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In vitro Transport of Fexofenadine·HCl in Deformable Liposomes Across the Human Nasal Epithelial Cell Monolayers

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ABSTRACT–Fexofenadine·HCl is non-sedating histamine H1 receptor antagonist that can be used for the treatment of seasonal allergic rhinitis. The objective of this study was to investigate whether the carriers of deformable liposomes can enhance the transepithelial permeability of fexofenadine·HCl across the *in vitro* ALI human nasal monolayer model. Characterization of this model was achieved by bioelectric measurements and morphological studies. The passage 2 and 3 of cell monolayers exhibited the TEER value of 2852 ± 482 ohm $\times$ cm^2 on 11 days of seeding and maintained high TEER value for 5 days. The deformable liposome of fexofenadine·HCl was prepared with phosphatidylcholine (PC) and cholic acid using extruder method. The mean particle size was about 200 nm and the maximum entrapment efficiency of 33.0% was obtained in the formulation of 1% PC and 100 μg/ml fexofenadine·HCl. The toxicity of the deformable liposome to human nasal monolayers was evaluated by MTT assay and TEER value change. MTT assay showed that it has no toxic effect on the nasal epithelial cells in 2-hour incubation when the PC concentration was below 1%. However, deformable liposome could not enhance the transepithelial permeability (P_{app}) and cellular uptake of fexofenadine·HCl. In conclusion, the *in vitro* model could be used in nasal drug transport studies and evaluation of transepithelial permeability of formulations.

Key words–Air-liquid interface, Passaged human nasal epithelial cells culture, Fexofenadine·HCl deformable liposome, Transport studies

Introduction

Fexofenadine·HCl (Figure 1), a non-sedating histamine H1 receptor antagonist, is an active metabolite of terfenadine and is known to have a better safety profile than terfenadine for treatment of seasonal allergic rhinitis. Lippert *et al.* reported that fexofenadine·HCl does not undergo significant biotransformation in human, because 95% of the dose is excreted either in the urine or feces after biliary excretion.[1] Fexofenadine ·HCl is commonly administrated by oral route with a dose of 60-120 mg per day.

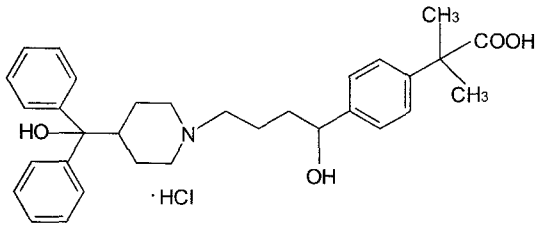

Figure 1–Chemical structure of fexofenadine·HCl.

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The nasal drug delivery route has attractive features, including the large surface area of the nasal cavity and the relatively high blood flow, which may achieve a rapid absorption and avoidance of hepatic first-pass effect.[2,3] Recently, *in vitro* models of human nasal epithelium offer the opportunity to study mechanisms related to drug absorption, metabolism and toxicity on cellular level.[4] Although *in vivo* animal models have been widely used for the investigation of nasal drug transport studies, *in vitro* nasal models are also recently employed in the studies of the transport and metabolic properties of the nasal mucosa using the excised nasal tissue model, nasal homogenates and cell culture model. Among these models, the nasal cell culture models have attracted the attention of pharmaceutical researchers as promising tools for defining transport mechanisms and testing novel strategies to enhance drug transport and absorption.

