Original Article

Evaluation of the Appropriate Time of Botulinum Toxin Type A Injection To Increase the Survival of Random Flaps in Rat

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ABSTRACT

BACKGROUND: Although many previous studies verified the role of BTX-A in the augmentation of flap survival and decreasing the rate of necrosis in random pattern cutaneous flaps, drug injection at different times has not been investigated in this regard. This study compares the effect of BTX- A injection time at 0, 3, and 7 days before surgery to determine the best and most effective injection time.

METHODS: In 20 male rats, divided into four equal groups, on different days of pre-operation (0, 3, or 7 days) BTX-A or saline was administered to the whole length of the flap. Random pattern dorsal skin flaps with 4:1 length-to-width ratios were elevated and returned to the original position. Flap survival was evaluated on day 10 and 20 after surgery and a histopathological examination were performed 20 days after flap elevation.

RESULTS: The BTX-A group had a greater survival mean compared with the saline group (87.92±16.30 vs 65.60±30.71 or 1.34 fold increase in survival rate) in flaps with a length-to-width ratio of 4:1 ($P = 0.05$), and If botulinum toxin is injected 7 days before flap elevation, this increase in survival will be 1.5 times (97.5±4.06 vs 65.60±30.71) compared to the saline group ($P = 0.02$).

CONCLUSION: Preoperative injection of botulinum toxin type A in random pattern skin flaps increases skin flap survival in rats, and the best time for injections is 7 days before flap elevation. We found a reduction in the proliferation of cutaneous myofibroblasts in flaps by injecting BTX-A. It could play a role in increasing blood flow and survival by reducing wound contraction, however, more studies should be conducted to determine the possible mechanism.

KEYWORDS
Botulinum toxin-A; Random flap; Cutaneous myofibroblasts injection time; survival

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INTRODUCTION

Clostridium botulinum is a Gram-positive, anaerobic bacterium known to produce seven serologically distinct types of toxin designated A through G, of which type A is the most potent. Botulinum toxin types A and B

are used medically and are available [1]. Currently, it is used for the treatment of blepharospasm, hemifacial spasm, survival dystonia, treatment of headaches, and facial rejuvenation [2].

Skin flaps are commonly used in plastic and reconstructive surgery to repair defects resulting from trauma, congenital anomalies, or tumor resection [3]. Unlike skin grafts, random flaps survive depending on the blood supply through a subdermal plexus rather than a skin perforator. Being dependent on various microvascular plexuses for their metabolic needs, they are prone to ischemic injury [3].

The random pattern skin flap length depends on the intravascular resistance of the supplying vessels and the perfusion pressure. Based on the donor site, random pattern skin flaps with length-to-width ratios greater than 2:1 to 3:1 will usually fail to some extent because of ischemia in the absence of additional interventions such as delayed procedures [3]. Surgical delay procedures are currently the gold standard for such techniques; however, they have some drawbacks. Notably, ≥1 surgical stage(s) is needed before performing the main procedure [4].

In contrast, nonsurgical methods for augmentation of the vascularity of random pattern skin flaps depend on many pharmacologic agents that have been used to decrease the rate of this ischemic injury, including sympatholytic, vasodilators, calcium channel blockers, prostaglandin inhibitors, anticoagulants, and glucocorticoids [3].

However, most of these agents should be applied systemically in high doses, which increases the incidence of systemic side effects [3].

According to Rohrich et al, an ideal pharmacologic agent for improving flap survival would have the following features: clinical availability, easy administration, a high therapeutic index, reproducible results, feasibility and cost-effectiveness, known mechanism of action, established bioavailability, and protective effect on flap necrosis. Still, today and after more than 30 years, no substance has been found to fulfill all the requirements [5].

Botulinum neurotoxin-A and B showed a potential role in improving flap survival in animal models [6].

The role of botulinum toxin type A in the augmentation of flap survival and decreasing necrosis rate in random pattern cutaneous flaps was verified [3]. But the BTX-A has been used at different times. Seventeen articles describe BTX- A injection before or during flap elevation or vascular anastomosis in a total of 266 animals. All studies have shown a beneficial effect of botulinum toxin administration in flap surgery or vascular anastomosis.

Neurotoxin-botulinum injection showed a reliable method for reducing vascular complications and increasing flap survival in animal models [6]. This study reviewed the effect of BTX-A on flap surgeries; including a series of BTX-A injections from one to thirty days and an average of 7.5 days before flap injections. A total of 4.4 units of BTX-A injection was associated with an increase in flap survival by 24.1% (± 12.25%) [6]. Another study examined a ratio of 3 to 1 on rats random flaps (P <0.001) [4]. Their results revealed, 24 IU/kg of BTX-A injection 2 weeks before flap elevation increased flap survivals in comparison to placebo (P <0.001) [4].

