

# Effects of bronchial thermoplasty and cryoablation on airway smooth muscle

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## Abstract

**Background:** The effectiveness of bronchial thermoplasty (BT) has been reported in patients with severe asthma. This study compared the effects of BT and cryoballoon ablation (CBA) therapy on the airway smooth muscle (ASM).

**Methods:** Eight healthy male beagle dogs were included in this experiment. In the preliminary experiment, one dog received BT treatment for both lower lobe bronchus, another dog received CBA treatment for 7 s on the upper and lower lobe of right bronchus, and 30 s on the left upper and lower lobe. The treatments were performed twice at an interval of 1 month. In subsequent experiments, the right lower lobe bronchus was treated with BT, and the left lower lobe bronchus was treated with CBA. The effects of treatment were observed after 1 ( $n = 3$ ) month and 6 months ( $n = 3$ ). Hematoxylin-eosin staining, Masson trichrome staining, and immunohistochemical staining were used to compare the effects of BT and CBA therapy on the ASM thickness, collagen fibers synthesis, and M3 receptor expression after treatment. One-way analysis of variance with Dunnett *post hoc* test was used to analyze the differences among groups.

**Results:** In the preliminary experiment, the ASM ablation effect of 30-s CBA was equivalent to that of 7-s CBA (ASM thickness:  $30.52 \pm 7.75 \mu\text{m}$  vs.  $17.57 \pm 15.20 \mu\text{m}$ ,  $P = 0.128$ ), but the bronchial mucociliary epithelium did not recover, and large numbers of inflammatory cells had infiltrated the mucosal epithelium at 1-month post-CBA with 30-s freezing. Therefore, we chose 7 s as the CBA treatment time in our follow-up experiments. Compared with the control group ( $35.81 \pm 11.02 \mu\text{m}$ ), BT group and CBA group ( $13.41 \pm 4.40 \mu\text{m}$  and  $4.81 \pm 4.44 \mu\text{m}$ , respectively) had significantly decreased ASM thickness after 1 month ( $P < 0.001$ ). Furthermore, the ASM thickness was significantly lower in the 1-month post-CBA group than in the 1-month post-BT group ( $P = 0.015$ ). There was no significant difference in ASM thickness between the BT and CBA groups after six months ( $9.92 \pm 4.42 \mu\text{m}$  vs.  $7.41 \pm 7.20 \mu\text{m}$ ,  $P = 0.540$ ). Compared with the control group ( $0.161 \pm 0.013$ ), the average optical density of the ASM M3 receptor was significantly decreased in 6-month post-BT, 1-month post-CBA, and 6-month post-CBA groups ( $0.070 \pm 0.022$ ,  $0.072 \pm 0.012$ ,  $0.074 \pm 0.008$ , respectively; all  $P < 0.001$ ). There was no significant difference in the average optical density of ASM M3 receptor between the BT and CBA therapy groups after six months ( $P = 0.613$ ).

**Conclusions:** CBA therapy effectively ablates the ASM, and its ablation effect is equivalent to that of BT with a shorter onset time. A neural mechanism is involved in both BT and CBA therapy.

**Keywords:** Airway smooth muscle; Bronchial thermoplasty; Cryoballoon ablation

## Introduction

At present, the number of patients with bronchial asthma is 300 million, and refractory asthma accounts for 17.4% of all cases of asthma.<sup>[1]</sup> The heterogeneity of airway inflammation in patients with severe asthma has led to the recognition of multiple distinct endotypes, allowing the use of novel specific biologics such as anti-immunoglobulin E and anti-interleukin 5 monoclonal antibodies.<sup>[2-6]</sup> Refractory asthma is not well controlled even with high doses of inhaled corticosteroids.

Bronchial thermoplasty (BT) is an endoscopic procedure that primarily targets airway remodeling by delivering temperature-controlled radiofrequency (RF) energy to the airway wall.<sup>[7]</sup> The United States Food and Drug Administration approved BT for the treatment of severe and persistent asthma, and its widespread use began on April 27, 2010.<sup>[8]</sup> However, some patients developed complications such as bronchiectasis and bronchial scar formation after BT, which are related to the formation of granulation tissue hyperplasia and scar contracture caused by thermal ablation.<sup>[9,10]</sup> Notably, a major advantage of cryoablation is that no scar is formed; however,

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cryoablation has not been used for ablation of airway remodeling.

In this study, we compared the efficacy of BT and cryoballoon ablation (CBA) techniques in airway smooth muscle (ASM) ablation using a beagle airway model.

## Methods

### Ethical approval

This study was approved and supervised by the Ethics Committee of Shanghai Tenth People's Hospital (No. 20KT75) and performed in accordance with the international ethics for animal use. Significant efforts were made to minimize the number of animals and reduce their suffering.

### Study design

Eight healthy male beagle dogs with a body weight of about 13 kg were purchased from Shanghai Jiaqian Biotechnology Co., Ltd. (Shanghai, China) and raised in the central laboratory animal room of our hospital. Two dogs underwent a preliminary experiment to observe the difference in ASM thickness, collagen fiber synthesis, and M3 receptor expression 1-month after BT and CBA in an effort to determine the most appropriate time for CBA. The both upper lobes were not treated with BT in one dog, these bronchi served as the control group. The right lower bronchus was used as the observation target for BT subacute injury. One month later, BT surgery was repeated on bronchus of the left lower lobe as the observation target of BT acute injury. In another dogs, the right lower bronchus and left lower bronchus were used as the observation targets of CBA subacute injury for 7 and 30 s freezing, and the right upper bronchus and left upper bronchus were used as the observation targets of CBA acute injury for 7 and 30 s. The two dogs were killed after the treatment.

