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Research paper

Effect of chitosan structure properties and molecular weight on the intranasal absorption of tetramethylpyrazine phosphate in rats

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ABSTRACT

The objective of this work was to assess and compare the absorption promoting effect of different molecular-weight chitosans, trimethyl chitosans and thiolated chitosans for intranasal absorption of 2,3,5,6-tetramethylpyrazine phosphate (TMPP). An in situ nasal perfusion technique in rats was utilized to test the rate and extent of TMPP absorption in situ. In vivo studies were carried out in rats and the pharmacokinetic parameters were calculated and compared with that of intravenous injection. All the chitosan derivatives investigated could enhance the intranasal absorption of TMPP significantly. However, thiolation could not improve the absorption-enhancing capacity of chitosan remarkably even when the thiolation ratio was as high as 152 μmol/g. In contrast, trimethylated chitosan exhibited stronger absorption-enhancing ability than the homopolymer chitosan. The permeation enhancing effect of chitosan increased with increasing molecular weight up to M_w 100 kDa. In vivo studies indicated that chitosan 100 kDa and TMC 50 kDa had comparable absorption-enhancing effect but chitosan 100 kDa functioned for more than 120 min versus 90 min for TMC. A good correlation was found between the in situ absorption data and plasma concentration in vivo for the polymers investigated. This study demonstrated that both chitosan structural features and chitosan molecular weight play a key role on promoting the intranasal absorption of TMPP. Taking safety reason into account, chitosan 100 kDa is the most promising as an intranasal absorption enhancer.

1. Introduction

Systemic drug delivery by the nasal route is currently receiving considerable attention because this route allows for a rapid onset of therapeutic effect, potential for direct-to-central nervous system delivery, avoidance of first-pass metabolism, and an easy way for drug administration [1]. To improve the intranasal delivery of challenging drugs, several strategies have been taken [2,3]. Among them, coadministration of chemical enhancers has been extensively exploited in recent years [4]. However, although numerous classical enhancers have been demonstrated to be able to promote the nasal absorption of biologically active peptides and proteins effectively, the successful application of these compounds is limited by their undesirable physiological effect on epithelial cells and irritation to the nasal mucosa [5,6]. Therefore, new absorption enhancers are expected to achieve safe permeation enhancement.

Chitosan, produced by partial deacetylation of chitin, has been found to be able to improve the intranasal absorption of peptides and reduce the clearance of liquid formulations from the nasal cavity through its bioadhesive characteristics, while causing negligible damage to the nasal mucosal membrane [7]. However, chitosan is only soluble in acidic milieu, in which the amino groups at the C-2 position are protonated [8]. To improve the poor water-solubility of chitosan, trimethyl chitosan was synthesized and has been proven to be a potent intranasal absorption enhancer of insulin in rats, especially at neutral pH, where chitosan salts are ineffective [9]. While the absorption-enhancing effect of chitosan was attributed to the capacity to open the tight junction and bioadhesive properties, thiolated chitosans were synthesized in order to further increase the bioadhesion of chitosan, and were found to have posed a significantly higher bioavailability of insulin after intranasal administration than that of unmodified chitosan [10]. The overall absorption-enhancing mechanism of thiolated chitosans is attributed to the formation of disulfide bonds between thiol moieties of thiomers and sulfhydryl groups of cysteine-rich subdomains of mucus glycoproteins providing prolonged residence time at the absorbing membranes [10]. However, the contribution of bioadhesion versus opening the tight junction in the absorption-enhancing effect of chitosan is unclear. So far, although numerous works have reported the promising potential of chitosan and its derivatives as safe and effective nasal absorption enhancers, the influence of chitosan

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structure properties on the intranasal absorption of drugs has not been elucidated systemically to the best of our knowledge.

2,3,5,6-Tetramethylpyrazine (TMPP) is a biologically active ingredient originally isolated from *Ligusticum wallichii* France in 1957 and currently used in China for the treatment of cardiovascular disease [11]. It was found to have significant therapeutic activity, including improving brain microcirculation, inhibiting thrombus formation, decreasing platelet aggregation, and improving blood viscosity [12]. It was reported recently that TMPP has appreciable blood–brain barrier (BBB) penetrability [13]. However, peroral absorption of TMPP is variable and incomplete, with a bioavailability of 10–30% and a short biological half-life of 0.5–2 h [14]. Accordingly, intranasal administration might represent a promising route to improve its bioavailability.

Therefore, taking TMPP as a model drug, influence of chitosan structure properties on the intranasal absorption of drugs was investigated using an in situ perfusion method and the absorption mechanism was discussed. Based on the in situ results, the selected chitosan formulations were further evaluated in vivo by measuring the blood concentration level after intranasal administration in rats.

2. Materials and methods

2.1. Materials

TMPP was a gift from Beijing Shuanghe Inc. Chitosan 400 kDa with a nominal degree of deacetylation of 85% was purchased from Weifang Kehai Chitin Co., Ltd. Carbamazepine (99.6% purity, internal standard) and acetic acid were obtained from Shandong Xinhua Pharmaceutical Company, Ltd. (Jinan, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) and *N*-hydroxysuccinimide (NHS) were purchased from Shanghai Medpep Co., Ltd. Methanol of liquid chromatographic grade was purchased from Tianjin Concord Tech Reagent Company (Tianjin, China). All other chemicals were of analytical grade.

