

The Effect of Hyperthermia as a Remote Hyperthermic Preconditioning (RHP) on Viability of Random Pattern Skin flap in Rat

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Background :

Vascular supply has a main role in flap survival. Nutrition was delivered to a tip of flap by interconnecting subdermal plexus. The nutrition brought by it's vascular supply should be sufficient to keep the viability of flap. In the context of flap viability, angiogenesis process is needed to maintain vascular blood supply because some pre-existing vessel was interrupted by elevation of cutaneous flap. Remote Hyperthermic Preconditioning (RHP) is a procedure that can stimulate VEGF expression and stimulate HSP-70 secretion. VEGF as a dominant growth factor for angiogenesis and HSP-70 as a protein chaperon play a predominant role to upregulation plasminogen activator and acceleration of recanalization microvascular for angiogenesis and anti apoptosis effect resulting improvement of flap viability.

Methods :

Random pattern skin flap was made by elevation along 1cm X 4 cm on abdominal skin of 32 adult Wistar Rats using mess and metzembaum. They were divided into two sample groups. One was underwent without RHP (as control group) and second group was underwent RHP (as treatment group) . RHP procedure was done by exposing right posterior extremity into 50⁰ C waterbath for 3 seconds. Clinical evaluation was done by measuring necrotic area with visitrax, and angiogenesis evaluation by immunohistochemically using VEGF antibody.

Result :

Remote hyperthermic preconditioning (RHP) decrease necrotic area and increase angiogenesis process.

Conclusion:

Remote hyperthermic preconditioning (RHP) procedure improve viability of skin flap rather than non RHP procedures.

Keywords : *Random pattern skin flap, remote hyperthermic preconditioning, HSP-70, VEGF, angiogenesis.*

Introduction

Skin flap is a one of procedures to cover a defects caused by trauma, infection or malignancy process. Along 2010 , Department of Plastic Surgery Dr. Soetomo General Hospital Surabaya had 46 cases of flap with a failure rate remains high. Between totally 46 cases, 14 cases among them was compromised and necrosis (30%) with 9 cases (64% of flap failure rate) were local flap including a skin flap. Flap failure may be caused by a tension, inappropriate technique, infection, ratio of length and width of the flap is limited (less than 2:1) and the supply of nutrients and poor oxygenation in the distal region. This requires a solution to increase the viability of flap.

Previews studies have been done to improve survival of tissue with preconditioning procedure. In 2010, Filho, et.al., showed that necrotic area in random skin flap can decrease by applied tourniquet for 10 minutes in posterior extremity of rat before elevation of flap. Another studies note that hyperthermic preconditioning can reduce neurologic deficit caused by spinal cord injury (Zhang P. Abraham S., et al., 2000) .

Our study using a supralethal and sublethal temperature as hyperthermic preconditioning thus improving microvascular circulation in rats (Wilkinson et al, 2009). Effects of local heat shock can provide systemic effects. It means that the remote effects of exposure to a particular tissue can provide the

effect of other tissue that are not directly exposed. Remote hyperthermic preconditioning was chosen as the conditions that need to be done before the elevation of the flap because the method is simple, relatively inexpensive and possible to be applied clinically.

Definition

Random pattern skin flap is comprised of cutis and subcutaneous tissue where survival obtained from the blood vessels in its subdermal plexus (Vedder, 2006). The main characteristic of random pattern skin flap have no dominant blood supply (Lync, 2004). Basic design by elevation of the rectangular skin and subcutaneous tissue with a ratio width: length of approximately 1: 2.

Indication

Skin flap usually used to close the defect if the vascularization of the wound base is inadequate and does not allow to do a skin graft (bone, tendon without the periosteum / paratenon is intact). The defects can be caused by a trauma, infection, or due process of malignancy. Defect closure is a very important procedure that aims to prevent infection, and of course further damage the return of function and esthetics.

Vascularization of Skin Flap

Survival of the flap is depend on nutrients that are brought to the distal end of the flap. When after flap was cut, nutrient and oxygen supply was insufficient in the distal of flap, subsequent process will promote compromisity and ischaemic of flap. Vascularization of the skin flap came from fasciocutaneous vascular plexus through the deep fascia perforator that could directly or indirectly. Blood vessels subcutaneous out on superficial fascia are divided into superficial and deep vasa subcutaneous fat layer. The blood vessels are formed by musculocutaneous and septocutaneous artery (Figures 1a and 1b).

Vascular dermis is the main blood supply to the skin in between the deep reticular dermis and subcutaneous fat. These blood vessels are responsible for dermal bleeding at the edge of the skin flap. Vascular dermis and sub-epidermal papillae are on the edge of the dermis and dermal-epidermal junction which will change the size of the arterioles into the capillarie

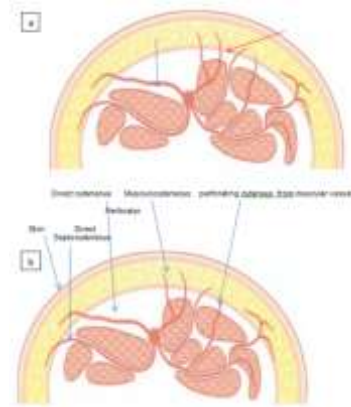


Figure 1a. Direct (blue arrows) and indirect perforator (red arrows); 1b. Perforators to the deep fascia (Quoted from Wei Mardini, 2009, Flaps and Reconstructive Surgery).

Flap drainage occurs through the veins under the skin or vein committantes. Veins do not close the valve on the distal based flap as there are gradations of denervation or decrease pressure on the venous valves and the alternative pathway (by pass) on the venous valves.

Arterioles of the terminal vascular system of direct cutaneous, musculocutaneous, or septocutaneous running on the layer beneath the skin and then branched into terminal arterioles that supply the subdermal plexus. Prekapiler sphincter controls the flow of blood to the capillaries, post capillary venule and later became a collecting venule. Arteriovenous will menganastomose between arterial and venous systems. The lymphatic system also plays a role in micro-circulation, especially to reduce edema in the flap.

Design of Flap

Flap design consists of two components, namely the donor and resipient site. Donor site is an area which will be performed and the flap elevation resipient site is the place where the flap will be placed (inset). Resipient site preparation is essential for determining the flap transfer. Color, texture, thickness and size of the function resipient site is required to achieve functional and cosmetic purposes.

Flap design is made to avoid tension on the pedicle and should consider the relaxed skin tension line (RSTL) which is the elastic properties of the skin and the pivot point of the flap. Flap frequently designed longer and wider than necessary. Donor site can be closed primary, with a skin graft or a flap from another location. Vascular circulation of skin should be

considered when we will elevate the flap.(Figure 2).

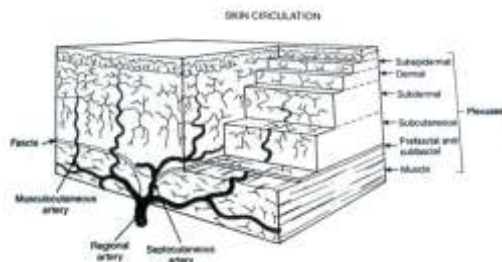


Figure 2. Vascular circulation of the skin. Vascular circulation is very important in the manufacture and design of flap survival, the techniques used to make the design must consider the anatomy of blood vessels and soft tissues underneath (From Mathes SJ, Nahai F, 1997, *Reconstructive Surgery: Principles, Anatomy and Technique*).

Regulation of blood flow to the flap consists of two levels, namely local and systemic. Systemic regulation of neural regulation (predominant) and humoral regulation. Depending on the neural regulation of sympathetic fibers and α -adrenergic receptor that affects vasoconstriction; β -adrenergic receptor that affect vasodilation. Humoral regulation occurs because of specific receptors that produce systemic vasoactive substances, such as epinephrine and norepinephrine on α -adrenergic receptor, serotonin, and prostaglandin F₂ α tromboxan A₂. This causes systemic vasoconstriction. Systemic vasodilation produced prostaglandin E₁ and I₂ (prostacyclin), histamine, leukotriene C₄ and Bradykinin and D₄.

Hemodynamic, anatomic and metabolic changes after the flap is elevated will determine viability of flap. A study states that although blood flow at the base of the pedicle has been protected after the elevation, the flow at the end of the flap is often decreased to 20% of normal, especially in the first 6-12 hours. Blood flow gradually increased to 75% of normal for 1-2 weeks and 100% at week-3 and 4 (Vedder, 2003).

After the flap is elevated, the proximal portion of the pedicle flap reduces blood flow due to local release of catecholamines and response to injury. In the distal region of local ischemia occurs, causing maximum vasodilation. Inadequate perfusion pressure of the distal part of proximal causes lack of blood flow. This lead to the failure of distal flap.

Kerrigan et al, stated that there are three conditions that lead to flap failure due to global ischemia; flap design is too large for intrinsic blood vessels, arterial and venous

thrombosis. This condition causes the tissue anaerobic metabolism with rapid depletion of oxygen levels, as well as glucose and ATP followed by increased levels of CO₂ and lactic acid.

