Effect of Absorption Enhancers on Ciliated Epithelium: A Novel *In Vivo* Toxicity Screening Method Using Rotifers

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INTRODUCTION

Nasal absorption of poorly absorbable compounds, such as peptides and proteins, can be improved by the coadministration of absorption enhancers like bile salts, fusidates, phospholipids and cyclodextrins (1–2). Many of these absorption enhancers are ciliotoxic as has been estimated by measuring their effect on mucociliary transport rate with the frog palate model and on the ciliary beat frequency of human adenoid and chicken embryonal trachea tissue (3–4). Membrane damaging effects have been demonstrated with the nasal epithelium of the rat by scanning electron microscopy, by histological studies and by the release of marker molecules from the nasal mucosa (5–6).

Toxicity testing using vertebrate animals to evaluate the safety of xenobiotics to humans has recently been heavily criticised because of ethical and financial considerations. The principal alternative to animal testing is *in vitro* testing. Many factors, as nervous control, systemic blood flow, reduced motility and heterogeneous cell populations, however, are absent in simple cell culture models.

The objective of this study was to develop a simple and inexpensive alternative test using non-vertebrates as model organism. We investigated if the rotifer *Brachionus calyciflorus* could be used as a test organism for screening the toxicity of absorption enhancers on ciliated epithelium. The main advantage of using rotifers as model organism is the ability to produce resting eggs which can be stored for months in dry conditions and hatched upon demand by simple manupilations (7). Rotifers are microscopic aquatic invertebrates with their lenght ranging from 40–2000 μm. The rotifer *B. calyciflorus* has been frequently used as a test organism in acute and short-chronic toxicity assessment for ecotoxicological evaluation (8–9). Rotifers reproduce predominantly parthenogenetic and are easy to culture (7). The ciliated corona, located at the anterior end of the animal, serves as a locomotory and food-collecting organ. In *B. calyciflorus* the corona consists of two concentric crowns of cilia: the outer crown is the cingilum and it serves for locomotion, the inner crown is the trochus, it sweeps food particles toward the mouth (7). The apical field is located between the two crowns and it contains microvilli.

In this study the effect of hydroxypropyl-β-cyclodextrin, β-cyclodextrin, didecanoyl-L-α-phosphatidylcholine, sodium-24,25-taurodihydrofusidate and sodium deoxycholate on the morphology of the ciliated corona of *B. calicyflorus* was investigated by scanning electron microscopy. We investigated the consequences of the morphological changes on the survival during acute (24-h) and on the survival, the reproduction and the population growth during short-time chronic (4 days) toxicity tests.

MATERIALS AND METHODS

Chemicals

Hydroxypropyl-β-cyclodextrin (HP-β-CD) was obtained from Janssen Biotech N. V. (Olen, Belgium). β-cyclodextrin (β-CD) was obtained from Roquette (Lestrem, France). Didecanoyl-L-α-phosphatidylcholine (DDPC) was a gift of Rhône-Poulenc Rorer (Köln, Germany). Sodium tauro-24,25-dihydrofusidate (STDHF) was provided by Leo Pharmaceutical Products Ltd. A/S (Ballerup, Denmark). Sodium deoxycholate (SDC) was purchased from Sigma-Aldrich NV/SA. (Bornem, Belgium). All other reagents were of analytical grade.

Rotifers

The cysts of *B. calicyflorus* originated from Medea, Boughzoul in Algeria. The cysts were hatched and the animals were continuously cultured in the laboratory at 25°C in synthetic freshwater. The synthetic freshwater medium (EPA), used as culturing and dilution medium consisted of 96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot H_2O$, 60 mg $MgSO_4$ and 4 mg KCl in 1 L distilled water (10). For the experiments with SDC and STDHF the medium contained only 96 mg $NaHCO_3$ in 1 L distilled water. The pH was adjusted to 7.4 with 0.1 N NaOH.

The algae *Scenedesmus obliquus* was used as food.

