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Research Paper

Nasal absorption and local tissue reaction of insulin nanocomplexes of trimethyl chitosan derivatives in rats

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Abstract

Objectives The objective of this work was to explore the potential and safety of trimethyl chitosan (TMC) and PEGylated TMC for improved absorption of insulin after nasal administration.

Methods The nasal absorption of insulin nanocomplexes of TMC or PEGylated TMC was evaluated in anaesthetized rats. Concomitantly, the histopathological effects of these nanocomplexes on rat nasal mucosa were studied using a perfusion fixation technique.

Key findings All insulin nanocomplexes containing TMC or PEGylated TMC showed a 34–47% reduction in the blood glucose concentration, when the insulin absorption through the rat nasal mucosa was measured indirectly. In addition, the relative pharmacodynamic bioavailability (F_{dyn}) of the formulations was found to be dependent upon the charge ratio of insulin and polymer, regardless of polymer structure. The F_{dyn} apparently decreased with increasing charge ratio of insulin : polymer. Although acute alterations in nasal morphology by the formulations were affected by the charge ratio of insulin and polymer, the formulation of insulin/PEGylated TMC nanocomplexes was shown to be less toxic to the nasal epithelial membrane than insulin/TMC nanocomplexes.

Conclusions PEGylated TMC nanocomplexes were a suitable absorption enhancer for nasal delivery of insulin.

Keywords insulin nanocomplexes; nasal delivery; nasal histology; PEGylation; trimethyl chitosan

Introduction

Over the past few decades, intranasal insulin delivery has been widely investigated as an alternative to subcutaneous injections for the treatment of diabetes. Nasal delivery systems have attracted interest due to unique features such as easy accessibility, the highly vascularized mucosal surface of the nasal mucosa, the reduced drug degradation in the nasal cavity due to the lower enzyme concentration (compared with the gastrointestinal tract), avoidance of liver first-pass metabolism, as well as the convenience of self-medication.[1] Furthermore, the pharmacokinetic profile of intranasal insulin can be similar to that obtained by intravenous injection and bears close resemblance to the 'pulsatile' pattern of endogenous insulin secretion during meal-times. This suggests that intranasal therapy has considerable potential for controlling post-prandial hyperglycaemia in the treatment of type I diabetes (insulin dependent diabetes, IDDM) and type II diabetes (noninsulin dependent diabetes, NIDDM).[2]

However, effective insulin absorption via the nasal route remains a challenge due to its enzymatic lability and high molecular weight.[3] Actually, the nasal route has been found suitable for drugs with molecular weights < 1 kDa without the use of adjuvants.[3] Several strategies have been explored to enhance the nasal absorption efficacy of insulin by the nasal route, including the use of surfactants, permeation enhancers, bioadhesive polymers and carrier systems.[2,4,5] Unfortunately, increased plasma insulin levels using surfactants and fatty acids often correlated with potential membrane irritation or damage as evidenced by protein release, epithelial disruption, excessive mucus discharge and ciliary toxicity.[6–8] Therefore, the design

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of intranasal insulin delivery avoiding unwanted local side effects has become a topic of intensive research.

N-Trimethyl chitosan (TMC), a derivative of chitosan which is well-soluble in a wide pH range (pH 1–14) showed mucoadhesive properties, depending on its degree of quaternization and degree of dimethylation.[9] Until now, it has received considerable attention in drug and gene delivery studies using several routes of administration.[10–16] Some in-vitro cytotoxicity studies in cell cultures showed that TMC was cytotoxic.[17–19] Jintapattanakit *et al*.[9] reported that the cytotoxicity of TMC depended on its degree of quaternization and dimethylation, which was observed when the ratio of degree of quaternization to degree of dimethylation was > 1. From in-vivo cytotoxicity studies, Haffejee *et al*.[20] reported that TMC did not show any toxic effects to nasal rat epithelium, while Florea *et al*.[14] reported that TMC showed mild irritation in pulmonary tissues of rats.

Recently, PEGylated TMC copolymers were developed in an attempt to improve solubility of chitosan as well as biocompatibility of TMC.[17] They have great potential of forming insulin nanocomplexes by spontaneous self-assembly under the appropriate pH conditions, which could enhance the uptake of insulin in Caco-2 cells.[21] Moreover, these copolymers exhibited beneficial properties as gene carrier systems for plasmid DNA.[22] These observations under in-vitro conditions prompted a more detailed investigation of the in-vivo safety and efficacy of PEGylated TMC copolymer as a nasal absorption enhancer of insulin.

Therefore, in this study, an in-vivo rat model was used to investigate the nasal absorption of insulin nanocomplexes based on TMC and PEGylated TMC copolymers. The influence of insulin/polymer (−/+) charge ratio and PEGylation on the nasal insulin absorption and the morphology of the rat nasal epithelium were studied. The ability to enhance the nasal absorption of insulin was investigated by determining the decrease in blood glucose concentration as a pharmacological response following nasal administration. Concomitantly, acute alterations in rat nasal morphology following treatment were determined after perfusion fixation. Light microscopy was utilized to determine changes in nasal morphology which might be consistent with increased insulin transport and/or toxicity.

