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The effects of water-soluble cyclodextrins on the histological integrity of the rat nasal mucosa

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Abstract

The aim of this study was to investigate the effect of highly water-soluble cyclodextrins (CDs) on the histological integrity of the nasal mucosa. In order to evaluate their effects, the in vivo single and repeated nasal exposure studies were performed using male Wistar rats. The rat nasal cavity was excised after an application of various CD solutions at different times. The morphological appearances of the rat nasal mucosae were analyzed with the light microscopic and the scanning electron microscopic studies. By utilizing 5-min exposure of each CD solution to the nasal mucosa, no tissue damage was visible for 1.5% w/v β-CD and 5 and 20% w/v hydroxypropyl β-CD (HP β-CD), and the effects were quite similar to controls. However, using 20% w/v randomly methylated β-CD (RM β-CD) showed severe damage on the integrity of nasal mucosa. The severity was similar to 1% w/v polyoxyethylene-9-lauryl ether or 1% w/v sodium deoxycholate. Meanwhile, 30 or 60 min exposure to 10% w/v HP β-CD or RM β-CD resulted in no obvious mucosal damage. In addition, in vivo repeated dosing of RM β-CD did not show any toxicity up to 20% w/v. These results suggest that at least, less than 10% w/v CD solutions do not induce gross tissue damage and can keep the histological integrity of the nasal mucosa.

Keywords: Nasal mucosa; Toxicity; β-Cyclodextrin; Hydroxypropyl β-cyclodextrin; Randomly melthylated β-cyclodextrin

1. Introduction

The nasal delivery appears to be a desirable alternative to the parenteral medication because of the existence of a rich vasculature (Drettner and Aust, 1974; Kunlien et al., 1985) and a highly permeable structure within the nasal membranes (Gizurarson, 1993). In addition, nasal application circumvents first-pass elimination (Chien et al., 1989) and/or degradation of drugs prone to enzymatic degradation in the GI tract, and may be employed routinely without any pain. Further-

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more, several studies demonstrated the existence of direct transport from the nasal cavity to the cerebrospinal fluid and proceeding to the brain (Sakane et al., 1991; Throne et al., 1995; Sakane et al., 1997; Chou and Donovan, 1997). Based on the literature, intranasal delivery seems to be a good candidate for those drugs, which act on the central nervous system, such as antiepileptic drugs. However, most of these drugs are generally hydrophobic, so the difficulty lies in the preparation of the intranasal liquid formulation.

In order to increase solubility for hydrophobic drugs and prepare liquid formulation, highly water-soluble cyclodextrins (CDs) are thought to be useful as a solubilizer. An increase in water solubility has been achieved in a large number of drugs using water-soluble CDs (Rjewski and Stella, 1996). In addition to their characteristics for use as a solubilizer, several CDs have already been recognized as mucosal membrane permeation enhancers for drugs (Hermens et al., 1990; Shao et al., 1992; Matsubara et al., 1995; Rjewski and Stella, 1996). Thus, the highly water-soluble CDs may be useful for preparation of the nasal dosage formulations, provided it dose not cause any severe damage to the nasal mucosa already induced by some type of absorption enhancers (Richardson et al., 1989; Hersey and Jackson, 1987). However, available resource data about the local effects of the water-soluble CD on the nasal mucosa by histological studies is limited. In addition, it is important to have the accurate information for concentrations, which are determined safe. Therefore, the histological effects of highly water-soluble CD, such as hydroxypropyl β-CD (HP β-CD) and randomly methylated β-CD (RM β-CD) on the nasal mucosae were evaluated in the in vivo single and repeated administration by light microscopic study. The scanning electron microscopy (Ennis et al., 1990) was also used for evaluating ultra-structural changes induced by exposure to the different solutions. Sodium deoxycholate (SDC) and polyoxyethylene-9-lauryl ether (laureth-9) are used as positive controls (Chandler et al., 1991), and their effects on the nasal mucosae were compared with CDs.

2. Materials and methods

2.1. Chemicals

Laureth-9 and SDC were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). β-CD, HP β-CD and RM β-CD were kindly supplied from Ensuiko Sugar Refining Co., Ltd (Kanagawa, Japan). All other chemicals were of analytical reagent grade.

2.2. Preparation of solutions

All solutions except for high concentration of RM β-CD solution were prepared by dissolving weighed amounts of the compound in sodium phosphate buffer (pH 7.4) and adjusted to the isotonic condition. The final concentrations were expressed in percentage weight-volume (% w/v). These solutions are listed in Table 1. Since solubility of β-CD is quite low (Uekama, 1981), only 1.5% w/v solution was prepared for β-CD. Twenty percent w/v of RM β-CD solution was prepared in purified water. The solution of SDC was adjusted at pH 8.5 in order to prevent gel formation (Blow and Rich, 1960). Each solution was freshly prepared prior to the experiments.