Recently, we have successfully established a passaged human nasal epithelial cell monolayer system using air-liquid interface (ALI) culture condition,[5] which developed a tight junction with the differentiation of cilia and mucin secreting cells. In drug transport study, however, the permeability of fexofenadine·HCl across the nasal cell monolayer was quite low, probably due to its hydrophilicity (log P=0.49). There have been many attempts to increase the permeability of

hydrophilic drugs using absorption enhancers, cyclodextrin, microsphere and liposome.[6-10] A novel type of deformable liposome was first introduced in 1992,[11] and was widely used to enhance the transdermal delivery of macromolecules, such as insulin and growth hormone.[12,13] Herein, we report the feasibility study on the penetration-enhancing effect of deformable liposome for fexofenadine·HCl using the nasal epithelial cell monolayer, together with the toxicity of adjuvant on nasal epithelium that might be the reason for the irritation.[14,15]

Experimental

Material

HEPES, D-glucose and cholic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phosphatidylcholine (S75) was obtained from Lipoid GMBH (Germany). Fexofenadine·HCl was the gift from Handok-Aventis Pharmaceutical Co. (Seoul, Korea). Cell culture reagents and supplies were obtained from GIBCO Invitrogen Co. (Grand Island, NY, U.S.A.). BEGM Bulletkit was obtained from Cambrex Bio Science Inc. (Walkersville, MD, U.S.A.). Transwells® (0.4 μm, 12 mm diameter, polyester) were obtained from Costar Co. (Cambridge, MA, U.S.A.). All other materials were of analytical grade and used as purchased.

Human Nasal Epithelial Culture Using ALI Condition

Nasal specimens were obtained during surgery from inferior turbinate mucosa of patients suffering from chronic sinusitis. The primary human nasal cell culture used in this study has been previously described in detail.[5] When nasal epithelial cells of passage 2 and 3 reached approximately 70-80% confluency, the cells were detached with 0.1% trypsin-EDTA and were seeded at densities of 2×10^5 to 3×10^5 cells/cm^2 on Transwell® insert with 0.5 ml in the apical side and 1.5 ml in the basolateral side of BEGM:DME/F12 (50:50) supplemented with hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), transferrin (10 μg/ml), epinephrine (0.5 μg/ml), triiodothyronine (6.5 μg/ml), gentamycin (50 μg/ml), amphotericin-B (50 μg/ml), retinoic acid (0.1 ng/ml), and epidermal growth factor (0.5 ng/ml human recombinant). After 24 hours, media were changed in both sides with BEGM:DME/F12 (50:50). The apical surface was directly exposed to ambient conditions after reaching confluence on day 3, while 0.8 ml of medium was added to the basolateral reservoir. The medium was changed daily.

Measurement of Bioelectric Parameters and Morphological Studies

After seeding, the transepithelial electrical resistance (TEER) was measured daily using an EVOM voltohmmeter device (WPI, Sarasota, FL, U.S.A.), and obtained by subtracting blank transwell and multiplying by the effective growth surface area.

For observing morphology of nasal epithelial cell monolayers, the monolayers were processed for scanning electron microscopy on day 14. The monolayer was fixed in 2.5% glutaraldehyde in PBS at 4°C for 1hr, rinsed in ice-cold PBS, and then fixed in 1.5% osmiumtetroxide in PBS at room temperature for 1 hr. The specimens were dehydrated through an alcohol series and allowed to air-dry overnight. The specimens were mounted on stubs with adhesive tape, sputter coated, and viewed in a Hitachi S-4200 scanning electron microscope (Hitachi, Japan).

Preparation and Characterization of Deformable Liposome of Fexofenadine·HCl

Deformable liposomes with various phosphatidylcholine (PC) contents were fabricated as reported in the literature.[11] Briefly, the soybean PC and cholic acid (7.35:1, w/w) were dissolved in the minimum amount of ethanol (2% v/v), and then mixed with fexofenadine·HCl in transport medium (drug amount 0.1%, 0.5% w/v). This suspension was sonicated 2 min and passed through a high-pressure extruder (Northern Lipids Inc., Vancouver, BC, Canada) with serial pore size membranes from 1.2 to 0.2 μm.

The size distribution of fexofenadine·HCl deformable liposome was determined using Nicomp 370 Submicron Particle Size Analyzer (NICOMP Instruments, U.S.A.). The liposome was diluted with transport medium and was put into the transparent cell in the system. The particle size distribution was processed with computerized inspection system.