Materials and Methods

Materials

TMC and PEGylated TMC copolymers were synthesized and characterized as previously described.[17] For abbreviation of the polymers, PEG(5k)$_{298}$-g-TMC400 and PEG(5k)$_{680}$-g-TMC400 refer to TMC macromolecules of 40% degree of quaternization derived from chitosan (MW 400 kDa, degree of deacetylation 84.7%) containing 298 and 680 chains of 5 kDa macrogol (PEG; polyethyleneglycol), respectively. The properties of the polymers have been summarized in Table 1.

Human recombinant insulin (28.5 IU/mg), trypsin (1060 BAEE unit/mg), PGO enzyme and *O*-dianisidine dihydrochloride were purchased from Sigma (St Louis, MO, USA). *N*-Benzoyl-L-arginine ethylester (BAEE) was purchased from Fluka (Schnelldorf, Germany). Tiletamine HCl/zolazepam HCl mixture was obtained from VIRBAC Laboratories (Zoletil 100, Carros, France). Xylazine was obtained from L.B.S. Laboratory Ltd, Part (X-Zine, Bangkok, Thailand). All other chemicals used were of analytical purity, except those for HPLC assay which were of HPLC purity.

Preparation of insulin/polymer nanocomplexes

Insulin/polymer nanocomplexes were prepared by self-assembly, utilizing the electrostatic interaction between the positively-charged polymers and negatively-charged insulin.[23] Briefly, insulin stock solution (3.5 mg/ml) and polymer solutions in various concentrations (1.0 and 3.5 mg/ml for TMC400 and 17.5 mg/ml for TMC400, PEG(5k)$_{298}$-g-TMC400 and PEG(5k)$_{680}$-g-TMC400) were prepared in isotonic 10 mM Tris buffer _87% (v/v) of 1.15×10^{-2} M HCl and 13% (v/v) of 0.1 M Tris(hydroxylmethyl)-aminomethane solution, supplemented with 147 mM NaCl at a pH of 7.4). An equal volume of insulin and polymer solutions were mixed resulting in nanocomplexes with defined ratios of insulin/polymer as indicated in Table 2. The mixture was then incubated for 20 min at room temperature. Complex size and zeta potentials were obtained by using a Zetasizer nano ZS (Malvern Instruments, UK) equipped with a 4 mW He-Ne laser at a wavelength of 633 nm at 25°C.

Stability of insulin against trypsin

The experiment was carried out according to the method described previously.[24] Briefly, 100 μl 3000 BAEE IU/ml trypsin solution was mixed with 900 μl insulin solution and insulin/polymer nanocomplexes containing 500 μg/ml insulin, and the mixtures were then incubated at 37°C. Three vials of mixture were taken out at predetermined times and an ice-cold 0.1% trifluoroacetic acid (TFA) solution was added to stop enzymatic degradation. The insulin concentration was then quantified by HPLC as described elsewhere.[23]

Table 1 Characteristics of trimethyl chitosan and macrogol-grafted trimethyl chitosan copolymers

Polymers	Degree of substitution (%)[a]	TMC content [%(w/w)]	Theoretical molecular weight (g/mol)	Dalton/charge[b]	Mass ratio [Ins] : [Pol][c]	Charge ratio [Ins] : [Pol][c]
TMC400	-	100.0	40 00 00	189	1 : 0.5	1 : 3
PEG(5k)$_{298}$-g-TMC400	12.0	22.7	18 90 000	1042	1 : 1.0	1 : 3
PEG(5k)$_{680}$-g-TMC400	27.4	10.6	38 00 000	2655	1 : 1.5	1 : 2

[a]Calculated by ^{1}H NMR measurement. [b]Calculated by a proportion of theoretical molecular weight to number of quaternized amino groups; Dalton per charge of insulin was 2900. [c]Optimized ratio in isotonic 10 mM Tris buffer, pH 7.4 for the formulation of nanocomplexes measured by turbidimetric titration.[23] Ins, insulin; PEG, macrogol; Pol, polymer; TMC, trimethyl chitosan.

Table 2 Characterization and main pharmacodynamic parameters after nasal administration of insulin solutions/nanocomplexes

Characterization of administered insulin solutions/nanocomplexes

Formulation[a]	Insulin concn (IU/kg)	Polymer concn (mg/kg)	Mass ratio [Ins] : [Pol]	Charge ratio [Ins] : [Pol]	Particle size (nm)	Zeta potential (mV)
Ins solution, s.c.	0.5	–	–	–	–	–
Ins solution, i.n.	4.0	–	–	–	–	–
Ins/TMC400 nanocomplexes (1 : 3), i.n.[b]	4.0	0.04	1.0 : 0.5	1 : 3	181 ± 2	7.4 ± 1.4
Ins/TMC400 nanocomplexes (1 : 6), i.n.	4.0	0.14	1.0 : 1.0	1 : 6	182 ± 5	14.9 ± 1.3
Ins/TMC400 nanocomplexes (1 : 30), i.n.	4.0	0.70	1.0 : 5.0	1 : 30	223 ± 3	13.9 ± 1.0
Ins/PEG(5k)$_{298}$-g-TMC400 nanocomplexes (1 : 14), i.n.	4.0	0.70	1.0 : 5.0	1 : 14	150 ± 1	10.3 ± 1.0
Ins/PEG(5k)$_{680}$-g-TMC400 nanocomplexes (1 : 6), i.n.	4.0	0.70	1.0 : 5.0	1 : 6	237 ± 7	10.0 ± 1.7

