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Effects of polyamidoamine (PAMAM) dendrimers on the nasal absorption of poorly absorbable drugs in rats

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ABSTRACT

The absorption enhancing effects of polyamidoamine (PAMAM) dendrimers with various concentrations and generations on the nasal absorption of fluorescein isothiocyanate-labeled dextran with an average molecular weight of 4400 (FD4) were initially studied in rats. PAMAM dendrimers with different generations improved the nasal absorption of FD4 and the absorption enhancing effects of PAMAM dendrimers were generation dependent. The rank order of absorption enhancement effects of PAMAM dendrimers was G3 > G2 > G1 > G0. The absorption enhancing effects of PAMAM dendrimers were shown to be concentration dependent for the same generation of PAMAM dendrimers. The nasal membrane toxicity of these PAMAM dendrimers was evaluated by measuring the release of protein and lactate dehydrogenase (LDH) in nasal cavity lavage fluid. PAMAM dendrimers with higher generations and concentrations caused some membrane damage to the nasal tissues, but it was much less than the damage caused by sodium deoxycholate as a positive control. Based on the consideration between the efficacy and safety of PAMAM dendrimers, 1% (w/v) G3 dendrimer with high effectiveness and low toxicity was considered to be a best absorption enhancer for improving the nasal absorption of FD4. 1% (w/v) G3 dendrimer also improved the nasal absorption of macromolecular compounds and drugs including FD10, FD70, insulin and calcitonin. Finally, we measured the zeta potentials of drug solutions with or without PAMAM dendrimers to elucidate their absorption enhancing mechanisms. The zeta potentials of model drug solutions changed to positive by the addition of 1% (w/v) G3 dendrimer. This changing might trigger the absorption enhancing effects of PAMAM dendrimers on the nasal absorption of FDs, insulin and calcitonin, as the first step of mechanisms. In conclusion, 1% (w/v) G3 dendrimer is a promising absorption enhancer for improving the nasal absorption of FDs, insulin and calcitonin without any membrane damage to the nasal tissues.
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1. Introduction

Nasal administration of macromolecular drugs including protein and peptide drugs has now been recognized as a very promising route for drug delivery of therapeutic compounds by the following reasons: (1) the nose has a large surface area available for drug absorption due to the coverage of the epithelial surface by numerous microvillus, (2) the subepithelial layer is highly vascularized, (3) the venous blood from the nose passes directly into the systemic circulation and therefore avoids the first-pass metabolism of drugs, (4) it offers lower doses, more rapid attainment of therapeutic blood levels, quicker onset of pharmacological activity, fewer side effects, high total blood flow per cm^3, porous endothelial basement membrane, and (5) non-invasiveness may maximize patient compliance (Ridley et al., 1995; Kissel and Werner, 1998; Turker et al., 2004). On the other hand, there are some disadvantages with nasal drug delivery including the low permeability of the mucosa to large molecules, enzymatic degradation, the very small absorption capability of the nasal cavity, limiting application to drugs that can be supplied in small doses, and the rapid mucociliary clearance, which shortens the period of time available for efficient absorption and causes low bioavailability (Harris, 1993; Jones et al., 1997; Behl et al., 1998). Therefore, absorption enhancers, proteolytic enzyme inhibitors and suitably designed formulations have been utilized to increase the bioavailability of poorly absorbable drugs in nasal delivery systems (Morimoto et al., 1995; Dondeti et al., 1996; Davis and Illum, 2003). The absorption enhancers, which increase the permeability of drugs through the epithelial membranes without causing any tissue damage, are especially useful for the delivery of peptide and protein drugs. Although non-ionic surfactants (Maitani et al., 2000; Ozsoy et al., 2000; Ahsan et al., 2001), bile salts (Betbeder et al., 2000), cyclodextrins (Matsubara et al., 1995), cationic polymers (Illum et al., 1994, 2000), fusidic acid derivatives (Lee et al., 1994), and phospholipids (Muramatsu et al., 1999) have been studied as nasal absorption enhancers, they usually caused some membrane damage to the nasal tissues. There-

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fore, the balance of their efficacy and toxicity is the key issue in the research and design of an effective and safe nasal absorption enhancer.

Polyamidoamine (PAMAM) dendrimers are a new class of artificial macromolecules that have shown excellent promise in biomedical applications due to their unique physical and chemical properties. Including a precise control on molecular size, shape and density, high solubility, a large number of surface functional groups and hydrophobic cavities can easily carry drugs or other guest molecules to penetrate through the membrane (Tomalia et al., 1985, 1990; Jansen et al., 1994; Milhem et al., 2000; Cheng and Xu, 2005). PAMAM dendrimers have successfully proved themselves as useful additives in different routes of drug administration including intravenous (Chauhan et al., 2004), oral (Wiwattanapatapee et al., 2000; D'Emanuele et al., 2004), transdermal (Wang et al., 2003), and ocular (Vandamme and Brobeck, 2005) delivery systems. However, few studies have been carried out to examine the effect of PAMAM dendrimer on the nasal absorption of drugs.

In this study, therefore, fluorescein isothiocyanate-labeled dextrans (FDs) with different average molecular weights (4000–70,000), insulin and calcitonin were chosen as models of poorly absorbable drugs and effects of PAMAM dendrimers on the nasal absorption of these drugs were examined in rats. In addition, we evaluated the effects of PAMAM dendrimers on the nasal membrane damage in rats. Furthermore, to clarify the absorption enhancing mechanisms of these PAMAM dendrimers, the zeta potentials of various model drug solutions with or without PAMAM dendrimers were also studied.

2. Materials and methods

2.1. Materials

Fluorescein isothiocyanate-labeled dextran with an average molecular weight of 4400 (FD4), fluorescein isothiocyanate-labeled dextran with an average molecular weight of 9100 (FD10), fluorescein isothiocyanate-labeled dextran with an average molecular weight of 71,600 (FD70), PAMAM G0, G1, G2, G3 dendrimers were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Insulin was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Calcitonin was supplied by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals and solvents were of analytical grade.

2.2. Preparation of drug solution

Dosing solutions containing different model drugs were prepared in isotonic phosphate buffer (PBS) at pH 7.4 to yield a final concentration of 50 IU/mL (insulin), 50 μg/mL (calcitonin), 100 mg/mL (FD4, FD10), and 200 mg/mL (FD70), respectively. In certain experiments, PAMAM dendrimers with various concentrations (0.1% (w/v), 0.5% (w/v), 1% (w/v), 2% (w/v) and 5% (w/v)) and different generations (G0, G1, G2 and G3) as absorption enhancers were added to different dosing solutions, and pH in these solutions was adjusted to 7.0–8.0, respectively.

