

# Botulinum Toxin Type A and Its Possible Mechanisms on Wound Healing

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## Abstract

Botulinum toxin type-A (BTX-A), a subtype from known seven types of botulinum neurotoxin (serotype A-G), is produced by a gram-positive bacterium, *Clostridium botulinum*. The toxin is now widely and efficiently used in treating a plethora of diverse symptoms and conditions. Recent evidence in the literature also shows that BTX-A exhibits a wide range of effects on non-neuronal cells. Its potential has markedly expanded to clinical applications other than the treatment of neurological and muscular conditions that are characterized by neuronal hyperactivity. A number of studies have shown BTX-A to improve the quality of scar outcome and prevent the formation of keloids and HTS. Although the mechanism of action of BTX-A on wound healing is still not clearly understood, lately there has been extensive research to grasp the underlying mechanisms of this multifunctional toxin. BTX-A seems to affect wound healing by a number of mechanisms that include action on tensile forces, inhibition of fibroblasts differentiation, downregulation of TGF- $\beta$ 1 and collagen expression. This review will explore the responses of Botulinum toxin type-A on wound healing and preventing pathological scars like HTS and keloids, and comprehend the overall effect BTX-A has on wound healing.

## Keywords

Wound Healing, Botulinum Toxin Type-A, Hypertrophic Scar, Keloid, Fibroblast, Myofibroblasts, Transforming Growth Factor  $\beta$ -1, Collagen, Metalloproteinases, Connective Tissue Growth Factor

## 1. Introduction

Scars, especially hypertrophic or keloids have been of particular interest in plas-

tic and reconstructive surgery due to their recurrences and the therapeutic dilemma in treating them successfully. Disfiguring scars can be very distressful to patients regarding their appearance, particularly when located in the noticeable areas like head and neck; and can have an economic burden and psycho-social impact on the patients [1] [2]. Wound healing in itself is a very complex process involving various components and synchronous events that interplay as a consequence of chemical signaling between the various cells involved. Slight imbalance in any of these fragile processes can result in extensive fibrosis and give rise to abnormal scarring like HTS and keloids. So, many of the treatment modalities available these days have focused on this principle to control and prevent extensive hyper-proliferation of scar tissues. The benefits of early intervention on the outcome of various types of scars have been documented in the literature, yet no consensus on prevention or optimal treatment regime for scars has been manifested till now [3]. Other than intralesional steroid injection, application of pressure garments, laser and radiation therapy, scar excision, or various other methods, there is a lesser known treatment of injecting botulinum toxin type-A intralesionally that has been successfully reported over the years in the literature. The first complete description of clinical botulism (“sausage poisoning”) published by Justinus Kerner was in the year 1820 [4] [5], and since then the application of botulinum toxin has been very wide and versatile. Hagenah *et al.* used BTX-A as a research tool to study the spinal cord physiology and published reports in 1977 [6]. Perception of botulinum toxin began to slowly change over the years, and in the early 1980s it took a turn when its therapeutic potential was finally realized. Over the years, it has been commonly used in medicine to treat chronic myofascial pain [7], headache [8], hyperhidrosis [9], urinary incontinence [10], various muscle spasms and diseases characterized by overactive muscle, or more recently in cosmetic applications [11] [12]. Several commercial toxins are available in the market under the brand names such as Botox and Dysport, among many others [13] [14].

### 1.1. BTX-A: Structure, and Mode of Action

Botulinum toxin-A released in its inactive form is a complex mixture of single polypeptide chain consisting of botulinum neurotoxin-A with a nontoxic non-hemagglutinin protein (NTNH) and other various associated non-toxic proteins (HA-hemagglutinin protein). When cleaved by its own proteases, it is converted into an active form consisting of a light chain (L; 50-kDa), and a heavy chain (H; 100-kDa), with 3 domains weakly held together by a peptide belt, a disulfide bond, and surface charges [15] [16]. Botulinum neurotoxin causes flaccid paralysis by binding pre-synaptically to high-affinity receptors on the cholinergic nerve terminals and thus inhibiting the release of acetylcholine from presynaptic neurons at the neuromuscular junction (NMJ) resulting in chemical denervation at the motor end plate [17].

