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Original Research Article

Effect and mechanism of action of botulinum toxin type A on hypertrophic scar *in vitro*

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Abstract

Purpose: *To examine the effect of botulinum toxin type A (BTXA) on the formation of hypertrophic scar and to unravel its mechanism of action.*
Methods: *HSF cells were isolated from hypertrophic scars and cultured. Immunohistochemistry (IHC) assays were performed to determine TGF-β1, FN, and Col1 expressions in hypertrophic scar and normal tissues while the expressions and phosphorylation of p38, ERK, JNK, as well as the expressions of α-SMA, Col1 and FN1 in HSF cells were evaluated by immunoblot techniques. CCK-8 and Transwell assays were used to assess the effect of BTXA on the viability and motility of HSF cells.*
Results: *BTXA suppressed MAPK pathway in hypertrophic scar fibroblasts ($p < 0.01$). It also suppressed excessive collagen deposits in hypertrophic scar through MAPK pathway ($p < 0.01$), and restrained HSF growth and motility via MAPK pathway ($p < 0.01$).*
Conclusion: *BTXA suppresses hypertrophic scarring via MAPK pathway and thus, can potentially be developed as a drug for the treatment of hypertrophic scarring.*

Keywords: *Hypertrophic scar, Botulinum toxin type A (BTXA), MAPK pathway, Collagen deposition, Fibroblasts*

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INTRODUCTION

Physical trauma can cause skin damage and scarring problems [1]. In developed countries, about 100 million people suffer scarring related problems each year [2]. Most superficial injuries do not leave significant scarring [3,4]. Both hyperplastic scars and keloids can cause a range of cosmetic and functional problems such as contracture, as well as self-reported itching and pain [5,6].

Botulinum toxin is a potent neurotoxin produced by the *Botulinum clostridium*, which has been proven to inhibit scar formation and improve wound healing [7]. Botulinum toxin type A (BTXA) is available for clinical use in treating hypertrophic scarring [7,8]. BTXA can reduce collagen deposition in hypertrophic scars by inhibiting phenotypic conversion of fibroblasts to myofibroblasts [9].

Dysregulation of TGF-β/Smad signaling is a major factor in the framework of scarring and

fibrosis, leading to abnormal collagen synthesis and deposition, higher proportions of collagen I/III and the formation of abnormally cross-linked collagen fiber bundles [10]. TGF-β plays a key role in producing the myofibroblast phenotype, which is responsible for large collagen deposition and wound contraction [11]. TGF-β1 regulates tissue homeostasis through a variety of cellular processes [12]. During wound healing, increased TGF-β1 promotes tissue regeneration, and a sustained increase in TGF-β1 activates a variety of intracellular signals such as Smads and the MAPK) pathway [13]. The activation of these pathways promotes the transcription of fibrosis-related molecules, resulting in a continuous positive feedback that leads to overproduction of matrix proteins [13]. This study was aimed to determine the role of BTXA in hypertrophic scars and unravel its mechanism of action.

METHODS

Skin samples

The hypertrophic scars and normal skin samples from patients undergoing plastic surgery were collected at the hospital. All procedures performed in the studies involving human participants received the approval of the Ethics Committee of The First Affiliated Hospital of Xinjiang Medical University (approval no. K202001-30), and complied with the guidelines of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects [14].

HSF cell isolation and culture, and drug treatment

Tissues were first minced into small pieces and incubated in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) containing 0.1 % collagenase type I (Sigma, St. Louise, Missouri, USA) at 37 °C for 4 h. The HSF cells were divided into groups as follows: (1) Control group (cells grown in culture medium without any treatment); (2) TGF-β1 group (The cells were treated with TGF-β1 (5 ng/ml, Sigma) in culture medium); (3) TGF-β1+BTXA group (The cells were treated with TGF-β1 (5 ng/ml, Sigma)) and BTXA (1U, Allergan, Ireland) in culture medium); (4) TGF-β1+BTXA +Anisomycin group (The cells were treated with TGF-β1 (5 ng/ml, sigma) and BTXA (1U, Allergan, Ireland), and Anisomycin (500 ng/mL) in culture medium).