2.2. Chitosan derivatives preparation and characterization

Chitosans of different molecular weights were prepared by depolymerization as described previously [15], and the obtained polymers were nominated based on their molecular weight. For example, chitosan with molecular weight of 400 kDa is abbreviated as CS 400, the same for the other polymers. Trimethyl chitosan (TMC) derivatives were prepared according to a two-step method with a quaternization degree of 40% [16] and their molecular weights were characterized using asymmetrical flow field-flow-fractionation method as described previously [17]. Thiolated chitosans were synthesized according to a previously reported method with little modification [18]. Briefly, cysteine was covalently attached to chitosan via the formation of an amide bond mediated by EDAC and NHS to obtain chitosan–cysteine conjugates. First, 1 g of chitosan was hydrated in 8 ml of 1 M HCl and then dissolved by the addition of demineralized water to obtain a 1% (w/v) polymer solution. The pH was adjusted to 4.0 by the addition of 1 M NaOH. Afterwards, 3 g cysteine, 2 g NHS and 2 g EDC in 10 ml of demineralized water was added under stirring. The pH was readjusted to 4.0. The reaction mixture was incubated for 6 h at room temperature under permanent stirring. The resulting polymer conjugate was precipitated by adjusting the system pH to 7.5 and washed several times with water, freeze-dried and stored at 4 °C until further use. Iodine titration was used to determine the thiol group content [18]. In vitro mucoadhesion of thiolated chitosans was studied [18] and compared with that of the unmodified chitosan. Firstly, 30 mg of lyophilized polymer–cysteine conjugates and controls were compressed into 5.0-mm diameter flat-faced discs. The compaction pressure was kept constant during the preparation of all discs. Discs described above were thereby attached to freshly excised intestinal porcine mucosa, which had been spanned on a stainless steel cylinder. Thereafter, the cylinder was placed in the dissolution apparatus according to the CP containing 100 mM PBS, pH 6, at 37 ± 0.5 °C. The fully immersed cylinder was agitated with 250 rpm. The detachment, disintegration and/or erosion of test discs were observed within a time period of 12 h. Properties of various chitosans employed in this study are listed in Table 1.

2.3. Preparation of intranasal formulations

All the chitosans applied were first dissolved in 0.5% (v/v) acetic acid saline solution to obtain desired concentrations. TMPP was dissolved into the above-mentioned solution (4 mg/ml) and pH of the solution was adjusted as required. Isotonicity was adjusted by sodium chloride. For in vivo studies, the formulations were prepared according to the following method: TMC 50, CS 50, CS 100 and H-CS(100)-Cys were dissolved in 0.5% (v/v) acetic acid saline solution to reach a concentration of 0.5% (w/v). Thereafter, TMPP was added to the above solutions (20 mg/ml) at pH 6. Intravenous injection solution was prepared by dissolving TMPP (4 mg/ml) in sterile saline solution and adjusting solution pH to 6.

2.4. Analytical method of TMPP

The content of TMPP was analyzed by a HPLC method. HPLC apparatus (Schimadzu, LC10-AS liquid chromatograph) connected to an ultraviolet variable wavelength detector (Model SPD-10A) with a C-18 reversed phase column (Bondapack, 5 μm, 4.6 mm × 200 mm, Shimadzu, Japan) and isocratic pump (Model LC10-AS, Shimadzu, Japan) was used. The mobile phase consisted of 55% of methanol and 45% of water. The flow rate was 1.0 ml/min. The UV detector wavelength was 295 nm and the oven temperature was 30 °C. The injection volume was 20 μl. The limit of detection and quantitation of TMPP was 0.08 ng and 0.2 ng, respectively. A linear relationship between As/Ai and concentration was found in the concentration range of 0.2–10 μg/ml (As/Ai = 0.3212 C + 0.0009, r = 0.9999, n = 6), where As is the peak area of TMPP and Ai is the peak area of the internal standard. The method and extraction recoveries of TMPP from plasma at three different concentrations were between 99.7–101.8% and 81.7–87.6%, respectively. The inter-day RSDs were less than 2.56% (n = 6), and the intra-day RSDs were less than 5.41% (n = 6).

2.5. Nasal perfusion studies in rats

The animal experiment was carried out in accordance with the Principles of Laboratory Animal Care (NIH Publication No. 86-23, revised 1985). Male Wistar rats (7 weeks old, 200 ± 20 g) were supplied by the Lab Animal Center of Shenyang Pharmaceutical University (Grade II, Certificate No. SYXK 2006-0064). The experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the Guide for Care and Use of Laboratory Animals. Rats were maintained at 22 ± 2 °C and 55 ± 5% relative humidity under a 12-h light–dark cycle for 4–6 days before experiments, with food and water available ad libitum.