Six dogs underwent a subsequent experiment to observe the differences in ASM thickness and M3 receptor expression after BT and CBA. BT surgery was performed on each bronchus of the right lower lobe in six dogs. CBA was performed on the left lower lobe bronchus. As the control group, the two upper lobes were not treated with BT or CBA therapy. Three dogs were killed 1 month after therapy, and the remaining three dogs were killed 6 months after therapy.

All dogs were put to death by intravenous injection of potassium chloride, bronchial tissues of each group were taken for hematoxylin-eosin staining and immunohistochemical staining, and those of the two dogs in the preliminary experiment were also taken for Masson trichrome staining.

During bronchoscopy and treatment, an intramuscular injection of 5% pentobarbital sodium (30–40 mg/kg) was administered in the hip of all the dogs. The treatment of each dog was carried out during examination using a bronchoscope (Mindhao Medical Technology Co., Ltd., Zhuhai, China) with an inner diameter of 2.6 mm.

### BT procedure

BT was performed using the Alair system (Boston Scientific, Marlborough, MA, USA). The system consists of a 460-kHz, low-power, monopolar RF generator (model ATS 115X1) and a four-electrode basket catheter (model ATS 2-5X1) [Supplementary Figure 1A, <http://links.lww.com/CM9/A713>]. The catheter contains electrodes on the thermocouple, which is used for temperature-controlled energy delivery. The electrode basket expands to contact the airway wall and then activates the RF generator to provide controlled RF energy to the airway wall for 10 s. In the present study, after each RF generator was activated, the catheter was immediately repositioned near the previous treatment site for the next activation. This process of treatment and repositioning of the catheter was repeated five times to produce continuous treatment for all target airways. Continuous local treatment was applied to all accessible airways to treat the entire bronchial tree with an airway diameter of >3 mm as close as possible. We performed a total of 27 to 34 activations in the preliminary experiment and 25 to 35 activations in subsequent experiments. Total operation time was 30 min, post-operative pulse oxygen saturation was 100%, no dyspnea or suffocation occurred, the recovery time was about 30 min.

### CBA procedure

The cryodevice (Shengjiekang, Ningbo, Zhejiang, China) mainly consists of three parts: the cryo-end (SR610), tube (SS610), and handle [Supplementary Figure 1B, <http://links.lww.com/CM9/A713>]. Among these parts, the tube body was mainly composed of a vacuum tube, an air inlet tube, and a return air tube; the handle was mainly composed of a protection tube, a vacuum connecting tube, an air inlet connecting tube, a return air connecting tube, a handle seat, and a joint. The cooling medium was liquid nitrogen, which could rapidly drop the end surface temperature. The effective length of the cryotube was 1.2 m (allowing it to enter the bronchoscope operating channel), and the length and diameter of the cryo-end were 15 and 4 mm, respectively.

The CBA method was performed as follows. After the catheter was connected to the cryotherapy equipment, the frozen end of the catheter was immersed in normal saline and the test button on the equipment was clicked to start the test. During the test, the user observed whether the liquid nitrogen leaked, whether normal ice ball formation occurred, and whether the tube body became frosted. After the test was completed, the catheter was extended into the working channel of the air intake endoscope, and the catheter tip was gently pushed until it was exposed in the field of view of the lens. With a clear view, the tip end protruded 2 cm below the level of the bronchoscope opening and was fitted to the bronchial mucosa. The freezing button on the freezing equipment was clicked to set the freezing time, and the confirm button was clicked to start the freezing process. When the freezing time was reached, the freezing process stopped and automatic reheating began. After the head end was completely thawed, the frozen end was separated from the tissue before the next freezing cycle. The

upper and lower lobe bronchus were treated for two and three cycles, respectively.

### **Hematoxylin-eosin staining and immunohistochemical assay**

Hematoxylin-eosin and immunohistochemically stained sections were fixed with 10% formalin at room temperature for 24 h and dehydrated with 70%, 85%, 95%, and 100% ethanol and xylene. The tissues were then embedded into 4- $\mu$ m-thick sliced sections. Following deparaffination in xylene, they were rehydrated in 100%, 95%, 85%, and 70% ethanol and distilled water. The sections were stained with hematoxylin for 5 min at room temperature and eosin for 2 min at room temperature; dehydrated with 70%, 85%, 95%, and 100% ethanol and xylene; and covered with a coverslip and mounting medium (Sangon Biotech Co., Ltd., Shanghai, China). The hematoxylin-eosin-stained sections were observed under an inverted light microscope. The sections were incubated overnight with anti-M3 antibodies (1:500) at a constant temperature of 4°C. All sections were processed using the peroxidase-anti-peroxidase method (Agilent Technologies, Santa Clara, CA, USA). The sections were washed with phosphate-buffered saline and incubated in 3,3'-diaminobenzidine tetrahydrochloride hydrate in 0.05 mol/L Tris buffer (pH 7.6) containing 0.03% hydrogen peroxide to observe the reaction of peroxidases. Next, the sections were washed with sterile water, counterstained with hematoxylin, and dehydrated; finally, a coverslip was added.

For the immunohistochemical analyses, the tissues were fixed in 10% formalin at room temperature for 24 h and dehydrated with 70%, 85%, 95%, and 100% ethanol and xylene. They were then embedded in 4- $\mu$ m-thick sliced sections. The tissue was deparaffinized in xylene and dehydrated in 100%, 95%, 85%, and 70% ethanol and distilled water. Next, the sections were soaked in 0.3% hydrogen peroxide to block the activities of endogenous peroxidases. After washing the sections with 10 mmol/L of citrate buffers (pH 6.0), they were autoclaved for 20 min at 121°C for antigen retrieval. Finally, the sections were washed.

The area of bronchial smooth muscle and the circumference of the bronchial lumen were measured in hematoxylin-eosin stained specimens using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). The measured smooth muscle areas were normalized to the selected intraluminal circumference to represent the thickness of the bronchial smooth muscle.

