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## **Administration of botulinum toxin a and vascular endothelial growth factor reduce norepinephrine levels and increase viability of modified mcfarlane flap in wistar rat**

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**Abstract**--Local hyperadrenergic states after a random flap elevation mediated by humoral vasoconstrictors can lead to flap necrosis. This study aimed to evaluate the effect of Botulinum Toxin A (BoNT-A) on reducing levels of Norepinephrine (NE) as the main humoral vasoactive substance, as well as its additive effect with Vascular Endothelial Growth Factor (VEGF) in increasing the viability of random flap. 28 Wistar Rats with Caudally Based Modified McFarlane Flap were divided into four groups. Group I received BoNT-A 3 days before flap elevation, group II received VEGF immediately after flap elevation, group III received a combination of BoNT-A and VEGF, group IV was a control group. Result in this study NE levels lowest was found in the BoNT-A+VEGF combination group ( $94.63 \pm 5.25$  ng/ml,  $p < 0.05$  in group I,  $p < 0.01$  in groups II and IV). The highest value of FVR was shown in the BoNT-A+VEGF combination group ( $93.37 \pm 2.19$  %,  $p < 0.05$  in group I,  $p < 0.01$  in groups II and IV). Meanwhile, the increase in flap viability in the VEGF group was not statistically significant. So Conclusion administration of BoNT-A in combination with VEGF can increase the viability of random flaps by decreasing NE levels.

**Keywords**--botulinum toxins, VEGF, norepinephrine, microsurgery, flap

**Introduction**

Random flap is one of the reconstruction modalities that is extensively used in plastic surgery. Based on their method of transfer, random flaps are usually local in nature and are usually used for simple defects by fulfilling the "replace like with like" principle so that they are more aesthetically superior than other types of flaps. Adequate vascularization is the most important determinant of flap viability where partial or complete necrosis of the flap can occur due to impaired flap perfusion. The random flap is a type of flap that does not have an anatomically recognized blood supply; therefore, it relies on the microvascular plexus for its metabolic needs; thus, this flap is highly susceptible to ischemic injury.

Several studies have shown that, after random flap elevation, blood flow to the most distal aspect of the flap is reduced to 20% of normal.(Roh *et al.*, 2020) Flap failure due to lack of vascularization is a common condition where one study by Kwok *et al.* (2017) stated that of about 1187 flaps that failed, 5.1% with head and neck flaps occupying the first position (7.7%).(Kwok and Agarwal, 2017) Under normal conditions, skin blood flow is mainly regulated by neural input; however, in the case of skin flaps, humoral vasoactive substances also play an important role. Immediately after flap elevation there is local humoral vasoconstrictor activity causing ischemic state that is most pronounced during the first 6 to 12 hours postoperatively due to a local "hyperadrenergic state" that persists for 18-36 hours after flap elevation. This ischemic state usually reverses after the 3rd day. If necrosis follows ischemic symptoms, the process is irreversible and flap failure occurs. This scenario is encountered mainly when the ratio of the length to width of the random flap is increased. Based on donor site, random flaps with length-to-width ratio >2:1 to 3:1 will usually fail to some extent due to ischemia in the absence of additional intervention (Ghanbarzadeh *et al.*, 2016).

Several interventions have been described to reduce the risk of flap failure due to this ischemia mechanism, such as procedure delay or ischemic preconditioning. However, this procedure is invasive and time consuming, which hinders its routine application in surgical practice. As a consequence, several pharmacological agents and growth factors were piloted to achieve comparable improvements in flap vascularity (Segreto *et al.*, 2019).

Botulinum toxin secreted from *Clostridium botulinum* consists of botulinum neurotoxin and various non-toxic proteins. Botulinum neurotoxin is a polypeptide chain consisting of a heavy chain (100 kDa) and a light chain (50 kDa), which is proteolyzed into two sub-chains during activation. Seven serotypes, namely A, B, C, D, E, F, and G, have been reported. Botulinum Toxin Type A (BoNT-A) is a variant that has been extensively studied and used clinically. The basic mechanism of BoNT-A is to block presynaptic release of acetylcholine. As a neurotoxin, BoNT-A blocks the neuromuscular junction, promoting muscle relaxation, which is the basis for the popular application of BoNT-A in the aesthetic field. However, there is no direct mechanistic evidence for its positive effect at the vascular level (Schweizer *et al.*, 2013).