Rats were surgically treated according to a previously described method [19]. A 5-ml solution was recirculated at 37 °C through the rat nasal cavity at a rate of 2.0 ml/min for 120 min (n = 5). Aliquots (50 μl) were sampled periodically and analyzed by HPLC assay. And an equal volume of isotonic saline of pH 6 was added in the meantime. The absorption-rate constants were calculated according to the absorption amount in the first 40 min using the following equation: $Q = Q_0 - k_a t$, where Q_0 and Q represent the initial and

Table 1
Properties of chitosan and its derivatives employed in this study

Polymer (kDa)	DD[a] (%)	Theoretical M_w[b] (kDa)	Determined M_w[c] (kDa)	Substitution
CS 50	89.9	50	56.3	
CS 100	85.4	100	109.1	
CS 200	85.2	200	202.5	
CS 400	85.1	400	402.2	
L-CS(50)-Cys		50.8		49.5[d]
H-CS(50)-Cys		51.3		151.2[d]
H-CS(100)-Cys				152.0[d]
TMC 50		50	52.0[f]	39.0[e]

[a] Degree of deacetylation, calculated by ^{1}H NMR analysis.
[b] Calculation based on the composition of the copolymer.
[c] Measured with capillary viscosimetry.
[d] Thiol group content (μmol/g), measured by iodine titration.
[e] Degree of quaternization (%), calculated by ^{1}H NMR analysis.
[f] Measured by asymmetrical flow field-flow-fractionation method as described in [17].

the remaining amounts of drug at time t, respectively, and k_a represents the absorption-rate constant [20].

2.6. In vivo studies in rats

Thirty healthy male Wistar rats weighing between 180 and 220 g were divided into six groups, CS 50 group, TMC 50 group, CS 100 group, H-CS(100)-Cys group, the control group and intravenous administration group as a control. The nasal formulations were administered using a microsyringe (Hamilton Bonaduz AG, Switzerland) attached via a needle to a short polyethylene tube inserted approximately 0.7 cm into one nostril [21]. For intravenous administration, a bolus injection was administered via the tail vein. For all the groups tested, at 0, 1, 3, 5, 10, 20, 30, 45, 60, 90 and 120 min after administration, blood samples of 0.25 ml were withdrawn and were harvested immediately by centrifugation at 4000 rpm for 15 min. Then plasma samples were stored at −20 °C immediately after collection until analysis. Plasma samples were processed with the following steps: 150 μl of methanol and 5 μl of internal standard (50 μg/ml carbamazepine in methanol) were added into 100 μl of serum samples. The mixture was vortexed for 2 min and centrifuged at 4000 rpm for 10 min. The supernatant was used for HPLC assay. Each point represents the mean of five rats.

2.7. Statistical and pharmacokinetic data analysis

The peak plasma concentration (C_{max}) and the time to reach the peak concentration (T_{max}) were determined directly from the plasma concentration–time curves. The absolute bioavailability (F) was calculated according to the following equation:

$$F = \frac{\mathrm{AUC(i.n.)} \times \mathrm{Dose(i.v.)}}{\mathrm{AUC(i.v.)} \times \mathrm{Dose(i.n.)}} \times 100\%.$$

The pharmacokinetic parameters were obtained using 3P97 program. Data are presented as means ± standard deviations of five experiments. The statistical significance was determined using one-way analysis of variance (ANOVA) followed by the Dunnett test. Probability values $P < 0.05$ were considered significant.

3. Results

Since the preliminary experiment demonstrated that TMPP was very stable in the nasal perfusate for over 2 h, any loss of the drug from the perfusate was regarded as its absorption across the nasal mucosa.

3.1. Effect of pH on intranasal absorption of TMPP

To optimize the solution pH, intranasal absorption of TMPP was investigated at pH 5, 6 and 7, respectively. As shown in Fig. 1, the absorption rate constant was quite low at pH 5 and significant increase was observed at pH 6 ($P < 0.05$), but no remarkable difference was found between pH 6 and pH 7 ($P > 0.05$). Since the pH of normal human nasal mucosa is in the range of 5.5–6.5, pH 6 was selected for the following studies.

3.2. Effect of perfusion concentration on intranasal absorption of TMPP

To investigate the absorption mechanism of TMPP across nasal mucosa, the effect of perfusion concentration on the nasal absorption was studied. Three concentration levels, 1, 2 and 4 mg/ml, were selected for this study. As shown in Fig. 2, irrespective of the initial concentration, the amount absorbed increased linearly with time in the initial 40 min, then the absorption rate slowed down and almost leveled off after 1 h. The absorption process follows zero-order kinetics in the first 40 min with correlation coefficients of 0.9986, 0.9996 and 0.9983 at 1, 2 and 4 mg/ml of perfusate concentrations, respectively. The absorption-rate constants increased with perfusate concentration and were found to be 0.042, 0.096 and 0.158 mg/min for 1, 2 and 4 mg/ml of perfusate, respectively. These results demonstrated that uptake of TMPP across nasal epithelium was dose-dependent. TMPP 4 mg/ml was selected for the following studies.