Table 1
Experimental condition in the in vivo exposure study

Compound	Concentration (% w/v)	Nasal exposure time (min)
Laureth-9	1	5
SDC	1	5
β-CD	1.5	5
HP β-CD	5	5
	20	5
	10	30
	10	60
RM β-CD	5	5
	20	5
	10	30
	10	60

2.3. Osmolarity

The osmotic pressures of the solutions were determined with osmotic pressure analyzer (Auto & STAT OM-6010, Kyoto Daiichi Science Co., Ltd, Kyoto, Japan).

2.4. Experimental procedure for the rats

2.4.1. In vivo nasal exposure experiment

The in vivo nasal experiment technique described by Hirai et al. (1981) was used in this study. Some modifications were made in order to adjust the exposure times of the various solutions. Male Wistar rats (Sankyo Labo Service Co., Tokyo, Japan), weighing 230–250 g, were subjected to overnight fasted prior to the experiments but allowed to drink water ad libitum. The rats were anesthetized by an intraperitoneal injection of 60 mg/kg sodium pentobarbital (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan). Then, the rats were tracheotomized to maintain respiration and the esophagus was closed by ligation onto the tracheal cannula. Animals were placed ventral side up on a heating board to maintain normal body temperatures. Next the test solutions were delivered to the nasal cavity (35 μl/sec) via inserted tracheal cannula (Intramedic Polyethylene Tubing, Clay Adams, NJ, USA) using a microsyringe that was attached to a blunt needle. The excess solution drained out from the nares, and the solution that did not flow out remained in the nasal cavity during the exposure period. The rats in the control group received only phosphate buffer. After 5, 30, or 60-min exposure period, the nasal cavity was flushed with isotonic saline solutions to remove the residual test solution. Then, after rats were sacrificed, the nasal septum and left and right lateral walls of nasal cavity were taken from each animal.

2.4.2. In vivo repeated nasal exposure experiment

Repeated dose (each 20 μl) of 5, 10, or 20% w/v RM β-CD solution and 1% w/v laureth-9 (a positive control) were given to the rats every 24 h for 7 consecutive days, via microsyringe through their right nostril. During the administration, the rats were anesthetized by diethylether, and were placed ventral side up. The animals were allowed to eat and drink ad libitum during the experimental period. After the last of the 24-h dosing, the rats were decapitated. The nasal tissue samples were taken for light microscopic study.

2.5. Histological studies

2.5.1. Light microscopy

The nasal tissue samples were gently flushed with neutral phosphate buffered formalin solution. The samples were immediately placed in the same formalin solution and fixed for 1 week. Following fixation, the specimens were decalcified and processed in a conventional manner. Three standard cross sections of the nasal septum were prepared and stained with haematoxylin and eosin (HE) for subsequent examination under light microscope. Neutral mucopolysaccharides were also demonstrated with periodic acid-Schiff staining (PAS) (Hayama, 1989). Photomicrographs were taken from standardized area on medial regions of nasal septum (Fig. 1A) using four grades of magnifications (40, 100, 200 and 400 ×).

2.5.2. Scanning electron microscopy

The above pre-fixation of the nasal tissue was carried out for 24 h at 4 °C. The samples were post-fixed with 1% w/v OsO_4, for 1 h at room temperature. Then the specimens were washed with phosphate buffer dehydrated in ethanol, dried with critical point drying method and sputter coated with platinum to a thickness of 25 nm. The samples were evaluated with a Hitachi S-800 electron microscope at 10 kV. Photomicrographs were taken from standardized area on medial region of the lateral wall of the nasal cavity, which stands midway between nasal vestibule and nasopharynx (Fig. 1B). Each standardized area was photographed incorporating three grades of magnifications (30, 1200 and ∼12000 ×).

3. Results

3.1. Osmolarity of CDs, SDC and laureth-9

Fig. 2 shows osmolarities of CD solutions (A) and SDC and laureth-9 solutions (B) at different