Pharmacodynamics of insulin in a healthy rat model

Formulation[a]	*n*	T_{min} (min)	C_{min} (% basal glucose)	$AOC_{0–240\ min}$ (% glucose·min)	F_{dyn} (%)
Ins solution, s.c.	6	113 ± 10	35.5 ± 8.4	10541 ± 1465	100.0 ± 13.9
Ins solution, i.n.	6	–	–	3279 ± 1470	3.9 ± 1.7
Ins/TMC400 nanocomplexes (1 : 3), i.n.[b]	6	92 ± 10	46.1 ± 6.4	7703 ± 1899*	9.1 ± 2.3*
Ins/TMC400 nanocomplexes (1 : 6), i.n.	6	101 ± 10	37.0 ± 8.1	9302 ± 2060*	11.0 ± 2.4*
Ins/TMC400 nanocomplexes (1 : 30), i.n.	6	103 ± 10	38.3 ± 5.0	10377 ± 1087*	12.3 ± 1.3*
Ins/PEG(5k)$_{298}$-g-TMC400 nanocomplexes (1 : 14), i.n.	6	98 ± 6	33.8 ± 6.2	9889 ± 1417*	11.7 ± 1.7*
Ins/PEG(5k)$_{680}$-g-TMC400 nanocomplexes (1 : 6), i.n.	6	120 ± 6	47.2 ± 8.0	8935 ± 1545*	10.6 ± 1.8*

Data are presented represent as mean ± SD. [a]Prepared in isotonic 10 mM Tris buffer, pH 7.4. [b]Insulin/TMC400 nanocomplex prepared at optimized insulin/polymer ratio. *$P < 0.05$ compared with that of intranasal insulin solution. i.n., intranasal; Ins, insulin; PEG, macrogol; Pol, polymer; s.c., subcutaneous; TMC, trimethyl chitosan.

Hypoglycaemic activity in healthy rats

Nasal absorption studies were performed with Wistar male rats obtained from the National Laboratory Animal Centre (Mahidol University, Nakhon Pathom, Thailand). Animal experiments were approved by the local ethical committees and were conducted according to the guidelines for animal welfare.

Animal preparation and dosing

Rats were acclimatized for one week before the study. Rats (approximately 250 g) were fasted for 18 h, but water was freely available. Six rats were assigned to each group. The rats were anaesthetized by intraperitoneal (i.p.) injection of a mixture of zoletil (50 mg/kg) and xylazine (10 mg/kg) and anaesthesia was maintained with 1:3 additional zoletil/xylazine as needed throughout the experiment. The rats were placed in a supine position.

The insulin solution and insulin/polymer nanocomplexes (~ 20 μl, insulin dose 4 IU/kg) were administered using a 50-μl Hamilton microsyringe, with a blunt needle inserted approximately 0.5 cm into the right nostril (marked on the tip) 30–45 min after the initial dose of anaesthetic agent. For a control, buffer without insulin was intranasally (i.n.) administered to the rats. Insulin solution was also subcutaneously (s.c.) administered to the rats (0.5 IU/kg) to allow comparison of nasal bioavailability to subcutaneous effect bioavailability (F_{dyn}).

Blood glucose measurement

Blood samples (~ 150 μl) were collected from the tip of the tails of anaesthetized animals in heparinized haematocrit tubes (Modulohm A/S, Herlev, Denmark) at −20, −10 min before formulation application and at 5, 15, 30, 45, 60, 90, 120, 180, 240 min after administration. The samples were then centrifuged at 3000 rev/min for 5 min to obtain serum samples and stored at −20°C until analysis. Blood glucose levels were determined with a blood glucose assay kit using the glucose oxidase method. Glucose content was calculated as a percentage of the mean value of the first three measurements of each animal. The values of blood glucose baselines ranged from 110 to 290 mg/dl and were normalized for each experiment at 100%. The blood glucose content at various times thereafter was calculated as a percentage of this initial value for each animal.

Analysis of data

In nasal drug delivery experiments, minimum blood glucose concentration (C_{min}) and time to minimum concentration (T_{min}) were determined directly from the pharmacodynamic time profile. Areas over the curve were calculated for the various groups for the times 0–240 min (AOC_{0-240}) using the linear trapezoidal method. Pharmacodynamic effect availability relative to subcutaneous administration (F_{dyn}) was calculated using the following equation:

$$F_{dyn} = \frac{AOC_{i.n} \times Dose_{s.c}}{AOC_{s.c} \times Dose_{i.n}} \times 100\% \quad (1)$$

Results are shown as the mean values (± SD) of six animals. Statistical analysis was performed by one-way analysis of variance, followed Scheffe *post hoc* for individual group comparisons with SPSS software version 11.5. The level of significance was set at $P < 0.05$.