The integrated optical density of the M3 receptor in bronchial smooth muscle was measured using immunohistochemical specimens of the muscarinic M3 receptor and normalized by the area of smooth muscle. The average optical density (AOD) (integrated optical density/area) of the M3 receptor in bronchial smooth muscle was then obtained.

### **Masson trichrome staining**

Masson trichrome staining was performed to detect collagen deposition and smooth muscle thickness. The

integrated optical density of the collagen fibers in the bronchial smooth muscle was measured in Masson-stained sections and normalized by the area of smooth muscle. The AOD of collagen in bronchial smooth muscle was then obtained.

### **Statistical analysis**

Each experiment was conducted at least in triplicate. All continuous data with normal distribution are presented as mean  $\pm$  standard deviation. One-way analysis of variance with Dunnett *post hoc* test was used to analyze the differences among the BT, 7-s cryoablation, 30-s cryoablation, and control groups in the preliminary experiment; it was also used to analyze the differences among the control, 1-month post-BT, 6-month post-BT, 1-month post-cryoablation, and 6-month post-cryoablation groups in subsequent experiments. The statistical analysis was carried out using the SPSS 23.0 software package, version 24.0 (IBM Corp., Armonk, NY, USA). A *P* value of  $<0.05$  was considered statistically significant.

## **Results**

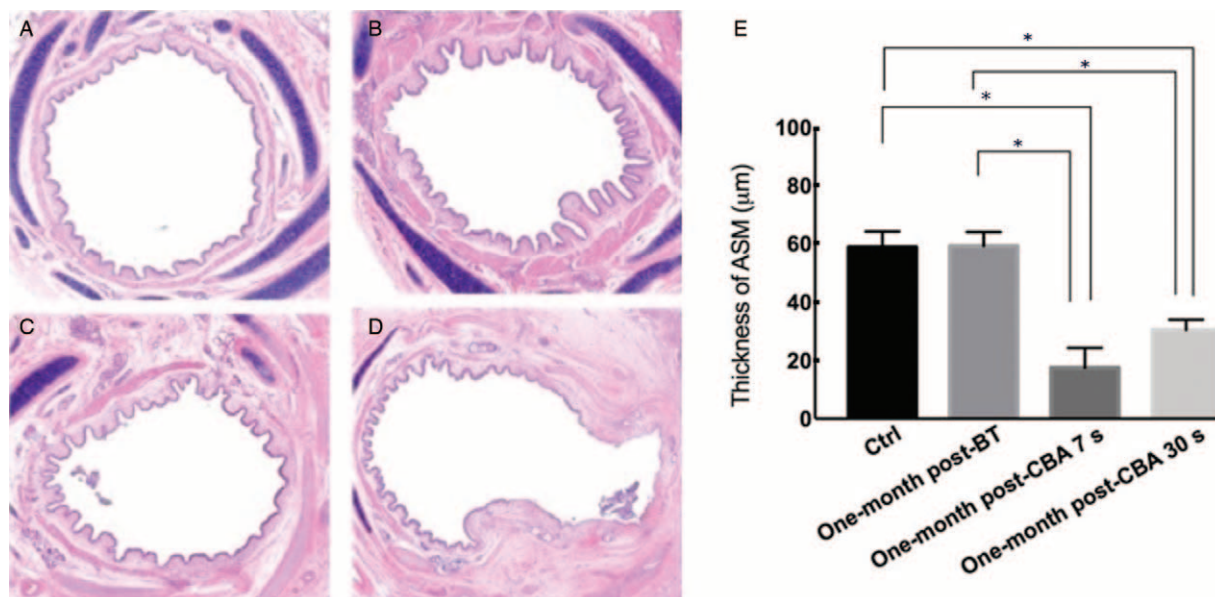
### **Safety observation**

All BT and CBA procedures were successfully completed without death. A small amount of mucosal bleeding occurred during CBA but improved after hemostasis with ice-cold normal saline. The CBA-treated dogs resumed eating and defecation within 24 h after cryosurgery without wound infection, coughing, or sputum production. These results indicated that the CBA procedure was safe.

During the freezing process in the bronchus, ice balls formed around the balloon with clear boundaries and adhered to the bronchial mucosa. After removing the frozen balloon, a clear boundary was observed between the frozen area and the surrounding unfrozen tissue, and the frozen area exhibited hyperemia and excessive edema.

### **Structural effects of BT and CBA**

Immediately after BT, the bronchial wall tissue showed destruction and exfoliation of epithelial cells at the site of contact with the RF probe. The mucosa was mildly edematous, and the submucosal capillaries were slightly congested compared with those of the control group [Supplementary Figure 2A, <http://links.lww.com/CM9/A713> and 2B, <http://links.lww.com/CM9/A713>]. Immediately after CBA, the bronchial wall tissue showed destruction and exfoliation of the epithelial cells at the contact point of the balloon, and the mucosa was obviously edematous. A large number of red blood cells had infiltrated the treatment area, the submucosal capillaries were congested, and the alveolar walls were congested [Supplementary Figure 2C, <http://links.lww.com/CM9/A713>]. There were no significant structural differences between freezing for 7 and 30 s. The bronchial mucociliary epithelium was intact at 1 month post-BT, and small numbers of inflammatory cells were seen. No submucosal congestion or edema was observed in the



**Figure 1:** Pathological changes of bronchus in the control (A), 1-month post-BT (B), 1-month post-7 s CBA (C), 1-month post-30 s CBA (D) groups in the preliminary experiment (hematoxylin and eosin staining, original magnification  $\times 10$ ). (E) ASM thickness changes in bronchus. \* $P < 0.05$ . ASM: Airway smooth muscle; BT: Bronchial thermoplasty; CBA: Cryoballoon ablation; Ctrl: Control.