The result of a similar study with the same ratio and a total injection of 2 units of BTX- A on rat random flaps confirmed an increase in flap survival compared to placebo (P <0.001) [3].

In these projects, BTX-A was injected at different times from 2 weeks[4], one week before surgery[6], and concurrent with flap elevation[3]. The ratio of 3 to 1 on random flaps increased survival flap or reduction of necrosis in the distal flap.

We compared the effect of BTX- A injection time at 0, 3, and 7 days before surgery on random flaps with a ratio of 4 to 1 to determine the best and most effective injection time.

MATERIALS AND METHODS

After obtaining the necessary permits and receiving the ethical code (IR.SBMU.MSP.REC.1400.067), 20 Sprague–Dawley male rats, average weighting of 350 g were fed appropriately in the animal laboratory of 15 Khordad Educational Hospital, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran 2021 and then randomly divided into four equal groups.

The first group received 0.4 ml subdermal BTX-A seven days before the operation (brand: Masport 500u Fig. 1), a total dose of 10 units injections in 8 points in an area of 2 x 8 cm at the dorsal level.

The second group received 0.4 ml subdermal BTX-A three days before the surgery in the same manner.

The third group received 0.4 ml subdermal BTX-A during the surgery.

The fourth group received 0.4 ml subdermal of normal saline at the same time with surgery at 8 points in an area of 2 x 8 cm (with a ratio of length to flap width of 4 to 1) at the dorsal level.

During the operation, the rats underwent general anesthesia with a combination of IM ketamine (75-95 mg/kg) and diazepam at standard doses.

Sterile preparation was done and one 8 cm ×2 cm caudally based fascio cutaneous random flap including the panniculus carnosus was raised completely and then sutured back to its primary site using 4/0 nylon sutures. The flap was marked using a marker pen with its base below the level of the iliac crest by 1 cm and designed to be rectangular, accurately over the center of the dorsum of the rat, with crushing any direct axial or muscular perforating cutaneous blood vessels supplying the flap and after releasing the flap from three sides (ventral and sides), Cut and ligated all the lateral vessels, to ensure a random skin flap (Fig. 3).

The group of surgeons who performed the experiments were not informed about each group.

Flap survival was evaluated on postoperative days 10 and 20. The flap was assessed clinically based on its appearance, color, and texture. Viable skin was soft, warm, and without alteration of skin color, whereas necrotic areas were brown-black, cold, and stiff.

The necrosis area was marked on a predefined transparent template that was placed on the flap templates, was then scanned and the necrosis area was calculated electronically using an image analysis system (Scion images (NIH-Scion Corporation, Frederick, MD)).

Figure 1: Brand of botulinum toxin A used in the study

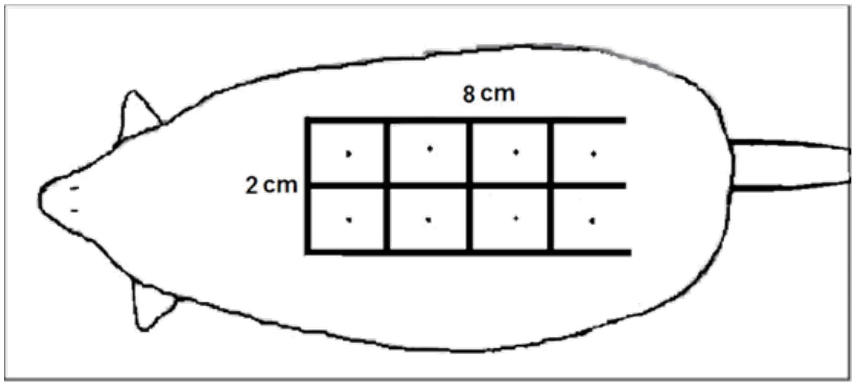

Figure 2: Design of flap and Injection sites of BTX-A

Histologic evaluation

The flaps were sent to the pathology lab for histological examinations after removing them from the rat's bodies on the 20th day after the surgery.
Sampling was performed in experimental and control groups and fixed with 10% formaldehyde. Specimens were taken from the center of the proximal, middle, and distal areas. HE (Hematoxylin & Eosin) staining was performed on paraffin-embedded flap tissues. Two pathologists examined samples for four indicators of ulcer presence, a proliferation of cutaneous myofibroblasts, neutrophil infiltration, and neovascularization.

