การประยุกต์ใช้สารโปรติโอไกลแคนจากกระดูกอ่อนของปลาเพื่อเร่งการหายของบาดแผลไหม้

นายสิทธิพล บุญมั่น



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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# APPLICATION OF PROTEOGLYCAN FROM FISH CARTILAGE FOR THE ACCELERATION OF BURN WOUND HEALING

Mr. Sitthiphon Bunman


A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Pharmacology
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| | |
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สิทธิพล บุญมั่น : การประยุกต์ใช้สารโปรตีโอไกลแคนจากกระดูกอ่อนของปลาเพื่อเร่งการหายของบาดแผลไหม้ (APPLICATION OF PROTEOGLYCAN FROM FISH CARTILAGE FOR THE ACCELERATION OF BURN WOUND HEALING) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ภัสราภา โตวิวัฒน์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: พรอนงค์ อร่ามวิทย์, นพดล ลาภเจริญทรัพย์, 129 หน้า.

แผลไหม้เป็นแผลที่พบได้บ่อยและมีค่าใช้จ่ายทางการแพทย์สูง ในแต่ละปีมีผู้ป่วยแผลไหม้สูงถึง 250,000 คน พบได้ทุกเพศทุกวัย ซึ่งในปัจจุบันยาทามาตรฐานที่นิยมใช้ในการรักษาแผลไฟไหม้คือ ครีมซิลเวอร์ซัลฟาไดอะซีนในความเข้มข้นร้อยละ 1 ซึ่งครีมนี้มีข้อดีในการต่อต้านเชื้อจุลชีพได้กว้าง สามารถควบคุมเชื้อรายีสต์แบคทีเรียแกรมบวกและแกรมลบรวมถึงแบคทีเรียดื้อยา ใช้ง่ายและราคาไม่แพง แต่มีข้อด้อยคือทำให้แผลหายช้าอันเนื่องมาจากซิลเวอร์จะไปยับยั้งกระบวนการสร้าง DNA มีการศึกษาพบว่า ภายในกระดูกอ่อนของปลาประกอบด้วยสารโปรตีโอไกลแคนซึ่งมีโครงสร้างโมเลกุลคล้ายกับ epidermal growth factor ซึ่งมีส่วนสำคัญต่อกระบวนการเร่งหายของบาดแผล ดังนั้นหากนำเอาโปรตีโอไกลแคนมาใช้ร่วมกับครีมซิลเวอร์ซัลฟาไดอะซีนร้อยละ 1 ในการรักษาแผลไฟไหม้น่าจะสามารถเร่งกระบวนการหายของแผลได้อย่างมีประสิทธิภาพ การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์ของโปรตีโอไกลแคนที่สกัดได้จากกระดูกอ่อนของปลาในการเร่งการหายของแผลไฟไหม้เมื่อใช้เป็นยาเดี่ยวหรือเมื่อใช้ร่วมกับครีมซิลเวอร์ซัลฟาไดอะซีนในความเข้มข้นร้อยละ 1 โดยทำการศึกษาในหนูทดลองที่ได้รับการทำให้เกิดแผลไหม้ในระดับสองแล้ววัดร้อยละการหายของบาดแผลในวันที่ 3, 7, 14, 21, 28 รวมถึงศึกษาจุลกายวิภาคศาสตร์ของเนื้อเยื่อผิวหนังในวันที่ 7, 14 และ 21 หลังจากการเหนี่ยวนำให้เกิดแผลไฟไหม้ โดยตรวจวิเคราะห์จำนวนของ multinucleated giant cells, macrophages cells, mast cells และหลอดเลือดที่สร้างใหม่จากเนื้อเยื่อของสัตว์ทดลองรวมถึงตรวจเอนไซม์ที่เกี่ยวข้องกับการทำงานของตับได้แก่ aspartate aminotransferase, alanine transaminase, alkaline phosphatase และตรวจวัดการทำงานของไตจาก blood urea nitrogen และ creatinine จากซีรัมเพื่อศึกษาความเป็นพิษของสารที่ใช้ทดสอบด้วย

ผลการศึกษาร้อยละการหายของแผลและร้อยละของ re-epithelialization พบว่า หนูกลุ่มที่ได้รับครีมซิลเวอร์ซัลฟาไดอะซีนร้อยละ 1, ครีมโปรตีโอไกลแคนร้อยละ 1 หรือ 2, ครีมซิลเวอร์ซัลฟาไดอะซีนร้อยละ 1 ร่วมกับโปรตีโอไกลแคนร้อยละ 1 และครีมซิลเวอร์ซัลฟาไดอะซีนร้อยละ 1 ร่วมกับโปรตีโอไกลแคนร้อยละ 2 มีการหายของแผลสูงกว่าอย่างมีนัยสำคัญทางสถิติเปรียบเทียบกับ cream base นอกจากนี้ยังพบว่าหนูกลุ่มที่ได้รับครีมซิลเวอร์ซัลฟาไดอะซีนร้อยละ 1 ร่วมกับโปรตีโอไกลแคนร้อยละ 2 จะมีร้อยละการหายของแผลสูงกว่าอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับหนูกลุ่มที่ได้รับครีมซิลเวอร์ซัลฟาไดอะซีนร้อยละ 1 และครีมโปรตีโอไกลแคนร้อยละ 1 ผลจากการศึกษาจุลกายวิภาคศาสตร์ของเนื้อเยื่อผิวหนังพบว่า จำนวนของ multinucleated giant cells และ macrophages ในวันที่ 7, 14, 21 และจำนวนของ mast cells ในวันที่ 7 และ 14 ของหนูที่ได้รับครีมในกลุ่มต่าง ๆ ไม่แตกต่างกันอย่างมีนัยสำคัญ เมื่อเปรียบเทียบกับกลุ่มควบคุม นอกจากนี้ยังพบว่าจำนวนของ multinucleated giant cells, macrophages และ mast cells สูงสุดในวันที่ 7 และลดลงในวันที่ 14 และ 21 ตามลำดับ การศึกษาจำนวนของหลอดเลือดที่สร้างใหม่พบว่าวันที่ 7 และ 14 พบว่า หนูกลุ่มที่ได้รับครีมซิลเวอร์ซัลฟาไดอะซีนร้อยละ 1 ร่วมกับ โปรตีโอไกลแคนร้อยละ 2 มีจำนวนของหลอดเลือดที่สร้างใหม่สูงกว่าอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับหนูกลุ่มที่ได้รับ cream base และครีมซิลเวอร์ซัลฟาไดอะซีนร้อยละ 1 จำนวนของหลอดเลือดที่สร้างใหม่สูงที่สุดในวันที่ 14 และจะลดลงในวันที่ 21 ตามลำดับ ในด้านความปลอดภัยจากการตรวจเอนไซม์วัดการทำงานของตับพบว่า ระดับเอนไซม์ aspartate aminotransferase, alanine transaminase และ alkaline phosphatase ไม่แตกต่างกันอย่างมีนัยสำคัญ เมื่อเปรียบเทียบกับหนูกลุ่มควบคุมส่วนตรวจวัดการทำงานของไตพบว่าระดับของ blood urea nitrogen และ creatinine ไม่แตกต่างกันอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับหนูกลุ่มควบคุม

การศึกษานี้แสดงให้เห็นว่าโปรตีโอไกลแคนเมื่อใช้เป็นยาเดี่ยวหรือใช้ร่วมกับครีมซิลเวอร์ซัลฟาไดอะซีนร้อยละ 1 สามารถเร่งกระบวนการหายของแผลได้เนื่องจากโปรตีโอไกลแคนจะไปเร่งกระบวนการสร้างเส้นเลือดใหม่ทำให้แผลหายเร็วขึ้นและในด้านความปลอดภัยไม่พบความผิดปกติของการทำงานของตับและไตหลังได้รับสารทดสอบ

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# # 5587242820 : MAJOR PHARMACOLOGY
KEYWORDS: PROTEOGLYCAN / FISH CARTILAGE / WOUND HEALING / BURN WOUNDS

SITTHIPHON BUNMAN: APPLICATION OF PROTEOGLYCAN FROM FISH CARTILAGE FOR THE ACCELERATION OF BURN WOUND HEALING. ADVISOR: ASST. PROF. FLG. OFF. PASARAPA TOWIWAT, Ph.D., CO-ADVISOR: ASSOC. PROF. PORNANONG ARAMWIT, Pharm.D, Ph.D., ASSOC. PROF. NOPPADOL LARBCHAROENSUB, M.D., 129 pp.

Burn wounds are common and the cost of treatment is normally intensive. There are over 250,000 burn patients yearly worldwide and may occur in any age groups in both genders. 1% silver sulfadiazine (SSD) is used as a standard treatment for burn wounds. The benefits of this medication is due to its broad-spectrum antimicrobial activities, which also has activity on mold, yeast, and bacteria, both gram positive and negative as well as drug-resistant bacteria; it can also be applied easily and inexpensive. However, the disadvantage of SSD is a delay in the wound-healing processes since SSD can inhibit DNA synthesis. According to the previous studies, fish bone cartilage composes of a variety of proteoglycans (PGs), which is an epidermal growth factor-like structure. The growth factor is essential for wound healing processes. Thus, using PG combined with 1% SSD for the treatment of burn wounds can accelerate wound healing cascade effectively. The objective of this study is to investigate the activity of PG extracted from fish cartilage alone or in combination with 1% SSD for facilitation of second-degree burn wound healing in an *in vivo* model. The percentage of wound healing was evaluated on day 3, 7, 14, 21 and 28 and histological analysis of animal tissues (number of multinucleated giant cells, macrophages, mast cells and neovascularization) on day 7, 14 and 21 after second degree burn inducing. Furthermore, liver function tests (aspartate aminotransferase, alanine transaminase and alkaline phosphatase levels) and kidney function tests (blood urea nitrogen and serum creatinine) were evaluated for toxicity of the compound.

The results showed that, the percentage of wound healing and re-epithelialization of treated groups (1% SSD, 1% PG, 2% PG, 1% SSD combined with 1% PG and 1% SSD combined with 2% PG) were significant higher compared to the control group (cream base-treated wounds). Moreover, the percentage of wound healing in the group received 1% SSD together with 2% PG was significant higher compared to the group received 1% SSD and 1% PG. The results of histological analysis showed that number of multinucleated giant cells and macrophages on day 7, 14 and 21 and mast cells on day 7 and 14 of treated groups (1% SSD, 1% PG, 2% PG, 1% SSD combined with 1% PG and 1% SSD combined with 2% PG) showed no significant difference compared to control group. Number of multinucleated giant cells, macrophage and mast cells reached the peak level on day 7 and then continuously decreasing on day 14 and 21, respectively. Evaluation of the number of neovascularization on day 7 and 14 showed that the group treated with 1% SSD combined with 2% PG had significant higher neovascularization compared to the control group and the group received 1% SSD. The highest number of neovascularization was found on day 14 and decreased on day 21, respectively. For safety evaluation, the level of aspartate aminotransferase, alanine transaminase and alkaline phosphatase showed no significant difference compared to control group. Kidney function test showed that the level of blood urea nitrogen and serum creatinine also showed no significant difference compared to control group.

The results from this study indicated that PG alone or in combination with 1% SSD can facilitate the wound healing by activation of neovascularization process. For safety evaluation, no abnormalities of liver and kidney function were found after exposure to the tested substance.

Field of Study: Pharmacology
Academic Year: 2014

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| | | |
|---|---|---|
| / | = | per |
| % | = | Percent |
| > | = | Greater than |
| < | = | Less than |
| $Ag^+$ | = | Silver ions |
| bFGF | = | Basic fibroblast growth factor |
| °C | = | Degree Celsius |
| cm | = | Centimeter |
| CS | = | Chondroitin sulfate |
| DNA | = | Deoxyribonucleic acid |
| CSPG | = | Chondroitin sulfate proteoglycan |
| DS | = | Dermatan sulfate |
| DSPG | = | Dermatan sulfate proteoglycan |
| EGF | = | Epidermal growth factor |
| EGFRs | = | Epidermal growth factor receptor |
| ER | = | Endoplasmic reticulum |
| FGF-2 | = | Fibroblast growth factor 2 |
| g | = | Gram |
| GAGs | = | Glycosaminoglycans |
| GalNAc | = | N-acetylgalactosamine |
| GlcNAc | = | N-acetylglucosamine |
| HA | = | Hyaluronan |
| hr | = | Hours |
| HS | = | Heparan sulfate |
| K | = | Lysine |

| | | |
|---|---|---|
| KS | = | Keratan sulfate |
| kDa | = | Kilodalton |
| ml | = | Milliliter |
| MRSA | = | Methicillin resistant *Staphylococcus aureus* |
| NO. | = | Number |
| *p* | = | *P*-value |
| PDFG | = | Platelet- derived growth factor |
| PG | = | Proteoglycan |
| R | = | Arginine |
| RER | = | Rough endoplasmic reticulum |
| s | = | Second |
| SC | = | Subcutaneous |
| $sq^2m$ | = | Square meter |
| SSD | = | Silver sulfadiazine |
| TBSA | = | Total body surface area |
| TGF-β | = | Transforming growth factor beta |
| μm | = | Micrometer |
| VEGF | = | Vascular endothelial growth factor |
| VRE | = | Vancomycin Resistant *Enterococci* |
| Y | = | Tyrosine |

# CHAPTER I

# INTRODUCTION

## Background and Rationale

A burn wound is a type of skin injury caused by tissue damage after exposure to heat, chemicals, electrical, or radiation. Burns are one of the most types of wound found in patients. Burn injury affects the patients both physically and psychologically. There are up to 250,000 burn patients each year. Burns can occur in all age groups, from babies to the elderly, and are a problem in both developed and developing country. The major causes of burn injury are classified as follows: flame exposure (55%), hot water exposure (scalds; 40%), and chemical and electrical exposure (5%). The incidence of burn injury is classified by age as follows: 16-64 years old (60%), 1-4 years old (20%), 5-14 years old (10%), and over 65 years old (10%) (S. Hettiaratchy & P. Dziewulski, 2004).

Burn wounds can affect the transformation of blood vessels, promote the excretion of cytokines and several types of inflammatory mediators, which result in vasodilation, increased vascular permeability and rapid formation of local edema (Kloppenberg, Beerthuizen, & ten Duis, 2001; Korthuis, Anderson, & Neil Granger, 1994; Mayers & Johnson, 1998). The severity of burn injury depends on the percentage of the total body surface area damage (% TBSA) and depths of skin layer damage (degree of burn depth). It has been indicated that more than 30% of total body surface area damaged could influence a change in the physical systems such as cardiovascular, respiratory, and immune systems (S. Hettiaratchy & P. Dziewulski, 2004). Burn wounds caused by heat exposure usually take a long time for recovery. If the patients have not been cured properly, they might have complications, resulting in delayed wound healing and may develop hypertrophic scars or scar contracture

(Papini, 2004), which could affect their personality and career. It is generally found that if the wounds heal quickly then fewer complications will be followed. Thus, the healing period is important to the patient's life.

Nowadays, there are many methods for treating burn wounds including medication, surgery or the new techniques such as amniotic membrane, cytokine and gene therapies (Atiyeh, Hayek, & Gunn, 2005) which depend on the severity level of the burns. Medications are the most common and easiest way to treat burns, and 1% of silver sulfadiazine (SSD) cream is used as a gold standard for the treatment of burn wounds. When SSD is exposed to the wound, it will ionize to $Ag^{+}$ and inhibit the enzymes involved in the electron transport chain and bacterial DNA replication process, resulting in killing of bacteria. SSD is absorbed through the skin to the blood vessels and then being transported to the liver, where the metabolism occurs and more than 50% is elimination via the kidney system in the original chemical form. A previous study showed that $Ag^{+}$ of SSD is toxic to the bone marrow, which leads to abnormal blood synthesis, including delayed wound healing (Atiyeh, Costagliola, Hayek, & Dibo, 2007) and also affects liver and kidney (Baldi, Minoia, Di Nucci, Capodaglio, & Manzo, 1988; Chaby et al., 2005). Thus, use of this drug in the patients that have hepatic or renal problems should be avoided (Baldi et al., 1988; Chaby et al., 2005; Trop et al., 2006). SSD is presented as cream, ointment coats on the bandage, or wound-covered silicone. The benefits of using this medication are that it has broad-spectrum antimicrobial activity which could control the amount of mold, yeast, and bacteria, including drug-resistant bacteria such as Methicillin-Resistant *Staphylococcus aureus* (MRSA) and Vancomycin-Resistant *Enterococci* (VRE). It can also be applied easily and has low price. However, the disadvantage of SSD is a delay in the wound-healing process (Atiyeh et al., 2007; Baldi et al., 1988; Singer & Dagum, 2008).

The epidermal growth factor (EGF) was discovered in 1962 and is composed of 53 amino acids which are arranged as polypeptides. It acts as an important substance in the body's fluid such as milk, saliva, gastric juice, tears and cerebrospinal fluid (Gregory, Walsh, & Hopkins, 1979; Hirata, Uchihashi, Nakajima, Fujita, & Matsukura, 1982; Ohashi et al., 1989). Previous study found that the epidermal growth factor (EGF) binds to the receptor called the "epidermal growth factor receptor" (EGFRs) on the cell surface to activate the re-epithelialization and wound-healing process (Yataes, Nanney, Gates, & king, 1991). At present, there are many cosmetic companies that have added EGF to their products in order to enhance collagenesis and wrinkle reduction (Brown et al., 1989). Besides the cosmetic usage, EGF can also be used clinically for enhancing the wound-healing process, especially for chronic wounds, and has been used to treat wounds in diabetic and cancer patients after radio therapy (Hong et al., 2009; Namviriyachote, 2012; Tuyet et al., 2009). EGF is an expensive agent because it can not be synthesized domestically in Thailand and has to be imported only. EGF can be produced using human recombinant technique, which is a high-cost process and prohibited in some countries.



Cartilage composed of more than 90% dry weight of proteoglycans (PGs), (Hardingham & Fosang, 1992) which is the core structure in extracellular matrix. PG in vertebates can be characterized by the type of glycosaminoglycans that binds to the core protein structure in 5 groups: hyaluronan (HA), chondroitin sulfate (CS), keratan sulfate (KS), dermatan sulfate (DS), and heparan sulfate (HS) (Perrimon & Bernfield, 2001). The PG that has been found in fish cartilage are chondroitan sulfate (CS) and dermatan sulfate (DS) (Knudson & Knudson, 2001; Neelam, 2013).

It has been found that PG plays an important role in the wound-healing process because its molecular stucture is similar to EGF from the arrangement of amino acids at KRKK $K_{93-97}$ $KKR_{103-105}$ and $RKYK_{110-113}$ (K: Lysine, R: Arginine and Y:

Tyrosine) (Figue 1 ) (Raab & Klagsbrun, 1997). It has bioactive mechanisms which are similar to EGF regarding the wound healing process such as promoting epidermal growth and regeneration of tissues and blood vessels (Knudson & Knudson, 2001; Perrimon & Bernfield, 2001). We then proposed that PG might have wound healing activity as EGF.

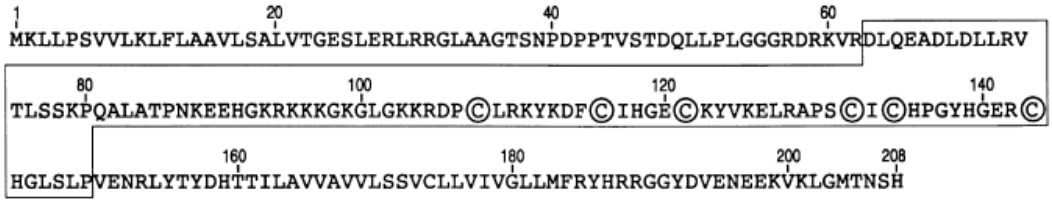



**Figure 1** Arrangement of amino acids at KRKK $K_{93\text{-}97}$ $KKR_{103\text{-}105}$ and $RKYK_{110\text{-}113}$ of Heparan sulfate PG which is similar to epidermal growth factor (Raab & Klagsbrun, 1997).

In 1992, Japanese researchers had first discovered and developed PG extraction from nasal tip cartilage of salmon (*Oncorhynus keta*) for commercial uses and named it "Proteoglycan-IPC" (Ichimaru Pharcos Co, LTD., Gifu, Japan). This material has been added to cosmetics to promote collagen synthesis and wrinkle relief.

Fishbone head is composed of cartilage, which contains a high amount of PG. In general, this part of the fish would be discarded or used as animal feed after fish processing such as Pla-Som. Therefore, it would be beneficial to use fish bone cartilage for extraction of PG for medical use and increase the value of agricultural waste as well.

The aim of this study is to investigate the activity of PG extracted from fish cartilage in facilitating burn wound healing in an *in vivo* model. In this study, percentage wound healing was evaluated on day 3, 7, 14, 21, 28 and histological analyses was conducted on day 7, 14 and 21 after burn wounds were induced in

order to evaluate the re-epithelialization and number of inflammation cells, such as multinucleated giant cells, macrophages, mast cells, and neovascularization.

The study was then designed to compare percentage wound healing of animals treated with cream base (control group), silver sulfadiazine (SSD) cream (positive control), PG cream or combination of PG with SSD cream in burn wounds. Moreover, this study also investigated toxicity to the liver and kidney of SSD, PG and combination of PG and SSD cream in animals by measuring the liver enzyme level, including aspartate aminotransferase, alanine transaminase, and alkaline phosphatase, and levels of blood urea nitrogen and creatinine.

**Research objectives**

1. To investigate the efficacy of cream base, silver sulfadiazine (SSD), PG extracted from fish cartilage, and combination of PG and SSD cream in treating burn wounds in an *in vivo* model.
2. To investigate the histology of wounds after receiving cream base, SSD, PG and combination of PG and SSD cream.
3. To investigate the safety profile of SSD, PG and combination of PG and SSD.

**Research hypotheses**

1. PG extracted from fish cartilage and combination of PG and SSD can facilitate burn wound healing by stimulating epithelialization and angiogenesis processes.
2. PG and combination of PG and SSD are less toxic to the liver and kidney compared to SSD alone.

**Benefits from the study**

Information regarding the efficacy of PG extracted from fish cartilage and combination of PG and SSD for the treatment of burn wounds will lead to the use of PG for substitution of EGF in the treatment of burn wounds. Information about liver and kidney safety profiles will also be obtained.

**Keywords**

Proteoglycan

Fish Cartilage

Wound healing

Burn wounds

# CHAPTER II

# LITERATURE REVIEW

## Skin

The skin is the largest organ of the body which is composed of many structures such as blood vessels, lymphatic vessels, nerve endings, sweat glands, and so on. The average size of the adult skin which covers the entire surface area is approximately 1.5–2 $sq^2m$ and weighs about 15 percentage of total body weight (Richardson, 2003). Its thickness ranges from 0.5 mm to 4 mm in depth according to the specific function; for example, the thicker areas of the skin tend to be found where there is more friction such as the palms of the hands or palms of the feet, and the thinnest area belongs to the eyelids, where the skin needs to be more flexible (2006).

## Structure of the skin

The structure of the skin consists of 3 layers. The outer layer is called the epidermis, and the second layer is the connective tissue part, which is called dermis; and the deepest layer is subcutaneous (SC) and is called the hypodermis. This layer consists of areolar and adipose tissue (Figure 2).