BT group compared with the control group [Figure 1A and 1B]. At 1 month post-CBA with 7-s freezing, the bronchial mucociliary epithelium was intact without submucosal edema. Large numbers of inflammatory cells had infiltrated the area, and the smooth muscle thickness was significantly reduced. No capillary congestion was observed [Figure 1C]. At 1 month post-CBA with 30-s freezing, the bronchial mucociliary epithelium did not recover and exhibited submucosal edema. Large numbers of inflammatory cells and red blood cells had infiltrated the area, and the smooth muscle mass was significantly reduced [Figure 1D].

### Effects of BT and CBA on ASM

In the preliminary experiment, there was a significant difference in the thickness of the ASM among the control ( $58.89 \pm 5.38 \mu\text{m}$ ), 1-month post-BT ( $59.28 \pm 4.86 \mu\text{m}$ ), 1-month post-CBA 7-s ( $17.57 \pm 15.20 \mu\text{m}$ ), and 1-month post-CBA 30-s ( $30.52 \pm 7.75 \mu\text{m}$ ) groups ( $F = 15.801$ ,  $P < 0.001$ ) [Figure 1A–E]. The ASM thickness was significantly lower in the 1-month post-cryoablation 7- and 30-s groups than in the control group ( $P < 0.001$ ), but there was no significant difference between the control and 1-month post-BT treatment groups ( $P = 0.958$ ). Furthermore, there was no significant difference in ASM thickness between the 1-month post-cryoablation 7- and 30-s groups ( $P = 0.128$ ). The ASM thickness was significantly lower in the 1-month post-cryoablation 7- and 30-s groups than in the 1-month post-BT group ( $P < 0.001$ ).

In the subsequent experiments, there was a significant difference in the ASM thickness among the control ( $35.81 \pm 11.02 \mu\text{m}$ ), 1-month post-BT ( $13.41 \pm 4.40 \mu\text{m}$ ), 6-month post-BT ( $9.92 \pm 4.42 \mu\text{m}$ ), 1-month post-CBA ( $4.81 \pm 4.44 \mu\text{m}$ ), and 6-month post-CBA ( $7.41 \pm 7.20 \mu\text{m}$ ) groups ( $F = 23.311$ ,  $P < 0.001$ ).

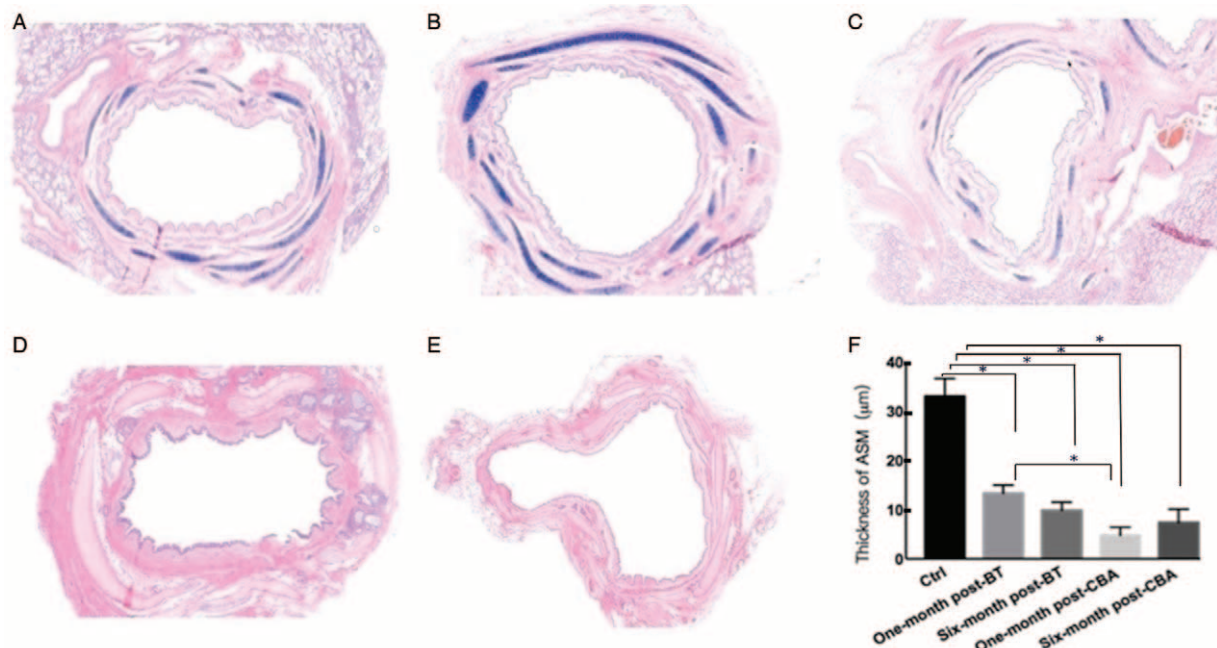
[Figure 2A–F]. The ASM thickness was significantly lower in the 1-month post-BT, 6-month post-BT, 1-month post-CBA, and 6-month post-CBA groups than in the control group (all  $P < 0.001$ ). The ASM thickness was significantly lower in the 1-month post-CBA group than in the 1-month post-BT group ( $P = 0.015$ ). However, there was no significant difference between the 1-month post-BT and 6-month post-BT groups ( $P = 0.283$ ). Furthermore, there was no significant difference between the 6-month post-CBA group and the 6-month post-BT group ( $P = 0.540$ ), or 1-month post-CBA group ( $P = 0.486$ ).