Immunohistochemistry (IHC) assays

Hypertrophic scars and normal skin samples from patients undergoing plastic surgery were fixed using 4 % PFA for 30 min, then blocked with 2 % BSA for 20 min. Subsequently, the antibodies were incubated with sections for 2 h. All sections were incubated with the FITC-labelled antibody for 1.5 h, followed by staining with DAPI for 3 min, and all sections were then examined under a microscope.

Western blotting

Proteins were separated by SDS-PAGE, and further transferred onto the PVDF membrane. The proteins were blocked with TBST containing 5 % milk for 1 h, and then the corresponding primary antibodies were added. Primary antibodies p38 (Abcam, ab32142; 1:1000), p-p38 (ab178867; 1:500), ERK (ab184699; 1:1000), p-ERK (ab201015; 1:1000), JNK (ab179461; 1:1000), p-JNK (ab307802; 1:1000), α-SMA (Sigma, SAB5500002; 1:1000), Col1 (ab270946; 1:500), FN1 (ab2413; 1:1000), β-actin (ab8226; 1:3000) were incubated for 2 h at room temperature, and then secondary antibodies were incubated for 1 h and photographed after chemiluminescence (Wuhan Google Co., LTD).

CCK-8 assay

The cells were incubated with CCK-8 for 4 h, and the absorbance was measured by a microplate reader (Becton, Dickinson, USA).

Transwell assay

The cells were allowed to migrate into the transwell for 24 h. The invaded cells on the upper chamber were stained with 2 % crystal violet, and images were captured. The effect on cell invasion was observed by counting stained cells.

Statistical analysis

GraphPad 5.0 software was used for statistical analysis. Data are presented as mean ± SD. One-way ANOVA followed by Tukey's post hoc test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of TGFβ1, FN, and Col1 in HSF tissues was upregulated

To confirm the effects of BTXA on the progression of hypertrophic scars, the hypertrophic scars and normal skin samples were taken from patients undergoing plastic surgery. The expression of three markers of hypertrophic scars, including TGF-β1, FN, and Col1, were detected through IHC assays. TGF-

β1, FN, and Col1 were all upregulated in hypertrophic scar tissues, further confirming the hyperplasia of HSF (Figure 1).

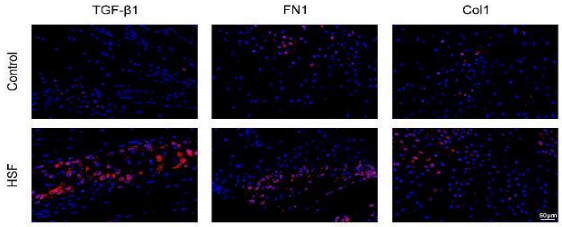

Figure 1: Expressions of TGF-β1, FN, and Col1 in HSF tissues were upregulated. IHC assays showed the expression of TGF-β1, FN, and Col1 in HSF tissues and normal tissues. Scale bar indicates 50 um. ***Note:*** Red panel indicates the expression of indicated proteins, while blue panel stained by DAPI indicate the nucleus

BTXA inhibits MAPK pathways in hypertrophic scar fibroblasts

Fibroblasts were then isolated from hypertrophic scar samples. The morphology of HSF is shown in Figure 2 A, which is typical of fibroblasts. TGF-β1 was used in the HSF cells and then treated with BTXA, along with the HSF cells, which was confirmed by immunoblot. The data confirmed that TGF-β1 treatment in HSF cells increased the phosphorylation of p38, ERK, and JNK, which were key regulators in the MAPK pathway (Figure 2 B - D). However, BTXA treatment obviously suppressed the phosphorylation levels of p38, ERK, and JNK (Figure 2 B - D). Therefore, BTXA suppressed the MAPK pathways in hypertrophic scar fibroblasts.