Norepinephrine (NE) is a non-peptide neurotransmitter that excites vasoconstrictor neurons and increases blood pressure by increasing vascular tone through activation of  $\alpha$ -adrenergic receptors. In vitro tests using the uterine artery and inferior vena cava in experimental animals showed that BoNT-A can inhibit the secretion of NE which is a local humoral vasoconstrictor and can cause an ischemic state of the flap.(Y. S. Kim *et al.*, 2009) Botulinum Toxin Type A (BoNT-A) acts as a vasodilator of preexisting major pedicles or microcirculation by influencing cutaneous autonomic sympathetic nervous system activity through selective suppression of sympathetic neurons. In other words, BoNT-A is predicted to increase flap survival by inhibiting perivascular smooth muscle contraction and thereby increasing blood flow to the flap. But despite these beneficial effects, BoNT-A has limitations as an angiogenic agent (Stone *et al.*, 2012).

Along with blood flow, flap vascularization is also dependent on neo-angiogenesis. The cause of flap necrosis is primarily due to changes in flap hemodynamics, either inadequate arterial flow, inadequate venous drainage, or a combination of both. The effects of a number of growth factors on neovascularization have been investigated in an attempt to increase flap viability. Some of these factors including transforming factor, fibroblast and endothelial cell-growth factor have shown a marked effect in increasing the viability of skin flaps. Vascular Endothelial Growth Factor (VEGF) is the most potent of these angiogenic agents and can also induce vasorelaxation (Huang *et al.*, 2012). Its receptors are found only on endothelial cells and are expressed under hypoxic conditions and after endothelial damage.(Zhang *et al.*, 2003)

Modified McFarlane Flap is a modification of the rat dorsal skin flap model originally described by McFarlane *et al* (1965) to study the failure of flaps with dimensions of 3x10 cm.(McFarlane, DeYoung and Henry, 1965) The length of the base flap is made narrower, resulting in lower vascular perfusion pressure to the distal flap, thus showing a larger area of skin necrosis and can be an adequate experimental model to assess the viability of skin flaps (Camargo *et al.*, 2014).

Although several studies have shown that botulinum toxin can increase the viability of skin flaps, research on changes in the sympathetic vasoconstrictive neurotransmitter in the randomized pattern skin flap model alone is very limited. In addition, there have been no studies studying the possible synergistic or additive effect between BoNT-A and VEGF in increasing the viability of random flaps. This study was conducted to prove that the administration of BoNT-A, VEGF and their combination can reduce NE levels and increase the percentage of viability of the Modified McFarlane Flap in Wistar rats.

## **Method**

### **Study Design and Sample Calculation**

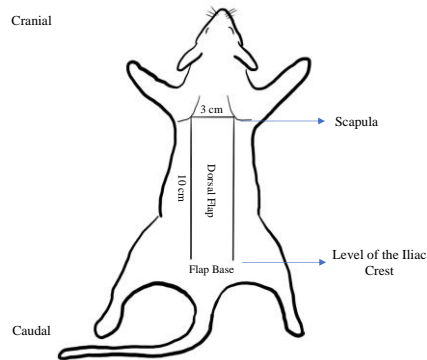
This study is a true-experimental *in vivo* laboratory study with the design of the randomized post test only control group. This research was conducted from December 2021 to February 2022 at the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University. The experimental protocols were approved by the Ethics Committee of the Faculty of Medicine, Udayana University with protocol number 2604/UN14.2.2.VII.14/LT/2021. The treatment and maintenance of the animals are following the Guide for the Care and Use of Laboratory Animals by monitoring the temperature of 25°C, 12 hours of light-dark cycle, 55% humidity, as well as standard food and drink.(National Research Council Committee for the Update of the Guide, 2011).