### Effects of BT and cryoablation on collagen fibers

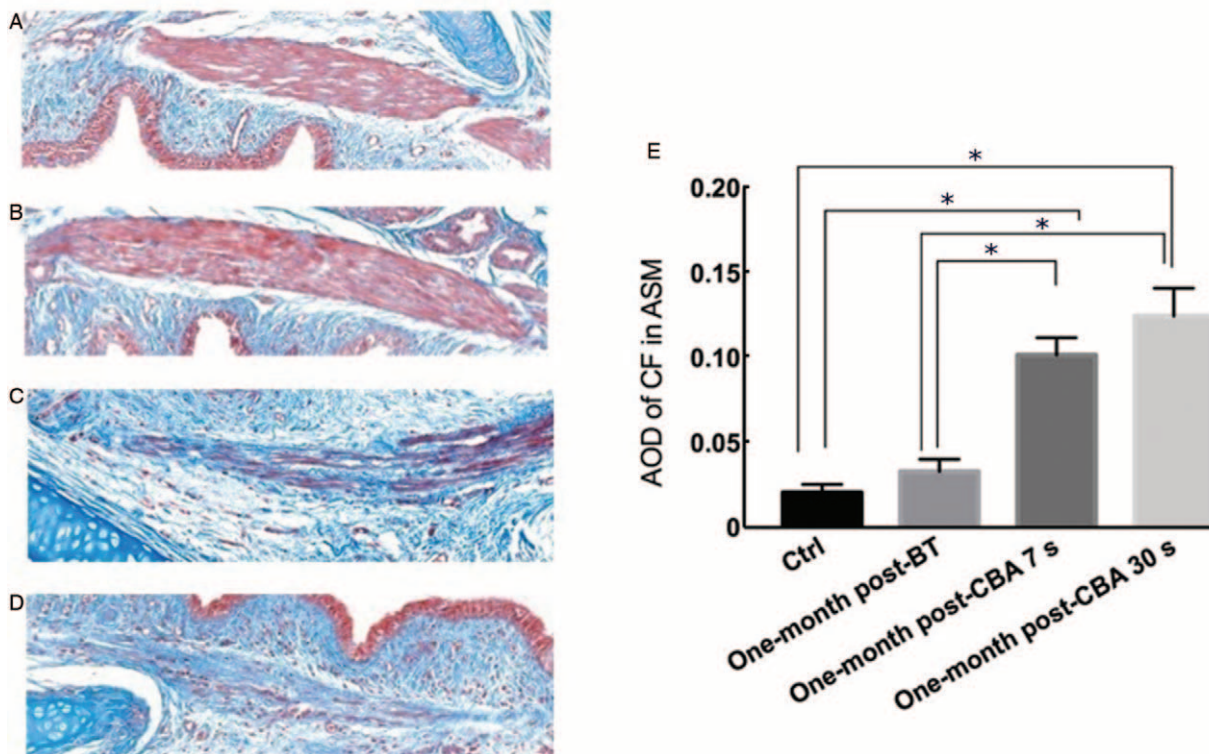
In the preliminary experiment, there was a significant difference in collagen fiber proliferation among the control ( $0.02 \pm 0.01$ ), 1-month post-BT ( $0.03 \pm 0.01$ ), 1-month post-CBA 7-s ( $0.10 \pm 0.01$ ), and 1-month post-CBA 30-s ( $0.13 \pm 0.02$ ) groups ( $F = 23.280$ ,  $P < 0.001$ ) [Figure 3A–E]. There was significantly more collagen fiber synthesis in the 1-month post-CBA 7- and 30-s groups than in the control group ( $P < 0.001$ ), but no significant difference was found between the 1-month post-BT treatment group and the control group ( $P = 0.171$ ). Furthermore, there was no significant difference between the 1-month post-CBA 7- and 30-s groups ( $P = 0.250$ ). The collagen fiber synthesis was significantly higher in the 1-month post-CBA 7- and 30-s groups than in the 1-month post-BT group ( $P < 0.001$ ).

### Effects of BT and CBA on M3 receptor

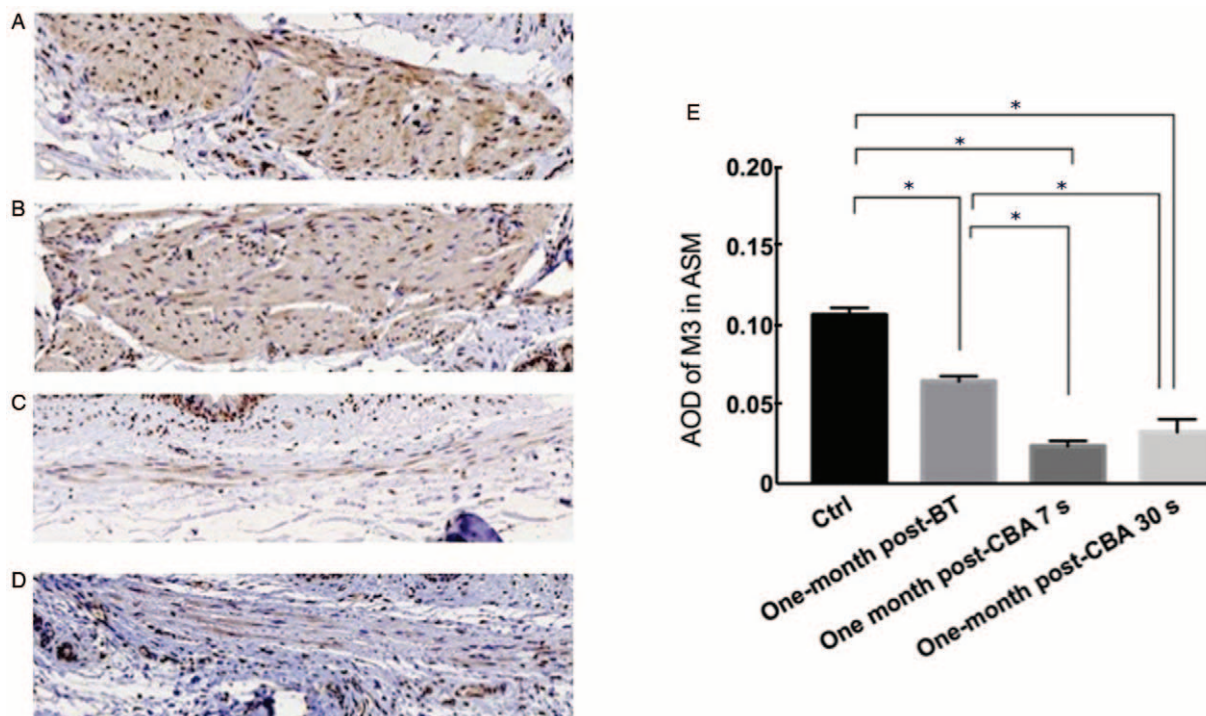
In the preliminary experiment, there was a significant difference in the AOD of the ASM M3 receptor among the four groups ( $F = 52.940$ ,  $P < 0.001$ ) [Figure 4A–E]. The AOD of the ASM M3 receptor expression was significantly lower in the 1-month post-BT ( $0.065 \pm 0.003$ ), 1-month



