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The potential effect of intradermal Botulinum Toxin Type-A (BTA) injection to increase extended random skin flap survival

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ABSTRACT

Background: Extended random skin flap failure often occurs due to insufficient vascularization. Several attempts have been tried to increase the viability of the flap, but none of these attempts have achieved maximal success. Previous research has shown that Botulinum toxin type A (BTA) increases the viability of cutaneous flaps by inhibiting muscle contraction along with increasing vasodilation and angiogenesis. This study examines the effect of intradermal BTA injection on extended random skin flaps viability.

Methods: In thirty-six Wistar rats, a rectangular random cutaneous flap (6 cm × 1.5 cm) was made on each dorsal rat area and then elevated the flap. The treatment group was intradermally injected evenly with BTA 8 IU and saline solution for the control group. Each rat flap viability was observed after five days. Primary outcome measures are survival flap area using Visitrak, microscopic assessment of the number and diameter of capillaries and Vascular Endothelial Growth Factor (VEGF) expression was analyzed using immunohistochemistry semi-quantitative scoring system. Data were analyzed using SPSS version 21 for Windows.

Results: The treatment group has better results in all observed parameters than the control group. However, Only the VEGF expression of proximal edge showed a statistically significant increase in the group treated with BTA injection (p=0.004).

Conclusion: This study showed that the BTA treatment has a positive effect on random skin flap survival and the reduction of distal necrosis in rats, so it has been proven that BTA has the potential to increase flap survival rates in small animal models. Further studies are needed to determine whether BTA treatment can produce similar findings in humans.

Keywords: BTA Injection, Myocutaneous Flap, Vascular Endothelial Growth Factor, Tissue survival.
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INTRODUCTION

Unlike most other surgical specialties, the field of plastic surgery does not claim to have any particular anatomical or functional area. Rather, the methods and techniques of a plastic surgeon's practice are applicable to all specialties and areas of anatomy.[1] Reconstructive surgery is the process of restoring the human body to a "whole", restoring form and function after the removal of a tumor, after an infection, after trauma, congenital, or acquired deformity. Since tumors, infections, trauma, or deformities have such a profound effect on a patient's life, health and well-being, reconstructive surgery has a powerful impact on literally rebuilding a patient's life.[1]

The concept of muscle and myocutaneous flap was first introduced by Mathes and Nahai in the 1970s and had a huge influence on the choice of reconstruction. Then, this concept was known as the concept of a reconstructive elevator or reconstructive ladder, a principle-based reconstructive choice algorithm.[1,2]

A flap is an important technique in reconstructing both the skin and the underlying tissue; therefore, the ability to create, design, perform, and handle a flap is the hallmark of a plastic surgeon. A good flap will aid reconstruction with good wound healing and restore the functions and skin aesthetics.[2,3] Necrosis is still considered one of the most important complications of reconstructive surgery. The random skin flap has different limitations, such as the length to width ratio limit and the size of the rotation. Unfortunately, necrosis of the distal part of the flap is not always avoidable by designing a suitable flap design. Several medicaments have been investigated to prevent flap ischemia; one of them is Botulinum toxin with vasodilating effect through inhibiting the acetylcholine and norepinephrine release as well as increasing the VEGF production.[3]

Botulinum toxin is known to be fatal, but its therapeutic use has been promoted since the 1980s. It is currently used in many fields for various therapeutic purposes, including treating blepharospasm and hemifacial spasms cervical dystonia, and has been shown to have therapeutic effects for headaches. In plastic surgery, botulinum toxin provides satisfactory results in rejuvenation.[3]

Since the FDA approved botulinum toxin A in 1989, more research has established the usefulness of botulinum toxin. The basic mechanism of botulinum toxin type A depends on the acetylcholine's presynaptic release. According to Çelik E et al., botulinum toxin type A inhibits muscle contraction and prevents muscle spasms after muscle flap surgery through a temporary denervation effect and increases muscle flap viability.[4]

Research evidence suggests botulinum toxin type A can cause vasodilation by affecting the skin's autonomic sympathetic nervous system through selective control of sympathetic neurons. Thus, it strengthens the hypothesis that botulinum toxin type A can improve flap survival through vasodilation based on previous studies.[5] Based on those mentioned above, this study aims to evaluate the effect of botulinum toxin type A intradermal injection on the viability of randomized expanded skin flaps in rats.

