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Full Length Research Paper

Effect of absorption enhancers on nasal delivery of basic fibroblast growth factor

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The present study was to screen an optimal absorption enhancer for enhancing the nasal absorption of basic fibroblast growth factor (bFGF), a promising therapeutic agent to neurodegenerative diseases. In this study, four absorption enhancers including chitosan, sodium caprate, poly-L-arginine (poly-L-Arg, 92.0 kDa) and dimethyl- -cyclodextrin were chosen to evaluate their toxicity using *in situ* toad palate model and *in vitro* Calu -3 cell model. Transport study of bFGF across Calu-3 cell monolayers in the absence or presence of chitosan was performed to determine the optimal concentration of chitosan. Pharmacokinetics study was conducted to monitor changes in the blood concentration of bFGF following nasal administration of bFGF solution with or without 0.5% (w/v) chitoson, in comparison with intravenous administration of bFGF alone. Of the absorption enhancers tested, chitosan at the concentration of 0.25 and 0.5% showed little toxicity to nasal cilia and Calu-3 cells, and exerted reversible effect on the reduction of transepithelial electrical resistance. Transport study showed that the apparent permeability coefficient (Papp) value was increased by about 16- and 2-fold, respectively with addition of 0.5 and 0.25% chitosan, compared with bFGF solution alone. Following nasal administration to rats, the formulation containing 0.5% chitosan significantly enhanced the absorption of bFGF with an area under curve ($AUC_{0\text{-}120min}$) nearly 1.4-fold that of bFGF solution ($p<0.05$). The absolute bioavailability of bFGF after intranasal administration was 5.35 and 7.53% for bFGF alone and 0.5% chitosan group, respectively. These results indicated that 0.5% chitosan is a safe and effective absorption enhancer for intranasal delivery of bFGF.

Key words: Basic fibroblast growth factor, absorption enhancer, chitosan, toxicity, pharmacokinetics, intranasal delivery.

INTRODUCTION

Basic fibroblast growth factor (bFGF) is a cationic peptide containing 154 amino acids with molecular weight of 18 kDa and isoelectric point of 9.6. bFGF is a clinical neurotrophic factor and also a strong mitogenic factor, which can protect neurons and promote nerve growth (Abe and Saito, 2001). Meanwhile, bFGF can treat cognitive impairment caused by traumatic brain injury, and prevent learning and memory decline due to aging (Sun et al., 2009; Zechel et al., 2010). Based on the aforementioned studies, it is a useful way to cure central nervous system (CNS) disorders especially neurodegenerative diseases with bFGF. Several researches have demonstrated the therapeutic function of bFGF on Alzheimer disease (Mark et al., 1997; Shi et al., 2002; Zhou et al., 2003; Bellucci et al., 2007). However, intracerebroventricular injection is applied in most of these studies, with complication and inconvenience. Therefore, non-invasive drug delivery methods are urgently needed.

Intranasal administration is a potential route for drug delivery to the brain that bypasses the blood-brain barrier. This route of administration is relatively more convenient than injection, thus improving patient compliance, and also allows more frequent administration due to its noninvasiveness (Okonko et al., 2009). Moreover, the plausibility of nose to brain pathway for the delivery of

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peptides and proteins, like nerve growth factor (Zhao et al., 2004; Vaka et al., 2009), cholera toxin B subunit-nerve growth factor (Zhang et al., 2008), insulin (Benedict et al., 2004; Djupesland, 2008; Henkin, 2010) and Insulin - like growth factor-1 (Liu et al., 2001; Thorne et al., 2004), has been demonstrated previously. However, the total amount of these macromolecules accessing the brain was reported to be low, because of their low membrane permeability, rapid mucociliary clearance from nasal cavity and susceptibility to degradation either within the lumen of nasal cavity or during passage across the epithelial barrier. Hence, it is necessary to explore ways to improve the efficacy of drug targeting to the brain following nasal administration.

The factors limiting drug uptake across the nasal epithelium could be counteracted with the use of absorption enhancers. Several absorption enhancers such as chitosan (Illum et al., 1994; Sinswat and Tengamnuay, 2003; Vaka et al., 2009, Nisha and Pramod, 2007) , dimethyl- -cyclodextrin (DM- -CD) (Shao et al., 1992; Merkus et al., 1999; Yang et al., 2004), poly-L-arginine (poly-L-Arg) (Natsume et al., 1999; Ohtake et al., 2002; Bertram et al., 2010) and sodium caprate (Mishima et al., 1987; Greimel et al., 2007) have been proved effective in intranasal delivery of proteins and peptides. Consequently, these absorption enhancers were chosen as potential candidates to enhance the nasal absorption of bFGF in this study. The conventional concentration of absorption enhancers was selected according to their enhancing efficacy profiles (Ohtake et al., 2002; Sinswat and Tengamnuay, 2003; Yang et al., 2004; Greimel et al., 2007; Khan et al., 2009).

