

Effect of Cellulose Powder on Human Nasal Epithelial Cell Activity and Ciliary Beat Frequency

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Keywords

Cellulose powder · Ciliary beat frequency · Proliferation · Human nasal epithelial cells

Abstract

Background: Cellulose powder (CP) has been reported as a safe and effective complementary treatment for allergic rhinitis (AR). Currently, CP has gained increasing application for clinical management worldwide, particularly in China. However, studies focusing on the effect of CP on normal human nasal epithelial cells (hNECs) and ciliary function are lacking. Here, we aimed to explore the adverse effects of CP on the activity and ciliary function of hNECs. **Methods:** We biopsied ethmoid sinus or middle turbinate tissues during surgical resection from control subjects who underwent endoscopic sinus surgery for diseases other than AR. Cells were isolated and passaged, followed by differentiation in an air-liquid interface (ALI). Flow cytometry and cell viability test (cell counting kit-8) were performed to detect the cytotoxicity of

CP (effects on cell proliferation) on normal hNECs. By using the ALI culture model, we investigated the effects of CP on ciliary beat frequency (CBF). **Results:** There was a significant reduction in hNEC count at high concentrations of CP (2.5 mg/mL) at days 3 and 7 (both $p < 0.05$). As the concentration increased, cell death increased progressively from day 3 to day 7. However, these effects were not evident at low concentrations (0.25 mg/mL, $p > 0.05$). High-dose CP (2.5 mg) significantly reduced the CBF ($p < 0.05$). At lower concentrations (0.25–2.5 mg/mL), CP initially increased but subsequently reduced the CBF of hNECs compared with control group. **Conclusions:** Cytotoxicity and the suppression of ciliary beat at high concentrations justify more prudent use of CP for the management of AR.

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Introduction

Allergic rhinitis (AR) is a common upper airway inflammatory disease that can significantly reduce quality of life, and it affects 10–40% of the population worldwide [1]. AR has also been reported as the most common chronic airway disease in children, contributing to absenteeism at school and sleep apnea, and resulting in an increased likelihood of asthma [2]. The total direct medical cost of AR has been estimated to about USD 3.4 billion and this is mostly attributable to prescription medications [3]. Furthermore, the epidemic of AR in some major cities of China has increased considerably. A high prevalence of AR (18.6–52.9%) and physician-diagnosed AR (10.5–31.4%) has also been seen in northern China where gross pollen levels are high [4]. The prevalence of sensitization to aeroallergens in patients with AR also increased from 69.7 to 76.6% in Guangzhou in 2005–2014 [5]. Therefore, AR has become a major public health issue that confers significant impact on socioeconomic burden and impairs daily quality of life. Exploration of effective treatment is warranted for the management of AR.

Currently, the major pharmaceutical management of AR includes topical steroids, antihistamines, leukotriene receptor antagonists, and immunotherapy [6]. However, side effects are common, particularly with maintenance therapy. For instance, the prolonged use (>2 weeks) of systemic corticosteroids may result in significant hypothalamic-pituitary-adrenal axis suppression, and inhaled corticosteroids have been associated with oral candidiasis and dysphonia [7]. Dry eyes, constipation, and dizziness are reportedly more common among patients treated with antihistamines, and anaphylaxis has been reported following allergen immunotherapy [8]. Therefore, research into a safe and effective treatment is warranted.

Cellulose powder (CP) may potentially bind to the nasal epithelial mucosal membrane following contact with moisture. The formation of a gel layer lining the nasal epithelia means that CP has the capacity of CP to protect the nasal mucosa from direct contact with aeroallergens that would readily trigger allergic inflammatory responses [9]. Administration of CP via Nasaleze™ (Kisska International Ltd., Keighley, UK), the most extensively applied device for intranasal dosing, is effective in restoring nasal epithelial mucus thickness and reducing sensitization to aeroallergens in patients with seasonal AR [10].

Recent clinical trials confirmed the effectiveness and safety of CP for the management of AR [9, 11, 12]. In contrast, another study demonstrated that CP conferred no significant effect on ameliorating dust mite-sensitized AR

children [13]. However, previous studies were limited by the relatively short duration of clinical observations and only patients' subjective rating being assessed. Currently, CP has gained increasing application for clinical management worldwide (particularly in China). Given the potential impact on clinical practice, exploration of the potential adverse effects of CP may better inform clinicians of the safety profiles that have not been hitherto sufficiently investigated.

