Cyclodextrins in Nasal Delivery of Low-Molecular-Weight Heparins: *In Vivo* and *in Vitro* Studies

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Purpose. To test the hypothesis that cyclodextrins reversibly enhance nasal absorption of low-molecular-weight heparins (LMWHs) and to investigate the mechanisms by which cyclodextrins enhance LMWH absorption via the nose.

Methods. Absorption of LMWHs was studied by measuring plasma anti-factor Xa activity after nasal administration of various LMWH formulations to anesthetized rats. *In vivo* reversibility studies were performed to investigate if the effects of cyclodextrins are reversible and diminish with time. The absorption-enhancing mechanisms of cyclodextrins were investigated in cell culture model. The transport of enoxaparin and mannitol, changes in transepithelial electrical resistance (TEER), and distribution of tight junction protein ZO-1 were investigated.

Results. Formulations containing 5% dimethyl-β-cyclodextrin (DMβCD) produced the highest increase in the bioavailability of LMWH preparations tested. *In vivo* reversibility studies with 5% DMβCD showed that the effect of the absorption enhancer at the site of administration diminished with time. Transport studies using 16HBE14o$^-$ cells demonstrated that the increase in the permeability of enoxaparin and mannitol, reduction in TEER, and the changes in the tight junction protein ZO-1 distribution produced by 5% DMβCD were much greater than those produced by β-cyclodextrin (βCD) or hydroxyl-propyl-β-cyclodextrin (HPβCD).

Conclusions. Of the cyclodextrins tested, DMβCD was the most efficacious in enhancing absorption of LMWHs both *in vivo* and *in vitro*. The study also suggests that cyclodextrins enhance nasal drug absorption by opening of cell-cell tight junctions.

KEY WORDS: bioavailability; cyclodextrins; enoxaparin; low-molecular-weight heparin; nasal absorption; reversibility; TEER; tight junctions; transport studies.

INTRODUCTION

In recent years, the nasal route has shown tremendous promise for systemic administration of drugs that are ineffective orally and need to be administered by injection. Because of rich vasculature, avoidance of first-pass effect, and ease of self-administration, the nasal route has been proposed as a convenient and safe route of administration for noninvasive delivery of those drugs (1). However, nasal drug delivery has been plagued with several disadvantages including toxicity of absorption promoters to nasal absorptive surface and relative impermeability of prospective drug candidates via nasal mucosa (2,3). To overcome the nasal mucosal barrier, drugs intended for nasal delivery need to be coadministered with absorption promoters including surfactants and cyclodextrins (2,3).

Cyclodextrins are oligosaccharides consisting of six to eight units of glucose in a cyclized ring with a central cavity that can accommodate other chemicals. Studies with various cyclodextrins have shown that dimethyl-β-cyclodextrin (DMβCD) was superior to hydroxyl-propyl-β-cyclodextrin (HPβCD), β-cyclodextrin (βCD), and α-cyclodextrin (αCD) in enhancing nasal absorption of various oligo- and polypeptides (4–6). Cyclodextrins are believed to enhance nasal absorption of peptides by inhibiting their enzymatic degradation, solubilizing membrane components, and/or opening tight junctions (7). However, little attention has been paid to the use of cyclodextrins in enhancing nasal absorption of macromolecules that are not peptide in nature, such as low-molecular-weight heparins.

Low molecular weight heparins (LMWHs) have routinely been used as an alternative to unfractionated heparin and proved to be as effective as unfractionated heparin in managing patients with deep vein thrombosis and pulmonary embolism (8). Nasal delivery of LMWH has recently been proposed as an alternative to invasive subcutaneous delivery because this delivery approach exhibits a quicker onset of action compared to subcutaneous LMWHs (9). However, because of its molecular weight (~5000 Da), excessive hydrophilicity, and strong negative surface charge, LMWHs are not absorbed from the nasal route without an absorption promoter. Because cyclodextrins enhance nasal absorption of macromolecules that have molecular weights comparable to that of LMWHs, it is reasonable to assume that cyclodextrins can also enhance nasal absorption of LMWHs. However, there is no experimental data in support of cyclodextrins' ability to enhance nasal absorption of LMWHs. Because LMWHs have negative surface charges, chemically they are polysaccharides, cyclodextrins may or may not be able to enhance nasal absorption of LMWHs by the similar mechanisms as that of polypeptides. Thus, it is important to investigate the mechanism by which cyclodextrins enhance nasal absorption of LMWHs. The objectives of this study were to i) investigate the efficacy of cyclodextrins in enhancing nasal absorption of LMWHs, ii) estimate the duration of action of cyclodextrin on nasal mucosa, and iii) investigate the mechanisms by which cyclodextrins enhance nasal absorption of LMWHs.

MATERIALS AND METHODS

Materials

Enoxaparin (Lovenox; 3000 U of anti-factor Xa activity per 0.3 ml), dalteparin (Fragmin; 2500 U of anti-factor Xa activity per 0.2 ml), and tinzaparin (Innohep; 20,000 U of anti-factor Xa activity per 1 ml) injections were obtained as sterile solutions from Aventis Pharmaceutical Products Inc. (Bridgewater, NJ, USA), Pharmacia & Upjohn Company (Kalamazoo, MI, USA), and Leo Pharmaceutical Products (Ballerup, Denmark), respectively. HPβCD and βCD were generous gifts from Wacker Biochem Corp (Eddyville, IA, USA) and from Roquette American, Inc. (Keokuk, IA, USA), respectively. ^{3}H-enoxaparin (specific activity of 250 μCi/mg) was purchased from American Radiolabelled Chemicals Inc. (St. Louis, MO, USA). DMβCD, ^{14}C-

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mannitol (specific activity of 53 mCi/mmol), paraformaldehyde, sodium citrate, Triton X-100, sodium azide, glycine, *p*-phenylenediamine, and anti-mouse IgG FITC were obtained from Sigma Chemicals Company (St. Louis, MO, USA). Modified Eagle's medium (MEM), fetal bovine serum (FBS), glutamine, penicillin and streptomycin solution, and trypsin EDTA solution were purchased from ATCC (Rockville, MD, USA). Transwells cell culture assembly with polycarbonate inserts (0.4-μm pore size, 1-cm^2 area) and 6-well plates were obtained from Corning Costar Corporation (Cambridge, MA, USA). Vitrogen-100 solution was obtained from Cohesion, Inc. (Palo Alto, CA, USA). Goat serum was obtained from Biocell Technology (Newport Beach, CA, USA). FITC-conjugated anti-ZO- antibody (mouse) was purchased from Zymed Laboratory Inc. (San Francisco, CA, USA).