The entrapment efficiency was measured by ultracentrifuge method.[16] Liposome-encapsulated fexofenadine·HCl was separated from free drug by centrifugation at 50,000rpm for 60 min at 4°C in an ultracentrifuge (Beckman Optima 100K Ultracentrifuge, Beckman Instruments Inc., Palo Alto, CA, U.S.A.) and washed twice with distilled water. After lysed with Triton X-100 (0.5%, w/v), the liposome-encapsulated fexofenadine ·HCl and free drug were determined by HPLC.

The Toxicity Studies of Liposome on Human Nasal Epithelial Cells by MTT

The viability of human nasal epithelial cells upon exposure to blank liposome was examined by the MTT colorimetric assay.[17] Briefly, 200 μl of 1×10^6 cells/ml was seeded into 96-well plates. After 48 hr cultured with BEGM, the cells further incubated for 2 hr with 200 μl blank liposome. Incubation

medium was withdrawn and the cells were washed twice with PBS. Aliquot (100 μl) of MTT solution (0.5 mg/ml) and 100 μl BEGM were added to each well. After 4 hr incubation, the supernatant was discarded and formazan crystals dissolved in DMSO followed by vigorous mixing. The optical density was determined by microplate reader at 560 nm (Emax, Molecular Devices Co., Sunnyvale, CA, U.S.A.).

Transport and Cellular Uptake Studies of Fexofenadine ·HCl deformable Liposome Across ALI Human Nasal Monolayers

The transport studies were performed by initially incubating the monolayers in transport medium (HBSS supplemented with 15 mM glucose and 15 mM HEPES, pH 7.4) for 20 min at 37°C. Each transport experiment was performed by adding 0.4 ml of transport medium containing fexofenadine·HCl liposome (100 μg/ml) in apical side, and 1.0 ml of blank transport medium in basolateral side. At predetermined time intervals, samples of 1.0 ml were withdrawn from the basolateral side and replaced with an equal volume of fresh transport medium. Permeation studies were conducted with the monolayers on 8-12 days after seeding. To monitor integrity of the nasal cell monolayers, the TEER value was measured at the beginning and end of each transport experiment.

To determine the cellular uptake amount of fexofenadine ·HCl after the transport studies for 2 hour, the monolayers were rinsed three times with ice-cold PBS and cut off from the transwells. The cells were trypsinized with 0.5% trypsin-EDTA for 30 min. Then the cell suspension was added with 2.0 ml methanol. After centrifugation at 4000 rpm for 10 min, the upper solution was evaporated under N_2. The sample residues were reconstituted with 150 μl water and determined by HPLC.

The cumulative amount of the compound was plotted as a function of time. The steady-state fluxes and the apparent permeability coefficients were estimated from the linear slope of the plot using the relation shown below:

$$P_{app} = \frac{dQ}{dt} \frac{1}{A \cdot C_0}$$

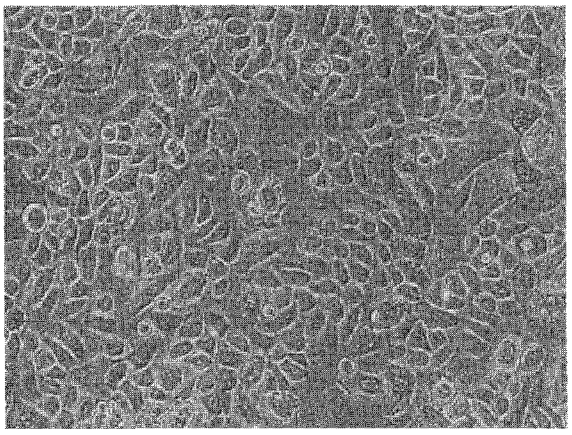
HPLC Assay of Fexofenadine·HCl

The samples of fexofenadine·HCl were directly analyzed using an isocratic HPLC method on a Shimadzu HPLC system (SPD-10AV, Shimadzu Co., Kyoto, Japan) equipped with a pump and an automatic injector (SPD-10AVVP). A reversed phase C-18 column (Lichrospher®100, RP-18, 125×4 mm, 5 μm, Merck Darmstadt, Germany) was used as stationary phase. Fexofenadine·HCl was measured in the mobile phase of phosphate buffer and acetonitrile (62:38,w/w) at a flow rate of 1.0 ml. UV detector was set at 220 nm. The retention time of fexofenadine·HCl was about 7.9 min.

