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

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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 spasmolytics. The ideal spasmolytic 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.

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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 spasmolytic 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 spasmolytic 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.

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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.

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 antispastic 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 nifedipine) 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

of clostridia.⁵⁶ The primary BTX mechanism of action is cholinergic neuromuscular transmission blockade through inhibition of the release of acetylcholine from nerve terminals.^{33,51,56} 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-B, -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.^{33,56} 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).⁵¹ 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.⁵¹

Currently, BTX is FDA approved for a very limited number of spastic 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.¹²

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).^{5,14,18,34,45,48} 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.^{14,18,34,48} Specifically, BTX improved microvascular anastomosis patency rates^{18,48} and counteracted vasospastic challenges from phenylephrine,^{14,34} potassium chloride (KCl),⁴⁵ norepinephrine (NE),⁴⁵ and cold,^{14,18,34} with these effects detectable as early as 30 minutes⁴⁵ after treatment. The spasmolytic potential of BTX in these studies was shown to be dependent on both dose and application time.^{5,45}

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 (Table 2) where the effects of BTX-A ($n = 14$) and BTX-B ($n = 1$) on flap viability, vasodilation, blood flow, angiogenesis, and inflammation were evaluated.^{1,6–8,10,22,32,36,39,40,49,50,54,57,62} 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,^{10,22,32,36,39,50,54,57} increased angiogenesis and angiogenic marker expression,^{36,39,40,49,50,54} improved blood flow,^{54,57} vasodilation,^{8,39,50,62} and reduced inflammation and inflammatory marker expression^{1,6} 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.^{10,36}

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.^{26,28,29} The first translational application of BTX-A for cerebrovascular bypass graft spasm prevention in humans was only recently published.⁶³

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.^{68,70} Norepinephrine (NE), produced in sympathetic nerves, is released from perivascular axon varicosities after sympathetic stimulation and binds to VSMC postsynaptic α and β adrenoreceptors. NE-induced VSMC contraction in most major arteries is thought to be primarily mediated by postsynaptic α_1 adrenoreceptor activation, with variable contribution from α_2 receptors (Fig. 2),²³ although the relative contributions of α_1 and α_2 adrenoreceptors to vasoconstriction depends on the anatomical location and segment of the vessel. In fact, in limb arteries the α_2 adrenoreceptor role in vasoconstriction increases when moving proximal to distal,²³ a finding that, when combined with the predisposition of the distal end of grafts to vasospasm, suggests a potentially important role of α_2 receptors in this process.⁵⁵

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.⁹ 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.^{9,44} 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.^{43,44} In light of these findings, it was

TABLE 1. Animal studies investigating BTX effects on in situ blood vessels and grafts

Authors & Year	BTX			Blood Vessels	Tx Time	Vasospastic Challenge	Time Points	Research Methods	Vasomotor Factors	
	Species	Type	Dose						Evaluated	Outcome
Clemens et al., 2009	Rat	A	10 IU/0.5 ml	Femoral	5 days preop	Phenylephrine & LE cold exposure	Vessel anast 5 days post-Tx, challenge after anast, eval 0 & 1 hr after challenge	Visual insp & vessel diam measurements	None	Patency maintained in 100% of vessels pretreated w/ BTX-A & only 44% pretreated w/ saline, sig shorter anast time & ease in BTX-A group vs saline
Murakami et al., 2009	Rat	C	1, 2.5, 5, 10, 20 IU/ml	Aortic rings	30 mins	KCl & NE	Isometric contractile responses measured post-Tx	Isometric contractile response measurement using myograph, hist exam using Elastica van Gieson stain	None	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
Arnold et al., 2009 ⁵	Rat	A	5 IU	Femoral	3 mins before skin closure	None	POD 1, 7, 14, & 28	Visual insp & vessel diam measurements	None	Max arterial diam after BTX Tx on POD 14, BTX-treated artery sig larger than ctrl on POD 14, BTX-treated vein sig larger than ctrl on POD 28
Fathi et al., 2010	Rabbit	A	20 IU/ml	Pst auricular arteries & marginal veins	7 days before vessel dissection	Cold exposure	Vessel measurement immediately after dissection, vessels then divided & re-anastomosed, exposed to cold challenge & vessel patency evaluated 1 hr later	Visual insp & vessel diam measurements	None	Artery & vein diams sig larger & patency rate higher in BTX-A pre-Tx group
Janz et al., 2011	Rat	B	100 IU	Femoral	Immediately after anast	Phenylephrine & LE cold exposure	Challenge at 12, 24, 48, 72, & 120 hrs after adventitia stripping, anast & Tx w/ subsequent patency eval	Visual insp & vessel diam measurements	None	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 ctrl
Park et al., 2014	Rat	B	1500 IU/0.3 ml	Femoral	3 days before vessel dissection	None	Vessel diams & blood flow measured 3 days after inject on dissection & after re-anast of vessels	Vessel diam & blood flow measurements using laser Doppler flowmetry	None	Vein & artery diam & peak blood flow velocity sig ↑ in BTX-B vs ctrls