Histological studies

A nasal irritation study was conducted to determine the response of rat nasal mucosa to the nasal insulin formulations. After the in-vivo absorption studies, three rats per group were randomly sampled and killed by exsanguination under zoletil/xylazine anaesthesia. An incision was made in the oesophagus to insert a silicone cannula into the posterior end of the nasal cavity. The nasal cavity of rats was flushed with 10% neutral-buffered formalin using a peristaltic pump for approximately 20 min. The animals were then decapitated and the mandibles, skin and soft tissue were removed from the skull. The heads were subsequently immersed in fresh fixative for 48 h, decalcified in formic acid–sodium citrate solution for two weeks, and processed as described by Chandler *et al*.[25] to yield complete cross-sections of the nasal cavity. The tissue was processed through to paraffin wax blocks using routine histological methods. Sections were cut serially at 5-μm thickness, mounted and stained with haematoxylin and eosin (HE). Cross-sections of the nasal cavity were examined using an Olympus CX31 microscope equipped with a 7.1 megapixel C7070 wide zoom Camedia camera (Olympus Corporation, Tokyo, Japan). Samples were evaluated by three levels of membrane damage (mild, moderate and severe) as described previously.[26]

Results

Nasal insulin formulations

The properties of insulin nanocomplexes used in this study have been summarized in Table 2. All complexes showed sizes in the range of 150–237 nm with a positive surface charge of 10.0–13.9 mV. Only the insulin/TMC400 nanocomplexes prepared at optimized insulin/polymer (−/+) charge ratio (Ins/TMC400 nanocomplex (1 : 3)) displayed opalescent suspension due to complete interaction between insulin and polymer, while the others were clear solutions of partial nanocomplexes, free insulin and free polymer.

Stability of insulin in the presence of trypsin

Since no significant difference in the protective effect of nanocomplexes prepared at different insulin/polymer ratios was observed ($P > 0.05$), only the protective effect of nanocomplexes prepared at the optimized insulin/polymer ratio (1 : 3) have been presented in Figure 1. The unprotected insulin in the control experiment was significantly degraded in the presence of trypsin and only 30% of the insulin remained intact after one hour. In contrast, partial protection of insulin from trypsin digestion was observed with the insulin/TMC400 and insulin/PEGylated TMC400 nanocomplexes ($P < 0.05$). The protective effect of all insulin/PEGylated TMC400 nanocomplexes was higher than that of the insulin/TMC400 nanocomplexes ($P < 0.05$) and the protective effect increased with increased macrogol substitution degree.

Hypoglycaemic activity

The blood glucose baselines of anaesthetized male Wistar rats before the application of the nasal formulations were in the range of 110–290 mg/dl. The hypoglycaemic effects and the corresponding pharmacodynamic parameters after nasal application of various insulin formulations vs a reference (s.c. administration) have been presented in Figure 2 and Table 2, respectively. Blood glucose levels were not reduced initially after administration of the isotonic Tris buffer as seen in the control; however, reduction with fluctuation of the blood glucose with time (< 15% of the basal level) was observed after 30 min ($P < 0.05$ at 45, 180, 240 min). Compared with isotonic Tris buffer, a slight decrease in blood glucose concentration of the intranasal insulin solution (4.0 IU/kg) was observed ($P > 0.05$). The subcutaneous reference of 0.5 IU/kg insulin yielded a C_{min} equal to 36% of the basal glucose concentration after 113 min.

As seen in Figure 2 and Table 2, nasal administrations of the insulin/TMC400 and insulin/PEGylated TMC400 nanocomplexes reduced blood glucose level significantly as compared with the control insulin solution ($P < 0.05$). All nanocomplexes decreased blood glucose level up to 37–47% of the basal blood glucose level at approximately 91–120 min after nasal instillation. The F_{dyn} over 4 h of all nanocomplexes

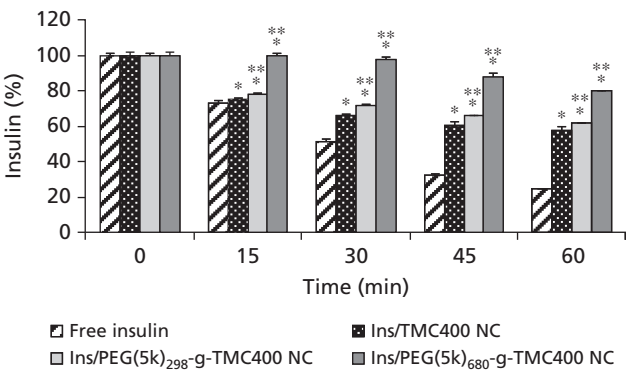

Figure 1 Effect of macrogol substitution degree on the enzymatic degradation of insulin by trypsin. Each value represents the mean ± SD of three experiments. The initial concentrations of insulin (Ins) and trypsin were 450 μg/ml and 300 BAEE IU/ml, respectively. All formulations prepared at optimal insulin/polymer (−/+) charge ratio. NC, nanocomplexes. $^{*}P < 0.05$ compared with the values of free insulin; $^{**}P < 0.05$ compared with the values of TMC400

