The effect of myrtol standardized on human nasal ciliary beat frequency and mucociliary transport time

Demin Han, M.D., Nan Wang, M.D., and Luo Zhang, M.D.

ABSTRACT

Background: *This study was designed to observe the effects of myrtol standardized (Gelomyrtol forte), a secretomucolytic phytomedicine, on both ciliary beat frequency (CBF)* in vitro *and mucociliary transport time (MTT)* in vivo.

Methods: *Changes in cultured human nasal CBF in response to immediate treatment with 75, 150, or 300 ng/mL of myrtol standardized and prolonged treatment (12 or 24 hours) with 300 ng/mL of myrtol standardized were quantified by using high-speed digital microscopy. In addition, MTT before and after oral application of myrtol standardized (three times a day, 900 mg/day, 10 days) was determined using the saccharine test, and the effects of this treatment regime on nasal patency was measured by acoustic rhinometry and active anterior rhinomanometry in 22 patients with nonallergic chronic rhinitis. Another 10 patients without medication, who had the same examinations twice with a 10-day interval, were involved as controls.*

Results: *Neither immediate nor prolonged treatment with myrtol standardized produced a distinguishable change in CBF. Meanwhile, only in patients with treatment, MTT, as well as a unilateral minimum cross-sectional area, the volume of 0–5 cm inside the nasal cavity, the unilateral nasal resistance at 75 Pa and total symptom visual analog score were significantly improved after treatment.*

Conclusion: *Based on these results we propose that a 10-day treatment with an herbal medicine, myrtol standardized, improves nasal mucociliary clearance as well as nasal patency in patients with chronic rhinitis. However, it has no impact on* ex vivo *CBF.*

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Key words: Ciliary beat frequency, herbal medicine, mucociliary transport time, myrtol standardized, nasal epithelial cells, phytotherapeutic, rhinitis

Mucociliary clearance (MCC), which moves mucus toward the nasopharynx, is a key host defense function of the nose and paranasal sinuses.[1] This function is influenced by ciliary beat frequency (CBF) as well as the properties of the upper mucus layer.[2–4] Although the relationship between CBF and mucociliary transport is still unclear, it has been generally accepted that CBF is an important determinant of MCC.[5] MCC is reported to be impaired in certain respiratory diseases, such as chronic rhinosinusitis, cystic fibrosis, chronic bronchitis, and asthma. Medication is administered to relieve these respiratory complaints. For example, in China, as well as Germany, myrtol standardized (Gelomyrtol forte), a secretomucolytic phytomedicine, is well established for the treatment of acute and chronic sinusitis, as well as bronchitis.[6,7] It is a compound reported to enhance airway mucus clearance and is commonly prescribed to patients with thick or excessive nasal mucus production, as well as for nasal congestion.

However, the influence of myrtol standardized on both CBF and mucociliary transport time (MTT) is not well understood. Although multicenter, placebo-controlled, double-blind, randomized studies have provided evidence that the compound could effectively treat respiratory inflammation, few have shown a clear effect of myrtol standardized on direct measures of CBF[8] and none have examined the drug's impact on nasal mucus clearance in patients with chronic rhinitis. The aim of the current study was to investigate the influence of myrtol standardized on nasal CBF, as assessed by *ex vivo* nasal tissue culture and high-speed (240 frames/s) digital microscopy. Moreover, we examined the influences of myrtol standardized treatment on both nasal MCC *in vivo*, as assessed by saccharine transport time, and nasal patency, as assessed by rhinomanometry and acoustic rhinometry.

Key Laboratory of Otolaryngology–Head and Neck Surgery, Capital Medical University, Ministry of Education of China, Beijing Institute of Otorhinolaryngology, Beijing, China, and Department of Otolaryngology, Head and Neck Surgery, Affiliated Beijing Tongren Hospital of Capital Medical University, Beijing, China

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Address correspondence and reprint requests to Luo Zhang, M.D., Beijing Institute of Otolaryngology, No. 17, HouGouHuTong, DongCheng District, Beijing 100005, China

E-mail address: dr.luozhang@gmail.com

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Beijing Institute of Otolaryngology.

