AN EVALUATION OF WOUND HEALING EFFICIENCY OF CHITIN–PAA–GTMAC HYDROGEL COMPARED WITH THE COMMERCIAL PRODUCT (INTRASITE™ GEL) IN WISTAR RATS MICROSCOPICALLY BY ROUTINE HISTOLOGICAL AND IMMUNOHISTOCHEMICAL TECHNIQUE

PATAMAPORN MOLEE

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (ANATOMY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2009

Thesis
entitled

AN EVALUATION OF WOUND HEALING EFFICIENCY OF CHITIN–PAA–GTMAC HYDROGEL COMPARED WITH THE COMMERCIAL PRODUCT (INTRASITE™ GEL) IN WISTAR RATS MICROSCOPICALLY BY ROUTINE HISTOLOGICAL AND IMMUNOHISTOCHEMICAL TECHNIQUE

Patamaporn Molee
.....
Miss Patamaporn Molee
Candidate

Kajee Pilakasiri
.....
Assoc. Prof. Kajee Pilakasiri,
Ph.D. (Anatomy)
Major-advisor

Jantima Roongruangchai
.....
Assoc. Prof. Jantima Roongruangchai,
D.D.S., Ph.D. (Anatomy)
Co-advisor

.....
COL Duangporn Phulsuksombati,
D.V.M., M.Sc. (Public Health)
Co-advisor

Arraya Sangiampong
.....
Assoc. Prof. Arraya Sangiampong,
M.Sc. (Anatomy)
Co-advisor

.....
Prof. Banchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

Jantima Roongruangchai
.....
Assoc. Prof. Jantima Roongruangchai,
D.D.S., Ph.D. (Anatomy)
Chair
Master of Science Programme in Anatomy
Faculty of Medicine Siriraj Hospital
Mahidol University

Thesis
entitled

AN EVALUATION OF WOUND HEALING EFFICIENCY OF CHITIN–PAA–GTMAC HYDROGEL COMPARED WITH THE COMMERCIAL PRODUCT (INTRASITE™ GEL) IN WISTAR RATS MICROSCOPICALLY BY ROUTINE HISTOLOGICAL AND IMMUNOHISTOCHEMICAL TECHNIQUE

was submitted to the Faculty of Graduate Studies, Mahidol Univarsity
for the degree of Master of Science (Anatomy)
on
October 9, 2009

Patamaporn Molee
.....
Miss Patamaporn Molee
Candidate

Jantima Roongruangchai
.....
Assoc. Prof. Jantima Roongruangchai,
D.D.S., Ph.D. (Anatomy)
Member

Chaiyaphruk Pilakasiri
.....
COL Chaiyaphruk Pilakasiri,
Ph.D. (Biology)
Chair

.....
COL Duangporn Phulsuksombati,
D.V.M., M.Sc. (Public Health)
Member

Kajee Pilakasiri
.....
Assoc. Prof. Kajee Pilakasiri,
Ph.D. (Anatomy)
Member

Siriporn Tanodekaew
.....
Miss Siriporn Tanodekaew,
Ph.D. (Polymer Chemistry)
Member

Arraya Sangiampong
.....
Assoc. Prof. Arraya Sangiampong,
M.Sc. (Anatomy)
Member

.....
Prof. Banchong Mahaisavariya,
M.D.
Dean
Faculty of Graduate Studies
Mahidol University

T. Kulthanan
.....
Clin. Prof. Teerawat Kulthanan,
M.D., F.I.M.S., F.R.C.S.T., F.I.C.S.
Dean
Faculty of Medicine Siriraj Hospital
Mahidol University

ACKNOWLADGEMENTS

I would like to express my deep gratitude to my major advisor, Associate Professor Dr. Kajee Pilakasiri for her kindness, valuable instructive advice, guidance, criticism, supervision and encouragement which enable me to carry out the study successfully.

I deeply thank my co-advisor; Associate Professor Dr. Jantima Roongruangchai, Associate Professor Arraya Sangiampong and COL Duangporn Phulsuksombati for their valuable advice, comments and helpful guidance in this thesis.

I am also very grateful to COL Dr. Chaiyaphruk Pilakasiri for being chair person of my thesis defense as well as his valuable advice, comments and helpful guidance in this thesis.

My sincere appreciation goes to LTC Noppadon Sangjun and personnel of the Department of Laboratory Animal, Armed Forces Research Institute of Medical Sciences for offering and providing facility, and accommodation as well as animal preparation and animal care that make this research possible.

My thank also goes to Dr. Siriporn Tanodekaew from National Metal and Materials Technology Center, Thailand for supplying the details and dressing materials used in this study as well as her valuable suggestions.

Special thank is extended to Mr. Kasem Koedpuech for his kindness, chemical, specimen and slide preparation. In addition, I thank all members of the microtechnique unit, Department of Anatomy, Faculty of Medicine Siriraj Hospital for their help and friendship.

My deep thanks go to Miss Duangkamol Sringernyuang, my nice and friendly lab partner for her support, encouragement and friendly that make my tough work easier throughout my graduate student life. Moreover, I thank all my friends in the Department of Anatomy for their kind supports.

I am very grateful to the Department of Anatomy, Faculty of Medicine Siriraj Hospital where chances and knowledge have been given to me.

Finally, I wish to express my deepest gratitude and appreciation to my family for their support and understanding throughout the entire course of this study.

This thesis was partially supported by Siriraj graduate thesis scholarship.

