ASSOCIATION BETWEEN PERIPHERAL WHITE BLOOD CELL COUNT AND AUTOLOGOUS SKIN FLAP HEALING RATE IN RABBITS

by

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ABBREVIATION

ADP	: Adenosine diphosphate
AECUSM	: Animal Ethics Committee, UniversitiSains Malaysia
CBC	: Complete blood count
DC	: Differential count
DPX	: Di-n-butyl phthalate in Xylene (a mounting medium)
ECM	: Extracellular matrix
FBC	: Full blood count
IgE	: Immunoglobulin E
IL-4	: Interleukin-4
K2EDTA	: Dipotassium ethylene diaminetetraacetic acid
NZW	: New Zealand White
ROS	: Reactive oxygen species
SD	: Standard deviation
SID	: Sample identification data
SPSS	: Statistical package for the social sciences
Th2	: T helper type 2 cells
UMK	: Universiti Malaysia Kelantan
USM	: UniversitiSains Malaysia
VEGF	: Vascular endothelial growth factor
WBC	: White Blood Cell

ABSTRAK

Kejayaan kemandirian flap kulit bergantung kepada kadar proses penyembuhan, yang mana ia mungkin boleh dikaitkan dengan peranan sel darah putih. Maka, tujuan kajian ini dijalankan adalah untuk mengkaji perkaitan antara pengiraan sel darah putih dan kadar penyembuhan flap kulit autologus pada arnab. Sejumlah empat ekor arnab jenis New Zealand White (NZW) yang berumur antara 9 hingga 15 bulan telah digunakan di dalam kajian ini. Kesemua arnab telah menjalani pembedahan flap kulit autologus pada bahagian abdomen ventral. Sebanyak 3 mililiter darah periferi telah diambil dari saluran arteri tengah di telinga pada hari sifar (sebelum pembedahan), pertama, ketiga, kelima, ketujuh dan kesepuluh selepas pembedahan. Pengiraan sel darah putih dan pengiraan secara manual pembezaan sel darah putih telah di ukur secara respektif menggunakan penganalisis Mythic 18 Vet dan pewarnaan Wright. Kadar penyembuhan flap kulit telah ditentukan oleh min peratusan kawasan nekrosis pada hari pertama, ketiga, kelima, ketujuh dan kesepuluh selepas pembedahan. Keputusan menunjukkan bahawa min peratusan kawasan nekrosis selepas pembedahan tiada perkaitan secara signifikasi dengan min pengiraan sel darah putih. Walaubagaimanapun, terdapat pengurangan pada peratusan kawasan nekrosis dan corak normalisasi pada pengiraan sel darah putih pada hari kesepuluh selepas pembedahan. Perubahan-perubahan ini boleh menandakan kejayaan proses penyembuhan luka. Kajian ini menunjukkan bahawa corak perubahan pada pengiraan sel darah putih mungkin boleh digunakan untuk menilai tahap perkembangan penyembuhan luka pada pembedahan flap kulit autologus.

ABSTRACT

Successful skin flap survival depends on the rate of healing process, which can be related to the roles of white blood cells (WBC). Therefore, the aim of this study was to investigate the association between WBC count and autologous skin flap healing rate in rabbits. A total of four New Zealand White rabbits, aged between 9 to 15 months, were used in this study. All rabbits underwent autologous skin flap surgery at the ventral abdomen. A total of 3 ml of peripheral blood were collected from the central ear artery on day 0 (before surgery), day 1, 3, 5, 7 and 10 post-operation. The WBC count and manual WBC differential count were measured using Mythic 18 Vet Analyzer and Wright's stain, respectively. The skin flap healing rates were determined by mean percentage of necrosis area at day 1, 3, 5, 7 and 10 post-operation. The results showed that the post-operative mean percentage of necrosis area was not significantly associated with the mean of WBC count. However, there was a decrease in the percentage of necrosis area and the WBC count showed normalization pattern on post-operative day 10. These changes could suggest successful wound healing process. This study showed that the pattern of WBC count changes could be used to evaluate the progress of wound healing in autologous skin flap surgery.

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 White blood cell

White blood cells (WBCs), also called leukocytes or leucocytes, are the cells of the immune system of all living mammals. The WBCs are derived from a multipotent cell in the bone marrow known as a hematopoietic stem cell. They are found throughout the body of mammals, including the blood and lymphatic system. The major functions of WBC include fighting infections, defending the body by phagocytosis against invasion of pathogens, producing and transporting antibodies in immune response (Etim, Williams, Akpabio & Offiong, 2014). There are five subtypes of normal peripheral WBCs which include neutrophils, lymphocytes, monocytes, eosinophils and basophils. These subtypes are distinguished by their physical and functional characteristics, for example the monocytes and neutrophils are phagocytic (Hato & Dagher, 2014; Hughland *et al.*, 2012), while the eosinophils along with basophils are important mediators of allergic responses (Ohnmacht & Voehringer, 2009).

