Role of botulinum neurotoxin–A in cerebral revascularization graft vasospasm prevention: current state of knowledge

Kristine Ravina, MD,1 Ben A. Strickland, MD,2 Robert C. Rennert, MD,4 Joseph N. Carey, MD,3 and Jonathan J. Russin, MD1,2

1Neurorestoration Center, 2Department of Neurological Surgery, and 4Division of Plastic and Reconstructive Surgery, Department of Surgery, Keck School of Medicine, University of Southern California, Los Angeles; and 3Department of Neurosurgery, University of California, San Diego, California

Graft stenosis and occlusion remain formidable complications in cerebral revascularization procedures, which can lead to significant morbidity and mortality. Graft vasospasm can result in early postoperative graft stenosis and occlusion and is believed to be at least partially mediated through adrenergic pathways. Despite various published treatment protocols, there is no single effective spasmolytic agent. Multiple factors, including anatomical and physiological variability in revascularization conduits, patient age, and comorbidities, have been associated with graft vasospasm pathogenesis and response to spasmodics. The ideal spasmodic agent thus likely needs to target multiple pathways to exert a generalizable therapeutic effect. Botulinum toxin (BTX)–A is a powerful neurotoxin widely used in clinical practice for the treatment of a variety of spastic conditions. Although its commonly described paradigm of cholinergic neural transmission blockade has been widely accepted, evidence for other mechanisms of action including inhibition of adrenergic transmission have been described in animal studies. Recently, the first pilot study demonstrating clinical use of BTX-A for cerebral revascularization graft spasm prevention has been reported. In this review, the mechanistic basis and potential future clinical role of BTX-A in graft vasospasm prevention is discussed.

https://thejns.org/doi/abs/10.3171/2018.11.FOCUS18514

KEYWORDS botulinum toxin; EC-IC bypass; graft patency; graft occlusion; revascularization; vasospasm

EARLY graft occlusion after cerebral revascularization occurs in approximately 5%–10% of cases despite advancements in surgical technique and pharmacological treatments.41,42,53 Although rare, graft vasospasm remains a formidable and potentially lethal complication that affects multiple surgical specialties,28 including plastic and reconstructive surgery, cardiac surgery, and neurosurgery. Patency and spasm rates vary by graft type, with arterial grafts currently preferred for revascularization procedures over venous grafts due to their lower risk of developing intimal hyperplasia and atherosclerosis, and greater long-term patency rates.11 Nevertheless, long-term failure of arterial grafts still occurs in 2%–18% of cases, and arterial conduits have a significantly greater risk of vasospasm. Whether from occlusions or flow-limiting vasospasm, perfusion deficits in downstream territories from graft compromise can have devastating clinical consequences.17,25,38

Despite extensive study to date, the exact cause of graft vasospasm remains unclear, and there is no single effective spasmodic agent for preventing graft vasospasm.27,55 While complex pharmacological antispastic protocols have been proposed in coronary bypass grafting (often involving systemic vasodilators), their use in cerebrovascular bypass is generally limited by the need to maintain cerebral perfusion in these patients.31

Botulinum toxin (BTX) is a potent neurotoxin widely used for the treatment of a variety of spastic and hyperactive autonomic conditions.12,33 The potent spasmodic properties of BTX have sparked interest in its possible application for the prevention of graft spasm. In this review, we examine the current state of knowledge regarding BTX.

ABBREVIATIONS BTX = botulinum toxin; CGRP = calcitonin gene-related peptide; eNOS = endothelial nitric oxide synthase; KCl = potassium chloride; MLC = myosin light chain; MLCP = MLC phosphatase; MYPT1 = myosin phosphatase targeting subunit 1; NE = norepinephrine; NO = nitric oxide; RhoA = ras homolog gene family member A; ROCK = rho-associated protein kinase; SNAP = synaptosomal-associated protein; VEGF = vascular endothelial growth factor; VSMC = vascular smooth-muscle cell.


INCLUDE WHEN CITING DOI: 10.3171/2018.11.FOCUS18514.
as it relates to graft vasospasm prevention, with a focus on revascularization conduit anatomy, physiology, and vasomotor mechanistic pathways.

**Background**

**Graft Vasospasm and Current Treatment Options**

Multiple factors affect vessel wall contractility and must be considered during graft selection and evaluation/optimization of potential spasmolytic agents. In addition to physical and chemical stimuli, vessel contractility is determined by anatomical features such as wall structure and the density and composition of receptors (Fig. 1), both of which can be affected by patient age and comorbidities such as atherosclerosis, hypertension, and diabetes.25,28,30,35,58 Although it is known that dynamic changes in blood vessel diameter are mediated by vascular smooth-muscle cells (VSMCs) and vessel elasticity is determined by the amount of elastic laminae in the vessel wall.24,25 Less is known about vasomotor receptor composition, which is only described in select arteries and may vary within different segments of the same artery.24,25,29,55 Despite their higher contractility, the common utilization of limb arteries in cerebrovascular bypass due to their ease of access and harvest makes the development of effective antispasmodic treatments critical.24,52

