In Vitro Evaluation of Nasal Mucociliary Clearance Using Excised Rat Nasal Septum

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Mucus on the nasal mucosa is translocated to the pharynx by ciliary beating, which is an important non-specific defense mechanism called mucociliary clearance (MC). MC is one of the important factors determining the rate and extent of drug absorption after nasal application. The purpose of this study is to evaluate MC using rat nasal septum under physiological condition in an *in vitro* system. The nasal septum was excised from rats anesthetized with urethane and the movement of fluorescent microspheres (FMS) applied on the nasal septum was observed with a fluorescence microscope. FMS were transported at a constant velocity in the same direction for a few minutes, but addition of 4% mucin solution on the nasal septum maintained MC for at least 90 min after excision. With our evaluation system established by modifying the method of Saldiva, MC was determined to be around 1 mm/min. Furthermore, the ciliostatic effect of benzalkonium chloride was observed, and it was confirmed that β -adrenergic antagonists and a cholinergic antagonist decreased MC, and that β -adrenergic agonists and a cholinergic agonist tended to increase MC, indicating that our system is valid and useful for evaluating MC function and the effect of drugs and pharmaceutical additives for nasal application on MC.

Key words mucociliary clearance; nasal absorption; mucus; cilia; nose

The nasal route has drawn much attention as an alternative to oral administration. Over the last two decades, there has been an increase in the number of drugs that have been reported for nasal administration. The advantages of nasal drug delivery include rapid absorption and a fast onset of action due to a relatively large absorption surface area and high vascularization, and avoidance of not only hepatic and intestinal first-pass metabolism, but also chemical and enzymatic degradation in the gastrointestinal tract.^{1,2)} In addition, nasal drug delivery is needle-free and can be performed without trained personnel, which facilitates self-medication and improves patient compliance compared to other parenteral routes.

The surface of respiratory mucosa is covered with mucus secreted from goblet cells. Some cells in nasal epithelium are ciliated. Superficial mucus is translocated to the pharynx by ciliary beating in order to clear noxious substances or infectious microorganisms trapped by the mucus layer. Infectious microorganisms are swallowed into the stomach and inactivated in the severe acidic environment. This function of respiratory mucosa is called mucociliary clearance (MC), and is very important for protection of the respiratory tract and avoidance of infectious diseases.^{1,3,4)} Since drugs applied into the nasal cavity are similarly cleared toward the pharynx by MC and swallowed into the stomach, MC is one of the important factors determining the rate and extent of drug absorption after nasal application.^{1,2)}

Many investigations on respiratory MC have been reported, where the transport rate of some kinds of dyes or particles on nasal and/or tracheal tissue was measured. Nasal or tracheal tissues from various animal species have been used, such as human,^{5–8)} rat nasal,^{9–13)} rat tracheal,¹⁴⁾ frog palate,^{7,9,15–17)} and bovine tracheal tissues.^{18,19)} In other *in vivo* studies,²⁾ the

clearance time of markers (dyes or particles) from the nasal cavity to the pharynx was evaluated by the collection of markers from the pharynx region. Recently, the *in vitro* and *in situ* measurements of MC have been reported, where the transport of particles on excised mucosa was observed directly and the movement velocity of particles was regarded as an index of MC.^{5,9,18,19)}

Although many types of evaluations on MC have been developed and reported, few reports describe the detailed relationship between MC function and nasal drug absorption. Furthermore, few evaluation systems on MC of the rat have been reported, although the rat is usually used in *in vivo* studies on drug absorption. Consequently, in order to reveal the relationship between nasal drug absorption and MC function experimentally in rats, an evaluation system on MC of the rat should be developed. The present study attempted to find out adequate conditions of an *in vitro* system to evaluate MC under physiological condition using excised nasal septums of the rat and to further clarify the effects of drugs, pharmaceutical additives, and the physicochemical properties of nasal formulations.

