

Effects of Intradermal Injection of Botulinum Toxin Type A in a Porcine Skin Model

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Abstract

Background

Intradermal injection (ID) of Botulinum toxin Type A (BTX-A) remains a relatively new technique for treating muscle spasm disorders and cosmetic indications. The effects and mechanisms of action are under investigated and still controversial.

Objectives

This study aimed to evaluate the effects of intradermal injection of botulinum toxin type A (BTX-A) on normal porcine skin using histopathological and immunohistochemical analyses.

Materials and Methods

A mixed-breed female pig was used. Both sides of the paraspinal region of the dorsum were randomly divided into 10 square areas. Five areas received intradermal injections of 0.05 mL botulinum toxin A (150 kDa; Dysport®, Ipsen, UK). Other five areas were injected with 0.05 mL of normal saline as controls. The observation of effected skins and 6 mm. full-thickness skin punch biopsies were done at 3, 7, 14, 21, 28 days after injection. Routine histopathology, modified Masson's trichrome stain (MS) and immunohistochemistry were performed to evaluate inflammatory response, blood vessel proliferation, fibroblast proliferation, collagen arrangement and density. The results showed that intradermal BTX-A injection induced transient mild inflammatory response with blood vessel proliferation. Grading MS stain of collagen density and arrangement showed no significant difference between BTX-A and control groups. Immunohistochemistry demonstrated increased fibroblast proliferation, as evidenced by Ki-67–positive fibroblastic nuclei, and increased intensity of collagen type I immunoreactivity.

Conclusions

Intradermal BTX-A injection in normal porcine skin demonstrated no local or systemic toxic effects and was associated with increased fibroblast proliferative activity and collagen type I expression.

Introduction

Botulinum Toxin (BTX) is a presynaptic neuromuscular blocking agent inducing selective and reversible muscle weakness [1]. Botulism toxin type A (BTX-A) was first introduced for treatment of disorders as strabismus and blepharospasm [2, 3]. BTX-A is clinically used in neurologic, ophthalmic, orthopedic and plastic surgery indications including hyperhidrosis, hyperfunctional facial lines and wrinkles as well as spastic disorders [4, 5, 6]. Currently, many off-label cosmetic adaptation of BTX-A are under evaluation and still controversial.

Some clinical uses showed a face-lifting effect due to increased collagen synthesis after intradermal injection of BTX-A to the mid and lower face; greater collagen synthesis, lower sebum production and smaller facial pore size may play an important role for the effects [7]. However, some other controversial hypotheses of face-lifting effect might be caused by percutaneous needle pricks themselves after intradermal injection [8, 9, 10]. Although many studies were launched and still controversial, no experimental trials have been conducted in laboratory animals to study the effect and mechanism of intradermal injection of BTX-A [11].

Dermal connective tissue matrix consists mainly of collagen and elastic fibers organized in a coherent pattern, principally bundles of collagen bordered by the elastic fibers. Type V collagen represents about 3% of dermal collagen and is found in nearly all connective tissue [12]. Several human studies demonstrated the correlation of intradermal BTX-A injection and the increasing of fibroblast and collagen synthesis [7, 11].

Antigen Ki-67 (MKi-67) is a protein encoded by the MKi-67 gene. Ki-67 protein is present during all active phases of the cell cycle but is absent from resting cells; therefore, Ki-67 is an excellent marker for determining the growth fraction of a given cell population [13]. The porcine skin is commonly used as the skin and wound healing study model because it has suitable properties such as mimicking human physiological function. Porcine skin is thicker and has less vascularity than human skin [14].

The porcine skin is commonly used as the skin and wound healing study model in medical experiments, because it has suitable properties such as the mimic physiological function or disease, availability, easy handling, long survival for functioning, fit with commonly used animal housing, and a suitable size for multiple samples. The porcine skin is hairless and has tight attachment between skin and subcutaneous like human. Cutaneous blood supply and wound healing characteristics in porcine skin are similar pattern to the humans. However, porcine skin is thicker and less vascularity than the human skin [14].

The purpose of present study aimed to conduct the study of the effect of intradermal injection of BTX-A on normal skin in the porcine model. Inflammatory reaction, number of blood vessels, collagen arrangement, collagen density, intensity of collagen type I and proliferation index of fibroblasts were observed and evaluated comparing to the controls after intradermal injection.

Materials and methods

Experimental Animals

A healthy female mixed-breed pig (Landrace, Large White and Duroc), weighing approximately 30 kg, was obtained from a licensed commercial swine farm in Thailand and transferred to the experimental facility of the Faculty of Veterinary Science, Chulalongkorn University, for this study. Permission for acquisition and research use was obtained from the supplier/farm owner before the animal was enrolled. The inclusion criteria for experimental processes were animals in good general health as determined by historical health records with normal physical examination and clinical pathology results. Exclusion

criteria for animals were those considered unsatisfactory for the study, including poor health, aggressive behavior, and abnormal blood results. The pig was acclimatized for 48 hours prior to housing in the experimental facility. Physical examination, complete blood count (CBC), and blood chemistry were evaluated for health checking before the surgical procedures. The pig was fed ad libitum with pig diet (CP-953, CPF Public Company Limited, Thailand) and tap water.

Experimental protocols

All procedures were performed according to protocols approved by Chulalongkorn University of Animal Care and Use (IACUC), Thailand (protocol no. 1673018). All guidelines for animal care and use from the applicable national, international and/or institutional protocols were followed. This study is described in accordance with the ARRIVE guidelines.