Materials

Myrtol standardized, obtained from by G. Pohl-Boskamp GmbH & Co. (Hohenlockstedt, Germany), was dissolved in Cremophor EL (Sigma-Aldrich-Fluka, Germany)[9] and diluted in Hanks' balanced salt solution (Sigma-Aldrich, St. Louis, MO) supplemented with 25 mM of HEPES (Sigma-Aldrich) (sHBSS, pH 7.4) to the final working concentrations with Cremophor EL concentrations of 0.1%. Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco-BRL (Carlsbad, CA).

Cell Culture

Primary cultures of human nasal epithelial cells were prepared as previously described.[10,11] Briefly, mucosa of the uncinate process was endoscopically obtained from five patients with chronic rhinosinusitis and cut into ~0.5-mm squares, plated on collagen-coated glass coverslips,[12] and cultured on DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C in 10% CO_2 for 6–8 days. To observe the prolonged effects of myrtol standardized, the 300-ng/mL myrtol standardized medium was added to the wells of the cell culture plate at the 6th day of culture and the cells were incubated for an additional 24 hours. CBF was sequentially measured before addition of the medication, and again 12 and 24 hours subsequently.

Measurement of CBF

Measurements of CBF were made according to a previously published method.[13,14] Generally, ciliated epithelial cells were viewed with an inverted microscope (Olympus IX71; Japan) equipped with a 40×, 1.3 N.A., Ph 4, oil-immersion objective. Imaging of cilia move-

ment was performed at 23°C and was achieved by directing the light forming the phase-contrast images into a high-speed CCD camera (TM-6710cL; Pulnix America, Sunnyvale, CA). The camera was used with a frame grabber (Meteor; Matrox Co., Quebec, Canada) and recording software (StreamPix 3.16.5; Norpix Corp., Quebec, Canada). The phase-contrast images of cilia movement were recorded at 240 frames/s. Images were evaluated using image analysis software (IPLab v3.65a; SCANALYTICS, Inc.). CBF was measured using a 3-second waveform (720 frames) that was generated by the variation in gray-level intensity of the phase-contrast images that resulted from the repetitive motion of cilia. The frequency of each ciliary beat cycle was calculated from the period of each cycle of the gray-intensity waveform by a beat-by-beat analysis.[13]

To observe the immediate effects of myrtol standardized on cilia movement, the concentration of the medication in the solution in the experimental chamber (400 μL) was adjusted using 1 mL of solution containing myrtol standardized with a concentration of 75, 150, or 300 ng/mL. The medication solution was added to the chamber by micropipette while an equal volume of the solution in the experimental chamber was removed simultaneously by suction. The process was completed within 20 seconds. The CBF was then measured every minute for the next 20 minutes.

Subject Recruitment

Thirty-two patients (14 male and 18 female patients) with nonallergic chronic rhinitis were recruited, who were divided into a treatment group (10 male and 12 female patients) and a control group (4 male and 6 female patients). The diagnosis was made based on two or more nasal symptoms (nasal congestion, rhinorrhea, sneezing/itching, and impairment of smell) of >1-hour duration most days and for longer than 3 months.[15] All patients underwent a skin-prick test (SPT) with histamine (positive control) and standardized extracts (ALK-AbellÓ, Hørsholm, Denmark) of the following allergens: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat and dog dander, mold mix, American cockroach, grass pollen mix, tree pollen mix, and weed pollens. The mean of the largest diameter of the wheal and its perpendicular diameter were recorded as the response to the SPT. The skin test index was the ratio between the response to an allergen and the response to histamine. None had a positive SPT (wheal diameter of ≥3 mm and the skin test index of ≥0.5). All patients were free from active lower airway infection, fever, or other active symptoms at the time of the study. The 22 patients in the treatment group were given myrtol standardized, 300 mg orally, three times a day (900 mg/day) for 10 days. For each subject, the nasal MTT and the nasal patency were investigated before and immediately after the 10-day application of the drug. Of the 10 patients in the control group, the MTT and the patency were measured twice with a 10-day interval.

Before and after the 10-day treatment or observation without medication, the severity of total symptoms was evaluated with a visual analog scale (VAS) using a 100-mm horizontal line without intermediate marks or signs. The left end was labeled "no symptom," and the right end was labeled "most severe symptom." The patients marked the scale with a cross to evaluate the severity of the disease. Informed consent was obtained for all individuals at the time of enrollment.