Although CP has been perceived as a nontoxic product, no study has actually investigated its effects on the nasal mucosal epithelium. In view of the fact that proper mucociliary clearance is vital to the maintenance of homeostasis of the nasal epithelium, an altered mucus thickness may influence mucociliary clearance. We hypothesized that intranasal administration of CP would impair nasal epithelial ciliary function and epithelial renewal.

In this study, we investigated how CP impaired the human nasal epithelial cells (hNECs) and the ciliary beat frequency (CBF). Our findings may call for a more prudent use of CP in clinical practice.

Methods

Specimen Collection

Eight healthy subjects (2 females and 6 males aged 23.9 ± 7.9 years) were recruited from the Department of Otorhinolaryngology, Head and Neck Surgery, at The First Affiliated Hospital of Sun Yat-sen University. We included healthy controls who underwent endoscopic sinus surgery for traumatic optic neuropathy, deviation of nasal septum, benign sinonasal tumors (nerve sheath tumor, neurofibroma, etc.), and noninflammatory sinonasal cysts. We biopsied the ethmoid sinus tissue or middle turbinate tissues during surgical resection. Exclusion criteria consisted of human immunodeficiency virus and hepatitis B and C virus infection, hypersensitivity to Nasaleze or other ingredients of CP, a history of chronic lower airway diseases, treatment with inhaled corticosteroids within 3 months, and participation in a clinical trial within 6 months. Human nasal samples were used for flow cytometry analysis, cell counting kit-8 (CCK8) analysis, and establishing air-liquid interface (ALI) cultures.

Cellulose Powder

CP was administered via the Nasaleze device, which contains a natural CP derived from vegetables and peppermint powder (and does not confer any known effect on human nasal epithelium).

hNECs and Cell Culture

hNECs were isolated from the ethmoid sinonasal or middle turbinate mucosa of control subjects. The nasal mucosa was incubated in 1% Dispase II (D4693–1G, Sigma Aldrich, USA) for 8–12 h at 4 °C, digested with 0.25% trypsin-ethylenediaminetetraacetic acid (Trysin-EDTA, Gibco Inc., USA) for 15 min at 37 °C, and neutralized with 10% FBS (Gibco Inc.). The digested tissue was

