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Nasal insulin delivery in the chitosan solution: in vitro and in vivo studies

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Abstract

The effects of chitosan concentrations, osmolarity, medium and absorption enhancers in the chitosan solution on nasal insulin delivery were studied in vitro and in vivo. The penetration of insulin through the mucosa of rabbit nasal septum was investigated by measuring the transmucosal flux in vitro, while the nasal absorption of insulin in vivo was assessed by the efficiency in lowering the blood glucose levels in normal rats. It was demonstrated that increasing concentrations of chitosan up to 1.5% (w/v) caused an increase in the permeability of insulin across the nasal mucosa. Insulin given intranasally in hypo- or hyperosmotic formulation showed a higher hypoglycemic effect than insulin delivered in isoosmotic formulation. Insulin formulation in chitosan solution prepared with deionized water brought to a higher relative pharmacological bioavailability (Fr) value than that prepared with 50 mM pH 7.4 phosphate buffer. A formulation containing both 1% chitosan and 0.1% ethylenediaminetetraacetic acid (EDTA), 5% polysorbate 80 (Tween 80) or 1.2% β-cyclodextrin (β-CD) did not lead to a higher Fr than insulin formulated with 1% chitosan alone. The formulation containing both 5% hydroxypropyl-β-cyclodextrin (HP-β-CD) and 1% chitosan was more effective at reducing blood glucose levels than the formulation containing 5% HP-β-CD or 1% chitosan alone. The studies indicated that chitosan concentrations, osmolarity, medium and absorption enhancers in chitosan solution have significant effect on the insulin nasal delivery. The results of in vitro experiments were in good agreement with that of in vivo studies.

Keywords: Nasal delivery; Insulin; Chitosan; Permeability; Bioavailability; Rats

1. Introduction

Chitosan [2-amino-2-deoxy-(1 → 4)-β-D-glucopyranan] is a mucopolysaccharide obtained by the deacetylation of chitin in crustaceans such as crabs and shrimps. Chitosan is soluble in organic acid (acetic acid) or inorganic acid (hydrochloric acid) and positively charged. The chemical properties of the polymer are determined by the degree of deacetylation, molecular weight and viscosity. Studies (Paul and Garside, 2000) showed that chitosan is non-toxic and its LD_{50} in mice exceeds 16 g/kg. Because of its biodegradability and biocompatibility, chitosan has been applied as a pharmaceutical excipient in oral, ocular, nasal, implant and transdermal drug delivery (Dodane and Vilivalam, 1998; Illum, 1998). Chitosan has been shown to have mucoadhesive properties because of its viscosity and interaction of the positively charged amino group with the negatively charged sites on the mucosa surface (Artursson et al., 1994; Luessen et al., 1996).

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Recent studies indicated that chitosan could enhance absorption of poorly absorbable drugs such as peptides and proteins (Luessen et al., 1996; Illum et al., 2000). The nasal delivery of chitosan was demonstrated to greatly enhance the absorption of insulin across the nasal mucosa of rats and sheep (Illum et al., 1994). In vivo evaluation in rabbits has proved that chitosan nanoparticles were able to improve the nasal absorption to a great extent than chitosan solution probably due to intensified contact of the nanoparticle with the nasal mucosa as compared to chitosan solutions (Fernandez-Urrusuno et al., 1999a,b). Later studies showed that chitosan nanoparticles were not as efficient as chitosan solution nor chitosan powder in terms of their nasal absorption promoting ability in rats and sheep (Dyer et al., 2002). Investigations have suggested that there are two effects of chitosan delivery systems on nasal mucosa. The mucoadhesive properties of the polymer can reduce the clearance rate of drugs from nasal cavity, thereby prolonging the contact time of chitosan delivery system with nasal epithelium. In addition, it has been shown that the interaction of the positively charged amino group of chitosan with the negatively charged sialic acid residues in mucus causes the transient opening the tight junctions and allows large hydrophilic compounds to be transported across the epithelium. The opening mechanism of the tight junctions has been demonstrated by a decrease in ZO-I proteins and the change in the cytoskeletal protein F-actin from a filamentous to a globular structure (Artursson et al., 1994; Schipper et al., 1997).

Most of studies utilized chitosan alone as absorption enhancer. Currently, it is not known if the combination of chitosan and other absorption enhancers, as well as some other factors could exhibits a synergistic effect in the nasal absorption of insulin. It is thought that EDTA affects the tight junctions interconnecting membrane cells by the removal of calcium and consequently increases paracellular transport (Cassidy and Tidball, 1967), and chitosan could also open the tight junctions. So it is interesting to study whether a nasal formulation containing both of these kinds of absorption enhancers could exhibit an additive or synergistic increase of the insulin absorption. Tweens with the ethylene oxide and a long hydrocarbon chain have been used to enhance the absorption of drugs in transdermal delivery systems (Breuer, 1979; Walters et al., 1987), and this kind of nonionic surfactants may penetrate into the intercellular matrix, increase the fluidity, and extract lipid components from biomembrane (Breuer, 1979; Walters et al., 1987). Cyclodextrins (CDs) could also extract the phospholipids and proteins from membrane (Shao et al., 1992). When chitosan interacts with the epithelial membrane, the tight junctions are opened, then Tweens or CDs could penetrate into the opened gaps between cells and extract the phospholipids in biomembrane. Thus, the tight junction proteins such as occludin (Furuse et al., 1993), claudin-1 and -2 (Furuse et al., 1998) are naked and may collapse after the removal of surrounding phospholipids, resulting in these fusion points untied. So the opening of the tight junctions may be strengthened by co-administration of chitosan and Tweens or CDs.

