Ultrasound-guided injection of botulinum toxin type A blocks cardiac sympathetic ganglion to improve cardiac remodeling in a large animal model of chronic myocardial infarction

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BACKGROUND Strategies to improve various cardiovascular diseases by blocking cardiac sympathetic ganglion have been increasingly available currently. Botulinum toxin type A (BTA), a typical neurotoxin, has been shown to block neural transmission in a safe and long-lasting manner.

OBJECTIVE The aim of the present preclinical study was to assess the efficacy of BTA microinjection to alleviate cardiac remodeling after chronic myocardial infarction (MI) by blocking cardiac sympathetic ganglion in a canine model.

METHODS Beagles were randomly divided into a control group (saline microinjection with sham surgery), an MI group (saline microinjection with MI), and an MI+BTA group (BTA microinjection with MI). Ultrasound-guided percutaneous BTA or saline injection into the left stellate ganglion (LSG) was performed followed by MI induction via left anterior descending artery occlusion (LADO) or sham surgery. After 30 days, electrocardiography, Doppler echocardiography, LSG function, neural activity, and ventricular electrophysiological detection were performed in all experimental dogs. At the end, LSG and ventricular tissues were collected for further detection.

RESULTS BTA treatment significantly inhibited LSG function and neural activity and improved heart rate variability. Additionally, BTA application alleviated ventricular remodeling, ameliorated cardiac function, and prevented ventricular arrhythmias after 30-day chronic LADO-induced MI.

CONCLUSION Ultrasound-guided percutaneous microinjection of BTA can block cardiac sympathetic ganglion to improve cardiac remodeling in a large animal model of chronic LADO-induced MI. Ultrasound-guided BTA microinjection has potential for clinical application as a novel cardiac sympathetic ganglion blockade strategy for MI.

KEYWORDS Ultrasound-guided injection; Botulinum toxin type A; Cardiac sympathetic ganglion; Myocardial infarction; Cardiac remodeling

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Introduction
Myocardial infarction (MI) retains a substantial footprint on global health, and chronic myocardial ischemia results in cardiac remodeling, causing structural and functional organic lesions that tend to induce ventricular arrhythmias (VAs) and sudden cardiac death in patients with MI.1 Given the important role of cardiac sympathetic hyperactivity in various adverse cardiovascular events, left stellate ganglion (LSG)
block have been used as therapeutic targets for MI. Initially, surgical LSG denervation has developed into the earliest nerve block strategy. However, the complex anatomical structure requires experienced thoracic surgeons to perform this approach and may result in some unwanted effects including unilateral hand dryness, abnormal sweating, and chest pain, all of which greatly limit the application of such denervation in clinical practice. LSG block induced by anesthetic agents is an alternative, less invasive, and reversible approach to sympathetic blockade. However, because of the short duration of local anesthetics, this approach necessitates multiple injections or is applied to temporarily stabilize conditions for further surgical denervation.

Botulinum toxin type A (BTA) is a neurotoxin that can inhibit the release of cholinergic transmitters, thus blocking neural transmission. Because of its safety, reversibility, and long-lasting activity, BTA was approved by the Food and Drug Administration in 1989 and has been widely used for the treatment of cosmetic and other disorders. Since preganglionic fibers of the sympathetic nerves are cholinergic and cholinergic neurotransmitters have been observed within the LSG, BTA has the potential to inhibit LSG hyperactivity.

In the present study, we performed a much more maneuverable and less invasive approach by using ultrasound-guided injection of BTA into the LSG and investigated whether BTA could sustainedly block cardiac sympathetic ganglion and thus play a cardioprotective role in a post-MI canine model.

Methods
Animal preparation and study protocol
Adult male beagles weighing 10 ± 2 kg were supplied by the Center of Experimental Animals at the Medical College of Wuhan University. All the experimental procedures were reviewed and approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University, and the study was performed in accordance with the National Institutes of Health. All beagles were anesthetized with 3% pentobarbital sodium (30 mg/kg body weight) and maintained at 60 mg/h during the experimental procedure. Animals were ventilated with room air by a positive pressure breathing machine (MAO01746, Harvard Apparatus, Holliston, MA). The left femoral vein and artery were catheterized to establish venous access and monitor arterial pressure. Normal saline was steadily infused at 100 mL/h to replace fluid losses. Body surface electrocardiography was recorded by a computer-based laboratory system (Lead 7000, Jinjiang, Chengdu, China).