In rabbits, the granules neutrophils appear bright pink to red color when stained with Romanowsky stains such as Wright's stain. This has resulted in the use of the term heterophils, amphophils, pseudoeosinophils and pseudoheterophils when referring to the rabbit neutrophils. Rabbit neutrophils have a diameter of 10-15 micrometers (μm) with a polymorphic nucleus that stains light blue to light purple. The cytoplasm stains light pink with the presence of large dark pink to red granules (Meredith & Flecknell, 2006). Neutrophils are highly motile, response to chemotaxis and react within the first two to three hours after the initial injury or damage to the body tissue, thus they serve as first line defence against invading pathogens and damage tissues. The neutrophil count

rapidly increased due to the chemical signals released by the endothelial cells at the injured area. These chemical signals initiate proliferation and migration of neutrophils to the affected area, thus increase the cells count in the peripheral blood circulation. Neutrophils are known as professional phagocytic cells which react instantly in the innate immune response. They engulf pathogen at the wound bed, produce antigen presentation and release cytokines and chemical such as hydrogen peroxide and reactive oxygen species (ROS) that kill pathogen and stimulate migration of monocytes to the infected areas to help in the wound healing process (Hato & Dagher, 2014).

Besides neutrophils, another subtype of WBC is lymphocytes. They are predominantly small in size (7-10 µm) but larger lymphocytes (10-15 µm) could also be seen in mammals. The nucleus of lymphocytes is round to oval in shape and stained dark purple-blue with Romanowsky stains. The cytoplasmic stains medium to dark blue and azurophilic granules may be present in large lymphocytes (Meredith & Flecknell, 2006). The lymphocyte count is predominant up to 70 percent of total WBCs in rabbit, aged less than four month old. The lymphocyte count may become equal to neutrophil count in adult rabbits. Lymphocytes can be divided into B lymphocyte, T lymphocyte and natural killer (NK) cells. The T lymphocytes can be further divided into T helper cells and T cytotoxic cells. Each of these cells has the ability to distinguish between normal cells and pathogen or infected cells and involved in either innate or adaptive immunity. Elevated levels of lymphocytes count are mostly related to viral infections and present of cancerous cells. NK cells can induce apoptosis in tumor cells and viral infected cells (Rystwej & Deptula, 2012).

The monocytes are the largest cells in the peripheral blood of rabbits, measuring 15-18 µm in diameter. They have large variably-shape nucleus with very fine chromatin

and stain light purple. The cytoplasm of monocyte is abundant and stained grey to blue-grey with Romanowsky stains. Few cytoplasmic vacuoles may be present in monocytes (Meredith & Flecknell, 2006). After activation and recruitment to the wound areas, monocytes differentiate into macrophages. Macrophages were highly motile and exert proinflammatory functions. Adequate monocytes recruitment and neovascularization at the site of injury increased flap survival. Macrophages have been recognized as critical regulator for angiogenesis (Khan *et al.*, 2013).

The eosinophils are slightly larger than neutrophils with diameter of 12-16 μm. The granules of eosinophils are larger than neutrophils and stained intensely pink or pink-orange. The nucleus is bilobed or has horse-shoe shaped and is stained purple color. The cytoplasm is stained light pink due to the present of eosinophilic granules (Meredith & Flecknell, 2006). Eosinophils are known for its anti-inflammatory action during parasitic infection, allergic reaction associated with asthmatic disease and gastro-intestinal disorders (Cadman *et al.*, 2013). Activated eosinophils release chemical component mainly peroxidase to kill parasites.

The basophils are the least common type of granulocyte and constitute less than 0.3 percent of circulating WBC in the peripheral blood of human with percentage of less than two percents; however the count can be up to three percents in rabbits. Rabbit basophils have similar size (10-15 μm) with neutrophils (heterophils). The nucleus usually lobulated and stained light purple with the cytoplasmic granules stained dark purple to purple-black using Romanowsky stains (Meredith & Flecknell, 2006). The basophils contain large cytoplasmic granules that stored histamine and heparin and the nucleus usually has two lobes. Histamine was vasodilator that promotes blood flow while heparin was an anticoagulant that prevents blood from clotting. Basophils

contribute to initiation and execution of type 2 immunity which is characterized by high levels of Immunoglobulin E (IgE) and increased number of T helper type 2 cells (Th2), eosinophils, mast cells and basophils. Basophils can be rapidly mobilized after helminth infection and recruited into the lymphoid and peripheral tissues, where they execute their functions. When activated, basophils release histamine, heparin, leukotrienes, proteolytic enzymes and various cytokines including interleukin-4 (IL-4), which contribute to inflammation. Overproductions of leukotrienes are known to cause contraction of the smooth muscle lining of the bronchioles causing inflammation in asthma and allergic rhinitis (Barton, 2008).

