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International Journal of Pharmaceutics 197 (2000) 53–67

international journal of pharmaceutics

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Chitosans as nasal absorption enhancers of peptides: comparison between free amine chitosans and soluble salts

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Received 15 June 1999; received in revised form 29 November 1999; accepted 30 November 1999

Abstract

A total of three free amine chitosans (CS J, CS L and CS H) and two soluble chitosan salts (CS G and CS HCl) were evaluated for their efficacy and safety as nasal absorption enhancers of peptides based on in situ nasal perfusion and subacute histological evaluation in rat. At 0.5% w/v, all chitosans were effective in enhancing the nasal absorption of [D-Arg^2]-Kyotorphin, an enzymatically stable opioid dipeptide. The enhancing effect of the free amine chitosans increased as the pH was decreased from 6.0 to 4.0 ($P < 0.05$). However, the pH effect was not significant for the two chitosan salts ($P > 0.05$), suggesting that their adjuvant activity may be less pH-dependent than the free amine form. CS J and CS G were subsequently selected for further studies. At only 0.02% w/v, their enhancing effect was already significant and comparable to that of 5% w/v hydroxypropyl-β-cyclodextrin (HP-β-CD). Both chitosans at 0.1% caused minimal release of total protein and phosphorus from the rat nasal mucosa, with the values similar to that of 5% HP-β-CD. At 0.5% the two chitosans also stimulated smaller release of lactate dehydrogenase, an intracellular enzyme used as marker of nasal membrane damage, than 1.25% dimethyl-β-cyclodextrin. Morphological evaluation of the rat nasal mucosa following 2-week daily administration indicated that the two chitosans (1.0%) produced only mild to moderate irritation. In conclusion, both the free amine and the acid salt forms of chitosans are effective in enhancing the nasal absorption of [D-Arg^2]-Kyotorphin and have potential for further studies as a safe and effective nasal absorption enhancer of peptide drugs.

Keywords: Free amine chitosans; Chitosan salts; Nasal absorption enhancers; Hydroxypropyl-β-cyclodextrin; Dimethyl-β-cyclodextrin; Protein release; Phosphorus release; Subacute histological evaluation

1. Introduction

Chitosan is a polymer obtained from deacetylation of chitin, a naturally-occurring structural polymer abundant in crab and shrimp shells. It is a cationic polysaccharide with linear chain con-

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0378-5173/00/$ - see front matter
PII: S0378-5173(99)00451-2

sisting of β-(1,4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) and 2-amino-2-deoxy-β-D-glucopyranose (GlcN) (Mathur and Narang, 1990). The greater the extent of deacetylation, the smaller is the proportion of GlcNAc in the polymer chain.

Recently, chitosan has been shown to enhance nasal and intestinal absorption of hydrophilic drugs like peptide hormones in both the in vitro and in vivo models (Artursson et al., 1994; Illum et al., 1994; Lueβen et al., 1996, 1997). According to the study using an in vitro Caco-2 cell model, its absorption enhancing mechanisms were reported to be a combination of mucoadhesion and an effect on the opening of the tight junctions (Artursson et al., 1994). Schipper et al. (1996), using the same in vitro model, reported that the structural properties of chitosans such as degree of acetylation and molecular weight, are very important for its absorption enhancement of hydrophilic drugs. They found that a low degree of acetylation (i.e. high percent deacetylation with greater charge density) and/or a high molecular weight appear to be necessary for chitosans to increase the epithelial permeability. Toxicity of chitosan also depends on its high charge density but appears to be less affected by the molecular weight.

Although this Caco-2 cell model has yielded a good deal of relevant information, the experimental conditions were far from the actual environment of the nasal cavity. In vivo absorption studies in animals have been carried out to evaluate the nasal absorption promoting activity of chitosan using insulin as a model peptide (Illum et al., 1994; Aspden et al., 1996). However, no correlation was found between the molecular weight nor the degree of acetylation of chitosan and its enhancing effect on the in vivo nasal absorption of insulin in rats (Aspden et al., 1996). Therefore, it appears that the results could vary among different experimental models and that more information is needed to further characterize the safety and efficacy of chitosan as a nasal absorption enhancer of peptides.

In addition, most of the studies utilized the salt forms of chitosan as the absorption enhancer, either hydrochloride or glutamate salt. Thus, it would be interesting to study the absorption enhancing effect of some free amine chitosans and compare the results with the soluble salt forms. The in situ nasal perfusion technique was utilized in this study due to its simple experimental set-up and its ability to characterize both the efficacy and safety of several nasal absorption enhancers (Tengamnuay and Mitra, 1990; Shao and Mitra, 1992; Shao et al., 1992). Furthermore, few longer-term histological studies of the effect of chitosans on the nasal mucosa are available. For example, Aspden et al. (1997) studied the histology of human inferior turbinates following daily nasal administration of 0.25% w/v chitosan glutamate to healthy volunteers for 7 days. A study with a longer exposure period and using higher concentration of chitosans would further characterize their safety profiles under a more rigorous condition.

Therefore, the objectives of this study were: (1) to evaluate the efficacy of chitosans as nasal absorption enhancers of peptides using the in situ rat nasal perfusion technique; (2) to study the effects of physicochemical factors such as chemical form (free amine versus salt form), pH, and chitosan concentration on their nasal absorption promoting activity; and (3) to study the possible deleterious effects of chitosans based on the release of various mucosal components and morphological evaluation of the nasal mucosa following 2-week daily nasal administration of chitosan solutions to intact rats.

2. Materials and methods

2.1. Chemicals

A total of three free amine chitosans (CS J, CS H and CS L) and two salt forms (CS G and CS HCl) were used. CS J was obtained from Kyowa Technos, Japan, whereas CS H and CS L were donated by Unicord Public, Bangkok, Thailand. Chitosan glutamate (CS G, Seacure© G 210+) and chitosan hydrochloride (CS HCl, Seacure© Cl 210) were gifts from Pronova Biopolymer, Drammen, Norway. The viscosity average molecular weight (M_v) of chitosans was determined from intrinsic viscosity ([η]) measurements using

Ostwald capillary viscometer (Thomas, Philadelphia, USA). Their specifications are given in Table 1. [D-Arg^2]-Kyotorphin, L-phenylalanine, bovine albumin (used in the protein assay), lactate dehydrogenase assay kits, hydroxypropyl-β-cyclodextrin (HP-β-CD) and dimethyl-β-cyclodextrin (DM-β-CD) were all obtained from Sigma, St Louis. Triple-distilled water was used to prepare buffers and enhancer solutions. All other reagents were of analytical grade and used as received. The enhancer concentrations were expressed as % w/v.