In the present work, firstly, the nasal ciliotoxicity and cytotoxicity study were conducted for the purpose of evaluating the safety of the absorption enhancers, using *in situ* toad palate model (Jiang et al., 1995) and Calu-3 cell model. Calu-3, the human lung adenocarcinoma cell line, has properties similar to the serous cells of the upper airway and has been used as an *in vitro* nasal platform to investigate microparticles and polymer gels for protein delivery (Witschi and Mrsny, 1999; Chemuturi et al., 2005; Seki et al., 2007). Secondly, the transport of bFGF across Calu-3 cell monolayers in the absence or presence of chitosan was investigated to determine the optimal concentration of chitosan. Finally, pharmacokinetics study was conducted to monitor changes in the blood concentration of bFGF using enzyme linked immunosorbent assay (ELISA) method following nasal administration of bFGF solution with or without 0.5% chitosan, in comparison with intravenous administration of bFGF alone.

MATERIALS AND METHODS

Materials

Calu-3 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Recombinant human basic fibroblast growth factor (bFGF) was obtained from Beijing SL Pharmaceutical Co., Ltd (Beijing, China). Lactate dehydrogenase (LDH) assay kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). Poly-L-Arg hydrochloride (MW 92.0 kDa), collagen solutions (Type I from rat tail), (3-[4, 5-dimethylthiazol-2-yl] -2, 5-diphenyltetrazolium) bromide (MTT), N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), sodium caparate and Triton® X-100 were obtained from Sigma- Aldrich Chemical Co. (St. Louis, MO, US). Non-essential amino acid solution 100×, penicillin streptomycin solution 100×, Dulbecco's Modified Eagle's Medium-Ham's F- 12 nutrient (DMEM/F-12 1:1), fetal bovine serum and trypsin/EDTA (0.25%/0.03% in phosphate buffer solution) were purchased from Invitrogen (CA, USA). DM- -CD was obtained from Waker Chemie AG (Munich, Germany). Chitosan in the form of hydrochloride salt (83% N-deacetylated, viscosity 46 mPa·s, Mw ~300 kDa) was a gift of Golden-Shell Biochemical Co. (Nanjing, China). Human bFGF ELISA development kit was purchased from Peprotech (NJ, USA). All other reagents were of analytical grade.

Animals

Male Sprague–Dawley (SD) rats weighing 180 to 230 g were obtained from Shanghai Sino- British Sippr/BK Lab Animal Ltd. (Shanghai, China) and housed in animal holdings with fixed dark and light cycle of 12 h at a constant temperature (25 ± 1°C). Toads weighing 20 to 30 g (male and female) were obtained from Nanjing Experimental Animal Center. The studies were approved by the Animal Ethics Committee of Fudan University, and every effort was made to reduce the stress of animals.

Nasal ciliotoxicity studies

In situ toad palate model was employed to evaluate the nasal ciliotoxicity of four absorption enhancers at test concentrations (Jiang et al., 1995) . Specific methods are as follows: 0.5 and 0.25% (w/v) chitosan, 5% (w/v) DM- -CD, 0.5% (w/v) poly-L-Arg and 0.5% (w/v) sodium caprate were dissolved in saline respectively and used as test solutions. The upper palate of toads was exposed and treated with test solutions for 240 min (ensuring that the maxillary mucosa of toad was fully submerged in solution). Then the test solution was washed away with saline, and one piece of mucosa about 3 × 3 mm was dissected out from the palate. The mucocilia was examined with a light microscope (UFX- DX, Nikon, Japan) at enlargements of 400 ×, and the duration of the ciliary movement (the time from drug administration to the ending of ciliary movement was recorded. Meanwhile, 1% (w/v) sodium deoxycholate and saline were severed as positive and negative controls, respectively (n = 4).

Cell experiments

Calu-3 cells culture

Calu-3 cells were maintained in 10 cm tissue culture dishes in DMEM/F12 medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids, penicillin (100 U/ml) and streptomycin (100 mg/ml). Each week, cells were passaged and cultured in a humidified atmosphere of 5% CO_2 /95% air at 37°C. For transport studies, the cells were seeded at an initial density of 5 × 105 cells/cm^2 on collagen- coated Transwell® polyester inserts (pore size 0.4 m, surface area 0.33 cm^2, Corning, NY, USA). After 48 h, an air -interface was created and the cells were maintained with 0.6 ml of culture medium in the basolateral chambers of Transwell® . The air-interface conditions stimulated differentiation of the cell monolayer to form polarized, bioelectrically ''tight'' epithelial monolayer. The passages used for the following experiments were

25 to 32. Transepithelial electrical resistance (TEER) values were measured with a Millicell ERS (Millipore, MA, USA).

MTT assay and lactate dehydrogenase (LDH) assay

To evaluate the cytotoxicity of the absorption enhancers, cell viability was determined by MTT assay. The absorption enhancers at concentrations mentioned previously were aseptically dissolved in Hank's balanced salt solution with 30 mM HEPES (HBSS/HEPES) of pH 7.4 to be used as test solutions. Calu- 3 cells were seeded into 96-well cell culture plates at a density of 5×10^4 cells/well. On the second day, the medium was replaced with 100 l of pre-warmed test solutions (n = 4). After exposure for 4 h, the cells were incubated with 100 l of MTT solution (1.25 mg/ml in phosphate buffer solution) for 4 h. Following the treatment, the medium was then removed and the formazan crystal, the metabolite of MTT, formed during these procedure was dissolved in sodium dodecylsulfate (SDS) solution (20% w/v) prepared in N, N-dimethyl formamide (DMF)/water (1:1 V:V) at pH 4.7 (pH adjusted with 1 M hydrochloric acid: acetic acid: Water 10:8:2 V:V:V) (Grenha et al., 2007; Matilainen et al., 2008). After thoroughly mixing, the plate was read at 570 nm using a SpectraMax multi -plate reader (Thermo, Shanghai, China) for optical density that is directly correlated with cell quantity. Survival rate was calculated from the relative absorbance at 570 nm and expressed as the percentage of control. In the MTT test, HBSS/HEPES and 5% SDS solution were used as negative and positive controls, respectively. The relative cell viability was then calculated as a percentage of the negative control from the absorbance values.

