences) analysis.

RESULTS AND DISCUSSION

We evaluated the effects of chemical and physical enhancements on *in vivo* transbuccal delivery of sCT by measuring the blood calcium level in rabbits. Although rabbits have non-keratinizatized buccal tissues, the transbuccal delivery of hydrophilic drugs will be hindered, as mentioned above, because of the main barriers such as mucus and intercellular lipids. That's why we used NAC (mucolytic agent), ethanol, and SDGC (lipid fluidity enhancers). In this study, we attempted various approaches to enhance the hypocalcemic effect of sCT; we used chemical enhancers (ethanol, NAC, and SDGC), iontophoretic physical enhancement, and a combination of both methods.

Chitosan is physiologically stable and a typical bioadhesive polymer for transbuccal delivery. Especially, optimal concentration of chitosan for mucoadhesiveness was reported as 2%; this concentration is advantageous for the drug release and the maintenance of viscosity in hydrogel [30]. Based on this literature, 2% chitosan hydrogel was used for muco-adhesive properties, which would increase the retention of the drug on the buccal mucosal surface and give the drug a chance to diffuse through buccal mucosa. Some hypothesize that chitosan acts as a enhancer by opening the tight junction just the same in the intestine. However, this seems unlikely because tight junctions are rare in the buccal epithelium and do not contribute to its barrier properties [29]. Therefore, the increased hypocalcemic effect is mostly attributed to chemical enhancers and iontophoresis, not enhancing effect of chitosan.

Effects of Chemical Enhancers on *In Vivo* Hypocalcemic Effect

We chose NAC, SDGC, and ethanol as chemical enhancers based on our previous study [5]. In that study, the *in vitro* transbuccal permeation of sCT was apparently NAC concentration dependent (5% > 2% > 1%), but the flux (J_s) of sCT, which was maximized at 1% of SDGC, did not show SDGC concentration dependency (1% > 2% ≒ 5%). Meanwhile a 10% ethanol solution combined with 5% NAC or 1% SDGC showed a great efficacy [5]. We therefore chose 5% NAC with 10% ethanol, and 1% SDGC with 10% ethanol for chemical enhancer compositions.

Ethanol is considered as a chemical enhancer for transmucosal delivery of hydrophilic drugs because it may solubilize lipids in the intercellular domains [29]. In the liquid formulation, the limit dose of ethanol is typically up to 10% in adult standard, but 10% ethanol exhibited no significant enhancement on *in vitro* transbuccal delivery of sCT [5]. Therefore, we focused on the combination effects of ethanol with other enhancers instead of the sole effect of ethanol as a chemical enhancer.

NAC, a well-known mucolytic agent, has been used as a chemical enhancer for transmucosal delivery because it can increase the permeation efficiency of hydrophilic macromolecules and minimize the damage of mucosa when used with other enhancers. [31]. Its action as an enhancer is caused by decreasing both viscosity and tenacity of mucus and saliva on the mucosa, thereby increasing the access of active ingredients to the epithelial membrane. However, the NAC group did not show any significant differences compared to negative control group (Fig. 3). Although NAC may reduce the viscosity of the mucus and saliva by rupturing the disulfide bonds of the high molecular glycoproteins [32], the effect of NAC is possibly hindered by continuous mucus and saliva secretion in buccal tissues of live rabbits. Moreover, NAC is known for promoting cellular glutathione (GSH) production, and GSH tends to remove the xenobiotics by raising the immune cell activation [33]. Therefore, in this *in vivo* experiment, the effects of NAC as a buccal permeation enhancer were much less than expected from the previous *in vitro* results [5].

SDGC, a bile salt, is one of the most widely used chemical enhancers for transbuccal drug delivery. It enhances the transbuccal delivery of hydrophilic compounds through intercellular spaces between the cells, which results from the interaction with polar intercellular lipids [29]. Its mechanisms of action are believed to be three steps: i) diffusion of SDGC into the buccal epithelium, ii) solubilization of polar intercellular lipids by the micelle formation of SDGC, and iii) alteration of the organized intercellular domains. On the other hand, a higher concentration (about 100 mM) of SDGC can activate transcellular pathway by diffusion into the cytoplasm [9]. Thus a high concentration of SDGC seems to enhance the buccal permeation via transcellular route, while lower concentrations seem to be advantageous in paracellular drug transport [6].