The experimental animals used in this study were male Wistar obtained from the Integrated Biomedical Laboratory Unit, Sub Animal Laboratory Unit, Department of Pharmacology and Therapy, Faculty of Medicine, Udayana University, aged 22 weeks, with bodyweight (BW) ranging from 300-400 grams. During the experiment and the analysis, no rats were excluded due to death or any illness. All samples (n = 28) were divided into four groups consisting of seven rats each. The sample size was determined by Federer's formula. All samples were randomly

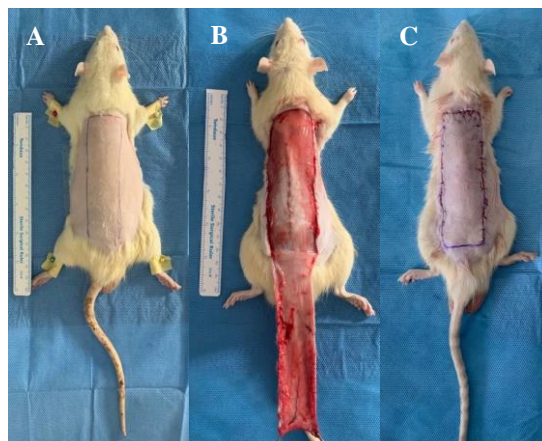
allocated in each group using a random number generator. The groups were divided into Group I that received pretreated BoNT-A 3 days before flap elevation, group II received VEGF immediately after flap elevation, group III received a combination of BoNT-A and VEGF and group IV was a control group.

### Flap Elevation Procedure

Prior to flap elevation, rats were anesthetized using Ketamine (100mg/ml) 2.5 ml, Xylazine (20 mg/ml) 2.5 ml and Acepromazine (10 mg/ml) 1 ml dissolved in 4 ml of sterile water (total volume 10 ml) and injected intramuscularly. Caudally Based Modified McFarlane Flap design (Figure 1) was made on the dorsal part of the rat vertically with the caudal part as the base of the flap. Starting from the midline, two equidistant points (1.5 cm each) are marked from the midline at the level of the iliac crest, the length of the flap is marked as two parallel lines 10 cm long starting from the base point that has been made previously resulting in a flap measuring 3 x 10 cm (Figure 1 and Figure 2A). Deepen the dissection of the flap to the panniculus carnosus layer and then perform flap elevation (Figure 2B). The flap was repositioned to its original position and sutured with an interrupted suture using 4-0 nylon monofilament (Figure 2C). The flap was closed using a transparent dressing and observed until the 7th day. During postoperative period, rats were placed in separate cages to prevent rats from biting each other.



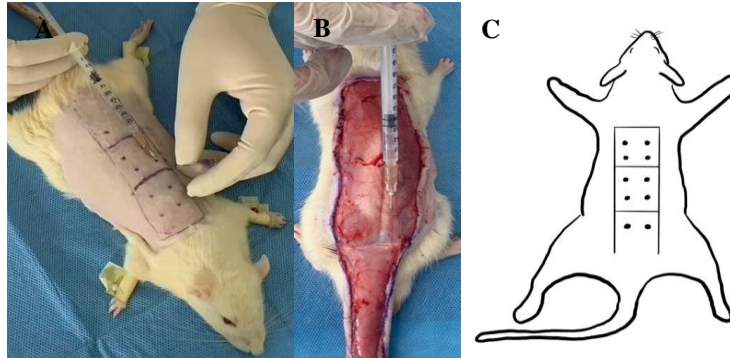
**Figure 1 :** Caudally Based Modified McFarlane Flap Design.



**Figure 2 :** Caudally Based Modified McFarlane Flap elevation procedure. A) Preoperative Flap Design. A caudally-based 3 x 10 cm flap was designed vertically on the dorsal rat, with the distal end at the lower margin of the scapula. B) Elevation of the flap. C) The flap is repositioned to its original position and sutured with interrupted suture using 4-0 nylon monofilament.

### BoNT-A and VEGF Injection

Vials of lyophilized BoNT-A (100 IU) were reconstituted in 10 mL of normal saline to a final concentration of 10 IU/mL. Preliminary tests were conducted before the study began. BoNT-A doses were assessed at 10, 20, 30, 40, 50 IU/flap where the percentage of flap viability on the 7th day after flap elevation was found to be the highest at doses  $\geq 20$  IU. When rats were given a dose of 50 IU, they died within a few days so it was decided to use a dose of 20 IU in this study. VEGF doses were assessed at 0.5, 1 and 2  $\mu\text{g}/\text{flap}$  where the highest percentage of flap viability on the 7th day after flap elevation was achieved at a dose of  $\geq 1$   $\mu\text{g}/\text{flap}$ . We used 165 amino acid isoforms of recombinant human VEGF suspended in Phosphate-Buffered Saline (PBS) (10  $\mu\text{g}$  in one vial diluted with 10 ml PBS with a final concentration of 1  $\mu\text{g}/\text{ml}$ ). Group I and III was pretreated with 20 Unit/2 ml/flap BoNT-A (Botox®, Allergan, Irvine, CA, USA) by intradermal injection 3 days before flap elevation distributed evenly in 10 regions of the flap area (Figure 3). Immediately after flap elevation, Group II and III were received 1  $\mu\text{g}/\text{ml}/\text{flap}$  VEGF (GeneTex Inc., San Fransisco, CA, USA) by subdermal injection distributed evenly in 10 regions of the flap area (Figure 3). Group IV received no treatment.