MATERIALS AND METHODS

Materials Fluorescent microspheres (FMS; Fluoresbrite® YG microspheres, size 6.00 μ m) were purchased from Polysciences, Inc. (Warrington, PA, U.S.A.). Mucin from pig stomachs, salbutamol sulfate, and terbutaline sulfate were supplied from Wako Pure Chemical Industries (Osaka, Japan). Benzalkonium chloride, propranolol hydrochloride, atenolol, acetylcholine chloride, and atropine were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 2,4-Dinitrophenol was

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obtained from Nacalai Tesque (Kyoto, Japan).

Preparation of Mucin Solution (MS) Mucin from pig stomachs was used in order to supplement the excised nasal septum with mucus. Mucin was suspended in Hank's balanced salt solution (HBSS) at the concentration of 4 w/v% and 10 w/v%, and stirred well. The resulting suspension was centrifuged at 13200rpm for 20 min twice to remove insoluble debris according to the method reported by Shah and Donovan^{18,19)} with minor modifications. The obtained supernatant was stored at 4°C and used as a MS.

In Vitro Evaluation System on Mucociliary Clearance Male Wistar rats weighing 250–350 g were used in all animal experiments. All animal studies were conducted under guidelines after approval by the local committee of Animal Care of Shujitsu University in accordance with the Principles of Laboratory Animal Care (NIH publication #85-23). Rat nasal septum was excised surgically under intraperitoneal urethane anesthesia (1.25 g/kg). To investigate the effect of anesthetics, pentobarbital (50 mg/kg) or inhalation of diethyl ether was applied instead of urethane. Since the maintenance of an appropriate humidity and temperature on nasal mucosa is necessary to keep ciliary function regularly,⁵⁾ the nasal septum was placed in a dish with a lid containing HBSS at 37°C just after excision to provide the surface of the septum with humidity. However, the isolated nasal septum was not bathed in HBSS to prevent HBSS from changing the fluid condition on the surface of the nasal septum, which is very important for precise and reproducible determination of MC. Immediately, the dish was mounted on the stage of the fluorescent microscope (AS LMD, Leica, Wetzlar, Germany) and 0.2 μ L of the FMS suspension was applied on the nasal septum with a microsyringe. Although no effect of marker size on mucociliary clearance

Fig. 1. Illustration Representing *in Vitro* Evaluation System of Nasal Mucociliary Clearance

has been reported,²⁰⁾ the effect of FMS size (3 to 6 μ m) on the measurement of MC was evaluated in the preliminary study, and no effect of the size was confirmed. For the observation with the fluorescent microscope, larger beads (6 μ m) are easier for us to observe their movement. Therefore, FMS with the size of 6 μ m was used in this system. The movement of FMS was observed with a fluorescent microscope 30 s after the instillation of the FMS suspension and photographs were taken serially by a charge coupled device (CCD) camera (CV-20M, Hitachi, Tokyo, Japan) at 10 s intervals for up to 5 min (Fig. 1). The movement velocity of FMS was calculated from the traveling distance of FMS determined from serial photographs. The maintenance of viability of isolated septum was confirmed by observing the ciliary beat frequency, which was kept to be $83 \pm 10\%$ of initial value until at least 90 min after incision. The effect of 2,4-dinitrophenol (1 mM) on the FMS movement was evaluated after pretreatment of excised septum for 30 min.

Fig. 2. Serial Photographs Indicating the Transport of Fluorescent Microspheres Applied on the Excised Rat Nasal Septum

(A) Photographs of the same section of the control nasal septum. Arrows represent the direction and distance of the transport of each microsphere for 10 s. (B) Photographs of the same section of the nasal septum pretreated with 2,4-dinitrophenol. No movement of spheres was observed.

Maintenance of Mucociliary Clearance In order to maintain mucociliary clearance, the effect of the addition of MS was investigated. After surgical excision, the nasal septum was placed in a lid dish containing HBSS for 0, 10, 30, 60, 90, 120, and 240 min at 37°C. Thereafter, 2.0 μ L of 4%, 10%, or 15% MS was added on the surface of the nasal septum, and the septum was incubated for 5 min. The FMS suspension (0.2 μ L) was applied on the surface and MC was measured as described above.