The mechanism of nerve terminal intoxication by the botulinum neurotoxin is divided into five major steps, 1) binding to neuronal cell membrane (dual-receptor

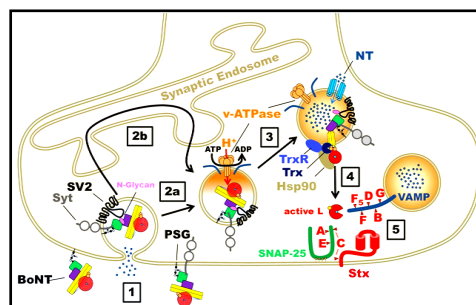
complex), 2) botulinum neurotoxin-receptor complex internalization within an endocytic compartment, 3) low pH driven translocation of L chain across the vesicle membrane, 4) Thioredoxin (Trx) catalyzes the reduction of a disulphide bond releasing L-chain into the cytosol, and 5) cleavage of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) substrate, and ensuing inhibition of membrane fusion and acetylcholine (ACh) release, thus leading to neuroparalysis as shown in **Figure 1** [18] [19]. Proximal axonal sprouting and muscle re-innervation by the formations of new NMJ leads to toxin recovery [20].

### 1.2. The H Chain

The H chain has two domains, HN—a translocation domain, and HC—a receptor-binding domain. The HC domain can be further divided into two distinct subdomains: 1) a carboxyl-terminal  $\beta$ -trefoil (HCC), and 2) an amino-terminal lectin-like jelly roll (HCN) [21]. The H chain provides specificity and binds the toxin to presynaptic receptors, and promotes L-chain translocation across the endosomal membrane. The HN domain has a central role in the translocation of the L chain across the membrane of the endocytic vesicle into the neuronal cytosol [16]. The HCC (binding domain), mediates neuro-specific binding of the toxin to a polysialoganglioside and also binds to a protein receptor, together they form a dual-receptor complex. This results in an accelerated and active interaction of the toxin with peripheral cholinergic nerve endings [22] [23] [24]. Although specific function of lectin-like HCN domain is yet unknown, evidence suggests it may promote attachment of BTX-A to the pre-synaptic membrane by interacting with certain anionic lipids [25].

### 1.3. The L Chain

The L chain is a metalloproteinase, possessing a zinc endopeptidase from the M27 family of peptidases, and is an active part of the toxin. It enters peripheral cholinergic nerve terminals where it cleaves the synaptosomal-associated protein SNAP-25 (SNARE protein family): a presynaptic membrane protein required for fusion of neurotransmitter containing vesicles [19] [26]. It impedes vesicle-plasma



**Figure 1.** Mechanism of intoxication by various *Botulinum neurotoxins*, A-G. (PSG, polysialoganglioside; Syt, synaptotagmin; SV, synaptic vesicle; Stx, syntaxin; Trx, thioredoxin; TrxR, thioredoxinreductase; VAMP, vesicle-associated membrane protein).

membrane fusion resulting in persistent but reversible inhibition of acetylcholine release from axon endings producing flaccid paralysis [17] [27]. The seven toxin types (A-G) have different tertiary structures and sequence differences, and different toxin types target different SNARE family members [28]. This specificity of action has made them effective therapeutic agents for various syndromes caused by overactive cholinergic nerve terminals. The scope of clinical applications of BTXA is rapidly growing and is now being substantially used to treat a broad range of clinical conditions [17].

## 2. Wound Healing

To understand the mechanisms of BTX-A on the outcome of scars, comprehending the process of wound healing is very crucial. When the skin is broken, an intricately regulated process involving multiple overlapping sequences of biochemical events are set into motion to repair the damage. The phases of wound healing are: 1) hemostasis, 2) inflammatory response and cellular migration, 3) proliferation (neo-angiogenesis, formation of granulation tissue and ECM, re-epithelialization), and 4) tissue remodeling. Upon wounding, initial vasoconstriction along with activation of the clotting cascade achieves hemostasis. Platelets that come in contact with the exposed sub-endothelium, tissue factor and collagen lead to further platelet aggregation and degranulation [29]. Platelets release various chemokines, growth factors (GFs like TGF, PDGF, etc.), and other pro-inflammatory mediators, which induce migration of a variety of cells into the wound site [30]. In the inflammatory phase, the actions on cell receptor by these cytokines, chemokines, GFs, proteases, pH gradients, pO<sub>2</sub> gradients modulate intracellular signaling cascade that results in cell proliferation, migration, and differentiation [27].

