Original Article

Effects of Botulinum Toxin Type A on the Axial Skin Flap Survival

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ABSTRACT

BACKGROUND

A suitable pharmacological substitute for the well-established surgical delay technique for axial skin flaps regarding increasing viability is elusive. We aimed to evaluate the effects of botulinum toxin type A (BTA) on the axial skin flap survival in a rat model.

METHODS

The present controlled experimental study was performed in Kerman University of Medical Science, Kermanshah, Iran during 2016-2017 on three groups of rats. Group 1 (control group) had no preconditioning while Groups 2 and 3 were preconditioned by the intradermal injection of normal saline (0.5 ml) in the cephalic end of the skin flap and the injection of the BTA (1.6 units Neuronex) reconstituted in normal saline, respectively. Two weeks after this intervention in each group, the flap was raised and kept in situ and a biopsy was simultaneously taken for evaluating neoangiogenesis, followed by evaluating flap necrosis after two weeks of following-up by photography.

RESULTS

Although BTA induced angiogenesis significantly, it failed to reduce the area of necrosis compared to the other groups.

CONCLUSION

BTA was effective in increasing angiogenesis in the axial skin flap although it was unable to reduce necrosis.

KEYWORDS
Botulinum toxin type A; Necrosis; Survival; Axial; Rat; Skin flap

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INTRODUCTION

Skin flap procedures, as the most common flaps, are routinely applied in plastic surgery to repair local tissue defects. Among all other forms of flaps, random flaps, especially Random Skin Flaps (RSFs) are widely performed in reconstructive surgery. Despite their advantages, RSFs rely on sub-dermal and dermal vascular plexuses which are unreliable for blood supply. In other words, these flaps, especially their distal parts are prone to ischemic necrosis and partial flap loss[1].
The normal skin blood flow is mainly regulated by the neural input.

However, humoral vasoactive substances such as nitric oxide (NO) play an important role in the case of a skin flap blood flow. The local loss of sympathetic input, as an immediate consequence of flap elevation, coupled with the unchallenged activity of humoral vasoconstrictors, leads to an ischemic state in random skin flaps, most pronounced during the first 6-12 h postoperatively[2].

Approximately, 6 to 12 h after flap elevation, 80% of the blood flow of the distal part of the flap is lost and the total flap circulation diminishes to 75% of the normal amount within one week or two weeks and returning to normal values lasting three to four weeks. Systemic and local factors such as hypotension, smoking, vasoconstrictors, dressings, positioning, and hematoma may exacerbate this phenomenon and contribute to flap necrosis. Theoretically, medical or surgical delay procedures are the most common interventions for reducing the risk of flap necrosis[3].

Nowadays, the surgical delay is accepted as the gold standard method for increasing flap viability although it has various disadvantages such as multistage surgery, increased fibrosis, along a decreased range of flap motion. Theoretically, the medical delay has many advantages over surgical delay although the lack of approved effective medication is considered as the main matter of medical delay. Previous researches have evaluated different medications such as anti-inflammatory agents, leukocyte aggregation and adhesion inhibitors, alpha-adrenergic antagonists, catecholamine release inhibitors, beta-agonist, direct vasodilators, and calcium channel inhibitors although there has been no consensus on any medication[4].

Botulinum toxin (BTX), as the polypeptide production of the bacterium Clostridium botulinum includes seven (A-G) serotypes[5]. The binding of botulinum toxin type A (BTA) to the presynaptic terminal of the neuromuscular junction causes the temporary block of acetylcholine release into the neuromuscular junction (chemical denervation) and the limited release of norepinephrine (chemical sympathectomy)[6]. Recently, the therapeutic indications of BTA have been expanded for axial hyperhidrosis, blepharospasm, facial spasms, cervical dystonia in addition to the spasms of the extremities and the aesthetic indications of facial wrinkles. In addition, a broad spectrum of other indications is present for migraine, achalasia, urinary bladder dysfunction, and anal fissure[5].

There is no evidence regarding the negative effect of BTA on adipose-derived stem cells, mature adipocytes, or fibroblasts. Previous researches have confirmed the usage of BTA for better wound healing and its application with the autologous fat graft for better survival. In vitro findings suggest that the medium concentration of BTA motivates the migration and angiogenesis of keratinocytes[7].

The administration of BTA significantly increases endothelial NO synthase expression while it has no effect on the level of NO, and neuropeptide-Y reduces the norepinephrine level[8].