Statistical evaluation

The data were analyzed using SPSS version 16 statistical software (Chicago, IL, USA). Each measurement is shown as a mean standard deviation. All differences between the measurements of the two groups were examined by an independent samples *t*-test and Non-parametric tests (Mann-Whitney Test and Wilcoxon's sign rank test). A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Two rats died on the second and ninth days after the surgery in the first and second groups respectively, and were excluded from the study.
Thus, 13 rats (72.2%) in this study received BTX-A, and 5 rats (27.8%) were in the control group and received only normal saline at the same time as flap removal. Among the rats who received BTX-A, 4 rats (22.2%) received BTX-A 7 days before surgery, 4 rats (22.2%) received BTX-A 3 days before surgery, and 5 rats (27.8%) received BTX-A at the same time as flap removal (Table 1).
The flaps status was assessed for survival 10 days after surgery (Fig. 4-7).
The mean percentage of flap survival after 10 days

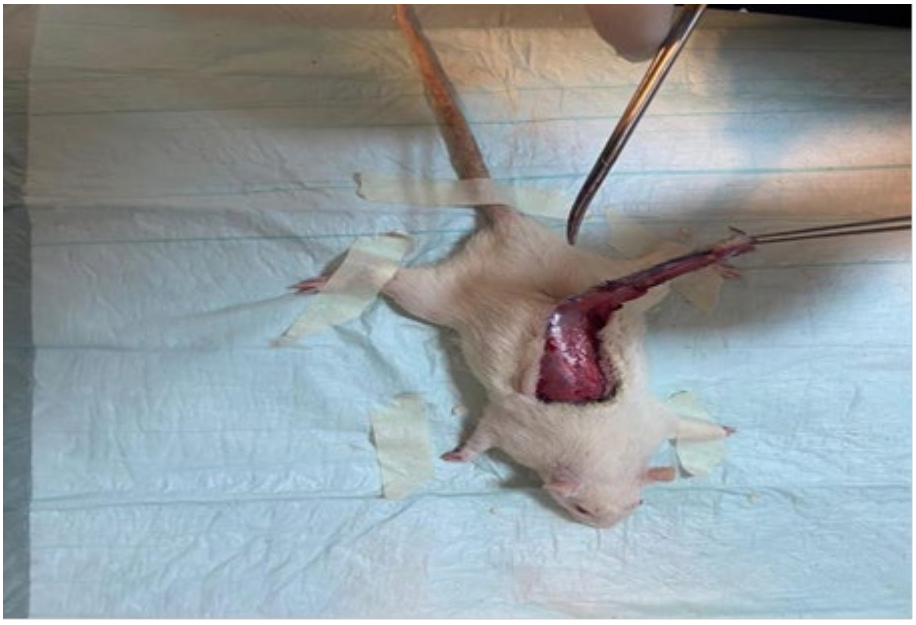

Figure 3: Release the flap from 3 sides and the bottom, and cut the vascular connections in these areas

Table 1: Comparison of Percentage of Flap Survival 10 days post-operation between group receiving BTX-A, and the group that received saline

Group	N	Mean	Std. Deviation	Std. Error Mean
Botulinum toxin type A	13	87.92	16.31	4.52
Saline	5	65.60	30.71	13.73
			$P = 0.05$	

was 90 percent in the groups that received BTX-A 3 or 7 days before the operation and 65.60 percent in the control group, which showed a significant difference $P = 0.015$ (Table 2).

The mean percentage of flap survival after 10 days was 97.5 percent in the groups that received BTX-A 7 days before the operation and 65.60 percent in the control group, which showed a significant difference $P = 0.02$ (Table 3,4).

We used non-parametric tests (Mann-Whitney U and Wilcoxon) to compare flap survival status in rats receiving BTX-A 7 and 3 days preoperatively. The results showed a significant difference between the two groups (P =0.05).

Evaluation of flap survival percentage, 20 days after surgery, in the Botox and normal saline groups were 97.81% and 96.8% respectively, which not showed any significant difference. $P = 0.686$.