As shown in Fig. 3, SDGC group reduced the blood calcium level compared to negative control group. Such a hypocalcemic effect is closely linked to the enhanced permeation of sCT by SDGC; this may primarily occur via the paracellular pathway because sCT has a strong positive

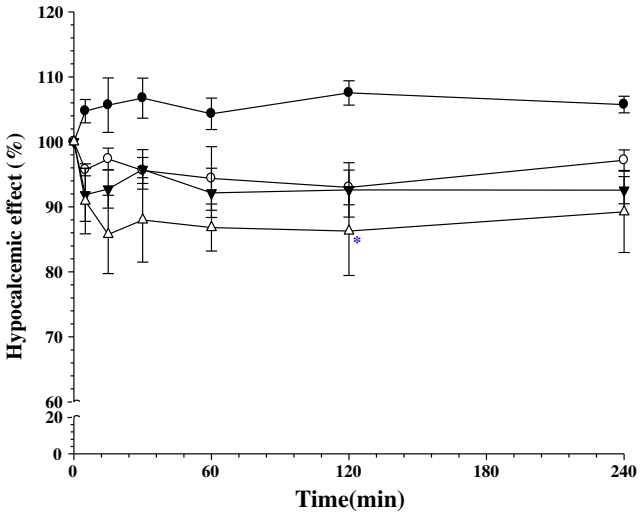


Fig. 3 Effect of chemical enhancers on the hypocalcemic effect of sCT ($n = 3$). ●: NC (negative control, no sCT formulation) group, ○: no enhancer group, ▼: NAC group, and ▵: SDGC group. * $0.05 < p < 0.1$.

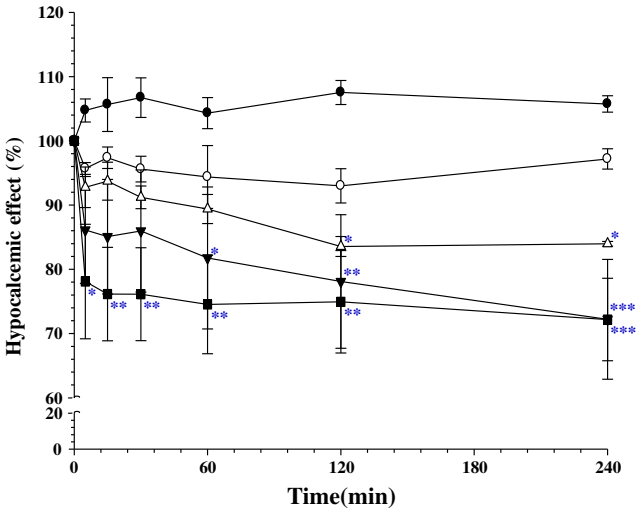


Fig. 4 Effect of electrical assistance on the hypocalcemic effect of sCT ($n = 3$). ●: NC (negative control, no sCT formulation) group, ○: no enhancer group, ▼: iontophoresis group, ▵: iontophoresis-NAC group, and ■: iontophoresis-SDGC group. * $0.05 < p < 0.1$, ** $0.01 < p < 0.05$, *** $p < 0.01$.

charge in the tested formulation, and also 1% SDGC (about 20 mM) activates the intercellular transport rather than intracellularly delivery. Furthermore, 10% ethanol also might help the permeation of sCT via paracellular route due to the interaction of ethanol with polar lipid molecules of buccal epithelium. Compared to passive delivery groups, only SDGC group showed significant hypocalcemic effect. Nevertheless, given significant difference was shown only at 2 h, it is debatable whether SDGC contributed to hypocalcemic effect of sCT.

Effect of Iontophoresis on *In Vivo* Hypocalcemic Effect

Iontophoresis is a widely used physical enhancing method to increase the delivery efficiency of hydrophilic charged small or macromolecular drugs locally or systemically. Main mechanism of iontophoresis is the activation of the paracellular pathway of a biomembrane with the combination of electrorepulsion, electro-migration, and electro-osmosis [12, 13, 34]. In this study, an Ag/AgCl electrode was used for the iontophoretic delivery of sCT because inactive electrodes such as carbon or platinum can cause proton production, which could induce tissue irritation and reduce the stability or permeation efficiency of drugs [26]. We used a safe 0.5 mA/cm^2 fixed anodal current to enhance the buccal permeation of sCT in live rabbits. As shown in Fig. 4, iontophoresis significantly enhanced the hypocalcemic effect of sCT compared to negative control group, and in particular the iontophoresis-SDGC group dramatically enhanced the onset of action and hypocalcemic effect of sCT. However, the iontophoresis-NAC group showed relatively low hypocalcemic effect compared to the iontophoresis group. Given the results in the previous section, we suggest that the sCT delivery efficiency of iontophoresis-NAC group is hindered by i) mucus and saliva secretion in a live rabbit buccal tissues and ii) xenobiotics scavenging of cellular glutathione (GSH) promoted by NAC [33].