The LDH test was performed using a commercial LDH assay kit to monitor the cell membrane damage (Lin et al., 2006). Calu-3 cells were seeded into a 96-well plate at a density of 5×10^4 cells per well overnight. After 4-h exposure to test solutions, 100 l of the cell culture supernatants were withdrawn. HBSS/HEPES and 1% (v/v) Triton®X-100 were used as negative and positive controls, respectively. LDH leakage was then determined according to the manufacturer's protocol and calculated as a percentage of the positive control.

TEER studies

Cell monolayer was initially washed twice and allowed to be equilibrated for 30 min with HBSS/HEPES buffer. Then the apical medium was replaced by test solutions mentioned previously in MTT assay and lactate dehydrogenase (LDH) assay. TEER value was measured 30 min before test solutions exposure and at 0, 15, 30, 60, 90, 120, 180 and 240 min after the administration. At the end of the experiment, the cells were rinsed two times with HBSS/HEPES, then replaced by fresh pre-warmed culture medium, and kept 48 h in the incubator to determine the recovery of the monolayer integrity (Florea et al., 2006; Salem et al., 2009). Background TEER value due to the differences among filters and inter-groups, were deducted by normalizing the measurement of 0 min to 100%, and all experiments were performed in triplicates.

***In vitro* permeation studies**

The test solutions were prepared by dissolving 0.25 or 0.5% chitosan in HBSS/HEPES solution with moderate stirring, and then mixed well with bFGF. The end concentration of bFGF was 50 g /ml. bFGF alone in HBSS/HEPES (50 g/ml) was used as control. The osmotic pressure of all solutions was 290 to 340 mOsm.

In transport studies, Calu-3 cell monolayers were equilibrated with 100 l HBSS/HEPES in the apical medium and 1 ml HBSS/HEPES in the basolateral chamber for 30 min. Afterwards, the apical medium was removed, and the cells were exposed to 100 l test formulations as well as control solution (n = 6). At 15, 30, 60, 90, 120, 180 and 240 min after administration, 110 l samples were withdrawn from the basolateral chamber, and were replaced by fresh HBSS/HEPES (Florea et al., 2006). Samples were assayed by using a human bFGF ELISA development kit according to the manufacturer's instruction.

Apparent permeability coefficients (Papp) were calculated by Equation (1), where dQ/dt is the flux of bFGF across the monolayers ($ng \cdot ml^{-1}$), C_0 is the initial concentration in donor chamber, and A is surface area of Transwell® polyester inserts.

$$\text{Papp} = \frac{dQ}{} \times \frac{1}{} \quad (1)$$

Pharmacokinetics studies of the bFGF formulations

Fifteen SD rats weighing 180 to 230 g were divided into three groups (n = 5/group). All of the animals were anesthetized by chloral hydrate (5% w/v, 350 mg/kg, intraperitoneal) and fixed in a supine position. For intravenous (i.v.) administration, bFGF (1 g/kg) diluted in saline was injected into the femoral vein rapidly. For intranasal (i.n.) administration, test formulations contained bFGF and 1% BSA (to reduce non-specific adsorption) were prepared without or with 0.5% chitosan. Then, 20 l of dosing solution were given to the nostril of each rat (40 g/kg) via a polyethylene 10 (PE 10) tube attached to a microlitre syringe. Blood was sampled from the tail vein at predetermined point, that is, 0.033, 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 8 and 12 h after administration. The serum samples, separated by centrifugation at 10,000 rpm for 5 min at 4°C, were analyzed by ELISA method.

Data analysis

Area under the blood concentration of bFGF versus time curve (AUC_{0-t}) was calculated by the trapezoidal method. The *in vivo* pharmacokinetic parameters was obtained using DAS version 2.0 (Bontz Inc., Beijing, China). All the data were expressed as mean ± standard deviation (SD). For multiple-group comparison, one-way ANOVA was used followed by Fisher's least significant difference post hoc test. Specific comparison between groups was carried out with an unpaired Student's t- test (two tailed). $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

There have been several studies as to intranasal administration of bFGF, which was reported to show effectiveness in inducing progenitor cell proliferation and neuroprotection after transient focal cerebral ischemia (Ma et al., 2008; Wang et al., 2008). To our knowledge, however, no investigation about optimizing the nasal absorption of bFGF and thus improving its therapeutical effect on CNS disorders was reported. Therefore, in this study, several candidate absorption enhancers were screened to facilitate nasal absorption of bFGF.