3.3. Effect of chitosan concentration

It is reported that 0.5–1.0% chitosan is very effective for intranasal absorption of desmopressin [22]. Hereby, taking chitosan 50 kDa as an example, the influence of chitosan concentration on the intranasal absorption of TMPP was investigated in the range of 0.1–1% (w/v). Control experiment was also conducted by perfus-

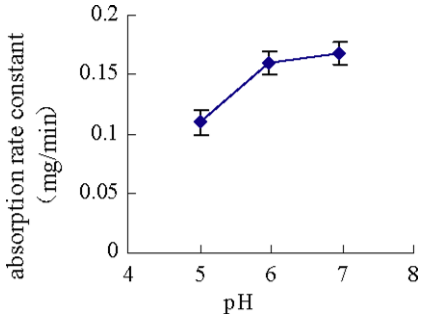

Fig. 1. Effect of pH on intranasal absorption of TMPP ($n = 5$).

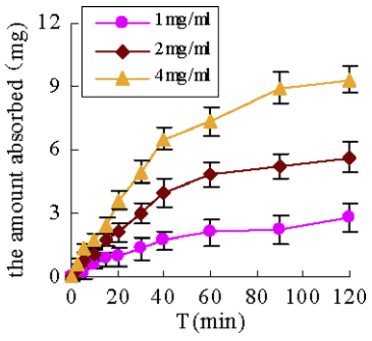

Fig. 2. Effect of perfusate concentration on intranasal absorption of TMPP ($n = 5$).

ing the TMPP solution without enhancers. As shown in Fig. 3a and Table 2, no apparent difference ($P > 0.05$) in absorption rate and extent was observed between CS group and the control group in the initial 40 min, irrespective of chitosan concentration. Compared to the control group, significant absorption increase was shown at 60 min and the absorption amount increased with increasing chitosan concentration, with an absorption increase of 16.4%, 23.9% and 22.7% for 0.1%, 0.5% and 1% chitosan group, respectively. A considerable difference was found between 0.1% and 0.5% chitosan group ($P < 0.05$). However, no statistical difference was found between 0.5% chitosan and 1% chitosan group ($P > 0.05$), indicating that the absorption-enhancing effect of chitosan was concentration-dependent and saturable.

Table 2
The absorption rate constants (k_a) of TMPP in vitro with different permeation enhancers (mean ± SD, $n = 5$)

Formulation	k_a (mg/min)
Control group (TMPP alone)	0.158 ± 0.02
0.5% CS (200 kDa)	0.160 ± 0.01*
0.5% CS (400 kDa)	0.158 ± 0.02*
0.5% CS(100 kDa)	0.159 ± 0.02*
0.1% CS (50 kDa)	0.159 ± 0.02*
0.5% CS (50 kDa)	0.160 ± 0.03*
1% CS (50 kDa)	0.158 ± 0.01*
0.1% L-CS-Cys (50 kDa)	0.159 ± 0.01*
0.5% L-CS-Cys (50 kDa)	0.158 ± 0.04*
1% L-CS-Cys (50 kDa)	0.160 ± 0.02*
0.5% H-CS-Cys (50 kDa)	0.159 ± 0.01*
0.1% TMC (50 kDa)	0.158 ± 0.04*
0.5% TMC (50 kDa)	0.160 ± 0.03*
1% TMC (50 kDa)	0.161 ± 0.01*

* Insignificant difference compared to the control group ($P > 0.05$).

3.4. Effect of thiolation

In order to elucidate the contribution of mucoadhesion on the absorption-enhancing effect of chitosan, two chitosan cysteine (CS-Cys) conjugates with different thiolation degree were synthesized (Table 1) and the adhesive properties were evaluated in vitro using the rotating cylinder method [18]. As shown in Fig. 4, the mucoadhesion of chitosan increased significantly with the increase of thiolation degree. Compared to the unmodified chitosan, the bioadhesion improved by 2.3 and 4 times for L-CS-Cys 50 and H-CS-Cys 50, respectively, at pH 6. Thereafter, the absorption-enhancing effect of L-CS-Cys 50, H-CS-Cys 50 and CS 50 was evaluated at the same concentration of 0.5%. As shown in Fig. 3b and Table 2, similar to chitosan, no significant enhancing effect was found in the initial 40 min for the two thiolated chitosans investigated ($P > 0.05$) compared to the control group and the enhancing effect was apparent after 60 min of perfusion. However, the difference between 0.5% L-CS-Cys 50 and 0.5% H-CS-Cys 50 was not remarkable ($P > 0.05$) and both of them generated an insignificant promoting effect compared to chitosan ($P > 0.05$), indicating that the absorption-enhancing effect of chitosan cannot be further improved by increasing bioadhesion in the range investigated.

3.5. Effect of trimethylation

To further increase the solubility of chitosan at neutral pH, trimethyl chitosans with a degree of quaternization of 40% were synthesized and their potential as intranasal absorption enhancers was investigated. As shown in Fig. 3c, a remarkable enhancing effect was found after the initial 30 min of perfusion with 0.1–1% TMC 50 ($P < 0.05$), compared to a lag time of 40 min in the case of chitosan and thiolated chitosans. A significant difference in absorption was found between 0.1% and 0.5% TMC 50 groups and the total absorption extent in 2 h was 71.3% and 84.3%, respectively ($P < 0.05$). However, the difference between 0.5% and 1% TMC 50

