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EFFECTS OF WHOLE BODY VIBRATION ON THE TREATMENT OF PRESSURE ULCERS IN MICE

Miss Nattaya Wano

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
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Thesis Title	EFFECTS OF WHOLE BODY VIBRATION ON THE TREATMENT OF PRESSURE ULCERS IN MICE
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ณัฐญา วาโน : ผลของการสั่นสะเทือนทั้งร่างกายต่อการรักษาแผลกดทับในหนูเม้าส์ (EFFECTS OF WHOLE BODY VIBRATION ON THE TREATMENT OF PRESSURE ULCERS IN MICE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. พญ.จุไรพร สมบุญวงค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. นพ.สมพล สงวนรังศิริกุล, 73 หน้า.

การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของการสั่นสะเทือนทั้งร่างกายต่อการสมานแผลกดทับในหนูเม้าส์ แผลกดทับระดับ 2 ถูกเหนี่ยวนำด้วยการบาดเจ็บแบบ Ischemia-reperfusion โดยการใช้แผ่นแม่เหล็ก 2 แผ่นประกบที่ผิวหนังด้านหลังของหนู จากนั้นแบ่งหนูสายพันธุ์ไอซีอาร์โดยการสุ่มออกเป็น 2 กลุ่มๆ ละ 16 ตัวได้แก่ 1) กลุ่มที่ไม่ได้รับการสั่นสะเทือนทั้งร่างกาย และ 2) กลุ่มที่ได้รับการสั่นสะเทือนทั้งร่างกาย โดยแต่ละกลุ่มแบ่งเป็น 2 กลุ่มย่อยเพื่อศึกษาผลการทดลองในวันที่ 7 และ 14 โปรแกรมการสั่นสะเทือนทั้งร่างกายอาศัยเครื่องสั่นด้วยความถี่ 45 เฮิร์ต 0.4 จี ครั้งละ 30 นาทีต่อวัน ต่อเนื่องกัน 5 วันต่อสัปดาห์ หลังสิ้นสุดการทดลองศึกษาอัตราการสมานแผล ศึกษาปริมาณคอลลาเจนในแผลวิเคราะห์ด้วย image analysis software หลังการย้อมแมสซองไตรโครม ระดับ TNF-α และ VEGF ในเนื้อเยื่อแผลศึกษาด้วยวิธี ELISA ศึกษาจุลพยาธิวิทยาของแผล และจำนวนนิวโทรฟิลในแผลด้วยการย้อมมาทอกซีลินและอีโอซิน ผลการทดลองพบว่า ในวันที่ 7 หนูกลุ่มที่ได้รับการสั่นสะเทือนทั้งร่างกายมีระดับ TNF-α ในเนื้อเยื่อแผล และจำนวนนิวโทรฟิลในแผลต่ำกว่ากลุ่มที่ไม่ได้รับการสั่นอย่างมีนัยสำคัญทางสถิติ แต่ไม่พบความแตกต่างของอัตราการสมานแผล ระดับคอลลาเจน และระดับ VEGF ในเนื้อเยื่อแผลเมื่อเทียบกับกลุ่มที่ไม่ได้รับการสั่น ในวันที่ 14 หนูกลุ่มที่ได้รับการสั่นสะเทือนทั้งร่างกายมีอัตราการสมานแผลและคอลลาเจนสูงกว่ากลุ่มที่ไม่ได้รับการสั่นและมีระดับ TNF-α ในเนื้อเยื่อแผล และจำนวนนิวโทรฟิลในแผลต่ำกว่ากลุ่มที่ไม่ได้รับการสั่นอย่างมีนัยสำคัญทางสถิติ แต่ไม่พบความแตกต่างของระดับ VEGF ในเนื้อเยื่อแผลเมื่อเทียบกับกลุ่มที่ไม่ได้รับการสั่น ผลการศึกษาจุลพยาธิวิทยาพบว่ากลุ่มที่ได้รับการสั่นมีกลุ่มเซลล์อักเสบลดลงในวันที่ 7 และเพิ่มการเกิด epithelialization ในวันที่ 14 เมื่อเทียบกับกลุ่มที่ไม่ได้รับการสั่น สรุปได้ว่าการสั่นสะเทือนทั้งร่างกายสามารถเร่งการสมานแผล เพิ่มระดับคอลลาเจนและ epithelialization รวมทั้งลดการอักเสบของแผลในโมเดลแผลกดทับในหนูทดลอง

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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NATTAYA WANO: EFFECTS OF WHOLE BODY VIBRATION ON THE TREATMENT OF PRESSURE ULCERS IN MICE. ADVISOR: ASSOC. PROF. JURAIPORN SOMBOONWONG, M.D., CO-ADVISOR: ASSOC. PROF. SOMPOL SAGUANRUNGSIRIKUL, M.D., 73 pp.

This study aimed to examine the effects of whole body vibration (WBV) on the healing of pressure ulcers (PrUs) in mice. Stage II PrUs were induced by ischemia-reperfusion injury using 2 cycles of external application of two magnet plates. Male Imprinting control region mice were randomly divided into two groups (n=16 each): untreated control and WBV-treated groups. Each group was subdivided into 2 subgroups for examination on days 7 and 14. WBV was performed using vibrator (frequency 45 Hz, peak acceleration 0.4 g) 30 min/day, 5 consecutive days/week. At the end of the study, wound closure rate was measured. Tissue collagen contents were analyzed by image analysis software in Masson's trichrome stained sections. Tissue TNF-a and VEGF were assayed using ELISA method. Skin histopathology and the amount of neutrophil infiltration were determined in hematoxylin & eosin stained sections. The results showed that on day 7, WBV-treated group exhibited a significant decrease in tissue TNF-a and neutrophil infiltration without any differences in wound closure rate, tissue VEGF and collagen deposition compared to control group. On day 14, WBV-treated group had a significant increase in wound closure rate and collagen deposition, and a decrease in tissue TNF-a and neutrophil infiltration, but tissue VEGF did not differ compared to the untreated controls. Histopathology of the ulcers in WBV-treated group revealed decreased inflammatory cells on day 7, and increased epithelialization on day 14 compared to controls. In conclusion, WBV facilitates the healing, increases collagen deposition and epithelialization and decreases wound inflammation in mice model of PrUs.

Field of Study: Medical Science
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

PrUs	Pressure ulcers
I/R injury	ischemia-reperfusion injury
WBV	Whole body vibration
IL-1β	Interleukin- 1β
IL-10	Interleukin- 10
TNF-α	Tumor necrosis factor-α
IL-6	Interleukin- 6
ICAM-1	Intercellular adhesion molecule- 1
VCAM-1	Vascular cell adhesion molecule- 1
ROS	Reactive oxygen species
TGF-β	Transforming growth factor-β
VEGF	Vascular endothelial growth factor
IGF-1	Insulin-like growth factor 1
CNS	Central nervous system
PNS	Peripheral nervous system
MMP	Matrix metallopeptidase
MMP-9	Matrix metallopeptidase-9
Cu	Copper
H&E	Hematoxylin- eosin
ICR	Imprinting control region
Hz	Hertz
HIF1α	Hypoxia-inducible factor 1α

NO	Nitric oxide
eNOS	Endothelial nitric oxide synthase
GH	Growth hormone
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
%CD	Percentage of collagen deposition
ELISA	Enzyme-linked immunosorbent assay
LIV	Low-intensity vibration

CHAPTER I

INTRODUCTION

Background and Rationale

Nowadays, many people worldwide are suffering from health problems caused by sedentary lifestyle and consumption of high energy diets or junk food. These factors increase the risk of many diseases such as cerebrovascular disease or stroke. A number of stroke patients mostly lack the ability to move and have a loss of body sensation as a result of brain damage. Thus, these patients are at high risk of developing several complications while bedridden for a long time. One of the most common complications of immobility is pressure ulcers (PrUs) or bed sore. In Thailand, PrUs usually occurs in patients with stroke, the incidence of which is about 1.7 – 47.6%[1, 2] and older patients tend to develop PrUs more than younger patients. Additionally, in the *United States*, the incidence of PrUs in patients admitted to Intensive Care Unit is 8.8 – 12.1%[3] and 2- 2.8% of the patients have PrUs after returning home(4). If not properly prevented and treated, PrUs can cause chronic skin damage, risk of wound infection, high mortality and high cost of medical care.

PrUs are localized injuries to the skin that usually occur over a bony prominence resulting from pressure and/or shear to the skin. PrUs are divided into four stages, depending on the depth of skin injuries(5). In stage I, the skin is intact and the redness of skin dose not fade when pressed (non-blanched). In stage II, there is a partial skin loss with shallow wounds, red skin, edema and blister formation. In stage III, skin damage extends into subcutaneous fat layer. Finally, at stage IV the sores extend into muscle, ligament, and bone[5]. The mechanism of skin injury involves ischemia-reperfusion injury (I/R injury), inducing the production of free radical and inflammatory mediators[6, 7]. In turn, the inflammatory mediators stimulate inflammatory cells to attach to vascular endothelium[8]. Histophathology of PrUs reveals microvascular occlusion by red blood cells, platelets, and fibrins, which leads to poor blood supply to the wound area resulting in delayed healing process. Delayed wound healing is also attributed to compression of the wounds. With

prompt treatment, stage I PrUs can be cured within five to ten days[9]. In the event that skin and small vessels are destroyed, the ulcers are susceptible to get infected and may progress into more severe stages easily. The prevention and treatment of PrUs include dressing, positioning, and antibiotics[10]. However, these modalities cannot decrease any inflammatory cytokines nor increase blood circulation to the PrUs area; both of which play an important role in wound healing process.

From the above data, it is interesting to find out other approaches to prevent and treat PrUs. It has been demonstrated that a single bout of local vibration can increase blood flow to skin and muscle. In 2005, Stewart and colleagues reported that a single bout of plantar vibration (frequency 45 Hz, acceleration 0.2 G, and time 15 min) in perimenopausal women immediately increased blood flow to calf, pelvic region, and thoracic region[11]. In 2007, Lohman and colleagues found that local vibration (frequency 30 Hz, acceleration 7 G, amplitude 5-6 mm, time 1 min, and 3 times) on the calf of healthy volunteers was able to increase blood circulation to calf skin immediately after WBV and continually increase over time of ten minutes[12]. In 2008, Maloncy-Hinds and colleagues found an increase in arm blood flow as measured at four and nine minutes after a single-bout vibration on the arm (frequency 30 and 50 Hz and time 5 min)[13].

Moreover, low-intensity whole body vibration (WBV) has been reported to accelerate the healing of stage I PrUs in patients and full excision wound in diabetic animals. According to Arachi and colleagues, vibration therapy (frequency 47 Hz, peak acceleration 1.78 m/s^2, time 15 min/times ,and 3 times/day) in older adult patients with stage I PrUs can increased the number of healed wounds as indicated by disappearance of red skin[9]. From the study by Weinheimer-Haus and colleagues, low-intensity WBV (frequency 45 Hz, acceleration 0.4 G, time 30 min/day, 5 days/week x2 weeks) was able to improve wound angiogenesis and the healing of full excision wound in diabetic mice. After 7 days of wound infection, WBV increased the levels of pro-healing insulin growth factor-1 (IGF-1) and pro-angiogenic endothelial growth factor (VEGF) in the wound, while decreasing inflammatory cytokine mRNA expression of tumor necrosis factor-alpha (TNF-α) in the wound

tissues[14]. After 14 days of wound infection, WBV increased the percentage of wound closure and macrophages in wound area.

As mentioned above, WBV treatment is effective for stage I PrUs. In addition, low-intensity WBV can accelerate wound healing, stimulate angiogenesis, and decrease wound inflammation in diabetic mice that were induced full excision wound. However, effects of WBV on PrUs and its underlying mechanisms have not been clearly understood.

Therefore, it is interesting to investigate whether low-intensity WBV can accelerate wound healing through a decrease in the levels of tissue TNF-α, neutrophil infiltration, and an increase in the levels of tissue VEGF and collagen deposition in pressure ulcer model in animals.

Research Question

Does WBV accelerate wound healing in pressure ulcer model in mice?

Research Objective

To examine the effect of whole body vibration on wound healing, the levels of tissue TNF-α and VEGF, neutrophil infiltration, and collagen deposition in pressure ulcers model in mice.

Hypothesis

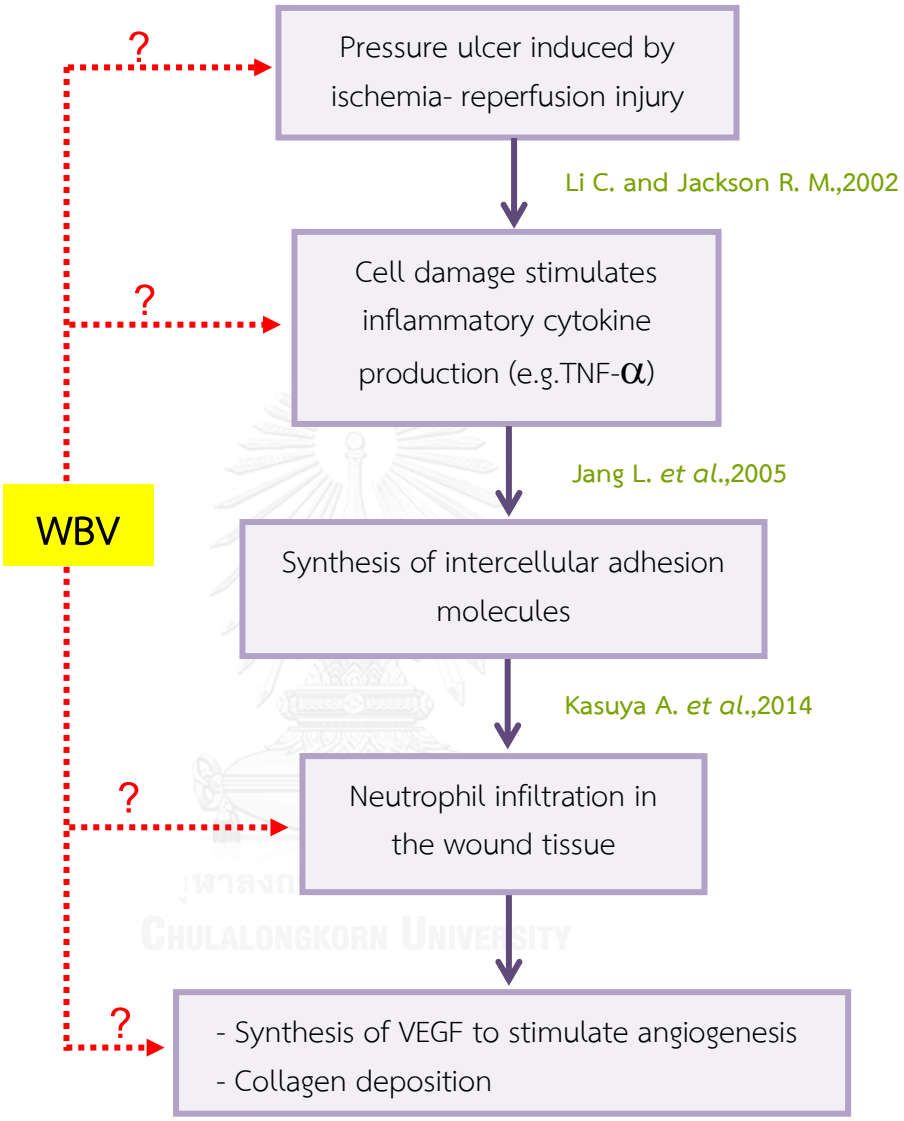
WBV can accelerate wound healing through a decrease in the levels of tissue TNF-α, neutrophil infiltration, as well as an increase in the levels of tissue VEGF and collagen deposition in pressure ulcer model in mice.

Expected Benefits and Application

The results obtained will give an understanding about the effects of whole body vibration on treatment of pressure ulcers as well as its mechanisms via modulation of pro-inflammatory cytokine TNF-α, VEGF, neutrophil infiltration, and collagen deposition.

This information will provide a basis for further studies and clinical applications for treatment in patients with pressure ulcers.

Conceptual framework



CHAPTER II

LITERATURE REVIEW

Definition and classification of pressure ulcers

Definition

Pressure ulcers (PrUs) are localized injuries to the skin resulting from compression and shearing stress. PrUs usually occur over bony prominences such as sacrum, spine, greater trochanter, sole and lateral malleolus[15].

