Comparison of Steroid and Botulinum Toxin Type A Monotherapy with Combination Therapy for Treating Human Hypertrophic Scars in an Animal Model

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Background: The authors evaluated the efficacy of a combined regimen of botulinum toxin type A (Botox) and a steroid (triamcinolone acetonide) for treating hypertrophic scars in comparison with the treatment with each drug alone.

Methods: Twenty excised human hypertrophic scar fragments obtained from surgically treated burn patients were divided into four groups: negative control (group A), triamcinolone alone (group B), Botox alone (group C), and a combination of triamcinolone and Botox (group D). These specimens were implanted into the backs of nude mice after intralesional injection from each group and were observed for 4 weeks. In total, 12 mice and 48 scars were studied. After 4 weeks, the hypertrophic scars were removed from the backs. The authors compared the scar weights, decorin staining, and the Cell Counting Kit-8 assay to evaluate treatment efficacy.

Results: Significant differences in scar weight reduction were observed among the four groups (group A, 10 percent; group B, 17 percent; group C, 23 percent; and group D, 30 percent; $p < 0.05$). Treatment groups (groups B, C, and D) showed strong decorin staining. Significant differences in reduction of fibroblast proliferation were observed among the four groups (group A, 0.58; group B, 0.44; group C, 0.21; and group D, 0.08; $p < 0.05$). Botox or triamcinolone intralesional monotherapy showed significant therapeutic efficacy compared with the control group. The combined therapy further exhibited a significant therapeutic effect compared with monotherapy.

Conclusion: This study indicates the potential of Botox and triamcinolone when combined for intralesional therapy in treating hypertrophic scars. (*Plast. Reconstr. Surg.* 140: 43e, 2017.)

After injury, Asians have a tendency to develop hypertrophic scars. Hypertrophic scars have abnormal collagen deposition and fibroblast activity, which commonly results in the loss of tissue function and disfigurement. Decorin is a native 118-kDa proteoglycan, which can inhibit fibroblast migration, proliferation, and fibroblast-mediated collagen contraction. Hypertrophic scars have decreased decorin expression compared with healthy skin. However, with the resolution of hypertrophic scarring, decorin expression increases to an almost normal level.[1]

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From previous literature, many drugs, such as steroids, calcium channel blockers, interferons, and 5-fluorouracil, have been suggested for the treatment of hypertrophic scars by means of intralesional injection.[2–4] However, there are variations in the therapeutic efficacy of each treatment, and they all have side effects. Thus, hypertrophic scar treatment continues to be a clinical challenge.

The standard and most commonly used treatment for hypertrophic scars is intralesional steroid injection. Steroids have diverse antiproliferative activities, including increased fibroblast apoptosis, augmented collagenase production, and decreased fibroblast proliferation. The International Advisory Panel on Scar Management recommends intralesional steroid injections for the treatment of keloids and hypertrophic scars.[5]

Botulinum toxin is derived from *Clostridium botulinum*. It is a potent neurotoxin that indirectly blocks neuromuscular transmission. There are various serotypes of botulinum toxin (A through F). Botulinum toxin type A (Botox; Allergan, Inc., Dublin, Ireland) has been used extensively to improve facial rhytides.[6] Holger et al. reported that botulinum toxin type A can minimize scarring in primates, as it prevents the contraction of muscles and skin during wound healing.[7] Xiao et al. showed that botulinum toxin type A can inhibit muscle tension and can affect the cell cycle distribution of fibroblasts derived from hypertrophic scars.[8,9] In another study by Xiao et al., botulinum toxin type A effectively inhibited the growth of fibroblasts derived from hypertrophic scars and influenced the expression of transforming growth factor (TGF)-β1.[10] According to these results, botulinum toxin type A may be a promising therapy for hypertrophic scars.

The process of scar formation is complex and not well understood. Each drug has a specific effect at different time points during scar formation. Combination therapy, using different drugs, has been suggested to target different processes during scar formation[11–13] and may strengthen the efficacy and reduce side effects.