2.2. In situ perfusion of the rat nasal cavity

2.2.1. Effects of pH medium, chitosan type and concentration on nasal absorption of [D-Arg^2]-Kyotorphin

[D-Arg^2]-Kyotorphin or L-Tyr-D-Arg ($C_{15}H_{23}N_5O_4$, MW 337.38, theoretical pI 8.75) is an analogue of kyotorphin (L-Tyr-L-Arg), a neural dipeptide which has a morphine-like activity by stimulating the release of endogenous met-enkephalin (Takagi et al., 1979a). It has a greater analgesic effect than the natural L-isomer due to its ability to resist degradation by peptidases (Takagi et al., 1979b). It had been shown to be enzymatically stable during nasal perfusion and was also poorly absorbed from the nasal perfusate, thereby making it an ideal model dipeptide to study the effect of absorption enhancers (Tengamnuay and Mitra, 1990).

Male Sprague–Dawley rats (250–300 g) were surgically treated according to the method of Hirai et al. (1981). The perfusing solutions consisted of 0.5 mM [D-Arg^2]-Kyotorphin and each of the five chitosans (concentration initially set at 0.5% w/v) in isotonic phosphate buffers with pH varying from 3.0 to 6.0. All chitosans were first dissolved in 1% v/v acetic acid to make stock solutions of 1.0% w/v. Following overnight swelling, the pH of each stock solution was adjusted to 3.0, 4.0, 5.0, or 6.0 by dropwise addition of either 1 N hydrochloric acid or 1 N sodium hydroxide. Isotonicity was further achieved by gradual addition of sodium chloride and was checked by osmometer (Osmomat 030-D, Gonotec, Germany) to be $\sim$290–310 mosmol/kg. The final perfusing solution was obtained by mixing equal volumes of each chitosan stock solution with a stock solution of 1 mM [D-Arg^2]-Kyotorphin in isotonic phosphate buffer of the same pH.

Then, 5 ml of each solution was recirculated at 37°C through the rat nasal cavity at a rate of 2.0 ml/min for 120 min ($n = 4$–6 rats per group). At appropriate time intervals, aliquots of the perfusate were withdrawn to analyze for the concentration of [D-Arg^2]-Kyotorphin remaining in the perfusate. An equal volume of the fresh buffer medium was added after each withdrawal to maintain the total perfusate volume. Control groups were performed by perfusing the rat nasal cavity with solutions of only the dipeptide in isotonic phosphate buffers of corresponding pH. After the optimum pH had been determined for each of the five chitosans, two chitosans with the most promising absorption enhancing activity

Table 1
Appearances and properties of the five chitosans

CS	Form	% Deacetylated	M_v[c] (kDa)	Percent acid content
J	Flake (free amine)	80.25[b]	1860	–
L	Flake (free amine)	97.2[a]	680[a]	–
H	Flake (free amine)	79.6[a]	900[a]	–
HCl	Powder (HCl salt)	>70[b]	910	10–20% HCl
G	Powder (glutamate salt)	>70[b]	800	35–45% Glutamic acid

[a] Obtained from results by Phaechamud, 1995.
[b] Obtained from the manufacturer.
[c] Viscosity average molecular weights (M_v) were determined from the intrinsic viscosity data ([η]) using Mark–Houwink equation, $[\eta] = KM_v^a$, where the proportionality constant (K) and the shape factor (a) of chitosans = 3.04×10^{-5} cm^3/g and 1.26, respectively (Roberts and Domszy, 1982).

were subsequently selected for further studies. The perfusion experiments were repeated at the chitosan concentrations of 0.02 and 0.1%. The results were then compared to that produced by 5.0% HP-β-CD in buffer pH 7.4.

Analysis of [D-Arg^2]-Kyotorphin remaining in the perfusates was achieved by a specific HPLC method (Sahamethapat, 1997). The system consisted of a solvent delivery pump (Waters 510, Millipore, MA) equipped with a variable wavelength UV detector set at 274 nm (Waters Model M 484), an autoinjector (Waters Model WISP 712), and an integrator (Waters 745 B Data Module). The stationary phase was a Spherisorb 10 ODS (2), 250 × 4.60-mm, stainless steel column (Phenomenex, CA). The mobile phase consisted of 1% v/v acetonitrile in 0.01 M sodium acetate pH 4.0 with a flow rate of 1.5 ml/min. An aliquot (25 μl each) of the perfusate was removed from the reservoir at 0, 30, 60, 90, and 120 min, immediately mixed with an equal volume of an internal standard solution (5 mM L-phenylalanine in the same buffer as the perfusate), and then injected to the HPLC. The standard solutions, which contained corresponding absorption enhancers, were similarly mixed with the internal standard prior to HPLC injection. The final volume and pH of the perfusate were regularly checked at the end of perfusion. By using isotonic buffer as the perfusing medium, changes in the final volume and pH of the perfusates were minimal.

2.2.2. Protein and phosphorus release study

Perfusion was also conducted to determine the effects of chitosan on the protein and phosphorus release from the rat nasal mucosa ($n = 3$ rats per group). Isotonic solutions of the two chitosans previously selected, each at 0.1% (no dipeptide) and at their respective optimum pH, were perfused in a similar manner. Aliquots of 200 μl were taken at appropriate time intervals to analyze for the rate and extent of protein and phosphorus release. After each withdrawal, the perfusate was also replaced with an equal volume of the fresh corresponding buffer. Control groups were conducted using only isotonic buffers (no enhancer and the dipeptide) as the perfusing solutions. The total protein contents in the perfusates were analyzed according to the method of Lowry et al. (1951), whereas the total phosphorus contents were assayed according to the method of Bartlett (1959) and Feldman et al. (1973). The phospholipid phosphorus contents were assayed according to the method of Zilversmit and Davis (1950). The effects of chitosans on the mucosal components release were also compared to that of 5.0% HP-β-CD in buffer pH 7.4. Isotonic lactate or acetate-borate buffers with the pH values corresponding to the optimum pH of each enhancer were selected as the perfusion media instead of the conventional phosphate buffers used in the previous part. This was to avoid the presence of any extraneous phosphate ions, which may interfere with the phosphorus analysis.

2.2.3. Lactate dehydrogenase release study

To further characterize the membrane irritating effect of chitosans at higher concentration and without the influence from the buffer, 0.5% solutions of two selected chitosans in isotonic saline (0.85% NaCl with pH adjusted to 6.0 with 1 N HCl) were similarly perfused through the rat nasal cavity for 30 min. At the end of the perfusion, the activity of lactate dehydrogenase (LDH; EC 1.1.1.27), a nasal epithelial intracellular enzyme, was quantitated according to the method of Cabaud and Wroblewski (1958). The results were compared with that of 5% HP-β-CD and 1.25% DM-β-CD, similarly perfused in the same medium ($n = 3$ rats per group).