Further, the injection of BTA before flap elevation increases angiogenesis via the hypoxia-inducible factor (HIF)1α/vascular endothelial growth factor (VEGF)-dependent angiogenesis[9] and significantly softens ischemia-reperfusion injuries[10].

Furthermore, the upregulating of the expression of ras homolog gene family, member A, ras-related C3 botulinum toxin substrate 1, and cell division control protein 42 after BTA injection augments VEGF and angiogenesis via the mitogen-activated protein kinase signaling pathway[11].

Autophagy, as a lysosomal-dependent catabolic pathway, significantly acts in maintaining cellular hemostasis. Additionally, it contributes to cell adaptation and survival. Thus, dysregulated autophagy leads to cell dysfunction or apoptosis. Further, BTA increases autophagy while it reduces apoptosis during ischemic reperfusion injuries[12].

The arterial and venous diameter and peak mean velocity of blood flow grow up by BTA application while it has no synergistic effect with topical vasodilators on vasodilation. Thus, it improves circulation while decreasing ischemic-reperfusion results, as well as the consequences of ischemia[13-15]. CD34 is a highly reliable marker for the actual angiogenesis of the surgical flap[9].

MATERIAL AND METHODS

Ethics Statement

All experimental procedures and animal maintenance protocols used in this research were reviewed and approved by Animals Research Ethics Committee of the Medical School of Kerman Medicine University.

Animals

The present controlled experimental study was performed in Kerman University of Medical Science,

Kermanshah, during 2016-2017 Iran. In general, 48 male Wistar albino, disease-free rats, weighing 250-350 g, were used in this experimental study. Each rat was individually placed in a polycarbonate cage at 20-22 °C temperature, received standard ventilation, was under a 12-hour light-dark cycle, and had free access to food and water.

Experimental Design

Three rats were used for training the team, adjusting the BTA dose, and setting up the protocols. Then, the 45 remaining rats were incidentally divided into three equal groups. Group 1 (control group) received no preconditioning while Groups 2 and 3 were preconditioned by the intradermal injection of normal saline (0.5 ml) in the cephalic end of the skin flap and the injection of the BTA (1.6 units NeuroNEXT) reconstituted in normal saline, respectively.

Surgery

To this end, 14 d after preconditioning, under general anesthesia, induced by the intramuscular injection of ketamine (80 mg/kg) and xylazine (3 mg/kg), the dorsal hair of each rat was shaved by an electrical shaver, and then cefazoline was administered as a single intramuscular injection. Next, under sterile conditions, a plastic surgeon who was unaware of the preconditioning status of each group elevated a cephalically-based axial fasciocutaneous flap at the dorsum of each rat respecting the width/length ratio of 1:4. Further, a small specimen (3*3mm) was biopsied from the most distal part of the flap to evaluate angiogenesis for studying the result of preconditioning. The flap was reinserted in its donor site and sutured to the surrounding skin. All rats were housed for 14 d under described circumstances (Figure 1). Next, the single daily dose of cefazolin was intramuscularly injected until the 4th postoperative day. After 14 d, digital photography was taken by Canon digital camera under general anesthesia. Finally, all rats were killed by injecting high dose intraperitoneal thiopental.

Image Analysis

The viability of the flaps was evaluated by a physician who was blind to the preconditioning status of each rat. Furthermore, the total surface area, the necrotic area, and the survival of each flap were measured by the AutoCAD system as millimeter square, followed by calculating the viable percentage of each flap.

Survival Flap Rate = (Total Flap Surface - Necrotic Surface Area) ÷ Total Flap Surface