1.2 White blood cell count (WBC count)

The WBC count is a test used to measure the total number of WBC in the blood circulation. The WBC count is a component in complete blood count (CBC) or full blood count (FBC). Another component of CBC is differential count (DC), which is a measurement of WBC subtypes. The number of each WBC subtype per 100 cells is counted and the results are expressed in percentage. The percentage of each subtype is multiplied by the WBC count to give the absolute count.

Stress and illnesses has an effect on the haematological parameters, especially the WBC parameters (Etim *et al.*, 2014), thus the CBC is definitely a good indicator for general health and disease monitoring. In this study, both male and female rabbits were used because there are no significant different in the WBC count between male and female rabbits (Milas *et al.*, 2009).

1.3 Autologous skin flap

Skin flap is a segment of skin harvested from a donor area and transferred to the recipient receptor area. Autologous skin flap means that the skin flap was taken and transfer from the same individual or patient. The used of autologous skin flap eliminate the possibility of skin flap rejection due to incompatibility between donor and recipient, thus provide more successful skin flap survival (Benichou *et al.*, 2011). It is a common treatment of choice for patients with large wound and tissue defect. Abdominal skin flap was used in this study because it can create bigger size of skin flap and has a well-defined vascular anatomy with rich blood supply. This type of skin flap is regularly used in reconstructive surgery. The abdominal skin flaps become a gold standard donor flap in autologous breast reconstruction surgery (Selber *et al.*, 2008).

During the procedure of harvesting the autologous skin flap, the flap is excised from the donor body. After the elevation of the excised tissue, the flap undergoes ischemia when the pedicle is clamped. Insufficient arterial blood supply reduces the normal metabolic activity of the flap tissue, which probably lead to necrosis and flap failure. The common causes of flap failure in autologous skin flap are mostly due to ischaemic reperfusion injury and vascular thrombosis (Kern & Sucher, 2013; Nguyen *et al.*, 2013). Therefore, the survival or healing of autologous skin flap predominantly depends on the integrity of the vasculature and vitality of the blood cells.

1.4 Healing of skin flap

The healing of skin flap is a complex biological process that can be divided into four phases, namely haemostasis, inflammation, proliferation and tissue remodelling. The final desired result in flap healing would be restoration of tissue integrity. Figure 1.1 shows the mechanism of skin flap healing process (Portou, Baker, Abraham & Tsui, 2015).