Beagles were randomized into a control group (n = 6, saline microinjection with sham surgery), an MI group (n = 6, saline microinjection with MI), and an MI + BTA group (n = 7, BTA microinjection with MI). BTA (50 U/0.25 mL) or an equal volume of saline was microinjected into the LSG percutaneously under ultrasonography guidance. MI was subsequently induced by left anterior descending artery occlusion (LADO) below the first diagonal with sterile thoracotomy, and sham surgery was performed by thoracotomy without LADO. Because of deaths caused by arrhythmias within 1 hour of MI induction, 19 dogs were eventually included in the 3 groups (Online Supplemental Figure S1). Thirty days after surgery, electrocardiography (ECG) and Doppler echocardiography were performed to evaluate heart rate variability (HRV) and cardiac function. Left thoracotomy was then performed to detect LSG function, neural activity, and ventricular electrophysiological parameters in all experimental dogs. Finally, LSG and ventricular tissues were collected for further detection. The detailed study protocol is outlined in Figure 1A.

Ultrasound-guided LSG injection
Before the formal experiment, we performed a preexperiment of ultrasound-guided LSG injection in blank dogs. A marker was firstly placed around the LSG after thoracotomy, and the ultrasound probe was moved percutaneously to identify the LSG and the adjacent structures including the carotid artery, left internal jugular vein, longus colli muscle, and vertebral artery (Online Supplemental Figure S2). The purpose of this operation is to physically help us to identify the LSG and its surrounding anatomy so that ultrasound-guided BTA/saline injection without thoracotomy can be accurately located in the LSG.

As shown in Figure 1B, for BTA/saline percutaneous injection in all experimental dogs without thoracotomy, the ultrasound probe was placed at the same body surface location and fixed where the LSG and adjacent structures could be observed. After the identification of anatomical location, a 22-G needle (Stimuplex D, B. Braun, Freiburg, Germany) was inserted into LSG tissue under ultrasound guidance, and 50 U/0.25 mL BTA (Botox®, Allergan, Irvine CA, USA) or an equal volume of saline was injected into the LSG. The LSG was visualized lifting off the anterior aspect of the longus colli muscle during injection. The dose of BTA was based on previous studies.

Measurements of LSG function and neural activity
After 30 days of chronic MI or sham surgery, all animals were subjected to left thoracotomy at the second intercostal space to expose the LSG. High-frequency electrical stimulation (20 Hz, 0.1 ms) by a stimulator (Grass Instrument S88, Astro-Med, West Warwick, RI) was then applied to the LSG, and the changes in maximal systolic blood pressure (SBP) in response to LSG electrical stimulation were defined as LSG function. We applied 6 incremental voltage levels of electrical stimulation—2.5, 5, 7.5, 10, 12.5, and 15 V—and compared the change in SBP after BTA or saline injection 30 days after chronic ischemia. Each high-frequency stimulation lasted <30 seconds, and the later measurement was not taken until BP returned to a normal level. In addition, LSG neural activity was evaluated by recording the firing amplitude and frequency. A pair of tungsten-coated microelectrodes was hooked up to the LSG, while a ground lead was attached to the chest wall. The LSG-generated electrical signals were recorded by a PowerLab data acquisition system (8/35, AD Instruments, New South Wales, Australia) with
power filters set at 300–1000 Hz and an amplification range of 30–50 times. Neural firing was characterized as deflections of a signal-to-noise ratio of >3:1 and manually determined as described previously.6

**Measurement of the ventricular effective refractory period**

After exposing the heart through the left fifth intercostal space, bipolar catheters were sutured at the left ventricular apical peri-infarct area, the left ventricular base, and the left ventricular median area (Online Supplemental Figure S3). The effective refractory period (ERP) values were measured by programmed stimulation with 8 S1 stimuli (S1-S1 interval was set to 330 ms cycle length) followed by a premature S2 stimulus. The stimulation amplitude was determined at twice the diastolic threshold, which was defined as the minimum voltage until ventricular capture occurred. The S1-S2 interval was initially decreased from 250 ms in 10 ms decrements until refractoriness was achieved, and then the decrement was

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**Figure 1**  Study protocol and verification of ultrasound-guided BTA microinjection. A: Study protocol. B: Schematic diagram of ultrasound-guided BTA/saline microinjection and ultrasound image of the LSG (arrowhead) with the ultrasound probe on the body surface in dogs without thoracotomy. Quantitative analysis of SNAP-25+ cells (C) (control and MI: n = 5; MI + BTA: n = 6) and TUNEL+ cells (D) (n = 6 per group) in the LSG (magnification, ×200). BTA = botulinum toxin type A; CA = carotid artery; ECG = electrocardiogram; IJ = internal jugular vein; LADO = left anterior descending artery occlusion; LC = longus colli muscle; LSG = left stellate ganglion; MI = myocardial infarction; ns = not significant; SNAP-25 = synaptosomal-associated protein 25; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; VA = vertebral artery. *P < .05.
adjusted to 2 ms decrements until refractoriness was reached again. The longest S1-S2 interval unable to capture the ventricle was defined as the ERP.