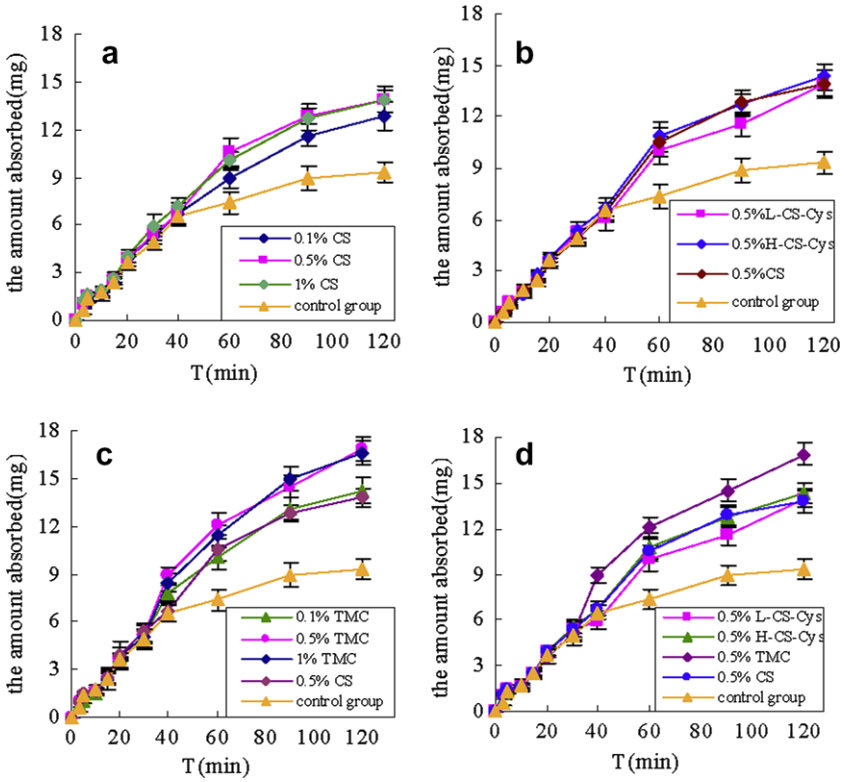

Fig. 3. Effect of chitosan structure on intranasal absorption of TMPP ($n = 5$). (a) CS 50, (b) CS-Cys 50, (c) TMC 50, (d) comparison.

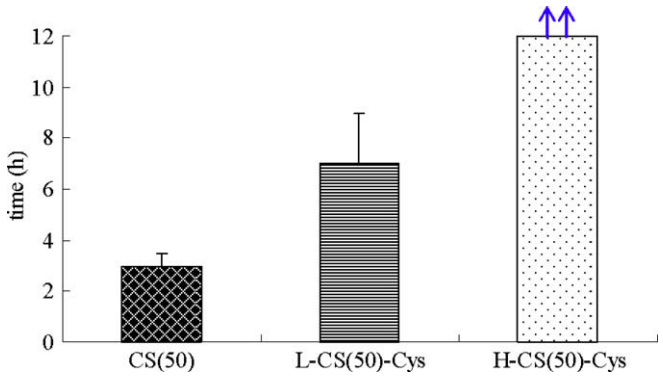

Fig. 4. Comparison of the mucoadhesive time of chitosan (50 kDa) and chitosan–cysteine conjugate (L-CS(50)-Cys, H-CS(50)-Cys) determined in in vitro adhesion studies on the rotating cylinder at pH 6, 37 °C.

groups was not remarkable ($P > 0.05$), implying that the absorption-enhancing effect of TMC was also concentration-dependent and saturable. On the other hand, comparable enhancing effect was found with 0.5% CS 50 and 0.1% TMC 50, indicating that TMC 50 possessed a stronger enhancing effect on intranasal absorption of TMPP than that of CS 50.

3.6. Effect of chitosan structure properties

To study the effect of chitosan structure properties on the intranasal absorption of TMPP, four types of chitosan with similar molecular weight, chitosan 50, TMC 50, L-CS(50)-Cys and H-CS(50)-Cys, were selected for comparison at 0.5% concentration level. As shown in Fig. 3d, compared to the control, all chitosans studied were capable of enhancing the nasal absorption of TMPP ($P < 0.05$). Among them, TMC was the most effective and the enhancing effect was significant compared with chitosan and thiolated chitosans ($P < 0.05$). No marked difference was found among chitosan and the two thiolated chitosans ($P > 0.05$). TMC had a lag time of 30 min to pose its enhancement compared to a lag time of 40 min for the other chitosans.

3.7. Effect of chitosan molecular weight

To elucidate the influence of chitosan molecular weight on the intranasal absorption of TMPP, chitosan 400, 200, 100 and 50 kDa were employed in the present study. It is clear from Fig. 5 that the permeation-enhancing effect of chitosan increased with increasing molecular weight up to M_w 100 kDa, thereafter, further increasing M_w to 400 kDa caused no remarkable increase in absorption. No significant difference in absorption was found among chitosan 400, 200 and 100 ($P > 0.05$).