Comparative Study of Different Administration Methods on Hypocalcemic Effect

sCT has generally been administered via parenteral route, but has a half-life of approximately 15-20 min [35]. Thus, frequent injections are needed to maintain its pharmacological effects *in vivo*. Such formulations of sCT are considered unfavorable to treat metabolic bone diseases, which need long-term therapy and patients are unwilling to undergo such a therapy regimen. For these reasons, alternative administration routes such as nasal, sublingual, pulmonary, and buccal, and sustained release strategies for sCT delivery have been extensively studied [36]. Among the alternative routes, buccal mucosa is less permeable than nasal mucosa and thus has relatively a slow onset of action [6]. In other words, buccal mucosa is more suitable transport route for a sustained delivery of sCT than nasal mucosa. In addition, retention of drug at the absorption site in buccal mucosa is longer compared to that in sublingual mucosa because it has an expanse of smooth muscle and comparatively motionless mucosa. Therefore, the buccal mucosa has been considered as an optimal route for the sustained delivery of peptide drugs [8].

We compared the hypocalcemic effect of the various administration routes including commercially available nasal route (Fig. 5). Intranasal or transbuccal delivery of sCT with no enhancing method decreased the blood calcium level compared to no sCT formulation but the effect was insignificant. Parenteral administration of sCT exhibited a fast and the strongest hypocalcemic effect; the values were significant

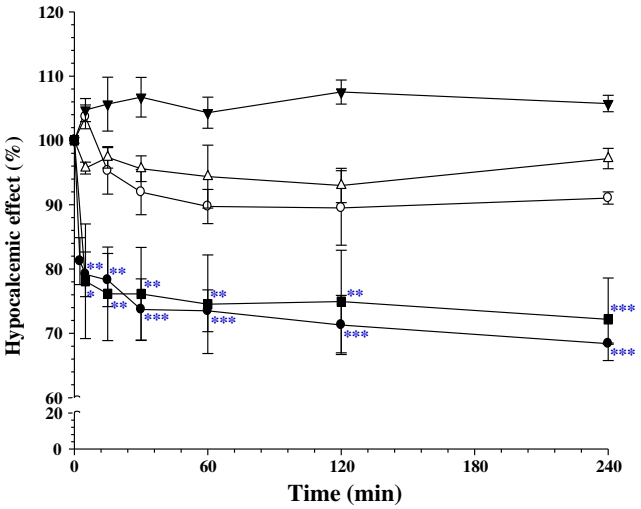


Fig. 5 The comparison of various administration routes on the hypocalcemic effect of sCT ($n = 3$). ●: intravenous administration of sCT with no enhancer, ○: intranasal administration of sCT with no enhancer, ▼: transbuccal delivery of NC (negative control, no sCT formulation) group, △: transbuccal delivery of no enhancer group, and ■: transbuccal delivery of iontophoresis-SDGC group. * $0.05 < p < 0.1$, ** $0.01 < p < 0.05$, *** $p < 0.01$.

($p<0.01$) compared to negative control group. On the other hand, iontophoresis-SDGC group showed not only fast onset of action almost like parenteral administration but also sustained hypocalcemic effect for 4 h. This supports the idea that iontophoresis could sustain the delivery of sCT, indicating that buccal delivery is suitable for sustained peptide drug delivery.

FT-IR Spectroscopic Study

It is important to understand the structural characteristics of the buccal mucosa as mucosa can act as a physical barrier in the transbuccal drug delivery, and its physical features are closely related to the permeation mechanisms [37]. With FT-IR spectroscopy, it is possible to know the status of a lipid bilayer, whether it is in the gel state (ordered) or the sol state (fluidized) [38]. The increase of sol state in the buccal epithelial lipids is strongly related to the decrease of barrier function of the lipids. Therefore, FT-IR study is a simple and useful method to evaluate the effects of the chemical and physical enhancements on the biophysical properties of the buccal mucosa.

The prominent peaks near 2,850 and 2,920 cm^{-1} represent the symmetric and asymmetric carbon-hydrogen (C-H) stretching, respectively [37]. The increase of fluidized lipids in the buccal mucosa can be explained by the following mechanisms: i) there is an increase of the gauche conformers within the lipid hydrocarbon chains, which results in increasing wave number in the symmetric and asymmetric C-H stretching absorbance peaks [39], ii) there is enhanced fluidity in the lipid acyl chains, which causes a broadening of the C-H stretching absorbance peaks [38]. Moreover, the increase of transbuccal drug flux can also be shown as the reduction in the C-H stretching frequency, which results from the extraction of the epithelial lipids by the permeation enhancing methods [37].

