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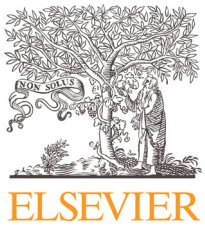

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Botulinum toxin type a intralesional monotherapy for treating human hypertrophic scar in a dose-dependent manner: In an animal model

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KEYWORDS
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Summary *Background:* The effect of Botulinum toxin type A (BTX-A) in treating or preventing a hypertrophic scar (HS) had been reported in clinical studies. However, the dose-effect relationship remains unclear.
Objective: To study the dose-effect relationship of BTX-A intralesional monotherapy treating human HS.
Methods: Six HS tissues were collected from six patients. Each tissue was segmented into 24 specimens and split into four groups: negative control (group A), 0.5U BTX-A (group B), 1U BTX-A (group C), and 2U BTX-A (group D). Six nude mice, each was prepared by implanting four specimens (one from each group) into the back for a total of 24 specimens. The process mentioned above were repeated six times. A re-entry operation was performed to obtain the specimens after 8 weeks. The weight of HS, the expression of decorin and TGF-β_1, the proliferation, and migration ability of hypertrophic scar fibroblasts (HSFBs) were compared among groups.
Results: The weight of HS, the expression of decorin and TGF-β_1, the proliferation, and migration ability of HSFBs showed significant differences in groups C and D as compared to group A; there has been no statistical significance in group B.

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Conclusion: BTX-A showed significant therapeutic efficacy when compared with the negative control group in a dose-dependent manner. BTX-A can reduce the weight of HS, upregulate the expression of decorin, downregulate the expression of TGF-β_1, and inhibit HSFBs proliferation and migration ability. This study indicates that BTX-A intralesional monotherapy treating HS should reach a threshold dose to achieve an effective treatment, and a high dose of BTX-A is more effective than a low dose.

Introduction

Asians have a tendency to develop hypertrophic scar (HS) after injury due to abnormal collagen deposition and hypertrophic scar fibroblasts (HSFBs) activity, which results in the loss of tissue function, psychological damage, and disfigurement. HS have decreased decorin and increased TGF-β_1 expression when compared with healthy skin.[1,2] TGF-β_1 is the most pivotal profibrotic cytokine related to fibrogenesis, which triggers fibroblasts to overproduce collagen types I, III, and fibronectin.[3] Decorin is a member of the small leucine-rich ubiquitous proteoglycan family that is a normal component of the extracellular matrix (ECM). It plays an important role in the regulation of intercellular contact, cell migration, and cell proliferation by the modulation of interactions between cell surface receptors and ECM.[4,5] Decorin binds to TGF-β_1, thereby inhibiting the biological activity of TGF-β_1 and then reducing the fibrous scar.[6-8]

There is a wide spectrum of measures to treat HS. Scar revision surgery is considered as a commonly therapy for treating hypertrophic scar,[9] but for the patients who wish to take conservative treatment, intralesional injection therapy is an option.[10-12] Triamcinolone is one of the most commonly used steroid for injection treating HS.[13,14] However, some complications of applying steroids are hard to accept by patients, such as severe pain during injection, ulcerate in injection-site, hyperpigmentation, telangiectasia, and skin atrophy.[15-18]

Botulinum toxin type A (BTX-A) is another option for intralesional injection, with milder complications and less side effects. Botulinum toxin is derived from clostridium botulinum. It is a potent neurotoxin that indirectly blocks neuromuscular transmission. Clinical studies on intralesional injecting BTX-A had indicated that less increase in scar width and improved discoloration of the scar in patients were observed[19,20] as well as significant improvement in cosmetic outcomes.[21,22] Both observer-dependent qualitative assessments and quantitative measurements were favorable in the BTX-A group. BTX-A could minimize scarring in primates, as it prevents the contraction of muscles and skin during wound healing.[23-25] However, the dose of BTX-A in treating HS in previous studies were mainly established on researchers' clinical experiment, and no evidence was given on the dose-effect relationship. Therefore, this study aims to compare the effects of different doses of BTX-A in intralesional monotherapy for treating human HS and wish to find a better dose standard for treating HS at the clinic.

Materials and methods

HS tissue and nude athymic mouse

HS tissue samples were collected from patients through scar revision surgery. None of these patients presented with a history of intralesional drug injection therapy. The acquisition of the tissues was subject to the consent of the patient. Institutional review board approval and informed consent from the patients were obtained. HS tissues were placed in antiseptic flasks, hydrated in saline solution, and processed within 1 h.

