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Immunohistochemical evaluation of the effect of Botox-A in the prevention of burn scar formation (An experimental study)

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ABSTRACT

Background: Burn scars hurt survivors' capacity to go about their everyday lives, lowering their standard of living and causing aesthetic and functional problems. Botulinum toxin A was investigated to enhance Burning scars by several researches.

Aims: To study the impact of botulinum toxin-A on burning scars in the skin by evaluating the level of immunohistochemistry cluster of differentiation 31 (CD31) and matrix metalloproteinases (MMP-9).

Materials and methods:10 Male albino rabbits weighing 1.5± 0.2 kg and aged 6-8 months each had injected (6 units of Botox/Kg = 0.6 milliliters volume) Botox groups and 0.6 ml normal saline (control groups) into subcutaneous with 5 cm between them the animals were divided into groups A and B, with five rabbits in each group. Botox injections were given at different times: group A after 24 hours, group B after 7 days, and then all animals were euthanized at 28 days. Immunohistochemical investigation of skin samples concentrated on two parameters represented by CD31 and MMP-9.

Results: Immunohistochemical findings show that Botox groups have improved Immunohistochemical scores and increased levels of CD31 and MMP-9 compared to control.

Conclusion: This study concludes that Botox may inhibit fibroblast proliferation and regulate extracellular matrix formation. This may prevent scar formation.

Keywords: burn scars, Botox, CD31, MMP9.

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1. Introduction

The skin makes up almost 15% of an adult's total body weight, making it the biggest organ in the body. 1, 2 In addition to preventing the body from losing too much water and helping with thermoregulation, it carries out several essential tasks, including providing defense against external physical, chemical, and biological threats. 3 The skin and its descendant components comprise the integumentary system the epidermis, dermis, and subcutaneous tissue are the three layers that make up the skin . 4 The outermost layer, the epidermis, is made up of a particular kind of cells called keratinocytes, which are responsible for producing the protective protein keratin, which is a long, thread-like strand, Collagen is essentially a fibrillar structural protein that makes up the middle layer, or dermis, the subcutaneous tissue, or panniculus, on which the dermis is located, comprises tiny lobes of fat cells called lipocytes. 2, 3

Skin injuries can result in scar development, which can have a variety of negative consequences, such as physical abnormalities and psychological issues, Scars are frequently seen after burns and other soft tissue trauma.5 An unsightly and functionally unsatisfactory scar that might hinder function is caused by an excessive amount of extracellular matrix mixed with poor remodeling of scar tissue. The scar is uncomfortable and itchy. 6

The abnormal scarring observed in postburn syndrome hypertrophic scar(HS) usually presents as an erythematous, itchy, and inelastic scar. 7 Burn patients must deal with pain and aesthetic issues in addition to the functional impairments caused by collagen deposition, which reorganizes and creates contractures if left untreated.8 The extracellular matrix (ECM), which has been produced in large amounts in hypertrophic scar tissue, differs in composition and structure from a normal dermis or mature scar, and this is what gives the tissue its physical characteristics.9

One of nature's most powerful biological poisons, botulinum toxin type A (BoNT/A), causes natural botulism in humans, a condition marked by dysautonomia and flaccid paralysis of the skeletal muscles when combined with serotypes B, E, and F. 10 The most widely utilized therapeutic protein nowadays is botulinum toxin, which is used to treat hyperkinetic movement disorders, autonomic problems, spasticity, and wrinkles in cosmetic procedures. 11 Primary hyperhidrosis, hemifacial spasm, blepharospasm, cervical dystonia, and neurogenic detrusor overactivity are among the conditions for which BoNT/A is approved. 12

2. Method

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This investigation was carried out at the Research Department of Surgery, Faculty of Veterinary, Mosul University, Iraq, from 1 March to 28 March 2024. Ethical approval was granted by both the local scientific committee and the research ethics board(UoM.Dent.24/1020 on 15/8/2024). All surgical interventions were conducted under the supervision of seasoned veterinary surgeons using established protocols. Ten healthy albino male rabbits aged 6-8 months and weighing 1.5±0.2 kg were included. Clean cages and a regular environment (25°C) were employed for them individually. They received equal quantities of grain, vegetables, and water. Their health was followed by the veterinarian during the experiment. The study includes 10 male albino rabbits divided into two main groups, five animals for each group according to time of injection of Botox after (24 hours,7 days). The animals received Botulinum toxin type-A on the study side (Botox) and normal saline (control side) with 5cm between the two burning wounds on the dorsum. All 10 albino rabbits are anesthesia at the same time after the rabbits are anesthetized by IM injections of ketamine 50 mg /kg general anesthetic agent and xylazine 5mg /kg sedative, analgesic solution complete anesthesia will be obtained within 10 minutes which keeps the animal anesthesia for about 2 hours after the animals are anesthetized the rabbit's hair will be shaving then 10% of povidone-iodine applied on the area of surgery then two identical square areas 1cm2 are marked on the dorsum of each rabbit study side and control side 5 cm between them to prevent diffusion. 13 Then lidocaine 0.2 mL with epinephrine was locally injected at the site of the burn to reduce pain. An instrument with an area of 1 cm2 and weighing 85 g was heated to 800C and placed on the selected shaved area of the rabbit's back for 14 seconds to get second-degree burns, as seen in Figure 1B). 14 Botox-A (Dermatox Canada,100 IU) vial was reconstituted with 10 mL of normal saline (0.9%). Hence, the volume of 0.1 mL is required to obtain 1IU of Botox-A, which was the dose chosen in our study See Table 1.