2.3. Animal experiments

All studies were carried out in accordance with the guideline of the animal ethic committee at Kyoto Pharmaceutical University. Male Wistar rats (Shimizu Laboratory Supplies), weighing 200–250 g, were fasted for 12 h before the experiments, but had free access to water. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg. A surgical operation for the *in vivo* nasal absorption study was investigated according to the modified method outlined by Hirai et al. (1981). Briefly, the rats were placed in the supine position, after the trachea had been exposed, a tracheal cannulation was performed to maintain respiration, and the trachea leading to the nasal cavity was ligated to prevent the liquid exuding from the surgical incision. Another polyethylene tube was inserted through the oesophagus to the posterior part of the nasal cavity to prevent drainage of the applied drug solution into the nasopharynx. After the surgical operation, 20 μL drug solution was administered into nasal cavity by means of a micropipette through the nostril. The jugular vein was exposed and blood samples (~0.2 mL) were collected into heparinized syringes at predetermined time intervals up until 120 min. Samples were immediately centrifuged at 12,000 rpm for 5 min to obtain the plasma fraction (100 μL), which was then kept in ice until determination. At the end of the experiments, the nasal cavity was washed with 10 mL PBS from the surgical incision to the posterior part of the nasal cavity through the cannula that placed in the esophagus. The nasal cavity lavage fluid was collected from the nostrils. The concentrations of protein and the activities of lactate dehydrogenase (LDH) were then determined after the fluids were centrifuged at 200 × *g* for 7 min at 4 °C.

In certain experiment, insulin and calcitonin solutions in PBS were intravenously administered into the caudal vein by bolus injection in order to calculate the pharmacological availability of drugs.

2.4. Analytical methods

The nasal absorption of insulin was estimated by measuring the plasma insulin levels and glucose levels, as reported previously (Yamamoto et al., 1994; Tozaki et al., 1997; He et al., 2007). The plasma concentrations of insulin were determined by Insulin-EIA TEST (Wako Pure Chemical Industries, Osaka, Japan) and the plasma glucose concentrations were determined by a glucose oxidase method (Glucose B Test Wako Kit, Wako Pure Chemical Industries, Osaka, Japan).

The nasal absorption of calcitonin was estimated by measuring the plasma calcium levels, as reported previously (Yamamoto et al., 1997, 2001; Tozaki et al., 1998, 2001; Fetih et al., 2006). The plasma calcium levels after calcitonin administration were determined by Calcium E Test (Wako Pure Chemical Industries, Osaka, Japan).

The nasal absorption of FDs was estimated by measuring the concentrations of FDs in plasma, as reported previously (Yamamoto et al., 1993, 2004; Gao et al., 2008a, 2008b). For determination of the FDs concentrations, 100 μL plasma was collected and added to an equal volume of 10% (w/v) Triton X-100. The fluorescence intensity of these samples was measured by a spectrofluorometer (Spectrafluor Plus, TECAN, Switzerland) at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The calibration curve over the concentration range of 0–20 μg/mL was used for the determination of FDs.

The concentrations of protein in the nasal cavity lavage fluid were determined using an assay kit DC protein assay kit (Bio-Rad Laboratories) using bovine serum albumin (BSA) as a standard. The LDH was determined using an assay kit LDH CII (Wako Pure Chemical Industries, Ltd.).

2.5. Data analyses

The peak concentration (C_{max}) and time to reach C_{max} (T_{max}) of these compounds were determined directly from their concentration–time profiles. The area under the curve (AUC) after administration of these compounds from each site was calculated by the trapezoidal rule from zero to the final sampling time.

In the case of insulin and calcitonin, the decrement of plasma glucose and calcium level (*D*%) after the intranasal administration of insulin and calcitonin with or without PAMAM dendrimer was also

calculated by a method as described previously using the following equation (Yamamoto et al., 1994; Fetih et al., 2005):

$$D\% = \left(1 - \frac{\mathrm{AUC}_{0\rightarrow 120}}{100\% \times 120\ \mathrm{min}}\right) \times 100$$

The area above the 100% line in the equation was ruled out for calculating the $\mathrm{AUC}_{0\rightarrow 120}$. The pharmacological availability (PA%) was calculated from the following equation:

$$\mathrm{PA}\% = \frac{D_{\mathrm{situ}}\%}{D_{\mathrm{iv}}\%} \times \frac{\mathrm{Dose}_{\mathrm{iv}}}{\mathrm{Dose}_{\mathrm{situ}}} \times 100$$

2.6. Measurement of zeta potential of drug solutions

For zeta potential measurements, 1% (w/v) G3 dendrimer was added to various model drug solutions. The freshly prepared samples solutions were dispensed into disposable tubes and zeta potential was measured in triplicate using a combination of the measurement techniques: Electrophoresis and Laser Doppler Velocimetry, called Laser Doppler Electrophoresis sometimes (Zetasizer Nano ZS, Malvern Instruments, Ltd., U.K.).

2.7. Statistical analyses

The results are expressed as the mean ± S.E. of three to five animals and statistical significance was performed by one-way analysis of variances (ANOVA) with $P < 0.05$ as the minimum level of significance.

3. Results

3.1. Effects of PAMAM dendrimers on the nasal absorption of FD4 in rats

Fig. 1 shows the plasma concentration–time profiles of FD4 after nasal administration of PAMAM dendrimers with different concentrations and generations to rats. As shown in Fig. 1(a), 1% (w/v) and 2% (w/v) G1 and 5% (w/v) G0 did not enhance the absorption of FD4, while we observed a slight increase in plasma concentration of FD4 when 5% (w/v) G1 was used as an absorption enhancer. In contrast, a marked increase in plasma concentration of FD4 was obtained in the presence of 1% (w/v) sodium deoxycholate as a positive control. The effect of G2 PAMAM dendrimer on the nasal absorption of FD4 was shown in Fig. 1(b). A significant increase in plasma concentrations of FD4 was seen in the presence of high concentrations of G2 dendrimer (2% (w/v) and 5% (w/v)). In contrast, we found no significant effect on the plasma concentrations of FD4 in the presence of low concentrations (0.1% (w/v), 0.5% (w/v) and 1% (w/v)) of G2 dendrimer. The effect of G3 PAMAM dendrimer on the nasal absorption of FD4 was also examined and was shown in Fig. 1(c). High concentrations of G3 dendrimer (1% (w/v), 2% (w/v) and 5% (w/v)) significantly increased the plasma concentrations of FD4 compared with control group, while low concentrations of G3 PAMAM dendrimer (0.1% (w/v) and 0.5% (w/v)) did not improve the nasal absorption of FD4. Overall, the absorption enhancing effect of G3 PAMAM dendrimer increased as its concentration increased.