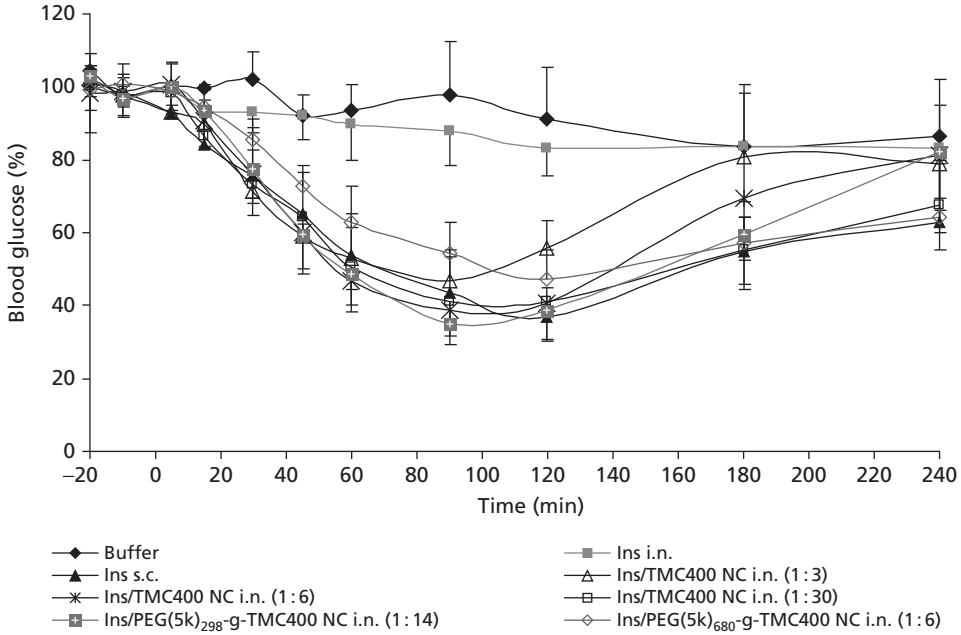

Figure 2 Blood glucose reduction in healthy rats. Doses of insulin (Ins) were 4 and 0.5 IU/kg for intranasal (i.n.) and subcutaneous (s.c.) administration, respectively. NC, nanocomplexes. Data are presented as mean ± SD of six experiments

was in the range of 9.1–12.3%, which was higher than that of the control group (3.9%) ($P < 0.05$). There was no significant difference in the F_{dyn} values of the various insulin nanocomplexes ($P > 0.05$); however, it was found that the F_{dyn} was dependent on insulin/polymer (−/+) charge ratio regardless of polymer structure. The F_{dyn} apparently decreased with increasing charge ratio of insulin/polymer as shown in Figure 3.

Histopathological effect of insulin nasal formulations on the nasal epithelium

Figure 4 depicts the distributions of epithelial lesions in the nasal cavity and microscopic appearances of nasal epithelium from the upper incisor root to the incisive papilla after 4-h exposure to each nasal insulin formulation. The epithelia of the control groups which were exposed to isotonic Tris buffer

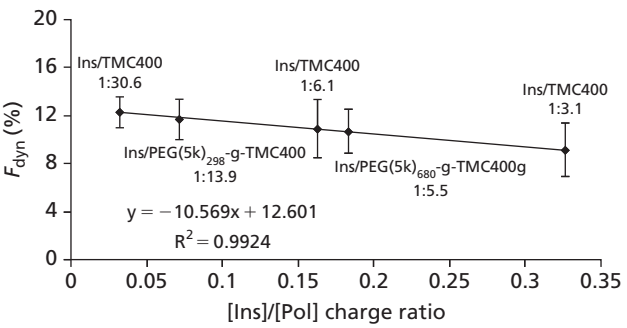

Figure 3 Correlation between insulin/polymer charge ratio of different insulin/polymer nanocomplexes and relative pharmacodynamic bioavailability. Data are presented as mean ± SD of six experiments. Ins, insulin; Pol, polymer

(photomicrographs not shown) and insulin solution (Figure 4a) showed no signs of damage or lesions. The epithelial cells remained unaffected in all sections studied. However, some rats demonstrated slight mucus secretion in both sides, which was considered to be a normal incidence since the rats were not kept in a tightly controlled, dust-free area.

Effect of insulin/polymer (−/+) charge ratio

Taking the insulin/TMC400 nanocomplexes as an example, it was observed that the damaging effects of insulin/TMC400 nanocomplexes to nasal epithelium increased with increasing ratio of TMC400. The nasal tissue exposed to the insulin/TMC400 nanocomplexes prepared at the optimized insulin/TMC400 ratio of 1 : 3 exhibited a moderate increase in goblet cell distention as compared with the undosed side, but this was restricted only to the septal region without flow into the dorsal meatus. However, slight subepithelial oedema was found on the nasoturbinate in the lateral meatus which might have been attributed to the dorsal position of the rat during the administration (Figure 4b). The administration of the 1 : 6 insulin/TMC400 nanocomplexes demonstrated a discharge of mucus, subepithelial oedema and considerable epithelial disruption. An epithelium was still maintained over all surfaces. The epithelial disruptions were observed on the septum, turbinates and lateral wall on the dosed side (Figure 4c). The exposure of the nasal tissue to the 1 : 30 insulin/TMC400 nanocomplexes resulted in severe damage, including subepithelial oedema, epithelial necrosis (pyknosis), haemorrhage and sloughing of epithelial cells. Complete loss of epithelium occurred in some places and large quantities of mucus were observed in the nasal cavity. Severe and widespread effects extended over the nasal cavity of the dosed side (Figure 4d).