Nude athymic mice (BALB/nu-nu; female; body weight, 25-30 g; and age, 16 weeks) (Vital River Laboratory Animal Technology Co. Ltd, Beijing, China) were first kept in a sterilized microisolator, and then placed in the air outlet of laminar flow ventilation in the isolation room. Each mouse was kept in a single cage with 12 h light/12 h darkness, allowed food and water ad libitum. The protocol of this study was approved by the Peking University Institutional Review Board.

Animal experiment

Six HS tissue samples were collected from six patients in this study. Each sample was segmented into 24 specimens (each specimen was 0.60 g with full thickness and the diameter was 9 mm) and split into four groups (each group containing six specimens) according to the dose of BTXA intralesional injected to each specimen (Figure 1): group A, control, 0.9% normal saline (0.05 ml); group B, 0.5U BTX-A (Lanzhou Institute of Biological Products Co., Ltd, China) (0.05 ml) (100 U/vial; 10 ml normal saline dilution, 10 U/ml); group C, 1U BTX-A (0.05 ml) (100 U/vial; 5 ml normal saline dilution, 20 U/ml); and group D, 2U BTX-A (0.05 ml) (100 U/vial; 2.5ml normal saline dilution, 40 U/ml). Six nude athymic mice, each was prepared by implanting four specimens (one from each group) into the back for a total of 24 specimens. (Figure 2) The process above were repeated six times. Eight weeks after the implantation, a re-entry operation was performed to obtain the specimens. The weight of specimen, the expression of decorin and TGF-β_1, and the proliferation and migration ability of HSFBs were recorded and compared among groups.

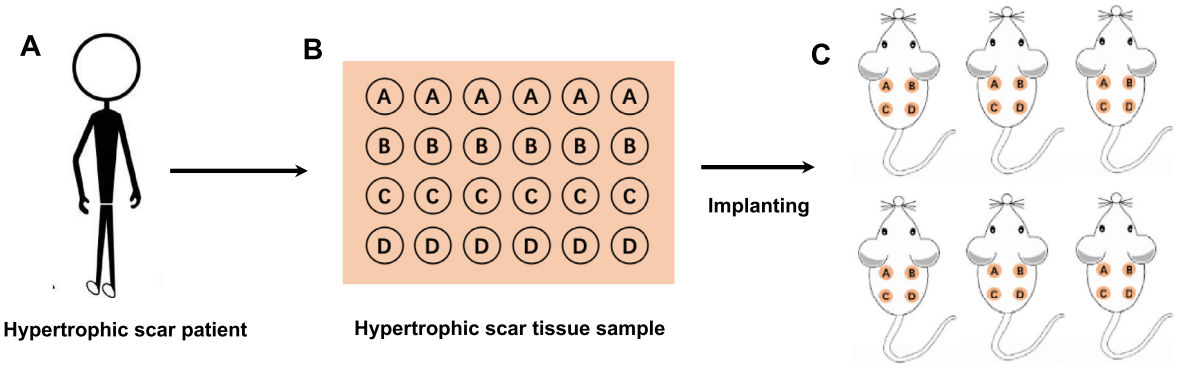

Figure 1 A flow chart for schematic illustration. Excised hypertrophic scar tissue sample form patient (A), the sample was segmented into twenty-four specimens (each specimen was 0.60g with full thickness and the diameter was 9mm) and split into four groups (each group containing six specimens) according to dose of BTXA intralesional injected to each specimen: group A, B, C and D (B), and a model was prepared by implanting four specimens, one from each group. In total, six mice models. (C). The process above were repeated six times (n=6).