Table 1 summarizes the pharmacokinetic parameters (C_{max}, T_{max}, AUC and absorption enhancement ratio) after nasal administration of FD4 with various generations and concentrations of PAMAM dendrimers. As shown in Table, the absorption enhancement ratios, which were calculated from the AUC values in the presence or absence of PAMAM dendrimers, were 1.0, 1.7, 2.6 and 3.4 for 5% (w/v) G0, 5% (w/v) G1, 5% (w/v) G2, and 5% (w/v) G3, respectively. These findings suggested that the absorption enhancing effect of PAMAM dendrimer on nasal absorption of FD4 was generation dependent. Therefore, the rank order of absorption enhancement effect of PAMAM dendrimers was G3 > G2 > G1 > G0. G0 dendrimer had no absorption enhancing effect in the nasal absorption of FD4 in this study. On the other hand, Table 1 also demonstrated the effects of different concentrations of PAMAM

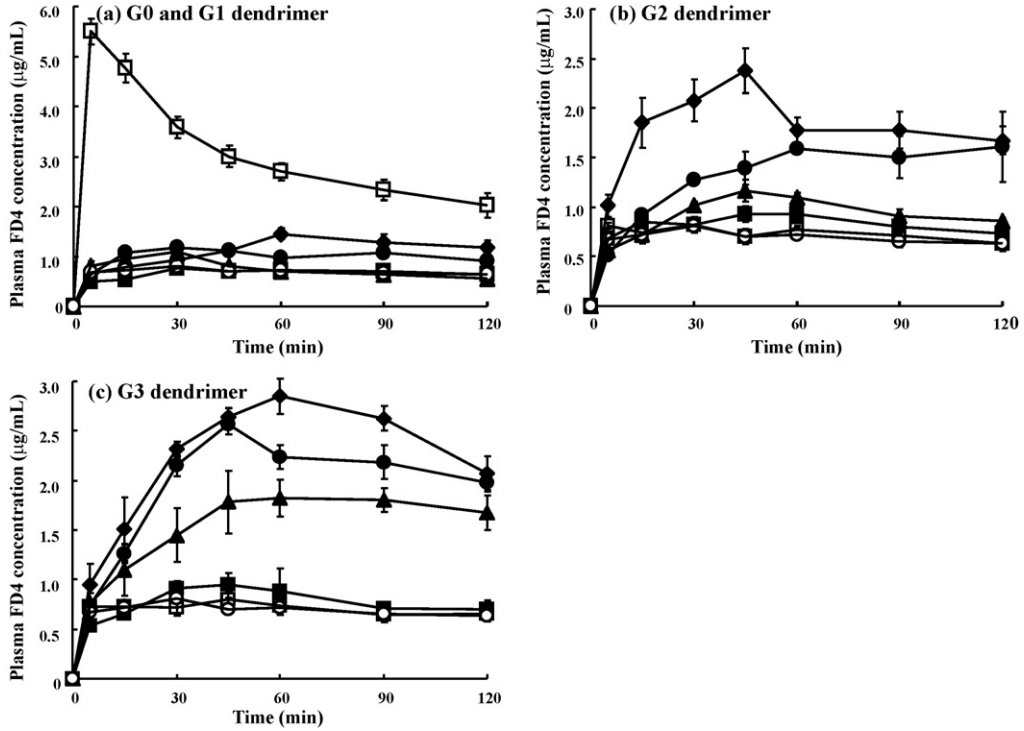

Fig. 1. Plasma concentration–time profiles of FD4 after nasal administration with various concentrations of G0 and G1 dendrimers (a), G2 dendrimer (b) or G3 dendrimer (c) to rats. Each point represents the mean ± S.E. of 3–5 experiments. Keys: (a) (○) Control, (■) 5% (w/v) G0, (▲) 1% (w/v) G1, (●) 2% (w/v) G1, (♦) 5% (w/v) G1, (□) 1% (w/v) Sodium deoxycholate. (b) (○) Control, (□) 0.1% (w/v) G2, (■) 0.5% (w/v) G2, (▲) 1% (w/v) G2, (●) 2% (w/v) G2, (♦) 5% (w/v) G2. (c) (○) Control, (□) 0.1% (w/v) G3, (■) 0.5% (w/v) G3, (▲) 1% (w/v) G3, (●) 2% (w/v) G3, (♦) 5% (w/v) G3.

Table 1
Pharmacokinetic parameters of FD4 after nasal administration with different concentrations and generations of PAMAM dendrimers to rats.

	C_{max} (μg/mL)	T_{max} (min)	$AUC_{0\rightarrow120}$ (μg·min/mL)	Enhancement ratio
Control	0.8 ± 0.0	16.7 ± 7.3	81.9 ± 3.6	1.0
5% G0 Dendrimer	0.8 ± 0.1	37.5 ± 7.5	79.2 ± 1.3	1.0
1% G1 Dendrimer	1.1 ± 0.0	25.0 ± 5.0	89.1 ± 3.3	1.1
2% G1 Dendrimer	1.2 ± 0.1	40.0 ± 5.0	120.2 ± 4.3*	1.5
5% G1 Dendrimer	1.5 ± 0.1	60.0 ± 0.0	134.8 ± 8.5**	1.7
0.1% G2 Dendrimer	0.8 ± 0.0	30.0 ± 2.4	83.0 ± 3.4	1.0
0.5% G2 Dendrimer	1.0 ± 0.1	40.0 ± 13.2	97.7 ± 5.8	1.2
1% G2 Dendrimer	1.2 ± 0.1	55.0 ± 5.0	110.7 ± 3.9*	1.4
2% G2 Dendrimer	1.9 ± 0.2	65.0 ± 13.2	160.2 ± 12.7**	2.0
5% G2 Dendrimer	2.4 ± 0.2	63.8 ± 18.8	215.7 ± 14.8**	2.6
0.1% G3 Dendrimer	0.8 ± 0.0	35.0 ± 3.4	83.1 ± 5.2	1.0
0.5% G3 Dendrimer	1.1 ± 0.1	45.0 ± 8.7	91.7 ± 8.6	1.1
1% G3 Dendrimer	2.1 ± 0.1	65.0 ± 13.2	187.9 ± 12.0**	2.3
2% G3 Dendrimer	2.6 ± 0.1	60.0 ± 15.0	237.6 ± 7.1**	2.9
5% G3 Dendrimer	2.9 ± 0.2	60.0 ± 0.0	274.2 ± 3.7**	3.4
1% NaDC	5.5 ± 0.3	5.0 ± 0.0	360.0 ± 25.0**	4.4