Activated by various GFs like TGF- $\beta$  family (TGF- $\beta$ 1,  $\beta$ 2, and  $\beta$ 3), IL family and angiogenesis factors (VEGF), the population of fibroblasts, keratinocytes, endothelial cells, and connective tissue greatly increases in the proliferative phase. Fibroblasts lay down ECM proteins including proteoglycans, fibronectins, and hyaluronic acid at the wound site and produce collagen and fibronectin, and eventually replace the temporary fibrin clot [31]. Thus formed granulation tissue is heavily vascularized by local angiogenesis [31] [32]. Different types of cells that are involved in the process of inflammation, neo-angiogenesis, and connective tissue synthesis adhere to, proliferate and differentiate on the collagen matrix produced and laid down by the fibroblasts. Whereas, myofibroblasts (transformed fibroblasts) connect to the surrounding protein collagen and fibronectin, assisting in wound contraction and promoting angiogenesis through matrix metalloproteinases (MMP) [33]. Remaining epithelial appendages near the wound and epithelial cells from around the wound border continue to repair the epidermis [34]. This phase continues for days and weeks [35].

The final step in wound healing is the remodeling phase and can take up to one or more years [29]. This stage in the wound healing needs a precise balance between the synthesis and degradation of ECM. Fibroblasts regulate both the

turnover and gradual breakdown of ECM by MMPs, and replacement of immature type III collagen by mature type I collagen [29] [30] [32]. As the scar matures over time, vascularity decreases and scar color changes, but only partially regains its original tensile strength. Although repair of the functional barrier is highly efficient, it does not always operate properly, and any disruption in the aforementioned processes will lead to uncontrolled wound healing resulting in two major pathological states: ulcerative defect or excessive scar formation like a hypertrophic scar or keloid [33] [35].

Hypertrophic scars (HTS) and keloids both are raised, thickened and firm scars formed due to overexpression of collagen during wound healing. Even though both can be pruritic, painful, it is also restricting and disfiguring, and there is a clear distinction between the two [36]. Unlike HTS, keloids are not contained within borders of the original injury, tend to overgrow into large benign tumors, can recur after excision, and are genetically predisposed. Often times HTS is self-limiting and can fade with time. However, studies have suggested hypertrophic scarring to be prevalent in darker skin populations and areas of skin subjected to tension [30] [36].

### **3. Effects and Potential Mechanisms of BTX-A on Wound Healing**

#### **3.1. Direct Inhibition of Active Fibroblasts and Increased Apoptosis**

Over the years, there have been several *in vitro* as well as *in vivo* studies that have concluded that BTX-A is not only a neurotoxin but tends to have a molecular effect on a cellular level, particularly the inhibition of fibroblasts. Shown in **Table 1** is a summary of studies which have indicated that BTX-A not only directly inhibits the proliferation but also affects the cell cycle distribution of fibroblasts derived from keloid, HTS, and scar contracture tissues when compared with normal fibroblasts [37]-[44]. Zhibo and colleagues conducted several studies to show the effect of BTX-A on fibroblasts derived from HTS and keloids. In one study, fibroblasts treated with BTX-A at the dose of 1 U/10<sup>6</sup> cells and 2.5 U/10<sup>6</sup> cells showed 58% and 61% in G0 to G1 phase, 8% and 9% in G2 to M phase, and 34% and 30% in S phase, respectively, showing a greater population of fibroblast arrested in G0 to G1 phase [40]. BTX-A effectively inhibited the proliferation of fibroblasts derived from HTS and also decreased CTGF protein thereby preventing excess collagen deposition. In another experiment, 64% of BTX-A treated fibroblast were in G0 to G1 phase compared to only 36% of control fibroblasts; a greater number of the control fibroblasts were in the proliferative phase (21% G2 to M; 43% in S) [45]. In yet another study by Zhibo *et al.*, keloids treated with intralesional BTX-A (70 to 140 U) per session at a 3-month interval for a maximum of 9 months showed striking outcomes. With no therapy failures and very high patient satisfaction 25% of subjects reported excellent, 41.6% good, and 33% fair [43]. Another similar study with 19 patients treated