Patamaporm Molee

AN EVALUATION OF WOUND HEALING EFFICIENCY OF CHITIN–PAA–GTMAC HYDROGEL COMPARED WITH THE COMMERCIAL PRODUCT (INTRASITE™ GEL) IN WISTAR RATS MICROSCOPICALLY BY ROUTINE HISTOLOGICAL AND IMMUNOHISTOCHEMICAL TECHNIQUE

PATAMAPORN MOLEE 5036459 SIAN/M

M.Sc.(ANATOMY)

THESIS ADVISORY COMMITTEE: KAJEE PILAKASIRI, Ph.D.(ANATOMY), JANTIMA ROONGRUANGCHAI, D.D.S., Ph.D. (ANATOMY), ARRAYA SANGIAMPONG, M.Sc.(ANATOMY), DUANGPORN PHULSUKSOMBATI, D.V.M., M.Sc. (PUBLIC HEALTH) SIRIPORN TANODEKAEW, Ph.D.(POLYMER CHEMISTRY)

ABSTRACT

The wound healing efficiency of chitin-PAA-GTMAC hydrogel compared with the commercial product (Intrasite™ Gel) for the treatment of full-thickness excision wounds in Wistar rats was evaluated. Three full-thickness wounds were created on the dorsal body wall of each animal. Each wound was covered with a different wound dressing, including chitin-PAA-GTMAC at the ratio of 1:4 and 1:10 along with the Intrasite™ Gel. Subsequently, the process of wound healing was evaluated macroscopically and microscopically. The former was determined by the size of the wound area while the latter was evaluated by the epidermal assessment and the number of the proliferating cell nuclear antigen (PCNA) positive cells at day 3, 7, 9, 12, 15 and 18 post-operation. The wound size was calculated by using a computer program. Skin wound tissues were processed for routine histological and immunoperoxidase techniques. The results demonstrated that the application of the chitin-PAA-GTMAC hydrogel in both ratios displayed a high healing rate and quality of wound healing. They resulted in a better pattern of epidermal development and more significantly average percentages of PCNA positive cells than those of Intrasite™ Gel. These results suggested that chitin-PAA-GTMAC might offer benefits in promoting wound healing and could be used for the treatment of full-thickness open wounds.

KEY WORDS: CHITIN-PAA-GTMAC / INTRASITE™ GEL / PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) / WOUND HEALING

127 pages

การประเมินการหายของแผลที่ปิดด้วยเจลปิดแผล Chitin-PAA-GTMAC เปรียบเทียบกับ เจล Intrasite™ ในหนูวิสตาร์ ด้วยเทคนิคทางจุลกายวิภาคและอิมมูโนฮิสโตเคมิสตรี

AN EVALUATION OF WOUND HEALING EFFICIENCY OF CHITIN–PAA–GTMAC HYDROGEL COMPARED WITH THE COMMERCIAL PRODUCT (INTRASITE™ GEL) IN WISTAR RATS MICROSCOPICALLY BY ROUTINE HISTOLOGICAL AND IMMUNOHISTOCHEMICAL TECHNIQUE

ปฐมาภรณ์ โมลี 5036459 SIAN/M

วท.ม. (กายวิภาคศาสตร์)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: ขจี ปิลกศิริ, ปร.ด.(กายวิภาคศาสตร์), จันทิมา รุ่งเรืองชัย, ท.บ., ปร.ด.(กายวิภาคศาสตร์), อารยา เสงี่ยมพงษ์, วท.ม.(กายวิภาคศาสตร์), ดวงพร พูลสุขสมบัติ, สพ.บ., วท.ม. (สาธารณสุขศาสตร์), สิริพร โตนดแก้ว, ปร.ด.(เคมีโพลิเมอร์)

บทคัดย่อ

การประเมินการหายของแผลที่ปิดด้วยเจลปิดแผล chitin-PAA-GTMAC เปรียบเทียบกับเจล Intrasite™ ในหนู วิสตาร์ โดยการทำแผล 3 แผลที่หลังของหนูจากนั้นใส่เจลปิดแผล chitin-PAA-GTMAC อัตราส่วน 1:4 และ 1:10 และ Intrasite™ ลงในแต่ละแผลตามลำดับ ศึกษาลักษณะและขนาดของแผลรวมทั้งลักษณะทางจุลกายวิภาค โดยศึกษาการสร้างและเจริญของชั้นหนังกำพร้าและจำนวนของ proliferating cell nuclear anigen (PCNA) positive cell ในวันที่ 3, 7, 9, 12, 15 และ 18 หลังจากการทำแผล โดยคำนวณขนาดของแผลด้วยโปรแกรมคอมพิวเตอร์ และนำชิ้นเนื้อบริเวณแผลไปย้อมด้วย hematoxylin & eosin และ immunoperoxidase ผลปรากฏว่าเจล chitin-PAA-GTMAC ทำให้แผลหายเร็วและมีคุณภาพดี มีการพัฒนารูปแบบของชั้นหนังกำพร้าได้ดีกว่าและมีเปอร์เซ็นต์เฉลี่ยของจำนวน PCNA positive cell มากกว่าแผลที่ปิดด้วยเจล Intrasite™ อย่างมีนัยสำคัญทางสถิติ จากผลการศึกษาสรุปได้ว่าเจล chitin-PAA-GTMAC มีประโยชน์ในการส่งเสริมการหายของแผล ซึ่งสามารถนำไปใช้ในการรักษาแผลเปิดที่มีความลึกถึงชั้นใต้หนังแท้ได้

127 หน้า

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF DIAGRAMS

CHAPTER I
INTRODUCTION

Skin is the biggest organ of human body and a barrier against environment. When it is destroyed by damage or disease, the moisture content, electrolyte and protein would be lost and the infection probability of the wound increases. If the initial wound is effectively covered with wound dressing, the infection and dehydration of the wound can be prevented. In addition, the wound dressing would promote the wound healing and decrease complication (Deng *et al*., 2007). The wound dressing must be preserving moisture, soft, good permeability, nontoxicity, able to protect the wound from secondary infection, promote tissue reconstruction process and removable without causing trauma to the wound (Lloyd *et al*., 1998; Wittaya-areekul *et al*., 2006; Deng *et al*., 2007). Chitin is one of the most abundant organic materials in nature. It can be easily prepared from the shells of crab, shrimp and squid pens. Because of its availability, biodegradability as well as biocompatibility, chitin and its derivatives have been used for a variety of applications such as water treatment, textile and paper, cosmetic, food and health supplements, agriculture and biotechnology (Goosen, 1997).

