Modification of Blood Vessel Diameter Following Perivascular Application of Botulinum Toxin-A

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Abstract The purpose of this study was to demonstrate that perivascularly applied botulinum toxin-A (BTX) increases the diameter of treated blood vessels in a rat femoral vessel exposure model. Six adult Sprague–Dawley rats were used and bilateral femoral artery and vein exposures were performed. Five units of BTX were applied to the experimental side and an equal volume of sterile saline was applied to the control side. Digital images of the vessels were obtained at the following time points: pretreatment, immediately posttreatment, and postoperative days (POD) 1, 14, and 28. Vessel diameters were equivalent at baseline and immediately following application of BTX and saline. The BTX artery was significantly larger than the control artery on POD 1 and 14. The BTX treated artery was significantly larger than all other vessels on POD 14 ($p < 0.05$) as well as all prior time points ($p < 0.01$). Direct perivascular application of BTX increases the diameter of rat femoral vessels as early as POD 1. The

affect is most robust on POD 14 where the artery was significantly larger than all other vessels at all time points. It is likely that the increased diameter of blood vessels results in an increased blood flow across the area of dilation. Such an increase in flow may serve to improve end-organ perfusion in microvascular procedures.

Keywords Botulinum toxin · Vessel · Dilation

Abbreviations

BTX	botulinum toxin-A
POD	postoperative day
SNAP-25	synaptosome-associated protein of 25,000 daltons
Ach	acetylcholine
SNARE	soluble NSF attachment receptor
NE	norepinephrine

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Introduction

Use of botulinum toxin type A (BTX) has increased in clinical practice in the last 20 years, predominately as a cosmetic agent. The substance received Food and Drug Administration (FDA) approval for use in humans in 1989 for the treatment of hemifacial spasm, strabismus, and blepharospasm (Botox Cosmetic, Allergan, package insert). Since that time, use of BTX for FDA-approved conditions and off-label uses has increased dramatically. BTX injection for the reduction of facial wrinkles was the most commonly performed cosmetic procedure in 2003 [3]. Besides its aesthetic uses, and on-label uses previously stated, BTX has found applications in the treatment of anal fissure [9], hyperhydrosis [12], bruxism [13], and bladder

dysfunction [11]. Several new indications have found clinical success in the recent past including treatment of migraine headache, achalasia, various forms of tremor, and tics [8]. There have been several scattered reports of using BTX as a vasodilatory agent [7, 15]. However, there are few if any basic science studies that directly document how BTX affects blood vessels.

The basic mechanism of action of the botulinum toxins (subtypes A–G) has been extensively studied [14]. For the A subtype, the toxin is bound to the presynaptic terminal of the neuromuscular junction and the light chain of the protein is taken up by the terminal. Within the terminal, the toxin itself binds to and cleaves synaptosome-associated protein of 25,000 daltons (SNAP-25), a protein responsible for the docking and subsequent release of acetylcholine (ACh). The failure of release of ACh reduces muscular contraction at the neuromuscular junction, thereby causing a temporary, partial denervation of the target muscle. Only recently has there been an effort to determine if both smooth and striated muscle are affected by BTX.

Experimental use of BTX for novel (i.e., non-cosmetic) uses has increased dramatically in the last 10 years. Early use of BTX in muscle flap surgery relied on chemical denervation to reduce muscular activity of transposed muscle [4]; the viability of the muscle flap was thought to benefit from reduced movement. Morris et al. [10] showed that BTX affects SNARE proteins and limits release of norepinephrine (NE) from the presynaptic terminal, thereby causing a chemical sympathectomy in the treated area. BTX has been shown to dilate the vascular bed of tumors in mice through inhibition of the neurogenic contractions of tumor vessels, thereby improving tumor perfusion and oxygenation [1]. These studies have shown that BTX does indeed have an effect on vascular smooth muscle, albeit through alternative mechanisms in addition to inhibition of ACh release. There now appears to be a consensus that BTX is emerging as a beneficial agent in any structure containing muscle.

The current study attempts to determine if direct perivascular application of BTX alters the diameter of vessels. This preliminary experimental approach to using BTX will form the basis for further studies of how this powerful pharmacologic agent may increase blood flow through segments of treated vessels, thereby improving perfusion of end organs.