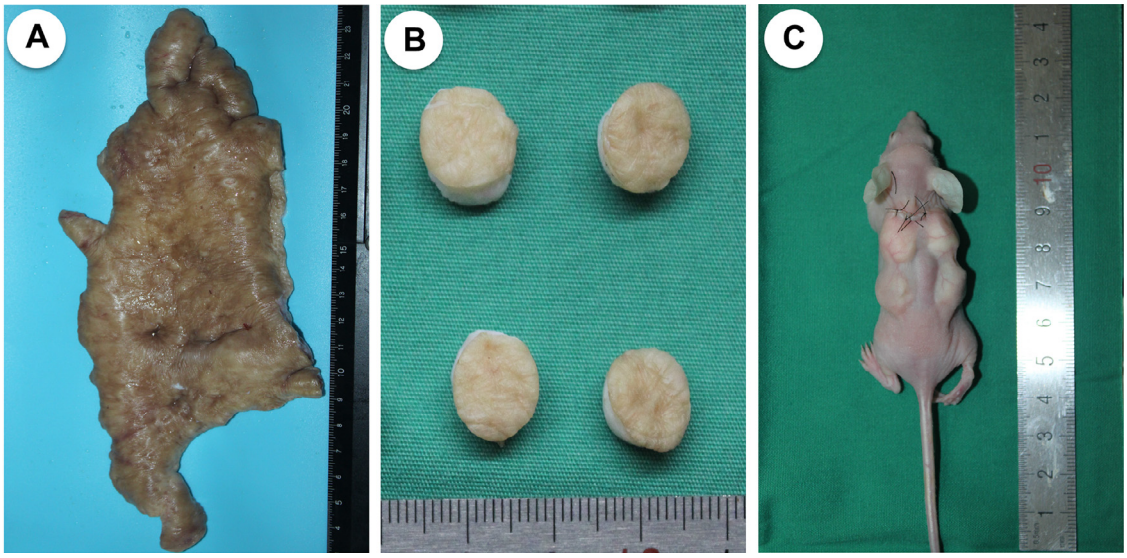

Figure 2 Excised human hypertrophic scar tissue (A), pre-implantation specimens (B) and post-implantation (C) images of nude athymic mice. Each nude athymic mice had four hypertrophic scar specimens implanted in its back with or without drug injections. Group A, control, 0.9% normal saline (0.05 ml); group B, 0.5U BTXA (0.05 ml) (100 U/vial; 10 ml normal saline dilution, 10 U/ml); group C, 1U BTXA (0.05 ml) (100 U/vial; 5 ml normal saline dilution, 20 U/ml); and group D, 2U BTXA (0.05 ml) (100 U/vial, 2.5ml normal saline dilution, 40U/ml).

Evaluation of therapeutic efficacy

Weight of HS

After eight weeks of implantation, the HS specimens were harvested from the back of nude athymic mice and the weights of explanted HS specimens were compared between groups.

Immunohistochemical staining

Immunohistological staining of the explanted HS specimens were performed to evaluate the difference in decorin and TGF-β_1 expression. After paraffin-embedded, the sections were stained with a primary antihuman decorin and TGF-β_1 antibody (R&D Systems, Inc., Minneapolis, Minn.) at 4°C overnight. On the following day, the sections were stained with a secondary antihuman decorin and TGF-β_1 antibody (Sigma Chemical Corp., St. Louis, Mo.) (1 mg antibody/ml) for 40 min. After rinsing, slides were counterstained with hematoxylin and observed under an optical microscope (EVOS FL Auto; Thermo Fisher Scientific, America).

Cell culture

The dermis of explanted HS specimens was fragmented into 5.0 mm^2 pieces. HSFBs were obtained by digesting these specimens with 0.2% collagenase (Sigma) for 3 h, and then they were cultured in 10 cm^2 culture dishes in 10 ml of

Dulbecco's Modified Eagle Medium (Sigma) with 10% fetal bovine serum (Sigma), streptomycin (100 μg/ml) (Sigma), and penicillin (100 IU/ml) (Sigma). The cultures were maintained in a humid incubator (5% carbon dioxide) at 37°C.

Western blot

HSFBs were seeded onto 6-well plates at a density of 1×10^6 cells per well. Decorin from the four groups was analyzed using western blotting. After discarding the medium, it was washed twice with prechilled PBS, lysed with 100 μl of cell lysate (RIPA Lysis and Extraction Buffer, Thermo Scientific, USA) containing 20 μl EDTA-free protease inhibitor cocktail (Thermo Scientific, USA) for 30 min on ice. The cells were gently scraped and centrifuged at 12,000 rpm for 10 min at 4°C. The protein concentrations of the supernatants were quantified using a BCA protein assay kit (TIANGEN BIOTECH, CHINA), and equal amounts of protein from each sample were loaded onto gels and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After separation was complete, the proteins in the gel were transferred onto polyvinylidene fluoride membranes (0.45 μm; Millipore, USA) and blocked with 5% Difco Skim Milk (BD, USA) for 2 h at room temperature. Membranes were subsequently incubated with the indicated primary antibodies at 4°C overnight. On the next day, membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBST, pH 7.4) and incubated with the appropriate secondary antibody at room temperature for 1 h. The immunoblots were detected by enhanced chemiluminescence (ECL, Beyotime Biotechnology, CHINA), and the band densities were analyzed using an ImageQuant LAS 4000 system (GE, USA).

HSFBs proliferation assay

Cell proliferation of HSFBs from the four groups was analyzed using Cell Counting Kit-8 (Sigma-Aldrich, Saint Louis, Mo.). The HSFBs suspension (100 μl) was distributed into a 96-well plate with 3000 cells/well in Dulbecco's Modified Eagle Medium and 10% fetal bovine serum and incubated for an additional 24 h. On the following day, 10 μl solution was added to each well and incubated for 2 h. Sodium dodecyl sulfate (10 μl of 1% weight/volume) was added to e ach well and the absorbance was measured at 450 nm using a microplate reader (EnSpire Multimode Plate Reader; PerkinElmer, America). All samples were read in and all of the reported experiments were performed 12 times.