Test Protocol

All the toxicity tests were conducted in sterile 24-well plates (multiwell, Falcon Becton Dickinson, Novolab, Geraardsbergen, Belgium). One ml of test medium was distributed to each well. The food density in the acute, the life table and the population growth experiments was $2*10^6$ cells·ml^{-1}. The 24-well plates were placed at 25°C ± 0.5°C in artificial daylight. To obtain test animals of a uniform age (a cohort), females bearing eggs were isolated from the cultures. After 5 hrs all the neonates were isolated and used for the experiments. Transfers were done under a dissecting microscope at 12 X with a glass micropipette so that the rotifers could be counted correctly.

Acute Toxicity Test

In order to obtain the 24-h LC_{50} for each absorption enhancer four different concentrations and one control were tested. For each treatment six replicates were used. The concen-

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trations were chosen so that the highest concentration caused 100% mortality and the lowest concentration caused 0% mortality (11). At the beginning of the experiment 5 neonate rotifers were transferred in 1 ml of test solution. After 24 hrs the rotifers were checked for mortality and the LC_{50} was calculated using a linear regression analysis. Based on these results four sublethal concentrations were chosen for each absorption enhancer in order to perform the short-time chronic toxicity tests. The experimental design for the life table and the population growth experiments consisted of one control and four treatments, each containing 12 replicates.

Life Table Experiment

One neonate rotifer was introduced in each of the wells containing 1 ml of test medium. The isolated rotifers were checked twice daily during four days for the number of attached eggs, offspring and mortality. The produced neonates were removed. Every 24 hrs the parental females were placed in a fresh 24-well plate with fresh test medium. The intrinsic rate of natural increase (r_m) in the life table experiments was calculated using the Euler-Lotka equation under stable age distribution (12):

$$\sum l_x m_x e^{-r_m x} = 1$$

The r_m value is affected by age at firts reproduction, frequency of reproduction, probability of survival from birth to a given age class (l_x), and by the expected number of offspring for a female in age class (m_x); x is the age in general (12). The average lifetime reproductive succes, $R_0 = \Sigma\, l_x m_x$, being the total number of neonates produced per female per lifetime was calculated

Population Growth Experiment

In the population growth experiments 5 neonates were introduced into each well containing 1 ml of test medium. The neonates are not removed and after a maturation period of 21 hrs at 25°C, these females gave birth to their first offspring (9). Daily the number of rotifers was counted and the rotifers were transferred to a fresh 24-well plate with fresh test medium. The population growth was measured until day four. The intrinsic rate of natural increase in the population growth experiments was calculated using the following equation (12): $r_m = (\ln N_t - \ln N_0)\cdot t^{-1}$ with N_0 = the initial number of rotifers into each well (5 rotifers) and N_t = the number of rotifers into each well after 4 days.

Scanning Electron Microscopy

For each absorption enhancer 120 rotifers were exposed during 48 hrs to the same concentrations as in the short time chronic toxicity tests. After exposure the rotifers were anaesthetized with CO_2 to prevent contraction of the corona. The animals were fixed overnight in 2% glutaraldehyde in 0.1 M Na-cacodylate (pH 7.4) at 4°C. Postfixation occured in 2% OsO_4 in 0.1 M Na-cacodylate at 4°C during 6 hrs. Afterwards the rotifers were dehydrated in EtOH, critical point dried and coated with gold. The morphology of the ciliated corona was examined using a scanning electron microscope (JEOL JSM 854). Changes in the appearance of membrane wounds, deformation or disappearance of the cilia and microvilli were identified. The morphological deformations of the cilia and the membrane integrity were scored from 1 to 5 and 1 to 7, respectively. The scores were attributed as follows for the ciliary morphology: 1 = normal, 2 = small deformations, 3 = gross deformations, 4 = few cilia disappeared and 5 = cilia totaly disappeared, for the membrane integrity 1 = normal, 2 = microvilli aggregated, 3 = microvilli unrecognizable, 4 = few small membrane wounds(<0.2 μm), 5 = a large number of small membrane wounds, 6 = few but large membrane wounds (>5μm) and 7 = a high number of large membrane wounds.