The extensive research on coronary artery bypass graft spasm possesses mixed relevance for cerebral revascularization.24,27,29,55 Given the multifaceted nature of vasospasm, variability of revascularization conduits, and pharmacological agents each targeting different pathways, a common conclusion of this work has been that there is no single ideal spasmolytic agent. Hence, complex spasmolytic protocols with combinations of different vasodilators applied pre-, intra-, and postoperatively, including both systemic and topical applications, have been developed. Besides emphasizing the importance of atraumatic graft harvest, these protocols typically contain papaverine or calcium channel antagonists (e.g., verapamil or nicardipine) and a nitrate (e.g., nitroglycerin).24,27 The utility of these protocols, which emphasize systemic vasodilator therapy, is limited in cerebrovascular bypass patients given the need for blood pressure/cerebral perfusion maintenance in the postoperative period.31,60 Additionally, the effects of intraoperative preventive and postoperative salvage vasodilators such as verapamil or papaverine are typically short-lived, and may require repeat interventions with increasing complication risks.7,19,37 Addressing these limitations, the ideal therapeutic agent would be safe, long-lasting, and quickly and easily applied intraoperatively for spasm prevention rather than treatment.

**BTX-A as a Spasmolytic Agent: Classic Concepts**

BTXs are potent biological toxins produced by anaerobic, gram-positive spore-forming bacteria from the *Clostridium* genus. There are 7 well-known, serotypically different BTXs (A–G) produced by 6 distinct groups

---

**FIG. 1.** Factors involved in vasoreactivity. Vasoreactivity is determined not only by external/systemic factors (presence of physical stimuli and vasoactive substances), but also by intrinsic anatomical and physiological contractile properties of blood vessels as dictated by the density and composition of receptors and vessel wall structure.
of clostridia. The primary BTX mechanism of action is cholinergic neuromuscular transmission blockade through inhibition of acetylcholine from nerve terminals. Specifically, BTX binds to a polysialoganglioside receptor on the presynaptic membrane and enters the nerve terminal via receptor-mediated endocytosis. The light chain of the BTX is a metalloproteinase that then cleaves one or more proteins from the soluble N-methylmaleimide sensitive factor attachment protein receptor complex that mediates synaptic vesicle exocytosis. BTX-A, -D, -F, and -G cleave vesicle-associated membrane protein, while BTX-A and -E cleave synaptosomal-associated protein–25 (SNAP-25), and BTX-C cleaves SNAP-25 and syntaxin. Although this BTX-mediated cholinergic neural transmission blockade is irreversible, the duration of BTX paralytic activity varies significantly depending on toxin type, dose, species, and type of cholinergic nerve terminals targeted (autonomic or skeletal muscle). BTX-A, one of the most commonly used subtypes, induces skeletal muscle paralysis that can be detected 2–3 days after injection and typically reaches its maximum at 1–2 weeks. This effect is gradually lost over time as nerve terminals are remodeled, with complete restoration of neural transmission to baseline levels in 3–4 months.

Currently, BTX is FDA approved for a very limited number of spas tic conditions, with the majority of the more than 50 reported therapeutic applications being unlabeled, including hemifacial spasm, hyperhidrosis, headache, urinary incontinence, and Raynaud’s phenomenon.

**BTX-A Utility in Vasospasm Prevention: Relevant Animal Studies**

To date, BTX use has been reported in 6 animal studies evaluating its effects on microvascular anastomosis patency, vessel diameter, and response to vasospastic challenge on in situ blood vessels or grafts (Table 1). In these studies, rat (n = 5/6) and rabbit (n = 1/6) models were used to evaluate various BTX types (n = 3 BTX-A, n = 2 BTX-B, and n = 1 BTX-C), doses (1–1500 IU), application methods (injection, direct application to the vessels in situ or in a tissue bath), and target vessels (femoral, aortic, and posterior auricular) with variable follow-up (30 minutes to 28 days). BTX efficacy was reported in all of these studies, demonstrating its utility for vasospasm prevention.

Specifically, BTX improved microvascular anastomosis patency rates and counteracted vasospastic challenges from phenylephrine, potassium chloride (KCl), nor epinephrine (NE), and cold. With these effects detectable as early as 30 minutes after treatment, the spasmolytic potential of BTX in these studies was shown to be dependent on both dose and application time.

Multiple reports on the effects of BTX in animal models of free and pedicled cutaneous, myocutaneous, and muscular flaps also support a potentially multifaceted mechanism of action. We identified 15 animal studies to date where the effects of BTX-A (n = 14) and BTX-B (n = 1) on flap viability, vasodilation, blood flow, angiogenesis, and inflammation were evaluated. In these studies, a rat model was most commonly used (n = 14/15) and there was significant variability in BTX dose (0.1–20 IU), application method (subdermal, intradermal, subcutaneous, intramuscular or perivascular injection, direct application to the vessels, or tissue bath), treated flaps (random cutaneous, abdominal or dorsal cutaneous, transverse rectus abdominis myocutaneous, cremaster, spinotrapezius, or gastrocnemius muscular), and evaluation time points (5 minutes to 21 days). Better flap survival rates and increased angiogenesis and angiogenic marker expression were observed in the BTX treatment groups as compared to controls. Interestingly, BTX-A also improved random cutaneous flap survival in rats after short- and long-term tobacco exposure, demonstrating its potential efficacy for the prevention of reconstructive and revascularization surgery complications in smokers.