Figure 6 shows the results of FT-IR studies to verify the effects of various permeation enhancing methods on the biophysical properties of the buccal epithelial lipids. We focused on the symmetric and asymmetric C-H stretching absorbance peaks, so the IR spectra were compared within the range of 2,800–3,000 cm^{-1}. There may be an increase in temperature on the buccal mucosa of the test groups applied with electricity, and the higher temperature might contribute to the increase of lipid fluidity [40]. The use of iontophoresis or chemical enhancer caused the broadening and reduction of C-H stretching absorbance peaks which resulted from the increase of lipid bilayer fluidity and the epithelial lipid extraction, respectively. Such effects in C-H stretching peaks were dramatically enhanced in iontophoresis-SDGC group; this formulation exhibited the strongest hypocalcemic effect as the transbuccal drug flux is closely related to the barrier function of buccal epithelial lipids (Fig. 4). It has been known that SDGC does not induce significant shifts in these stretching peaks [29]; however, at higher concentrations (>100 mM), the significant shifts were shown due to extraction of cellular membrane lipids [5, 41]. In fact, in the *in vitro* transbuccal drug permeation study using porcine buccal mucosa, the test groups containing 5% SDGC (about 100 mM) caused the peak shifts towards higher wave numbers significantly [5]. In the present *in vivo* study, however, a significant difference in wave numbers among samples was not observed because we used 1% SDGC lower than 5% and live rabbits which maintain homeostasis (Table II). This demonstrates that enhancing effect of 1% SDGC is attributed to lipid extraction, which is irrelevant to alteration of order of membrane lipids [29]. In addition, iontophoresis and chemical enhancers contributed to decrease in the barrier function of lipids, considering broadening and reduction of C-H stretching absorbance peaks.

Histological Study

The epithelial lipid and mucus of the buccal mucosa are considered as the main barriers to transbuccal drug delivery, especially for hydrophilic and macromolecular drugs [6, 9, 10, 42] and it seemed that they are important to maintain the electric resistance (**Ω**) of the tissues. Generally, the penetration enhancing methods change the viable structures of the target tissues, and they sometimes induce irreversible tissue damage. Thus, the histological study is inevitable to evaluate the tissue integrity [43]. To evaluate the safety of the enhancing methods on the viable tissues, hematoxylin/eosin (H&E) staining was used.

Under visual examination of the buccal tissues right after the permeation study, no changes in integrity or color of the mucosal surfaces were observed in most of the test groups.

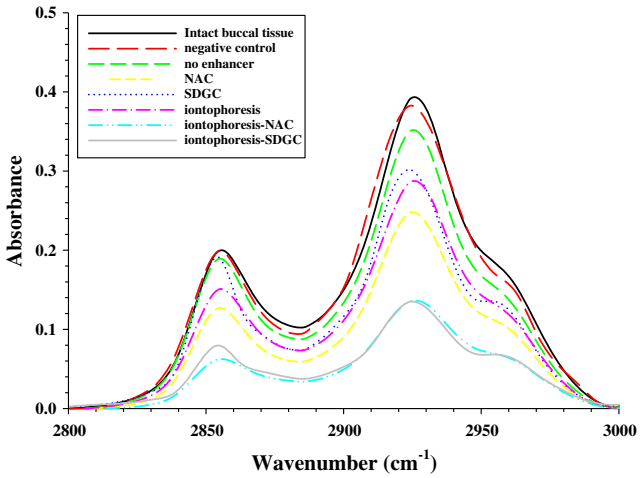


Fig. 6 FT-IR spectra of the excised rabbit buccal mucosa. Symmetric and asymmetric C-H bond stretching absorbances were observed after the application of each enhancing method group.