**Figure 2:** Pathological changes of bronchus in control (A), 1-month post-BT (B), 6-month post-BT (C), 1-month post-7 s CBA (D), and 6-month post-7 s CBA (E) groups in the subsequent experiments (hematoxylin and eosin staining, original magnification  $\times 10$ ). (F) ASM thickness changes in bronchus.  $*P < 0.05$ . ASM: Airway smooth muscle; BT: Bronchial thermoplasty; CBA: Cryoballoon ablation; Ctrl: Control.



**Figure 3:** Masson changes of bronchus in control (A), 1-month post-BT (B), 1-month post-7 s CBA (C) and 1-month post-30 s CBA (D) groups in the preliminary experiment (Masson trichrome staining, original magnification  $\times 200$ ). (E) AOD changes of CF in ASM.  $*P < 0.05$ . AOD: Average optical density; ASM: Airway smooth muscle; BT: Bronchial thermoplasty; CBA: Cryoballoon ablation; CF: Collagen fiber; Ctrl: Control.



**Figure 4:** M3 expression changes in ASM in control (A), 1-month post-BT (B), 1-month post-7 s CBA (C), and 1-month post-30 s CBA (D) in the preliminary experiment (immunohistochemical staining, original magnification  $\times 200$ ). (E) AOD changes of M3 expression in ASM. \* $P < 0.05$ . AOD: Average optical density; ASM: Airway smooth muscle; BT: Bronchial thermoplasty; CBA: Cryoballoon ablation; Ctrl: Control.

post-CBA 7-s ( $0.024 \pm 0.003$ ), and 1-month post-CBA 30-s ( $0.033 \pm 0.008$ ) groups than in the control group ( $0.107 \pm 0.005$ ) (all  $P < 0.001$ ). Furthermore, there was no significant difference in the AOD of the ASM M3 receptor expression between the 1-month post-CBA 7- and 30-s groups ( $P = 0.327$ ). The AOD of the ASM M3 receptor expression was significantly lower in the 1-month post-CBA 7- and 30-s groups than in the 1-month post-BT group (both  $P < 0.001$ ).

In the subsequent experiments, there was a significant difference in the AOD of the ASM M3 receptor among the control ( $0.161 \pm 0.013$ ), 1-month post-BT ( $0.157 \pm 0.024$ ), 6-month post-BT ( $0.070 \pm 0.022$ ), 1-month post-CBA ( $0.072 \pm 0.012$ ), and 6-month post-CBA ( $0.074 \pm 0.008$ ) groups ( $F = 56.041$ ,  $P < 0.001$ ) [Figure 5A–F]. The AOD of the ASM M3 receptor expression was significantly lower in the 6-month post-BT, 1-month post-CBA, and 6-month post-CBA groups than in the control group ( $P < 0.001$ ). Furthermore, there was no significant difference in the AOD of the ASM M3 receptor expression between the 1-month post-BT and control groups ( $P = 0.716$ ). The AOD of the ASM M3 receptor expression was significantly lower in the 6-month post-BT, 1-month post-CBA, and 6-month post-CBA groups than in the 1-month post-BT group (all  $P < 0.001$ ). There was no significant difference between the 6-month post-CBA group and the 6-month post-BT group ( $P = 0.613$ ), or 1-month post-CBA group ( $P = 0.679$ ).

## Discussion

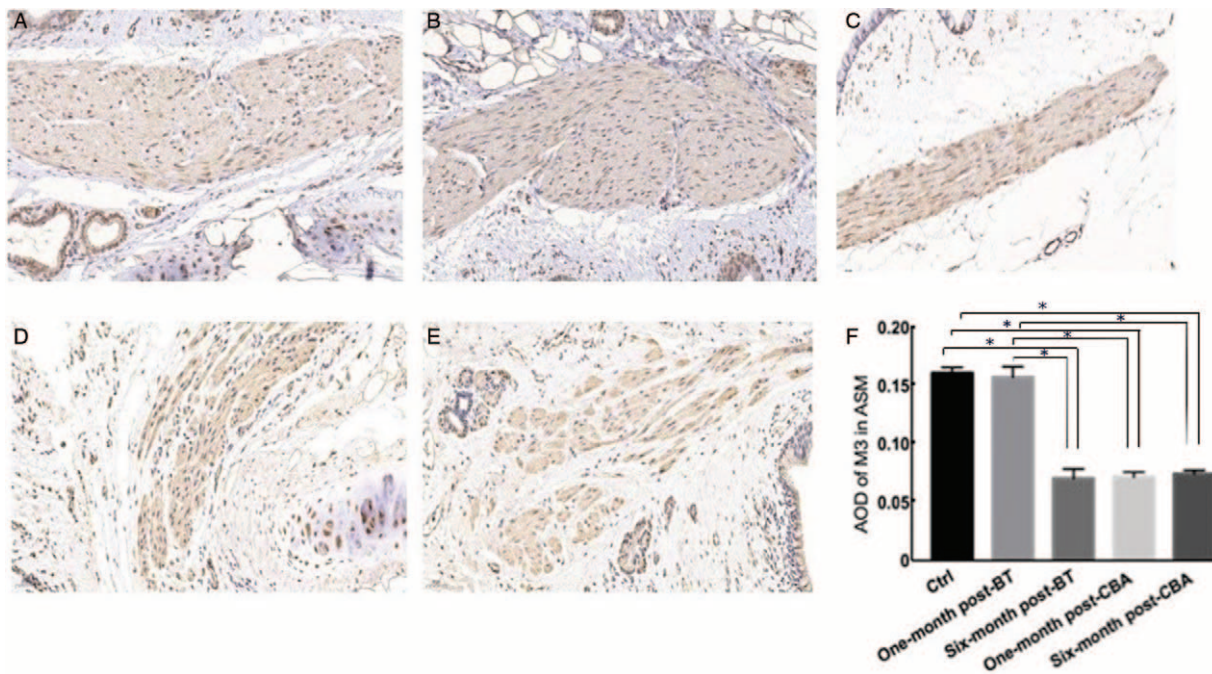
In the preliminary experiment, the CBA group was divided into two subgroups (7- and 30-s freezing time). Our data