**Figure 3 :** A) BoNT-A Injection (Botox®, Allergan, Irvine, CA, USA). B) VEGF Injection (GeneTex Inc., San Fransisco, CA, USA). C) Injection were distributed evenly in 10 regions of the flap area.

### Tissue Sampling and Norepinephrine Analysis

Norepinephrine (NE) levels (ng/L) was measured 1 day after flap elevation was performed where a flap tissue sample of 1x1 cm at the margin of viable and necrotic skin was taken for examination (Figure 4). A viable flap is indicated by a portion of the flap tissue that is soft, color matched to healthy skin, Capillary Refill Time (CRT) < 2 seconds, warm and bleeding when cut with a surgical blade. On the other hand, the necrotic area of the flap is shown with brown/blackish skin color, hard and does not bleed when cut with a surgical blade. The tissue

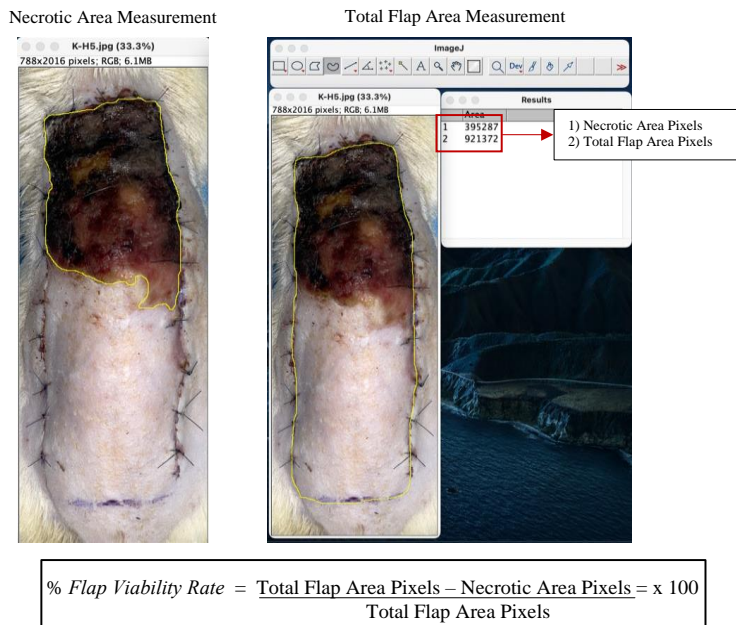
sample was put into a 1.5 ml microtube containing 500  $\mu$ L of PBS and stored at -20°C. Following centrifugation at 12.000 r.p.m. for 10 minutes, the supernatant was removed and stored at -20°C. NE levels (ng/L) was determined with Rat Norepinephrine Enzyme-Linked Immunosorbant Assay (ELISA) Kit (Bioenzy, BZ 08187311-EB, Indonesia) according to the manufacturer's instructions. Briefly, samples were diluted with 120  $\mu$ L standard diluent as per manufacturer's instructions and then loaded onto 96-well plates. Following addition of the conjugate and substrates, the reaction was stopped with the stop solution and the optical density read on a microplate reader at 450 nm wave length.



**Figure 4 :** Tissue Sample.

### Flap Viability Rate

Flap Viability Rate (FVR) is the Viable Flap Area (Total Pixel Area – Necrosis Area)/Number of Pixels Total Flap Area  $\times$  100% where the percentage of flap survival is calculated on the 7th day after flap elevation using Image J Software (National Institutes of Health, Bethesda, MD) (Figure 5). Flaps were observed and documented on day 1, 3, 5 and 7 postoperatively. Digital photos were taken using a 12-megapixel digital camera (iPhone 12 Pro Max, Apple Inc., California) with the camera at a distance of 12 cm from the flap.