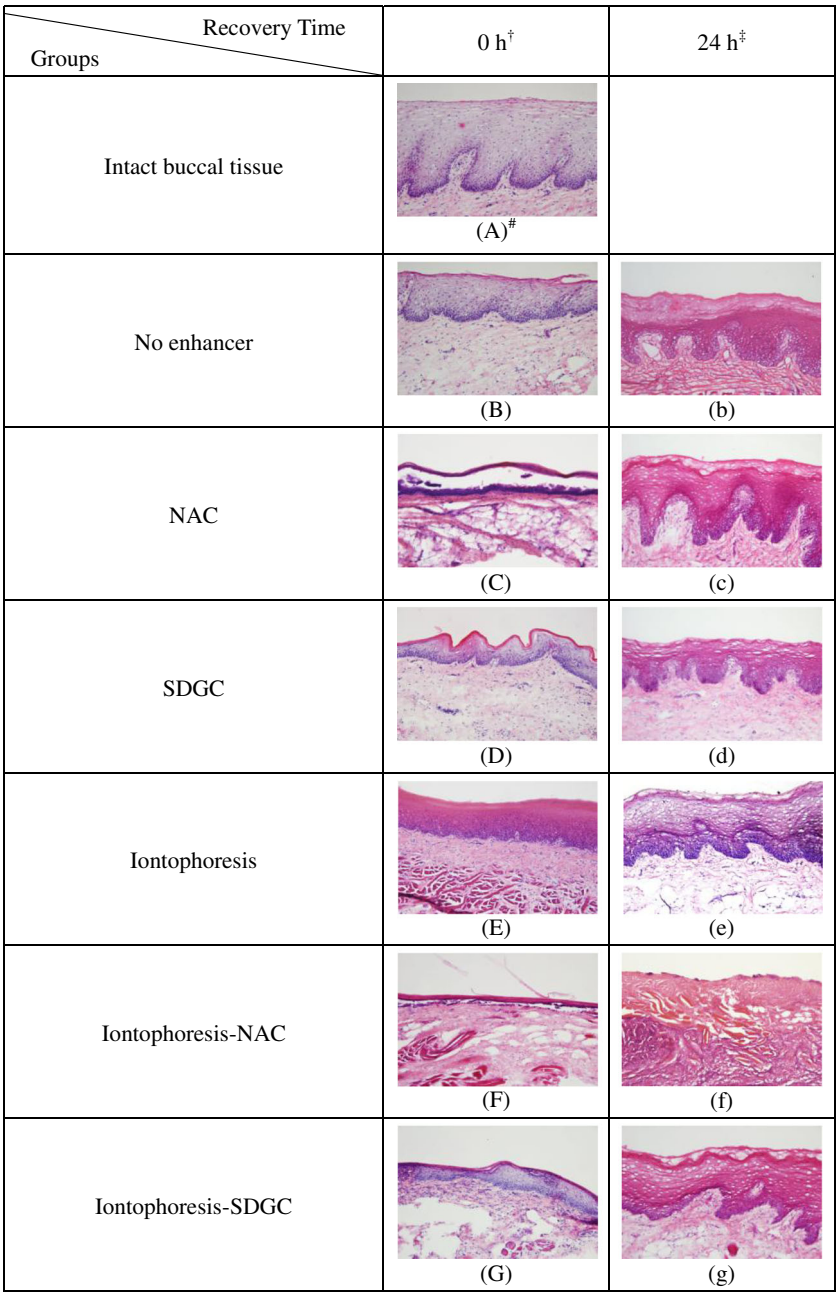
However, the two NAC groups had a quite rough mucosal surface and obviously showed desquamation after the permeation study (data not shown). The photomicrographs of H&E stained tissue samples observed by light microscopy provided the detailed information for the histomorphological alterations of the buccal mucosa (Fig. 7). The mucosal integrity of the no-enhancer group was similar with the untreated intact tissue, and only a slight decrease of thickness in the buccal mucosa was observed (Fig. 7b). However, the enhancing method groups exhibited some damage to the buccal structure and the desquamation of the surface layer of rabbit buccal epithelium. The NAC group showed the serious damage; loss of buccal mucosa layer and dermal-epidermal junction, even the occurrence of dermal edemas (Fig. 7c). These damage deteriorated more in iontophoresis-NAC group (Fig. 7f). Although the damage induced by iontophoresis-NAC group seemed to recover slightly after 24 h (Fig. 7f), the degree of recovery was feeble unlike NAC group (Fig. 7c). The other groups except two NAC groups showed remarkable recovery profiles as well as relatively low tissue damage. The slight damage of dermal-epidermal junction was shown in iontophoresis group but it reversibly recovered after recovery time (Fig. 7e, e). NAC and iontophoresis act as the permeation enhancing methods by reducing the viscosity of mucus of the mucosa and extracting or fluidizing the buccal epithelial lipid. The electric resistance of the buccal mucosa decreased due to these effects, and in particular the resistance decrease of the buccal mucosal layer by NAC was dose dependent [5, 43]. The combination of iontophoresis and NAC can reduce the electric resistance of the buccal mucosa below 10% of the initial value [5]. In general, the electric resistance reflects the effect of electric current on the integrity of viable tissues. Nugroho *et al.* reported that iontophoresis could reduce electric resistance of tissue membrane [44], and Kottgen reported that NAC could affect to the electrical properties of epithelial cells by reducing trans-epithelial voltage (V_m) and increasing whole-cell conductance (G_m) [45].