Preparation of Formulations for *in Vivo* Studies

Stock solutions (10%) of three cyclodextrins, βCD, HPβCD, and DMβCD, were prepared by dissolving the reagents in normal saline. On the day of experiment, formulations for nasal absorption studies were prepared by mixing enoxaparin with saline or appropriate concentrations of different cyclodextrins. The concentrations of cyclodextrins used in the formulations were 1.25, 2.5, and 5% (w/v). The strength of the final formulation was such that each 20 μl of the solution contained an amount of enoxaparin sodium equivalent to 100 U of anti-factor Xa activity. Formulations for subcutaneous administration were prepared to contain 100 U of anti-factor Xa activity in each 100 μl of solution, and formulations for intravenous administration contained 100 U of anti-factor Xa activity in each 200 μl of solution. The nasal formulations of dalteparin and tinzaparin were prepared as described above.

Nasal Absorption Studies in Rats

Male Sprague-Dawley rats (Charles River Laboratories, Charlotte, NC, USA) weighing between 250–350 g were used for *in vivo* absorption experiments. Prior to the experiment, the animals were anesthetized by intramuscular injection of an anesthetic cocktail containing xylazine (30 mg/kg), ketamine (30 mg/kg), and acepromazine (10 mg/kg). Anesthesia was maintained with additional intramuscular injections of anesthetic solution as needed throughout the experiments. An aliquot of 16.7–23.3 μl formulation (330 U/kg body weight) was instilled to the left nare of rat using a pipetter with a disposable plastic tip. For subcutaneous administration, formulations were administered as a single 83.3–116.7 μl (330 U/kg) injection under the back skin, and for intravenous administration, 166.6–233.4 μl (330 U/kg) bolus injection was administered via the tail vein. After nasal and subcutaneous administration, blood samples (~300 μl) were collected from the tip of the rat tail at 0, 30, 60, 120, 240, and 360 min in citrated microcentrifuge tubes and placed on ice. However, in case of intravenous administration, blood was collected from the orbital vein. Subsequently, the plasma was separated by centrifugation (1600 × *g* for 5 min), and the plasma samples thus obtained were stored at −20°C until further analysis.

Reversibility Studies

In vivo reversibility studies were performed as reported earlier (9). For these experiments, formulations were administered in two phases. In the first phase, 20 μl of formulation containing 5% DMβCD, without enoxaparin, was administered to the left nare at time zero. In the second phase, formulations containing only enoxaparin, equivalent to 330 U/kg of anti-factor Xa, were administered to the same nostril immediately afterwards (time zero) or at 60, 120, 240, 360 min after the first phase of administration. In these sets of experiments, blood samples were collected at 0, 5, 15, 30, 45, 60, 90, and 120 min as described above.

All animal studies were approved by the institutional animal care and use committee and were conducted in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*.

Enoxaparin Degradation in 16HBE14o$^-$ Cell Extracts

16HBE14o$^-$ (human bronchial epithelial cells) cells were a gift from Dr. Dieter Gruenert (University of Vermont, Burlington, VT, USA). They were grown in MEM, supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified 37°C incubator with 5% CO_2. 16HBE14o$^-$ cell homogenates were prepared as described previously (10). Briefly, 16HBE14o$^-$ cells were plated in 75 cm^2 culture flask (coated with fibronectin/collagen solution) until 85–90% confluency. Subsequently, the cells were washed with ice-cold saline (pH 7.4), scraped, homogenized in 3 ml ice-cold media at 13,000 rpm for 10 min at 4°C (Ultra-Turrax 725 Basic, IKA-WERKE, Staufen, Germany), and centrifuged at 12,000 rpm for 10 min at 4°C (Avanti J-25I, Beckman Instruments Inc. Palo Alto, CA, USA). The resultant supernatant was used as 16HBE14o$^-$ cell extracts. Protein content (0.50 ± 0.03 mg/ml) of the extracts was determined by Bradford method using a Coomassie blue dye assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) (10). Increasing concentrations of enoxaparin (0.5 to 5000 U/ml) were incubated at 37°C for 5, 12, and 24 h in saline or cell extracts. The resulting solutions were analyzed for anti-factor Xa activity by a colorimetric assay using Coatest Heparin Kit (Diapharma Group Inc., West Chester, OH, USA).

Measurement of Anti-factor Xa Activity

Anti-factor Xa activity present in plasma samples and cell homogenates was determined by a colorimetric assay using the Chromogenix Coatest Heparin Kit (Diapharma Group, Inc., West Chester, OH, USA). The anticoagulant effect of heparin, both unfractionated and low-molecular-weight heparin, is achieved through their ability to bind antithrombin and inhibit clotting activity of factor-Xa. In this study, anti-factor Xa activity was used as a surrogate marker for LMWH absorption. In fact, pharmacokinetic/pharmacodynamic (PK/PD) studies involving unfractionated heparin and LMWHs typically involve replacement measurements such as anti-factor Xa activity or anti-factor IIa activity (9,11). This indirect method of using pharmacodynamic response to assess absorption and bioavailability of LMWHs has widely been used to monitor therapeutic efficacy of LMWHs. A linear relationship between the area under the curve (plot of anti-factor Xa vs time) and injected dose was observed when 20, 40, 60, and 80 mg of enoxaparin was administered subcutaneously to human subjects (12).

In Vitro Transport and Transepithelial Electrical Resistance Studies in 16HBE14o$^-$ Cell Monolayers

16HBE14o$^-$ cells were plated in 75 cm^2 culture flask (coated with fibronectin/collagen solution) and subcultured after achievement of 85–90% confluency. Media were changed every 2 days, and the passages used for the experimentation were between 29 and 40. The inserts of the Transwells (0.4-μm pore size, 1-cm^2 area) were coated with Vitrogen-100 solution.

For the transport studies, 16HBE14o$^-$ cells were seeded at a density of 25,000 cells/well onto collagen-coated polycarbonate Transwells (0.4-μm pore size, 1-cm^2 area) and the media were changed every day. The integrity of the confluent cell monolayers was evaluated by measuring transepithelial electrical resistance (TEER) using an EVOM epithelial voltohmeter (World Precision Instruments, Sarasota, FL, USA). The monolayers with TEER values between 400 and 600 Ω·cm^2 were used in the experiments.