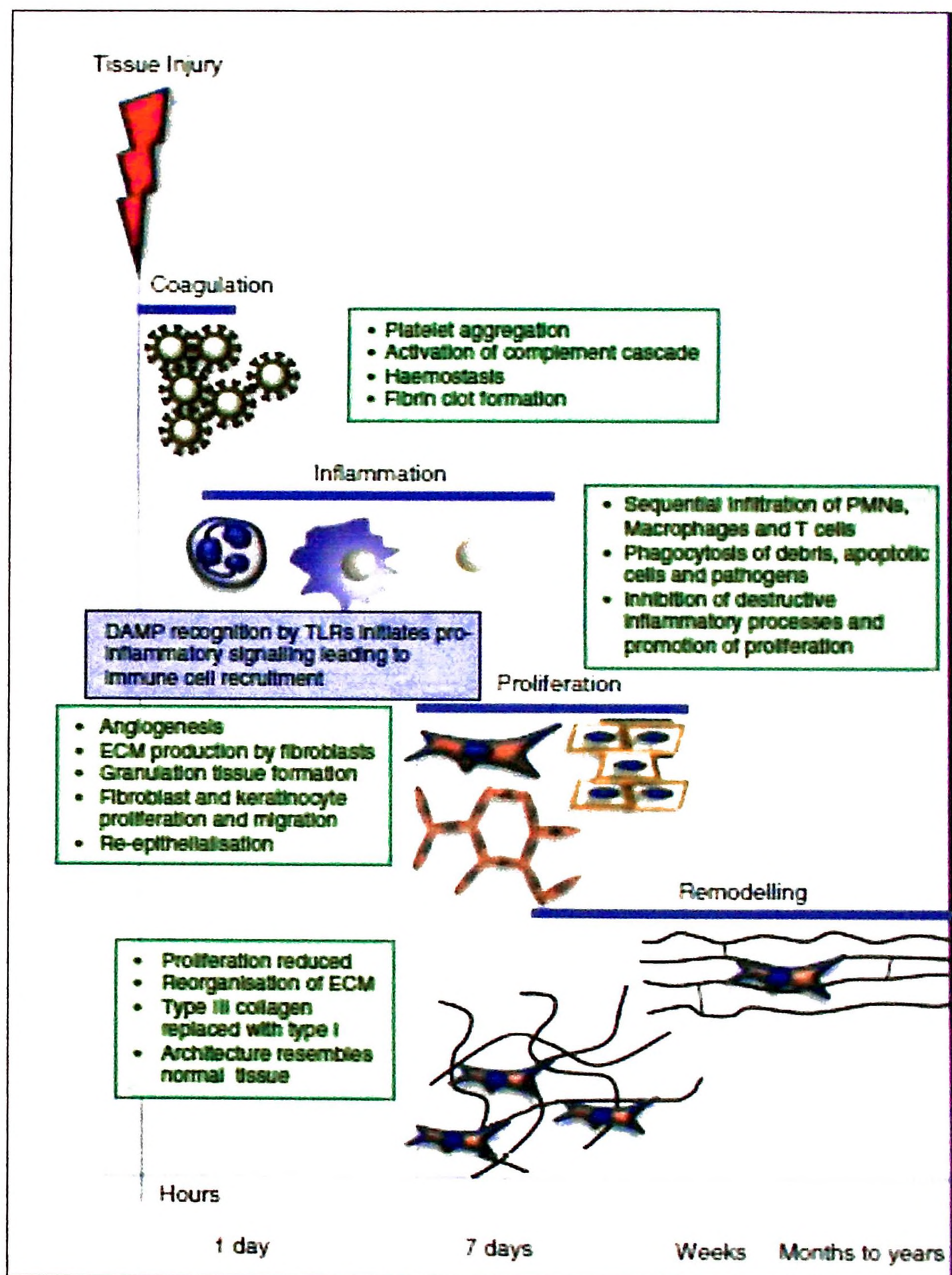

Figure 1.1: Mechanism of skin flap healing process (Portou *et al*., 2015).

1.4.1 *Haemostasis*

Haemostasis is a process of stopping the bleeding at the wounded tissue. It occurs when there is a leakage of blood from an injured blood vessel. There are three phases involved in haemostasis process. Firstly is the vasoconstriction, followed by formation of platelet plug and finally involves formation of fibrin clot (Portou *et al.*, 2015).

Vasoconstriction is the narrowing of blood vessels which reduced the blood flow at the injured vessel, thus reducing the blood loss. This process is triggered by various chemical signal released by injured endothelial cell and platelets. A glycoprotein known as Von Willebrand factor present in plasma will stimulate the formation of platelet plugs. Circulating platelets adhere to the injured vessels to form a temporary platelet plug and then degranulate and released more chemical signals such as adenosine diphosphate (ADP), serotonin and thromboxane A2. Further released of these signals causing more platelet to stick to the injured vessels and released more chemical signal to form a stable platelet plug. Once the platelet plug was formed, the clotting factor presence in plasma will converted fibrinogen to fibrin to form a collagen fiber that cover and replace the platelet plug forming the stable blood clot (Golebiewska & Poole, 2014).

1.4.2 *Inflammation*

Inflammation was the response of tissues to an injury. It is a protective response that involves immune cells, blood vessels and cell mediators. The main purpose is to eliminate the cause of injury such as bacteria or virus, remove dead or injured cell by phagocytosis and initiate tissues repair. The classical symptoms of acute inflammation

are pain (*dolor*), heat (*calor*), redness (*rubor*), swelling (*tumor*) and loss of function (*functiolaesa*) (Ricciotti& FitzGerald, 2011). The endothelial cells released of chemokines and signalling message from pain receptors at the wound bed initiate the inflammation response. The acute phase of inflammation start with the infiltration of leucocytes predominantly by neutrophils, also known as polymorphonuclear cells (PMNs) followed by monocytes that mature or transform into inflammatory macrophages. Release of cytokines from the inflammatory cells induces proliferation and recruitment of more inflammatory cells to the infected area causing the sign of classical symptoms of inflammation. When the inflammation has been resolved, the granulocyte mainly neutrophils were eliminated and macrophages and lymphocytes return to its normal count and functions. Failure of acute inflammation to resolve may lead to complication such as autoimmunity, chronic dysplastic inflammation and excessive tissue damage (Mohammed *et al.*, 2014; Nathan, 2002).