For nasal administration, the effect of drugs and additives on nasal mucosa should be learned at an early stage of nasal preparation development. Many absorption enhancers such as L- -lysophosphatidylcholine and sodium glycocholate enhanced the nasal absorption of

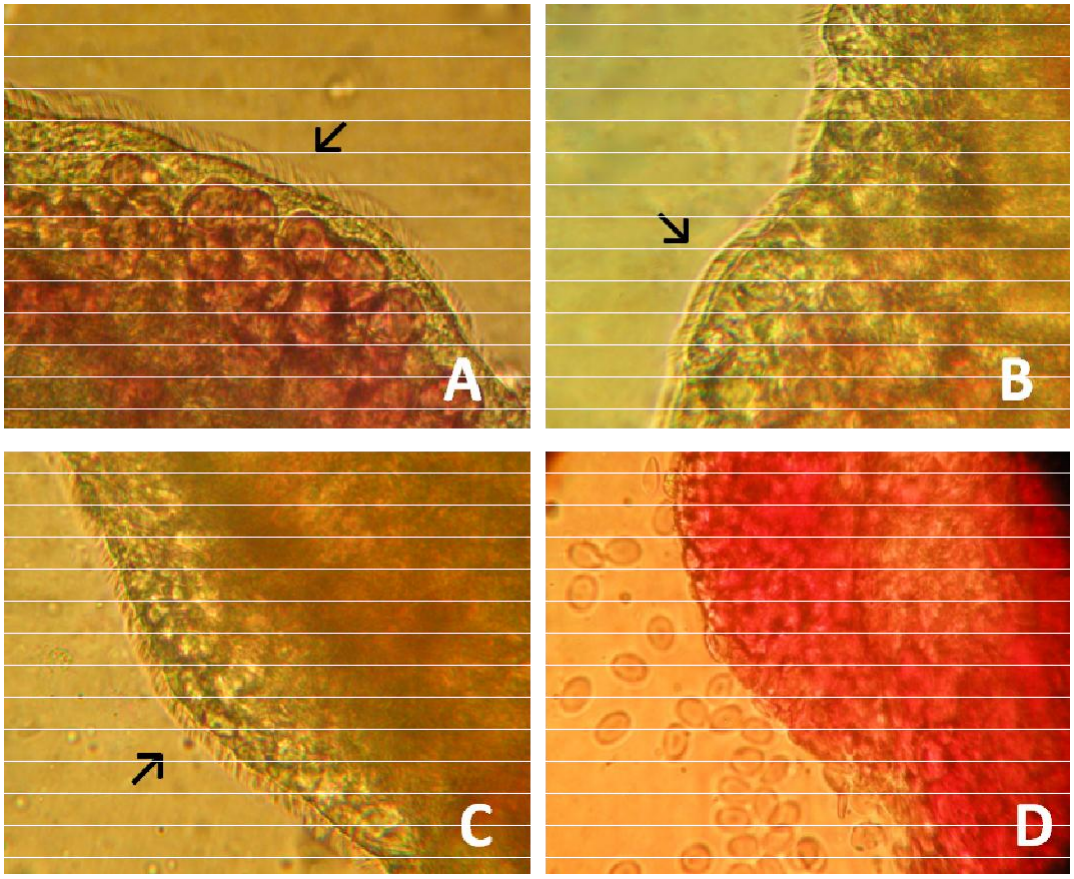

Figure 1. Optical microscopic images of (A) negative control, (B) 0.25% chitosan, (C) 0.5% chitosan and (D) positive control. Cilia indicated by arrow (10 × 40 magnification, n = 4).

peptide drugs accompanied by severe nasal membrane damage (Marttin et al., 1998; Merkus et al., 1999; Natsume et al., 1999). Therefore, in this study, we investigated the toxicity of four candidate absorption enhancers at test concentrations at first.

Nasal ciliotoxicity

Nasal Cilia are mobile fingerlike appendages extending from the surface of the nasal epithelial cells which move in a well-organized and coordinated way to propel the overlying mucus layer toward the throat. They contribute to the body's primary nonspecific defense mechanism by removing potentially hazardous substances. Since the ciliary movement is a major indicator for mucociliary clearance in the upper airways, it is important to evaluate the possible effects of the absorption enhancers on ciliary morphology (Hermens et al., 1990; Ugwoke et al., 2000). For the present study, the toad palate model is considered to be the optimal choice for the study of cilia toxicity, as the results can give nice reproducibility and the experimental technique is easy to be mastered (Jiang et al., 1995). Optical microscopic observation of toad palate showed that there were a great number of cilia with a fast beating rate on the edge of the mucosa treated with 0.25 and 0.5% chitosan solution for 4 h (Figures 1B and C). However, for toads treated with 0.5% sodium caprate, 0.5% poly-L-Arg or 5% DM- -CD, it was found that partial cilia fell off from the edge of the mucosa and partial cilia were stasis (data not shown). For all the mucosa treated with test solutions, the ciliary movement lasted for more than 11.7 h, slightly lower than that of the negative control (14.57 h) and notablely longer than that of the positive control (only 15 min). It is worth noting that the percentages of duration of ciliary movement compared with the negative control after treated with 0.25 and 0.5% chitosan solution were above 90%, indicating no ciliotoxicity of chitosan. However, significant differences can be found between the other three absorption enhancers and the negative control (Figure 2), suggesting that 0.5% sodium caprate, 0.5% poly-L-Arg and 5% DM- -CD cause mild to moderate nasal ciliotoxicity