On the day of experiment, ^{3}H-enoxaparin and ^{14}C-mannitol were dissolved in culture media and mixed with βCD, HPβCD, or DMβCD stock solution in saline. The final concentration of βCD, HPβCD, or DMβCD used in the experiments was 5%. The pH of the solutions was 7.4. Prior to the initiation of transport experiments, 1.5 ml fresh media was added to the basolateral side and 0.5 ml of prewarmed media containing ^{3}H-enoxaparin and ^{14}C-mannitol were added to the apical side in the presence or absence of different concentrations of βCD, HPβCD, or DMβCD. Monolayers for transport experiments were kept in a 37°C/5% CO_2 incubator. Samples (100 μl) were withdrawn from the basolateral chamber at various time intervals (0, 15, 30, 45, 60, 90, 120 min). The basolateral chamber was replenished with fresh media (100 μl) after each sampling. The amount of ^{14}C-mannitol and ^{3}H-enoxaparin transported across cell monolayers was determined by counting the samples in a Beckman LS 6500 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA, USA). The apparent permeability coefficient (P_{app}) was calculated as described earlier (13). TEER was recorded during the experiments at various time intervals (0, 30, 60, 120 min) and was plotted as a fraction of initial TEER vs. time.

Immunocytochemistry Experiments

Immunocytochemical studies were carried out as described previously with slight modifications (14,15). Confluent 16HBE14o$^-$ cell monolayers were pretreated with 5% solution of any of the three cyclodextrins or culture media alone for 2 h. Following treatment, the monolayers were fixed with 4% paraformaldehyde for 15 min at room temperature. The cover slips with cell monolayers were rinsed twice with 25 mM glycine buffer and then permeabilized with 0.1% Triton X-100 for 15 min at room temperature.

The primary antibody (FITC-conjugated anti-ZO-1 antibody) was diluted in saline-sodium citrate (SSC) antibody buffer (containing 1% BSA, 2% goat serum) for a working concentration of 5 μg/ml. After fixing and permeabilization of 16HBE14o$^-$ cells, the cells were incubated in the diluted primary antibody solution at room temperature for 2 h. After incubation with the primary antibody, the cells were washed three times with SSC wash buffer (0.05% Triton X-100), and then incubated with the secondary antibody solution (anti-mouse IgG FITC conjugate, 1:50 working dilution) for 45 min at room temperature. The stain was removed, and the cells were washed three times again with SSC wash buffer (0.05% Triton X-100). The cover slips were cautiously cut out with a scalpel and transferred to slides, where they were embedded in *p*-phenlenediamine medium and sealed. The slides were air dried for 30 min at room temperature in dark and then observed under the fluorescence microscope.

Image photographs were taken using a 40× (oil immersion) objective on an UltraVIEW Imaging System Olympus IX70 fluorescent microscope with a fluorescent filter at 485 nm and OlymPix CCD camera (Perkin Elmer Life Sciences Ltd., Fremont, CA, USA).

Pharmacokinetic and Statistical Analysis

Standard noncompartmental analysis (Kinetica, version 4.0, Innaphase Corp., Philadelphia, PA, USA) was performed for LMWH absorption profiles. Absolute and relative bioavailabilities were estimated by comparing $AUC_{0\rightarrow360}$ for nasally administered LMWH with that of intravenously and subcutaneously administered LMWH, respectively. One-way ANOVA was used to compare the data. When the differences in the means were significant, post hoc pairwise comparisons were conducted using Newman-Keuls multiple comparison (GraphPad Prism, version 3.03, GraphPad Software, San Diego, CA, USA). Differences in p values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Nasal Absorption Studies

The potency and efficacy of cyclodextrins in enhancing nasal absorption of LMWH were investigated by formulating enoxaparin, a widely used LMWH preparation available as subcutaneous injection in the U.S. market, with three cyclodextrins, βCD, HPβCD, and DMβCD. When enoxaparin was formulated with 1.25% βCD and administered nasally to anesthetized rats, there was little or no increase in the absorption of enoxaparin compared to enoxaparin formulated in saline (Fig. 1A). However, when 2.5% or 5% βCD was added to the enoxaparin formulation, there was a significant increase in plasma anti-factor Xa level ($p < 0.05$), indicating that biologically active enoxaparin was absorbed from the rat nose. A plasma anti-factor Xa level of 0.2 U/ml is considered to produce antithrombic effect in rodent model (9). There was nearly a 1.5-fold increase in C_{max} when the concentration of βCD was increased from 1.25% to 2.5% (Table I). The increase in LMWH absorption is also evident from the increase in $AUC_{0\rightarrow360}$ values for formulations containing enoxaparin plus βCD (Fig. 1D). However, anti-factor Xa level and AUC value produced by 2.5% βCD formulation were not significantly different from that obtained from 5% βCD formulations ($p > 0.05$). This similarity between the extent of absorptions produced by 2.5% and 5% βCD was probably because of the solubility limit (1.8%) of βCD (16). Because both 2.5% and 5% βCD formed supersaturated solutions, the number of βCD molecules available for promoting absorption of the drug were the same in both formulations. Like formulations containing 1.25% βCD, there was no increase in anti-factor Xa level when enoxaparin was formulated with 1.25% or 2.5% HPβCD (Fig. 1B). When the concentration of HPβCD was increased to 5%, there was an increase in anti-factor Xa level