Transwell migration assay

Migration ability of HSFBs from the four groups was analyzed using Transwell (8 μm pore-sized, 6.5 mm diameter) (Corning, NY, USA) invasion assay. Dulbecco's Modified Eagle Medium (Sigma) with 10% fetal bovine serum (Sigma) (700 μl) is placed in a lower chamber (24-well plate). The HSFBs suspension (200 μl, 1×10^5 cells) with no fetal bovine serum was distributed into an insert with a semi-permeable membrane, which is suspended in the lower chamber. ECM gels are formed in situ on the membrane and allowed HSFBs to migrate toward 10% fetal bovine serum in the lower chamber, incubated for an additional 24 h. The cultures were maintained in a humid incubator (5% carbon dioxide) at 37°C. The cells were fixed with 95% ethanol; the cells were removed from the basement membrane of the lower chamber; and hematoxylin and eosin staining was done. Observations were made and pictures were taken under a 20x microscope (EVOS FL Auto; Thermo Fisher Scientific, America),[26,27] using Image J software for cell counts. Four fields of view were randomly selected for each group, the average was set, and three repetitions were set.

Statistical analysis

In this study, all statistical analyses were performed using Graphpad Prism 7.0. Statistical differences were evaluated by the analysis of variance, where a value of $p<0.05$ was considered statistically significant.

Results

Weight of HS

Measured the weight of harvested HS specimens and calculated the percentage with original weight. Group A (0.9% normal saline) showed no significant reduction when compared with the original. The highest decrease was in group D (2U BTX-A). Significant differences were observed in the weight reduction of the HS specimens among the four groups (group A, 100.67 ± 11.13 percent; group B, 87.67 ± 8.79 percent, $p>0.05$; group C, 73.67 ± 6.05 percent, $p<0.05$; and group D, 67.67 ± 1.11 percent, $p<0.05$) (Figure 3 and Supplement 1).

Decorin expression

The explanted HS specimens were immunohistochemically stained for decorin expression. The specimens from all groups showed positive decorin staining. (Supplement 2)

Western blot was used as a quantitative method to analyze decorin from the four groups. HSFBs cultured from group A (0.9% normal saline) had a lower rate of decorin expression than that of the treatment groups (groups B, C, and D). Significant differences in decorin expression of HSFBs were observed among the four groups (group A, 1.11 ± 0.14; group B, 1.27 ± 0.07, $p>0.05$; group C, 1.51 ± 0.11, $p<0.05$; and group D, 1.63 ± 0.17, $p<0.05$). (Figure 4 and Supplement.1)

TGF-β_1 expression

The explanted HS specimens were immunohistochemically stained for TGF-β_1 expression. The specimens from all groups showed positive TGF-β_1 staining. (Supplement 3) Western blot was used as a quantitative method to analyze TGF-β_1 from the four groups. HSFBs cultured from group A (0.9% normal saline) had a higher rate of TGF-β_1 expression

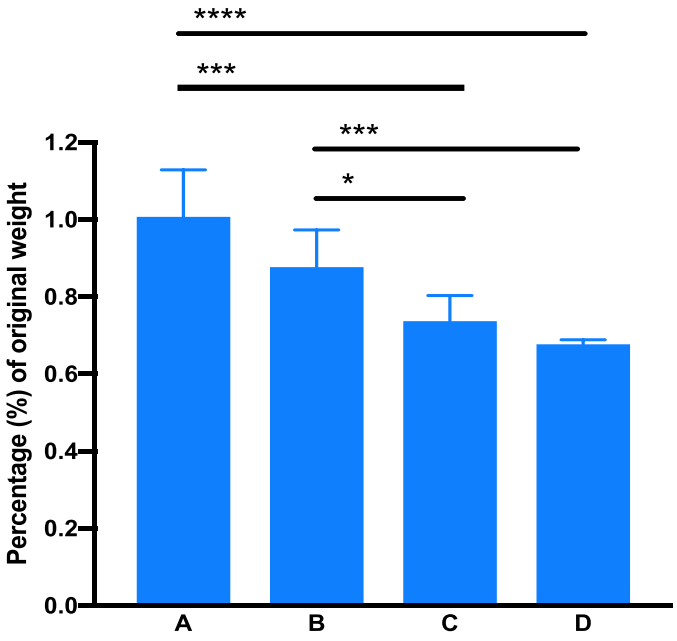

Figure 3 Percentage of harvested hypertrophic scar specimen weight eight weeks after implantation. Hypertrophic scar specimens weight reduction was observed in the four groups (group A, 100.67±11.13 percent; group B, 87.67±8.79 percent; group C, 73.67±6.05 percent; and group D, 67.67±1.11 percent). Bar marked with an asterisk show significant differences in weight reduction between treatment groups. GroupA, control, 0.9% normal saline; group B, 0.5U BTXA; group C, 1U BTXA; and group D, 2U BTXA. **** $p < 0.0001$; *** $p < 0.001$; * $p < 0.05$.