Anast = anastomosis; ctrl = control; diam = diameter; eval = evaluation; hist = histological; inject = injection; insp = inspection; LE = lower extremity; POD = postoperative day; pst = posterior; sig = significant(y); subcut = subcutaneous; Tx = treatment; ↑ = increase(d).

TABLE 2. Animal studies investigating BTX effects on free and pedicled flaps

Authors & Year	Species	BTX		Application	Flap	Tx Time	Vasospastic Challenge	Time Points	Research Methods	Vasomotor Factors Evaluated	Outcome
		Type	Dose								
Kim et al., 2009	Rat	A	1.5 IU/0.05 ml	Single intradermal inject in central portion of proximal 3rd of flap after its elevation	Random cutaneous flap	On flap elevation	None	Flap evaluated on POD 1, 3, 5, & 7	Gross & laser-induced fluorescein fluorescence; optical flap eval; surviving flap area measurements; hist eval using H&E; detection of CD31, VEGF, & iNOS mRNA expression using RT-PCR	VEGF, iNOS	Sig better flap survival rate in BTX A-treated group vs ctrl; hist eval showed more prominent vasodilation & endothelial proliferation in BTX-A Tx group vs ctrl; higher CD31, VEGF, & iNOS gene mRNA expression rates in BTX-A group vs ctrl
Arnold et al., 2009 ⁷	Rat	A	NR	Direct Tx to vessels for several mins before closure	Ventral pedicled island cutaneous flap, inferior epigastric vessels	On flap elevation	None	On POD 4 flap evaluated for necrosis & ischemia	Visual insp & percentage area of flap necrosis calculations	None	No sig differences of necrosis area btwn Tx groups & adventitia intact vs disrupted groups; papaverine Tx group showed least ischemic changes & least amount of abnormal perfusion vs ctrl & BTX-A Tx groups
Stone et al., 2012	Rat	A	4, 6, & 10 IU	Tissue bath	Cremaster muscle microvasculature	After muscle dissection & placement in tissue bath	None; sympathetic contribution evaluated by adding α adrenergic blockers prazosin & rauwolfscine; phenylephrine used to evaluate successful adrenergic blockade	Vessel diams measured immediately after BTX-A Tx & every 5 mins up to 20 mins post-Tx; sympathetic contribution evaluated after adding pharmacologic agents & evaluating diam every 5 mins up to 20 mins	Microvascular measurements using live video microscopy, direct systemic arterial pressure measurements using femoral catheter	None	BTX-A Tx caused sig more prominent arteriolar vasodilation vs ctrls w/ sig dose & time effects; max vasodilation at 10 mins post-Tx w/ 6 & 10 IU doses being more effective than 4 IU; BTX-A vasodilatory effects likely mediated by sympathetic adrenergic blockade

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TABLE 2. Animal studies investigating BTX effects on free and pedicled flaps