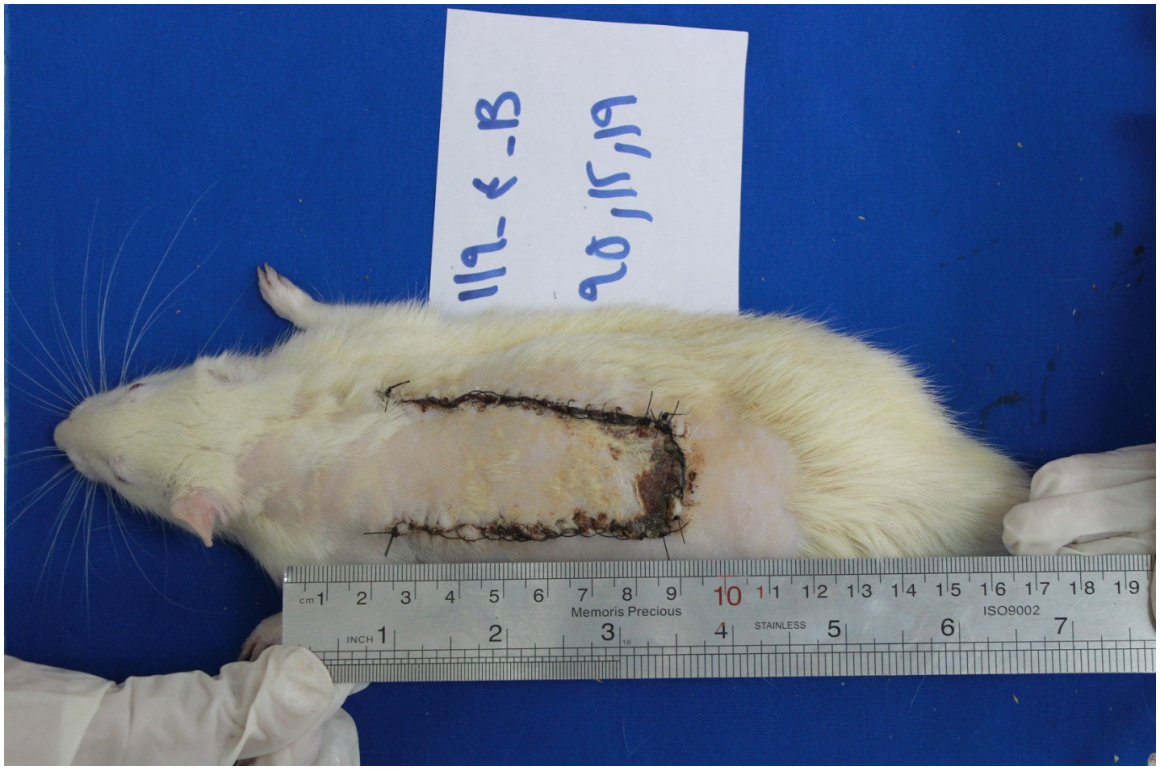

Figure 1: Flap photography on the 14th day after flap harvest

Histological Staining

The specimens were fixed with 10% neutral formalin solution and embedded in paraffin. Then, multiple 4-µm-thick 10% formalin-fixed, paraffin-embedded tissue sections were prepared and stained with hematoxylin-eosin (H&E). The sections were deparaffinized in xylene three times, each process lasting 10 min, and subsequently rehydrated by graded alcohols. Endogenous peroxidase activity was blocked by treating the sections with a blocking solution. For antigen retrieval, the sections were treated while boiling in citrate buffer (pH9.0) in a microwave oven. Then the sections were cooled down at room temperature for 1.5 h. After rinsing in distilled water and TBS successively, sections were incubated afterward with primary antibodies against CD34 (ZYTOMED systems, clone: QBEnd/10,Cat.No.BMS045,Ready-to-use,without dilution), at 60 min. After each step, slides were rinsed with TBS buffer for 3 min. A pathologist blinded to the treatment group checked the amount of angiogenesis in tissue slides staining by H&E as well as CD34.

Concerning the number of neovessels in high power fields, the results of angiogenesis were divided into mild (≤ 5 neovessels/hpf), moderate (5 < neovessels/hpf ≤ 10), severe (>10 neovessels/hpf) groups[4] (Figures 2-4).

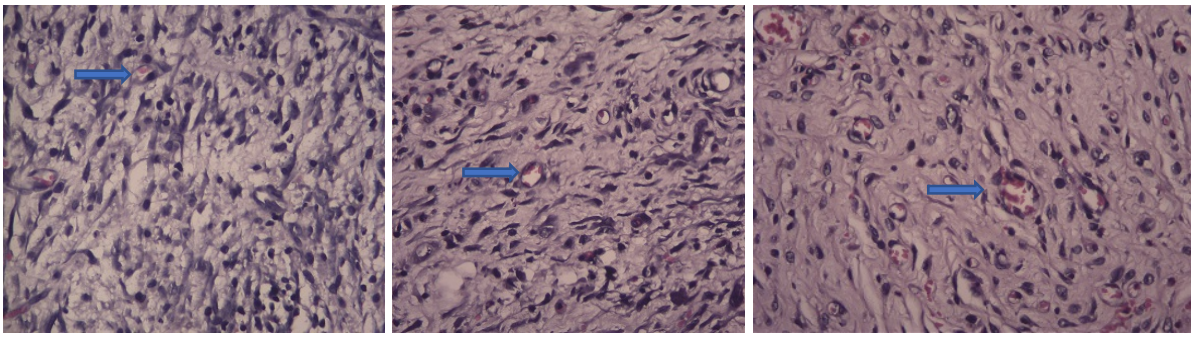

Figure 2: H&E staining (×400) for angiogenesis on 14th day postinjection (mild, moderate, severe)