Cytotoxicity

The vitality of Calu-3 cells after exposure to different

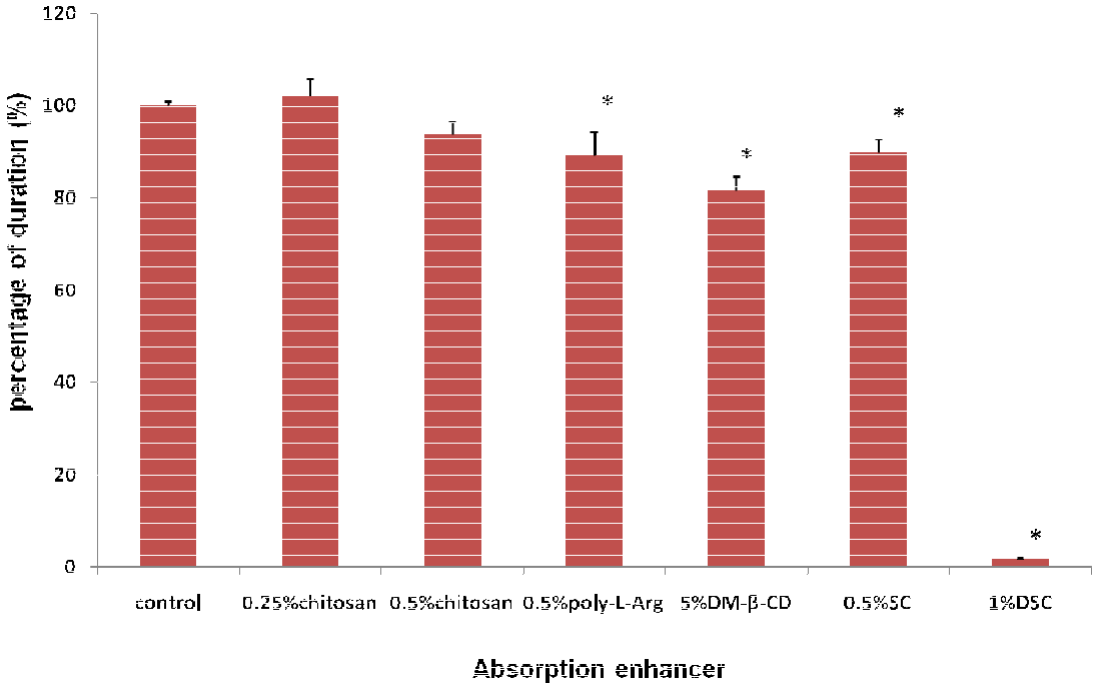

Figure 2. Percentage of duration of ciliary movement for mucosa treated with saline (as negative control), 0.25 and 0.5% chitosan, 5% DM- -CD, 0.5% poly-L-Arg, 0.5% sodium caprate (SC) and 1% sodium deoxycholate (SDC, as positive control) (mean±SD, n = 4). *p<0.05, compared with negative control. Percentage of duration (%) = duration of test solutions / duration of negative control.

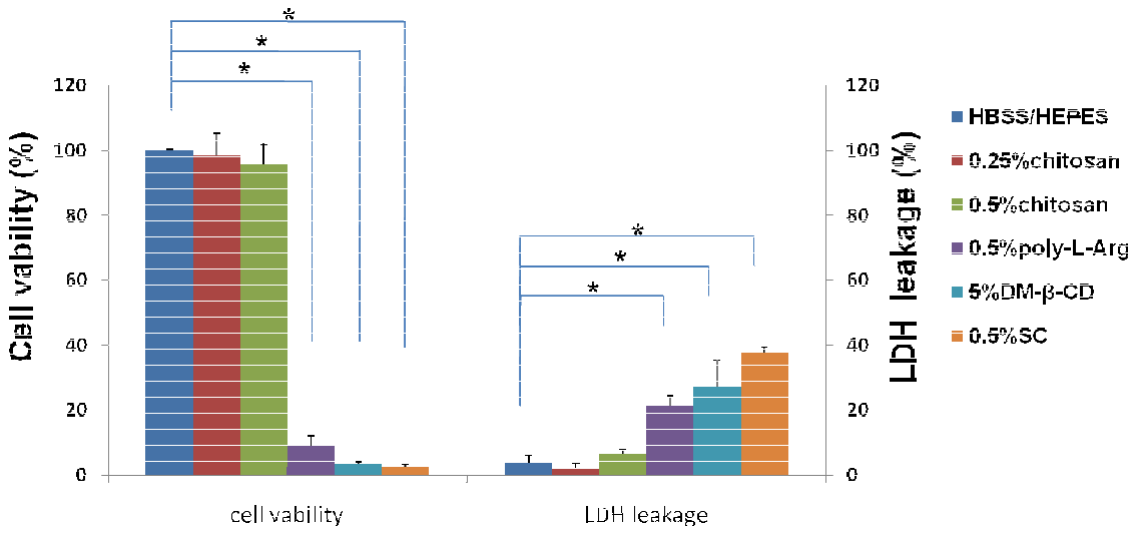

Figure 3. Cell viability of Calu-3 cells after exposure to the absorption enhancers for 4 h (left), calculated as a percentage of the negative control (HBSS/HEPES); the lactate dehydrogenase (LDH) leakage in culture medium after 4-h exposure to four absorption enhancers (right), calculated as a percentage of the positive control (1% Triton®X-100). (mean±SD, n = 4). *p<0.05, compared with HBSS/HEPES group. SC, sodium caprate.

absorption enhancers was shown in Figure 3. Compared with negative control, 0.25% and 0.5% chitosan had no toxicity to cells (*p*>0.05). In contrast, 0.5% sodium caprate, 0.5% poly-L-Arg and 5% DM- -CD diminished the cell viability by about 90%, indicating distinct cytotoxic effect (p<0.01, compared with negative control).