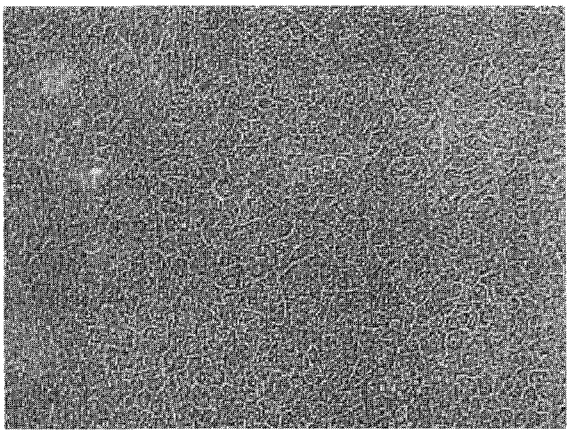
Results and Discussion

Bioelectric Parameters and Morphology of ALI Human Nasal Monolayers

The passaged human nasal cells with serum-free BEGM were successfully cultivated without collagen coating T-flask, as shown in Figure 2A (passage 2). The passaged culture appeared a cuboidal epithelium-like morphology with the contact inhibition. When seeded at the density of $2 \times 10^5 - 3 \times 10^5$ cells/cm^2 in serum-free ALI culture condition, the nasal epithelium developed the optimal barrier properties (Figure 2B). The ALI cell monolayers of passage 2 and 3 appeared to reach



(A)



(B)

Figure 2–Morphology of human nasal epithelial cell under inverted microscope. (A), Passage 2 nasal epithelial cell in T-flask (×200), (B), Passage 2 human nasal monolayer on transwell using ALI condition (×100).

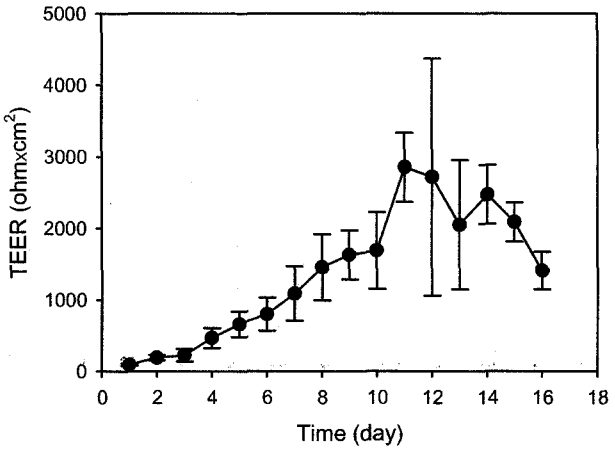


Figure 3–Time-TEER course of human nasal monolayer using ALI condition.

Figure 4–Scanning electron microscopy of passage 2 human nasal epithelium on 14 days using ALI culture condition.

confluence and began to exhibit a measurable transepithelial resistance from day 3. As shown in Figure 3, TEER-time course profile was observed with the mean maximum TEER value of 2852 ± 482 ohm $\times$ cm^2 (n=9) appearing on 11 days after seeding. Moreover, morphological studies of SEM were performed to observe the development of cilia. After 2 weeks of ALI culture, the markedly increased number of matured ciliated cells was observed (Figure 4). The nasal epithelial monolayers grown under ALI condition seem to improve the delivery of oxygen across the thin film of liquid to the cells, and may change the cellular respiration to a more aerobic nature.[5,18] Thus, the culture conditions critically affect the morphological and functional feature of human nasal cells and the expression of differentiated phenotype. ALI culture condition seems to be a suitable model for *in vitro* nasal drug transport studies, which provides the advantage of minimizing the cost and limiting controversial use of animals.