Classification

Pressure ulcers can be classified, depending on the depth of skin injury, into four stages[5, 8] as follows (figure 2-1):

1. Stage I PrUs

The damaged area is red and edematous without skin loss. The histopathology shows infiltration of inflammatory cells and fibroblasts at the ulcer edge, along with occlusion of many large blood vessels near the surface.

2. Stage II PrUs

There is a loss of epidermal and dermal layers of skin and the damaged area may extend to the upper layer of subcutaneous fat. Blistering also occurs accompanying with inflammation that leads to soreness, edema, redness and warmness. Histopathological finding of the ulcer reveals a dense fibrin lattice interspersed with neutrophils and macrophages. Moreover, there are dense collagen bundles in interstitial matrix of ulcer.

3. Stage III PrUs

In this stage, the area of damage extends to subcutaneous fat layer, but muscle and bone remain normal. Severe inflammation takes place at and around the

wound site. According to histopathological study, the ulcer edge is composed of dense fibrinous regions (40-60% of fibrin) interspersed with inflammatory cells.

4. Stage VI PrUs

The damage further extends into ligament, muscle and bone and the ulcer is further enlarged. Osteomyelitis may be found in this stage. Histopathology shows thin ulcer edge, fat droplets in granulation tissue, fibroblasts cells, and inflammatory cells in the subcutaneous fat area.

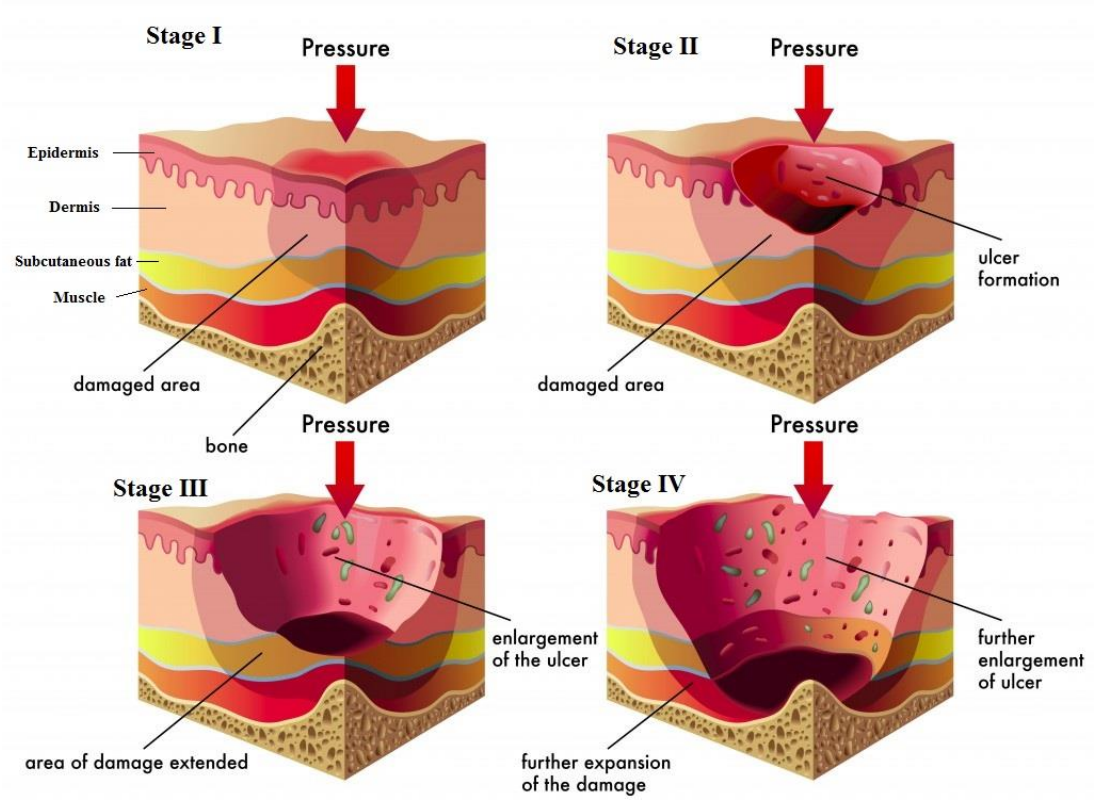


Figure 2- 1 The staging of pressure ulcer[16]

Pathophysiology of pressure ulcer

PrUs are injures to the skin caused by repeated I/R injury.

Figure 2-2 illustrates the pathophysiology of PrUs during prolonged period of skin compression and/or compression with pressure of greater than 4.3 kilopascals leading to damage the microcirculation of skin. These mechanical insults leads to a

loss of blood supply to skin tissue. As a result of ischemia, there is a lack of oxygen and energy used for cellular biological *processes,* particularly protein synthesis that plays an important role in repair process after tissue injury. In addition, heavy, prolonged skin compression causes accumulation of metabolic waste products[17] that damage normal cells and tissues[15, 18-21].

Reperfusion process occurs after mobilization or positioning, which results in a rapid return of blood circulation into the affected area. The wounded tissues use energy through an aerobic metabolism process again. Subsequently, high amount of free radicals are generated in the cells, which leads to a decrease in the levels of antioxidant and, hence oxidative stress[22]. This causes a damage of many cell structures such as DNA, and protein and lipid (caused by protein and lipid peroxidation). Moreover, the damaged cells are stimulated to produce inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6)[6, 7, 23] These cytokines induce the expression on vascular endothelium of adhesion molecules such as intercellular adhesion molecules (ICAM-1), vascular cell adhesion molecules (VCAM-1)[24]. These adhesion molecules also stimulate the infiltration of neutrophils and macrophages at the wound sites. Repeated I/R injury of PrUs induce chronic wound inflammation and microvascular occlusion with red blood cells, platelets, and fibrins. These conditions lead to a decreased blood flow in wound area, resulting in delayed wound healing process in PrUs patients. Most of the patients with PrUs lack the ability to move and are susceptible to repeated compression or during friction[15]. Therefore, appropriate care is needed in order to prevent progressive severity of ulcer.

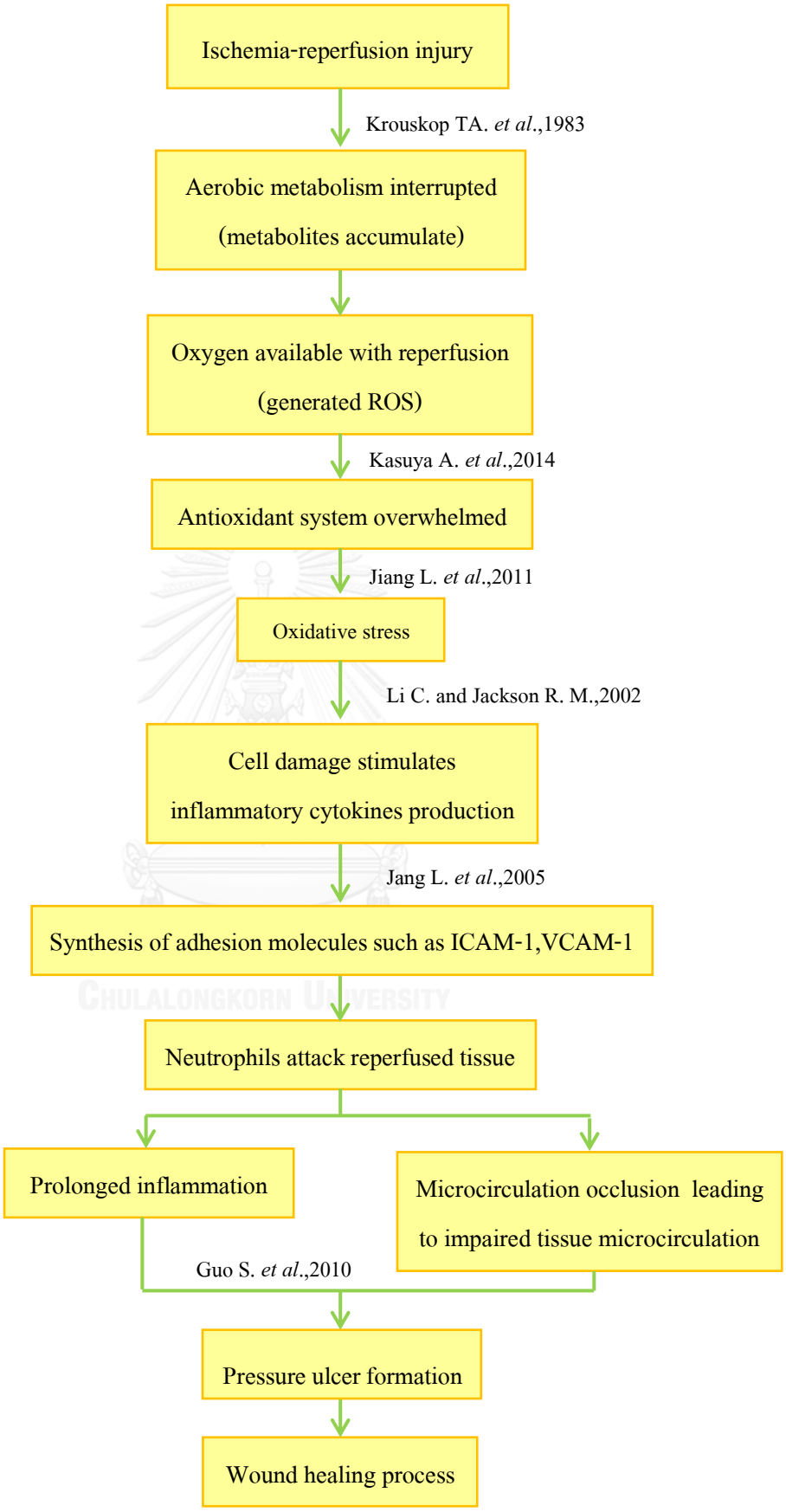


Figure 2- 2 The pathophysiology of pressure ulcer

Healing process[25]

Healing process is defined as an intricate *process* of the body that repairs itself after skin or tissue injury. The healing process is divided into three phases: inflammatory phase, proliferative phase and remodeling phase.

1. Inflammatory phase

This phase lasts three to five days after injury. A number of macrophages in the wound produce inflammatory cytokines such as TNF-α, IL-1β, IL-6, and other cytokines, induce vasodilatation and creating soreness, redness, and warmness. Besides, these cytokines stimulate the production of adhesion molecules that induce the chemotaxis of inflammatory cells (e.g. neutrophils, macrophages) to the wound sites. The inflammatory cytokines can induce wound edema because of increased vascular permeability.

Prolonged inflammation of the wound will lead to delayed healing process such as those occurred in diabetic condition and repeated I/R injury induced PrUs.

2. Proliferative phase

This phase begins on day five to seven after injury and is characterized by the formation of granulation tissue consisting of new blood vessels and collagen deposition. Wound macrophages produce many growth factors such as VEGF and TGFβ. VEGF increases new capillary formation that help to supply adequate nutrients needed for repair process. Moreover, the growth factors stimulate re-epithelialization on surface layer of skin. Furthermore, fibroblasts are stimulated to migrate into the wound and synthesize extracellular matrix, the major of which is collagen.

Efficient angiogenesis and collagen deposition are required for rapidly healed wounds.

3. Remodeling phase

This phase lasts several weeks to a year. Myofibroblasts differentiation from fibroblasts is a critical component of the healing process for decreasing wound size.

Additionally, there are many matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases enzymes synthesize and degrade collagen, respectively, which for balance collagen deposition and increase wound strength.

Therapeutic principles of pressure ulcer

Generally, treatment of pressure ulcer can be divided into two parts: systemic treatment and local treatment.

1. Systemic treatment

Systemic treatment is aimed at correcting the internal body conditions that affect wound healing process. For example, good nutrition should be provided according to nutrition guideline: Some diseases/ conditions such as anemia should be treated, if any.

2. Local treatment

Local treatment involves localized methods that prevent the progression of PrUs severity as described below.

2.1 Positioning

Patient position be changed every hour to reduce external forces to ensure sufficient blood supply to the wound site**.**

2.2 Debridement and dressing

The wounds should be taken care appropriately with debridement to reduce interruption of wound healing. Furthermore, dressing is a standard strategy that uses normal saline for cleaning wounds every day to keep normal healing process.

2.3 Other modalities

There are several studies to develop of treatment modalities of PrUs such as air-fluidized bed, electrical stimulation, and skin graft surgery[10].

Whole body vibration (WBV)

WBV is an oscillatory mechanical stimulation using vibration generator that consists of parameters setting and platform. The parameter setting includes setting of frequency, amplitude, peak acceleration, and duration. Moreover, the platform is divided into two forms of motion: vertical motion and horizontal motion as shown in figure 2-3.

The common frequency ranges are 20-50 Hz. Furthermore, squatting on the vibrator is a common position in human subject because this position can decrease transmitted forces to head, neck and jaw. If there are a lot of forces in those areas, subject will felt uncomfortable and dizzy. Therefore, standing in a squat position is commonly used in whole body vibration exercise. On the other hand, in *vivo* study in animals such as rodent, anatomical position in a standing position does not produce any forces transmitted to head and other structures because rodent stands parallel to the ground. In addition, researchers cannot force rodent to stand like the human. Thus, vibration while animal stands relaxed is called whole body vibration[26].

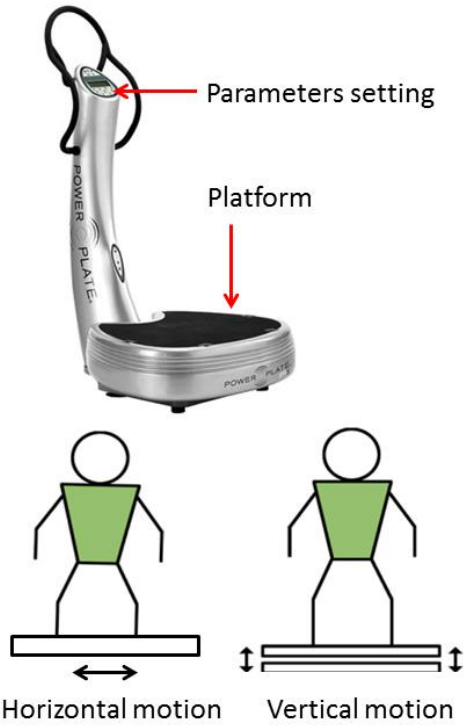


Figure 2- 3 The vibrator and motion of platform[27]

The action mechanisms of vibration on neuromuscular system are schematized in figure 2-4. **V**ibration causes to change in length of muscle- tendon complex which can stimulate reflex muscle contraction is called tonic vibration reflex[28].Moreover, vibration produces deformation of the soft tissue which leads to activation of muscle spindles and enhances stretch-reflex loop. These effects can stimulate α-motor neuron and increase synchronization of motor units, leading to increased neuromuscular performance and electromyography during vibration[29, 30]. Moreover, vibration wave from WBV can activate higher centers through primary and secondary endings of muscle spindles and γ-motor neuron[31]. The primary-secondary somatosensory cortex and supplementary motor area in central nervous system are higher centers that play an important role in control and planning of pattern of motion before body movement[31, 32]. However, effects of vibration depend on parameters setting[33]. The difference effects of parameters are not well understood and still needs further investigations.

