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 loss¹. 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.

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.

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, and 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.

Botulinum toxin (BTX), as the polypeptide production of the bacterium Clostridium botulinum includes seven (A-G) serotypes. 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). 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.

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. 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.

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

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. 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.

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. CD34 is a highly reliable marker for the actual angiogenesis of the surgical flap.

**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.

\[
\text{Survival Flap Rate} = \left( \frac{\text{Total Flap Surface} - \text{Necrotic Surface Area}}{\text{Total Flap Surface}} \right) 
\]

**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) groups² (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 (Table 2) 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$^{17}$.

| Group        | Total flap surface area (cm²) | Survived flap surface area (cm²) | 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                           |
| Number       | 15                            | 15                               | 15                              |
| Mean         | 18.6918                       | 13.8915                          | 73.1941                         |
| **Normal saline** |                       |                                  |                                 |
| Number       | 15                            | 15                               | 15                              |
| Mean         | 18.3025                       | 10.5994                          | 59.0143                         |
| Stand. Deviation | 2.97194                  | 4.58118                          | 23.65352                        |
| **Control**  |                               |                                  |                                 |
| Number       | 15                            | 15                               | 15                              |
| Mean         | 17.6981                       | 12.0281                          | 67.8647                         |
| Stand. Deviation | 4.24396                  | 5.15155                          | 20.92417                        |
| **Total**    |                               |                                  |                                 |
| Number       | 45                            | 45                               | 45                              |
| Mean         | 26.98                         | 25.84                            | 99.61                           |

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
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\(^8\). 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 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      | 1.660 | 0.202 |
| **Survived flap surface** |              |     |             |       |       |
| Between groups         | 85.535         | 2   | 42.767      |       |       |
| Within groups          | 1082.159       | 42  | 25.766      | 1.660 | 0.202 |
| **Survival percentage** |                |     |             |       |       |
| Between groups         | 1786.922       | 2   | 893.461     | 2.147 | 0.129 |
| Within groups          | 17477.193      | 42  | 416.124     |       |       |

### 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** |       |         |               |                   |
| Mild          | 7         | 46.7    | 46.7          | 100.0             |
| **Total**     | 15        | 100.0   |               |                   |
| **Control**   |           |         |               |                   |
| Mild          | 5         | 33.3    | 33.3          | 33.3              |
| Moderate      | 10        | 66.7    | 66.7          | 100.0             |
| **Total**     | 15        | 100.0   |               |                   |

### Table 4: Mean of angiogenesis in groups

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

### 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 |           |
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 in TRAM flap while a decrease in CD31 positively stained vessel density in 2nd and 4th zones 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\(^2\).

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.

**REFERENCES**

1. Cristina PC, Alfredo LJ, Cláudia NB, Miriam L, et al. Botulinum toxin type A on cutaneous flap viability in diabetic and tobacco-exposed rats. *Acta Cirúrgica Brasileira* 2015; Vol. 30 (9): 639 – 645.

2. Ghanbarzadeh K, Tabatabaie OR, Salehifar E, Amanlou M, et al. Effect of botulinum toxin A and nitroglycerin on blood flow of ischemic DBA/2J mice. *Int J Exp Pathol* 2015; 96 (4): 92-100.

3. Hikmet K, Burak K, Muzaffer C, Ahmet T, Gürcan A. Prevention of unfavourable effects of cigarette smoke on flap viability using botulinum toxin in random pattern flaps: An experimental study. *Plast Surg* 2015; 23 (3):177-182.

4. Iranpour M, Khodarahmi A, Khodarahmi N, Shafiee M, et al. Montelukast for Medical Delay in Flap Surgery. *World J Plast Surg* 2020; 9 (1): 48-54.

5. Akihiko U, Kazuya Y, Buddhini P, Sachiko O, et al. Protective effect of botulinum toxin A after cutaneous ischemia-reperfusion injury. *Scientific Reports* 2015;5:9072. doi: 10.1038/srep09072: 1-7.

6. Peter BA, Taolin F, Somjade JS, Georgios Z, et al. Inflammatory Response and Survival of Pedicled Abdominal Flaps in a Rat Model after Perivascular Application of Botulinum Toxin Type A. *Plast Reconstr Surg* 2014;133(4):491e-498e.