BTX- A Preparation

A concentration of 70 units/mL abobotulinumtoxinA was prepared by reconstituting a 500-unit vial of Dysport® (Ipsen, UK) with 7 mL of sterile, preservative-free 0.9% sodium chloride, sterile, preservative – free 0.9% Sodium Chloride and stored in a refrigerator (2° to 8° C) prior to use.

Experimental design and anesthesia protocol

Both sides of the paraspinal region of the dorsum were divided into 10 square areas of 3 cm wide and 3 cm long with 5 cm distance apart (Figure S1a) by simple flipping a coin randomization. Aseptic protocols were done before the injection. The injected skin area was clipped and scrubbed with 1% chlorhexidine solution with spraying the 10% povidone iodine and 70% isopropyl alcohol for three times. The demographic design for injection was shown in figure S1. Five areas were intradermal injected with 0.05 mL of BTX-A and the others were injected with 0.05 mL of normal saline as control (Figure S1a). The observation of injected areas and 6 mm full-thickness skin punch biopsies were done at 3, 7, 14, 21 and 28 days after injection.

The pig was withdrawn food and water for 8 hours and 6–12 hours, respectively to prevent aspiration. The pig was premeditated with intramuscular administration of 2 mg/kg xylazine HCl mixing with 4 mg/kg of tiletamine and zolazepam (Zoletil, Virbac animal health Inc., France) (IM), and 0.5 mg/kg morphine sulphate. General anesthesia was induced with intravascular injection of 6–8 mg/kg and maintained with 1–3% isoflurane and oxygen after intubation. Oxygen saturation, heart rate, respiratory rate and rectal temperature were routinely measured and were monitored throughout the experiment. The administration of 10 mL/kg/hr crystalloid fluid was done via cephalic vein or marginal ear vein during anesthesia. During the recovery period, the pig was placed on the soft floor and kept warm with warm air blowing machine. For preventing infection, the pig was received enrofloxacin (Baytril™, Bayer health care, pharmaceuticals, Germany) 5 mg/kg (PO) pre-operative and post-operatively. The

intramuscular injection of 0.5 mg/kg morphine sulphate and 4 mg/kg tramadol HCl (TramCare, Advacare pharma., USA) (PO) for pain management and continued twice daily until the end of the experiment.

Special staining and immunohistochemistry protocols

Modified Masson's trichrome staining protocol was modified from Kiernan [15]. Deparaffinization was done by submerging the tissue sections into the series of absolute xylene and ethanol. The slide then was submerged in warmed Bouin's solution at 60° C for 45 minutes. To differentiate nuclei, the tissue was immersed in modified Wiegert's haematotoxin for 8 minutes. In order to stain cytoplasm and erythrocytes, slide was put into anionic dyes, acid fuchsin (C.I. 42590, Merck, Germany) for 5 minutes. The slide was treated with phosphomolybdic acid solution for 10 minutes as a mordant and methyl blue (C.I. 42780, Merck, Germany) solution for 5 minutes to stain fibroblast and collagen. After that, slide was treated with 1% acetic acid solution for 1 min. Slide then was dehydrated into a series of alcohol dipped into absolute xylene. The slide was mounted with a cover slip using DPX Mounting.

Collagen type I

Immunohistochemical analysis of collagen type I was performed on paraffin- embedded tissues. Briefly, the section was deparaffinized the series of absolute xylene and ethanol. The slide then was immersed in phosphate buffered saline (pH6) and then put in the microwave for 5 minutes. The section was treated with 3% H₂O₂ solution to blocked the endogenous peroxidase activity, and washed in phosphate buffered saline. The slide was incubated with primary antibody for collagen type I (Rabbit polyclonal Anti-Collagen I antibody) overnight at 4°C. Envision was used for detection of the immunoreactive staining. The section was reacted with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. The positive cytoplasmic staining of collagen type I was shown as brown color.

Ki-67

Immunohistochemical analysis was performed on the Leica Microsystems Bond max System (Leica Microsystems, Bannockburn, IL). Slides were incubated for 60 minutes at 60°C and treated with Bond Dewax Solution. Epitope retrieval was performed by incubating the slides in Bond Epitope Retrieval Solution 2 for 30 minutes at 100°C. Primary antibody was applied for 40 minutes at room temperature followed by 3 consecutive rinses with Bond Wash Solution. 3% hydrogen peroxide was then applied for 5 minutes to peroxide block and rinsed 3 times with Bond Wash Solution. Polymer Poly-HRP IgG (Leica Microsystems) was applied for 8 minutes and rinsed 3 times with Bond Wash Solution. The diaminobenzidine chromogen was applied for 4 minutes and then the slide was counterstained with hematoxylin for 5 minutes. Ki- 67 showed nucleus staining as a positive brown color.

Histopathological Evaluations

Full-thickness skin biopsies, 6 mm diameter, were performed. 5 tissue samples from each control and treatment group were collected on days 3, 7, 14, 21 and 28 after injection. The specimens were fixed in 10% neutral buffer formalin for at least 24 hours before routine histological processes. The samples were cut into 4–6 μ m thickness and stained with hematoxylin-eosin for histological evaluation.

For studying the tissue effects of BTX-A in the skin, microscopic findings of inflammatory response, blood vessel proliferation, and collagen arrangement and density were observed and evaluated by a pathologist with blind technique under the light microscopy on high power fields ($\times 400$). The severity of inflammatory cell infiltration and blood vessel proliferation was semiquantitatively assessed by counting inflammatory cells (Fig. 1A) and blood vessels (Fig. 2A) in 50 randomly selected high-power fields ($\times 400$) per group. The fibroblastic proliferation was determined by counting a number of immunohistological positive nucleus of Ki-67 in nucleus of fibroblasts (Fig. 3A) that infiltrated at injected areas in random 50 histological areas at high power field in each group.