In biomedical area, it was found that chitin possessed high activity as a wound healing accelerator (Usami *et al.,* 1998). Many types of chitin-based materials adapted for uses in wound dressing application have been patented. However, low water sorption ability of chitin which yields an inefficient exudate removal from the wound surface limits its utility as a wound dressing. The chemical structure of chitin has been modified to overcome this undesirable characteristic. Among many attempts, grafting of various monomers containing hydrophilic groups onto chitin chains seems to be a promising method to enhance its water sorption ability. Grafted products gain a hydrogel characteristic with a great water retention capacity whereas the beneficial properties of chitin such as biodegradability and bioactivity still remain. In fact, there have been very few publications on the grafting of hydrophilic monomers onto chitin,

probably due to the insolubility of chitin in water and common organic solvents (Tanodekaew *et al.,* 2004).

1.1 Skin (School of Anatomy and Human Biology - The University of Western Australia, 2006)

The skin is the outer covering of the body. In humans, it is the largest organ of the integumentary system made up of multiple layers of mesodermal tissues, and guards the underlying muscles, bones, ligaments and internal organs.

1.1.1 Skin components

Skin has mesodermal cells, pigmentation, or melanin, provided by melanocytes, which absorb some of the potentially dangerous ultraviolet radiation (UV) in sunlight. It also contains DNA-repair enzymes that help reverse UV damage, and people who lack the genes for these enzymes suffer high rates of skin cancer. One form predominantly produced by UV light, malignant melanoma, is particularly invasive, causing it to spread quickly, and can often be deadly. Human skin pigmentation varies among population in a striking manner. This has led to the classification of people on the basis of skin color.

The skin is the largest organ in the human body. For the average adult human, the skin has a surface area of between 1.5-2.0 square meters (16.1-21.5 sq ft.), most of it is between 2-3 mm (0.10 inch) thick. The average square inch (6.5 cm^2) of skin holds 650 sweat glands, 20 blood vessels, 60,000 melanocytes, and more than a thousand nerve endings.

1.1.2 Functions

Skin performs the following functions:

1.1.2.1 Protection: an anatomical barrier from pathogens and damage between the internal and external environment in bodily defense; Langerhans cells in the skin are part of the adaptive immune system.

1.1.2.2 Sensation: contains a variety of nerve endings that react to heat and cold, touch, pressure, vibration, and tissue injury; see somatosensory system and haptics.

1.1.2.3 Heat regulation: the skin contains a blood supply far greater than its requirements which allows precise control of energy loss by radiation, convection and conduction. Dilated blood vessels increase perfusion and heat loss while constricted vessels greatly reduce cutaneous blood flow and conserve heat. Erector pili muscles are significant in animals.

1.1.2.4 Control of evaporation: the skin provides a relatively dry and semi-impermeable barrier to fluid loss. Loss of this function contributes to the massive fluid loss in burns.

1.1.2.5 Aesthetics and communication: others see our skin and can assess our mood, physical state and attractiveness.

1.1.2.6 Storage and synthesis: acts as a storage center for lipids and water, as well as a means of synthesis of vitamin D by action of UV on certain parts of the skin.

1.1.2.7 Excretion: sweat contains urea, however its concentration is 1/130 that of urine, hence excretion by sweating is at most a secondary function to temperature regulation.

1.1.2.8 Absorption: Oxygen, nitrogen and carbon dioxide can diffuse into the epidermis in small amounts, some animals using their skin for their sole respiration organ. In addition, medicine can be administered through the skin, by ointments or by means of adhesive patch, such as the nicotine patch or iontophoresis. The skin is an important site of transport in many other organisms.

1.1.2.9 Water resistance: The skin acts as a water resistant barrier so essential nutrients aren't washed out of the body.

1.1.3 Skin layers (Figure 1)

Skin is composed of three primary layers:

1.1.3.1 the epidermis, which provides waterproofing and serves as a barrier to infection

1.1.3.2 the dermis, which serves as a location for the appendages of skin

1.1.3.3 the hypodermis (subcutaneous adipose layer).

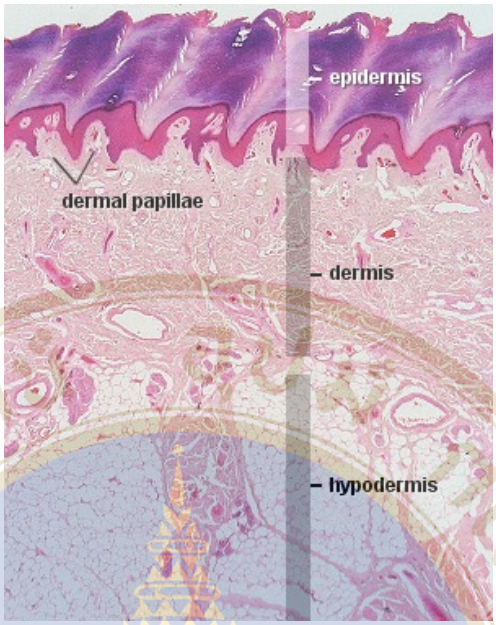


Figure 1 The micrograph shows the human skin layers which is composed of epidermis, dermis and hypodermis. H&E staining (School of Anatomy and Human Biology - The University of Western Australia, 2006).

1.1.3.1 Epidermis (Figure 2)

The epidermis is a keratinised stratified squamous epithelium. The main function of the epidermis is to protect the body from harmful influences from the environment and against fluid loss. Five structurally different layers can be identified:

- **The stratum basale** is the deepest layer of the epidermis (closest to the dermis). It consists of a single layer of columnar or cuboidal cells which rest on the basement membrane. Basal cells are the stem cells of the epidermis. Their mitotic activity replenishes the cells in more superficial layers as these are eventually shed from the epidermis. The renewal of the human epidermis takes about 3 to 4 weeks.