**Figure 5 :** Calculation of Necrotic Area and Total Flap Area Pixels with Image J Software.

### Statistical Analysis

Data are presented as mean  $\pm$  SD. One-way analysis of variance with post hoc comparisons using the least significant difference (Post Hoc) test was used to determine the statistical significance between groups using IBM SPSS 23.0 (SPSS® Inc, Chicago). A p-value of less than 0.05 was considered significant.

### Results and Discussion

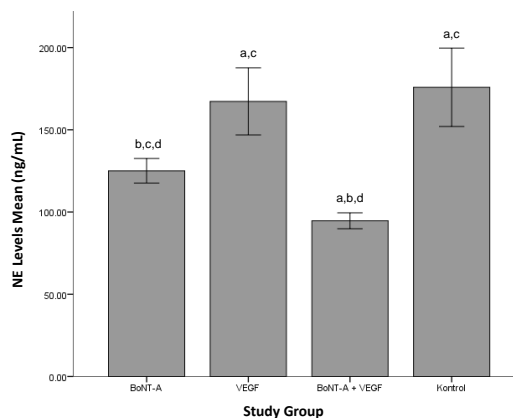
The mean of Norepinephrine (NE) levels and Flap Viability Rate (FVR) can be seen in Table 1.

**Table 1 : The Mean of NE Levels and FVR**

	Group I	Group II	Group III	Group IV
<b>NE (ng/L) Levels Mean <math>\pm</math> SD</b>	125,09 $\pm$ 8,07	167,25 $\pm$ 22,05	94,63 $\pm$ 5,25	175,84 $\pm$ 25,76
<b>FVR (%) Mean <math>\pm</math> SD</b>	81,51 $\pm$ 6,87	62,32 $\pm$ 5,01	93,37 $\pm$ 2,19	49,44 $\pm$ 11,98

### NE Levels Mean Analysis

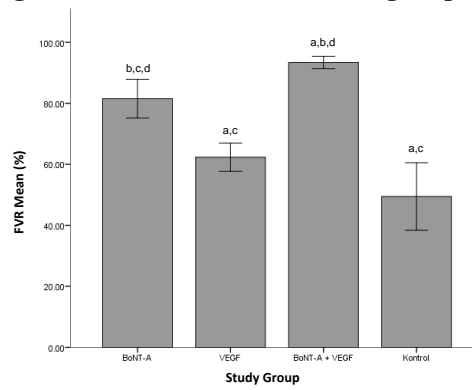
There was a statistically significant NE levels mean difference between group I compared to other groups. Statistically significant NE levels mean differences were also found between group III and all other groups with group III being the lowest. On the other hand, there was no significant difference between group II and group IV (Figure 6).



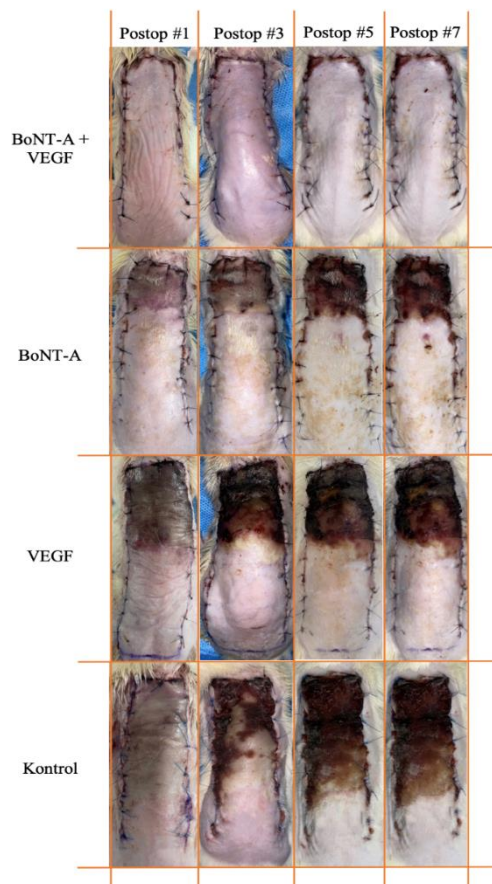
**Figure 2:** a, significantly different from the BoNT-A treatment; b, significantly different from VEGF treatment; c, significantly different from the treatment of BoNT-A + VEGF; d, significantly different from the control (p < 0.05).

### FVR Mean Analysis

Figure 3 showed significant differences between groups I and III with all other groups, including each other, with group III being the highest (Figure 7). However, there was no statistically significant difference between group II and group IV.