In each group of tissues that stained with Masson's trichrome stain, the collagen arrangement and density were evaluated by the color intensity and arrangement of the collagen infiltrated in the affected areas in random 5 areas of 10x magnification power fields. The collagen density grading was determined as follow; Grade 0 (absent): negative staining, Grade I (mild): Pale blue staining of the collagen networks, Grade II (moderated): Blue staining of the collagen networks and Grade III (intense): Dark blue staining of the collagen mesh work (Fig. 4A, 4B, 4C and 4D). The collagen arrangement grading was evaluated as follows; Grade I: unsystematic arrangement: Grade II moderate connection of the collagen bundles and Grade III: continuous and regular arrangement of the collagen fibers (Fig. 5A, 5B and 5C).

To study and evaluate collagen type I in the injected areas, the color intensity of immunohistochemical positive areas of collagen was evaluated in the random 5 areas at high power field in each group. The grading of collagen type I intensity was determined as follow; Grade 0 (absent): negative staining, Grade I (mild): Pale brown staining of the collagen networks, Grade II (moderated): Brown staining of the collagen networks and Grade III (intense): Dark brown staining of the collagen networks (Fig. 6A, 6B and 6C).

Statistical analysis

The number of injected skin sites and biopsy specimens collected from the pig was determined a priori, and data were analyzed using non-parametric statistical methods. To compare differences between BTX-A-treated and control sites at each observation time point, paired data were analyzed. Quantitative and semi-quantitative variables, including numbers of inflammatory cells, blood vessels, Ki-67-positive fibroblastic nuclei, collagen arrangement grades, collagen density grades, and collagen type I immunoreactivity grades, were summarized as median values with interquartile ranges (IQRs). Paired comparisons between treatment and control groups were performed using the Wilcoxon signed-rank test. A P value < 0.05 was considered statistically significant. All statistical analyses were conducted using SPSS for Windows (version 22.0; IBM Corp., Armonk, NY, USA).

Results

Gross appearance

After intradermal injection, skin of both control and BTX-A groups showed immediately redness, hemorrhage and edema. The severity of the lesions of both the control and BTX-A groups decreased on day 1 after injection. On day 3, both groups showed no remarkable lesion on the injected sites (Figure S1b).

Histopathological appearances

The histopathological finding showed that both control and BTX-A treated groups after injection had the infiltration of inflammatory cells, mainly lymphocytes and macrophages around the blood vessel in the dermis (Fig. 1A and 1B).

The Severity of Inflammation

The control group showed less infiltration of total inflammatory cells than the BTX-A group on days 3, 14 and 21. There was statistically significant difference of total inflammatory cells between the control and the BTX-A groups on day 21 post-operative days (Fig. 1C).

Blood Vessel Proliferation

There was a statistically significant difference of the number of blood vessels between the control and the BTX-A groups on day 3 post-operative day ($p < 0.05$). On day 7, 14, 21 and 28 after injection, the average number of blood vessels in the BTX-A group trended to higher than controls. However, there was no significant difference between control and treatment groups (Fig. 2B).

Fibroblasts Proliferation

There were statistically significant differences of the number of Ki-67 positive nucleus of fibroblasts between the control and the BTX-A groups on day 7 and 21 post-operative days (Fig. 3B).

Special Stains for Collagen Density and Arrangement

Collagen density

There were no significant differences of the intensity of collagen density between the control and BTX-A groups of all time points (Fig. 4D).

Collagen arrangement

There were no statistically significant differences ($p > 0.05$) of the collagen arrangement grades between the control and BTX-A groups of all observation times (Fig. 5D).

Immunohistochemistry

Collagen Type I

There was statistically significant difference of the intensity of collagen type I between the control and the BTX-A groups at day 7 post-operative days (Fig. 6D).

Discussion

Intradermal (ID) injection of Botulinum Toxin Type A (BTX-A) represents an evolving therapeutic frontier, offering a relatively novel alternative for cosmetic and dermatological applications [8]. Despite its growing clinical use, many off-label adaptations remain under investigation, and the precise physiological effects of ID administration continue to be a subject of academic debate. While human clinical studies have noted improvements in facial skin laxity, sebum production, and pore size [7, 16, 17], objective experimental data verifying these mechanisms in a controlled animal model have been lacking.

Our study is the first to utilize a porcine skin model to evaluate the pathological and immunohistochemical effects of ID BTX-A. The porcine model was selected due to its significant physiological and anatomical similarities to human skin, including thickness, hair density, and wound healing characteristics [14]. Our results indicate that ID BTX-A administration is safe and well-tolerated in this model, with no observed mortality or systemic adverse reactions. Pathological assessments revealed that while BTX-A induced neovascularization and mild inflammatory responses, these did not differ significantly from control groups, suggesting low antigenicity and a favorable safety profile for therapeutic use.

In contrast to the conventional intramuscular (IM) route, which carries risks of muscular paralysis, weakness, and respiratory failure [18, 19], the ID route appears to localize effects to the superficial dermis and epidermis. Previous studies on IM injections have reported significant alterations in muscle fiber composition and increased mast cell infiltration [20, 21]. Conversely, our ID investigation found only mild inflammatory responses characterized by lymphocytes and macrophages, notably lacking mast cell infiltration. This suggests that the ID route avoids the deeper myogenic complications associated with standard BTX-A therapy, providing a more localized and potentially safer delivery method [22, 23, 24].