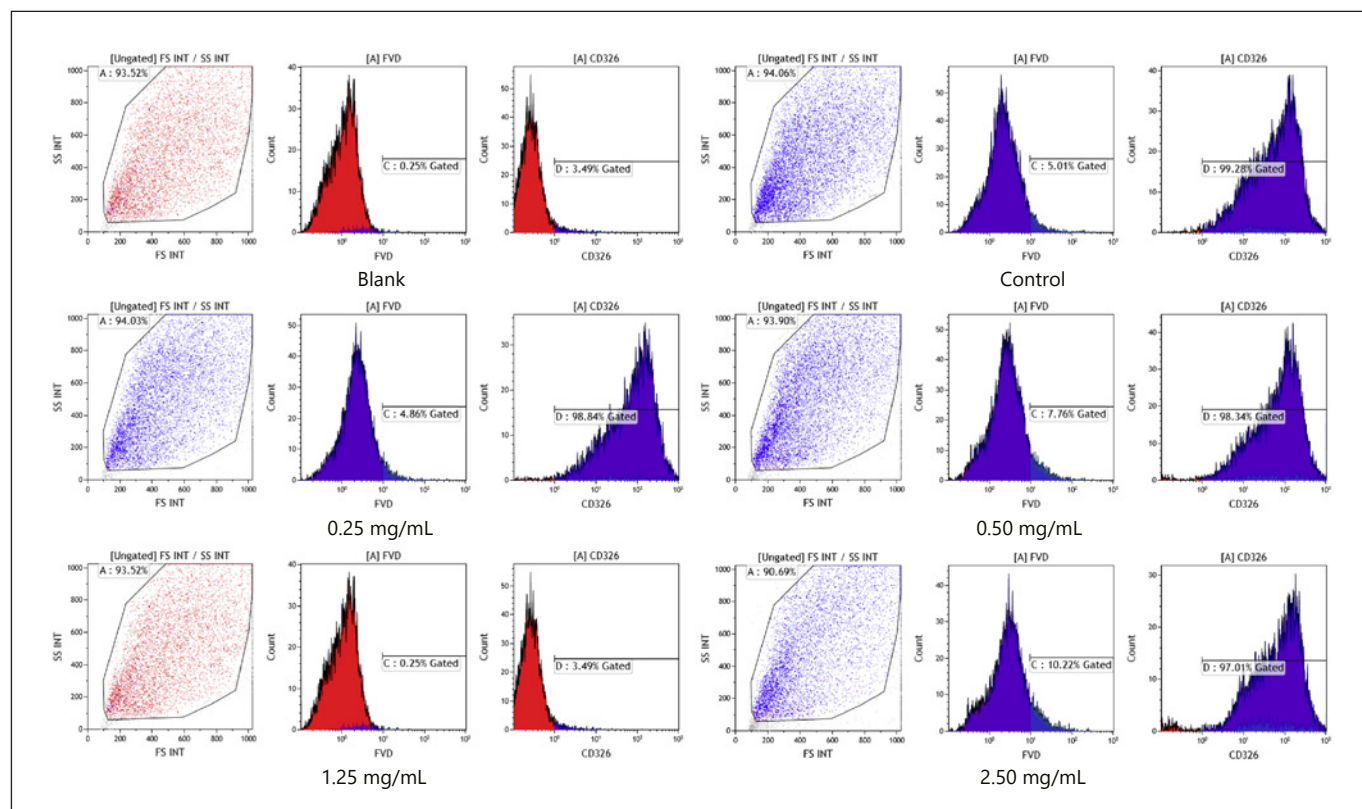


Fig. 1. Effects of different concentrations of cellulose powder (CP) (blank: negative control group, control: positive control group, 0.25, 0.5, 1.25, and 2.5 mg/mL) on normal human nasal epithelial cells (hNECs) at day 3. The percentage of FVD staining represented dead cells and CD326 staining indicated live cells. Flow cytometry results showed that increased concentrations of CP were associated with a progressively higher proportion of FVD-stained cells and a lower percentage of CD326-stained cells at day 3.

passed through a 100- μ m cell strainer (FALCON, Biosciences Inc., USA) to remove the undigested tissue for the collection of hNECs. The hNECs were subsequently incubated in bronchial epithelial cell basal medium (Lonza Inc., USA).

Flow Cytometry

The hNECs (3×10^5 cells) were harvested and washed twice with 1 mL PBS (HyClone Inc., USA). CD326 (2.5 μ L; EpCAM, an epithelial marker of hNECs; eFluor 660, Ebioscience Inc., USA) and PBS (50 μ L) were added for incubation at 4 °C for 30 min, followed by the addition of 450 μ L PBS and 0.5 μ L fixable viability dye (FVD, a marker of dead hNECs; eFluor 450, Ebioscience Inc.) at 4 °C for 20 min. The hNECs were washed twice with 2 mL PBS. The supernatant was discarded and cell pellets were resuspended in 300 μ L PBS. Flow cytometric analysis was performed by using a flow cytometer (Gallios, Beckman Coulter Inc., Miami, FL, USA).

Cell Counting Kit-8 Assay

hNECs were seeded on a 96-well plate (1,500 cells/well). After treatment with 0.25 mg/mL CP for 12 h, they were washed with PBS for subsequent colorimetric assay with 10 μ L CCK8 (Dojindo Laboratories Co. Ltd., Japan) to determine cell viability. The 96-

well plate was incubated at 37 °C for 2 h. The optical density was detected at a wavelength of 450 nm with a microplate reader (BioTek, SynergyHT, USA).