Increase in glucose consumption peak at day 3 and then back to normal on day 7. Anaerobic metabolism causes an increase in the production of toxic superoxide radicals can react directly with the endothelial membrane causing lipid peroxidation process, which will damage the membrane protein, boost the cells permeability and cause cell dysfunction. Another mechanism is through the chemotactic metabolite of O₂, which is superoxyde anion that causes neutrophil migration into reperfused area and cause tissue damage. Rapid intravascular neutrophil accumulation, there was a progressive decrease in perfusion, causing no-reflow phenomenon. Additionally the activity of neutrophils can cause endothelial injury and microvascular occlusion (Fig. 3).

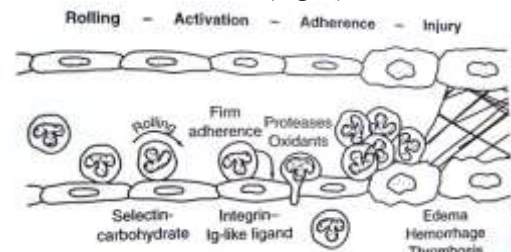


Figure 3. Activity of neutrophils. Diagram showing the incidence of injury sustained in neutrophils and microvascular (From Mathes SJ, Nahai F, 1997, *Reconstructive Surgery: Principles, Anatomy and Technique*).

On flap which is survive, blood flow will increase gradually. Neovascularization will start on day-3 to day-7 in which angiogenic growth factors play a major role in this process. In normal, endothelial cell was in a quiescent state which only be stimulated to proliferate by angiogenic growth factor. It is this process which is the main factor to enhance flap viability (Baker, 2007). Early neovascularization in rabbits and pigs started on day-4, and the rat began on day-3.

Resepient site is very important for survival of flap. If the flap is placed in an optimal recipient site then on the second day will form a layer of fibrin between the flap and the recipient. Vascular tangle will be interconnected between the flap and resepient site resulting in revascularization processes that occur in day-7 in humans and rats.

Method of Moving Flap

Skin flap can be moved and placed both local and distant.

Local Flap

Used as a cover defects that are close to the location of the donor tissue. The basis of the type of local flap is the most dominant type of movement.

1. Advancement flap

This flap is driven directly to the front (forward) direction away from the perpendicular to the pedicle in the absence of collateral movements. (Figure 4).



Figure 4. VY flap is one of the advancement flap (From Grabb and Smith's 5th ed., 1997, Basic Techniques and Principles in Plastic Surgery).

2. Rotation Flap

A flap that semisirkuler, rotates through a certain axis (pivot point) towards the direction of the defect to be closed. This flap design is often used to close the defect in almost all parts of the body, because the technique is easy and can cover a relatively wide defect.

The design diameter of the rotation flap is made as large as possible. Secondary defect or defect on the donor can be closed primary or skin graft done. Double rotation flap used to close the defect in addition to the wide well to avoid tension at the closure of the defect and the donor. Incision or incisions cut back on the diameter of the triangular pivot point needs to be done to increase the rotation toward the direction of the closure of the defect (Fig. 5).

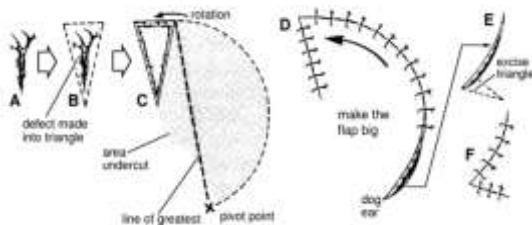


Figure 5. Rotation flap (Quoted from Grabb and Smith's Plastic Surgery 5th ed, 1997, Basic Techniques and Principles in Plastic Surgery).

3. Transposition Flap

This flap is moved laterally by means of a pivot point toward the nearby defect.

Design is usually rectangular with a ratio of flap is longer than the defect. Donor site can be closed with a primary or skin graft (Fig. 6a, b, c, d).

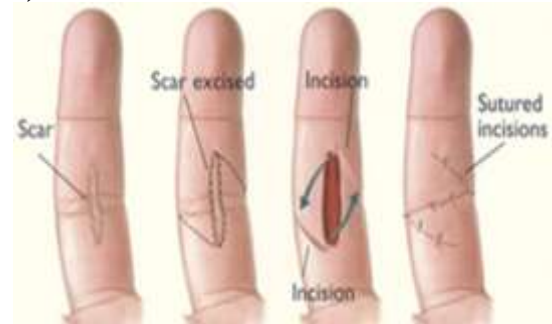


Figure 6a. Z-plasty (Quoted from Loverock MD, 2011, My Medical Journey, Basic of Z-plasty).

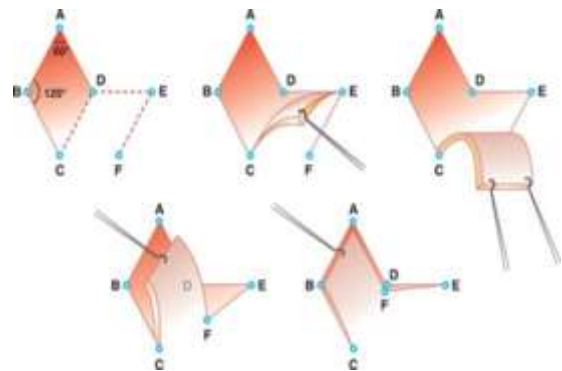


Figure 6b. Rhomboid (Limberg) flap (Quoted Patrick Knipper, 2007, Reconstructive Surgery in Difficult Circumstances, Coverage of Knee).

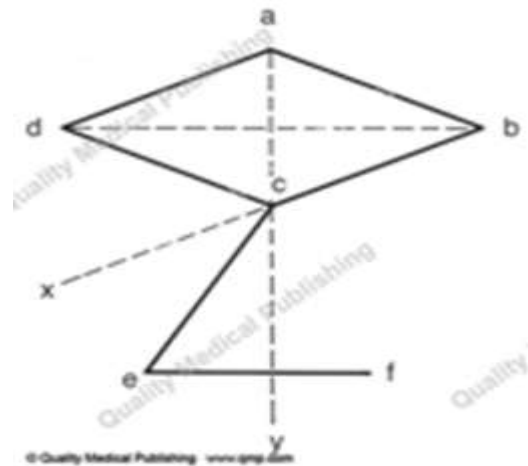
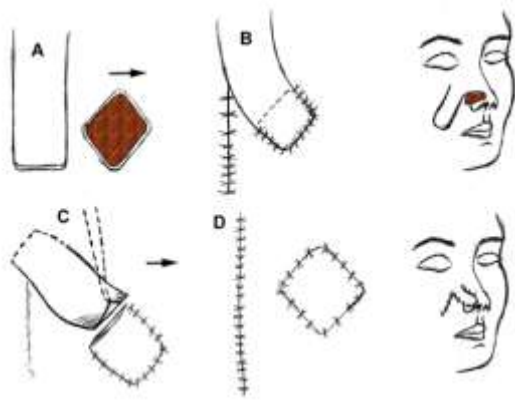


Image 6c. Defourmentel Flap (Quoted from Artmart, 2011, Surgical Technique, Reconstructive Surgery).



6D image. Interpolation Flap
(Quoted from thir Sau, 2011, Flap Classification, Oral Surgery and Maxillofacial)

Distant Flap

Closing defect which located far from the donor site can be used distant flaps. Defect removal can be done directly, with the tubing or with microvascular techniques.

1. Tubbing Flap

Tubbing flap is the technique of moving the flap from the donor site to the recipient site where the lateral edge of the flap is then sutured met (figure 7).



Figure 7. Groin Flap
(Quoted from Rebecca Yu, 2007, Two New flap).

2. Direct flap

Moving the flap to the donor directly to the defect can be closed is known as direct flap. This flap is maintained for 1 to 3 weeks later the flap excision (Fig. 8).



Figure 8. - Groin flap for reconstruction in the hand
(Quoted from Wei Mardini, 2009, Flap and Reconstructive Surgery).

Flap microvascular

Microvascular flap technique by making a vascular anastomosis of donor and recipient using microvascular surgery (Fig. 9). Size of the donor flap can be tailored to the size of the defect would be reconstructed. The composition of the skin flap may be its subcutis, muscle and bone can also be included.



Figure 9. Anterolateral Thigh Flap
(Quoted from Martin Jigenburg, 2008, Toronto Microsurgeon, Antero Lateral Thigh Flap).

Monitoring Skin Flap

After the flap is designed and placed on resipient site, carried out monitoring the viability of the flap to find out early signs of failure and prevent further necrosis.

Clinical observations are the best way to use today, where the pale color is a sign of arterial insufficiency is a sign of failure, while the bluish vein system. Other clinical signs such as capillary refill time (CRT) and temperature are also important flap. Pin prick tests performed to determine the viability of the flap. Test objectivity as pH monitoring, dopler laser, ultrasound and transcutaneous dopler oxygen tension (PO₂) can also be used for early detection of flap viability.

Remote Hyperthermic Preconditioning

Preconditioning is an endogenous mechanism after stimulation from outside of body that protects body cells against injury ischemia / reperfusion (Filho et al, 2009). To create a state of cellular stress can be either ischemic preconditioning, heat shock, endotoxin, adenosine, α 1 adrenergic agonists, reactive oxygen species (ROS) or opioids.

Reperfusion injury is a process of restoration of blood flow to the organ after a certain period of no flow ischemia, which can cause cell damage. Critical ischemia is a particular period of the cell to withstand the process of ischemia so that no damage of the endothelial and parenchymal. Critical ischemia in the organ is not the same, depending on the organ and the nature of cells arranged.