In a previous study, we demonstrated that the combined use of a steroid and calcium channel blocker injections on excised human hypertrophic scars improved the expression of decorin of the scar tissue in an animal model.[14] Furthermore, in an animal study, we evaluated the effect of a cocktail regimen containing a steroid, calcium channel blocker, and interferon for hypertrophic scar treatment, which exhibited significant therapeutic efficacy over that of a single high dose of steroids.[15]

The objective of this study was to compare the therapeutic effects of botulinum toxin type A and a steroid alone with a combination of both on hypertrophic scars using an animal model implanted with human scar tissue. We compared scar weights, decorin staining, and Cell Counting Kit-8 (Sigma-Aldrich, Saint Louis, Mo.) assay to evaluate treatment efficacy.

MATERIALS AND METHODS

Study Design

Twenty excised human hypertrophic scar fragments, obtained from surgically treated burn patients, were placed in antiseptic flasks, hydrated in saline solution, and processed within 2 hours. Institutional review board approval and informed consent from the patients were obtained. Each human hypertrophic scar fragment was then divided into multiple small specimens (0.50 g with full thickness). In total, 48 specimens with intralesional injections of drugs were subcutaneously implanted into the backs of 12 nude athymic mice (BALB/nu-nu; National Applied Research Laboratories, Taipei, Taiwan). The 48 specimens were divided into four study groups: group A, control, 0.9% normal saline (0.05 ml/g); group B, triamcinolone acetonide (0.05 ml/g) (triamcinolone suspended injection, 40 mg/ml/vial; Tai Yu Chemical & Pharmaceutical Co., Zhudong, Taiwan); group C, botulinum toxin type A (0.05 ml/g) [botulinum toxin type A purified neurotoxin complex (Botox); 100 U/vial; 2.5 ml normal saline dilution, 40 U/ml]; and group D, triamcinolone acetonide (0.025 ml/g) and botulinum toxin type A (0.025 ml/g) (100 U/vial, 2.5 ml normal saline dilution, 40 U/ml) (Fig. 1).

Evaluation of Therapeutic Efficacy

Hypertrophic Scar Tissue Weight

Four weeks after implantation, the hypertrophic scars were harvested from the backs of the mice. The weights of the explanted hypertrophic scars were compared between the treatment and control groups.

Decorin Expression

Immunohistologic staining of specimens was performed to evaluate the difference in decorin expression. Implanted hypertrophic scars were harvested and paraffin-embedded. Sections of hypertrophic scar were stained with a primary antihuman decorin antibody (R&D Systems, Inc., Minneapolis, Minn.) at 4°C overnight. On the following day, the specimens were stained with a

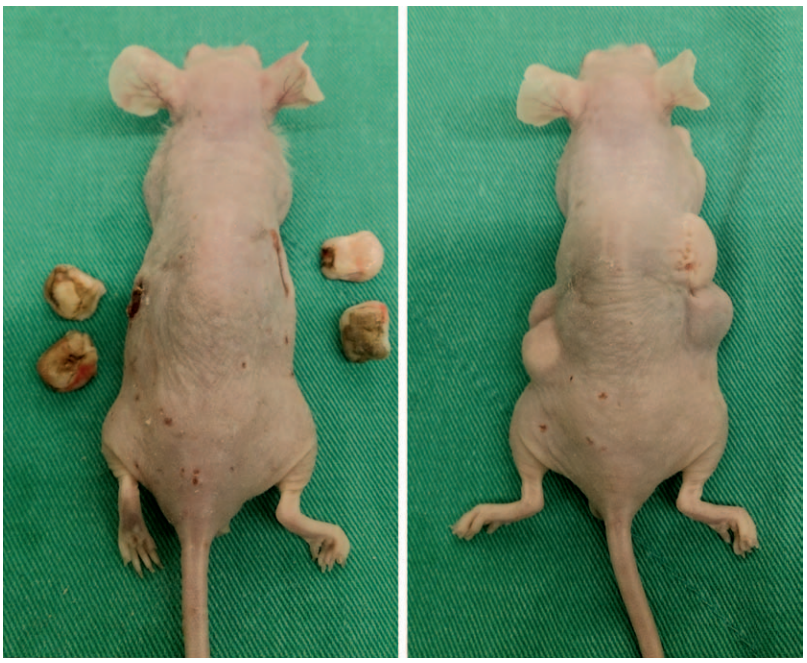

Fig. 1. Preimplantation (*left*) and postimplantation (*right*) images of nude mice. Each nude mouse had four human hypertrophic scars implanted in its back with or without drug injections. Group A, control, normal saline 0.9% (0.05 ml/g); group B, triamcinolone (0.05 ml/g); group C, botulinum toxin type A (0.05 ml/g); and group D, triamcinolone (0.025 ml/g) and botulinum toxin type A (0.025 ml/g).