**Figure 3** : a, significantly different from the BoNT-A treatment; b, significantly different from VEGF treatment; c, significantly different from the treatment of BoNT-A + VEGF; d, significantly different from the control ( $p < 0.05$ ).



**Figure 8** : Representative photos of the BoNT-A+VEGF, BoNT-A, VEGF and Control groups (from top to bottom) on days 1, 3, 5 and 7 post-action (from right to left). The BoNT-A+VEGF group had the highest percentage of flap viability.

In this study, it was found that the administration of BoNT-A either in combination with VEGF or not, had a significant effect on decreasing NE levels on the first day after elevation Caudally Based Modified McFarlane Flap Wistar rats with the lowest NE level was in the BoNT-A+VEGF combination group. The results also showed a significantly higher percentage of flap viability / Flap Viability Rate (FVR) in the administration of BoNT-A either in combination with VEGF or not 7 days after elevation Caudally Based Modified McFarlane Flap Wistar rats with the highest FVR value in the BoNT-A+VEGF combination group. There was an increase in flap viability in the VEGF group but not statistically significant.

### **Caudally Based Modified McFarlane Flap**

The dorsal skin flap model of the rats was originally described by McFarlane et al (1965) to study skin flap necrosis and its prevention.(McFarlane, DeYoung and Henry, 1965) This flap design has been modified several times to achieve a significant skin flap necrosis model. In this study, we used a modified 3 x 10 cm caudally-based flap model, which was perfused randomly by the subdermal plexus. Inadequate blood supply to the distal part of the flap creates varying degrees of ischemia, which allows for investigation of flap physiology, angiogenesis, and flap survival.(Zhang *et al.*, 2003) This flap model was previously introduced by Camargo, *et al* (2014) who found the Modified McFarlane Flap with flap dimensions of 3x10 cm showed a more significant area of necrosis (10 x larger than the original McFarlane Flap) and could be an adequate experimental flap model.(Camargo *et al.*, 2014).

The caudal part of the flap was chosen as the base because when the cranial portion becomes the base flap, the flap could be an axial flap as there are small vascular bundles that accompanying the skin of the flap from the cranial to the caudal portion.(Camargo *et al.*, 2014) Disadvantages of this cranial-based flap also include the varying anatomic location of the flap base when positioned at the level of the scapula. The position of the forelimbs of mice can affect the anatomical height of the scapula, depending on the angle formed between the forelegs and the torso, the fixed position of the flap base can change because the scapula can be shifted up to one centimeter.(Briggs, 1987).

Another controversy regarding the use of animals as skin flap models is the selection of animal species. The character of rat skin is different from that of humans, especially in the layer after the dermis. In rats, this layer is muscular (panniculus carnosus) and strongly attached to the dermis layer as well as connected to the deep fascia via loose areolar tissue. In humans, this layer is the panniculus adiposus (superficial fat layer) which is more firmly attached to the deep fascia. However, considering from the supply of blood vessels, these layers are comparable because both have larger blood vessels. In both rats and humans, random flaps must include the panniculus containing blood vessels to ensure flap viability. However, compared to these differences, the advantage of using small animals in this study is more convenience (McFarlane et al., 1965)

### **Effects of BoNT-A on Sympathetic Neurotransmitters**

After flap elevation, significant changes occur in the circulatory system of the skin. First, with the breakdown of the sympathetic nervous system, the humoral neurotransmitters that cause vasoconstriction are released including NE. Blood vessels to the flap are cut off and perfusion pressure drops significantly in the distal flap, resulting in an ischemic state.(Y. S. Kim *et al.*, 2009; Krammer *et al.*, 2015) In this study, BoNT-A was used as a pharmacological adjuvant therapy to increase flap viability. The mechanism is thought to affect the cutaneous autonomic sympathetic nervous system via suppression of sympathetic neurons. It was hypothesized that BoNT-A could inhibit neurotransmitter release in perivascular sympathetic vessels based on the fact that BoNT-A is known to block acetylcholine release from presynaptic located in striated muscle.(Morris, Jobling

and Gibbins, 2002) This can cause mainly vasodilation which increases blood volume and pressure.(T. K. Kim *et al.*, 2009)