Materials and Methods

The experimental protocol was evaluated and accepted by the University of Virginia's Animal Care and Use Committee prior to initiation of the experiments. Six adult Sprague–Dawley rats were used. Animals were anesthetized using

standard inhalational techniques with isoflurane. After satisfactory inhalational anesthesia, the bilateral groins were clipped free of hair at the level of the inguinal ligament. The area was prepped and draped in the usual sterile fashion utilizing betadine and sterile towels. The skin overlying the femoral vessels was injected with 1% lidocaine and the skin incised with a no. 15 scalpel blade. The femoral vessels lie just beneath the dermis in this location. At this point, the femoral vessels (both artery and vein) were exposed bilaterally. The vessels were dissected free from surrounding tissues and from one another; the adventitial layer remained intact, as this serves as the sympathetic innervation for the vascular smooth muscle. A solid-color background material was placed behind the vessels and a micrometer placed in the field. A high-resolution digital image was obtained and stored. BTX (Allergan Corp.) was reconstituted according to the manufacturer's recommendations (2 ml sterile saline added to vial for final concentration of 10 U/0.2 cc) and stored at 4°C prior to use. BTX was obtained from our hospital's pharmacy and was reconstituted as it is recommended for cosmetic use. Five units of the reconstituted BTX was applied directly to both the artery and the vein. The choice to use 5 U of BTX was decided upon based on an assumption of presumed effect; as this was our initial study with using BTX for manipulation of vessel diameter, there were no prior dose–response curve experiments.

Application of the agent was limited to a small segment of the vessels, and this area was marked with proximal and distal 8–0 suture material to the surrounding soft tissue to facilitate identification of the treated areas during subsequent evaluations. An equal volume of sterile saline was applied in a similar manner to the contralateral side and this location marked with sutures. The vessels were imaged once again before skin closure to determine if there was any immediate effect of the topical agents. BTX and saline exposure time was 3 min, after which the skin was closed using subcuticular 5–0 nylon. The overlying skin was covered with a surgical cyanoacrylate glue and the animal allowed to recover.

At days 1, 14, and 28, the animals were anesthetized with isoflurane and the groin incisions reopened. Identification of the manipulated areas was accomplished by visualizing the previously placed suture material. After placement of a background material behind the vessels, a digital photograph was taken and stored. Note that a micrometer was placed in the field to allow for calibration during the analysis of the data. The skin was once again closed following imaging on days 1, 7, and 14. Following acquisition of the digital images on day 28, the animals were given a lethal dose of pentobarbital via intraperitoneal route.

Animals were monitored using standard postsurgical techniques which included full recovery in our laboratory which is staffed by a full-time veterinary technician. Pain

control was achieved with subcutaneous Butorphanol at the appropriate dose. Once the animals recovered, they were returned to the vivarium and followed every 8 h or more frequently if necessary.

Comparison of vessel diameter in vivo was made at each time point. The digital images acquired before application of the solutions were used as a baseline on both the treated and untreated sides. The images were imported into an image analysis program (Adobe Photoshop, Adobe Systems, Inc.). Calibration of the measurement tool was made by measuring a small segment of the micrometer placed into the field of each acquired image. This allowed for accurate measurements within each of the images. Images acquired at days 1, 14, and 28 were compared to the original images; the contralateral sides served as an internal control across the entire study. A two-tailed Student's *t* test with Bonferroni post hoc analyses was performed.

Results

All animals survived throughout the duration of the study, and there was no obvious muscular paralysis of the abdominal wall or lower extremities. Additionally, there were no wound healing difficulties in any of the animals.

At all analysis time points, there was no indication that any of the vessels were clotted or otherwise damaged. Although

Figure 1 Comparison of pretreatment artery and BTX-treated artery on day 14. Increase in artery diameter 14 days after treatment with BTX. The vessel shown in **a** and **b** is the same vessel with equivalent scales in each image. Arrows indicate location of marking sutures in **b**.