The two SDGC-containing groups also showed low tissue damage (Fig. 7d and g), but iontophoresis-SDGC group showed some dermal edemas unlike SDGC group. The histopathological damage of the biological barriers caused by bile salt are reversible and also dose dependent [46, 47]. According to the Oh's *in vitro* transbuccal permeation study using porcine buccal tissue, 5% SDGC caused minor negligible damage to the buccal mucosa [5]. It means that 1% SDGC used in the present study would be safe to the buccal mucosa, and an efficient enhancing method for paracellular drug transport because 1% SDGC activates the intercellular transport rather than intracellularly delivery. The iontophoresis-SDGC group exhibited the fastest onset of action and the strongest hypocalcemic effect; furthermore, its histological damage were not serious and rapidly recovered. These results might be explained by the action mechanisms of SDGC. SDGC applied to the buccal mucosa accumulates within buccal tissues, and drug flux increases due to its interaction with intercellular or membrane lipids; especially, low concentration of SDGC (c.a. 10 mM) mainly interacts with intercellular lipids [5, 29, 46]. In these processes, the SDGC may simply increase the lipid extraction of buccal mucosa without interruption of membrane lipid organization. Such effects of SDGC might contribute to the prevention of the severe dermal edema and

Table II Effects of The Enhancing Methods on The Symmetric and Asymmetric C-H Bond Stretching Absorbance Peaks of Rabbit Buccal Epithelium Measured with Ft-Ir Spectrophotometer ($N = 3$)

Groups	Intact	NC	No enhancer	NAC	SDGC	Iontophoresis	Iontophoresis-NAC	Iontophoresis-SDGC
C-H symmetric stretching absorbance 2,850 cm^{-1}	2,854.9 ± 2.1	2,854.7 ± 2.5	2,854.5 ± 2.1	2,854.7 ± 2.1	2,855.3 ± 1.4	2,855.2 ± 1.4	2,855.1 ± 1.6	2,855.7 ± 1.1
C-H asymmetric stretching absorbance 2,920 cm^{-1}	2,924.4 ± 2.3	2,924.0 ± 3.3	2,924.2 ± 2.8	2,924.1 ± 2.6	2,925.4 ± 1.2	2,925.3 ± 0.9	2,925.2 ± 1.2	2,925.8 ± 0.3

NC Negative Control (no sCT formulation); *NAC* *N*-acetyl-L-cysteine; *SDGC* Sodium deoxyglycocholate



† buccal tissues of right after *in vivo* experiment of 4h
‡ recovered buccal tissues for 24h after *in vivo* experiment of 4h
# untreated buccal tissue with no experiment

Fig. 7 Microphotographs of rabbit buccal tissues. All samples were formalin-fixed, embedded in OCT, cross-sectioned, and stained with hematoxylin-eosin for optical evaluation. Samples were observed at the magnification of × 200. (A): Intact, untreated. (B) and (**b**): treatment with no enhancer group. (C) and (**c**): treatment with NAC group. (D) and (**d**): treatment with SDGC group. (E) and (**e**): treatment with iontophoresis group. (F) and (**f**): treatment with iontophoresis-NAC group. (G) and (**g**): treatment with iontophoresis-SDGC group.

desquamation of the buccal mucosa even when combined with iontophoresis. These results can be explained and supported by the results of previous Oh's study; where iontophoresis and NAC markedly reduced electric resistance after *in vitro* buccal delivery, while no significant changes of electric resistance was observed in SDGC even when combined with iontophoresis [5]. Coincidentally, iontophoresis-SDGC group showed just a little damage to buccal tissues, and this damage virtually recovered after 24 h. Although a more precise mechanism hypothesis should be explained why SDGC was harmless to buccal tissues, such effects of SDGC may be helpful for the transbuccal peptide drug delivery along with iontophoresis.

In this study, we confirmed that iontophoresis-SDGC group facilitated fast onset of action within 15 min ($p<0.05$) as well as sustained efficacy on transbuccal sCT delivery; moreover, tissue damages by this enhancing method are minor, reversible, and rapidly recovered. For applying these promising results clinically, essential would be some adjustments such as minimizing electric current and application time, designing appropriate micro-device, and optimizing formulation composition.

CONCLUSION

We investigated the effects of chemical enhancers, iontophoresis, and combination of them on *in vivo* hypocalcemic effect of sCT, and evaluated the damage/recovery profiles of the buccal mucosa. The test groups applied with iontophoresis exhibited a significant hypocalcemic effect compared to the negative control group, and in particular the iontophoresis-SDGC group showed the most helpful strategy for transbuccal sCT delivery; it showed fast onset of action and sustained effect compared to other groups. Although the FT-IR study exhibited changes in the barrier function of the buccal epithelial lipids, these small structural changes of the buccal tissues by iontophoresis-SDGC group rapidly recovered. In conclusion, we found the combination of SDGC and anodal iontophoresis to be a safe and optimal strategy to enhance transbuccal sCT delivery.