Measurement of Nasal MTT

The nasal MTT was determined by the saccharine test.[16] Briefly, the test was performed in a supine position at 23–25°C and a humidity of 20–24% by placing under direct visualization a 5-mg particle of saccharine, measuring ~0.5 mm, ~10 mm posterior to the anterior tip of one inferior turbinate. The subjects were told to refrain from sneezing, sniffing, drinking, or eating and were told to swallow approximately every 30 seconds.[10] A stopwatch was started and the time taken between the application of the saccharine and the sensation of a sweet taste was defined as MTT. If the subject was unable to taste any sweetness after 30 minutes, an additional particle of saccharine was placed on the anterior aspect of the subject's tongue to exclude taste loss. In all determinations, the subjects had intact taste.

The Evaluation of Nasal Patency

Before the examination, the subjects sat quietly in the laboratory (temperature of 24 ± 1°C and humidity of 70 ± 1%) for 20 minutes and remained sitting upright. Minimum cross-sectional area (MCA) and the volume of 0–5 cm inside the nasal cavity (V5) were measured by acoustic rhinometry (Eccovision Acoustic Rhinometer; Hood Labs).[17] Subsequently, the unilateral nasal resistance at 75Pa (R75) was measured by active anterior rhinomanometry (ATMOS 300 Rhinomanometer; ATMOS MedizinTechnik GmbH & Co., Lenzkirch, Germany). The examination was completed within 6 minutes by the same researcher to maintain a constant congestive state.

Data Analysis and Statistics

Data were expressed as mean ± SEM. Pre- and postmedication MTT, as well as nasal patency comparisons, were made using the paired Student's test. Other statistical analyses were performed using one-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA on ranks. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effects of Myrtol Standardized CBF

To observe the immediate CBF changes in response to myrtol standardized, four groups of cells were treated with 75, 150, and 300 ng/mL of myrtol standardized and 0.1% Cremophor EL, respectively. The basal CBF (CBF before treatment) for each of the four groups was 12.9 ± 0.9 Hz ($n = 10$), 13.7 ± 0.7 Hz ($n = 10$), 12.9 ± 0.6 Hz ($n = 10$), and 12.5 ± 0.6 Hz ($n = 7$), respectively. There was no significant difference in basal CBF within groups.

The immediate CBF changes in response to increasing concentrations of myrtol standardized are summarized in Fig. 1. On treatment with 75 ($n = 10$), 150 ($n = 10$), or 300 ng/mL ($n = 10$) of myrtol standardized, no distinguishable change in CBF relative to the basal CBF was identified within 20 minutes (Fig. 1, *A–C*, respectively). The 0.1% Cremophor EL was used as a negative control to rule out either the influence of the turbulence caused by changing the solution in the cell chamber or the effects of the vehicle. On treatment with 0.1% Cremophor EL, no distinguishable change in CBF relative to the basal CBF ($n = 7$) was identified within 20 minutes (Fig. 1 *D*).

To observe the CBF changes in response to prolonged treatment with myrtol standardized, cells were incubated with 300 ng/mL of myrtol standardized for 24 hours. Before incubation, the basal CBF was 11.4 ± 0.3 Hz ($n = 15$). No significant change in CBF was found after 12-hour (11.7 ± 0.4 Hz) or 24-hour incubation (11.8 ± 0.4 Hz; Fig. 2).

Effect of Myrtol Standardized on MTT

The treatment group and control group were well balanced for demographic features (Table 1). All of the subjects were able to taste sweetness within 30 minutes. Of 22 patients in the treatment group, the mean MTT before and after treatment with myrtol standardized was 883 ± 61 seconds and 715 ± 63 seconds, respectively. The difference was significant ($p < 0.001$). Of 10 patients in the blank control group, there was no significant difference in the mean MTTs with a 10-day interval, which were 926 ± 52 seconds and 1001 ± 50 seconds, respectively.

