

Novel Options for the Pharmacological Treatment of Chronic Anal Fissure – Role of Botulin Toxin

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Abstract: A chronic anal fissure (CAF) is commonly referred to as an ischemic ulcer. For many years it was thought that sphincterectomy produces anal sphincter relaxation, enhances microcirculation and promotes CAF healing. The latest studies have shown that fissure healing does not appear to be dependent on reduction in mean resting anal pressure. Our description of the process of CAF healing is based on understanding the balance between nitric oxide (NO) concentration and a level of oxidative and nitroxidative stress in wounds, which is responsible for contraction of smooth muscles (also anal sphincters), endothelial/skeletal muscle cell remodelling and proliferation. Pharmacological sphincterotomy with botulinum toxin (BTX) has an effect on motor endplate but it also has an influence on nitric oxide synthase (NOS) and other agents. Hypoxia in contracted anal sphincters induces vasoconstriction, in part, by decreasing endothelial NOS expression. *Clostridium botulinum* C3 exoenzyme - Rho-kinase inhibitor reverses this vasoconstriction. CAF is a site where the haemostatic mechanisms are activated. Rho inactivator C3-transferase from *Clostridium botulinum* abolished thrombin - stimulated endothelial cell contraction. Attenuated biotransformation of Glyceryl trinitrate (GTN) by mitochondrial aldehyde dehydrogenase and suppression of cGMP-dependent protein kinase expression may play a key role in understanding the problem of synergistic action of BTX and GTN. BTX and GTN are different forms of pharmacological sphincterectomies. This mechanism could explain the potentiating effect of BTX action after NO donors application for CAF. The application of BTX releases the blockage in GTN bioactivation in smooth muscle cells and suppresses basal continuous sympathetic activity, causing modulation of anal sphincters. It is responsible for CAF healing.

Key Words: Botulinum toxin, anal fissure, pharmacological sphincterectomy, Rho kinase, nitric oxide.

INTRODUCTION

The idea of using botulinum neurotoxin (BTX) therapeutically was suggested nearly 200 years ago when Justinus Kerner, a German physician and poet published the first studies of botulism [1]. He performed lab tests on himself and animals with BTX. He thought, as it is thought today, that the toxin interrupts motor signal transmissions in the peripheral and autonomic systems. In 1993, the German doctors Jost and Schimrigk applied BTX for the first time for the therapy of anal fissure [2]. As this therapy may be a substitute for surgical sphincterotomy it is also named pharmacological sphincterectomy [3].

In this brief review we present the pharmacological approaches to the therapy of anal fissure with the use of BTX and discuss potential biochemical mechanisms that may be involved in its beneficial therapeutic effects.

WHAT IS AN ANAL FISSURE AND WHY DOES PHARMACOLOGICAL SPHINCTEROTOMY WORK?

Anal fissure is a linear or rocket-shaped ulcer in the distal anal canal, which most often occurs in the posterior midline (in about 90% of all cases). Generally patients with anal fissure have anal pain after defecation (which may last up to several hours). The anal pain may be associated with blood

appearing on the stool or toilet paper [4, 5]. The most consistent finding in typical fissures is the spasm of the internal anal sphincter [4-7]. It is a widely held view that the anal pain is caused by spasm-induced ischemia of the sphincter. The evidence for this is the association of spasm relief with the relief of pain and fissure healing. However, it is not clear if anal sphincteric hypertonia and ischemia are the cause of anal fissure. After a period of 2-3 months, the fissure acquires chronic features comprising induration at the edges, a sentinel tag of skin or/and hypertrophied anal papillae [5].

Post mortem morphometric studies revealed that the blood supply of the anoderm at the posterior midline is significantly lower than that of the other sides of the anal canal [8]. Using a laser Doppler flow meter, Schouten *et al.* [9] demonstrated that an anodermal blood flow is negatively correlated with the resting anal pressure. These findings form the basis for the understanding of fissure predilection in the posterior midline of the anal canal and the rationale for the use of drugs that reduce sphincter spasm to treat chronic anal fissure (CAF) [4-9].