Results are expressed as the mean ± S.E. of 3–5 experiments.
* $P < 0.05$, compared with the control group.
** $P < 0.01$, compared with the control group.

dendrimers on the nasal absorption of FD4 in rats. As shown in Table 1, we found that the absorption enhancing effects of PAMAM dendrimers on the nasal absorption of FD4 increased as the concentration increased for the same generation of PAMAM dendrimer. As is evident from the Table, the absorption enhancement ratios of various concentrations of G3 dendrimer with concentrations of 0.1% (w/v), 0.5% (w/v), 1% (w/v), 2% (w/v) and 5% (w/v) were 1.0, 1.1, 2.3, 2.9 and 3.4, respectively. In addition, all generations and concentrations of PAMAM dendrimers could increase the T_{max} of FD4, although some dendrimers did not promote the C_{max} and AUC values of FD4 after their nasal absorption. These findings suggest that PAMAM dendrimers could prolong the absorption time of FD4 after nasal administration in rats.

3.2. Effects of PAMAM dendrimers on the membrane damage to the nasal tissues

In order to confirm the safety of PAMAM dendrimers, we next evaluated the nasal membrane damage in the presence of various generations and concentrations of PAMAM dendrimers by measuring the amounts of total protein and the activities of LDH in nasal cavity lavage fluid. As shown in Fig. 2(a), high concentrations of PAMAM dendrimers including 2% (w/v) G2 and G3, 5% (w/v) G2 and G3 significantly increased the amounts of total protein, but its amounts were much less than that caused by 1% (w/v) sodium deoxycholate as a positive control. On the other hand, the other kinds of PAMAM dendrimer did not enhance the toxic marker compared with the control group.

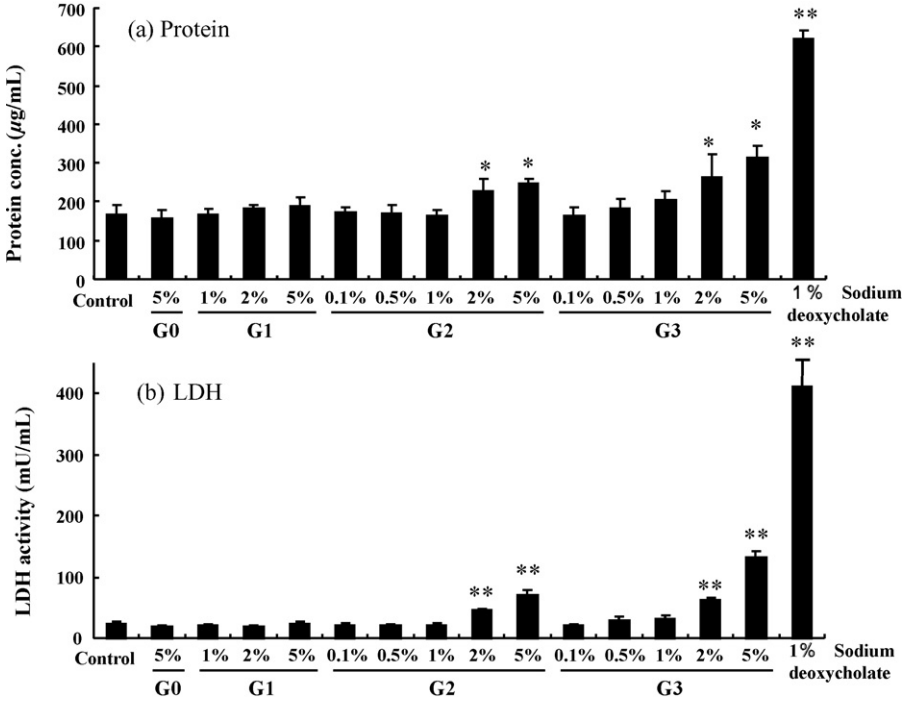

Fig. 2. The total protein levels (a) and LDH activities (b) in nasal cavity lavage fluid at 2 h after nasal administration of various generations and concentrations of PAMAM dendrimers to rats. Each point represents the mean ± S.E. of 3–5 experiments. ** $P < 0.01$, * $P < 0.05$, compared with the control groups.

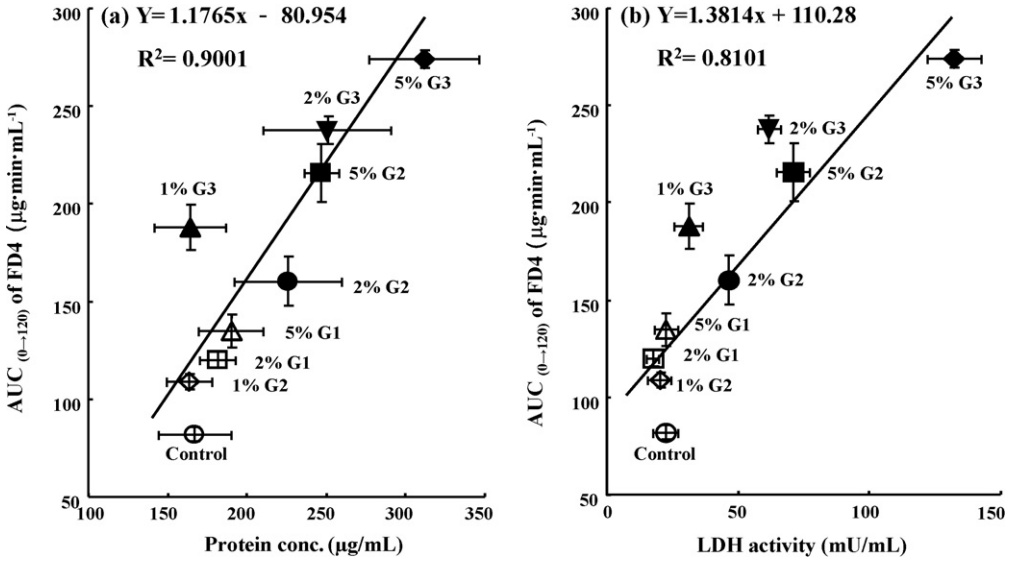

Fig. 3. Relationship between AUC of FD4 (effectiveness) and the amounts of protein (a) or the release of LDH (b) (toxicity) after nasal administration of FD4 with or without PAMAM dendrimers in rats. Keys: (○) Control, (□) 2% (w/v) G1, (Δ) 5% (w/v) G1, (◊) 1% (w/v) G2, (●) 2% (w/v) G2, (■) 5% (w/v) G2, (▲) 1% (w/v) G3, (▼) 2% (w/v) G3, (♦) 5% (w/v) G3.