2.3. Histological evaluation

A total of seven groups of rats were used ($n = 3$ rats per group). The first group received no treatment (intact control) whereas groups 2, 3 and 4 (buffer control) respectively received once-daily nasal administration of only the isotonic buffers with the pH values corresponding to the optimum pH for the two previously selected chitosans and HP-β-CD. The buffer types were the same as in the protein and phosphorus release study to allow for direct comparison of the enhancers' safety profiles. Groups 5 and 6 were treated with once-daily nasal administration of the two chitosans (each at 1.0% and at their corresponding optimum

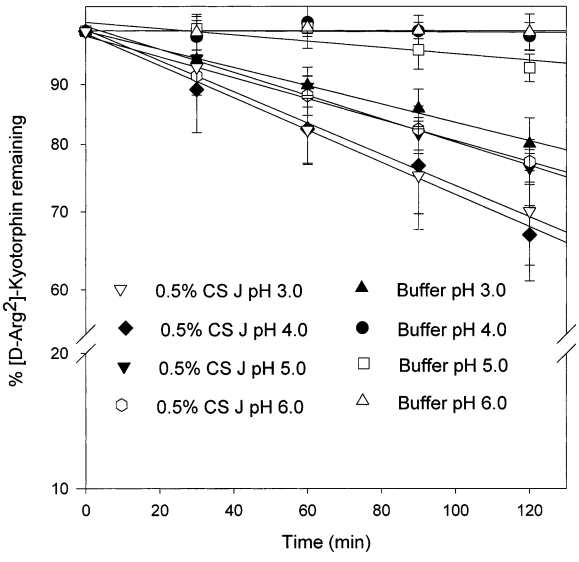

Fig. 1. Semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining in the perfusates versus time after nasal perfusion of the rat with 0.5% CS J at various pH. The control groups consist of only the dipeptide in isotonic phosphate buffers. Values are means ± S.D. ($n = 4–6$).

pH). The last group (group 7) was daily administered with a solution of 5.0% HP-β-CD in buffer pH 7.4. The administered volume was 30 μl in all cases and was applied to the right nostril only. The animals were sedated with a low dose (~ 45 mg/kg) of Thiopental i.p. before each dosing to facilitate nasal administration. After 2 weeks, the rats were sacrificed and processed to obtain slides of the nasal mucosal tissues for light microscopic examination according to the method of Chandler et al. (1991). In addition to conventional haematoxylin and eosin (HE) staining, some slides were stained with alcian blue to identify acidic mucopolysaccharides in mucus and goblet cells.

2.4. Statistical evaluation

One-way analysis of variance (ANOVA) was performed to compare different independent groups at 5% significance level. If significance was detected, the data were further compared using Duncan's new multiple range test at the same significance level. All data were presented as means ± S.D.

3. Results and discussion

3.1. Effect of chitosan type (free amine versus salt forms), pH and concentration on their nasal absorption enhancing efficacy

In situ perfusion experiments revealed that all chitosans studied were capable of enhancing the nasal absorption of [D-Arg^2]-Kyotorphin as compared to controls ($P < 0.05$). The absorption pattern, as seen from the loss of the dipeptide from the perfusate, appeared to follow first order process ($r^2 = 0.90–0.99$), indicating that the substance was transported across the nasal mucosa by passive diffusion. Fig. 1 shows semilogarithmic plots of percent [D-Arg^2]-Kyotorphin remaining in the perfusate versus time after nasal perfusion of the dipeptide in the presence of 0.5% CS J in isotonic phosphate buffers pH 3.0–6.0. Control experiments were also conducted by perfusing only the dipeptide solutions without chitosans at their respective pH.

3.1.1. Free amine chitosans and pH effect

Fig. 1 reveals that the enhancing effect of CS J, which is in a free amine form, is dependent on pH. The absorption was found to increase with decreasing pH, reaching maximum absorption around pH 3.0 and 4.0. After 120-min perfusion at pH 3.0 and 4.0, the concentration of [D-Arg^2]-Kyotorphin decreased to 70.1 ± 9.0 and 66.9 ± 3.9%, respectively, of the initial level, equivalent to ~ 30% absorption. At higher pH conditions (pH 5.0 and 6.0), the dipeptide concentration reduced to only 76.4 ± 2.2 and 77.3 ± 3.4%, which were equivalent to ~ 23% absorption. Perfusion of the control groups at pH 4.0, 5.0, and 6.0 did not result in any substantial absorption. This indicated that, in the absence of CS J, the permeability of the rat nasal mucosa to this dipeptide was very low (Fig. 1).

Perfusion of other free amine chitosans (CS H and CS L) also resulted in a similar pH-dependent absorption enhancement. Fig. 2 is a histogram summarizing the pH effect of various chitosan solutions (0.5%) on the percent dipeptide remaining at 120 min (or at 60 min for CS L). Since both CS H and CS L could not dissolve at pH 6.0 and

above, the perfusion experiments were conducted only at pH 3.0, 4.0 and 5.0. Fig. 2 shows that when the pH was decreased from 5.0 to 3.0, the absorption enhancing activity of CS H and CS L also increased. For example, the average percent dipeptide remaining at 120 min for CS H dropped from $89.8 \pm 1.9\%$ at pH 5.0 to 82.5 ± 2.6 and $62.8 \pm 2.2\%$ at pH 4.0 and 3.0, respectively.

It is interesting to note that perfusion of the control group at the more acidic pH 3.0 also resulted in some absorption of the dipeptide ($\sim$ 20% at 120 min; Fig. 1), which was significant when compared to other control groups at higher pH values ($P < 0.05$). It is possible that the highly acidic condition of this buffer may have caused some deleterious effects to the nasal mucosa and resulted in an increased permeability to the dipeptide. Ohwaki et al. (1985, 1987) also observed an increase in nasal absorption of secretin in rats at an acidic pH of 2.94, with the corresponding structural changes in the nasal epithelial cells upon exposure to this pH solution. As a result, the perfusion data at pH 3.0 were excluded from statistical comparison to avoid contributing effect from the buffer at this pH.