7. Alfred G, Johanna K, Melanie S, Elisabeth B, et al. Botulinum Toxin A: Dose-dependent Effect on Reepithelialization and Angiogenesis. *Plast Reconstr Surg Glob Open* 2016;4:e837: 1-7.

8. Tai S, Bok KJ, Insik Y, Dae HL, et al. Effect of botulinum toxin A on vasoconstriction and sympathetic neurotransmitters in a murine random pattern skin flap model. *Wound Repair Regen* 2016; 20 (5): 75-85.

9. Tae HP, Song HL, Yun J, Young SL, et al. Presurgical Botulinum Toxin A Treatment Increases Angiogenesis by Hypoxia-Inducible Factor-1α/Vascular Endothelial Growth Factor and Subsequent Superiorly Based Transverse Rectus Abdominis Myocutaneous Flap Survival in a Rat Model. *Ann Plast Surg* 2016;76: 723–728.

10. Yun JP, Jang WL, Yoseph Ch, Tae HP. Botulinum toxin A increases allograft tolerance in an experimental transplantation model: a preliminary study. *Bioscience Reports* 2018; 38 BSR20171721: 1-8.

11. Tae HP, Ji H, Choong HC, Dong KR. Botulinum Toxin A Upregulates Rac1, Cdc42, and RhoA Gene Expression in a Dose-Dependent Manner: In Vivo and in Vitro Study. *J Craniofac Surg* 2016;27: 516–520.

12. Yanyu S, Huang L., Jiankun C, Chao C. Botulinum toxin type A induces protective autophagy in human dermal microvascular endothelial cells exposed to an in vitro model of ischemia/reperfusion injury. *Exp Ther Med* 2018; 16:4379–4386.

13. Roberto GA, Somjade JS, Samantha RS, Peter BA, et al. Microcirculatory Effects of Botulinum Toxin A in the Rat Acute and Chronic Vasodilation. *Ann Plast Surg* 2017;79: 82–85.

14. Tae HP, Yun JP. The Effect of Botulinum Toxin A on Ischemia-Reperfusion Injury in a Rat Model. *Biomed Res Int* 2017;2017:1074178.

15. Huang L. Beneficial effect of botulinum toxin A on secondary ischaemic injury of skin flaps in rats. *Br J Oral Maxillofac Surg* 2018; 56:144-147.

16. Miao C, Xiucun L, Zhenmin J, Xu G. Visualizing the Pharmacologic Preconditioning Effect of Botulinum Toxin Type A by Infrared Thermography in a Rat Pedicled Perforator Island Flap Model. *Plast Reconstr Surg* 2019; 144: 1016e.

17. Umut Z, Neşe KÖ, Zekiye H. Effect of Botulinum Toxin-A Injected to Muscle Tissue on Perfusion and Survival of Fasciocutaneous Single Perforator-pedicled Propeller Flap in Rats. *Balkan Med J* 2020;37:84-90.

18. Wael M El S, Ahmed EEA, Wael MS, Emad MH, et al. Effect
of Perivascular Injection of Botulinum Toxin Type A versus Lidocaine in Survival of Random Pattern Flaps in a Rat Model. Plast. Reconstr Surg 2019; 143: 527e.
19. Sung YK, Song HL, Boram I, Yun JP, et al. The Protective Effects of Botulinum Toxin A Against Flap Necrosis After Perforator Twisting and Its Underlying Molecular Mechanism in a Rat Model. Ann Plast Surg 2016;77: 242–248.
20. Cristina PC, Felipe AF, Michel HML, Luiza CMS, et al. The positive effect of Botulinum toxin type A on the viability of random flap in tobacco exposed in rats. Acta Cirúrgica Brasileira 2016; Vol. 31 (11): 720- 723.
21. Gökhan T, Nebil Y, Hakan S, Ali CA, et al. Increasing the survival of transverse rectus abdominis musculocutaneous flaps with a Botulinum toxin-A injection: A comparison of surgical and chemical flap delay methods. J Plast Reconstr Aesthet Surg 2016; 69, 944-951.
22. Tae HP, Dong KR, Yosep C, June-kyu K. The Effects of Botulinum Toxin A on Survival of Rat TRAM Flap With Vertical Midline Scar. Ann Plast Surg 2015;74: 100-106.