

Lowering of sodium deoxycholate-induced nasal ciliotoxicity with cyclodextrins¹

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KEY WORDS deoxycholic acid; cyclodextrins; erythrocyte membrane; nasal mucosa; mucociliary clearance; differential scanning calorimetry

ABSTRACT

AIM: To lower the nasal ciliotoxicity of sodium deoxycholate (SDC) in combination with cyclodextrins (CD). **METHODS:** The erythrocyte hemolysis test was carried out to evaluate the damaging effect of SDC on the erythrocyte membrane. The *in situ* toad palate model and scanning electron microscope technique were used to investigate the nasal ciliotoxicity of SDC solution in combination with CD. The inclusion effect between SDC and β -cyclodextrin (β -CD) was studied by differential thermal analysis (DTA) and X-ray diffractometry. **RESULTS:** The hemolysis test showed that β -CD and dimethyl- β -cyclodextrin (DM- β -CD) could effectively protect the erythrocyte membrane against damage by SDC at the molar ratios of 1:1 and 2:1. When SDC combined with β -CD or DM- β -CD at a molar ratio of 1:2 or 1:3, the ciliotoxicity of SDC was greatly alleviated and the percent lasting time of the ciliary movement increased to 50% or above. Scanning electron microscope investigations showed that SDC combined with β -CD at a molar ratio 1:2 had no marked damage on the rat nasal mucosa after nasal administration thrice a day for a week. DTA and X-ray diffractometry investigations showed that SDC formed an inclusion with β -CD. **CONCLUSION:** Combining β -CD or DM- β -CD with SDC can greatly lower the hemolytic effect and ciliotoxicity of SDC and the optimal molar ratio of SDC to CD is 1:2. Such protection provided by CD is due to the inclusion effect between SDC and CD.

INTRODUCTION

The surge of new peptide and protein drugs urges the development of innovative delivery systems. Of all the available parental routes such as buccal, nasal, ocular, rectal, and vaginal, and the nasal route is perhaps the most prospective one. However many peptide and protein drugs commonly show low nasal bioavailabilities, which do not meet the clinical demands. When peptide and protein drugs are administered with a variety of absorption enhancers they can transport through the nasal membrane faster and more effectively⁽¹⁾. But many *in vitro* or *in vivo* tests proved that most of these promoters had a damaging effect on the nasal membrane^(2,3). So the potential for these enhancers to cause mucosal damage and irritation is a concern, and limits their clinical use, especially in the chronic therapy. In nasal administration, cyclodextrins (CD) can be used as absorption enhancers of peptide and protein drugs, which have low nasal bioavailability⁽⁴⁾. And it is reported that CD could decrease the toxicity of some absorption enhancers including glycodeoxycholate, Laureth-9, and lysophosphatidylcholine^(5,6).

In this study, we used three *in vitro* and *in situ* methods to investigate the nasal ciliotoxicity of sodium deoxycholate (SDC), which was a powerful absorption enhancer in nasal administration, in combination with two kinds of CD. We also studied the inclusion effect between β -CD and SDC to explain the protection mechanism. The major aim of this study was to lower the serious nasal ciliotoxicity of SDC thus improving the application value of this nasal absorption enhancer.

MATERIALS AND METHODS

Drugs and reagents SDC was purchased from Sino-American Biotechnology Company. β -CD and DM- β -CD were obtained from Tokyo Food Institution, Japan.

Erythrocyte hemolysis Blood from healthy

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human subjects was mixed with the anticoagulant heparin. Blood sample was centrifuged and the plasma was removed. The erythrocytes were then washed three times with phosphate buffer saline (PBS, pH 7.0). The erythrocyte stock solution was prepared by diluting 1 mL of packed cells with 7 mL PBS, and it was used within 12 h. The test solution was prepared by dissolving SDC with or without CD at various molar ratios in PBS. Erythrocyte stock solution 0.125 mL was added into 5 mL test solution. After 10 min incubation at 37 °C, the mixture was cooled immediately and centrifuged for 2 min at $540 \times g$. The optical density (OD) of the supernatant was measured at 540 nm to determine the percentage of hemolysis. The total hemolysis was estimated by incubating the cells with distilled water and basal value for hemolysis was estimated by incubating the cells with PBS. The hemolysis of each test solution was determined in duplicate.