**Table 1.** Effects of botulinum toxin type-A. Summary of studies included.

First Author (year)	Study type/Details	Type of Scar/Cell	Toxin Application	Results
Xiao Z <i>et al.</i> (2009) [44]	Prospective uncontrolled trial. 6 months follow-up	Active HTS from 19 patients	Intralesional BTX-A injection of 2.5 U per cubic cm of lesion, once monthly for 3 months	All patients showed acceptable improvement and high rate of therapeutic satisfaction. Scores for erythema, itching sensation, and pliability post BTX-A injections were significantly lower than prior to BTX-A injections ( $p < 0.01$ ).
Xiao Z <i>et al.</i> (2011) [40]	<i>In-vitro</i> /cell culture study	HTS derived FBs from 8 different patients	3 groups; cells treated with BTX-A in concentrations of 1 U/10 <sup>6</sup> cells vs. 2.5 U/10 <sup>6</sup> cells vs. control	Proliferation of FBs treated with BTX-A was slower than of FBs without BTX-A ( $p < 0.01$ ). Compared with FBs without BTX-A, BTX-A at 1 U/10 <sup>6</sup> cells decreased expression of CTGF by 49.2% $\pm$ 12.5% ( $p < 0.01$ ), and BTX-A at 2.5 U/10 <sup>6</sup> cells, decreased CTGF expression by 56.9% ( $p < 0.01$ ).
Zhibo X <i>et al.</i> (2008) [45]	<i>In-vitro</i> /cell culture study	FBs cultured from HTS of 8 different patients	FBs in 1 U/10 <sup>6</sup> BTX-A vs. control FBs	Significant differences in cell cycle distribution between experimental (64% in G0 - G1, 6.4% in G2-M, 29% in S phase), compared to control FBs (36% in G0 - G1; majority in proliferative phase; 21% in G2-M, 43% in S), ( $p < 0.01$ ).
Jeong HS <i>et al.</i> (2015) [37]	<i>In-vitro</i> /cell culture study	HTS derived FBs vs. normal mature scar derived FBs	FBs treated with BTX-A 4 U/ml vs. control	FB proliferation in both normal mature scar and hypertrophic scar tissue decreased significantly after treatment with 4 U/ml BTX-A ( $p < 0.001$ ). $\alpha$ -smooth muscle actin mRNA and proteins also decreased in BTX-A treated group compared to control (TGF- $\beta$ 1 only) of FBs derived from HTS, but not FBs derived from normal mature scars. FBs to myofibroblasts differentiation decreased in FBs of HTS after BTX-A treatment.
Xiao Z Qu G. (2012) [39]	<i>In-vivo</i> experiment (animal model)	HTS of 8 different rabbits ears.	Rt. ear injected with BTX-A (0.5 U per cubic cm, once a month for 3 months), Lt. ear as control	Thicknesses of HTS in BTXA group were lower than in control groups ( $P < 0.01$ ). Collagen fibers were thicker and arrangement of fibers was disordered in control group than in BTXA group.
Xiao Z <i>et al.</i> (2010) [42]	<i>In-vitro</i> /cell culture study	HTS tissue obtained from 8 different patients	FBs treated with BTX-A concentration of 1 U/10 <sup>6</sup> cells, 2.5 U/10 <sup>6</sup> cells, and control	TGF- $\beta$ 1 concentration per cell in FBs without BTX-A was higher than in FBs with BTX-A ( $p < 0.01$ ). Significant difference noted in TGF- $\beta$ 1 production per cell between FBs treated with 1 U/10 <sup>6</sup> cells of BTX-A and FBs treated with 2.5 U/10 <sup>6</sup> cells of BTX-A ( $p < 0.01$ ).
Hao R <i>et al.</i> (2018) [46]	<i>In-vitro</i> study	Human Keloid FBs vs. Normal FBs	FBs treated with different concentrations of BTX-A (0.01, 0.1, 1 and 10 U/L)	Viability of Keloid FBs decreased with increasing BTXA dose. BTXA inhibited proliferation, and S phase of Keloid FBs. MMP-1, -2 RNA and protein showed high expression, but TGF- $\beta$ 1 and MMP-9 showed low expression than control.
Chen M <i>et al.</i> (2016) [47]	<i>In-vitro</i> /cell culture study	Scar contracture tissue from 10 patients	BTX-A concentrations of 1 U/10 <sup>6</sup> cells, 2.5 U/10 <sup>6</sup> cells, and control	FBs without BTX-A treatment had higher proliferation than groups with BTX-A; proliferation of FBs significantly inhibited by BTX-A ( $p < 0.05$ ). BTX-A also inhibited protein of $\alpha$ -SMA and myosin II in FBs treated with BTX-A compared to FBs without BTXA ( $p < 0.05$ ).
Liu DQ <i>et al.</i> (2017) [72]	<i>In-vivo</i> experiment (animal model)	HTS of 18 different rabbit ears	4 groups: 12 ears as BTX-A (0.5, 1.0, 1.5, 2.0 IU), 12 ears as triamcinolone acetate (TAC) group, 12 ears phosphate-buffered saline (PBS), and healthy skin as control	Mean hypertrophic index of HTS with BTX-A (2.0 IU) were lower than that of control ( $p < 0.05$ ). BTX-A and the TAC group showed significantly less expression of collagen fibrils compared to PBS. BTXA (2.0 IU) and TAC significantly reduced FBs compared to control group.
Lee BJ <i>et al.</i> 2009 [73]	Prospective randomized experimental study	Surgical skin wounding on the dorsum of rat	10 U, 0.5 mL BTXA injected in one wound and normal saline injected into adjacent wound as control	Significant differences in wound size at 3rd and 4th week between BTXA and control ( $p < 0.05$ ). Less inflammatory cells in BTXA group than control at 2nd week ( $p < 0.05$ ). BTXA group showed less FBs and fibrosis than control at 4th week ( $p < 0.05$ ). BTX-A group had strong collagen density than control at 8th week ( $p < 0.05$ ). At 4th week, BTX-A group had lower TGF- $\beta$ 1 expression than control ( $p < 0.05$ ).