Although these animal studies provide valuable insights into the potential benefit of BTX in revascularization surgery, marked differences in innervation and response to spasmogens have been demonstrated between human and animal blood vessels. The first translational application of BTX-A for cerebrovascular bypass graft spasm prevention in humans was only recently published.

**Potential Mechanisms of BTX A–Mediated Vasospasm Prevention**

**BTX and Adrenergic Vascular Innervation**

Understanding the spasmolytic effects of BTX requires a mechanistic understanding of vasoconstriction. Postganglionic sympathetic axons form a plexus at the adventitia-media border in most arteries, arterioles, and veins across animal species, including humans. Nor epinephrine (NE), produced in sympathetic nerves, is released from perivascular axon varicosities after sympathetic stimulation and binds to VSMC postsynaptic α and β adrenergic receptors. NE-induced VSMC contraction in most major arteries is thought to be primarily mediated by postsynaptic α adrenergic receptor activation, with variable contribution from α receptors (Fig. 2). Although the relative contributions of α and α adrenergic receptors to vasoconstriction depends on the anatomical location and segment of the vessel. In fact, in limb arteries the α adrenergic role in vasoconstriction increases when moving proximal to distal, a finding that, when combined with the predisposition of the distal end of grafts to vaso spasm, suggests a potentially important role of α receptors in this process.

The affinity of BTX for cholinergic transmission blockade has been found to be related to the abundance of high-affinity acceptor sites on cholinergic neurons. Although noncholinergic nerve terminals have a relatively low number of these acceptor sites, BTX can still be taken up via low-affinity sites if its concentration is high enough. Moreover, while BTX-A cleavable SNAP-25 has been found in sympathetic axons innervating guinea pig blood vessels, NE-mediated vascular constriction was shown to be reduced after BTX-A administration with only partial cleavage of SNAP-25. In light of these findings, it was
<table>
<thead>
<tr>
<th>Authors &amp; Year</th>
<th>Species</th>
<th>BTX Application</th>
<th>Blood Vessels</th>
<th>Tx Time</th>
<th>Vasomotor Challenge</th>
<th>Time Points</th>
<th>Research Methods</th>
<th>Vasomotor Factors Evaluated</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clemens et al., 2009</td>
<td>Rat A</td>
<td>10 IU/0.5 ml in perivascular space</td>
<td>Femoral</td>
<td>5 days preop</td>
<td>Phenylephrine &amp; LE cold exposure</td>
<td>Vessel anast 5 days post-Tx, challenge after anast, anast eval 0 &amp; 1 hr after challenge</td>
<td>Visual insp &amp; vessel diam measurements</td>
<td>None</td>
<td>Patency maintained in 100% of vessels pretreated w/ BTX-A &amp; only 44% pretreated w/ saline, sig shorter anast time &amp; ease in BTX-A group vs saline</td>
</tr>
<tr>
<td>Murakami et al., 2009</td>
<td>Rat C</td>
<td>1, 2.5, 5, 10, 20 IU/ml tissue bath</td>
<td>Aortic rings</td>
<td>30 mins</td>
<td>KCI &amp; NE</td>
<td>Isometric contractile responses measured post-Tx</td>
<td>Isometric contractile response measurement using myograph, hist exam using Elastica van Gieson stain</td>
<td>None</td>
<td>Complete inhibition of KCl-induced graft spasm w/ 5 IU/ml BTX-C Tx; dose-dependent NE-induced graft spasm prevention by BTX-C w/ max effect at 5 IU/ml; BTX-C effects on NE exposure longer lasting than w/ papaverine</td>
</tr>
<tr>
<td>Arnold et al., 2009</td>
<td>Rat A</td>
<td>5 IU direct application to vessels</td>
<td>Femoral</td>
<td>3 mins before skin closure</td>
<td>None</td>
<td>POD 1, 7, 14, &amp; 28</td>
<td>Visual insp &amp; vessel diam measurements</td>
<td>None</td>
<td>Max arterial diam after BTX Tx on POD 14, BTX-treated artery sig larger than cntrl on POD 14, BTX-treated vein sig larger than cntrl on POD 28</td>
</tr>
<tr>
<td>Fathi et al., 2010</td>
<td>Rabbit A</td>
<td>20 IU/ml subcutaneous injection around vessels</td>
<td>Pst auricular arteries &amp; marginal veins</td>
<td>7 days before vessel dissection</td>
<td>Cold exposure</td>
<td>Vessel measurement immediately after dissection, vessels then divided &amp; re-anastomosed, exposed to cold challenge &amp; vessel patency evaluated 1 hr later</td>
<td>Visual insp &amp; vessel diam measurements</td>
<td>None</td>
<td>Artery &amp; vein diams sig larger &amp; patency rate higher in BTX-A pre-Tx group</td>
</tr>
<tr>
<td>Janz et al., 2011</td>
<td>Rat B</td>
<td>100 IU anast tissue bath &amp; direct injection in perivascular region 2 cm proximal to anast</td>
<td>Femoral</td>
<td>Immediately after anast</td>
<td>Phenylephrine &amp; LE cold exposure</td>
<td>Challenge at 12, 24, 48, 72, &amp; 120 hrs after adventitia stripping, anast &amp; Tx w/ subsequent patency eval</td>
<td>Visual insp &amp; vessel diam measurements</td>
<td>None</td>
<td>Vessel thrombosis rate sig lower in BTX-B group at all time points except 120 hrs, when there were no thrombotic events; BTX-B produced sig greater ↑ in vessel diam vs cntrl</td>
</tr>
<tr>
<td>Park et al., 2014</td>
<td>Rat B</td>
<td>1500 IU/0.3 ml perivascular injection</td>
<td>Femoral</td>
<td>3 days before vessel dissection</td>
<td>None</td>
<td>Vessel diams &amp; blood flow measured 3 days after injection on dissection &amp; after re-anast of vessels</td>
<td>Vessel diam &amp; blood flow measurements using laser Doppler flowmetry</td>
<td>None</td>
<td>Vein &amp; artery diam &amp; peak blood flow velocity sig ↑ in BTX-B vs cntrls</td>
</tr>
</tbody>
</table>