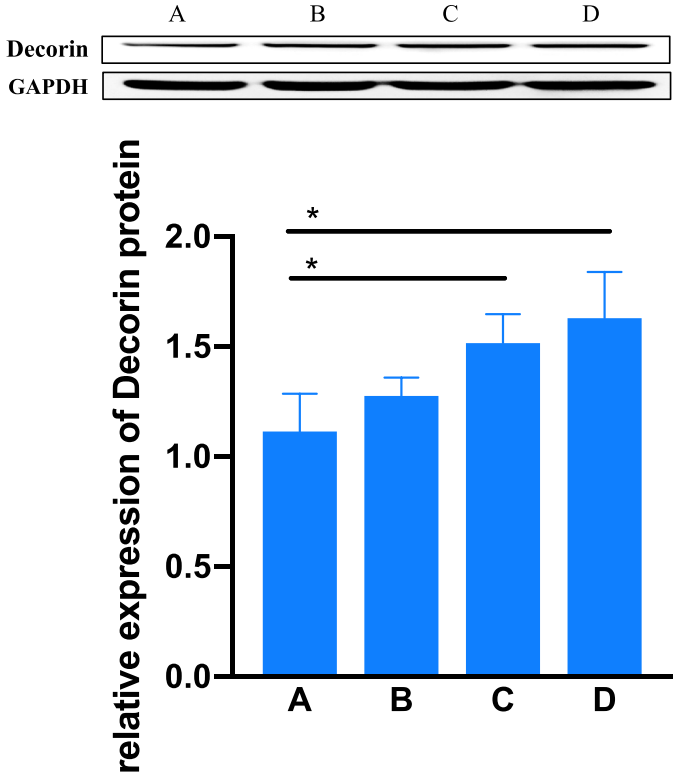

Figure 4 . Decorin expression of HSFBs were analyzed in the four groups (group A, 1.11±0.14; group B, 1.27±0.07; group C, 1.51±0.11; and group D, 1.63±0.17). Bar marked with an asterisk show significant differences in decorin enhancement between treatment groups. GroupA, control, 0.9% normal saline; group B, 0.5U BTXA; group C, 1U BTXA; and group D, 2U BTXA. * $p < 0.05$.

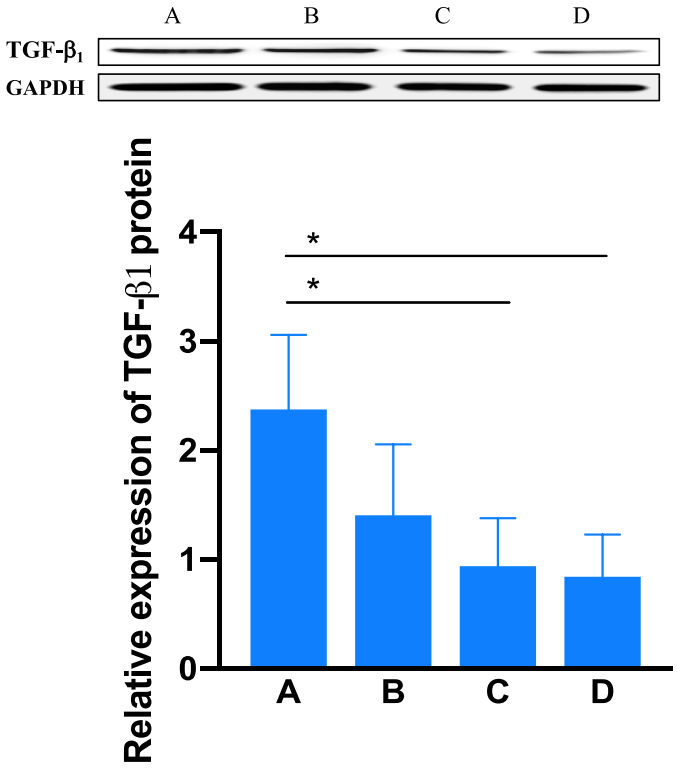

Figure 5 TGF-b1 expression of HSFBs were analyzed in the four groups (group A, 2.28±0.51; group B, 1.23±0.55; group C, 0.82±0.37; and group D, 0.74±0.33). Bar marked with an asterisk show significant differences in TGF-b1 decrease between treatment groups. GroupA, control, 0.9% normal saline; group B, 0.5U BTXA; group C, 1U BTXA; and group D, 2U BTXA. * $p < 0.05$.