Remote system is a preconditioning in which a particular organ-stressing process can provide an effect on other organs that do not affect by stressing directly (Filho et al, 2009). This procedure has the advantage that no target organ damage / disturbance resulting from treatment during the treatment process.

Protection of preconditioning is time dependent process that will arise and disappear at a certain time interval. Early preconditioning will occur in the size of the minutes and are transient (temporary) and not affected by de novo protein synthesis but is mediated by systems such as adenosine receptor, adrenoceptor, protein kinase C (PKC), protein tyrosine kinase (PTK), mitochondrial K-ATP channels and calcium.

Delayed preconditioning takes time to achieve a complete induction, the process will run slowly and through the mechanism of protein synthesis de novo. Cellular cascade process consists of three stages, namely initiation (trigger) to ischemic stressors, protein expression within 24-72 hours which would protect cells from ischemic and to translate the trigger signal is captured at the beginning of a process to synthesize a mediator. Trigger on a

delayed preconditioning include: reactive oxygen species (ROS), nitric oxide, opioids, heat shock protein (HSP), COX-2 and manganese (Mn) SOD.

Heat Shock Protein

Exposing tissue with suprafisiologis and sublethal temperature in specific time can result in changes in cellular biosynthesis. Hyperthermia is a one of preconditioning in order to improve the regulation of heat stroke proteins (HSP). HSP will stimulate upregulation of plasminogen activator resulting in increase recanalization of tromboembolized microvasculature, and counteract the function plasminogen activator inhibitor (PAI) thus HSP will induces endogenous hiperfibrinolysis result of reducing failures of flap.

HSP was first discovered in 1962 as a clump of chromosomes in the heat-shock exposure, consisting of molecular chaperones that will expression rapidly after exposure to high temperatures and reaches a maximum level a few hours after that. HSP was found in all organisms and are classified in groups cytoplasmic protein with a molecular weight 20-120kDa.

Non surgical methods of heating include the provision of hot water, ultrasound, microwave, paraffin wax bath, hot water blanket, radiant hit devices, high temperature hydrotherapy and combination therapy. Invasive method of heating include using a variety of heating devices, infusion with a solution that is heated in the peritoneal cavity or blood ekstrakorporeal heating via heat exchanger (Bachynsky et al, 2009).

In experimental animals, the inferior extremities of rats put in waterbath with a given temperature and time period. With this treatment is expected that the local heat shock that occurs will trigger the release of heat shock protein (HSP) are chaperones to the size of a 70 kilo Dalton to play a role in the homeostasis of intracellular survival (Morimoto, 1996; Rynlander, 2005). In particular, based on location is divided into 2, which is contained in an organelle HSP (HSP 78, -75, -60, and -10), and the class of HSP 110, -90, -73, -72 and -10 contained the cell nucleus and cytosol.

Exposing tissue on heating device should considere about time and high of temperature. Because both of these can prevent damage of cell and supposed tissue for stimulating HSP. There are specific protocol of thermal preconditioning for maximal induction of HSP (table 1).

SPTable 1. Thermal preconditioning protocols for maximal induction of HSP 70 expression

T ₁ E ₁				T ₂ E ₂			
Model Type	Temperature (°C)	Exposure time (minutes)	Citation	Model Type	Temperature (°C)	Exposure time (minutes)	Citation
Animal: mouse	41	15	Pegues et al. (2007)	Cell: BAEC	40	2 minutes	Rynlander et al. (2005)
Animal: Rat	40-42.5	15	Leger et al. (2006)	Animal: Rat	39	2 minutes	Schulz et al. (2002)
Cell: rat cardiac	40	15-40	Hugh et al. (1997)	Cell: NIH-3T3	39	27 minutes	Q-Guang et al. (2004)
Cell: NHDF	40	15-40	Wilms et al. (2004)	Cell: BAEC	39	1 minute	Rynlander et al. (2005)
Cell: BAEC	44	33	Rynlander et al. (2007)	Cell: RPE	39	2 minutes	Shi et al. (2001)
Tissue: RAFT	44	33	Wilms et al. (2004)	Cell: SREB	55	4 minutes	Bowman et al. (1997)
Cell: NIH-3T3	44	41	Beckham et al. (2004)	Cell: SREB	56	1 minute	Ortiz et al. (2001)

BAEC, bovine aortic endothelial cell; NHDF, normal human dermal fibroblast; NHEK, normal human epithelial keratinocytes; NIH-3T3, murine embryo fibroblast cell line; RAFT, engineered skin equivalent model; RPE, retinal pigment endothelial cell; THES, high temperature short exposure; TLEL, low temperature long exposure (Quoted from Bowman et.al, 1997, System and Method for Conditioning Animal Tissue).

HSP70 and -27 exhibit properties typical of the rise in temperature, ischemia and oxidative stress and can increase cellular protection, inhibition of cell death (Topping et al, 2001; Rynlander, 2005). $\geq 5-6^{\circ}\text{C}$ increase in temperature can induce the upregulation of HSP 70 (Beckham et al, 2004).

Subsequent research showed that local hyperthermia is more effective in preventing flap necrosis than the provision of monophosphoryl lipid A as an increase in stress tolerance against hypoxic cells were more affected by HSP70 (Rynlander, 2005; Cotaldo et al, 2007).

Angiogenesis

Angiogenesis is a target to be achieved in improving flap viability with the treatment of this RHP. The process of angiogenesis can be occurred in the second path, through the mobilization of endothelial precursor cells (EPCs) and pre-existing vessels (Fig. 10) (Peichev et al, 2000; Kumar 2009).

There are three groups precursors of endothelial cells involve in neovascularization, namely EPCs derived from bone marrow, the endothelial cells of blood vessel walls that circulate in the peripheral blood (circulating endothelial cell (CEC)) and endothelial

outgrowth cells (EOC). CEC and EOC cell cultures obtained from peripheral blood mononuclear cells in the appropriate medium (Nababan et al, 2007).

During embryogenesis, which is bipoten hemangioblast can differentiate into stem cells hemopoitik (Hematopoietic Stem Cell (HSC)) and the EPC. In vivo, endothelial cells can be derived from the HSC, myeloid progenitors, granulocyte-macrophage progenitor and mesenchymal stem cell (Bailey et al, 2006). EPC differentiation of cells derived from monocytes migrate into the gap perivascular and secrete proangiogenic cytokines, such as Vascular Endothelial Growth Factor (VEGF), Human Growth Factor (HGF), granulocyte-colony stimulating factor (G-CSF) and granulocyte-Macrophage Colony Stimulating Factor (GM-CSF).

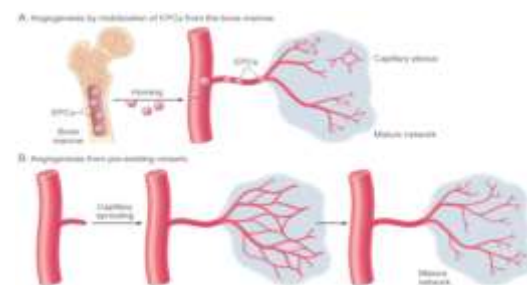


Figure 10. The process of angiogenesis. A. Endothelial Precursor Cells (EPCs) are mobilized from bone marrow and migrate into the lesion site. EPCs differentiate and form a mature vascular by linking the existing blood vessels. B. Angiogenesis of pre existing vessels, endothelial cells become motile and proliferate to form capillaries. Maturation process involves pericyte and vascular smooth muscle cells form a layer periendothelial (Quoted from Conwy EM, et al., 2001, Molecular Mechanism and Blood Vessels Growth).

Process of angiogenesis can be stimulated by stimulus of hypoxic conditions. Optimal microenvironment conditions play important role in this process. Activation process of angiogenesis is vascular endothelial derived from the old (pre-existing vessels) and endothelial spindle-shaped in the form of new blood vessels Cobble stone (Nababan, 2007).

Angiogenesis derived from pre-existing vessels originated from the role of vasodilation and increased permeability of existing blood vessels, extracellular matrix degradation and endothelial migration. Vasodilating effect of nitric oxide response will increase the permeability of blood vessels resulting in increased induction of VEGF in endothelial old. Proteolytic degradation occurs in the membrane of blood vessels by

metalloproteinase and damage interconnecting between cells (Kumar, 2011).

Endothelial cells then migrate causing angiogenesis in a new stimulated place. Proliferation of endothelial cells occurs at the leading front of cells that have migrated. Endothelial cell maturation occurs later in the control of growth inhibition and remodeling in capillary tubes. Periendotel immediately supports vein endothelial tubes and the maturation process.

Biomolecular Aspects of VEGF

VEGF is produced by macrophages, a predominant factor in the process of angiogenesis to increase blood flow and skin flap survival. ⁽⁷⁾. VEGF is a disulfide-linked dimeric glycoprotein with a molecular weight of 45kDa base that binds heparin and is structurally related to platelet derived growth factors ⁽²⁴⁾. VEGF consist of several groups, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E. The greatest role in angiogenesis is VEGF-A. Gen of VEGF-A consists of 8 exons separated by 7 introns on chromosome 6p21.3.

Among of many growth factors, VEGF including the most important factor in angiogenesis process. Biological effects of VEGF may result from binding to its receptor (Table 2). Receptor in the presence of hypoxic cells showed an autocrine regulatory mechanism of VEGF produced by hypoxic cells or experiencing stress. ⁽²⁵⁾.