ALI Culture and CBF Assessment

hNECs (2.5×10^5 cells/well) were seeded on 24-well plate inserts (Corning HTS Transwell®, USA) at an ALI by using PneumaCult™-Ex and ALI medium (STEMCELL Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's protocol. To determine changes of ciliary activity, hNECs were placed in an incubation chamber until they reached a mature stage, and CBF measurements were taken with Sisson-Ammons video analysis software (SAVA, Bruce-Ammons Co. Ltd., USA) [14]. In addition, the CBF was detected following the administration of different concentrations of CP. Ten random fields were selected for CBF assessment, and subsequently averaged to represent the mean value for individual samples.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism v6.0 (GraphPad Inc., USA). Continuous variables were presented as mean $\pm$ standard deviation. In this study, we sought to compare the

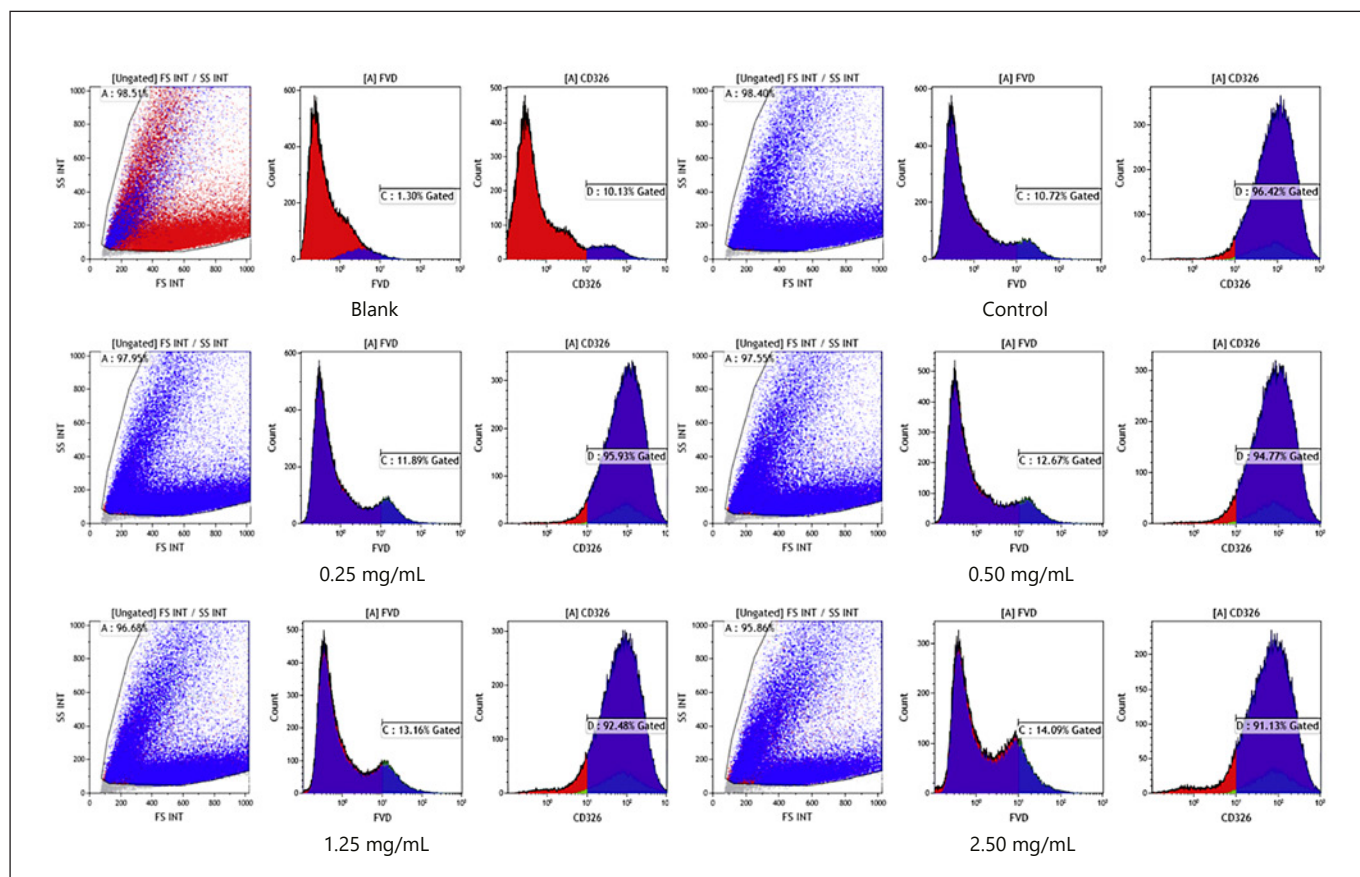


Fig. 2. Effects of different concentrations of cellulose powder (CP) (blank: negative control group, control: positive control group, 0.25, 0.5, 1.25, and 2.5 mg/mL) on normal human nasal epithelial cells (hNECs) at day 7. The percentage of FVD staining represented dead cells and CD326 staining represented live human nasal epithelial cells. Increased concentrations of CP were also associated with a progressively higher proportion of FVD-stained cells and a lower percentage of CD326-stained cells at day 7.