Figure 2 Comparison of saline- (**a**) and BTX-treated (**b**) arteries on day 14. Artery diameter is significantly increased in the BTX-treated artery. Scales in the two images are equivalent.

there were no quantitative evaluations of flow in these vessels, gentle manipulation of all arteries and veins demonstrated adequate flow. Representative images of the treated arteries are shown in Figs. 1 and 2. Note that the micrometer is visible in each image, which served to calibrate the measuring device in the image analysis software. The vessel imaged in Fig. 1a, b is the same vessel; note the suture material placed proximally and distally to the vessel segment as indicated by the arrows (Fig. 1). There is a clear increase in vessel diameter in this example. BTX-treated arteries were compared to saline-treated vessels within the same animal (Fig. 2). Note again that the BTX-treated artery is clearly larger than the saline-treated artery in this example.

The mean and standard deviation of each time point were calculated for statistical comparison within and between each group (Table 1). There was an overall trend of arterial dilation in the BTX-treated group from baseline through day 14 with a decrease in arterial diameter at day 28 (Fig. 3). Maximal arterial dilation in the BTX-treated group occurred on day 14 with a mean arterial diameter of 0.837 mm, which was significantly different from the day 14 control artery group ($p < 0.05$). Additionally, the BTX-treated arteries at day 14 were significantly larger ($p < 0.01$) compared to all previous time points.

There was an overall trend towards dilation of the saline-treated arteries (Fig. 3). There was a slight insignificant decrease in artery diameter on postoperative days (POD) 1, with subsequent increase in mean diameter on days 14 and

Table 1 Average diameters at various time points for BTX- and saline-treated vessels.

	Baseline	Day 0	Day 1	Day 14	Day 28
Artery BTX	0.516±0.091	0.536±0.046	0.627±0.058	0.837±0.122	0.706±0.085
Artery control	0.485±0.141	0.535±0.096	0.500±0.105	0.610±0.112	0.651±0.142
Vein BTX	0.529±0.109	0.598±0.114	0.636±0.106	0.727±0.048	0.622±0.137
Vein control	0.533±0.056	0.546±0.117	0.590±0.106	0.691±0.169	0.510±0.122

Average vessel diameters (mm±SD) at baseline and POD 0, 1, 14, and 28 for BTX- and saline-treated groups
BTX botulinum toxin-A

28. There were no significant differences between the saline-treated arteries at the various time points.

Venous diameters in both BTX- and saline-treated groups increased from baseline through day 14 with a decrease in diameter on day 28 (Fig. 4). The BTX-treated veins were larger than the saline-treated veins at each point of analysis. Only the day 28 BTX-treated vein was significantly larger than the saline-treated vein ($p<0.01$).

Discussion

The current study has shown a clear difference in vessel diameter in rat femoral vessels treated with BTX and saline, with the BTX-treated vessels being significantly larger than the saline-treated vessels in many cases. It stands to reason that an increase in vessel diameter would result in an increase in blood flow through the dilated segment related

to Poiseuille's law, given by the equation $Q=(\Delta P)(r^4)/nL$, where Q is the rate of blood flow in a vessel, ΔP is the difference of pressure between the ends of the vessel, r is the vessel radius, n is the viscosity of blood, and L is the length of the vessel. It should be noted that the calculation of flow through small diameter vessels using Poiseuille's equation tends to break down due to the variability of blood viscosity and the elasticity of the vessel walls [2]. Nonetheless, application of this equation to small vessels remains a reasonable way to estimate flow.

The effect of BTX on striated and smooth muscle has been extensively studied as it relates to inhibition of ACh release [5, 6, 8, 15]. As would be expected of such a powerful neuromodulating/neuroinhibitory substance, there are many additional effects BTX exerts on both the autonomic and adrenergic nerves related to maintenance of vascular tone. Morris and colleagues have shown that BTX prevents vasoconstriction of vascular smooth muscle of

Figure 3 Average diameter of BTX- vs. saline-treated arteries. There is a trend towards dilation of both treatment groups beginning on day 0 to 1, with a maximal dilation on day 14. Statistical significance was found on day 14 between the BTX-treated arteries compared to saline-treated arteries ($p<0.05$) as well as between day 14 BTX-treated arteries compared to all previous time points of BTX-treated arteries ($p<0.01$). Vessel diameters decreased for both groups on day 28.