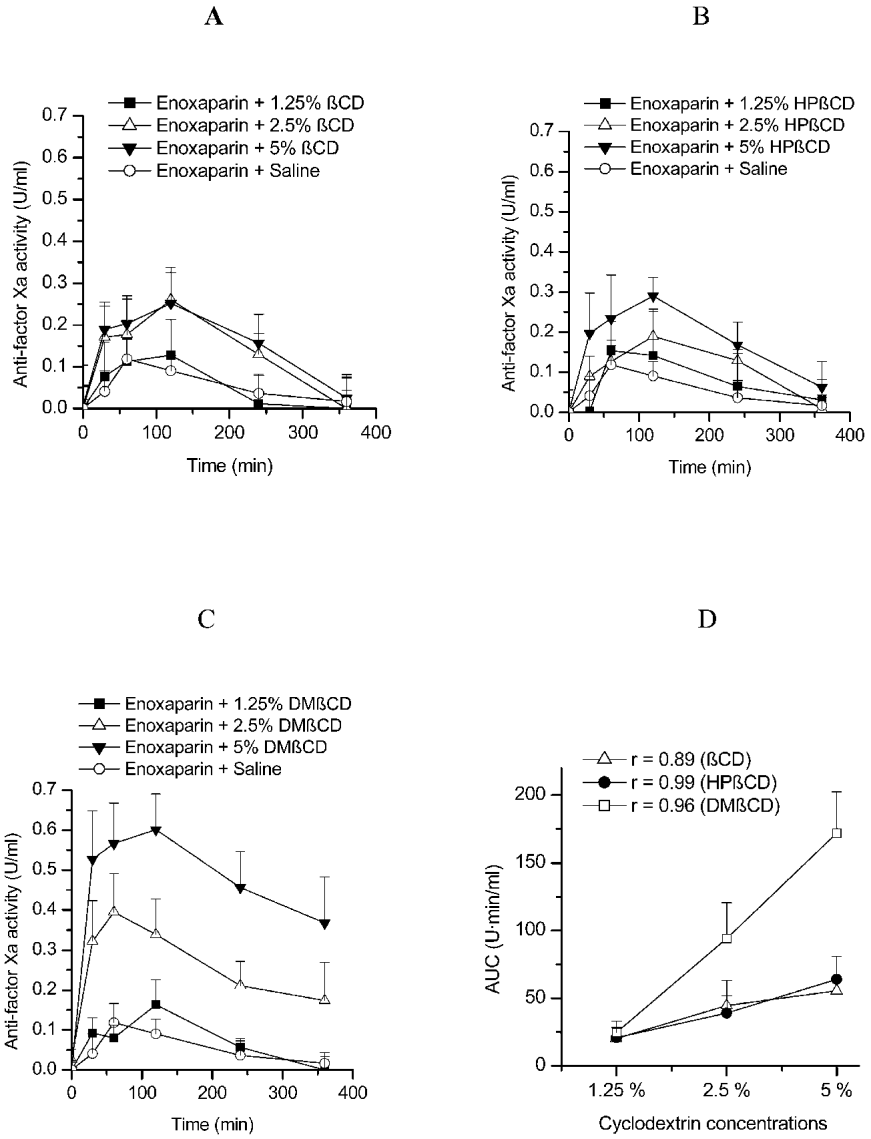

Fig. 1. Changes in anti-factor Xa activity after nasal administration of enoxaparin (330 U/kg) in saline or in the presence of increasing concentrations of (A) βCD, (B) HPβCD, or (C) DMβCD; (D) changes in $AUC_{0\rightarrow360}$ for anti-factor Xa activity-time curve with increasing concentrations of cyclodextrins. Data represent mean ± SD, n = 3 to 5.

that was barely enough to produce therapeutic effect in rodent model (>0.2 U/ml).

Unlike βCD and HPβCD, DMβCD produced a substantial increase in the nasal absorption of enoxaparin when it was formulated with 2.5% or 5% DMβCD (Fig. 1C). When enoxaparin was formulated with 2.5% DMβCD and administered nasally, there was a 4-fold increase in $AUC_{0\rightarrow360}$ compared to the formulation containing enoxaparin plus 1.25% DMβCD. Similarly, 5% DMβCD produced a 8-fold increase in the $AUC_{0\rightarrow360}$ compared to formulation containing enoxaparin in saline (Table I). Furthermore, the data presented in Fig. 1D show a good correlation between the concentration of DMβCD in the formulation and the extent of absorption (r = 0.96), indicating a dose-dependent increase in enoxaparin absorption. The pharmacokinetic data presented in Table I also confirm the superiority of DMβCD over βCD and HPβCD in enhancing nasal absorption of enoxaparin. Similar to the data presented in Fig. 1, both absolute and relative bioavailabilities were increased when enoxaparin was formulated with 5% of any of the cyclodextrins compared to the control (Table I). Maximum increase in the bioavailability was produced by formulation containing 5% DMβCD.

The most potent cyclodextrin, DMβCD, was further formulated with two other LMWH preparations, tinzaparin and dalteparin, to assess if this agent also enhances nasal absorption of LMWHs that have slightly different chemical and pharmacokinetic features. Like enoxaparin in saline, formulation containing dalteparin plus saline or tinzaparin plus saline failed to produce an anti-factor Xa level that can elicit a therapeutic effect (Fig. 2). However, formulation of tinzaparin and dalteparin in 5% DMβCD lead to a rapid and substantial increase in anti-factor Xa level. Comparing the graphs presented in Fig. 2 and $F_{relative}$ values in Table II, one can observe that there are no significant differences among the

Table I. Pharmacokinetic Parameters for Formulations Containing Enoxaparin (330 U/kg) in Saline or in Different Cyclodextrins at Various Concentrations

Absorption enhancer	Concentration	C_{max} (U/ml)	T_{max} (min)	$AUC_{0\rightarrow360}$ (U·min/ml)	$F_{absolute}$ (%)	$F_{relative}$ (%)
None	—	0.15 ± 0.04	66 ± 33	21.3 ± 4.0	4.8 ± 0.9	5.1 ± 1.1
	1.25%	0.17 ± 0.02	70 ± 54	20.2 ± 9.4	4.6 ± 2.0	4.9 ± 2.2
βCD	2.5%	0.26 ± 0.10	120	44.5 ± 21.4	10.0 ± 4.8	10.7 ± 5.4
	5%	0.26 ± 0.09	100 ± 45	55.4 ± 13.2*	12.5 ± 2.7*	13.4 ± 3.1*
HPβCD	1.25%	0.14 ± 0.07	100 ± 69	20.8 ± 12.5	4.7 ± 2.8	5.0 ± 2.9
	2.5%	0.19 ± 0.07	160 ± 89	38.9 ± 16.5	8.8 ± 3.8	9.4 ± 4.0
	5%	0.30 ± 0.04*	90 ± 67	64.0 ± 21.9*	14.4 ± 4.9*	15.4 ± 5.4*
DMβCD	1.25%	0.16 ± 0.07	120	24.5 ± 8.7	5.5 ± 1.9	5.9 ± 2.1
	2.5%	0.39 ± 0.16*	60	94.2 ± 34.0*	21.2 ± 7.6*	22.7 ± 8.3*
	5%	0.61 ± 0.13*	90 ± 67	171.8 ± 44.7*	38.7 ± 9.8*	41.4 ± 10.7*
Subcutaneous	—	1.19 ± 0.12	200 ± 80	414.8 ± 15.8	93.0 ± 3.6	—
Intravenous	—	3.66 ± 0.24	0	446.0 ± 38.4	100	—

Data represent mean ± SD, n = 3 to 5.
* $p < 0.05$ compared to the formulation without enhancer (control).

anti-factor Xa levels produced by three LMWHs. Differences in the C_{max} and $F_{relative}$ values for three LMWH formulations with 5% DMβCD are not statistically significant ($p > 0.05$). It is important to note that these LMWHs have slightly different chemical and pharmacokinetic features although they are derived from the same unfractionated heparin (17). Despite the slight differences in the molecular weight and chemistry, LMWH preparations formulated in DMβCD did not show any appreciable differences in the nasal absorption profile, suggesting that the size and chemistry of the molecules perhaps do not play much role in their absorption. This result agrees with the fact that cyclodextrins probably do not interact directly with the drug; rather these agents exert their effects on the nasal membrane. It is unlikely that cyclodextrins enhance absorption of LMWH by forming inclusion complex with the drug because LMWH is too hydrophilic and bulky to be included in the cyclodextrin cavity.