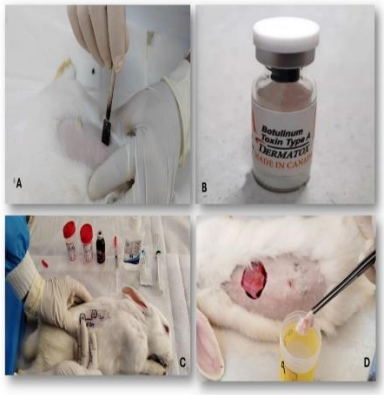

Figure 1

Dose (unit)	Diluted volume
100 U	10ml
50 U	5ml
10 U	1ml
1 U	0.1 ml

Table 1: Dose preparation of Dermatox

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Group A: injected 24 after the burning procedure with Botox (subcutaneously by using a 1 ml insulin syringe), half of the quantities were at the corners of the square, and another half at the center of the square. The control side was injected with normal saline 0.9%. (figure1C)
Group B: injected 7 days after the burning procedure with Botox(subcutaneously by using a 1 ml insulin syringe), half of the quantities were at the corners of the square and another half at the center of the square. The control side was injected with normal saline 0.9%. (figure 1C)

Sample collection:
After 28 days, all animals are sacrificed after anesthetized with ketamine and xylazine. Skin samples were collected and preserved in containers 10% formalin till immunohistochemical investigations CD31 and MMP9 were performed. (see Figure 1D). The criteria for determining CD31 and MMP9 depended on a semi-quantitative score, ranked as score 0 for the absence of staining, score 1 for weak staining, score 2 for moderate staining, and score 3 for strong staining. 15, 16

Procedure of Immunohistochemistry:
There are numerous stages in IHC, These steps include fixing the sample correctly, preparing the paraffin blocks, retrieving the antigen, selecting the antibody and reagents, incubating, washing, and counterstaining. 17

Statistical analysis
We used the Graph Pad Prism software program to conduct our statistical study. Since the data consisted of finite scores that did not follow a normal distribution, non-parametric statistical tests were employed for all statistical analyses (Mann-Whitney U test) to compare the medians of the scores. The Mann-Whitney U test was used to compare the median score of two groups(control and Botox) on the same day.

4. Results

Clinical evaluation for the rabbits used in this study showed that there were no complications following surgery, including infection or bleeding. They all made a full recovery during the .healing process

To ensure fairness, standard scores were then calculated. A digital camera was used to inspect each slide under a light microscope with oil lenses with magnification powers of 4X and 10X. .Two markers are investigated: CD31 and MMP9

In the case of CD31, the control side of Group A showed negative expression of the CD31 marker, while the Botox-injected side showed strong expression immunoreactivity against the CD31 marker as illustrated in Figure 2.

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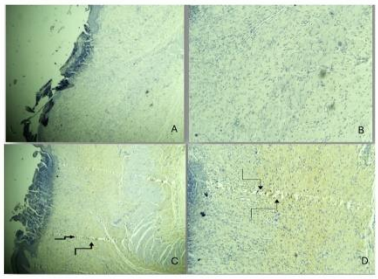

Figure 2

Similarly, the control side of Group B showed weak expression of the CD31 marker, while the Botox injected side showed strong expression immunoreactivity against the CD31 marker as demonstrated in Figure 3. The median scores for CD31 expression in both groups A and B is shown in Table 2.

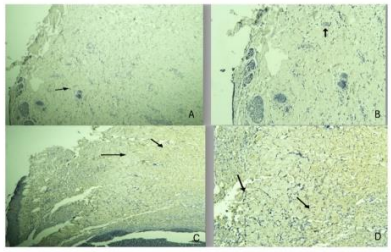

Figure 3

Groups	Subgroup	Median CD31	Median mmp9
Group A	AC	0	1
	AB	3	2
Group B	BC	1	0
	BB	3	1

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Table 2: Table (2): MMP9 and CD31 expression intensity in the skin specimens. The intensity median scores of MMP9 and CD31 immunohistochemistry expression from control and Botox groups. The scores represent 0 (- negative expression), 1 (weak positive expression), 2 (moderate .positive expression), and 3 (strong positive expression)

The Mann-Whitney U test results indicated there is a highly statistically significant difference ($p< 0.05$) between (the control and Botox) in group A. Also, a statistically significance difference between the control and Botox in group B See Table 3

Groups	Subgroup*	Median MMP-9 score (interquartile range)	*P* - value	Median CD31 score (interquartile range)	*P* - value
A	AC	1(0.5-2)	0.4841	0(0-0)	0.0079**
	AB	2(1-2.5)		3(1.5-3)	
B	BC	0(0-0)	0.0476*	1(0-1)	0.0317*
	BB	1(0.5-1)		3(1.5-3)	
*The Mann-Whitney U test was used to compare the value of MMP9 and CD31between groups (control and Botox) at p <0.05					

Table (3): Comparisons of statistical analysis of the intensity median scores of MMP-9 and CD31 immunohistochemistry expression scores between the control and Botox groups on the same day.