Anast = anastomosis; cntrl = control; diam = diameter; eval = evaluation; hist = histological; inject = injection; insp = inspection; LE = lower extremity; POD = postoperative day; pst = posterior; sig = significantly; subcut = subcutaneous; Tx = treatment; ↑ = increase(d).
TABLE 2. Animal studies investigating BTX effects on free and pedicled flaps

<table>
<thead>
<tr>
<th>Authors &amp; Year</th>
<th>Species</th>
<th>Type</th>
<th>Dose</th>
<th>Application</th>
<th>Flap</th>
<th>Tx Time</th>
<th>Vasomotor Factors Evaluated</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kim et al., 2009</td>
<td>Rat</td>
<td>A</td>
<td>1.5 IU/0.05 ml</td>
<td>Single intradermal injection in central portion of proximal 3rd of flap after its elevation</td>
<td>Random cutaneous flap</td>
<td>On flap elevation</td>
<td>Flap evaluated on POD 1, 3, 5, &amp; 7</td>
<td>VEGF, iNOS</td>
</tr>
<tr>
<td>Arnold et al., 2009</td>
<td>Rat</td>
<td>A</td>
<td>NR</td>
<td>Direct Tx to vessels for several mins before closure</td>
<td>Ventral pedicled island cutaneous flap, inferior epigastric vessels</td>
<td>On flap elevation</td>
<td>None</td>
<td>Visual inap &amp; percentage area of flap necrosis calculations</td>
</tr>
<tr>
<td>Stone et al., 2012</td>
<td>Rat</td>
<td>A</td>
<td>4, 6, &amp; 10 IU</td>
<td>Tissue bath</td>
<td>Cremaster muscle microvasculature</td>
<td>None; sympathetic contribution evaluated by adding α-adrenergic blockers prazosin &amp; rauwolscine; phenylephrine used to evaluate successful adrenergic blockade</td>
<td>Vessel diams measured immediately after BTX-A Tx &amp; every 5 mins up to 20 mins post-Tx; sympathetic contribution evaluated after adding pharmacologic agents &amp; evaluating diam every 5 mins up to 20 mins</td>
<td>Microvascular measurements using live video microscopy, direct systemic arterial pressure measurements using femoral catheter</td>
</tr>
</tbody>
</table>

CONTINUED ON PAGE 6 »
### TABLE 2. Animal studies investigating BTX effects on free and pedicled flaps

<table>
<thead>
<tr>
<th>Authors &amp; Year</th>
<th>Species</th>
<th>BTX</th>
<th>Application</th>
<th>flap</th>
<th>Tx Time</th>
<th>Vasospastic Challenge</th>
<th>Time Points</th>
<th>Research Methods</th>
<th>Vasomotor Factors Evaluated</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schweizer et al., 2013</td>
<td>Mouse A &amp; B</td>
<td>0.1 IU &amp; 0.5 IU</td>
<td>Subcut injection near vascular pedicle</td>
<td>Axially perfused dorsal skin flap</td>
<td>Preconditioning group, 24 hrs before op; Tx group, intraoperatively</td>
<td>None</td>
<td>POD 1, 3, &amp; 5 blood flow &amp; tissue oxygenation measurements; microdialysis performed on POD 1 &amp; development of tissue necrosis evaluated on POD 5</td>
<td>Blood flow measurements using laser Doppler flowmetry; tissue partial O$_2$ tension measured using microprobes; microdialysis measurements of tissue metabolism (glucose, lactate/pyruvate ratio, glycerol); tissue necrosis evaluation using laser Doppler camera; immunofluorescent IHC expression of TUNEL, eNOS, &amp; RhoA</td>
<td>eNOS, RhoA</td>
<td>BTX Tx groups had sig ↑ blood flow vs cntrl; sig improved tissue oxygenation, higher glucose &amp; lower lactate levels were observed in BTX Tx groups vs cntrl; flap viability was sig ↑ in BTX groups as were levels of eNOS &amp; RhoA immunoexpression; no difference between BTX types &amp; application time</td>
</tr>
<tr>
<td>Akcal et al., 2013</td>
<td>Rat</td>
<td>A</td>
<td>3.5 IU IM inject in midportion of flap, perivascular inject in connective tissue around vessels</td>
<td>Gastrocnemius muscle flap</td>
<td>Pre-Tx 7 days before flap elevation &amp; global flap ischemia induction then 7-day reperfusion period</td>
<td>None</td>
<td>7 days post-reperfusion, 14 days post-Tx</td>
<td>Macro- &amp; microscopic eval of angiogenesis, amount of lymphocyte infiltration, edema, &amp; myocyte damage; IHC eval of CGRP, FLT-4, VEGF, &amp; substance P expression</td>
<td>VEGF, substance P</td>
<td>Degree of muscle flap injury not sig different among Tx groups; sig ↑ no. of fibroblasts in BTX-A Tx groups; no sig general differences in CGRP, VEGF, &amp; substance P immunoexpression among the groups; CGRPR1 expression marked in thick-walled vessels of BTX-A groups; VEGF &amp; substance P expression marked in thin-walled vessels of BTX-A groups; perivascular BTX-A application showed less necrosis, inflammation, &amp; edema than IM application group</td>
</tr>
<tr>
<td>Arnold et al., 2014</td>
<td>Rat</td>
<td>A</td>
<td>2 IU/ml, 1 ml Inject in soft tissue around base of pedicle</td>
<td>Pedicled ab flap elevated on superficial inferior epigastric vessels</td>
<td>On flap elevation</td>
<td>None</td>
<td>POD 1, 2, &amp; 7 mRNA expression of TNFα, IL-1, &amp; VEGF using RT-PCR</td>
<td>VEGF</td>
<td>↓ IL-1 mRNA expression on POD 2; ↓ TNFα mRNA expression on POD 2 &amp; 7</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 2. Animal studies investigating BTX effects on free and pedicled flaps

