

N-acetylcysteine effects on sinonasal cilia function

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Abstract

Objective: To evaluate the pharmacological effects of the N-acetylcysteine (NAC) on human respiratory epithelial cultures specifically addressing electrolyte transport and cilia beat frequency.

Methods: Well-differentiated human bronchial epithelial cultures grown at an air liquid interface were treated on the apical or basolateral surface with varying concentrations of NAC. The best NAC concentration for ideal cilia beat frequency was found. The effects of NAC were evaluated on cilia beat frequency. After the effect of N-acetylcysteine on beat rate was found, its efficiency was investigated by ATP or IBMX to understand its mechanism of action. Changes in ciliary beat frequency were determined using the Sissons-Ammons video analysis system.

Results: Maximal stimulatory effect on cilia function was evident at 10 mg/ml NAC concentration. After wash up, cilia movement were increased very dramatically. This increase of cilia beat frequency was even higher after NAC plus IBMX and NAC plus ATP washings.

Conclusion: Apical application of NAC prominently stimulates cilia beat frequency and after wash up, cilia movement was increased very dramatically. After NAC use by washing with PBS in clinical efficacy can be enhanced.

Keywords: N-acetylcysteine, sinonasal, cilia.

Özet: N-asetil sisteinin sinonazal siliyer atım frekansına etkileri

Amaç: N-asetil sisteinin (NAC) insan respiratuvar epitelyal hücre kültüründe, başta elektrolit transportu ve siliyer atım frekansı olmak üzere farmakolojik etkilerinin değerlendirilmesi.

Yöntem: Hava-sıvı arayüzünde üretilmiş, iyi diferansiyeli edilmiş insan bronşiyal epitelyal hücre kültüründe farklı konsantrasyonlarda NAC uygulaması yapıldı. İdeal siliyer atım frekansını sağlayan NAC konsantrasyonu saptandı. N-asetil sisteinin atım hızına etkisi saptandıktan sonra, etkinliği ATP veya IBMX ile araştırılarak etki mekanizması ortaya konmaya çalışıldı. Siliyer atım frekansı Sissons-Ammons video analiz sistemi ile saptandı.

Bulgular: Siliyer fonksiyonlarda maksimum stimülasyonun 10 mg/ml NAC konsantrasyonunda olduğu saptandı. Yıkama sonrası siliyer hareketler dramatik olarak arttı. Bu artış NAC artı IBMX ve NAC artı ATP yıkamalarından sonra daha da artış gösterdi.

Sonuç: N-asetil sisteinin apikal kullanımı, siliyer atım frekansını belirgin düzeyde artırmaktadır. N-asetil sistein sonrası PBS ile yıkamak ile klinik etkinlik elde edilebilir.

Anahtar sözcükler: N-asetil sistein, sinonazal, siliya.

The mucociliary transport system plays an important role in the clearance of excessive mucus and inhaled foreign materials from the respiratory tract. Airway epithelium's ciliary structure is a part of the natural defense system that protects the respiratory system by propelling debris laden mucus. The mucus flow is generated by the vigorous asymmetric beating of the cilia, which can be named as effective strokes and recovery strokes.^[1] During normal mucus clearance,

inhaled pathogens and particles become trapped in the mucus layer and are then expelled before they colonize the airways.^[2] The airway secretions which line the respiratory tract form a biphasic layer composed of an aqueous 'sol' layer and a more superficial 'gel' layer. The sol layer is also described as the 'periciliary' layer or 'airway surface fluid', where the cilia beat and relax. The lubricant sol layer enables the gel mucus present at the tips of the cilia to be

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Received: October 13, 2015; **Accepted:** November 5, 2015

Online available at:
www.entupdates.org
doi:10.2399/jmu.2015003002
QR code:



transported by the ciliary beating of the ciliated cells. The mucus layer has a variable range of thickness, and mucus is kept away from ciliated airway epithelia by the presence of a $\sim 7 \mu\text{m}$ periciliary liquid layer which, as the name suggests, surrounds the beating cilia and acts as a lubricant to keep mucus away from the epithelial cell surface. All layers consisting mucus layer make up the airway surface liquid (ASL).^[3] The rate of mucociliary clearance is strongly influenced by the degree of hydration of the ASL and mucus layers.^[4] Mucus hydration is regulated by epithelial ion transport processes from both the superficial epithelium and the submucosal glands.^[3,4]

Many techniques have been used to improve mucociliary clearance in patients with a variety of chronic mucus hypersecretory conditions. The goal is to hydrate airway surfaces by stimulating secretion and/or inhibiting absorption.