A significant finding in this study was the evidence of increased vessel proliferation, suggesting that BTX-A may play a role in stimulating angiogenesis. This aligns with previous research indicating that BTX-A can enhance neovascularization in wound healing and skin graft scenarios [25].

Furthermore, while traditional histological staining showed no significant change in overall collagen density or arrangement, immunohistochemical analysis provided deeper mechanistic insights. We observed a marked increase in fibroblast proliferation, as evidenced by Ki-67 expression in fibroblastic nuclei, alongside increased intensity of Collagen Type I staining. These findings suggest that BTX-A actively stimulates the dermal regenerative process. This corroborates the work of Chang [7], who

observed neocollagen synthesis in human biopsies, and Oh [11], who demonstrated that BTX-A upregulates Type I collagen expression while inhibiting degradation in vitro.

Collectively, these data provide a robust experimental foundation for the clinical observations of "face-lifting" effects, attributing them to active dermal remodeling rather than mere muscular relaxation. For the veterinary community, this porcine model not only validates the safety of ID BTX-A but also establishes a clear histopathological benchmark for future comparative dermatological research.

This study has certain limitations. The experiment was conducted in a single porcine subject; however, the use of multiple intradermal injection sites with paired intra-animal controls minimized inter-individual variability and allowed robust comparative analysis. In addition, collagen remodeling was assessed primarily using histochemical and immunohistochemical methods, and future studies incorporating quantitative molecular analyses may further elucidate extracellular matrix dynamics. Finally, the observation period was limited to 28 days, and longer-term studies may be required to assess chronic dermal remodeling. Despite these limitations, the study provides controlled experimental evidence supporting the biological effects and safety of intradermal BTX-A injection in normal skin.

Conclusion

In conclusion, intradermal injection of botulinum toxin type A in normal porcine skin resulted in mild and transient inflammatory responses without evidence of local or systemic toxicity. Although no significant alterations in overall collagen density or arrangement were detected by Masson's trichrome staining, increased fibroblast proliferation and collagen type I immunoreactivity were observed. These findings support the biological activity and relative safety of intradermal BTX-A and provide experimental evidence for its potential applications in dermatologic and cosmetic procedures.

Abbreviations

BTX-A

botulinum toxin type A

CBC

complete blood count

CE-CAC

Center of Excellence for Companion Animal Cancer

DAB

diaminobenzidine tetrahydrochloride

H&E

hematoxylin and eosin

IACUC

Institutional Animal Care and Use Committee

ID

intradermal injection
IHC
immunohistochemistry
IM
intramuscular
IQR
interquartile range
MS
modified Masson's trichrome stain.

Declarations

Ethics approval and consent to participate

All procedures were performed according to protocols approved by Chulalongkorn University Animal Care and Use Committee (IACUC), Thailand (protocol no. 1673018). All applicable national, international, and institutional guidelines for animal care and use were followed, and the study is reported in accordance with the ARRIVE guidelines. The pig used in this study was obtained from a licensed commercial swine farm in Thailand with permission for acquisition and research use from the supplier/farm owner. No privately owned client animal was enrolled in the study.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization: Pasakorn Brikshavana, Sumit Durongpongton, and Theerayuth Kaewamatawong. Formal analysis: Panadda Darapong. Methodology: Panadda Darapong and Pasakorn Brikshavana. Investigation: Panadda Darapong, Pasakorn Brikshavana, and Theerayuth Kaewamatawong. Writing -

original draft: Panadda Darapong and Theerayuth Kaewamatawong. Writing - review and editing: Theerayuth Kaewamatawong. All authors read and approved the final manuscript.