with BTX-A at monthly intervals for 3 months resulted in remarkable improvement in symptoms of erythema, itching, pliability, and high patient satisfaction [44].

More recently, Hao *et al.*, compared the viability of keloid fibroblast to normal fibroblasts by treating both with increasing dose of BTX-A. At lowered BTX-A concentrations, no significant changes were noted but when the dose was increased to 1 U/L, keloid fibroblast showed fragmentation, increased S phase, increased cell apoptosis and a significant decrease in the number of adherent cells [46]. Other several studies have also reported similar inhibitory effects of BTX-A on keloid or scar-derived fibroblasts. The number of viable fibroblasts was significantly decreased when the fibroblasts derived from both HTS and normal mature scar tissue was treated with BTX-A (0 or 4 units/ml) ( $p < 0.001$ ), suggesting that BTX-A inhibited the proliferation of fibroblasts [37].

In another study, control fibroblasts from normal tissue showed very high proliferation on the 7th day, whereas fibroblasts from scar contracture tissue treated with BTX-A  $2.5 \text{ U}/10^6$  cells showed nucleus pyknosis and significant cellular apoptosis ( $p < 0.05$ ) [47]. BTX-A not only seems to arrest the proliferation of fibroblasts but also escalates the apoptotic activity, leading to an overall decline in the fibroblast population in the granulation tissue hence controlling the extent of fibrosis.

### 3.2. Downregulation of TGF- $\beta$ 1 Expression/SMAD Signaling Pathway

Dysregulation of cytokine TGF- $\beta$ 1 seems to be the central driving force in the pathogenesis of HTS and keloids. TGF- $\beta$ 1 is a multifunctional growth factor expressed by most cells in wound healing and play a prominent part in signaling cellular proliferation, differentiation, apoptosis and adhesion, stimulation and deposition of ECM, including collagen type I, collagen type III, and fibronectin [42] [48] [49]. Over-expressions of TGF- $\beta$ 1 and - $\beta$ 2 along with flawed SMAD activity, and decreased expression of TGF- $\beta$ 3 increase collagen production and ECM deposition leading to the pathogenesis of abnormal scars [50] [51] [52] [53] [54]. TGF- $\beta$ 1 and - $\beta$ 2 activates fibroblasts, while TGF- $\beta$ 3 is a receptor antagonist and leads to diminished fibroblast activity. In TGF $\beta$ 1/SMAD signaling pathway, TGF- $\beta$ 1 binds to two different trans-membrane receptor serine/threonine kinases and activates the cytoplasmic SMAD proteins. Phosphorylation of receptor-activated SMAD2 and SMAD3 by TGF- $\beta$  receptor type I results in the formation of a complex with Co-SMAD to produce SMAD4 [55]. Through this mechanism TGF- $\beta$ 1 activates collagen gene transcription and differentiates fibroblasts into myofibroblasts. Aberration in these processes culminates to abnormal scar pathogenesis.