Authors & Year	Species	BTX			Application	Flap	Tx Time	Vasospastic Challenge	Time Points	Research Methods	Vasomotor Factors Evaluated	Outcome
		Type	Dose	BTX								
Schweizer et al., 2013	Mouse	A & B	0.1 IU & 0.5 IU		Subcutaneous near vascular pedicle	Axially perfused dorsal skin flap	Preconditioning group, 24 hrs before op; Tx group, intraoperatively	None	POD 1, 3, & 5 blood flow & tissue oxygenation measurements; microdialysis performed on POD 1 & development of tissue necrosis evaluated on POD 5	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 evaluated using laser Doppler camera; immunofluorescent IHC expression of TUNEL, eNOS, & RhoA	eNOS, RhoA	BTX Tx groups had sig ↑ blood flow vs ctrl; sig improved tissue oxygenation, higher glucose & lower lactate levels were observed in BTX Tx groups vs ctrl; flap viability was sig ↑ in BTX groups as were levels of eNOS & RhoA immunorepression; no difference btwn BTX types & application time
Akcal et al., 2013	Rat	A	3.5 IU		IM inject in midportion of flap, perivascular inject in connective tissue around vessels	Gastrocnemius muscle flap	Pre-Tx 7 days before flap elevation & global flap ischemia induction then 7-day reperfusion period	None	7 days post-reperfusion, 14 days post-Tx	Macro- & microscopic eval of angiogenesis, amount of lymphocyte infiltration, edema, & myocyte damage; IHC eval of CGRP, FLT-4, VEGF, & substance P expression	VEGF, substance P	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, & substance P immunorepression among the groups; CGRP ⁺ expression marked in thick-walled vessels of BTX-A groups; VEGF & substance P expression marked in thin-walled vessels of BTX-A groups; perivascular BTX-A application showed less necrosis, inflammation, & edema than IM application group
Arnold et al., 2014	Rat	A	2 IU/ml, 1 ml		Inject in soft tissue around base of pedicle	Pedicled abdominal flap elevated on superficial inferior epigastric vessels	On flap elevation	None	POD 1, 2, & 7	mRNA expression of TNF α , IL-1, & VEGF using RT-PCR	VEGF	↓ IL-1 mRNA expression on POD 2, ↓ TNF α mRNA expression on POD 2 & 7

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TABLE 2. Animal studies investigating BTX effects on free and pedicled flaps

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

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TABLE 2. Animal studies investigating BTX effects on free and pedicled flaps

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

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TABLE 2. Animal studies investigating BTX effects on free and pedicled flaps

Authors & Year	Species	BTX		Application	Flap	Tx Time	Vasospastic Challenge	Time Points	Research Methods	Vasomotor Factors Evaluated	Outcome
		Type	Dose								
Huang, 2018	Rat	A	5 IU	Subcut in- ject near vascular pedicle	Ab skin flap raised on epigastric artery	12 hrs before flap el- evation & ischemia- reper- fusion induction	None	5 days after 2 ischemia-re- perfusion chal- lenges & areas of ischemia & necrosis were evaluated	Measurement of necrotic & ischemic flap areas	None	No sig differences on necrosis btwn Tx & ctrl groups; extent of arterial & venous ischemia was sig smaller in BTX-A group vs ctrls

Ab = abdominal; CD31 = cluster of differentiation 31; Cdc42 = cell division control protein 42; CGRP1 = 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; TNF α = tumor necrosis factor- α ; TUNEL = terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; WB = Western blot; $\uparrow$ = increase(d); $\downarrow$ = decrease(d).

hypothesized that BTX-A might also be inhibiting adrenergic neurotransmission via a mechanism distinct from SNAP-25 cleavage.⁴⁴

A recent study utilized α_1 and α_2 receptor blockers to indirectly examine the mechanism of BTX A-mediated vasodilation in rat cremaster muscle microvasculature.⁶² Sympathetic 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,⁵⁴ 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.^{20,46,71} The first report of BTX-A in patients with Raynaud's phenomenon was published in 2004.⁶⁴ Since then, multiple case series have demonstrated that direct perivascular BTX-A injections can provide rapid and long-lasting digit vasospasm relief.^{46,47,69} 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.⁴⁷ Notably, the effect of BTX-A in this setting appears to be prolonged, ranging from 13 to 59 months after a single injection.⁴⁷ 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.⁶² This differs from classic BTX-A characteristic effects, which have a known delay of several days and a duration of approximately 3–4 months.^{46,47,51} Insights from the current literature on BTX-A indicate that the mechanism of vasospasm prevention is likely different from the classic paradigm.

BTX-A and RhoA/ROCK Pathway

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,^{16,59,61,72} but also in graft vasospasm via similar mechanisms.²⁸ Takagi et al. investigated the effects of the ROCK inhibitor fasudil on radial artery graft vasospasm prevention.⁶⁵ 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.⁶⁵ 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 α_{2c} adrenoreceptors from the Golgi apparatus to the plasma membrane, and inhibition of myosin light chain phosphatase (MLCP; Fig. 2).⁶¹ Findings from Smith et al.⁶¹ 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.^{1,39,50} 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.⁶³ 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 grafts is only 10 IU/ml.⁶³ As a comparison, recommended doses of BTX-A used in injection form for spastic 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),⁷³ 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.

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Disclosures

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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.

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