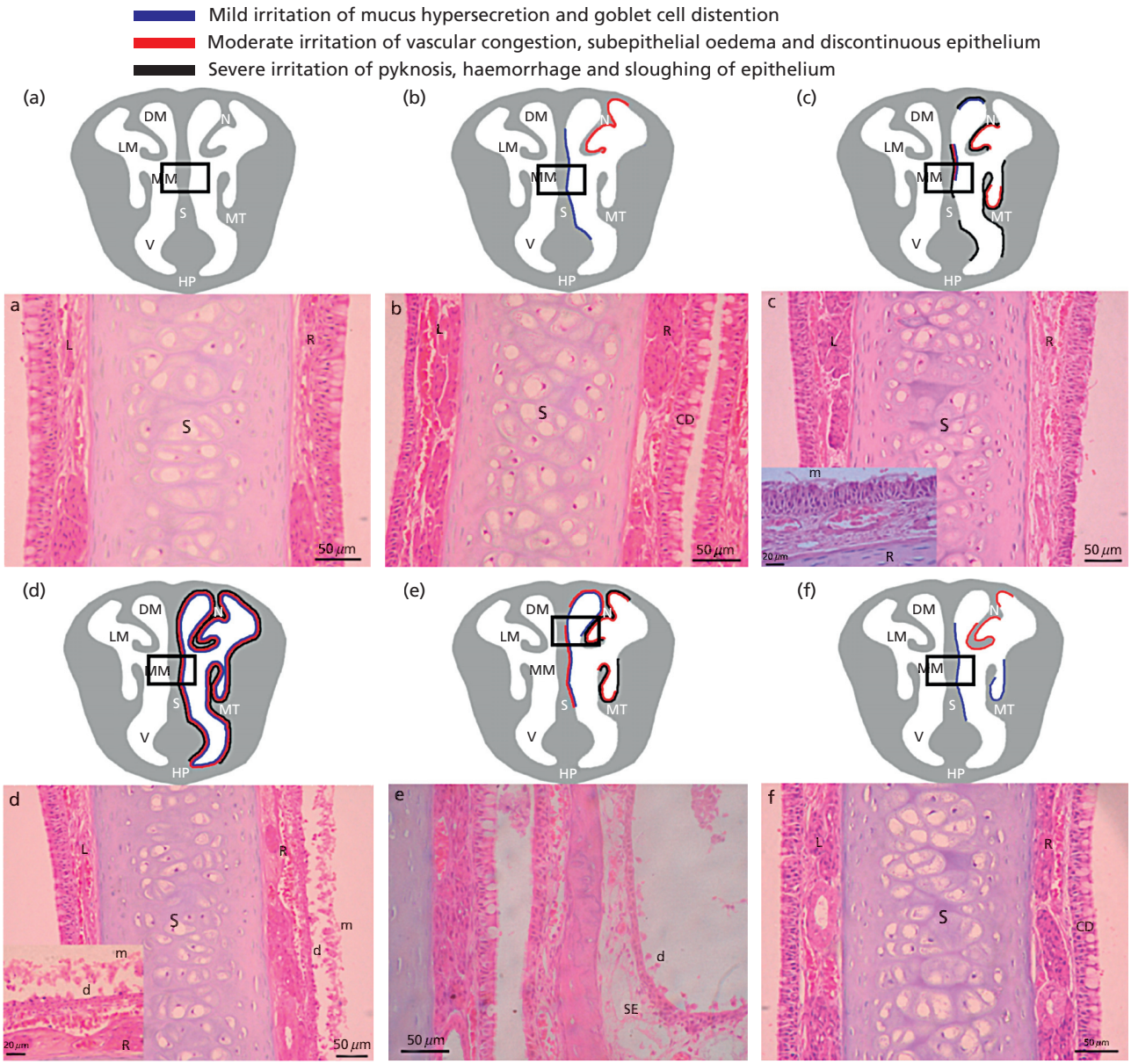

Figure 4 Diagram of location of lesions and photomicrographs of a vertical section of the rat nasal cavity. Staining was with haematoxylin and eosin (20 ×). (a) Treated with insulin solution; (b) treated with Ins/TMC400 nanocomplexes (1 : 3); (c) treated with Ins/TMC400 nanocomplexes (1 : 6), insert shows higher magnification (40 ×) for dosed site; (d) treated with Ins/TMC400 nanocomplexes (1 : 30), insert shows higher magnification (40 ×) for dosed site; (e) treated with Ins/PEG(5k)$_{298}$-g-TMC400 nanocomplexes (1 : 14); (f) treated with Ins/PEG(5k)$_{680}$-g-TMC400 nanocomplexes (1 : 6). d, disrupted epithelium; DM, dorsomedial meatus; GD, goblet cell distension; HP, hard palate; L, untreated side; LM, lateral meatus; m, mucus; MM, middle meatus; MT, maxilloturbinate; N, nasoturbinate; R, treated side; S, nasal septum; SE, subepithelial oedema; V, ventral meatus

Effect of PEGylation

In accordance with the insulin/TMC400 nanocomplexes, the damaging effect of insulin/PEGylated TMC to nasal epithelium was dependent upon insulin/polymer (−/+) charge ratio. However, the incidence and severity of the insulin/PEGylated TMC nanocomplexes were much lower than that of the insulin/TMC400 nanocomplexes. Compared with the 1 : 6 insulin/TMC400 nanocomplexes (Figure 4c), the 1 : 6 insulin/PEG(5k)$_{680}$-g-TMC400 nanocomplexes (Figure 4f) appeared to exhibit a mild level of epithelial damage with slight mucus secretion and goblet cell distension. Small lesions of vascular congestion and subepithelial oedema, and signs of moderate damage were observed on the nasoturbinate. In addition, the nasal epithelium exposed to the 1 : 14 insulin/PEG(5k)$_{298}$-g-TMC400 nanocomplexes (Figure 4e) demonstrated similar histological changes as found in the nasal epithelium exposed to the 1 : 6 insulin/TMC400 nanocomplexes (Figure 4c), even though the positive charge ratio of the insulin/PEG(5k)$_{298}$-g-TMC400 nanocomplexes was higher than that of the insulin/TMC400 nanocomplexes.

