Original Research

Botulinum toxin type A suppresses arterial vasoconstriction by regulating calcium sensitization and the endothelium-dependent endothelial nitric oxide synthase/soluble guanylyl cyclase/cyclic guanosine monophosphate pathway: An *in vitro* study

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Impact statement

Raynaud syndrome (RS), usually caused by cold or mental stress, may lead to cyanosis and reactive hyperemia accompanied by pain or paresthesia. Although a variety of drugs have been used to alleviate the symptoms, the effects have not been satisfactory, so there is an urgent need to explore new alternative treatments. The present study investigated the mechanism underlying the effects of botulinum toxin type A (BTX-A) on arterial vasoconstriction, which may provide new approaches for RS. We found that BTX-A suppresses arterial vasoconstriction by regulating smooth muscle calcium sensitization and the endothelial nitric oxide synthase (eNOS)/soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway. These findings clarify the underlying mechanism of the effects of BTX-A on arterial vasoconstriction and provide new theoretical support for the use of BTX-A on RS. It may also help us to develop new medicines which regulate calcium sensitization and the eNOS/sGC/cGMP pathway against RS.

Abstract

Botulinum toxin type A (BTX-A) is a potent neurotoxin that causes relaxation of striated muscle by inhibiting the release of acetylcholine at the neuromuscular junction. Some studies have suggested that BTX-A treatment for Raynaud syndrome is safe and effective with few adverse reactions. However, the underlying mechanism remains unclear. In the present study, we used both arterial rings isolated from rabbits and human microvascular endothelial cells (HMEC-1) to evaluate the mechanism underlying the effects of BTX-A on arterial vasoconstriction induced by 5-hydroxytryptamine. The roles of calcium sensitization and the endothelial nitric oxide synthase (eNOS)/soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway were investigated. BTX-A caused a concentration-dependent decrease in the contraction of endothelium-intact arteries and significantly reduced calcium sensitization in the arteries. Inhibitors of eNOS (L-NAME) and sGC (methylene blue) both significantly abolished the vasodilatory action of BTX-A. Furthermore, BTX-A increased eNOS activity and the cGMP level in dose- and time-dependent manners and increased eNOS and sGC protein levels in a time-dependent manner in HMEC-1. Taken together, these findings indicate that BTX-A suppresses arterial vasoconstriction by regulating smooth muscle calcium sensitization and the eNOS/sGC/cGMP pathway.

Keywords: Botulinum toxin type A, artery vasoconstriction, smooth muscle cell, calcium sensitization, endothelial nitric oxide synthase, soluble guanylyl cyclase, cyclic guanosine monophosphate

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Introduction

Raynaud syndrome (RS), usually caused by cold or mental stress, was first described by the French scholar Raynaud in 1862. The main clinical manifestations of RS include cyanosis and reactive hyperemia accompanied by pain or paresthesia.[1,2] A survey conducted in the USA found that the incidences of RS in women and men are approximately 9.6 and 5.8%, respectively.[1,3–5] The main sites of the disease include the fingers, toes, ears, nose, and tongue. The onset duration usually varies between 20 min to several hours.[6,7] RS can be divided into primary and secondary diseases according to the aetiology. The former is also called

Raynaud's disease, which accounts for 80% of RS cases; it is normally caused by changes in vascular function and results in no irreversible tissue damage.[1] Secondary RS is known as Raynaud's phenomenon, which is secondary to other diseases, including mixed connective tissue disease (86%), systemic lupus erythematosus (31%), rheumatoid arthritis (22%), and Sjogren's syndrome (13%).[8] In addition, atherosclerosis and some drugs or poisons can also cause Raynaud's phenomenon.[8] The frequency of RS onset is higher than that of Raynaud's disease and may be accompanied by pain, skin ulcers, and gangrene.[1,8]

Although a variety of drugs have been used to alleviate the symptoms, the effects have not been satisfactory. Commonly used drugs include calcium channel blockers, α1 receptor antagonists, angiotensin converting enzyme inhibitors, angiotensin II receptor antagonists, 5-hydroxytryptamine (5-HT) reuptake inhibitors, phosphodiesterase inhibitors, prostaglandins, and endothelin receptor antagonists.[8] Among them, calcium channel blockers are the most frequently used and can significantly reduce the onset frequency of RS. However, most drugs are ineffective for patients with severe symptoms. Some drugs may reduce the functions of the heart, liver, kidney, and other organs, and should not be applied for a long duration. Thoracic sympathectomy can be used for patients with intolerance to drug therapy, but this treatment may be accompanied by different degrees of trauma, pneumothorax, hemothorax, and Horner's syndrome. Moreover, the recurrence rate is as high as 55–82.1%.[9] As a result, there is an urgent need to explore new alternative treatments.

Botulinum toxin (BTX) is produced by anaerobic bacteria of the genus *Clostridium* and is the most toxic protein in the world (mouse LD50 values can be as low as 1 ng/kg).[10] BTX can be divided into seven serotypes (A, B, C, D, E, F, and G) based on antigenicity. Types A, B, E, and F cause human BTX poisoning, whereas types C and D cause mammalian and avian poisoning. Among these serotypes, BTX toxin type A (BTX-A) is used most frequently in clinical applications via intramuscular or subcutaneous injection.[11] BTX-A is a potent neurotoxin that causes relaxation of striated muscle by inhibiting the release of acetylcholine (ACh) at the neuromuscular junction. It also acts on the autonomic nervous system, inhibiting the secretion of glands and release of smooth muscle ACh.[12] After the US Food and Drug Administration approved the use of BTX-A in clinical practice in 1989, it was rapidly developed and applied in the plastic surgery field. At the same time, broader applications of BTX-A have been constantly explored in clinical practice.[13] However, the specific mechanism is still unclear, and its relationship with vascular endothelial cells needs further study.

Under the action of vascular shearing force, endothelial cells produce vasoactive substances such as ACh. Endothelial nitric oxide synthase (eNOS) in endothelial cells produces nitric oxide (NO), which diffuses freely into smooth muscle cells, producing cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) and finally inhibiting vasoconstriction via cGMP-dependent protein kinase G. Soluble guanylyl cyclase (sGC), which is completely intracellular, is the only known receptor for NO that is involved in vasodilation. NO induces an approximately 200-fold increase in sGC activity. Similar to the contraction mechanism of striated muscle, contraction of smooth muscle is also dependent on myofilaments under the regulation of actin–myosin interactions, which require Ca^{2+} for excitation and ATP for its energy supply. Previous studies have shown that BTX-A regulates arterial vasoconstriction, but whether the underlying mechanism involves eNOS, sGC, cGMP, or Ca^{2+} needs to be clarified.