1.4.3 *Proliferation*

The proliferation stage starts when the haemostasis has been achieved and the inflammation process was balanced. This process start to repaired the damage at the affected areas by complex processes that occur simultaneously which incorporate angiogenesis, the formation of granulation tissue, collagen deposition, epithelialization and wound retraction. Angiogenesis is a process of forming new blood vessels at the avascular area of the wound. This involved neovascularization and repair of damaged blood vessels which was triggered by release of vascular endothelial growth factor (VEGF) and other cytokines by macrophages or monocytes. Macrophage has long been recognized as critical regulators of angiogenesis (Khan *et al.*, 2013).

The remodelling of the extracellular matrix (ECM) and new capillaries network was formed during the angiogenesis process. However, the new capillary vessels are still weak and permeable which prone to cause tissue oedema. At the same time, fibroblasts migrated to the wound area and release extracellular matrix protein to produce collagen and fibronectin. Granulation tissue was formed when fibrous tissues replace the clot at the affected area. These tissues connect with the surrounding collagen and fibronectin and assist in wound contraction. Epithelialization started when the wound trigger the embryonic process which allows the epithelial cells to migrate from the edge of the wound to the wound surface. The epithelial cells then attached to the matrix below and start proliferation process in order to repopulate the wound area with new epithelial cells and complete the wound repair. Wound retractions usually begin at about seven days after injury. The wound contracted due to interaction between actin and myosin which pull the cell bodies closer together to decrease the size of the wound (Harper, Young & McNaught, 2014).

1.4.4 *Tissue remodelling*

The final stage of wound healing results in development of normal epithelium and maturation of the scar tissue. This process begins at about 2 to 3 weeks and can take up to 2 years to regain the structure similar to the normal tissue. This phase is characterised by reduced proliferation and inflammation, active re-organization of the ECM, reduction in the vascularity and the scar change color from red to pink to grey with time as the scar matured (Portou *et al*., 2015).

1.5 Involvement of white blood cells in the healing of skin flap

Khan and colleagues (2013) recently reported that neutrophils are the first WBCs to be recruited to the wound bed within 2 to 3 hours of injury and the count of neutrophils at the injury site reaches the highest peak at 24 to 48 hours. Besides the neutrophils, monocytes are also reported as playing an active role during the initial inflammatory phase of tissue injury. The monocytes are converted to macrophages around three to five days after the initial injury. Both of these cells exert proinflammatory functions, such as antigen presentation, phagocytosis and production of inflammatory cytokine along with growth factor at the early inflammatory phase (Khan *et al.*, 2013). During acute inflammation phase, circulating lymphocytes are activated and differentiated into T and B cells. The lymphocytes eventually produce antigen recognition receptor. The lymphocytes may mature into memory cells for further action in the chronic phase of inflammation if the invading pathogen still present. The eosinophils and basophils are usually involved in allergic reaction and these cells might get activated if there is presence of allergen on the wound area.

2.0 STUDY OBJECTIVES AND HYPOTHESES

2.1 General objective

To investigate the association between WBC count and autologous skin flap healing rate in rabbits.

2.2 Specific objectives

a) To demonstrate the pattern of total WBC count changes during healing of autologous skin flap in rabbits.

b) To demonstrate the changes of WBC differential count during healing of autologous skin flap in rabbits.

c) To demonstrate the changes of skin necrosis during healing of autologous skin flap in rabbits.

d) To determine the association between WBC count and skin necrosis during healing of autologous skin flap in rabbits.

2.3 Research hypotheses

Null hypothesis (H_O)

There is no association between white blood cells count with autologous skin flap healing rate in rabbits.

Alternative hypothesis (H_A)

There is an association between white blood cells count with autologous skin flap healing rate in rabbits.

3.0 MATERIALS AND METHODS

This study was conducted following the animal ethics guidelines and approved by Animal Ethics Committee, Universiti Sains Malaysia (AECUSM). The flow chart of the study protocol was shown by Figure 3.1.