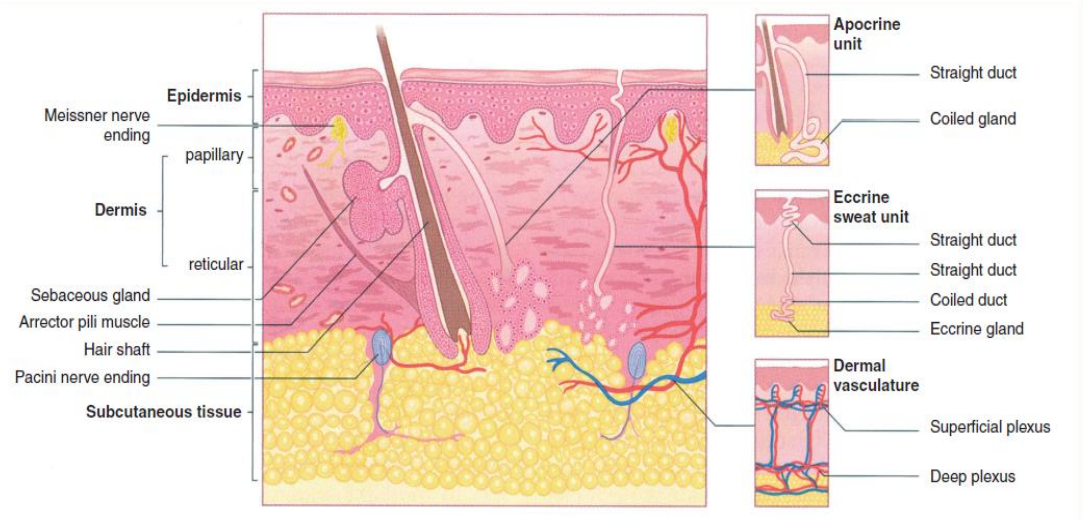



**Figure 2** Structure of the skin (James, Berger, & Elston, 2006).

## Epidermis

The epidermis or cuticle is a stratified squamous epithelial tissue which is mainly composed of keratinocytes and dendritic cells (Murphy, 1997). This layer contains large amounts of melanocyte, which is a melanin-producing cell. This type of cell will be transferred to the dermis layer, which is composed of 5 layers. First is the stratum corneum layer, a death cells which located at the outer layer. It is a flat-shaped, does not have any nucleus, and contains keratin. The stratum lucidum is a translucent layer which is found in thick skin like the palms and soles. Stratum granulosum consists of 3-5 sublayers which is generated for the thick skin area. It is flat and square in shape and contains rough granules, which are an intermediate of keratin. This type of skin layer might not be present in thin skin areas. The stratum mucosum or Malphiglan layer is composed of many layers of cells, separated into 2 types: Stratum spinosum and Stratum germinativum. The shape of the Stratum spinosum varies from cubic to polyhedron-like and flat. This cell layer consists of many stratums, depending on the thickness of the epidermis layers. The inner layer

of the epidermis is the stratum germinativum, which is located at the junction of the dermis and epidermis. It is rod-shaped and has a high proliferation rate because it has to compensate for the exfoliated dead cells (James et al., 2006; Murphy, 1997). Furthermore, Melanocyte, which is a melanin pigment producing cell, can be found in this layer.

Within the epidermis layers, the capillaries are not present and therefore all of the nutrients are transferred to each layer through the diffusion from the capillaries in the dermis. This is the reason that the upper cells supposed to death from starvation and then exfoliate away from the surface as scurf. Actually, the skin turn-over would be around 12-14 days if the upper layer is exposed to any cold, and the epidermis proliferation, which occurs during bedtime or relaxation, would change.

**Dermis**

The Dermis or corium is located under the epidermis layers and it consists of connective tissues. The layer thickness depends on the body area, with the thickness part belonging to the palm of the hand, which is around 3 mm in depth. The Dermis is separated into 2 layers. The papillary layer, detached to the dermis that contains a curve shaped called dermal papilla. This type of layer could be found in the palm of the hand, the soles of the feet, and also the lips, penis, and nipples in contrast to the eyelids and scrotum. The other is the reticular layer, which contains many fibres (collagen elastic and reticular fibres), capillaries and nerves. The size and amount of collagen elastic and reticular fibres increase with age. In this layer, we also find capillaries, lymph ducts, sensory nerve endings, and other components which are transformed from the dermis such as sweat glands, sebaceous glands, and hair

follicles, except for the palms and the soles, which do not contain any sebaceous glands or hair follicles (James et al., 2006).

**Subcutaneous layer**

This is deep apart from the dermis layer, called the subcutaneous or hypodermis layer or superficial fascia, and this layer consists of loose connective tissue and adipose tissue which promote skin movement. Furthermore, this layer also contains capillaries and nerves. The amount of adipose tissues can be used to distinguish the dermis and subcutaneous, except for the area which has no the adipose tissues such as the eyelid, scrotum, and penis.

**Function of the skin**

**Maintaining body temperature**

Body temperature is controlled by sensing the outer of environmental temperature via the nervous system and then adjusting to vasodilatation, increase blood flow to skin and sweat production accordingly to try and maintain the body temperature, estimated at around 37 °C (2006). It does this by producing more sweat from the sweat glands, which evaporates and cools, and by blood vessel dilation to reduce the temperature.

**Protection against infection and ultraviolet rays**

The stratum corneum is a tightly-interlocking layer of dead cells that guard against infections entering via the skin. It is also mostly waterproof, preventing the loss or entry of fluids. The acidic pH balance controls and inhibits the growth of bacteria on skin. Trauma to the skin creates an opportunity for invasion by micro-organisms and results in an inflammatory response characterized by redness,

swelling, localized heat, pain and pyrexia (Richardson, 2003). The skin protects the body from harmful ultraviolet (UV) rays. The pigment melanin is produced in special cells called melanocytes, which are found at the base of the epidermis. Melanin production is influenced by sunlight.

**Sensation**

The skin is the body's largest sensory organ and its sensory (or afferent) nerve receptors detect a number of different stimuli: mechanical, such as pressure or vibration or stretching; and thermal, in terms of heat and cold (2006).

**Absorption and excretion**

Although the skin is almost waterproof, some lipid soluble materials can penetrate it. Small molecules of carbon dioxide, oxygen, nitrogen can be absorbed through the skin. In addition to water, sweat, and a number of waste products, including sodium chloride and urea, can be excreted through the skin.



**Secretion**

Sebum is secreted from the sebaceous glands; the sebum balances the skin acid mantle. Sebum makes the skin oily when there is over production. It also moisturizes the skin, keeping it soft and supple and therefore prevents cracks and openings in the skin.

**Vitamin D synthesis**

The skin can be synthesis vitamin D, when are contact UV (ultraviolet) light or sunlight. The UV light converts fatty substance into vitamin D.

**The wound and definition of a wound**

A wound is an injury to a part of the body, especially one in which a break is made in the skin or tissue. There are various types of wounds, including an incised wound, a lacerated wound, abrasions, contusions, ulcers, and burn wounds (Somboonwong, Kankaisre, Tantisira, & Tantisira, 2012).

**Wound-healing process**

Wound healing is process to heal of physical after body or tissue injuried, this process involving soluble mediators such as cytokines or growth factors, blood cells such as platelet and white blood celsl, extracellular matrix, and parenchymal cells. Wound healing has four phases: hemostasis, inflammation, tissue proliferation and tissue maturation or remodeling that overlap in time (Figure 3) (Li, Chen, & Kirsner, 2007; Singer & Dagum, 2008).

**Hemostasis phase**

Skin injury can cause vasoconstriction, which occurs immediately in order to stop the bleeding. Afterwards, platelet aggregation will emerge, accompanied with the release of some clotting factors from the cells which stimulate the coagulation and complement cascades through intrinsic and extrinsic coagulation pathways. Then, prothrombin is triggered to be assembled as thrombin as with fibrinogen, which is transformed to fibrin to stop the bleeding at the wound site. It is not only the platelet release clotting factor that is involved here; they also provide cytokine and growth factors such as PDFG (Platelet- Derived Growth factor), EGF (Epidermal Growth Factor) and TGF-β (Transforming Growth Factor-β), Insulin-like growth factor-1, and platelet factor-IV, which facilitate the wound-healing process (Enoch & Leaper, 2005).

**Inflammatory phase**

This phase occurs within 24 h after the skin is injured and continues to several days or weeks depending on the level of the injury. After blocking the bleeding wound, the aggregated platelets will secrete an chemo-attractant to activate the inflamed cells in order to start the wound-healing process. Specific enzymes are secreted from the cell membrane to stimulate prostaglandins and leukotrienes synthesis. Afterwards, the wound-surrounded tissues and capillary endothelial cells will secrete the histamines which permeated the capillary wall and increase the interstitial space, resulting in fluid flows from the capillaries to the interstitial space. This process causes edema, which has 2 phases: in the initial phase, edema will occur immediately after mast cells secrete histamine, and leukotrienes, which cause vasodilation. Clotting products such as kinas and thrombin also enhance capillary permeability.

The second phase is caused by the penetration of the fluid through the capillaries. Moreover, there is activation of the mediator combined with peptides called cytokine, which function as cellular immune response enhancers, responding to the antigen and then white blood cells such as macrophage, neutrophil and monocytes are leaked driven to the interstitial space surrounded the wound. Neutrophils will drive away all of the dead cells using protease enzymes, which degrade the white blood cells. The monocytes are transformed to macrophage, which defends against dead cells and bacteria. After macrophage release the several types of growth factor which are angiogenesis factor stimulate the endothelial cells of wound surrounded capillaries resulting in the generation of new capillaries to the wound, and also produce some growth factor such as PDFG, EGF and TGF-$\beta$ which promote the synthesize of fibroblast which then move to the center of the wound for proliferation (Enoch & Leaper, 2005; Mayers & Johnson, 1998).

**Proliferation phase**

This phase occurs from 3 days up to 2-4 weeks after the injury and might overlap with the end of the inflammatory phase. There is the movement of the fibroblast, which results in the generation of tissues for filling in the injured skin (Enoch & Leaper, 2005). Fibroblast functions as a collagen producer, which causes the wound to adhere. Vitamin C, oxygen and iron are essential for the production of collagen, which is processed in the fibroblast cells through the synthesis of tripeptides.

**Neovascularization**

After the inflammation phase, local factor in the wound of microenviromental as decrease of oxygen tension, decrease of pH and increase lactate actually initate release some growth factor to contribute new capillary (LaVan & Hunt, 1990). This process is called neovascularization or angiogenesis and hypoxia induce macrophages secreted angiogenic growth factor such as VEGF, basic Fibroblast Growth Factor (bFGF) and TGF-β (Battegay, 1995; Tonnesen, Feng, & Clark, 2000).



**Re-epithelialization**

The process of epithelialization is stimulated by the presence of EGF and TGF-β, which are produced by being activated at the wound site by macrophage, platelets, and keratinocytes (Yataes et al., 1991). The keratinocytes are migrate cover on the skin surface, and are proliferated and migrate across the wound. When the keratinocytes cover the wound completely with new epidermal cells, the wound is closed completely.

**Maturation or remodeling phase**

The maturation or remodeling phase takes place after the wound is healed. At the beginning, there is redness, swelling. and itchiness. Protein degradation of the unused protein and also collagen rearrangement are processed here. Actually, this phase takes around 6 months but in some cases it could continue to 2 years

All of the wound-healing processes could be summarized as follows: there are natural repairing processes which are complicated, continue, and overlap in order to complete the wound-healing process. For chronic wounds, these processes cannot continue consecutively; the process would be arrested at some phase. Chronic wounds are caused by repeated trauma, ischemia or necrotic tissue or infection, or a disease such as diabetes, including defective white blood cell activity, the arrest of all cells transformation processes, a decrease of fibroblast level resulting in collagen synthesis deficiency, when the growth factor is broken down which blocks the proliferation phase.

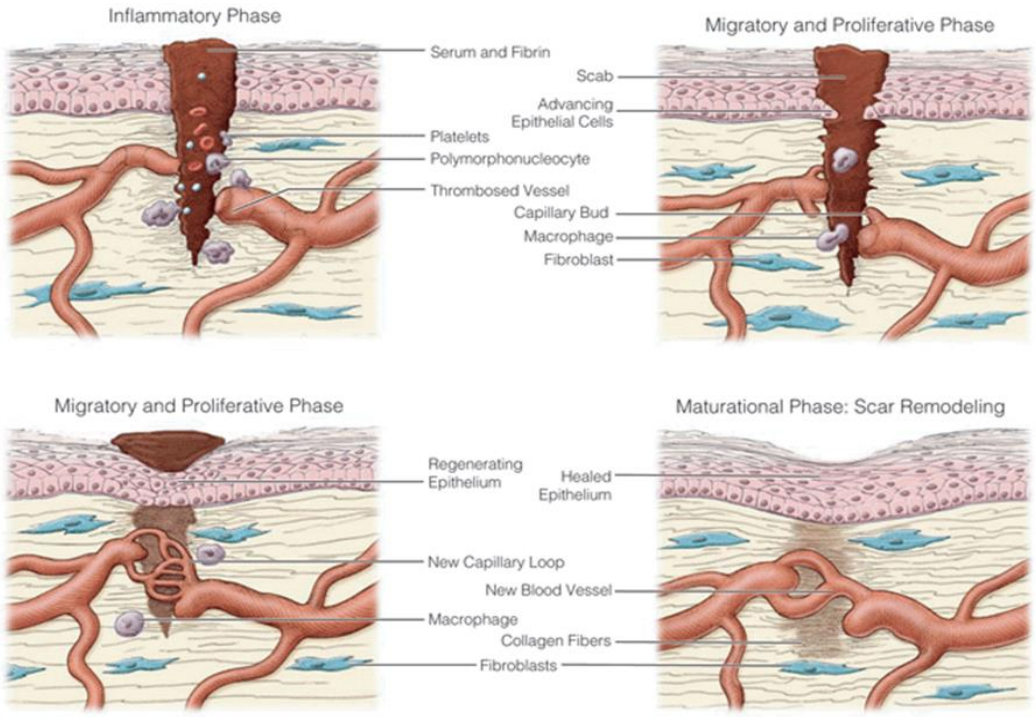



**Figure 3** The wound-healing process has four phases: hemostasis, inflammation, tissue proliferation, and tissue maturation or remodeling, which overlap in time (Lida).

**Type of wound healing (Enoch & Leaper, 2005)**

**Primary intention**

The wound is initially cleaned, and the wound edges are brought together and closed approximated 12-24 hours after its creation (e.g. surgical wound, clean minimal abrasion). Thus, there is only minimal scarring.

**Secondary intention**

The wound appears to be dirty or caused by a severe accident resulting in a mortal wound and being deeply injured. This type of wound has to be filled via the generation of new tissue. Afterward, there tissue formation covers the wound.

**Tertiary intention**

Here the wound healing is delayed from primary closure or secondary suture. Tissue transplant occurs after the tissue generation.

**The burn wound and wound healing**

**Burns**

Burns are a type of injury to the flesh or skin caused by heat, electrical, friction, chemical reagents or radiation energy. This type of injury affects the transformation of blood vessels which results in vasodilatation and promotes the excretion of cytokines and several types of inflammatory mediators (Korthuis et al., 1994; Mayers & Johnson, 1998).



**Pathophysiology of thermal burns**

**Local response**

The skin functions as the protective barrier which protects the body from the outer environment, preventing dehydration, and controls the body temperature. The injurious from thermal damaged depends on temperature and exposure period. When the temperature rises to 45°C, protein coagulation occurs. Douglas Jackson has classified thermal injury into 3 zones. The first zone is called the “zone of coagulation,” which is permanently damaged; the second is the “zone of stasis,”

which can change to the “zone of coagulation” if the blood cannot flow through the cells caused by receiving more or less fluids. The last is the “zone of hyperemia.” A burn in this zone can return to normal skin. Thus, the aim of healing is to prevent a change from the zone of stasis to the zone of coagulation (Figure 4).

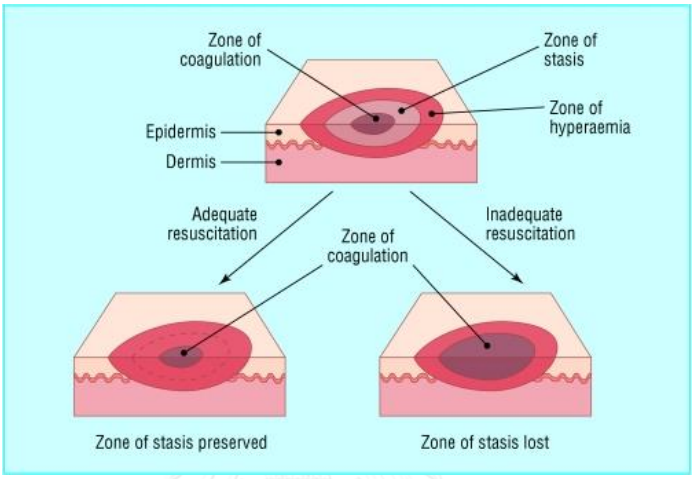



**Figure 4** Jackson’s burn zones and the effects of adequate and inadequate resuscitation (S. Hettiaratchy & P Dziewulski, 2004).

Within the zone of stasis, there are several inflammatory mediators and vasoactive mediators, such as prostaglandins, histamine and bradykinin. In addition, an increase in blood vessel permeability can cause wound edema. Moreover, oxygen-free radicals, for example, xanthine oxidase, can cause wound edema.

### Systemic response

Over 30% of burns have a systemic inflammatory response to several cytokines and mediators, resulting in dehydration and hypovolemic, which can affect the oxygen transferred to the cells and subsequently can cause the arrest of the blood flow and a decrease in the cardiac muscle pressing rate from the tumor

necrotic factor. In the case of severe burns, there is a decrease in pulmonary function without inhalation injury which comes from bronchoconstriction caused by histamine, serotonin, thromboxane $A_2$, and also from the lung function and chest decreased.

Hettiaratchy and Dziewulski 2004, found that the syndrome composed of a decrease in mesenteric blood, mucosal integrity and capillary leak can allow the bacteria into portal circulation and then cause infection in the blood, lungs, skin, and urinary tract. This group of syndromes could be prevented by feeding enough fluid, nutrients and glutamine via blood vessels to promote enterocyte synthesis for curing mucosal integrity.

Regarding the immunological system, it has been found that neutrophil chemotaxis, phagocytosis and intercellular bacterial killing decrease in line with cellular-mediated immunity and also lymphocyte activation suppressive mediators (Figure 5).

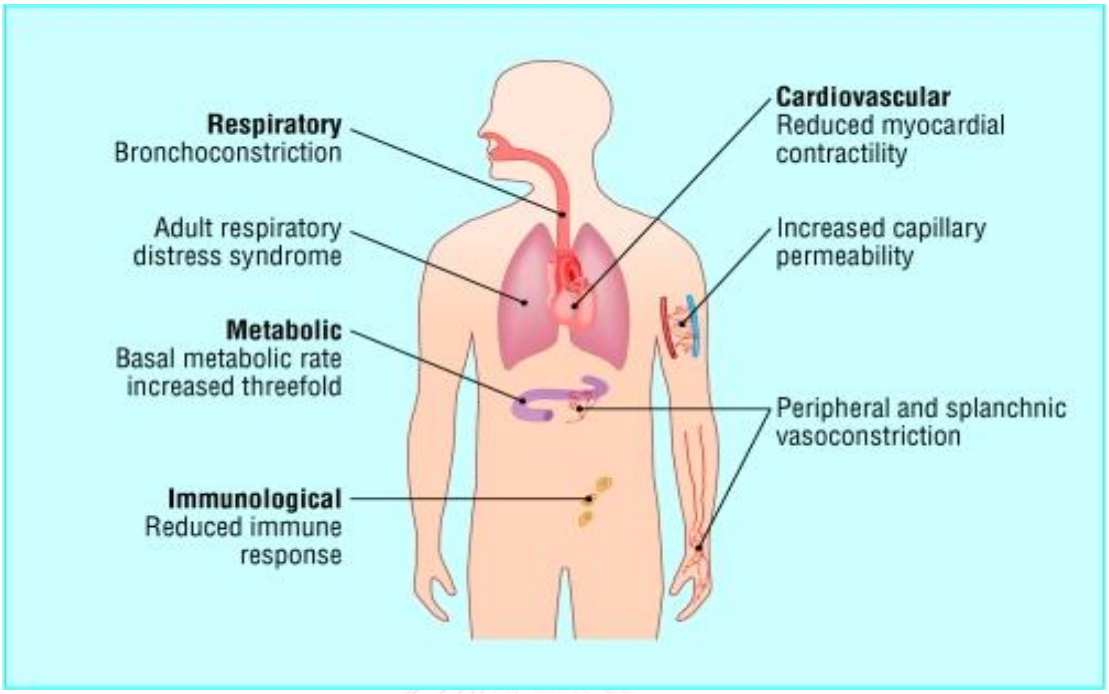



**Figure 5** Systemic changes that occur after burn injury (S. Hettiaratchy & P. Dziewulski, 2004).

### Assessment of severity of burn wound

The degree of a burn is considered from the depth of the damaged tissue, which depends on the temperature and prolonged exposure period, divided into 3 classifications (Figure 6) (Cameron AM, 2010; S. Hettiaratchy & P Dziewulski, 2004; Hettiaratchy & Papini, 2004).

### Classification of burn injury depth

#### First-degree burns or superficial burns

Superficial burns are not severe; there is partially-damaged skin only in the epidermal layer. The wound is dry and has not erupted but there might be some swelling and a bit of pain, and the surrounding skin will be red. This type of wound can heal automatically within 7-14 days without any scar. Most patients with this

type of would can carry on with their daily life as usual without staying in the hospital. Superficial burns are caused by sunlight, steam, flames, and so on.

**Second-degree burns or partial-thickness burns**

Superficial second-degree burns or superficial partial-thickness burns partially damage the epidermis and some parts of the dermis layers. The wound is red or dark pink and also has some blisters, swelling, blister serum, and causes pain. Exposure to hot metal or flaming fluid and also diluted chemicals can cause second-degree burns, but they can heal themselves in 14 to 21 days.

For deep second-degree burns or deep dermal partial, there is more damage deep in to the dermis layer but the pores and sweat glands are not damaged. The wound is a dry, pale color, and there is the presence of partial blisters, serum leakage, and severe pain. The causes of injury are as previously mentioned. Actually, it typically takes over 21 days to get better and there is always scarring and tissue contraction. This type of wound can be cured by medication or skin graft transplantation or a combination of these methods.



**Third-degree burns or full-thickness burns**

Here, all of the epidermis and dermis, including the pores and sweat glands, are completely damaged. Moreover, the degree of damage might be deep into the fat layer, fascia, muscle, and bone. The wound is dry, yellow or brown to dark colors. There might be some dark-brunt crust present that is thick and hard textured. Patients will not feel any pain or just a bit of pain due to the nerve ends and nerve nets being damaged. A third-degree burn is caused by exposure to high heat or high voltage electric current or highly-concentrated chemicals for a long period. Furthermore, this type of burn cannot be healed by the human system itself, and

therefore surgery or skin transplantation (skin graft) is essential and post burning may be complication scar contraction.

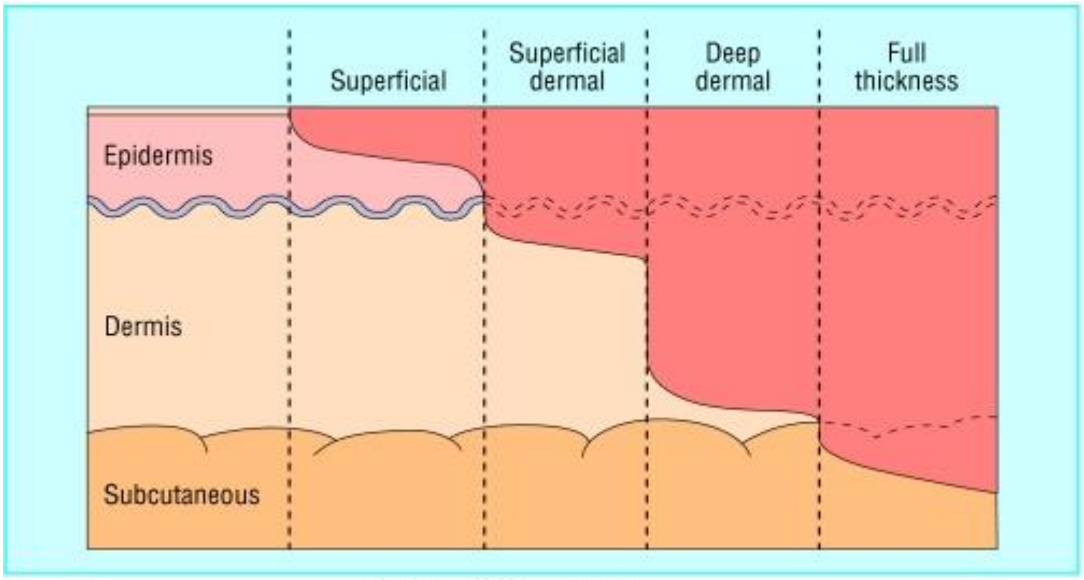



**Figure 6** Diagram of the different three-degree deep burn wounds (Hettiaratchy & Papini, 2004).



**Assessment of the severity area of burn wounds**

To determine the area damaged, the total body surface area (TBSA) is defined as 100%, followed by 3 determination methods (S. Hettiaratchy & P Dziewulski, 2004; Hettiaratchy & Papini, 2004).