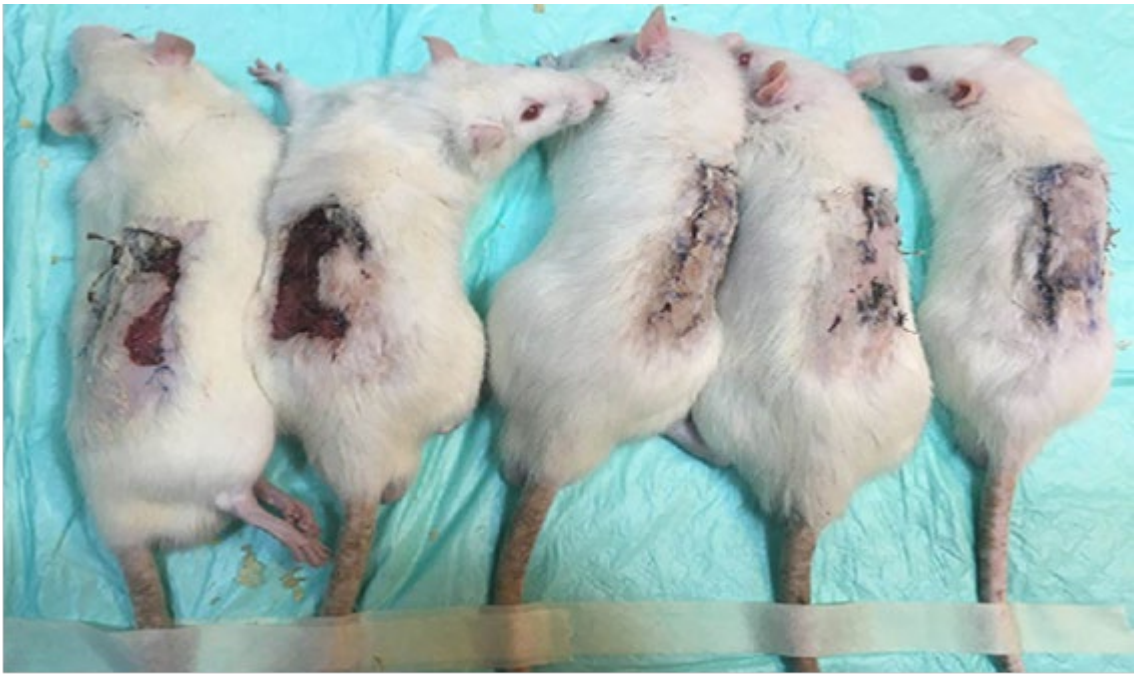

Figure 4: Flap status 10 days after surgery in the saline group at the same time as flap lifting

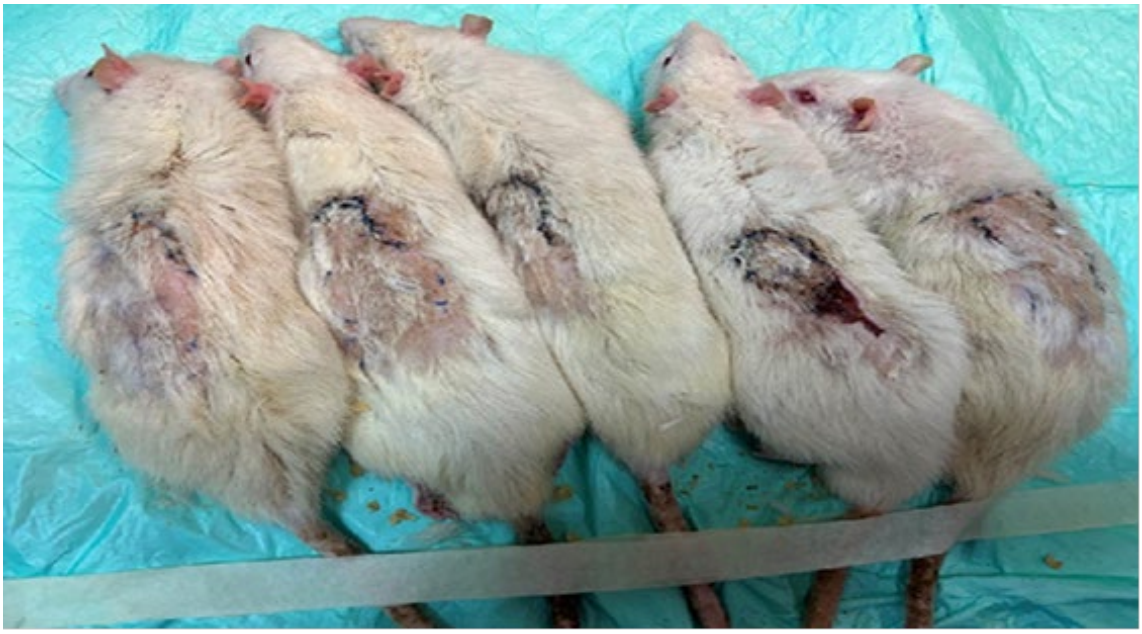

Figure 5: Flap status 10 days after surgery in the BTX-A group at the same time as flap lifting