Statistical analyses on percent [D-Arg^2]-Kyotorphin remaining in the nasal perfusate at 120 min ($\%T_{120}$) revealed that CS J exhibits significantly greater absorption enhancing effect at pH 4.0 than at pH 5.0 or 6.0 ($P < 0.05$). Due to lack of perfusion data at pH 6.0, statistical comparison of the nasal absorption enhancing effect of CS H was made between pH 4.0 and 5.0 only. Similar to CS J, its absorption enhancing activity is significantly greater at pH 4.0 ($P < 0.05$). On the other hand, the pH effect could not be tested for CS L. Like CS H, it could not dissolve at pH 6.0. Furthermore, it was observed to precipitate from the solution after 60-min perfusion at pH 5.0, resulting in a premature termination of the experiment. Thus, pH 4.0 was left to be the only pH available for the activity of CS L. However, visual comparison of the percent dipeptide remaining at 60 min (before precipitation occurred) also re-

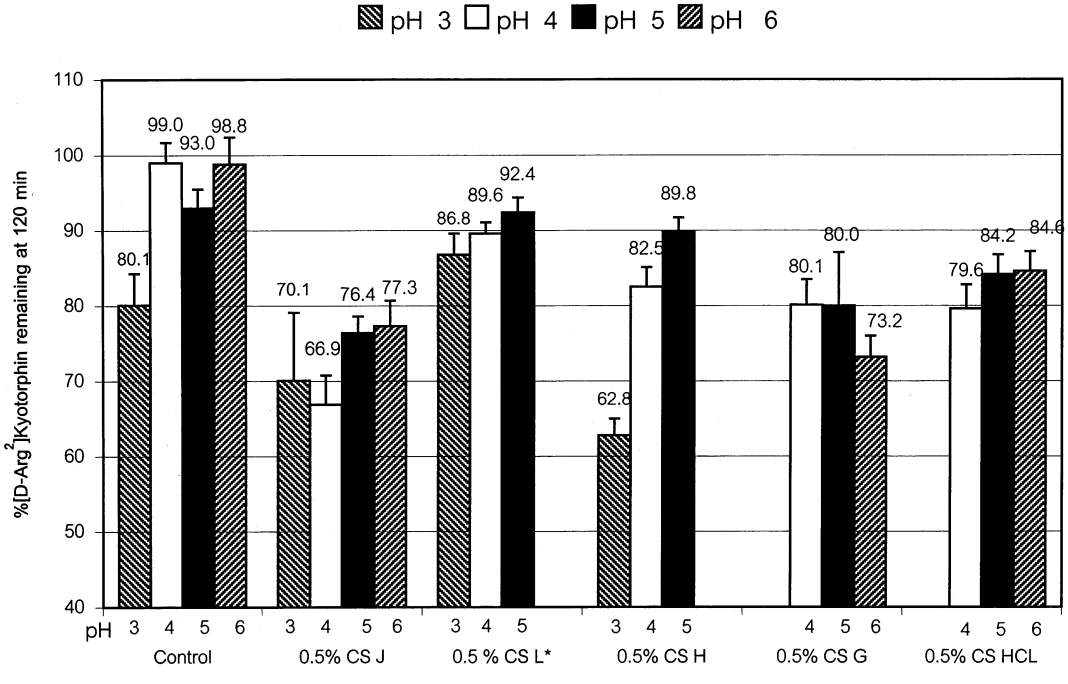

Fig. 2. Effect of pH (3–6) of the perfusion medium (isotonic phosphate buffer) on the percent [D-Arg^2]-Kyotorphin remaining after 120-min perfusion. Data are means $\pm$ S.D. ($n = 4$–6). * Percent dipeptide remaining at 60 min was used for comparison of CS L since precipitation of CS L occurred after 60-min perfusion at pH 5.

vealed that the enhancing effect of CS L appears to be slightly greater at pH 4.0 ($89.6 \pm 1.5\%$) than at pH 5.0 ($92.4 \pm 2.0\%$; Fig. 2). Therefore, pH 4.0 was considered to be the optimum pH for the three free amine chitosans employed in this study.

The reasons for the precipitation of CS L after perfusion at pH 5.0 and the failure of CS L and CS H to dissolve at pH 6.0 are not presently known. However, some impurities in the form of suspended matter were observed in the solutions of CS L and CS H and could not be removed. On the other hand, no suspended particles were observed with solutions of CS J, CS G or CS HCl.

Comparison of $\%T_{120}$ at this optimum pH 4.0 revealed that CS J (66.9%) was a more effective enhancer than CS L (78.8%) and CS H (82.5%) ($P < 0.05$). The greater enhancing effect of CS J might be due to its much higher molecular weight than CS L and CS H (Table 1). This observation was in agreement with that of Schipper et al. (1996), who suggested that a high degree of deacetylation and/or high molecular weight appeared to be necessary for chitosans in enhancing the in vitro epithelial permeation of hydrophilic molecules in a Caco-2 cell model. For example, CS J and CS H had a similar degree of deacetylation ($\sim 80\%$). However, the average molecular weight of CS J (1860 kDa) was $\sim$2-fold higher than CS H (900 kDa). This may impart a greater charge density per molecule for CS J and subsequently allow its molecule to have better interaction with the nasal mucosa than CS H. On the other hand, CS L had the highest degree of deacetylation (97.2%) and was supposed to be highly charged at the same optimum pH. However, its much lower average molecular weight (680 kDa) may have compromised its ability to interact with the nasal epithelium and thus resulted in a lower absorption enhancing efficacy than CS J.

In addition, Lehr et al. (1992), using an in vitro mucoadhesion test, reported a general trend that the mucoadhesive property of chitosans and other pharmaceutical polymers increases with their viscosity and molecular weight. Therefore, the greater adjuvant activity of the high molecular weight CS J could also be attributed to its possible better mucoadhesive property in addition to the reported mechanism of direct interactions with the apical cell membrane and subsequent opening of the tight junctions (Schipper et al., 1997). Nevertheless, more studies are needed to confirm the above observations.

3.1.2. Chitosan salts and pH effect

Nasal perfusion of 0.5% CS HCl, a soluble chitosan salt, also showed the same trend of increasing dipeptide absorption as the pH was lowered (Fig. 2). The average $\%T_{120}$ decreased from $84.6 \pm 2.6\%$ at pH 6.0 to $79.6 \pm 3.2\%$ at pH 4.0. However, these values were not significantly different ($P > 0.05$). For CS G, the ranking of the pH effect seems to be in reverse order to that of the free amine chitosans and CS HCl (Fig. 2). The average $\%T_{120}$ after nasal perfusion with 0.5% CS G at pH 6.0, 5.0 and 4.0 were found to be 73.2 ± 2.8, 80.0 ± 7.1 and $80.1 \pm 3.4\%$, respectively. However, this was also not significant ($P > 0.05$). Thus, based on the available in situ perfusion data, it appears that the absorption enhancing activity of the soluble salt form of chitosans may be less pH-dependent than that of the free amine chitosans.

