The Potential of Chitosan in Enhancing Peptide and Protein Absorption across the TR146 Cell Culture Model—An *in Vitro* Model of the Buccal Epithelium

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Purpose. To investigate the potential of chitosan (CS) to enhance buccal peptide and protein absorption, the TR146 cell culture model, a model of the buccal epithelium, was used.

Methods. The sensitivity of TR146 cells to several CS solutions (different salts with different MW) was investigated by using the MTS/PMS assay. Permeability studies were performed to determine the enhancing effect of CS glutamate (1, 20, 40, 60, and 100 μg/mL) on the permeability of ^{3}H-mannitol and fluorescein isothiocyanate labeled dextrans (FD) with various MW (4.4–19.5 kD) across the cell culture model.

Results. Sensitivity of TR146 cells to CS solutions depended on the concentration, the pH, and the type of CS salt. CS glutamate solutions (pH 6.0) were found to be the least harmful. CS glutamate was able to increase the permeability of model substances with MW up to 9.5 kD across the cell model. An enhancing effect was found for CS concentrations of 20 μg/mL and higher, correlating with a decrease in TEER values. The 20 μg/mL CS concentration had a negligible effect on the enzyme activity of the cells as determined by the MTS/PMS assay.

Conclusions. CS glutamate is effective in enhancing the transport of macromolecules across the buccal TR146 cell culture model. Therefore, it might be a promising vehicle for peptide and protein buccal administration.

KEY WORDS: buccal permeability enhancement; chitosan; peptide and protein absorption; TR146 cell culture model.

INTRODUCTION

Nowadays, several mucosal surfaces, such as the buccal, nasal, and pulmonary mucosae, are being extensively explored as alternative noninvasive routes for the systemic administration of macromolecular drugs. Among them, buccal mucosa is receiving a great deal of attention because of the extraordinary easy access of the drug to the absorption site and good patient acceptance. The buccal route has the advantage that it avoids hepatic first-pass metabolism and gastrointestinal degradation, which follow oral administration. Furthermore, the buccal epithelium contains less proteolytic enzymes than the epithelium of the gastrointestinal tract (1).

Unfortunately, peptide and protein absorption is limited because of low membrane permeability due to high molecular weight (MW) and hydrophilic nature. Therefore, for successful absorption of peptides and proteins, the use of permeability enhancers is required (2). Relatively few studies have been carried out on the effect of absorption enhancers on peptide and protein transport across the buccal mucosa (2–4). An ideal permeability enhancer must be nonirritant and nontoxic; nevertheless, in practice, drug absorption enhancement is generally accompanied by mucosal damage. However, the rapid cellular recovery after local irritation, inherent to the buccal epithelium, constitutes a very important additional benefit of this absorption route.

The mucoadhesive polysaccharide chitosan (CS) has behaved in an exceptional way. It has promoted insulin absorption through nasal mucosa (5,6) and permeation of peptide/protein drugs across cultured Caco-2 cells (7–10). It has been suggested that the enhancing mechanism of CS is a combination of mucoadhesion and a transient widening of the junctions between epithelial cells (7). Taking this into account, CS is very promising for buccal peptide drug administration.

Based on the potential suitability of CS for transmucosal peptide and protein administration, the aim of the present study was to investigate the enhancing effect of this polymer on the buccal permeability of macromolecular drugs. For this purpose, fluorescein isothiocyanate labeled dextrans (FD) of different MW were chosen as protein models and ^{3}H-mannitol as a marker of paracellular transport. The TR146 cell culture model was selected as an *in vitro* model of the human buccal epithelium (11). The cell line originates from a human buccal metastasis (12) and after culturing, it forms a stratified epithelium resembling the buccal epithelium (11). The TR146 cell culture model seems to be a valid *in vitro* model of the human buccal epithelium regarding its physical barrier characteristics (13).