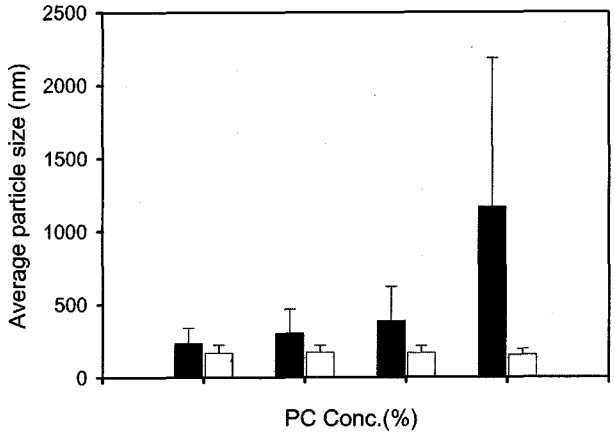


Figure 5–Relationship between particle size of Fexofenadine·HCl deformable liposome and phosphatidylcholine concentration. (■), Without filtration, (□), After filtration of 0.2 μm membrane.

Characteristics of Fexofenadine·HCl Deformable Liposomes

After passing through 0.2 μm pore size, fexofenadine·HCl liposome showed an average volume-weighted diameter of 200 nm, as shown in Figure 5. The deformable liposome was automatically formed when fexofenadine·HCl solution was added to the ethanolic lipid. Without homogenization of extruder, the particle size increased from 231.3 to 1166.6 nm with the enhancement of PC concentration from 0.1% to 1.0%. However, there was no significant difference among all the liposome formulations after filtration through the 0.2 μm membrane, which was confirmed by the narrow size distribution after passing through the extruder (Figure 6).

The entrapment efficiency of fexofenadine·HCl liposome in various formulations was shown in Table I. It is generally known that the entrapment efficiency of hydrophilic drugs in liposome is lower than that of lipophilic drugs. The maximum entrapment efficiency of fexofenadine·HCl in the deformable liposome was obtained to be 33.0% with 1% PC and 100 μg/ml loading dose. The entrapment efficiency increased with the increase of PC concentration. However, due to the limited capacity of the inner aqueous phase of the liposome, the entrapment efficiency with 500 μg/ml fexofenadine·HCl was lower than that with 100 μg/ml, although the absolute amount of fexofenadine·HCl in the liposome was higher.

Toxicity of Deformable Liposome on ALI-cultured Human Nasal Cell Monolayers

The nasal epithelium represents an important defense system, e.g. against mucosal infections.[14] Thus, the toxicity of liposome on the nasal epithelium was investigated by measuring the change of TEER value and MTT assay as indicators