Effect of Various Drugs on Mucociliary Clearance For the validation of the system, the effects of drugs known to change MC were evaluated. Benzalkonium chloride (BZC), a preservative often used for nasal dosage forms,^{21,22} propranolol (PPL) and atenolol (ATL), β -adrenergic antagonists,^{23,24} salbutamol (SBM) and terbutaline (TBL), β -adrenergic agonists,^{25–27} acetylcholine (ACH), a cholinergic agonist,^{14,27,28} and atropine (ATRP), a cholinergic antagonist,²⁹ were employed to examine their effects on MC. Each drug was dissolved in HBSS at two different concentrations. Under urethane anesthesia, 40 μ L of the drug solution was instilled into the rat nasal cavity by a microsyringe at a depth of 10-mm from the nostril. The nasal septum was excised surgically 15 min after drug administration, and MC was measured as described above.

Statistical Analysis Statistical significance of the differences of the means was determined by Dunnett's test by utilizing statistical software, JMP® (SAS Institute Japan, Tokyo, Japan).

RESULTS

Mucociliary Clearance Determined with *in Vitro* Evaluation System

Figure 2A shows typical images of FMS movement, which were taken through the fluorescent microscope at 10 s intervals. FMS on the surface of the nasal septum moved at a constant velocity in the same direction for a few minutes. In addition, when the excised nasal septum was pretreated with 2,4-dinitrophenol (1 mM) for 30 min, FMS did not move at all (Fig. 2B). Since microscopic observations by CCD camera confirmed that ciliary beating was completely terminated, the movement of FMS would be ascribed to the translocation of the mucus driven by ciliary beating. In this study, the mean moving velocity of FMS, V_{FMS} , which is calculated from serial photographs, was used as an index of MC.

Effect of Anesthetics on Mucociliary Clearance First of all, to select an adequate anesthetic, the effect of anesthetics on MC was investigated (Fig. 3). In comparison with the results obtained from control rats stunned by a blow (no anesthesia), all anesthetics decreased V_{FMS} . Significant decreases were caused by diethyl ether and sodium pentobarbital. Since the effect of urethane on V_{FMS} was the smallest, urethane was selected and utilized as an anesthetic in the following studies.

Maintenance of Mucociliary Clearance Figure 4 shows the change in V_{FMS} as a function of time after septum excision. In case of no supplementation with mucin (control), V_{FMS} just after excision was 1.12 ± 0.09 mm/min, but the value of V_{FMS} drastically decreased time-dependently. First of all, saline was added on the surface of nasal septum before the application with FMS to examine the effect of dehydration on MC. As a result, FMS were not moved at all although it was confirmed that ciliary beating was maintained normally.

Fig. 3. Effects of Various Anesthetics on Mucociliary Clearance

Nasal septums were excised from control rats stunned by a blow or from rats anesthetized with urethane, diethyl ether, or sodium pentobarbital. Transport rates of fluorescent microspheres were evaluated based on serial photographs taken through the fluorescent microscope. Results are expressed as the mean of three to five independent experiments with the vertical bar showing S.E. * $p < 0.05$, compared with control.

Fig. 4. Effects of Mucin Solution on Transport Rates of Fluorescent Microspheres

After incubation at 37°C for the designated times, 4% or 10% mucin solution (MS) was added on the nasal septum and transport rates of fluorescent microspheres were determined based on photographs serially taken. Results, indicated as % of initial, are expressed as the mean of three to five independent experiments with the vertical bar showing S.E. keys: ○, control (no addition of MS); △, 4% MS; ■, 10% MS. ** $p < 0.01$; * $p < 0.05$, compared with the initial rate at each condition.