MATERIALS AND METHODS

Materials

The following chemicals were purchased from commercial suppliers and used as received: Chitosan (CS): SC 123, SC 223, SC 320, SC G110, SC G210, SC Cl210, Protasan Cl110, Protasan UP Cl113, SC L110, and SC L210 (Pronova Biopolymer A.S., Drammen, Norway) (CS characteristics are shown in Table I); Hanks' balanced salt solution without phenol red (HBBS) and Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated foetal bovine serum, penicillin (90 U/mL) and streptomycin (90 μg/mL) (Gibco BRL, Paisley, UK); morpholino-ethanesulphonic acid anhydrous (MES) (Applichem GmbH, Darmstadt, Germany); D-mannitol-[(1-^{3}H-(N)], phenazine methosulphate (PMS), and fluorescein isothiocyanate labeled dextrans (FD) FD4, FD10, and FD20 with average MW (kD)/FITC contents (molecule FITC/molecule glucose) of 4.4/0.004, 9.5/0.008, and 19.5/0.006, respectively (Sigma Chemical Co., St. Louis, MO); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Madison, WI); Ultima Gold™ MV scintillation cocktail (Packard Instruments BV, Groningen, The Netherlands); Falcon® 12-well tissue culture plates and cell culture inserts (0.9 cm^2, 0.4 μm pore size, polyethylene terephthalate membranes) (Becton

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Dickinson Labware, Franklin Lakes, NJ); Costar® flat-bottomed 96-well cell culture cluster (tissue culture treated, polystyrene) (Corning Inc., Corning, NY).

Methods

Cell Culture

The TR146 cells were kindly provided by Imperial Cancer Research Technology (London, UK) and cultured as previously described (11,14). For the MTS/PMS assay, the TR146 cells were seeded in a 96-well culture plate at a density of 3×10^4 cells/well and cultured for 22–24 h. For the permeability studies, the seeding density was 2.1×10^4 cells/filter, and culturing was carried out over 28–30 days. Passages 2.6.3–2.6.10 (mycoplasma free) were used.

Preparation of CS Solutions

CS salts were dissolved in Milli-Q water and CS base in 3% v/v acetic acid at 5% w/v by magnetic agitation and centrifuged (Hettich Centrifuge; Bie & Bernsten A/S, Denmark) at 6000 g for 15 min to remove insoluble impurities. Just before the experiments, the CS solutions were diluted with HBSS/MES (20 mM MES in HBSS, adjusted to pH 6.0 with NaOH) to the appropriate concentrations. Unless otherwise noted, experiments were conducted with CS solutions at pH 6.0. Osmolality values were ~300 mOsm in all the solutions.

MTS/PMS Assay

The dehydrogenase activity in the TR146 cells was used to estimate the cells sensitivity to different CS solutions and measured according to the MTS/PMS assay optimized for the TR146 cell line (15). The enzyme activity in untreated cells was set at 100%, and the effect of the CS solutions was measured as the relative decrease in enzyme activity, *i.e.,* the IC50 was defined as the concentration of CS inhibiting cell viability with 50%. The assay was performed by using concentrations of CS ranging from 0.001 to 10 mg/mL at pH values of 5.5 and 6.0. Analysis of five replicates was conducted.

Permeability Studies with the TR 146 Cell Culture Model

The permeability experiments were performed at 37°C and 150 rpm by using a temperated horizontal shaker (Edmund Bühler, Bodelshausen, Germany). Donor solution (0.9 mL) consisting of test substance in HBSS/MES (pH 6.0) was applied to the apical side of the cell layers, and the inserts were moved to wells containing 2.1 mL receptor solution (consisting of the same buffer). Immediately, 100-μL samples were withdrawn from both the receptor solution and the donor solution. Hereafter, samples were collected from the basal side and replaced with the used buffer, every 15 min for 1 h and every 30 min up to 4 h. At the end of the experiments, 100-μL samples were taken from the donor and the receptor solutions. In experiments with enhancer, the CS concentrations tested were 1, 20, 40, 60, or 100 μg/mL. The concentrations of the test substances were 9.25 kBq/mL ^{3}H-mannitol, 0.45 mM FD4, and 0.50 mM FD10 and FD20. Studies were carried out in six replicates using two different passages of cells.

Before and after each permeability experiment, transepithelial electrical resistance (TEER) was measured by using an Endohm™ culture cup connected to an EVOM™ volt-ohmmeter (World Precision Instruments, Herts, UK). The mean initial TEER value was $339 \pm 89\ \Omega \times cm^2$ ($n = 137$).

Release Studies from CS Solutions

These studies were performed in the same way as the permeability experiments but with the use of inserts without cells; sampling was performed every 10 min for 1 h followed by 30-min intervals for 1 h ($n = 3$).

Analytical Procedures

For the MTS/PMS assay, the optical density (OD) values were recorded at the wavelength maximum of 490 nm by using a Microplate reader (Labsystems Multiskan MS, Helsinki, Finland).

Samples (100 μL) of radioactive labeled test substance were mixed with 2 mL scintillation cocktail and quantified by using a Tri-Carb® 2100 liquid scintillation analyser (Packard Instruments, Meriden, CT).