In different studies, BTX-A has shown to decrease the levels of TGF- $\beta$ 1 induced phosphorylation of SMAD2 activity in fibroblasts derived from scar contractures [56] [57]. Since TGF- $\beta$ 1 is also secreted by fibroblasts, and BTX-A's ability to directly inhibit the growth and enhance apoptosis of fibroblasts further

decreases secretion of TGF- $\beta$ 1 from these cells and in turn reduces the final fibroblasts turnover. Zhibo *et al.* found that with BTX-A treatment inhibition of fibroblasts proliferation derived from HTS also coincided with down-regulation of TGF- $\beta$ 1 protein expression, highlighting the molecular mechanism of BTX-A in HTS [42]. In *in-vitro* studies, exogenous addition of neutralizing antibodies to TGF- $\beta$ 1 and TGF- $\beta$ 2 reduced neovascularization, fibronectin, collagen type III and type I deposition in the early wound healing [54]. It also improved the structure of the neo-dermis resembling more closely to that of the normal dermis. Likewise, exogenous administration of TGF- $\beta$ 3 peptide had similar effects, whereas wounds treated with TGF- $\beta$ 1 or TGF- $\beta$ 2 had more extracellular matrix deposition [54]. TGF- $\beta$ 1 has been regarded as a dominant cytokine closely linked to the regulation of scar pathogenesis, and the action of BTX-A is effective and significant in the inhibition of HTS and keloid formation through the interference of TGF- $\beta$ /SMAD signaling pathway.

### 3.3. Inhibition of Fibroblast to Myofibroblast Differentiation

In the late proliferative phase, via TGF- $\beta$ 1, fibroblasts differentiate to a specialized proto-myofibroblasts, which in response to the cellular changes and mechanical tension forms myofibroblasts. These contractile cells show specific attributes of both fibroblasts and smooth muscle cells. Induced by TGF- $\beta$ 1, myofibroblast along with stress fibers containing  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) enhances increased proliferation, migration, deposition of ECM, and further production of cytokines. It also facilitates wound contraction and thus plays a primary role in the maturation of granulation tissue [58] [59] [60] [61]. Myosin II has a role in the cell deformation, adhesion, and along with actin can regulate the cell contraction process [62] [63] [64]. The contractility of fibroblasts corresponds to the expression of  $\alpha$ -SMA, and by lessening the expression of  $\alpha$ -SMA and myosin II, the contraction of scars can be greatly reduced [65] [66]. TGF- $\beta$  is a powerful mediator of myofibroblast differentiation that directly boots myofibroblast maturity by the inducement of  $\alpha$ -SMA expression, and also function as a stimulant of EMT CTGF, which acts downstream of TGF- $\beta$  to heighten TGF- $\beta$ -mediated response [32] [54] [65] [67].

Several animal models and cell culture studies have exhibited that inhibition of TGF- $\beta$ 1 signaling at all levels of the pathway effectively prevents myofibroblast formation and development of fibrosis. These studies show that BTX-A inhibits TGF- $\beta$ 1, and by blocking this pathway BTX-A successfully inhibits the differentiation of myofibroblasts and their expression of  $\alpha$ -SMA and myosin II proteins [37] [47]. In a study conducted by Jeong *et al.*, BTX-A treated HTS fibroblasts showed a significant decrease in  $\alpha$ -SMA transcription,  $\alpha$ -SMA protein levels, and  $\alpha$ -SMA staining [37]. Another similar study showed a significant decrease in the expression of  $\alpha$ -SMA mRNA levels in fibroblasts derived from HTS when treated with BTX-A [47]. Since  $\alpha$ -SMA is a marker for myofibroblast, the decreased level of  $\alpha$ -SMA in all these studies clearly show the inhibitory effect of BTX-A on TGF- $\beta$ 1 signaling and subsequent prevention of myofibroblast diffe-



rentiation by downregulating TGF- $\beta$ 1 expression. These can partly explain the molecular mechanism and effect of BTX-A on fibroblasts thus curtailing abnormal scarring.