Botulinum Toxin Type A (BoNT-A) has a potent vasodilating effect exerted primarily by blocking the release of cholinergic neurotransmitters. Synaptosomal Associated Protein-25 (SNAP-25) is a SNAP Receptor protein (SNARE) that is responsible for the release of neurotransmitters. BoNT-A inhibits the release of cholinergic neurotransmitters by proteolysis of SNAP-25 among other SNAREs.(Foran *et al.*, 2003) Furthermore, BoNT-A interacts with the Rho/Rho kinase system and, by interfering with Ca<sup>2+</sup> influx, results in relaxation of the smooth muscle in the vessel wall. The mechanism by which BoNT-A inhibits muscle contraction involving the initial uptake of BoNT-A to the nerve terminal is usually delayed for 1 to 4 days, which is the basis for administering BoNT-A 3 days before flap elevation which was performed in this study.(Setler and Riley, 2002).

Schweizer, *et al* (2013) also described increased flap blood flow and survival by similar mechanism.(Schweizer *et al.*, 2013) Kim, *et al* (2009) proposed a sympatholytic mechanism for BoNT-A, and reported an 8.3% increase in random flap survival in BoNT-A intervention group.(T. K. Kim *et al.*, 2009) Arnold, *et al* (2014) described that BoNT-A increased flap survival due to its anti-inflammatory and vasoactive effects indicated by decreased levels of Interleukin-1 (IL-1) and Tumor Necrosis Factor (TNF)- $\alpha$  2 days after flap elevation.(Arnold *et al.*, 2014; Ghanbarzadeh *et al.*, 2016).

In this study, the lowest NE levels were found in the BoNT-A and VEGF combination group, followed by the BoNT-A group only. VEGF itself is an endogenous stimulator of angiogenesis, one of which can increase blood vessel permeability due to vasodilation. Vasodilation is caused by the release of Nitric Oxide which has a deactivating effect on catecholamine hormones, especially NE, but not with Neuropeptide-Y (NPY).(Kolo *et al.*, 2019) However, further research is needed to confirm the synergistic effect of BoNT-A and VEGF combination on vasodilatation effect.

### **VEGF Administration to Increase Flap Viability**

VEGF is a heparin binding glycoprotein that is secreted as a 45 kDa homodimer and a potent stimulator of endogenous angiogenesis and vascular permeability. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and Placenta Growth Factor (PlGF). VEGF-A is the most dominant protein. One of the variants of VEGF-A is VEGF<sub>165</sub> which is the main isoform in all cells and tissues.(Kryger *et al.*, 2000) When tissue is hypoxic or endothelial damaged, VEGF protein expression is increased. In this study, the increase in flap viability in VEGF group was not significantly significant. There are several possible causes that could explain this: first, VEGF does not persist in the target organ for a sufficient time after injection considering that the half-life is only 30-45 minutes under normal conditions.(Papapetropoulos *et al.*, 1997) The study by Kryger *et al* (2000) proved that VEGF can be effective when administered in a variety of methods, including subdermal, subfascial and systemic injection, as well as when applied topically to randomized bed flap recipients. The most effective method of

delivery in this study was to administer VEGF systemically in multiple high-concentration boluses. Given the relatively short half-life of VEGF in vivo, it is logical that maintaining adequate VEGF levels at high concentrations is effective for achieving maximal results.(Kryger *et al.*, 2000; Yang *et al.*, 2012).

In this study, VEGF was administered subdermally immediately after flap elevation. Contrary to the results of this study, Kryger *et al* (2000) stated that subdermal administration of VEGF was considered the most practical and could provide significant results at a dose of 1 ml (1 µg/ml) subdermally in the thick fascia layer of Wistar rats immediately after flap elevation was performed.(Kryger *et al.*, 2000; Yang *et al.*, 2012) Variations in the effect of VEGF on flap viability have been demonstrated by a number of studies. Although several studies demonstrated a positive effect of VEGF administration on flap viability by various routes(Kryger *et al.*, 2000; Li *et al.*, 2000; T. K. Kim *et al.*, 2009; Vourtsis *et al.*, 2012; Yang *et al.*, 2012), several studies show the opposite. Research by Bolukbasi, *et al* (2014) showed that there was no effect of topical VEGF administration at a dose of 2 µg on skin tissue healing and described the effectiveness of VEGF administration depending on the route and dose of administration.(Bolukbasi *et al.*, 2014).