The purpose of this paper was to evaluate the effects of chitosan concentrations, osmolarity, medium and some absorption enhancers in chitosan solution on the insulin permeation across the rabbit nasal mucosa in vitro and the serum glucose concentrations after nasal administration of insulin to normal rats. Moreover, the correlation between the in vitro and in vivo studies was also investigated.

2. Materials and methods

2.1. Materials

Crystalline porcine zinc insulin (27.5 IU/mg) was purchased from Xuzhou Biochemical Company (People's Republic of China), and ^{125}I-insulin was obtained from the China Institute of Atomic Energy. EDTA, Tween 80, β-CD and HP-β-CD were supplied by Sigma (St. Louis, MO, USA). Chitosan (non-salt form) was purchased from Qingdao Haihui Company (People's Republic of China). The molecular weight of chitosan is about 100,000 Da and the deacetylation degree is 85%. The blood glucose assay kit was a product of Zhongsheng High-Tech Bioengineering Company (People's Republic of China). All other reagents were of analytical grade.

2.2. Preparation of insulin formulations

Chitosan was dispersed in deionized water, and hydrochloric acid was added into the above sys-

tem under agitation until chitosan was dissolved completely. The pH of this solution was about 4.0. ^{125}I-insulin or unlabeled insulin was dissolved in chitosan solutions to prepare stock solution. As the controls, ^{125}I-insulin or unlabeled insulin was directly dissolved in pH 4.0 aqueous solutions without chitosan.

The concentrations of chitosan in stock solution were 0.5, 1 and 1.5% (w/v), respectively. Different amount of sodium chloride was added into the stock solution containing 1% chitosan to achieve hypoosmolarity (50 mOsm), isoosmolarity (292 mOsm) or hyperosmolarity (612 mOsm). The 50 mM pH 7.4 phosphate buffer was also used as the medium of the test solutions, besides the deionized water. EDTA (0.1%), Tween 80 (5%), β-CD (1.2%) or HP-β-CD (5%) was dissolved in the stock solution containing 1% chitosan respectively. As the controls, each of the absorption enhancers was dissolved in the insulin solution at the same concentrations, respectively without chitosan. The insulin formulations used in this studies are summarized in Table 1.

2.3. Tissue preparation

The rabbit nasal mucosa was prepared as described by previous studies (Carstens et al., 1993; Hosoya et al., 1994). Male Japanese white rabbits (the Experimental Animal Center of Health Science Center of Peking University) weighing 2.5–3.0 kg were used in this study. They were fasted overnight and sacrificed by air embolism. The skin around the nasal region was removed and septum made visible by removal of the lateral wall of nasal cavity. The nasal septum was carefully isolated with a scissors and placed in ice-cold Ringer's solution (NaCl 125 mM, KCl 5 mM, $CaCl_2$ 1.4 mM, NaH_2PO_4 1.2 mM, $NaHCO_3$ 10 mM and D-glucose 11 mM). Then two mucosae were carefully stripped from the nasal septum using round-edged tweezers. They were immediately mounted between the two halves of Valia-Chien diffusion chamber. The effective diffusion area of the mucosa was 0.126 cm^2. Each chamber was filled with 5 ml of insulin preparation or Ringer's solution. Temperature was kept at 37 °C during the experiments.

Table 1
Summary of insulin formulations administered to rats

Impacting factor	No.	Chitosan (%)	Insulin (IU/ml)	Osmolarity	Absorption enhancer
Chitosan concentration	1	–	200	Hypo	–
	2	0.5	200	Hypo	–
	3	1.0	200	Hypo	–
	4	1.5	200	Hypo	–
Osmolarity	5	1.0	200	Hypo	–
	6	1.0	200	Iso	–
	7	1.0	200	Hyper	–
Medium	8	1.0	200	Water	–
	9	1.0	200	Phosphate buffer	–
Absorption enhancer	10	1.0	200	Hypo	–
	11	–	200	Hypo	0.1% EDTA
	12	1.0	200	Hypo	0.1% EDTA
	13	–	200	Hypo	5% Tween 80
	14	1.0	200	Hypo	5% Tween 80
	15	–	200	Hypo	1.2% β-CD
	16	1.0	200	Hypo	1.2% β-CD
	17	–	200	Hypo	5% HP-β-CD
	18	1.0	200	Hypo	5% HP-β-CD
Control (physiological saline)	–	–	–	Iso	–

No.: number of insulin formulation; hypo: hypoosmolarity; iso: isoosmolarity; hyper: hyperosmolarity.

2.4. In vitro permeation studies

Prior to the experiment, the diffusion chambers were filled with Ringer's solution and kept at 37 °C for 0.5 h. Then the Ringer's solution on the donor side was replaced by the ^{125}I-insulin solutions. At the beginning, 20 μl aliquot of sample was taken from the donor (mucosal) side. During the experiment, 3 ml of sample was removed from the acceptor (serosal) side at fixed intervals (0.5, 1, 2, 3, 4 h) and the same volumes were added into the acceptor side. The samples containing ^{125}I-insulin were determined with a γ-counter. Each study was repeated five times.

2.5. Calculation of the permeability coefficient

Flux data were plotted as the cumulative amount of ^{125}I-insulin that diffused from the mucosal to the serosal of epithelium versus time. The permeability coefficient (P) was calculated using the following equation:

$$P = \frac{\mathrm{d}Q/\mathrm{d}t}{C_0 A}$$

where $\mathrm{d}Q/\mathrm{d}t$ represents the permeability rate, and C_0 stands for the initial concentration in the donor chamber, while A is the effective surface area of the mucosa.