### 3.4. BTX-A on the Expression of Collagen, MMP-1, MMP-2, and MMP-9

Collagen is a long fibrous protein displaying outstanding endurance and is one of the main components of ECM. Fibroblasts in the proliferative phase synthesize collagen and together with elastin and soft keratin, they provide tensile strength and elasticity to tissues. With regard to normal fibroblasts, there is a 3-fold increase of collagen in HTS and a 20-fold increase in keloids, leading to a larger and abnormal scar [68]. Histologically, HTS has more of collagen type III in a wavy, regular pattern arranged parallel to the skin surface, whereas keloids have a haphazard pattern, or nodular, whorls of collagen type I and type III [69].

TGF- $\beta$ 1 has demonstrated to up-regulate the expression of different types of collagen in cultured fibroblasts, and excessive collagen is a key feature of HTS and keloid. In one recent study, keloid fibroblasts treated with BTX-A (0.5 unit/ $10^5$  cells) significantly decreased collagen type III mRNA expression ( $p < 0.05$ ) compared to no treatment control [70]. Collagen type III expression also decreased significantly in BTX-A of 0.5 unit/ $10^5$  cells with TGF- $\beta$ 1 compared to TGF- $\beta$ 1 alone [70]. In another cell culture study, collagen type I $_{\alpha 1}$  was significantly decreased ( $p < 0.05$ ) by BTX-A but collagen type III expression was not affected in the same study [57]. In other animal model studies, characteristics of collagen in HTS were compared with controls to assess the effects of BTX-A on hyper-proliferative scars [39] [71] [72] [73]. Scar thickness and deposition of collagen was examined histologically. HTS tissue administered with BTX-A showed a decrease in the number of collagen fibrils with more organized fibers, absent of granulomatous or inflammatory infiltrates. The control had thicker collagen fibers with a haphazard arrangement. The BTX-A treated group also showed diminished expression of TGF- $\beta$ 1 in one of the animal model studies [73].

MMPs are matrix metalloproteinases that are regulated by TGF- $\beta$ 1 and have an important role in the regulation of synthesis and breakdown of ECM. MMP-1 (collagenase) and MMP-2 (gelatinase-A) degrade ECM during tissue remodeling and reduction in the synthesis of these molecules may explain the lack of scar regression seen in abnormal scarring [74]. In one study, when keloid fibroblasts were treated with BTX-A, it resulted in a substantial increase in the expressions of MMP-1 and MMP-2 genes in comparison to normal fibroblasts, but the expressions of TGF- $\beta$ 1 gene and MMP-9 declined with increasing toxin dose [46]. Other studies showed a significant increase in MMP-1 mRNA expression and MMP-2 activity in keloid fibroblasts compared with the control group ( $p < 0.05$ ) after treatment with BTX-A even without the addition of exogenous TGF- $\beta$  [70]. In another experimental study, fibroblasts treated with BTX-A with or without

the combination of TGF- $\beta$ 1 significantly increased MMP-2 and matrix MMP-9 expression when compared to control group ( $p < 0.05$ ), but MMP-2 decreased significantly after treatment with only TGF- $\beta$ 1 [57]. These studies suggest that BTX-A inhibits TGF- $\beta$ 1 and as a result increases the expression of MMP-1 and MMP-2 which then prevents excess collagen and ECM deposition, resulting in less fibrosis. BTX-A thus downregulates collagen expression and upregulates MMPs to reduce overtly synthesized ECM.

### 3.5. BTX-A Inhibits Connective Tissue Growth Factor (CTGF) Expression

Connective tissue growth factor (CTGF) is a pro-fibrotic and a downstream regulator of some of the responses of TGF- $\beta$ 1 functions. Induction of this complex molecule by TGF- $\beta$ 1 produce prolonged signaling of collagen mRNA expression, resulting in excessive collagen deposition, cellular adhesion, and growth leading to a state of sustained fibrosis [75] [76] [77] [78]. Therefore, blocking CTGF can prevent the synergistic effect of CTGF and TGF- $\beta$ 1 on hyper-proliferative scars. CTGF is excessively expressed in dermal fibrotic lesions like keloids, HTS, scleroderma, fibro-sarcomas, and as well as in lung, gingival, and liver fibrosis [79]-[84]. The elevated level of CTGF expression observed in fibrotic lesions is one of the best molecular markers of the fibrotic phenotype.