Nasal ciliotoxicity The test solutions were prepared by dissolving the required amount of compounds in the control solution (saline). Nasal ciliotoxicity studies were carried out in *in situ* toad palate model^[7]. In brief, the upper palate of toad (35 g \pm 4 g, ♂ ♀, Experimental Animal Center, Fudan University) was treated with 0.5 mL of test solution for 1 h, then rinsed with saline. The palate was dissected out and the mucocilia was examined with an optical microscope and the lasting time of the ciliary movement was calculated as: Percent lasting time of ciliary movement = $t_{\text{Test solution}}/t_{\text{Saline}} \times 100\%$, $t_{\text{Test solution}}$ and t_{Saline} stands for the lasting time of ciliary movement after the mucocilia is contacted with test solution and saline, respectively.

Scanning electron microscopy observations Sprague-Dawley rats ($n = 15$, 248 g \pm 33 g, ♂ ♀, Grade II, Certificate No 005, Experimental Animal Center, Fudan University) were randomly divided into 3 groups with five rats in each group. Test solution 100 μL was administered to one side of the rat nasal nostril thrice a day for a week. Group A was administered 0.75 % SDC solution combined with β -CD at molar ratio of 1:2. Group B and C were administered saline and 0.75 % SDC as negative and positive control, respectively. Twenty-four hours after the last administration, rats were killed and the nasal septum mucosa was removed and its surface was washed with cold saline. The tissue sample was fixed with 2.5 % glutaraldehyde solution and 1 % osmic acid. Then, the

sample was dehydrated by a series of ethanol dilutions, replaced by *n*-amyl acetate, dried at critical pointer of carbon dioxide, coated with gold by an ion coater, and examined by a scanning electron microscope (S-520, HITACHI, Japan).

Differential thermal analysis (DTA) The powder of SDC- β -CD inclusion was precipitated by acetone. DTA curves were recorded by a CDR-1 DTA machine. Each sample (inclusion, physical mixture at molar ratio of 1:2 and pure components) was scanned at a speed of 10 °C/min, in the scanning range of $\pm 100 \mu\text{V}$.

X-ray powder diffractometry X-ray of the samples (inclusions, physical mixture at molar ratio of 1:2, and pure components) were recorded using a D-500 X-ray Diffractometer (SIEMENS) under the following conditions: Cu radiation, angular speed 2°(2 θ)/min.

RESULTS

Erythrocyte hemolysis When the three compounds were investigated separately, it was observed that the hemolysis started at a concentration of 0.03 % SDC 0.72 mmol/L, 0.2 % DM- β -CD 1.72 mmol/L, 1.18 % β -CD 10.4 mmol/L. Therefore, the order of hemolytic potency of these compounds was SDC > DM- β -CD > β -CD. β -CD completely protected the erythrocytes from the hemolytic effect of SDC, when SDC 0.9 mmol/L and 1.8 mmol/L was mixed with β -CD at molar ratios of 1:1 and 1:2, respectively (Fig 1). DM- β -CD completely protected the erythrocytes from the hemolytic effect of SDC when SDC 0.9 mmol/L and 1.8 mmol/L was mixed with DM- β -CD at a molar ratio of 1:1. As the molar ratio of SDC to DM- β -CD was 1:2, the erythrocytes were observed to be partially protected from the hemolytic effect of SDC (Fig 2).