**HRV analysis**
A body surface ECG was recorded by a PowerLab data acquisition system (8/35). A 5-minute ECG recording at baseline and after 30 days was performed to analyze the HRV by commercially available software (LabChart 8.0, AD Instruments). The low-frequency (LF) component (0.04–0.15 Hz), the high-frequency (HF) component (0.15–0.4 Hz), and the ratio of LF to HF (LF/HF) were calculated as previously described. The results are presented using the normalized units of LF and HF and LF/HF.

**Echocardiography**
Doppler echocardiography was performed to evaluate cardiac function by measuring left ventricular (LV) end-diastolic volume, LV end-systolic volume, and LV ejection fraction. The method is detailed in the Online Supplement.

**Measurements of VA inducibility and ventricular fibrillation threshold**
The methods of VA inducibility and ventricular fibrillation (VF) threshold measurements are detailed in the Online Supplement.

**Myocardial infarct size determination**
The myocardial infarct size determination is described in the Online Supplement.

**Histological staining**
The histological staining for synaptosomal-associated protein 25 (SNAP-25) and Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining are described in the Online Supplement.

**Blood sampling**
Serum norepinephrine (NE) level assessments are described in the Online Supplement.

**Statistical analysis**
All experimental data are expressed as mean ± SD and were tested for normal distribution by using the Kolmogorov-Smirnov test. An unpaired Student t test and 1-way analysis of variance were used to analyze the above data. All the data were analyzed and graphed using GraphPad Prism software version 8.0.2 (La Jolla, CA). Values at P < .05 were considered statistically significant.

**Results**
**Verification of the efficacy and safety of BTA injection**
SNAP-25 is the essential protein for cholinergic neurotransmitter release, and a decrease in its expression indicates

cholinergic neural blockade. Figure 1C shows a quantitative analysis of SNAP-25+ cells in the 3 groups in which BTA microinjection significantly reduced SNAP-25 expression in neural cells compared to that in the MI group, indicating that neural transmission in the LSG was successfully blocked. The expression of SNAP-25 was comparable between the MI group and the control group. Additionally, the quantitative analysis of TUNEL staining indicated that there was no significant difference in LSG cell apoptosis among the 3 groups, thus verifying the safety of BTA with respect to LSG cell apoptosis (Figure 1D).

**BTA injection significantly inhibited LSG function and neural activity**
Figure 2A shows the maximal SBP change in response to LSG electrical stimulation. As shown in Figures 2B–2G, 30 days of myocardial ischemia induced a significant elevation in LSG function at all 6 levels of LSG electrical stimulation. However, animals in the MI + BTA group showed a reversal of this elevated LSG function. Figure 2H shows representative images of LSG neural activity recorded after 30 days of remodeling in the 3 groups. As shown in Figure 2I, the MI group exhibited increased LSG neural activity compared with that in the control group while BTA treatment significantly reduced the excessive firing of neurons manifested as decreased frequency and amplitude.

**Blocking effect of BTA injection on the cardiac autonomic nervous system**
HRV has been widely used as an index to evaluate autonomic nervous system function. It was performed by analyzing the power spectral variables, including LF and HF components, in the ECG and calculating the normalized units of LF and HF and LF/HF. No obvious difference was observed in LF, HF, LF/HF, and serum NE levels among the 3 groups at baseline, indicating that their initial autonomic balance was relatively uniform. Compared to the control group, 30-day MI induced sympathetic activation, which led to higher values of LF and LF/HF and lower values of HF. However, BTA injection significantly attenuated MI-induced increases in LF and LF/HF and decreases in HF after 30 days. In addition, the MI-induced elevation in serum NE levels was significantly reversed in the MI + BTA group (Figure 3).

**BTA injection significantly improved ventricular ERP, VA inducibility, and VF threshold**
Ventricular ERP was used to evaluate ventricular electrophysiological properties. Figure 4A shows a representative ERP measurement. A comparison of the MI group with the control group indicated that MI shortened the ventricular ERP in all 3 sites of the LV. However, BTA injection inhibited these changes, as demonstrated by prolonging the ventricular ERP to the normal range (Figure 4B).