Reversibility Study

In vivo reversibility studies have been proposed as a useful method to determine if enhancers cause increased drug permeation via a reversible way (9). In this study, DMβCD was used because this cyclodextrin derivative was the most potent in enhancing nasal LMWH absorption. When enoxaparin was administered at time zero immediately after DMβCD administration, there was a significant increase in anti-factor Xa activity (Fig. 3). This absorption profile is comparable to that obtained in nasal absorption study with formulations containing 5% DMβCD plus enoxaparin. However, when enoxaparin was applied 1, 2, 4, and 6 h after DMβCD administration, there was a significant decrease in the C_{max} and $AUC_{0\rightarrow120}$ compared to enoxaparin administered immediately after DMβCD administration ($p < 0.05$). The differences between C_{max} (0.188 ± 0.020 U/ml) and $AUC_{0\rightarrow120}$ (16.4 ± 4.7l U·min/ml) values for enoxaparin administered 6 h after DMβCD administration and C_{max} (0.129 ± 0.085 U/ml) and $AUC_{0\rightarrow120}$ values (12.3 ± 2.9 U·min/ml) for the control group (enoxaparin plus saline) were not statistically significant ($p > 0.05$). These data indicate that the effect of DMβCD on nasal membrane diminishes with time, and increased nasal epithelial permeability returns to restrictive barrier properties after 6 h. The reversibility data also suggest that acute exposure of DMβCD is unlikely to produce any irreversible nasal mucotoxicity. In addition, it is important to note that this study was performed in anesthetized rats. In conscious animal, the duration of action and time for the epithelium to return to normal physiological state could be shorter than that observed in anesthetized animal, because in conscious animal, as pointed out by Marttin *et al.* (18), nasally administered drug formulation will be diluted by the mucus and subsequently removed by the mucociliary clearance within a brief period of time. The data obtained from reversibility studies agrees with the toxicological studies of cyclodextrins reported by others. It has been shown that nasal irritation caused by DMβCD was negligible compared to conventional absorption promoting agents (16). Nasal spray containing 6.2% DMβCD was found to be very well tolerated, and no adverse effects were observed when the formulations were administered twice a day for 6 months to human subjects (19, 20). Others have also investigated the effects of cyclodextrins on the histological integrity of nasal mucosa. For example, Asai *et al.* (21) showed that water-soluble cyclodextrins do not affect the histological integrity of rat nasal mucosa even when they were used at a concentration of 10% (w/v). Studies with transmission electron microscopes have also confirmed that DMβCD causes no alteration in nasal epithelial membrane at a concentration ranging from 1-5% (22).

Enoxaparin Degradation in 16HBE14o$^-$ Cell Extracts

To evaluate if enoxaparin undergoes nonspecific or enzymatic degradation of LMWHs during nasal administration, enoxaparin was incubated with 16HBE14o$^-$ cell extracts. Data on metabolic stability study, presented in Table III, shows no differences between the anti-factor Xa activity of the different concentrations of enoxaparin incubated in 16HBE14o$^-$ cell extracts and enoxaparin incubated in saline. These results indicate that no metabolic degradation/depolymerization of the LMWH chain occurred; thus, the biological activity of enoxaparin was not lost, and enoxaparin did not degrade due to nonspecific or enzymatic degradation of the drug during a 24-h period. However, it is important to

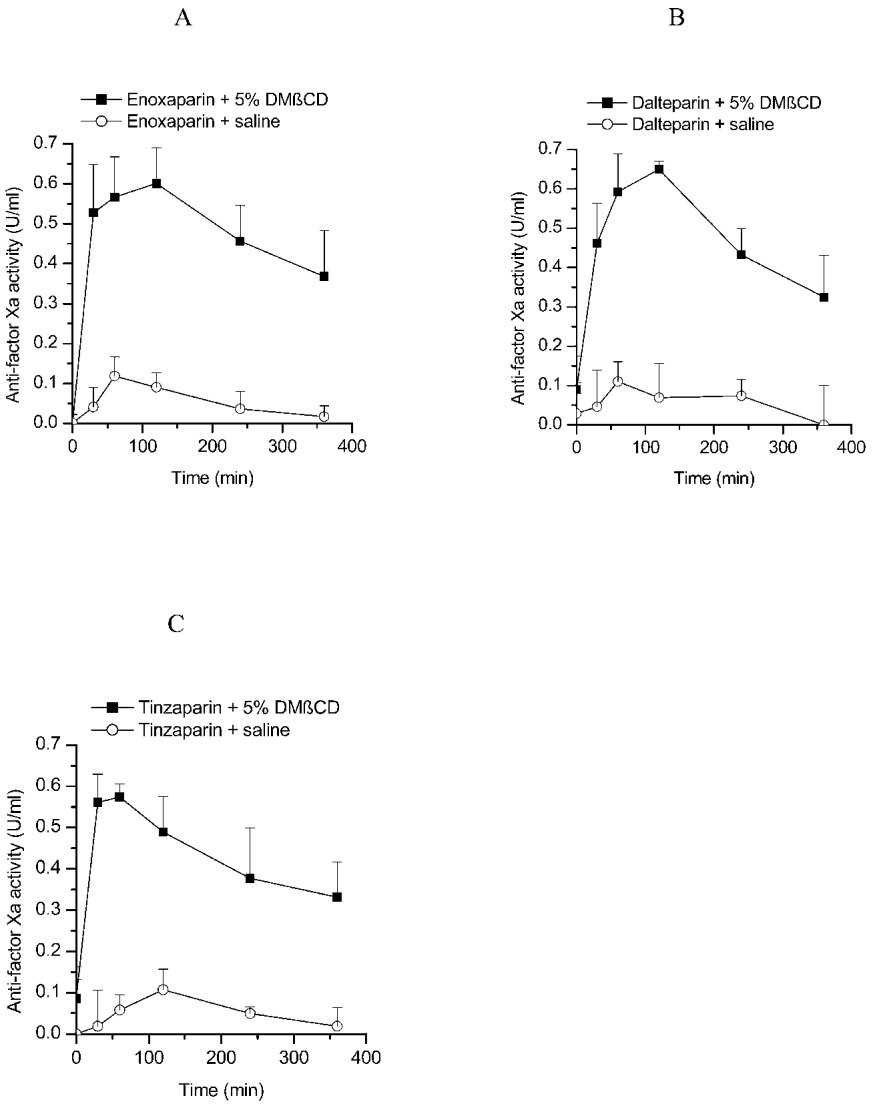

Fig. 2. Changes in anti-factor Xa activity after nasal administration of 330 U/kg of (A) enoxaparin, (B) dalteparin, or (C) tinzaparin formulated with or without 5% DMβCD. Data represent mean ± SD, n = 3 to 5.

note that significant interspecies variations were observed between the absorption profiles of peptide drugs obtained in animal model and human subjects (7). One of the reasons for interspecies differences is perhaps peptide drugs undergo extensive enzymatic degradation and rate of degradation varies from species to species. A significant interspecies variability is unlikely to occur in cyclodextrin-based formulation of LMWH, as this drug does not undergo enzymatic degradation as shown above. However, this data does not rule out the possibility of degradation of LMWH by the extracellular enzymes present on the nasal epithelial surface.