The reason for the difference in the pH effect observed between the free amine chitosans and the salt forms could be due to the difference in their chemical nature. CS J, CS H, and CS L exist in a free amine form which normally requires an acidic condition for ionization to occur. Chitosan is a basic polymer with an intrinsic pK_a value of ~ 6.5 independent of the degree of deacetylation (Schipper et al., 1996). As the pH is lowered below its pK_a, the fraction of the ionized groups in the chitosan molecules increases. Subsequent hydration and uncoiling of the chitosan molecules will allow them to have greater contact with the nasal mucosa (Artursson et al., 1994). CS G and CS HCl, on the other hand, are already in a soluble salt form. It may not need that much acidity to hydrate or dissolve. Lehr et al. (1992) found that CS G was the most readily soluble of all chitosans studied. It is possible that both CS G and CS HCl are still able to assume the highly ionized, elongated shape which helps maintain their adjuvant activities at higher bulk pH values. The enhancing effect of CS G was even observed

to be slightly greater at pH 6.0 than at pH 4.0 or 5.0. Indeed, a synthetic quaternary salt of chitosan (*N*-trimethyl chitosan chloride) was shown to have a marked increase in basicity, charge density and solubility even at more neutral pH values (Kotzé et al., 1997).

Data in Table 1 indicate that the approximate molecular weights of CS G (800 kDa) and CS HCl (910 kDa) are in the same range with CS L (680 kDa) and CS H (900 kDa). No distinct relationship was observed between the approximate molecular weight and the nasal absorption enhancing effect of the four chitosans at their respective optimum pH (pH 4.0 for CS H, CS L and CS HCl and pH 6.0 for CS G). However, the molecular weights of the two chitosan salts were also half of CS J, with resulting lower absorption enhancing activity based on comparison of $\%T_{120}$ under their optimum pH (Fig. 2). Thus, the data seem to suggest that chitosans should differ in their molecular weight by at least twofold in order to have a clearly differentiating effect on the nasal absorption enhancement of this dipeptide.

It is interesting to note that 0.5% CS G at pH 6.0 appeared to be more effective than 0.5% CS HCl regardless of its pH conditions. The average value of $\%T_{120}$ for CS G at pH 6.0 (73.2%) was significantly lower than that of CS HCl at pH 4.0 (79.6%), pH 5.0 (84.2%), or pH 6.0 (84.6%) ($P < 0.05$, Student's *t*-test). This seems to be in contrast to the previous findings by Kotzé et al. (1998, 1999). They found that at pH 6.2, CS HCl (0.25–1.50% w/v) was always more effective than CS G in lowering the transepithelial electrical resistance and in increasing the in vitro transport of hydrophilic markers (mannitol and PEG-4000) across the Caco-2 cell monolayers. They attributed the better efficacy of CS HCl to its higher concentration of equivalent chitosan base per weight basis as compared to CS G, assuming the same degree of deacetylation (Kotzé et al., 1998). However, their molecular weights were not available for comparison.

The reason for the better efficacy of CS G at pH 6.0 over CS HCl is not presently known. The molecular weight of CS G is even slightly lower than that of CS HCl (800 vs. 910 kDa). Since the exact values of their degree of deacetylation were not available from the manufacturer, it is possible that this particular batch of CS G may have higher degree of deacetylation and molecular charge density than CS HCl. However, more experiments are needed to confirm this hypothesis. Evidently, the roles of molecular weight, degree of deacetylation and charge density need to be explored in more detail to fully understand their effects on the nasal absorption enhancing activity of these cationic polymers.

Since CS J appeared to give the highest absorption enhancing effect among the free amine chitosans, especially at pH 4.0, it was subsequently selected as a representative of the free amine group for further characterization at this optimum pH. On the other hand, CS G was found to give better enhancing activity than CS HCl, with the optimum effect observed at pH 6.0. Therefore, it was chosen as a model of the soluble chitosan salts for further studies at this pH.

3.1.3. Effect of chitosan concentration

The absorption enhancing effect of CS J and CS G, at their respective optimum pH of 4.0 and 6.0, was found to increase as the chitosan concentration was increased from 0.02 to 0.5% (graph not shown). Perfusion was not performed at concentrations higher than 0.5% since the chitosan solutions became too viscous to obtain reliable perfusion. At the lowest concentration of 0.02%, the values of $\%T_{120}$ for CS J ($84.1 \pm 1.8\%$) and CS G ($80.4 \pm 3.9\%$) were already significant when compared to their corresponding control groups ($P < 0.05$). Furthermore, the effect was equivalent to that produced by 5% HP-β-CD, which gave average $\%T_{120}$ of $82.4 \pm 2.3\%$ ($P > 0.05$). At 0.5% the absorption enhancing activities of both chitosans were highest and became significantly greater than 5% HP-β-CD ($P < 0.05$).

HP-β-CD is a cyclodextrin derivative which has been extensively studied for its promoting effect on epithelial drug transport. It was chosen as a reference enhancer in this study due to its relatively high safety profile and the availability of data for direct comparison (Shao et al., 1992). The concentration was therefore fixed at 5% and the perfusion experiments were conducted at pH 7.4 since it was reported to be the pH of the rat

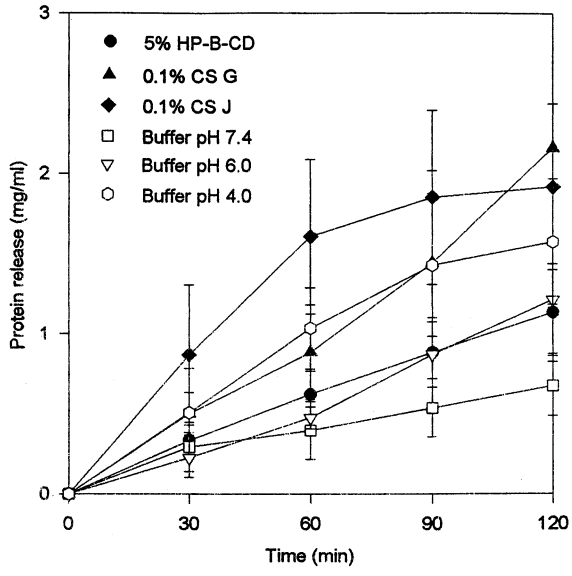

Fig. 3. Release profiles of total protein in the rat nasal perfusates containing 0.1% CS J, 0.1% CS G and 5% HP-β-CD. The perfusion media were isotonic lactate buffer (pH 4.0), and isotonic acetate-borate buffers (pH 6.0 and 7.4), respectively. Values are means ± S.D. ($n = 3$).