N-acetylcysteine (NAC) is a widely used mucolytic agent, with known anti-oxidative and anti-apoptotic properties (references). However, some clinical and pharmacological effects of NAC are still unclear. In addition to thinning the mucus, animal studies suggest that NAC also stimulates ciliary beating frequency at low concentrations, while it inhibits the same function at higher concentrations (need references here). Thus, in this study we investigated the *in vitro* effect of NAC on human sinonasal ciliary activity. Ciliary beating frequency increased arithmetically at the best concentration of NAC and decreased after washout. NAC has an effect on some ligands and receptors and by this way it can change mucociliary clearance.

Materials and Methods

Surgical technique

Air-liquid interface cultures

Human sinonasal epithelium from patients who underwent chronic rhino sinusitis operation was cultured according to the described protocol below.

Briefly, tissue was harvested which grown on 6.5-mm diameter permeable filter supports (Corning Life Sciences, Lowell, MA, USA) submerged in culture medium. The media was removed from the surface at the 4th day after reaching confluence of the cells which was fed via the basal chamber. Differentiation and ciliogenesis occurred in all cultures within 10 days to 14 days.

Ciliary beat frequency analysis

Images were visualized using a 20 \times lens on an inverted scope (Leica Microsystems, Inc., Bannockburn, IL, USA). Image data was captured using a Model A602f-2 Basler area

scan high-speed monochromatic digital video camera (Basler AG, Ahrensburg, Germany) at a sampling rate of 100 frames per second with a resolution of 640 \times 480 pixels. The video images were analyzed using the Sisson-Ammons video analysis (SAVA) system version 2.1 (Ammons Engineering, Mt. Morris, MI, USA).¹⁸

For each experiment, a large area of beating cilia was detected with the inverted microscope. The digital image signal was then routed from the camera directly into a digital image acquisition board (National Instruments Corp., Austin, TX, USA) within a Dell XPS 710 Workstation (Dell, Roundrock, TX, USA) running Windows XP Professional (Microsoft, Redmond, WA, USA) operating system. Images were captured, compressed, and stored to disk. Files were reloaded and analyzed with virtual instrumentation software highly customized to perform ciliary beat frequency (CBF) analysis. All of the recordings in the present experiments were made at 200 \times magnification. Experiments were all performed at ambient temperature (22 °C).

Whole field analysis was performed with each point measured representing one cilium.

For each sample, the reported frequencies represent the arithmetic means of these values, followed by standard deviations. As our data includes thousands of individual points (cilia), very small changes in CBF result in a statistical significance due to the high power of the study. Thus, statistical analysis of the arithmetic means derived from each culture was performed using two-tailed unpaired t-tests to ensure reproducibility.

Experimental paradigm

Well-differentiated human bronchial epithelial cultures grown at an air liquid interface were treated on the apical or basolateral surface with varying concentrations N-acetylcysteine. First step was to estimate the best concentration for cilia beat frequency. Cell cultures of four chronic rhino sinusitis patients were used to find the best NAC concentration in this study. In the second step, the effect of NAC were evaluated on cilia beat frequency. The effects which occurred at cilia during 20 minutes after wash up the cell with DPS using best concentration of NAC were studied. After wash up, beat frequency of cilia was increased dramatically and decreased gradually in 10 minutes.

In the third step, four different chronic rhinosinusitis cell cultures (transwells) were used. The efficiency of NAC was investigated with adenosine triphosphate (ATP)

or 3'-isobutyl-1-methylxanthine (IBMX) using four transwells for better understanding on NAC mechanism. First transwell was used for showing ATP effect, second transwell was used for showing "ATP + NAC" effect, third transwell was used for showing IBMX effect and fourth transwell was used for showing "IBMX + NAC" effect.

Four transwells were prepared from each patient's material. Two transwells were used to demonstrate the effects of NAC and ATP, while two transwells were used to demonstrate the effects of NAC and IBMX. IBMX effects on cilia were examined in 10 minutes in two transwells. After ten minutes with IBMX, NAC was added to the first transwell, while Dulbecco's phosphate buffered saline (DPBS) was added to the second transwell. Following next ten minutes, both transwells were washed up. ATP effects on cilia were examined in 10 minutes in two transwells. After ten minutes with ATP, NAC was added to the third transwell, while DPBS was added to the fourth transwell. Following next ten minutes, both transwells were washed up. These mentioned steps were repeated for all four patients.