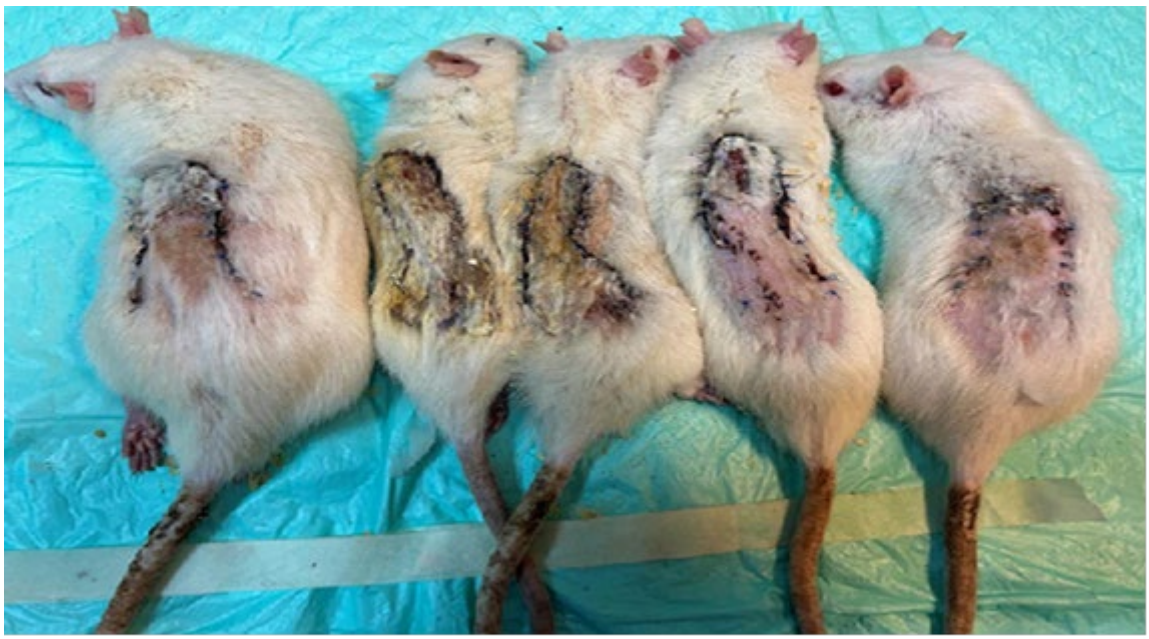

Figure 6: Flap status 10 days after surgery in the BTX-A, 3 days before flap lifting

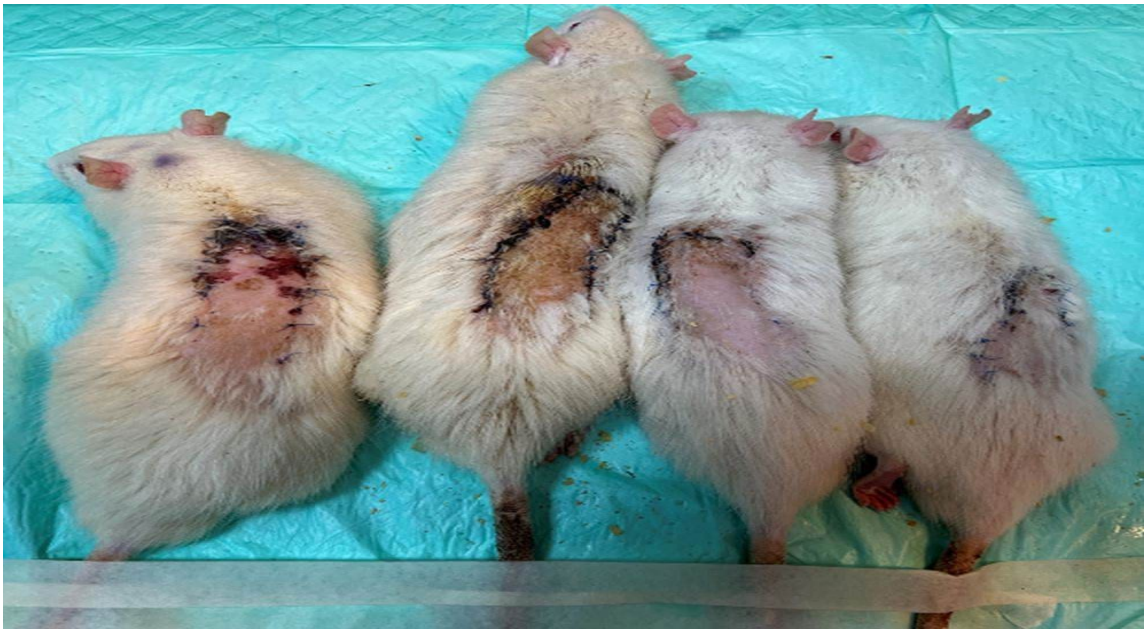

Figure 7: Flap status 10 days after surgery in the BTX-A group, 7 days before flap lifting

The results of histological examinations 20 days after surgery are as follows:

There were no significant differences in the results of ulcer existence, the proliferation of cutaneous myofibroblasts, neutrophil infiltration, and neovascularization in the BTX-A, and the Normal saline groups, but the proliferation of cutaneous myofibroblasts in the group who received BTX-A seven days before the surgery showed significantly different (Table 5,6).