Effect of Myrtol Standardized on Nasal Patency

Nineteen of the 22 patients (9 male and 10 female patients) were able to complete the pre- and posttreatment objective examination of nasal patency. The mean value of unilateral MCA after treatment

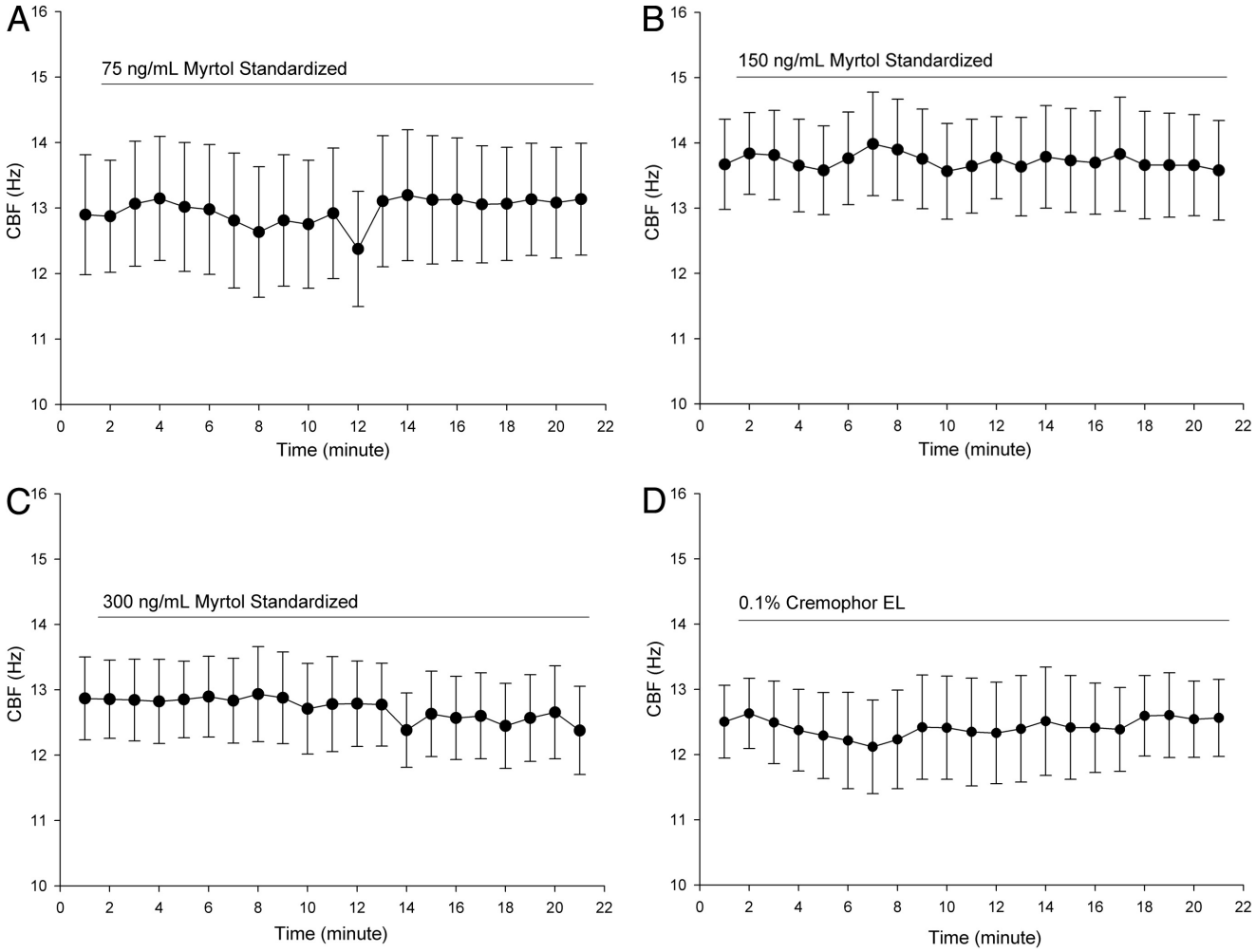

Figure 1. Immediate effect of myrtol standardized and Cremophor EL on human nasal ciliary beat frequency (CBF). (A) Seventy-five (n = 10), (B) 150 (n = 10), and (C) 300 ng/mL (n = 10) of myrtol standardized and (D) 0.1% Cremophor EL (n = 7) did not induce significant change in CBF compared with basal CBF.