Changes in ciliary beat frequency were determined using the Sissons-Ammons Video Analysis system. Images were captured, compressed, and stored to disk. Files were reloaded and analyzed with virtual instrumentation software highly customized to perform CBF analysis. After CBF was analyzed, arithmetic mean and standard deviation were calculated.

Results

N-acetylcysteine concentration was titrated to identify the optimal concentration for our experimental paradigm for

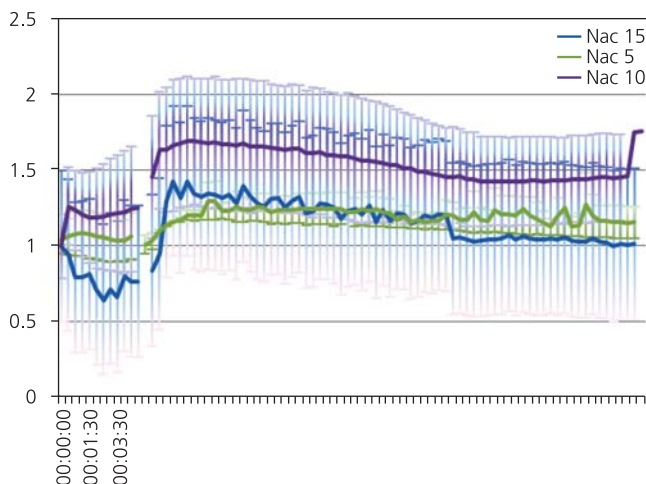


Fig. 1. N-acetylcysteine effects on sinonasal cilia function.

effects on cilia function. CBF was analyzed following application of NAC at 0.5 mg/ml, 5 mg/ml, 7.5 mg/ml, 10 mg/ml, 15 mg/ml, and 50 mg/ml. We found at 50 mg/ml that NAC was ciliotoxic with the cessation of cilia beating 2 minutes following application. Maximal stimulatory effect on cilia function was evident at 10 mg/ml NAC concentration. At first step, we found 10 mg/ml for maximal stimulatory effect. Afterwards, CBF was analyzed at 5 mg/ml, 10 mg/ml, and 15 mg/ml NAC concentrations for five minutes, and then cilia were washed up two times with DPBS. After wash up, cilia movement increased very dramatically (Fig. 1).

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CBFs measured after IBMX, after IBMX+NAC, and after washout were shown in Fig. 2. CBFs measured after IBMX, after IBMX+ DPBS, and after washout were shown in Fig. 3. CBF measured after ATP, after ATP+ NAC, and after washout were shown in Fig. 4. CBF measured after ATP, after ATP+ DPBS, and after washout were shown in Fig. 5.

Comparison of ATP-NAC and ATP-DPBS effects on CBF was shown in Table 1. Comparison of IBMX-NAC and IBMX-DPBS effects on CBF was shown in Table 2.

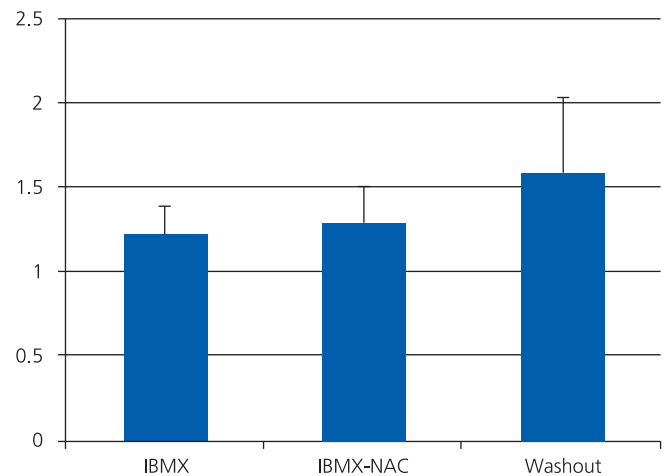


Fig. 2. IBMX and NAC effects on sinonasal cilia function.