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Figures

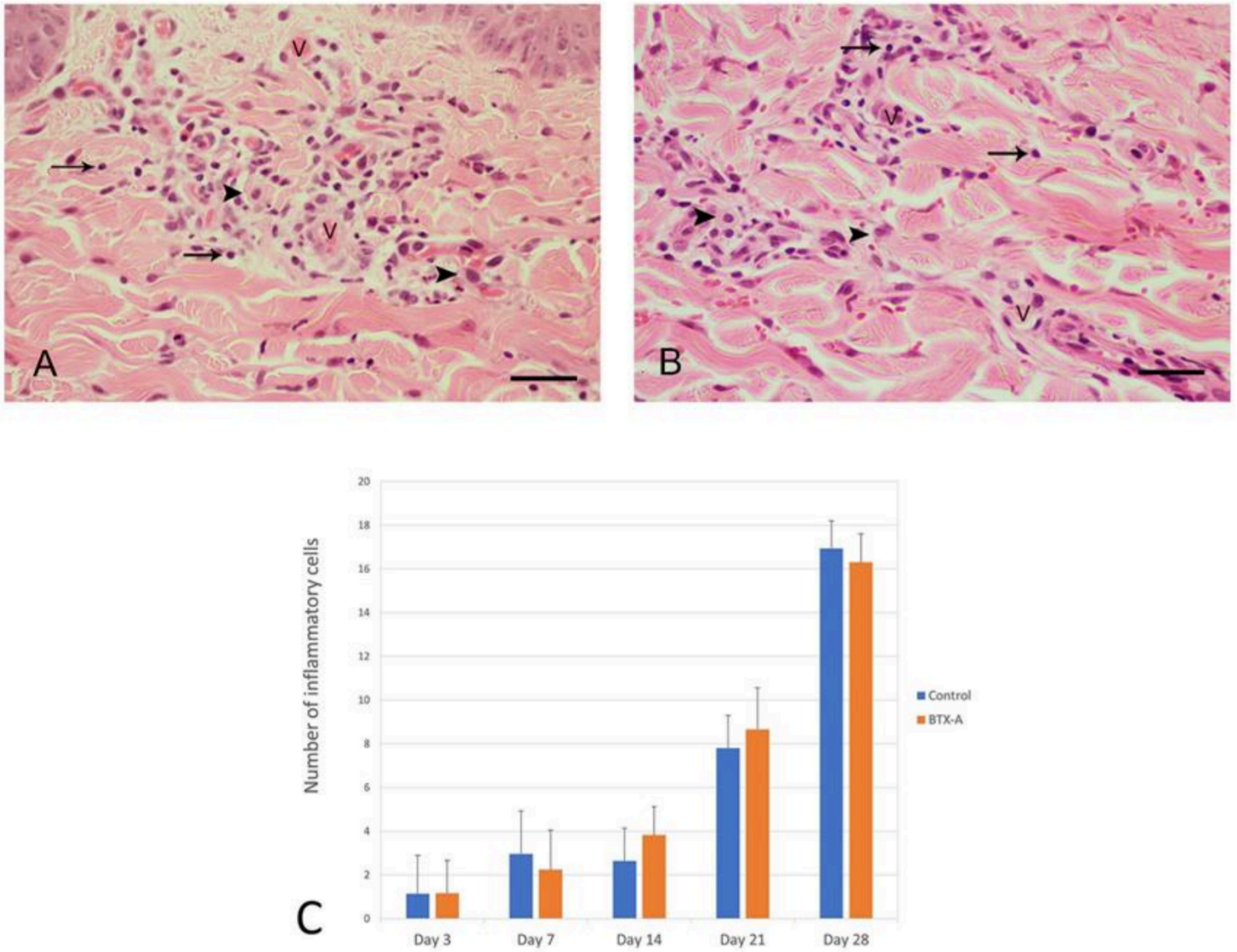


Figure 1

Inflammatory cells infiltration, mainly lymphocytes (arrows) and macrophages (arrowheads), was found around blood vessel (v) in control group (A) and BTX-A treated group (B) at day 3 after injection, H&E, 100x. The bar chart demonstrated grading of the intensity of collagen type I in the BTX-A and control groups; $p<0.05$ (C).

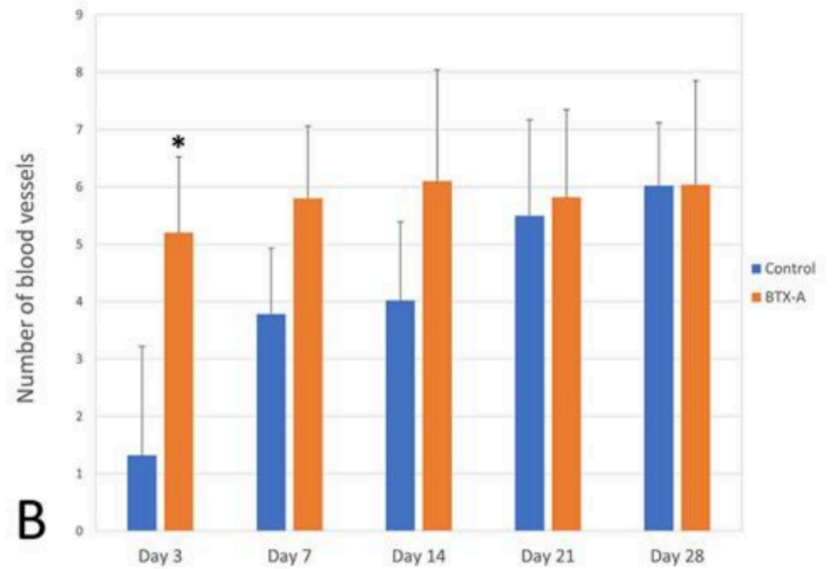
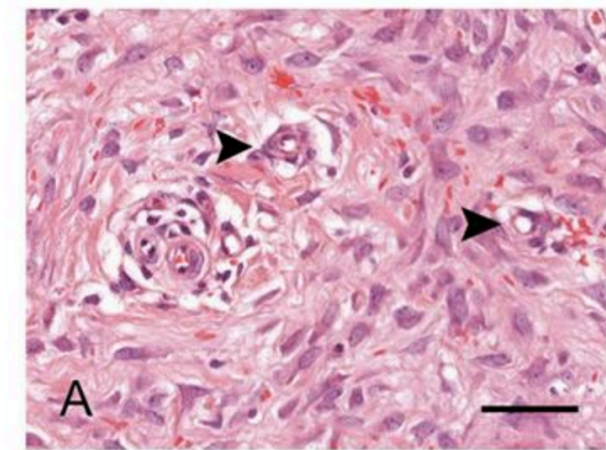


Figure 2

The distribution of blood vessels (arrowheads) was observed in the dermis **(A)** at day 3 after injection, H&E, 400x. The bar chart demonstrated the number of blood vessels in the BTX-A and control groups; $p < 0.05$ **(B)**.