indicate that frozen balloon ablation did not benefit from the delayed treatment time but instead exhibited an increase in the post-operative repair time and an increased risk of post-operative complications.

Our study showed that BT and CBA therapy significantly reduced the ASM thickness; the ASM thickness was significantly lower in the 1-month CBA group than in the BT group. The onset time of CBA was shorter than that of BT treatment, indicating that CBA is faster and more effective than BT. The therapeutic effects of BT and CBA continued throughout the 6-month period, and the effects were equivalent. The ablated ASM was replaced by fibrous tissues.

The expression of the ASM muscarinic M3 receptor was decreased 6-month after BT and 1-month after CBA, and the down-regulation effect of CBA was equivalent to that of BT. In addition to directly causing necrosis of ASM cells, BT and CBA also participate in the therapeutic mechanism: reversing airway remodeling by down-regulating smooth muscle muscarinic M3 receptor expression and thereby reducing airway resistance.

In the pathogenesis of asthma, excessive contraction of bronchial smooth muscle leads to airway stenosis. Smooth muscle spasm is the main cause of wheezing and airway obstruction. Bronchial smooth muscle is not only an important effector in acute asthma attack but also participates in airway remodeling.<sup>[3]</sup> Airway remodeling is mainly characterized by airway wall thickening, increased mucous glands and goblet cells, increased



**Figure 5:** M3 expression changes in ASM in control (A), 1-month post-BT (B), 6-month post-BT (C), 1-month post-7 s CBA (D), and 6-month post-7 s CBA (E) groups in the subsequent experiments (immunohistochemical staining, original magnification  $\times 200$ ). (F) AOD changes of M3 expression in ASM.  $^*P < 0.05$ . AOD: Average optical density; ASM: Airway smooth muscle; BT: Bronchial thermoplasty; CBA: Cryoballoon ablation; Ctrl: Control.

vascular formation, and most importantly bronchial smooth muscle hypertrophy. Airway remodeling is the pathological basis for fixed airflow limitation and impaired lung function. The ASM layer is thicker in patients with refractory asthma than in those with moderate asthma, and the muscle mass in the airway tissue is significantly increased. Moreover, the smooth muscle is closer to the epithelium, reflecting smooth muscle cell migration to the epithelial surface. The airway remodeling is negatively correlated with the forced expiratory volume in 1 s (FEV<sub>1</sub>).<sup>[4]</sup> It can cause expiratory dyspnea in patients with asthma.

The characteristic pathological change in patients with asthma is airway remodeling, which involves ASM hyperplasia and hypertrophy, epithelial cell hyperplasia, mucosal subepithelial fibrosis, interstitial hyperplasia, and vascular proliferation.<sup>[5]</sup> These pathological changes can lead to airway stenosis. Increased airway tract resistance provides a theoretical basis for BT. The basic principle of BT is that the catheter passes through the bronchoscope’s operating channel and opens at a designated site; the electrodes are in close contact with the wall, which in turn controls the release of energy. At the time of action and required temperature, a high-frequency electromagnetic wave (350–500 kHz) is delivered into the tissue, and after the electromagnetic conversion, the charged ions in the tissue oscillate and generate heat energy. After the local temperature reaches the preset level, the cells are dissolved, intracellular proteins are denatured, water inside and outside the cells is lost, and tissue undergoes coagulative necrosis; this reduces the number of ASM cells and weakens airway contractility. Danek *et al*<sup>[6]</sup> first demonstrated the idea of ablation of the ASM to reduce airway

hyperresponsiveness in a canine model. The results showed that BT can selectively act on the ASM for permanent ablation, reducing the number of ASM cells. This effect can be maintained for 3 years after treatment.

In previous studies of lung biopsy performed before and 3 months after treatment, the proportion of the ASM in three different treatment sites was observed. The results showed that the ASM decreased by 48.7% to 78.5%.<sup>[7]</sup> Therefore, ASM ablation by BT can improve the reversible airflow obstruction, chronic inflammation, and high reactivity of the airway and can reduce the frequency and severity of acute attacks. In the present study, the thickness of the ASM was not significantly reduced at 1-month after BT treatment. Considering the short treatment time in the present study, the observation time should be extended in subsequent experiments.