<table>
<thead>
<tr>
<th>Authors &amp; Year</th>
<th>BTX Type</th>
<th>Species</th>
<th>Dose</th>
<th>Application</th>
<th>Flap Type</th>
<th>Tx Time</th>
<th>Vasospastic Challenge</th>
<th>Time Points</th>
<th>Research Methods</th>
<th>Vasmotor Factors Evaluated</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kucukkaya et al., 2014</td>
<td>A</td>
<td>Rat</td>
<td>0.5 IU</td>
<td>Inject in open wound &amp; graft area</td>
<td>Dorsal cutaneous flap</td>
<td>On flap elevation</td>
<td>None</td>
<td>Graft area evaluated on POD 5, 15, 30, &amp; 60</td>
<td>Macro- &amp; microscopic exam using simple stains</td>
<td>None</td>
<td>Sig ↑ in neovascularization &amp; fibroblast density, ↓ in no. of hair follicles, sweat, &amp; sebaceous glands in BTX-A group vs cntrls; nonsig ↑ in mononuclear cell infiltration in BTX-A group vs cntrls; lesser degree of wound contraction observed in BTX-A vs cntrls</td>
</tr>
<tr>
<td>Karayel et al., 2015</td>
<td>A</td>
<td>Rat</td>
<td>2 IU</td>
<td>Administration in center of proximal 3rd of flap</td>
<td>Random cutaneous flap</td>
<td>1 wk before flap elevation</td>
<td>Cigarette smoke</td>
<td>Flap evaluated for viability daily for 14 days; hist eval on POD 7</td>
<td>Clinical necrotic area eval &amp; measurements, hist exam using H&amp;E stain</td>
<td>None</td>
<td>Sig ↓ necrotic area in BTX-A Tx group undergoing tobacco exposure along w/ ↑ angiogenesis &amp; reduced epithelial damage</td>
</tr>
<tr>
<td>Park et al., 2015</td>
<td>A</td>
<td>Rat</td>
<td>20 IU</td>
<td>Subdermal injects distributed evenly through flap</td>
<td>Transverse rectus abdominis myocutaneous flap</td>
<td>5 days after initial midline incision, 5 days before flap elevation</td>
<td>None</td>
<td>Flap survival evaluated on POD 0, 1, 3, &amp; 5</td>
<td>VEGF mRNA expression level detection using RT-PCR</td>
<td>VEGF</td>
<td>BTX-A group showed sig improved flap survival, larger artery &amp; vein lumen area, higher CD31 immunoeexpression in certain flap areas, lower CD31 &amp; higher VEGF mRNA expression</td>
</tr>
<tr>
<td>Camargo et al., 2016</td>
<td>A</td>
<td>Rat</td>
<td>20 IU</td>
<td>Multiple intradermal injects to cover flap area</td>
<td>Random dorsal cutaneous flap</td>
<td>7 days before flap elevation</td>
<td>Cigarette smoke</td>
<td>2 &amp; 4 mos tobacco exposure; eval on POD 7</td>
<td>Necrotic flap area measurements, carboxyhemoglobin measurement in blood samples</td>
<td>None</td>
<td>BTX-A ↑ random flap viability in tobacco-exposed rats at 2 &amp; 4 mos</td>
</tr>
<tr>
<td>Ghanbarzadeh et al., 2016</td>
<td>A</td>
<td>Rat</td>
<td>24 IU/kg</td>
<td>Intradermal injects distributed throughout flap</td>
<td>Random dorsal cutaneous flap</td>
<td>2 wks before flap elevation</td>
<td>None</td>
<td>Necrosis evaluated on POD 7</td>
<td>Necrotic flap area measurements</td>
<td>None</td>
<td>BTX-A Tx sig reduced distal flap necrosis vs cntrls &amp; topical nitroglycerine application groups</td>
</tr>
</tbody>
</table>