3.8. In vivo studies

While previous studies indicated that the cytotoxicity of TMCs increased with increasing molecular weight [16], thus, based on the in situ results and taking the safety into account, the following four polymers, 0.5% CS 50, 0.5% TMC 50, 0.5% CS 100 and 0.5% H-CS(100)-Cys, were selected for in vivo studies to elucidate the influence of chitosan molecular weight, chitosan structure and increased mucoadhesion on the intranasal absorption of TMPP. Plasma concentrations of TMPP after intranasal administration and intravenous injection are depicted in Fig. 6 and main pharmacokinetic parameters are listed in Table 3. The pharmacokinetic profiles can be described with a two-compartment model. Interestingly, the lag time observed in situ was not apparent in vivo for all the chitosan-related groups. After intranasal administration, TMPP

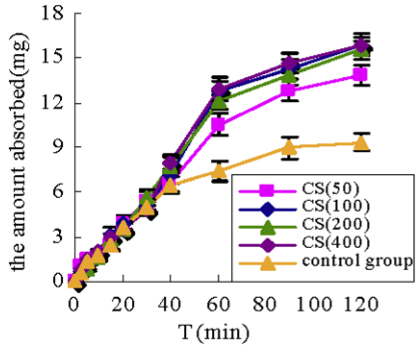

Fig. 5. Effect of chitosan molecular weight on intranasal absorption of TMPP ($n = 5$).

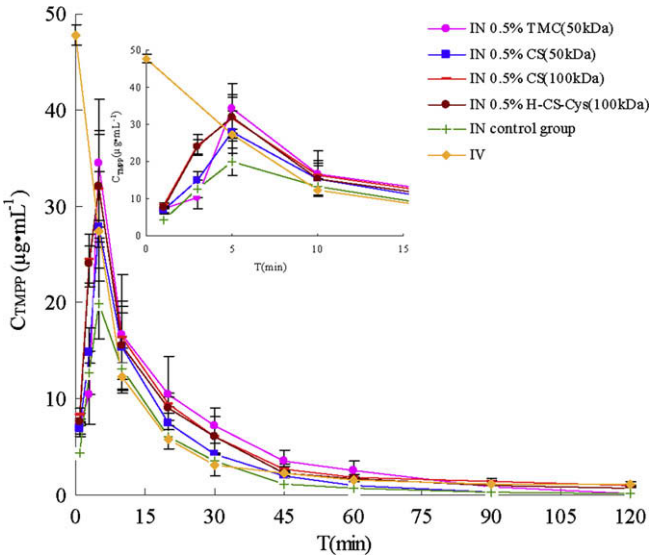

Fig. 6. Mean plasma concentration–time profiles of TMPP after nasal application of 0.5% CS 50, 0.5% TMC 50, 0.5% CS 100 and 0.5% H-CS(100)-Cys formulations in rats, compared with those of polymer-free group and intravenous injection group. Indicated values are the mean of five experiments ($n = 5$).

was absorbed into the blood quickly with T_{max} of approximately 5 min for all the groups investigated. Compared to the polymer-free group, significant enhancing effect was observed for the 0.5% CS 50 formulation in the first 60 min ($P < 0.05$), whereas the remarkable enhancing effect lasted for more than two hours for the 0.5% CS 100 groups ($P < 0.05$). In agreement with in situ results, statistical difference was found between the 0.5% CS 50 and 0.5% CS 100 group ($P < 0.05$), indicating that chitosan molecular weight influences its absorption-enhancing ability and chitosan 100 is stronger than chitosan 50 as an absorption enhancer. With the same molecular weight and concentration, TMC 50 exhibited a stronger absorption-enhancing effect than that of chitosan 50 in the first 90 min ($P < 0.05$), implying that chitosan structure properties influence their absorption-enhancing effect. Anyhow, the enhancing effect lasted for only 90 min for both of them. Comparable absorption-enhancing effect was noticed for 0.5% chitosan 100 group and 0.5% TMC 50 group in the first 90 min, thereafter, no considerable difference was found between TMC and control groups. In contrast, the significant enhancing effect of chitosan 100 group lasted for more than 120 min. Also, the fact that no considerable difference in in vivo absorption was found between chitosan 100 and H-CS(100)-Cys groups further demonstrated that the contribution of mucoadhesion is minimal. As shown in Table 3, the absolute bioavailability of 0.5% TMC 50 kDa group was 73.6%,

Table 3
Main pharmacokinetic parameters after intranasal (i.n.) administration of 0.5% CS (50 kDa, 100 kDa), 0.5% H-CS(100)-Cys, 0.5% TMC (50 kDa) and the control formulations, as well as after intravenous (i.v.) injections of TMPP to rats (mean ± SD, $n = 5$)

Formulation	I.n. control group	I.n. 0.5% CS 50	I.n. 0.5% CS 100	I.n. 0.5% TMC 50	I.n. 0.5% H-CS-Cys 100	I.v.
TMPP dose (mg/kg)	3.2	3.2	3.2	3.2	3.2	3.2
C_{max} (μg/ml)	19.94 ± 4.2	27.88 ± 3.6	31.76 ± 7.8	34.42 ± 5.3	32.1 ± 4.9	
T_{max} (min)	3.74	6.35	5.15	3.99	5.37	
Lag time (min)	0.16	0.57	0.31	0.19	0.28	
AUC (μg min/ml)	336.1 ± 12.7	421.6 ± 10.3	503.2 ± 16.4	541.4 ± 21.9	478.9 ± 23.1	735.7 ± 27.1
Absolute bioavailability (%)	45.7 ± 3.2	57.3 ± 8.7*	68.4 ± 3.1*,△	73.6 ± 6.5*,△	65.1 ± 5.2*	

* Significant difference compared to intranasal control group ($P < 0.05$).
△ Significant difference compared to CS 50 group ($P < 0.05$).