than that of the treatment groups (groups B, C, and D). Significant differences in TGF-β_1 expression of HSFBs were observed among the four groups (group A, 2.28 ± 0.51; group B, 1.23 ± 0.55, $p>0.05$; group C, 0.82 ± 0.37, $p<0.05$; and group D, 0.74 ± 0.33, $p<0.05$). (Figure 5 and Supplement.1)

HSFBs proliferation ability

HSFBs proliferation ability among the four groups was analyzed using the Cell Counting Kit-8 assay. HSFBs cultured from group A (0.9% normal saline) had a higher rate of proliferation than that of fibroblasts cultured from treatment groups (groups B, C, and D). Significant differences in optical density were observed among the four groups (group A, 0.65 ± 0.04; group B, 0.63 ± 0.06, $p>0.05$; group C, 0.52 ± 0.04, $p<0.05$; and group D, 0.48 ± 0.01, $p<0.05$). (Figure 6 and Supplement.1)

HSFBs migration ability

HSFBs migration ability among the four groups was analyzed using the transwell assay. HSFBs cultured from group A (0.9% normal saline) had a higher rate of migration ability than that of the treatment groups (groups B, C, and D). Significant differences in HSFBs migration ability were observed among the four groups (group A, 89.5 ± 10.36; group B, 77.25 ± 2.38, $p>0.05$; group C, 64.5 ± 1.50, $p<0.05$; and group D, 44.5 ± 3.28, $p<0.05$). (Figures 7-8 and Supplement.1)

Discussion

To our knowledge, this is the first study on dose-effect relationship of BTX-A intralesional monotherapy in the treatment of human HS in an animal model. HS specimens were implanted into a subcutaneous pocket in nude athymic mice that were used to simulate the progression of scar tissue in human patients, which has been previously validated as a reliable model with which to test drug efficacy.[28,29] BTX-A was confirmed to reduce the proliferation and migration of human scar fibroblasts.[30] In this study, significant differences in HSFBs migration ability were observed in a dose-dependent manner. This finding stands with the previous clinical studies from another aspect on how the BTX-A might act on preventing unfavorable scars.

In this study, the weight of HS specimens, the proliferation and migration ability of HSFBs in group B were all reduced, while the expression of decorin was upregulated and the expression of TGF-β_1 was downregulated, but with no statistical significance as compared to negative control. This indicated that BTX-A should reach a threshold dose (1 U/0.60 g/9 mm at least) to initiate an effective clinical treatment. On the other hand, groups C and D, with a higher dose BTX-A, showed a significant difference in reducing the weight of HS, upregulating the expression of decorin, and downregulating the expression of TGF-β_1. Thus inhibiting the proliferation and migration ability of HSFBs as compared to group A and B, indicating that BTX-A intralesional injection might take effect in a dose-dependent manner. Decorin as a soluble dermatan sulfate proteoglycan that can bind

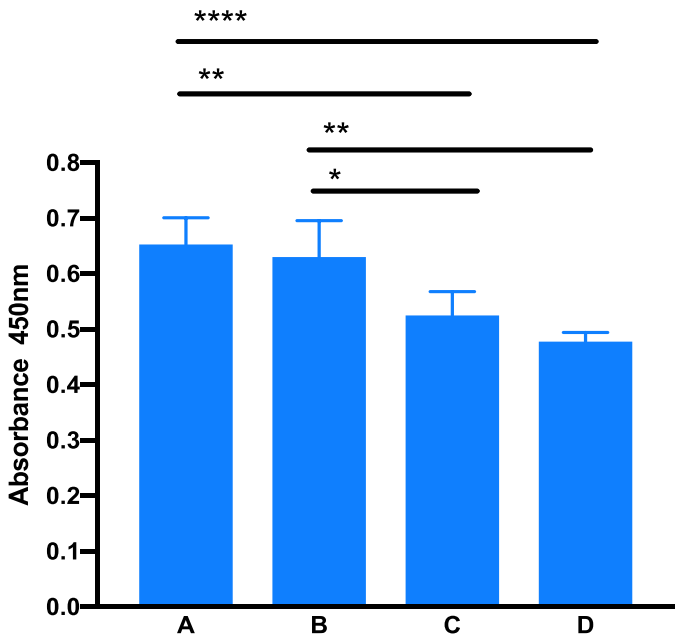

Figure 6 . The proliferation ability of HSFBs were analyzed in the four groups (group A, 0.65±0.04; group B, 0.63±0.06; group C, 0.52±0.04; and group D, 0.48±0.01). Bar marked with an asterisk show significant differences in HSFBs proliferation reduction between treatment groups. GroupA, control, 0.9% normal saline; group B, 0.5U BTXA; group C, 1U BTXA; and group D, 2U BTXA. **** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$.