Table 1. Flow cytometry results at day 3 with different concentrations of CP

	FVD stain	<i>p</i>	CD326 stain	<i>p</i>
Blank	0.26±0.17%	0.033	1.34±1.87%	<0.001
Control	7.78±2.46%	–	98.31±2.24%	–
0.25 mg/mL	10.74±5.90%	0.364	98.30±1.89%	0.963
0.5 mg/mL	13.16±4.68%	0.057	98.02±1.92%	0.484
1.25 mg/mL	9.93±3.64%	0.104	96.77±2.33%	0.211
2.5 mg/mL	12.70±3.27%	0.050	96.78±1.76%	0.067

All groups are compared with the control group separately and the variables are presented as mean ± standard deviation.

Table 2. Flow cytometry findings at day 7 treated with different concentrations of CP

	FVD stain	<i>p</i>	CD326 stain	<i>p</i>
Blank	1.06±0.77%	<0.001	6.94±5.71%	0.003
Control	10.58±1.20%	–	97.07±2.40%	–
0.25 mg/mL	11.24±1.99%	0.306	96.74±2.58%	0.108
0.5 mg/mL	12.53±1.07%	0.002	95.74±3.22%	0.106
1.25 mg/mL	12.10±3.08%	0.305	93.92±4.30%	0.104
2.5 mg/mL	14.16±0.21%	0.047	91.71±3.59%	0.017

All groups are compared with the control group separately and the variables are presented as mean ± standard deviation.

effects of different concentrations of CP on epithelial cell death, so differences between groups were analyzed by using one-way ANOVA and then unadjusted with the least significant differences test. Analysis of the effects of CP at multiple time points was performed by using one-way ANOVA for comparison of the means, followed by the Bonferroni correction to adjust for multiple testing. $p < 0.05$ was considered statistically significant, unless otherwise stated.

Results

Cytotoxicity of CP on hNECs at Day 3

In our pilot study, we tested the effects on CP at the concentration range of 1.5–3.5 mg/mL, so for this study, we initially selected the moderate concentration of 2.5 mg/mL as the upper limit. To determine the cytotoxicity of different concentrations of CP (blank: negative control group, control: positive control group, 0.25, 0.5, 1.25, and 2.5 mg/mL) on normal hNECs, we cultured the same count of hNECs (3×10^5 per well for duplicate wells) in different plates and cocultured with different concentrations of CP on the apical aspect for 3–7 days. We determined the activity of cells when growing steadily at day 3, to examine whether CP could significantly affect the proliferation of hNECs.

Increased concentrations of CP were associated with a progressively greater dead cell count at day 3 (Fig. 1). The experiment was repeated in triplicate, and there was evidence of an increase in the percentage of cells on FVD staining (Table 1). The proportion of dead cells increased significantly when treated with 2.5 mg/mL CP ($p < 0.05$). However, the percentage of CD326-stained cells did not differ statistically in the control group and all treatment groups at day 3 (Table 1).

Cytotoxicity of CP on hNECs at Day 7

To evaluate the effects of prolonged exposure to CP on hNECs, we further cultured the identical amount of hNECs (3×10^5 per well, with duplicate wells) in different plates, followed by coculture with different concentrations of CP for up to 7 days.

Similarly, greater concentrations of CP resulted in an increased number of dead hNECs at day 7 (Fig. 2). The experiment was repeated in triplicate, and there was evidence of a higher percentage of FVD-stained cells. This increase in FVD staining was significantly greater when treated with 0.5 mg/mL ($p = 0.002$) and 2.5 mg/mL CP ($p = 0.047$). Meanwhile, we noted a progressive decline in the percentage of CD326-stained cells between the control group and the 2.5-mg/mL group ($p = 0.017$) at day 7 (Table 2).