Figure 4 Average diameter of BTX- vs. saline-treated veins. A trend towards dilation of both treatment groups was seen beginning on day 1 with a maximal dilation seen on day 14. There were no significant differences identified between the two treatment groups. Again, diameters decreased on day 28.

guinea pig uterine arteries at the level of the neuromuscular junction by blocking NE release [11]. Interestingly, this inhibition of release was only seen with low-frequency electrical stimulation (1 Hz) and was seen to a much lesser degree with higher frequency stimulation (10 Hz).

In their recent study, Kim et al. [7] showed that injection of BTX to the proximal one third of a random cutaneous flap in the rat translated to a 8.3% increase in flap survival over a saline control group after a period of 7 days. Additionally, they showed that the diameter of vessels is larger and the number of immature vessels is greater in the BTX-treated group using histologic analysis techniques. This group hypothesizes that the mechanism of action of BTX affects the autonomic nervous system through suppression of sympathetic neurons in the dermal plexus which would result in vasodilation and an increase in blood volume and pressure. Using reverse transcriptase polymerase chain reaction techniques, they also showed an increase in endothelial cell markers vascular endothelial growth factor and CD31 in the BTX-treated group. These cell markers underlie endothelial proliferation and serve to increase the number of immature vessels in the flap.

Although the model used in the study of Kim et al. was substantially different from our model, the two studies complement each other in an attempt to understand the mechanism underlying vascular dilation seen with BTX application. There appears to be a clear increase in vessel dilation following direct application of BTX, and this effect persists for several days to weeks. Kim et al. showed a

maximal flap survival benefit (presumably from vascular dilation) at 7 days following application; there was no additional benefit seen beyond 7 days in their study. Our data show that maximal dilation occurs 14 days after application of BTX, and although we did not measure vessel diameter on day 7, it is reasonable to assume that both arteries and veins would continue their trend of dilation at this time point. The mechanism underlying this dilation may involve both a direct smooth muscle effect via paralysis of smooth muscle through inhibition of ACh release and a loss of effective sympathetic input. We hypothesize that perivascular application of BTX directly to the major vessels containing smooth muscle, supplying a distinct angiosome, may provide both smooth muscle relaxation and inhibition of sympathetic input. Further actions of BTX through additional unknown pathways cannot be excluded and in fact may be likely considering the widespread effect of BTX on multiple systems.

Results from this study show that both arteries and veins become dilated beginning on POD 1, are maximal at POD 14, and begin to return to baseline by POD 28 for both BTX- and saline-treated groups. Statistical comparisons of these groups showed that the BTX-treated group was significantly larger when compared to the saline-treated group. The increase in vessel diameter in the saline-treated group may be due to several different factors including manipulation of the vessels, local inflammatory factors from the surgery, and interruption of the sympathetic input to the vessels during dissection of the arteries from the

veins as well as from the surrounding tissues. Our data support the concept that application of BTX provides as sustained dilatatory effect on the vessels compared to saline-treated controls over the measured time period.

The current study represents our initial efforts into elucidating the mechanism of BTX on vascular smooth muscle. Using a rat ischemic island pedicle island flap model, we are currently investigating if perivascular application of BTX to the dominant pedicle increases the area of flap survival. Further investigation is needed to fully understand the time course of the effect of BTX on these flaps in an attempt to provide a maximal benefit for flap survival. We also are planning a dose–response curve as our choice of initial dose was empiric.

Conclusion

Review of the studies related to vessel dilation with BTX in addition to the current study reveals that the use of BTX in reconstructive and microvascular procedures remains promising. Although the precise mechanism of action of BTX on blood vessels and smooth muscle has yet to be completely elucidated, evidence to support the use of BTX in reconstructive procedures is emerging. Increasing arterial and venous flow through pedicled cutaneous flaps may offer a direct survival benefit to these flaps. An increase of flow may allow for larger length-to-width ratios when designing random flaps for wound coverage as well as providing a survival benefit to the distal-most aspect of pedicled flaps. Further experimental and clinical studies are needed to determine the dosages and limits of BTX application and how its use may afford the reconstructive surgeon an additional modality when treating patients requiring complex wound coverage.

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