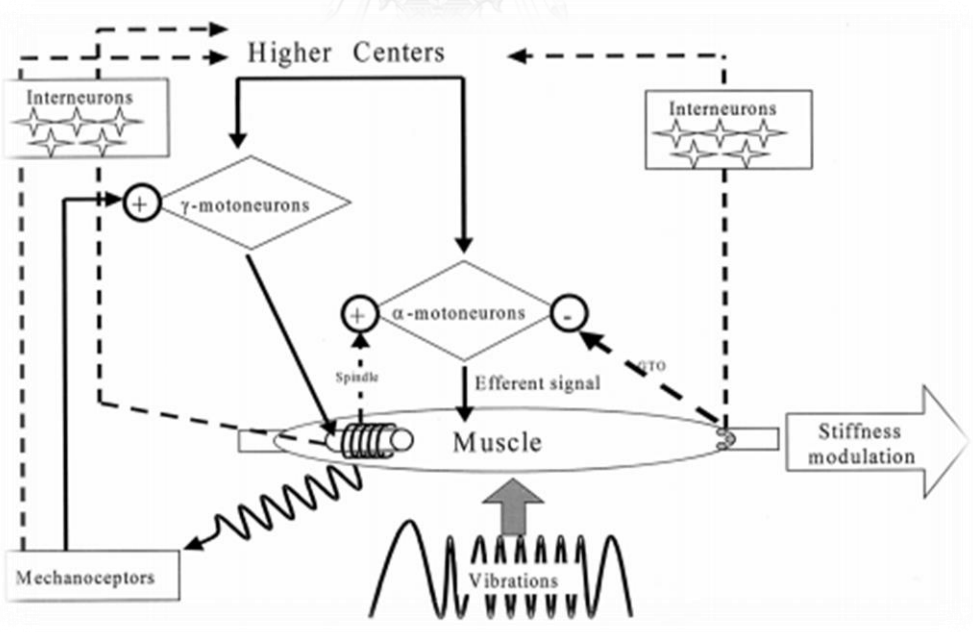


Figure 2- 4 The action mechanism of vibration on neuromuscular system[31]

The benefits of whole body vibration

Vibration has long been used as a therapeutic measure since Greek and Roman periods. During the nineteenth century, vibrator was used to activate and rehabilitate the body[34]. Later, Russian scientists Nazarov and Spivak were the first to use whole body vibrator for athletic training. They found that WBV increased muscle strength and flexibility in athletes. Thus, exploring the effects of WBV is of interest and whole body vibrator began to be used as exercise equipment[35, 36].

A number of research evidence of the effects of WBV on body systems is as follows

1. Musculoskeletal system

WBV is well-recognized among athletes for improving their performance. Studies have shown that WBV facilitates musculoskeletal growth.

In 2006, Fagnani and colleagues found that WBV with squatting (vertical motion of platform, frequency 35 Hz, amplitude 4 mm, 3 times/wk X8 weeks) increased the strength of quadriceps femoris muscle, jumping ability, and muscle flexibility in female athletes as assessed by sit- and- reach test[37].

In 2013, Komrakova and colleagues demonstrated that WBV with vertical motion of platform (frequency 30 and 50 Hz) increased density and size of cortical and callus bone by stimulating osteocalcin secretion, and increased total muscle weight in ovariectomized rats[38].

2. Nervous system

Previous studies demonstrated that whole body vibrators were applied for improvement of neuromuscular performances in neurovascular patients who lacked ability of movement.

In 2013, Wirth and colleagues showed that 12-wk WBV increased density of synaptic terminals at thoracic spine, and improved urinary bladder function at 6th and 12th weeks in spinal cord injured rats that start WBV at day 14 after injury[39].

In 2014, Miyara and colleagues found that post stroke patients who sat with the both calves on vibrator (frequency 30 Hz, amplitude 4-8 mm, time 3 min) exhibited an increased active and passive range of motion in ankle dorsiflexion and knee extension, and improved walking speed and cadence[40].

3. Endocrine system

It has been found that WBV can elevate the levels of anabolic hormones for musculoskeletal growth.

In 2010, Cardinale and colleagues examined that a single bout WBV while squatting (vertical motion of platform, frequency 30 Hz, and time 10 min) activated autonomic nervous system to increase plasma insulin like growth factor 1 (IGF-1) in older individuals[41]. Moreover, WBV increased plasma growth hormone and testosterone, and decreased plasma cortisol in healthy men[42].

A study in type II diabetic patients was done in 2013 by Sanudo and colleagues who found that WBV during squatting (frequency 12-16 Hz, time 12-20 min, and 12 weeks) was able to decrease blood glucose levels and body weight[43].

4. Cardiovascular system

The effects of WBV on cardiovascular system are described below.

In 2001, Ryan and colleagues found that a 10- min plantar vibration with frequency 47 Hz can increase the amount of fluid in upper dermis and epidermis of layer of sole in sixteen subjects with stage I PrUs assessed by ultrasonography[44].

In 2005, Stewart and colleagues examined the effects of WBV in post-menopausal women. They found that a single bout of plantar vibration (frequency 45 Hz, peak acceleration0.2 G, time 15 min) can increase blood circulation in calf, pelvic, and thoracic region after 30-min vibration[11].

The study of Lohman and colleagues in 2007, they reported a single bout of calf vibration (frequency 30 Hz, amplitude 5-6 mm, peak acceleration 7 G, time 1 min, 3 times) at calf of skin increased calf skin blood flow in healthy volunteers immediately until 10 min[12].

Maloncy- Hinds and colleagues (2008) reported a single bout of arm vibration (frequency 30 and 50 Hz, time 5 min) increased arm skin blood flow in healthy volunteers at 4 and 9 min[13].

According to Figueroa and colleagues (2012), WBV with static semi-squatting (frequency 25-30 Hz, amplitude 1-2 mm, WBV and rest 30-60 sec, 3 times/week for 6 weeks) and slow, dynamic exercise in obese adults decreased stiffness of arteries in legs, aortic systole pressure, sympathovagal balance, and strengthened quadriceps femoris muscle[45].

The related research of whole body vibration on wound healing

Currently, the effects of WBV on wound healing have been found only in two studies: the healing of stage I of PrUs in older patients, and that of full excision wounds in diabetic animals.

Arachi and colleagues (2010) reported that vibration therapy accelerates healing of stage I PrUs in older adult patients. A nonrandomized blinded, controlled trial was conducted in thirty one stage I PrUs inpatients who were divided in 2 groups: 1) control group receiving standard dressing care, 2) vibration group (frequency 47 Hz, peak acceleration 1.78 m/s^2, time 15 min/times ,and 3 times/day). The results showed a significant increase in the number of healed ulcers, the healing rate, the mean of relative changes per day in wound area, and intensity of redness in vibration group when compared with the control group[9]. It is proposed that shear stress originated from vibration stimulates vascular endothelial cells to produce nitric oxide which activates vascular dilatation that leads to increased blood flow in the wound area[46].

In 2014, Weinheimer- Haus and colleague examined whether low-intensity vibration (LIV) improved angiogenesis and excisional wound healing in 12-14-week-old of diabetic (db/db) mice. The mice were divided into two groups: LIV (30 min/day, 5 days/week for 2 weeks) and no vibration. The results showed that wound closure, re-epithelialization, granulation thickness, angiogenesis, macrophage accumulation, MCP-1, VEGF and IGF-1 were significantly increased in LIV group when compared to the control group. On the other hand, neutrophil accumulation and TNF-α were significantly decreased in LIV group compared to the control group[14].

However, there are no information about the effect of WBV on the healing of PrUs. Therefore, it is interesting to investigate these effects and the molecular mechanism.

Effect of whole body vibration on oxidative stress

In 2013, Naghii and colleague studied in normal rats that were employed WBV with vertical motion (frequency 10-50 Hz , amplitude 1-10 mm, cycle time 5 min/time, rest 1-2 min, 3 times, for 8 weeks). There were no significant changes in the levels of oxidative stress products (malondialdehyde and uric acid), and levels of antioxidants (plasma CU, zinc, superoxide dismutase, glutathione peroxidase, catalase, total antioxidant capacity and vitamin C) in blood samples. Thus, the researchers concluded that WBV induced no oxidative stress in rats. Therefore, WBV is considered a safe exercise training method[47].

Side effects and safety of whole body vibration

It has been demonstrated that subjects who undergo WBV while holding a standing posture may be feel uncomfortable in cervical bone and jaw. Therefore, squat during WBV program is required in order to decrease an impact force and transmission force to the cervical bone and jaw.

In animals, Weinheimer-Haus and colleagues (2014) found low intensity WBV did not induce inflammation of excision wound in diabetic mice, as evidenced by IL-1β and MMP-9. Furthermore, these program can decrease TNF-α in wound[14]. Naghii and colleagues (2013) reported that WBV with frequency 10-50 Hz did not stimulate oxidative stress production in rat model[47].

The WBV program used in the present study was done according to Weinheimer-Haus *et.al.* (2014) using frequency 45 Hz and peak acceleration 0.4 G. This frequency did not produce any trauma to animals.

CHAPTER III

MATERIALS AND METHODS

This study was an animal experiment research design. It was aimed to examine whether WBV can accelerate wound healing in stage II PrUs model in mice. In addition, the effects of WBV on the levels of TNF-α and, VEGF, neutrophil infiltration, and collagen deposition in the wound tissues are investigated. All protocols and procedures employed in this study were reviewed and approved by the committee of Animal Care, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (Approval No. 01/58).

Reagents

Reagents used in the study are as follows:

Deionized or distilled water

10% formalin buffer solution (Sigma-Aldrich, St. Louis, Missouri, USA)

Isoflurane, USP (Attane, Bethlehem, PA, USA)

Normal saline

Protease inhibitor cocktails (Sigma Chemical, Saint Louis, Missouri, USA)

Radio-immunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology, Danvers, MA, USA)

Animals

Thirty two male ICR mice (8 weeks old; body weight 35-40 g) were purchased from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Nakornpathom, Thailand. The experimental procedures were conducted according to

the guidelines for experimental animals by the National Research Council of Thailand, and approved by the Committee of animal care, Faculty of Medicine, Chulalongkorn University. The mice were housed one per plastic cage in a room at 25 °C with 12:12 hour light-dark cycle and were fed ad libitum with normal chow and water. They were allowed one week to get acclimated to the facility before the start experiment. Body weight was recorded at 4:00 pm everyday during experimental period.

Experimental protocol

The animals were randomly divided into two groups as follows:

Group 1 (PrU group, n = 16): the mice were subjected to stage II PrUs induced by I/R injury using the method modified from Assis de Brito et al[48]. This method involved 2 cycles of external application of two magnet plates (compression force 50 mmHg, diameter 10 mm) on dorsal skinfolds for 16 hours and then the magnets were removed for 8 hours, producing bilateral ulcers (figure 3-1).

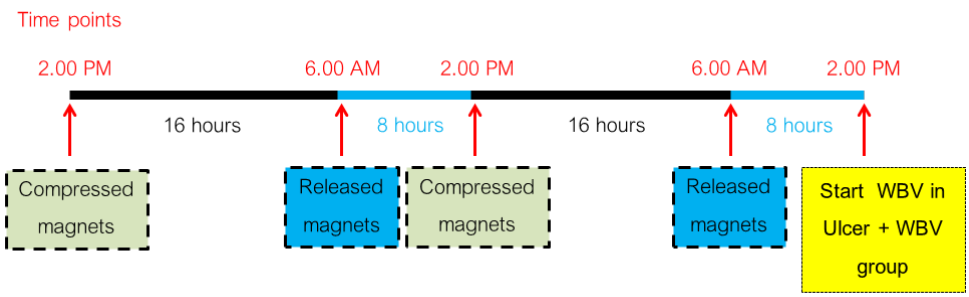


Figure 3- 1 Diagram of ischemia-reperfusion injury induction

Group 2 (PrU + WBV group, n = 16): the mice were subjected to PrUs induced by I/R injury as described above. Immediately after the completion of 2-cycle I/R injury, they were engaged in a WBV program using vibrator (frequency 45 Hz, peak acceleration 0.4 g, vertical motion), 30 min/day for 5 consecutive days/week (figure 3-1).

The ulcers were cleaned with normal saline once a day and were photographed photos for measuring wound area before starting experiment.

Each group was subdivided into two subgroups for studying on days 7 and 14 after ulcer formation. The parameters studied include rate of wound healing, levels of tissue TNF-α and VEGF, histopathology of the wound tissues, collagen deposition and the amount of neutrophil infiltration.

At the end of the experiment, the mice in all groups were euthanized using inhaled 5% isoflurane (3-5 min). The areas of left- and right- sided wounds were measured at the end of the experiment (day 7 or 14) to compare with those measured on the day of wound formation (day 0). The average areas of both- sided wounds were used to calculate the rate of wound healing. The wound tissues were removed circular incision away from edge of wound about 2 mm and depth about 3 mm.

Left- sided wound frozen at -80 ºC for TNF-α and VEGF analysis and right-sided wound fixed in 10% formalin solution for further paraffin processing and embedding. H&E staining was performed for analysis of skin histopathology and for measuring the amount of neutrophil infiltration. Masson's trichrome staining was done for analysis of collagen contents in the wound tissues. The levels of tissue TNF-α and VEGF were assayed using ELISA method. Diagram of experimental design is shown in figure 3-2.

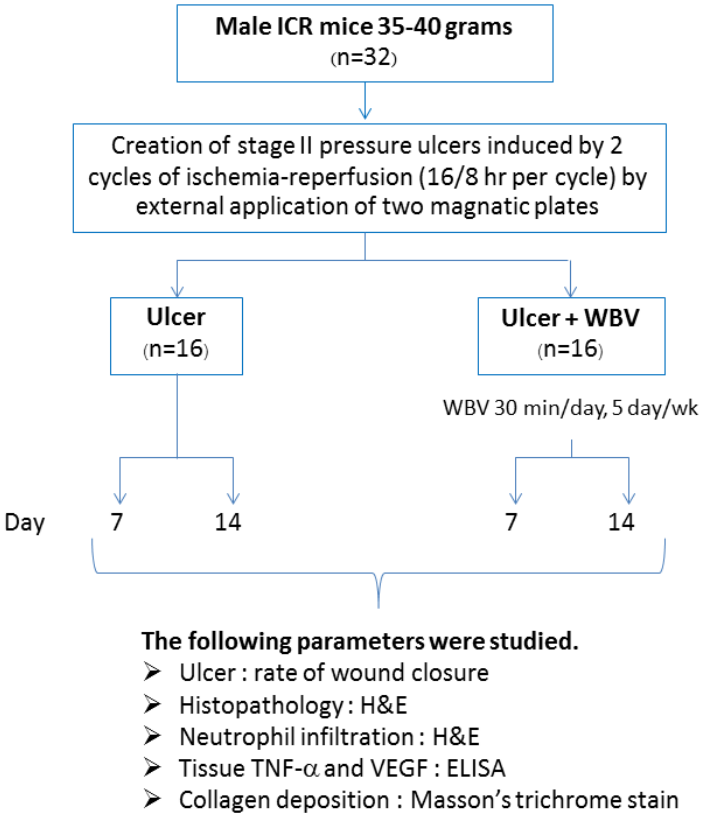


Figure 3- 2 Diagram of experimental design

Pressure ulcer induction

Pig is the most ideal animal for PrUs research because pig skin is more similar to human. However, rat and mouse PrUs models induced by I/R injury are commonly used.

In previous studies, PrUs were induced by insertion of a magnet plate under the skin and another overlaid on the skin of animal. However, the current studies have used external application of two magnets to compress the skin. This is a non-invasive method that reduces the risk of wound infection.

Mice were subjected to pressure ulcer induced by ischemia-reperfusion injury using the method modified from Assis de Brito et al[48]. Mice were anesthetized with 5% of an inhaled isoflurane for 3-5 min. Their dorsal skin was disinfected with 70%

isopropyl alcohol. All 3 layers of dorsal skin including epidermis, dermis and hypodermis were pulled. The skin was marked with a marking pen at 2 cm below occipital bone to localize the site of magnet application, with the distance of approximately 5 mm between the two magnets. Magnet disks sized 3 mm of thickness, 1.7 g of weight, and magnet pressure 300 Gauss that skin was compressed 50 mmHg of pressure of magnet as shown in figure 3-3. The mice's dorsal skin was compressed for 16 hours and was subsequently released for 8 hours. Two PrUs were formed after repeating 2 cycles of I/R injury[48-50] as shown in figure 3-3. The PrUs of mice were taken care by normal saline. Food consumption and body weight were recorded everyday during experimental period.