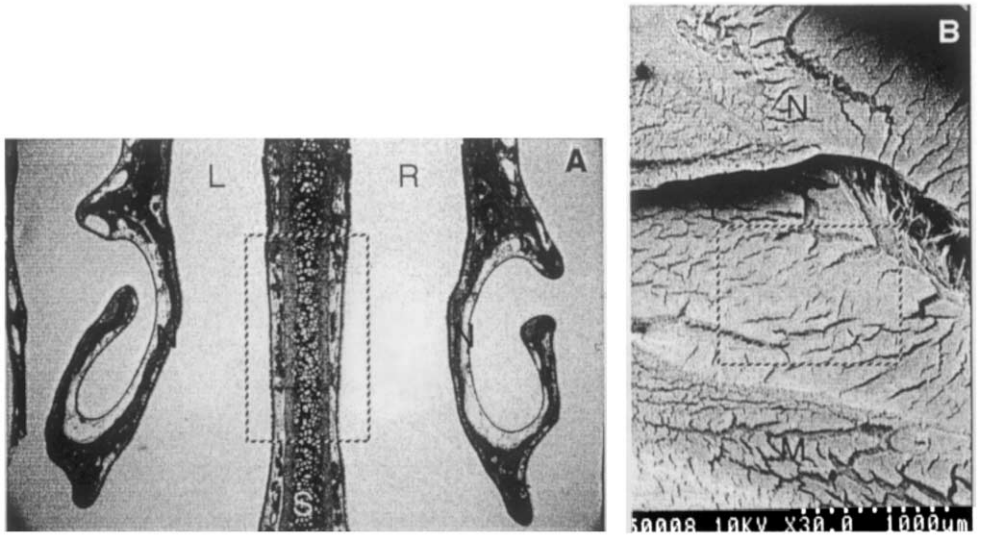

Fig. 1. (A) Typical light microscopic photo of a vertical section through medial region of rat nasal cavity showing the left (L) and right (R) side. The nasal septum (S) is covered with ciliated pseudostratified columnar epithelium (E), and the nasoturbinate (N) is also shown. All light micrographic photos were taken from the medial region of the nasal septum that is designated by rectangles (HE 40 ×). (B) Typical scanning electron microscopic photo of the lateral wall of the right nasal cavity of rat showing the nasoturbinate (N) and maxilloturbinate (M). All scanning electron micrographic photos were taken from the medial region of the lateral wall that is designated by rectangles (SEM 30 ×).

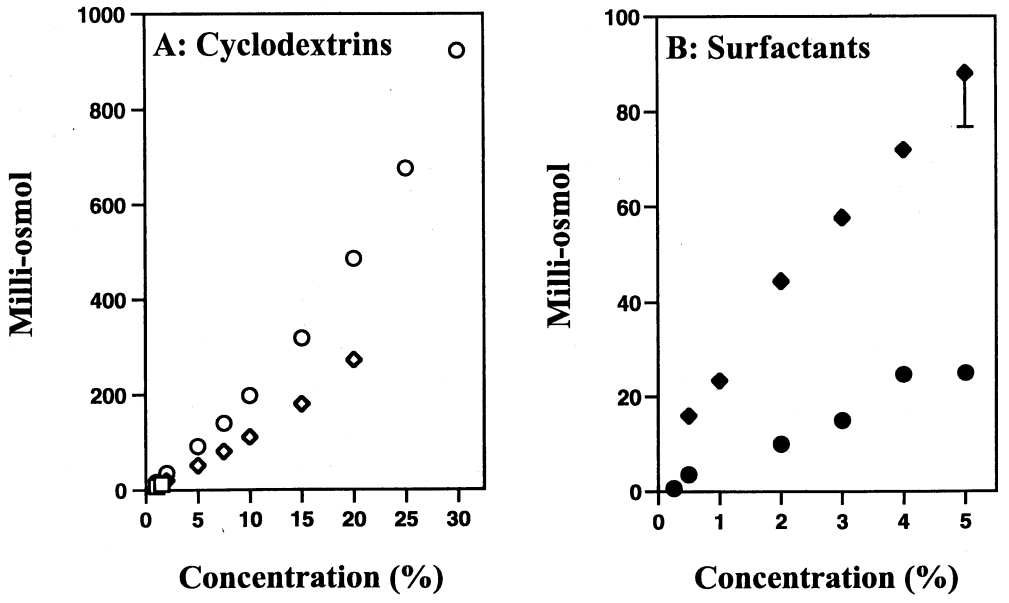

Fig. 2. (A) The osmolarities of β-CD(□), HP β-CD (◇) and RM β-CD (○) in purified water at 25 °C. (B) The osmolarities of SDC (◆) and laureth-9 (●) in purified water at 25 °C. Data are means ± SD ($n = 3$).

concentrations. The osmolarities of SDC and laureth-9 showed a linear relationship with concentrations. On the other hand, there was not a linear relationship between the osmolarities of CDs and concentrations. The osmolarities of test solutions gave hypotonicity except for RM β-CD solutions at the concentration more than 15% w/v. All hypotonic sample solutions were adjusted to the isotonic condition with a phosphate buffered solution.

3.2. In vivo nasal exposure experiment

The medial region of nasal septum from control rats was covered with typical respiratory epithelium (Fig. 3A1 and B1). It was ciliated pseudostratified columnar epithelium, and cilia densely populated. Obviously severe damage to the mucosa was observed from exposing to 1% w/v SDC or laureth-9 even for as little as 5 min (Fig. 3A2 and A3). The epithelia of the treated groups exhibited epithelium disruption and complete loss of some parts of the epithelium. In contrast, the findings of the epithelia exposed to 1.5% w/v β-CD for 5 min were similar with those of the controls (Fig. 3A4 and B2). With the exception of the slight increase of mucous secretion, no severe effects were observed in groups with exposure to HP β-CD for 5 min even at a high concentration (20% w/v) (Fig. 3A5, A6 and B3). While the significant changes were observed in the epithelium of the groups exposed to RM β-CD for 5 min (Fig. 3A7 and A8), it was recognized by PAS staining that the changes of the epithelium were caused by the increased mucous secretions (Fig. 3B4). In the group exposed to 20% w/v RM β-CD, the slight reduction in epithelium thickness was also observed.