CONTINUED ON PAGE 8
# TABLE 2. Animal studies investigating BTX effects on free and pedicled flaps

<table>
<thead>
<tr>
<th>Authors &amp; Year</th>
<th>Species</th>
<th>BTX Type</th>
<th>Dose</th>
<th>Application</th>
<th>Flap</th>
<th>Tx Time</th>
<th>Vasospastic Challenge</th>
<th>Time Points</th>
<th>Research Methods</th>
<th>Vasomotor Factors Evaluated</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Park et al., 2016</td>
<td>Rat</td>
<td>A</td>
<td>10 IU</td>
<td>Subdermal inject</td>
<td>Transverse rectus abdominis myocutaneous flap</td>
<td>5 days before flap elevation</td>
<td>None</td>
<td>Flap survival area evaluated on POD 5, gene expression analysis evaluated on POD 1, 3, &amp; 5</td>
<td>Surviving flap area measurements, RhoA, Rac1, &amp; Cdc42 mRNA expression quantification using RT-PCR</td>
<td>RhoA, Rac1, &amp; Cdc42 (angiogenesis regulators) mRNA expression sig higher in BTX-A group vs cntrls in all zones of flap</td>
<td></td>
</tr>
<tr>
<td>Human dermal fibroblasts in vitro</td>
<td>A</td>
<td>1, 5, &amp; 10 IU</td>
<td>Added to cell culture</td>
<td>NA</td>
<td>1 day before gene expression analysis</td>
<td>NA</td>
<td>Gene expression analysis 1 day after BTX-A Tx</td>
<td>RhoA, Rac1, &amp; Cdc42 mRNA expression quantification using RT-PCR</td>
<td>RhoA mRNA expression sig higher in 1 IU BTX-A group while all 3 gene mRNA expression sig upregulated in 5 &amp; 10 IU groups vs cntrls with most prominent effects observed in 10 IU group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aru et al., 2017</td>
<td>Rat</td>
<td>A</td>
<td>10 IU/ml, 0.2 ml</td>
<td>NR</td>
<td>Spinotrapezius muscle flap arteriole</td>
<td>2 wks before flap elevation</td>
<td>None</td>
<td>Gene expression analysis 1 day after flap elevation</td>
<td>Microcirculation assessment using transillumination through microscope</td>
<td>No change</td>
<td></td>
</tr>
<tr>
<td>Roh et al., 2017</td>
<td>Rat</td>
<td>A</td>
<td>29 IU/kg</td>
<td>Inject in center of flap</td>
<td>Random dorsal cutaneous flap</td>
<td>3 days before flap elevation</td>
<td>None</td>
<td>Flap survival &amp; blood flow eval, HPLC analysis of amount of NE, WB expression of eNOS &amp; NPY &amp; calorimetric assay of NO performed on POD 3 &amp; 7; on POD 7, IHC eval of VEGF &amp; PECAM/CD31 immunoreaction</td>
<td>Surviving flap area measurements, in vivo microcirculation assessment using laser Doppler flowmetry, VEGF &amp; CD31 IHC expression, NE level measurements using HPLC, NPY &amp; eNOS protein expression analysis using WB, calorimetric assay of NO end products</td>
<td>VEGF, NE, eNOS, NO</td>
<td>BTX-A Tx sig improved flap survival &amp; ↑ blood flow in all flap areas on POD 3 &amp; in distal flap area on POD 7 vs cntrls; sig higher CD31 immunoreaction was found in BTX-A groups vs cntrls; expression of NE in BTX-A group was sig lower immediately after flap elevation &amp; on POD 3 vs cntrls; eNOS protein level was sig higher in BTX-A group immediately after flap elevation &amp; on POD 3; expression of NPY &amp; levels of NO were not affected by BTX-A</td>
</tr>
</tbody>
</table>

CONTINUED ON PAGE 9 »
hypothesized that BTX-A might also be inhibiting adrenergic neurotransmission via a mechanism distinct from SNAP-25 cleavage. A recent study utilized $\alpha_1$ and $\alpha_2$ receptor blockers to indirectly examine the mechanism of BTX-A–mediated vasodilation in rat cremaster muscle microvasculature. Sym pathetic blockade seemed to be the likely mechanism, as BTX-A and adrenergic antagonist administration caused similar levels of vasodilation when applied separately, while BTX application after adrenergic antagonist administration caused no additional vasodilation. Another, more recent study evaluated the effects of BTX-A injection on the survival of random dorsal cutaneous flaps in a rat model, and reported significantly improved flap survival, increased blood flow, and elevated expression of angiogenesis markers and endothelial nitric oxide synthase (eNOS) in the BTX-A–treated as compared to the control group. Interestingly, flap tissue NE levels, as measured by high-performance liquid chromatography assay, were found to be significantly reduced immediately and 3 days after flap elevation in the BTX group (3 and 6 days after BTX-A treatment, respectively), suggesting that BTX-A–mediated blockade of NE release might be contributing to its vasodilatory effects (Fig. 2). Although the reduction of NE found 3 days after BTX-A treatment in this work is likely mediated in part via SNAP-25 cleavage, earlier time points after BTX-A treatment were not investigated, leaving open questions regarding the mechanisms of early BTX-A effects seen when applied to cerebral revascularization and Raynaud’s phenomenon (discussed below). Although these studies provide valuable insights into the possible mechanisms of BTX-A vascular spasmolytic activity, they may not be directly translatable to humans given the arterial graft variability and differences in small animal versus human vascular innervation.