Data Analysis

For the short time chronic toxicity tests statistically significant differences between the controls and the treatments were determined using a one-way ANOVA. The homogenity of variances was tested using the Bartlett test. If the variances of the controls and the treatments were found not to be equal, the data were transformed. To further compare the effects of the different treatments a multiple comparison among pairs of means was performed using a Tukey Kramer Test with $p < 0.05$ as significance level. For all the statistical analysis the computer program SYSTAT was used.

RESULTS

Acute Toxicity Tests

The 24-h LC_{50} value for HP-β-CD, β-CD, STDHF, SDC, and DDPC were: 2.387%, 0.348%, 0.048%, 0.00446% and 0.00244% (w/v), respectively.

Life Table Experiments

The effect of absorption enhancers on the net-reproduction (R_0) and intrinsic rate of natural increase (r_m) are presented in Table I. Concentrations of 1% HP-β-CD and above decreased the R_0 value significantly. The r_m value was only decreased significantly at a concentration of 2% HP-β-CD. The use of β-CD resulted in a slight but non-significant decrease of the net-reproduction and the intrinsic rate of natural increase. Rotifers exposed to 0.006% STDHF showed a decrease of the r_m value. The exposure to 0.012% and 0.024% STDHF, 0.0015% and 0.003% SDC and 0.0006% and 0.0012% DDPC resulted in a significantly reduced R_0 and r_m in comparison with the controls.

Population Growth Experiments

The effect of different concentrations of the absorption enhancers on the intrinsic rate of natural increase of the population growth is presented in Table I. Fig. 1 shows as an example the reduction in the population growth for increasing concentrations of SDC. All the concentrations of SDC resulted in a significant decrease of the r_m value after the fourth day. The population growth curves of rotifers exposed to increasing concentrations of DDPC were very similar as for the different SDC concentrations. Exposure to 0.0006% and 0.0012% DDPC resulted in a significant decrease of r_m after the fourth day. The effect of increasing concentrations of HP-β-CD, β-CD and STDHF on the population growth was not as severe as

Table I. Effects of Increasing HP-β-CD, β-CD, STDHF, SDC and DDPC Concentrations on the Net-Reproduction (R_0) and the Intrinsic Rate of Natural Increase (r_m) in the Life Table Experiments and on the Intrinsic Rate of Natural Increase (r_m) in the Population Growth Experiments

Absorption enhancer	Concentration (%; w/v)	Life table experiments		Population growth experiments
		R_0	r_m	r_m
HP-β-CD	Control	10.70 ± 1.37	0.98 ± 0.12	0.54 ± 0.03
	0.5	8.45 ± 2.98	0.95 ± 0.14	0.50 ± 0.03
	1	7.76 ± 2.39[a]	0.84 ± 0.23	0.38 ± 0.07[a]
	1.5	5.75 ± 2.53[a]	0.79 ± 0.29	0.34 ± 0.10[a]
	2	2.50 ± 2.68[a]	0.40 ± 0.43[a]	0.22 ± 0.15[a]
β-CD	Control	12.42 ± 5.09	1.15 ± 0.39	0.50 ± 0.07
	0.04	11.42 ± 5.58	1.05 ± 0.52	0.49 ± 0.05
	0.08	11.42 ± 6.27	1.02 ± 0.45	0.50 ± 0.07
	0.16	8.58 ± 4.94	0.97 ± 0.19	0.46 ± 0.05
	0.32	8.92 ± 4.94	0.91 ± 0.45	0.37 ± 0.05[a]
STDHF	Control	10.20 ± 2.78	1.11 ± 0.16	0.48 ± 0.09
	0.003	7.18 ± 6.55	0.79 ± 0.58	0.50 ± 0.09
	0.006	5.58 ± 4.48	0.62 ± 0.40[a]	0.41 ± 0.06
	0.012	4.42 ± 4.50[a]	0.52 ± 0.54[a]	0.37 ± 0.12[a]
	0.024	3.00 ± 3.69[a]	0.47 ± 0.49[a]	0.27 ± 0.19[a]
SDC	Control	9.75 ± 2.52	1.17 ± 0.27	0.60 ± 0.07
	0.000375	7.58 ± 5.24	1.12 ± 0.56	0.44 ± 0.12[a]
	0.00075	5.67 ± 4.59	0.88 ± 0.62	0.36 ± 0.11[a]
	0.0015	2.33 ± 1.93[a]	0.39 ± 0.42[a]	0.07 ± 0.11[a]
	0.003	3.25 ± 2.68[a]	0.52 ± 0.47[a]	−0.13 ± 0.15[a]
DDPC[b]	Control	8.36 ± 3.74	0.76 ± 0.30	0.55 ± 0.06
	0.00015	7.33 ± 3.74	0.80 ± 0.21	0.47 ± 0.08
	0.0003	4.08 ± 3.80	0.45 ± 0.44	0.46 ± 0.11
	0.0006	2.92 ± 3.15[a]	0.33 ± 0.39[a]	0.37 ± 0.12[a]
	0.0012	0.17 ± 0.39[a]	0	−0.08 ± 0.09[a]