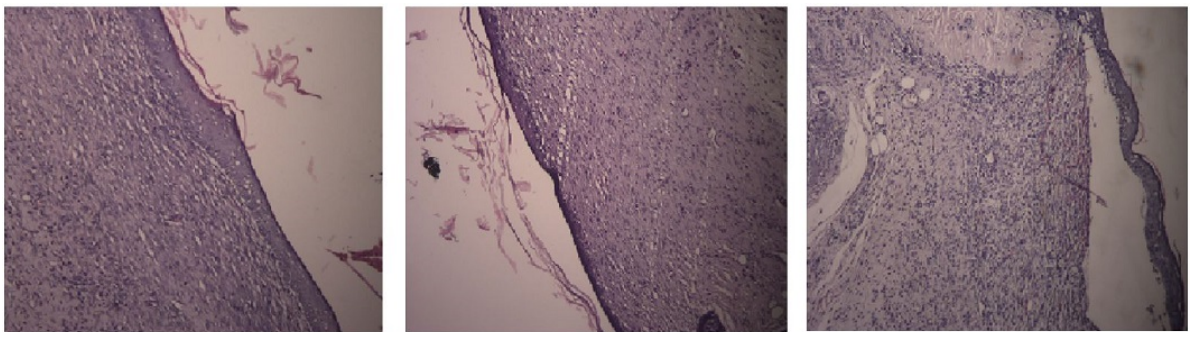

Figure 3: H&E staining (×100) for angiogenesis on 14th day postinjection (mild, moderate, severe)

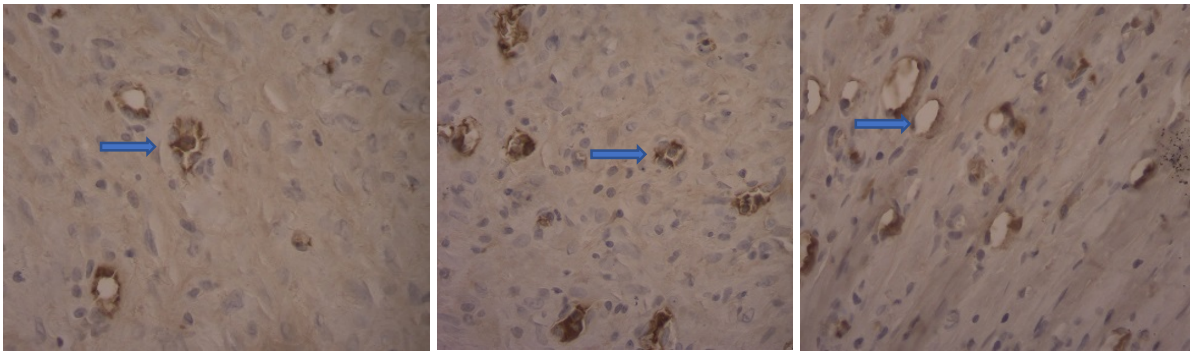

Figure 4: CD34 IHC staining (×400) for angiogenesis on 14th day postinjection (mild, moderate, severe)

Statistical Analysis

Statistical analyses were performed using SPSS (version 16, Chicago, IL, USA), and a $P<0.05$ value was regarded as statistically significant. The Kruskal-Wallis and the analysis of variance (ANOVA) tests were used for more precious data evaluations.

RESULTS

Table 1 presents the total surface area, viable area, and flap survival percentage. Although the flap survival percentage was not equal between the groups, the results of the ANOVA test (Table2) revealed no significant differences between the groups. Eventually, botulinum toxin type A (BTA) had no positive effect on reducing flap necrosis ($P=0.129$).

The angiogenesis rates of all groups are provided in Table 3. A significant difference was found between the groups regarding angiogenesis. According to the results of Kruskal-Wallis test (Tables 4 and 5; BTA significantly induces angiogenesis.

DISCUSSION

Although some studies have focused on the effect of BTA on some kinds of flaps such as muscular, random skin, perforated skin, and transverse rectus myocutaneous (TRAM) flap, no data are available regarding evaluating BTA preconditioning on the axial flap. For example, Miao Chen et al demonstrated the effect of BTA injection in improving flap survival although they failed to find any difference among the time of injection, 2,3, or 4 wk before the surgery. Finally, they reported the increased number of chock vessels after BTA administration, which was meaningfully time-dependent[16].