2.6. In vivo studies

In vivo studies were performed as earlier reported (Chandler et al., 1991). Briefly, male Sprague–Dawley rats (the Experimental Animal Center of the Health Science Center of Peking University) weighing 250–300 g were fasted overnight and anaesthetized by intraperitoneal injection of ethyl carbamate (1.35 g/kg). The rats were tracheotomised to divert the airflow from the nasal passages and aid breathing. The oesophagus was closed by ligation onto the tracheal cannula. The left carotid artery and the right external jugular vein were cannulated for blood sampling and fluid (physiological saline) replacement, respectively. The insulin preparation (200 IU/ml) were delivered through the right nostril using a PVC tube connected to a microliter syringe to give an insulin dose of 10 IU/kg. The preparation administered nasally was about 12–15 μl, depending on the weight of the rat. Blood samples (0.2 ml) were taken at various time intervals up to 5 h after administration and the total volume of blood removed from each rat was about 1.5 ml.

The samples were centrifuged to obtain serums and the blood glucose levels were determined immediately with blood glucose assay kits using the glucose oxidase method.

As a control, the physiological saline solution without insulin was also intranasally administered to the rats. Insulin solution was subcutaneously (s.c.) administered to the rats (1 IU/kg) to calculate the relative pharmacological bioavailability (Fr). Each group contains five rats.

2.7. Data analysis

The area above the serum glucose levels time curves (AAC) were calculated using the trapezoidal rule. The relative pharmacological bioavailability (Fr) was calculated according to the formula (Shen et al., 1999):

$$\mathrm{Fr} = \frac{\mathrm{AACi.n.} \times \mathrm{Doses.c.}}{\mathrm{AACs.c.} \times \mathrm{Dosei.n.}} \times 100\%$$

The "i.n." and "s.c." represent "intranasal" and "subcutaneous", respectively.

Student's t-test and ANOVA were used to determine statistical significance. Differences were considered to be significant for values of $P < 0.05$.

3. Results

The effects of chitosan concentrations, osmolarity of the solution, medium as well as some absorption enhancers on the insulin permeation across the rabbit nasal mucosa in vitro are shown in Figs. 1 and 2, respectively. The permeation coefficients of insulin across the rabbit nasal epithelium in vitro under different conditions are listed in Table 2.

When the concentrations of the chitosan increased from 0 to 1.5%, the amount of insulin across the mucosa increased consequently. There were significant differences in permeation coefficients between three chitosan concentrations ($P < 0.05$). It was shown that the permeability coefficient of insulin with 1.5% chitosan is almost 25-fold higher than that without chitosan. In the hypoosmotic or hyperosmotic solution, there was a significant permeability increase

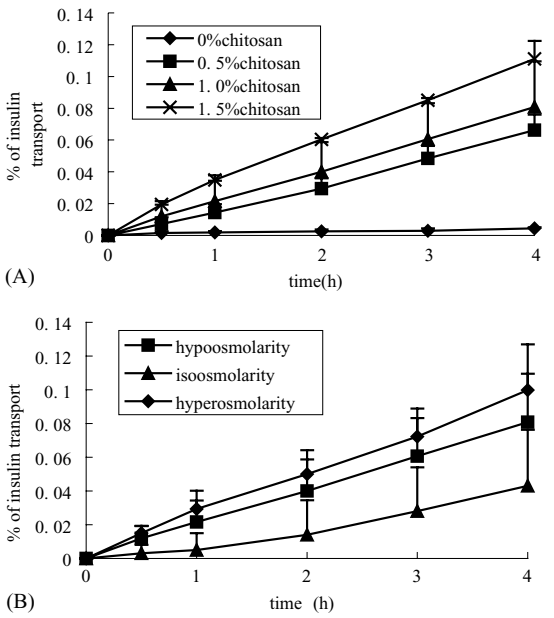

Fig. 1. Effect of concentrations (A) and osmolarity (B) of chitosan solution on the transport of insulin across the rabbit nasal membrane in vitro. Bars represent the S.D. of five experiments.

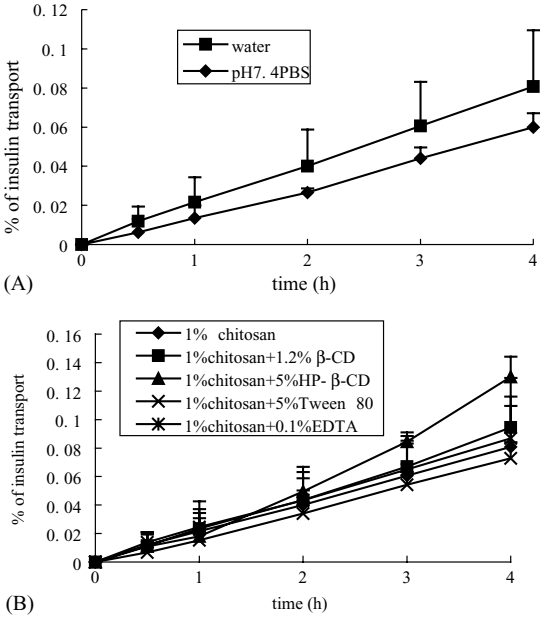

Fig. 2. Effect of medium (A) and other absorption enhancers (B) on the transport of the insulin from chitosan solution across the rabbit nasal membrane in vitro. Bars represent the S.D. of five experiments.