In Vitro Transport and TEER Studies in 16HBE14o$^-$ Cell Monolayers

Transport of enoxaparin was conducted in 16HBE14o$^-$ cell culture model. This cell line was chosen because of its ability to form well-defined tight junctional complexes and its morphological similarity to nasal epithelial cells. In fact, the nasal cavity, nasopharynx, larynx, trachea, and bronchi are lined with pseudostratified, ciliated, columnar epithelium with many goblet cells (23). Furthermore, little or no differences were observed when morphology, ciliary activity, histology, and functions of cultured nasal epithelial cells were compared with that of bronchial epithelial cells (24). Additionally, these cultured cells resemble those *in vivo* cells and

Table II. Pharmacokinetic Parameters for Nasal Formulation of Enoxaparin, Dalteparin, and Tinzaparin (330 U/kg)

	C_{max} (U/ml)		$F_{relative}$ (%)	
LMWHs	Saline	5% DMβCD	Saline	5% DMβCD
Enoxaparin	0.15 ± 0.04	0.61 ± 0.13*	4.8 ± 0.9	41.4 ± 10.7*
Dalteparin	0.12 ± 0.08	0.65 ± 0.04*	4.7 ± 1.0	41.4 ± 6.0*
Tinzaparin	0.12 ± 0.03	0.60 ± 0.09*	4.5 ± 2.8	36.9 ± 4.2*

Data represent mean ± SD, n = 3 to 5.
* $p < 0.05$ compared to the same low-molecular weight heparins (LMWHs) formulated with saline (control).

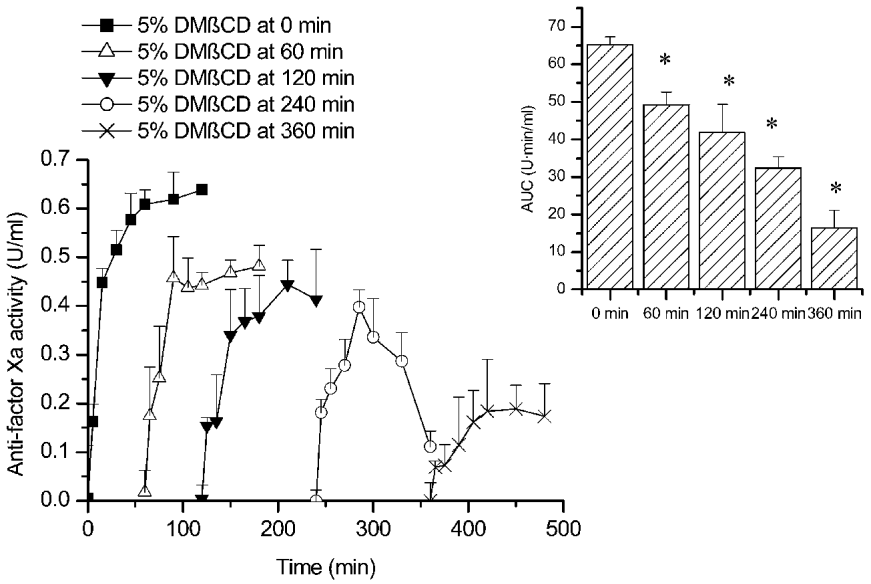

Fig. 3. Changes in plasma anti-factor Xa activity following nasal administration of 330 U/kg enoxaparin either at 0 min (■), 60 min (Δ), 120 min (▼), 240 min (O), or 360 min (×) postadministration of 5% DMβCD. Inset shows changes in $AUC_{0\rightarrow120}$ for anti-factor Xa activity-time curve with the different time points. Data represent mean ± SD, n = 3 to 5. *p < 0.05 compared to the administration of enoxaparin at 0 min.

have been used as *in vitro* model for evaluation of nasal drug delivery (13, 25).

The transport of ^{3}H-enoxaparin across Transwell-plated 16HBE14o^{-} cell monolayers was studied in the presence or absence of various cyclodextrins. When ^{3}H-enoxaparin was placed in the apical chamber in the absence of cyclodextrins, there was a little increase in the permeation of the drug from the apical to basolateral chamber (Fig. 4A). However, ^{3}H-enoxaparin transports were increased when 5% βCD or HPβCD was added to the apical side (Table IV). The highest increase in enoxaparin permeability was observed when 5% DMβCD was added to the apical side of the membrane ($p < 0.05$). The permeation of ^{14}C-mannitol in the presence or absence of cyclodextrins across 16HBE14o^{-} cells was assessed concomitantly with ^{3}H-enoxaparin transport (Fig. 4B). Like transport studies with ^{3}H-enoxaparin, the addition of cyclodextrins enhanced ^{14}C-mannitol flux across the 16HBE14o^{-} cell layer and the greatest increase in mannitol permeability was observed when DMβCD was added as permeability enhancer ($p < 0.05$) (Table IV).

TEER measurements were used in the study in order to assess the effects of three cyclodextrins on the tight junctional stability and paracellular pathway of 16HBE14o^{-} cells. TEER across the cell monolayers was measured following a 120-min treatment with cyclodextrins in the apical chamber (Fig. 4C). In the absence of any enhancer, there was no change in TEER during the course of the entire measurement period. In contrast, addition of formulations containing 5% DMβCD resulted in significantly diminished TEER values even after 30 min ($p < 0.05$), suggesting the opening of tight junction and increase in paracellular permeability. However, 5% βCD or 5% HPβCD did not show much effect on the TEER values ($p > 0.05$). Such a finding is entirely consistent with previous work in Caco-2 model. The TEER of the Caco-2 monolayers was not affected by treatments with cyclodextrins (including βCD and HPβCD) other than DMβCD (26).