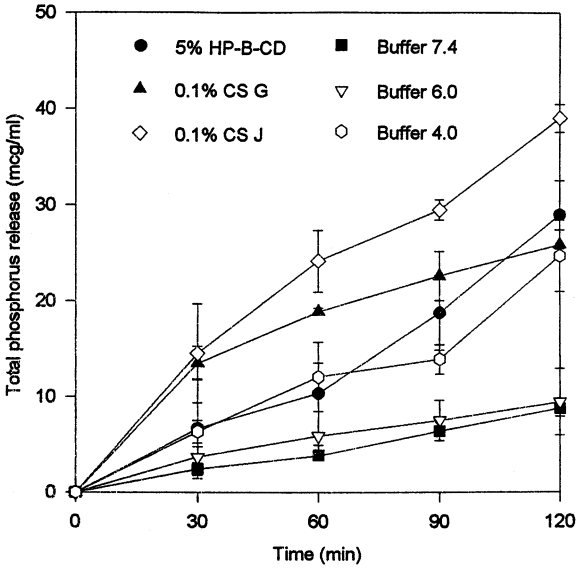

Fig. 4. Release profiles of total phosphorus in the rat nasal perfusates containing 0.1% CS J, 0.1% CS G and 5% HP-β-CD. The perfusion media were isotonic lactate buffer (pH 4.0), and isotonic acetate-borate buffers (pH 6.0 and 7.4), respectively. Values are means ± S.D. ($n = 3$).

nasal mucosa (Hirai et al., 1981). Thus, on a weight basis, CS J and CS G appear to be more effective than HP-β-CD in enhancing the nasal absorption of [D-Arg^2]-Kyotorphin.

3.2. Effect of chitosans on the release of mucosal components from the rat nasal mucosa

The purpose of this part of the study was to determine if the two selected chitosans, under their optimum pH conditions, would have any deleterious effects on the rat nasal epithelium as judged from the release of mucosal protein, phosphorus and lactate dehydrogenase into the perfusate.

3.2.1. Total protein, total phosphorus and phospholipid phosphorus release

Figs. 3–5 respectively depict the release profiles of total protein, total phosphorus and phospholipid phosphorus following nasal perfusion with solutions of CS J (0.1% in isotonic lactate buffer pH 4.0), CS G (0.1% in isotonic acetate-borate buffer pH 6.0), HP-β-CD (5% in isotonic acetate-borate buffer pH 7.4) and their corresponding buffers. Although the chitosan concentration of 0.02% could have been used in this study since its adjuvant activity was already comparable to 5% HP-β-CD, the higher concentration of 0.1% (5-fold) was used instead to provide increased assurance of their safety or toxicity under a more severe condition.

Perfusion with lactate buffer pH 4.0, acetate-borate buffers pH 6.0 and 7.4 resulted in very low contents of total protein released at 120 min, with the mean values ranging from 0.68 to 1.27 mg/ml, respectively. These values agree quite well with that of Pujara et al. (1995), who reported that the average total protein release at the end of 120-min perfusion of isotonic phosphate buffers with pH between 3 and 10 was only $\sim$0.40 mg/ml. However, when the pH became more acidic or more basic, there was a marked increase in the total protein release as a result of possible membrane damage. Furthermore, the same authors found that the type and concentration of the buffer species also had a differentiating effect on the release of the total protein and other mucosal

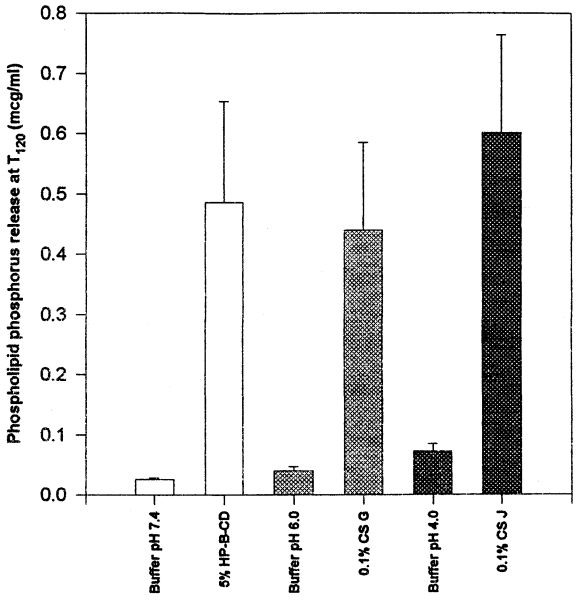

Fig. 5. Histogram of phospholipid phosphorus contents in the perfusates at 120 min after nasal perfusion with 0.1% CS J, 0.1% CS G, 5% HP-β-CD and their control buffers. Values are means ± S.D. ($n = 3$).

components. Therefore, in order to accurately appraise the membrane-irritating potential of different absorption enhancers, the contribution of the buffer effect should be taken into account, particularly when comparison was made under different buffer and pH conditions as employed in this study.

As seen from Fig. 3, inclusion of 0.1% CS J, 0.1% CS G and 5% HP-β-CD caused a slightly further increase in the protein release ($\sim$1.13–2.16 mg/ml at 120 min). To obtain a more accurate comparison of the enhancer effect, the average protein release data of the corresponding buffer were subtracted from the individual data of each enhancer at the same time points in order to correct for the buffer effect. The corrected values of the total protein release rate were found to be between 3.96 and 7.51 μg/ml per min (Table 2). These values were not significantly different among the three enhancers (ANOVA; $P > 0.05$).

Similar observations also apply to the total phosphorus release data. Since phosphorus is an important component of the cell membrane, its release from the nasal mucosa can be used as an indicator of membrane damage caused by the enhancers and buffers (Shao et al., 1992). Fig. 4 reveals that CS J caused significantly greater release of total phosphorus at 120 min (39.0 μg/ml) than HP-β-CD (28.9 μg/ml) and CS G (25.9 μg/ml) ($P < 0.05$). However, this could be due to contribution of the lactate buffer pH 4.0 used as the medium for CS J. This buffer alone was able to stimulate significantly greater release of total phosphorus (24.7 μg/ml) than the acetate-borate buffers pH 6.0 (9.5 μg/ml) and pH 7.4 (8.8 μg/ml) ($P < 0.05$). Therefore, the total phosphorus release data of each enhancer were similarly corrected for the buffer effect. In agreement with the

Table 2
Comparison of the experimental results of the present study with the data of Shao et al. (1992)