THE BOTULINUM TOXINS AND OTHER TYPES OF PHARMACOLOGICAL SPHINCTERECTOMY

The Botulinum Toxins

All organisms capable of producing botulinum neurotoxins (BTX) are classified as *Clostridium botulinum* (a gram positive, obligate anaerobic, spore-forming, rod-shaped), although they are diverse and show different phenotypic characteristics [10]. BTX is an endopeptidase which blocks

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acetylcholine release at the neuromuscular gap of alpha motor neurones, at gamma neurones in muscle spindles and in all parasympathetic and cholinergic postganglionic sympathetic neurones [11, 12]. There are seven sub-types (designated A–G) antigenically and serologically distinct but structurally similar [10, 13]. The preparations of BTX-A marketed in the United States (BOTOX®, by Allergan; Irvine, Calif), the United Kingdom and Europe (Dysport, by Speywood-Vaccine and Research Laboratory-Porton Down; Salisbury, UK), and Japan (CS-BOT) have different potency. It is therefore important that the specific market brand of BTX is defined both in academic publications and in recommendations for clinical practice [14]. In addition BTX B (Myobloc, Elan Pharmaceuticals, USA) is commercially available.

The BTX molecule is synthesized as a single chain (150 kDa). The heavy chain of BTX provides cholinergic specificity - after BTX injection it binds to glycoprotein structures specifically found on cholinergic nerve terminals. Endocytosis of plasma membrane containing toxin-receptor complexes introduces the BTX within endosomes into the presynaptic neurone. This is binding and internalization of the toxin into cholinergic neurons. Internalisation is independent of calcium concentration [10, 13]. The disulfide bond between light and heavy chains is cleaved by a protease. The light chain (~50 kDa) of the toxin crosses the hydrophobic barrier of the endosomal membrane into the cytoplasm of the nerve terminal. After this translocation the light chain of BTX (which is a zinc (Zn²⁺) endopeptidase similar to tetanus toxin with proteolytic activity) cleaves one or more proteins (SNAP-25, synaptobrevin or syntaxin) at a neuromuscular junction involved in the vesicle transport pathway of acetylcholine. Thus it prevents these vesicles from anchoring to the presynaptic membrane of neurons thereby inhibiting acetylcholine release. BTX does not block synthesis or storage of acetylcholine, only its release [13].

BTX relaxes smooth muscles although nerve endings and smooth muscle cells usually do not form junctions of the neuromuscular synapse type. Acetylcholine released from nerve endings stimulates adjacent myocytes but the action of BTX on smooth muscles is not well known [12]. The internal anal sphincter (IAS) is composed of smooth muscle in contrast to the external anal sphincter (EAS), which is of skeletal muscle. Injection of BTX into the IAS or the EAS relaxes the anal sphincters, enhancing microcirculation at the fissure site and promotes fissure healing [4, 5, 7].

Clostridium botulinum also produces other exotoxins including adenosine 5'-diphosphate (ADP)-ribosyltransferases, termed exoenzyme C3. This is produced as a single polypeptide of 26 kDa and inactivates members of the Rho GTPase family [15]. Rho GTPases (small ~21 kDa signaling G proteins) affects multiple biological functions due to binding to a range of intracellular effectors. This modulates actin cytoskeleton reorganization, cell growth, proliferation, differentiation, regulation of NO production and cellular oxidative stress [16]. A small GTPase encoded by the gene RhoA (RhoA) plays an important role in smooth muscle contraction maintenance of the basal tone in the IAS [17]. The inhibition of RhoA by *Clostridium botulinum* C3 exoenzyme has potential therapeutic value for chronic anal fissure.

Nitric Oxide Precursors

Nitric oxide (NO) acts through the stimulation of the soluble guanylate cyclase with subsequent formation of a cyclic guanosine-3',5'-monophosphate (GMP). It activates protein kinases and leads ultimately to the dephosphorylation of the myosine light chain [18]. NO donors such as glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN) applied topically or orally, lead to IAS relaxation [4, 5, 19-29]. NO is also the neurotransmitter mediating neurogenic relaxation of the IAS in humans [30]. Topical application of artificial NO precursors (ISDN and GTN) frequently has side effects (headache, dizziness, flushing) [19-29]. The natural precursor of NO L-arginine also reduces anal canal pressure and results in fissure healing, even in patients who have not responded to artificial NO precursors [31].

Brisinda *et al.* [29] reported that BTX-A is more effective than topical GTN ointment for treating CAF. Madalinski *et al.* [32, 33] report patients who did not respond to initial GTN ointment treatment nor show any improvement after having been injected BTX-A, but showed considerable improvement after repeated administration of GTN.