The assessment of FD was performed with a Jasco Model 821-FP intelligent spectrofluorometric detector (Jasco, Japan Spectroscopic Co., Tokyo, Japan). The detector was set at excitation/emission wavelengths of 491 nm/520 nm, and samples (100 μL) were diluted with 700 μL of HBSS/MES before analysis.

Data Analysis

Enzyme activity of TR146 cells toward CS was determined relative to cells exposed to HBSS/MES and calculated according to Eq. (1):

$$\text{Rel. enzyme activity}\ (\%) = (OD_1 - OD_2)/\ (OD_3 - OD_4) \times 100\% \quad (1)$$

where OD_1 is the OD of wells with cells and CS solution; OD_2 is the OD of wells without cells and with CS solution; OD_3 is the OD of wells with cells and without CS, and OD_4 is the OD of wells without cells and without CS.

The release rate constant (k_t) values for drug release from the CS solutions were calculated according to Eq. (2):

$$Q = k_t \times t^{1/2} \quad (2)$$

where Q is the amount of drug released and k_t is the release rate constant.

The apparent permeability coefficient (P_{app}) for the permeability of the test substances across the TR 146 model was calculated according to Eq. (3):

$$P_{app} = dQ/dt \times 1/(A \times C_0) \quad (3)$$

where dQ/dt is the steady-state rate of permeation, A is the diffusion area (cm^2), and C_0 is the initial concentration of test substance in the donor compartment.

The cellular permeability coefficient (P_c) was determined according to Eq. (4):

$$1/P_{app} = 1/P_c + 1/P_{ins} \quad (4)$$

where P_{ins} is the permeability across inserts, which represents the permeability coefficient across the unstirred water layer and the terephthalate membrane.

The enhancement ratio (ER) for each test solution was determined as described in Eq. (5):

$$ER = P_c\ (\text{enhancer})/P_c\ (\text{control}) \quad (5)$$

where P_c (enhancer) and P_c (control) are the cellular permeability coefficients of the test compounds across the TR146 model in the presence and in the absence of CS, respectively.

The TEER is expressed as a percentage of the initial value.

Statistical Evaluation

Data are presented as means ± standard deviation (SD) (n) where n is the number of replicates. Statistical differences were investigated by using one-way ANOVA followed by the Student-Newman-Kreuls method for multiple comparisons. Differences between group means were judged significant at $P < 0.05$.

RESULTS AND DISCUSSION

MTS/PMS Assay

Although CS has been widely described in literature as biocompatible, biodegradable, and nontoxic, few studies have focused on this subject. The studies referred to make it clear that there is an existing controversy about CS toxicity and the factors that influence it. In general, these factors are the type of CS salt, the MW, and, basically, the degree of deacetylation (8,16). To our knowledge, no studies have been performed for CS toxicity on buccal cell lines.

In an attempt to obtain more information about CS toxicity, we used the MTS/PMS assay to investigate the effects of several CS salts with different MW and similar degrees of deacetylation on intracellular dehydrogenase activity in the TR146 model. It should be emphasized that the MTS/PMS assay was carried out by using TR146 cells that were in the exponential growth phase, which might be more sensitive to CS solutions than the multilayered and well-differentiated TR146 cell culture model grown on permeable inserts for 4 weeks.

In Fig. 1, the relative enzyme activity of cells exposed to various concentrations of CS glutamate (SC G110) at pH 5.5 and 6.0 was plotted, as a representative example of the typical

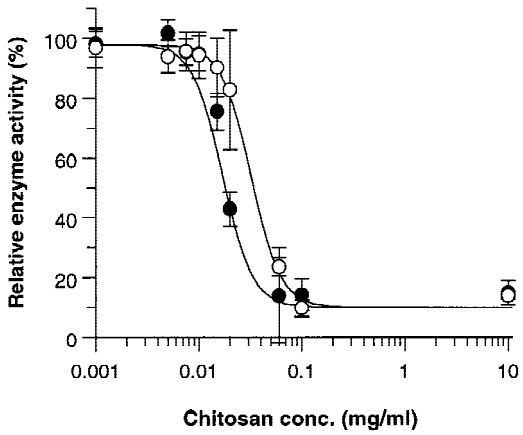

Fig. 1. The sensitivity of TR146 cells toward various concentrations of chitosan glutamate (SC G110) at pH 5.5 (●) and 6.0 (○), determined by the MTS/PMS assay (means ± SD, n = 5).