**Palmer surface area**

Using the patient's hand with closed fingers determined as 0.8% of the total body surface area is the easiest method to measure a small burn wound (< 15% of total surface area) or very large burns (> 85%, when unburnt skin is counted).

**William's rule of nines**

This method is used for adult patients by dividing all of the skin area into a small part which is determined as 9% of the total body surface area as listed in the following:

| | |
|---|---|
| Head and neck | 9 % |
| Arm (each side) | 9 % (total 18 %) |
| Chest surface | 9 % |
| Abdominal surface | 9 % |
| Back surface (upper part) | 9 % |
| Back surface (lower part) | 9 % |
| Front of leg (each side) | 9 % (total 18 %) |
| Back of leg (each side) | 9 % (total 18%) |
| Reproductive organ | 1 % |
| Total | 100 % |

The advantages of using this method are simplicity and accuracy, but it cannot be used with children because their total body surface area is quite different from adults and it also depends on age (Figure 7).

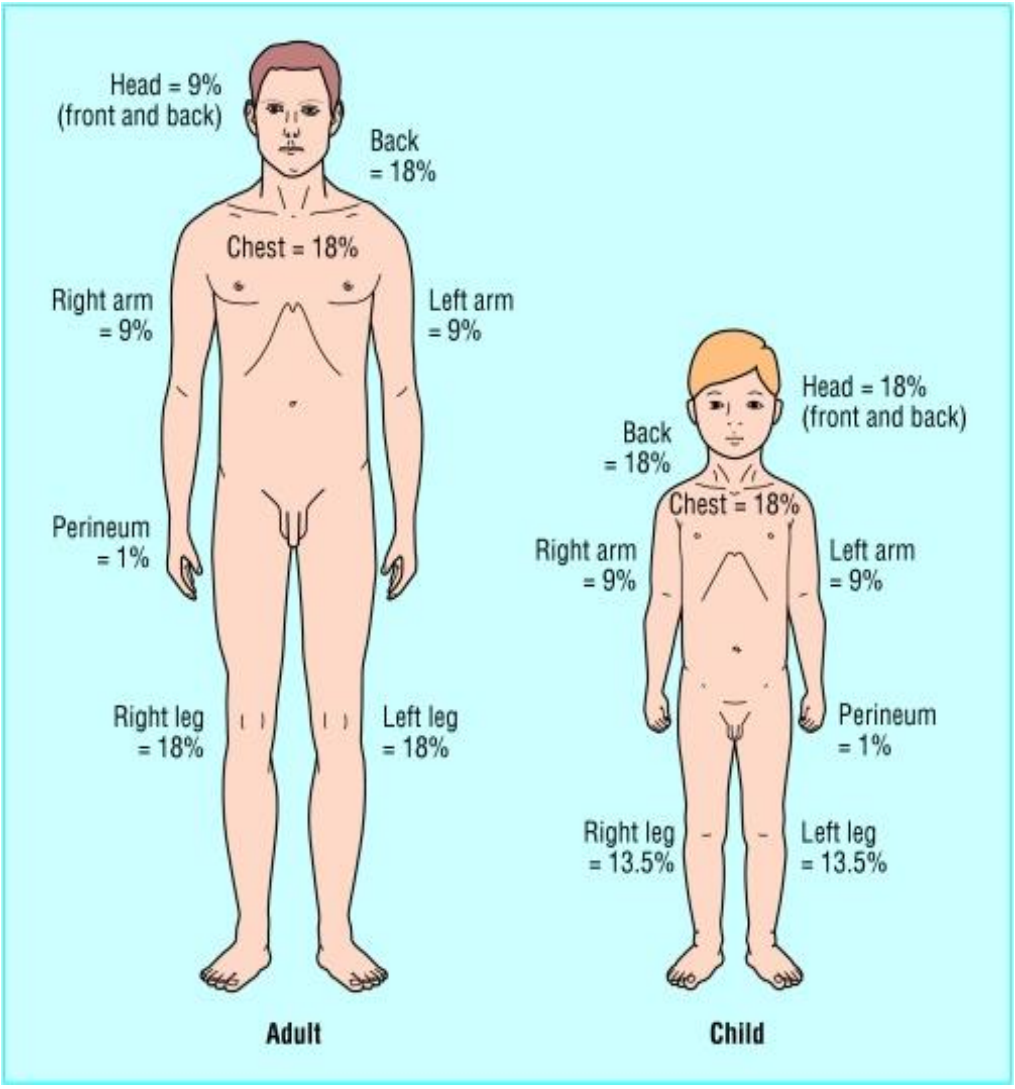
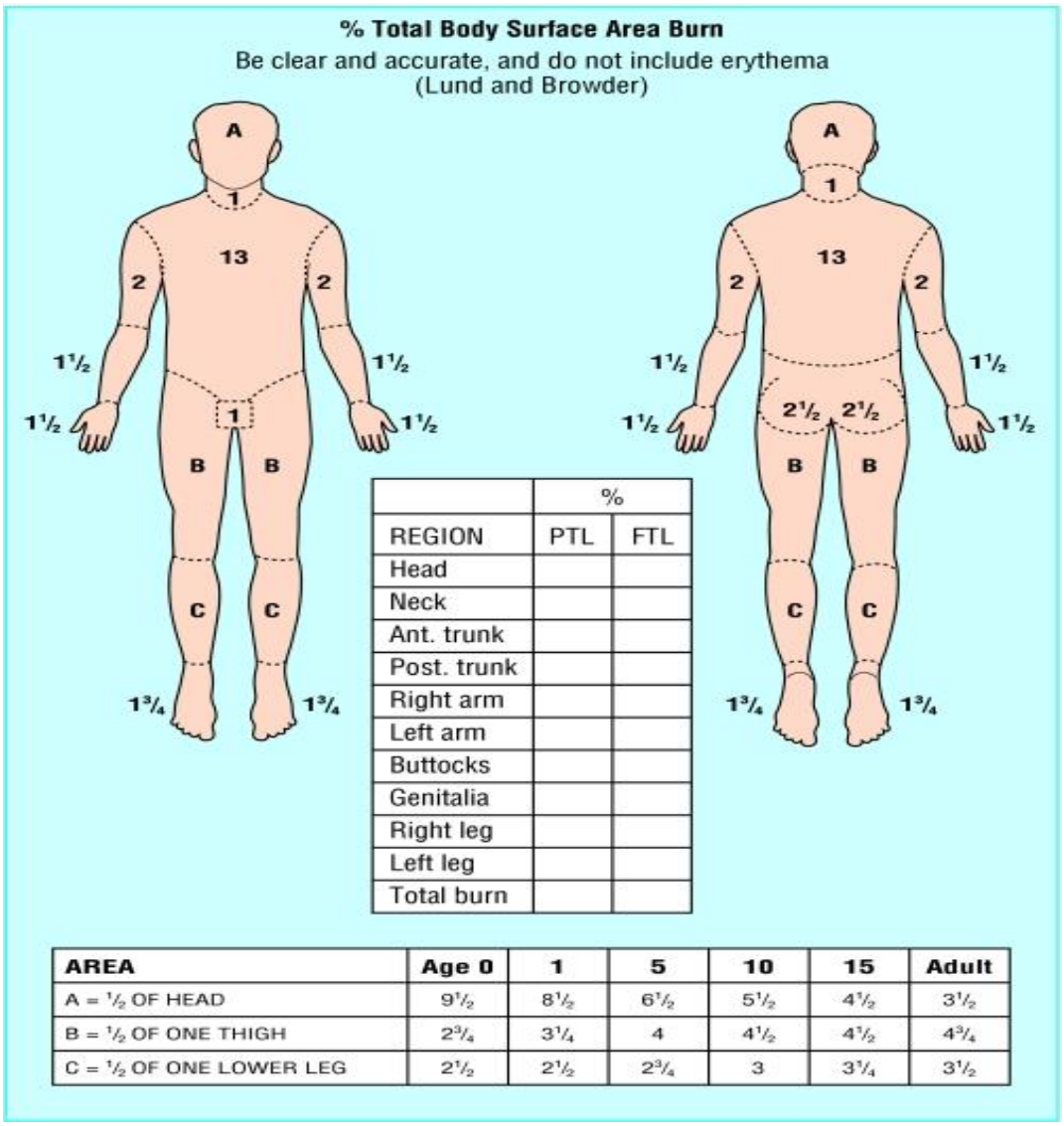





**Figure 7** William's rule of nines (Hettiaratchy & Papini, 2004).

**Lund and Browder method**

The Lund and Browder method is more reliable and can be compared with the previous-described methods. It uses a grid to display the size of the wound, which depends on the patient's age. This method is usually used for infants to 15 year old children because infants have a bigger head compared with their legs and they will be longer when they grow up (Figure 8).




| | % | |
|---|---|---|
| REGION | PTL | FTL |
| Head | | |
| Neck | | |
| Ant. trunk | | |
| Post. trunk | | |
| Right arm | | |
| Left arm | | |
| Buttocks | | |
| Genitalia | | |
| Right leg | | |
| Left leg | | |
| Total burn | | |

| AREA | Age 0 | 1 | 5 | 10 | 15 | Adult |
|---|---|---|---|---|---|---|
| A = ½ OF HEAD | 9½ | 8½ | 6½ | 5½ | 4½ | 3½ |
| B = ½ OF ONE THIGH | 2¾ | 3¼ | 4 | 4½ | 4½ | 4¾ |
| C = ½ OF ONE LOWER LEG | 2½ | 2½ | 2¾ | 3 | 3¼ | 3½ |

**Figure 8** Lund and Browder method (Hettiaratchy & Papini, 2004).

**Assessment severity grade of burn wound**

The classification of severity grade according to the American Burn Association (ABA) is divided into 3 grades as follows (Singer & Dagum, 2008).

**Mild or minor burns**

This category is for burn wounds which have the damaged area less than 15% in adults, less than 5% in children, less than 2% in the elderly for third-degree burns, or less than 2% of total body surface area with second-degree burns. This estimate does not include burn wounds on the face or eyes and ears, or reproductive organs, which are classified as first-degree burns. Patients can recover as outpatients.

**Moderate burns**

At this categorized, the skin has to damaged 10 – 20% in adults, 5 – 10% in children or the elderly, 2 – 5% for third-degree burn or 15 – 25% of total body surface area with second-degree burn injuries. Patients have to get specific treatment from a specialist.



**Severe or major burns**

Over 20% of adults, less than 10% of children and the elderly, less than 5% for third degree burns or 25% of total body surface area damaged by second degree burns, including burn wounds on the chest surface, face, ears, hands, feet and reproductive organs, are classified in this grade. The cause of severe injury here might be from high voltage electric current and also inhalation injury. Patients have to have specific treatment and observation from expert physicians.

**Management of burn wound injuries** (Murkhtar & Jone, 2003; Papini, 2004)

The goals of burn treatment could be survival of the patient with rapid healing of the wound, with minimal scarring and abnormal pigmentation. The most important treatment of a burn wound is to reduce the morbidity and mortality and the prevention of organ function damage after burns. The primary goal in the management of the wound is to achieve protective infection, rapid healing with optimal functional, decrease morbidity and mortality. This is best accomplished by preventing infection and providing an environment that optimizes healing of the wound.

**Pharmacological therapy**

Burn wound are rapid to bacterial growth with the potential for invasive infection. A topical antibiotic is commonly used for burn wounds. A topical antibiotic should be applied to prevent and treat infection with second and third degree burns (Jull, Rodgers, & Walker, 2008; Malik, Malik, & Aslam, 2010; Singer & Dagum, 2008).

Silver sulfadiazine (SSD) is the standard treatment used for prophylaxis against infection but is generally not used for superficial burns such sunburn. It should be applied with a sterile-gloved hand at a thickness of $^{1}/_{16}$ inch. Burned area should be covered with cream at all times (Lockhart, Rushworth, Azmy, & Raine, 1983). Treatment with SSD may delay wound healing (Singer & Dagum, 2008) and increase the frequency of dressing changes, resulting in increased pain.

The action of SSD is driven by inhibition respiratory enzymes and components of the microbial electron transport system, as well as impairing some DNA function. The inhibitory action of silver can be attributed to its strong interaction with thiol groups present in cell respiratory enzymes in the bacterial cell. The mechanism of action is upon the bacterial cell wall and cell membrane (Atiyeh et al., 2007; Singer &

Dagum, 2008). SSD is a bactericidal for many gram-negative, gram-positive bacteria and yeast such as active against *Pseudomonas aeruginosa*, *Pseudomonas maltophilia*, *Enterobacter* species, *Klebsiella* species, *Serratia* species, *Escherichia coli*, *Proteus mirabilis*, *Morganella morganii*, *Providencia rettgeri*, *Proteus vulgaris*, *Providencia* species, *Citrobacter* species, *Acinetobacter calcoaceticus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus* species, *Candida albicans*, *Corynebacterium diphtheriae*, and *Clostridium perfringens* (Lockhart et al., 1983).

Pharmacokinetic studied of SSD revealed that there is a concern about the silver absorption via skin from partial- and full-thickness burn wounds (5% body surface area), where most of the silver is associated with area of burn wound surface and very little is absorbed into deeper layers (Atiyeh et al., 2007). SSD is metabolized via the liver but the mechanism is still unknown. It has been estimated that half-life elimination is around 10 hours, prolonged by renal impairment, reaching a peak in serum at around 3 - 11 days of continuous therapy as well as excretion through the urine (approximately 50% as unchanged drug) (Lockhart et al., 1983).

Adverse effects of tropical therapy when applied to skin such as skin rashes, especially patient allergy to sulfonamide group. Should be avoided, when used within patient especially patient hepatic impairment and renal impairment. Previous study has shown that $Ag^{+}$ in SSD is toxic to liver tissue and the kidneys (Baldi et al., 1988; Lockhart et al., 1983) and also causes bone marrow toxicity, which leads to abnormalities in blood synthesis, including delayed wound healing (Atiyeh et al., 2007).

**Non-pharmacological therapy**

Burn wound dressing is an alternative way to assist with the healing of burn wounds. At the present, there are many burn wound dressing products that are widely, used such as Acticoat, Urgotul SSD, Aquacel $Ag^{+}$, Mepitel, Askina Calgitrol $Ag^{+}$ (Atiyeh et al., 2005; Singer & Dagum, 2008). All of the above have a good effect on burn wounds if they are suitably used for each degree of burn, especially for superficial partial and thick burns. In the case of severe burns, surgery and skin graft transplantation would yield a better result.

Skin graft transplantation is one of the most popular ways to respond to deep partial burn wounds and third-degree wound healing. This method was used in the $18^{th}$ century by British surgeons but it was not quite successful in that age. In 1870, George David Polock successfully carried out skin graft transplantation with burn wound patients and this method has been continually developed until now (Jose, 2010).

**New technologies for burn wound closure and healing**

Up to the present, there has been an attempt to develop several techniques and products which could enhance the wound-healing processes in terms of the healing period, and reducing the death rate and also complications, resulting in shorter stays in the hospital and reducing the incapacity rate after wound healing. Thus, there has been the development of wound dressing materials for wound healing process enhancement.

Later, after the development pioneer period, temporary wound dressing used biological materials which were able to control the number of pathogens or covered significant of blood vessels. This kind of material could reduce the wound size, protecting the wound from pathogens and promoting the wound-healing processes.

After the wound gets better, the physician can further consider or search for other recovery methods in order to recover the organ function or to obtain a good appearance later on, for example, with large burn wounds or necrotizing fasciitis. As in the case of biological dressing, human allograft (cadaver skin) or xenograft (e.g. pig skin) could be used in burn wound dressing. Allograft might be accompanied with the biological dressing but at around 5-10 days after the dressing, depending on the immune system of the recipient (Glat, 1997).

Today, there is the development of silicone polymers or composite membranes by using skin graft theory. This material could be used for temporary wound covering. Nowadays, scientists have developed tissue cultures in order to be used as skin structure, resulting in better healing results; however, research is still in the development phases as with other techniques, such as dermal regeneration templates (artificial skin substitutes), culture dermal, fibroblast cultures, including cytokine and gene therapy (Atiyeh et al., 2005).

**Proteoglycan**

Proteoglycans (PGs) is a hybrid molecule composed of a central core protein by bonding it with polysaccharides (glycosaminoglycans or GAGs) with a covalent bond (Prydz & Dalen, 2000), previously called mucopolysaccharide due to its physical property (jelly-like, sticky and viscosity). GAGs can be divided into 5 groups: chrondroitin/chorndroitan sulfate, keratin/keratin sulfate, dermatin/dermatan sulfate, heparin/heparan and hyaluronan. All of these are polysaccharides, which link disaccharides, which are always N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc), for the other group belongs to the uronic acid group, such as glucuronic acid or iduronic acid. Some types of PG are composed of the sulfate group and react with amino sugar, like glycosaminoglycansintermediate

between tissue cells (Prydz & Dalen, 2000) by using specific serine as a linkage between GAGs and PG. PG can be found in intracellular cells, the cell surface and the extracellular matrix which has a significant role in cellular function such as cell to cell, cell to matrix adhesion, cell proliferation, cell division, and morphogenesis and regulation of signaling molecules via GAG chains.

**Composition of proteoglycan**

PG could be separated into two parts: core protein and glycosaminoglycans. Core protein is 10 – 500 kDa proteins which have been synthesized in the rough endoplasmic reticulum (RER). It is a long chain of serine which links with glycosaminoglycans. Previous study has revealed that around 30 protein core contain one serine which functions as a linkage with glycosaminoglycans (Iozzo, 1998). Glycosaminoglycans has been synthesized from the endoplasmic reticulum (ER)-Golgi pathway. It is a long chain polysaccharide which has over 100 kDa in size linked to a core protein at the serine position.



**Location of proteoglycan**

**Intracellular proteoglycan**

PG has been found mainly in cellular granules, for example, mast cells, granulocytes, NK cells, lymphocytes, platelets, macrophages, eosinophils, and cytotoxic T-lymphocytes. Moreover, cellular granules contain several substances such as protease enzymes and histamines.

Examples of cellular proteoglycan function

- Chondroitin sulfate PG acts as a composition in hematopoietic cells which are involved in anticoagulants synthesis such as heparin.

- Serglycin in storage granules has an important role in blood coagulation, cancer cell elimination, and wound-healing processes.

- Several studies found that PG and glycosaminoglycans are involved in the cell cycle by transporting substances to the cell surface, assisting with the growth factor of cell surface activities such as fibroblast growth factor 2 (FGF-2), fibroblast growth factor-16 (FGF) and epidermal-like growth factor (Deepa, Umehara, Higashiyama, Itoh, & Sugahara, 2002).

**Cell membrane proteoglycans**

This type of PG has been found in fibroblast and epidermal cells, and functions as a receptor and signaling transduction by binding with several types of growth factors such as the FGF and hepatocyte growth factors. It can activate the cell cycle, promote the cell division and generate blood vessels (Couchman, 2010).

**Extracellular proteoglycan**

This PG has a large spindle structure which results in an elastic texture and stabilized tissues when they are exposed to pressure and control the activity of growth factors.

**Cartilage proteoglycans**

From previous study, it was revealed that cartilage is composed of PG of over 90% dry weight (Hardingham & Fosang, 1992), which is the main structure in the extracellular matrix. Types of PG that have been found in cartilage is chondroitin sulfate, dermatan sulfate and heparin sulfate (Mathews & Lozaityte, 1958) and aggrecan, which function as core protein (Doege, Sasaki, Kimura, & Yamada, 1991). Besides aggrecan, other types of PG which have been found in cartilage are decorin, biglycan, and perlecan.

**The efficacy of proteoglycan on wound healing**

In 2013, Neelam and her group studied the effect of *Melampodium divaricatum* (Pers.) on L929 fibroblast cells by using PG, which is extracted from salmon nasal tip cartilage as a standard because its property like epidermal growth factor. The results showed that leaves crude extract and salmon PG extract could promote fibroblast cell activation by activating the proliferation and migration processes, including collagen synthesis, which is involved in wound-healing processes (Neelam, 2013).

Regarding the immunohistochemical and biochemical of dermatan sulfate proteoglycan (DSPG) and chondroitin sulfate proteoglycan (CSPG) in the wound healing processes of guinea pig. This studied showed that CSPG has a more significant role in the wound healing processes as compared to the DSPG and GAGs chain. Furthermore, this study also revealed that DSPG synthesis in granulation tissue of guinea pig wounds was higher than in the normal mouse (Yeo, Brown, & Dvorak, 1991).

The study of growth factor activation indicated that the binding between heparan sulfate and soluble growth factors, including cytokines, has an effect on the activation of FGF-2, resulting in cell division and promoting collagen synthesis (Rapraeger, Krufka, & Olwin, 1991; Yayon, Klagsbrun, Esko, Leder, & Ornitz, 1991). Moreover, the researchers also found that heparan sulfate could bind to several types of growth factors such as the EGF-like growth factor and the hepatocyte growth factor (Aviezer & Yayon, 1994; Reichsman, Smith, & Cumberledge, 1996).

One study investigated the effect of PG on promoting wound healing processes by binding with several growth factors, such as TGF- β, FGF-2, the VEGF family and PDGF, and as a result of cell division, promoted the wound healing

processes and enhanced the expression of many syndecans, for example, syndecan-1, syndecan-3 and syndecan-4, involved in wound healing processes (Gallo, 2000).

**Wound healing in the rat model**

Burn wounds can be studied both of *in vivo* and *in vitro*, like cell cultures such as fibroblastic cells and keratinocyte; however, *in vivo* is the most popular method. Experiments have been carried out with pigs, rabbits, and mice. For the study of the wound-healing process, rats were used as the study model. The Wister rat and Spraque Dawley rat are widely used for this field (Santos, 1996). In this study, the researchers selected the Wister rat because it could tolerate to pain better than the Spraque Dawley rat.

Using an anesthesia agent or paregoric are the most practical ways for pain recovery in order to reduce the pain of tested animals during burn wound induction. The general anesthesia, which has been used in this study, is sodium pentobarbital or a mixture of ketamine and xylazine. For the inhalation agents which are widely used, such as ether, isoflurane or combination with injection such as ketamine with methoxyflurane or sodium pentobarbital with isoflurane (Santos, 1996).

There are several methods of burn wound induction such as acid or base induction, but these are not the principle way for burn wound induction because it is difficult to control the degree of burn. Thermal induction is the most practical method of burn wound induction (Santos, 1996) by using a heated iron which is 1-2 cm in diameter at 50-100 °C placed on the rat skin, and the exposure period, including temperature, depends on the type of study. In this study, 2 cm (diameter) electric hot plate at 90 °C was placed on rat skin for 10 sec to induce second degree burn (Pereira Ddos et al., 2012).

The measurement of wound area estimation was displayed in centimeter square units for evaluating the difference between before and after treatment. There are various types of wound area measurements, but the most proper method is Photography, combined with ImageJ® (National Institutes of Health) and Visitrak® (Smith & Nephew - United kingdom). Photography combined with ImageJ® could estimate the wound area without touching the burn wound, which can overcome the contamination problem. For Visitrak®, it could be operated easily as compared to Photography combined with ImageJ®. Furthermore, it could estimate the wound area in terms of width, length and depth, including wound temperature. However, this method provided a high cost of operation because its measured plate could not be recycled by sterilization but this method of accuracy for measurement were non-significant difference compared to Photography, combined with ImageJ® (Chang, Dearman, & Greenwood, 2011).