The results of LDH determination shown in Fig. 2(b) also demonstrated that high concentrations of G2 and G3 dendrimers (2% (w/v) and 5% (w/v)) significantly caused the nasal membrane damage by increasing the LDH activities. In contrast, G0 and G1 dendrimers and low concentrations (0.1% (w/v), 0.5% (w/v) and 1% (w/v)) of G2 and G3 dendrimers did not cause any membrane damage to the nasal tissues. On the other hand, there was a remarkable increase in the activity of LDH after nasal administration of 1% (w/v) sodium deoxycholate as a positive control. These findings suggested that the toxicity of PAMAM dendrimers to the nasal tissues might be related to their generations and concentrations.

3.3. Relationship between effectiveness and toxicity of PAMAM dendrimers in rats

Fig. 3 indicates the relationship between effectiveness and toxicity after nasal administration of FD4 in presence and absence of PAMAM dendrimers in rats. As shown in Fig. 3(a), there exists a almost linear correlation between the AUC values of FD4 and the amounts of protein. Similarly, a linear correlation also exists between the AUC values of FD4 and the activities of LDH (Fig. 3(b)). This finding suggested that the toxicity of PAMAM dendrimers increased as their absorption enhancing effects increased. From the evaluation in Fig. 3, we can consider that 1% (w/v) G3 dendrimer with a great absorption enhancing effect and low toxicity was a best absorption enhancer for improving the nasal absorption of FD4. Based on this finding, we investigated the absorption enhancing effect of 1% (w/v) G3 dendrimer on the nasal absorption of other poorly absorbable drugs in the subsequent studies.

3.4. Effect of G3 PAMAM dendrimer on the nasal absorption of FD10 and FD70 in rats

Fig. 4 shows the plasma concentration profiles of FD10 and FD70 after nasal administration with 1% (w/v) G3 dendrimer to rats. In addition, the pharmacokinetic parameters (C_{max}, T_{max}, AUC and absorption enhancement ratios) of these drugs after nasal administration in the presence or absence of 1% (w/v) G3 dendrimer are also summarized in Table 2. We found that the plasma concentration of FD10 increased by the co-administration with 1% (w/v) G3 dendrimer. Similarly, a significant increase in plasma concentration of FD70 was also observed by the addition of 1% (w/v) G3 dendrimer (Fig. 4). As shown in Table 2, C_{max}, T_{max} and AUC of FD10 and FD70 increased by the addition of 1% (w/v) G3 dendrimer, suggesting that this dendrimer could increase the absorption of these drugs and prolong their absorption time after their nasal administration. To sum up, 1% (w/v) G3 dendrimer could enhance the nasal absorption of macromolecular compounds including FD10 and FD70.

3.5. Effect of G3 PAMAM dendrimer on the nasal absorption of insulin in rats

Fig. 5 shows the effect of 1% (w/v) G3 dendrimer on the nasal absorption of insulin by measuring both plasma glucose and insulin

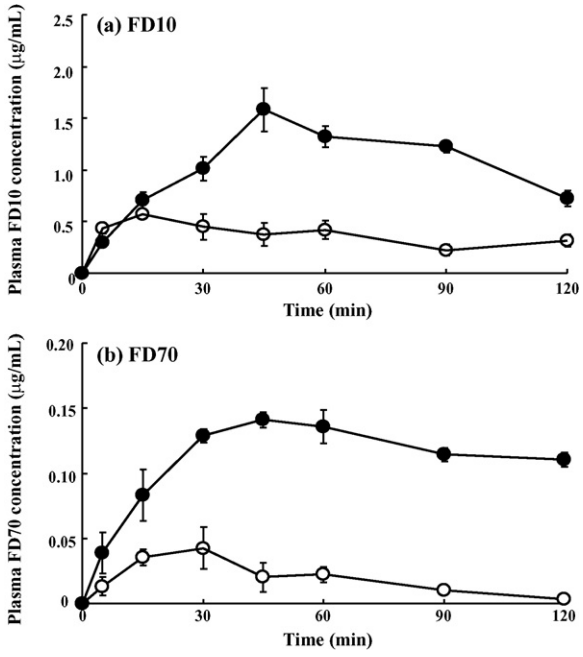

Fig. 4. Plasma concentration–time profiles of FD10 (a) and FD70 (b) after nasal administration with 1% (w/v) G3 dendrimer to rats. Each point represents the mean ± S.E. of 3–5 experiments. Keys: (○) Control, (●) 1% (w/v) G3 dendrimer.

Table 2
Pharmacokinetic parameters of FD10 and FD70 after nasal administration with 1% G3 dendrimer to rats.

	C_{max} (μg/mL)	T_{max} (min)	$AUC_{0\rightarrow120}$ (μg·min/mL)	Enhancement ratio
FD10				
Control	0.57 ± 0.05	20.0 ± 5.0	43.7 ± 7.2	1.0
1% G3 Dendrimer	1.43 ± 0.02	52.5 ± 3.4	127.4 ± 5.1**	2.9
FD70				
Control	0.05 ± 0.01	20.0 ± 5.0	3.9 ± 0.9	1.0
1% G3 Dendrimer	0.14 ± 0.01	45.0 ± 3.4	22.8 ± 1.2**	5.9

Results are expressed as the mean ± S.E. of 3–5 experiments.
** $P < 0.01$, compared with the control group.

concentrations in rats. As shown in Fig. 5(a), in the case of PBS solution without insulin, the plasma glucose profiles nearly showed a steady level around 100%. For the treatment of control group (insulin without 1% (w/v) G3 dendrimer), the plasma glucose level showed a tenuous decrement. In contrast, a significant hypoglycemic effect was observed after nasal administration of insulin with 1% (w/v) G3 dendrimer. The maximum effect (the lowest glucose concentration of 72.3% relative to baseline) occurred at 90 min in the rats. Table 3 summarizes the pharmacodynamic parameters (*D*% and PA%) of insulin after nasal administration of insulin with 1% (w/v) G3 dendrimer. *D*% and PA% of insulin significantly increased in the presence of 1% (w/v) G3 dendrimer, compared with the control.