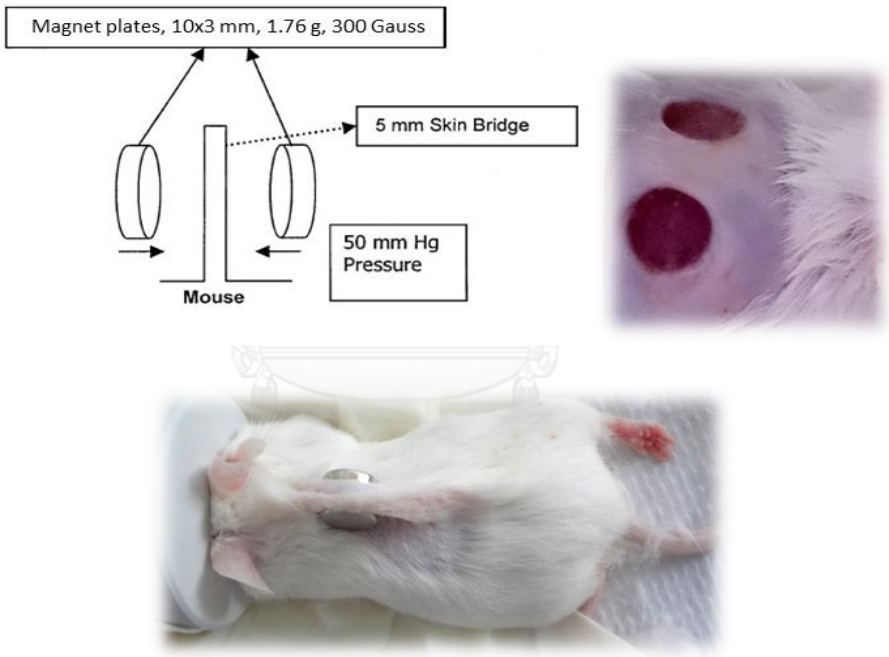


Figure 3- 3 Pressure ulcer induction

Whole body vibration program

WBV program was started immediately after the completion of 2-cycle I/R injury in WBV group. Mice were placed on a vibrating plate of vibrator. To prevent escape of mice from the vibrator, the mice were covered with a clear rectangular plastic cap with ventilation holes (figure 3-4**)**. The vibrator was set to oscillate at a

vertical motion (frequency 45 Hz and peak acceleration 0.4 G or approximately 30 microns). The parameter setting was calibrated every time before starting WBV using computer software as shown in figure 3-4. The vertical motion of plate at this frequency and acceleration helped decrease crushing forces that may cause injury to the animals. WBV was performed 30 min/day and 5 days/week for 2 weeks as previously described by Weinheimer-Haus et al[14].

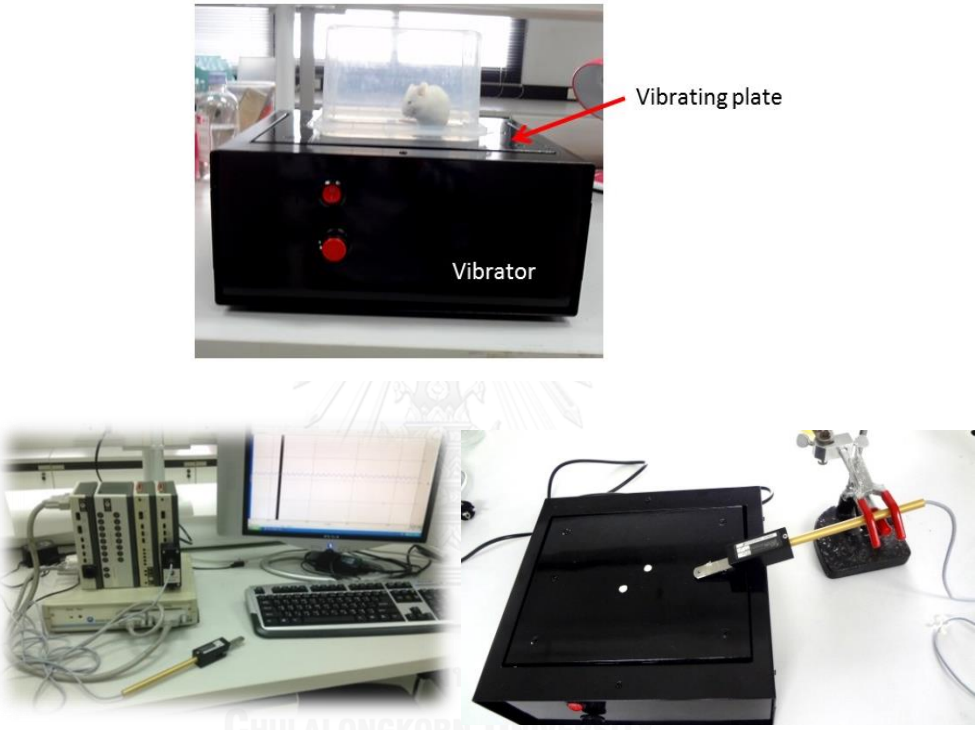


Figure 3- 4 Whole body vibrator

Measurement of wound closure rate

The photographs of both wounds of each mouse were taken by digital camera (SONY Cyber- shot; Sony, Tokyo, Japan) at days 0, 7 or 14. Then, the wound area was analyzed by digital image software analysis (ImageJ, National Institutes of Health). The picture was scaled by setting known distances and unit of length. The distance between the wound edges was defined by the distance between the first hair follicle at each end of the wound site and transverse epithelium of wound site, and the wound edges were drawn by pencil icon in the software program to

measure wound area as shown in figure 3-5. The average values of both wound areas were calculated. Finally, the percentage of wound closure (%WC) was calculated using the following formula:

$$\%WC = \frac{\text{Area of original wound} - \text{Area of actual wound}}{\text{Area of original wound}} \times 100\%$$

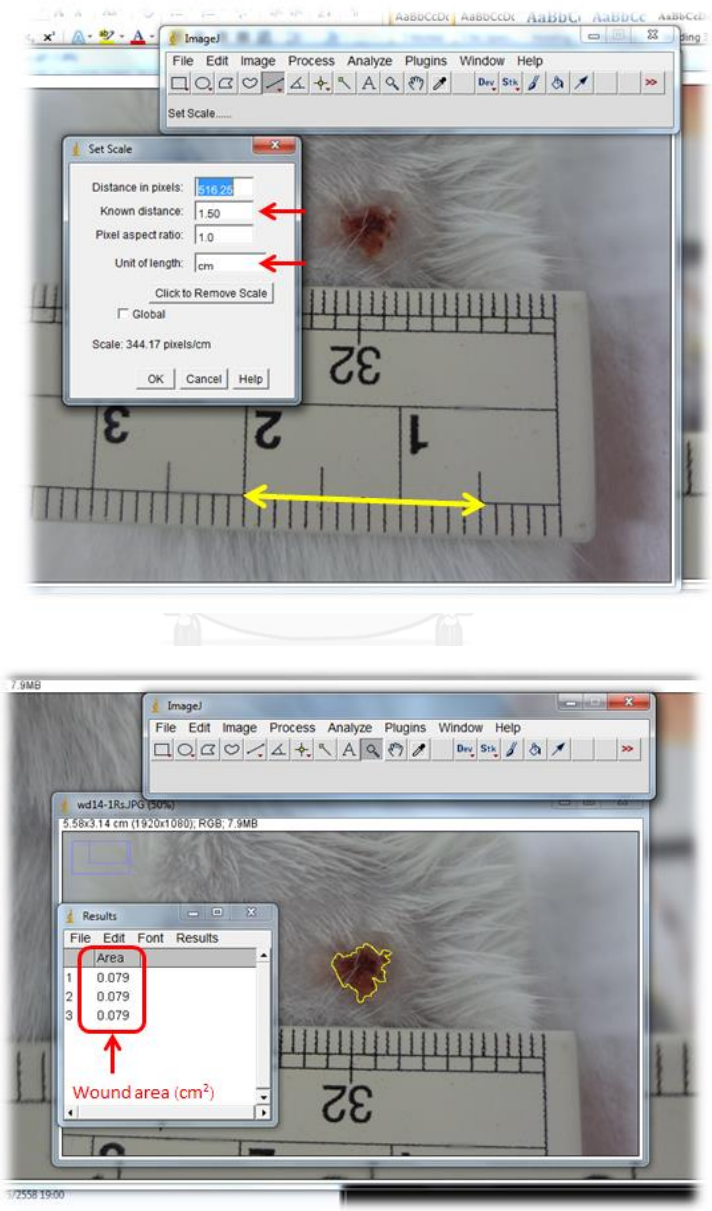


Figure 3- 5 Measurement of the wound area (cm^2) using ImageJ

The study of skin histopathology

The left side of wound tissue was removed by circular incision about 2 mm away from wound edge with the depth of about 3 mm. Next, the wound tissue was fixed in 10% formalin solution for 24 hours and cut into four pieces. Next, all skin were done paraffin processing, embedding, and cutting at 1 μm thickness[51]. Then, tissue H&E staining was performed for analysis of skin histopathology with light microscope at a power of 100x**.**

Measurement of neutrophil infiltration

The neutrophil infiltration was assessed by counting cells in H&E- stained slide. Eight frames of each slide were photographed by using microscope (Olympus BX53) at 400 power as shown in figure 3-6. The number of neutrophils (cells/frame) infiltrated in dermal layer of wound area was counted and the average of the numbers was calculated.

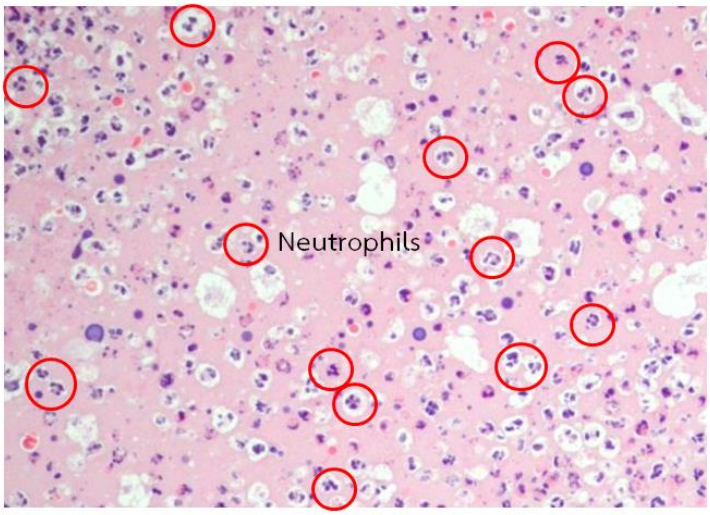
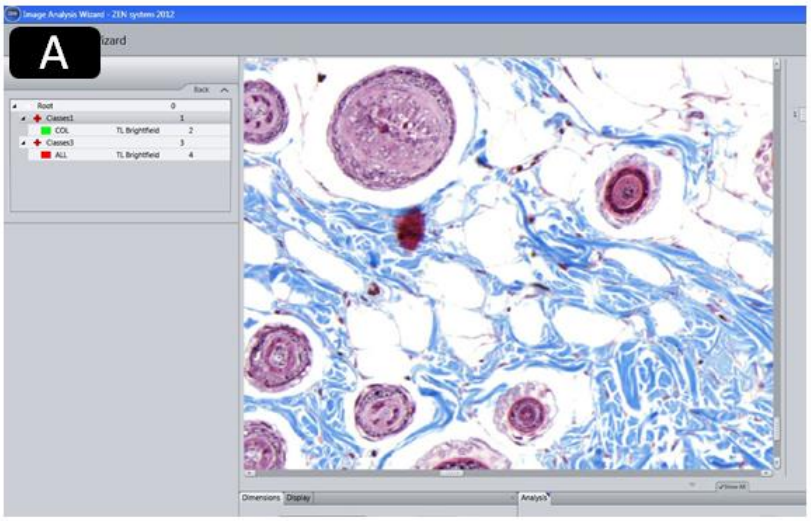


Figure 3- 6 The morphology of neutrophil cells in wound area. Photographs of H&E-stained section were taken at 400 power.

Assessment of collagen deposition

The wound tissues were removed and fixed in 10% formalin solution for further paraffin processing and embedding. Masson's trichrome staining was performed for identification of collagen deposition (International Medical Equipment; IMEB, San Marcos, CA, USA). Furthermore, trichrome staining was captured the entire specimen area of the microscope slide at 20 power by scanner (Axio Scan Z1; Carl Zeiss, Jena, Germany) as shown in figure 3-7A. The percentage of blue collagen - stained area relative to the total area of the wound were analyzed by image analysis software ZEN (Carl Zeiss, Jena, Germany)[14]. The threshold intensity of blue stained area in each image was represented by green color (figure 3-7B) and the whole area of sectional skin was set by red color (figure 3-7C) for calculation percentage of collagen deposition. Moreover, only clearly stained pixels were chosen for threshold intensity. The percentage of collagen deposition (%CD) was calculated using the following formula:

$$\%CD = \frac{\text{Blue collagen-stained area}}{\text{Total area of the wound bed}} \times 100\%$$



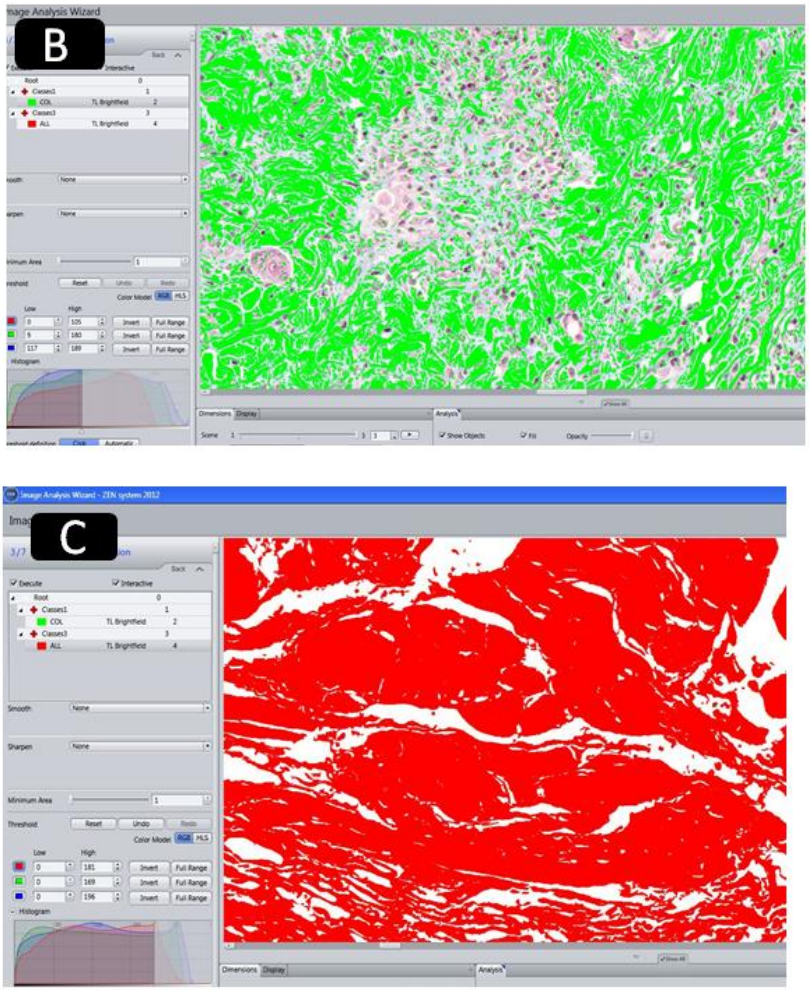


Figure 3- 7 Analysis of collagen deposition (%) using Image analysis software (Carl *Zeiss*)

Measurement of tissue VEGF level

Right- sided tissue samples were harvested from each mouse at day 7 and 14 after inducing PrUs and then frozen at -80 °C. 50 mg of tissue sample was homogenized in 50 mL of RIPA lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with protease inhibitor cocktails (Sigma Chemical Co, Saint Louis, Missouri, USA), sonicated and centrifuged at 10,000 rpm for 10 min. Lastly, the supernatant of each sample was collected for analysis of VEGF levels by ELISA method (R&D Systems, Minneapoli, MN, USA)[52].

All reagents and samples were placed at room temperature before analysis. Next, 50 µL of assay diluent RD1N, standard, control, and sample were added to each well. Plate frame was gently tapped for 1 minute to mix the reagents. Then, the plate was covered with the adhesive strip and incubated for 2 hours at room temperature. Each well were aspirated and washed with wash buffer (400 µL) for repeating the process four times for a total of five washes by autowasher (wellwash; ThermoFisher scientific, Rockford, IL, USA). Next, the plate was added with 100 µL of mouse VEGF conjugate to each well and was incubated for 2 hours at room temperature again. After repeating the wash process by autowasher, each well was added with 100 µL of substrate solution and were incubated for 30 minutes at room temperature in a dark area. Besides, 100 µL of stop solution were added to each well and were gently tapped the plate to ensure thorough mixing. Finally, the optical density of each well was determined at wavelength 450 nm within 30 minutes by using a microplate reader (Varioskan™ Flash; ThermoFisher scientific, Rockford, IL, USA).