# CHAPTER III

# METERIALS AND METHODS

## Drugs and chemical substances

- Cream base
- Silver sulfadiazine (SSD; Sigma-Aldrich, St-Louis, MO, USA)
- Proteoglycan (PG; Garguar Lab, Co., Ltd., Thailand)
- Sodium pentobarbital (Nembutal; Tariqbrian Ltd, USA)
- 0.9% NSS (Thai Nakorn Patana Ltd, Thailand)
- Alcohol 70% (Siribuncha Ltd, Thailand)
- Hematoxylin (Bio-optica, Italy)
- Eosin (Bio-optica, Italy)
- Formaldehyde (FORMALIN®, Vidhyasom Co., Ltd, Thailand)
- Paraffin (Tyco Healthcare Group LP, USA)

## Instuments

- Analytical Balance (Mettler Toledo, USA)
- Electrical hot-plate (diameter 2 cm)
- Centrifuge (Thermo scientific, Germany)
- Slide
- Cover glass
- Color photograph
- UV spectrophotometer (Instrumentation laboratory, Milano Italy)
- Visitrack® (Smith & Nephew, UK)
- Stainless surgical blade NO. 15 (Swann Morton, Sheffield, UK )
- Surgical set (Lab Systems co., Ltd)

- Needle, Disposable NO. 18x1 (Nipro, Japan)
- Needle, Disposable NO. 25x1 (Nipro, Japan)
- Needle, Disposable NO. 25x5/8 (Nipro, Japan)
- Needle, Disposable NO. 26x1/2 (Nipro, Japan)
- Syringe 1 ml (Nipro, Japan)
- Syringe 10 ml (Nipro, Japan)
- Disposable sterile dressing set (Lab Systems co., Ltd)
- Gauze sterile 1x1 inch (Lab Systems co., Ltd)
- Conform sterile size 1 inch. (Lab Systems co., Ltd)
- Micropore plaster (3M™ Micropore™ co., Ltd)
- Blade (Gillette super click, Germany)
- Aspartate aminotransferase assay kit, NO.0018257540 (Instrumentation laboratory, Milano Italy)
- Alanine aminotransferase assay kit, NO.0018257440, (Instrumentation laboratory, Milano Italy)
- Alkaline phosphatase assay kit, NO. P/N 0018259740, (Instrumentation laboratory, Milano Italy)
- Creatinine assay kit, NO. 018257240 (Instrumentation laboratory, Milano Italy)
- Blood urea nitrogen assay kit (Instrumentation laboratory, Milano Italy)
- Sterile wooden tongue depressor (Twoforussupply co., Ltd, Thailand)

**Experimental animals**

Male Wistar rats weighing 250-300 g were purchased from the National Laboratory Animal Centre, Mahidol University, Salaya, Thailand. The animals were housed in the Laboratory Animal Unit of the Faculty of Pharmaceutical Sciences, Chulalongkorn University under standard conditions of temperature 25±2°C, 50-60% humidity, and a 12 h/12 h light/dark cycle. Rats were kept under laboratory conditions for one week prior to the start of the experiments and allowed food and water *ad libitum*. At the end of each experiment, the animals were sacrificed with carbon dioxide asphyxiation. Animal experiments in this study were carried out in accordance with the Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes of the National Research Council of Thailand. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand (Protocol Approval No. 13-33-011) (Appendix A).

**Cream preparation**

SSD powder, PG solution, stearic acid, glyceryl monostearate, isopropyl myristate, sodium lauryl sulfate, glycerin, triethanolamine, uniphen P-23 and germaben II-E were used to formulate PG, SSD or SSD + PG creams. SSD powder and/or PG solution were dissolved in warm water and then mixed with other ingredients during the cream-forming process.

**Methodology**

**Animal preparation**

The animals were randomly divided into 6 groups of 10 animals, the control group, the positive control group, and treatment groups. The control group received

cream base (no therapeutic effect). The positive control group received 1% silver sulfadiazine (1% SSD, standard treatment). Treatment groups received 1% proteoglycan (1% PG), 2% proteoglycan (2% PG), combination of 1% silver sulfadiazine and 1% proteoglycan (1% SSD + 1% PG), or combination of 1% silver sulfadiazine and 2% proteoglycan (1% SSD + 2% PG).

**Electrical hotplate inducing second-degree burn wounds**

The effect of PG from fish cartilage for treatment of burn wounds was investigated using the method of Somboonwoung *et al*. (2000), which was modified from Zawacki (Zawacki, 1974a, 1974b). The animals were anesthetized by intraperitoneally injection with sodium pentobarbital (60 mg/kg). The hair on the back of each animal was shaved and second-degree burn wounds were induced by placing an electrical hot plate diameter 2 cm set at the temperature of 90 °C on selected skin area of the back of rats for 10 s (Figure 9, 10, and 11 ) (Sener, Sehirli, Satiroglu, Keyer-Uysal, & B, 2002). The wound surface area of each animal was measured immediately after burning and on day 3, 7, 14, 21 and 28 post-burn with a Visitrack® machine (Smith & Nephew, United Kingdom; Figure 12). Percentage wound healing was calculated using the method described by Kumar et al. (2006); Danielle et al. (2012); Wu et al. (2012) (Pereira Ddos et al., 2012; Senthil, Sripriya, Vijaya, & Sehgal, 2006; Wu, Luo, Gu, & Xu, 2012). The wounds were applied with 1 g of cream base, 1% SSD cream, 1% PG cream, 2% PG cream, combination of 1% SSD + 1% PG cream or combination of 1% SSD + 2% PG cream once daily and covered with sterile gauzes. The wounds were cleaned on day 0, 2, 4, 6, 9, 12, 15, 18, 21, 24 and 27 with 0.9% normal saline solution and covered with sterile gauzes. At the end of each experiment, the animals were sacrificed with carbon dioxide asphyxiation.

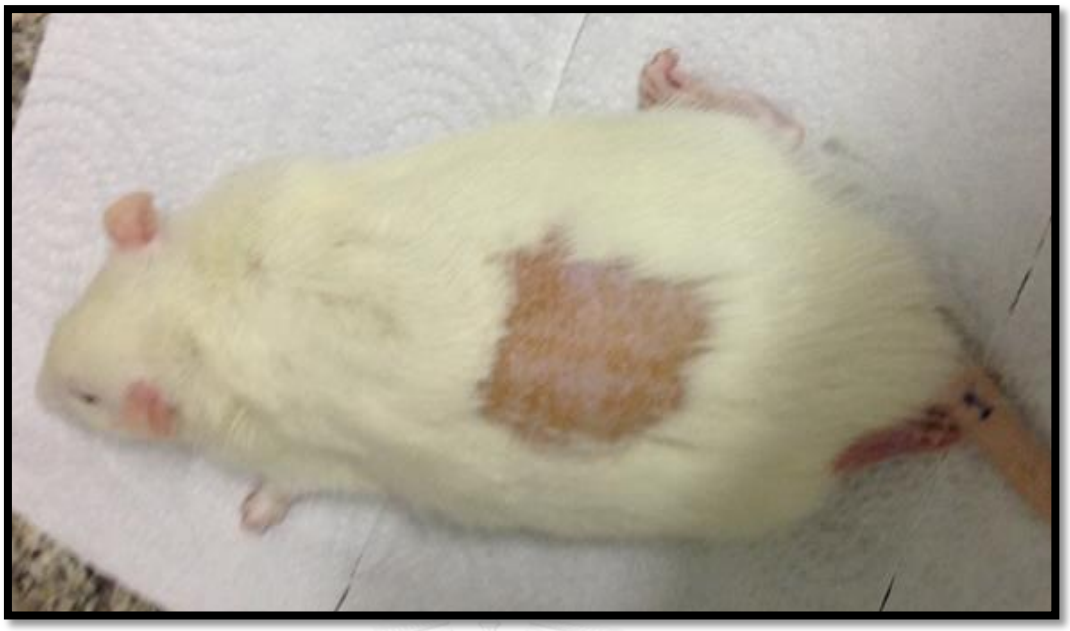


**Figure 9** Hair on the back of a rat was removed before inducing the burn wound with an electrical hot plate.


**Figure 10** An electrical hot plate.

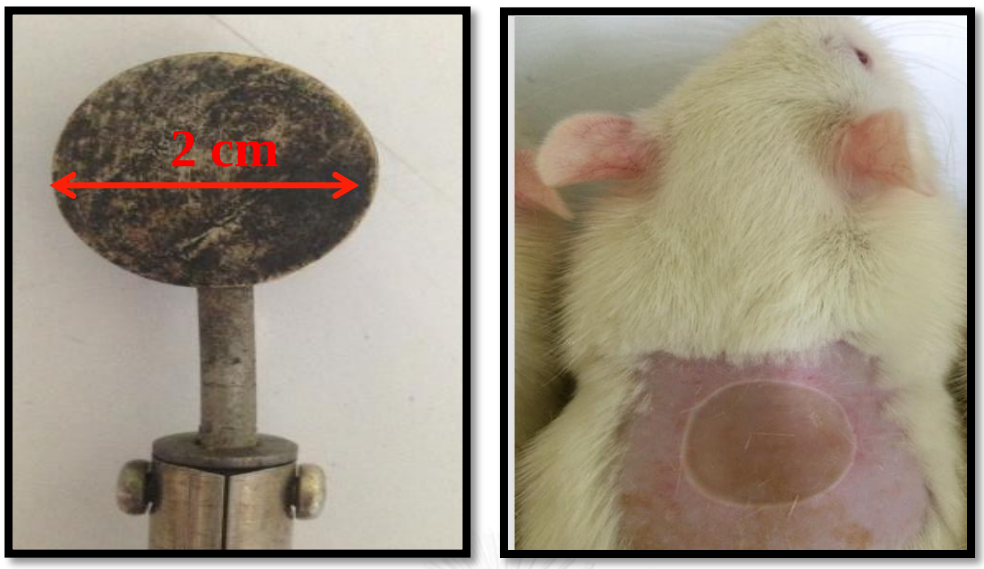



**Figure 11** A second-degree burn wound was induced by placing an electrical hot plate (temperature 90 °C, diameter 2 cm) on the selected skin area of the back of a rat for 10 s.

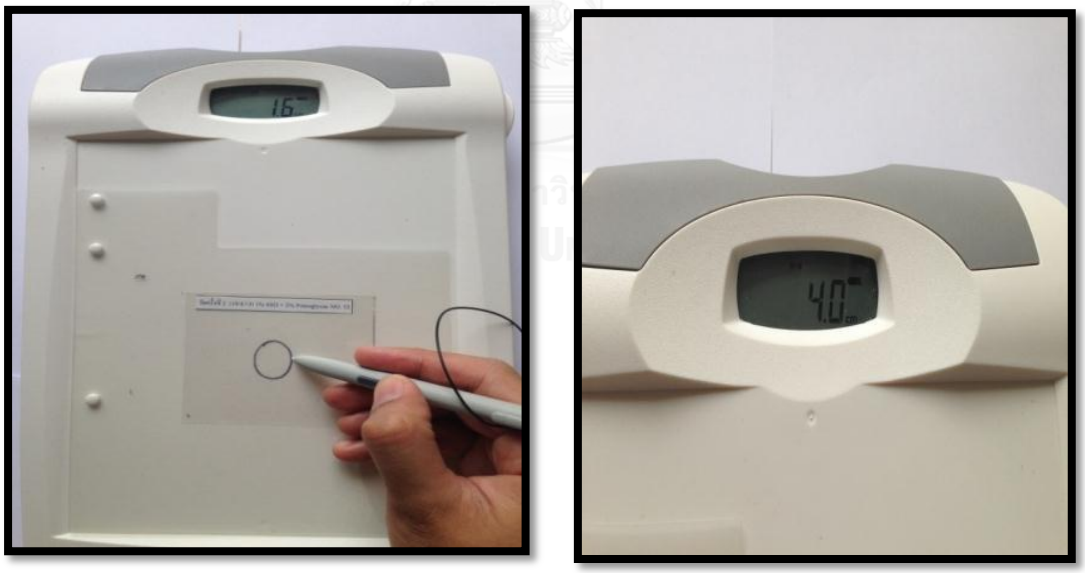


**Figure 12** The total surface area of a burn wound was measured using the Visitrack® machine. Copy a picture of a burn wound on a sterile plastic sheet and place on the Visitrack® board then draw a picture of the wound with the pen. The total surface area of the wound was calculated and shown on the monitor ($cm^2$).

**Evaluation of burn wound healing**

The total surface area of burn wounds were measured with sterile visitrack® plastic sheets immediately after burning and on day 3, 7, 14, 21 and 28 post-burnusing the visitrack® machine. Percentage wound healing was calculated using the following formula previously described by Kumar *et al*. (2006); Danielle *et al.* (2012); Wu *et al.* (2012):

$$\% \text{ Wound healing on day x} = \frac{(\text{Area on day 0} - \text{Area on day x})}{\text{Area on day x}} \times 100$$

**Histological analysis**

The small excision size of skin tissue (2.5 x 2.5 cm) from the burn area were taken for histological studies on day 7, 14 and 21 post-burn. Tissues were fixed in 10% formalin buffer solution for at least 24 h and embedded in paraffin. The sections of 5 μm thickness were cut and stained with hematoxylin and eosin dye. A light microscope with x4 and x10 objective lens was used to evaluate histological changes of the tissues. These tissues samples were evaluated for percentage of re-epithelialization, number of cells/fields, such as multinucleated giant cells, macrophages, mast cells and neovascularization compared with the controls. Percentage of re-epithelialization of the wound was calculated for each time point from the measured distance between epithelium tips of the middle of the wound using the following formula:

$\%$ Epithelialization $day_n$ = area of epithelium $day_n$/total wound area $day_n$ x 100 (Schallberger, Stanley, Hauptman, & Steficek, 2008).

**Safety evaluation**

**Blood collection**

Blood collection from the rat was performed with aseptic technique. The rat was restrained with a plastic restrainer and its tail was cleaned with 70% alcohol. The tail was heated with warm water to facilitate vasodilation of the tail vein. A 26 gauge needle with 1 mL syringe was inserted at a 45 degree angle into the tail vein and applied slight negative pressure which helps to determine if the needle was entered into the vein (Figure 13). Collect 0.5 mL of blood and put into a microtube (Diehl et al., 2001; Koch, 2006).

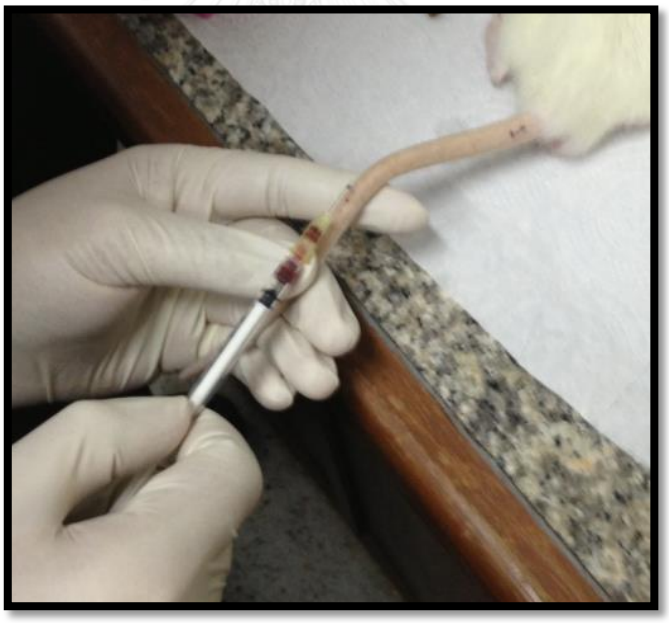


**Figure 13** Blood collection from the lateral vein of a rat.

**Preparation of plasma**

The whole blood in the test tube was centrifuged at 3,000 rpm for 10 minutes. The whole blood was separated into two layers: upper layer which contains plasma (approximately 55% of whole blood) and lower layer which contains erythrocytes (approximately 45% of whole blood). Plasma was stored at 2–8°C and used within 24 h for further analysis (Henry, 1979; Thavasu, Longhurst, Joel, Slevin, & Balkwill, 1992).

**Evaluation of liver function tests**

The liver function test of rats was evaluated by measuring three hepatic enzyme levels including aspartate aminotransferase, alanine transaminase and alkaline phosphatase. Twenty microliters of plasma were used for measurement of each enzyme level. The activities of aspartate aminotransferase, alanine transaminase and alkaline phosphatase were determined using assay kits which are commercially available diagnostic laboratory tests and light UV spectrophotometer (I lab 150, Instrumentation laboratory, Italy).



**Evaluation of renal function tests**

The renal function test of rats was evaluated by measuring levels of blood urea nitrogen and creatinine. Twenty microliters of plasma were used for measurement of each parameter. The concentrations of blood urea nitrogen and creatinine were determined using the assay kits and a UV spectrophotometer (I lab 150, Instrumentation laboratory, Italy).

**Data treatment and statistical analysis**

Results are expressed as means ± S.D. Data were analyzed using one-way analysis of variance (ANOVA), followed by a Bonferroni *post hoc* test using SPSS for Windows, ver. 17. Values of $p<0.05$ were considered to be statistic significant.

# CHAPTER IV

# RESULTS

## Percentage wound healing

The wound healing was calculated on day 3, 7, 14, 21 and 28 post-burn. On day 3 post-burn, rats treated with both 1% PG and 2% PG significantly ($p<0.05$, $p<0.01$, respectively) showed higher percentages of wound healing when compared to controls (receiving cream base). Additionally, 2% PG showed a higher percentage of wound healing than 1% PG. The effect of 1% PG on the increase in the percentage of wound contraction was comparable to the standard drug (1% SSD).

On day 7 post-burn, the effects of all cream containing PG seem to be higher than the standard drug alone. From day 7 to day 28 post-burn, all groups of rats (1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG) showed significant higher percentage of wound healing when compared to the control group ($p<0.05$, $p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$, respectively for day 7 and $p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$, $p<0.001$, respectively for day 14, 21, and 28). The effects of 1% PG, 2% PG, combination of 1% SSD + 1% PG, and combination of 1% SSD + 2% PG were comparable to 1% SSD. Wound healing processes measured as percentage wound healing for all treated groups were completed on day 28, while the control group was not yet completed (Table 1).

**Table 1** Percentage wound healing on day 3, 7, 14, 21 and 28 post-burn. N = 10 for all groups.

| Groups | % Wound healing | | | | |
|---|---|---|---|---|---|
| | Day 3 | Day 7 | Day 14 | Day 21 | Day 28 |
| Control (Cream base) | 4.51 ± 1.69 | 14.06 ± 4.25 | 35.00 ± 10.23 | 66.80 ± 8.50 | 92.83 ± 19.71 |
| 1% SSD | 6.71 ± 3.09 | **(+)** 38.68 ± 18.30 | **(+++)** 69.33 ± 8.55 | **(+++)** 88.45 ± 4.96 | **(+++)** 100 ± 0.00 |
| 1% PG | **(+)** 5.80 ± 4.09 | **(+++)** 40.89 ± 10.93 | **(+++)** 69.25 ± 7.59 | **(+++)** 89.78 ± 4.05 | **(+++)** 100 ± 0.00 |
| 2% PG | **(++, $)** 11.60 ± 7.70 | **(+++)** 42.47 ± 11.55 | **(+++)** 74.04 ± 6.71 | **(+++)** 93.28 ± 1.81 | **(+++)** 100 ± 0.00 |
| 1% SSD + 1% PG | 8.30 ± 2.00 | **(+++)** 45.63 ± 13.63 | **(+++)** 77.39 ± 6.69 | **(+++)** 92.39 ± 4.65 | **(+++)** 100 ± 000 |
| 1% SSD + 2% PG | 9.39 ± 3.73 | **(+++)** 54.01 ± 13.96 | **(+++)** 80.22 ± 10.70 | **(+++)** 95.62 ± 2.85 | **(+++)** 100 ± 0.00 |

$^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ significantly different compared to cream base.

$^{\$}p<0.05$ significantly different compared to 1% PG.