- **The stratum spinosum,** the cells become irregularly polygonal. The cells are often separated by narrow, translucent clefts. These clefts are spanned by spine-like cytoplasmatic extensions of the cells (hence the name of the layer and of its cells: spinous cells), which interconnect the cells of this layer. Spines of cells meet end-to-end or side-to-side and are attached to each other by desmosomes. In addition to the usual organelles of cells, EM shows membrane-bound lamellar granules in the cytoplasm of the spinous cells.

- **The stratum granulosum** consists, in thick skin, of a few layers of flattened cells. Only one layer may be visible in thin skin. The cytoplasm of the cells contains numerous fine grains, keratohyalin granules. The keratohyalin is not located in membrane-bound organelles but forms "free" accumulations in the cytoplasm of the cells. The cells begin to release the contents of the lamellar granules. The lipids contained in the granules come to fill the entire interstitial space, which is important for the function of the epidermis as a barrier towards the external environment.
- **The stratum lucidum** consists of several layers of flattened dead cells. Nuclei already begin to degenerate in the outer part of the stratum granulosum. In the stratum lucidum, faint nuclear outlines are visible in only a few of the cells. The stratum lucidum can usually not be identified in thin skin.
- **In the stratum corneum**, cells are completely filled with keratin filaments (horny cells) which are embedded in a dense matrix of proteins. Individual cells are difficult to observe because (1) nuclei can no longer be identified, (2) the cells are very flat and (3) the space between the cells has been filled with lipids, which cement the cells together into a continuous membrane. In the electron microscopy, the cell membranes appear thickened and interdigitate with those of neighbouring cells. Closest to the surface of the epidermis, the stratum corneum has a somewhat looser appearance. Horny cells are constantly shed from this part of the stratum corneum.The protection of the body by the epidermis is essentially due to the functional features of the stratum corneum.

Variations in the thickness of the epidermis (~0.1 mm in thin skin, 1 mm or more in thick skin) are mainly the result of variations in the thickness of the stratum corneum, although the other layers also vary in thickness. Cells of the epidermis of the skin will at some time of their life keratinise and are collectively also called keratinocytes.

The epidermis contains no blood vessels, and cells in the deepest layers are nourished by diffusion from blood capillaries extending to the upper layers of the dermis. The main type of cells which make up the epidermis are Merkel cells, keratinocytes, with melanocytes and Langerhans cells also present. The epidermis can be further subdivided into the following strata (beginning with the outermost layer): corneum, lucidum (only in palms of hands and bottoms of feet), granulosum, spinosum, basale. Cells are formed through mitosis at the basale layer. The daughter cells (see cell division) move up the strata changing shape and composition as they die due to isolation from their blood source.

The cytoplasm is released and the protein keratin is inserted. They eventually reach the corneum and slough off (desquamation). This process is called keratinization and takes place within about 27 days. This keratinized layer of skin is responsible for keeping water in the body and keeping other harmful chemicals and pathogens out, making skin a natural barrier to infection.

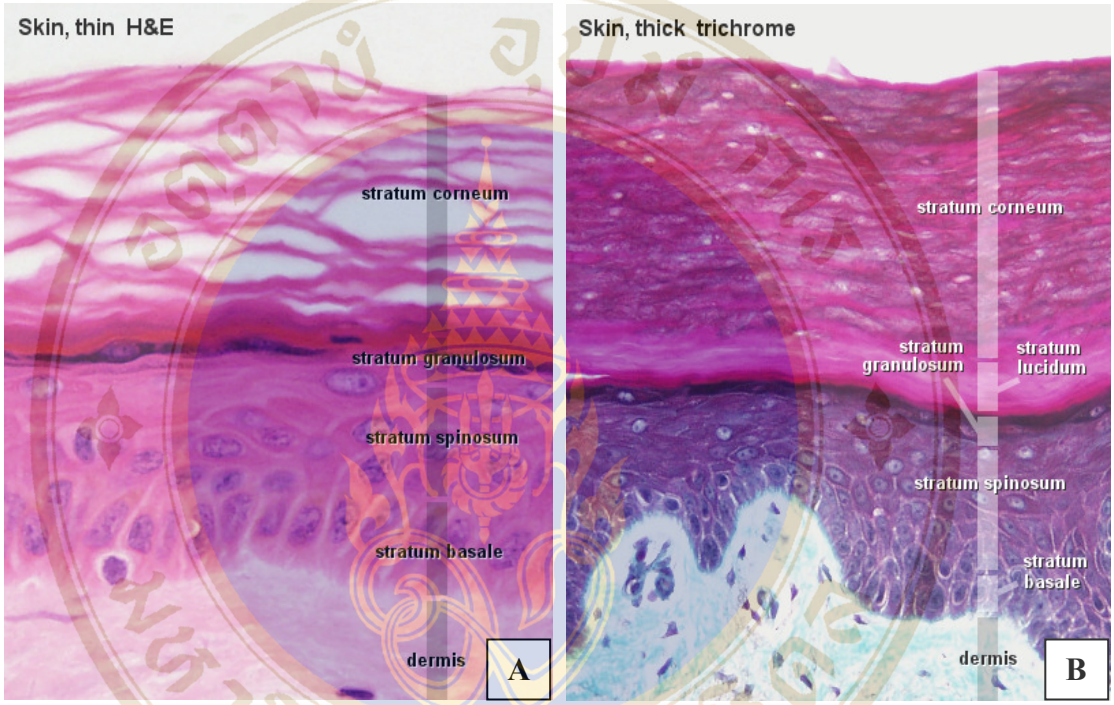


Figure 2 The micrographs show the human skin which is composed of the layers of epidermis. A shows the thin skin and B shows the thick skin. H&E (A) and Trichrome (B) staining (School of Anatomy and Human Biology - The University of Western Australia, 2006).