Table 2
The permeation coefficient (P) of insulin across the rabbit nasal epithelium in vitro under different conditions (mean ± S.D., $n = 5$)

Impacting factor	Formulation	P ($\times 10^{-7}$) (cm s^{-1})
Chitosan concentration	0% chitosan	1.25 ± 0.19
	0.5% chitosan	18.31 ± 2.75[a]
	1.0% chitosan	22.36 ± 7.93[a]
	1.5% chitosan	30.70 ± 3.11[a]
Osmolarity	Hypoosmolarity (1.0% chitosan)	22.36 ± 7.93[b]
	Isoosmolarity (1.0% chitosan)	11.90 ± 9.21
	Hyperosmolarity (1.0% chitosan)	27.63 ± 7.45[c]
Medium	Water (1.0% chitosan)	22.36 ± 7.93
	pH 7.4 phosphate buffer (1.0% chitosan)	16.60 ± 1.98[d]
Absorption enhancer	1.0% chitosan	22.36 ± 7.93
	0.1% EDTA + 1.0% chitosan	24.01 ± 8.13
	5% Tween 80 + 1.0% chitosan	20.18 ± 3.08
	1.2% β-CD + 1.0% chitosan	26.15 ± 9.55
	5% HP-β-CD + 1.0% chitosan	35.95 ± 3.89[e]

[a] $P < 0.05$ between 0.5, 1.0 and 1.5% chitosan concentration.
[b] $P < 0.05$ vs. isoosmolarity.
[c] $P < 0.05$ vs. isoosmolarity.
[d] $P < 0.05$ vs. water.
[e] $P < 0.05$ vs. 1.0% chitosan.

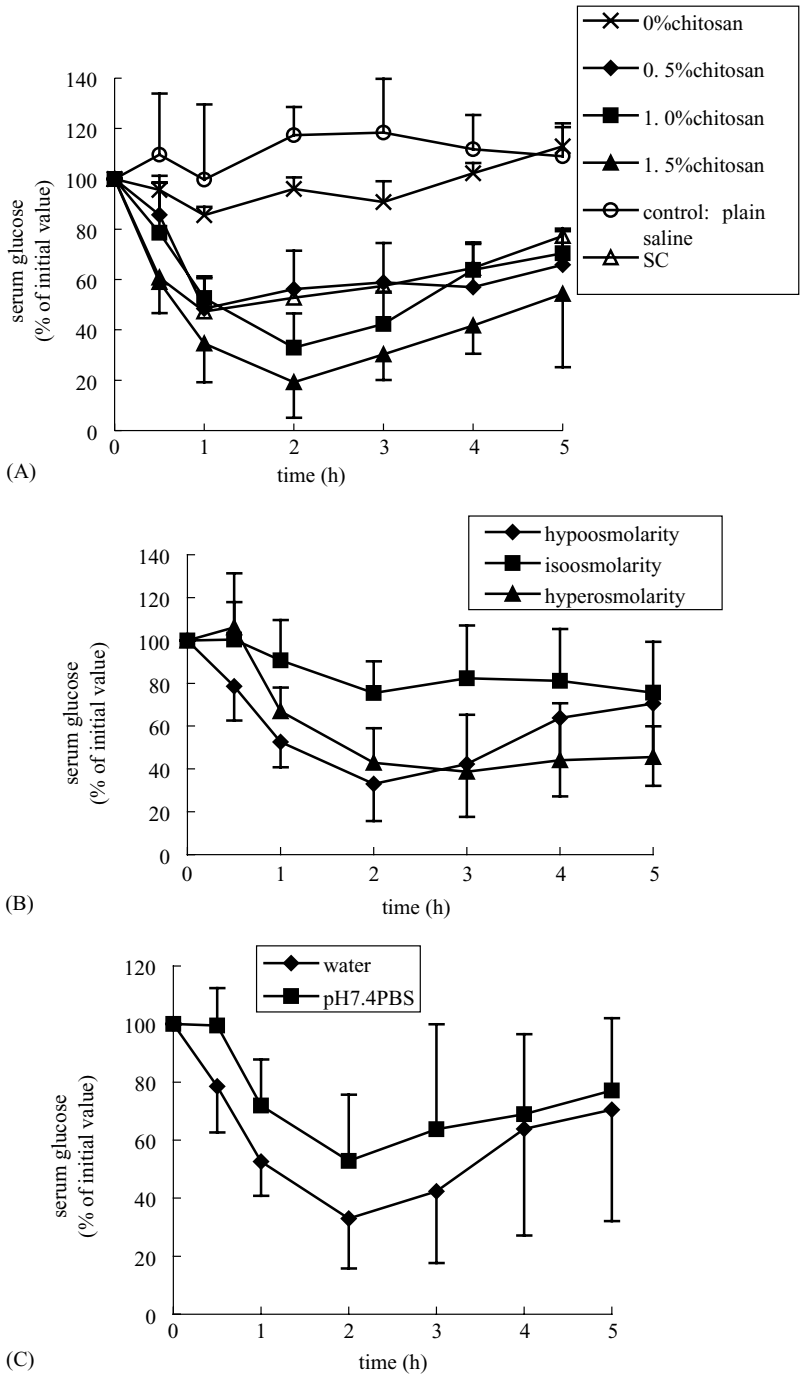

Fig. 3. Effect of concentrations (A), osmolarity (B) and medium (C) of the chitosan solution on the mean serum glucose concentrations after nasal administration of 10 IU/kg insulin to rats. Bars represent the S.D. of five experiments.

($P < 0.05$) of insulin across the mucosa, compared to that in isoosmotic solution.

An increase in the permeability coefficient of insulin in the chitosan formulation prepared with deionized water was demonstrated, as compared to that with pH 7.4 phosphate buffer. There was a remarkable increase in the permeability of insulin in the formulation containing both 5% HP-β-CD and 1% chitosan, compared to that of the control formulation containing 1% chitosan alone. No significant difference ($P > 0.05$) in the permeability coefficient was seen between the formulation containing 0.1% EDTA as well as 1% chitosan and the control formulation. The similar results with 0.1% EDTA were observed when 1.2% β-CD or 5% Tween 80 was combined with 1% chitosan.