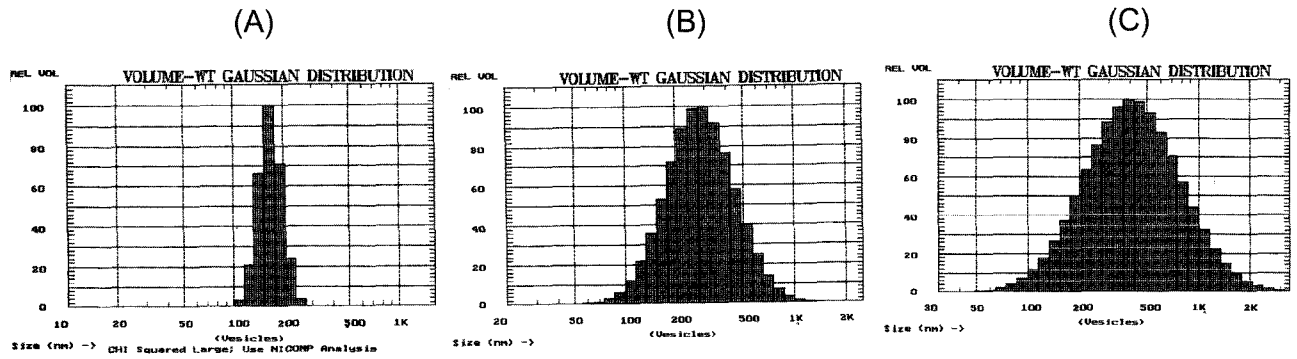


Figure 6–Particle size distribution of fexofenadine·HCl deformable liposome with 0.5% PC concentration after filtration of different pore size membranes. (A), 0.2 μm, (B), 0.6 μm, (C), without filtration.

Table I–*Entrapment Efficiency of Fexofenadine·HCl Deformable Liposome in Different Formulations (n=3)*

PC Conc. (%)	Entrapment efficiency[a] (%)		Absolute amount in liposome (μg Fexofenadine·HCl/mg lipid)	
	100 μl/ml	500 μl/ml	100 μl/ml	500 μl/ml
0.2%	9.56±0.49	4.26±0.26	4.78±0.24	10.66±0.66
0.5%	22.52±0.83	10.61±0.12	4.50±0.17	10.48±0.28
1.0%	33.04±4.18	22.65±0.24	3.30±0.42	11.32±0.12

[a]All the formulations were filtered with 0.2 μm membrane.

for tight junction functionality and membrane disruption.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is a tetrazolium salt that is oxidized by mitochondrial dehydrogenases in living cells to give a dark blue formazan product. Damaged or dead cells show reduced or no dehydrogenase activity.[17] The amount of formazan would depend on the living cell number. MTT assay to assess the cytotoxicity of absorption enhancers towards Caco-2 cells has been recently reported.[19] In this study, the cell viability after incubating blank liposome with various concentrations of PC for 2 hours was evaluated by MTT assay. As shown in Table II, the viability of cells decreased as PC concentration increased in a dose-dependent manner. Liposome with 5% PC concentration showed high cytotoxicity with the viability of only 37.6%, while more than 90% of nasal cells were viable after exposure to low concentration (< 1%) of PC. Thus, the deformable liposomes were prepared with less than 1% of PC for further fexofenadine ·HCl transport studies. The TEER values of nasal membrane decreased less than 25% after applying liposome containing 100 μg/ml of fexofenadine·HCl, and recovered to the initial value within 2 hours (data not shown), which suggests that incorporation of fexofenadine·HCl into the liposome would not cause significant changes in tight junction permeability or membrane integrity.

Effect of Liposome on the Transport and Cellular Uptake of Fexofenadine·HCl

In the bioelectric and morphological studies, it was confirmed that intact tight junction was developed in ALI cultured nasal cell monolayers, which can be used for transport studies. In preliminary studies, fexofenadine·HCl was stable in transport medium and the integrity of the monolayer checked by the change of TEER was maintained for 120 min of transport studies (data not shown).

As shown in Figure 7(A), the transport profiles of fexofenadine ·HCl (100 μg/ml) across the ALI nasal cell monolayer were linear for up to 120 min in the two directions. There was no significant difference in the P_{app} value between two directions (Table III), which was consistent with the previous results.[5] Transport profiles of fexofenadine·HCl incorporated in deformable liposomes were shown in Figure 7(B). The P_{app} value of fexofenadine·HCl showed a decreasing trend with the increase of PC concentration in the liposome, although it was not significantly different (Table III). Moreover, there was no sig-

Table II–*The Cytotoxic of Blank Deformable Liposome on Human Nasal Epithelial Cells Viability by MTT Method*

PC Conc. (%)	0.1	0.2	0.5	1.0	2.0	5.0
Viability (%) (Mean±SD)	92.9±6.5	93.9±3.0	94.3±3.5	94.8±8.0	76.4±7.7	37.6±1.5