Nasal ciliotoxicity Optical microscope results showed that there were a great number of cilia beating actively on the edge of mucosa that was treated with saline for 1 h, and the lasting time of ciliary movement of saline was (2040 \pm 51) min. Cilia beat was slower and then stopped when treated with 0.5 %, 0.75 %, and 1.0 % SDC, indicating that SDC, alone had a damaging effect on the cilia movement, so the percent lasting time of ciliary movement was 0 % in all. The effects of 3.2 % β -CD and 5 % DM- β -CD on the ciliary movement were moderate (percent lasting time was 56.2 % \pm 2.9 % and 58 % \pm 3 %, respectively). DM- β -CD 10 % reduced cilia movement partially, and

the percent lasting time decreased to 41 % ± 18 %. But this effect was much less than that found in SDC solution.

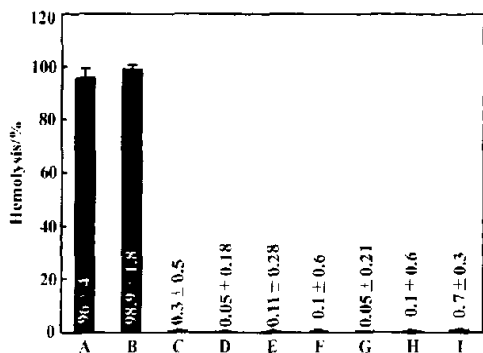


Fig 1. Effect of various molar ratios of β -CD on hemolysis caused by SDC. A: SDC 0.9 mmol/L; B: SDC 1.8 mmol/L; C: β -CD 0.9 mmol/L; D: β -CD 1.8 mmol/L; E: β -CD 3.6 mmol/L; F: SDC (0.9 mmol/L): β -CD = 1:1; G: SDC (0.9 mmol/L): β -CD = 1:2; H: SDC (1.8 mmol/L): β -CD = 1:1; I: SDC (1.8 mmol/L): β -CD = 1:2.

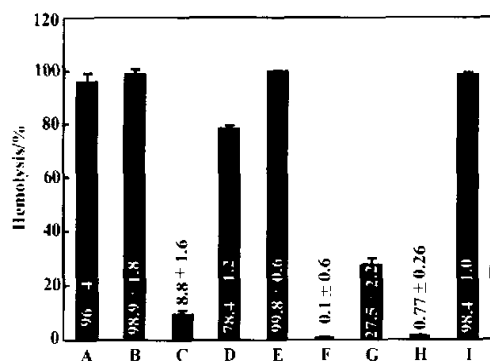


Fig 2. Effect of various molar ratios of DM- β -CD on hemolysis caused by SDC. A: SDC 0.9 mmol/L; B: SDC 1.8 mmol/L; C: DM- β -CD 0.9 mmol/L; D: DM- β -CD 1.8 mmol/L; E: DM- β -CD 3.6 mmol/L; F: SDC (0.9 mmol/L): DM- β -CD = 1:1; G: SDC (0.9 mmol/L): DM- β -CD = 1:2; H: SDC (1.8 mmol/L): DM- β -CD = 1:1; I: SDC (1.8 mmol/L): DM- β -CD = 1:2.

The damaging effects exhibited by the three concentrations of SDC solutions were lowered when they were combined with CD at different molar ratios, and the lasting time of ciliary movements were prolonged markedly (Tab 1). The molar ratio of SDC to β -CD or DM- β -CD influenced the improvement in cilia movement greatly. β -CD and DM- β -CD completely protected the

mucosa from the damaging effects of 0.5 %, 0.75 %, and 1.0 % SDC when they were mixed with SDC at the molar ratios of 2:1 and 3:1. Optical microscopy results showed that the edge of the mucosa were integrated with all the cilia beating actively. However, at the molar ratios of 1:1, the CD only partially protected the mucosa from damage. It was observed that some cilia fell off from the edge of the mucosa, and the remaining was still beating, but the lasting time of ciliary movement decreased.