The results of LDH leakage experiment were also shown in Figure 3. LDH, a stable protein present in the

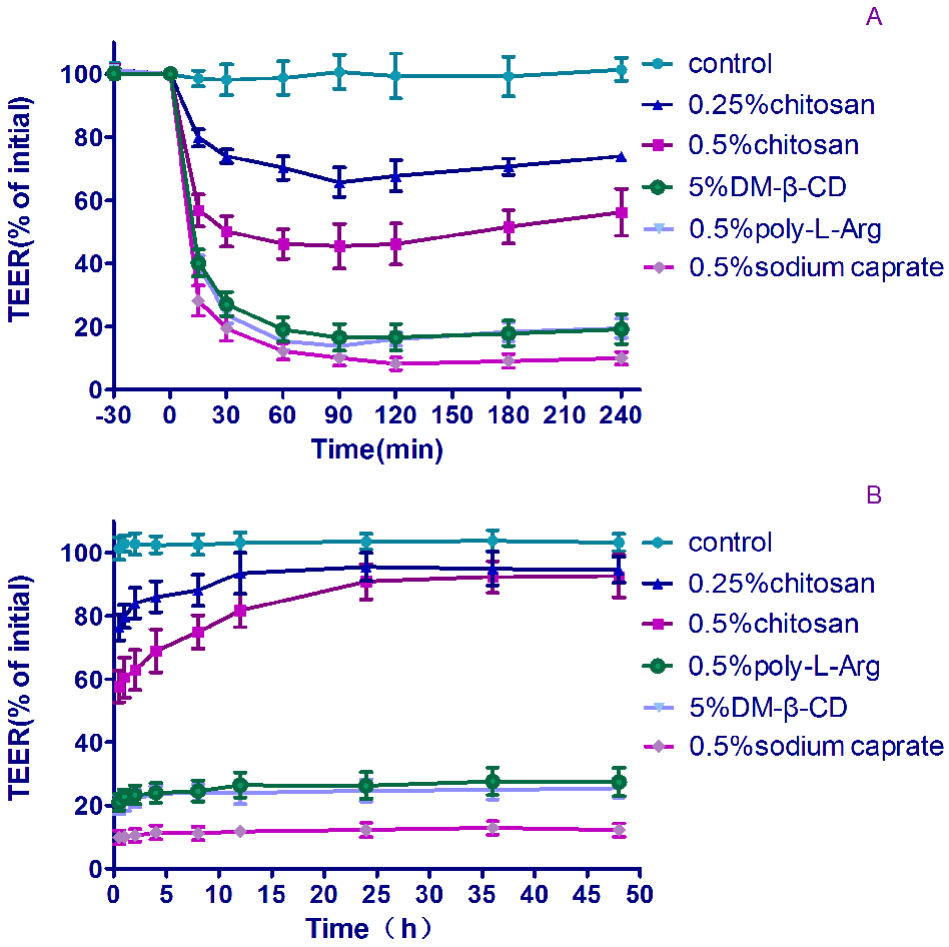

Figure 4. The TEER values of absorption enhancers across Calu-3 cell monolayers in apical to basolateral direction (mean±SD, n = 6) . The TEER value for every monolayer at time zero was normalised to 100%. (A) Changes in TEER values measured before and after the administration; (B) the TEER recovery of Calu-3 monolayers after the test solutions were removed.

cytoplasm of normal cells, can be released in the event of cell membrane damage. As a result, the degree of cell damage can be determined by detecting the LDH level in cell culture medium (Lin et al., 2006). Both 0.25 and 0.5% chitosan solution resulted in a little LDH leakage from the Calu-3 cells, without significant differences from the effect of HBSS/HEPES. However, after exposure to 0.5% poly-L-Arg, 5% DM- -CD and 0.5% sodium caprate for 4 h, LDH releases were increased to 21.22±3.09, 27.07±8.42 and 37.59±1.87%, respectively, significantly higher than the negative control ($p<0.01$), suggesting that these absorption enhancers damaged the cell membrane integrity.

TEER reduction and recovery

TEER measurements were used in the study to assess the effect of four absorption enhancers on the tight junction stability of Calu-3 cells. TEER across the cell monolayer was measured following a 4-h treatment with absorption enhancers in the apical chamber (Figure 4A). In the absence of any enhancer, there was no change in TEER during the course of the entire measurement period. The TEER was reduced by approximately 91, 81 and 80% in cells treated with 0.5% sodium caprate, 0.5% poly-L-Arg and 5% DM- -CD, respectively. The rapid drop occurred within 60 min of exposure, after which TEER remained relatively constant for the duration of the experiment (4 h). On the other hand, 0.25% chitosan induced a decrease by 27% in TEER value after 4 h treatment. As to 0.5% chitosan, the TEER value promptly descended to 45% and gradually ascended to 56%.