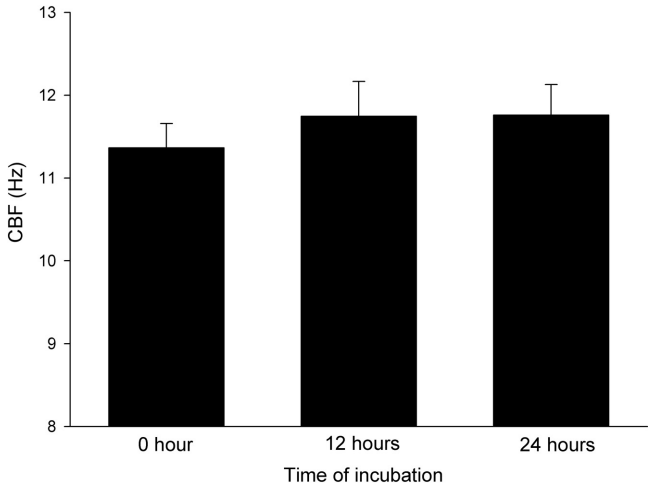

Figure 2. Prolonged effect of myrtol standardized on human nasal ciliary beat frequency (CBF). CBF did not significantly change after the cells were incubated with 300 ng/mL of myrtol standardized for 12 (11.7 ± 0.4 Hz) or 24 hours (11.8 ± 0.4 Hz) compared with basal CBF (11.4 ± 0.3 Hz, n = 15).

(0.56 ± 0.03 cm^2, n = 38 sides) was significantly higher than that before treatment (0.42 ± 0.03 cm^2, n = 38 sides; $p < 0.001$; Fig. 3 *A*). Similarly, there was a significant difference between the V5 measured before and after the treatment (pretreatment, 3.71 ± 0.23 cm^3, versus posttreatment, 4.43 ± 0.18 cm^3; $p < 0.01$; Fig. 3 *B*). The change in the mean value of unilateral R75 after treatment (2.00 ± 0.16 $Pa/cm^3 \cdot s^{-1}$, n = 38 sides) compared with that before treatment (2.67 ± 0.19 $Pa/cm^3 \cdot s^{-1}$, n = 38 sides) was also significantly different ($p < 0.05$; Fig. 3 *C*).

Of the 10 patients in the control group, the mean values of unilateral MCA (pretreatment, 0.45 ± 0.05 cm^2, versus posttreatment, 0.47 ± 0.04 cm^2), V5 (pretreatment, 3.83 ± 0.32 cm^3, versus posttreatment, 3.77 ± 0.21 cm^3), and unilateral R75 (pretreatment, 3.00 ± 0.25 $Pa/cm^3 \cdot s^{-1}$ versus posttreatment, 2.93 ± 0.21 $Pa/cm^3 \cdot s^{-1}$) did not change significantly after 10-day observation without treatment.

Evaluation of Clinical Efficacy

Of the 22 patients in treatment group, the reduction in the mean value of VAS was statistically significant after the treatment (pretreatment, 5.8 ± 0.3, versus posttreatment, 5.4 ± 0.3; $p = 0.046 < 0.05$). Among them, 21 patients requested further medication because of the residual symptoms. Consequently, they were treated with topical steroids. Of the 10 patients in the control group, the mean value of VAS was identical after 10-day observation without treatment (pre-

Table 1 **Demographic and clinical characteristics of study participants**

	Treatment Group	Control Group
No. of patients	22	10
Treatment	Myrtol standardized	None
Age (yr)	38.5 ± 2.7	42.2 ± 2.6
Sex (male/female)	10/12	4/6
VAS	5.8 ± 0.3	5.9 ± 0.6
MTT (s)	883 ± 61	926 ± 52
MCA (38 sides, cm^2)	0.42 ± 0.03	0.45 ± 0.05
V5 (38 sides, cm^3)	3.71 ± 0.23	3.83 ± 0.32
R75 (38 sides, $Pa/cm^3 \cdot s^{-1}$)	2.67 ± 0.19	3.00 ± 0.25

Data are mean ± SEM or number of patients.
MCA = mucociliary clearance; MTT = mucociliary transport time; VAS = vicual analog scale.

treatment, 5.9 ± 0.6, versus posttreatment, 5.9 ± 0.5). Topical steroids were prescribed to them.