Lysy *et al.* [23] applied topical ISDN and 20 units of Botox as therapy for CAF and described similar effects as mentioned by Madalinski *et al.* [32, 33]. In their opinion combined BTX-A treatment and local application of nitrates can be more effective than BTX-A alone [23]. However, Madalinski *et al.* [32, 33] suggested that monotherapy with higher doses of BTX-A (50 and 100 units of Botox) can be more effective, than combination of lower doses of BTX-A and topical nitrates. Higher efficacy of CAF treatment with larger doses of BTX-A than those used by Lysy *et al.* [23] has also been shown later by Brisinda *et al.* [34] in 2002. Witte and Klaase [35] showed that BTX-A may be successful therapy for CAF that does not respond to NO donors.

Calcium Channel Antagonists

Voltage-dependent calcium channels (VDCC) are ion pores (multi-subunit complexes) in the plasma membrane of electrically excitable cells that regulate the entry of extracellular calcium into electrically excitable cells and serve as signal transduction centers. Contractions of smooth muscles are regulated by the cytosolic Ca²⁺ level. Calcium blockers (diltiazem and nifedipine) act by inhibiting the flow of Ca²⁺ into the sarcoplasm, with consequent saving of oxygen and decrease in the mechanical contraction of the muscle fibers [27, 36]. Calcium channel blockers are as effective as topical GTN in reducing persistence of fissure at 30 days to 6 months [37].

Other Types of Pharmacological Sphincterotomy

Potassium channels play an essential role in the membrane potential of smooth muscle, and in regulating contractile tone. It is known that endothelium-dependent vasodilators (e.g. NO, prostacyclin I₂) may activate potassium channels in cell membrane by allowing K⁺ efflux out of the cell. This causes a decrease potential and hyperpolarization of cell membrane closes voltage-gated calcium channels in the cell membrane, leading to smooth muscle relaxation [38]. A potassium channel agonist – minoxidil, easily absorbed

through the skin causes IAS relaxation but has little therapeutic value in CAF healing [39].

Angiotensin II is produced locally in the rat IAS and causes potent contraction of the sphincter. Inhibitors of angiotensin-converting enzyme that inhibit the production of angiotensin II, relax smooth muscle and reduce both mean anal resting and maximum resting pressures [40].

It has been reported that phosphodiesterase inhibitors may represent a new pharmacotherapy for the treatment of anal fissure. *In vitro*, all phosphodiesterase inhibitors (phosphodiesterase-1-5 inhibitors – (vinpocetine, adenine hydrochloride, trequinsin, rolipram, zaprinast, dipyridamole) cause a dose-dependent reduction in the tone of the IAS [41]. Phosphodiesterase-5 inhibitors block the breakdown of cAMP and cGMP and stimulate Protein Kinase G-phosphorylation [42]. Topical administration of sildenafil (a phosphodiesterase-5 inhibitor) significantly reduces anal sphincter pressure in patients with CAF [43].

Cholinomimetic (bethanechol) and an alpha-adrenoreceptor antagonist (indoramin) also reduce anal resting pressure [36, 44, 45]. Although indoramine reduces anal resting pressures in patients with CAF and normal subjects by a mean of 35.8% and 39.9% respectively, double-blind randomized trials do not show a significant therapeutic effect in CAF [44, 45].

INTERNAL ANAL SPHINCTER TONE MODULATION

The autonomic nervous system and enteric neural stimulation regulate the IAS tone. Calmodulin, caldesmon, calponin and myosin have an influence on Ca^{2+} regulation in the cytosol and on muscle contraction but the precise mechanism responsible for prolonged contraction of smooth muscle tone is unclear. It is widely known that a decrease in cytosolic Ca^{2+} causes relaxation of the smooth muscle [46]. This process is induced by the stimulation of beta2 adrenoreceptors. It results in a return of Ca^{2+} to the sarcoplasmic reticulum, which is mediated by cyclic adenine-3'5'-monophosphate (cAMP). Stimulation of neurons releasing NO causes a similar effect although this is mediated by cGMP. When the direct influx of extracellular Ca^{2+} through the membrane of Ca^{2+} channels is blocked, IAS relaxation is observed. And vice versa, direct influx of calcium through calcium channels causes smooth muscle contraction. Also the agonist alpha₁-adrenoreceptors induces the contraction of the smooth muscle per release of Ca^{2+} from the intracellular sarcoplasmic reticulum [47]. It is worthwhile noting that the ultimate neural modulation of the IAS contraction is caused by a “play” of different numerous enteric neurotransmitters, such as noradrenaline (NA), acetylcholine (Ach), Prostaglandin E₂ (PGE₂), Prostaglandin F₂ (PGF₂), 5-hydroxytryptamine (5-HT), and dopamine. A thorough identification of different pathways and the understanding of this modulation is extremely difficult in part due to the fact that several of the transmitters may be released from the same neurons.