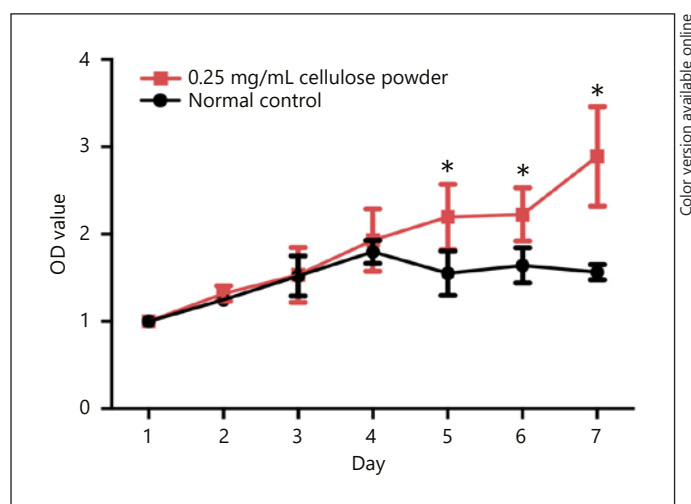


Fig. 3. CCK8 experiment. Effect of 0.25 mg/mL cellulose powder on the growth rate of normal human nasal epithelial cells (hNECs) at day 7. hNECs were placed on a 96-well plate, and OD value was measured by CCK8 kit at the same time point on days 1–7 (5 repetitions per day, data presented as mean $\pm$ standard deviation, * $p < 0.05$ compared with control group).

Effects of CP on Proliferation of hNECs

Our previous findings revealed that 0.25 mg/mL corresponded to the maximal tolerable concentration of CP for hNECs. To further validate this point, we examined the effect of CP at 0.25 mg/mL on cellular proliferation by performing a CCK8 assay (Fig. 3). Reassuringly, 0.25 mg/mL CP conferred no significant cytotoxicity on hNECs in terms of cell proliferation.

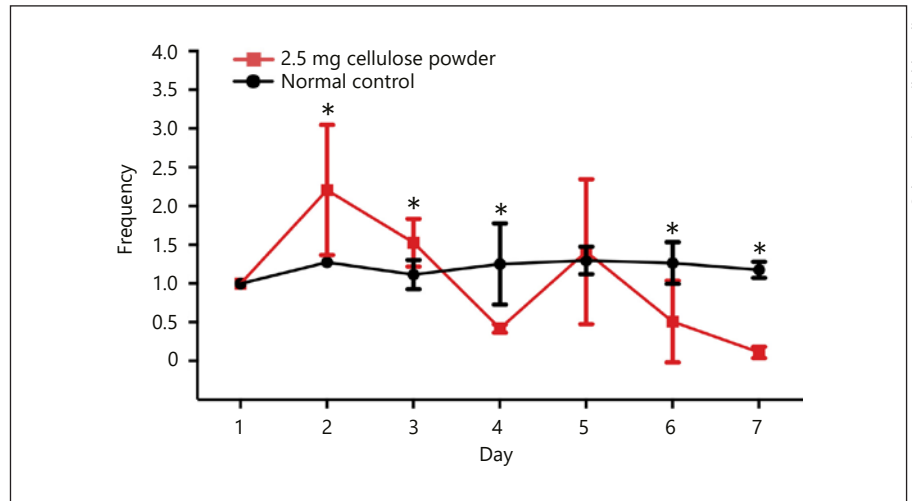
Effect of CP on CBF of hNECs

Next, we sought to further explore the effect of CP on ciliary motility. A single dose of CP was administered (1.5–3.5 mg) in the above experimental setting, so we selected an intermediate dose of 2.5 mg for initial testing. We found that direct treatment with 2.5 mg of CP in the apical aspect (the surface of cilia in the upper chamber) resulted in a temporary increase in the CBF (until day 3), followed by a progressive decrease thereafter. Interestingly, the decrease in CBF was not tempered, even after elution with PBS at day 4 (Fig. 4).