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REFERENCES

1. Veuillez F, Kalia YN, Jacques Y, Deshusses J, Buri P. Factors and strategies for improving buccal absorption of peptides. Eur J Pharm Biopharm. 2001;51:93–109.
2. Yu S, Zhao Y, Wu F, Zhang X, Lu W, Zhang H, *et al.* Nasal insulin delivery in the chitosan solution: in vitro and in vivo studies. Int J Pharm. 2004;281:11–23.
3. Jeon SO, Hwang HJ, Oh DH, Seo JE, Chun KH, Hong SM, *et al.* Enhanced percutaneous delivery of recombinant human epidermal growth factor employing nano-liposome system. J Microencapsul. 2012;29:234–41.
4. Lee HE, Lee MJ, Park CR, Kim AY, Chun KH, Hwang HJ, *et al.* Preparation and characterization of salmon calcitonin-sodium triphosphate ionic complex for oral delivery. J Control Release. 2010;143:251–7.
5. Oh DH, Chun KH, Jeon SO, Kang JW, Lee S. Enhanced transbuccal salmon calcitonin (sCT) delivery: effect of chemical enhancers and electrical assistance on in vitro sCT buccal permeation. Eur J Pharm Biopharm. 2011;79:357–63.
6. Hoogstraateand AJ, Wertz PW. Drug delivery via the buccal mucosa. Pharm SciTechnol Today. 1998;1:309–16.
7. Chen LL, Chetty DJ, Chien YW. A mechanistic analysis to characterize oramucosal permeation properties. Int J Pharm. 1999;184:63–72.
8. Shojaei AH. Buccal mucosa as a route for systemic drug delivery: a review. J Pharm Pharmaceut Sci. 1998;1:15–30.
9. Hoogstraate AJ, Wertz PW, Squier CA, Geest AB, Abraham W, Garrison MD, *et al.* Effects of the penetration enhancer glycodeoxycholate on the lipid integrity in porcine buccal epithelium in vitro. Eur J Pharm Sci. 1997;5:189–98.
10. Sudhakar Y, Kuotsu K, Bandyopadhyay AK. Buccal bioadhesive drug delivery — a promising option for orally less efficient drugs. J Control Release. 2006;114:15–40.
11. Pillaiand O, Panchagnula R. Transdermal iontophoresis of insulin. J Control Release. 2003;88:287–96.
12. Giannola LI, De Caro V, Giandalia G, Siragusa MG, Campisi G, Florena AM, *et al.* Diffusion of naltrexone across reconstituted human oral epithelium and histomorphological features. Eur J Pharm Biopharm. 2007;65:238–46.
13. Jacobsen J. Buccal iontophoretic delivery of atenolol.HCl employing a new in vitro three-chamber permeation cell. J Control Release. 2001;70:83–95.
14. Patel MP, Churchman ST, Cruchley AT, Braden M, Williams DM. Electrically induced transport of macromolecules through oral buccal mucosa. Dent Mater. 2013;29:674–81. official publication of the Academy of Dental Materials.
15. Patel MP, Churchman ST, Cruchley AT, Braden M, Williams DM. Delivery of macromolecules across oral mucosa from polymeric hydrogels is enhanced by electrophoresis (iontophoresis). Dent Mater. 2013;29:e299–307. official publication of the Academy of Dental Materials.
16. Chaturvedula A, Joshi DP, Anderson C, Morris RL, Sembrowich WL, Banga AK. In vivo iontophoretic delivery and pharmacokinetics of salmon calcitonin. Int J Pharm. 2005;297:190–6.
17. Azria M. Possible mechanisms of the analgesic action of calcitonin. Bone. 2002;30:80S–3S.
18. Visser EJ. A review of calcitonin and its use in the treatment of acute pain. Acute Pain. 2005;7:185–9.
19. Chang SL, Hofmann GA, Zhang L, Deftos LJ, Banga AK. Transdermal iontophoretic delivery of salmon calcitonin. Int J Pharm. 2000;200:107–13.
20. Kitano M, Maitani Y, Takayama K, Nagai T. Buccal absorption through golden hamster cheek pouch in vitro and in vivo of 17β-estradiol from hydrogels containing three types of absorption enhancers. Int J Pharm. 1998;174:19–28.
21. Shojaei AH, Chang RK, Guo X, Burnside BA, Couch RA. Systemic drug delivery via the buccal mucosal route. Pharm Technol. 2001;25:70–81.
22. Lee KC, Lee YJ, Song HM, Chun CJ, DeLuca PP. Degradation of synthetic salmon calcitonin in aqueous solution. Pharm Res. 1992;9:1521–3.
23. Gandhiand RB, Robinson JR. Permselective characteristics of rabbit buccal mucosa. Pharm Res. 1991;8:1199–202.
24. Wang X, Zheng C, Wu Z, Teng D, Zhang X, Wang Z, *et al.* Chitosan-NAC nanoparticles as a vehicle for nasal absorption enhancement of insulin. J Biomed Mater Res B Appl Biomater. 2009;88:150–61.
25. J.D. Smart. The role of water movement and polymer hydration in mucoadhesion. Bioadhesive drug delivery systems: fundamentals, novel approaches and development:11–23 (1999).
26. Kalia YN, Naik A, Garrison J, Guy RH. Iontophoretic drug delivery. Adv Drug Deliv Rev. 2004;56:619–58.
27. Dondeti P, Zia H, Needham TE. In vivo evaluation of spray formulations of human insulin for nasal delivery. Int J Pharm. 1995;122:91–105.
28. Schipper NGM, Verhoef JC, Romeijn SG, Merkus FWHM. Methylated β-cyclodextrins are able to improve the nasal absorption of salmon calcitonin. Calcif Tissue Int. 1995;56:280–2.
29. Nicolazzo JA, Reed BL, Finnin BC. Buccal penetration enhancers–how do they really work? J Control Release. 2005;105:1–15.
30. Şenel S, İkinci G, Kaş S, Yousefi-Rad A, Sargon MF, Hıncal AA. Chitosan films and hydrogels of chlorhexidine gluconate for oral mucosal delivery. Int J Pharm. 2000;193:197–203.
31. Matsuyama T, Morita T, Horikiri Y, Yamahara H, Yoshino H. Enhancement of nasal absorption of large molecular weight compounds by combination of mucolytic agent and nonionic surfactant. J Control Release. 2006;110:347–52.
32. Rochat T, Lacroix JS, Jornot L. N-acetylcysteine inhibits Na+ absorption across human nasal epithelial cells. J Cell Physiol. 2004;201:106–16.
33. Gillissenand A, Nowak D. Characterization of N-acetylcysteine and ambroxol in anti-oxidant therapy. Respir Med. 1998;92:609–23.