VEGF is a major receptor tyrosine kinase, VEGFR-1 consists of FLT-1 VEGFR-2 KDR flk-1 and VEGFR-3 FLT-4. All three have seven immunoglobulin like domains in the extracellular, a transmembrane section next to the intracellular tyrosine kinase.

Table 2. Vascular Endothelial Growth Factor ⁽²³⁾

Protein	Family members: VEGF (VEGF-A), VEGF-B, VEGF-C, VEGF-D
	Dimeric glycoprotein with multiple isoforms
	Targeted mutations in VEGF result in defective vasculogenesis and angiogenesis
Production	Expressed at low levels in a variety of adult tissues and at higher levels in a few sites, such as podocytes in the glomerulus and cardiac myocytes
Inducing Agent	Hypoxia
	TGF- β
	PDGF
	TGF- α
Receptors	VEGFR-1
	VEGFR-2 (connected to endothelial cells)
	VEGFR-3 (lymphatic endothelial cells)
	Targeted mutations in the receptors result in lack of vasculogenesis
Functions	Promotes angiogenesis
	Increases vascular permeability
	Stimulates endothelial cell Migration
	Stimulates endothelial cell proliferation
	VEGF-C selectively induces hyperplasia of lymphatic vasculature
	Up-regulates endothelial expression of plasminogen activator, plasminogen activator inhibitor-1, tissue factor, and interstitial collagenase

VEGFR-1 binds to VEGF-A and B; expression in trophoblast cells, monocytes, mesangial cells and kidney cells are reactive astrocytes after stimulated VEGF. VEGFR-2 binds VEGF-A, C, D and E; hematopoietin expression in stem cells, progenitor cells megakariosit and retina. VEGFR-3 binds to VEGF-C and D; expression in lymph vessels (Table 3).

Table 3. VEGF Receptor

Receptor	Ligand	Function
VEGFR-1 (Flt-1)	VEGF-A121	Promotes cell migration
	VEGF-A165	Organization of blood vessels
	VEGF-B	Homeostasis of macrophages and monocytes
	PlGF-1 PlGF-2	
VEGFR-2 (KDR, Flk-1)	VEGF-A121	Angiogenesis, differentiation of endothelial cells
	VEGF-A145	Promotes cell migration
	VEGF-A165	Enhancement of vascular permeability
	VEGF-C	
	VEGF-D	
VEGFR-3 (Flt-4)	VEGF-A145	Remodeling of primary capillary circulation
	VEGF-A165	Endothelial cell survival and development
	VEGF-A183	Regulation of growth and maintenance of lymphatic system
	PlGF-2 VEGF-B167	
Flt-1	VEGF-A165	Development of vascular system
Flt-2	PlGF-2	
	VEGF-A183	Organization of peripheral nervous system Development of vascular network

(Quoted from : Felmeden, 2003, Angiogenesis: basic pathophysiology and implication for disease. Eur Heart J; 24: 592).

From three types of these receptors VEGFR-2 is the major receptor that can cause biological effects of VEGF. VEGFR-2 task is to stimulate the mobilization of EPCs from the bone marrow and increases proliferation and differentiation. This mechanism is also supported by the FGF-2 (Peichev, 2000). Stabilization of new blood vessel formation is helped by Ang1 and Ang2, PDGF and TGF- β in this case is the recruitment of pericyte, smooth muscle cells and deposition of extracellular matrix proteins.

Interaction of VEGF with VEGFR 1 and 2 have a difference in the location of the bonds between VEGF with VEGFRnya. VEGF-1 binds to VEGF amino acids at the end of the VEGF monomer. VEGFR-2 at the beginning of the amino acid monomer of VEGF. VEGF binds to VEGFR on the second and third loop section like immunoglobulin domain (Figure 11).

The complex process of angiogenesis begins when cells in a tissue hypoxic conditions respond by increasing production of VEGF. Once secreted, VEGF binds to VEGFR Surface residing on vascular endothelial cells. Association of VEGF-VEGFR pathway would stimulate intracellular signaling cascade that initiates the process of angiogenesis.

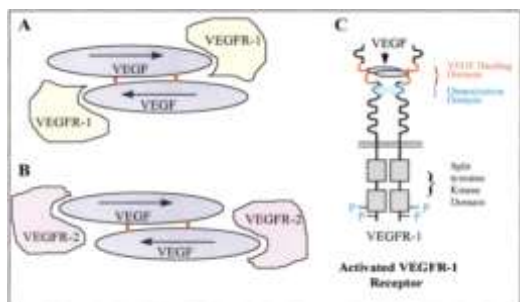


Figure 11. Interaction with the VEGF receptor tyrosine kinase signaling (VEGFR)
(Quoted from Neufeld et al. Vascular Endothelial Growth Factor (VEGF) and its Receptors. FASEB J 13: 14).

When cells experience hypoxia, VEGF mRNA expression is induced due to increased transcription of VEGF mRNA and decreased VEGF mRNA degradation. Hypoxia induces the Hypoxia Inducible Factor (HIF-1). HIF-1 binds to Arylhydrocarbon Nuclear Receptor Translocator (Arnt) in the cell nucleus to activate gene transcription of VEGF mRNA. VEGF to form, which will bind to the protein-Hur. Bond is what will prevent the degradation of VEGF mRNA caused by hypoxia.

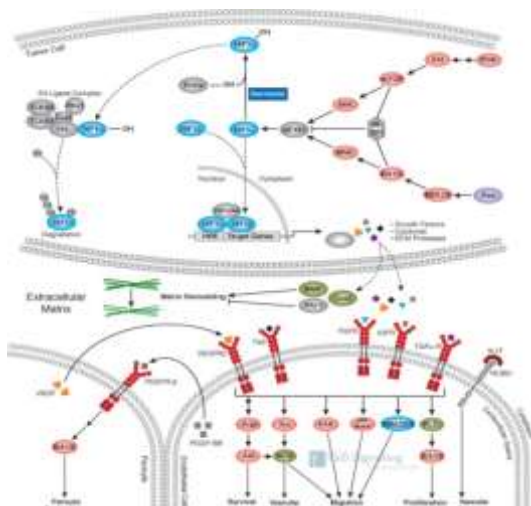


Figure 12. VEGF pathway (quoted from <http://www.cellsignal.com/reference/pathway/Angiogenesis>).

Thus HIF-1 is a regulator of VEGF gene transcription, mRNA stability Hur is a regulator of VEGF. Some inflammatory cytokines such as IL-1 and TNF- α can induce HIF-1. VEGF mRNA will come out from the core to VEGF, has IRES (internal ribosome entry site) that will maintain the effectiveness of VEGF protein in hypoxic conditions. Hypoxia also causes Ifs (Initiation Factor) which can induce IRES to function. But there is no explanation how it could happen.

Chaperones protein ORP 150 plays a role in intracellular transport of VEGF protein of the endoplasmic reticulum to the Golgi apparatus for secreted out of cells. The process is the production of VEGF as the first stage of the process of angiogenesis.

The next track cell migration and cell survival beginning of VEGF binding to VEGFR-2 tyrosine phosphorylation causes an increase of Focal Adhesion Kinase (FAK), which was instrumental in the process of cell migration-assisted matiks Fluid Shear Stress and Integrin (FSS), whereas the increase Phosphatidylinositol 3 kinase (PI3K) activates the antiapoptotic kinase (AKT) is assisted by an increase in antiapoptotic proteins (Bcl-2, A1).

Upon binding of VEGF-VEGFR activation of phospholipase C occurs (PLC- γ) and inositol 1,4,5 mebuat triphosphatase (IP3). IP3 increases the cytoplasmic calcium, which then support the production of NO. PLC- γ will increase the production of diacylglycerol (DAG). DAG then activates protein kinase C (PKC), this will really signal-regulated kinase induces extracellular (ERK1 / 2) and stimulates endothelial cell proliferation. ERK $\frac{1}{2}$ serves to form arachidonic acid (AA) and cyclooxygenase-1 (COX-1) which affects the synthesis of PGI₂. This is what will increase the permeability of blood vessels.

METHODS

The skin flap was made on the abdominal skin of rats (*Rattus norvegicus*). All procedures were performed on male Wistar rats strain weighing between 250-300 grams and were divided into 2 groups: control group and treatment group. Anesthesia was induced by intramuscular of ketamine (50mg/kg). The rats were housed individually in an environmentally controlled room with 12h of light and darkness and were offered standar rat chow and water ad libitum. The study was conducted to determine the necrotic area and the amount of angiogenesis in random pattern skin flap in the treatment of Remote Hyperthermic preconditioning (RHP) as compared with the flap that is elevated with no previous treatment.

K : control group without treatment RHP

P : treatment group, performed RHP

Research procedures that have been made are as follows :

1. Both groups of shaved fur on the belly, made the design of a long rectangular incision length of 4 cm and 1 cm wide (Fig. 13).



Figure 13. Incision design

2. Disinfection with povidone iodine 10% solusio and Savlon 1: 30
3. Incision is performed using mess no.15 and small metzembaum. Linear incision was made of 4 x 1 cm under cutis and then elevated to form a flap in the distal random pedicle (Figure. 14).