Therefore, in the present study, we used both arterial rings isolated from rabbits and human microvascular endothelial cells (HMEC-1) to investigate the mechanism underlying the effects of BTX-A on arterial vasoconstriction. The roles of calcium sensitization and the eNOS/sGC/cGMP pathway were also investigated.

Materials and methods

Preparation of arterial rings

First, 136 four-month-old male New Zealand white rabbits (2–2.5 kg, obtained from the laboratory animal center of Tongji University School of Medicine) were used in the study. Rabbit is a common model to study the arterial ring, as the diameters of arterial rings isolated from rabbits were bigger than those of arterial rings isolated from rats or mice, making the experiments easier to perform and the experimental error smaller. The study was approved by the Animal Experiment Administration Committee of Tongji University School of Medicine (No. K17-154). All the animal experimental procedures followed the Guide for the Care and Use of Laboratory Animals, 8th Edition (National Academies Press, Washington, DC, 2010).

The rabbits were first anesthetized by intramuscular injection of xylazine hydrochloride (0.2 mL/kg). The abdominal aortas of the rabbits were extirpated, and 3–4 mm long ring slices were prepared by separating the lipids and connective tissues in a Petri dish filled with Krebs–Henseleit solution (K–H) solution (120.0 mM NaCl, 25.0 mM $NaHCO_3$, 5.0 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.4 mM NaH_2PO_4, and 11.0 mM glucose) bubbled with 95% O_2 and 5% CO_2. The temperature was maintained at $37 \pm 0.5^{\circ}C$. One end of the aorta slice was fixed in the tissue bath. The bath was changed for every experiment. Changes in arterial tension were recorded isometrically by the Multi Myograph System (Danish Myo Technology, Aarhus, Denmark). Each arterial ring was stretched gradually to a resting tension of 1.8–2 mN during a 90 min equilibration period to obtain optimal tension. The K–H solution in the chamber was replaced every 20 min, and tension was readjusted to 1.8–2 mN.

Vascular reactivity test

After the equilibration period, 60 mM KCl was added to the bath, and the contractile force was recorded. The vascular rings were washed four times with K–H solution at 5 min intervals to restore basal tension. A change in the vascular tone amplitude <3 mN was considered indicative of poor

contractibility, and the arterial rings were discarded. The bath was changed for every experiment.

Endothelial integrity test

Small forceps were inserted into the lumen, and the arterial ring was rolled to denude the endothelium. Endothelial integrity of the arterial rings was confirmed by at least 80% relaxation in response to ACh (10^{-6} mol/L) at the 5-HT plateau (10^{-5} mmol/L). Successful removal of the endothelium was confirmed by the lack of a vasorelaxant response to ACh.

Effect of 5-HT on arterial rings

5-HT was used to produce an evoked response for vasoconstriction. 5-HTwas added to intact and endothelium-denuded rings cumulatively, and tension was expressed as a percentage of the initial contraction of 5-HT (10^{-5} mol/L). A cumulative concentration–response curve for 5-HT was obtained.

Effect of BTX-A on vasoconstriction induced by 5-HT

BTX-A was purchased from Lanzhou Biological Products Institute (Lanzhou, China). It was received as lyophilized powder. Before it was used for experiments, the BTX-A powder was diluted using 1 ml of normal saline (0.9%) per 100 IU, for a concentration of BTX-A solution of 100 IU/mL. The solution was then added into the K–H solution or the RPMI-1640 to archive the concentration of 1, 2.5, 5, 10, 20, or 40 IU/mL. The rings were incubated with BTX-A at 1, 2.5, 5, 10, 20, and 40 IU/mL for 30 min, and the change in tension was measured. To determine the effect of BTX-A on contractility, a 5-HT concentration–response curve was obtained while maintaining the dosage of BTX-A at 10, 20, or 40 IU/mL. The time and concentrations selected for this study are based on other publications in the field.[14,15] Similar concentration of BTX-A was used in other cells.[14] Even in RS patients, 30 min of infiltration with BTX-A showed a very good perception of response in some RS patients.[15] After the BTX-A treatment was done, it was washed out within 1 min.

Effects of different treatments and the presence of endothelium on the inhibition of vasoconstriction by BTX-A

5-HT was used to produce an evoked response for vasoconstriction. Cumulative concentrations of 5-HT were added to intact rings incubated with BTX-A (20 IU/mL) in the presence or absence of the eNOS inhibitor L-NAME (10^{-4} mol/L) or the sGC inhibitor methylene blue (MB, 5×10^{-6} mol/L). In endothelium-denuded rings, cumulative concentrations of 5-HT were added without any other treatment after incubation with BTX-A (20 IU/mL).

Myogenic vasoconstriction and smooth muscle calcium measurements

Arterial rings were mounted in an arteriograph chamber, as described previously.[16] Myogenic activity was compared between Control (n = 8) and BTX-A (n = 8) arterioles by measuring their diameters and tones at pressures of 40–80 mmHg. Arterial rings in the Control groups received no treatment; they were kept in 37°C K–H solution (120.0 mM NaCl, 25.0 mM $NaHCO_3$, 5.0 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.4 mM NaH_2PO_4, and 11.0 mM glucose) bubbled with 95% O_2 and 5% CO_2. Arterial rings in the BTX-A groups were treated with BTX-A dissolved in 37°C K–H solution (20 IU/mL). To investigate the relationship between myogenic tone and calcium in arterial rings, a calcium-sensitive dye (Fura 2-AM) was used as described previously.[17] Briefly, the arterial rings were pressurized to 40 mmHg and equilibrated for 30 min to allow spontaneous development of myogenic tone. Changes in the smooth muscle diameter and calcium level were evaluated by stepwise increases using IonWizard software (IonOptix, Westwood, MA, USA) at pressures ranging from 40 to 80 mmHg.

Measurement of calcium sensitivity in arterial rings

Calcium sensitivity in arterial rings was determined using the method described by Cipolla *et al.*[18] Briefly, the arterial rings were mounted in an arteriograph filled with HEPES-buffered physiological saline solution. The rings were pressurized to 40 mmHg and equilibrated for 30 min. The arterial rings were permeabilized with *Staphylococcus aureus* α-toxin (800 U/mL) in relaxing solution at room temperature for 20 min. The *S. aureus* α-toxin was washed from the bath, and the arterial rings were equilibrated in relaxing solution at 37°C for 30 min. The bath was changed for every experiment. The vasoactive response to calcium was determined by replacing the relaxing solution with activating solution containing known concentrations of free ionic calcium. The inner diameters were measured at each calcium concentration. The arterial rings in Control were kept in the HEPES-buffered physiological saline solution, while the arterial rings in BTX-A were kept in HEPES-buffered physiological saline solution with BTX-A (20 IU/mL). The same volume of saline as the BTX-A group was added into the bath of Control.