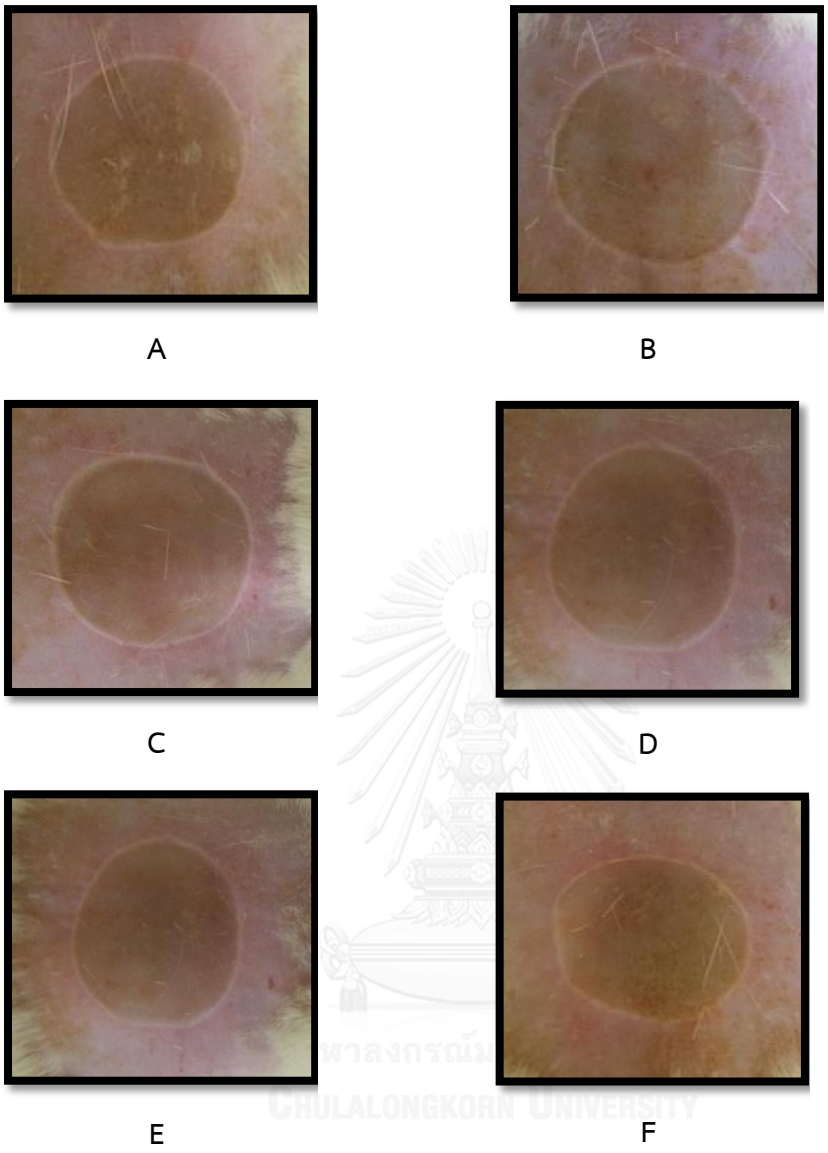



**Figure 14** General appearance of the burn wounds on day 0.

A = Cream base

B = 1% SSD

C = 1% PG

D = 2% PG

E = 1% SSD + 1% PG

F = 1% SSD + 2% PG

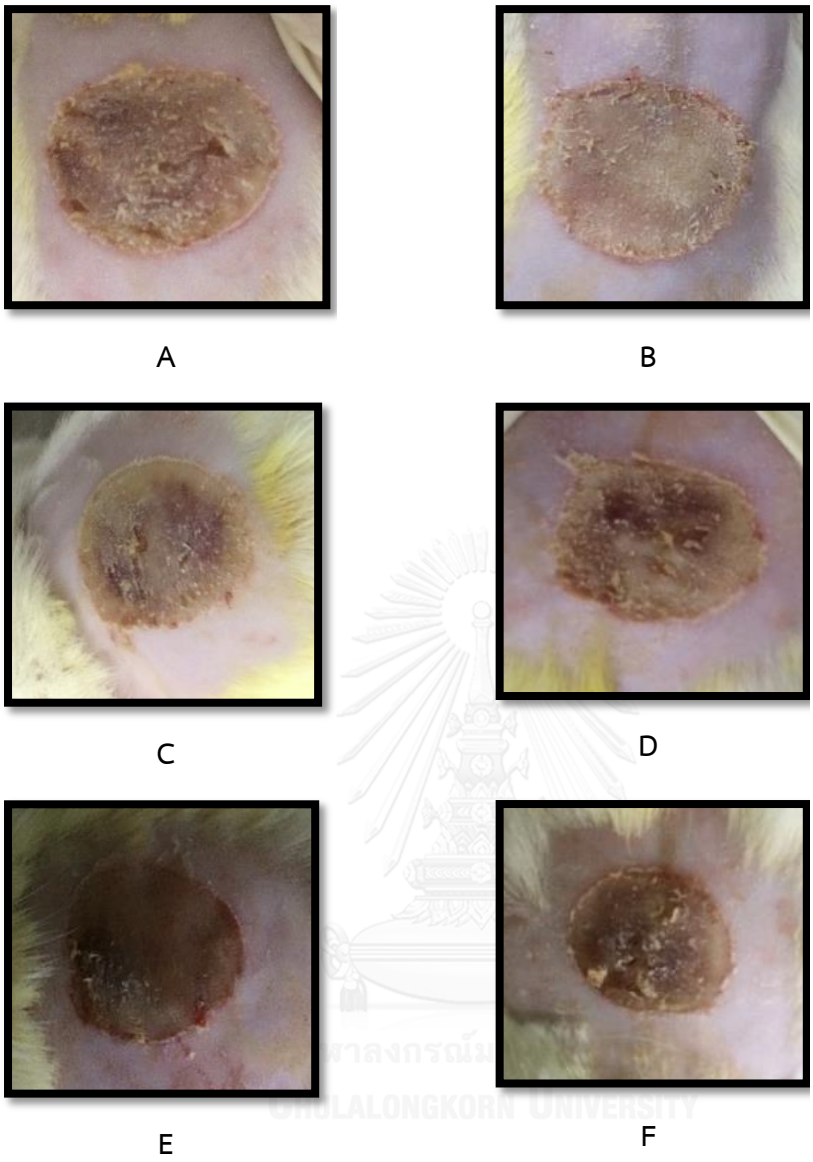



**Figure 15** General appearance of the burn wounds on day 3 post-burn.

A = Cream base

B = 1% SSD

C = 1% PG

D = 2% PG

E = 1% SSD + 1% PG

F = 1% SSD + 2% PG

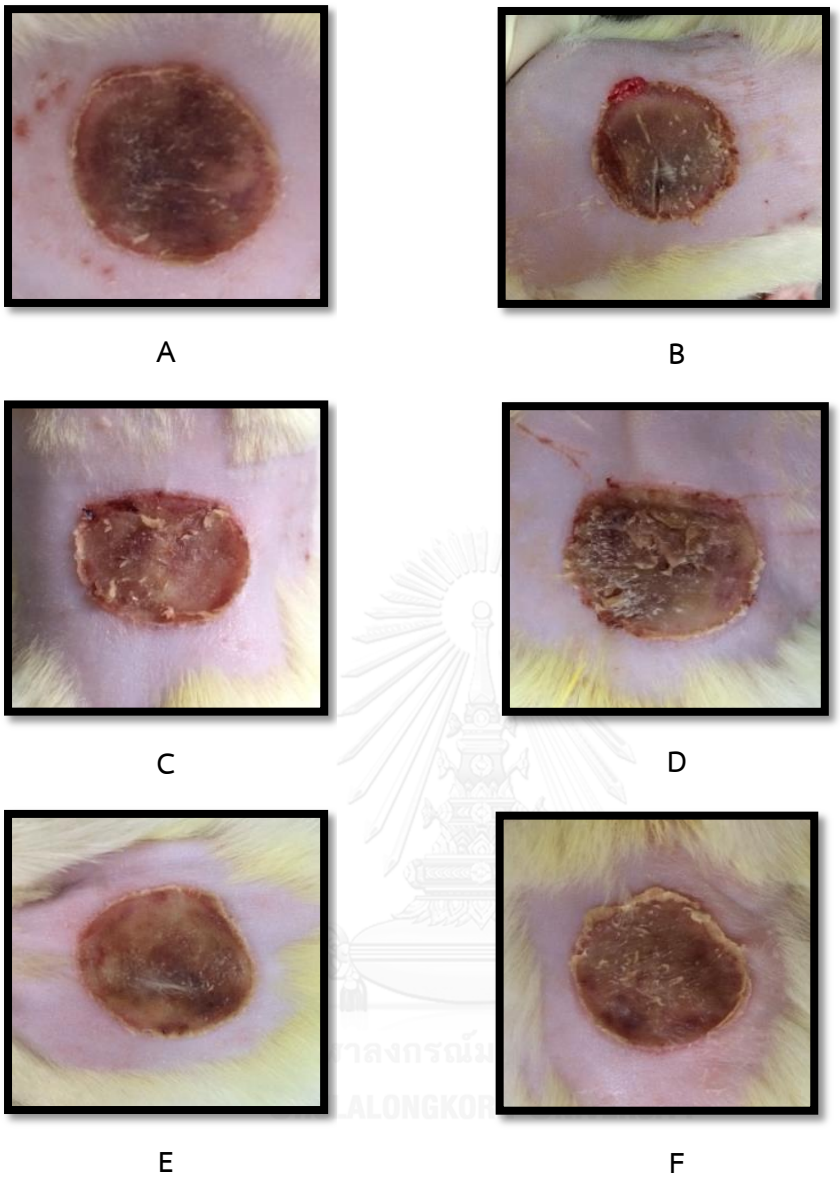



**Figure 16** General appearance of the burn wounds on day 7 post-burn.

A = Cream base

B = 1% SSD

C = 1% PG

D = 2% PG

E = 1% SSD + 1% PG

F = 1% SSD + 2% PG

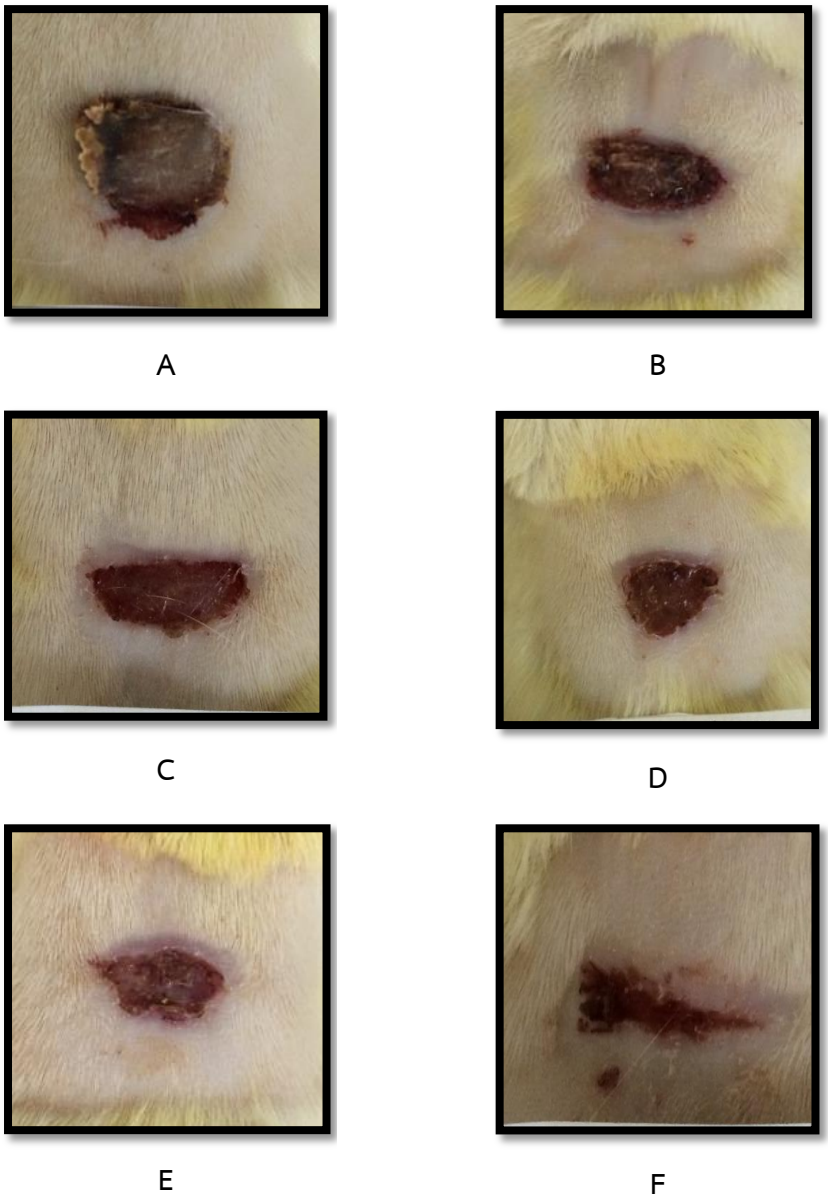



**Figure 17** General appearance of the burn wounds on day 14 post-burn.

A = Cream base

B = 1% SSD

C = 1% PG

D = 2% PG

E = 1% SSD + 1% PG

F = 1% SSD + 2% PG

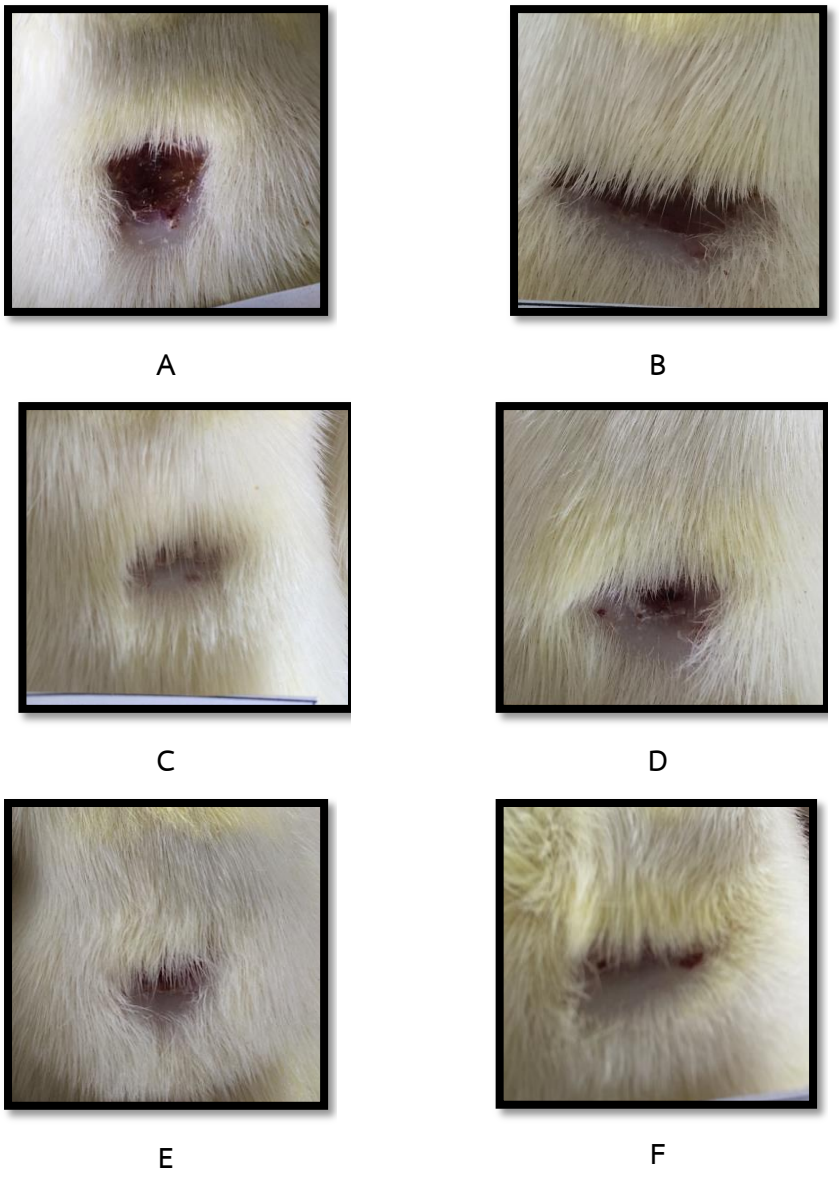



**Figure 18** General appearance of the burn wounds on day 21 post-burn.

A = Cream base

B = 1% SSD

C = 1% PG

D = 2% PG

E = 1% SSD + 1% PG

F = 1% SSD + 2% PG

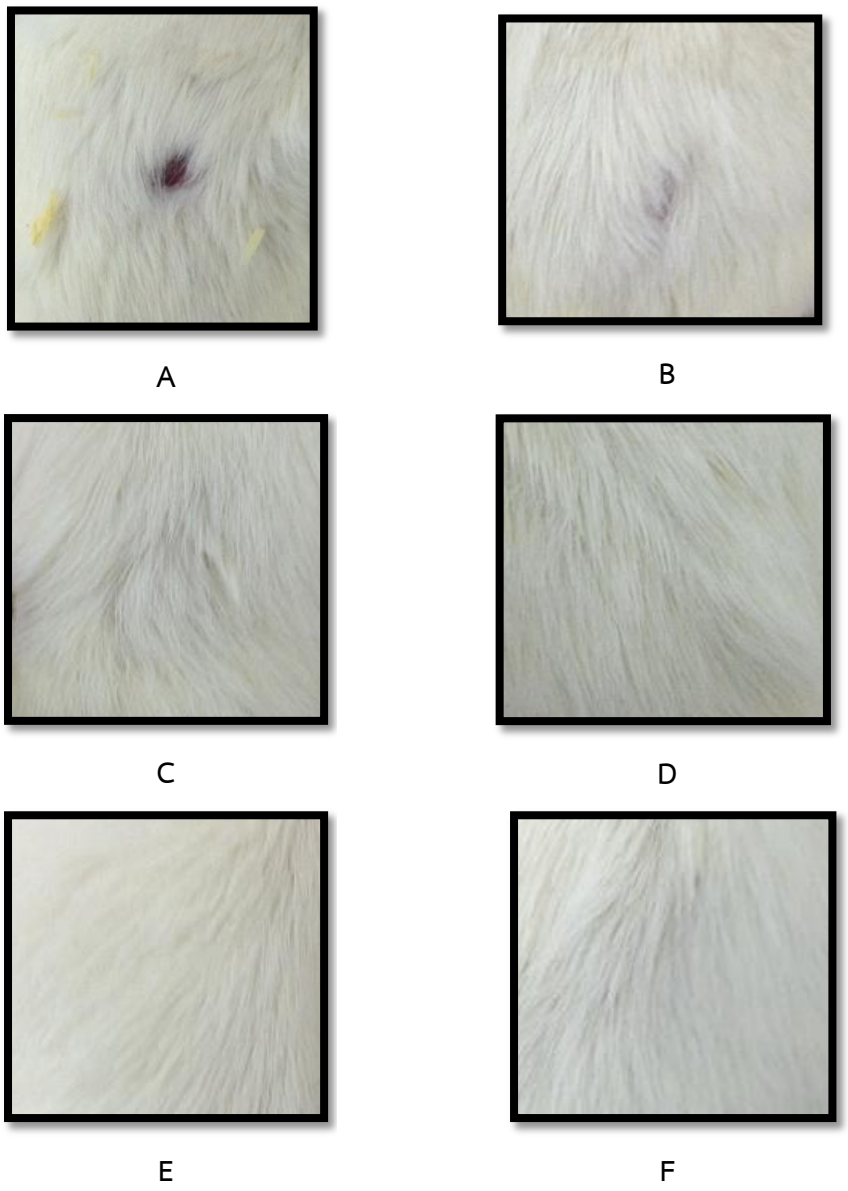



**Figure 19** General appearance of the burn wounds on day 28 post-burn.

A = Cream base

B = 1% SSD

C = 1% PG

D = 2% PG

E = 1% SSD + 1% PG

F = 1% SSD + 2% PG

**Histological analysis**

After burning, skin samples from each group were evaluated for percentage of re-epithelialization, number of cells/field such as multinucleated giant cells, macrophage, mast cells, and neovascularization on day 7, 14 and 21.

**Percentage of re-epithelialization**

On day 7 post-burn, percentage of re-epithelialization of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not statistically different when compared to the control group.

On day 14 post-burn**,** percentage of re-epithelialization of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were significantly higher when compared to the control group ($p<0.05$, $p<0.05$, $p<0.01$, $p<0.05$, and $p<0.01$, respectively). Rats treated with combination of 1% SSD + 2% PG showed the highest percentage of re-epithelialization. In all groups, keratinocytes seem to proliferate and migrate across the wounds when observed under the light microscope.

On day 21 post-burn, percentage of re-epithelialization of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were significantly higher when compared to the control group ($p<0.01$, $p<0.01$, $p<0.01$, $p<0.05$, and $p<0.01$, respectively). The skin surface of all rats treated with combination of 1% SSD + 2% PG was almost completely covered with new epidermal cells (Figure 20).

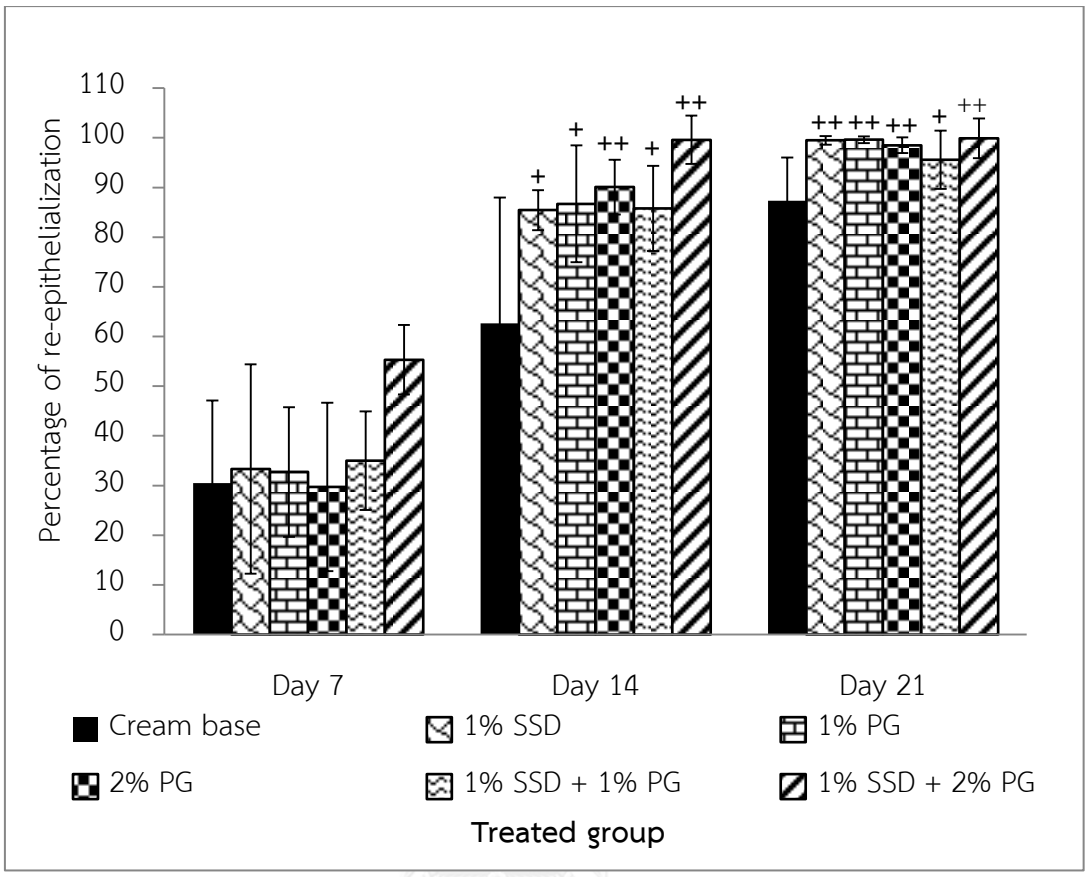



**Figure 20** Percentage of re-epithelialization on day 7, 14 and 21 post-burn**.**

N = 6 for all groups.



$^{+}p<0.05$, $^{++}p<0.01$ significantly different compared to cream base.

**Number of multinucleated giant cells/field**

On day 7, 14 and 21 post-burn, Number of multinucleated giant cells/field of the wounds treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG showed no significant difference compared to the control group. Number of multinucleated giant cells of all groups were highest on day 7 and then decreased on day 14 and 21 (Figure 21).

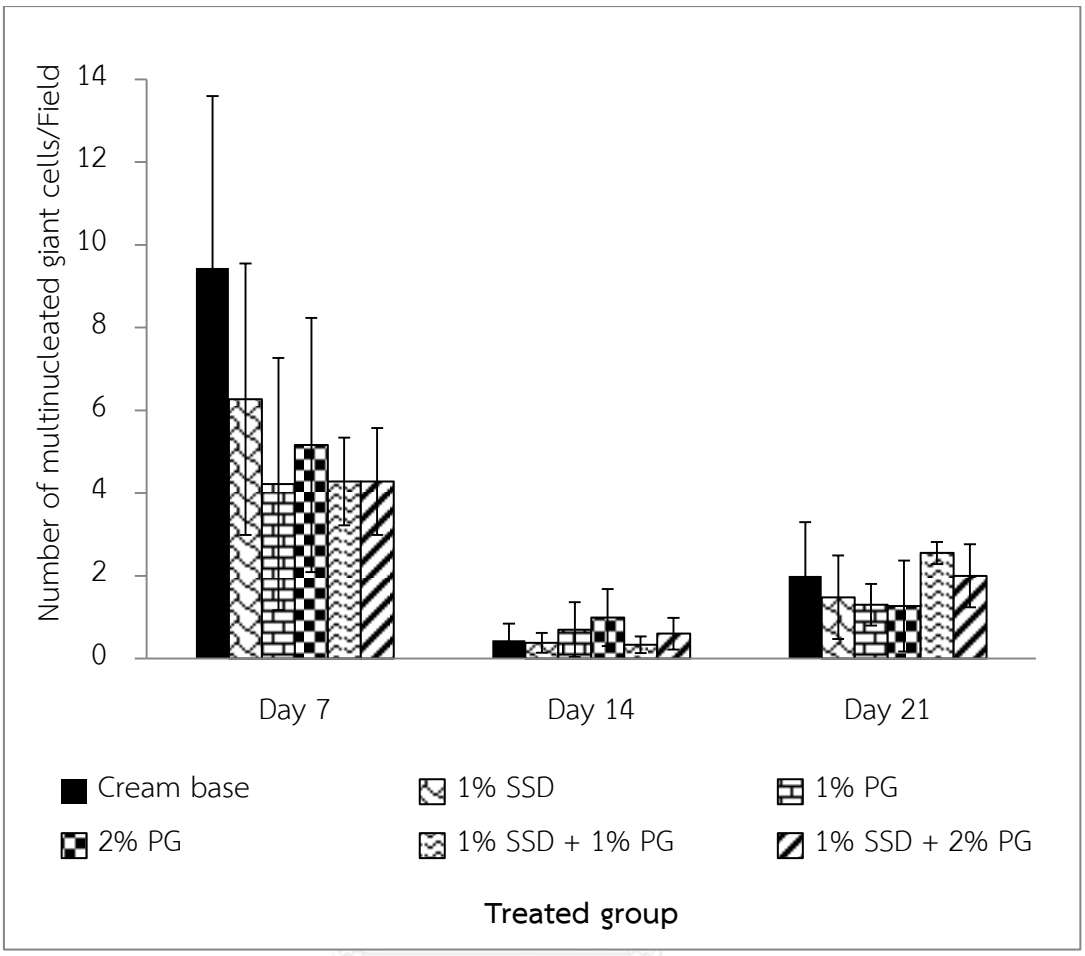



**Figure 21** Number of multinucleated giant cells/field on day 7, 14 and 21 post-burn. N = 6 for all groups.

**Number of macrophages/field**

On day 7, 14 and 21 post-burn, number of macrophages/field of the wounds treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG showed no significant difference when compared to the control group. Number of macrophages was highest on day 7 and then decreased on day 14 and 21 (Figure 22).