secondary antihuman decorin antibody (Sigma Chemical Corp., St. Louis, Mo.) (1 mg antibody/ml) for 40 minutes. After rinsing, slides were counterstained with hematoxylin. Samples positive for decorin expression (Alphaphot2; Nikon, Tokyo, Japan) appeared dark brown to black.

Fibroblast Proliferation

The implanted hypertrophic scars were harvested from the backs of the mice 4 weeks after implantation. The dermis of the hypertrophic scars from each group was fragmented into 5.0-mm^2 pieces. Fibroblasts were obtained by digesting these specimens with 0.2% collagenase (Sigma) for 3 hours, 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.

After 10 days of culture, the activity of the fibroblasts from the four groups was analyzed using a proliferation assay, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl) -2H-tetrazolium,monosodium salt] (Cell Counting Kit-8). The fibroblast suspension (100 μl) was distributed into a 96-well plate with 5000 cells/well in Dulbecco's Modified Eagle Medium and 10% fetal bovine serum and incubated for an additional 24 hours. On the following day, 10 μl of Cell Counting Kit-8 solution was added to each well and incubated for 4 hours. Sodium dodecyl sulfate (10 μl of 1% weight/volume) was added to each well and the absorbance was measured at 450 nm using a microplate reader (Infinite M200 PRO; Tecan Group Ltd., Männedorf, Switzerland). All samples were read in triplicate and all of the reported experiments were performed 12 times.

Statistical Analysis

In this study, all statistical analyses were performed using IBM SPSS Version 22.0 (IBM Corp., Armonk, N.Y.). Statistical differences were evaluated by analysis of variance, where a value of $p < 0.05$ was considered statistically significant.

RESULTS

Hypertrophic Scar Tissue Weight

Four weeks after implantation, the weight of the excised human hypertrophic scars was measured

as a percentage of the original weight calculated. In group A (no drug), there was a natural degradation process of the hypertrophic scars and the weight was reduced to 90 percent of the original. The highest decrease in hypertrophic scar weight was noted in group D (triamcinolone, 0.025 ml/g; and botulinum toxin type A, 0.025 ml/g), which was reduced to 70 percent of the original weight. The hypertrophic scar weight was reduced to 83 percent and 77 percent in groups B (triamcinolone, 0.05 ml/g) and C (botulinum toxin type A, 0.05 ml/g), respectively. Significant differences were observed in the weight reduction of the hypertrophic scars among all four groups (group A, 10 percent; group B, 17 percent; group C, 23 percent; and group D, 30 percent) (Fig. 2).

Decorin Expression

The expression of decorin is lower in hypertrophic scars than in normal dermis. Four weeks after implantation, the hypertrophic scars were harvested from the backs of the mice and immunohistochemically stained for decorin expression. Specimens positive for decorin appeared dark brown to black. All treatment groups (groups B, C, and D) showed strong decorin staining. The staining of group D was stronger than that of groups B and C as shown in 32 of 48 samples. In group A (no drug), there was low and patchy decorin staining, indicating saline treatment of hypertrophic scars (Fig. 3).