Note: Data are presented as the mean ± SD, (n = 12).
[a] Significant different from the control, $p < 0.05$.
[b] DDPC or a degradation product hereof.

for SDC. Concentrations of 1%, 1.5% and 2% HP-β-CD, 0.32% β-CD and 0.012% and 0.024% STDHF significantly reduced the intrinsic rate of natural increase after the fourth day.

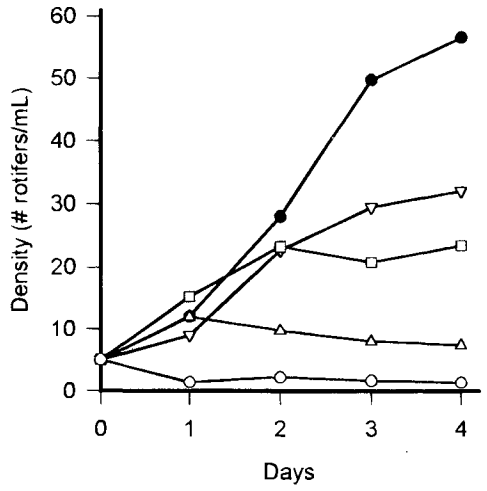

Fig. 1. Effect of increasing SDC concentration (%; w/v) on the population growth of rotifers. (●) control, (▽) 0.000375 %, (□) 0.00075 %, (Δ) 0.0015 %, and (○) 0.003 % SDC. Mean values (n = 12).

Scanning Electron Microscopy

The effect of exposure to different concentrations of absorption enhancers on the membrane integrity and ciliary morphology of rotifers is presented in Table II. In comparison with the control animals the changes observed ranged from no changes, to deformation of the cilia and microvilli, disappearance of the microvilli and cilia and appearance of membrane wounds. The rank order of increasing morphological damage following a 48-hr exposure was: DDPC < HP-β-CD < β-CD < STDHF < SDC.

The effect of a 0.0012% DDPC and 0.024% STDHF on the morphology of the ciliated corona of rotifers is presented in Fig. 2 and Fig. 3. Rotifers treated with increasing DDPC concentrations showed no dramatic changes in comparison with the controls. Some of the rotifers exposed to increasing HP-β-CD concentrations exhibited strong aggregation and disappearance of the microvilli. The effect of increasing concentrations of β-CD, STDHF and SDC on the corona of the rotifers was much more severe. All the rotifers treated with increasing concentrations of STDHF and SDC showed deformation of the cilia of the trochus. This effect was most severe for rotifers exposed to SDC. With increasing concentrations of β-CD the microvilli of the apical field disappeared and small membrane wounds were seen (<0.2 μm). STDHF and SDC had a similar effect but the membrane wounds were larger (5–8 μm).