Effects of chitosan concentrations, osmolarity of the test solutions, medium and some absorption enhancers on the mean serum glucose concentrations after nasal administration of 10 IU/kg insulin to normal rats are depicted in Figs. 3–5, respectively, while the concomitant changes in relative pharmacological bioavailability (Fr) are presented in Table 3.

As a control, nasal administration of insulin without chitosan failed to reduce the blood glucose levels, but obviously hypoglycemic effect of insulin was seen when the formulation containing different concentration of chitosan was delivered into the rat nasal as shown in Fig. 3A. The Fr for the three chitosan concentrations are significantly different ($P < 0.05$). There was an effect of the concentration increase of chitosan on the pharmacological effect of insulin (as seen in Table 3). The nadir of glucose levels were obtained 1 h after the administration of the insulin formulation containing 0.5% chitosan, while the minimum glucose levels were achieved 2 h after insulin was given together with 1 or 1.5% chitosan to the rats. The time to reach minimum glucose levels was remarkably delayed at higher concentration of chitosan compared to that at lower concentration. There were significant differences in Fr and nadirs between 1 and 1.5% chitosans ($P < 0.05$).

Insulin in isoosmotic formulation showed a relative weak effect on lowering the blood glucose contents as given in Fig. 3B. However, the administration of insulin solution, either hypo- or hyperosmolarity, resulted in a significant decrease in blood glucose concentrations ($P < 0.05$). The relative pharmacological bioavailabilities (Fr) of three insulin formulations

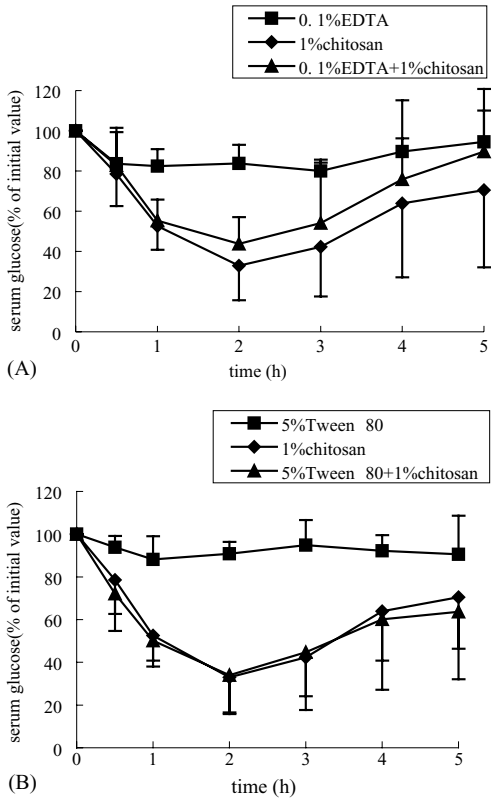

Fig. 4. Effect of EDTA or/and chitosan (A), Tween 80 or/and chitosan (B) on the mean serum glucose concentrations after nasal administration of 10 IU/kg insulin to rats. Bars represent the S.D. of five experiments.

were consistent with their permeability coefficients in vitro.

The effect of reducing blood glucose levels of the insulin formulation prepared with deionized water was more effective than that prepared with pH 7.4 phosphate buffer as illustrated in Fig. 3C, and there are significant differences between them in Fr ($P < 0.05$).

As shown in Fig. 4A, the blood glucose levels after nasal administration of 10 IU/kg insulin to rats decreased but not significant when EDTA was used as enhancer alone at the concentration of 0.1%. Nasal delivery of insulin with 1% chitosan resulted in an obvious decrease in serum glucose levels, with a minimum value of 32.95% of the initial glucose concentration at 2 h after administration. The minimal glucose levels, 43.78% of the initial value, was obtained 2 h after

Table 3
The relative pharmacological bioavailability (Fr) after nasal administration of 10 IU/kg insulin to rats under different conditions (mean ± S.D., $n = 5$)

Impacting factor	No.	Formulation	Fr (%)
Chitosan concentration	1	0% chitosan	0.89 ± 0.63
	2	0.5% chitosan	9.77 ± 3.26[a]
	3	1.0% chitosan	11.35 ± 5.32[a]
	4	1.5% chitosan	15.41 ± 5.43[a]
Osmolarity	5	Hypoosmolarity	11.35 ± 5.32[b]
	6	Isoosmolarity	4.09 ± 3.95
	7	Hyperosmolarity	11.42 ± 5.49[c]
Medium	8	Water	11.35 ± 5.32
	9	pH 7.4 phosphate buffer	7.52 ± 5.69[d]
Absorption enhancer	10	1.0% chitosan	11.35 ± 5.32
	11	0.1% EDTA	3.61 ± 1.49
	12	0.1% EDTA + 1.0% chitosan	8.88 ± 4.54
	13	5% Tween 80	1.98 ± 1.72
	14	5% Tween 80 + 1.0% chitosan	11.81 ± 3.91
	15	1.2% β-CD	3.43 ± 2.64
	16	1.2% β-CD + 1.0% chitosan	12.61 ± 7.19
	17	5% HP-β-CD	7.67 ± 3.56
	18	5% HP-β-CD + 1.0% chitosan	16.08 ± 3.28[e]
s.c. (1 IU/kg)	–	–	100 ± 0.47

s.c.: subcutaneous administration; No.: number of insulin formulation (the compositions can be referred to Table 1).
[a] $P < 0.05$ between 0.5, 1.0 and 1.5% chitosan concentration.
[b] $P < 0.05$ vs. isoosmolarity.
[c] $P < 0.05$ vs. isoosmolarity.
[d] $P < 0.05$ vs. water.
[e] $P < 0.05$ vs. 1.0% chitosan.

the administration of the insulin formulation containing 1% chitosan and 0.1% EDTA. These differences were not significant ($P > 0.05$).