Fibroblast Proliferation

Fibroblast proliferation among the four groups was analyzed using the Cell Counting Kit-8 assay. Fibroblasts cultured from group A (no drug) had a higher rate of proliferation compared with fibroblasts cultured from treatment groups (groups B, C, and D). Significant differences in fibroblast proliferation were observed among the four groups (group A, 0.58; group B, 0.44; group C, 0.21; and group D, 0.08; $p < 0.001$). [**See Figure, Supplemental Digital Content 1,** which demonstrates Cell Counting Kit-8 assay of fibroblasts cultured from the implanted hypertrophic scars. Significant differences in fibroblast proliferation were observed among the four groups (group A, 0.58; group B, 0.44; group C, 0.21; and group D,

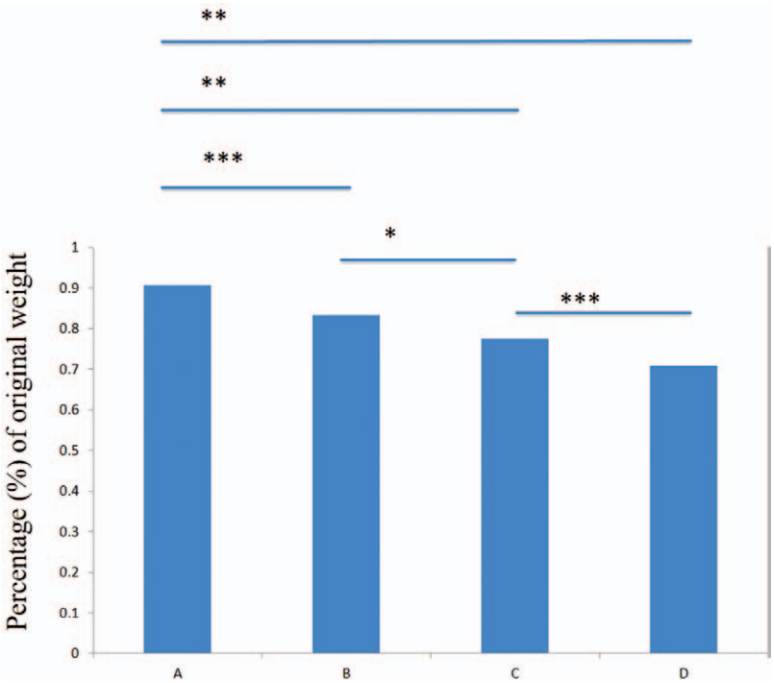

Fig. 2. Percentage of residual hypertrophic scar weight 4 weeks after implantation. Hypertrophic scar weight reduction was observed in the four groups (group A, 10 percent; group B, 17 percent; group C, 23 percent; and group D, 30 percent). Bars marked with an *asterisk* show significant differences in weight reduction between treatment groups. Group A, control, normal saline 0.9% (0.05 ml/g); group B, triamcinolone (0.05 ml/g); group C, botulinum toxin type A (0.05 ml/g); and group D, triamcinolone (0.025 ml/g) and botulinum toxin type A (0.025 ml/g). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