Therefore, to maintain mucociliary clearance, the effect of addition of MS on MC was investigated. After addition of 4% MS, V_{FMS} was unchanged for 90 min after septum excision. Even 240 min later, around 70% of the initial V_{FMS} was maintained. Continuous observation also confirmed that the moving velocity of FMS was not changed for up to 10 min after FMS application. MS (10%) significantly decreased V_{FMS} at 60 min after septum excision, but it recovered at 120 min. The application of 15% MS significantly suppressed V_{FMS} to $40.9 \pm 6.1\%$ ($n=3$, $p < 0.01$) of control at time 0. These results suggest that the addition of 4% MS on the surface of the excised nasal septum maintained MC for a long period of time after septum excision. Therefore, in the following studies 4% MS was used to maintain MC.

Effects of Various Drugs on Mucociliary Clearance Drugs with different modes of pharmacological action were used to investigate their effects on MC. V_{FMS} after

Fig. 5. Effects of Benzalkonium Chloride on Mucociliary Clearance

The nasal septum was excised 15 min after nasal application of benzalkonium chloride. Transport rates of fluorescent microspheres were determined from photographs serially taken. Benzalkonium chloride (0.10%) completely blocked mucociliary transport, which is described as "stasis" in the figure. Results are expressed as the mean of three to five independent experiments with the vertical bar showing S.E. Keys: ■, 0.02%; ■, 0.10%. * $p < 0.05$, compared with controls.

Fig. 6. Effects of Propranolol and Atenolol, β -Adrenergic Antagonists, on Mucociliary Clearance

The nasal septum was excised 15 min after nasal application of propranolol (PPL) or atenolol (ATL). Transport rates of fluorescent microspheres were determined from photographs serially taken. Results are expressed as the mean of three to five independent experiments with the vertical bar showing S.E. Keys: ■, 10⁻⁶ M; ■, 10⁻⁴ M. ** $p < 0.01$; * $p < 0.05$, compared with controls.

pretreatment with BZC, a preservative often used for nasal formulations, at a concentration of 0.02% (0.68 ± 0.08 mm/min) was significantly lower than the non-treated control (1.22 ± 0.20 mm/min) (Fig. 5). Pretreatment with 0.1% BZC completely blocked MC (Fig. 5). These results coincide with previous reports that BZC had a ciliostatic effect on nasal epithelium.^{21,22} Figure 6 indicates the effects of pretreatment with PPL or ATL, a β -adrenergic antagonist, on V_{FMS} . Results clearly show that PPL or ATL significantly decreased V_{FMS} and that the decrease in V_{FMS} by both drugs was dependent on the drug concentrations. It was reported that ciliary beat was activated by β -adrenergic agonists,²⁵⁻²⁷ suggesting that a β -adrenergic antagonist would decrease MC due to the suppressed ciliary beat, which was confirmed by our present results. Then, the effects of β -adrenergic agonists, SBM and TBL, were investigated (Fig. 7). Pretreatment with each β -adrenergic agonist on V_{FMS} tended to increase V_{FMS} , but no effect was observed at higher concentrations of each agonist.

A cholinergic agonist (ACH) and antagonist (ATRP) were also examined in terms of their effects on V_{FMS} (Fig. 8). Pretreatment with 10⁻⁴ M ACH increased V_{FMS} by 55%, while no significant change was observed by pretreatment with 10⁻⁶ M ACH. In the case of ATRP, V_{FMS} at a higher concentration of

Fig. 7. Effects of Salbutamol and Terbutaline, β -Adrenergic Agonists, on Mucociliary Clearance

The nasal septum was excised 15 min after nasal application of salbutamol (SBM) or terbutaline (TBL). Transport rates of fluorescent microspheres were determined from photographs serially taken. Results are expressed as the mean of three to five independent experiments with the vertical bar showing S.E. Keys: ■, 10⁻⁵ M; ■, 10⁻³ M.

Fig. 8. Effects of Acetylcholine, a Cholinergic Agonist, and Atropine, a Cholinergic Antagonist, on Mucociliary Clearance

The nasal septum was excised 15 min after nasal application of acetylcholine (ACH) or atropine (ATRP). Transport rates of fluorescent microspheres were determined from photographs serially taken. Results are expressed as the mean of three to five independent experiments with the vertical bar showing S.E. Keys: ■, 10⁻⁶ M; ■, 10⁻⁴ M. * $p < 0.05$, compared with controls.