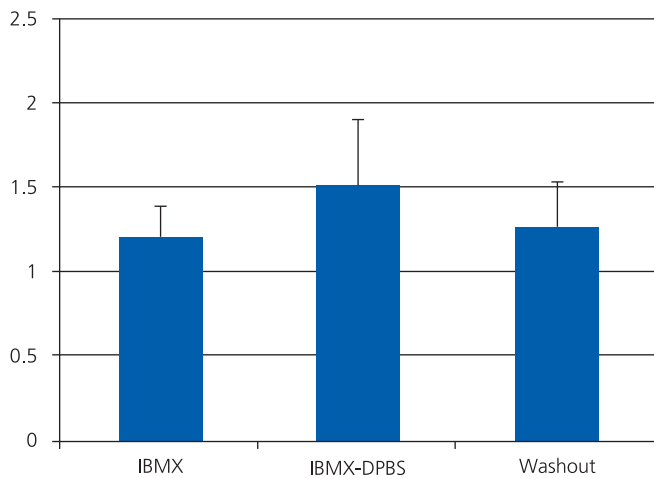


Fig. 3. IBMX and DPBS effects on sinonasal cilia function.

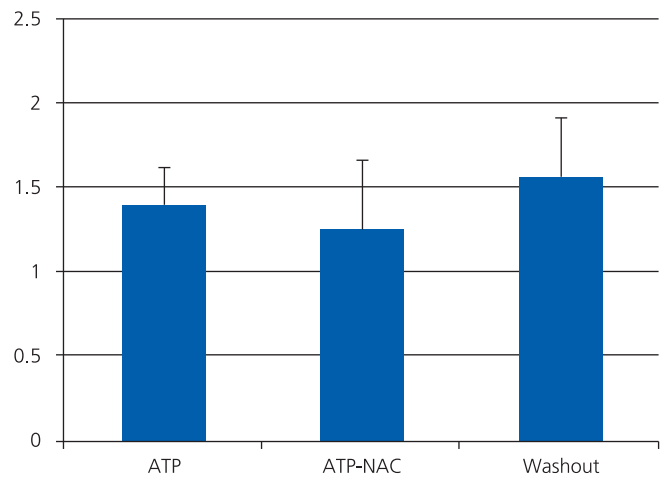


Fig. 4. ATP and NAC effects on sinonasal cilia function.

Discussion

Inhaled pathogens in the nose and paranasal sinuses are cleared by the sinonasal respiratory epithelium. Proper ciliary beating and the biological properties of ASL, which consists of cross-linked glycoproteins, are the main properties of respiratory epithelial mucociliary clearance (MCC).^[5] The mucus flow is mediated by the ciliary beat frequency. Mucus transportability by the cilia is impaired when it becomes more rigid and more viscous.^[5]

N-acetylcysteine has been frequently used as supplementary medication for various diseases i.e. chronic obstructive pulmonary disease, respiratory distress syndrome, and CF.^[6] Cysteine, the main glutathione precursor, is the reduced

form of NAC.^[7] The wide use of NAC depends on its capability to interrupt disulfide bonds and to decrease mucus viscosity; however, recent literature emphasizes the anti-oxidant properties of NAC. A few studies emphasized the ion transport ability of NAC in the respiratory epithelium.^[8,9]

Furthermore, GSNO (S-nitrosoglutathione), a derivative of glutathione, is shown to improve Cl₂ (chloride) efflux from CF airway epithelial cells via CFTR.^[10-12] Consequently, it seemed rational to examine the effect of NAC, which is an indirect glutathione precursor, on Cl₂ efflux from epithelial cells of the respiratory tract.^[13]

N-acetylcysteine initiated a straight dose- and time-dependent reduction in ciliary beat frequency. This decrease

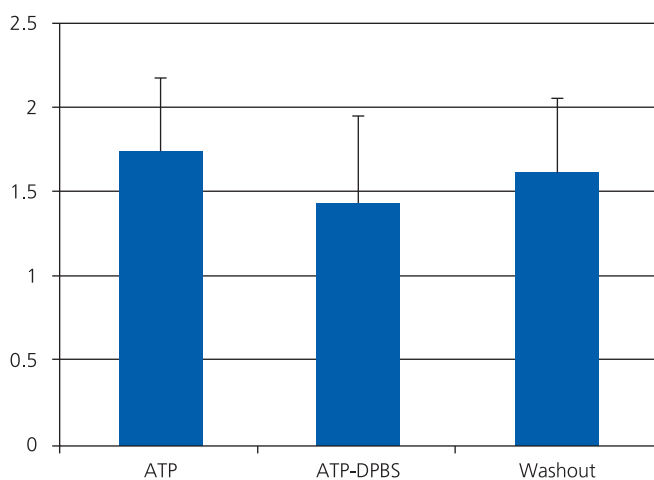


Fig. 5. ATP and DPBS effects on sinonasal cilia function.