MATERIAL AND METHODS

The experimental animal used in this study was 36 healthy male rats (*Rattus novergicus*) of Wistar strain, approximately 3 months old and weighing between 250-300 grams. The observation was conducted on the control and treatment groups. Rats were anesthetized using ketamine-xylazine 20 mg/kgBW intramuscular in the vastus lateralis muscle. Then, 6 cm-long and 1.5 cm-wide rectangular incisions were made in the shaved back of each rat. We performed disinfection with a 10% solution of povidone-iodine and Savlon 1:30.

A linear incision was made involving the panniculus carnosus and then elevated to form a flap with a pedicle on the cranial side using a surgical blade number 15 and small Metzenbaum scissors. The donor wound and the flap were closed with a sterile transparent dressing (Tegaderm). The flap formed is placed back over the donor wound, closed with a transparent dressing so that there is no vascular encounter between the flap and the donor surface. Sew the ends of the flap using a simple suture Nylon 4.0 thread. The flap was closed with a transparent dressing before being covered with gauze and leucoplast, and the flap area was measured using Visitrak. All rats were given Penicillin Procaine injection of 100 mg/kgBW intramuscularly.

Rats in the control group were given 0.8 mL saline intradermal injection of NaCl 0.9%. On the other hand, the treatment group received intradermal Botulinum toxin type A (BOTOX®) injection. BOTOX® in this experiment is manufactured by Allergan, Irvine, CA, USA that about 8 IU (0.8 mL). Each injection was evenly distributed in four zones, namely zone A, zone B, zone C, and zone D: 0.2 mL per zone. Each zone was injected intradermally using a 10 cc syringe, with a 30G needle at four injection spots with a dosage for each spot of 0.05 mL. Furthermore, the rats were kept in their cages and given the same dietary intake.

On the fifth day, the bleeding point was examined on the flap with an incision parallel to lines C and D with a 3 mm distance. Then, a clinical assessment of the necrotic area was carried out using Visitrak. The compromise to the necrotic area is defined as a bluish, grey to the blackish area on the distal flap surface. Incisions were made with a 3 mm distance parallel to line C and line D from caudal to proximal; pinpoint bleeding was observed to confirm the viable area. Rats were decapitated, then 6 cm x 1.5 cm skin flaps were excised and spread on filter paper. The edges of the flap were fixed with a needle and then the viable area was measured. We took 0.5 cm x 0.5 cm specimens from zone A and B's distal edges in the viable area to assess VEGF expression, vascular density, and vascular diameter by microscopic examination. The specimens were put into bottles containing 10% formalin buffer for tissue fixation then immunohistochemical staining was carried out with VEGF antibodies.

Expression of VEGF was assessed by a chromogenic brown color which appears to be found both on the endothelial wall, fibroblast cells and inflammatory cells in the healing area. Expression of VEGF data was obtained by modified Remmele method, where the Remmele scale index is presented as Immuno Reactive Score (IRS), which is the result of multiplication of the percentage of Positive Immunoreactive Cells (PIC) with Colour Intensity Scores (CIS) on immunoreactive cells based on the previous study.[5]

Specimens were examined under a 200 times magnification microscope to assess VEGF expression and 400 times magnification to assess vascular density and diameter. The collected data were then analyzed for normality using the Shapiro-Wilk normality test and mean difference using independent t-test in SPSS version 21.0 for Windows.