Figure 14. Flap Incision

4. And the donor site is closed with a transparent dressing (Tegaderm) sterile. Formed flap is put back on the closed donor wound with a transparent dressing to prevent a meeting between the flap with the vascular surface of the donor (Figure 15).



Figure. 15. Donor wound and flap

5. Flap covered with transparent dressing and wrapped with gauze and covered with leucoplast (Figure 16).



Figure. 16 Flap is covered with gauze and leucoplast

6. On a treatment group, a right posterior extremity was dipped/exposed in a water bath with a temperature of 50⁰ C for 3 seconds before flap elevation. Whereas in the control group performed without this procedure (Figure 17).



Figure 17. Waterbath with a temperature of 50⁰ C and the exposing of RHP

7. Both groups were clinically observed a visitrax and histopathologic observations using immunohistochemical staining with VEGF antibody. These observations are carried out on day-5 after flap elevation (Fig. 18 a, b and c).



(a) necrotic area (b) visitrax (c) paraffin block

8. Research started with preliminary study using one rat in control group and treatment group with the same procedure above. We need to know how this procedure affect a rat and to predict necrotic area of flap. We didn't perform imunnohistochemical examination in preliminary study.

Preliminary Study Results

After day-5 of elevation flap, group in a control rat and a treatment rat with RHP obtained results:

1. Both rats in conditions of life and active moving.
2. Donor wound in both rats didn't found sign of infections.
3. Flap in each group of rat appear bluish to blackish areas with different size
 - Control : 2.3 cm²
 - RHP treatment : 1.1 cm²

Flap in rats showed extensive necrotic areas that differ between the control group and with RHP treatment groups (Figure 19 a and b). Based on the results of this study, we performed research using subsequently 16 rat as control rats and 16 rat treated with a RHP procedure similar with preliminary study. Results of flap in the day-5 were evaluated with visitrax and immunohistochemical examination using a VEGF antibody.

Preliminary Study Result :



Figure 19 (A) : control rat

(B): treatment rat with RHP

Area Of Necrotic

Examination of necrotic area performed on day-5. Inspection done by determining the boundary / demarcation of the area colored blue, gray to black found on the edge of the flap in control group rat and treatment groups rat (Figure 20 a and b).

Necrotic area was measured starting from a tip of the flap (4 cm from pedicle) . After being cut from the pedicle, this area is spread out on a paper measures 4 x 1 cm drawn properly on transparent mica with black marker. Copy of the pattern is then measured the extent of using visitrax (Table 5). Broad scale is used cm².

The results of flap pieces were fixed with buffered formalin and 10% in the bottle to

be processed in histopathology at the Anatomical Pathology Laboratory.

Table 5. The result of the necrotic area

Specimen No.	Area Of Necrotic (cm ²)	
	Control	Treatment
1.	2,1	0,4
2.	2,0	1,1
3.	2,5	0,5
4.	2,4	1,1
5.	1,5	1,3
6.	1,6	1,1
7.	1,8	0,9
8.	3,1	0,9
9.	1,6	0,7
10.	2,5	0,6
11.	1,5	1,0
12.	3,3	1,1
13.	3,4	0,7
14.	2,7	2,7
15.	2,7	1,2
16	1,5	2,5

Statistical tests performed with Kolmogorov-Smirnov test for normality of distribution view. In the analysis of the normality test of data from two samples are free to use One sample Kolmogorov-Smirnov statistics show the value of Z is 0.620 and 0.996 with p value is 0.836 and 0.275, all of which > $\alpha = 0.05$. Thus these data have a normal distribution (Table 6.7, 8).

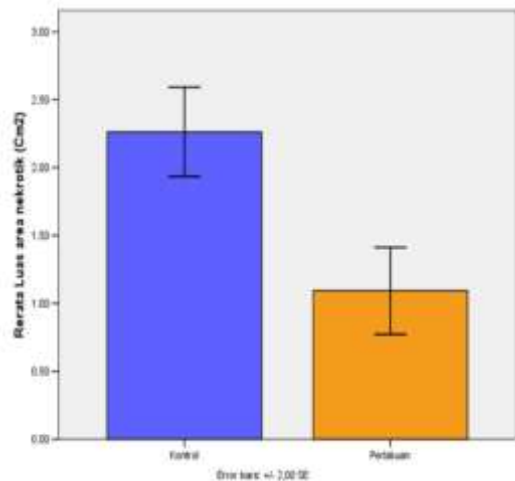
Table 6. Kolmogorov-Smirnov Test

One-Sample Kolmogorov-Smirnov Test			
		Luas area nekrotik Kontrol	Luas area nekrotik Perlakuan
N		16	16
Normal Parameters a,b	Mean	2,2625	1,0938
	Std. Deviation	,65917	,64132
Most Extreme Differences	Absolute	,155	,249
	Positive	,155	,249
	Negative	-,124	-,140
Kolmogorov-Smirnov Z		,620	,996
Asymp. Sig. (2-tailed)		,836	,275

a. Test distribution is Normal.

b. Calculated from data.

The mean necrotic area of the treatment group was 1.1 + 0.6 cm, smaller than the control group of 2.3 + 0.7 cm. T2 test results obtained free sample price p < 0.0001 means that there are significant differences in mean necrotic area between the control and treatment groups on day-5.



Graph 1. The relationship between the average necrotic area of the control and treatment groups

Table 7. The mean necrotic area of the control group and treatment group

Variable	Group Sample		P
	Control	Treatment	
Area Of Necrotic	2,3 ± 0,7	1,1 ± 0,6	< 0,0001

Table 8. Lavene test / t 2 free sample

Independent Samples Test										
	Levene's Test for Equality of Variances				t-Test for Equality of Means					
	F	Sig.	t	df	t Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	Lower	Upper	
Equal variances assumed	1.818	.200	5.803	30	.000	1.16675	-.22962 .00000			
Equal variances not assumed			5.803	29.307	.000	1.16675	-.22962 .00000			



Fig. 20(A) Necrotic area a control day 5th



Fig. 20(B) Necrotic area with RHP day 5th

Evaluation of Angiogenesis

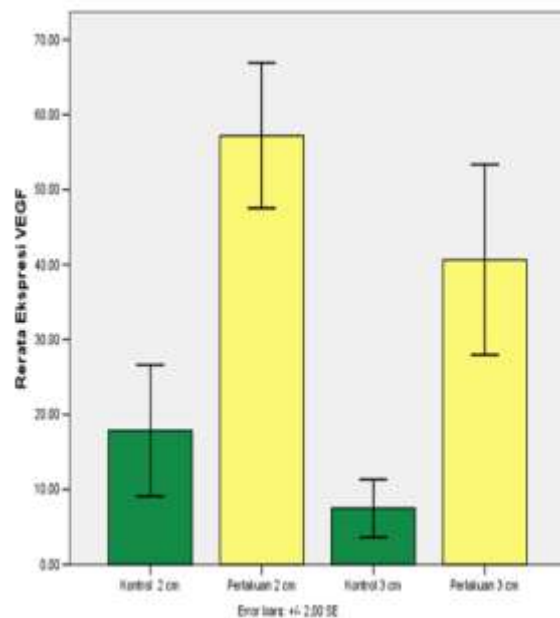
Evaluation of angiogenesis in the day-5 carried out using an immunohistochemical examination of VEGF antibody with 400x magnification. The number of active vessels that have angiogenesis is determined from the number of blood vessels (expressed VEGF) on endothelial brownish color. Calculations performed on the entire surface / thick slices perpendicular to the flap on the pedicle axis is 2 cm, 3 cm and 4 cm from the pedicle (Tables 9 and 10).

Table 9. Quantification results (control) : Blood Vessels with VEGF expressed

Specimen No.	distance slice from pedicle		
	2 cm	3 cm	4 cm
1.	12	0	0
2.	0	1	0
3.	0	0	0
4.	0	10	0
5.	17	22	0
6.	29	10	0
7.	50	2	0
8.	0	0	0
9.	50	20	0
10.	38	10	0
11.	36	11	0
12.	1	0	0
13.	14	19	0
14.	13	10	0
15.	11	5	0
16.	15	0	0

Table 10. Quantification results (treatment): Blood Vessels with VEGF expressed

Specimen No.	distance slice from pedicle		
	2 cm	3 cm	4 cm
1.	101	59	0
2.	48	43	0
3.	58	79	0
4.	33	20	0
5.	31	19	0
6.	76	33	0
7.	68	31	0
8.	60	54	0
9.	78	44	0
10.	39	87	0
11.	52	39	0
12.	60	49	0
13.	76	69	0
14.	49	0	0
15.	32	24	0
16.	54	0	0



Graph 2. Average number of blood vessels expressed VEGF slices 2 and 3 cm from the pedicle in the control group and RHP

Table 11. Kolmogorov Smirnov Test

		Ekspresi VEGF 2 cm Kontrol	Ekspresi VEGF 3 cm Kontrol	Ekspresi VEGF 2 cm Perlakuan	Ekspresi VEGF 3 cm Perlakuan
N		16	16	16	16
Normal Parameter a =	Mean	17.8750	7.5000	57.1075	40.6250
	Std. Deviation	17.53805	7.72673	19.35362	25.37157
Most Extreme Differences	Absolute	.207	.199	.130	.072
	Positive	.207	.199	.130	.072
	Negative	-.154	-.106	-.088	-.072
Kolmogorov-Smirnov Z		.830	.797	.519	.298
Asymp. Sig. (2-tailed)		.487	.558	.901	1.000

a. Test distribution is Normal.
b. Calculated from data.