1.1.3.2 Dermis (Figure 3)

The dermis is the layer of skin beneath the epidermis that consists of connective tissue and cushions the body from stress and strain. The dermis is tightly connected to the epidermis by a basement membrane. It also harbors many Mechanoreceptor/nerve endings that provide the sense of touch and heat. It contains the hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels. The blood vessels in the dermis provide nourishment and waste removal to its own cells as well as the Stratum basale of the epidermis.

Component of dermis

The dermis is structurally divided into two areas: a superficial area adjacent to the epidermis, called the papillary region, and a deep thicker area known as the reticular region.

- **Papillary region**

The papillary region is composed of loose areolar connective tissue. It is named for its fingerlike projections called papillae that extend toward the epidermis. The papillae provide the dermis with a "bumpy" surface that interdigitates with the epidermis, strengthening the connection between the two layers of skin.

In the palms, fingers, soles, and toes, the influence of the papillae projecting into the epidermis forms contours in the skin's surface. These are called friction ridges, because they help the hand or foot to grasp by increasing friction. Friction ridges occur in patterns (see: fingerprint) that are genetically and epigenetically determined and are therefore unique to the individual, making it possible to use fingerprints or footprints as a means of identification.

- **Reticular region**

The reticular region lies deep in the papillary region and is usually much thicker. It is composed of dense irregular connective tissue, and receives its name from the dense concentration of collagenous, elastic, and reticular fibers that weave throughout it. These protein fibers give the dermis its properties of strength, extensibility, and elasticity.

Also located within the reticular region are the roots of the hair, sebaceous glands, sweat glands, receptors, nails, and blood vessels.

1.1.3.3 Hypodermis (Figure 3B and 3C)

The hypodermis is not part of the skin, and lies below the dermis. Its purpose is to attach the skin to underlying bone and muscle as well as supplying it with blood vessels and nerves. It consists of loose connective tissue and elastin. The main cell types are fibroblasts, macrophages and adipocytes (the hypodermis contains 50% of body fat). Fat serves as padding and insulation for the body.

Microorganisms like Staphylococcus epidermidis colonize the skin surface. The density of skin flora depends on region of the skin. The disinfected skin surface gets

recolonized from bacteria residing in the deeper areas of the hair follicle, gut and urogenital openings.

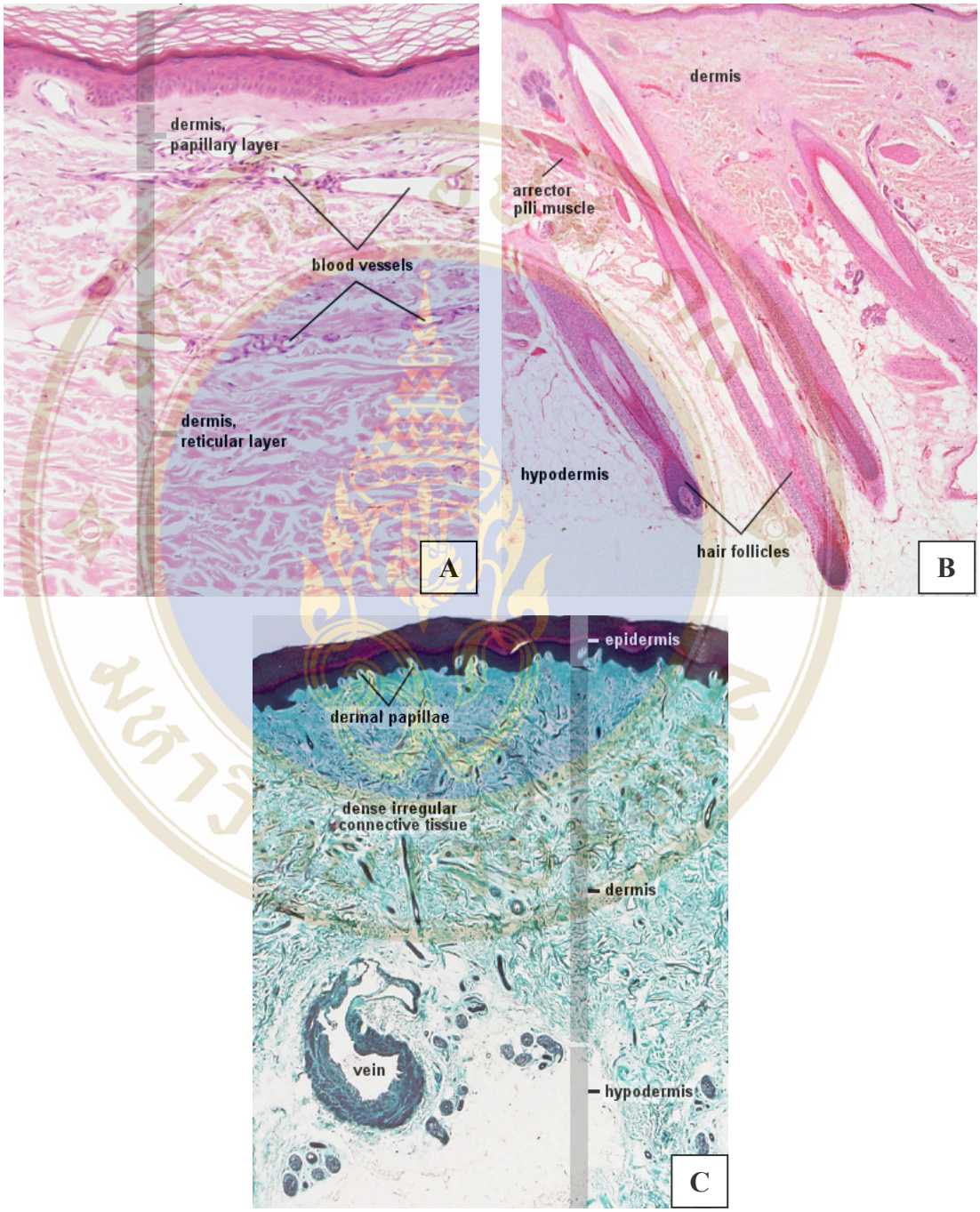


Figure 3 The micrographs show the human skin. A shows the dermis layers (H&E staining), B (H&E staining) and C (Trichome staining) show the coponents of dermis and hypodermis (School of Anatomy and Human Biology - The University of Western Australia, 2006).