RESULTS

This study analyzed the effect of intradermal Botulinum Toxin A injection on extended random skin flap in Wistar rats by assessing the capillary number, capillary diameter, VEGF expression score and viable flap area. Two rats in the control group and one rat in the treatment group died. The total number of samples studied in the control group was 16 individuals, while in the treatment group there were

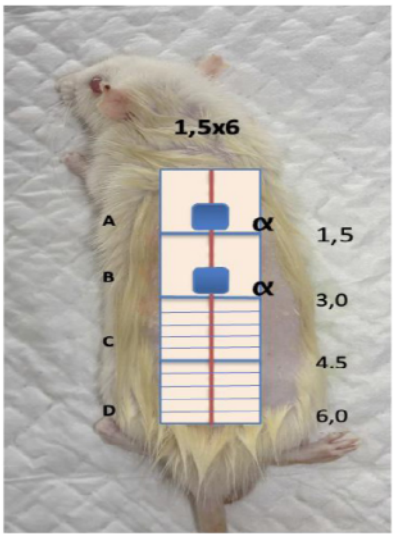

Figure 1.
The clinical examination zones of flap viability. A symbol of α indicates our suturing marking for pathology anatomy superior and inferior marking. Values are presented as the distance of the distal part of each flap zone from the pedicles in centimeters (cm). The clinical examination zones (A, B, C, and D zones) are defined by line A, line B, line C, and line D. Incision is made with a distance of 3 mm parallel to line C and parallel to line D from the caudal to proximal. Blue boxes indicated the area of the samples were taken.

17 individuals. Samples were only taken at zone A's distal edge and zone B's distal edge (Figure 1).

The distal edge of zone A is the proximal edge, and the distal edge of zone B is the distal edge. In taking viable flap area sample data, the author looked at the whole macroscopic (zone A, zone B, zone C and zone D) of the extended random skin flap. The first study was microscopically assessed at the capillary diameter and the number of capillaries of the skins of the rats using Haematoxylin Eosin (HE) staining (Figure 2).

The capillary number value is presented as count per 5 fields of view and capillary diameter is presented as ìm. Capillary number data is the total number of capillaries found in five different FVs, while capillary diameter data is the mean diameter value of 25 capillaries found at five different FVs. All data on this examination were obtained at 1000 times magnification. Code A is a sample taken from the proximal edge of the extended random skin flap, while code B is taken from the distal edge of the extended random skin flap. According to the Table 1, the mean capillary number and median capillary diameter tended to be higher in the treatment group instead of not statistically significant ($p>0.05$).

This study demonstrated an increase in the average number of capillaries and capillary diameter in the group treated with botulinum toxin type A injection in the proximal flap and distal flap areas. Furthermore, this study was evaluated the microscopic expression of VEGF using Immunohistochemical (IHC) examination, as shown in Figure 3. VEGF expression value is presented as Immuno Reactive Score (IRS) and viable skin area is presented as cm^2. Tables 1 and 2 show greater capillary number, diameter, and VEGF expression in the proximal edge and distal edge and viable skin area (Figure 3). The treatment group has greater results in all observed parameters than the control group but statistically significant only in the proximal edge of VEGF expression ($p<0.05$) (Table 2).