The nasal septum exposed to 10% w/v HP β-CD for 30 and 60 min showed slight changes including a reduction in the epithelium height and mucous secretion (Fig. 4A1, A2 and B1). The changes of the epithelium exposed to 10% w/v RM β-CD for 30 and 60 min were similar with those of the epithelium exposed to 5% w/v RM β-CD for 5 min (Fig. 4A3, A4 and B2). Increased mucous secre-

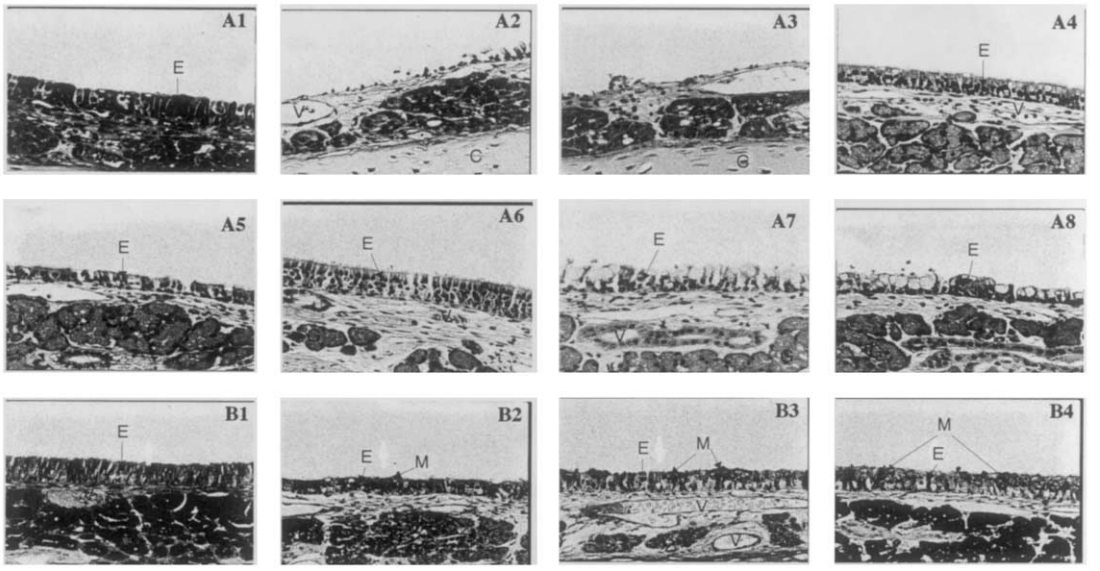

Fig. 3. (A) The light microscopic photos of a HE stained vertical sections of the medial region of nasal septum of rat after a 5 min exposure of isotonic phosphate buffer solution (A1), 1% w/v SDC (A2), 1% w/v laureth-9 (A3), 1.5% w/v β-CD (A4), 5% w/v HP β-CD (A5), 20% w/v HP β-CD (A6), 5% w/v RM β-CD (A7) and 20% w/v RM β-CD (A8). (B) The light microscopic photos of a PAS stained vertical sections of the medial region of nasal septum of rat after a 5 min exposure of isotonic phosphate buffer solution (B1), 1.5% w/v β-CD (B2), 5% w/v HP β-CD (B3) and 5% w/v RM β-CD (B4). E, ciliated pseudostratified columnar epithelium; C, cartilage; G, glandular tissue; M, discharged mucous V; vascular sinus (400 ×).