1.2 Sources of chitin

Chitin is essentially extracted from crustaceous shells (exoskeletons), insects, mushrooms, and cell wall of fungi (Rinaudo, 2006). From a practical viewpoint, the shells of crustaceans such as shrimps, crabs and squid pens are the major sources. They are available as waste from the seafood processing industry and used for commercial production of chitin (Figure 4). These shells contain 15-40% chitin and other two components, proteins (20-40%) and calcium carbonate (20-50%) as well as a small quantity of pigments. The rough estimate of chitin and calcium carbonate contain various sources are listed in Table 1 (Kurita, 2006).

Table 1 Content of chitin and calcium carbonate in different sources (Kurita, 2006).

Source	Chitin (%)	$CaCO_3$ (%)
Shrimp cuticle	30-40	20-30
Squid pen	20-40	Negligible
Krill cuticle	20-30	20-25
Crab cuticle	15-30	40-50
Fungi cell wall	10-25	Negligible
Insect cuticle	5-25	Negligible
Clam/oyster shell	3-6	85-90

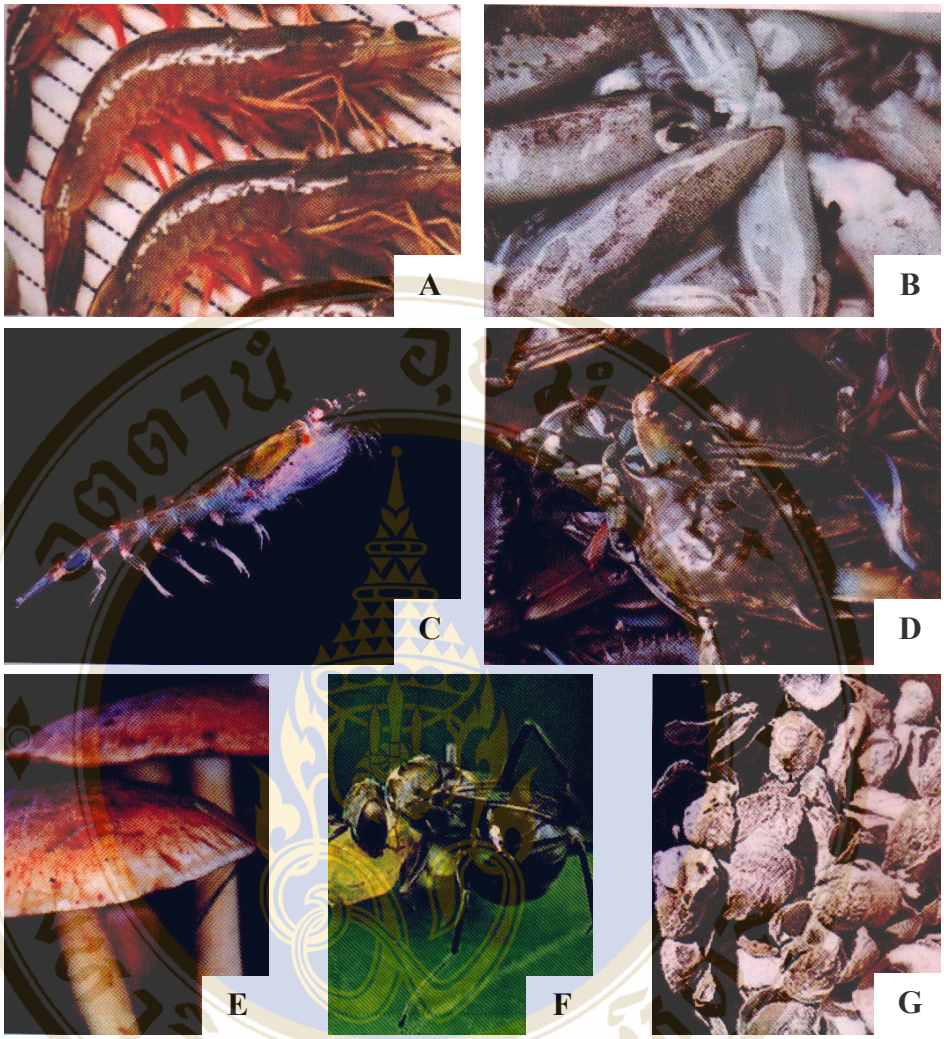


Figure 4 Source of chitin.
A, Shrimps; **B**, Squid pens; **C**, Krill; **D**, Crabs; **E**, Mushrooms
F, Insect; **G**, Oyster shells.
(A: Alibaba, 2006; B: Buf, 2006; C: Hempel and Hempel, 1996; D: Wpopp, 2004; E: Wunneesuthi, 2006; F: Dada, 2006; G: Ncfisheries, 2007)

1.3 Crystallography

Three crystalline forms are known for chitin: α-, β- and γ-chitin. The most abundant and easily accessible form is α-chitin, where the molecules are aligned in an antiparallel fashion as disclosed by X-ray diffraction studies (Figure 5). This molecular arrangement is favorable for the formation of strong intermolecular hydrogen bonding, and α-chitin is the most stable form of the three crystalline variations. Squid pens also contain chitin that is classified as β-chitin. This material is distinguished from the ordinary α-chitin in crustacean shell such as crab and shrimp, according to the difference in crystalline structure. In β-chitin, the molecules are packed in parallel arrangement, leading to weaker intermolecular forces is thus assumed to be less stable than α-chitin (Blackwell *et al*., 1980; Kurita, 2001). Kurita *et al*. (1993) demonstrated that β-chitin exhibits much higher reactivity in deacetylation than α-chitin (Kurita, 1993). γ-chitin having an antiparallel and parallel structure (Jang, 2004). γ-chitin is less stable than α-chitin but more stable than β-chitin. It is found in mushroom and fungi (Surawattanawan, 2003).