Test for normality data distribution (Kolmogorov-Smirnov) indicate the data has a normal distribution. To examine differences in the average number of blood vessels expressed VEGF in slices 2 cm and 3 cm from the pedicle between the control and treatment groups tested the independent sample t-test (table 12.13).

Table 12. Lavene Test / independent sample test for the number of VEGF expression area of 2 cm and 3 cm

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference
									Lower Upper
Ekspresi VEGF 2 cm	Equal variances assumed	.038	.847	-6.021	30	.000	-39.31250	6.52948	-52.64748 -25.97752
	Equal variances not assumed			-6.021	29.714	.000	-39.31250	6.52948	-52.65287 -25.97213
Ekspresi VEGF 3 cm	Equal variances assumed	12.075	.002	-4.996	30	.000	-33.12500	6.63066	-46.66661 -19.58339
	Equal variances not assumed			-4.996	17.760	.000	-33.12500	6.63066	-47.06900 -19.18100

Table 13. The mean number of blood vessels expressed VEGF area of 2 cm and 3 cm

Variables (blood vessels expressed VEGF)	Group Samples		P
	Control	RHP	
2cm slices of the pedicle	17,9 ± 17,5	57,2 ± 19,4	< 0,0001

The mean number of blood vessels expressed VEGF slices 2 cm from the pedicle in the treatment group +19.4 57.2 more than the control group by 17.9 +17.5. With independent sample t-test tests obtained p-value = 0.000 (p <0.0001), meaning that there are significant differences the number of blood vessels expressed VEGF on flap incision is 2 cm from the pedicle between the control and treatment groups on day-5.

The mean number of blood vessels expressed VEGF slices 3 cm from the pedicle in the treatment group of 40.6 +25.4 more than the control group at 7.5 +7.7. With independent sample t-test tests obtained p-value = 0.000 (p <0.0001), meaning that there are significant differences the number of blood vessels expressed VEGF on flap incision is 3 cm from the pedicle between the control and treatment groups on day-5 (tables 14 and 15).

Table 14. The mean number of blood vessels expressed VEGF slices of 3 cm

variables (blood vessels expressed VEGF)	Group Samples		P
	Control	Control	
3 cm slices of the pedicle	7,5 ± 7,7	40,6 ± 25,4	< 0,0001

Table 15. Lavene test / independent sample test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference
									Lower Upper
Ekspresi VEGF 2 cm	Equal variances assumed	.038	.847	-6.021	30	.000	-39.31250	6.52948	-52.64748 -25.97752
	Equal variances not assumed			-6.021	29.714	.000	-39.31250	6.52948	-52.65287 -25.97213
Ekspresi VEGF 3 cm	Equal variances assumed	12.075	.002	-4.996	30	.000	-33.12500	6.63066	-46.66661 -19.58339
	Equal variances not assumed			-4.996	17.760	.000	-33.12500	6.63066	-47.06900 -19.18100

These three areas (2cm, 3m, and 4 cm from pedicle) were examined separately using immunohistochemical techniques to calculate the blood vessels expressed VEGF (Figures 21 and 22). But in 4 cm area from pedicle shows only necrotic area in both control and treatment

groups without angiogenesis process (Figure 23).

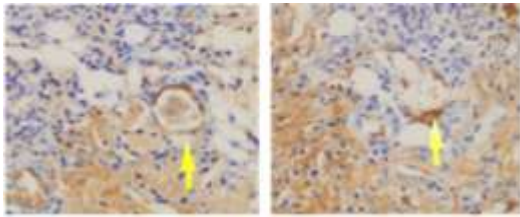


Figure 21. Active blood vessels (angiogenesis) that expressed VEGF (magnification 400x)

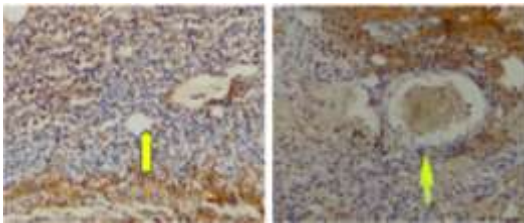


Figure 22. Blood vessels that was not expressed VEGF (magnification 400 X)

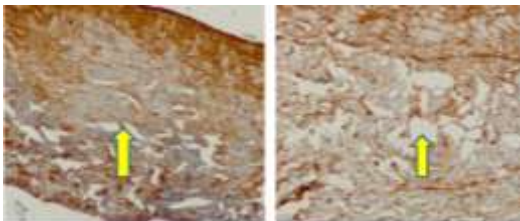


Figure 23. Necrotic areas on slices 4 cm from the pedicle showed a loose network structure and cracking without angiogenesis

DISCUSSION

Random pattern skin skin flap flap is comprised of cutis and subcutaneous tissues with survival obtained from the blood vessels in the plexus subdermalnya own (Vedder, 2006) with the main feature does not have a dominant supply of blood flow (Lync, 2004). Basic design is the elevation of the rectangular flap of skin and subcutaneous tissue with a ratio length: width of approximately 2:1.

Preconditioning is an endogenous mechanism after stimulation from outside of body that protects body cells against injury ischemia / reperfusion (Filho et al, 2009). To create a state of cellular stress can be either ischemic preconditioning, heat shock, endotoxin, adenosine, $\alpha 1$ adrenergic agonists, reactive oxygen species (ROS) or opioids. In recent years the concept of preconditioning

began to expand along with the growing knowledge of self defense attempt to make the body before having a pathological condition. Preconditioning modalities is needed to improve the viability of the flap, one of which is the delay procedure. Changes in the vascular anatomy of the flap with longitudinal reorientation of small blood vessels in the subdermal plexus causes the growth of new blood vessels (neovaskuler).

Remote preconditioning system is a method of preconditioning in which a process stressing the particular organ can affect other organs that do not stressing (Filho et al, 2009). This procedure has the advantage that no target organ damage / disturbance resulting from treatment during the treatment process. A given stimulus will provide a systemic response so as to achieve the therapeutic target specific organs can be performed on the organ to another stimulus. Improvement of blood supply and oxygen are needed to improve survival rates in the random-pattern skin flap in the hope can be applied in humans to maintain the viability of skin flap.

Hyperthermia is one of preconditioning procedure by exposing supranormal and sublethal temperatures. The exposure was carried out at a certain temperature and certain long. The higher the temperature, which is the time required will be even shorter, whereas the lower temperature, which is the time required for exposure will be even longer to reach the condition of preconditioning. It has systemic effects, namely: (1) increased regulation of Plasminogen Activator in vivo, (2) acceleration of microvascular recanalization of thromboembolism vessels, (3) improve the regulation of heat stroke proteins that will induce endogenous hyperfibrinolysis.(4) anti apoptosis

Hyperthermic preconditioning for the provision of heat shock on the network prior to flap in a certain time and temperature on the state suprafisiologis sublethal to result in temporary changes in cellular biosynthesis through the induction of a variety of proteins including heat shock proteins (HSPs). HSPs consist of molecular chaperones that will expression rapidly after exposure to high temperatures and reaches a maximum level a few hours after that. HSP is found in all organisms, ranked in the cytoplasmic protein with a molecular weight of 20-120kDa. Molecular chaperones play a role in the homeostasis of intracellular survival (Morimoto, 1996; Rynlander, 2005). HSP70 and -27 exhibit properties typical of the rise in temperature,

ischemia and oxidative stress. Increased expression of Hsp70 can enhance cellular protection, inhibition of cell death (Topping et al, 2001; Rynlander, 2005). Increasing 5-6 °C from normal body temperature can induce Hsp-70 (Beckham et al, 2004). HSP-70 would facilitate the body's cells in the mechanism of angiogenesis.

Angiogenesis is the growth of new blood vessels that can occurs in the second path, through the mobilization of endothelial precursor cells (EPCs) and pre-existing vessels (Fig. 11) (Peichev et al, 2000; Kumar 2009). VEGF A has a main role in the process of angiogenesis. Of VEGF-A gene consists of 8 exons separated by 7 introns on chromosome 6p21.3. Of the many growth factors, VEGF including the most important factor in the process of angiogenesis. Biological effects of VEGF may result from binding to its receptor (Table 2). Receptor in the presence of hypoxic cells showed an autocrine regulatory mechanism of VEGF produced by hypoxic cells or experiencing stress (Lamallice, 2007).

Recent developments in mind that remote hyperthermic preconditioning (RHP) has a real effect on increased angiogenesis mediated by the role of HSP-70. The process of angiogenesis that occurs will retain the nutrients and oxygen supply to the flap structure that has been elevated, especially the distance end of the pedicle flap. Increased angiogenesis will have a positive effect on skin flap viability. This leads to research that concluded that remote hyperthermic preconditioning has an important role in increasing the viability of random pattern skin flap.

To prove the effect of remote hyperthermic preconditioning (RHP) has an influence on increasing the viability of random pattern skin flap studies using experimental animals the male Wistar rat strain carried a skin flap elevation procedure based distally on the abdomen by tangential excision using mess no 15 and small metzembaum.