Reversibility study has been proposed as a useful tool to determine if enhancers cause the monolayer integrity damage via a reversible way. In this study, we further

monitored the TEER value of cell monolayers till 48 h after washing out the dosing solution. After 48 h recovery procedure, diminished TEER value of 0.25 and 0.5% chitosan group nearly recovered to baseline level (approximately 90% of the initial) (Figure 4B), indicating the chitosan treatment caused only a transient disruption of the cell monolayer's barrier property and this effect was reversible. However, TEER of cell monolayers treated with other three absorption enhancers remained at low level during the entire recovery period, and some cells were found floating at the end of the experiment, suggesting that sodium caprate, poly-L-Arg and DM- -CD at test concentrations resulted in severe damage on monolayer integrity and the effects were irreversible.

In the present study, the *in situ* toad palate model and *in vitro* cell model were employed to find safe absorption enhancers for intranasal delivery of bFGF. According to the results from these two models, among four absorption enhancers, chitosan was the safest, while 0.5% sodium caprate, 0.5% poly- L-Arg and 5% DM- -CD showed mild to moderate nasal ciliotoxicity and severe cytotoxicity. More severe toxic effects *in vitro* could be attributed to the fact that in MTT experiment, the cells were totally immersed in absorption enhancer solutions, while the ciliated epithelium *in situ* is protected by the mucus barrier and mucociliary clearance, and was exposed to the test solutions only apically. In addition, the epithelial cells of the nasal mucosa were constantly replaced by cells from the basement membrane *in vivo*, a situation which could not be mimicked *in vitro* (Dimova et al., 2005).

In consideration of nasal cilitoxicity and cytotoxicity, chitosan at concentrations of 0.25 and 0.5% were finally chosen as absorption enhancer in the subsequent cell transport experiment.

bFGF transport across Calu-3 cells

Paracellular transport experiments with marker fluorescein sodium which could permeate across Calu-3 cell monolayers have been previously performed in our lab. Apparent permeability coefficient (Papp) value was 2.46×10^{-7} cm/s, similar to the previous report (Forbes, 2000), indicating Calu-3 cells had formed a confluent sheet.

In this study, the Papp values of bFGF in the absence or presence of chitosan were shown in Table 1. The Papp of control group was $0.67\pm0.18\times10^{-8}$ cm/s, and cumulative transport amount at 4 h was only 0.039% (w/w) of the applied dose. The addition of 0.25 and 0.5% chitosan dramatically enhanced bFGF permeation across the Calu-3 cell layer. Especially, compared with solution alone, the Papp value and the cumulative transport amount had been increased nearly 16-fold and 12-fold with 0.5% chitosan, respectively.

Based on the transport results, 0.5% chitosan exhibited stronger permeation facilitation on bFGF than 0.25% chitosan. These findings are in agreement with the results previously published (Artursson et al., 1994), so chitosan at the concentration of 0.5% was used in subsequent pharmacokinetic study.

Pharmacokinetics studies of the bFGF formulations

The potency and efficacy of chitosan in enhancing nasal absorption of bFGF were investigated by formulating bFGF with 0.5% chitosan. The preparation supplemented with 1% BSA to minimize bFGF loss by non-specific absorption. The concentrations of bFGF with time in blood following Intravenous (i.v.) and Intranasal (i.n.) administration to rats are given in Figure 5 and the main pharmacokinetic parameters were illustrated in Table 2. The i.v. injection of bFGF solution resulted in immediate appearance of the peptide in blood with a rapid decline showing no detected concentration 1 h after the administration. On the other hand, nasal administration of bFGF solution alone resulted in a much lower blood bFGF level. The peak concentration was reached at about 40 min. Inclusion of 0.5% chitosan in the formulation caused higher Cmax and extended tmax (about 60 min). The $AUC_{0\text{-}120h}$ of the bFGF formulation following i.n. administration was 335.34±22.10 ng min/ml, increased to 472.2±52.36 ng min/ml as a result of 0.5% chitosan added. The absolute bioavailability was 5.35 and 7.53% for intranasal bFGF in the absence and presence of 0.5% chitosan, respectively. Although the absolute nasal bioavailability seemed to be low when compared to i.v. administration, the $AUC_{0\text{-}120h}$ values of 0.5% chitosan group was significantly greater than the control intranasal group ($p< 0.05$, Table 2).

It was reported that the mechanism of chitosan on absorption enhancement may include the properties of mucoadhesion and reversible opening of tight junctions among cells, as the result of the interaction between its positively charged amino groups with the negatively charged sialic acid residues in mucus, which lengthened nasal residence time of drugs and led to improvement in the transport of large hydrophilic compounds across the epithelium (Illum et al., 1994).