The effects of BTX-A on CTGF in hypertrophic scar still are largely unknown. However, a study exhibited that BTX-A adequately suppressed the growth of fibroblasts derived from HTS in 8 different patients, which in turn bring about a significant decrease in CTGF expression [40]. Compared to fibroblasts without BTX-A treatment, HTS derived fibroblasts when treated with BTX-A at  $1.0 \text{ U}/10^6$  cells reduced CTGF expression by  $49.2\% \pm 12.5\%$  ( $p < 0.01$ ), and with BTX-A at  $2.5 \text{ U}/10^6$  cells reduced CTGF expression by  $56.9\%$  ( $p < 0.01$ ) [40]. These results displayed that BTX-A had significant effects on the down-regulation of CTGF expression. So, the capacity of BTX-A to reduce CTGF expression and inhibit fibroblast proliferation may be associated with clinical improvement in the hypertrophic scar.

### 3.6. Relieves Wound Tension

Skin-tension is a known cause that acts on the wound borders during the healing process and prolongs the inflammatory reactions that aggravate fibrosis. The mechanical tension of skin closure is converted chemically into complex signaling that releases cytokines, particularly TGF- $\beta$ 1, that directly influence the quality of a scar [85] [86]. The mechanical stress detected by the mechanoreceptors in cells stimulates myofibroblast contraction that activates latent TGF- $\beta$ 1 in the ECM [87] [88]. Thus, this mechano-chemical stimulation accelerates the induction of cell proliferation, collagen synthesis and upregulation of expressions of TGF- $\beta$ 1, integrin- $\beta$ 1, and cytoskeleton p130Cas [87] [89] [90]. It is known that wound edges perpendicular to the relaxed skin tension lines (RSTL) pull the wound muscles and tissues with an opposing force that impedes the normal

wound healing process, and leads to sustained inflammation and fibrosis leading to the formation of HTS and keloids.

BTX-A, when injected locally, causes temporary paralysis of the muscles and tissues in the wound edges and reduces the dynamic muscle tension to enhance the healing process by blocking the mechanotransduction [88] [90] [91] [92]. In the year 2000, Gassner *et al.*, used BTX-A on primates to show the effect it had on wound tension [93]. Gassner *et al.*, in another study showed that BTX-A immobilized wound edges when injected locally in facial wounds resulting in a better cosmetic outcome compared to the controls [94]. Gassner conducted several studies and showed promising results of the effect of chemo-immobilization on wounds by injecting BTX-A locally [94] [95] [96]. Other studies have also effectively concluded that BTX-A injection can relax the wound edges in the anatomic locations prone to tensile stress by temporary paralysis of the muscles resulting in a far better outcome than in the controls [97] [98].

#### 4. Conclusion

In this manuscript, the authors have studied responses of botulinum toxin type-A on wound healing and preventing pathological scars like HTS and keloids, and comprehended the overall effect BTX-A has on wound healing. The mechanisms of BTX-A on the prevention of HTS and keloids are still not understood very well but the staggering amount of results of *in vivo* and *in vitro* experiments has indeed identified a number of effects of BTX-A on non-neuronal cells in the skin. This multifunctional toxin seems to have a direct action on dermal fibroblasts, TGF- $\beta$ 1 expression and wound tension, and if effectively applied in the very early stages of wound healing can regress abnormal scarring and prevent HTS and keloids. However, there is much to be investigated regarding the mechanism of actions of BTX-A on wound healing and doors are just opening for further studies.

#### Conflicts of Interest

None declared.

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## **Abbreviations**

BTX-A: Botulinum Toxin Type-A; NMJ: Neuromuscular Junction; ECM: Extracellular Matrix; HTS: Hypertrophic Scar; TGF  $\beta$ -1: Transforming Growth Factor  $\beta$ -1; MMP: Matrix Metalloproteinase; CTGF: Connective Tissue Growth Factor; EMT: Epithelial-Mesenchymal Transition