Tab 1. Effect of SDC saline solutions combined with β -CD or DM- β -CD at various molar ratios on percent lasting time of ciliary movement (%) in the *in situ* toad palate. $n = 5$. $\bar{x} \pm s$. ^b $P < 0.05$, ^c $P < 0.01$ vs SDC: β -CD = 1:1 group. ^d $P > 0.05$ vs SDC: β -CD = 1:2 group. ^e $P < 0.05$ vs SDC: DM- β -CD = 1:1 group. ^f $P > 0.05$ vs SDC: DM- β -CD = 1:2 group.

Molar ratio of SDC to CD	Percent of lasting time/%		
	0.5 %	0.75 %	1.0 %
SDC: β -CD = 1:1	47 ± 9	5.5 ± 1.4	25 ± 17
SDC: β -CD = 1:2	73 ± 13 ^b	64 ± 13 ^c	51.3 ± 1.5 ^c
SDC: β -CD = 1:3	74 ± 14 ^{bd}	68 ± 10 ^{bd}	-
SDC: DM- β -CD = 1:1	57 ± 6	27 ± 3	26.0 ± 2.3
SDC: DM- β -CD = 1:2	65.0 ± 2.9 ^b	72 ± 9 ^b	58 ± 6 ^b
SDC: DM- β -CD = 1:3	77 ± 7 ^b	67.6 ± 2.3 ^{bi}	68 ± 8 ^b

Scanning electron microscopy As shown in Fig 3, cilia on the nasal mucosa treated with 0.75 % SDC solution combined with β -CD at molar ratio of 1:2 were similar with that of the negative control (physiologic saline). In contrast, the cilia on the nasal mucosa treated with 0.75 % SDC alone were seriously denuded. The result suggested that 0.75 % SDC combined with β -CD at molar ratio of 1:2 did not damage the rat nasal mucosa after nasal administration thrice a day for one week.

Inclusion effect between β -CD and SDC The DTA thermograms obtained for SDC- β -CD system are shown in Fig 4. SDC crystals show the characteristic exothermic peak at 200 °C. The DTA curve of β -CD exhibits a big endothermic peak at 100 °C due to water loss. These two peaks were evident in the DTA thermogram of the physical mixture. The exothermic peak of SDC disappeared in the thermogram of the solid inclusion, while a new exothermic peak appeared at 298 °C. X-ray diffraction curves (Fig 5) of the SDC- β -