Calculation of results

The average values of optical density of the duplicated readings for each standard and sample were calculated. The standard curve was created by Microsoft Excel. The calibration graph and equation of fitted linear line were shown in figure 3-8.

mVEGF (pg/ml)	Optical Density			
	I	II	Average	Corrected
0	0.072	0.077	0.0745	-
7.8	0.120	0.104	0.112	0.0375
15.6	0.150	0.143	0.1465	0.072
31.3	0.207	0.201	0.204	0.1295
62.5	0.350	0.344	0.347	0.2725
125	0.651	0.602	0.6265	0.552
250	1.160	1.160	1.160	1.0855
500	2.088	2.077	2.0825	2.008

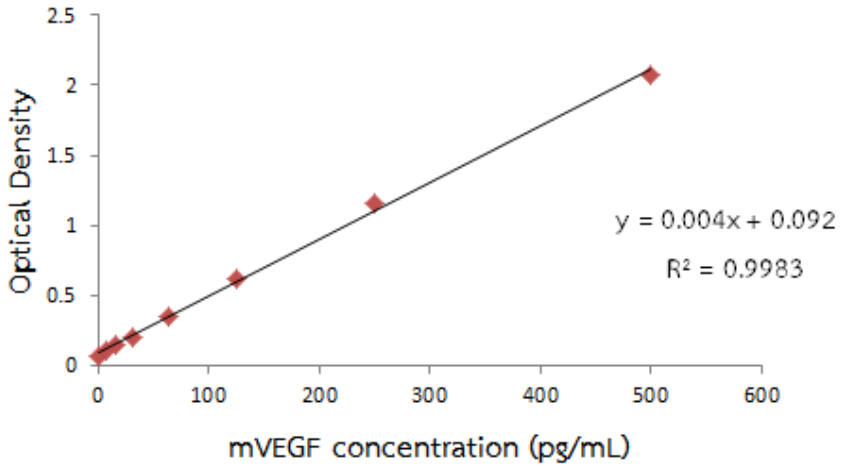


Figure 3- 8 Calculation of optical density for VEGF analysis

Measurement of tissue TNF-α level

Right- sided tissue samples were collected from each mouse at day 7 and 14 after inducing PrUs and then frozen at - 80 °C. 50 mg of tissue sample was homogenized in 50- mL RIPA lysis buffer (Cell Signaling Technology, Danvers, MA, USA) with protease inhibitor cocktails (Sigma Chemical, Saint Louis, Missouri, USA), sonicated and centrifuged at 10,000 rpm for 10 min. Finally, the supernatants of each

sample were used to analyze TNF-α levels by ELISA method (R&D Systems, Minneapoli, MN, USA).

All reagents and samples were placed at room temperature before analysis. Next, 50 µL of assay diluent RD1-63, standard, control, and sample were added to each well. Plate frame was gently tapped for 1 minute to mix the reagents. Then, the plate was covered with the adhesive strip and incubated for 2 hours at room temperature. The each well were aspirated and washed with wash buffer (400 µL) for repeating the process four times for a total of five washes by autowasher (wellwash; ThermoFisher, Rockford, IL, USA). Next, the plate was added 100 µL of mouse TNF-α conjugate to each well and was incubated for 2 hours at room temperature again. After repeating the wash process by autowasher again, each well were added 100 µL of substrate solution and were incubated for 30 minutes at room temperature and dark area. Besides, 100 µL of stop solution were added to each well and were gently tapped the plate to ensure thorough mixing. Finally, the optical density of each well was determined at wavelength 450 nm within 30 minutes by using a microplate read (Multiskan EX; ThermoFisher, Rockford, IL, USA).

Calculation of results

The average optical density of duplicated reading for each standard and sample were calculated. The standard curve was created by Microsoft Excel. The calibration graph and equation of fitted linear line were shown in figure 3-9.

mTNF-α (pg/ml)	Optical Density			
	I	II	Average	Corrected
0	0.080	0.079	0.0795	-
10.9	0.128	0.134	0.131	0.0515
21.9	0.174	0.180	0.177	0.0975
43.8	0.296	0.296	0.296	0.2165
87.5	0.503	0.461	0.482	0.4025
175	0.934	0.904	0.919	0.8395
350	1.697	1.572	1.6345	1.555
700	2.897	3.047	2.972	2.8925

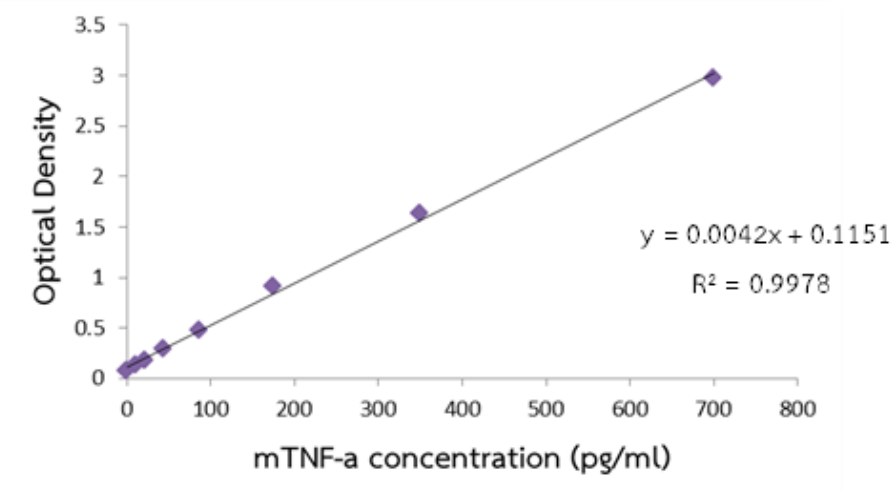


Figure 3- 9 Calculation of optical density for TNF-α analysis

Determination of the total tissue protein

The total protein in the supernatants of all tissue samples were measured by micro bicinchoninic protein assay kit (Thermo Fisher scientific, Rockford, IL, USA) to find out the real quantity of protein in tissue each sample for calculation levels of tissue VEGF and TNF-α in pg per mg protein.

Firstly, 150 µl of all reagents and samples were added into a microplate well with duplicate standard and sample. Then, the plate was added 150 µl of the bicinchoninic working reagent and mixed thoroughly on a plate shaker for 30 seconds. Next, the plate was covered with sealing tape and incubated at 37°C for 2 hours. Finally, the optical density was determined at wavelength 562 nm using a microplate reader (Varioskan™ Flash; Thermo Fisher scientific, Rockford, IL, USA).

Calculation of results

The average optical density of the duplicated reading for each standard and samples were calculated. The standard curve was created by Microsoft Excel. The calibration graph and equation of fitted linear line were shown in figure 3-10.

Protein (µg/ml)	Optical Density			
	I	II	Average	Corrected
2000	2.400	2.420	2.410	2.303
1500	2.10	1.90	2.00	2.013
1000	1.40	1.40	1.40	0.873
750	0.897	0.899	0.898	0.791
500	0.715	0.713	0.714	0.607
250	0.500	0.502	0.501	0.394
125	0.337	0.337	0.337	0.23
25	0.168	0.166	0.167	0.06
0	0.108	0.106	0.107	-

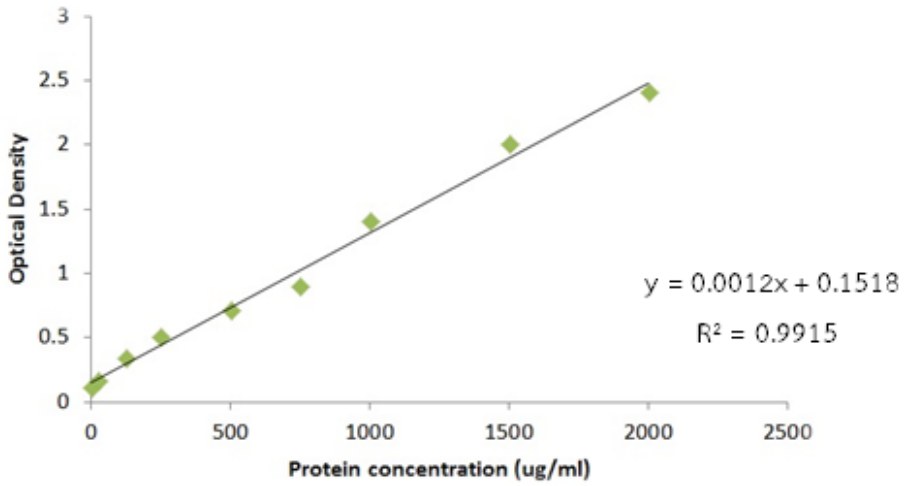


Figure 3- 10 Calculation of optical density for total protein analysis

The levels of tissue VEGF and TNF-α were adjusted for the total tissue protein and expressed in pg/mg protein unit.

Statistical analysis

Data were presented as mean and standard deviation (SD). One way analysis of variance (one-way ANOVA) and Mann-Whitney U test were used to compare among all groups of animals. Differences were considered statistically significant at $p<0.05$.

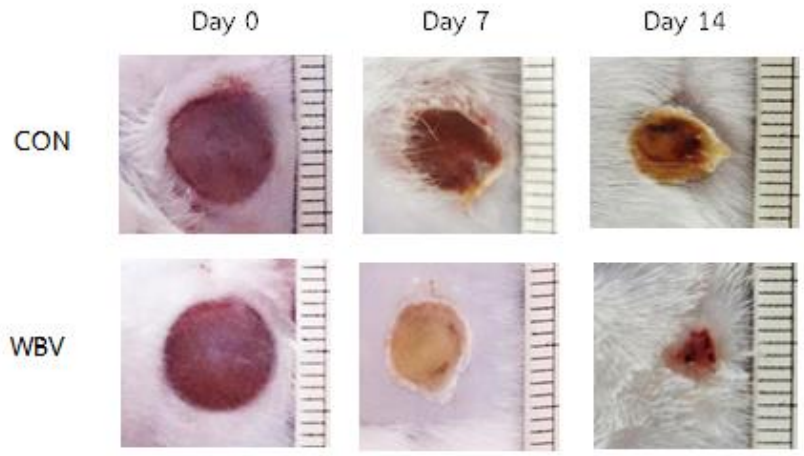
CHAPTER IV

RESULTS

Effects of WBV on wound closure rate

On day 0, there were no significant differences in the wound areas induced by I/R injury among any groups.

On day 7, no significant differences in wound closure rate were found between CON and WBV groups (CON= 15.54±9.79, WBV= 24.18±9.79 %). However, on day 14, wound closure rate significantly increased in the WBV group (CON= 55.25±22.13, WBV= 89.99±3.53 %) compared to the CON group (figure 4-1).



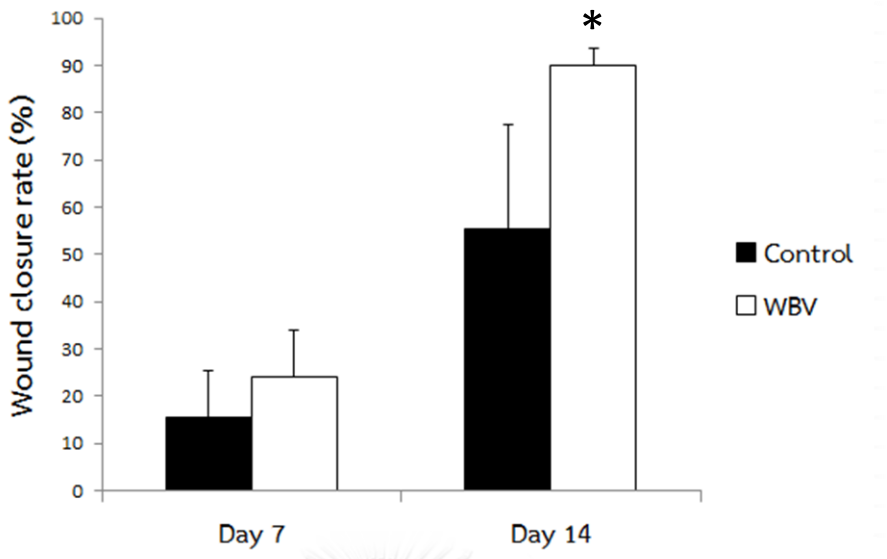


Figure 4- 1 Percentage of wound closure rate was shown as average areas both sides of wound on days 7 and 14. CON: no vibration group; WBV: vibration group. Data are expressed as mean ± SD. Star indicates a significant difference from CON ($p \leq 0.01$).

Effects of WBV on the neutrophil infiltration

Neutrophil infiltration was expressed as average number of neutrophils per frame in both groups.

Figure 4-2 shows histopathology of neutrophil infiltration in the wound areas on day 7 and 14 in H&E-stained section.

Neutrophil infiltration significantly decreased in the WBV group on day 7 (CON= 46.1±15.4, WBV= 25.6±4.3 cells/frame) and day 14 (CON= 19.2±4.0, WBV= 8.8±3.0 cells/frame) (figure 4-3).

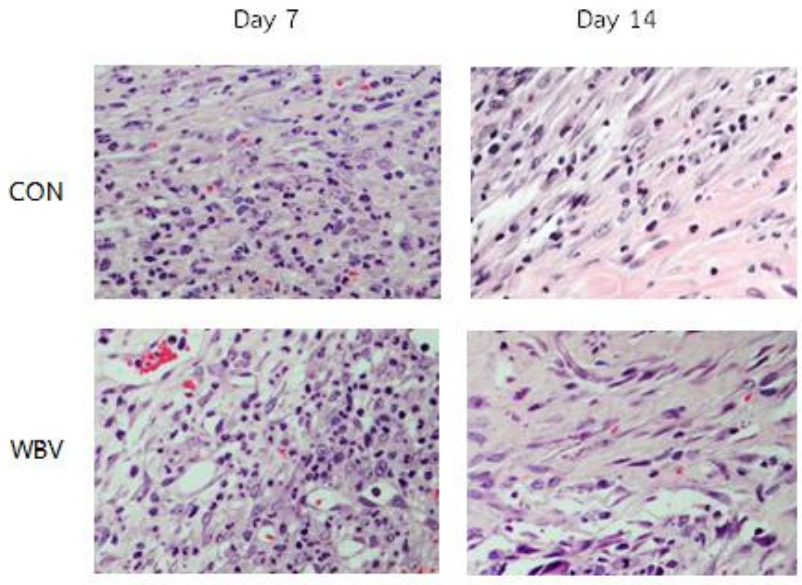


Figure 4- 2 Histopathology showing neutrophil infiltration in the wound areas on day 7 and 14 in H&E-stained section (400x). CON: no vibration group; WBV: vibration group.

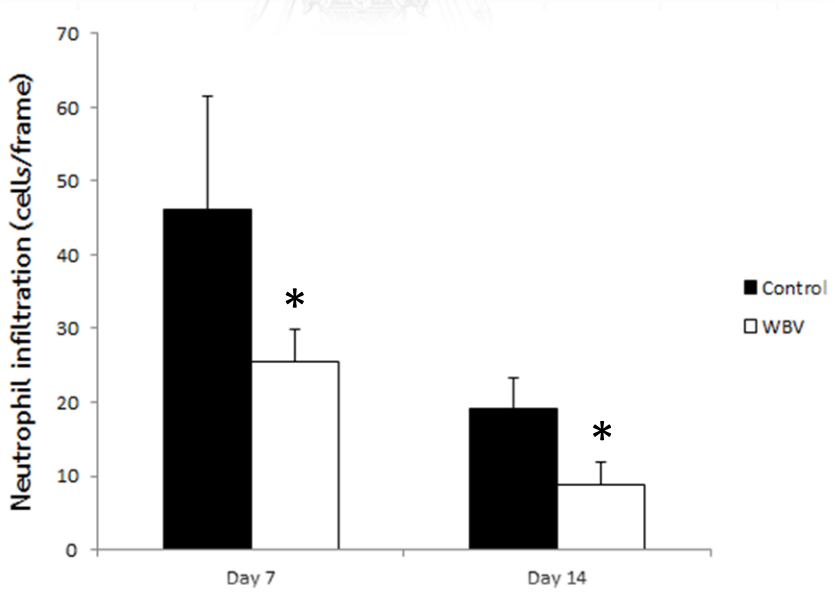


Figure 4- 3 Neutrophil infiltration was expressed as average number of neutrophils per frame on day 7 and 14. CON: no vibration group; WBV: vibration group. Data are expressed as mean ± SD. Star indicates a significant difference from CON ($p < 0.01$).

Effects of WBV on the skin histopathology

On day 7 in CON group, histopathological finding of the ulcer in epidermis layer revealed a large ulceration, fibrin, and bacterial colony. Ulcer bed was covered by keratin debris. Moreover, the inflammatory exudate showed numerous lymphocytes, monocytes and macrophages in dermis layer. There was a mild inflammation reaction in hypodermis layer (figure 4-4a).