We observed similar results in the case of plasma insulin data after nasal administration of insulin, which was indicated in Fig. 5(b), as was the case of plasma glucose data. In the presence of 1% (w/v) G3 dendrimer as an absorption enhancer, the plasma concentration of insulin significantly increased. The highest plasma concentration of insulin was also observed at 90 min. Pharmacokinetic parameters (C_{max}, T_{max}, AUC and absorption enhancement ratio) of insulin after nasal administration with 1% (w/v) G3 dendrimer to rats were also summarized in Table 3. All the results

Table 3
Pharmacodynamic and pharmacokinetic parameters of insulin after nasal administration with 1% G3 dendrimer to rats.

	PBS	Control	1% G3 dendrimer
Pharmacodynamic parameters			
D%	–	7.1 ± 0.5	18.8 ± 1.7
PA%	–	4.4 ± 0.3	11.6 ± 1.0**
Enhancement ratio	–	1.0	2.7
Pharmacokinetic parameters			
C_{max} (mU/mL)		28.5 ± 3.0	79.5 ± 0.1
T_{max} (min)		25.0 ± 10.0	90.0 ± 0.0
$AUC_{(0\rightarrow120)}$ (mU·min/mL)		2772.6 ± 262.4	6492.0 ± 264.8**
Enhancement ratio		1.0	2.3

Results are expressed as the mean ± S.E. of 3–5 experiments.
** $P < 0.01$, compared with the control group.

above indicated that 1% (w/v) G3 dendrimer could enhance the nasal absorption of insulin in rats.

3.6. Effect of G3 PAMAM dendrimer on the nasal absorption of calcitonin in rats

Fig. 6 shows the plasma concentration–time profiles of calcitonin after nasal administration with 1% (w/v) G3 dendrimer to rats. To confirm whether G3 dendrimer itself could influence the plasma calcium level, 1% (w/v) G3 dendrimer solution without calcitonin was also administered to rats. As indicated in Fig. 6, neither 1% (w/v) G3 dendrimer nor PBS solution reduced plasma concentration of calcium after their nasal administration. On the contrary, when calcitonin was co-administrated with 1% (w/v) G3 dendrimer as an absorption enhancer, a prominent reduction of plasma calcium concentration was observed compared with nasal administration of calcitonin without any enhancer. The pharmacodynamic parameters of calcitonin after nasal administration with 1% (w/v) G3 dendrimer were calculated. The PA% of calcitonin after its nasal

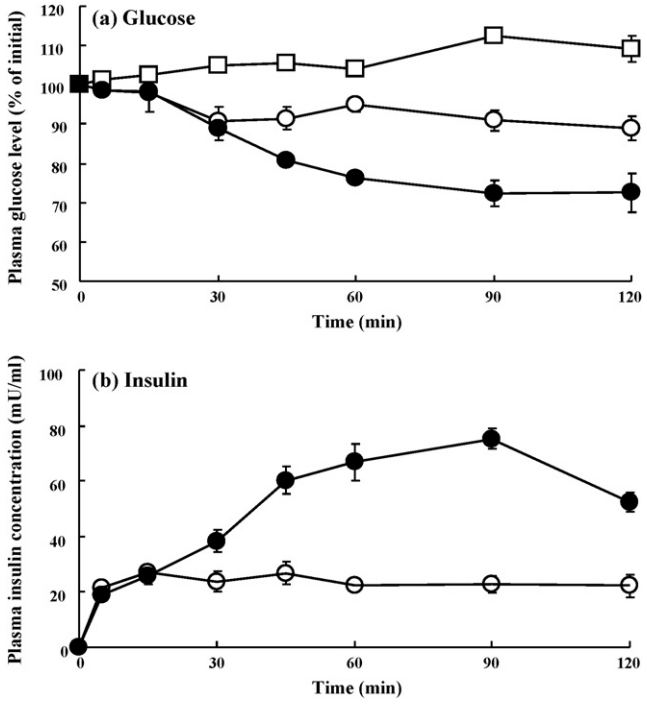

Fig. 5. Effect of 1% (w/v) G3 dendrimer on the nasal absorption of insulin in rats. (a) Glucose levels in plasma; (b) plasma insulin concentrations-time profiles after nasal administration of insulin. Each point represents the mean ± S.E. of 3–5 experiments. Keys: (□) PBS, (○) Control, (●) 1% (w/v) G3 dendrimer.

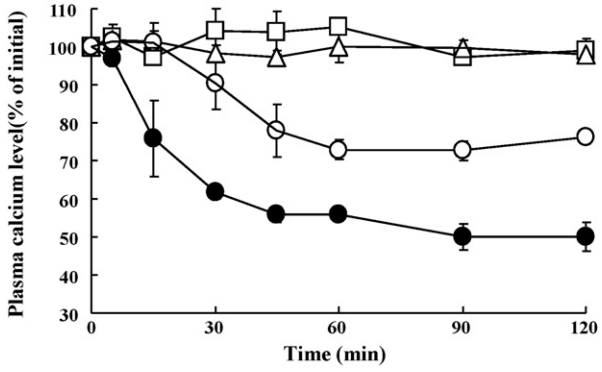

Fig. 6. Effect of 1% (w/v) G3 dendrimer on the nasal absorption of calcitonin in rats. Each point represents the mean ± S.E. of 3–5 experiments. Keys: (□) PBS, (Δ) 1% (w/v) G3 dendrimer only, (○) Control (calcitonin only), (●) Calcitonin with 1% (w/v) G3 dendrimer.