DISCUSSION

Secretomucolytic phytomedicines, such as myrtol standardized and BNO-101 (Sinupret) are well established for the treatment of sinusitis,[18] as well as bronchitis[6,7] in Germany, China, and other countries. Although it has been widely used as a "mucoactive" agent for the symptoms of respiratory infections, its underlying mechanisms have not been clarified. Our study examined the effects of myrtol standardized on MCC as measured *in vivo* by the saccharine test and on ciliary motility as measured *ex vivo* by CBF. We found that myrtol standardized treatment improved MCC, as well as clinical symptoms in patients with chronic nonallergic rhinitis, but had no significant effect on ciliary motility *ex vivo*. Myrtol standardized is a phytotherapeutic distillate with primarily secretolytic and secretomotoric properties accompanied by anti-inflammatory and bronchodilatory effects[9] and mainly consists of three monoterpenes: (+)α-pinene, *d*-limonene, and 1,8-cineole.[6] Each capsule contains as its active substance 300 mg of myrtol, standardized to provide at least 75 mg of limonene, 75 mg of cineole, and 20 mg of α-pinene.

By using techneitium-99m sulfur colloid as the radiopharmaceutical, Behrbohm *et al.*[19] reported increases in mucociliary transport velocity in the maxillary sinuses of five healthy subjects who had taken myrtol standardized orally, as measured with a γ-camera. That result hints at the stimulatory effect on MCC induced by myrtol standardized. By using a saccharine test, we also found that nasal mucociliary transport velocity was increased by a 10-day myrtol standardized treatment in patients with chronic rhinitis. Additionally, the nasal patency, as well as clinical symptoms of patients, was improved after this treatment. It is not surprising to find that there was little significant change in each examination in the control group. These clinical benefits may be caused by the effects of myrtol standardized and/or its metabolites on the biophysical properties of the mucus, as well as its anti-inflammatory effects and vasoconstriction. It was reported that myrtol standardized, especially 1.8-cineole, inhibits the activity of 5-lipoxygenase of human basophil and eosinophil leukocytes and the formation of leukotriene C_4.[9] The medication might reduce infection-induced oxidative attack and damage because of its effects on inflammatory processes.[20]

Based on a pharmacokinetic study, the main components of myrtol standardized are absorbed ~100% into the body.[21] Being carried by the bloodstream, it reaches the deepest branches of the bronchial tubes and sinuses and finally penetrates the airways. Although we, together with others, confirmed that the compound is able to improve nasal MCC, limited *ex vivo* data supported the hypothesis that myrtol standardized might stimulate the mucociliary transport rate by directly increasing the CBF. During the 9th congress of the Asian Pacific Society of Respirology, Kwok *et al.*[8] briefly reported that human nasal