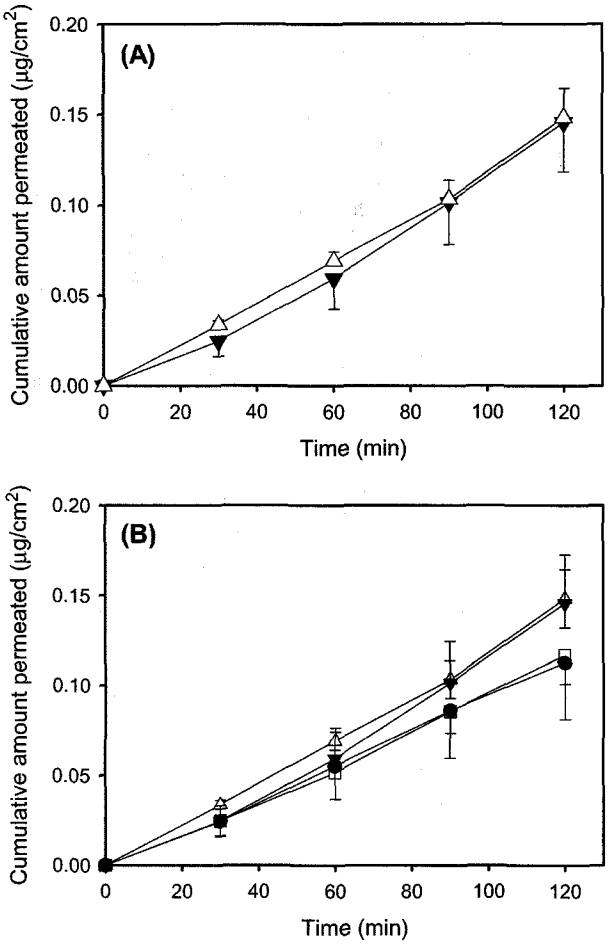


Figure 7–Transport profile of fexofenadine·HCl across ALI human nasal monolayers. (A) Transport profile of 100 μg/ml fexofenadine·HCl in apical to basolateral direction and reverse direction. (▼), Apical to basolateral direction, (△), Basolateral to apical direction. (B) Transport profile of fexofenadine·HCl deformable liposome in different PC concentration in apical to basolateral direction. (▼), Control (without PC), (△), 0.2% PC, (□), 0.5% PC, (●), 1.0%PC.

Table III–*P_{app} and Cellular Uptake of Fexofenadine·HCl Incorporated in the Fexofenadine·HCl Deformable Liposome (100 μg/ml) Across ALI HNE Monolayer*

PC Conc. (%)	P_{app} (10^{-7} cm/s)		Uptake amount After 2 hr transport (ng/cm²)
	Apical to basolateral	Basolateral to apical	
Control	2.25±0.44	2.10±0.32	135.9±12.3
0.2%	1.92±0.32	-	157.9±25.4
0.5%	1.73±0.15	-	145.7±9.2
1.0%	1.64±0.45	-	125.4±7.5

Each data represents the mean±S.D. (n>6).

nificant difference among the cellular uptake of fexofenadine·HCl in different liposome formulations. These results suggest that liposome formulations did not alter the cellular uptake of fexofenadine·HCl, while slightly decreasing the overall permeability of fexofenadine·HCl. Further studies are under way in this laboratory to understand the underlying transport mechanism of deformable liposome.

Conclusions

The passaged ALI human nasal epithelial cell monolayer was successfully established for drug transport studies, which developed tight junction and the differentiation of cilia with similar morphological characteristic *in vivo*. The feasibility of monolayer for transport studies of fexofenadine·HCl was investigated using the deformable liposome. The P_{app} value and cellular uptake of liposome were not significantly different from that of solution, which indicated that the deformable liposome formulation could not enhance the transepithelial permeability of hydrophilic drugs, such as fexofenadine·HCl. However, these results show that human nasal epithelial cell monolayer model seems to be useful to investigate *in vitro* drug transport and mechanism studies, and is feasible to predict the *in vivo* nasal permeability.

Acknowledgements

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