Insights From BTX-A Utility in the Treatment of Raynaud’s Phenomenon

Raynaud’s phenomenon is characterized by an exaggerated vasospastic response to cold or emotional stress stimuli in cutaneous arterioles. The first report of BTX-A in patients with Raynaud’s phenomenon was published in 2004. Since then, multiple case series have demonstrated that direct perivascular BTX-A injections can provide rapid and long-lasting digit vasospasm relief. Immediate pain relief was reported in more than 80% of patients, while improved digit perfusion (as evaluated by laser Doppler scans) was achieved within 30 minutes of injection in about 70% of patients, with a striking 300% flow improvement as early as 15 minutes after injection. Notably, the effect of BTX-A in this setting appears to be prolonged, ranging from 13 to 59 months after a single injection. Direct vasodilatory effects of BTX have also been reported in a rat model as early as 5 minutes after treatment, with a maximum effect seen at 15 minutes. This differs from classic BTX-A characteristic effects, which have a known delay of several days and a duration of approximately 3–4 months. Insights from the current literature on BTX-A indicate that the mechanism of vasospasm prevention is likely different from the classic paradigm.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Species</th>
<th>BTX Type</th>
<th>Dose</th>
<th>Application</th>
<th>Flap Type</th>
<th>Time Points</th>
<th>Research Methods</th>
<th>Vasomotor Factors Evaluated</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huang,</td>
<td>2018</td>
<td>Rat</td>
<td>A 5 IU</td>
<td>Subcut in-</td>
<td>Ab skin flap</td>
<td>5 days after 2</td>
<td>Measurement of</td>
<td>Ischemia-reperfusion induction</td>
<td>Area necrosis &amp; ischemia &amp; areas of necrosis &amp; ischemia &amp; areas</td>
<td>No sig differences on necrosis betwn Tx &amp; cntrl groups; extent of arterial &amp; venous ischemia was sig smaller in BTX-A group vs cntrls.</td>
</tr>
</tbody>
</table>

Ab = abdominal; CD31 = cluster of differentiation 31; CIK42 = cell division control protein 42; CRGPR = CGRP receptor 1; FLT-4 = Fms-related tyrosine kinase 4; HPLC = high-performance liquid chromatography; IHC = immunohistochemistry; IL-1 = interleukin-1; IM = intramuscular; iNOS = inducible nitric oxide synthase; mRNA = matrix RNA; NA = not applicable; NPY = neuropeptide Y; NR = not reported; PECAM = platelet endothelial cell adhesion molecule; Rac1 = Rac family small GTPase 1; RT-PCR = real-time polymerase chain reaction; TUNEL = terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling; WB = Western blot; ↑ = increase(d); ↓ = decrease(d).
Growing evidence also demonstrates ras homolog gene family member A (RhoA)/rho-associated protein kinase (ROCK) pathway involvement in not only hypertension pathogenesis via increased inflammation, VSMC contractility, and endothelial dysfunction from negative regulation of the nitric oxide (NO) pathway, but also in graft vasospasm via similar mechanisms. Takagi et al. investigated the effects of the ROCK inhibitor fasudil on radial artery graft vasospasm prevention. Their findings showed an increase of in situ free blood flow with a corresponding decrease of myosin phosphatase targeting subunit 1 (MYPT1) and myosin light chain (MLC) phosphorylation that strongly counteracted the effects of major vasoconstrictors (NE and 5-hydroxytryptamine). Enhanced ROCK activity was also identified in skeletonized, spastic radial arteries as compared to nonmanipulated, nonspastic arteries, indicating that mechanical stimulation–induced vasospasm is likely mediated via the ROCK pathway as well. Moreover, ROCK pathway inhibition via abolished MYPT1 and MLC phosphorylation yielded a greater increase of in situ free blood flow as compared to that produced by the widely used antispastic agent verapamil, which showed no effect on ROCK. Currently, however, the clinical use of fasudil is approved only in Japan and China.

The two main mechanisms of ROCK-mediated upregulation of VSMC contractility are calcium-dependent translocation of \( \alpha_{2c} \) adrenoreceptors from the Golgi apparatus to the plasma membrane, and inhibition of myosin light chain phosphatase (MLCP; Fig. 2). Findings from Smith et al. also demonstrate that ROCK activity (specifically, phosphorylation of MYPT1) and ROCK expression itself can change independently, as MYPT1 phosphorylation activity can increase while ROCK expression remains unchanged in hypertensive as compared to normotensive subjects. This underlines the distinction between ROCK expression and the functionality of its downstream effec-
tors, and provides a potential explanation for the recent controversial findings of BTX A–mediated Rho kinase upregulation in murine myocutaneous and skin flaps.49,57 In these and numerous other animal studies, improved flap survival, increased tissue perfusion, vasodilation, and angiogenesis after BTX-A pretreatment has been demonstrated.6,8,39,49,57,66 Hence, the reported upregulation of Rho kinase expression in response to BTX-A seems to be contradictory to its well-reported vasodilatory effects in animal models and Raynaud’s phenomenon treatments in humans.20,46,64 Accordingly, Schweizer et al. hypothesized that increased RhoA immunoexpression after BTX-A administration could result from a compensatory overexpression in response to functional inhibition.57 however, no targeted investigations of BTX A–specific effects on RhoA downstream effectors in the ROCK pathway have been reported.