When insulin formulation containing 5% Tween 80 alone was delivered into the rat nasal, a slight decrease of the glucose levels was seen as shown in Fig. 4B, as compared to the formulation containing 1% chitosan. The formulation containing 5% Tween 80 and 1% chitosan was as effective as the formulation containing 1% chitosan alone, in terms of hypoglycemic effect. The serum glucose levels after administration of these two formulations fell to 33.94 and 32.95% of the initial levels, respectively, at about 2 h. There was no significant difference ($P > 0.05$) in Fr between these two samples. The decrease in serum glucose levels showed a similar pattern when insulin was administered with 1.2% β-CD or/and 1% chitosan (Fig. 5A).

The nasal administration of insulin formulation containing 5% HP-β-CD alone produced a obvious decrease in blood glucose levels, reaching minimal value of 57.62% of the initial concentration at 0.5 h as demonstrated in Fig. 5B. Insulin formulation containing 5% HP-β-CD and 1% chitosan was more efficient at lowering glucose levels than the formulation containing 1% chitosan alone. For the late two formulations, the minimum values of 21.44 and 32.95% of the initial glucose concentrations were reached, respectively, 2 h after administration. There was a significant difference ($P < 0.05$) in Fr between those two formulations.

Taking the permeability coefficient (P) as X-axis values and relative pharmacological bioavailability (Fr) as Y-axis values, the correlation between in vitro and in vivo evaluation of different insulin formulations was obtained, as shown in Fig. 6, with a correlation coefficient of 0.9472, suggesting a good correlation between in vitro permeation enhancing effect and in vivo pharmacological effect.

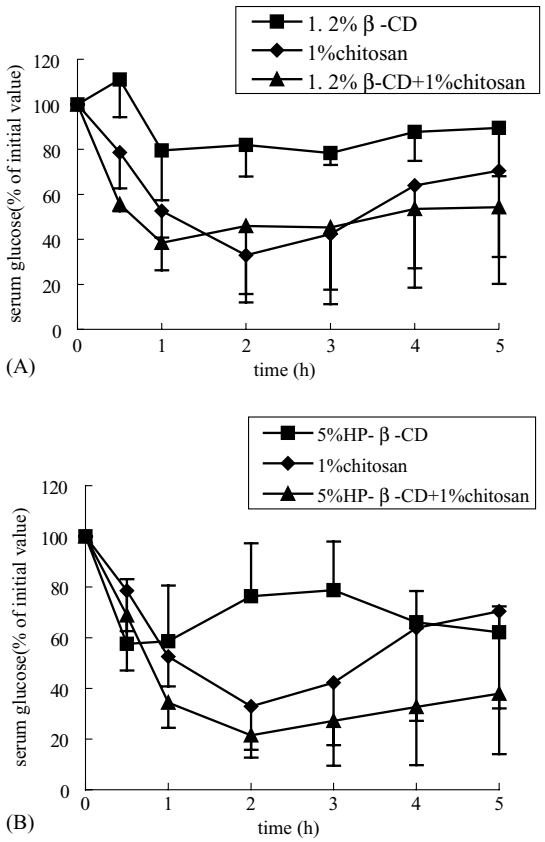

Fig. 5. Effect of β-CD or/and chitosan (A), HP-β-CD or/and chitosan (B) on the mean serum glucose concentrations after nasal administration of 10 IU/kg insulin to rats. Bars represent the S.D. of five experiments.

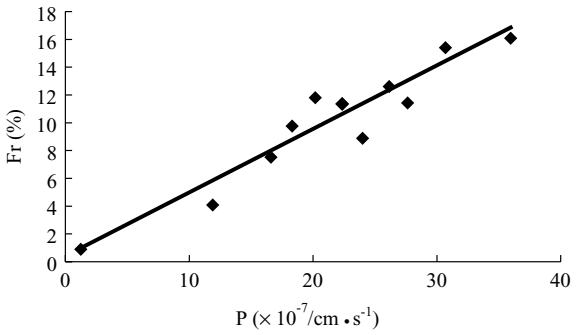

Fig. 6. The correlation between permeability coefficient (*P*) and relative pharmacological bioavailability (Fr) of different formulations.