Discussion

The goal of this study was to identify suitable nanocomplexes that had the required balance between activity in enhancement of insulin absorption and safety for intranasal delivery of insulin. To avoid the variations which may occur in diabetic rats, a healthy rat model was used to study the intranasal absorption enhancing and histological effect of the insulin nanocomplexes.

An in-vivo study performed previously in our laboratories showed that insulin/PEG(5k)$_{40}$-g-TMC100 nanocomplexes prepared at optimized insulin/polymer ratio did not enhance nasal insulin absorption.[21] However, studies performed in an in-vitro mucus-secreting HT29-MTX-E12 cell culture model demonstrated that the charge ratio between insulin and polymer of TMC400 and PEGylated TMC400 in the formulation played an important role in the enhancement of insulin uptake.[27] The nanocomplexes prepared at optimized insulin/polymer ratio did not enhance insulin uptake due to complete release of insulin from the nanocomplexes after adhering to the mucus layer. On the other hand, insulin uptake was enhanced by the nanocomplexes prepared at a higher polymer ratio. It was observed that after the nanocomplex mixture of nanocomplexes, free polymer and free insulin adhered on the mucus layer, the negative charge of mucin would reduce the positive charge of polymer and allow them to capture more unassociated insulin, forming complexes on the mucus layer, and resulting in a decrease in release of insulin from the nanocomplexes.[27]

To investigate whether the effect of insulin/polymer (−/+) charge ratio could have similar consequences *in vivo*, nasal insulin nanocomplexes were prepared at different insulin/polymer (−/+) charge ratio (Table 2). The doses of insulin for both intranasal and subcutaneous administration, were selected based on the expected pharmacological effect and the respective route of administration, and concentrations of polymer were not more than those used in in-vivo experiments.[14,28,29] Recently, Yu *et al.*[30] reported that osmolarity of a nasal formulation affected nasal insulin absorption. Moreover, hypo- or hyper-osmotic formulations might possibly cause lesions on the nasal epithelium. To avoid such effects, all nasal insulin formulations in this study were prepared in isotonic Tris buffer.

Although nasal delivery avoids the hepatic first-pass effects, the enzymatic barriers of the nasal mucosa may cause a presystemic first-pass effect. It was reported that insulin was not degraded by aminopeptidase-M, an enzyme predominantly appearing in the nasal tissue.[31] Lang *et al.*[32] found that chymotrypsin- and trypsin-like endopeptidases were responsible for the initial cleavage of human calcitonin in excised bovine nasal mucosa. The subsequent metabolic degradation of the primary metabolites followed the typical sequential pattern of aminopeptidase-M activity. Indeed, trypsin and chymotrypsin are the major proteolytic enzymes cleaving various bonds within the insulin molecule. Therefore, the potential role of TMC and PEGylated TMC nanocomplexes in protecting insulin from trypsin digestion was investigated.

Comparing the control of insulin, the preparation of insulin in the form of nanocomplexes could protect insulin from trypsin digestion and the nanocomplexes of PEGylated TMC400 could protect insulin from the degradation by trypsin more efficiently than those of unmodified TMC. This was probably due to a consequence of the steric effect of macrogol segments that hindered the enzyme access to the protein.[33,34] Those results were in agreement with the observation by Mao *et al.*,[23] suggesting that PEGylated TMC copolymers could improve the stability of insulin in nanocomplexes at high temperature due to hydrophilic macrogol chains.

Studies of nasal insulin absorption were conducted in anaesthetized male Wistar rats. The absorption of insulin was evaluated by monitoring the hypoglycaemic effect after intranasal administration of insulin formulations. It is well documented in the literature that the values of the blood glucose baselines are in the range of 75–105 mg/dl for healthy rats and 350–500 mg/dl for diabetic rats.[28] However, in this experiment the blood glucose baselines were in the range 110–290 mg/dl. This was probably a consequence of the stress effect due to the anaesthetic agents. Ahsan *et al.*[35] observed a similar phenomenon with the use of a ketamine/xylazine mixture.

In the control group, which intranasally received isotonic Tris buffer, the reduction in fluctuation of blood glucose with time was observed. This could be attributed to the hypoglycaemic effect of the endogenous insulin in the rats. Compared with isotonic Tris buffer, the insulin solution partially decreased the blood glucose level ($P > 0.05$), which was in accordance with data reported by Mayor and Illum[36] indicating that the slight decrease in blood glucose concentration could be from an effect of the anaesthetic agent on the mucociliary clearance.

All insulin nanocomplexes enhanced the nasal absorption of insulin to a greater extent than insulin solution, which was found to be dependent upon the charge ratio of insulin and polymer (Figure 3). The enhancement of insulin absorption in the nanocomplexes prepared at higher polymer ratio is likely to be related to the adhesion of the unassociated free polymer to the mucus, causing the capture of free insulin on the mucus and subsequently enhancing insulin absorption by adsorptive endocytosis.[21,27] Another possibility to explain the increased bioavailability of insulin was that the positive charge density of a higher free polymer concentration could make it apparently more effective in the interaction with the epithelium and in modulating the tight-junctional barrier, resulting in enhancement of insulin absorption.[14] Similar observations were published by Simon *et al.*[28] who reported that insulin/amine modified poly(vinyl alcohol)-*graft*-poly(L-lactide) nanocomplexes containing a threefold higher polymer concentration increased insulin bioavailability more effectively than nanocomplexes containing optimal polymer concentration. Illum *et al.*[37] and Yu *et al.*[30] observed an influence of chitosan concentration on insulin bioavailability.