BTX-A and Other Vasoactive Substance Release

Evidence supporting other mechanistic pathways for BTX-mediated spasmolytic effects has also been reported. Calcitonin gene-related peptide (CGRP) is a known microvascular dilator found in sensory and motor nerve endings,20,67 and CGRP upregulation in rat cholinergic nerve terminals and neurons after BTX-mediated muscular paralysis has been found.67 Substance P is a neuropeptide involved in neurogenic inflammation and endothelium-dependent NO-mediated vasodilation.20 Although no significant CGRP and Substance P overall immunoexpression differences in BTX A–treated muscular flaps as compared to controls has been demonstrated, prominent CGRP expression was specifically found in thick-walled vessels, while Substance P expression was limited to the thin-walled vessels of BTX A–treated tissues.1 Expression of vascular endothelial growth factor (VEGF), a factor in NO-mediated angiogenesis and vasodilation, has also been shown to be increased following BTX administration in multiple animal studies.1,20,50 Although more detailed investigation is necessary, these findings indicate that the role of BTX in vasodilation is likely multifactorial.

BTX-A Clinical Use and Safety

BTX is currently FDA approved for therapeutic and cosmetic indications and has demonstrated an excellent safety profile in labeled as well as unlabeled applications.3,15 Serious adverse events such as death, generalized muscle weakness, dysphagia, respiratory insufficiency, and anaphylaxis after BTX injections have been rarely reported and are typically associated with excessively high toxin doses and serious predisposing comorbidities in affected patients.15 Generalized side effects have been associated with retrograde axonal migration of BTX from the site of injection.2–4 The proposed ex-vivo perivascular toxin application by soaking the graft in a reconstituted BTX-A solution intraoperatively during cerebral bypass does not involve injections.65 Moreover, after approximately 30 minutes of soaking in BTX-A solution, the graft is thoroughly flushed and washed to remove any residual toxin, thus minimizing risks of local and systemic spread.

While more extensive studies determining optimal dosing and exposure times for BTX-A use are certainly necessary, the concentration of the toxin proposed in cerebral revascularization is only 10 IU/ml.63 As a comparison, recommended doses of BTX-A used in injection form for spas tic disorders reaches up to 100 IU/ml, with total dosages of 360 IU per treatment session.3

Similar to numerous currently practiced unlabeled BTX-A and other off-label medication uses,12,13 BTX-A use in graft vasospasm is unlabeled and will likely remain so given the costly and time-consuming nature of obtaining FDA approval for new indications of currently approved drugs.21,73 Unlabeled drug use, defined as prescribing currently available medications for an indication that does not have FDA approval (including unapproved patient populations, doses, or administration forms),23 is nonetheless at the treating physician’s discretion for indications that they believe are in the patient’s best interests based on credible evidence.21,73

Conclusions

Animal studies suggest that BTX effects on the vasculature likely reach beyond its classic cholinergic paradigm and involve multiple pathways. The optimal application conditions and therapeutic outcomes for BTX use in revascularization patients remain ill defined. Elucidating the role of BTX-A in graft spasm prevention will aid in determining whether and how the findings from animal studies are translated to clinical practice. This knowledge will aid in optimizing patients, conduit selection, and application conditions for BTX treatment. Based on experimental data and initial clinical reports of BTX-A for spasmolysis, the applications for BTX-A will likely extend to cardiac revascularization as well as reconstructive surgery.

References

24. Ghanbarzadeh K, Tabatabaie OR, Salehifar E, Amanlou M, Khorasani G: Effect of botulinum toxin A and nitroglycerin in dermatology: the perspective of the patient, the physician, and the pharmaceutical companies. *[epub ahead of print],* 2018
28. He GW, Yang CQ: Radial artery has higher receptor-mediated contractility but similar endothelial function compared with mammary artery. *Ann Thorac Surg* **113:**1346–1352, 1997
38. Khot UN, Friedman DT, Pettersson G, Smedira NG, Li J, Ellis SG: Radial artery bypass grafts have an increased occurrence of angiographically severe stenosis and occlusion compared with left internal mammary arteries and saphenous vein grafts. *Circulation* **109:**2086–2091, 2004
Disclosures
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions
Conception and design: all authors. Acquisition of data: Russin, Ravina, Carey. Analysis and interpretation of data: all authors. Drafting the article: Russin, Ravina, Rennert. Critically revising the article: Russin, Ravina, Strickland. Reviewed submitted version of manuscript: all authors. Administrative/technical/material support: Russin, Carey. Study supervision: Russin.

Correspondence
Jonathan J. Russin: University of Southern California Neuroreconstruction Center, The Keck School of Medicine, Los Angeles, CA. jonathan.russin@med.usc.edu.