Table I. Physicochemical Properties of Chitosan and Estimated IC_{50} (μg/mL) Values Obtained for the Different Types of Chitosan at pH 5.5 and 6.0

Type of chitosan salt	Chitosan code	MW (kD); DD[a] (%)	IC_{50}[b] (pH 6.0)	IC_{50} (pH 5.5)
Glutamate	SC G110	150; >80	33.8 ± 2.1[c]	17.8 ± 0.3[c]
	SC G210	300; >80	57.3 ± 2.2	20.9 ± 1.1
Acetate	SC 123	50; >80	18.4 ± 0.4	14.4 ± 1.3
	SC 223	150; >80	19.5 ± 0.5	9.7 ± 0.5
	SC 320	300; >80	18.3 ± 0.4	9.6 ± 0.5
Hydrochloride	P Cl110	110; 87	21.3 ± 0.8	n.d
	PUP Cl113	110; 87	18.0 ± 0.9	n.d
	SC Cl210	100–150; 81	28.9 ± 0.8	n.d
Hydrolactate	SC L110	100–150; 86	30.0 ± 0.7	n.d
	SC L210	200–250; 82	28.1 ± 0.1	n.d

[a] DD = degree of deacetylation.
[b] IC_{50} values were estimated by using a four-parameter logistic model: $y = 1/(1 + e^{(x-a)/b)})$.
[c] Values are means ± SD, n = 5.
n.d = not determined.

curves obtained for the different types of CS. A clear concentration-dependent effect on the intracellular dehydrogenase activity was observed for all the CS assayed. The IC_{50} values obtained for the different CS samples tested at pH 5.5 and pH 6.0 are listed in Table I. Our results suggest that CS glutamate salts are the least toxic, having the highest IC_{50}. CS toxicity could be ranked in the order CS acetate > CS hydrochloride > CS hydrolactate > CS glutamate. The effect of the type of salt on enzymatic activity is not surprising taking into account that the nature of the counterion may influence the interaction of the protonated amine groups of the CS with negatively charged cellular components. Other investigators have reported similar behavior related to the type of salt on CS toxicity (16) using the MTS/PMS assay. However, staining with trypan blue, Kotzé and coworkers (10,17) found that after prolonged incubation with hydrochloride or glutamate salts of CS (5 and 15 mg/mL), the Caco-2 cell line remained undamaged and functionally intact.

For the MW, it was found that the effect of CS MW on CS toxicity was dependent on the type of CS and, in addition, it was more noticeable for experiments performed at pH 6; for CS glutamate and CS hydrochloride, the higher the MW, the lower the toxicity, but no differences were observed for the acetate and hydrolactate salts. In agreement with our results, in a study performed by using B16F10 cells (a murine melanoma cell line), it was found that CS toxicity decreased as its MW increased (16).

Therefore, for the salt and MW effects, it is difficult to come to a conclusion, because it seems that the most important factor affecting CS toxicity, in this study, was the pH of the CS solution. In fact, lower pH values led to lower IC_{50} values and, hence, higher toxicity. This finding concurs with a previous study in which the key factor in CS toxicity was attributed to its degree of deacetylation and, consequently, the positive charge density of the polymer (8). CS has an apparent pKa of about 6.5; thus, at the lower pH the CS molecule becomes more ionized, it uncoils and assumes a more elongated shape (18), increasing its possibility for intimate contact with the epithelial membrane. It must be pointed out that the TR146 cells are not sensitive to pH changes in the range 5–9 (14).

Table II. Cellular Permeability Coefficients (P_c) for Permeability of ^{3}H-Mannitol, FD4, FD10, and FD20 across the TR146 Cell Culture Model in the Absence or Presence of Different Chitosan Concentrations

Chitosan concentration (μg/mL)	P_c ($\times 10^7$) ± SD (cm/s)			
	^{3}H-mannitol	FD4	FD10	FD20
Control	30.92 ± 5.27	7.85 ± 2.22	2.95 ± 1.18	1.75 ± 0.65
1	38.51 ± 15.0	9.83 ± 2.63	5.15 ± 0.93*	1.89 ± 0.59
20	85.02 ± 10.69*	22.52 ± 6.60*	5.32 ± 0.44*	3.03 ± 1.25
40	80.39 ± 9.66*	17.52 ± 4.48*	4.81 ± 1.25*	2.72 ± 1.03
60	88.23 ± 25.88*	18.39 ± 3.71*	4.27 ± 0.75	2.08 ± 0.57
100	83.78 ± 10.78*	18.15 ± 5.90*	4.27 ± 1.48	2.28 ± 0.60

* Denotes significant difference from control. $P < 0.05$. Values are means ± SD, n = 6.