In addition, our results demonstrated that the nanocomplexes prepared at optimized insulin/polymer ratio enhanced nasal insulin absorption, which was inconsistent with Mao *et al.*[21] who indicated that the nanocomplexes prepared at optimized insulin/polymer ratio did not enhance nasal insulin absorption in diabetic rats. This discrepancy could have been because of the low molecular weight polymer used. Besides, it should be noted that the pharmacodynamic activity was

investigated in healthy rats in this study. Indeed, in the diabetic rat, deficiency of insulin results in severe changes in metabolism, decreased activity of the sympathetic nervous system, dehydration, glycosuria and osmotic diuresis.[38] Therefore, the responses of diabetic rats to insulin formulation might differ from that of healthy rats.[39]

Although many studies have reported the correlation between level of insulin absorbed and the hypoglycaemic response, the hypoglycaemia of healthy rats could be influenced by the endogenous insulin in the rat, which could be seen from the decrease in blood glucose concentration in the rat groups treated with nasally applied isotonic Tris buffer.[40,41] This finding implied that the changes in blood glucose concentration might have been a synergism of administered insulin and rat insulin. Therefore, the efficacy of the insulin nanocomplexes on the hypoglycaemia after nasal administration should be confirmed by insulin blood level and corresponding pharmacokinetic data.

It has often been suggested that the use of intranasal penetration enhancers to increase membrane permeability could be accompanied by histological damage to the nasal tissue. From a practical point of view, morphological integrity of nasal epithelium after nasal administration of each formulation needed to be examined.

The damaging effect of insulin/TMC400 nanocomplexes prepared at insulin/TMC400 charge ratio of 1 : 6 and 1 : 30 could be explained by its high positive charge and mucoadhesive properties of free TMC in the formulations. Recently, Amidi *et al*.[12] studied the influence on cilia beat frequency of TMC solution/nanoparticles on excised chicken embryo trachea and found that TMC exhibited a cilio-inhibiting effect. Therefore, it is reasonable to assume that the damaging effect of insulin/TMC400 nanocomplexes prepared at higher optimal polymer ratio resulted from retention of free polymer on the nasal mucosa and the toxicity of TMC400 could be reduced due to the complexation with insulin. However, an inverse finding was found by Haffejee *et al*.[20] indicating that TMC did not show any toxic effects on the morphology of rat nasal epithelium. This discrepancy can probably be explained by the different molecular weight, concentration and degree of quaternization of the polymers employed.

The results of the histopathological study revealed that PEGylated TMC copolymers were safer for use as permeation enhancers *in vivo* in the nose than unmodified TMC. The less damaging effect of PEGylated TMC nanocomplexes could be from the properties of macrogol. As a biocompatible, nontoxic, nonimmunogenic and water soluble polymer, macrogol is often used in nasal drug delivery to improve biocompatibility and transmucosal transport.[42–45] For instance, nasal administration of 1% w/v bile salt enhancer sodium glucocholate exhibited a strong damaging effect to nasal epithelium.[46] However, when nasally administering 1% w/v sodium glucocholate with macrogol-600, slight histological changes of the nasal epithelium were observed.[42] Similarly, Wu *et al*.[44] studied cytotoxicity of *N*-((2-hydroxy-3-trimethylammonium) propyl) chitosan chloride (HTCC) solution and hydrogel prepared from HTCC, macrogol and α-β-glycerophosphate on nasal epithelium by confocal laser scanning microscopy. The results showed that HTCC-macrogol-α-β-glycerophosphate hydrogel did not damage the epithelial cells, while HTCC solution exhibited some distortions of cystoskeleton structure and cell divisions.

Taking the pharmacodynamic data and histopathological results into consideration, it was found that the hypoglycaemic response of the insulin nanocomplexes did not consistently correlate with acute alterations in nasal morphology. This implied that increased insulin absorption was not accompanied by disruption of the nasal epithelium. The charge ratio between insulin and polymer plays an important role for insulin absorption enhancement and histological damage of the nasal tissue. Therefore, to use TMC and PEGylated TMC as the nasal insulin absorption enhancer, the charge ratio of insulin and polymer in the formulation should be optimized between activity and safety.

Conclusions

It has been demonstrated that co-administration of insulin and TMC400/PEGylated TMC400 in the form of nanocomplexes enhanced the nasal absorption of insulin to a greater extent than insulin solution. The charge ratio between insulin and polymer in the formulation influenced both the F_{dyn} and nasal epithelial integrity, whereas the polymer structure affected only the nasal epithelial integrity. The F_{dyn} and the severity of acute alterations in rat nasal morphology increased with an increase in positive charge ratio of polymer. Moreover, TMC appeared to cause more damage to nasal epithelium than PEGylated TMC. Further investigation in diabetic rats to confirm the suitability of the nasal insulin formulations identified in this study need to be conducted. Studies involving more frequency and duration in exposure are also necessary to assess the safety of formulations before clinical application.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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