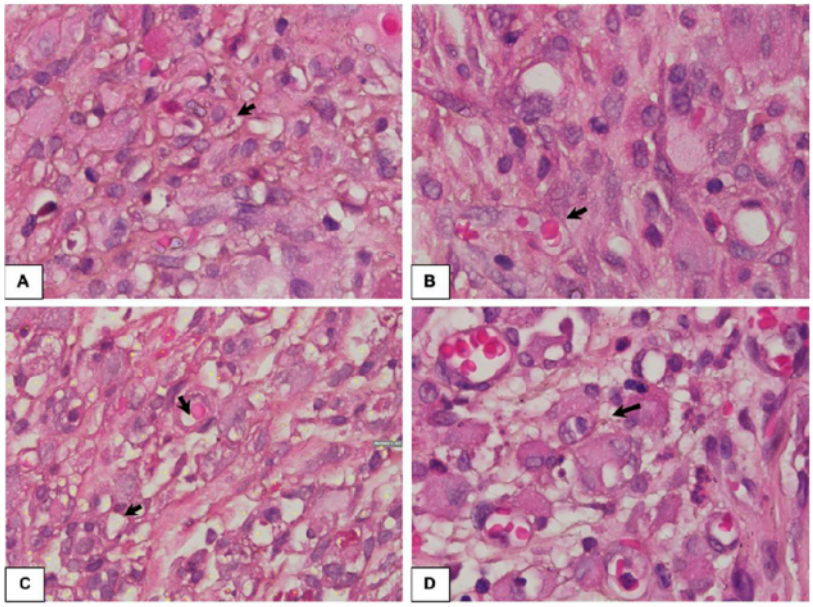

Figure 2. Comparison of the number of capillaries. The picture showed that the total number of capillaries in five different FVs indicated that the C treatment group was the highest, and the B control group was the lowest, while the A control group and D treatment group were relatively the same (HE staining;1000x; Nikon H600L microscope; 300megapixel DS Fi2 camera) (A. The proximal part of the flap (the control group); B. The distal part of the flap (the control group); C. The proximal part of the flap (the treatment group); D. The distal part of the flap (the treatment group); and the arrows show the capillaries and the capillaries diameters).

DISCUSSION

This study evaluated the viable macroscopic area of the extended random skin flap area on the rat's backs using the Visitrak tool. After assessing bleeding points in the necrotic area. There was no statistically significant mean difference in viable flap area between the control group and the group injected with botulinum toxin type A. In this study, the percentage increase in viable area in the group with botulinum toxin type A injection was 11.1%, higher than the percentage of the viable area from the study conducted by Kim TK et al., which obtained 8.3%. However, this was not an exact comparison because our study and Kim et al. used different types of rats, botulinum toxin type A doses and the flap techniques.[6]

The advantage of this study was that the tissue specimen collection technique was carried out uniformly in each zone A and B on the flap of the botulinum toxin type A injection group and the control group. In contrast, previous studies did not elaborate on tissue specimen sampling area uniformity.[5,6]

Upon evaluation, the mean proximal edge capillary diameter in the treatment group was higher than in the control group. There was a mean difference in the diameter increase at the proximal and distal edges in the group with botulinum toxin type A injection of 0.83% and 1.88%, respectively. Although this was not statistically significant, this result was in accordance with the theory that botulinum toxin type A could increase blood vessel diameter through its muscle relaxant mechanism by inhibiting the release of acetylcholine.

In evaluating the number of capillaries, there was an increase in the number of capillaries at both the proximal and distal edges in the treatment group. There was a difference in the increase in capillaries at the proximal and distal edges in the group with botulinum toxin type A injection of 14.33% and 27%, respectively. Although this increase was not statistically significant, it has been theoretically proven

Table 1. Observation results of capillary number and diameter

Group	Sample	Capillary Number				Capillary Diameter			
		Proximal edge		Distal edge		Proximal edge		Distal edge	
		Mean±SD	p	Mean±SD	p	Median (Min-Max)	p	Median (Min-Max)	p
Control	16	338.00±124.00	0.300[a]	264.00±90.00	0.123[a]	5.85 (4.90-7.10)	0.692[b]	5.84 (4.50-6.70)	0.640[b]
Treatment	17	386.00±138.00		334.00±155.00		6.01 (4.40-9.70)		6.01 (4.40-8.40)	

The capillary number value is presented as count per 5 fields of view; Capillary diameter value is presented as ìm; [a]Independent T-Test; [b]Mann-Whitney Test; Min: Minimum; Max: Maximum; *Statistically significant if p-value less than 0.05.