Effect of Different Concentrations of CP on the CBF of hNECs

Having demonstrated that high-dose CP could impair the CBF of hNECs, considering that the area for contact between nasal epithelia and CP in the Transwell chambers could be limited, we further sought to examine the

Fig. 4. Ciliary beat frequency (CBF) of normal human nasal epithelial cells (hNECs) after treatment with 2.5 mg of cellulose powder (CP) on day 1–7. The hNECs were placed in 24-well Transwell chambers until they reached a well-differentiated stage, and treated with 2.5 mg CP. The CBF of hNECs was determined from day 1 to day 7 (3 repetitions per day, data are presented as mean $\pm$ standard deviation). * $p < 0.05$ compared with control group.



effects of CP at lower concentrations by diluting the CP. We next treated the well-differentiated ciliated cells with different concentrations (control, 0.25, 0.5, 1.25, and 2.5 mg/mL) of CP in the apical aspect of cultured hNECs (upper chamber) for 7 days. When administered at all concentrations, treatment with CP consistently resulted in a temporary increase in the CBF, followed by a progressive decrease when compared with the control group (Fig. 5).

Discussion

CP has been marketed as a preventative medication for exacerbations of AR in clinical practice worldwide, particularly in China. Despite previous reports delineating the therapeutic effects of CP in the prevention of flare-ups of AR, no study has meticulously examined the potential side effects on the nasal epithelium, particularly when administered at higher doses or concentrations. In this study, we investigated the effects of CP on hNECs which can act as a physical barrier against aeroallergens and pathogens. We found that high-dose (but not low-dose) CP had a significant adverse impact on the proliferation of hNECs. Our findings have provided evidence of the side effects of high-dose CP. Interestingly, CBF (a critical parameter reflecting ciliary motility) could also be affected by different dosages of CP. Administration of CP at greater concentrations and dosages consistently led to a temporary increase, followed by a slow but progressive decline, in the CBF. All these crucial findings may call for more prudent use of CP in future clinical practice.

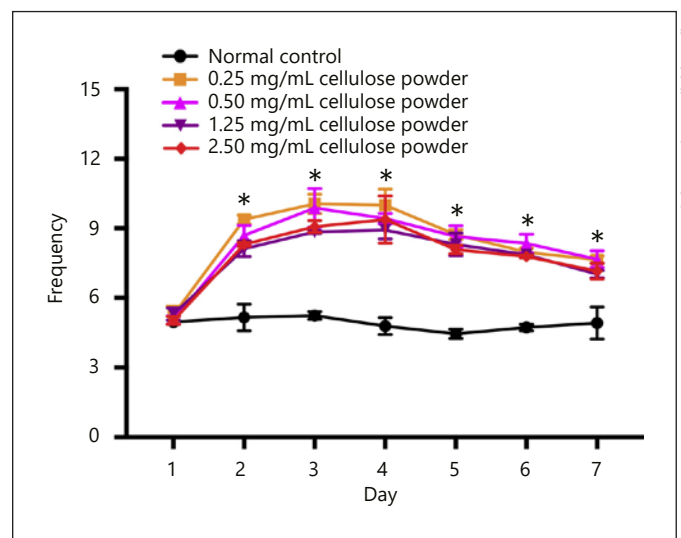


Fig. 5. The ciliary beat frequency (CBF) of normal human nasal epithelial cells (hNECs) treated with different concentration of cellulose powder from day 1 to day 7. The hNECs were placed in 24-well Transwell chambers till a mature stage and treated with different concentrations (control, 0.25, 0.5, 1.25, and 2.5 mg/mL) of CP. The CBF was determined from day 1 to day 7. * $p < 0.05$ compared with control group.

Since the 1990s, CP has gained extensive clinical application in the preventative management of AR and hay fever worldwide [15, 16]. However, few studies have directly explored the impact of CP on mucociliary clearance. An altered nasal epithelial microenvironment (i.e., increased thickness of the airway surface lining fluid layer, bacterial or viral infection, and malfunction of the cil-

iated cells) may contribute to defective mucociliary clearance and CP may exert its pharmacological actions via direct contact with the nasal mucosa. Caution should be therefore be exercised when using CP due to the potential impact on mucociliary clearance. Consistent with this, a previous study documented that a decrease in mucociliary clearance facilitated proliferation of the pathogen in the airway epithelium [17].