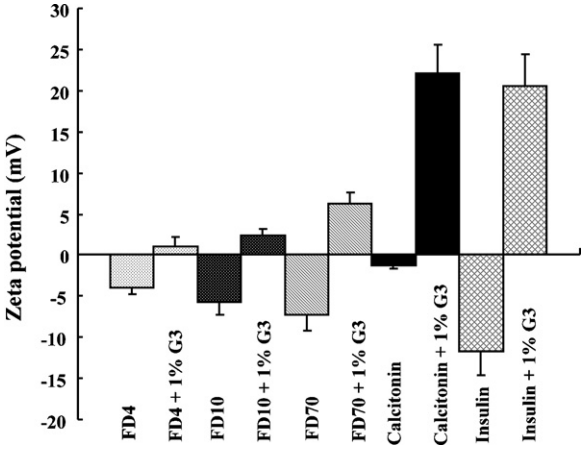

Fig. 7. Zeta potentials of various model drug solutions with or without 1% (w/v) G3 dendrimer. Each point represents the mean ± S.E. of 3 experiments.

administration in the control study was 6.9%, but it achieved to 13.9% after nasal administration of calcitonin with 1% (w/v) G3 dendrimer and increased two times compared with the control. Overall, these findings demonstrated that 1% (w/v) G3 dendrimer had the absorption enhancing effect on the nasal absorption of calcitonin in rats. The decrement of plasma calcium level was not due to 1% (w/v) G3 dendrimer itself, since it did not change the plasma calcium concentration.

3.7. Measurement of zeta potentials of drug solutions

Finally, we measured the zeta potentials of drug solutions to elucidate the absorption enhancing mechanisms of PAMAM dendrimers in this study. Fig. 7 shows the zeta potentials of various model drug solutions with or without 1% (w/v) G3 dendrimer, including FD4, FD10, FD70, calcitonin and insulin. In this study, 1% (w/v) G3 dendrimer was chosen as an absorption enhancer, because it had absorption enhancing effect for improving the absorption of the entire model drugs as described above. To avoid the colored effect in drug solution for the determination of zeta potential, FDs solutions were diluted in this study. As revealed in Fig. 7, the zeta potentials of all model drug solutions were negative. When 1% (w/v) G3 dendrimer was added to various solutions, the zeta potential changed to positive, suggesting that the charge of various model drugs became neutral by 1% (w/v) G3 dendrimer.

4. Discussion

Although in some reports, PAMAM dendrimer-drug complexes or conjugates have already been evaluated in different routes of drug administration systems, such as intravenous (Chauhan et al., 2004; Asthana et al., 2005), oral (Wiwattanapatapee et al., 2000; D'Emanuele et al., 2004), transdermal (Wang et al., 2003), and ocular (Vandamme and Brobeck, 2005) drug delivery systems, few studies have been carried out to examine the effects of PAMAM dendrimers as novel absorption enhancers on the nasal absorption of poorly absorbable drugs. In this study, therefore, we examined the absorption enhancing effects of PAMAM dendrimers on the nasal absorption of FDs with different average molecular weights (4000–70,000), insulin and calcitonin in rats.

We found that the nasal absorption of FD4 was significantly enhanced by the addition of PAMAM dendrimers with various generations and concentrations (Fig. 1 and Table 1) and the absorption enhancing effects were generation and concentration dependent in these studies. El-Sayed et al. (2002) reported that PAMAM dendrimers enhanced the paracellular permeability across Caco-2 cell monolayers, since the dendrimers increased the permeability of ^{14}C-mannitol, a typical paracellular marker compound, in a generation-, concentration- and incubation time-dependent fashion. More recently, Lin et al. (2010) reported that PAMAM dendrimers increased the small intestinal absorption of drugs including 5(6)-carboxyfluorescein, calcitonin and FD4 in a concentration and generation dependent manner. Therefore, our present results were well correlated with their previous findings.

The relative variance of bioavailability is very important, when we try to improve the nasal absorption of poorly absorbable drugs. If the relative variance (CV) of bioavailability with some absorption enhancers was very high, it is very difficult to develop such kind of absorption enhancer in clinical application. However, as shown in Table 1, the CV values of AUC with PAMAM dendrimers were quite small and we found the data with little variation in the presence of PAMAM dendrimers. Therefore, from the standpoint of variance of AUC and bioavailability, PAMAM dendrimers had very favorable characteristics and these PAMAM dendrimers with the optimal generation and concentration could be useful to improve the nasal absorption of poorly absorbable drugs.

For *in vivo* applications, safe absorption enhancers are needed as suitable nasal delivery systems for peptide and protein drugs. It is important to evaluate the potential local toxicity of absorption enhancers. Recently, the membrane damage of these absorption enhancers is usually estimated by measuring the biological markers including protein and LDH released from the nasal tissues in nasal cavity lavage fluid. Generally, protein is an index of membrane damage, because protein is one of the components of biological membrane and is released if the biomembrane is damaged. LDH is the cytosolic enzyme and its presence in nasal cavity lavage fluid is generally regarded as evidence of cell membrane damage. As shown in Fig. 2, we found that G2 and G3 dendrimers with high concentrations (2% (w/v) and 5% (w/v)) increased the amount of protein and LDH released from the nasal tissue in nasal cavity lavage fluid. In contrast, G0, G1 and low concentrations (0.1% (w/v), 0.5% (w/v) and 1% (w/v)) of G2 and G3 dendrimers did not affect the release of protein and LDH, indicating that they did not cause any mucosal damage to the nasal tissues. In addition, we can also consider that the toxicity of PAMAM dendrimers on nasal absorption was generation and concentration dependent. This tendency is fairly consistent with the previous report by El-Sayed et al. (2002). After Caco-2 cell monolayers were incubated with PAMAM dendrimers, LDH studies demonstrated that PAMAM dendrimers increased their cytotoxicity and their toxicity was depend on their generation number, concentration and incubation time, We found a linear correlation between the AUC values and the amounts of protein or the activities of LDH for PAMAM dendrimer on nasal absorption of FD4 in Fig. 3, suggesting that the toxicity of PAMAM dendrimers proportionally increased with increasing their absorption enhancing effects. After an assessment on effectiveness and toxicity of PAMAM dendrimers, 1% (w/v) G3 dendrimer with great enhancement and low toxicity was selected as a suitable absorption enhancer in the subsequent studies. The effect of 1% (w/v) G3 dendrimer on the nasal absorption of other drugs including FD10, FD70, insulin and calcitonin was then studied and the results are summarized in Figs. 4–6 and Tables 2 and 3. Taken together, 1% (w/v) G3 was confirmed as the best absorption enhancer on the nasal absorption of poorly absorbable drugs. Saovapakhiran et al. (2009) reported that G3 PAMAM dendrimer was employed to investigate the influence of PAMAM dendrimer surface properties on cellular internalization and intracellular trafficking in the human colon adenocarcinoma HT-29 cell line. In addition, it was reported that G3 dendrimer was less cytotoxic than both G4 and G5 PAMAM dendrimers (Jevprasesphant et al., 2003a,b) and was chosen in preference to G2, G1 and G0 dendrimers, because the latter possessed less apparent permeation