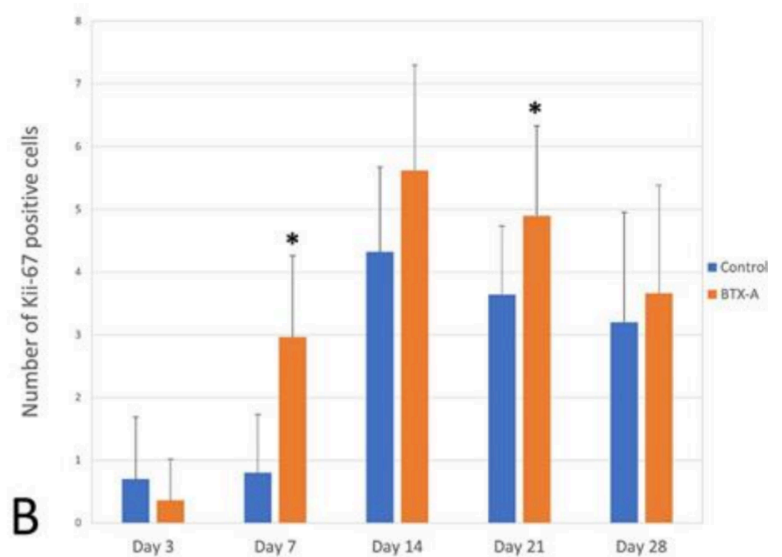
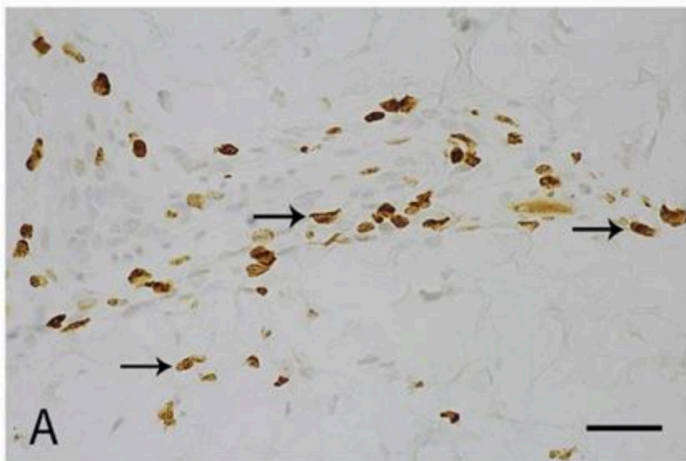


Figure 3

Immunohistological positive nucleus of Ki-67 of fibroblasts (arrows) in the injected areas **(A)**, 200x. The bar chart demonstrated the number of Ki-67 positive nuclei of fibroblasts in the BTX-A and control groups; $p < 0.05$ **(B)**.

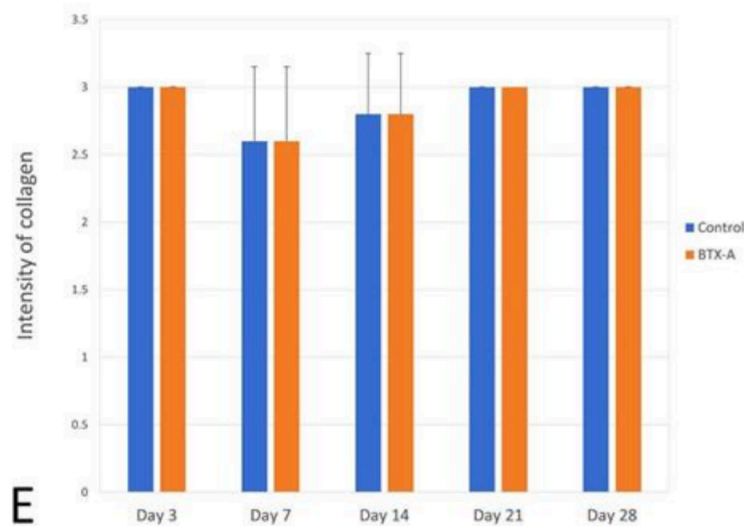
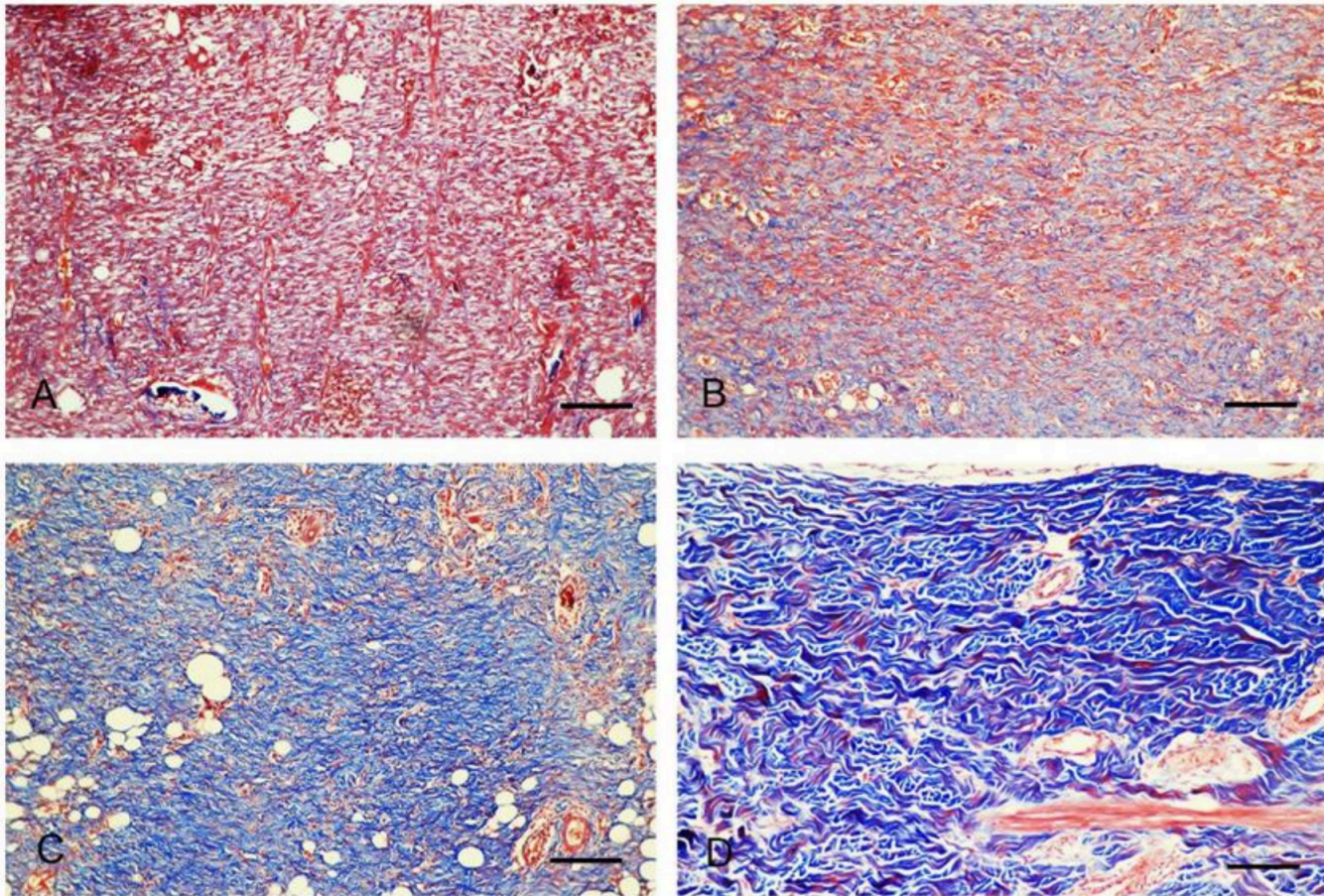