Detection of eNOS activity in HMEC-1

The HMEC-1 line was purchased from the ATCC (Manassas, VA, USA) and used in this experiment. The cells were pre-incubated with 0, 1, 2.5, 5, 10, 20, or 40 IU/mL BTX-A in RPMI-1640 supplemented with 10% fetal bovine serum (Beyotime, Shanghai, China) for 30 min or with 20 IU/mL BTX-A for 0, 10, 20, 30, or 60 min. eNOS activity was evaluated by determining the conversion ratio of [^{3}H]L-arginine to [^{3}H]L-citrulline using a liquid scintillation counter (Packard Inc., Prospect, CT, USA), using the method described by Kimura *et al.*[19] The cell viability was measured with MTT assay following the manufacture instruction (Beyotime, Shanghai, China) after HMEC-1 cells were treated with 0, 1, 2.5, 5, 10, 20, or 40 IU/mL BTX-A in RPMI-1640 supplemented with 10% fetal bovine serum for 30 min or with 20 IU/mL BTX-A for 0, 10, 20, 30, or 60 min.

Measurement of cGMP levels in HMEC-1

HMEC-1 (passages 3–6) showing >99% positive immunostaining of α-smooth muscle actin were pre-incubated with 0, 1, 2.5, 5, 10, 20, or 40 IU/mL BTX-A in RPMI-1640 supplemented with 10% fetal bovine serum (Beyotime, Shanghai, China) for 30 min or with 20 IU/mL BTX-A for 0, 10, 20, 30, or 60 min. The cGMP levels in HMEC-1 were determined using the Parameter™ Cyclic GMP Assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Western blot

Total proteins (100 μg) extracted from cells were first subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After the membranes were blocked with 5% non-fat milk powder in 0.05% Tween-20 PBS buffer for 1 h, they were incubated with a primary antibody against eNOS, sGC, or GAPDH overnight at 4°C. The next day, the membranes were rinsed with 0.05% Tween-20 in PBS three times and incubated with a secondary antibody conjugated to horseradish peroxidase (cat. no. G-21040; 1:10,000; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. The membranes were rinsed with 0.05% Tween-20 in PBS three times. A chemiluminescence reagent (GE Healthcare Life Sciences, Little Chalfont, UK) was added to the membranes and incubated for 2 min. The developed film was analyzed using a chemiluminescence ChemiDoc™ imager (Bio-Rad Laboratories, Inc.), Bio-Rad Laboratories, Inc., Hercules, CA, USA), and ImageJ 1.43 software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical analyses were performed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA) and one-way analysis of variance, followed by the Student–Newman–Keuls *post hoc* test. Differences were considered significant at $p < 0.05$.

Results

Effect of BTX-A on aortic ring resting tension and 5-HT-induced vasoconstriction

Figure 1 shows the effects of BTX-A on arterial ring resting tension and 5-HT-induced vasoconstriction. BTX-A had no direct effect on the resting tension of the aorta in the absence of the endothelium but caused a concentration-dependent decrease in the contraction of endothelium-intact arteries (Figure 1(a)). As the concentration of BTX-A increased, the effect on contraction decreased. BTX-A at 10, 20, and 40 IU/mL had a significant effect on 5-HT-induced vasoconstriction compared with in the absence of BTX-A (Figure 1(b)). Regarding the effect of the interaction between BTX-A and the endothelium on vasoconstriction, only the presence of both endothelium and BTX-A significantly inhibited vasoconstriction (Figure 1(c)). No significant difference was observed among the endothelium-only (no BTX) group, no endothelium or BTX-A group, and the BTX-A-only (no endothelium) group.

Effect of BTX-A on myogenic tone and calcium

Figure 2 shows the relationship between myogenic tone and the smooth muscle calcium level in arterial rings from the Control and BTX-A groups. The arterial rings were larger in diameter in the BTX-A group than Control group as a result of decreased myogenic tone (Figure 2(a) and (b)), but there was no difference in the calcium level of arterioles between the Control and BTX-A groups (Figure 2 (c)). When the arterial rings were treated with different concentrations of calcium, the tone of arterioles was lower in the BTX-A group than Control group for the same calcium level (Figure 2(d)). The rate of tone increase induced by calcium was lower in the BTX-A group.

Calcium sensitization of permeabilized arterioles after the BTX-A treatment

Figure 3 shows the calcium sensitization of permeabilized arterioles after BTX-A treatment. The myogenic tone in arterioles and calcium sensitivity were decreased significantly in

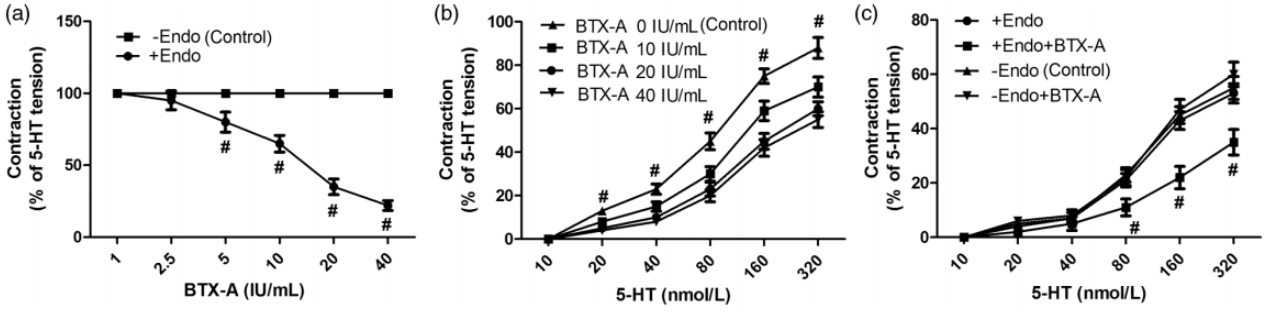

Figure 1. Effect of BTX-A on aortic resting tension and 5-HT-induced vasoconstriction. (a) The effect of BTX-A on arterial resting tension in the absence versus presence of the endothelium; −Endo group was used as Control. (b) The effect of BTX-A on 5-HT-induced vasoconstriction; BTX-A 0 IU/mL was used as Control. (c) The effect of the interaction of BTX-A and the endothelium on vasoconstriction; −Endo group was used as Control. −Endo: arterial ring without endothelium; + Endo: arterial ring with endothelium. + Endo + BTX-A: arterial ring with endothelium treated with 20 IU/mL BTX-A; −Endo + BTX-A: arterial ring without endothelium treated with 20 IU/mL BTX-A. # $p < 0.05$ compared with the −Endo group or with the 0 IU/mL BTX-A group. N = 8.
BTX-A: botulinum toxin type A; 5-HT: 5-hydroxytryptamine.