Enhancer	Rate of total protein release, μg/ml per min		Rate of total phosphorus release, μg/ml per min	
	Result of Shao[a]	Result of present study[b,c]	Result of Shao[a]	Result of present study[b,c]
5% DM-β-CD	132.40 ± 23.38	–	0.734 ± 0.217	–
5% Dextrose	1.09 ± 0.82	–	0.038 ± 0.019	–
5% HP-β-CD	7.46 ± 1.87	9.37 ± 2.76 (4.05 ± 2.76)	0.171 ± 0.010	0.233 ± 0.019 (0.150 ± 0.035)
0.1% CS G	–	17.65 ± 5.90 (7.51 ± 5.95)	–	0.203 ± 0.019 (0.127 ± 0.019)
0.1% CS J	–	15.72 ± 4.19 (3.96 ± 2.75)	–	0.310 ± 0.005 (0.120 ± 0.005)
Buffer pH 7.4	–	5.32 ± 1.31	–	0.072 ± 0.005
Buffer pH 6.0	–	10.23 ± 3.31	–	0.076 ± 0.027
Buffer pH 4.0	–	10.63 ± 1.75	–	0.190 ± 0.032

[a] Perfusion medium was normal saline. Values are means ± S.D. ($n = 3$).
[b] Values are means ± S.D. ($n = 3$–4 rats).
[c] Values in parentheses were obtained after subtraction of the corresponding buffer effect.

protein release, ANOVA on the release rates of total phosphorus after buffer correction showed no significant difference among CS J, CS G and HP-β-CD, with the mean values ranging from 0.12 to 0.15 μg/ml per min (Table 2, $P > 0.05$). The same result was also observed for the extent of phospholipid phosphorus release at 120 min (Fig. 5). The three enhancers were found to cause equivalent and minimal release of this marker following buffer correction, with the corrected values in the range of 0.43 to 0.53 μg/ml ($P > 0.05$).

HP-β-CD was shown to be one of the least irritating nasal absorption enhancers based on the mucosal components release and ciliotoxicity studies (Shao et al., 1992; Merkus et al., 1993; Marttin et al., 1995). The similar effects on the protein and phosphorus release observed with 0.1% CS J and 0.1% CS G may suggest that the two chitosans could be as safe as 5% HP-β-CD.

Table 2 also compares the effects of the three enhancers and their buffers on the protein and phosphorus release rates with the data of Shao et al. (1992). The values for HP-β-CD obtained in this study were small regardless of buffer correction and comparable to their results. Interestingly, the release rates for CS J and CS G were two to seven times smaller than those reported for 5% dimethyl-β-cyclodextrin (DM-β-CD), even without subtraction of the buffer effect. DM-β-CD is an effective nasal absorption enhancer of many peptide drugs (Verhoef et al., 1994). However, its potential membrane irritating effect may greatly compromise its usefulness in clinical application.

3.2.2. Lactate dehydrogenase release

To directly compare the safety of the two chitosans with DM-β-CD, rats were nasally perfused with these enhancers for 30 min and the extent of lactate dehydrogenase (LDH) release was measured. The perfusion medium was isotonic saline in all groups to allow for comparison of the enhancer effect without influence from different buffers and pH conditions. The perfusion pH was set at 6.0 because LDH loses its activity at pH below 4 (Pujara et al., 1995) and chitosans could not readily dissolve beyond pH 6. To see the effect more clearly, the chitosan concentration was also increased from 0.1 to 0.5%.

Perfusion with 1.25% DM-β-CD resulted in LDH release at 30 min of 475.83 ± 94.10 U/ml, which was nearly twice that of 0.5% CS J (259.44 ± 71.39 U/ml) and 0.5% CS G (273.28 ± 130.96 U/ml). On the other hand, perfusion of 5% HP-β-CD caused minimum release of this enzyme (41.28 ± 6.79 U/ml), which was equivalent to that of pure saline (45.54 ± 31.33 U/ml).

Since LDH is an intracellular enzyme, its leakage into the nasal perfusate is an indicator of membrane irritation. DM-β-CD has a strong membrane-solubilizing effect. Shao et al. (1992) reported that nasal perfusion of a higher concentration of DM-β-CD (5% in isotonic saline) induced a much greater extent of LDH release. After 30 min the average LDH concentration in the perfusate was ~ 2000 U/ml and reached over 6000 U/ml after 90 min. DM-β-CD was also found to be extremely hemolytic even at low concentration (Yoshida et al., 1988). In our study, one rat showed slight bleeding of the nasal mucosa after perfusion with 1.25% DM-β-CD, as evidenced from the light orange coloration of the perfusate. No change in the perfusate color was detected with other groups.

Since 5% HP-β-CD induced less LDH release than 0.5% chitosans, it was considered to be the least membrane irritating. However, it is also a very weak nasal absorption enhancer and failed to produce significant absorption enhancement in many cases (Merkus et al., 1993). At the conventional concentration of 5%, its absorption enhancing effect on [D-Arg^2]-Kyotorphin was significantly less than that of 0.5% CS J and CS G. Therefore, the results indicate that the two chitosans appear to be less membrane irritating than DM-β-CD and possess greater activity than HP-β-CD in enhancing the nasal absorption of this dipeptide. Studies are being conducted to compare the in vivo absorption-enhancing efficacy of CS J and CS G with that of DM-β-CD and HP-β-CD using a larger peptide (salmon calcitonin) as a model.

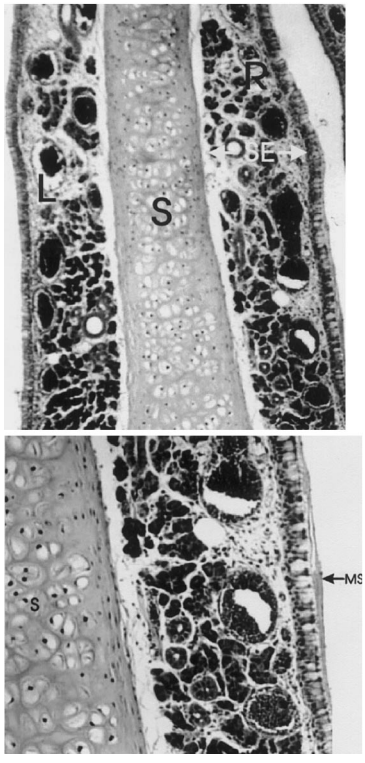

Fig. 6. Light photomicrograph taken from the anterior cross-section of the rat nasal cavity following 14-day exposure to 5% HP-β-CD. Top figure (× 80) shows slight subepithelial edema (SE) of the epithelial lining of dorsal medial meatus in the dosed side (R). Bottom figure (× 100) illustrates some intraluminal mucus secretion (MS) coated on the epithelial surface of the dosed side. HE stain; L, undosed side; S, nasal septum.