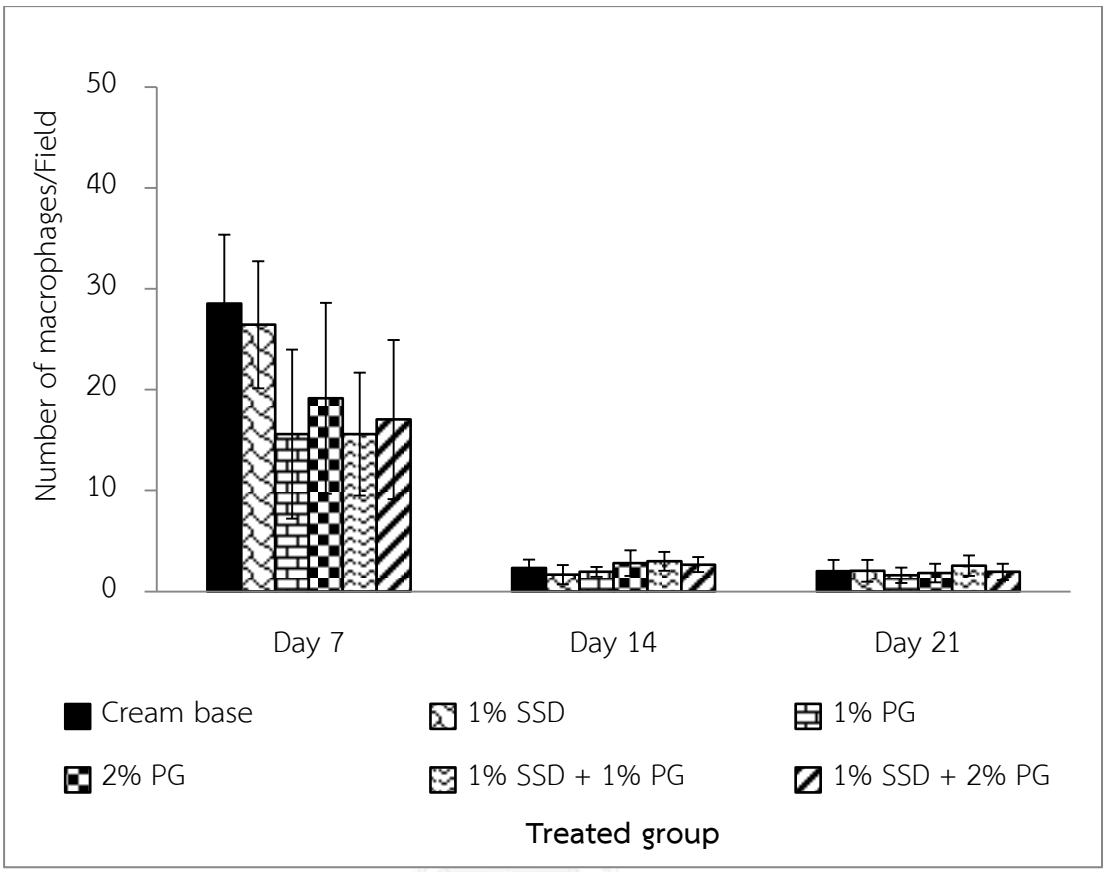



**Figure 22** Number of macrophages/field on day 7, 14 and 21 post-burn.

N = 6 for all groups.

**Number of mast cells/field**

On day 7 and 14 post-burn, number of mast cells/field of the wounds treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG showed no significant difference when compared to the control group. However, on day 21 post-burn, number of mast cells/field of the wounds treated with cream base was significantly ($p<0.01$) higher than other groups (Figure 23).

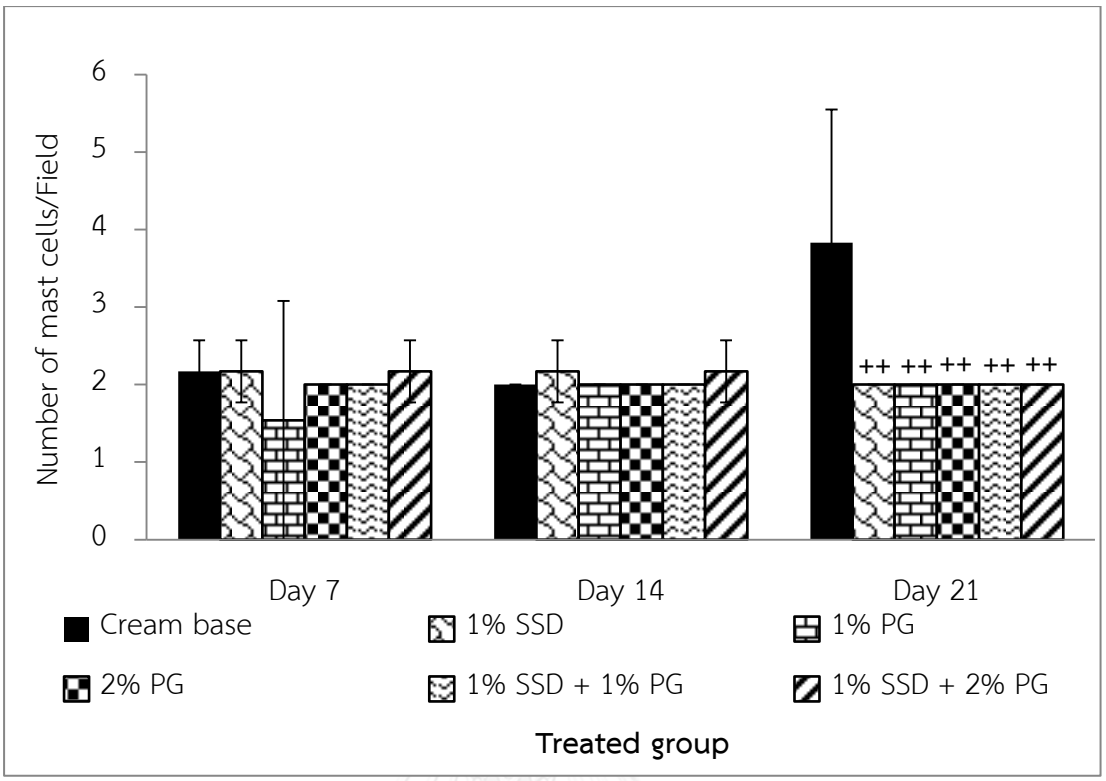



**Figure 23** Number of mast cells/field on day 7, 14 and 21 post-burn. N = 6 for all groups.

$^{++}p<0.01$ significantly different compared to cream base.

**Neovascularization**

On day 7 post-burn, number of vessels/field of the wounds treated with combination of 1% SSD + 2% PG was significantly higher when compared to the control group, 1% SSD, 1% PG, 2% PG ($p<0.05$, $p<0.01$, $p<0.05$ and $p<0.01$, respectively).

On day 14 post-burn, number of vessels/field of the wounds treated with combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were significantly higher when compared to the control and 1% SSD group ($p<0.01$ and $p<0.01$, respectively). Number of vessels/field was highest on day 14. On day 21 post-burn, number of vessels/field of all groups tend to decrease and showed no significant difference when compared to the control group (Figure 24).

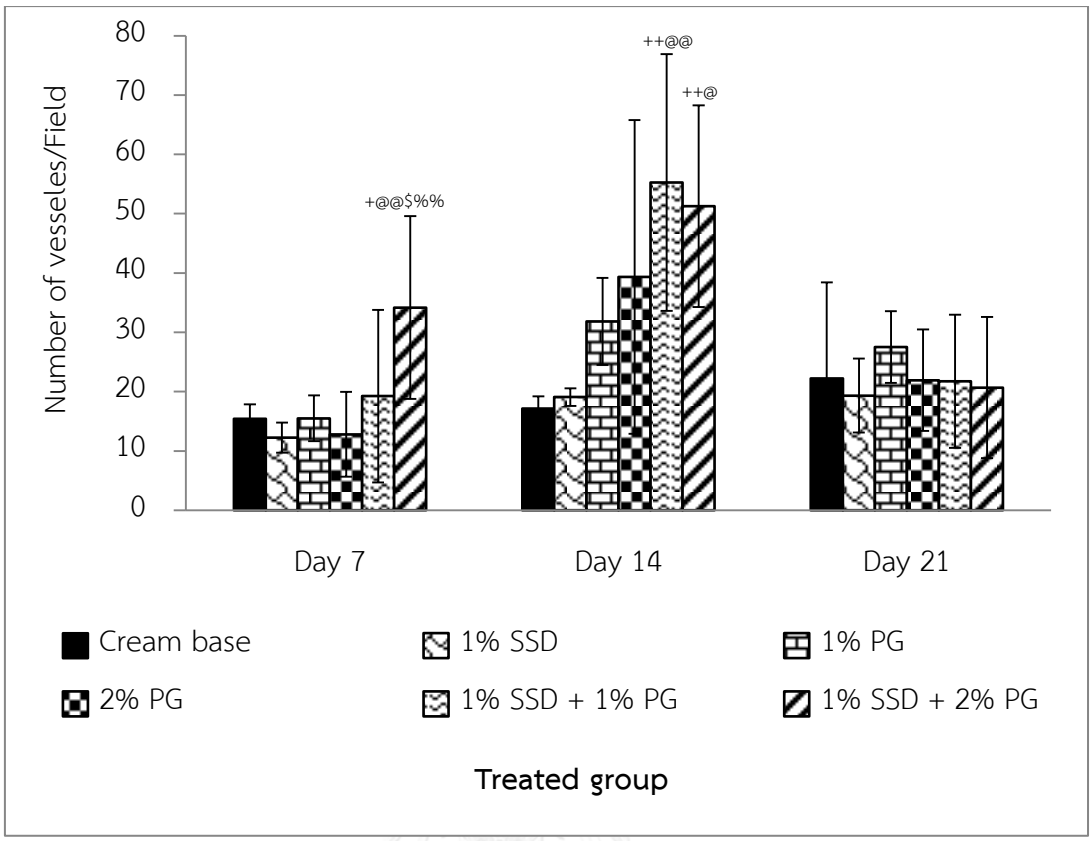



**Figure 24** Number of vessels/field on day 7, 14 and 21 post-burn. N = 6 for all groups.



$^{+}p<0.05$, $^{++}p<0.01$ significantly different compared to cream base.

$^{@}p<0.05$, $^{@@}p<0.01$ significantly different compared to 1% SSD.

$^{\$}p<0.05$ significantly different compared to 1% PG.

$^{\%\%}p<0.01$ significantly different compared to 2% PG.

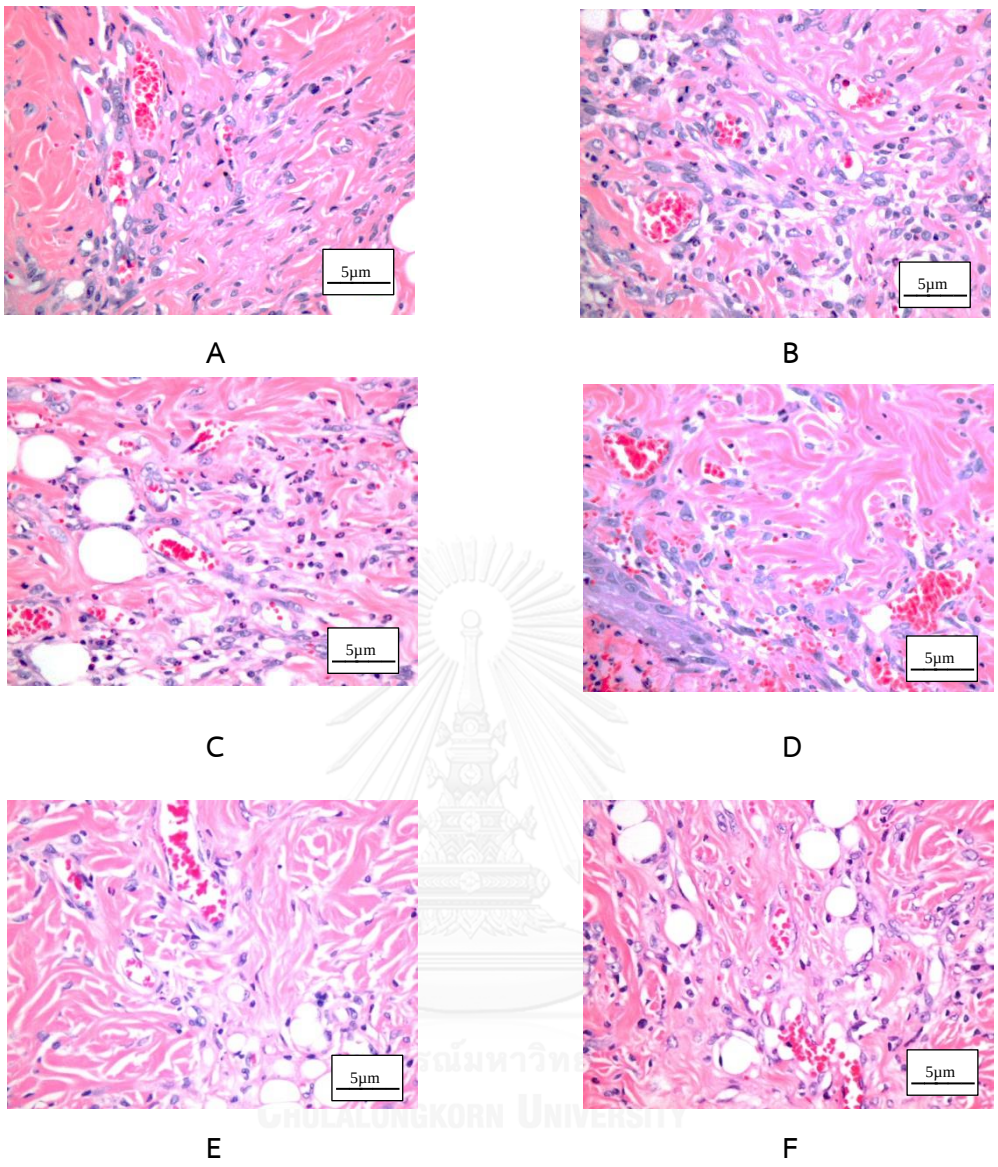



**Figure 25** : Histological appearance on day 7 post-burn.

A = Cream base

B = 1% SSD

C = 1% PG

D = 2% PG

E = 1% SSD + 1% PG

F = 1% SSD + 2% PG

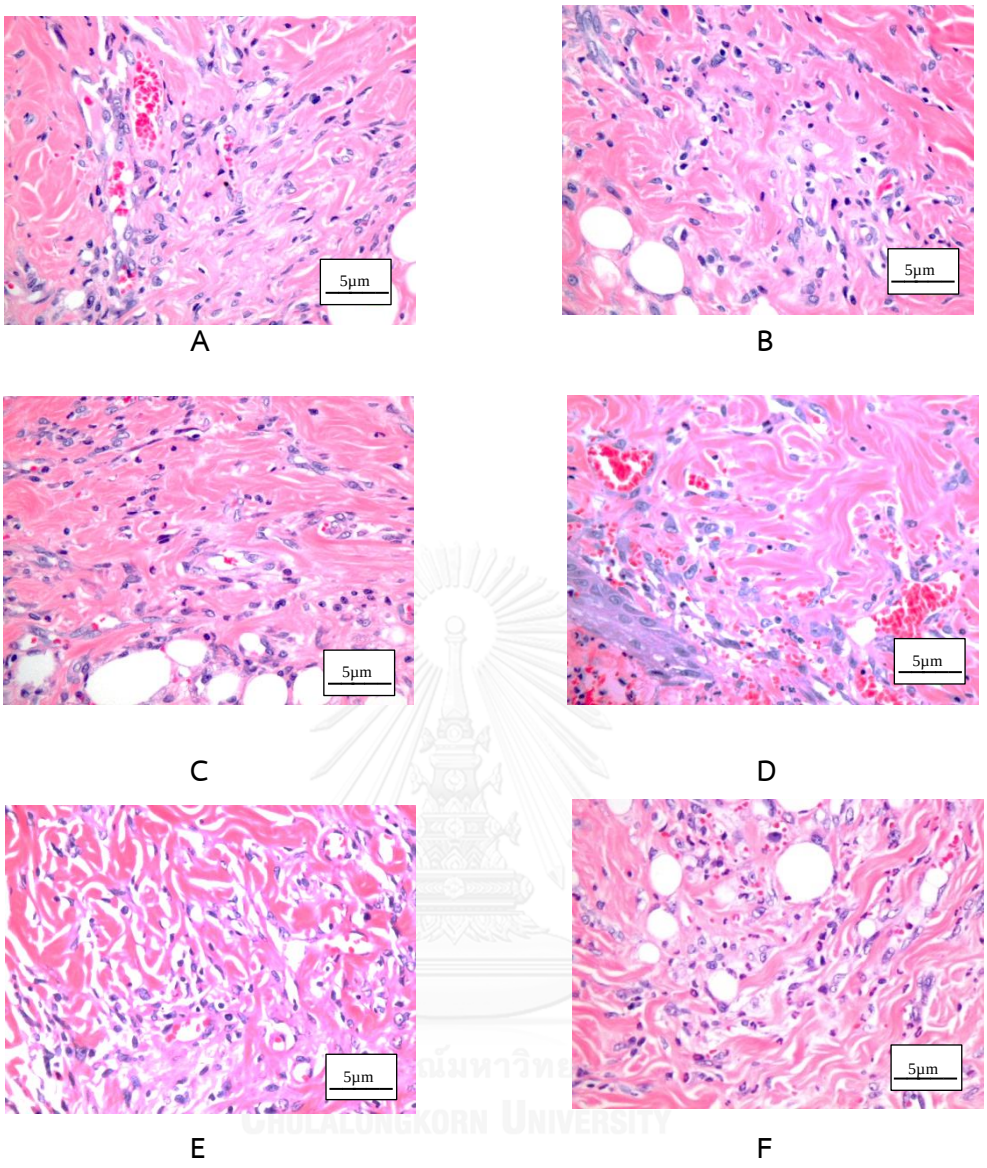



**Figure 26** Histological appearance on day 14 post-burn.

A = Cream base

B = 1% SSD

C = 1% PG

D = 2% PG

E = 1% SSD + 1% PG

F = 1% SSD + 2% PG

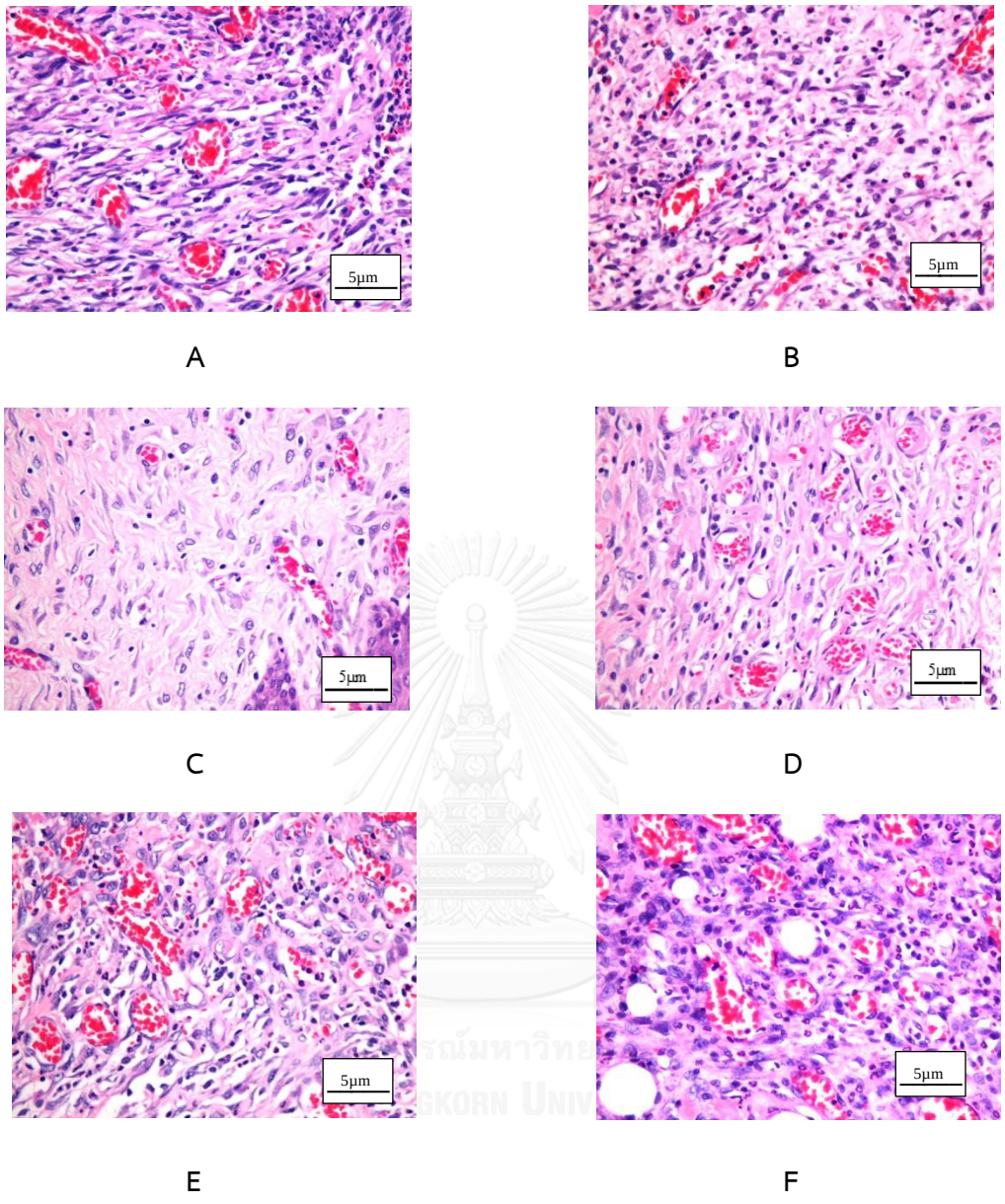



**Figure 27** Histological appearance on day 21 post-burn.

A = Cream base

B = 1% SSD

C = 1% PG

D = 2% PG

E = 1% SSD + 1% PG

F = 1% SSD + 2% PG

**Evaluation of liver function tests**

Serum samples from each group were evaluated for aspartate aminotransferase, alanine transaminase and alkaline phosphatase levels on day 7, 14, 21 and 28 post-burn.

**The level of aspartate aminotransferase enzyme**

On day 7, 14 and 28 post-burn the levels of aspartate aminotransferase enzyme of the serum of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group.

On day 21 post-burn, the levels of aspartate aminotransferase enzyme of the serum of rats treated with 1% SSD, 1% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group but were significantly lower than their own baselines ($p<0.001$, $p<0.001$, $p<0.01$ and $p<0.001$, respectively) (Table 2).