In addition, we assessed the effect of BTA injection on the improvement of VA susceptibility after chronic myocardial
ischemia by examining VA inducibility and VF threshold. In the MI group, beagles exhibited elevated VA inducibility and a decreased VF threshold. However, BTA significantly improved these outcomes (Figures 4C and 4D).

**BTA injection significantly reversed post-MI cardiac dysfunction**

Doppler echocardiography and triphenyltetrazolium chloride staining of LV tissue were performed to assess LV function and myocardial infarct size. Figure 5A shows representative images of the infarct area in the MI group and MI + BTA group. As shown in Figure 5B, BTA treatment significantly decreased the myocardial infarct size compared with that in the MI group. Representative apical 4-chamber and 2-chamber views for volume calculations using the biplane Simpson’s method are shown in Figure 5C. As shown in Figures 5D–5G, the LV ejection fraction was significantly lower and the LV end-diastolic volume and LV end-systolic volume were significantly higher in the MI group.
than in the control group. BTA treatment significantly reversed LV dysfunction due to MI. These results of the echocardiographic evaluation showed that dogs in the MI + BTA group exhibited improved LV systolic and diastolic function compared with that in the MI group.

Discussion

Major findings

Our present study provided direct evidence for a potentially beneficial role of BTA microinjection into the LSG in the inhibition of cardiac sympathetic ganglion, thus protecting LADO-induced chronic MI. We found that increased LSG function and neural activity induced by MI were significantly inhibited by BTA application. The sympathetic components of HRV and serum NE levels were suppressed. In addition, BTA treatment markedly attenuated the size of the MI, alleviated LV remodeling and ameliorated the decreased LV function evaluated by echocardiography. Meanwhile, it spurred a considerable reduction in VA susceptibility by prolonging ventricular ERP, reducing VA inducibility, and increasing VF threshold. Notably, no significant difference in neural apoptosis was observed between the MI + BTA group and the other groups.

Blocking effects of BTA application on the sympathetic nervous system

Botulinum neurotoxin, including BTA, is a family of toxic bacterial proteins composed of light chain and heavy chain. The heavy chain recognizes receptors on the surface of nerve endings and mediates light chain entry into the cytoplasm through endocytosis to block neurotransmission by cleaving SNAP-25, which is a neuronal substrate protein required for cholinergic neurotransmitter exocytosis. Caleo et al demonstrated that BTA can be effective in treating movement disorders such as spasticity and dystonia, and BTA acts by cleaving SNAP-25 at the neuromuscular junction, thus blocking synaptic transmission and weakening overactive muscles.

All preganglionic neurons, including LSG preganglionic nerves, are cholinergic, and recent preclinical and clinical studies have shown that BTA has the potential to block peripheral sympathetic ganglion. For example, in a case report, Park et al injected BTA into the stellate ganglion of a patient with craniofacial hyperhidrosis and reported obviously reduced sweating. In the present study, we found that microinjection of BTA into the LSG could effectively decrease SNAP-25 expression and inhibit its neural function and activity. Furthermore, the
modulatory effects of BTA treatment on serum NE levels and HRV described previously, which are markers of autonomic nerves, confirm the same findings.

The cardiac sympathetic nervous system regulates cardiac remodeling and VAs
Altered autonomic activity is an important mechanism for myocardial remodeling and the development of VAs, and autonomic remodeling after MI often presents as cardiac sympathetic nerve sprouting and hyperinnervation. MI can deteriorate the neural structural remodeling of the LSG and the myocardial infarct site reflected by the density of sympathetic nerves and their neural activity, which also exacerbates the instability of ventricular electrophysiology and the occurrence of VAs.11 As LSG hyperactivity can precede most malignant VAs, multiple methods of cardiac sympathetic nerve activity suppression through stellate ganglion blockade have proven to be valid in preventing cardiac remodeling and VAs in various clinical studies, including surgical denervation and local anesthetic drug blockade. However, there may be some side effects or the need for multiple injections associated with local anesthetics that impede its clinical use.12 In the present study, we propose that BTA microinjection, as a modulatory rather than a destructive strategy for LSG, can inhibit the cardiac sympathetic nervous system and thus improving cardiac remodeling and VA susceptibility after LADO-induced MI in a safer and longer-lasting manner (Figure 6).