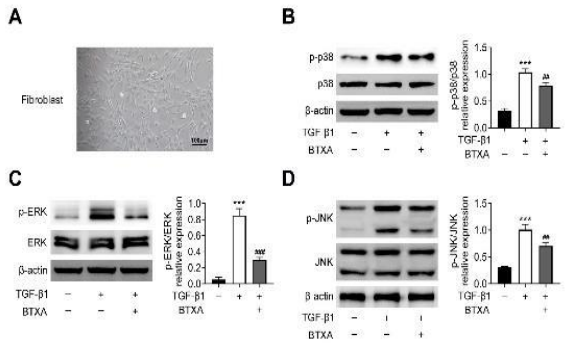

Figure 2: BTXA inhibited MAPK pathways in hypertrophic scar fibroblasts. (A). Morphology of isolated HSF cells in phase contrast microscopy; (B-D) Immunoblot showed the expression and phosphorylation levels of p38; (B) ERK; (C) and JNK; (D) in HSF cells upon the treatment of TGF-β1, BTXA, and anisomycin. Data were represented as mean ± SD. ***P < 0.001, TGF-β1 vs control, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001, TGF-β1+BTXA vs TGF-β1

BTXA inhibits hypertrophic scar collagen deposition through MAPK pathway

Through Immunoblot, TGF-β1 treatment significantly increased the expression of collagen deposition markers, including α-SMA, Col1 and FN1, suggesting the promotion of hypertrophic scar (Figure 3 A - C). Interestingly, BTXA treatment reversed the expression of α-SMA, Col1 and FN1 caused by TGF-β1 treatment in HSF cells (Figure 3A-C). Anisomycin (JNK and p38 activator) were used to activate MAPK pathway. Anisomycin treatment further reversed the expression of α-SMA, Col1 and FN1 caused by BTXA treatment in HSF cells (Figure 3 A - C). BTXA therefore inhibited hypertrophic scar collagen deposition through the MAPK pathway.

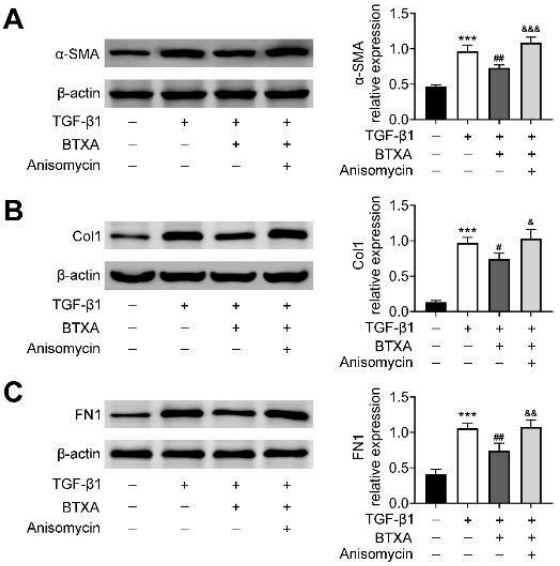

Figure 3: BTXA inhibits hypertrophic scar collagen deposition through MAPK pathway. (A-C). Immunoblot showed the expression and phosphorylation levels of α-SMA (A), Col1; (B), and FN1; (C) in HSF cells upon TGF-β1, BTXA, and Anisomycin. Data are mean ± SD. ***P < 0.001, TGF-β1 vs control, $^{\#}p$ < 0.05, $^{\#\#p}$<0.01, TGF-β1+BTXA vs TGF-β1; $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01, $^{\&\&\&}p$ < 0.001 TGF-β1+BTXA + anisomycin vs TGF-β1+BTXA

BTXA inhibited HSF viability and motility via MAPK pathway

CCK-8 assay results show that TGF-β1 stimulated the viability of HSF cells (Figure 4 A). BTXA treatment suppressed the viability of HSF cells (Figure 4 A). Furthermore, Anisomycin treatment reversed the suppression of HSF cell viability caused by BTXA treatment (Figure 4 A). Moreover, Transwell data indicate that TGF-β1 induced the migration of HSF cells, and BTXA treatment suppressed HSF cell migration (Figure 4 B and C). However, Anisomycin treatment induced HSF cell migration (Figure 4 B and C).

Thus, BTXA inhibited HSF viability as well as motility through MAPK pathway.