ATRP (10⁻⁴ M) was significantly lower than that of the control, but pretreatment with a lower concentration of ATRP (10⁻⁶ M) did not change V_{FMS} at all (Fig. 8).

DISCUSSION

Since MC clears drugs applied into the nasal cavity, the rate and extent of drug absorption after nasal application would be highly dependent on MC.^{1,2} Recently, many approaches utilizing viscous solutions and mucoadhesive formulations^{12,13,30-32} have been conducted to increase nasal residence time of the formulation. These approaches were intended to reduce MC, which is believed to result in an increase in nasal drug absorption. However, few studies have referred to the relationship between nasal residence time and nasal drug absorption. In order to clarify this relationship, an *in vitro* system which makes it possible to easily and precisely determine MC function has been highly anticipated. Therefore, we tried to establish an *in vitro* evaluation system based on the method reported by Saldiva *et al.*⁹ utilizing rats in the present study, since rats are often used for preclinical studies of new drug candidates.

Various methods have been developed to evaluate MC of airway epithelium, in most of which MC rates were determined *in vivo* or *in vitro* utilizing dyes as nonabsorbable markers or particles as mucus translocation markers. Various tissues such as nasal, tracheal, and palate tissue from various species such as human, bovine, rat, and frog have been utilized. Mason *et al.*⁵⁾ reported that the MC rate of humans was 4.2 mm/min on average, ranging from 2.7 to 5.7 mm/min. On the other hand, it was reported that human MC ranged from 1.0 up to 20 mm/min.³³⁾ Shah and Donovan¹⁸⁾ reported that MC of bovine trachea was 5.2 to 9.0 mm/min. In the case of rat nasal epithelium, MC was reported to be 2 to 3 mm/min.⁹⁾ As referred to above, these findings on MC provided by many investigations indicate large variations and differences in MC dependent on the method and/or species.

Saldiva *et al.*⁹⁾ dropped 2.0 μ L of marker suspension on the surface of nasal septum. However, since the area of excised rat nasal septum is approximately 100 mm², we thought that 2.0 μ L would be too large as an applying volume of marker suspension because mucus layer thickness of rat nasal epithelium under physiological conditions is 0.5 to 5.0 μ m.²⁾ Applying too much volume onto the surface of the septum might result in the dilution of the mucus layer, and cause a fluctuation in the condition of nasal mucosa. Furthermore, since Saldiva *et al.*⁹⁾ observed the movement of markers just after application, it is possible that MC was affected by the flow caused by instillation of a large volume of suspension. Therefore, to prevent these effects on MC, 0.2 μ L was set as a proper volume of marker suspension based on our preliminary studies and observations started 30 s after instillation of the marker suspension.

After instilling FMS on the excised rat nasal septum, FMS was transported unidirectionally at a constant velocity during measurement (Fig. 2A) and microscopic observations clarified that the movement of FMS was dependent on ciliary activity based on the inhibitory effect of 2,4-dinitrophenol on ciliary beating (Fig. 2B). Since anesthetics generally reduce MC and prolong nasal residence time of the formulation administrated to the nasal cavity,³⁴⁾ the influence of anesthesia on MC was, first of all, investigated using three anesthetic agents and urethane was selected because it had the least effect on MC (Fig. 3).