Feasibility of ultrasound-guided BTA injection for LSG block
As an Food and Drug Administration–approved agent, the pharmacological action of BTA involves reducing cholinergic neurotransmission rather than damaging the nerves, and BTA has been proven to be safe for application. Maiaru et al13 injected BTA into the spinal cords of a mouse model to block specific pain-processing neurons and obtained long-lasting pain relief without toxic side effects. In addition, studies have revealed that the systemic median lethal dose of BTA

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Figure 4  Effects of BTA injection on ventricular ERP, VA inducibility, and VF threshold. Representative images (A) of ERP measurement and ERP values at 3 epicardial sites (B) in the 3 groups. Effects of BTA injection on VA inducibility (C) and VF threshold (D) (control and MI: n = 6; MI + BTA: n = 7). ERP = effective refractory period; LVA = left ventricular apex; LVB = left ventricular base; LVM = left ventricular median; VA = ventricular arrhythmia; VF = ventricular fibrillation; other abbreviations as in Figure 1. *P < .05.
The intramuscular injection in apes was 39 U/kg. In the clinical setting, the therapeutic dose of intramuscular BTA is 20–500 U and several long-term follow-up clinical studies using BTA to block sympathetic ganglion observed no adverse BTA-related reactions. Additionally, ultrasound-guided techniques have been widely used in clinical punctures for the diagnosis and treatment of multiple diseases. Ultrasound-guided cardiac sympathetic modulation via transtracheal or transesophageal approaches has been developed to further reduce surgical injury. Notably, percutaneous ultrasound-guided puncture diagnosis and treatment, including stellate ganglion block, is routine and safe, even at the bedside for outpatients in the clinic and the percutaneous approach greatly improves the convenience of the operation. In the present study, we provided a much more maneuverable and less invasive approach to modulate LSG activity by using ultrasound-guided injection of BTA in a large animal model. Our local injection dose (50 U total) was far below the median lethal dose, and TUNEL staining showed no significant difference in neural apoptosis within the LSG among the 3 groups. This evidence ensured that the approach in the present study was much more feasible and minimally invasive, making its use suitable for clinical practice.

Meanwhile, the effect of BTA is sustained. Carroll et al found that compared with the standard lumbar sympathetic block with bupivacaine in patients with refractory complex
regional pain syndrome, BTA plus local anesthetic application significantly prolonged the sympathetic blocking effect to 253 days. In a series of studies of the blocking effect of BTA in the cardiac autonomic nervous system, although atrial electrophysiological indicators recovered within 3 weeks, the residual effects of cardiac autonomic nervous system suppression persisted much longer. Moreover, some long-term nerve interventions including intermittent vagal nerve stimulation have been shown to cause LSG neuronal damages including fibrosis and apoptosis, which may reduce sympathetic tone and translate into a more durable result. In the present study, we provided the evidence that BTA could effectively inhibit LSG activity for 4 weeks by blocking neurotransmission by cleaving SNAP-25 with the absence of apoptosis. In future studies, longer-term persistence of BTA blocking and whether it could lead to LSG neuronal damage need further investigation.

**Study limitations**

There are several limitations of this research. First, we explored only 30-day chronic MI models in this study, the long-lasting effects of BTA in improving MI over months or years need to be verified. Second, we used only the dog model of MI to verify the effects of BTA, different animal models need to be explored in the future. Third, as LSG regulation is more related to LV physiology, which is mainly dominated by the left anterior descending coronary artery, we chose the intervention mode of BTA injection into the LSG and found it effectively improve LADO-induced MI. However, more treatments of other types of MI (eg, right coronary artery lesion-induced MI) need to be explored in future studies. Fourth, the catheter-based transvascular MI model is worthy of consideration in future studies because of its advantages of relatively low invasiveness and valuable clinical prospects. Last, we mainly focused on exploring the effects of BTA on post-MI LV remodeling on the 30th day; however, 30-day ECG monitoring during the ischemic period on a regular basis may be necessary, which will be explored in future studies.

**Clinical implications**

In recent years, the global market for BTA has grown to millions of dollars. In this study, ultrasound-guided percutaneous microinjection of BTA as a modulatory rather than a destructive strategy for cardiac sympathetic ganglion provides a feasible minimally invasive approach for the treatment of LADO-induced MI. The advantages described above indicate that such BTA treatment might be beneficial to reduce the VA burden, the need for hospitalization, and even the necessity of catheter ablation or surgery for some patients.

**Conclusion**

Ultrasound-guided percutaneous microinjection of BTA into the LSG can effectively suppress cardiac sympathetic hyperactivity and prevent cardiac remodeling after chronic
LADO-induced MI in a large animal model. Thus, it may potentially become a highly effective and safe adjuvant approach for treating MI.

References