Immunocytochemistry Experiments

Tight junctions are formed from macromolecular complexes of proteins, such as ZO-1, claudin, and occludin, and have a continuous distribution around the periphery of epi-

Table III. Anti-Factor Xa Activity of Enoxaparin Incubated in 16HBE14o^{-} Cell Homogenates and Saline for 5, 12, and 24 h

	Final enoxaparin concentration (U/ml)					
	5 h		12 h		24 h	
Initial enoxaparin concentration (U/ml)	In cell homogenates	In saline	In cell homogenates	In saline	In cell homogenates	In saline
5000	4852 ± 311	5074 ± 111	4967 ± 244	5096 ± 33	5208 ± 8	4803 ± 74
500	523 ± 12	486 ± 16	534 ± 4	531 ± 4	542 ± 4	542 ± 7
50	51.6 ± 0.7	49.1 ± 1.1	51.9 ± 0.3	52.6 ± 2.0	55.8 ± 1.9	54.3 ± 0.6
5	5.2 ± 0.1	5.0 ± 0.2	5.4 ± 0.2	5.3 ± 0.1	5.5 ± 0.1	5.3 ± 0.1
0.5	0.52 ± 0.04	0.52 ± 0.01	0.51 ± 0.01	0.54 ± 0.04	0.54 ± 0.01	0.53 ± 0.02

Data represent mean ± SD, n = 3.

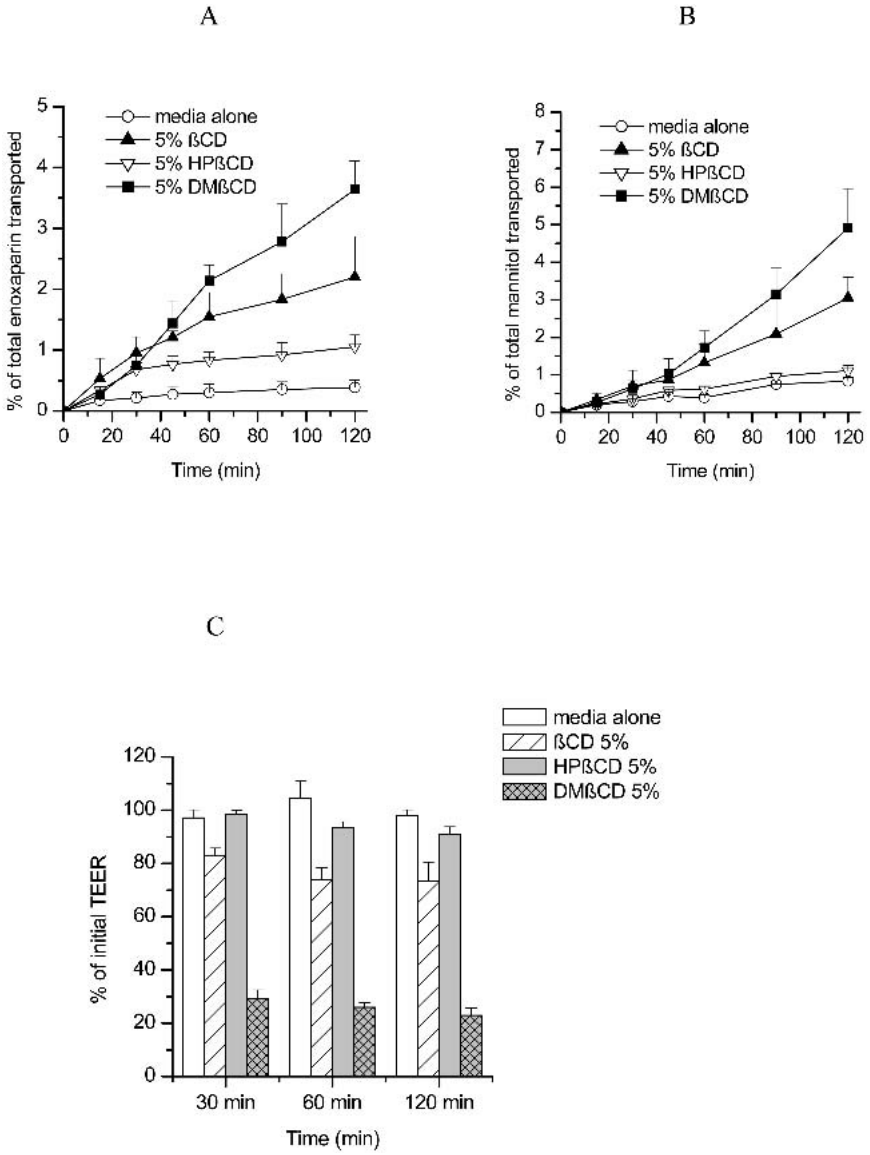

Fig. 4. Percentage of (A) ^{3}H-enoxaparin or (B) ^{14}C-mannitol transported across 16HBE14o^{-} cell monolayers in the absence or presence of 5% βCD, HPβCD, or DMβCD; (C) changes in the TEER of 16HBE14o^{-} cell monolayers with time in the absence or presence of 5% βCD, HPβCD, or DMβCD. Data represent mean ± SD, n = 3.

thelial cells. 16HBE14o^{-} cell line has been found to form polarized layers with well-defined tight junctional barriers. This is evident by the presence of tight junction proteins like the ZO-1 (14). Figure 5 shows that confluent monolayers of 16HBE14o^{-} cells exhibit well-defined, peripheral band, staining of the tight junction protein ZO-1. Tight junctions in these cells appeared as near-continuous rings localized to the periphery of each cell (Fig. 5A). When 16HBE14o^{-} cells were treated with 5% βCD or 5% HPβCD, only local and subtle modifications of the ZO-1 pattern were seen, yet the cells maintained a consistent cuboidal morphology (Figs. 5B and 5C). However, exposure to 5% DMβCD caused tight junctional belts to become thinner, and less overall ZO-1 immunoreactivity was observed (Fig. 5D). Additionally, epithelial cell morphology seemed altered after exposure to 5% DMβCD, resulting in a smaller cell size and an irregular, faint peripheral band pattern for ZO-1 protein (Fig. 5D). The immunocytochemical experiments support the conclusion that 5% DMβCD alters the paracellular barrier in confluent 16HBE14o^{-} cells by forcing a reorganization of ZO-1 protein in cell-cell contact sites.