Table II. Influence of Increasing Enhancer Concentrations on the Ciliary Morphology and the Membrane Integrity After a 48 h Exposure

Treatment		Ciliary morphology	Membrane integrity	n
CONTROL		1	1.17 ± 0.38	18
HP-β-CD	0.5%	1.10 ± 0.45	1.35 ± 0.75	19
	1%	1	1.63 ± 1.09	16
	1.5%	1	1.19 ± 0.40	16
	2%	1	2.60 ± 0.94	19
β-CD	0.04%	1.77 ± 1.24	2.40 ± 0.70	9
	0.08%	1.18 ± 0.60	2.36 ± 0.74	10
	0.16%	3.10 ± 1.45	2.50 ± 1.73	8
	0.32%	3.50 ± 0.71	4.50 ± 0.71	7
STDHF	0.003%	1	4.50 ± 2.28	13
	0.006%	1.82 ± 0.40	4.27 ± 2.20	11
	0.012%	2	3.50 ± 2.00	8
	0.024%	3.00 ± 1.73	5.33 ± 2.08	10
SDC	0.000375%	2.20 ± 0.63	5.10 ± 1.45	10
	0.00075%	3.57 ± 0.94	5.93 ± 1.64	14
	0.0015%	3.29 ± 0.76	6.00 ± 1.41	7
	0.003%	4.20 ± 1.01	6.33 ± 1.05	15
DDPC[a]	0.00015%	1	1.30 ± 0.47	18
	0.0003%	1	1.40 ± 0.50	20
	0.0006%	1.17 ± 0.58	1.25 ± 0.45	17
	0.0012%	1	1.20 ± 0.42	8

Note: Date are expressed as the mean ± SD. n is the total number number of rotifers scored for each treatment.
[a] DDPC or a degradation product hereof.

DISCUSSION

The objective of this study was to develop an alternative *in vivo* test using aquatic non-vertebrates as model organism to evaluate the effect of absorption enhancers on ciliated epithelium. The rotifer *B. calyciflorus* was chosen as model organism because it has been frequently used in toxicity tests. Morphological deformations of the cilia of the corona might affect the swimming and feeding behaviour of *B. calyciflorus* and consequently influence life table data which determine the population parameters (7). When comparing the results of the acute and short-time chronic toxicity tests with data from the literature we can conclude that a same rank order of increasing toxicity was found except for DDPC: HP-β-CD < β-CD < STDHF < SDC < DDPC. 5% HP-β-CD and 1.8% β-CD showed a minor effect on the ciliary beat frequency of chicken embryonal tracheal tissue (13). Exposure of human erythrocytes to 0.043% SDC caused ± 90% haemolysis after 30 minutes (14). Previous mucociliary transport and ciliotoxic studies have demonstrated that the mucus transport rate was irreversibly affected after the application of 1% STDHF and 1% SDC (3). Exposure of human adenoid tissue to 0.5% STDHF and 0.3% SDC caused a fast and irreversible ciliostasis (4). Similar results were obtained in our study. Rotifers exposed to 0.5% STDHF and 0.3% SDC immediately stopped swimming. The ciliairy beat frequency was irreversibly halted. Rotifers treated with 0.3% STDHF and 0.1% SDC showed a reduced swimming speed after 5 minutes. From the results of the acute and chronic toxicity tests DDPC seemed the most toxic absorption enhancer. This could be due to the enzymatic degradation of DDPC by endogenously phospholipase A_2, reported to be present in the corona of *B. calyciflorus* (15). DDPC can be metabolized enzymatically by phopholipase A_2 with the formation of lysophosphatidylcholine (LPC) and capric acid. LPC is known to be ciliotoxic, immediately after the application of 1% LPC the mucociliary transport was irreversibly halted (3). When human erythrocytes where exposed to 0.0047% LPC during 30 minutes 50% haemolysis occured (14).