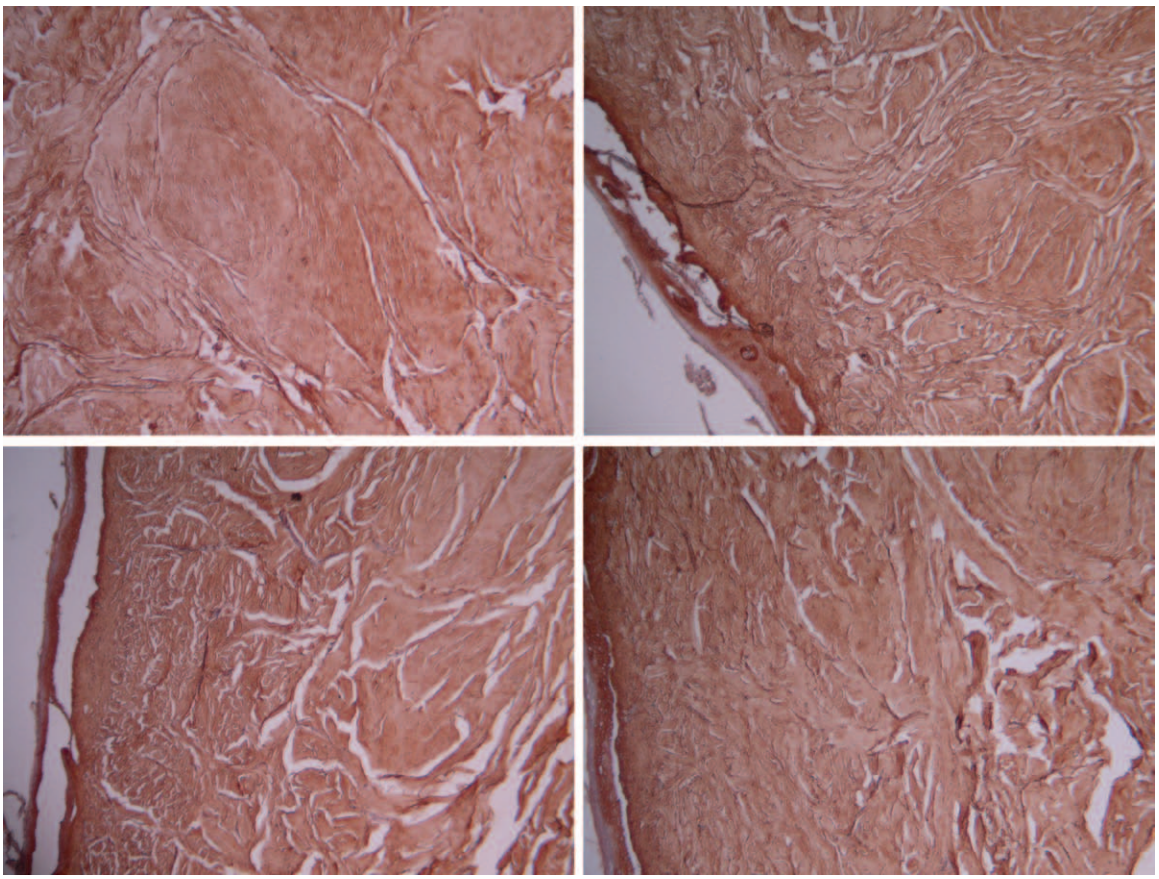

Fig. 3. Decorin expression in hypertrophic scar tissue 4 weeks after treatment. These photographs were obtained at 40× magnification (group A, *above*, *left*; group B; *above*, *right*; group C, *below*, *left*; group D, *below*, *right*). Treatment groups (B, C, and D) showed strong decorin staining, whereas the staining in group D is stronger than in groups B and C. In group A (no drug), there is low and patchy decorin staining, indicating saline treatment of hypertrophic scars. Group A, control, normal saline 0.9% (0.05 ml/g); group B, triamcinolone (0.05 ml/g); group C, botulinum toxin type A (0.05 ml/g); and group D, triamcinolone (0.025 ml/g) and botulinum toxin type A (0.025 ml/g).

0.08). ***Significant differences ($p < 0.001$) in optical density between treatment groups. Group A, control, normal saline 0.9% (0.05 ml/g); group B, triamcinolone (0.05 ml/g); group C, botulinum toxin type A (0.05 ml/g^3); and group D, triamcinolone (0.025 ml/g) and botulinum toxin type A (0.025 ml/g), ***http://links.lww.com/PRS/C200***.] These results indicate that the proliferation of fibroblasts was inhibited with the combined triamcinolone and botulinum toxin type A treatment more than that in any other group.

DISCUSSION

To our knowledge, this is the first study to compare a steroid and botulinum toxin type A monotherapy with a combination therapy for treating human hypertrophic scars in an animal model. Human hypertrophic scars implanted into a subcutaneous pocket in nude athymic mice 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.[16–18]

There is no universally accepted treatment for hypertrophic scars or keloids, but the most commonly used treatment is intralesional steroid injection.[19] Steroids can block fibroblast proliferation, decrease collagen formation, augment collagenase production, and reduce contraction. Triamcinolone is one of the most commonly used steroids, and its intralesional administration has shown a 50 to 100 percent improvement in clinical efficacy.[20] Injecting a uniform quantity of triamcinolone into the hypertrophic scar and not the surrounding subcutaneous tissue is quite challenging because the scar tissue is dense and resistant to receiving fluid. Laser-assisted delivery of steroids potentially offers a safe and effective therapy for hypertrophic scars.[21]

However, there are multiple adverse effects, including injection-site pain, skin atrophy, telangiectasia, and pigmentary changes, which are not acceptable by most patients. Because there are many side effects of steroids, we considered reducing the dosage and combining it with other drugs, such as botulinum toxin type A, for clinical use.