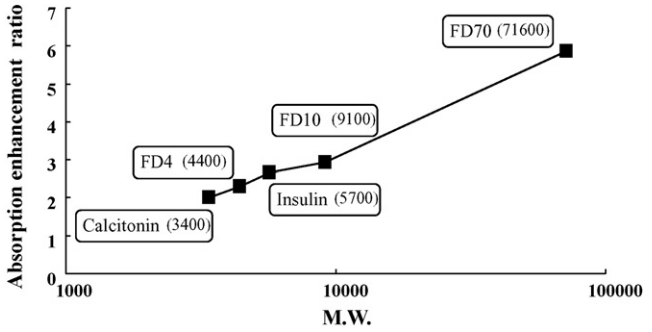

Fig. 8. Correlation between the absorption enhancement ratios of 1% (w/v) G3 dendrimer for improving the nasal absorption of drugs and their molecular weight. FD4, FD10, FD70, insulin and calcitonin were used as models of drugs.

through cell monolayers than G3 dendrimer (Jevprasesphant et al., 2003b).

In our previous data, we found that the nasal absorption of drugs decreased with increasing molecular weights of model drugs (Yamamoto et al., 1993). Similarly, McMartin et al. (1987) studied the bioavailability of many compounds using the literature values and found that the absorption of drugs across the nasal membrane fell off sharply at molecular weights greater than 1000. Our present results are consistent with these previous reports. As shown in Tables 1 and 2, the AUC values of FD4, FD10 and FD70 after the nasal absorption were 187.9, 43.7 and 3.9, respectively. The nasal absorption of these drugs decreased with increasing their molecular weights. On the other hand, Fig. 8 shows the relationship between the absorption enhancement ratios of drugs with 1% (w/v) G3 dendrimer and the molecular weights of FD4, FD10 and FD70 on the nasal absorption of drugs. As shown in this figure, the absorption enhancing effect of 1% (w/v) G3 dendrimer generally increased as the molecular weight of drugs increased. We previously reported that the absorption enhancing effect of sodium deoxycholate (as a positive control in the present study) showed the same situation until the molecular weight of drugs increased to 1000, but suddenly decreased when the molecular weight achieve to 10,000 (Yamamoto et al., 1993). The reason for the differences on the absorption enhancing actions between G3 dendrimer and sodium deoxycholate is not clarified at present. However, since 1% (w/v) G3 dendrimer showed the different T_{max} values compared with those in the case of sodium deoxycholate, such difference in characters of absorption enhancing effect might affect the results.

The mechanisms by which PAMAM dendrimers enhanced the nasal absorption of FDs, insulin and calcitonin are not fully understood. Based on the data in Fig. 7, we hypothesized that 1% (w/v) G3 dendrimer enhanced the nasal absorption of FDs, insulin and calcitonin by changing the molecule's negative surface charge to positive and increasing their permeability across the cell membrane. Since the surface of the mucus membrane involving sialic acid is negatively charged, the coulombic repulsion effect generated between the negatively charged cell membrane and the negatively charged drug perhaps contributes in part to the poor absorbability of FDs, insulin and calcitonin (in the absence of 1% (w/v) G3 dendrimer) from the nasal epithelium. The change in drug molecular charge to positive by the addition of 1% (w/v) G3 dendrimer may diminish the coulombic repulsion effect and consequently increase drug absorption across the nasal epithelium. Furthermore, as the amino group contents of PAMAM dendrimers with various generations and concentrations would influence the zeta potentials of drug solutions, this phenomenon might be related to the absorption enhancing effects of PAMAM dendrimers on the nasal absorption of FD4 in this study.

The poor absorption of drugs from the nasal cavity can be associated with another two major factors, including possible (enzymatic) degradation in the nasal cavity/tissue and rapid clearance from the absorption site. The first factor is of little consequence for most drugs so far studied to date (Illum, 2003). However, mucociliary clearance is an interesting problem in nasal drug delivery. In humans, the clearance of mucus from the nasal cavity by the mucociliary clearance mechanism has a half-time of about 15 min. Thus, some drugs may not have sufficient time to be absorbed before clearance occurs. As shown in the entire Tables in present study, all generations and concentrations of PAMAM dendrimers could increase the T_{max} of model drugs, although some dendrimers did not promote the C_{max} and AUC values of FD4, FD10, FD70, insulin and calcitonin after their nasal absorption. PAMAM dendrimers might be able to slow down the clearance process of drugs from the nasal cavity or inhibit the degradation of drugs in nasal cavity. These actions of PAMAM dendrimers can therefore be advantageous in enhancing the nasal absorption of poorly absorbable drugs by prolonging the absorption time.

Another possible mechanism for improving the nasal absorption of poorly absorbable drugs by PAMAM dendrimers, especially 1% (w/v) G3 dendrimer is that these dendrimers might allow the drugs traverse nasal epithelium via an endocytosis pathway, thereby improving the absorption of FD4, FD10, FD70, insulin and calcitonin from the nasal cavity, since a lot of *in vivo* studies already demonstrated that PAMAM dendrimers were capable to internalize and traffick across the nasal epithelium via an endocytosis pathway (El-Sayed et al., 2002, 2003; Kitchens et al., 2007, 2008; Seib et al., 2007). Alternatively, it has been suggested that PAMAM dendrimers decreased the transepithelial electrical resistance (TEER) values by loosening tight junction of Caco-2 cells (Kolhatkar et al., 2007; Kitchens et al., 2008). This finding indicates that the absorption enhancing effect of PAMAM dendrimers may be due to the opening the tight junction in the epithelium, thereby increasing the transport of drugs via a paracellular pathway, although the structure of tight junction in the nose might be different from that in Caco-2 cells. Therefore, some more detailed studies are required to elucidate the absorption enhancing mechanisms in the future.

5. Conclusion

In conclusion, the present findings indicated that PAMAM dendrimers, especially 1% (w/v) G3 dendrimer were effective to increase the nasal absorption of macromolecules including FDs, insulin and calcitonin without any membrane damage. These adjuvants may be very useful for improving the nasal absorption of many macromolecular drugs, especially peptide and protein drugs.

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