3.3. Effects of chitosans on morphological integrity of the rat nasal mucosa

Following 2-week daily nasal administration to intact rats ($n = 3$ rats/group), the nasal tissues were processed for gross examination under light microscope. Evaluation criteria were set up by classifying the observed morphological changes into three levels of irritation, i.e. mild, moderate and severe. For example, mucus hypersecretion and increased goblet cell activity (cell distention) were considered to be symptoms of only mild level whereas vascular congestion and subepithelial edema were considered to be moderate. The signs for severe irritation included observation of epithelial necrosis, sloughing of epithelial cells and/or hemorrhage. The administration volume (30 μl) used in this study was sufficiently small to allow for direct comparison of the nasal mucosa between the dosed (right) side and the undosed (left) side of the nasal cavity. Evaluation was made in all cross-sections of the excised nasal cavity. However, the anterior cross-sections between the upper incisor root and the incisive papilla (region II in Chandler et al., 1991) were used as a primary site for comparison. This was due to the presence of the nasal septum and clear division of the cavity into the right and left sides, the latter being used as a self-control.

All the untreated rats showed intact ciliated respiratory epithelium and normal goblet cell appearance (photomicrographs not shown) whereas the buffer-treated groups (pH 4.0, 6.0 and 7.4) demonstrated only a mild level of irritation with slight intraluminal mucus secretion and increased goblet cell activity (photomicrographs not shown). Exposure to 5% HP-β-CD pH 7.4 (Fig. 6) and 1% CS G pH 6.0 (Fig. 7) also resulted in mild level of mucus hypersecretion and goblet cell distention. However, the effects were more pronounced than their buffer control groups. Subepithelial edema, a sign of moderate irritation, was also observed with 5% HP-β-CD but the extent was very limited. The mucosal irritating effects of 1% CS J (pH 4.0) appeared to be

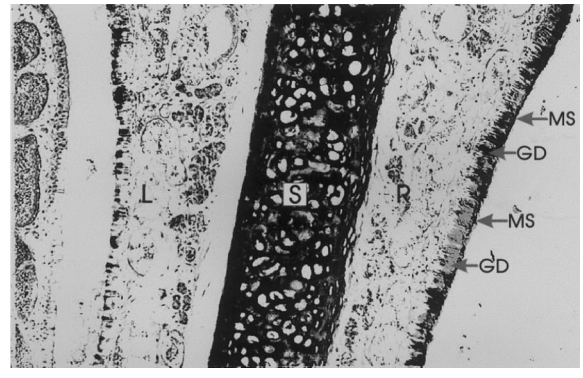

Fig. 7. Light photomicrograph taken from the anterior cross-section of the rat nasal cavity following 14-day exposure to 1% CS G. Strong Alcian blue positive staining of the goblet cells and intraluminal mucus secretion in the dosed side (R) indicates extensive goblet cell distention (GD) and intermediate extent of mucus secretion (MS). HE stain; L, undosed side; S, nasal septum. × 100.

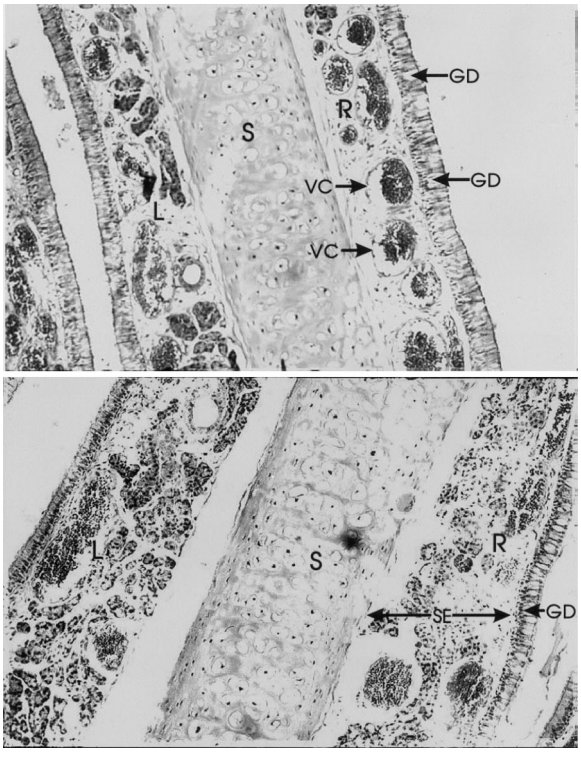

Fig. 8. Light photomicrograph taken from the anterior cross-section of the rat nasal cavity following 14-day exposure to 1% CS J. Top figure shows some vascular congestion (VC) and goblet cell distention (GD) in the epithelial lining of the dosed side (R). Bottom figure illustrates slight subepithelial edema (SE) on the dosed side. HE stain; L, undosed side; S, nasal septum. × 100.

slightly greater than 1% CS G and 5% HP-β-CD. Apart from intermediate extent of mucus secretion and goblet cell distention, a few signs of moderate irritation such as vascular congestion and subepithelial edema were also observed (Fig. 8). However, none of the severe signs such as appearance of epithelial necrosis, sloughing of epithelial cells and hemorrhage were detected in any of the rats. All of the morphological changes observed here are reversible despite the relatively high chitosan concentration of 1% used in this study. Normal cilia were also present in all groups. Our results thus agree with previous studies by Aspden et al. (1997), who reported that daily nasal administration of 100 μl chitosan glutamate solution (0.25% w/v) to healthy volunteers for 7 days had no significant effects on nasal histology and in vivo mucociliary clearance times.

4. Conclusion

According to the in situ rat nasal perfusion, all the chitosans tested are effective in enhancing the nasal absorption of a model dipeptide [D-Arg^2]-Kyotorphin. The enhancing effect of the free amine chitosans (CS J, CS H, CS L) is pH-dependent and increases with decreasing pH whereas the salt forms tested (CS G and CS HCl) appear to be less pH-dependent. Based on % dipeptide remaining at 120 min, the ranking of the enhancing effect of five chitosans (all at 0.5% under their respective optimum pH condition) was CS J > CS G > CS L ~ CS HCl ~ CS H. Results from the mucosal component release and subacute morphological studies further substantiate that both the free amine and salt forms of chitosans may have promising potential for use as safe and effective nasal absorption enhancers of poorly absorbed drugs. More studies are being conducted to confirm their efficacy and safety in vivo.

Acknowledgements

This project was supported by a grant from the Rachadapisek Research Fund of Chulalongkorn University, Bangkok, Thailand. The authors wish to thank the technical assistance of Prapasri Sinswat and Arisara Muangkum for determination of the average molecular weight and LDH studies of chitosans.

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