In this study, addition of chitosan in bFGF solution increased the bioavailability of bFGF by 1.4-fold after intranasal administration, which was not as significant as other reports. Sinswat and Tengamnuay (2003) proved that chitosan elevated the bioavailability of calcitonin from 1.22% to 2.45% (2-fold) and Dyer et al. (2002) demonstrated that the bioavailability of insulin was promoted from 0.5 to 3.6% (7.2-fold) in the presence of 0.5% chitosan. The relatively lower enhancement in nasal absorption is probably because the higher bioavailability of bFGF alone (5.35%) than that of insulin and calcitonin. bFGF is a cationic peptide, which may interact with anionic cell membrane and therefore improve its permeability through nasal mucosa and transport to the

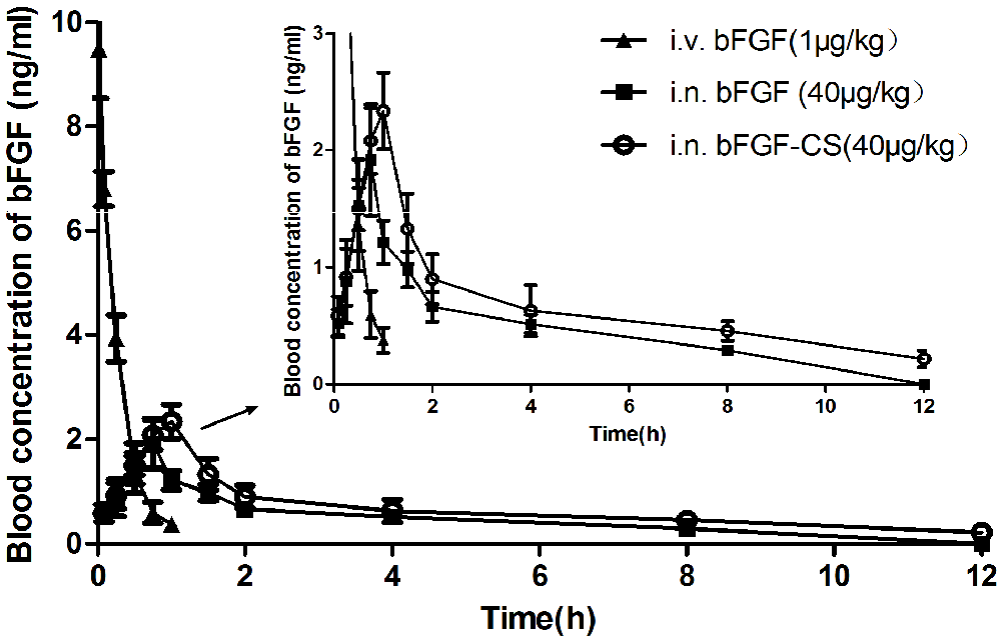

Figure 5. Concentration-time profiles of bFGF in blood following intranasal (i.n.) administration of bFGF solution with or without 0.5% chitosan, in comparison with intravenous (i.v.) administration of bFGF solution to rats (mean±SD, n = 5). bFGF-CS, bFGF solution with 0.5% chitosan.

Table 1. Effect of chitosan on bFGF transport across Calu-3 cell monolayers (mean±SD, n = 6).

Formulation	*Papp* × 10^{-8} (cm/s)	*ER*[a]	bFGF transport (%)[c]	*ER*[b]
Control	0.67±0.18	–	0.039±0.005	–
0.25% chitosan	1.71±0.51*	2.57	0.088±0.023*	2.24
0.5% chitosan	10.09±3.54*	16.31	0.465±0.123*	11.83

[a] P_{app} enhancement ratio= $P_{app\ test\ solution}$/ $P_{app\ control}$. [b] Transport (%) enhancement ratio = bFGF transport (%) $_{test\ solution}$ / bFGF transport (%)$_{control}$. [c] Cumulative transport amount of the applied dose. *p<0.01, compared with control group

Table 2. Pharmacokinetic parameters of intact bFGF in blood after intranasal (i.n.) administration of bFGF to rats, with or without 0.5% chitosan, in comparison with intravenous (i.v.) administration (mean±SD, n = 5).

Dose and route of administration	C_{max} (ng/ml)	T_{max} (min)	$AUC_{0\text{-}12h}$ (ng.min/ml)	%*F*abs[a]	%*F*rel[b]
i.v. bFGF (1 g/kg)	--	--	156.72±16.48	100.00	--
i.n. bFGF (40 g/kg)	1.92±0.48	39.00±8.22	335.34±22.10	5.35	100.00
i.n. bFGF-CS (40 g/kg)	2.33±0.32	57.00±6.71	472.2±52.36*	7.53	140.82

[a] Absolute nasal bioavailability; [b] Relative nasal bioavailability; * p<0.01, compared with i.n. bFGF group.

systemic circulation.

Conclusion

It was concluded that chitosan is a safe and effective absorption enhancer for nasal delivery of large molecule drugs such as bFGF. The present study provided basic information for the research on the nasal delivery of bFGF. The brain uptake and pharmacodynamic experiments need to be further studied.

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Abbreviations: bFGF, Basic fibroblast growth factor; **CNS**, central nervous system; **Poly-L-Arg**, poly-L-arginine; **DM- -CD,** dimethyl- -cyclodextrin; **MTT, (3-[4,** 5-dimethylthiazol-2-yl]-2, 5-diphen**yltetrazolium)** bromide; **LDH**, lactate dehydrogenase; **ELISA,** enzyme linked immunosorbent assay; **TEER,** transepithelial electrical resistance; HEPES, N-[2-hydroxyethyl] piperazine- N' -[2-ethanesulfonic acid]; **HBSS/HEPES**, Hank's balanced salt solution with 30 mM **HEPES; Papp**, apparent permeability coefficients; **AUC, area under curve; SD, standard deviation; bFGF-CS, bFGF solution with** 0.5% chitosan;

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