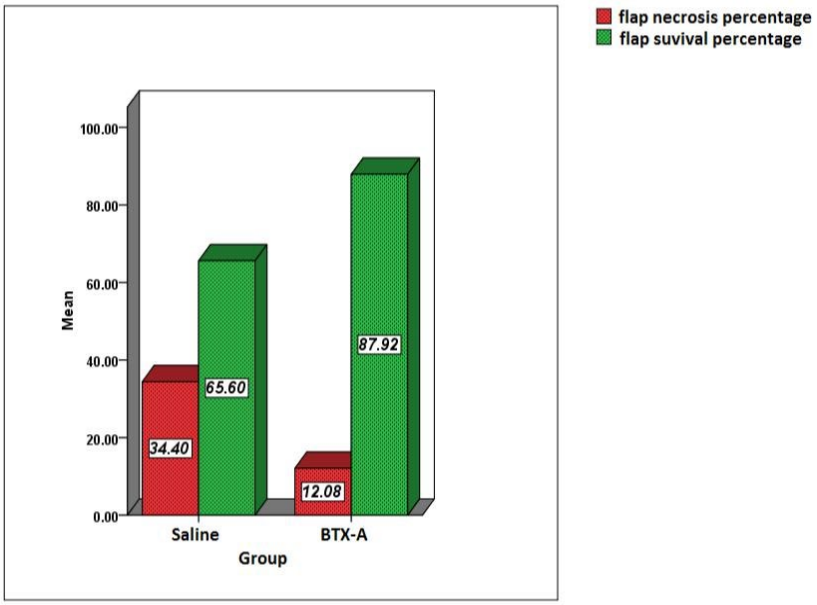

Figure 8: Mean Percentage of Flap Survival and necrosis 10 days post operation in the BTX-A, and the saline groups

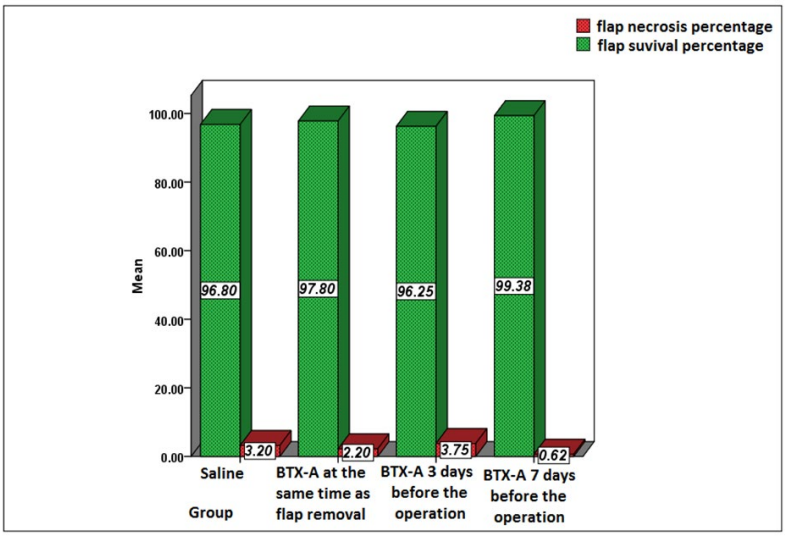

Figure 9: Mean Percentage of Flap Survival and necrosis 20 days post operation in BTX-A groups according to the time of receiving BTX

Table 2: Comparison of Percentage of Flap Survival 10 days post-operation between group receiving BTX-A 3 or 7 days before the operation and the group that received saline

Group	N	Mean	Std. Deviation	Std. Error Mean
BTX-A 3 or 7 days before the operation	8	90.00	11.08	3.92
Saline	5	65.60	30.71	13.73
	$P = 0.015$			

Table 3: Comparison of Percentage of Flap Survival 10 days post operation between group receiving BTX-A 7 days before the operation and the group that received saline

Group	N	Mean	Std. Deviation	Std. Error Mean
BTX-A 7 days before the operation	4	97.50	4.06	2.03
Saline	5	65.60	30.71	13.73
	$P = 0.02$			