Botulinum toxin type A is used not only in aesthetic medicine but also in reconstructive scar prophylaxis. Clinical trials have reported that botulinum toxin type A injection into dermal tissue can improve cheiloplasty scarring.[22–24] Zelken et al. preoperatively injected botulinum toxin type A in frontalis muscles, which enhanced the forehead flap donor-site scar appearance.[25] Kim et al. demonstrated positive results for the injection of botulinum toxin type A for the treatment of fresh thyroidectomy scars.[26] However, the underlying mechanisms of botulinum toxin type A in hypertrophic scar prophylaxis are not well understood. Xiao et al. reported that botulinum toxin type A could inhibit muscle tension, affect the cell cycle distribution of fibroblasts, and suppress fibroblasts proliferation by inhibiting the expression of TGF-β1.[8–10] In 2015, Jeong et al. reported that botulinum toxin type A directly inhibits fibroblast-to-myofibroblast differentiation in vitro.[27] The results mentioned above are consistent with our experiment. In this study, botulinum toxin type A monotherapy was significantly more effective in hypertrophic scar weight reduction, decorin staining, and fibroblast suppression than triamcinolone alone and control treatment.

Although botulinum toxin type A, on administration into scar tissues, also induces pain at the injection site, the adverse effect of a botulinum toxin type A injection is very low compared with that of triamcinolone. Botulinum toxin type A does not cross the blood-brain barrier and there is no current evidence of teratogenicity.[28] Common complications include ecchymosis and headaches, which can be tolerated by most patients.[29] However, there is a risk of botulinum toxin spreading from the injection site and weakening the adjacent muscles. The U.S. Food and Drug Administration investigated a report on adverse reactions in patients treated with botulinum toxin, which suggested spread of the toxin beyond the injection site.[30] The combined use of botulinum toxin type A and triamcinolone for hypertrophic scar treatment may help reduce the dosage of each drug and decrease their side effects. The results of this study revealed that intralesional combination therapy (a half dose of botulinum toxin type A and a half dose of triamcinolone) is significantly more effective than a full dose of botulinum toxin type A or triamcinolone alone in hypertrophic scar weight reduction, decorin staining, and fibroblast suppression in an animal model. Both triamcinolone and botulinum toxin type A have the potential to block fibroblast proliferation and reduce scar size. On the basis of these results, we hypothesize that there may be a synergistic effect between botulinum toxin type A and triamcinolone in fibroblast suppression. We plan to perform an additional study to compare triamcinolone and botulinum toxin type A monotherapy with combination therapy for patients with hypertrophic scars.

The greatest difference between other studies and our study is that this was an in vivo study. In this study, human hypertrophic scars were implanted in a subcutaneous pocket in mice after an intralesional drug injection to simulate the progression of hypertrophic scars in human patients, but there are several limitations to this study. The hypertrophic scar specimens from different patients increased the variation in groups A, B, C, and D. There is no animal weight comparison in this study, which could interfere with the resulting hypertrophic scar tissue weight after 4 weeks of implantation. Although the results revealed that botulinum toxin type A and the combination therapy have the potential for scar inhibition, the underlying mechanisms require further study. We cannot explain why botulinum toxin type A alone inhibits fibroblast proliferation more than triamcinolone monotherapy. Further studies need to be conducted to find the molecular network of botulinum toxin type A and triamcinolone in fibroblasts derived from hypertrophic scars.

CONCLUSIONS

Botulinum toxin type A or triamcinolone intralesional monotherapy showed significant therapeutic efficacy in hypertrophic scar treatment compared with the control group. Furthermore, the combined intralesional injection of botulinum toxin type A and triamcinolone exhibited a significant therapeutic effect over triamcinolone or botulinum toxin type A alone. This study indicates the potential of botulinum toxin type A and triamcinolone intralesional combined therapy for use in treating hypertrophic scars after surgery, burns, or trauma.

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