Table 2. Observation Results of VEGF Expression and Viable Skin Area

Group	Sample	VEGF Expression				Viable Skin Area	
		Proximal edge		Distal edge			
		Mean±SD	p	Mean±SD	p	Median (Min-Max)	p
Control	16	7.50±1.60	0.004*	7.40±1.80	0.564	3.10 (2.00-6.60)	0.087
Treatment	17	9.30±1.70		7.80±1.70		4.50 (2.40-7.80)	

VEGF: Vascular Endothelial Growth Factor; VEGF expression value is presented as Immuno Reactive Score (IRS); Viable skin area value is presented as cm^2; [a]Independent T-Test; [b]Mann-Whitney Test; Min: Minimum; Max: Maximum; *Statistically significant if p-value less than 0.05.

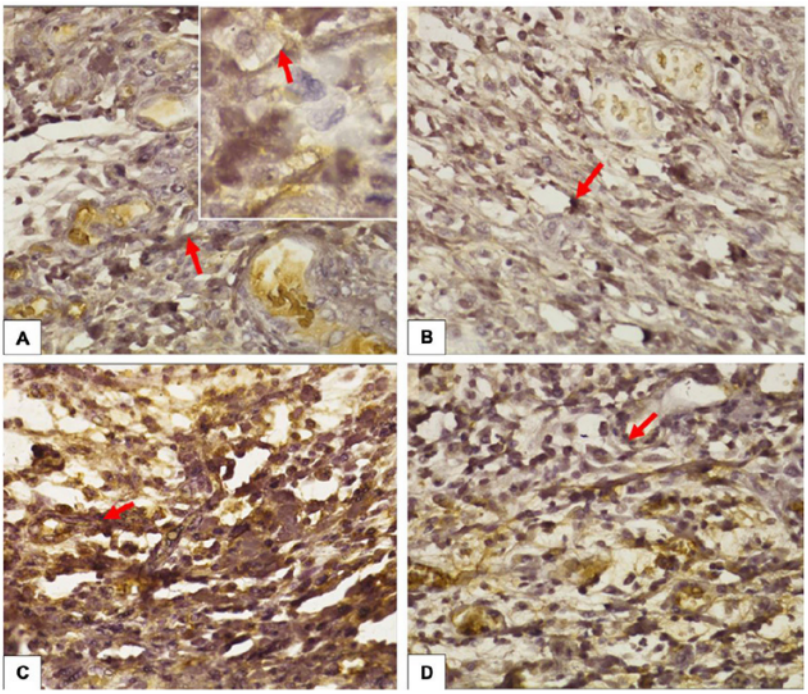

Figure 3. Comparison of VEGF expressions between treatment groups. Expression of VEGF is marked with a chromogenic brown color which appears to be found both on the endothelial wall (arrow), fibroblast cells and inflammatory cells in the healing area (immunohistochemical staining, 400x magnification; 1000x inlet; Nikon H600L microscope; 300 Megapixel DS Fi2 camera) (A. The proximal part of the flap (the control group); B. The distal part of the flap (the control group); C. The proximal part of the flap (the treatment group); and D. The distal part of the flap (the treatment group)).

that botulinum toxin type A injection can increase capillary volume by stimulating the angiogenesis process.[7]

This theory is also supported by an increase in the finding of the VEGF expression in the group treated with botulinum toxin type A injection compared to the control group in the proximal flap and distal flap areas, respectively 24.63% and 5.01%, with the statistical test in the proximal area showed a significant increase. This is in accordance with the theory that botulinum toxin type A can increase VEGF, whereas VEGF as an angiogenic agent can increase angiogenesis to increase flap survival.[7]

Our study suggested a large standard deviation at the proximal edges due to one sample in the treatment group with a capillary number of 99 capillaries. In contrast, the number of capillaries in the other sample was only three to five times larger. In contrast to the findings, the mean diameter in the sample was the largest mean diameter among the other samples in the treatment group.