In the treatment group, 1 hour prior to the elevation of the right posterior extremity flap rats dipped in waterbath with a temperature of 50°C for 3 seconds in accordance with hyperthermic preconditioning protocol. Excision performed was limited to the skin over the panniculus carnosus. Donor wound is closed with a transparent dressing, placed over the donor flap is then covered with a transparent dressing. After that donor and flap wound was closed with sterile gauze and wrapped round with lekoplast.

In the control group performed without exposing in waterbath before flap elevation. Flap would observed day-to-5 post-flap elevation.

Clinical observations were made by calculating the area of necrotic area using visitrax, and histopathological evaluation using immunohistochemical techniques with VEGF antibody. Necrotic area was measured using the pattern on mica paper with permanent ink. This pattern of necrotic area was measured using a visitrax with cm² scale.

Histopathological observations made by cutting a flap perpendicular to the longitudinal axis at a distance of 2cm, 3cm, and 4cm from the base of the pedicle. The slice was made of paraffin block, then cut of staining with immunohistochemistry using VEGF antibody. Selected observations of day-to-5 because the demarcation of the necrotic area is apparent clearly and prominent angiogenesis process has been seen on day-5.

Table 7 shows that the average size of the necrotic area in the treatment group (with RHP) is smaller than in the control group (without treatment RHP). In the control group an average of necrotic area was 2.6 cm², it means that skin flap in rats viable control in the area between 1 cm and 2 cm (4 cm - 2.6 cm = 1.4 cm) of the pedicle. So the design for the flap that can still viable is the ratio of length: width of about 1:1 to 2: 1. This design is accordance with the flap design commonly used in clinical applications. While in the treatment group, an average RHP extensive necrotic area was 1.1 cm², it means skin flap in rats RHP viable treatment groups in the area between 2cm and 3cm (4 cm - 1.1 cm = 2.9 cm) of the pedicle. So the design for the flap that can still viable with ratio of length: width of about 2: 1 to 3:1.

Increased area of viable skin flap between the control and treatment groups for RHP (2.9 cm - 1.4 cm): 1.4 cm X 100% = 100.07%. Increased viability of the area by more than 2-times by treatment with RHP.

To examine differences in necrotic area between the control and treatment groups, at day-5 tested samples t-test independent homogeneous variance. The result obtained p-value = 0.000 means that there are significant differences between the necrotic area of the control group and treatment group on day-5. By the RHP before treatment can reduce flap necrotic area on random pattern skin flap.

In Table 13 show that the average number of angiogenesis is indicated as active vascular endothelial staining brown with antibody to VEGF-5 treatment groups (with

RHP) more than in the control group (without treatment RHP). To examine differences in the average number of angiogenesis between the control and treatment groups, at day-5 tested samples t-test independent homogeneous variance.

On 2 cm slice of the results obtained $p = 0.000$, it mean that there are significant differences between control group and the amount of angiogenesis treatment groups on day-5. On 3 cm slices of the results obtained $p = 0.000$, which means there are significant differences between control group and the amount of angiogenesis treatment groups on day-5.

On slices of 4 cm (end from the pedicle flap) did not look any blood vessels expressed VEGF, it shows only necrotic tissue and a loose network structure and cracking. Angiogenesis needs a specific stimulus like VEGF. In this area (4 cm from pedicle) found VEGF on all the field, it cause by hypoxic condition can stimulate VEGF expression, but to create angiogenesis in pre-existing vessel it needs a role of reseptor (VEGFR) to receive VEGF. Appropriate microenvironment absolutely needed in this process. But severe hypoxic and lack of nutrition in this area cause failure of this process. So we couldn't find active vessel expressed VEGF.

Previous research suggests that ischemic preconditioning by femoral artery occlusion prior to elevation flap can increase flap viability is also proved in our study.

From a results above indicate that the macroscopic parameters of necrotic area RHP proved that the treatment can reduce the necrotic area. Increasing viable area was proved. In a microscopic amount of active blood vessel angiogenesis has also increased the flap with RHP treatment. Viability of the flap which is an indicator of the success of the flap, the macroscopic and microscopic in our study proved to increase with RHP treatment.

Although this study shows positive results on random pattern skin flap viability of the treated group, there are still limitations in this research. It is about time for exposing RHP was only 3 seconds.

Exposure to temperatures 50°C for 3 seconds, including one of hyperthermic preconditioning protocol (THES), but from the literature mentioned that hyperthermic preconditioning protocol with exposure to lower temperatures for a long time (TLEL) provide a more optimal results. Flap viability study can only be increased up to 3 cm area of the pedicle. RHP with THES exposure can improve the

design length: width flap of 2: 1 to 3: 1, but if it done by $T_L E_L$ protocol, it is possible to provide a better viability results in area 4 cm from the pedicle. This limitation is due to engineering difficulties that long exposure of the legs of rat.

CONCLUSIONS

1. RHP can reduce necrotic area on random pattern skin flap in rat
2. RHP can improve angiogenesis on random pattern skin flap in rat
3. RHP can improve viability of random pattern skin flap in rat.

Advice

1. Further research using $T_L E_L$ protocol (using lower temperature with longer time exposure).
2. RHP can be recommended to be a clinical procedure before elevation skin flap to cover a wide wound.

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Appendix

Appendix 1: Figure all flap in evaluation on day-5

Control Group



Appendix 2: Figure all flap in evaluation on day-5

RHP treatment group



Appendix 3: Measurements of necrotic area using visitrax

Control Group



Appendix 4: Results of measurements of the necrotic area using visitrax

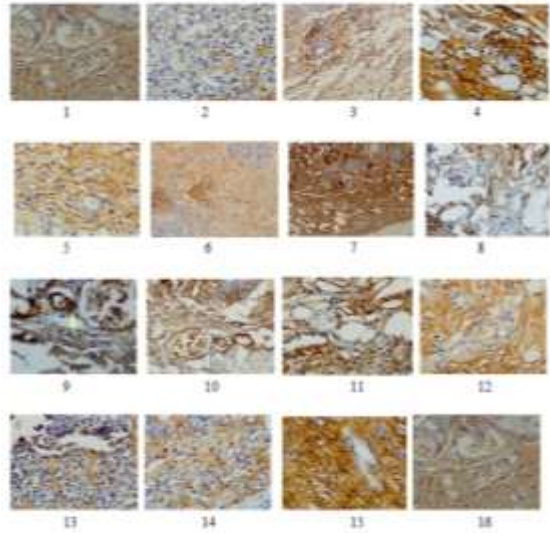
Treatment Group



Appendix 5: Microscopic pictures of immunohistochemical examination using VEGF antibody

Control group:

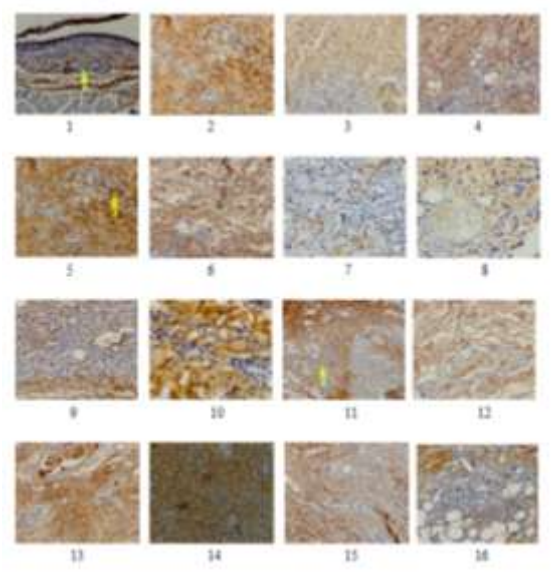
2 cm from the pedicle



Appendix 6: Microscopic pictures of immunohistochemical examination using VEGF antibody

Control group:

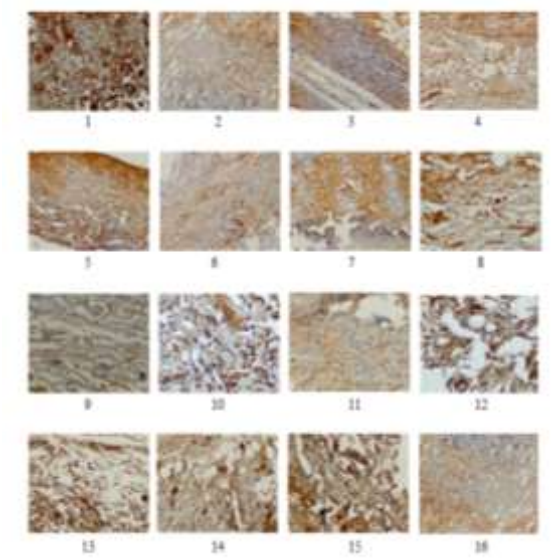
3 cm from the pedicle



Appendix 7: Microscopic pictures of immunohistochemical examination using VEGF antibody

Control group:

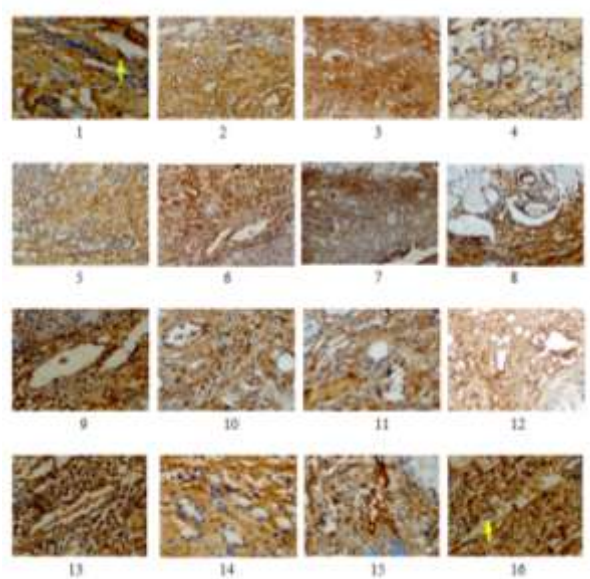
4 cm of pedicle



Appendix 8: Microscopic pictures of immunohistochemical examination using VEGF antibody