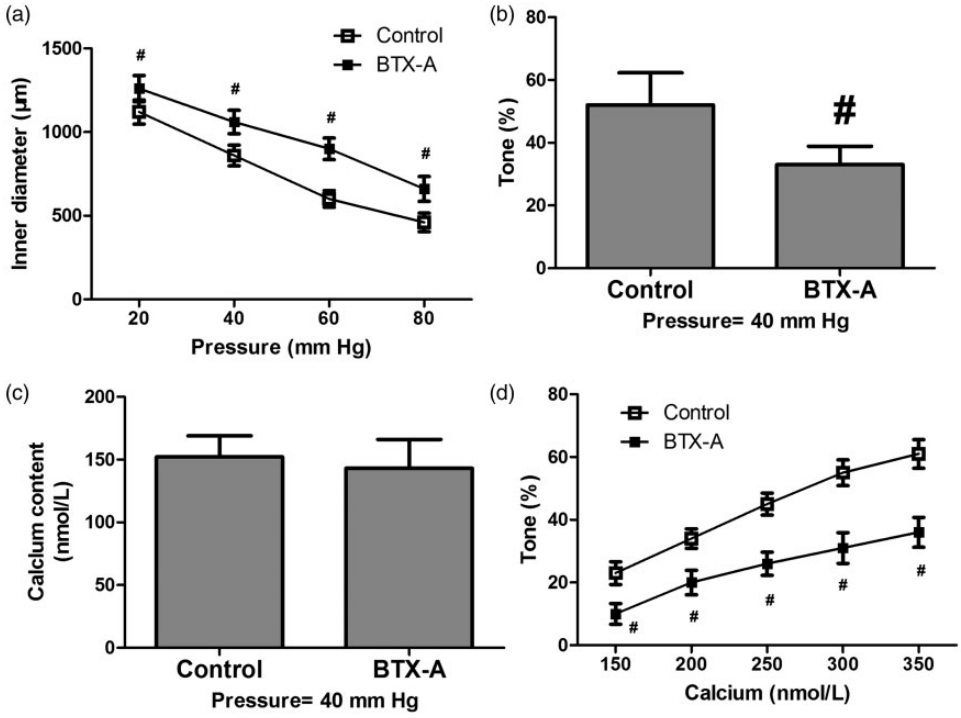

Figure 2. Effect of BTX-A on arterial ring diameter and myogenic tone. Differences in arterial ring diameter (a), myogenic tone (b), and calcium content (c) between the Control and BTX-A groups. (d) Difference in myogenic tone between the Control and BTX-A groups in the presence of calcium. # $p < 0.05$ compared with the Control group. $N = 8$.
BTX-A: botulinum toxin type A.

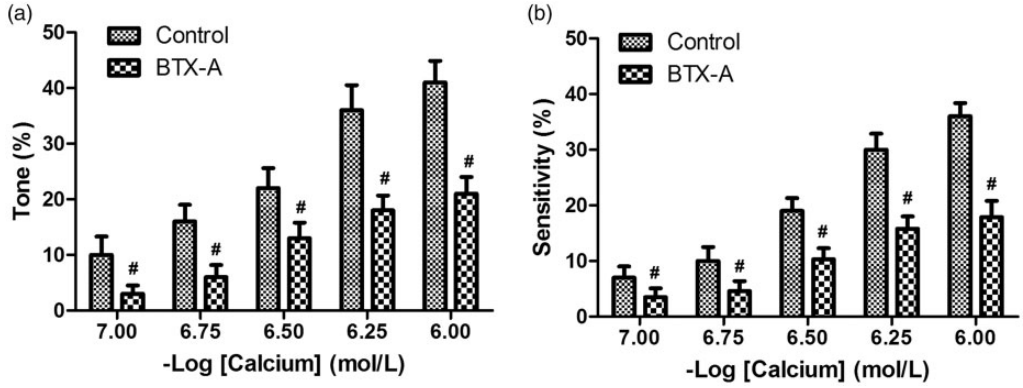

Figure 3. Calcium sensitization of permeabilized arterioles after BTX-A treatment. Myogenic tone in arterioles (a) and calcium sensitivity (b) in the Control and BTX-A groups. A decrease in calcium sensitivity was detected after BTX-A treatment. The effective concentration that produced half maximal constriction (EC50) was significantly higher for arterioles in the BTX-A group than the Control group. # $p < 0.05$ compared with the Control. $N = 8$.
BTX-A: botulinum toxin type A.

the BTX-A group compared with the Control (Figure 3(a) and (b)). The effective concentration that produced half maximal constriction (EC50) was significantly higher for arterioles in the BTX-A group than in the Control group.

Effects of eNOS and sGC inhibitors on the vasodilatory action of BTX-A

Figure 4 shows the effect of inhibitors of eNOS (L-NAME) and sGC (MB) on the vasodilatory action of BTX-A. Treatment with inhibitors of eNOS (L-NAME) and sGC (MB) significantly abolished the vasodilatory action of BTX-A (Figure 4(a) and (b)). Treatment with these chemicals alone had no effect on the vasoconstriction caused by 5-HT. Figure 4c shows the changes in vascular diameter caused by BTX-A, L-NAME, and MB. Under treatment with 80 nmol/mL 5-HT, BTX-A significantly increased, while co-treatment with L-NAME or MB significantly decreased, vascular diameter, indicating that the vasodilatory action of BTX-A was abolished by L-NAME and MB (Figure 2(c)).

BTX-A increased eNOS activity and cGMP levels in HMEC-1

To further explore the involvement of eNOS and cGMP in the vasodilatory action of BTX-A, we measured eNOS

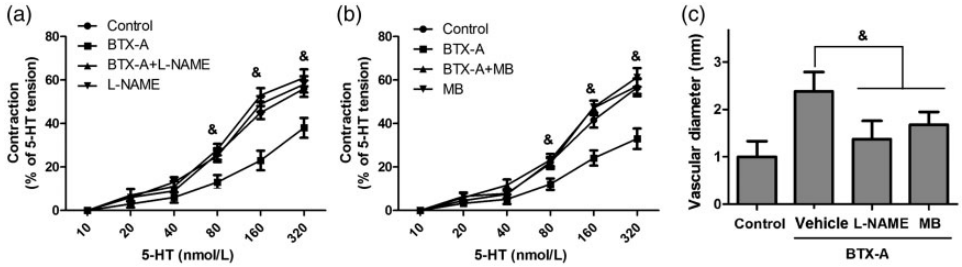

Figure 4. Effect of inhibitors of eNOS and sGC on the vasodilatory action of BTX-A. The effects of an inhibitor of eNOS (L-NAME) (a) and an inhibitor of sGC (MB) (b) on the vasodilatory action of BTX-A. (c) The effects of BTX-A, L-NAME, and MB on vascular diameter. $^{\&}$ $p < 0.05$ compared with the BTX-A group. $N = 8$.
BTX-A: botulinum toxin type A; 5-HT: 5-hydroxytryptamine; MB: methylene blue.