4. Discussion

The influence of chitosan concentrations on the effect of insulin both in vitro and in vivo in our studies indicated that chitosan concentration is one of the impacting factors influencing the enhancement of drugs to pass through the membrane, probably due to the mucoadhesive properties and high viscosity produced by the chitosan solutions, which make the drugs stay in the nasal cavity for a long time and be cleared slowly by mucocilia from nasal mucosa. But in this rat model, the mucociliary clearance mechanism is impaired hence the mucoadhesiveness has less importance in this studies. On the other hand, chitosan may open the tight junctions between cells due to the interaction of the positively charged amino group of it with the negatively charged sialic acid residues in mucus, leading to the transport increase of large hydrophilic compounds across the epithelium, as it was mentioned in the introduction. Studies (Artursson et al., 1994) demonstrated that an increase in chitosan concentrations resulted in an increase in the permeability coefficient of ^{14}C-mannitol with a plateau level between 0.25 and 0.5%, using a human intestinal cell line (Caco-2) as the model epithelial cell layer. It was shown by earlier studies (Lehr et al., 1992) that the minimum concentration of chitosan resulting in strong bioadhesiveness on pig intestinal mucosa was 1% (w/v). Previous studies (Illum et al., 1994) reported that the lowest plasma glucose levels were obtained after nasal delivery of insulin with 0.2% chitosan to rats, and due to the increased viscosity no increased absorption enhancement was observed when chitosan concentration was higher than 0.2%. However, in our studies the increase in chitosan concentrations from zero to 1.5% resulted in a continuous decrease in blood glucose concentrations. The minimal blood glucose level was significantly delayed for the insulin formulation containing 1 or 1.5% chitosan as compared to that containing 0.5% chitosan, which could be explained by the longer contact time between the drug and nasal membrane when higher chitosan concentration was presented. There were significant differences ($P < 0.05$) in both Fr and nadir between 1 and 1.5% chitosans in our studies. The differences between 1 and 1.5% chitosans can be explained by the following reasons. One reason is that maybe there is a correlation between the capacity of opening tight junctions

and chitosan concentrations. In addition, maybe there is also a correlation between the membrane damage and chitosan concentrations (1 and 1.5%). The previous reports (Artursson et al., 1994; Illum et al., 1994) investigated the chitosan concentrations only up to 1% and the concentrations higher 1% were not studied. The second and third reasons remain to be investigated.

In present studies, insulin delivered intranasally in the hypo- or hyperosmotic formulation was more effective in both transport increase of insulin across the rabbit nasal membrane and reducing blood glucose levels of normal rats than that in isoosmotic formulation.

The effect of hypoosmotic environment on the in vitro and in vivo studies could be possibly explained by the so-called "regulatory volume decrease (RVD) response" and "solvent drag". Firstly, the hypoosmotic environment may result in cell swelling and in most cases cell swelling will lead to cell shrinkage back to the original volume. The RVD response is triggered by the opening of volume-sensitive anion (Cl^-) and cation (K^+) channels in cell membrane accompanying with the efflux of KCl and the loss of water, and consequently promotes the transport of insulin across the biomembrane. Earlier studies (Coransanti et al., 1990) reported that a hypotonic solution cause rat hepatocytes to swell to adapt their intraosmotic pressure to tonicity of surrounding medium. These cells change their volumes and exhibit an ability to return toward their resting (isotonic) volumes. Other studies (Noach et al., 1994) demonstrated that a rapid and fully reversible drop in the transepithelial electrical resistance (TEER) and an increase in transport of fluorescein-Na or fluorescein-isothiocyanate-labeled dextran were observed when a hypotonic solution was applied at the apical side of the Caco-2 monolayers. Another mechanism called "solvent drag" shown in earlier study (Pappenheimer and Reiss, 1987) suggests that a hypotonic solution will induce fluid flow from the hypotonic to the isotonic side of a cell layer, which will enhance hydrophilic molecules to transport the epithelial cell layer.

The effect of hyperosmotic environment on the epithelial membrane seems controversial. Studies (Sakiya et al., 1981) reported that the absorption of quinine from the small intestine decreased with increasing osmotic pressure. An increase of TEER was observed when some epithelial mucosae were exposed to hyperosmotic condition, which is probably due to the collapse of the epithelial intercellular space (Madara, 1983). The same findings were also reported in other studies (Ritter et al., 1991). Earlier study showed that insulin delivered intranasally in a hyperosmotic gel system had no significant effect on reducing blood glucose levels (Pereswetoff-Morath and Edman, 1995). In contrast, the studies (Noach et al., 1994) demonstrated that a drop in TEER could be seen in Caco-2 cell test during the application of an apical hypertonic solution. The finding in our study is also interesting and the reason is not clear, probably due to the "regulatory volume increase (RVI) effect" or the lesion on the nasal epithelium caused by the hyperosmotic condition. Regulatory volume increase (RVI) could occur in response to hypertonic shrinkage of cells and is generally mediated by salt and water entry into cells (Coransanti et al., 1990). In addition, the absorption of insulin can be also driven by the difference of the osmotic pressure. In our study, this hyperosmolarity would not lead to a salt out of insulin and the insulin is stable in this environment.

The different effect of insulin formulations in different medium (deionized water and pH 7.4 phosphate buffer) could be explained by the inference that the anion (HPO_4^{2-} and $H_2PO_4^-$) in the phosphate buffer would aggregate around the positively charged amino group ($-NH_3^+$) of chitosan and prevent the contact between the chitosan and negatively charged sites of nasal epithelium. It is also well known that in solution with high ionic strength, chitosan collapses because of the neutralization of the positive charges and becomes less efficient in the absorption enhancement. Even in some cases, a salting out effect could occur.

When EDTA was added into insulin solution without chitosan, zinc ions could be chelated by EDTA, leading to insulin deaggregation from hexamer to dimers as was evidenced from a circular dichroism study (Liu et al., 1991). Obviously, it is easier for the smaller molecular dimension to pass though the epithelial membrane, compared to the larger one. However, the smaller species of insulin will have higher degradation rate than the larger ones. Fig. 4A indicated that insulin released from the formulations was in its active form, showing that EDTA did not influence the stability of insulin. On the other hand, it is thought that EDTA affects the tight junctions

interconnecting membrane cells and consequently increases paracellular or pore transport (Cassidy and Tidball, 1967). But the negatively charged carboxyl groups of EDTA would interact with the positively charged amino groups of chitosan, when EDTA was mixed with chitosan, which may prevent either agent from contacting with nasal mucosa and also inhibit the deaggregation of insulin. Therefore, co-administration of EDTA and chitosan may result in a weak mutual inhibition in their abilities of absorption enhancement, which properly explained the fact in our studies that co-administration of these two absorption enhancers was not as effective as chitosan alone in lowering blood glucose levels.