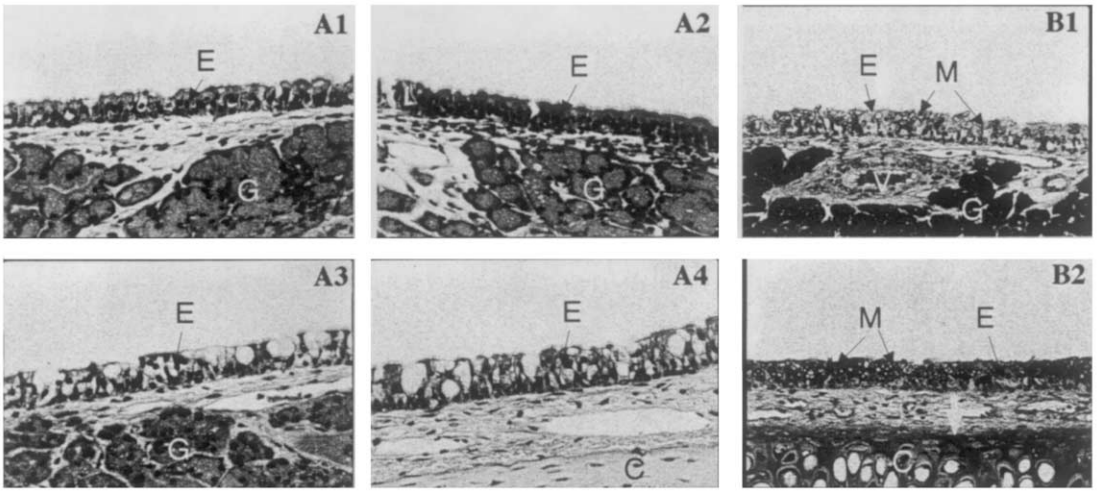

Fig. 4. (A) The light microscopic photos of a HE stained vertical section of the medial region of nasal septum of rat after a 30 min exposure (A1), 60 min exposure (A2) of 10% w/v HP β-CD solutions, and 30 min exposure (A3), 60 min exposure (A4) of 10% w/v RM β-CD solutions. (B) The light microscopic photos of a PAS stained vertical section of the medial region of nasal septum of rat after a 30 min exposure of 10% w/v HP β-CD solutions (B1) and 30 min exposure of 10% w/v RM β-CD solutions (B2). E, ciliated pseudostratified columnar epithelium; C, cartilage; G, glandular tissue; M, discharged mucous; V, vascular sinus (400 ×).

tions were also observed, however the reductions in the epithelium height were not shown (Fig. 4A3 and A4).

Fig. 5A1 and A2 showed the medial region of the right lateral wall of nasal cavity exposed to isotonic phosphate buffer (control) analyzed by scanning electron microscopy. The scanning electron micrographic photos obtained from the medial region on lateral wall of nasal cavity clearly and more precisely show morphological change of the nasal epithelium. As shown in these figures, the ciliated cells densely populated in controls. The morphological appearances of the epithelium exposed to 1.5% w/v β-CD for 5 min were the same as those of the controls (Fig. 5B1 and B2). Similarly, there was no difference in the morphological appearances between controls and those groups treated with 5 or 20% w/v HP β-CD for 5 min (Fig. 6A1 and A2). In addition, no gross change of the epithelium was seen after 30 or 60 min exposure of 10% w/v HP β-CD (Fig. 7A1 and A2). Furthermore, an exposure to 5% w/v RM β-CD for 5 min did not show any discernible changes on the epithelium (Fig. 6B1). Even the epithelium treated with 10% w/v RM β-CD for 30 or 60 min, no gross change was observed by the scanning electron micrographic photos (Fig. 7B1 and B2). However, the treatment of 20% w/v RM β-CD for 5 min induced severe morphological change (Fig. 6B2). Cilia depletion occurred and large cracks were seen.

3.3. *In vivo repeated nasal exposure*

Fig. 8Al showed the vertical section from the medial region of rat nasal cavity at 5 min after single dosing of 20 μl of pH 7.4 isotonic phosphate buffered solution into the right side of the nasal cavity. The right side of nasal septum was covered with ciliated pseudostratified columnar cells (Fig. 8A2). Seven repeated doses of 20 μl of 1% w/v laureth-9 resulted in the epithelium disruption on septum and nasoturbinate surfaces on the administered side of the nasal cavity (Fig. 8B1). On the other hand, 7 repeated doses of 20 μl 5, 10 and 20% w/v RM β-CD did not induce the epithelium disruption on the administered side. The nasal septum of the administered side showed no change compared with the control side (Fig. 9A1, B1 and C1). The increased mucous secretion, which was observed in the in vivo nasal exposure experiments

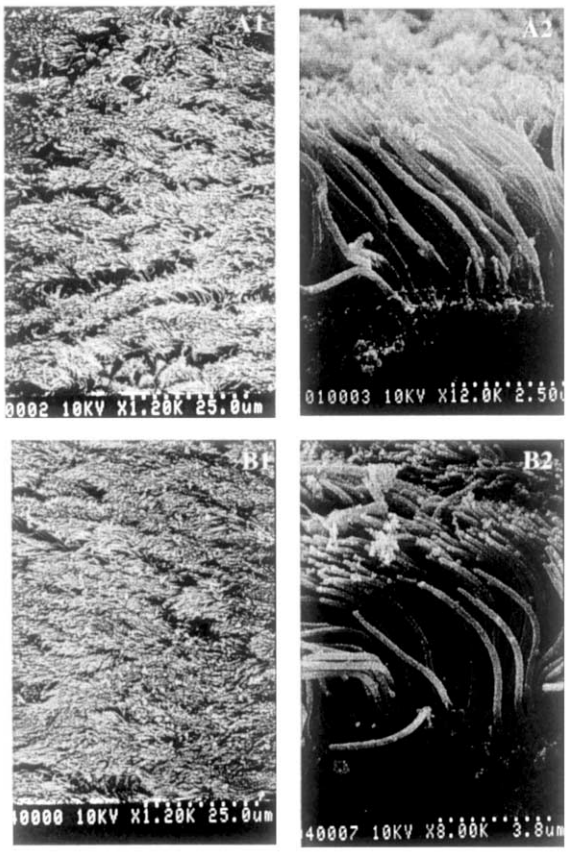

Fig. 5. The scanning electron microscopic photos of the medial region of lateral wall of rat nasal cavity after 5 min exposure of isotonic phosphate buffer solution (A1: 1200 ×, A2: 12 000 ×) and 1.5% w/v β-CD solutions (B1: 1200 ×, B2: 8000 ×).