DOUBTS ABOUT THERAPY FOR ANAL FISSURE

Should the BTX be Injected into the Internal or External Anal Sphincter?

Many authors claim that BTX-A should be injected into the IAS [4,23,29,48,49]. Jost and Schimrigk [50] questioned

BTX-A injection into the IAS in relation to the IAS thickness. The point of IAS thickness in MRI and USG has been raised for discussion (in dorsal projection is 1.96 ± 0.61 mm) [51,52]. Moreover, Jost [53] showed in an electromyographic examination the effect of the BT-A injection on the relaxation of the EAS. However, Italian authors [29,48,49] described the precision of BTX-A administration into the IAS through manometry, observing no distinct influence on the EAS relaxation and currently most practitioners hold the view that injection of BTX-A into the IAS is possible [4,23,29,48,44].

BTX-A diffuses from the injection point, crossing bone and fascia, over a distance of 30 – 45 mm. The increased toxin diffusion occurs when either the dose or the volume of the injection is increased [54].

Why Sphincterectomy Heals Anal Fissure?

Resting anal pressure reduction has been the focus of therapy for chronic anal fissure. Many observations during the last ten years showed that BTX-A reduces the resting anal pressure and this has led us to assume that this is the mechanism responsible for anal fissure healing [4-6,9,23,29,34-37,39,48-50]. However, recent studies have questioned this. Ho and Ho [55] reported that fissure healing did not appear to be dependent on the reduction in the mean of resting anal pressure. Also, fissures associated with normal anal pressures heal after lateral internal sphincterotomy [55]. Furthermore, Pascual *et al.* [56] showed that when manometric and endosonographic findings were compared, no statistically significant differences could be observed between healing and none healing anal fissures. Thornton *et al.* [57] described a group of patients in whom no correlation between clinical outcome (fissure healing or deterioration in continence) and subsequent significant reduction in maximum anal resting pressure was observed, (although it fell only by 17 percent). Before these studies an alternative hypothesis has been suggested by Madalinski and Chodorowski [58]. According to this hypothesis a key requirement for anal fissure healing is increased “stretchability” of anal sphincters, through known chemical pathways. Sufficient distension of the IAS after BTX-A injection (especially during defecation) reduces the risk of trauma and its complications during defecation [58]. When the endothelium has been traumatized, the platelet products ADP, ATP, 5-HT, platelet activation factor, and thrombin and substance P cause smooth muscle contractions. These substances, normally in the vascular system, cause relaxation of normal blood vessels by releasing NO and prostacyclin I₂ (PGI₂) [58,59]. In this way, the eruption of tissue in the fissure region and release of the contraction vessel mediators may tend to arrest fissure healing [58]. We also know that BTX-A therapy has an effect on striated muscle tone and muscle distention (“stretchability”) [60].

A New Look at Pathogenesis of Anal Fissure and Therapy with Botulinum Toxin

Various bacterial toxins can induce contraction of endothelial cells *via* the Rho/Rho-kinase pathway as a result of myosine light chain phosphorylation [61]. Reduction of NOS activity, insufficient cGMP generation (low-level stimulation of soluble guanylate cyclase) which can occur in diseases

such as diabetes and heart failure can be responsible for poor anal sphincters “stretchability” and represent a primary cause of biochemical changes in anal fissure [62]. Increased a “metabolic economy” and insufficient ATP generation lead to insufficient relaxation of the anal sphincters during defecation predisposing to fissure development. We also think that due to this reason in case of intensive work of anal sphincters during diarrhoea and activation of Rho-kinase by bacterial toxins the risk of anal fissure increases in patients with diarrhea. It develops in 4–7% of patients with acute diarrhea [63].

There is known that inflammatory pain is inhibited by BTX-A treatment. This effect is independent of muscle-relaxation. During tissue damage inflammatory mediators, such as substance P and glutamate, activate primary sensory neurons and produce pain [64, 65]. There is evidence that BTX-A inhibits the release of substance P *in vitro* [66-68].