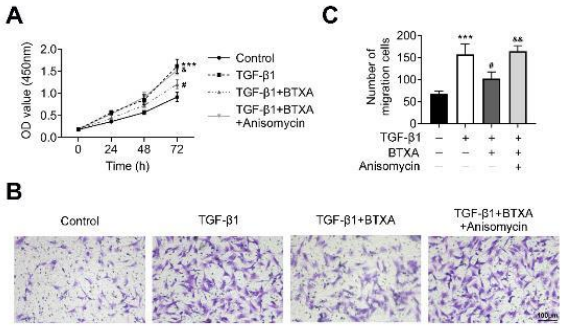

Figure 4: BTXA inhibited HSF viability as well as motility through MAPK pathway. (A) Viability of HSF cells upon treatment with TGF-β1, BTXA and anisomycin. (B, C). Migration capacity of HSF cells following TGF-β1, BTXA and anisomycin treatments, respectively. (B). Migration cell numbers (C). Data are presented as mean ± SD. ***P < 0.001, TGF-β1 vs control, $^{\#}p$ < 0.05, TGF-β1+BTXA vs TGF-β1. $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01, TGF-β1+BTXA + anisomycin vs TGF-β1+BTXA

DISCUSSION

Hypertrophic scar is a kind of skin lesion [15]. After infection, the remaining epidermal cells are damaged, making the wound deeper, and thus takes longer to heal [15]. Inflammatory factors promote fibroblast proliferation, and repeated infection causes the abnormal proliferation of granulation tissue [16]. Hypertrophic scar is usually caused by excessive proliferation of fibroblasts in an unsupervised and uncontrolled state during the process of repair and proliferation [15]. Therefore, the inhibition of excessive proliferation and myofibroblast transformation is very important to the cure for the disease. In this study, BTXA inhibited hypertrophic scars by inhibiting MAPK pathway.

Botulinum toxin (BTX) is a neurotoxin containing macromolecular protein produced by *Botulinum clostridium* [7]. Based on the toxin antigenicity, it is divided into A-F as well as G7 subtypes [7]. Botulinum toxin type A (BTXA) is the most widely used for seborrheic dermatitis, hyperhidrosis of the hands and feet, axillary hyperhidrosis, as well as migraine, eyelid muscle spasm, dystonia and so on. It can also act in the neuromuscular junction, inhibiting the release of acetylcholine and transmission of excitation to the neuromuscular junction, resulting in the paralysis of muscle fibers, so that the muscle cannot produce effective contractile movement [17]. BTXA can reduce collagen deposition in hypertrophic scars by inhibiting phenotypic conversion of fibroblasts to myofibroblasts.

BTXA inhibits the formation of collagen fibers and the proliferation of fibroblasts, and significantly reduces the number of fibroblasts in the G2-M phase of mitosis [18], decreases TGF-β1 and increases the expression of RNA and protein of matrix metalloproteinase-1 (MMP-1) and MMP-2. On the other hand, some scholars have proven that the formation of a scar is related to the mechanical tension of tissue, and botulinum toxin can relax muscle, reduce the tension of tissue, and directly inhibit the differentiation of fibroblasts into myofibroblasts, which is the main factor of scar contracture, all so as to improve the scar [18].

In this study, hypertrophic scar fibroblasts were isolated using hypertrophic scar, and normal skin samples taken from patients undergoing plastic surgery. The expressions of TGF-β1, FN1 and Col1 in hypertrophic scar tissue were determined by immunohistochemical assay. Furthermore, the effect of the immunofluorescence and immunoblot tests on MAPK pathway was assessed. MAPK pathway is thought to play an important role in the process of hypertrophic scar formation. Through several experiments, it was shown that BTXA inhibits excessive collagen deposition and cell contraction, thus inhibiting hypertrophic scar.

There is a lack of unified norms and ideal and stable treatment, but the only thing that can be confirmed is that combination therapy is often better than single therapy. Next, the therapeutic effect of BTXA combined with other drugs on hypertrophic scar can be further explored, and the related molecular mechanism can be further explored [2].

CONCLUSION

BTXA inhibits hypertrophic scar by suppressing MAPK pathway. Therefore, it can potentially be used as an effective therapeutic agent for the management of this scar but this has to be ascertained in clinical trials.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Juan Ma, Xianglin Dong, Bo Zhang designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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