Another study evaluated the effect of the muscular injection of BTA on the perforator flap. The perfusion area was larger immediately after flap elevation and its necrosis was lower after the 8th day compared to the other areas[17].

Table 1: Mean, standard deviation, minimum and maximum of the total surface area, viable surface area and survived percentage of flap in all groups of study at 14th day

Group		Total flap surface area (cm_2)	Survived flap surface area (cm_2)	Survived flap surface percentage
Botulinum	Number	15	15	15
	Mean	16.0999	11.5934	71.3856
	Stand. Deviation	5.27825	5.01271	15.69726
	Minimum	9.27	5.03	38.71
	Maximum	26.98	20.58	96.33
Normal saline	Number	15	15	15
	Mean	18.6918	13.8915	73.1941
	Stand. Deviation	3.96590	5.58414	21.03516
	Minimum	12.23	5.00	40.19
	Maximum	25.94	25.84	99.61
Control	Number	15	15	15
	Mean	18.3025	10.5994	59.0143
	Stand. Deviation	2.97194	4.58118	23.65352
	Minimum	11.86	2.01	8.89
	Maximum	22.61	18.42	83.54
Total	Number	45	45	45
	Mean	17.6981	12.0281	67.8647
	Stand. Deviation	4.24396	5.15155	20.92417
	Minimum	9.27	2.01	8.89
	Maximum	26.98	25.84	99.61

Table 2: Compression of the survival area as well as survival percentage of flap among three groups by ANOVA statistical test

Variable		Sum of squares	df	Mean square	F	Sig.
Total flap surface	Between groups	58.604	2	29.302	1.677	0.199
	Within groups	733.889	42	17.474		
	total	792.494	44			
Survived flap surface	Between groups	85.535	2	42.767	1.660	0.202
	Within groups	1082.159	42	25.766		
	total	1167.694	44			
Survival percentage	Between groups	1786.922	2	893.461	2.147	0.129
	Within groups	17477.193	42	416.124		
	total	19264.115	44			

Table 3: Degree of angiogenesis on the 14th day of study

Group		Frequency	Percent	Valid percent	Cumulative percent
Botulinum	Mild	5	33.3	33.3	33.3
	Moderate	8	53.3	53.3	86.7
	Severe	2	13.3	13.3	100.0
	Total	15	100.0	100.0	
Normal Saline	Without angiogenesis	8	53.3	53.3	53.3
	Mild	7	46.7	46.7	100.0
	Total	15	100.0	100.0	
Control	Mild	5	33.3	33.3	33.3
	Moderate	10	66.7	66.7	100.0
	Total	15	100.0	100.0	

In their study on evaluating the effect of BTA on random skin flaps with various width-to-length ratios, BTA did not affect the width-to-length ratio of 1:1 although it improved the survival of the width-to-length ratio of 1:2 and 1:3 flaps[18].

A significant increase in the survival percentage of perforator flaps was observed after BTA application despite 180- or 360-degree perforator twisting[19]. However, the present study reported no significant improvement effect of BTA on the survival of the axial skin flap. In another study, the use of BTA improves the viability of the random flap in tobacco-exposed rats[20].

Additionally, the chemical delay effect of BTA on TRAM flap was compared with and without a surgical delay[21]. The results revealed significant increased vascular density and diameter of the

Table 5: Compression of angiogenesis among groups by Kruskal-Wallis test

Variable		N	Mean Rank
Angiogenesis	Botulinum	15	30.00
	Normal Saline	15	10.33
	Control	15	28.67
	Total	45	

Table 4: Mean of angiogenesis in groups

Variable	Angiogenesis
Chi-square	23.956
Df	2
Asymp. Sig.	0.000

arterial vessels while decreased necrotic areas of the flap in a surgical and chemical delayed flap concerning the non-delayed flap with no difference between these two delay methods.

BTA causes a significant decrease in the relative messenger RNA (mRNA) expression of the CD31 n TRAM flap while a decrease in CD31 positively stained vessel density in 2nd and 4thzones of TRAM flap. On the other hand, it leads to an increase in the relative mRNA expression of VEGF and the survival of this flap[22].

In addition, BTA significantly increases the relative mRNA expression of CD34 and VEGF, as well as the relative protein expression of CD34, VEGF, and HIF-1α and the survival of the animal TRAM flap[6]. Accordingly, the findings support the positive effect of BTA on CD34 density and the angiogenesis of the axial skin flap.

CONCLUSION

Although botulinum toxin type A preconditioning increases the angiogenesis of axial skin flaps, it cannot grow up their survival.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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