Permeability Studies

From the permeability and release profiles of ^{3}H-mannitol, FD4, FD10, and FD20, the apparent permeability coefficient (P_{app}), the cellular permeability coefficient (P_c) and absorption enhancement ratio (ER) after incubation with different concentrations of CS glutamate at pH 6.0 were calculated. As expected, the highest cellular permeability was obtained for ^{3}H-mannitol, whereas decreasing permeability was observed as the MW increased for FD4, FD10, and FD20 (Table II). Decreased permeability rates of hydrophilic substances with increasing MW have also been reported for TR146 cell culture and *in vitro* porcine models (19). Results from the permeability studies showed that CS glutamate increased the permeability of all the model substances ($P < 0.05$) except FD20. In previous studies conducted in the same cell culture model (19), it was found that sodium glycocholate (GC), a bile salt, was able to significantly increase the permeability of ^{14}C-mannitol and the same FD as used in the present study. The enhancing effect of GC was related to the MW of the test compounds in a parabolic way, the highest ER value being observed for FD10 (about 80-fold increase). This is not comparable to the present results, which could give an indication of different enhancing mechanisms between classical enhancers (such as bile salts) and CS. The former study disclosed that the increase in FD permeability across the TR146 cell culture model in the presence of GC was a result of increased paracellular permeation as well as induced transcellular penetration into the superficial cells of the epithelial model. The mechanism of action of CS for enhancing the permeability of macromolecules has been reported to deal mainly with the paracellular pathway (9,10,17).

Figure 2 depicts the ER values obtained for permeability of ^{3}H-mannitol and FD across the TR146 cell culture model in the presence of several CS glutamate concentrations. The enhancing effect was found for CS concentrations of 20 μg/mL or more. It was observed that the most effective CS concentration for absorption enhancement was 20 μg/mL, because an increase in polymer concentration did not result in further increases in the ER and even resulted in a decrease in the ER for FD10 and FD20. This phenomenon could be explained by the decrease in release rate constants (k_t) (Table III), which indeed was attributed to the increase in viscosity of the CS solution. When CS concentration is increased, the solutions become more viscous, and the diffusion of the test substances is hindered, especially that of those with high MW.

In general, the results suggest that the plateau permeation levels were reached between 1 and 20 μg/mL. This slight dependence on concentration of the CS effect concurs with other studies performed both in Caco-2 cells and *in vivo* models, where a plateau level was shown for CS glutamate, although at higher concentrations (between 2.5 and 5 mg/mL) (7,17).

TEER Measurements

Measurement of the TEER is believed to be a good indication of the tightness of the junctions between cells. Results from our studies showed that CS glutamate at concentrations of 20 μg/mL and higher was able to drastically reduce the TEER of the TR146 cell culture model to around 30% in comparison with untreated cells. Furthermore, the effect on the TEER values seemed to be saturable because no major differences were found when increasing the CS glutamate concentration, as shown in Fig. 3. This observation was previously reported for experiments in Caco-2 cell monolayers (7,17).

It is interesting to note that the clear concentration-dependent effect on dehydrogenase activity was not reflected in either TEER or permeability results. Indeed, the most effective CS concentration for absorption enhancement (20 μg/mL) was considered minimally harmful at pH 6.0, because it corresponded to a relative enzyme activity of 82% as determined in the MTS/PMS assay. This finding suggests that the enhancing effect of CS on permeation across TR146 cells was

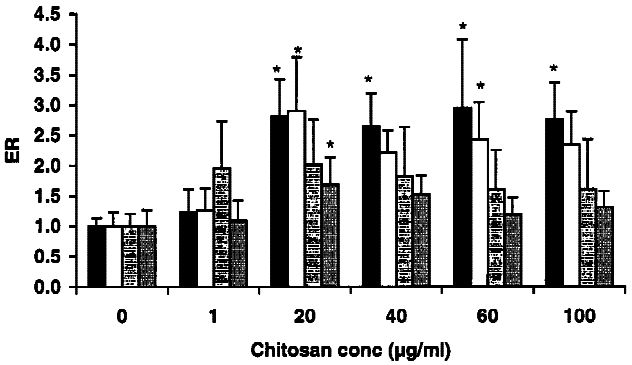

Fig. 2. Enhancement ratio (ER) values of (■) ^{3}H-mannitol, (□) FD4, (▤) FD10, and (▩) FD20 permeation across the TR146 cell culture model treated with different chitosan concentrations relative to untreated cells (means ± SD, n = 5–6). *Denotes the difference from untreated cells. $P < 0.05$.