The rank order of increasing morphological damage following a 48-hr exposure to absorption enhancers was: DDPC < HP-β-CD < β-CD < STDHF < SDC. This rank order is in good agreement with published data on the toxic effect of absorption enhancers in other models. Rat nasal mucosa treated with 1% STDHF for 1 hr caused cell loss, epithelium rearrangment and considerable reduction of epithelium height (16). When human intestinal epithelial cells (Caco-2) where exposed to 0.175% STDHF for 4 hrs they showed aggregated and shortened microvilli, areas with decreased microvilli density sometimes in combination with small membrane wounds (<0.5 μm) (17). After a 5 min exposure of the rat nasal mucosa to 1% STDHF some fusion of the cilia was seen and exposure to 1%

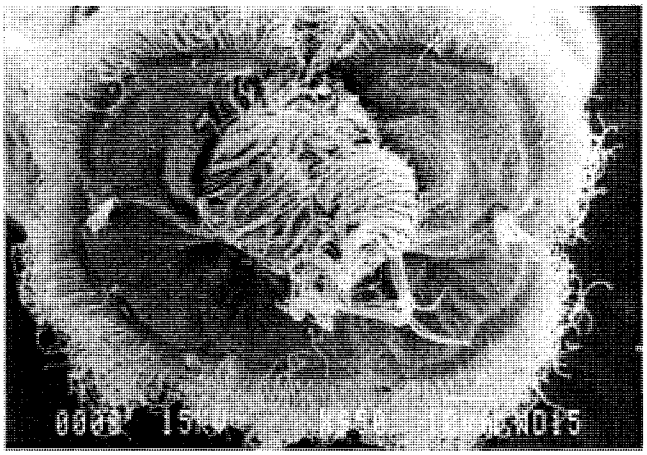

Fig. 2. The corona of a rotifer treated with 0.0012% DDPC. The ciliary morpholgy is normal (score = 1). In the upper right corner of the apical field the microvilli are aggregated (score = 2).

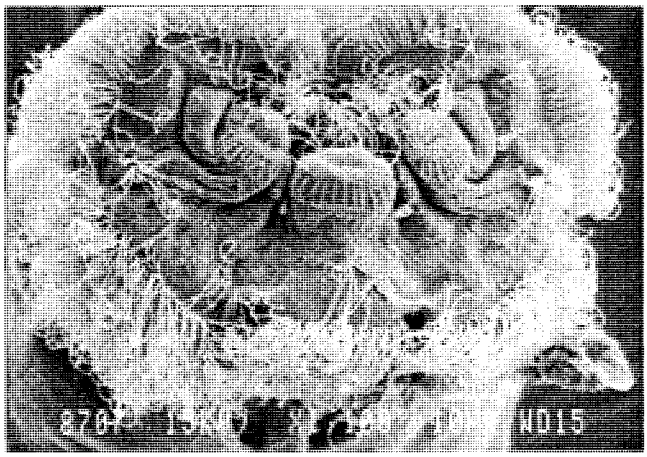

Fig. 3. The corona of a rotifer exposed to 0.024% STDHF. The cilia of the throchus are totaly disappeared (score = 5). In the apical field a few large membrane wounds are observed (score = 6).

SDC resulted in severe deformations of the cilia and the mucosal surface integrity (5). Similar results were obtained for rotifers exposed to STDHF and SDC. There seems to be a good correlation between the morphological changes due to the absorption enhancers and the results of the acute and short-chronic toxicity tests in rotifers except for DDPC. It can be concluded that the use of the acute, short chronic toxicity tests in combination with scanning electron microscopy could be considered as an interesting alternative for screening the toxicity of absorption enhancers on ciliated epithelium. There are several advantages of conducting the rotifer tests: rotifers are easy to culture, the described screening tests are easy to conduct, and the tests made statistical interpretations of the results possible.

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