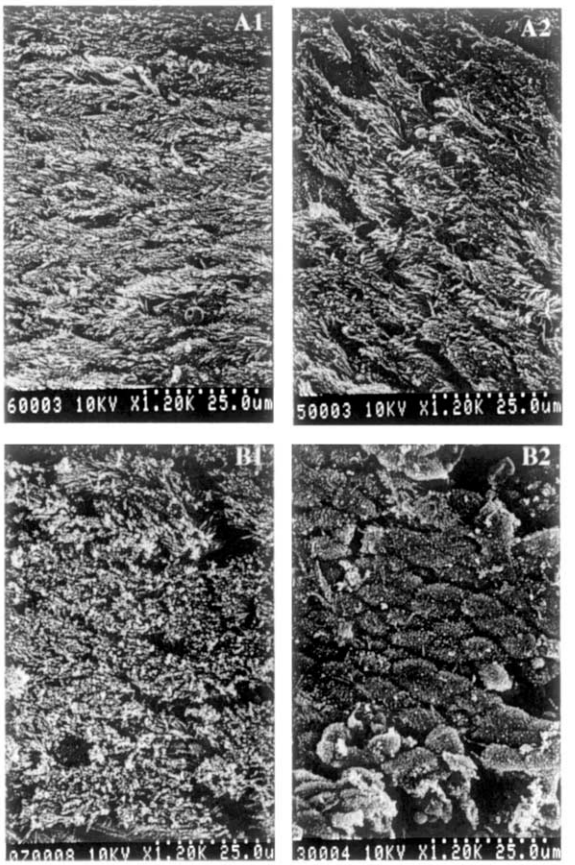

Fig. 6. The scanning election microscopic photos of the medial region of lateral wall of rat nasal cavity after 5 min exposure of 5% w/v HP β-CD (A1), 20% w/v HP β-CD solution (A2), 5% w/v RM β-CD (B1) and 20% w/v RM β-CD solution (B2) (1200 ×).

for 5 min, were not shown with the repeated dosing of RM β-CD (Fig. 9A2, B2 and C2).

4. Discussion

The primary objective in this study was to clarify the effects of highly water-soluble CDS on the histological integrity of the rat nasal mucosa, in order to ascertain an acceptability of these materials as solubilizer for the nasal liquid formulation. The CDS induced lysis of membranes by removal of membrane components, such as cholesterol from the cells, phospholipids, and in particular phosphatidylcholine and sphingomyelin, from the outer half of the membrane bilayer (Irie and Uekama, 1997) and proteins (Ohtani et al., 1989). If these untoward effects occurred extensively, the mucosal membrane would be subjected to easy destruction. On the other hand, the mechanisms of cytotoxicity induced by CDs are yet to be clearly understood. Thus, it is considered that the investigations of extracting membrane components, such as cholesterol, phospholipids and so on are insufficient for the practical and specific evaluation of histological effects on a limited area. We employed, therefore, histological analysis in this study in order to evaluate cytotoxic effect of CDs directly. The in vivo rat nasal exposure experiments were useful

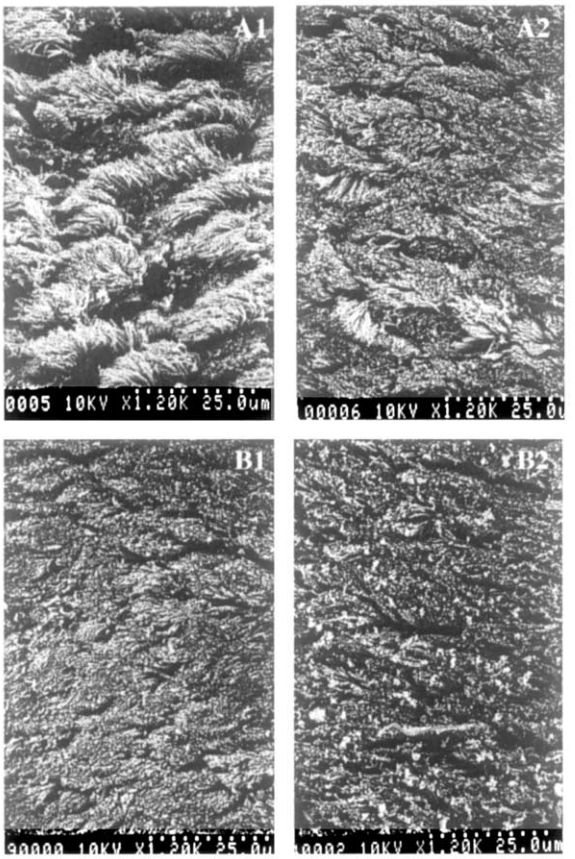

Fig. 7. The scanning electron microscopic photos of the medial region of lateral wall of rat nasal cavity after exposure to 10% HP β-CD solution for 30 min (A1) and 60 min (A2), and 10% RM β-CD solution of 30 min (B1) and 60 min (B2) (1200 ×).

for the critical and absolute evaluation of the toxic effects on the rat nasal mucosa induced by the liquid formulations, though the method is relatively excessive when compared to an actual clinical condition. The in vivo repeated nasal exposure experiments in rat were more practical, and therefore possible to assess for an acute toxicity in the clinical condition. In this study, the administration of test solution was performed to only one side of nostril; therefore, the effects on the nasal mucosa were easily compared to the other non-treated nasal epithelia.