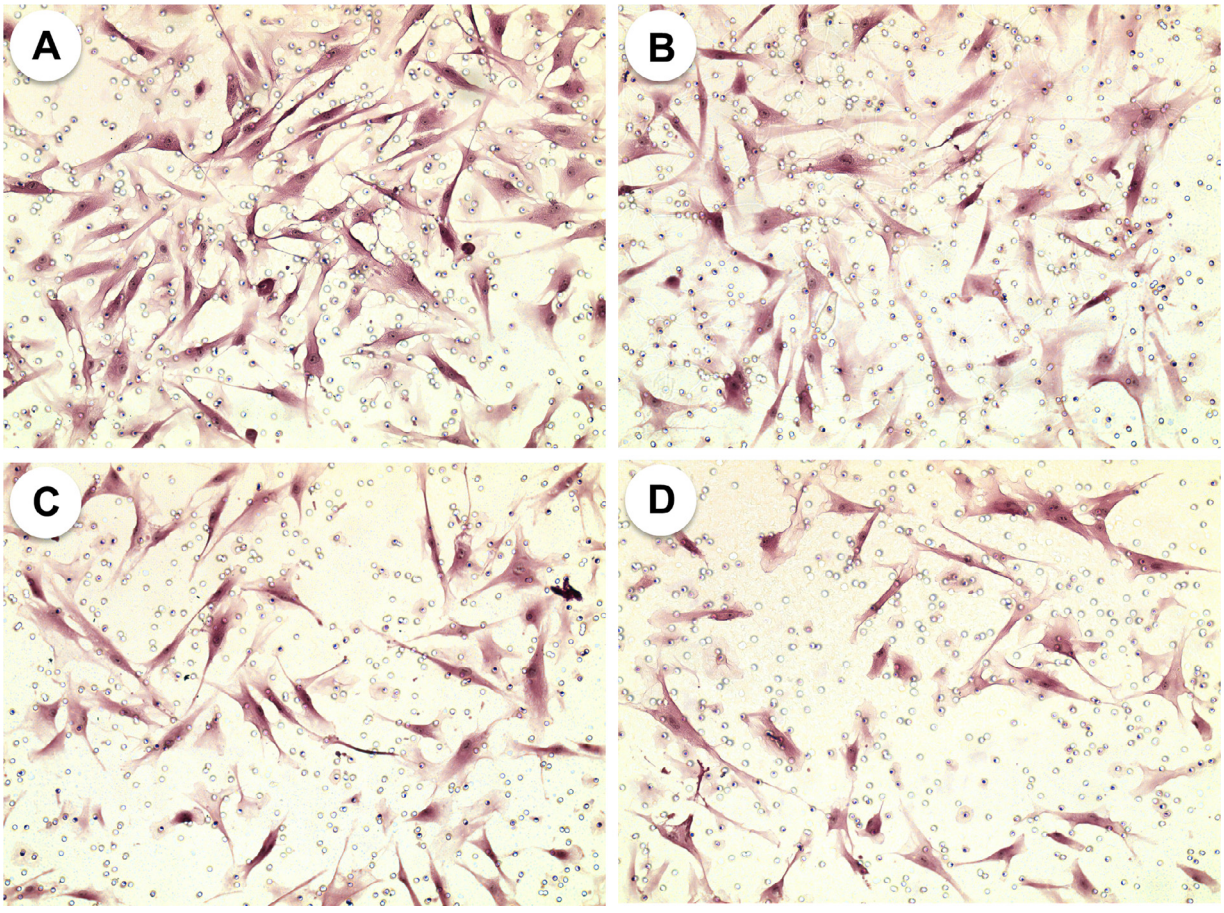

Figure 7 . Light microscopic photographs of HSFBs migration. These photographs were obtained at 20× magnification. GroupA, control, 0.9% normal saline (A); group B, 0.5U BTXA (B); group C, 1U BTXA (C); and group D, 2U BTXA (D).