Anal fissure leads to microvascular damage and activation of hemostatic processes. Thrombin increases endothelial permeability. Receptor activation by thrombin in endothelial cells leads to an early increase in stress fibre formation followed by cortical actin accumulation and cell rounding. Chronic contraction of anal sphincters *per se* may lead to local hypoxia which has an influence on endothelial nitric synthase (eNOS) expression [69, 70]. There are evidences that the inhibition of Rho by *Clostridium botulinum* C3 exoenzyme reverses hypoxia-induced by decrease in eNOS expression [69] and abolishes thrombin-stimulated endothelial cell contraction [71, 72]. Due to these reasons inhibition of RhoA by *Clostridium botulinum* C3 exoenzyme can put this method therapy of CAF at an advantage. We propose the hypothesis that RhoA is also relevant to BTX CAF healing.

Jones OM, *et al.* [73] showed that major effect of BTX on IAS may relay on blockade of sympathetic (noradrenaline mediated) neural output. Stimulation of α_2 -adrenergic receptors can induce Ca^{2+} sensitization of smooth muscle through both RhoA and phospholipase C (PLC). PLC induces two second messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The binding of IP_3 to receptors on the sarcoplasmic reticulum release of Ca^{2+} from it to cytosol and followed by a Ca^{2+} -calmodulin interaction stimulates phosphorylation of the light chain (MLC) kinase – thus the molecular interaction of myosin with actin are enable [46, 73, 74]. The other second messenger, DAG, activates protein kinase C (PKC), which has also contraction-promoting effect because it phosphorylates MLC kinase, calmodulin-dependent protein kinase II and various ion transporters and channels [46, 73, 74]. Activation of α_2 -adrenergic receptors can also induce activation of Rho-GTP by Rho-guanine nucleotide exchange factors. This activated RhoA-GTP activates Rho kinase, which phosphorylates the myosin-binding subunit of MLC phosphatase and inhibits its activity and thus leads to contraction [46, 73, 74]. We are convinced that when we decrease adrenergic activation by BTX applying, we diminish concentration of noradrenaline – an agonist of numerous G protein-coupled receptors, and diminish activation of RhoA. We suspect that decrease of RhoA activation can be essential for CAF healing. It can especially important when NO production is inhibited.

Prolonged smooth muscle contraction may be understood by remodelling or cytoskeletal rearrangement. Actin-myosin interactions may be crucial only for the initial development of force but “stucking” of actin filaments may be responsible for maintain smooth muscular tension. We think that after applying BTX there is decrease in activity of the RhoA/Rho kinase signaling and agonists induced Ca^{2+} signaling what is important for disruption of actin filaments [46, 73, 74].

Madalinski *et al.* [32, 33] and Witte and Klaase [35] showed that the BTX-A causes fissure healing even when there is a lack in effect after applying NO donors. NO donors can relax smooth muscle due to their effect on cytosolic cGMP levels and subsequently activation a cGMP-dependent protein kinase (PKG). Attenuated biotransformation of GTN by mitochondrial aldehyde dehydrogenase (mtALDH) and/or suppression of cGMP-dependent protein kinase expression may explain described poor reaction CAF for applying of NO donors [75].

We also know that acute and chronic NOS inhibition enhances α_2 -adrenoreceptor-stimulated RhoA and Rho kinase *in vitro* [74]. There is a question why BTX action (decrease in activity of the RhoA/Rho kinase signaling pathway) is so important that later applying NO donors acts better than before applying of BTX and why the NO-cGMP-PKG pathway which can deinhibit RhoA/ROK and myosin phosphatase *in vitro* by accelerating dephosphorylation of CPI-17 was insufficient in described cases by Madalinski *et al.* [32, 33].

CONCLUSIONS

Deliberations on BTX-A and the small GTPase Rho may be significant not only for the therapy of anal fissure. The same reactions play an important role not only in heart diseases but also in cancer because - overexpression of RhoC, correlates in various human cancers with high metastatic ability and poor prognosis [76].

Up to now explanations of the BTX effect on fissure healing were based on well known facts that the toxin inhibits the release of acetylcholine at the terminals of cholinergic neurons at the neuromuscular junction. In this paper for the first time alternative biochemical pathways have been described to create a biochemical model for fissure healing. It takes time to understand it. We hope that further investigation will help us reveal the other chemical dependences, which may have a wider influence than anal fissure healing.

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