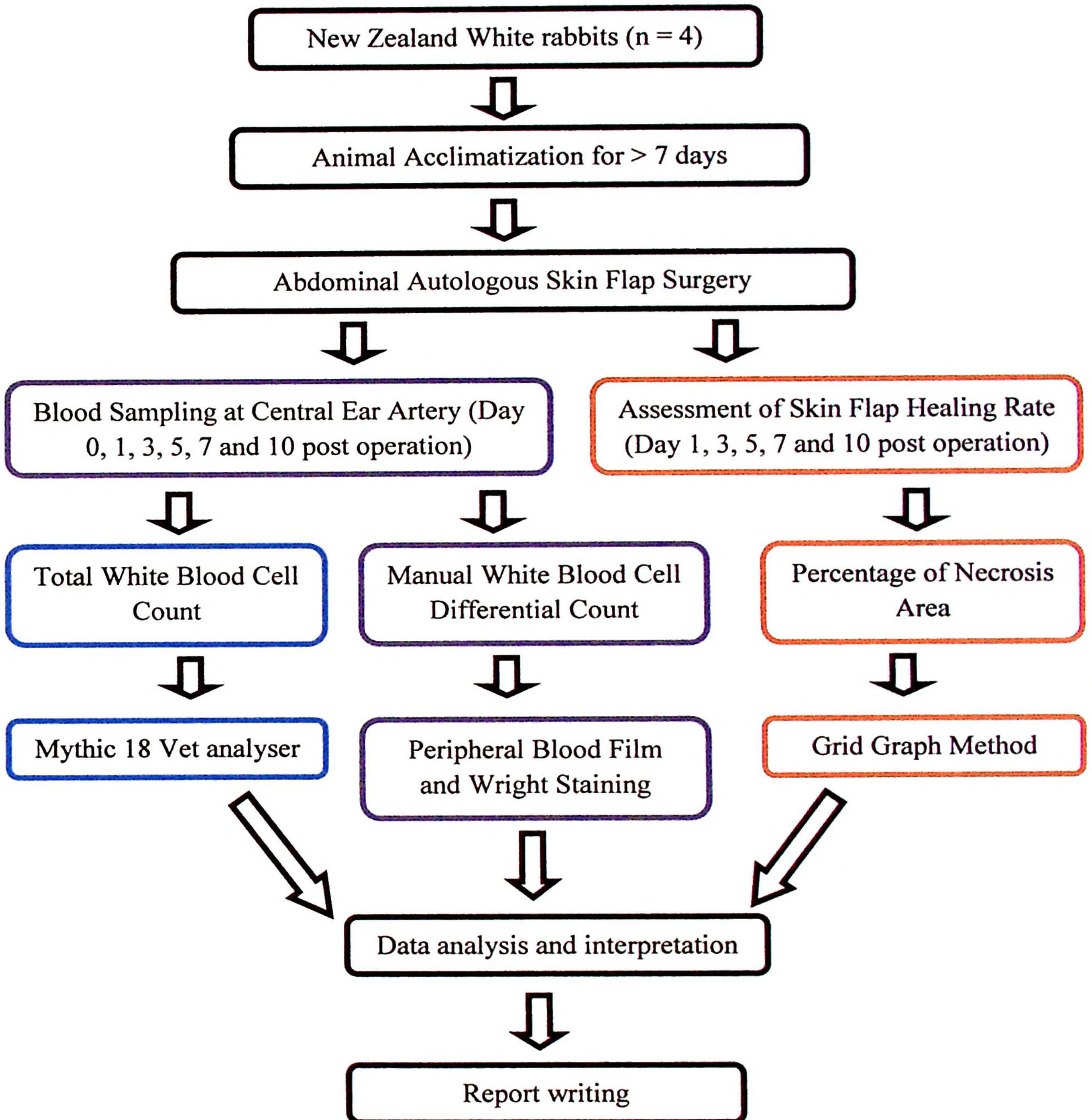

Figure 3.1: Flow chart of study protocol.

3.1 The animals

A total of four healthy New Zealand White (NZW) rabbits (*Oryctolagus cuniculus*), two male and two female with the mean body weight of 2.65 kilogram (2.0 to 3.3 kilogram) and aged between 9 to 15 months were used as an experimental animals. The rabbits were chosen for this study due to the size of the skin area that allows for easy observation and bigger flap size. In comparison to rodents, the rabbits are easier to handle and frequent blood collection with bigger volume is feasible in rabbits (Joslin, 2009). Both male and female rabbits were used in this study because there was no significant difference in the WBC count between male and female rabbits as reported by Milas *et al.* (2009).

The animals were housed individually and were acclimatized for at least seven days before surgical procedures. The acclimatization procedure is important to familiarize the animals to the experimental conditions and prevent stress to the animals which can greatly affect the WBC count results (Joslin, 2009). Commercialized food pellet and tap water were given ad-libitum during the whole study duration. Food pellet was given to the animals to prevent selective feeding (Meredith & Flecknell, 2006).

3.2 Blood sampling

At each sampling time, the blood collection was done right after the rabbit was anaesthetized prior to surgery at as baseline and on subsequent days at day 1, 3, 5, 7 and 10 post-operation. The rabbit ear area was shaved and cleaned with 70 % alcohol and 3 millilitre of blood were collected from the central ear artery (Figure 3.2) using 25-gauge needles. The blood samples were collected using K2EDTA container (Phillips, Coiner, Smith, Becker & Leong, 1998) and stored in transportation box with ice, where the samples were brought to the Hematology Laboratory of Faculty of Veterinary Medicine, Universiti Malaysia Kelantan (UMK) and tested for FBC using Mythic 18 Vet (Orphee SA, France).