Table 4: Comparison of Percentage of Flap Survival 10 days post operation between group receiving BTX-A 7 days before the operation, and the group that received saline using non parametric tests

Ranks			
Group	**N**	**Mean Rank**	**Sum of Ranks**
BTX-A 7 days before surgery	4	7.25	29.00
Saline	5	3.20	16.00
Total	9		

Test Statistics	
Mann-Whitney U	1.000
Wilcoxon W	16.000
Z	-2.214
Asymp. Sig. (2-tailed)	.027
Exact Sig. [2*(1-tailed Sig.)]	.032a

a. Not corrected for ties b. Grouping Variable

Table 5: Comparison of the histopathological results 20 days after surgery in the BTX-A 7 days pre operation and saline groups

Group		N	Mean Rank	Sum of Ranks	Test Statistics b				
					Mann-Whitney U	Wilcoxon W	Z	Asymp. Sig. (2-tailed)	Exact Sig. [2*(1-tailed Sig.)]
Ulceration	Saline	5	5.20	26.0	9.00	19	-.283	.777	.905a
	BTX-A 7 days pre operation	4	4.75	19.0					
	Total	9							
Neutrophil infiltration	Saline	5	5.40	27.0	8.00	18	-.537	.592	.730a
	BTX-A 7 days pre operation	4	4.50	18.0					
	Total	9							
Neovascularization	Saline	5	5.60	28.0	7.00	17	-.782	.434	.556a
	BTX-A 7 days pre operation	4	4.25	17.0					
	Total	9		27.0					

a. Not corrected for ties b. Grouping Variable

Table 6: Comparison of the presence of proliferation of cutaneous myofibroblasts in the flap pathology sample 20 days after surgery in rats receiving BTX-A 7 days pre-operation with the control group

Group	N	Mea n	Std. Deviation	Std. Error Mean
BTX-A 3 or 7 days before the operation	4	1.25	.50	.25
Saline	5	1.80	1.30	.58
	P =0.075			

DISCUSSION

Traditionally, surgical delay has been used to prevent necrosis of larger flaps. Surgical delay is believed to enhance flap survival mainly via two ischemia-related mechanisms: durable vasodilation and angiogenesis. Given the drawbacks of 2-stage surgical preconditioning, recent work has focused on alternative nonsurgical delay techniques. Pharmacological delayed agents were suggested by Pang et al in 1989. Since then, numerous attempts to explain the effect of topically applied drugs on flap survival rates, including random cutaneous flaps [7].

BTX-A can promote flap healing by interacting with Ca2+ influx, relaxing the smooth muscle in vessel walls, and causing neoangiogenic, sympatholytic, and anti-inflammatory effects [8-11].

Manipulating different doses of Botox noticed that a total of 4.4 units will increase the survival of the flap by 24.1% in a period between 1 and 30 days (an average of 7.5 days) before flap surgery [6].

In previous studies, the ratio of 3 :1 has been considered as a random flap size which received different doses of Botox- A (24 IU / kg and 2 weeks before surgery, and 2 Units simultaneously with the flap surgery), and their results were associated with increased survival of the flap compared to placebo [3,4] .

In previous studies to investigate the effect of BTX-A on the random flaps survival with a length-to-width ratio of 3:1 [1,2,4] or 2:1 were used [3] While the recent study compares the effect of BTX- A injection time at 0, 3, and 7 days before surgery on random flaps with a ratio of 4 to 1 to determine the best and most effective injection time.

Comparison of flap survival after 10 days in our study showed:

BTX- A injection was associated with an increase in flap survival by an average of 21.9% (87.92 ± 16.30 vs. 65.60 ± 30.71 or a 1.34-fold increase in survival). BTX-A injection in the interval of 3 or 7 days before surgery was associated with an increase in flap survival by an average of 24.4%.(90 ± 11.08 vs. 65.60 ± 30.71). BTX-A injection 7 days before the operation indicates an increase in flap survival by an average of 31.9% (97.54± 4.06 vs. 65.60 ± 30.71 or 1.5-fold increase in survival rate) *P*=0.02. BTX-A injection 7 days before surgery had a greater effect on the flap survival compared with the group that received BTX-A 3 days before surgery. *P* = 0.05.

In this study, different from other studies without previous similarities, the flaps were re-examined 20 days after surgery.

The mean percentage of survival flaps twenty days after surgery was 97.8 percent in the intervention group and 96.8 percent in the control group, which showed no significant difference between the two groups. *P* = 0.686.