**Table 2** The level of aspartate aminotransferase enzyme on day 7, 14, 21 and 28 post-burn. N = 10 for all groups.

| Groups | The level of aspartate aminotransferase enzyme (U/L) | | | | |
|---|---|---|---|---|---|
| | **Baseline** | **Day 7** | **Day 14** | **Day 21** | **Day 28** |
| Control (Cream base) | 119.10 ± 8.94 | 118.80 ± 17.52 | 97.20 ± 21.19 | 98.10 ± 14.91 | 139.11 ± 43.38 |
| 1% SSD | 126.30 ± 16.57 | 101.90 ± 15.09 | 100.80 ± 24.10 | **(***)** 84.60 ± 12.94 | 164.20 ± 86.28 |
| 1% PG | 129.70 ± 19.47 | 114.30 ± 29.08 | 101.60 ± 18.37 | **(***)** 91.80 ± 12.98 | 151.10 ± 35.32 |
| 2% PG | 134.40 ± 21.34 | 114.30 ± 30.74 | 124.70 ± 26.80 | 108.90 ± 27.84 | 146.11 ± 46.59 |
| 1% SSD + 1% PG | 123.10 ± 9.12 | 115.00 ± 27.90 | 110.70 ± 32.28 | **(**)** 90.50 ± 9.95 | 143.20 ± 30.85 |
| 1% SSD + 2% PG | 137.30 ± 28.51 | 99.70 ± 14.71 | 126.20 ± 46.04 | **(***)** 99.90 ± 15.36 | 180.87 ± 92.51 |

***p*<0.01, ****p*<0.001 significantly different compared to baselines.

**The level of alanine transaminase enzyme**

On day 14 post-burn, the level of alanine transaminase enzyme of the serum of rats treated with 2% PG was significantly higher ($p<0.05$) than baseline. However, on day 7, 21 and 28 post-burn, the levels of alanine transaminase enzyme of the serum of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group (Table 3).

**Table 3** The level of alanine transaminase enzyme on day 7, 14, 21 and 28 post-burn. N = 10 for all groups.

| Groups | The level of alanine transaminase enzyme (U/L) | | | | |
|---|---|---|---|---|---|
| | Baseline | Day 7 | Day 14 | Day 21 | Day 28 |
| Control (Cream base) | 33.80 ± 9.07 | (*) 52.10 ± 16.91 | 34.90 ± 8.46 | 43.40 ± 8.25 | 52.30 ± 24.33 |
| 1% SSD | 32.80 ± 10.11 | 43.80 ± 4.61 | 42.40 ± 11.08 | 38.60 ± 5.21 | 56.00 ± 23.45 |
| 1% PG | 39.30 ± 10.91 | 52.60 ± 20.06 | 43.30 ± 9.12 | 37.00 ± 2.36 | 57.22 ± 27.61 |
| 2% PG | 41.20 ± 11.79 | 45.60 ± 8.85 | (+) 50.50 ± 11.28 | 45.40 ± 15.43 | 46.90 ± 19.53 |
| 1% SSD + 1% PG | 38.00 ± 8.86 | 50.80 ± 10.36 | 42.80 ± 9.34 | 39.30 ± 7.48 | 41.40 ± 14.68 |
| 1% SSD + 2% PG | 39.60 ± 6.47 | 43.80 ± 10.85 | 44.00 ± 9.50 | 40.50 ± 6.55 | 47.42 ± 10.93 |

*$p<0.05$ significantly different compared to baseline.

+$p<0.05$ significantly different compared to cream base.

**The level of alkaline phosphatase enzyme**

On day 7 post-burn, the levels of alkaline phosphatase enzyme of the serum of rats treated with 1% SSD, 1% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not different when compared to the control group but the levels of alkaline phosphatase enzyme of all groups were significantly higher than their own baselines ($p<0.01$, $p<0.001$, $p<0.001$, $p<0.001$ and $p<0.01$, respectively).

On day 14 post-burn, the levels of alkaline phosphatase enzyme of the serum of rats treated with 1% SSD, 1% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were significant higher compared to the control group ($p<0.05$, $p<0.05$, $p<0.05$ and $p<0.001$, respectively). Moreover, the level of alkaline phosphatase enzyme of rats treated with combination of 1% SSD + 2% PG cream was significant higher than baseline ($p<0.05$).

On day 21 post-burn, the level of alkaline phosphatase enzyme of rats treated with cream base and combination of 1% SSD + 2% PG were significant higher than their baselines ($p<0.001$ and $p<0.05$, respectively).

However, on day 28 post-burn, the levels of alkaline phosphatase enzyme of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significantly different when compared to the control group (Table 4).

**Table 4** The level of alkaline phosphatase enzyme on day 7, 14, 21 and 28 post-burn. N = 10 for all groups**.**

| Groups | The level of alkaline phosphatase enzyme (U/L) | | | | |
|---|---|---|---|---|---|
| | **Baseline** | **Day 7** | **Day 14** | **Day 21** | **Day 28** |
| Control (Cream base) | 138.30 ± 20.30 | **(**)** 202.10 ± 50.65 | 183.00 ± 53.43 | **(**)** 219.50 ± 53.66 | 153.80 ± 25.57 |
| 1% SSD | 137.30 ± 25.52 | **(***)** 220.70 ± 24.89 | **(*)** 194.80 ± 40.19 | 200.80 ± 50.71 | 160.90 ± 44.17 |
| 1% PG | 152.20 ± 28.96 | **(***)** 231.50 ± 29.89 | **(*)** 211.50 ± 44.02 | 200.40 ± 64.95 | 156.30 ± 33.01 |
| 2% PG | 154.10 ± 36.75 | 206.40 ± 46.25 | 200.00 ± 32.00 | 198.10 ± 61.64 | 148.80 ± 27.12 |
| 1% SSD + 1% PG | 146.30 ± 26.68 | **(***)** 221.10 ± 46.45 | **(*)** 205.90 ± 35.00 | 208.10 ± 37.93 | 158.70 ± 20.47 |
| 1% SSD + 2% PG | 143.60 ± 32.99 | **(***)** 244.30 ± 35.15 | **(***, +)** 237.30 ± 43.38 | **(*)** 210.60 ± 27.68 | 160.20 ± 55.91 |

*$p<0.05$, **$p<0.01$, ***$p<0.001$ significantly different compared to baseline.

$^{+}p<0.05$ significantly different compared to cream base.

**Evaluation of kidney function tests**

Serum samples from each group were evaluated for levels of blood urea nitrogen and creatinine on day 7, 14, 21 and 28 burning.

**Blood urea nitrogen**

On day 7 post-burn, the levels of blood urea nitrogen of rats treated with 1% PG and combination of 1% SSD + 2% PG were significantly higher than the control group ($p<0.05$ and $p<0.01$, respectively). Moreover, on day 7 and day 14 post-burn, the level of blood urea nitrogen of rats treated with combination of 1% SSD + 2% PG were also significantly ($p<0.05$) higher than 1% SSD.

On day 21 post-burn, the levels of blood urea nitrogen of rats treated with cream base and 1% PG were significantly higher than their own baselines ($p<0.01$ and $p<0.01$, respectively) (Table 5).

**Table 5** The level of blood urea nitrogen post-burn on day 7, 14, 21 and 28 post-burn. N = 10 for all groups.

| Groups | The level of blood urea nitrogen (mg%) | | | | |
|---|---|---|---|---|---|
| | Baseline | Day 7 | Day 14 | Day 21 | Day 28 |
| Control (Cream base) | 21.50 ± 2.07 | 22.50 ± 4.25 | 23.40 ± 1.96 | (**) 37.34 ± 3.76 | 26.50 ± 3.66 |
| 1% SSD | 34.10 ± 6.14 | 24.51 ± 6.73 | 29.90 ± 3.35 | 41.13 ± 12.91 | 27.80 ± 3.74 |
| 1% PG | 24.40 ± 7.20 | (+) 35.32 ± 9.79 | 27.90 ± 3.35 | (**) 38.89 ± 7.37 | 27.70 ± 3.74 |
| 2% PG | 32.00 ± 5.66 | 33.81 ± 10.10 | 27.20 ± 4.32 | 36.55 ± 8.26 | 26.70 ± 2.26 |
| 1% SSD + 1% PG | 30.40± 5.64 | 29.46 ± 10.51 | 28.20 ± 2.25 | 38.31 ± 9.09 | 26.60 ± 2.67 |
| 1% SSD + 2% PG | 30.20 ± 5.07 | (++, @) 37.59 ± 10.94 | 27.90 ± 2.81 | (@) 28.35 ± 10.55 | 29.90 ± 3.35 |

**$p<0.01$ significantly different compared to baseline.

$^{+}p<0.05$, $^{++}p<0.01$ significantly different compared to cream base.

$^{@}p<0.05$ significantly different compared to 1% SSD.

**Creatinine**

On day 7 post-burn, the levels of creatinine of rats treated with 1% PG and combination of 1% SSD + 1% PG were significant higher than the control group ($p<0.05$ and $p<0.05$, respectively). However, on day 14, 21 and 28 post-burn, the levels of creatinine of rats treated with 1% SSD, 1% PG, 2% PG, combination of 1% SSD + 1% PG and combination of 1% SSD + 2% PG were not significant different compared to the control group (Table 6).

**Table 6** The level of creatinine on day 7, 14, 21 and 28 post-burn. N = 10 for all groups.

| Groups | The level of creatinine (mg%) | | | | |
|---|---|---|---|---|---|
| | **Baseline** | **Day 7** | **Day 14** | **Day 21** | **Day 28** |
| Control (Cream base) | 0.54 ± 0.05 | 0.48 ± 0.04 | 0.50 ± 0.05 | 0.53 ± 0.05 | 0.54 ± 0.16 |
| 1% SSD | 0.51 ± 0.05 | 0.50 ± 0.07 | 0.57 ± 0.05 | 0.60 ± 0.12 | 0.57 ± 0.13 |
| 1% PG | 0.54 ± 0.05 | **(+)** 0.58 ± 0.05 | 0.56 ± 0.05 | 0.59 ± 0.05 | 0.51 ± 0.51 |
| 2% PG | 0.54 ± 0.05 | 0.53 ± 0.05 | 0.55 ± 0.05 | 0.54 ± 0.05 | 0.53 ± 0.08 |
| 1% SSD + 1% PG | 0.55 ± 0.05 | **(+)** 0.57 ± 0.05 | 0.55 ± 0.05 | 0.55 ± 0.05 | 0.49 ± 0.07 |
| 1% SSD + 2% PG | 0.53 ± 0.07 | 0.55 ± 0.05 | 0.55 ± 0.05 | 0.52 ± 0.04 | 0.72 ± 0.07 |



$^{+}p<0.05$ significantly different compared to cream base.

# CHAPTER V

# DISCUSSION

Wound healing is a process in which the skin repairs itself after injury. This process involves soluble mediators such as cytokines, growth factors, and cells such as platelets and white blood cells. Wound healing is a complex process involving epidermal regeneration, keratinocyte migration, fibroblast proliferation, neovascularization, and collagen synthesis (Enoch & Leaper, 2005; Li et al., 2007).

In this study, epidermal regeneration and keratinocyte migration were analyzed histologically, and calculated as the percentage of re-epithelialization and wound healing on days 3, 7, 14, 21, and 28 post-burn. On day 3, rats treated with PG cream had a faster wound healing rate than those treated with 1% SSD as a standard positive control. The burn wounds in all groups look dry and red around the wound edge from inflammatory process. There were mild degree of swelling of all layers (epidermis and dermis) of all groups but discharge was apparent only in the control group.

On days 7 and 14 post-burn, re-epithelialization was well developed in rats treated with 1% SSD or PG. The percentages of wound healing in all PG groups were comparable to that in the 1% SSD group. The characteristic of burn wounds were dry. The swelling and redness around the wound edge was decreased and discharge was not apparent in the control group. Wound edges on day 7 and 14 post-burn were contracted as a result of keratinocyte migration and fibroblast proliferation (Battegay, 1995; Enoch & Leaper, 2005; Li et al., 2007; Pastar et al., 2014). The wound size on day 14 post-burn was apparently smaller than day 7.

On day 21, rats treated with 1% SSD + 2% PG cream had the fastest wound healing rate. The wounds were covered with new tissue and new hair. Application of 1% SSD or PG facilitated and accelerated healing of burn wounds, as observed on the last day of the study (day 28). Results from histological observation were in accordance with the percentages of wound healing obtained by calculation.

The presence of multinucleated giant cells and macrophages is an indicator of wound inflammation and healing. Macrophages trigger the wound healing process by eliminating pathogens and dead tissue through phagocytosis. Furthermore, these cells activate secretion of growth factors and other cytokines that promote wound healing (Guo & Dipietro, 2010; Mahdavian Delavary, van der Veer, van Egmond, Niessen, & Beelen, 2011). The levels of macrophages and multinucleated giant cells increase in the inflammatory phase and decrease in the proliferative and remodeling phases (Li et al., 2007). From the results, the number of multinucleated giant cells and macrophages started to decrease after day 14 post-burn, indicating reduced inflammation of the wounds.

The number of mast cells increases in the presence of inflammation, allergy or irritation (Theoharides et al., 2012). The number of mast cells in all groups on days 7, 14, and 21 were similar to those in the control group, indicating that the formulations did not cause irritation or allergy. On day 21, the number of mast cells in the control group was significantly higher than that in the other groups, but was within the normal range (25 cells/$mm^2$) (Aldenborg & Enerback , 1986).

The inflammatory phase in wound healing is followed by a proliferation phase involving neovascularization and collagen synthesis, and PG promotes neovascularization by binding to VEGF receptors (Gallo, 2000). The number of vessels/field in all treated groups reached its highest level on day 14 and then decreased on day 21. Rats treated with 1% SSD + 2% PG cream and 1% SSD + 1% PG cream had the highest number of vessels/field on days 7 and 14, respectively,

compared to the other groups, indicating the efficacy of PG in promoting neovascularization. The remodeling phase starts after the proliferation phase, as indicated by a decrease of collagen and fiber synthesis and blood vessel regeneration (Broughton, Janis, & Attinger, 2006; Enoch & Leaper, 2005). Our results showed that the remodeling phase is present on day 21 and wound healing was nearly completed at this time. Overall, the study showed that PG can accelerate and facilitate burn wound healing in rats, partly by stimulating neovascularization. A combination of 1% SSD + 1% or 2% PG cream seem to be the effective formulations for treating burn wounds.

The level of liver enzymes including aspartate aminotransferase and alanine transaminase in the blood increase when liver cells were damaged injured or inflamed from alcohol and drugs. Aspartate aminotransferase has also been found in other cells besides liver cells such as cardiac cells and striated muscle cells. Thus, the level of this enzyme was not specific to liver dysfunction but also a disease like acute myocardial infarction. Therefore, alanine transaminase level is more specific than aspartate aminotransferase level as an indicator for the liver function test (Kanai, Honda, Uehara, & Matsumoto, 2008). On Day 21, the level of aspartate aminotransferase in rats treated with 1% SSD and PG increased significantly compared to baselines but still within the normal range (82-127 U/L) (Diloke, Mathurot, Rapee, & Kanchana, 2011). The level of alanine transaminase only in rats treated with 2% PG increased significantly compared to baselines but still within the normal range (36-64 U/L) (Diloke et al., 2011).

Alkaline phosphatase is an enzyme in epithelial cells of gall bladder. The level of this enzyme increases when patient has an obstruction of bile duct, small biliary tract, stones, and tumors in the liver. Alkaline phosphatase is also found in other organs such as bone, placenta and intestine (Salama, Bilgen, Al Rashdi, & Abdulla, 2012). On day 7, the level of alkaline phosphatase in rats treated with

cream base increased significantly compared to baselines which may be due to dehydration from second degree burn. The level of alkaline phosphatase of rats treated with 1% SSD and PG also increased on day 14 and 21 post-burn and decrease to normal range (82-112 U/L) on day 28 (Diloke et al., 2011). The increase of all three liver enzymes levels of less than 3 folds are indicated as acceptable levels in clinical setting (Koolhaas, 2010; Thrall, Weiser, Allison, & Campbell, 2012; Zhang et al., 2004). However, these enzymes levels should be monitored continuously if these preparations will be used for open wounds for long time.

Blood urea nitrogen (BUN) and creatinine are the waste products from the metabolism process which are excreted by the kidney to maintain the homeostasis. High level of BUN and creatinine indicates an impairment of renal function. Baseline levels of BUN of all groups were higher than normal levels (15.6-20.1 mg/dl) reported by Diloke, Mathurot, Rapee, & Kanchana (2011), which was probably due to different housing environment of the animals. The level of BUN of rats treated with SSD appeared to decrease, while BUN level of rats treated with PG appeared to increase compared to baselines on the early stage of wound healing (day 7). On day 7 post-burn, the level of BUN in rats treated with 1% PG was significantly higher compared to cream base and in rats treated with 1% SSD + 2% PG was significantly higher compared to cream base and 1% SSD. The levels of BUN of all groups reached their maximum levels on day 21 or 28 and were decreased to baselines at the end stage of wound healing (day 28). The decreased in BUN levels after day 21 post-burn was in accordance with other studies (Barisoni & Bertolini, 1981; Sirinoot, Pornprom, & Pornanong, 2011). The results indicated that the increase levels of BUN may partly be due to PG.

Baseline levels of creatinine in all groups were higher than normal levels (0.37-0.47 mg/dl) reported by Diloke et al. (2011) which also probably due to

different housing environment. On the early stage of wound healing (day 7 post-burn), the levels of creatinine in rats treated with 1% PG and combination of 1% SSD + 1% PG cream were significantly higher compared to cream base. However, the levels of creatinine of both groups were decreased to baselines at the end stage of wound healing (day 28). These results were in agreement with previous reports of Barisoni & Bertolini (1981) and Sirinoot et al. (2011). The results indicated that the increase levels of creatinine may partly be due to PG. Altogether, these results indicated that the increase in BUN and creatinine levels were partly due to PG.

PG is an essential component in the extracellular matrix (Perrimon & Bernfield, 2001). Research displayed that PG has a similar structure to epidermal growth factor (Aviezer & Yayon, 1994; Neelam, 2013; Raab & Klagsbrun, 1997). Previous study found that PG can be bound to several types of growth factors, for example, TGF- β, FGF-2, VEGF family and PDGF resulting in cell division, cell proliferation and activate neovascularization (Gallo, 2000; Perrimon & Bernfield, 2001). It has been reported that PG found in cartilage was important for the wound healing process (Hardingham & Fosang, 1992; Knudson & Knudson, 2001). This study demonstrated that PG extracted from fish cartilage can accelerate and facilitate burn wound healing without causing toxic effects to the liver and kidney. PG has no antimicrobial effect but 1% SSD does. Therefore, the combination of 1% SSD with PG may have synergistic effect in enhancing wound healing process with antimicrobial activity.

# CHAPTER VI

# CONCLUSION

The results suggest that PG extracted from fish cartilage can accelerate and facilitate wound healing in rats. The combination of 1% SSD and 1% or 2% PG seem to have high efficacy in accelerating and facilitating wound healing in rats. The combination of 1% SSD and 1% or 2% PG is safe for long term use. Further investigation of the safety profile of PG and the combination of 1% SSD and PG in detail is required.

**The future research could comprise of several objectives as listed below:**

(1) To better understand the mechanism of PG that involved in wound healing effects.

(2) To investigate the potential use of PG in combination with other anti-microbial drugs.

(3) To elucidate side effects and toxic effects of PG at high dosage or after chronic use.

These studies may provide important clues to help understand the mechanism underlying the wound healing effects of PG and further support the use of such compounds in clinical setting.

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# APPENDIX

# Appendix A

## Certification of Project Approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

## Chulalongkorn University Animal Care and Use Committee

| Certificate of Project Approval | ☐ Original ☐ Renew |
|---|---|
| **Animal Use Protocol No. 13-33-011** | **Approval No. 13-33-011** |

**Protocol Title**
Application of proteoglycan from fish cartilage for the treatment of burn wounds

**Principal Investigator**
PASARAPA TOWIWAT, Ph.D.

**Certification of Institutional Animal Care and Use Committee (IACUC)**
This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

| Date of Approval | Date of Expiration |
|---|---|
| March 4, 2013 | March 4, 2015 |

**Applicant Faculty/Institution**
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan BKK-THAILAND. 10330

| Signature of Chairperson | Signature of Authorized Official |
|---|---|
| Name and Title<br>**THONGCHAI SOOKSAWATE, Ph.D.**<br>**Chairman** | Name and Title<br>**NARUEPORN SUTANTHAVIBUL, Ph.D.**<br>**Associate Dean (Research and Academic Service)** |

*The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.*

*This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*

# Appendix B

## The steps to extraction of PG from fish cartilage

**The steps to extraction of PG from fish cartilage are as follow;**

A. Have the fish bone macerated in 4% acetic acid at 4° C and stirs up continuously for about 48 hours and then filtered with filter paper.

B. Put the solution in (A) and centrifuge at 10,000 rpm at 4° C for 20 minutes.

C. Put sodium chloride dissolved in ethanol until getting saturated and then make the substance of clause (B) macerated for 24 hours and centrifuge at 10,000 rpm at 4° C for 20 minutes.

D. After centrifuged, the sediment is the PG which can be purified by removing the dissolve in 4% acetic acid again.

E. Have it dialyzed through the dialysis bag pore size about 1,000 KDa.

F. Have it freeze-dried until became fine powder, then sieve to get the size as you prefer.

# Appendix C

## Data of Percentage wound healing

**Table 7** Percentage wound healing on day 3 post-burn.

| Treatments | % Wound healing |
|---|---|
| Cream base | 4.51 ± 1.68 |
| 1% SSD | 6.71 ± 3.09 |
| 1% PG | 5.80 ± 4.09 |
| 2% PG | 11.60 ± 7.70 |
| 1% SSD + 1% PG | 8.30 ± 2.00 |
| 1% SSD + 2% PG | 9.39 ± 3.73 |

Values means ± Standard deviation; N = 10 for all groups

**Table 8** Percentage wound healing on day 7 post-burn.

| Treatments | % Wound healing |
|---|---|
| Cream base | 14.06 ± 4.25 |
| 1% SSD | 38.68 ± 18.30 |
| 1% PG | 40.89 ± 10.93 |
| 2% PG | 42.47 ± 11.55 |
| 1% SSD + 1% PG | 45.63 ± 13.63 |
| 1% SSD + 2% PG | 54.01 ± 13.96 |

Values means ± Standard deviation; N = 10 for all groups

**Table 9** Percentage wound healing on day 14 post-burn.

| Treatments | % Wound healing |
|---|---|
| Cream base | 35.00 ± 10.23 |
| 1% SSD | 69.33 ± 8.55 |
| 1% PG | 69.25 ± 7.59 |
| 2% PG | 74.04 ± 6.71 |
| 1% SSD + 1% PG | 77.39 ± 6.69 |
| 1% SSD + 2% PG | 80.22 ± 10.70 |

Values means ± Standard deviation; N = 10 for all groups

**Table 10** Percentage wound healing on day 21 post-burn.

| Treatments | % Wound healing |
|---|---|
| Cream base | 66.80 ± 8.50 |
| 1% SSD | 88.45 ± 4.96 |
| 1% PG | 89.78 ± 4.05 |
| 2% PG | 93.28 ± 1.81 |
| 1% SSD + 1% PG | 92.39 ± 4.65 |
| 1% SSD + 2% PG | 95.62 ± 2.85 |

Values means ± Standard deviation; N = 10 for all groups

**Table 11** Percentage wound healing on day 28 post-burn.

| Treatments | % Wound healing |
|---|---|
| Cream base | 2.83 ± 19.71 |
| 1% SSD | 100 ± 0 |
| 1% PG | 100 ± 0 |
| 2% PG | 100 ± 0 |
| 1% SSD + 1% PG | 100 ± 0 |
| 1% SSD + 2% PG | 100 ± 0 |

Values means ± Standard deviation; N = 10 for all groups

# Appendix D

## Data of Histological analysis

## (Percentage of epithelialization)

**Table 12** Percentage of epithelialization on day 7 post-burn.

| Treatments | % epithelialization |
|---|---|
| Cream base | 30.49 ± 16.16 |
| 1% SSD | 33.31 ± 21.08 |
| 1% PG | 32.73 ± 13.50 |
| 2% PG | 29.71 ± 16.94 |
| 1% SSD + 1% PG | 35.01 ± 9.44 |
| 1% SSD + 2% PG | 55.33 ± 7.00 |