Although a high concentration of water-soluble CD would have an advantage with solubilization, it was reported that hypotonic and/or hypertonic solutions caused structural change of the nasal epithelial cells when compared to the controls (Ohwaki et al., 1985, 1987; Pujara et al., 1995). In order to exclude such an additional effect on the nasal mucosa, the osmolarities of water-soluble CDs, SDC and laureth-9 were measured and were adjusted to an isotonic condition by phosphate buffer. The histological effects on the rat nasal mucosa after exposures to active controls (SDC and laureth-9) were consistent with the published data (Richardson et al., 1989; Hersey and Jackson, 1987). They were thought to be extensively toxic. In contrast there were no significant differences between 5 min exposure for β-CD, HP β-CD and controls (Fig. 3A1, A4, A5 and A6). In addition, the unusual long time (30 and 60 min) exposure for 10% w/v HP β-CD showed no structural changes of the epithelia in the nasal mucosa (Fig. 4A1 and A2). On the other hand, the exposures for RM β-CD showed significant changes of the epithelia in HE staining, especially at 20% w/v concentration (Fig. 3A7 and A8). However, it was appeared from PAS staining that the changes of the epithelium were caused by the increased mucous secretions (Fig. 3B4), therefore, the observed morphological changes were thought not to be structural but due to irritation. The long time (30 and 60 min) exposures for 10% w/v RM β-CD also showed an increase mucous secretion (Fig. 4A3 and, A4). However, it was thought that the morphological changes in the epithelia exposed to 10% w/v RM β-CD (long time) were milder than those exposed to 5% w/v RM β-CD. During the exposure period, the local concentration of RM β-CD was subjected to possible dilution through secreted mucous. Thus, the irritant effect of 10% w/v RM β-CD might not appear so severely.

The safety of β-CD, HP β-CD, RM β-CD (except for the case of 20% w/v RM β-CD) was also confirmed from the scanning electron micrographic photos obtained from medial region of lateral wall of nasal cavity (Fig. 1B). The ciliated cells densely populated (Fig. 5B1 and B2, Fig. 6A1, A2 and B1) in these treated epithelia. The exposure to 20% w/v RM β-CD showed obvious cellular changes such as depletion of cilia and large cracks (Fig. 6B2). The 30 or 60 min exposures to 10% w/v HP β-CD or RM β-CD showed no gross

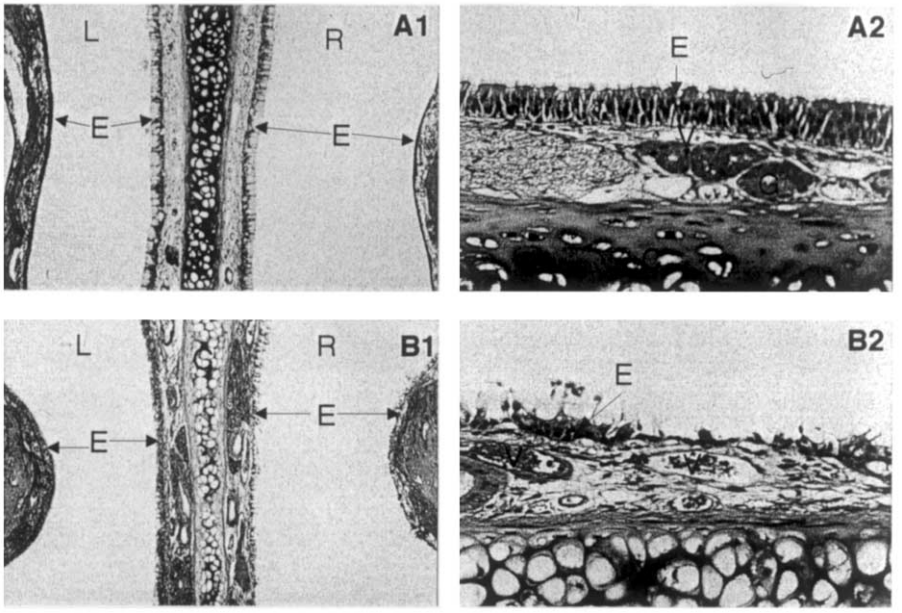

Fig. 8. The light microscopic photos of a HE stained vertical section of the medial region of the rat nasal cavity (A1 and B1: 40 ×) and septum (A2 and B2: 400 ×). A: 5 min after single dosing of 20 μl isotonic phosphate buffer solution into the right side (R) of nasal cavity. B: 24 h after 7 times repeated dosing of 20 μl laureth-9 (1% w/v) into the right side (R) of nasal cavity.

structural changes, however, it was appeared that some fused cilia might be covered with mucous secretions from exposing to RM β-CD for 60 min (Fig. 7B2). It seemed likely that the increased mucous secretion could block the untoward effect of 10% w/v RM β-CD to the cilia.