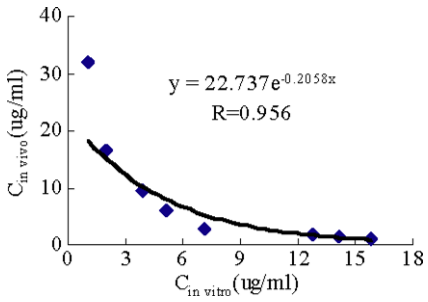

Fig. 7. The relationship between TMPP plasma concentration in vivo and drug absorption in situ for CS 100 group.

compared to 57.3%, 68.4%, 65.1% for 0.5% CS 50, 0.5% CS 100 and 0.5% H-CS(100)-Cys groups, respectively. Based on these results and in conjugation with the safety profile of chitosan and TMC [16], it is reasonable to conclude that chitosan 100 is a preferred intranasal absorption enhancer.

In addition, the correlation between in situ results and in vivo absorption data was investigated. Fig. 7 exemplified the relationship between TMPP plasma concentration in vivo and TMPP absorption in situ and a good exponential correlation was established for CS 100 kDa. Similarly, the correlation was also found for chitosan 50 and TMC 50 kDa, with the following equation: $Y = 31.715e^{-0.38X}$, $r = 0.987$ and $Y = 36.75e^{-0.2903X}$, $r = 0.972$, respectively. This result implies that in situ perfusion method could be used as an effective way to predict intranasal absorption in vivo.

4. Discussion

The intranasal absorption of TMPP was found to be pH-dependent. This can probably be explained by the fact that, similar to other biomembranes, nasal mucosa also exerts a lipophilic screen-like action to the transport of drugs. It is hypothesized that the main transport mechanism of lipophilic drug across nasal mucosa is by lipoidal pathway [1]. The pK_a of TMPP is 3.5, when the system pH changed from 5 to 6, TMPP existing in the unionized form increased by 100 times, and the increased lipophilicity of TMPP facilitated its transport across the nasal mucosa through the lipophilic cell pathway. This is in good agreement with our experimental results. However, drug transport via transcellular pathway was still limited, demonstrated by the low bioavailability of TMPP solution after intranasal administration (45.7% as shown in Table 3). Therefore, it was essential to develop some other transport route, such as paracellular route provided by chitosan and its derivatives. As indicated in the result part, compared to the control, all chitosans studied were capable of enhancing the nasal absorption of TMPP ($P < 0.05$), showing that paracellular transport of TMPP was indeed enhanced. Among them, TMC was the most effective and the enhancing effect was significant compared with chitosan and thiolated chitosans ($P < 0.05$). No marked difference was found among chitosan and the two thiolated chitosans ($P > 0.05$).

The intranasal absorption of polymer-free TMPP solution was dose-dependent and leveled off after 40 min. We observed the similar saturation phenomenon with the intranasal absorption of analgin [23]. However, application of chitosan and its derivatives could significantly enhance the intranasal absorption after 40 min.

Chitosan is thought to be able to disrupt intercellular tight junctions and the function was dose-dependent and molecular weight-dependent [24]. Also, our study indicated that the absorption-enhancing effect of chitosan was concentration-dependent and saturable. It was noticed that chitosan exerted a remarkable enhancing effect on the absorption of TMPP just after 40 min and no further absorption increase was attained when chitosan concentration higher than 0.5% was applied. Similarly, Sinswat and Tengamnuay [25] indicated that the effect of chitosan on nasal absorption of salmon calcitonin was concentration-dependent from 0.25% to 1.0% and leveled off at 1.25%. The absorption-enhancing mechanism of chitosan was suggested to be a combination of mucoadhesion and an effect on the opening of tight junctions [26]. Therefore, it is reasonable to assume that its effect on the opening of tight junctions in the cell membrane needs some time to work and 0.5% CS is sufficient to open all the available tight junctions on the nasal mucosa. Also, this result indicated that the most likely absorption-enhancing mechanism of chitosan in this study is opening the tight junctions and the contribution of mucoadhesion from increased chitosan concentration is marginal. In accordance with the previous report [24], our study showed that the permeation-enhancing effect of chitosan was also molecular weight-dependent and chitosan 100 kDa is sufficient to achieve the highest enhancement. Based on this result, it is reasonable to assume that a threshold chain length is essential to open the tight junctions effectively. The fact that no considerable difference was found among chitosan 400, 200 and 100 kDa groups supports the suggestion that if the chains of chitosan with an excessive high M_w are too long, the extent of interpenetration is strongly reduced [27]. Similarly, it was reported that chitosan/siRNA formulations prepared with low M_w (approximately 10 kDa) showed almost no knockdown of endogenous-enhanced green fluorescent protein in H1299 human lung carcinoma cells, whereas those prepared from higher M_w (64.8–170 kDa) showed greater gene-silencing and the highest gene-silencing efficiency was achieved using chitosan/siRNA nanoparticles with higher M_w (114 and 170 kDa) chitosans [28]. Again, the fact that no statistical difference was found among chitosan 100, 200 and 400 kDa groups implies that increasing bioadhesion or viscosity has only marginal contribution to the intranasal absorption in the range studied.