In WBV group, histopathology revealed similar characteristics of lesion to the CON group but lower degree in severity (figure 4-4b).

On day 14 in CON group, histopathological study of the ulcer found a decrease in polymorphonuclear cell infiltrate, muscle atrophy, and blood vessels. Furthermore, there were epithelialization and a small size of hemorrhagic crust (figure 4-4c).

In WBV group, histopathology of the wound was similar characteristics to the CON group. However, WBV group was higher wound epithelialization and collagen bundles than another group (figure 4-4d).

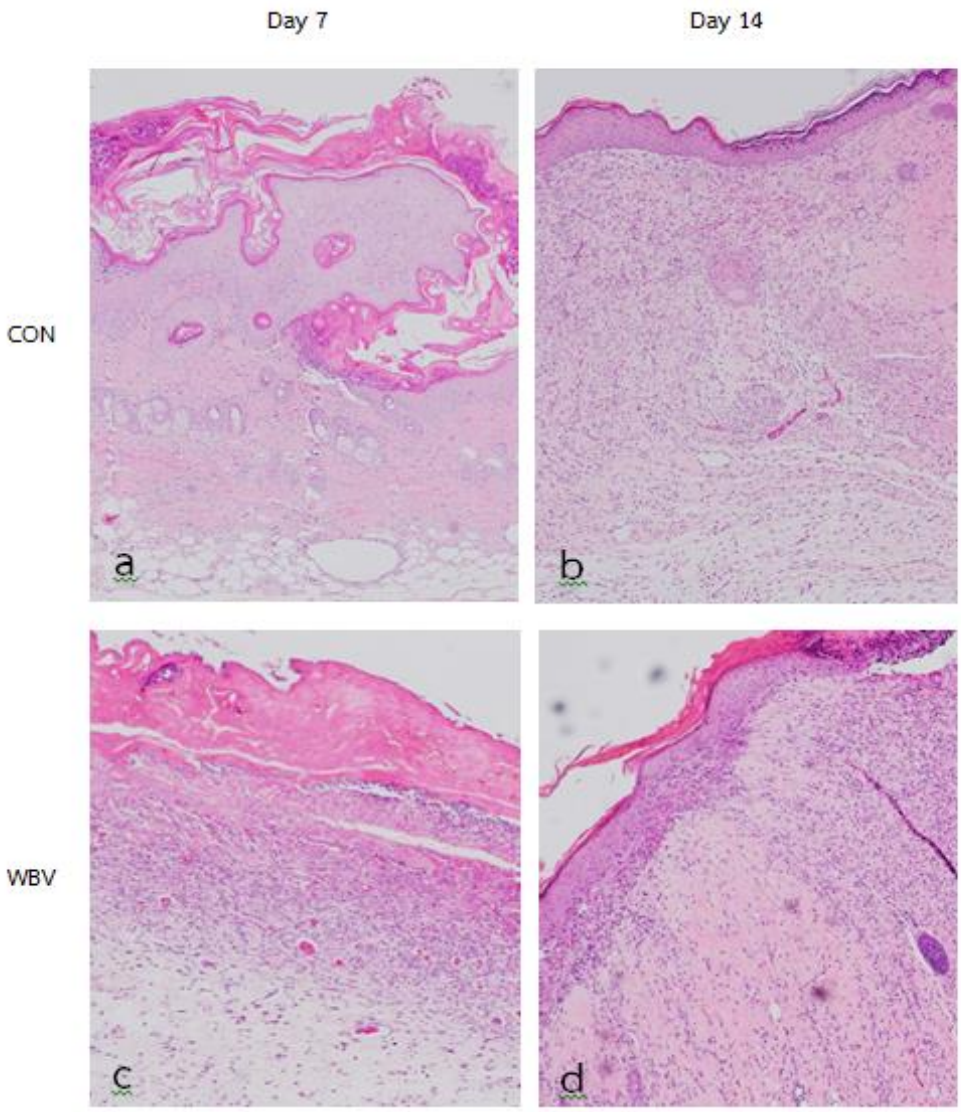


Figure 4- 4 The histopathology of pressure ulcers on day 7 and 14. CON: no vibration group; WBV: vibration group.

Effects of WBV on the tissue VEGF level

The level of tissue VEGF showed no significant differences between CON and WBV on day 7 (CON= 581±123.43, WBV= 723.90±152.35 pg/mg protein) and day 14 (CON= 318.10±86.08, WBV= 238.19±58.37 pg/mg protein) (figure 4-5).

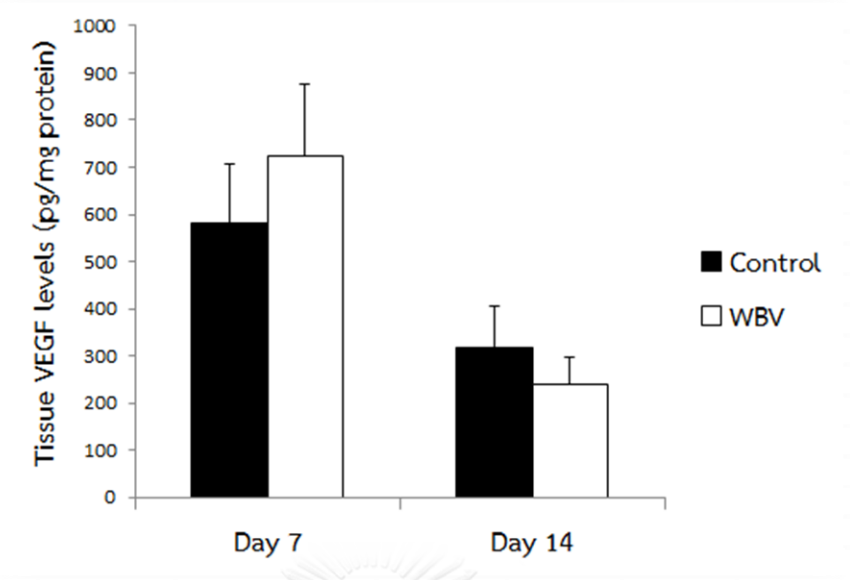


Figure 4- 5 Tissue VEGF levels on day 7 and 14. CON: no vibration group; WBV: vibration group. Data are expressed as mean ± SD. Star indicates a significant difference from CON ($p < 0.05$).

Effects of WBV on the tissue TNF-α level

Tissue TNF-α was significantly decreased in the WBV group on day 7 (CON= 994.06±110.69, WBV= 420.16±111.67 pg/mg protein) and day 14 (CON= 772.65±245.21, WBV= 30.25±17.68 pg/mg protein) when compared to the CON group (figure 4-6).

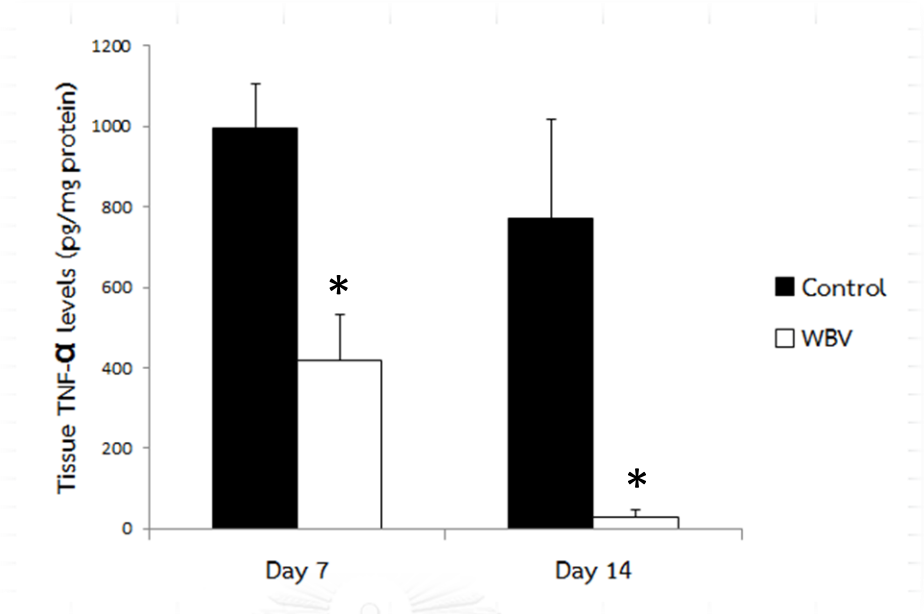


Figure 4- 6 Tissue TNF-α levels on day 7 and 14. CON: no vibration group; WBV: vibration group. Data are expressed as mean ± SD. Star indicates a significant difference from CON ($p < 0.01$).

Effects of WBV on the collagen deposition

Collagen deposition had no significant differences between CON and WBV groups on day 7 (CON= 15.81±1.96, WBV= 17.88±5.76 %). However, collagen deposition significantly increased in the WBV group on day 14 (CON= 31.58±4.29, WBV= 48.98±3.20 %) when compared to the CON group. (figure 4-7)

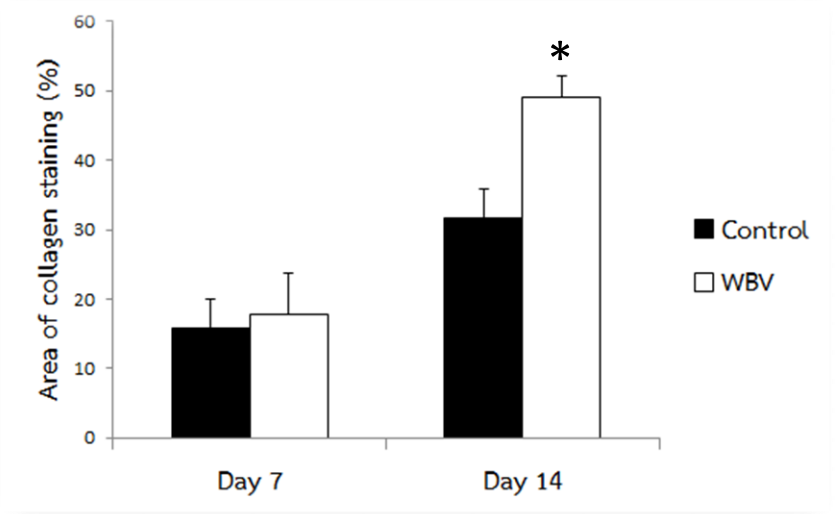


Figure 4- 7 Percentage of area of collagen staining on day 7 and 14. CON: no vibration group; WBV: vibration group. Data are expressed as mean ± SD. Star indicates a significant difference from CON ($p < 0.01$).

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Pressure ulcers (PrUs) are localized skin injures that are mostly found in bedridden elderly patients resulting from pressure and shear force[5]. PrUs can cause chronic skin damage and increased risk of wound infection, high mortality and high cost of medical care. Because the benefits of WBV have been found in some previous studies, WBV may be another choice for enhancing wound healing process.

The objectives of the present study were to examine the effect of whole body vibration on wound healing as well as the levels of tissue TNF-α, tissue VEGF, neutrophil infiltration, and collagen deposition in pressure ulcers model in mice.

The main findings of this study were that WBV significantly increased wound closure rate, collagen deposition and decreased the level of tissue TNF-α, neutrophil infiltration in stage II PrUs mice. In particular, there was a significant decrease in tissue TNF-α and neutrophil infiltration on day 7 and 14. Furthermore, a significant increase in wound closure rate and collagen deposition was found on day 14. However, tissue VEGF did not change at both time points.

Effect on the wound closure and collagen deposition

In this study, it was found that in WBV group, the wound closure and collagen deposition were significantly increased on day 14 when compared to CON group. Similarly, a previous study by Weinheimer-Haus et al. showed that low-intensity vibration increased wound closure on days 7 and 15, and granulation tissue on day 7 in excisional wound in diabetic mice[14]. Although Weinheimer-Haus found no significant difference in collagen deposition in both groups, the increased amount of granulation tissue in vibration group may imply an increased in type III collagen because granulation tissue is composed of tissue matrix consisting of type III collagen

that is synthesized by fibroblasts, and a network vessels of blood vessels. The conflicting results on collagen deposition in the present study and that of Weinheimer-Haus et al. are probably due to the host condition and the wound severity. Weinheimer-Haus et al. used diabetic mice that had low ability of collagen synthesis. Moreover, the full-thickness excisional wound involves all layers of skin that was more severe than stage II PrUs in this study. Consistent with this study, Arachi and colleagues (2010) reported that vibration therapy accelerated healing of stage I PrUs in older adult patients, as evidenced by a significant increase in the number of healed ulcers, the healing rate, the mean of relative changes per day in wound area, and intensity of redness in vibration group when compared with the control group(9).

Healing process is defined as an intricate *process* of the body that repairs itself after skin or tissue injury. Inflammatory phase lasts three to five days after injury. A number of macrophages in the wound produce inflammatory cytokines**.** Proliferative phase begins on day five to seven after injury and is characterized by the formation of granulation tissue consisting of new blood vessels and collagen deposition. Lastly, Remodeling phase lasts several weeks to a year. Myofibroblasts differentiation from fibroblasts is a critical component of the healing process for decreasing wound size. Additionally, there are many matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases enzymes help balance collagen deposition[25]. This study found that collagen deposition was increased on day 14 in remodeling phase.

The enhancement of wound healing and collagen deposition may be explained by two mechanisms. Firstly, WBV stimulated hypothalamic neuro-secretory center[53] by increasing the level of growth hormone (GH)[41] and local level of IGF-1[41, 42]. Moreover, GH activated liver to produce IGF-1. Circulating and local IGF-1 activated the collagen synthesis by stimulating fibroblast proliferation[54, 55]. Furthermore, IGF-1 stimulated keratinocyte proliferation and migration leading to improved re-epithelialization[56]. Therefore, the more collagen deposition, the more improvement of wound healing (figure 5-1).

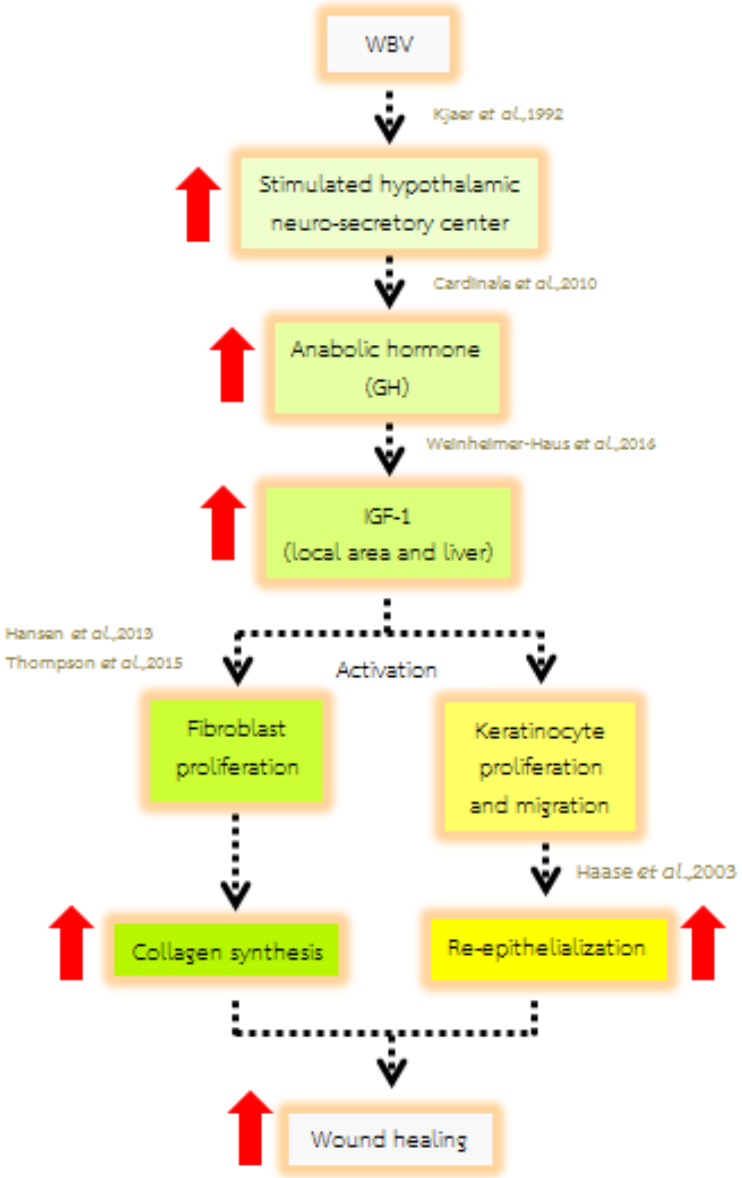


Figure 5- 1 Propose mechanism of WBV on the healing through an increased in collagen deposition.