It is conclusive that botulinum toxin types A still works with its vasodilating effect. According to theory, the revascularization process in the presence of an angiogenic stimulus is preceded by a vasodilation process of blood vessels.[7] In these experimental rats, there was an

increase in the VEGF expression score. In this case, the botulinum toxin type A also acts as an angiogenic stimulus which increases the VEGF.

In this study, specimens taken on the fifth day showed an increase in the number and diameter of capillaries compared to the control group, although statistically significant. However, there was a significant increase in VEGF. This finding indicates that there is still a proliferation process on the fifth day, and there is a potential for a significant increase in the number and diameter of capillaries with a longer observation time. It is in accordance with the theory that neovascularization on the flap starts on the third to the seventh day. Early neovascularization in mice was obtained on the third day.[7]

The viable area in the treatment group was larger than the control group. Although it was not statistically significant, there was an 11.11% mean difference in the group receiving botulinum toxin type A injection. An increase in viable area was in accordance with the theory that botulinum toxin type A could increase flap survival with several mechanisms leading to inhibition of acetylcholine release in the smooth muscle of the blood vessels to prevent vasoconstriction, and increasing VEGF is in accordance with the recent theory that VEGF can increase flap survival because it is an angiogenic agent that supports the endothelial proliferation process.[7-9]

A previous study showed a variation in botulinum toxin type A dosage with an average total dose of 10.28±6.51 IU.[9] The dose used in this study was 8 IU. Ghanbarzadeh K et al. found that botulinum toxin type A injection significantly reduced the necrotic area on the flap compared to the controls. This is different from our research because Ghanbarzadeh et al. used different numbers of mice, the trademark botulinum toxin type A and different flap preparation techniques.[10] Ghanbarzadeh K et al. Used 72 mice, botulinum toxin type A under the brand Dysport and botulinum toxin type A injection performed 2 weeks before the flap was elevated.[10] In contrast, the onset of action of botulinum toxin type A appears on day 1 after flap elevation and the maximum is on day 14th.[9,10]

Arnold PB et al. also found no significant difference in the flap necrosis area in the group injected with botulinum toxin type A compared to the control. Arnold et al. used different types of mice and had a shorter observations period.[11] It may take a longer observation time to observe more significant differences. Rat skin is different from human skin, mainly due to a muscle layer of panniculus carnosus under the mouse skin. Therefore, direct comparisons between mouse and human skin flaps are difficult.

Intradermal injection of botulinum toxin type A in the extended random skin flap of Wistar rats increased the number and diameter of capillaries at the proximal and distal edge; and survival area, also the number and diameter of the capillary count at the proximal tip of the flap, although not statistically significant. Intradermal injection of botulinum toxin type A in the extended random skin flap of Wistar rats also significantly increased VEGF expression score at the proximal and distal edges, the proximal tip of the flap, and the flap increases were statistically significant. Botulinum toxin type A has the potential to increase flap survival rates, in which it can increase the number and diameter of capillary blood vessels, VEGF expression score, and viable area in the extended random skin flap.

CONCLUSION

This study showed that the BTA treatment has a positive effect on random skin flap survival and the reduction of distal necrosis in rats, so it has been proven that BTA has the potential to increase flap survival rates in small animals models. Further studies are needed to determine whether BTA treatment can produce similar findings in humans.

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CONFLICT OF INTEREST

The author reports no conflicts of interest in this work.

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ETHICS CONSIDERATION

This research was reviewed and approved by the Animal Care and Use Committee (ACUC) of Veterinary Faculty, Universitas Airlangga, Surabaya, Indonesia, with reference number 2.KE.002.01.2020.

AUTHOR CONTRIBUTIONS

All authors are equally responsible for the initial conceptualization, study design, intellectual content, literature search, clinical studies, experimental studies, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing, review of the earliest draft of the manuscript, and guarantor.

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