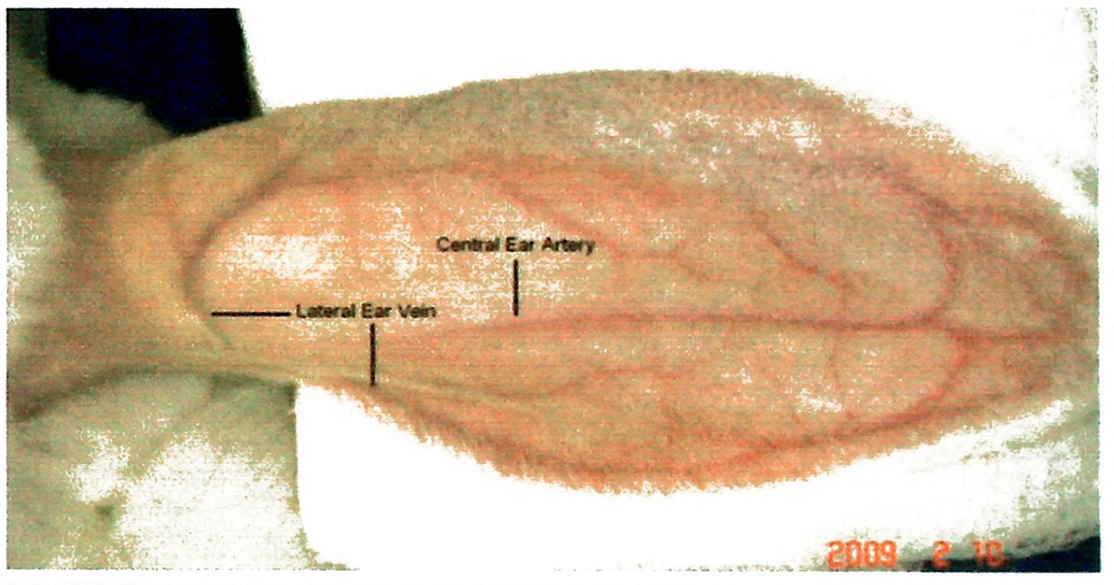

Figure 3.2: Rabbit ear with central ear artery and lateral ear vein identified (Joslin, 2009).

3.3 Preparation for skin flap surgery

The rabbit was weighted and anaesthetized with 5 mg/kg xylazine and 45 mg/kg ketamine mixture intramuscularly. The abdominal areas were shaved using electrical clipper from the lower chest to the inguinal folds (Figure 3.3). The anaesthetized rabbit were placed in the dorsal recumbent position with all four limbs facing upwards on the operating table. The abdominal area was marked with marker pen from midline to the left side of the rabbit abdominal skin to create a left paramedian flap with the size of 14 centimeters in length and 5 centimeters in width (Figure 3.4). A well fitted face mask was placed on the mouth and nosed of the rabbit to deliver isofluorane gas for maintenance of anaesthesia during operating procedure. An intravenous line was set up and 12 ml/kg/hour of normal saline were administered through the marginal ear vein. Pulse oxymeter was placed on the rabbit ear vein to monitor the heart rate and oxygen saturation (Figure 3.5). The ventral abdominal skin of the rabbit was prepared with povidone iodine spray and draped with a sterile draping paper sheet and aseptic techniques were applied throughout the surgery procedure.

Figure 3.3: Anaesthetic drugs (ketamine and zylazine) that were given to the rabbit before surgical flap procedure.