With several polyoxyethylene chains and a long hydrocarbon chain, Tweens have been used to enhance the absorption of drugs in transdermal delivery system in previous studies (Breuer, 1979; Walters et al., 1987). There are two possible mechanisms by which drug absorption is increased using this kind of nonionic surfactants. Firstly, the surfactant may penetrate into the stratum corneum (SC), extract lipid components from it, disrupt the lipid arrangements, increase the fluidity, and increase the water content of the protein in the barrier, leading to a higher solubility for drugs. Secondly, the penetration of the surfactant into the SC intercellular matrix accompanied by the interaction and binding with keratin filaments may result in a disruption of the corneocyte. However, Tween 80 in the nasal delivery system in our investigations did not result in a significant absorption enhancement as compared to that in transdermal drug delivery system. The result did not reach the expected effect that Tween could work synergistically with chitosan in nasal delivery system and this is probably due to the difference between the structures of skin barrier and nasal membrane.

Cyclodextrins (CDs) could enhance nasal absorption of therapeutic compounds such as peptides and proteins. Merkus et al. (1999) have summarized the absorption enhancing effects of cyclodextrins in nasal drug delivery. But their mechanisms of absorption enhancement are not very clear. It may be attributed to some of the following reasons: (i) complexation with lipophilic penetrants, resulting in an increased water solubility for these lipophilic compounds (Schipper et al., 1990), (ii) direct epithelial membrane disruption effect by extraction of phospholipids and proteins, (iii) inhibition of proteolytic enzyme activity, (iv) dissociation of insulin oligomers. Studies (Shao et al., 1992) showed that the relative effects of various cyclodextrins on causing insulin hexamer dissociation follow the descending order of DM-β-CD (5%) > α-CD (5%) > HP-β-CD (5%) > γ-CD (5%) > β-CD (1.8%). The order of protein release rates from biomembrane follows in a descending manner: DM-β-CD (5%) > α-CD (5%) > β-CD (1.8%) > HP-β-CD (5%) > γ-CD (5%) and the rate of total phosphorus release follows the same rank order correlation as that of total protein release. β-CD and HP-β-CD are chosen as absorption promoters in this studies due to their relatively lower toxicity and irritation compared to dimethyl-β-cyclodextrin (DM-β-CD) (Yoshida et al., 1988). In present study, co-administration of 1.2% β-CD and 1% chitosan did not give rise to a higher Fr value of insulin than delivery with 1% chitosan alone. Co-administration of 5% HP-β-CD and 1% chitosan was more effective both in reducing blood glucose levels and in increasing the permeability coefficient than administration of 1% chitosan alone, suggesting that 5% HP-β-CD was more effective in the absorption enhancement for insulin than 1.2% β-CD. It was very interesting to demonstrate the synergistic effect between the two absorption-enhancing agents, HP-β-CD and chitosan, in our in vitro and in vivo studies. The result in present studies could be explained by following reasons. CDs could extract the phospholipids and proteins from membrane by forming a new lipid inclusion compartment in the aqueous phase (Shao et al., 1992). When chitosan interacts with the epithelial membrane, the tight junctions are opened, then HP-β-CD could penetrate into the opened gaps between cells and extract the phospholipids from biomembrane. Thus, the tight junction proteins such as occludin (Furuse et al., 1993), claudin-1 and -2 (Furuse et al., 1998) are naked and may collapse after the removal of surrounding phospholipids, resulting in these fusion points untied. Moreover, it has been shown that the interior of the tight junction pores are highly hydrated and negatively charged. A relative modest alteration in the relative concentration of specific species of ions within the volume of the ZO pores would result in substantial alterations in tight junction resistance leading to opening of the pore (Madara, 1989). The naked ZO pores caused by HP-β-CD could be affected more easily by the com-

position and concentration of specific species of ions in the pores. In other words, the opening of tight junctions by chitosan with HP-β-CD becomes easier than that without HP-β-CD. In addition, the dissociation of insulin and the inhibition of proteolytic enzyme activity could strengthen the absorption enhancement of insulin. So HP-β-CD could work synergistically with chitosan and the absorption enhancement of co-administration of chitosan and HP-β-CD was more effective than that of HP-β-CD or chitosan used alone. β-CD could also extract the phospholipids and release proteins from biomembrane, but the capacity of releasing proteins is greater than that of HP-β-CD (Shao et al., 1992), so the number of glycoproteins on the cell membrane exposed to β-CD is smaller than that exposed to HP-β-CD. Thus, the interaction between positively charged chitosan and negatively charged glycoproteins exposed to β-CD would be weakened to a larger extend, compared to that exposed to HP-β-CD. In addition, 1.8% β-CD was less effective in dissociating insulin hexamers than 5% HP-β-CD (Shao et al., 1992). So the enhancing effect of co-administration of chitosan and β-CD was less than that of co-use of chitosan and HP-β-CD.

This study showed that the combination of chitosan and HP-β-CD is the most effective in enhancing the absorption of insulin in nasal delivery system. The results of in vitro experiments were in good agreement with those performed in vivo, so the in vitro test could be used to evaluate the nasal delivery of insulin.

5. Conclusions

The results in this study indicated that the chitosan concentrations, osmolarity, medium and absorption enhancers in chitosan solution have significant effect on nasal insulin delivery. The maximum hypoglycemic effect was achieved when insulin was administered in a formulation containing both HP-β-CD and chitosan.

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