Values means ± Standard deviation; N = 6 for all groups

**Table 13** Percentage of epithelialization on day 14 post-burn.

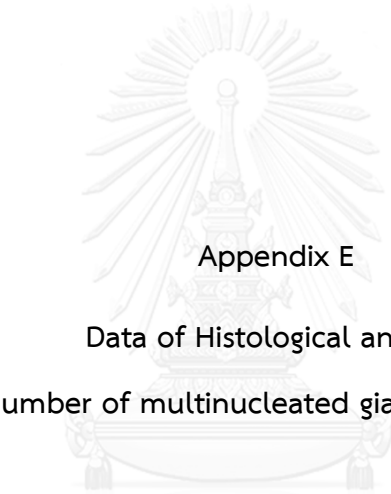
| Treatments | % epithelialization |
|---|---|
| Cream base | 62.67 ± 25.08 |
| 1% SSD | 85.42 ± 4.01 |
| 1% PG | 86.70 ± 11.74 |
| 2% PG | 90.07 ± 5.50 |
| 1% SSD + 1% PG | 85.77 ± 8.57 |
| 1% SSD + 2% PG | 90.99 ± 4.87 |

Values means ± Standard deviation; N = 6 for all groups

**Table 14** Percentage of epithelialization on day 21 post-burn.

| Treatments | % epithelialization |
|---|---|
| Cream base | 87.35 ± 8.70 |
| 1% SSD | 99.47 ± 0.88 |
| 1% PG | 99.62 ± 0.67 |
| 2% PG | 98.50 ± 1.59 |
| 1% SSD + 1% PG | 95.60 ± 5.86 |
| 1% SSD + 2% PG | 99.83 ± 0.40 |

Values means ± Standard deviation; N = 6 for all groups



# Appendix E

## Data of Histological analysis

## (Number of multinucleated giant cells/field)

**Table 15** Number of multinucleated giant cells/field on day 7 post-burn.

| Treatments | Multinucleated giant cells/ field |
|---|---|
| Cream base | 9.44 ± 4.16 |
| 1% SSD | 6.27 ± 3.28 |
| 1% PG | 4.22 ± 3.05 |
| 2% PG | 5.16 ± 3.07 |
| 1% SSD + 1% PG | 3.10 ± 1.06 |
| 1% SSD + 2% PG | 1.99 ± 1.29 |

Values means ± Standard deviation; N = 6 for all groups

**Table 16** Number of multinucleated giant cells/field on day 14 post-burn.

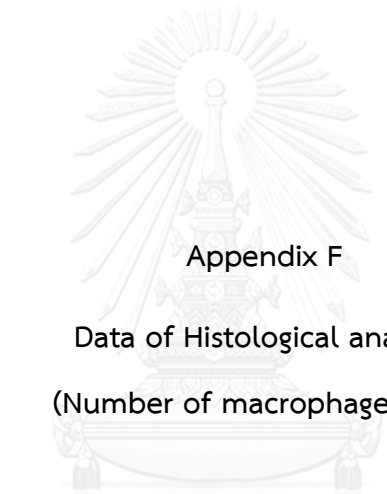
| Treatments | Multinucleated giant cells/field |
|---|---|
| Cream base | 0.44 ± 0.42 |
| 1% SSD | 0.38 ± 0.24 |
| 1% PG | 0.70 ± 0.66 |
| 2% PG | 0.99 ± 0.69 |
| 1% SSD + 1% PG | 0.33 ± 0.20 |
| 1% SSD + 2% PG | 0.60 ± 0.38 |

Values means ± Standard deviation; N = 6 for all groups

**Table 17** Number of multinucleated giant cells/field on day 21 post-burn

| Treatments | Multinucleated giant cells/field |
|---|---|
| Cream base | 2.00 ± 1.39 |
| 1% SSD | 1.48 ± 1.01 |
| 1% PG | 1.30 ± 0.50 |
| 2% PG | 1.10 ± 0.45 |
| 1% SSD + 1% PG | 2.55 ± 0.27 |
| 1% SSD + 2% PG | 1.99 ± 0.76 |

Values means ± Standard deviation; N = 6 for all groups



# Appendix F

## Data of Histological analysis

## (Number of macrophages/field)

**Table 18** Number of macrophages/field on day 7 post-burn.

| Treatments | Macrophages/field |
|---|---|
| Cream base | 28.55 ± 6.81 |
| 1% SSD | 26.44 ± 6.31 |
| 1% PG | 15.60 ± 6.38 |
| 2% PG | 19.16 ± 8.38 |
| 1% SSD + 1% PG | 15.60 ± 6.09 |
| 1% SSD + 2% PG | 17.05 ± 7.88 |

Values means ± Standard deviation; N = 6 for all groups

**Table 19** Number of macrophages/field on day 14 post-burn.

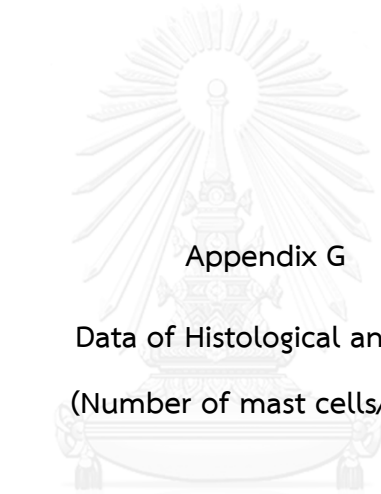
| **Treatments** | **Macrophages/field** |
|---|---|
| Cream base | 2.33 ± 0.84 |
| 1% SSD | 1.66 ± 0.96 |
| 1% PG | 1.94 ± 0.49 |
| 2% PG | 2.81 ± 1.26 |
| 1% SSD + 1% PG | 2.99 ± 0.94 |
| 1% SSD + 2% PG | 2.60 ± 0.74 |

Values means ± Standard deviation; N = 6 for all groups

**Table 20** Number of macrophages/field on day 21 post-burn.

| Treatments | Macrophages/field |
|---|---|
| Cream base | 2.33 ± 1.09 |
| 1% SSD | 2.05 ± 1.08 |
| 1% PG | 1.59 ± 0.76 |
| 2% PG | 1.83 ± 0.93 |
| 1% SSD + 1% PG | 2.55 ± 1.02 |
| 1% SSD + 2% PG | 1.94 ± 0.82 |

Values means ± Standard deviation; N = 6 for all groups





# Appendix G

## Data of Histological analysis

## (Number of mast cells/field)

**Table 21** Number of mast cells/field on day 7 post-burn.

| Treatments | Mast cells/field |
|---|---|
| Cream base | 2.16 ± 0.40 |
| 1% SSD | 2.16 ± 0.40 |
| 1% PG | 3.00 ± 1.54 |
| 2% PG | 2.00 ± 0.00 |
| 1% SSD + 1% PG | 2.00 ± 0.00 |
| 1% SSD + 2% PG | 2.16 ± 0.40 |

Values means ± Standard deviation; N = 6 for all groups

**Table 22** Number of mast cells/field on day 14 post-burn.

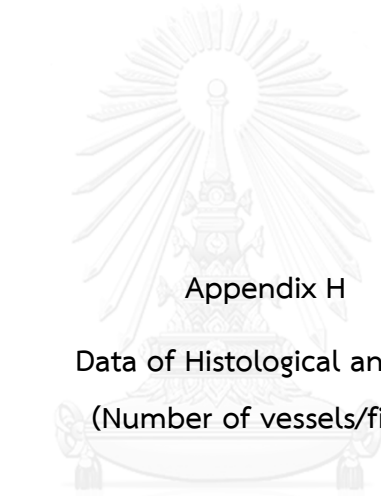
| **Treatments** | **Mast cells/field** |
|---|---|
| Cream base | 2.00 ± 0.00 |
| 1% SSD | 2.16 ± 0.40 |
| 1% PG | 2.00 ± 0.00 |
| 2% PG | 2.00 ± 0.00 |
| 1% SSD + 1% PG | 2.00 ± 0.00 |
| 1% SSD + 2% PG | 2.16 ± 0.40 |

Values means ± Standard deviation; N = 6 for all groups

**Table 23** Number of mast cells/field on day 21 post-burn.

| Treatments | Mast cells/field |
|---|---|
| Cream base | 3.83 ± 1.72 |
| 1% SSD | 2.00 ± 0.00 |
| 1% PG | 2.00 ± 0.00 |
| 2% PG | 2.00 ± 0.00 |
| 1% SSD + 1% PG | 2.00 ± 0.00 |
| 1% SSD + 2% PG | 2.00 ± 0.00 |

Values means ± Standard deviation; N = 6 for all groups



# Appendix H

## Data of Histological analysis

## (Number of vessels/field)

**Table 24** Number of vessels/field on day 7 post-burn.

| Treatments | Vessels/field |
|---|---|
| Cream base | 15.37 ± 2.41 |
| 1% SSD | 12.25 ± 2.52 |
| 1% PG | 15.50 ± 3.86 |
| 2% PG | 12.78 ± 7.15 |
| 1% SSD + 1% PG | 19.25 ± 14.15 |
| 1% SSD + 2% PG | 34.16 ± 15.41 |

Values means ± Standard deviation; N = 6 for all groups

**Table 25** Number of vessels/field on day 14 post-burn.

| Treatments | Vessels/field |
|---|---|
| Cream base | 17.16 ± 2.01 |
| 1% SSD | 19.08 ± 1.49 |
| 1% PG | 31.83 ± 7.33 |
| 2% PG | 39.33 ± 26.46 |
| 1% SSD + 1% PG | 55.25 ± 21.64 |
| 1% SSD + 2% PG | 51.25 ± 17.01 |

Values means ± Standard deviation; N = 6 for all groups

**Table 26** Number of vessels/field on day 21 post-burn.

| Treatments | Vessels/field |
|---|---|
| Cream base | 22.25 ± 16.17 |
| 1% SSD | 19.33 ± 6.22 |
| 1% PG | 27.50 ± 6.51 |
| 2% PG | 21.91 ± 8.57 |
| 1% SSD + 1% PG | 21.75 ± 11.23 |
| 1% SSD + 2% PG | 20.66 ± 11.91 |

Values means ± Standard deviation; N = 6 for all groups

# Appendix I

Data of Liver function test

(Aspartate aminotransferase enzyme)

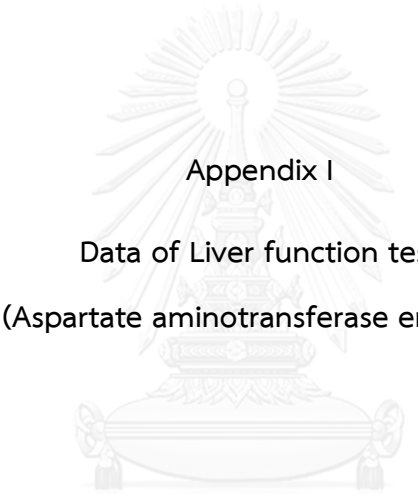


**Table 27** The level of aspartate aminotransferase enzyme post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 119.10 ± 8.94 |
| 1% SSD | 126.30 ± 16.57 |
| 1% PG | 129.70 ± 19.47 |
| 2% PG | 134.40 ± 21.34 |
| 1% SSD + 1% PG | 123.10 ± 9.12 |
| 1% SSD + 2% PG | 137.30 ± 28.52 |

Values means ± Standard deviation; N = 10 for all groups

**Table 28** The level of aspartate aminotransferase enzyme on day 7 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 118.80 ± 17.52 |
| 1% SSD | 101.90 ± 15.09 |
| 1% PG | 114.30 ± 29.08 |
| 2% PG | 114.30 ± 30.74 |
| 1% SSD + 1% PG | 115.00 ± 27.90 |
| 1% SSD + 2% PG | 99.70 ± 14.71 |

Values means ± Standard deviation; N = 10 for all groups

**Table 29** The level of aspartate aminotransferase enzyme on day 14 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 97.20 ± 21.19 |
| 1% SSD | 100.80 ± 24.10 |
| 1% PG | 101.60 ± 18.37 |
| 2% PG | 124.70 ± 26.80 |
| 1% SSD + 1% PG | 110.70 ± 32.28 |
| 1% SSD + 2% PG | 126.20 ± 46.04 |

Values means ± Standard deviation; N = 10 for all groups

**Table 30** The level of aspartate aminotransferase enzyme on day 21 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 98.10 ± 14.91 |
| 1% SSD | 84.60 ± 12.50 |
| 1% PG | 91.80 ± 12.98 |
| 2% PG | 108.90 ± 27.84 |
| 1% SSD + 1% PG | 90.50 ± 9.95 |
| 1% SSD + 2% PG | 99.90 ± 15.36 |

Values means ± Standard deviation; N = 10 for all groups

**Table 31** The level of aspartate aminotransferase enzyme on day 28 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 139.11 ± 43.38 |
| 1% SSD | 164.28 ± 86.28 |
| 1% PG | 115.10 ± 35.32 |
| 2% PG | 164.11 ± 46.59 |
| 1% SSD + 1% PG | 143.20 ± 30.85 |
| 1% SSD + 2% PG | 180.87 ± 92.59 |

Values means ± Standard deviation; N = 10 for all groups

# Appendix J

## Data of Liver function test

## (Alanine transaminase enzyme)

**Table 32** The level of alanine transaminase enzyme post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 33.80 ± 9.07 |
| 1% SSD | 32.80 ± 10.11 |
| 1% PG | 39.30 ± 10.91 |
| 2% PG | 41.20 ± 11.79 |
| 1% SSD + 1% PG | 38.00 ± 8.86 |
| 1% SSD + 2% PG | 39.60 ± 6.47 |

Values means ± Standard deviation; N = 10 for all groups

**Table 33** The level of alanine transaminase enzyme on day 7 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 52.10 ± 16.91 |
| 1% SSD | 43.80 ± 4.61 |
| 1% PG | 52.60 ± 20.06 |
| 2% PG | 45.60 ± 8.85 |
| 1% SSD + 1% PG | 50.80 ± 10.36 |
| 1% SSD + 2% PG | 43.80 ± 10.85 |

Values means ± Standard deviation; N = 10 for all groups

**Table 34** The level of alanine transaminase enzyme on day 14 post-burn.

| Cream base | Enzyme activity (U/L) |
|---|---|
| Cream base | 34.90 ± 8.46 |
| 1% SSD | 42.40 ± 11.08 |
| 1% PG | 43.30 ± 9.12 |
| 2% PG | 50.50 ± 11.28 |
| 1% SSD + 1% PG | 42.80 ± 9.34 |
| 1% SSD + 2% PG | 44.00 ± 9.50 |

Values means ± Standard deviation; N = 10 for all groups

**Table 35** The level of alanine transaminase enzyme on day 21 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 43.40 ± 8.25 |
| 1% SSD | 38.60 ± 5.21 |
| 1% PG | 37.00 ± 2.36 |
| 2% PG | 45.40 ± 15.43 |
| 1% SSD + 1% PG | 39.30 ± 7.48 |
| 1% SSD + 2% PG | 40.50 ± 6.55 |

Values means ± Standard deviation; N = 10 for all groups

**Table 36** The level of alanine transaminase enzyme on day 28 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 52.30 ± 24.33 |
| 1% SSD | 56.23 ± 23.45 |
| 1% PG | 57.22 ± 27.61 |
| 2% PG | 46.90 ± 19.55 |
| 1% SSD + 1% PG | 41.40 ± 14.68 |
| 1% SSD + 2% PG | 47.24 ± 10.93 |

Values means ± Standard deviation; N = 10 for all groups

# Appendix K

## Data of Liver function test

## (Alkaline phosphatase enzyme)

**Table 37** The level of alkaline phosphatase enzyme post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 138.30 ± 20.30 |
| 1% SSD | 137.30 ± 25.52 |
| 1% PG | 152.20 ± 28.96 |
| 2% PG | 154.10 ± 36.75 |
| 1% SSD + 1% PG | 146.30 ± 26.68 |
| 1% SSD + 2% PG | 143.60 ± 32.99 |

Values means ± Standard deviation; N = 10 for all groups

**Table 38** The level of alkaline phosphatase enzyme on day 7 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 202.10 ± 50.65 |
| 1% SSD | 220.70 ± 24.89 |
| 1% PG | 231.50 ± 29.89 |
| 2% PG | 206.40 ± 46.25 |
| 1% SSD + 1% PG | 221.10 ± 46.45 |
| 1% SSD + 2% PG | 244.30 ± 35.15 |

Values means ± Standard deviation; N = 10 for all groups

**Table 39** The level of alkaline phosphatase enzyme on day 14 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 183.00 ± 53.43 |
| 1% SSD | 194.80 ± 40.19 |
| 1% PG | 211.50 ± 44.02 |
| 2% PG | 200.00 ± 32.00 |
| 1% SSD + 1% PG | 205.90 ± 35.00 |
| 1% SSD + 2% PG | 237.30 ± 43.38 |

Values means ± Standard deviation; N = 10 for all groups

**Table 40** The level of alkaline phosphatase enzyme on day 21 post-burn.

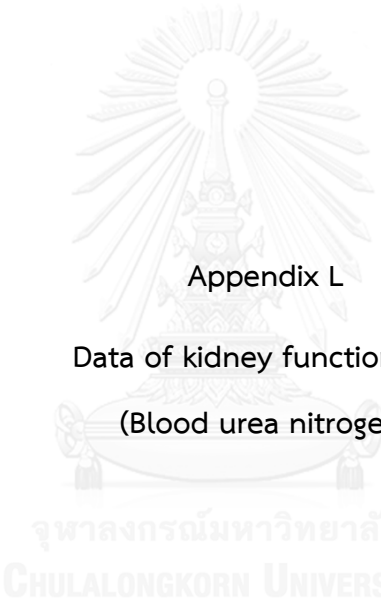
| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 219.50 ± 53.66 |
| 1% SSD | 200.80 ± 50.71 |
| 1% PG | 200.40 ± 64.95 |
| 2% PG | 198.10 ± 61.64 |
| 1% SSD + 1% PG | 208.10 ± 37.93 |
| 1% SSD + 2% PG | 210.60 ± 27.68 |

Values means ± Standard deviation; N = 10 for all groups

**Table 41** The level of alkaline phosphatase enzyme on day 28 post-burn.

| Treatments | Enzyme activity (U/L) |
|---|---|
| Cream base | 153.80 ± 25.57 |
| 1% SSD | 160.90 ± 44.17 |
| 1% PG | 156.30 ± 33.01 |
| 2% PG | 148.80 ± 27.12 |
| 1% SSD + 1% PG | 158.70 ± 20.47 |
| 1% SSD + 2% PG | 160.20 ± 55.91 |

Values means ± Standard deviation; N = 10 for all groups




# Appendix L

## Data of kidney function test

## (Blood urea nitrogen)

**Table 42** The level of BUN post-burn

| Treatments | BUN (mg%) |
|---|---|
| Cream base | 21.50 ± 2.07 |
| 1% SSD | 34.10 ± 6.14 |
| 1% PG | 24.40 ± 7.20 |
| 2% PG | 32.00 ± 5.66 |
| 1% SSD + 1% PG | 30.40 ± 5.64 |
| 1% SSD + 2% PG | 30.20 ± 5.07 |

Values means ± Standard deviation; N = 10 for all groups

**Table 43** The level of BUN on day 7 post-burn.

| Treatments | BUN (mg%) |
|---|---|
| Cream base | 22.50 ± 4.25 |
| 1% SSD | 24.51 ± 6.73 |
| 1% PG | 35.32 ± 9.79 |
| 2% PG | 33.81 ± 10.10 |
| 1% SSD + 1% PG | 29.46 ± 10.51 |
| 1% SSD + 2% PG | 37.59 ± 10.94 |

Values means ± Standard deviation; N = 10 for all groups

**Table 44** The level of BUN on day 14 post-burn.

| Treatments | BUN (mg%) |
|---|---|
| Cream base | 23.40 ± 1.96 |
| 1% SSD | 29.90 ± 3.35 |
| 1% PG | 27.90 ± 3.35 |
| 2% PG | 27.20 ± 4.32 |
| 1% SSD + 1% PG | 28.20 ± 2.25 |
| 1% SSD + 2% PG | 27.90 ± 2.81 |

Values means ± Standard deviation; N = 10 for all groups

**Table 45** The level of BUN on day 21 post-burn.

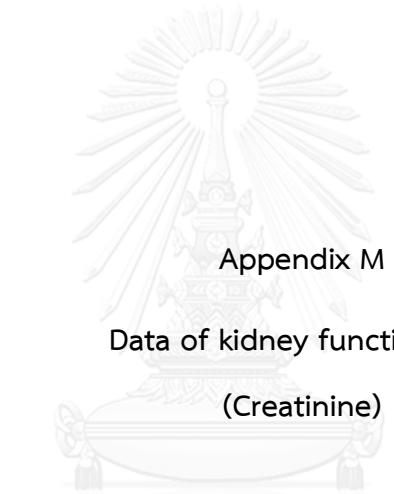
| Treatments | BUN (mg%) |
|---|---|
| Cream base | 37.34 ± 3.76 |
| 1% SSD | 41.13 ± 12.91 |
| 1% PG | 38.89 ± 7.37 |
| 2% PG | 36.55 ± 8.26 |
| 1% SSD + 1% PG | 38.31 ± 9.09 |
| 1% SSD + 2% PG | 28.35 ± 10.55 |

Values means ± Standard deviation; N = 10 for all groups

**Table 46** The level of BUN on day 28 post-burn.

| Treatments | BUN (mg%) |
|---|---|
| Cream base | 26.50 ± 3.66 |
| 1% SSD | 27.80 ± 3.74 |
| 1% PG | 27.70 ± 3.74 |
| 2% PG | 26.70 ± 2.26 |
| 1% SSD + 1% PG | 26.60 ± 2.67 |
| 1% SSD + 2% PG | 29.90 ± 3.35 |

Values means ± Standard deviation; N = 10 for all groups



# Appendix M

## Data of kidney function test

## (Creatinine)

**Table 47** The level of creatinine post-burn.

| Treatments | Creatinine (mg%) |
|---|---|
| Cream base | 0.54 ± 0.05 |
| 1% SSD | 0.51 ± 0.05 |
| 1% PG | 0.54 ± 0.05 |
| 2% PG | 0.54 ± 0.05 |
| 1% SSD + 1% PG | 0.55 ± 0.05 |
| 1% SSD + 2% PG | 0.53 ± 0.07 |

Values means ± Standard deviation; N = 10 for all groups

**Table 48** The level of creatinine on day 7 post-burn.

| Treatments | Creatinine (mg%) |
|---|---|
| Cream base | 0.48 ± 0.04 |
| 1% SSD | 0.50 ± 0.07 |
| 1% PG | 0.58 ± 0.05 |
| 2% PG | 0.53 ± 0.05 |
| 1% SSD + 1% PG | 0.57 ± 0.05 |
| 1% SSD + 2% PG | 0.55 ± 0.05 |

Values means ± Standard deviation; N = 10 for all groups

**Table 49** The level of creatinine on day 14 post-burn.

| Treatments | Creatinine (mg%) |
|---|---|
| Cream base | 0.50 ± 0.05 |
| 1% SSD | 0.57 ± 0.05 |
| 1% PG | 0.56 ± 0.05 |
| 2% PG | 0.55 ± 0.05 |
| 1% SSD + 1% PG | 0.55 ± 0.05 |
| 1% SSD + 2% PG | 0.55 ± 0.05 |

Values means ± Standard deviation; N = 10 for all groups

**Table 50** The level of creatinine on day 21 post-burn.

| Treatments | Creatinine (mg%) |
|---|---|
| Cream base | 0.53 ± 0.05 |
| 1% SSD | 0.60 ± 0.12 |
| 1% PG | 0.59 ± 0.05 |
| 2% PG | 0.54 ± 0.05 |
| 1% SSD + 1% PG | 0.55 ± 0.05 |
| 1% SSD + 2% PG | 0.52 ± 0.04 |

Values means ± Standard deviation; N = 10 for all groups

**Table 51** The level of creatinine on day 28 post-burn.

| Treatments | Creatinine (mg%) |
|---|---|
| Cream base | 0.54 ± 0.16 |
| 1% SSD | 0.57 ± 0.13 |
| 1% PG | 0.51 ± 0.51 |
| 2% PG | 0.53 ± 0.08 |
| 1% SSD + 1% PG | 0.49 ± 0.07 |
| 1% SSD + 2% PG | 0.72 ± 0.07 |

Values means ± Standard deviation; N = 10 for all groups

# VITA

| | |
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