At present, two methods are available for airway ablation: BT and cryoablation. In the clinical setting, treatment methods with less local tissue stimulation should be selected to reduce the formation of granulation tissue hyperplasia and scar contracture and avoid long-term complications. Local tissue stimulation in BT treatment is significantly enhanced compared with that in cryotherapy, increasing the incidence of granulation tissue proliferative stenosis. Compared with BT treatment, it is uncertain whether cryotherapy can reduce bronchial smooth muscle. The mechanism of damage involves freezing-induced formation of ice crystals inside and outside the cell, which dehydrates the cells and changes the concentration of electrolytes and pH, thereby causing the cells to be poisoned and die. Melting after freezing, especially slow natural melting, causes small ice crystals in the cells to

accumulate into large ice crystals, which can also cause cell destruction and death. The intracellular ice crystals recrystallize, which denatures the lipoprotein complex and breaks the cell membrane. It also changes the osmotic pressure of the cells, leading to hemorrhage caused by microvascular injury, inflammatory changes, ischemia, fibrosis, and apoptosis.<sup>[9]</sup> The efficacy of freezing is related to the minimum temperature, freezing rate, thawing rate, number of freeze-thaw cycles, and tissue water content. Cold-sensitive tissues include tumors, granulation tissue, skin, mucosa, nerves, and endothelium; in contrast, fat, cartilage, connective tissue, and fibrous tissue are cryoresistant.<sup>[10]</sup> This can prevent the occurrence of sputum formation or damage to the tracheal cartilage, providing a theoretical basis for the safety of cryotherapy. Compared with RF, cryotherapy can reduce the incidence of bronchial scars. This phenomenon may be related to the increased type III/I collagen ratio and the inhibition of fibroblast growth after cryotherapy. The prominent feature of cryotherapy is no scar formation after healing of frozen damaged mucosa.<sup>[11]</sup>

As a bronchoscopic interventional therapy, cryotherapy has been applied to central airway obstruction with a significant curative effect. It can effectively relieve central airway obstruction, improve symptoms of dyspnea, and improve the FEV<sub>1</sub>. Moreover, with respect to the forced vital capacity,<sup>[12,13]</sup> cryotherapy can reduce or eliminate 93% of hemoptysis caused by malignant tumors.<sup>[14]</sup> In a study comparing the efficacy of percutaneous thoracic cryotherapy (PTC) and RF ablation in patients with inoperable lung cancer, 43.3% received RF ablation treatment and 66.7% received PTC treatment. For <3-cm space-occupying lesions in the lung, the patients achieved complete ablation rates of 76.2% and 85.7% by RF ablation and PTC, respectively. The survival and progression-free survival rates were significantly higher in the complete ablation group than in the partial ablation group. In terms of surgical complications, only one case of self-limiting hemoptysis and one case of pneumothorax occurred in the PTC group; no death or serious complications occurred.<sup>[15]</sup> In the present study, we also found that frozen balloon treatment can ablate the ASM and that its ablation effect is faster and more effective than that of BT.

In addition to its therapeutic effect on the ASM, BT also has an effect on bronchial nerves. Pretolani *et al*<sup>[16]</sup> observed neuroendocrine cells of the airway submucosa and reported that nerve fibers were associated with a decrease in the ASM and mucosal epithelial layer after 3 months of BT treatment. Interestingly, the right middle lobe of patients who did not undergo BT also showed reduction of epithelial neuroendocrine cells, which in turn decreased abnormal structures associated with airway stenosis and airway hyperresponsiveness. The respiratory system is innervated by autonomic nerves, including the sympathetic, parasympathetic, and non-adrenergic non-cholinergic nervous systems.<sup>[17]</sup> Parasympathetic nerves play a major role in bronchoconstriction. Neuroreceptors are classified into muscarinic receptors and nicotinic receptors. Muscarinic (M) receptors are expressed by structural cells in the airways, predominantly in the ASM, airway epithelium, and airway fibroblasts. The

distribution of muscarinic receptor subtypes throughout the bronchial tree is mainly restricted to muscarinic M1, M2, and M3 receptors. The M3 receptor is mainly expressed in the ASM, and its excitability can directly lead to ASM contraction. The M3 receptor is also present in the submucosal glands of the airway, and its excitability can promote mucus secretion in the airway.<sup>[18]</sup> Higher expression of cholinergic M3 receptors is present in the bronchial mucosa of patients with severe asthma than in patients with mild to moderate asthma.<sup>[19]</sup> In patients with severe asthma who respond to allergen exposure, excessive release of acetylcholine leads to airway remodeling via increased expression of muscarinic receptor M3.<sup>[20]</sup> Additionally, the expression of ASM M3 receptor is positively correlated with the expression of nerve growth factor and type III procollagen, but is negatively correlated with the value of FEV<sub>1</sub>, indicating that the M3 receptor is involved in airway remodeling of asthma in many aspects.<sup>[21]</sup> By inhibiting expression of the M3 receptor in a chronic asthma model, smooth muscle thickening and collagen deposition around the bronchi are significantly reduced, thereby inhibiting airway remodeling.<sup>[22]</sup> The present study showed that BT and frozen balloon treatment can down-regulate the M3 receptor of the ASM, thereby attenuating contraction of the ASM and reversing airway remodeling.

This is the first study to demonstrate that cryoablation therapy has a remarkable curative effect on smooth muscle. Furthermore, the effects of BT and cryoablation therapy on the ASM M3 receptor from the viewpoint of the neural mechanism will be further investigated. However, the subject of this study was not an asthma model, and the post-operative observation time was only 6 months; these factors need to be considered in future research.

In conclusion, cryoablation treatment has an effect on bronchial smooth muscle ablation. Its ablation effect is equivalent to that of BT and its onset time is short. The ablated ASM is replaced by collagen fibers. Expression of the muscarinic M3 receptor of the ASM is reduced after cryoablation. The ablation effect of frozen balloon therapy is not dose-dependent, which provides a theoretical basis for the treatment of bronchial asthma with frozen balloon therapy.

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### Conflicts of interest

None.

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