It has been reported that mucus secretion on mucosa immediately stopped after tissue excision, while ciliary beating continued for a longer period of time.²⁹⁾ Therefore, it is possible that the change in mucus volume on mucosa would influence MC of drugs applied on the excised rat nasal septum. In fact, it was found that MC decreased dependent on the time after excision (Fig. 4). First of all, the effect of saline on the surface of nasal septum was examined to estimate the effect of dehydration on MC. However, FMS applied on nasal septum was not moved at all in addition with saline, although microscopic observation by CCD camera confirmed the maintenance of ciliary beating. These results suggest that the maintenance of aqueous phase on the nasal surface can keep the ciliary beating, but not MC function. Therefore, to maintain MC function, the effect of MS addition on the excised septum was examined. It was found that the addition of 4% MS made it possible for V_{FMS} to be constant for 120 min after excision (Fig. 4). However, the effect of 10% MS was unstable (Fig. 4), and the addition of 15% MS significantly decreased V_{FMS} just after

excision of the nasal septum. Since the viscoelastic properties of mucus are important for mucociliary function,^{10,35–37)} the mucin concentration used would be critical for maintaining MC function. It is also known that the concentration of glycoprotein in the mucin of respiratory mucosa is 3%.²⁾ Therefore, 4% MS would give adequate viscoelasticity to mucus and be better for maintaining normal mucociliary function. MC determined by our *in vitro* system was 1.22 ± 0.20 mm/min, which is comparable with, but smaller than that reported by Saldiva *et al.* (2–3 mm/min).⁹⁾ The reduction or increase in V_{FMS} by a 10% MS could be due to the high viscoelasticity of the 10% MS, but the reason why MC recovered again remains to be clarified.

Some drugs and formulation additives are known to affect the function of MC.³⁴⁾ In the present study, several compounds with different pharmacological actions, the effects of which on MC have been previously reported, were investigated for validating our *in vitro* system. It is known that BZC shows a ciliostatic effect on ciliary cells due to cytotoxicity.^{21,22)} We confirmed that pretreatment with BZC attenuated MC in a concentration-dependent manner and that ciliostasis was observed when 0.1% BZC was applied (Fig. 5). These results coincide with reports by Mallants *et al.*²¹⁾ and Hofmann *et al.*²²⁾

Airway epithelial cells express functional β -adrenergic receptors (β ARs), and β -adrenergic agonists and antagonists are known to affect ciliary function through the β ARs signal pathway.^{25,26,38)} The β ARs signal stimulates mainly adenylate cyclase, which increases the intracellular level of cAMP, resulting in the stimulus of ciliary function.^{25,26,38)} It would also be the case for nasal mucosa, because β ARs are also expressed in nasal mucosa.³⁹⁾ As shown in Fig. 6, β -adrenergic antagonists, PPL and ATL, significantly decreased MC. The effect of β -adrenergic agonists, SBM and TBL, was not significant, but the increasing tendency of MC was observed at a lower concentration of β -adrenergic agonists. Further investigations are necessary to clarify the effect of β -adrenergic agonists on MC, but these results suggested that MC of nasal mucosa could also be regulated via the β ARs signal pathway. Mucociliary function is also stimulated via the cholinergic signal pathway.²⁸⁾ Second messengers such as intracellular calcium, cGMP, and cAMP have been shown to participate in the process of ciliary activation.²⁸⁾ Results shown in Fig. 8 indicate that a cholinergic agonist (ACH) tended to increase and antagonist (ATRP) decreased MC function.

As shown in Figs. 7 and 8, β -adrenergic and cholinergic agonists showed some effect on V_{FMS} in a concentration-independent manner, but the effect was not statistically significant. According to our preliminary observation on ciliary beat using a microscope and a high-speed camera, the effect of these β -adrenergic and cholinergic agonists on the ciliary beat frequency, which is a direct index of ciliary activity, was similar with those on V_{FMS} . Therefore, it might be difficult to observe the stimulating effect beyond normal ciliary beating due to the limitation of ciliary movement. We plan to investigate the relationship between ciliary beating and MC including the effect of ciliary stimulating agents in the next project.

As shown above, the results obtained in the present study coincided with those reported previously, clearly indicating that our *in vitro* MC evaluation system utilizing isolated rat nasal septum is valid for estimating nasal MC function.

CONCLUSION

We successfully evaluated rat nasal MC under physiological condition with an *in vitro* system established by modifying Saldiva's method. This system can be used to evaluate MC and to estimate the effects of drugs and pharmaceutical additives for nasal application.

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