Figure 4

Collagen density grading using color intensity of the collagen fiber; Grade 0 (Absent; No blue color staining) **(A)**, Grade I (Mild; Pale blue staining of the collagen networks) **(B)**, Grade II (Moderate; blue staining of the collagen networks) **(C)** and Grade III (Intense; Dark blue staining of the collagen networks **(D)**, Masson's trichrome stain, 100x. The bar chart demonstrated grading of the intensity of collagen type I in the BTX-A and control groups; $p < 0.05$ **(E)**.

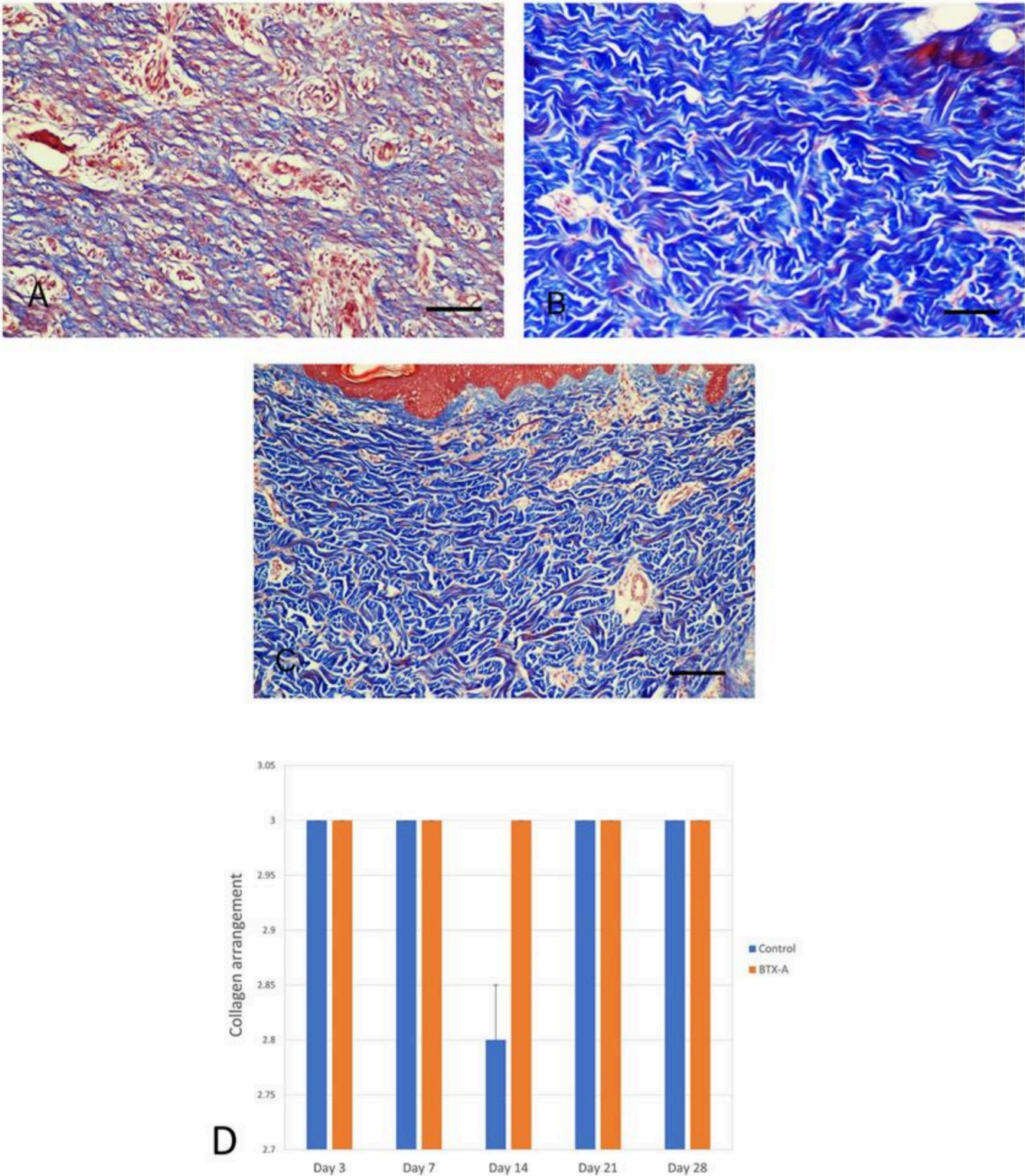


Figure 5

Collagen arrangement grading using arrangement of the collagen fiber; Grade I (unsystematic arrangement) **(A)** Grade II (moderate connection of the collagen and regularly arrangement) **(B)** and Grade III (continuously