In order to further elucidate the contribution of mucoadhesion on the absorption-enhancing effect of chitosan, the influence of two thiolated chitosans on the intranasal absorption of TMPP was studied and compared with that of the unmodified chitosan.

Both of them generated an insignificant promoting effect compared to chitosan in in situ model ($P > 0.05$), indicating that the absorption-enhancing effect of chitosan cannot be further improved by increasing bioadhesion in the range investigated. Similar phenomenon has been reported previously. Harris et al. [29] indicated that although the addition of a viscous agent to nasal formulations may produce a more sustained effect, it delays the onset of activity and no enhancement is achieved in the total bioavailability. Johansson et al. [30] reported that no significant differences in effects were observed when the test formulations of different viscosities were compared after intranasal administration. Anyhow, in order to avoid the limitation of in situ model, in vivo study was further performed to demonstrate the conclusion drawn from in situ study. While in situ study indicated that the absorption-enhancing effect of chitosan was molecular weight-dependent and chitosan 100 kDa was the potential candidate, thiolated chitosan 100 (H-CS(100)-Cys, 152 μmol/g) was synthesized and its influence on TMPP absorption in vivo was investigated. In great agreement with the in situ result, in vivo data further demonstrated that the intranasal absorption of TMPP cannot be improved by the increase of adhesion in the range studied.

An earlier study demonstrated that chitosan-4-thiobutylamidine microparticles generated 3.6-fold increase in the intranasal absorption of insulin compared to that of the unmodified chitosan microparticles and the permeation-enhancing effect of the thiomer/glutathione system has been ascribed to the inhibition of the enzyme protein tyrosine phosphatase in addition to the prolonged residence time [31]. Similarly, it has been demonstrated recently that the absorption-enhancing mechanism of thiolated chitosan across intestinal mucosa is by the inhibition of intestinal P-glycoprotein (P-gp) [32]. Since TMPP is a small molecular-weight drug substance and stable against enzymes, no apparent absorption-enhancing effect was observed. This study implies that the main absorption-enhancing mechanism of chitosan for TMPP was opening the tight junctions on the nasal mucosa and the contribution of mucoadhesion was minimal.

In this study, comparable enhancing effect was found with 0.5% CS 50 and 0.1% TMC 50, indicating that TMC 50 possessed a stronger enhancing effect on intranasal absorption of TMPP than that of CS 50. Similarly, in comparison to native chitosan, the superiority of TMC as an adjuvant for inducing immune responses to ovalbumin via the nasal route was demonstrated [33]. This can probably be explained by the charge density difference of the polymers. Chitosan has an intrinsic pK_a value of 6.5 [4] and only approximately 24% of its primary amino groups are positively charged at pH 6, whereas all primary and quaternized amino groups of TMC are protonated [26]. The increased charge density of TMC at physiological pH promotes its interaction with the negatively charged cell membrane, leading to stronger and faster opening of the tight junctions to allow for drug absorption [9], whereas chitosans were less charged and therefore less effective. However, our previous study demonstrated that trimethyl chitosans were quite toxic due to their high positive charge density and their cytotoxicity was molecular weight-dependent [16]. Therefore, for the safety reason, it is recommended to select TMCs with molecular weight less than 100 kDa as absorption enhancers. However, cytotoxicity tests showed that TMC produced an extremely significant decrease of the CBF (ciliary beat frequency) of chicken embryo trachea [34], although Hamman et al. [35] indicated that TMC did not induce visible local toxicity effects on the nasal epithelial cells. On the contrary, 1% chitosan caused no injury to nasal mucosal cilia compared to the control [36]. Thus, judging from both the safety and efficacy viewpoints, chitosan 100 kDa may possess superior characteristics as a safe and effective nasal absorption enhancer.

5. Conclusions

In this work, the absorption-promoting effect of different molecular weight chitosans, trimethyl chitosans and thiolated chitosans for intranasal absorption of 2,3,5,6-tetramethylpyrazine phosphate was studied using an in situ nasal perfusion model in rats. All the chitosans tested are effective in enhancing the intranasal absorption of TMPP. However, compared to chitosan-alone group, thiolation could not improve the absorption-enhancing capacity of chitosan even when the thiolation ratio was as high as 152 μmol/g. The permeation-enhancing effect of chitosan is molecular weight-dependent and above M_w 100 kDa, further increasing M_w causing no remarkable increase in absorption. Trimethyl chitosan was found to be a stronger absorption enhancer. This study indicated that structural features of chitosan play a key role on promoting the intranasal absorption of TMPP and the main absorption mechanism is by opening the tight junctions. Increase of mucoadhesion has only marginal contribution in the range studied. In vivo studies further demonstrated that chitosan 100 kDa and TMC 50 had comparable absorption-enhancing effect. A good correlation was established between in situ absorption data and in vivo plasma concentration.

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