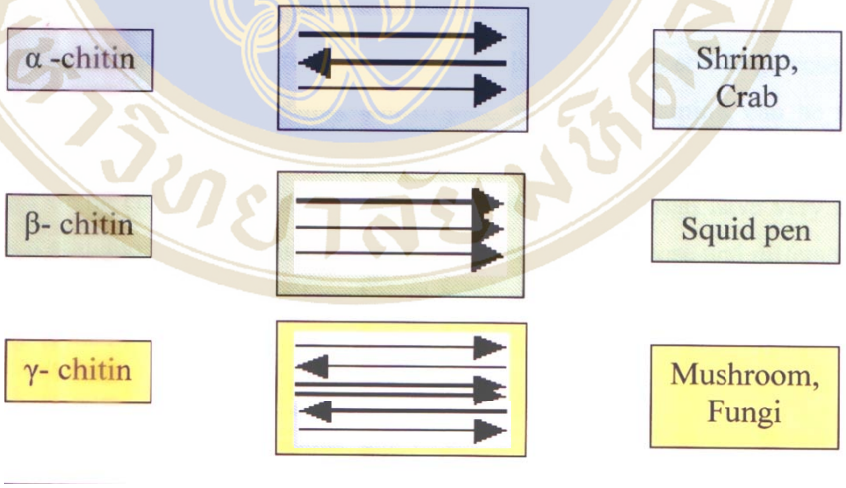


Figure 5 Crystalline forms of α-chitin, β-chitin and γ-chitin disclosed by X-ray diffraction studies. (Adapted from Surawattanawan, 2003)

1.4 History of chitin

Chitin was first found in mushrooms in 1811 by Professor H. Braconnot while he was Professor of Natural History and Director of the Botanical Gardens at the Academy of Sciences in Nancy, France. In the 1830's, it was isolated in insects and named chitin. Professor C. Rouget discovered chitosan in 1859, and over the next century, much fundamental research took place on these compounds. An intense interest in new applications grew in the 1930s and early 1940s, as evidenced by almost 50 patents; however, the lack of adequate manufacturing facilities and competition from synthetic polymers hampered commercial development. Renewed interest in the 1970s was encouraged by the need to better utilize shellfish shells. Scientists worldwide began to chronicle the more distinct properties of chitin and its derivatives and understand the potential of these natural polymers. Since then, numerous research studies have been undertaken to find ways to use materials (Chitin: A Natural Product for the 21st Century, 2008).

1.5 Chemistry, physical properties and biological function of chitin

Chitin $(C_8H_{13}O_5N)_n$ is a long-chain polymer of a N-acetylglucosamine, a derivative of glucose, and it is found in many places throughout the natural world. It is the main component of the cell wall of fungi, the exoskeleton of arthropods, such as crustaceans (like the crab, lobster and shrimp) and the insects, including ants, beetles and butterflies, the radula of mollusks and the beaks of the cephalopods, including squid and octopuses. Chitin has also proven useful for several medical and industrial purposes. Chitin is a biological substance which may be compared to the polysaccharide cellulose and to the protein keratin. Although keratin is a protein, and not a carbohydrate like chitin, both keratin and chitin have similar structural functions.

Chitin is a polysaccharide and is synthesized from units of N-acetylglucosamine (more completely, N-acetyl-D-glucos-2-amine). These units form covalent β-1,4 linkages (similar to the linkages between glucose units forming cellulose). Chitin may therefore be described as cellulose with one hydroxyl group on each monomer substituted with an acetylamine group. This allows for increased

hydrogen bonding between adjacent polymers, giving the chitin-polymer matrix increased strength (Figure 6).

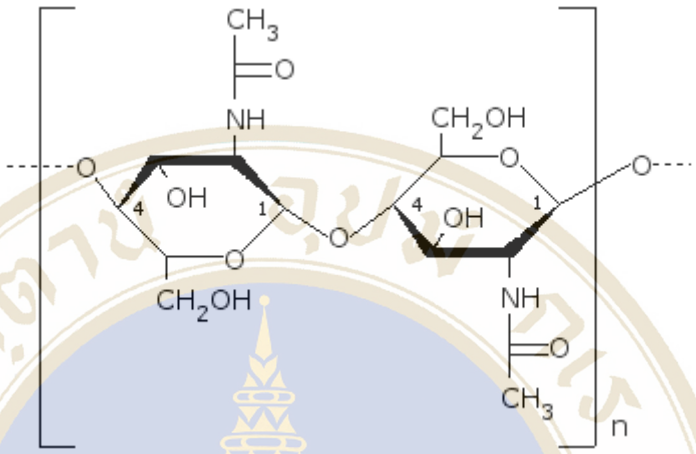


Figure 6 Structure of the chitin molecule, showing two of the N-acetylglucosamine units that repeat to form long chains in β-1,4 linkage (Wikipedia, 2008).

In its unmodified form, chitin is translucent, pliable, resilient and quite tough. In arthropods, however, it is often modified, becoming embedded in a hardened proteinaceous matrix, which forms much of the exoskeleton. In its pure form it is leathery, but when encrusted in calcium carbonate it becomes much harder. The difference between the unmodified and modified forms can be seen by comparing the body wall of a caterpillar (unmodified) to a beetle (modified) (Campbell, 1996).

Chitin is one of many naturally occurring polymers. Its breakdown may be catalyzed by enzymes called chitinases, secreted by microorganisms such as bacteria and fungi, and produced by some plants. Some of these microorganisms have receptors to simple sugars from the decomposition of chitin. If chitin is detected, they then produce enzymes to digest it by cleaving the glycosidic bonds in order to convert it to simple sugars and ammonia (Campbell, 1996).