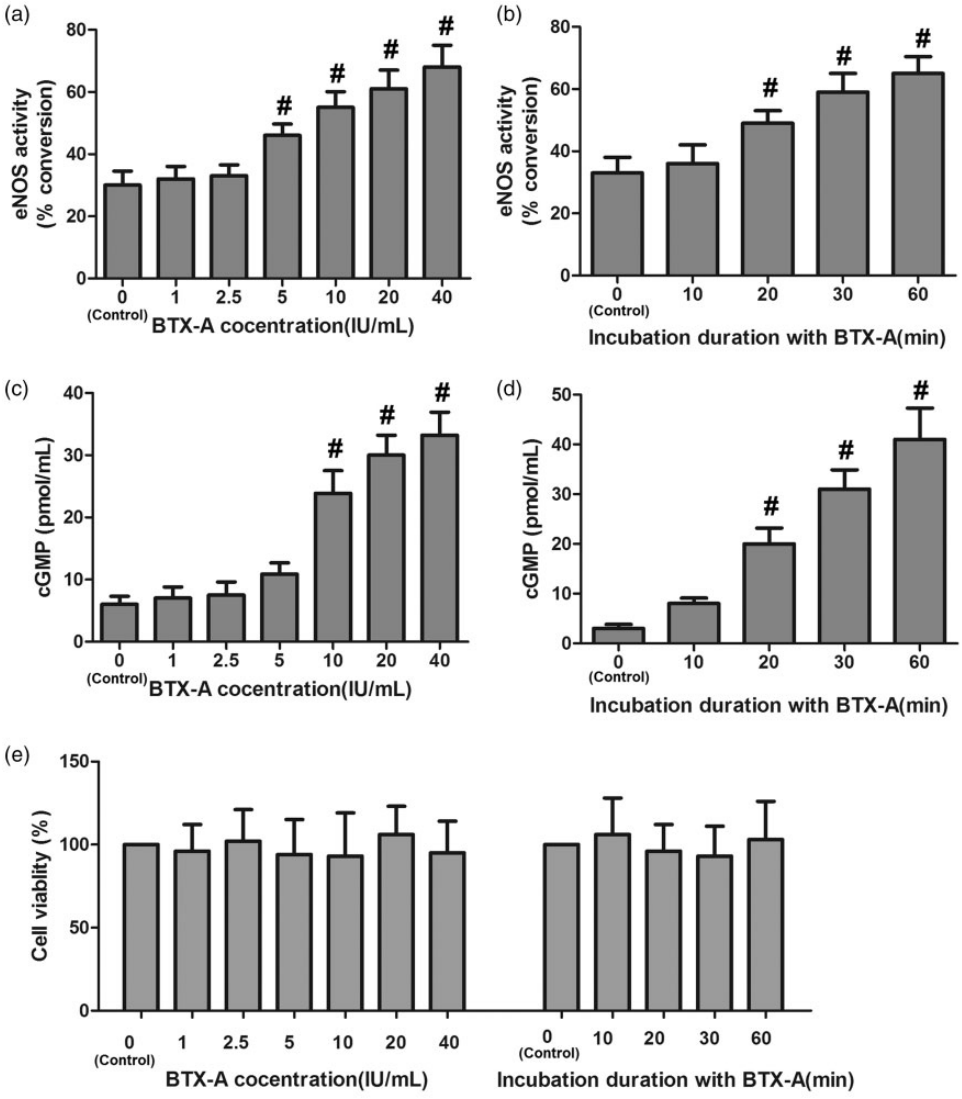

Figure 5. Effects of BTX-A on eNOS activity and cGMP levels in HMEC-1. Effects of the BTX-A concentration and treatment duration on endothelial eNOS activity were shown in Figure 5(a) and (b). Effects of the BTX-A concentration and treatment duration on cGMP level were shown in Figure 5(c) and (d). In Figure 5(a) and (c), BTX-A 0 IU/mL was used as Control; in Figure 5(b) and (d), 0 min was used as Control. $^{\#}$ $p < 0.05$ compared with the 0 IU/mL BTX-A (a and c) or 0 min ((b) and (d)) group. $N = 8$.
BTX-A: botulinum toxin type A; cGMP: cyclic guanosine monophosphate; eNOS: endothelial nitric oxide synthase.

activity and cGMP levels in HMEC-1. The eNOS activity was measured by calculating the percentage of [^{3}H]L-citrulline converted from [^{3}H]L-arginine. BTX-A increased endothelial eNOS activity in dose- and time-dependent manners (Figure 5(a) and (b)) and significantly increased the cGMP level at a concentration over 5 IU/mL (Figure 5 (c) and (d)). BTX-A increased the cGMP level in dose- and time-dependent manners. The cell viability was measured

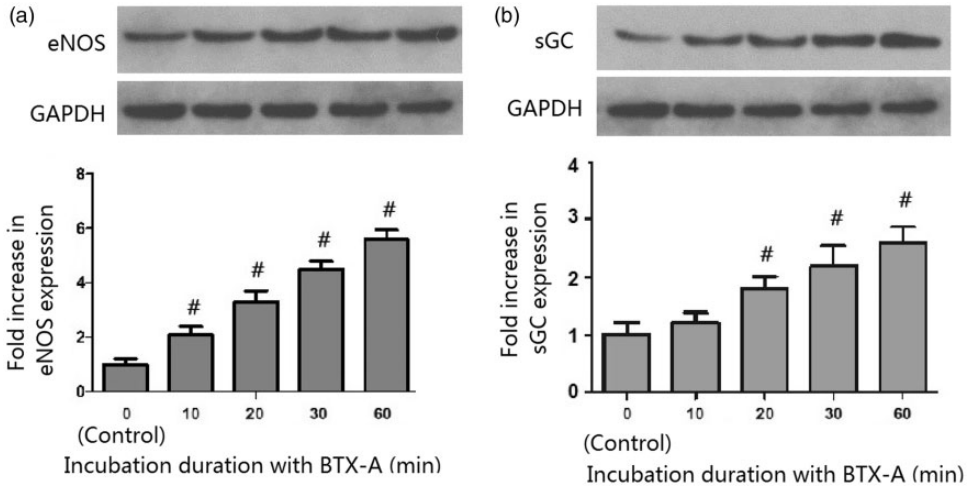

Figure 6. Effects of BTX-A on eNOS and sGC protein levels in HMEC-1. Effects of the BTX-A treatment duration on eNOS (a) and sGC (b) protein levels. 0 min was used as Control. # $p < 0.05$ compared with the 0 min group. $N = 8$.
eNOS: endothelial nitric oxide synthase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; sGC: soluble guanylyl cyclase.

with MTT assay. As shown in Figure 5(e), all the treatment with BTX-A did not affect the cell viability.