Table 1. Comparison of ATP-NAC and ATP-DPBS effects on sinonasal cilia function.

	ATP-NAC/ATP-DPBS	0.047828
Entry	ATP-NAC wo/ATP-DPBS wo	0.044302
Exit	ATP-NAC wo/ATP-DPBS wo	0.106019

Table 2. Comparison of IBMX-NAC/IBMX-DPBS effects on sinonasal cilia function.

	IBMX-NAC/IBMX-DPBS	0.066444
Entry	IBMX-NAC wo/IBMX-DPBS wo	0.401604
Exit	IBMX-NAC wo/IBMX-DPBS wo	0.000623

was first shown at 2 mg/ml and reached significant levels at 20 mg/ml. Total cessation of ciliary movement was initiated within 15 s and at 200 mg/ml; however, this effect was completely reversible within 15 min of perfusion with medium alone. Although NAC had no effect on the ciliary beating pattern, it appears to have a completely reversible inhibitory effect on the ciliary beating frequency of human epithelium.^[14]

Chloride efflux measured with the fluorescent probe MQAE revealed that treatment with NAC increased Cl₂ efflux from CFBE cells in a dose-dependent manner, with the concentration of 10 mM producing the highest effect. Anions have an important role in the regulation of ASL volume, viscosity and pH.

In this experiment, the effect of NAC on human nasal cilia in tissue was studied. Sample of human sinonasal epithelium of patients who underwent chronic rhino sinusitis operation was cultured according to previously described protocol. Cell cultures of four chronic rhino sinusitis patients were used to find the best NAC concentration. Maximum effect on cilia was determined at 10 mg/ml NAC concentration. Cilia were examined at different NAC concentrations for five minutes, and later washed up two times with DPBS at second step. After wash up, ciliary movement increased dramatically. After this increase, ciliary movement decreased gradually in ten minutes. The reason for this increase on CBF was questioned. It was postulated that some cell cycles might be activated after the wash up procedure.

In the third step, ATP and IBMX were added to NAC transwell. IBMX is a calcium channel agonist. Extracellular nucleotides not only ATP, but also ADP and AMP, are key components of the signaling network regulating airway clearance. They are released by the epithelium into ASL to stimulate cilia beating activity, mucus secretion and airway hydration.^[15] The reaction $2ADP \leftrightarrow ATP + AMP$, providing energy for the beating of cilia.^[16]

Dynamic regulation of respiratory CBF is regulated by fluxes in intracellular calcium Ca²⁺. P2X receptors (P2XR) are extracellular ATP-gated, Ca²⁺-permeable, nonselective cation channels. CBF significantly increased four times over baseline from 5.99 ± 3.16 Hz to 22.4 ± 4.33 Hz in the presence of zinc chloride (50 micromoles) and calcium chloride (3 mM).^[17]

In the third step of this experiment both ATP and IBMX increased CBF after wash up returned to normal. If NAC was added to these transwell mediums, dramatic

increase was noted after wash up. This can be attributed to the positive effect of NAC on Cl₂ efflux by a direct effect on cystic fibrosis transmembrane conductance regulator protein and/or alternative chloride channels.^[18] IBMX agonist of NAC enhancing Cl₂ efflux from CFBE cells indicates that not only CFTR, but also alternative Cl₂ channels are responsible for the increase in Cl₂ efflux observed after treatment with NAC.^[18] Then, intracellular Cl⁻ concentration in bronchial epithelial cell was significantly decreased. It was concluded that further studies were needed to show the exact reason of this dramatic increase which probably was caused by the blockage of Ca⁺⁺ and Cl⁻ channel activity cells.

Conflict of Interest: No conflicts declared.

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Please cite this article as: Kapusuz Gencer Z, Saydam L, Cohen NA, Cingi C. N-acetylcysteine effects on sinonasal cilia function. *ENT Updates* 2015; 5(3):87–92.