34. Barry BW. Novel mechanisms and devices to enable successful transdermal drug delivery. Eur J Pharm Sci. 2001;14:101–14.
35. Huwyler R, Born W, Ohnhaus EE, Fischer JA. Plasma kinetics and urinary excretion of exogenous human and salmon calcitonin in man. Am J Physiol. 1979;236:E15–19.
36. Millest AJ, Evans JR, Young JJ, Johnstone D. Sustained release of salmon calcitonin in vivo from lactide: glycolide copolymer depots. Calcif Tissue Int. 1993;52:361–4.
37. Swarnakar NK, Jain V, Dubey V, Mishra D, Jain NK. Enhanced oromucosal delivery of progesterone via hexosomes. Pharm Res. 2007;24:2223–30.
38. Panchagnula R, Salve PS, Thomas NS, Jain AK, Ramarao P. Transdermal delivery of naloxone: effect of water, propylene glycol, ethanol and their binary combinations on permeation through rat skin. Int J Pharm. 2001;219:95–105.
39. Takahashi K, Sakano H, Yoshida M, Numata N, Mizuno N. Characterization of the influence of polyol fatty acid esters on the permeation of diclofenac through rat skin. J Control Release. 2001;73:351–8.
40. Bhatia KS, Gao S, Singh J. Effect of penetration enhancers and iontophoresis on the FT-IR spectroscopy and LHRH permeability through porcine skin. J Control Release. 1997;47:81–9.
41. Hoogstraate AJ, Senel S, Cullander C, Verhoef J, Junginger HE, Bodd HE. Effects of bile salts on transport rates and routes of FITC-labelled compounds across porcine buccal epithelium in vitro. J Control Release. 1996;40:211–21.
42. Meaneyaand C, O'Driscoll C. Mucus as a barrier to the permeability of hydrophilic and lipophilic compounds in the absence and presence of sodium taurocholate micellar systems using cell culture models. Eur J Pharm Sci. 1999;8:167–75.
43. Prasad R, Anand S, Koul V. Biophysical assessment of DC iontophoresis and current density on transdermal permeation of methotrexate. Int J Pharm Investig. 2011;1:234–9.
44. Nugroho AK, Li L, Dijkstra D, Wikström H, Danhof M, Bouwstra JA. Transdermal iontophoresis of the dopamine agonist 5-OH-DPAT in human skin in vitro. J Control Release. 2005;103:393–403.
45. Kottgen M, Busch AE, Hug MJ, Greger R, Kunzelmann K. N-Acetyl-L-cysteine and its derivatives activate a Cl- conductance in epithelial cells. Pflugers Arch. 1996;431:549–55.
46. Dhiman MK, Dhiman A, Sawant KK. Transbuccal delivery of 5-fluorouracil: permeation enhancement and pharmacokinetic study. AAPS PharmSciTech. 2009;10:258–65.
47. Zhang J, Niu S, Ebert C, Stanley TH. An in vivo dog model for studying recovery kinetics of the buccal mucosa permeation barrier after exposure to permeation enhancers: apparent evidence of effective enhancement without tissue damage. Int J Pharm. 1994;101:15–22.