The airway mucosa functions as the first-line defense component against invading allergens and pathogens through the epithelium barrier and mucociliary clearance. Therefore, medications that directly act on the nasal mucosa might affect epithelial barrier function and alter the mucociliary clearance. This is particularly important in the milieu of the significant remodeling of nasal mucosa in patients with AR [18, 19], which indicates an impaired epithelial proliferation capacity and mucociliary clearance. A previous study showed that cellular activities such as DNA replication may depend on oxygen concentration, so hypoxia might result in a decrease in the rate of cell proliferation [20]. In other studies, it was found that mucus hypersecretion can lead directly to the obstruction of the airways, resulting in poor mucociliary clearance and poor tissue oxygenation [21, 22]. These findings have lent support to the fact that high concentrations of CP may considerably impair the airway epithelial function by forming a thickened gel on the epithelial surface. In contrast, when administered at lower concentrations or doses, CP may form only a thin layer of hydrogel, so it is not surprising that its effect on epithelial proliferation may not be noteworthy, consistent with our findings. Our results have demonstrated that high doses of CP may not only contribute to cytotoxicity of hNECs, but also impair the epithelial mucociliary clearance.

To our knowledge, cilia are involved in some important physiologic processes and ciliary function mainly manifests as changes in CBF. The CBF may depend considerably on many factors, particularly environmental conditions such as temperature, pH value, medications, and toxins [23]. Ideally, the drug for nasal delivery should be inert within the applied concentration ranges, and not cause harm to the epithelium, particularly ciliary motility [24]. Recently, some studies documented that many preservatives may have cumulative adverse effects on the CBF and result in irreversible ciliostasis, even after they have been withdrawn and the nasal mucosa has been rinsed [25, 26]. Ciliary motility may recover once the stimuli (such as inflammatory mediators, pathogens, and allergens) are removed [27]. Sanderson et al. [28] demon-

strated that the CBF increased transiently by $\geq 20\%$ after mechanical stimulation of the cellular surface or the cilia, and that these events comprised a short lag, an increase, and a recovery phase. Therefore, upon stimulation, the CBF may initially accelerate for clearance of the invading pathogens or allergens, and then subsequently return to normal levels once the stimuli are effectively removed. Nonetheless, continuous stimulation by inflammation, pathogens, and allergens may have predisposed the CBF to be significantly suppressed [29].

There are clear clinical implications to our study findings. In patients with AR, epithelial barrier function might have been significantly abrogated by the exaggerated nasal epithelial remodeling. Preventative agents such as CP at high doses or concentrations may further aggravate the defect airway barrier function by limiting cellular self-repair and the beating of the cilia. Our study calls for more prudent application of CP in clinical practice, by providing objective evidence of the maximal safe dose or concentration. In addition, given the adverse effects of the prolonged use of CP, we do not recommend long-term maintenance therapy with CP for AR sufferers.

Our study has notable limitations. First, we did not sample the inferior turbinate mucosa for control. However, there have been publications supporting the use of ethmoid sinonasal or middle turbinate mucosa as the control tissue [30–33]. Second, the assessment of whether CP affects the proliferation and function of the relatively normal epithelium (which has a capacity for self-repair) may provide greater insight for clinical relevance, so the nasal epithelial cells were derived solely from healthy subjects. This meant, however, that in view of chronic inflammation and/or infection in the epithelium of AR patients, our study was not capable of determining which of the epithelial cells are less affected or not affected by allergic inflammation. Third, we only focused on the proliferation and functional changes of epithelial cells. Further investigation focusing on cell morphology and structure after CP application may help to elucidate the potential impact of CP on hNECs. Fourth, as ciliary mobility depends on CBF (e.g., a low CBF may only require hydrolysis of the ATP and a high CBF is governed by signaling of the second messengers [34]), further investigations that explore the mechanisms underlying the changes in CBF caused by CP are needed. Furthermore, the limited sample sizes constrained our analysis of the repeatability of our findings.

In conclusion, CP confers a significant negative impact on cell activity and ciliary mobility at a high dosage

or concentration. CP should be applied more prudently, taking into consideration its impact on mucociliary clearance. Maintenance therapy with or prolonged use of high-dose CP is not warranted.

Acknowledgements

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Statement of Ethics

Ethics approval was obtained from the hospital institutional review board. The independent ethics committee for clinical research and animal trials of the First Affiliated Hospital of Sun Yat-sen University approved all procedures (No. [2017] 164). All patients signed a written informed consent.

Disclosure Statement

The authors declare that they have no relevant conflicts of interest.

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