In the case of MMP-9, the control side of Group A showed weak expression of the MMP-9 marker, while the Botox injected side showed moderate expression immunoreactivity against the MMP-9 marker as illustrated in Figure 4.

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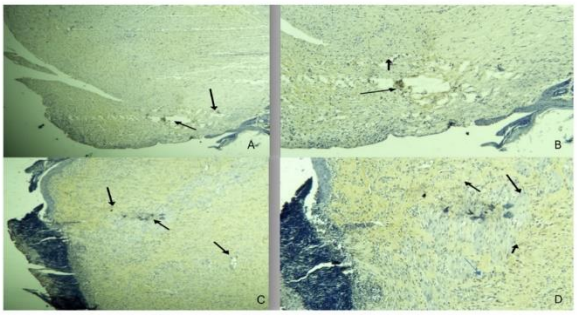

Figure 4

Similarly, the control side of Group B showed negative expression of the MMP-9 marker, while the Botox injected side showed weak expression immunoreactivity against the MMP-9 marker as demonstrated in Figure 5. The median scores for MMP-9 expression in both groups A and B are shown in Table 2.

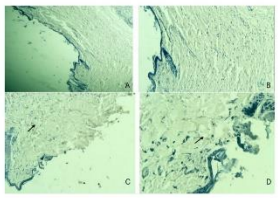

Figure 5.

The Mann-Whitney U test results indicated there is no statistically significant difference (P-value > 0.05) between the control and Botox sides in groups (A). However, there is a significant difference between (the control and Botox) in group (B) (P-value <0.05 see Table 3).

5. DISCUSSION

Advancements in acute burn treatment have allowed people to endure extensive burns that were previously fatal. Currently, up to 70% of individuals acquire hypertrophic scars following burns. The functional and emotional consequences pose a significant rehabilitation challenge, diminishing quality of life and hindering societal reintegration. 18

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Strategies to enhance the healing ability of burn wounds involve specialized wound care and surgical interventions to reduce the formation of hypertrophic scarring. Most of the time, these methods don't work, and the scar stays changed, even though the best indications, timing, and therapy combinations are still unknown. The need for new therapies is very high, and upcoming efforts to improve outcomes and quality of life should include improving wound healing to reduce or avoid hypertrophic scarring. 19

Toxin type A (BoNT/A) is one of the strongest biological toxins known to science. It is one of the four types of botulism and causes skeletal muscles to become weak and the person to lose control of their body. Research in the early 1800s revealed that an unknown toxin in tainted food was the cause of botulism symptoms. This led to the idea of using a small amount of this chemical to treat hyperactive nerve disorders. Researchers discovered and separated different types of BoNT during the 20th century. 12

Intralesional BTX-A injection can diminish scar volume and suppress collagen secretion and fibroblast production. 20, 21

According to the results of the current study, the expression levels of the CD31 marker were significantly increased on the Botox-injected side compared to the control side for both groups A and B were injected after 24 hours and 7 days, respectively. This is attributed to the influence of Botox on the expression of vascular endothelial growth factor (VEGF) and platelet endothelial cell adhesion molecule-1 (PECAM-1)CD31.22 These effects facilitate the development of new blood vessels 23, 24, enhancing blood circulation 25 and accelerating the healing process. 26 Furthermore, Botox's impact on the circulatory system is associated with enhanced vasodilation, as the toxin relaxes vascular smooth muscle cells and suppresses sympathetic input. 27

On the other hand, the current study revealed that MMP-9 levels were increased on the Botox-injected side compared to the control side for both groups A and B were injected after 24 hours and 7 days, respectively. This is related to the anti-inflammatory, fibroblast activity modulation, and the inhibitory effects on the production of fibroblasts caused by intralesional Botox injection. 28, 29 These effects lead to the enhancement of extracellular matrix remodeling and suppression of the synthesis of type I and III collagen while enhancing the production of matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9. 26, 30 This agrees with a previous study, which approved that Botox modulates the expression levels of matrix metalloproteinase MMP-9 and extracellular matrix remodeling. 26, 30.

6. CONCLUSIONS

The current study concludes that Botox-A improved the skin healing capacity in a burning wound rabbit model. Furthermore, it has a positive effect on MMP9 that regulates extracellular matrix remodeling and CD31 and angiogenesis and enhanced blood vessel formation.

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