Secondly, vibration has a mechanical effect on microskeleton in endothelial cells and produced shear stress at the endothelial cell surface[57]. Shear stress in turn stimulates local NO production through an activated endothelial nitric oxide synthase (eNOS). NO causes relaxation of smooth muscle in small arteries or

vasodilation. So, vascular vasodilation results in an increased blood flow which led to improved wound healing (figure 5-2).

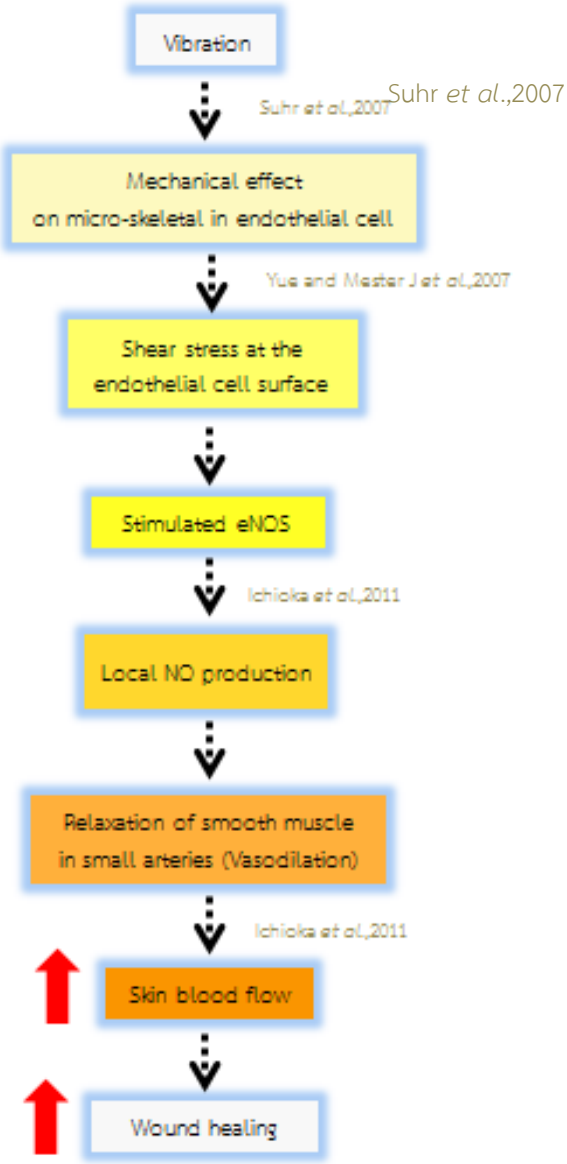


Figure 5- 2 Proposed mechanism of WBV on the healing through an increased blood flow.

Effect on the tissue TNF-α level, neutrophil infiltration and skin histopathology

The tissue TNF-α level and amount of neutrophil infiltration was decreased in WBV group on days 7 and 14 as compared to CON group. According to the skin histopathology study, WBV group had decreased inflammatory cells on day 7 when compared to the CON group. This eventually occurred in proliferative phase. A previous study found that WBV can decrease TNF-α mRNA expression and neutrophil accumulation in excisional wound in mice[14]. Besides, Rodriguez-Miguelez and colleague (2015) reported that 8-wk WBV increased interleukin-10 (IL-10) mRNA and decreased plasma TNF-α level in elderly subjects through toll-like receptor 2 and 4 signaling pathways[58].

The mechanism may be explained by that WBV program affected on down-regulation of the toll-like receptor 2 (TLR2) and TLR4, which controlled pro-inflammatory cytokines and anti-inflammatory cytokines production[59]. WBV increased IL-10 mRNA and decreased plasma TNF-α level, leading to inhibition of inflammation[58]. (figure 5-3).

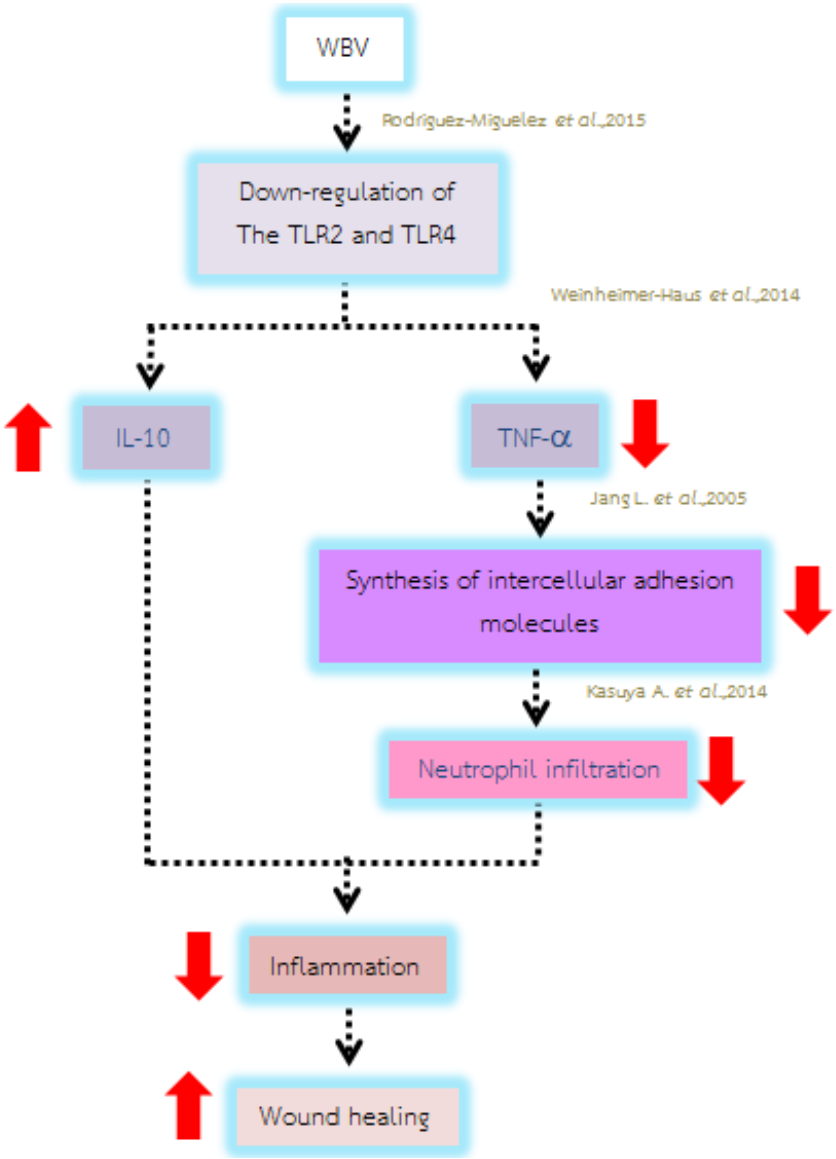


Figure 5- 3 Propose mechanism of WBV on the healing through a decreased inflammation.

Effect on the tissue VEGF level

In the present study, tissue VEGF levels were not significantly different among any groups on day 7 and 14. Similarly, in the study in healthy men, WBV did not elevate the angiogenic stimulus (serum concentration of VEGF) when applied during resistance exercise for 6 weeks[60].

In contrast, the study of Weinheimer-Haus et al. (2014) showed that WBV increased tissue VEGF level in diabetic mice[14]. In addition, Suhr et al. (2007) reported

that 30 Hz WBV in healthy male subjects increased VEGF serum levels in WBV group when compared to no vibration group[57]. They proposed the mechanism for increasing serum VEGF to be mechanical effects from WBV induced shear stress on endothelial cell surface. This reason led to increased blood flow via NO production[46].

The time course of VEGF mRNA expression was maximally about day 3 to day 7 post-injury in full-thickness wound[63,64]. This may be an explanation why no differences in tissue VEGF were found in this study that examined the level of tissue VEGF on day 7 and 14. Interestingly, this study also demonstrated that tissue VEGF levels tended to decrease with time.

However, tissue VEGF level in WBV group in the present study supports the previous finding about the reduction of hypoxia-inducible factor 1 alpha (HIF1α) by 47 Hz WBV (15 min/day for 2 weeks) in PrUs[61]. HIF1α was a master transcriptional regulator of cellular response to hypoxia. Generally, HIF1α was increased in necrotic tissue or low oxygen area, stimulated VEGF to produce new capillaries for supplying oxygen and nutrient for tissue[62].

Therefore, tissue VEGF levels in PrUs mice in WBV group were not significantly different among any groups, which may result from VEGF expression in a specific time point and a decreased of HIF1α.

Conclusion

The present finding can be summarized as follows (figure 5-4):

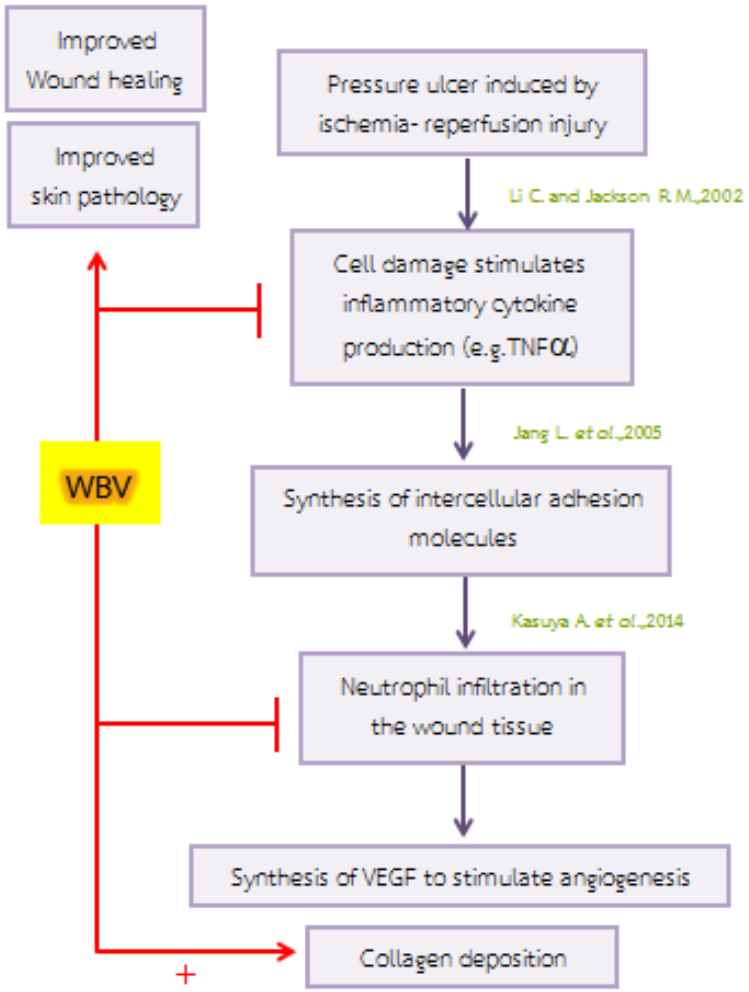


Figure 5- 4 Summary of effects of WBV on the treatment of PrUs in mice.

1. 45 Hz WBV accelerated wound healing, and increased collagen deposition on day 14 in PrUs mice.
2. WBV used in this study decreased inflammation through a decrease in the tissue TNF-α level and neutrophil infiltration on day 7 and 14 in pressure ulcers model in mice. However, this program did not significantly increase in the levels of tissue VEGF.

3. WBV program improved skin morphology in stage II of Prus mice.

The proposed mechanism by which effects of WBV on enhancement of wound healing through a decrease in the levels of tissue TNF-α, neutrophil infiltration, and increase in collagen deposition in pressure ulcers model in mice (figure 5-5).

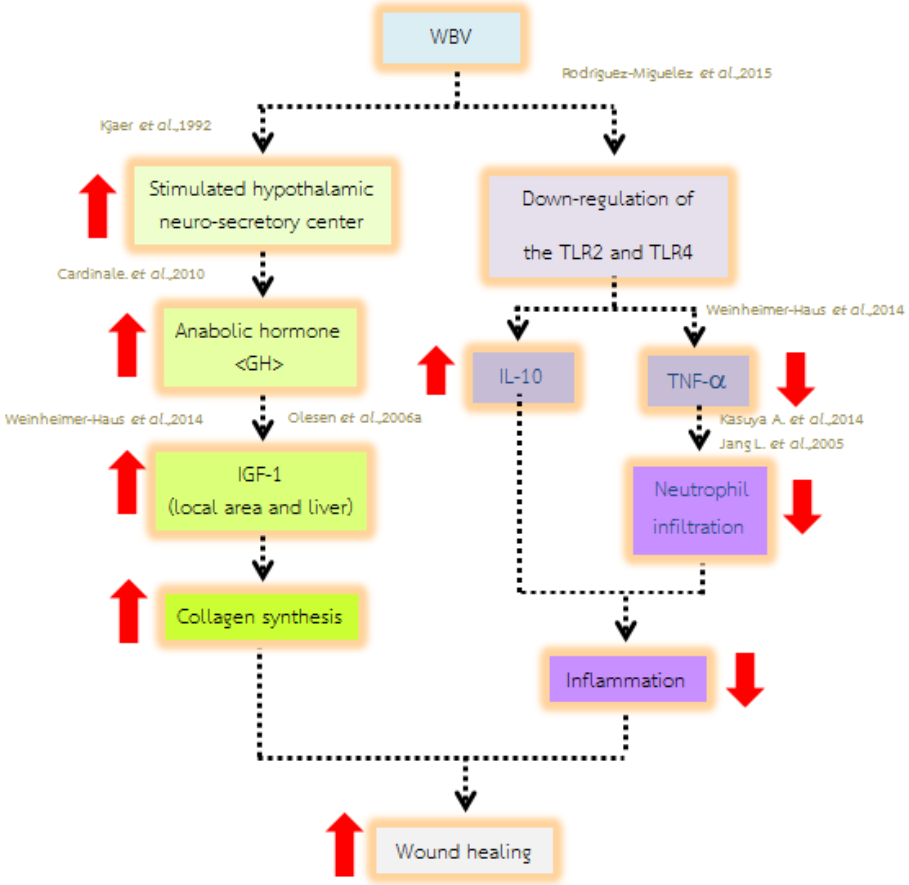


Figure 5- 5 Propose mechanism of WBV on the healing and inflammation.

WBV program in this study can increase wound closure rate and collagen deposition. Furthermore, WBV can decrease tissue TNF-α and neutrophil infiltration in PrUs wound. This event facilitates wound healing and reduces inflammation, in I/R injury-induced stage II PrUs in mice.

The information obtained from this study provides a basis for clinical use of WBV as a therapy in patients with PrUs. Further investigations to explore microvascular responses, including blood flow and capillary vascularity, is warranted.