BTX-A increased eNOS and sGC protein levels in HMEC-1

To confirm the effects of BTX-A on eNOS and sGC protein levels in HMEC-1, we treated the cells with 20 IU/mL BTX-A for different durations (0–60 min) and measured eNOS and sGC protein levels by Western blot. BTX-A increased the eNOS and sGC protein levels in a time-dependent manner (Figure 6(a) and (b)).

Discussion

The present study used both arterial rings isolated from rabbits and HMEC-1 to study the mechanism underlying the effects of BTX-A on arterial vasoconstriction. The roles of calcium sensitization and the eNOS/sGC/cGMP pathway were also investigated. Our results demonstrate that BTX-A caused a concentration-dependent decrease in the contraction of endothelium-intact arteries. BTX-A inhibited vasoconstriction significantly only when the endothelium was present. It is important to note that we used an evoked response for vasoconstriction by 5-HT, it is not spontaneous, and that BTX-A has effects on both condition of evoked vasoconstriction and no evoked vasoconstriction model. Figure 1 showed that under vasoconstriction induced by 5-HT, BTX-A significantly caused a concentration-dependent decrease in the contraction of arteries. Figure 2 shows under no evoked vasoconstriction, the diameter of arterial rings was larger in the BTX-A group than Control group. Treatment with inhibitors of eNOS (L-NAME) and sGC (MB) both significantly abolished the vasodilatory action of BTX-A. The arterial rings were larger in diameter in the BTX-A group due to decreased myogenic tone. When the arterial rings were treated with different concentrations of calcium, arterioles in the BTX-A group had less tone compared with those in the Control group at each level of calcium. The increase in tone induced by calcium was lower in the BTX-A group. A decrease in calcium sensitivity was detected after BTX-A treatment. BTX-A increased eNOS activity and cGMP levels in HMEC-1 in dose- and time-dependent manners. Furthermore, BTX-A increased eNOS and sGC protein levels in HMEC-1 in a time-dependent manner.

Some studies have indicated that BTX-A may offer a new option for treating RS. Although the mechanism is not clear, it has been confirmed that BTX-A treatment for RS is safe and effective with few adverse reactions, such as intrinsic weakness and dysesthesia, which disappeared within 2–5 months.[20] BTX-A was first used to treat RS in 2004. Subsequently, several clinical studies confirmed that BTX-A significantly alleviates finger artery spasms in patients with RS. BTX-A increases blood flow, promotes ulcer healing, and relieves pain without obvious complications.[21–24] In a three-year follow-up study, Medina *et al.* revealed that after 30 min of infiltration with BTX-A, it showed a very good perception of response in some RS patients. After the patients were treated with BTX-A for one month, more than half of them have maintained a very good response throughout the study.[15] In the present study, using arterial rings isolated from rabbits, we showed that BTX-A caused a concentration-dependent decrease in the contraction of endothelium-intact arteries (Figure 1). BTX-A significantly inhibited vasoconstriction only when the endothelium was present. The arterial rings were larger in diameter due to decreased myogenic tone in the BTX-A group. All of these results reveal that BTX-A directly suppresses arterial vasoconstriction, and that the effect is dependent on the endothelium.

Previous studies reported that BTX-A inhibits the release of ACh from the presynaptic membranes of motor nerve terminals, and this process usually takes 1–14 days. In contrast, injecting BTX-A into patients with RS is effective immediately and exerts long-term effects. This phenomenon indicates that BTX-A may treat RS via a different mechanism, but previous studies have suggested multiple explanations. One reported mechanism is that BTX-A

inhibits the release of vascular smooth muscle norepinephrine vesicles as well as supplementation of the alpha-adrenergic energy receptor, thereby reducing the activity of sensitive C-fiber pain receptors and vascular smooth muscle contraction.[25,26] BTX-A may reduce pain in patients with RS by inhibiting ectopic sodium channels or the release of pain-causing substances, such as glutamate, substance P, and calcitonin gene-related peptide.[27]

Because of the importance of calcium and the eNOS/sGC/cGMP pathway in the regulation of arterial vasoconstriction, we explored the possible involvement of calcium sensitization and the eNOS/sGC/cGMP pathway in this mechanism. The arterial rings in the BTX-A group were larger in diameter and had less myogenic tone compared with those in the Control group (Figure 2). When the arterial rings were treated with different concentrations of calcium, the arterioles exhibited lower myogenic tone in the BTX-A group than Control group. The increase in tone induced by calcium was lower in the BTX-A group. The myogenic tone in arterioles and calcium sensitivity decreased significantly in the BTX-A group compared with the Control group (Figure 3). The EC50 was significantly higher for arterioles in the BTX-A than Control group. The relationship between BTX-A and calcium has been examined in previous studies. Ali *et al.*[28] showed that a single unilateral BTX-A injection into albino rats induced a significant decrease in the serum calcium level. Calcium plays an important role in RS. Calcium channel blockers are the most widely used drugs to manage RS and could significantly reduce the frequency and severity of RS.[29–31] The present study suggests that the mechanism underlying the protective effect of BTX-A on RS may be regulation of calcium sensitization and not just the calcium level.

Smooth muscle cells are effectors that regulate vasoconstriction and relaxation. Under the action of a vascular shearing force, endothelial cells produce vasoactive substances, such as ACh and NO, which play important roles in the regulation of vascular tone via regulation of eNOS in endothelial cells. NO diffuses freely into smooth muscle cells, activates sGC to synthesize cGMP from GTP, and finally induces vasorelaxation.[32,33] On the other hand, elevated levels of cGMP and cAMP allow relaxation of smooth muscle cells.[33] To further examine the role of the eNOS/sGC/cGMP pathway in the vasorelaxant effect of BTX-A, first we examined the effects of inhibitors of eNOS (L-NAME) and sGC (MB) on the vasodilatory action of BTX-A. Inhibitors of eNOS (L-NAME) and sGC (MB) both significantly abolished the vasodilatory action of BTX-A. Treatment with BTX-A significantly increased, whereas co-treatment with L-NAME or MB significantly decreased, vascular diameter, indicating that the vasodilatory action of BTX-A was abolished by L-NAME and MB. Next, we measured eNOS activity and cGMP levels in HMEC-1. As shown in Figure 5, BTX-A increased both endothelial eNOS bioactivity and cGMP levels in dose- and time-dependent manners. Furthermore, BTX-A increased eNOS and sGC protein levels in HMEC-1 in a time-dependent manner. These results suggest that BTX-A suppresses arterial vasoconstriction via the eNOS/sGC/cGMP pathway, and that BTX-A induces relaxation via the endothelial-dependent pathway as mediated by the eNOS/sGC/cGMP pathway. Previous studies have shown that eNOS consists of oxygenase and reductase domains with a Ca^{2+}/calmodulin-binding domain. Calcium binds to calmodulin and then to eNOS, thereby activating enzymatic activities. eNOS catalyzes the production of NO by oxidizing L-arginine, which diffuses into vascular smooth muscle cells, stimulates sGC, and increases intracellular cGMP levels. cGMP, in turn, activates cGMP-dependent protein kinase G, resulting in reduced intracellular calcium levels and relaxation of smooth muscle cells.[34] Increased levels of cGMP and subsequent activation of cGMP-dependent protein kinase A may result in vasodilation.[35,36] These statements are consistent with our results and may explain the underlying mechanism of action of BTX-A on smooth muscle cells.