Table III. Release Rate Constants (k_t) for Release of ^{3}H-Mannitol, FD4, FD10, and FD20 from Several Chitosan Solutions Measured in Inserts without Cells

Chitosan concentration (μg/mL)	k_t ($\times 10^2$) ± SD (μmol/min$^{1/2}$)			
	^{3}H-mannitol	FD4	FD10	FD20
Control	25.12 ± 1.07	0.77 ± 0.04	0.74 ± 0.11	0.37 ± 0.05
1	24.14 ± 0.67	0.65 ± 0.12	0.74 ± 0.13	0.41 ± 0.06
20	20.71 ± 0.32	0.60 ± 0.01	0.47 ± 0.04*	0.21 ± 0.03*
40	24.23 ± 0.81	0.66 ± 0.04	0.48 ± 0.04*	0.20 ± 0.05*
60	21.81 ± 1.65	0.61 ± 0.03	0.51 ± 0.08*	0.18 ± 0.02*
100	21.82 ± 0.39	0.55 ± 0.03	0.44 ± 0.02*	0.18 ± 0.01*

* Denotes the difference from control. $P < 0.05$. Values are means ± SD, n = 6.

not related to cellular damage but to decreased epithelial integrity and, consequently, to the cell-cell adhesion, as can be deduced from the TEER results.

The mechanism for permeability enhancement by CS has been reported in Caco-2 cells to be related to the polymer binding to the epithelial cell membrane through a charge-dependent mechanism, resulting in F-actin depolimerization, disbandment of the tight junction protein ZO-1, and consequently, the opening of tight junctions (9). However, it is well known that for buccal mucosa the permeability barrier is not based on tight junctions (20); therefore, there must be another mechanism of action responsible for enhancement instead of the action on tight junctions. In the buccal epithelium, the main permeability barrier for hydrophilic substances is believed to be situated in the intercellular region of the superficial layers of the epithelium, where membrane-coating granules extrude their lipid and glycolipid contents (21). In addition, the cells of the oral epithelium are surrounded by an intercellular matrix, composed mainly by carbohydrate-protein complexes, which may play a role in cellular adhesion (22). Furthermore, the polycationic nature of CS has been shown to lead to strong interactions with lipids having the opposite charge (23). Therefore, a possible mechanism of action of CS improving transport of drugs across the buccal mucosa could be attributed to its interference with the lipid organization in the buccal mucosa, as has been previously suggested by Senel *et al* (24). As an alternative hypothesis, CS could interact with the proteoglycan matrix by ionic interactions, leading to a loosening of the intercellular filaments. Further studies will be conducted to investigate the mechanism of CS as a buccal absorption enhancer.

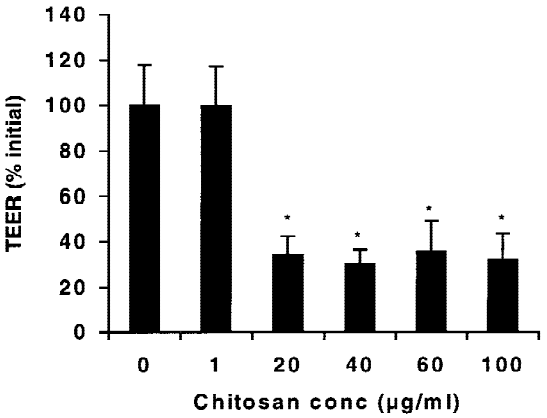

Fig. 3. Reduction in transepithelial electrical resistance (TEER) after 4-h incubation with several concentrations of chitosan, expressed as percent of initial values (means ± SD, n = 6). *Denotes significant difference from untreated cell layers. $P < 0.05$.

CONCLUSIONS

Our results show the ability of CS glutamate to increase the permeability of large hydrophilic compounds across the TR146 cell culture model, a model of the buccal mucosa. Furthermore, the maximal enhancing effect was found at minimally harmful CS concentrations as determined by the MTS/PMS assay. In addition to its mucoadhesive properties, our results suggest the usefulness of CS for peptide and protein buccal administration.

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