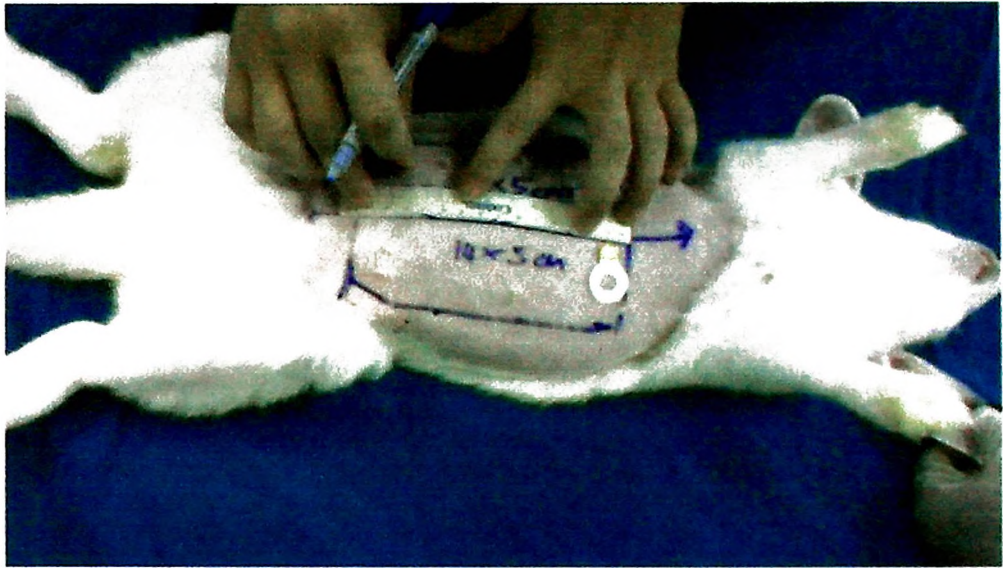

Figure 3.4: The ventral abdominal areas of the rabbit were marked with marker pen to create a left paramedian flap of 14x5cm in length and width.

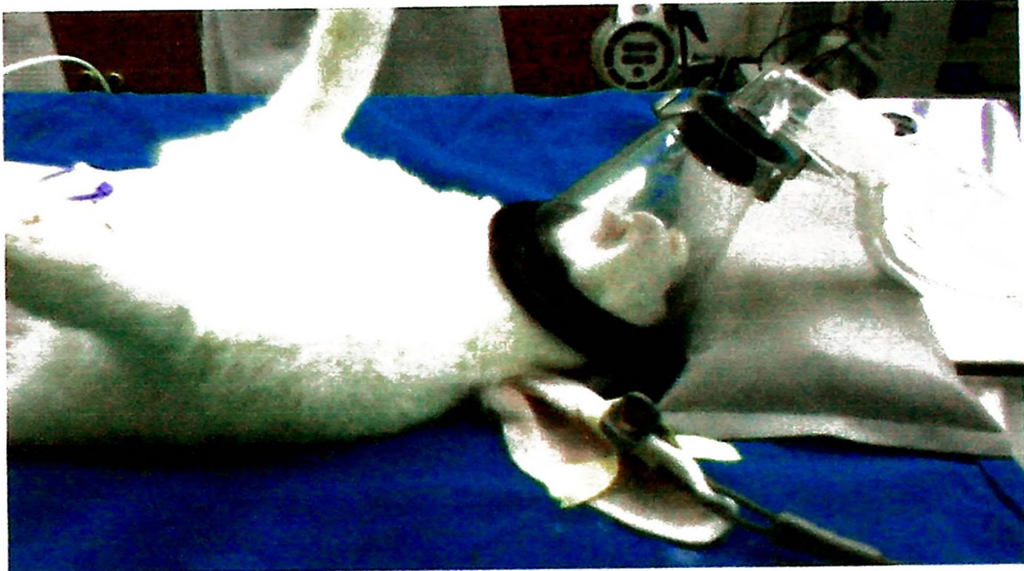

Figure 3.5: Pulse oxymeter and face mask were placed on the rabbit to monitor oxygen saturation level and maintenance of anaesthesia.

3.4 Autologous skin flap surgery

The autologous skin flap surgery was conducted by a qualified plastic and reconstructive surgeon. The ventral abdomen of the New Zealand White rabbits was dissected under surgical loupe with 2.5 magnifications and using microsurgical instruments (Figure 3.6). The incisions were made through the midline of the abdominal skin, thin layer of subcutaneous tissue and the panniculus carnosus muscle. The incision was then made on the superior margin and the lateral margin of the flap. The superficial inferior epigastric vessel system was identified along with the superficial circumflex iliac and thoracodorsal vessels. The superficial external pudendal vessels were also identified during the incisions. The continuation or anastomoses of the superficial inferior epigastric (SIE) vessels with the lateral thoracic vessel, the superficial circumflex iliac and the external pudendal was then ligated using an electronic vessel sealer. The single pedicle flap was raised caudally to reveal the vascular anatomy of superficial inferior epigastric vessels which originated from the femoral vessels and incisions was carefully made at the inferior margin of the flap and only left with one blood vessel supply from the superficial inferior epigastric vessels. The flap consisted of skin, subcutaneous tissue, panniculus carnosus muscle and deep fascia.

The flap was then repositioned and sutured back to the original position with 4/0 absorbable polyglycolic acid sutures at the midline margin, superior margin and lateral margin. The SIE vessels at the inferior margin were then clamped for 2 hours using microvascular clamps (Figure 3.7) to mimic the ischemic period of free skin flap transfer. After 2 hours, the clamps were removed and the flap at the inferior margin was sutured back to the original position. The sutured area was then cleaned with 70% alcohol and covered with sterile gauze. Bandage was placed around the body and the

front foot of the rabbits to protect the abdominal area (Figures3.8). Antimicrobial with dose of 10mg/10kg enrofloxacin was administered intramuscularly after completion of the surgical procedure and was given for three consecutive days.