In general, when BTX-A is used to treat RS, it is injected at the local sites of palm, which can alleviate pain, improve blood flow, and elevate temperature of the fingers.[37] Using this route of delivery for RS in clinic, the BTX-A could directly act on the microvessel in palm, which is similar to our organ bath direct application in the present study. However, it should be noted that local administration of BTX-A may also produce systemic effects. It was reported that an intramuscular injection of BTX-A produced a significant decrease in the calcium level with a significant increase in the phosphorus, alkaline phosphatase, C-reactive protein, and tumor necrosis factor α levels in serum.[28] It also induced pronounced alteration of heart rate variability.[38] The effect of BTX-A on blood pressure was not observed, but a study showed that BTX-B slightly decreased systolic blood pressure.[39] However, it is also reported that heart rate and blood pressure remained stable after a patient received an unintended intrathecal injection of BTX-B.[40] These results remind us that we should pay attention to potential systemic effect of BTX-A in clinical. In addition, Tanyeli *et al.*[41] compared the vasodilator effects of BTX-A and papaverine on human radial artery grafts and found that BTX-A inhibits both ET-1- and 5-HT-induced contractions in human radial artery. This study reminds us that the arterial vasodilatating effect of BTX-A may be used in broader clinical situations, such as coronary artery bypass graft surgery.

The present study might have potential for translational research but it also has some limitations compared with direct studies in humans. First, this is an *in vitro* study using arterial rings isolated from rabbits and HMEC-1. The experimental condition in the *in vitro* study was well-controlled, but in the human body, the situation is far more complicated. Whether the smooth muscle calcium sensitization and the eNOS/sGC/cGMP pathway are still the main mechanism of BTX-A in human body is not clear. Future work should examine the role of smooth muscle calcium sensitization and the eNOS/sGC/cGMP pathway in clinical trials regarding the protection of BTX-A against RS. Second, age and gender influence clinical outcomes, perhaps in response to many medications, including BTX-A. In the present study, we used four-month-old male New Zealand white rabbits to isolate the arterial

rings, so the fact of age and gender was not analyzed in the study, which should be further explored in future study.

In conclusion, the present study revealed that BTX-A caused concentration-dependent vasodilation of endothelium-intact arteries. BTX-A significantly reduced calcium sensitization in the arteries. Furthermore, inhibitors of eNOS and sGC both significantly abolished the vasodilatory action of BTX-A, and BTX-A increased eNOS activity and cGMP levels in HMEC-1 in dose- and time-dependent manners. Taken together, these findings indicate that BTX-A suppressed arterial vasoconstriction by regulating smooth muscle calcium sensitization and the endothelium-dependent pathway via the eNOS/sGC/cGMP pathway.

Author contributions: LH conducted the experiments and prepared the manuscript; YF performed the data analysis and interpretation; WL prepared the figures; LJ performed the References research; ZN designed the study and revised the manuscript.