Comparing the presence of ulcers, proliferation of cutaneous myofibroblasts, neutrophil infiltration, and neovascularization in flap pathology sample 20 days after surgery in rats receiving BTX-A, there was no significant difference with the control group (*P* = 0.954, *P* = 0.381, *P* = 0.835, *P* = 0.424 respectively).

The number of neutrophil infiltration is an indicator of infection that may lead to flap necrosis.

In our study, there was no significant difference in any of the groups in terms of neutrophil infiltration, which can increase the accuracy of the evaluation of flaps in the absence of infection.

Then, the comparison of three indicators of ulcer presence, neutrophil infiltration, and neovascularization in the flap pathology sample 20 days after surgery in rats that received BTX-A 7 days before surgery was performed with the control group, which showed no significant difference. (*P* = 0.905, *P* = 0.730, *P* = 0.556, respectively). However, in comparing the mean proliferation of cutaneous myofibroblasts, there was a significant difference with the control group *P* = 0.075

BTX-A by blocking TGF-alpha signaling, prevents the differentiation of fibroblasts into myofibroblasts,

thus reducing excessive fibrosis and wound retraction [12]. In a study higher fibroblast density and inflammation were noted in the control group than in the BTX-A group which is consistent with our study [13]. Regarding the mechanism of action of BTX-A, it is believed that the increase in subcutaneous blood flow increases the survival flap, which is documented by the results of the skin Doppler laser perfusion assessment. Serial measurement of blood perfusion before BTX-A injection, one week after the injection, immediately after raising the flap, and one week after raising the flap, it was proven that blood flow to the distal region was maintained and that no reduction in blood flow in the middle region was observed for 1 week [2]. After BTX-A injection one week after flap lifting, even the distal area showed an increase in blood flow compared to the normal range [2].

These results may be due to vascular contraction affected by the sympathetic system caused by stressful conditions during flap lift. In the control group, blood flow could not be maintained due to the collapse of arteries, narrow veins, and capillaries in the distal region. As shown in the histological results, samples stained with hematoxylin and eosin from the midline of the flap in the BTX-A, injected group showed larger arteries and small veins with an increasing number of narrow capillaries [2].

Another study[14] found that at low concentrations, when we compared the effects of BTX-A with papaverine (on the contraction inhibition) of 5-hydroxytryptamine, it was observed that BTX-A was more effective than papaverine after surgery (P <0.05). Both BTX-A and papaverine inhibit the maximum contractile effect of endothelin-1 equally at the start of injection, but the inhibitory effect of BTX-A in the second hour is much stronger (P <0.05) [14].

In our study, the effect of BTX- A on survival flap at 4: 1 was determined at the same time as surgery, but not as much as in another study[14].

In a study on the effect of botulinum toxin A on cutaneous flaps in diabetic and tobacco-exposed rats, it was found that botulinum toxin A increased survival of cutaneous flaps in control and Diabetic rats on the seventh day after surgery and ina group of rats who were diabetic and received BTX-A, an increase in arterial diameter, an increase in lumen diameter and an increase in the ratio of lumen to wall thickness were observed[15].

Preoperative use of BTX-A in the emergency department could help reduce traumatic stress and subsequent thrombosis and improve trauma management. In one sudy by Dr Hassanpour and Dr Tarahomi they explored the bilateral femoral arteries of 10 rats using a microscopic technique. The injury was caused by crushing the artery using a bulldog clamp Randomly [16].

BTX-A was injected into one leg of each mouse. The other side received normal saline as its control. After 24 hours, the distal femoral arteries were closed at the site of the trauma and severed between the affected and closed areas. The arteries injected with BTX-A had pulsed bleeding without thrombosis [16].

The limitation of the study is not using advanced methods to measure flap necrosis, such as gene expression. We did not evaluate the effects of different doses of BTX-A. Under the rat skin, the panniculus carusus muscle layer is present, which makes rat skin different from human skin. Therefore, it is difficult to compare the skin flaps of rats and humans directly. In addition, the number of participants in each group was small. As well, no evaluation was conducted of the blood flow in the flaps before and after surgery. This may be addressed in future studies. BTX-A has not been studied in humans to determine if it affects the viability of flaps. It is essential to use it in human studies to improve the results of flap surgery, which is a major component of reconstructive surgery.

CONCLUSION

Preoperative injection of BTX- A in randomly patterned dermal flaps increases flap survival in rats, and the best time to inject is 7 days before flap lift. In this study, we saw a reduction in the proliferation of cutaneous myofibroblasts in flaps injected with BTX-A, which may increase blood flow and survival by reducing flap and wound contraction, but more studies are needed to determine the possible mechanism.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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