Another possible cause are the dose of VEGF may be insufficient so that local concentrations are inadequate and the injections that were distributed evenly on the proximal, middle and distal part of the flap are no more effective than concentrating the injection only in the distal areas where the perfusion is most impaired.(Alon *et al.*, 1995; Papapetropoulos *et al.*, 1997; Vourtsis *et al.*, 2012). Although the preliminary study found that a dose of 1 µg/flap was able to provide effective results, further research is needed with different doses and routes of administration and a longer examination time, considering that revascularization occurs as early as 7 days after flap elevation.(Chu *et al.*, 2010)

### **Combination of BoNT-A and VEGF to Increase Flap Viability**

Botulinum Toxin Type A (BoNT-A) has limitations as an angiogenic agent (Stone *et al.*, 2012). Instead, it acts as a vasodilator of preexisting major pedicles or microcirculation. In other words, BoNT-A can improve flap viability by blocking perivascular smooth muscle contraction and vasodilation leading to increased blood flow to the flap. However, the correlation of BoNT-A with VEGF and angiogenesis is controversial. Research by Kim *et al* (2009) showed that after administration of BoNT-A, there was an increase in VEGF and CD31, followed by endothelial proliferation, due to changes in blood volume and pressure.(T. K. Kim *et al.*, 2009; Park *et al.*, 2016) In this study, a synergistic effect between BoNT-A and VEGF was expected due to an increase in VEGF mRNA in the BoNT-A group.(Y. S. Kim *et al.*, 2009).

This is different from the study by Arnold, *et al* (2014) which showed a decrease in VEGF expression in the group BoNT-A. The difference in the contrast results is thought to be because in Kim, *et al* (2009) study, tissue sampling included areas of necrosis which indicated a more severe ischemic state of the tissue that triggers endogenous VEGF production.(Arnold *et al.*, 2014) Research by Zhang, *et al* (2003) also did not find an increase in endogenous VEGF in the distal part of the

flap which is the most ischemic area. VEGF expression was found to be highest in the proximal flap so that in this study it was concluded that VEGF expression did not increase in ischemia with a certain degree of severity due to too low oxygen supply and the formation of free radicals.(Zhang *et al.*, 2003)

Park, *et al* (2017) showed that administration of Botulinum Toxin Type A (BoNT-A) can increase the expression of VEGF and produce protection against ischemic reperfusion injury depending on increased angiogenesis. Another study also concluded that administration of BoNT-A increased VEGF expression in a mouse model of transplantation or the Transverse Rectus Abdominus Myocutaneous (TRAM) flap in a mouse model.(Park *et al.*, 2016, 2018) The concentration of BoNT-A used in this study was 10 IU/mL/flap. Furthermore, Gugerell, *et al* (2016) found that a high BoNT-A concentration of 20 IU/mL/flap could reduce VEGF expression and inhibit angiogenesis.(Gugerell *et al.*, 2016) In addition, Harper, *et al* (2008) highlighted that VEGF has a proangiogenic isoform and an antiangiogenic isoform, and the antiangiogenic VEGF<sub>xxx</sub> isoform might be transferred to the proangiogenic VEGF<sub>xxx</sub> isoform via factor splicing. Thus, the balance of VEGF isoforms and BoNT-A concentrations may lead to varying results of the BoNT-A and VEGF correlations.(Harper and Bates, 2008; Zhou *et al.*, 2020).

In this study, the combination of Botulinum Toxin Type A (BoNT-A) and VEGF gave a maximum effect on the concentration of BoNT-A 20 IU/flap which potentially result in a decrease in endogenous VEGF expression (Gugerell *et al.*, 2016), so that this became the other reason for researchers to give exogenous VEGF. A significant increase in flap viability as well as a decrease in NE was demonstrated in the combination group compared to the single administration of BoNT-A and VEGF. This beneficial effect may be due to the synergistic effect of exogenous VEGF with existing endogenous VEGF due to administration of BoNT-A or due to post-flap elevation ischemia. The angiogenic effect of VEGF increases flap viability after BoNT-A-induced vasodilation by decreasing NE.(Gugerell *et al.*, 2016)

## **Conclusion**

In this experimental model, the administration of BoNT-A alone or of its combination with VEGF can increased flap viability as well as decrease NE levels compared with the other group. BoNT-A in combination with VEGF had more superior effect on both NE levels and flap viability compared with the other group. VEGF alone had no significant effect in increasing flap viability.

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