and regularly arrangement of the collagen fibers) **(C)**, Masson's trichrome stain, 100x. The bar chart demonstrated grading of the Grading of collagen arrangement in the BTX-A and control groups; $p < 0.05$ **(D)**.

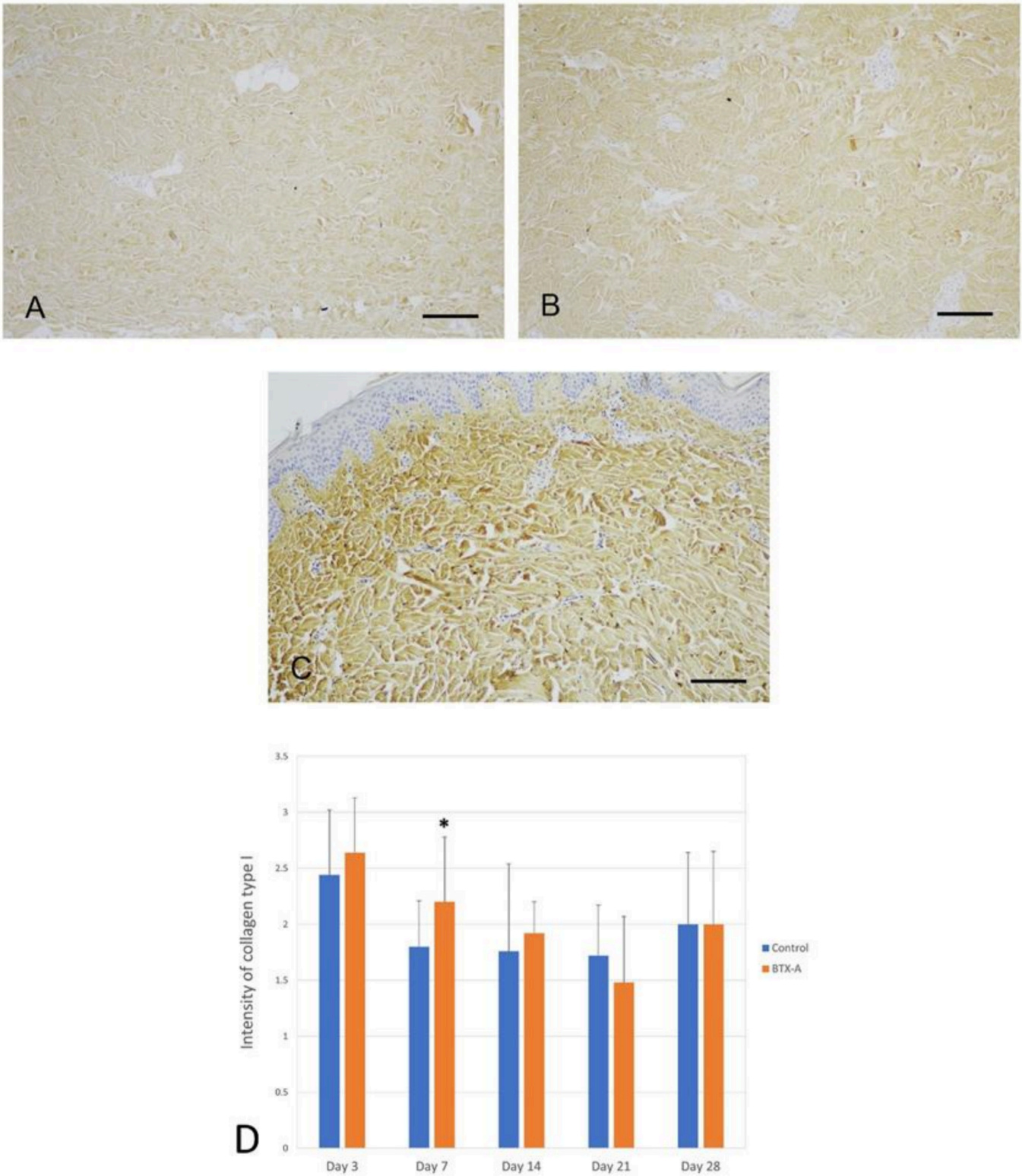


Figure 6

Collagen Type I grading using color intensity; Grade 0 (Absent, No color staining), Grade I (Mild Pale brown staining of the collagen networks) **(A)** Grade II (Moderated Brown staining of the collagen networks) **(B)** and Grade III (Intense Dark: brown staining of the collagen networks) **(C)**, 100x. The bar chart demonstrated grading of the intensity of collagen type I in the BTX-A and control groups; $p < 0.05$ **(D)**.

Supplementary Files

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