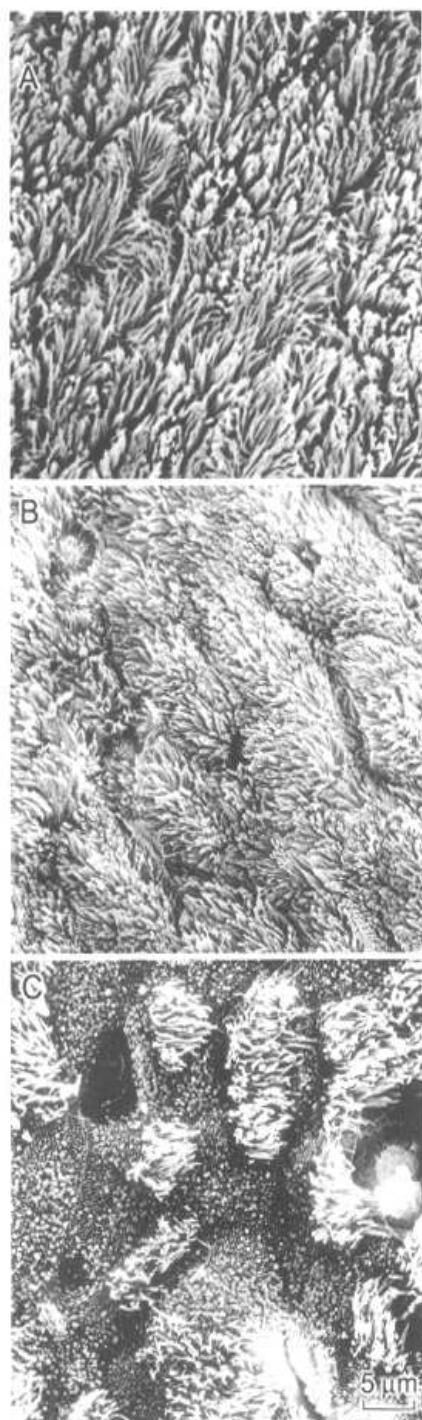


Fig 3. Scanning electron micrographs of the rat nasal mucosa one week after nasal administration of test solution thrice a day. $\times 2000$. A) 0.75 % SDC combined with β -CD at molar ratio of 1:2; B) Saline; C) 0.75 % SDC.

CD system showed the amorphous state of the sample. The disappearance of the SDC signals while presented in the physical mixture showed the existence of a new solid phase having a lower crystallinity than SDC and β -CD.

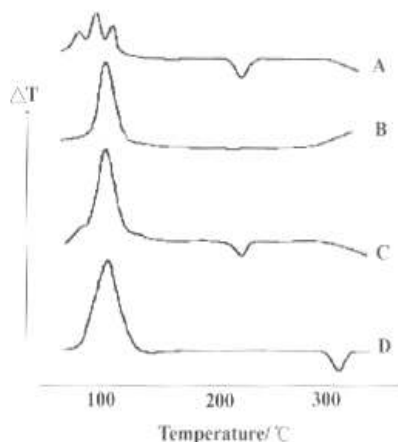


Fig 4. DTA thermograms of SDC- β -CD systems. A) SDC; B) β -CD; C) Physical mixture; D) Inclusion.

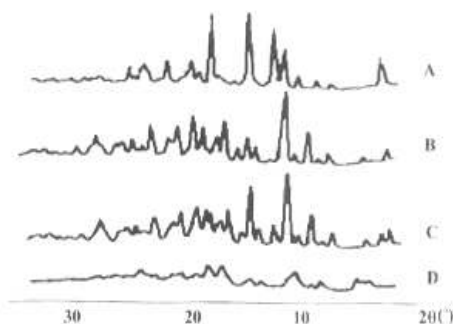


Fig 5. X-ray diffractory curves of SDC- β -CD systems. A) SDC; B) β -CD; C) Physical mixture; D) Inclusion.

DISCUSSION

There are some hypotheses to explain the hemolytic effect caused by sodium deoxycholate. One explanation is that at concentrations lower than the critical micellar concentration, the solubilizing effect of sodium deoxycholate is solely due to their incorporation into cell membranes and solubilization of membrane components. The cyclodextrin-induced hemolysis is probably a result of rapid and reversible formation of inclusion in which the lipophilic components of erythrocyte membranes are partially included into the cyclodextrin cavity^[8]. The solubilizing potency of the cyclodextrins depends on the

size and the hydrophobicity of the inclusion cavity in which specific lipids can be included. It is possible that the observed decrease in the hemolytic activity of β -CD as compared with DM- β -CD may be due to the decreased ability of β -CD to remove membrane components of the erythrocytes.

SDC 0.9 mmol/L and 1.8 mmol/L caused complete hemolysis, while in combination with CD especially β -CD the percent hemolysis of SDC decreased to about 0%. This can be explained by the inclusion effect between SDC and β -CD. DTA and X-ray diffractory results showed SDC could form inclusion with β -CD, thus it could not be adsorbed into the membrane of erythrocytes and cause hemolysis. DM- β -CD 1.8 mmol/L on its own would have been expected to lyse the erythrocytes; however, when it was mixed with SDC at molar ratio 1:1, the erythrocytes were fully protected (Fig 2). It is possible that SDC forms a 1:1 inclusion of DM- β -CD, hence making the cavity unavailable for interaction with the membrane components of the erythrocytes and when the molar ratio of SDC to DM- β -CD was 1:2, excessive DM- β -CD in the solution caused hemolysis again. Because the DM- β -CD is soluble in water and many kinds of organic solvents, it is difficult to obtain the solid form of SDC-DM- β -CD inclusion, so we did not study the inclusion effect between SDC and DM- β -CD.