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APPENDIX

Wound closure rate

Wound area at day 0

Descriptives[a]

	group			Statistic	Std. Error
pre wc	1	Mean		.643667	.0139370
		95% Confidence Interval for Mean	Lower Bound	.610711	
			Upper Bound	.676622	
		5% Trimmed Mean		.645481	
		Median		.641167	
		Variance		.002	
		Std. Deviation		.0394198	
		Minimum		.5657	
		Maximum		.6890	
		Range		.1233	
		Interquartile Range		.0507	
		Skewness		-.951	.752
		Kurtosis		1.398	1.481
	2	Mean		.658979	.0076371
		95% Confidence Interval for Mean	Lower Bound	.640920	
			Upper Bound	.677038	
		5% Trimmed Mean		.658421	
		Median		.656833	
		Variance		.000	
		Std. Deviation		.0216011	
		Minimum		.6333	
		Maximum		.6947	
		Range		.0613	
		Interquartile Range		.0325	
		Skewness		.423	.752
		Kurtosis		-1.149	1.481

a. day = 7

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
pre wc	1	8	7.50	60.00
	2	8	9.50	76.00
	Total	16		

a. day = 7

Test Statistics[b,c]

	pre wc
Mann-Whitney U	24.000
Wilcoxon W	60.000
Z	-.840
Asymp. Sig. (2-tailed)	.401
Exact Sig. [2*(1-tailed Sig.)]	.442[a]

a. Not corrected for ties.

b. day = 7

c. Grouping Variable: group

Descriptives[a]

	group			Statistic	Std. Error
pre wc	1	Mean		.673071	.0073795
		95% Confidence Interval for Mean	Lower Bound	.655014	
			Upper Bound	.691128	
		5% Trimmed Mean		.673329	
		Median		.678333	
		Variance		.000	
		Std. Deviation		.0195244	
		Minimum		.6483	
		Maximum		.6932	
		Range		.0448	
		Interquartile Range		.0397	
		Skewness		-.320	.794
		Kurtosis		-2.217	1.587
	2	Mean		.656222	.0071594
		95% Confidence Interval for Mean	Lower Bound	.639713	
			Upper Bound	.672732	
		5% Trimmed Mean		.655090	
		Median		.647500	
		Variance		.000	
		Std. Deviation		.0214782	
		Minimum		.6335	
		Maximum		.6993	
		Range		.0658	
		Interquartile Range		.0321	
		Skewness		.972	.717
		Kurtosis		.554	1.400

a. day = 14

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
pre wc	1	7	10.71	75.00
	2	9	6.78	61.00
	Total	16		

a. day = 14

Test Statistics[b,c]

	pre wc
Mann-Whitney U	16.000
Wilcoxon W	61.000
Z	-1.642
Asymp. Sig. (2-tailed)	.101
Exact Sig. [2*(1-tailed Sig.)]	.114[a]

a. Not corrected for ties.

b. day = 14

c. Grouping Variable: group

Wound closure rate (%)

Descriptives[a]

	group			Statistic	Std. Error
%wc	1	Mean		15.537734	3.4604059
		95% Confidence Interval for Mean	Lower Bound	7.355174	
			Upper Bound	23.720294	
		5% Trimmed Mean		15.629561	
		Median		17.904710	
		Variance		95.795	
		Std. Deviation		9.7875059	
		Minimum		1.0066	
		Maximum		28.4160	
		Range		27.4095	
		Interquartile Range		18.1354	
		Skewness		-.269	.752
		Kurtosis		-1.274	1.481
	2	Mean		24.175698	3.4603049
		95% Confidence Interval for Mean	Lower Bound	15.993377	
			Upper Bound	32.358019	
		5% Trimmed Mean		24.237274	
		Median		24.210318	
		Variance		95.790	
		Std. Deviation		9.7872203	
		Minimum		11.0181	
		Maximum		36.2249	
		Range		25.2067	
		Interquartile Range		19.9617	
		Skewness		-.037	.752
		Kurtosis		-1.723	1.481

a. day = 7

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
%wc	1	8	6.75	54.00
	2	8	10.25	82.00
	Total	16		

a. day = 7

Test Statistics[b,c]

	%wc
Mann-Whitney U	18.000
Wilcoxon W	54.000
Z	-1.470
Asymp. Sig. (2-tailed)	.141
Exact Sig. [2*(1-tailed Sig.)]	.161[a]

a. Not corrected for ties.

b. day = 7

c. Grouping Variable: group

Descriptives[a]

	group			Statistic	Std. Error
%wc	1	Mean		59.642656	7.4812666
		95% Confidence Interval for Mean	Lower Bound	41.336656	
			Upper Bound	77.948656	
		5% Trimmed Mean		60.282875	
		Median		68.499450	
		Variance		391.785	
		Std. Deviation		19.7935710	
		Minimum		28.9342	
		Maximum		78.8272	
		Range		49.8929	
		Interquartile Range		37.8732	
		Skewness		-1.042	.794
		Kurtosis		-.826	1.587
	2	Mean		82.712381	7.3557032
		95% Confidence Interval for Mean	Lower Bound	65.750099	
			Upper Bound	99.674663	
		5% Trimmed Mean		85.219339	
		Median		89.698117	
		Variance		486.957	
		Std. Deviation		22.0671096	
		Minimum		24.5298	
		Maximum		95.7697	
		Range		71.2399	
		Interquartile Range		6.1009	
		Skewness		-2.872	.717
		Kurtosis		8.431	1.400

a. day = 14

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
%wc	1	7	5.00	35.00
	2	9	11.22	101.00
	Total	16		

a. day = 14

Test Statistics[b,c]

	%wc
Mann-Whitney U	7.000
Wilcoxon W	35.000
Z	-2.593
Asymp. Sig. (2-tailed)	.010
Exact Sig. [2*(1-tailed Sig.)]	.008[a]

a. Not corrected for ties.

b. day = 14

c. Grouping Variable: group

Neutrophil infiltration

Descriptives[a]

	group			Statistic	Std. Error
neutrophil	1	Mean		46.112500	5.4603911
		95% Confidence Interval for Mean	Lower Bound	33.200727	
			Upper Bound	59.024273	
		5% Trimmed Mean		45.363889	
		Median		40.800000	
		Variance		238.527	
		Std. Deviation		15.4443182	
		Minimum		31.9000	
		Maximum		73.8000	
		Range		41.9000	
		Interquartile Range		25.8500	
		Skewness		.857	.752
		Kurtosis		-.470	1.481
	2	Mean		25.562500	1.5364543
		95% Confidence Interval for Mean	Lower Bound	21.929363	
			Upper Bound	29.195637	
		5% Trimmed Mean		25.302778	
		Median		25.200000	
		Variance		18.886	
		Std. Deviation		4.3457492	
		Minimum		20.5000	
		Maximum		35.3000	
		Range		14.8000	
		Interquartile Range		2.9250	
		Skewness		1.764	.752
		Kurtosis		4.472	1.481

a. day = 7

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
neutrophil	1	8	12.13	97.00
	2	8	4.88	39.00
	Total	16		

a. day = 7

Test Statistics[b,c]

	neutrophil
Mann-Whitney U	3.000
Wilcoxon W	39.000
Z	-3.046
Asymp. Sig. (2-tailed)	.002
Exact Sig. [2*(1-tailed Sig.)]	.001[a]

a. Not corrected for ties.

b. day = 7

c. Grouping Variable: group

Descriptives[a]

	group			Statistic	Std. Error
neutrophil	1	Mean		18.342857	1.3426798
		95% Confidence Interval for Mean	Lower Bound	15.057438	
			Upper Bound	21.628276	
		5% Trimmed Mean		18.158730	
		Median		16.800000	
		Variance		12.620	
		Std. Deviation		3.5523969	
		Minimum		15.0000	
		Maximum		25.0000	
		Range		10.0000	
		Interquartile Range		4.5000	
		Skewness		1.210	.794
		Kurtosis		1.015	1.587
	2	Mean		10.622222	2.0204357
		95% Confidence Interval for Mean	Lower Bound	5.963089	
			Upper Bound	15.281355	
		5% Trimmed Mean		10.191358	
		Median		10.000000	
		Variance		36.739	
		Std. Deviation		6.0613072	
		Minimum		4.0000	
		Maximum		25.0000	
		Range		21.0000	
		Interquartile Range		5.3000	
		Skewness		1.820	.717
		Kurtosis		4.467	1.400

a. day = 14

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
neutrophil	1	7	12.07	84.50
	2	9	5.72	51.50
	Total	16		

a. day = 14

Test Statistics[b,c]

	neutrophil
Mann-Whitney U	6.500
Wilcoxon W	51.500
Z	-2.648
Asymp. Sig. (2-tailed)	.008
Exact Sig. [2*(1-tailed Sig.)]	.005[a]

a. Not corrected for ties.

b. day = 14

c. Grouping Variable: group

Tissue VEGF level

Descriptives[a]

	group			Statistic	Std. Error
VEGF	1	Mean		581.579930	43.6404013
		95% Confidence Interval for Mean	Lower Bound	478.386779	
			Upper Bound	684.773082	
		5% Trimmed Mean		584.640604	
		Median		629.980080	
		Variance		15235.877	
		Std. Deviation		123.4336948	
		Minimum		412.7241	
		Maximum		695.3436	
		Range		282.6195	
		Interquartile Range		252.7390	
		Skewness		-.561	.752
		Kurtosis		-1.744	1.481
	2	Mean		723.901270	53.8638717
		95% Confidence Interval for Mean	Lower Bound	596.533453	
			Upper Bound	851.269087	
		5% Trimmed Mean		716.422504	
		Median		648.344124	
		Variance		23210.533	
		Std. Deviation		152.3500357	
		Minimum		583.2918	
		Maximum		999.1285	
		Range		415.8367	
		Interquartile Range		250.5603	
		Skewness		.935	.752
		Kurtosis		-.407	1.481

a. day = 7

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
VEGF	1	8	7.25	58.00
	2	8	9.75	78.00
	Total	16		

a. day = 7

Test Statistics[b,c]

	VEGF
Mann-Whitney U	22.000
Wilcoxon W	58.000
Z	-1.051
Asymp. Sig. (2-tailed)	.293
Exact Sig. [2*(1-tailed Sig.)]	.328[a]

a. Not corrected for ties.

b. day = 7

c. Grouping Variable: group

Descriptives[a]

	group			Statistic	Std. Error
VEGF	1	Mean		316.412920	35.0892035
		95% Confidence Interval for Mean	Lower Bound	230.552732	
			Upper Bound	402.273108	
		5% Trimmed Mean		317.193527	
		Median		324.327689	
		Variance		8618.765	
		Std. Deviation		92.8373061	
		Minimum		186.7530	
		Maximum		432.0219	
		Range		245.2689	
		Interquartile Range		177.4153	
		Skewness		-.377	.794
		Kurtosis		-1.362	1.587
	2	Mean		248.381474	20.8605349
		95% Confidence Interval for Mean	Lower Bound	200.276994	
			Upper Bound	296.485954	
		5% Trimmed Mean		249.280655	
		Median		249.626494	
		Variance		3916.457	
		Std. Deviation		62.5816047	
		Minimum		150.6474	
		Maximum		329.9303	
		Range		179.2829	
		Interquartile Range		106.7605	
		Skewness		-.271	.717
		Kurtosis		-.573	1.400

a. day = 14

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
VEGF	1	7	10.43	73.00
	2	9	7.00	63.00
	Total	16		

a. day = 14

Test Statistics[b,c]

	VEGF
Mann-Whitney U	18.000
Wilcoxon W	63.000
Z	-1.430
Asymp. Sig. (2-tailed)	.153
Exact Sig. [2*(1-tailed Sig.)]	.174[a]

a. Not corrected for ties.

b. day = 14

c. Grouping Variable: group

Tissue TNF-**α** level

Descriptives[a]

	group			Statistic	Std. Error
tnf alpha	1	Mean		994.055930	39.1355806
		95% Confidence Interval for Mean	Lower Bound	901.514987	
			Upper Bound	1086.596873	
		5% Trimmed Mean		995.283525	
		Median		977.700951	
		Variance		12252.749	
		Std. Deviation		110.6921377	
		Minimum		813.5235	
		Maximum		1152.4916	
		Range		338.9681	
		Interquartile Range		162.7715	
		Skewness		.197	.752
		Kurtosis		.218	1.481
	2	Mean		420.155589	39.4808962
		95% Confidence Interval for Mean	Lower Bound	326.798105	
			Upper Bound	513.513074	
		5% Trimmed Mean		417.777787	
		Median		396.888505	
		Variance		12469.929	
		Std. Deviation		111.6688377	
		Minimum		300.0864	
		Maximum		583.0252	
		Range		282.9388	
		Interquartile Range		212.9646	
		Skewness		.576	.752
		Kurtosis		-1.380	1.481

a. day = 7

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
tnf alpha	1	8	12.50	100.00
	2	8	4.50	36.00
	Total	16		

a. day = 7

Test Statistics[b,c]

	tnf alpha
Mann-Whitney U	.000
Wilcoxon W	36.000
Z	-3.366
Asymp. Sig. (2-tailed)	.001
Exact Sig. [2*(1-tailed Sig.)]	.000[a]

a. Not corrected for ties.

b. day = 7

c. Grouping Variable: group

Descriptives[a]

	group			Statistic	Std. Error
tnf alpha	1	Mean		794.524125	96.8663564
		95% Confidence Interval for Mean	Lower Bound	557.500690	
			Upper Bound	1031.547561	
		5% Trimmed Mean		789.952121	
		Median		784.096802	
		Variance		65681.637	
		Std. Deviation		256.2842894	
		Minimum		532.4114	
		Maximum		1138.9329	
		Range		606.5215	
		Interquartile Range		579.4041	
		Skewness		.426	.794
		Kurtosis		-1.634	1.587
	2	Mean		95.730071	65.7071425
		95% Confidence Interval for Mean	Lower Bound	-55.790872	
			Upper Bound	247.251013	
		5% Trimmed Mean		71.410440	
		Median		29.040622	
		Variance		38856.857	
		Std. Deviation		197.1214274	
		Minimum		9.6802	
		Maximum		619.5333	
		Range		609.8531	
		Interquartile Range		38.7208	
		Skewness		2.960	.717
		Kurtosis		8.819	1.400

a. day = 14

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
tnf alpha	1	7	12.57	88.00
	2	9	5.33	48.00
	Total	16		

a. day = 14

Test Statistics[b,c]

	tnf alpha
Mann-Whitney U	3.000
Wilcoxon W	48.000
Z	-3.021
Asymp. Sig. (2-tailed)	.003
Exact Sig. [2*(1-tailed Sig.)]	.001[a]

a. Not corrected for ties.

b. day = 14

c. Grouping Variable: group

Collagen deposition

Descriptives[a]

	group			Statistic	Std. Error
collagen	1	Mean		15.813914	.6935917
		95% Confidence Interval for Mean	Lower Bound	14.173830	
			Upper Bound	17.453997	
		5% Trimmed Mean		15.809418	
		Median		15.881130	
		Variance		3.849	
		Std. Deviation		1.9617737	
		Minimum		12.9085	
		Maximum		18.8002	
		Range		5.8917	
		Interquartile Range		3.3162	
		Skewness		-.034	.752
		Kurtosis		-.704	1.481
	2	Mean		17.875052	2.0364020
		95% Confidence Interval for Mean	Lower Bound	13.059727	
			Upper Bound	22.690377	
		5% Trimmed Mean		17.714764	
		Median		17.126652	
		Variance		33.175	
		Std. Deviation		5.7598145	
		Minimum		10.3844	
		Maximum		28.2509	
		Range		17.8666	
		Interquartile Range		8.7218	
		Skewness		.702	.752
		Kurtosis		.145	1.481

a. day = 7

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
collagen	1	8	7.75	62.00
	2	8	9.25	74.00
	Total	16		

a. day = 7

Test Statistics[b,c]

	collagen
Mann-Whitney U	26.000
Wilcoxon W	62.000
Z	-.630
Asymp. Sig. (2-tailed)	.529
Exact Sig. [2*(1-tailed Sig.)]	.574[a]

a. Not corrected for ties.

b. day = 7

c. Grouping Variable: group

Descriptives[a]

	group			Statistic	Std. Error
collagen	1	Mean		30.985904	1.6092956
		95% Confidence Interval for Mean	Lower Bound	27.048100	
			Upper Bound	34.923709	
		5% Trimmed Mean		30.785385	
		Median		28.780432	
		Variance		18.129	
		Std. Deviation		4.2577960	
		Minimum		27.3880	
		Maximum		38.1931	
		Range		10.8051	
		Interquartile Range		7.4049	
		Skewness		1.239	.794
		Kurtosis		-.310	1.587
	2	Mean		47.505328	1.7761378
		95% Confidence Interval for Mean	Lower Bound	43.409547	
			Upper Bound	51.601109	
		5% Trimmed Mean		47.794815	
		Median		47.829925	
		Variance		28.392	
		Std. Deviation		5.3284133	
		Minimum		35.7477	
		Maximum		54.0522	
		Range		18.3045	
		Interquartile Range		5.9672	
		Skewness		-1.290	.717
		Kurtosis		2.667	1.400

a. day = 14

Ranks[a]

	group	N	Mean Rank	Sum of Ranks
collagen	1	7	4.29	30.00
	2	9	11.78	106.00
	Total	16		

a. day = 14

Test Statistics[b,c]

	collagen
Mann-Whitney U	2.000
Wilcoxon W	30.000
Z	-3.123
Asymp. Sig. (2-tailed)	.002
Exact Sig. [2*(1-tailed Sig.)]	.001[a]

a. Not corrected for ties.

b. day = 14

c. Grouping Variable: group

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