Treatment group

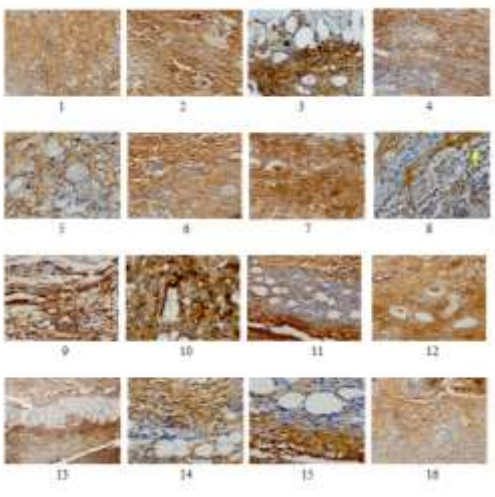
2 cm from the pedicle:



Appendix 9: Microscopic pictures of immunohistochemical examination using VEGF antibody

Treatment group

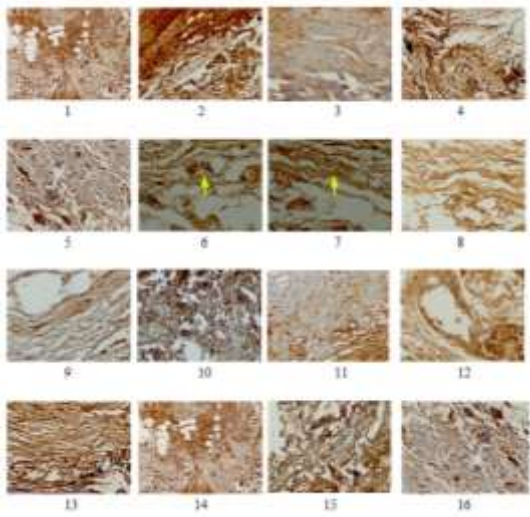
3 cm from the pedicle:



Appendix 10: Microscopic pictures of immunohistochemical examination using VEGF antibody

Treatment group

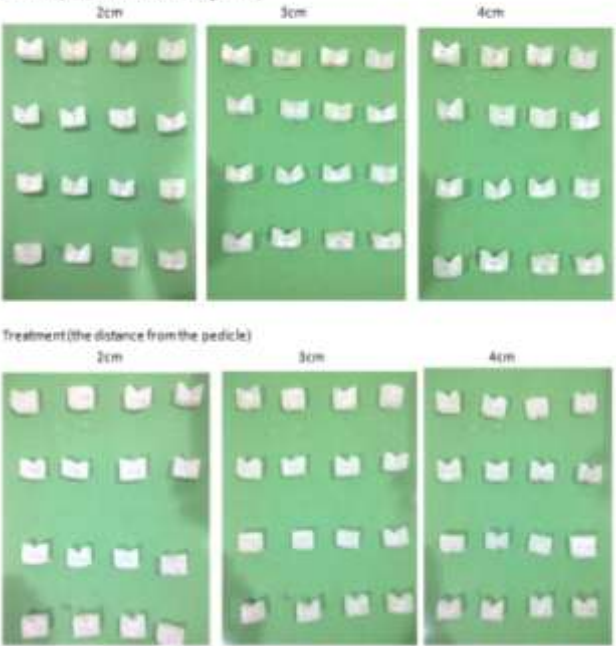
4 cm of pedicle:



Appendix 11: paraffin block to create a slide immunohistochemical examination



Appendix 12: photographs of paraffin blocks



Appendix 13:

HASIL PEMERIKSAAN MIKROSKOPIS PATOLOGI ANATOMI
TERHADAP SEDIAAN / POTONGAN JARINGAN KULIT ABDOMEN TIKUS
DENGAN PULASAN IMMUNOHISTOKIMIA
MENGUNAKAN ANTIBODI VEGF

Jumlah Pembuluh Darah yang Terkapresi VEGF

1. Kelompok Kontrol

No. Sampel	Lokasi Potongan dari Pedikel		
	2 cm	3 cm	4 cm
1.	12	0	0
2.	0	1	0
3.	0	0	0
4.	0	10	0
5.	17	22	0
6.	29	10	0
7.	50	2	0
8.	0	0	0
9.	50	20	0
10.	38	10	0
11.	36	13	0
12.	1	0	0
13.	14	19	0
14.	13	10	0
15.	11	5	0
16.	13	0	0

Mengubah,

dr. Dyan Fauziah, Sp PA (K)

HASIL PEMERIKSAAN MIKROSKOPIS PATOLOGI ANATOMI TERHADAP SEDIKAAN / POTONGAN JARINGAN KULIT ABDOMEN TIB DENGAN PULASAN IMMUNOHISTOKIMIA MENGGUNAKAN ANTIBODI VEGF			
Jumlah Positivitas Darah yang Terkapsasi VEGF			
1.5. Kelompok Perikutan			
No. Sampel	Lokasi Potongan dan Profil		
	2 cm	3 cm	4 cm
1.	101	59	0
2.	48	43	0
3.	58	79	0
4.	33	20	0
5.	31	19	0
6.	76	33	0
7.	68	31	0
8.	60	54	0
9.	78	44	0
10.	39	87	0
11.	32	39	0
12.	60	49	0
13.	76	69	0
14.	49	0	0
15.	32	24	0
16.	54	0	0

Mengirimkan,



Dr. Diah Fauziah, Sp.PA (K)

BACKGROUND
VEGF, a homodimeric glycoprotein of relative molecular mass 45,000, is the only cytokine that specifically acts on endothelial cells. It may be a major regulator of tumor angiogenesis in vivo. Vascular endothelial growth factor is a cytokine primarily for vascular endothelial cells. It is, however, structurally related to platelet-derived growth factor. VEGF shows homology with the PDGF A chain and B chain, including conservation of all 8 cysteines found in PDGFs. VEGF gene contains 8 exons. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH1-mediated sensitization and inflammation in the lung. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Vascular endothelial growth factor (VEGF) stimulate neurogenesis in vitro and in vivo.

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Appendix 14: Product Information Sheet

Product Information Sheet	
Polyclonal Anti-Vascular endothelial growth factor VEGF	
Catalog No. PA1002	Immunogen
Lot No. 200207	A synthetic peptide corresponding to a sequence near the N-terminus of human VEGF, identical to the related rat sequence.
Ig type: rabbit IgG	Purity
Size: 100µg/ml	Immunogen affinity purified
Specificity	Application
Human, mouse, rat	Western blot
No cross reactivity with other proteins	At 2.5-10µg/ml with the appropriate system to detect VEGF in rats and humans (immunohistochemistry)
Recommended application	At 1-2µg/ml to detect VEGF in formalin fixed and paraffin embedded tissues. Blocking the sections is required.
Western blot	(Other applications have not been tested)
Immunohistochemistry	Optimal dilutions should be determined by end user.
1:1000	Controls
	1 with rat contains 50ng BSA, 0.5mg NaCl, 10mg MgCl ₂ , 0.1mg Thiomersal, 0.5mg NaH ₂ PO ₄ .
	Reconstitution
	0.2ml of distilled water will yield a concentration of 500µg/ml.
	Storage
	At -20°C for one year. After reconstitution, at 4°C for one month. It can also be aliquoted and stored frozen at -20°C for longer time.
	Relative detection systems
	Boehr provides a series of assays reacted with primary antibodies. Antibody can be assayed by chemoluminescence kit EL1002 or WB kit EL1002 in kit.

70-73/41

0.7-0.7 70-73/41

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Appendix 15: Ethical Clearance

 KOMITE ETIK PENELITIAN KESERAHAN FAKULTAS KEDOKTERAN UNIVERSITAS AIRLANGGA SURABAYA KETERANGAN KELAIKAN ETIK ("ETHICAL CLEARANCE") No. 004/EC/KEPK/FAK/2012 KOMITE ETIK PENELITIAN KESERAHAN FAKULTAS KEDOKTERAN UNIVERSITAS AIRLANGGA SURABAYA, TELAH MEMPELAIKAN SECARA SUKSES RANCANGAN PENELITIAN YANG DIBERIKAN, SAKA DENGAN INI MENYATAKAN BAHWA PENELITIAN TERSEBUT: Pengaruh Hipertensi Sebagai Faktor Hipertensi Preconditioning (RHP) Terhadap Survival Rancan - Patensi Skin Flap Pada Tikus PENELITI UTAMA Agil Mulya Astadita, dr. UNIT LEMBAGA / TEMPAT PENELITIAN Laboratorium Biokimia Fakultas Kedokteran Universitas Airlangga DINYATAKAN LAIK ETIK. 7 Februari 2012 Prof. M. Agil (Ketua Komisi Etik), SpB, SpDB	
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