In the study of the in vivo repeated nasal exposure, 1% w/v laureth-9 caused nasal epithelium disruption (Fig. 8B1 and B2). The findings were similar to those studied in the in vivo exposure experiments. However, 7 day in vivo repeated administration of RM β-CD solutions (5, 10 and 20% w/v) were well tolerated. After repeated exposure to RM β-CD, there were no structural changes compared to controls (Fig. 9). These results were consistent with a previous report, in which 2% dimethyl β-CD were administered to human twice daily over several months (Hermens et al., 1991). In the in vivo situation, dilution and mucociliary clearance contributed to further decrease in local concentrations of the applied compounds (Gizurarson, 1993) and may therefore reduce the effects on the nasal membrane. The methylated derivatives of CDs are most effective as solubilizer. Indeed, dimethyl β-CD and RM β-CD are effective at low concentrations ranging between 2 and 5% (Marttin et al., 1995), therefore, it seems that RM β-CD can be also used safely at the effective concentration in the clinical condition.

5. Conclusions

The present study indicates that less than 20% w/v solutions of HP β-CD and 10% w/v RM β-CD do not induce gross tissue damage, and in vivo situation, repeated RM β-CD doses do not cause irritation to the nasal mucosa. Therefore, the highly water-soluble CDs are thought to be good candidates for the solubilizers of intranasal formulations.

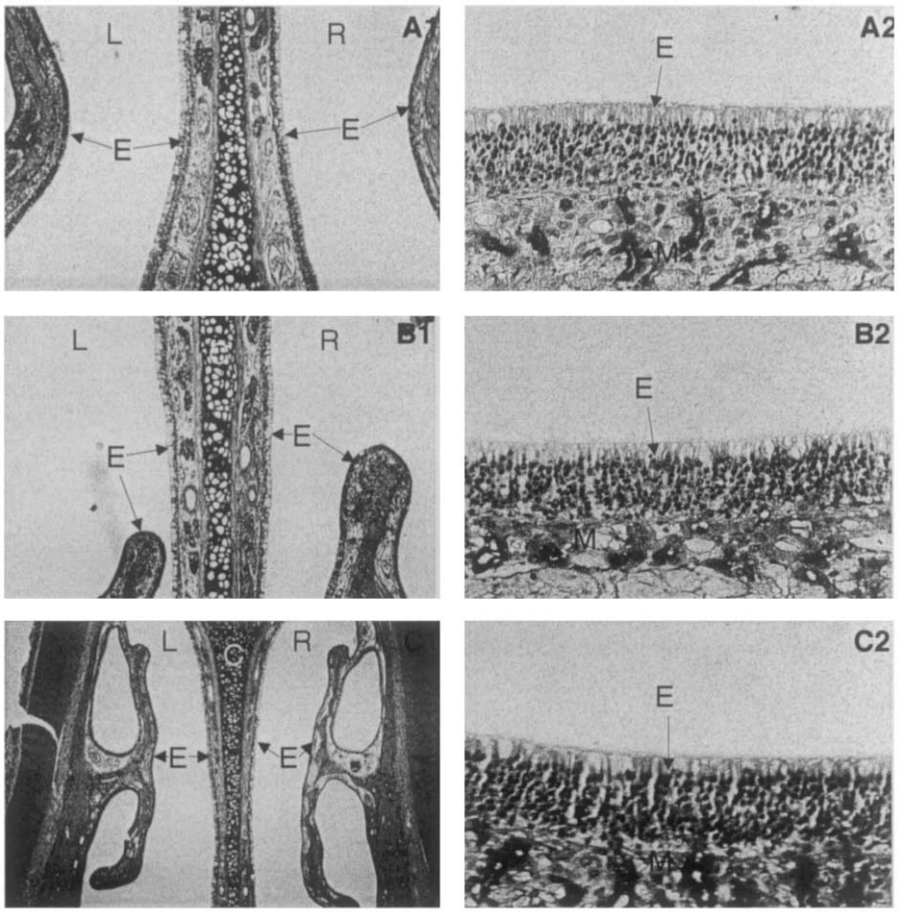

Fig. 9. The light microscopic photos of a HE stained vertical section of the medial region of the rat nasal cavity at 24 h after 7 times repeated dosing of 20 μl 5% w/v (A1), 10% w/v (B1) and 20% w/v RM β-CD (C1) into the right side (R) of nasal cavity (40 ×). The light microscopic photos of a PAS stained vertical section of the medical region of rat nasal septum at 24 h after 7 times repeated dosing of 20 μl 5% w/v (A2), 10% w/v (B2) and 20% w/v RM β-CD (C2) into the right side (R) of nasal cavity (400 ×).

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