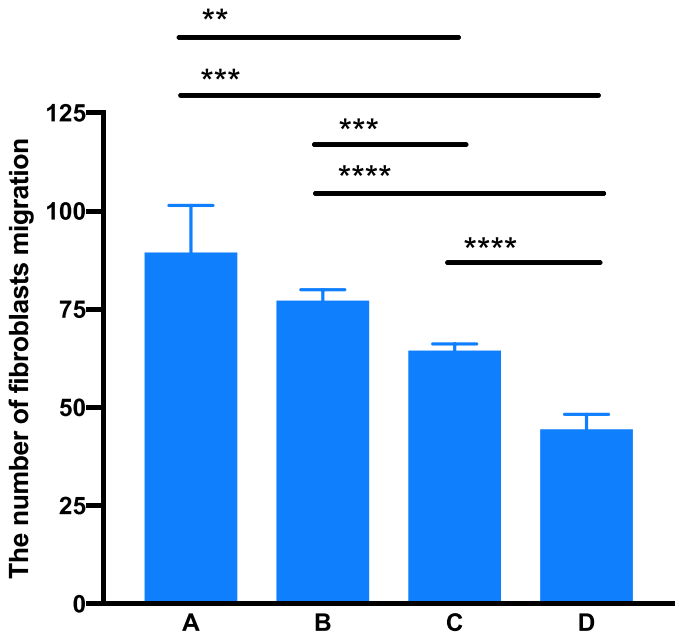

Figure 8 . The number of HSFBs migration were analyzed in the four groups (group A, 89.5±10.36; group B, 77.25±2.38; group C, 64.5±1.50; and group D, 44.5±3.28). Bar marked with an asterisk show significant differences in HSFBs migration reduction between treatment groups. GroupA, control, 0.9% normal saline; group B, 0.5U BTXA; group C, 1U BTXA; and group D, 2U BTXA. **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$.

and sequester TGF-β_1, which results in reduced biological activity of TGF-β_1. In this study, we found that the expression of decorin in HS tissue gradually increased with increasing dose of BTX-A, meanwhile the expression of TGF-β_1 in HS tissue gradually decreased.

It is usually very difficult to obtain a large piece of HS tissue from one patient that is sufficient for multiple intervention groups to repeat. Therefore, other studies can only design a study based on HS tissues from a wide variety of patients in a study group, which might increase the variation among groups. In this study, 24 specimens from the same HS tissue sample were split into four study groups, thus it significantly reduces the variation among groups. Moreover, we also repeated the process more than six times using six HS tissue samples that were collected from six patients.

Injecting a uniform quantity of triamcinolone precisely into the HS lesion without infiltrating the surrounding subcutaneous tissue is quite challenging because scar tissue is dense and resistant to tension. Inaccurate injection to the surrounding normal tissue might cause significant complications such as skin atrophy. The accompanying pain at the injection site will also be severe. Previous studies suggested that botulinum toxin could spread beyond the injection site.[31] However, the diffusing feature of BTX-A might have an additional benefit on releasing tension surrounding the scar by paralyzing underlying muscles while causing little complications. When used in intralesional injection, BTX-A neither passes hepatic metabolism nor crosses the blood-brain barrier; reports on serious systemic adverse reactions are rare, and there is no current evidence of teratogenicity.[32] However, high dose or frequent injections of botulinum toxin poses a risk to produce antibodies that result in decrease in efficacy or ineffectiveness in some patients when reinjected. Therefore, finding out the optimal BTX-A dose for effectively treating HS while reducing the dose of injection and injection times helps to avoid potential adverse side effects, which is of great importance.

The results in this study revealed that BTX-A intralesional monotherapy treats human HS in a dose-dependent manner. But the maximum dose of BTX-A in this study was 2 U, limited by the body weight of nude athymic mice. While the maximum dose of BTX-A for humans in a single use could reach 300-600 U, and the maximum dose for a single injection point could reach 60 U, [33-35] animal test based on larger body weight animals needs to be carried out to discover the dose-effect relationship of higher doses, and to further decide the optimal dose for treating HS in humans. Although the results revealed that BTX-A could inhibit the migration of HSFBs, further studies need to be conducted to investigate the underlying mechanisms.

Conclusions

BTX-A showed significant therapeutic efficacy when compared with the negative control group in a dose-dependent manner. BTX-A can reduce the weight of HS, upregulate the expression of decorin, downregulate the expression of TGF-β_1, and inhibit HSFBs proliferation and migration ability. This study indicates that BTX-A intralesional monotherapy treating HS should reach a threshold dose to achieve an effective treatment, and a high dose of BTX-A is more effective than a low dose.

Declaration of Competing Interest

None declared.

Funding

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Ethical approval

Approved by Peking University Institutional Review Board (LA2019097).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bjps.2021.03.062.

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