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REFERENCES

1. Herrick AL. The pathogenesis, diagnosis and treatment of raynaud phenomenon. *Nat Rev Rheumatol* 2012;**8**:469–79
2. Prete M, Fatone MC, Favoino E, Perosa F. Raynaud's phenomenon: from molecular pathogenesis to therapy. *Autoimmun Rev* 2014;**13**:655–67
3. Ingegnoli F, Boracchi P, Gualtierotti R, Lubatti C, Meani L, Zahalkova L, Zeni S, Fantini F. Prognostic model based on nailfold capillaroscopy for identifying Raynaud's phenomenon patients at high risk for the development of a scleroderma spectrum disorder: PRINCE (prognostic index for nailfold capillaroscopic examination). *Arthritis Rheum* 2008;**58**:2174–82
4. Suter LG, Murabito JM, Felson DT, Fraenkel L. The incidence and natural history of Raynaud's phenomenon in the community. *Arthritis Rheum* 2005;**52**:1259–63
5. Katada Y, Tanaka T. Images in clinical medicine. Lingual Raynaud's phenomenon. *N Engl J Med* 2012;**366**:e12
6. Pope JE. The diagnosis and treatment of Raynaud's phenomenon: a practical approach. *Drugs* 2007;**67**:517–25
7. Wigley FM. Clinical practice. Raynaud's phenomenon. *N Engl J Med* 2002;**347**:1001–8
8. Valdovinos ST, Landry GJ. Raynaud syndrome. *Tech Vasc Interv Radiol* 2014;**17**:241–6
9. Maga P, Kuzdzal J, Nizankowski R, Szczeklik A, Sladek K. Long-term effects of thoracic sympathectomy on microcirculation in the hands of patients with primary Raynaud disease. *J Thorac Cardiovasc Surg* 2007;**133**:1428–33
10. Montecucco C, Molgó J. Botulinal neurotoxins: revival of an old killer. *Curr Opin Pharmacol* 2005;**5**:274–9
11. Trindade De Almeida AR, Secco LC, Carruthers A. Handling botulinum toxins: an updated literature review. *Dermatol Surg* 2011;**37**:1553–65
12. Bhidayasiri R, Truong DD. Expanding use of botulinum toxin. *J Neurol Sci* 2005;**235**:1–9
13. Jin L, Kollewe K, Krampfl K, Dengler R, Mohammadi B. Treatment of phantom limb pain with botulinum toxin type A. *Pain Med* 2009;**10**:300–3
14. Zanotti S, Kapetis D, Gibertini S, Salerno F, Ciusani E, Colombo C, Gronchi A, Morandi L, Mantegazza R, Molteni F, Mora M. Botulinum toxin type A affects the transcriptome of cell cultures derived from muscle biopsies of controls and spastic patients. *Toxicol In Vitro* 2018;**50**:124–36
15. Medina S, Gómez-Zubiaur A, Aldeolivas-Casillas N, Polo-Rodríguez I, Ruíz L, Izquierdo C, Guirado C, Cabrera A, Trasobares L. Botulinum toxin type A in the treatment of Raynaud's phenomenon: a three-year follow-up study. *Eur J Rheumatol* 2018;**5**:224–9
16. Cipolla MJ, Sweet JG, Gokina NI, White SL, Nelson MT. Mechanisms of enhanced basal tone of brain parenchymal arterioles during early postischemic reperfusion: role of ET-1-induced peroxynitrite generation. *J Cereb Blood Flow Metab* 2013;**33**:1486–92
17. Gokina NI, Park KM, McElroy-Yaggy K, Osol G. Effects of rho kinase inhibition on cerebral artery myogenic tone and reactivity. *J Appl Physiol* 1985;**98**:1940–8
18. Cipolla MJ, Chan SL, Sweet J, Tavares MJ, Gokina N, Brayden JE. Postischemic reperfusion causes smooth muscle calcium sensitization and vasoconstriction of parenchymal arterioles. *Stroke* 2014;**45**:2425–30
19. Kimura M, Sudhir K, Jones M, Simpson E, Jefferis AM, Chin-Dusting JP. Impaired acetylcholine-induced release of nitric oxide in the aorta of male aromatase-knockout mice: regulation of nitric oxide production by endogenous sex hormones in males. *Circ Res* 2003;**93**:1267–71
20. Motegi S, Yamada K, Toki S, Uchiyama A, Kubota Y, Nakamura T, Ishikawa O. Beneficial effect of botulinum toxin A on Raynaud's phenomenon in Japanese patients with systemic sclerosis: a prospective, case series study. *J Dermatol* 2016;**43**:56–62
21. Jahn R, Fasshauer D. Molecular machines governing exocytosis of synaptic vesicles. *Nature* 2012;**490**:201–7
22. Wheeler A, Smith HS. Botulinum toxins: mechanisms of action, antinociception and clinical applications. *Toxicology* 2013;**306**:124–46
23. Neumeister MW, Chambers CB, Herron MS, Webb K, Wietfeldt J, Gillespie JN, Bueno RA Jr, Cooney CM. Botox therapy for ischemic digits. *Plast Reconstr Surg* 2009;**124**:191–201
24. Neumeister MW. Botulinum toxin type A in the treatment of Raynaud's phenomenon. *J Hand Surg Am* 2010;**35**:2085–92
25. Matic DB, Lee TY, Wells RG, Gan BS. The effects of botulinum toxin type A on muscle blood perfusion and metabolism. *Plast Reconstr Surg* 2007;**120**:1823–33
26. Morris JL, Jobling P, Gibbins IL. Differential inhibition by botulinum neurotoxin a of cotransmitters released from autonomic vasodilator neurons. *Am J Physiol Heart Circ Physiol* 2001;**281**:H2124-32
27. Cui M, Khanijou S, Rubino J, Aoki KR. Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. *Pain* 2004;**107**:125–33
28. Ali DM, Abdelzaher WY, Abdel-Hafez S. Evaluation of the rivastigmine role against botulinum toxin-A-induced osteoporosis in albino rats: a biochemical, histological, and immunohistochemical study. *Hum Exp Toxicol* 2018;**37**:1323–35
29. Bakst R, Merola JF, Franks AG Jr, Sanchez M. Raynaud's phenomenon: pathogenesis and management. *J Am Acad Dermatol* 2008;**59**:633–53
30. Thompson AE, Pope JE. Calcium channel blockers for primary Raynaud's phenomenon: a meta-analysis. *Rheumatology* 2005;**44**:145–50
31. Thompson AE, Shea B, Welch V, Fenlon D, Pope JE. Calcium-channel blockers for Raynaud's phenomenon in systemic sclerosis. *Arthritis Rheum* 2001;**44**:1841–7

32. Raghavan SA, Dikshit M. Vascular regulation by the L-arginine metabolites, nitric oxide and agmatine. *Pharmacol Res* 2004;**49**:397–414
33. Sandoo A, van Zanten JJ, Metsios GS, Carroll D, Kitas GD. The endothelium and its role in regulating vascular tone. *Open Cardiovasc Med J* 2010;**4**:302–12
34. Chang GJ, Lin TP, Ko YS, Lin MS. Endothelium-dependent and -independent vasorelaxation induced by CIJ-3-2F, a novel benzylfuroquinoline with antiarrhythmic action, in rat aorta. *Life Sci* 2010;**86**:869–79
35. Flacco N, Segura V, Perez-Aso M, Estrada S, Seller JF, Jiménez-Altayó F, Noguera MA, D'Ocon P, Vila E, Ivorra MD. Different beta-adrenoceptor subtypes coupling to cAMP or NO/cGMP pathways: implications in the relaxant response of rat conductance and resistance vessels. *Br J Pharmacol* 2013;**169**:413–25
36. Nelson CP, Rainbow RD, Brignell JL, Perry MD, Willets JM, Davies NW, Standen NB, Challiss RA. Principal role of adenylyl cyclase 6 in K(+) channel regulation and vasodilator signalling in vascular smooth muscle cells. *Cardiovasc Res* 2011;**91**:694–702
37. Iorio ML, Masden DL, Higgins JP. Botulinum toxin A treatment of Raynaud's phenomenon: a review. *Semin Arthritis Rheum* 2012;**41**:599–603
38. Romanov A, Pokushalov E, Ponomarev D, Bayramova S, Shabanov V, Losik D, Stenin I, Elesin D, Mikheenko I, Strelnikov A, Sergeevichev D, Kozlov B, Po SS, Steinberg JS. Long-term suppression of atrial fibrillation by botulinum toxin injection into epicardial fat pads in patients undergoing cardiac surgery: three-year follow-up of a randomized study. *Heart Rhythm* 2019;**16**:172–7
39. Chinnapongse R, Gullo K, Nemeth P, Zhang Y, Griggs L. Safety and efficacy of botulinum toxin type B for treatment of sialorrhea in Parkinson's disease: a prospective double-blind trial. *Mov Disord* 2012;**27**:219–26
40. Carroll I, Fischbein N, Barad M, Mackey S. Human response to unintended intrathecal injection of botulinum toxin. *Pain Med* 2011;**12**:1094
41. Tanyeli O, Duman I, Dereli Y, Gormus N, Toy H, Sahin AS. Relaxation matters: comparison of in-vitro vasodilatory role of botulinum toxin-A and papaverine in human radial artery grafts. *J Cardiothorac Surg* 2019;**14**:15

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