The results of *in vitro* transport study presented above agree with the data on the effect of cyclodextrin on nasal absorption of peptide drugs reported earlier (4–6) and nasal absorption of LMWHs. Like the data presented in this paper, DMβCD also enhances nasal absorption of peptide drugs more efficaciously and expeditiously than any other cyclodextrins. The difference in the potency of cyclodextrins as nasal absorption promoters is partly due to the fact that different cyclodextrins have different rates of membrane solubilization. DMβCD is found to release more biochemical marker from the nasal mucosa than any other cyclodextrins (27,28). In fact, incorporation of methyl substituents significantly increases the solubility of cyclodextrins, and when the number

Table IV. Permeability of ^{3}H-Enoxaparin and ^{14}C-Mannitol Across 16HBE14o^{-} Cell Monolayer in the Presence or Absence of 5% βCD, HPβCD, or DMβCD

	$P_{app} \times 10^{-7}$ (cm/s)	
Formulations	Enoxaparin	Mannitol
No enhancer	3.27 ± 0.99	6.96 ± 0.87
5% βCD	18.48 ± 5.55*	25.71 ± 4.59*
5% HPβCD	8.51 ± 2.22*	9.35 ± 1.19
5% DMβCD	30.64 ± 3.68*	48.08 ± 12.53*

Data represent mean ± SD, n = 3.
* $p < 0.05$ compared to the formulation without enhancer (control).

of methyl groups reaches to 13–14, the solubility reaches to a maximum (29,30). Therefore, solubilization of membrane components by cyclodextrins varies with the solubility of cyclodextrins and degree of methylation. Furthermore, a recent study with confocal laser scanning microscopy showed that methylated cyclodextrins produces increase in absorption via the paracellular route (7,31). In the current study, the degree of reduction in TEER and increase in mannitol/enoxaparin permeability caused by three cyclodextrin was in the following order: DMβCD > βCD > HPβCD. In fact, a statistically significant correlation was observed after analysis of regression between the reduction in TEER and increase in permeability (Table V). Moreover, the immunocytochemistry studies suggests that DMβCD has more effects on altering the paracellular barrier in confluent 16HBE14o^{-} cells by forcing a reorganization of ZO-1 protein in cell-cell contact sites. These data, coupled with studies by Shao *et al.* (27) and Martin *et al.* (28), suggest that cyclodextrins possibly enhance nasal absorption of enoxaparin by two mechanisms: solubilization of membrane components and opening of tight junctions. However, it is not yet clear whether the possible opening of tight junctions is triggered by the solubilization membrane component or whether tight junction opening and solubilization occur simultaneously and independently from each other. Previously, it has been suggested that the opening of tight junctions by methylated-β-cyclodextrins is closely associated with cholesterol release (7).

In conclusion, all three cyclodextrins can enhance nasal absorption of enoxaparin. Of the cyclodextrins tested, DMβCD was the most efficacious in enhancing nasal enoxa-

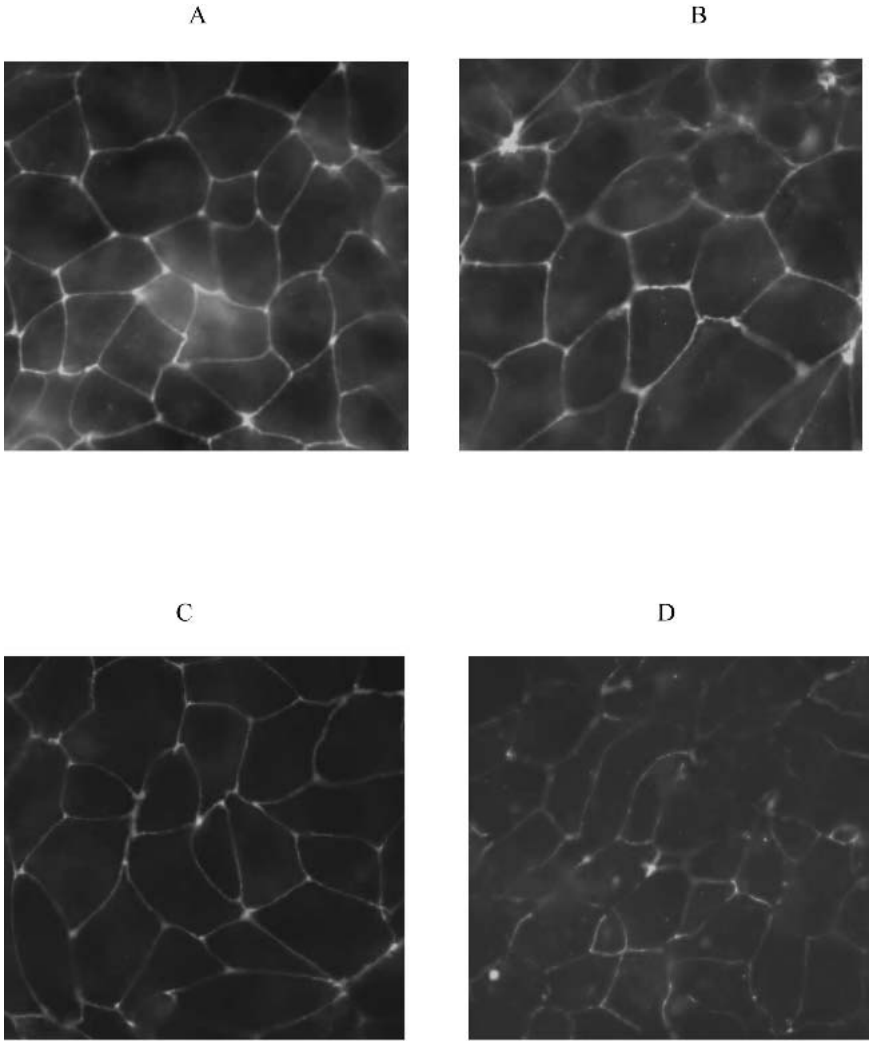

Fig. 5. Immunofluorescence images showing immunolocalization of ZO-1 in the 16HBE14o^{-} cells in the (A) control or presence of (B) 5% βCD, (C) 5% HPβCD, or (D) 5% DMβCD.

Table V. Correlation Between Reduction in TEER and Increase in Enoxaparin Permeability in the Presence or Absence of 5% βCD, HPβCD, or DMβCD

	Deduction in TEER (%)	Enoxaparin $P_{app} \times 10^{-7}$ (cm/s)	Correlation coefficient
Control	0	3.27 ± 0.99	
5% βCD	26.7 ± 7.2	18.48 ± 5.55	$r^2 = 0.9512$
5% HPβCD	9.1 ± 2.9	8.51 ± 2.22	($p < 0.05$)
5% DMβCD	77.1 ± 2.8	30.64 ± 3.68	

Data represent mean ± SD, n = 3.
TEER, transepithelial electrical resistance.

parin absorption. DMβCD is also efficacious in nasal absorption of dalteparin and tinzaparin despite the slight differences in their chemical and pharmacokinetic properties. The mechanism of enoxaparin absorption perhaps involves a combination of two effects; that is, solubilization of membrane components and opening of tight junctions. Because the effect of DMβCD on nasal mucosa is reversible after 6 h, DMβCD-based nasal formulation of LMWHs could provide important clinical utility for deep vein thrombosis and pulmonary embolism.

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