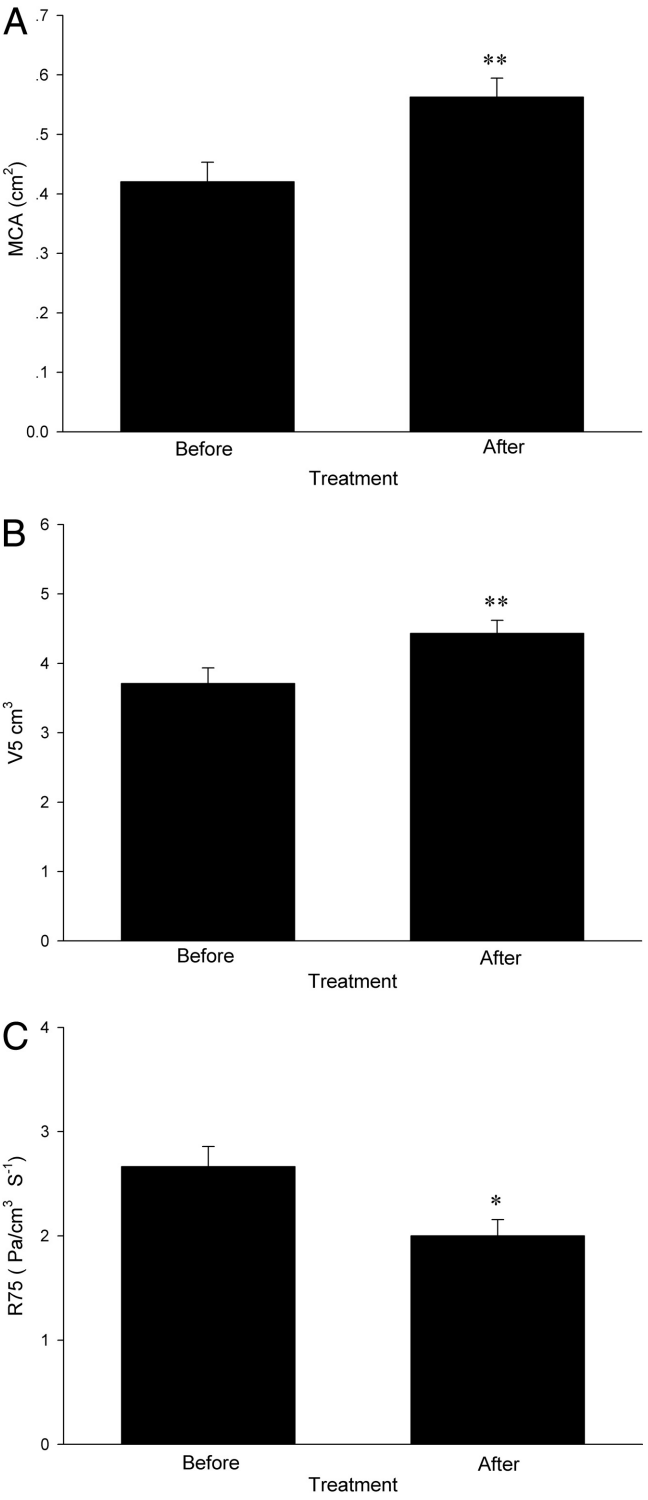

Figure 3. *Pre- and posttreatment objective examination of nasal patency (n = 19). After the treatment, (A) unilateral MCA and (B) V5 were significantly higher than those before the treatment, respectively, and (C) the unilateral R75 after the treatment was significantly reduced. **p < 0.01; *p < 0.05.*

CBF (13 healthy inferior turbinates) was increased after incubation with 100 or 250 ng/mL of myrtol standardized suspended in M199. In contrast, culture medium without the agonist also produced this stimulatory effect. In addition, statistical analysis showed no significant difference after a 4-hour incubation between CBF obtained with 100 or 250 ng/mL of myrtol standardized. In the current study, we examined both the immediate and the prolonged influences of myrtol standardized on CBF. We chose Cremophor EL to completely dissolve myrtol standardized, an essential oil, as reported by Beuscher *et al*.[9] It is a nonionic sobubilizer and emulsifier with the main component being glycerol-polyethylene glycol ricinoleate. The maximum concentration of myrtol standardized in the study was 300 ng/mL, which was higher than the plasma peak concentration (C_{max}; <240 ng/mL) for the uncrushed oral capsules.[20] Our results showed that myrtol standardized, within a range of 75–300 ng/mL, had no significant immediate stimulatory effect on cultured human nasal CBF from patients with chronic rhinosinusitis. Moreover, the compound with a concentration of 300 ng/mL had no significant prolonged stimulatory effect on CBF after incubation of up to 24 hours. However, our data did not rule out the possibility that the metabolites of myrtol standardized might have direct effects on CBF.

In summary, by using digital high-speed imaging combined with beat-by-beat CBF analysis and the saccharine test, respectively, we have found that myrtol standardized, within a range of concentrations from 75 to 300 ng/mL, has no significant immediate or prolonged effect on *ex vivo* CBF in patients with chronic rhinosinusitis. On the other hand, a 10-day myrtol standardized treatment improves nasal MCC as well as nasal patency in patients with chronic rhinitis, which may caused by the influences on mucus rather other ciliary activity.

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