We found that the lasting time of ciliary movement became longer with increase in the molar ratio of CD to SDC. But difference was not significant between SDC; CD = 1:2 and SDC; CD = 1:3 group (Tab 1). This was due to the moderate effect of CD on ciliary beating⁽⁹⁾. With the increase of the concentration of CD, the ciliotoxicity of CD began to appear, especially of DM- β -CD. Combining with the scanning electron microscopy results, we conclude that the optimal molar ratio for lowering ciliotoxicity of SDC is 1:2 (SDC:CD).

Erythrocyte hemolysis is commonly used as a model to investigate membrane interactions. In research of nasal drug delivery systems the effect of enhancers on the erythrocyte membrane partially correlates with their effect on nasal mucosa. A good relationship has been established between the effect of absorption promoters on nasal ciliary beating and their influence on nasal tissue integrity. One of the models used for studying the effects of drugs on nasal ciliary movement is the toad palate model. Toad palate is a robust tissue giving reproducible results and the experimental technique is

easy⁽¹⁰⁾. The order of the hemolytic effect and nasal ciliotoxicity of the three compounds tested in this study was SDC >> DM- β -CD > β -CD. But there still existed discrepancy on the optimal molar ratio of SDC to CD for lowering ciliotoxicity of SDC. Such discrepancy can be explained by the difference between the *in vitro* and *in situ* studies. This *in vitro* hemolytic model cannot imitate the physical conditions *in vivo* well, so it is useful for screening compounds with respect to their effect on nasal epithelium, but the results of this methods is not fully predictive of the ultimate effects on the nasal mucosa and mucociliary movement *in vivo*. Compared with the hemolytic model, this toad palate model is closer to the real physical conditions, so the results are more predictive. In the present study, the result of the scanning electron microscopy also confirmed it.

We conclude that combining β -CD or DM- β -CD with SDC can greatly lower the hemolytic effect and ciliotoxicity of SDC, and the optimal molar ratio of SDC to CD was 1:2. Such protection provided by CD is due to the inclusion effect between SDC and CD.

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环糊精降低脱氧胆酸钠诱导的鼻纤毛毒性¹

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关键词 脱氧胆酸; 环糊精类; 红细胞膜; 鼻粘膜; 粘膜纤毛清除; 差示扫描量热法

目的: 用环糊精改善鼻腔吸收促进剂去氧胆酸钠(SDC)的鼻纤毛毒性。**方法:** 分别用红细胞溶血实验、蟾蜍上腭模型和扫描电镜观察环糊精对 SDC 细

胞膜破坏作用和鼻纤毛毒性的影响。SDC 和 β -环糊精(β -CD)之间的包合作用采用差示热分析法和 X-衍射法进行验证。**结果:** 当 SDC 与 β -CD 或二甲基- β -环糊精(DM- β -CD)的摩尔比为 1:1 和 1:2 时能完全掩盖 SDC 的溶血作用。用蟾蜍上腭模型评价时, 加入摩尔比 1:2 或 1:3 的 SDC 与 β -CD 或 DM- β -CD 的混合液使 SDC 的相对纤毛持续运动时间由原来的 0% 提高至 50% 以上。大鼠鼻腔连续给予 1:2 摩尔比的 SDC- β -CD 溶液一周后, 扫描电镜显示粘膜纤毛无明显改变。差示热分析法和 X-衍射结果显示 SDC 能与 β -CD 形成包合物。**结论:** 加入 β -CD 或 DM- β -CD 能显著降低 SDC 的溶血作用和鼻纤毛毒性, 两者的最佳比例是 SDC 与环糊精摩尔比 1:2。环糊精的这种保护作用与形成包合物有关。

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