Chemically, chitin is closely related to chitosan (a more water-soluble derivative of chitin). It is also closely related to cellulose in that it is a long unbranched chain of glucose derivatives. Both materials contribute structure and strength, protecting the organism (Campbell, 1996).

1.6 Etymology of chitin

The English word "chitin" comes from the French word "chitine", which first appeared in 1836. These words were derived from the Latin word "chitōn", meaning mollusk. That is either influenced by, or related to the Greek word *khitōn*, meaning "tunic" or "frock", the Central Semitic word "*kittan", the Akkadian words "kitû" or "kita'um", meaning flax or linen, and the Sumerian word "gada" or "gida" (Wikipedia, 2008).

1.7 Applications of chitin

1.7.1 Industry

Chitin is used industrially in many processes. It is used in water purification, and as an additive to thicken and stabilize foods and pharmaceuticals. It also acts as a binder in dyes, fabrics, and adhesives. Industrial separation membranes and ion-exchange resins can be made from chitin. Processes to size and strengthen paper employ chitin (Campbell, 1996).

1.7.2 Medicine

Chitin's properties as a flexible and strong material make it favourable as surgical thread. Its biodegradability means it wears away with time as the wound heals. Moreover, chitin has some unusual properties that accelerate healing of wounds in humans. Chitin has even been used as a stand-alone wound-healing agent. Chitin is used to make Chitosan, a product with numerous commercial and possible biomedical uses. Its most famous use is as a weight loss product (Campbell, 1996).

Occupations associated with high environmental chitin levels, such as shellfish processors, are prone to high incidences of asthma. Recent studies have suggested that chitin may play a role in a possible pathway in human allergic disease. Specifically, mice treated with chitin develop an allergic response, characterized by a build-up of interleukin-4 expressing innate immune cells. Treatment with a chitinase enzyme abolishes the response (Campbell, 1996).

1.7.3 Agriculture

Most recent studies point out that chitin is a good inductor for defense mechanisms in plants. It was recently tested as a fertilizer that can help plants develop healthy immune responses, and have a much better yield and life expectancy (Campbell, 1996).

1.8 How to extract chitin from the crustacean?

While there are many existing extraction methods of the chitin from the crustacean shells, the principles of chitin extraction are relatively simple. The proteins are removed by a treatment in a dilute solution of sodium hydroxide (1-10%) at high temperature (85-100°C). Shells are then demineralized to remove calcium carbonate. This is done by treating in a dilute solution of hydrochloric acid (1-10%) at room temperature. Depending on the severity of these treatments such as temperature, duration, concentration of the chemicals, concentration and size of the crushed shells, the physico-chemical characteristics of the extracted chitin will vary. For instance, the three most important characteristics of the chitin i.e., degree of polymerization, acetylation and purity, will be affected. Shell also contains lipids and pigments. Therefore, a decolorizing step is sometimes needed to obtain a white chitin. This is done by soaking in organic solvents or in a very dilute solution of sodium hypochlorite. Again, these treatments will influence the characteristics of the chitin molecule (Beaulieu, 2008).

1.9 Chitin–PAA (Chitin–polyacrylic acid)

Chitin grafted with poly acrylic acid (chitin–PAA) was prepared with the aim of obtaining a hydrogel characteristic for wound dressing application. The chitin–PAA films were synthesized at various acrylic acid feed contents to investigate its effect on water sorption ability. Acrylic acid (AA) was first linked to chitin, acting as the active grafting sites on the chain that was further polymerized to form a network structure. The evidences of grafting were found from FTIR and solid state ^{13}C-NMR spectra. The TGA results exhibited the high degradation temperature of the grafted

product suggesting the formation of a network structure. The degree of swelling (DS) of chitin–PAA films was found in the range of 30–60 times of their original weights depending upon the monomer feed content. The chitin–PAA film with 1:4 weight ratio of chitin:AA, possessed optimal physical properties. The cytocompatibility of the film was investigated with a cell line of L929 mouse fibroblasts. The morphology and behavior of the cells on the chitin–PAA film were determined after different time periods of culture up to 14 days. The L929 cells proliferated and attached well onto the film. These results suggested that the 1:4 chitin–PAA has a potential to be used as a wound dressing (Tanodekaew *et al.*, 2004).

Acrylic acid (AA), one of well known hydrogel forming monomers, has been widely applied in graft copolymerization of chitosan to increase its hydrophilicity. The detail of hydrogel forming of chitosan and poly acrylic acid (PAA) has been previously described. Some of chitosan–PAA hydrogels involved the use of glutaraldehyde for crosslinking purpose. Although, it strengthened the gel, glutaraldehyde was considered as a toxic chemical. The toxicity of the end products, therefore, is the concerned issue when toxic chemicals have been employed, especially in the preparation of medical products. In the present paper, a novel procedure to prepare acrylic grafted chitin (chitin–PAA), to impart a hydrogel characteristic for wound dressing, is reported. Since acrylic acid was directly grafted onto chitin, a costly and time consuming process to produce soluble chitin derivatives such as chitosan, employed as a starting material, was unnecessary (Tanodekaew *et al.*, 2004).

1.10 Preparation of chitin–PAA hydrogels

The chitin–PAA was prepared by a two-step reaction, as shown in Figure 7. Chitin was first mixed with acrylic acid, yielding a heterogeneous mixture. Under acid-catalyzed reaction, the esterification was occurred to form ester linkages between carboxylic groups of acrylic acid and hydroxyl groups of chitin. Chitin–AA, the paste-like mixture containing active grafting sites was obtained. In fact, the esterification by reacting acrylic acid with chitin is not easily occurred. Regardless of the heterogeneous aqueous reaction which is a result of water insolubility of chitin, the

chemical structure of chitin itself hinders the accessibility of reacting acrylic acid monomer. However, chitin used in this experiment is in the β-form which chains arrange in a parallel fashion with relatively weak interchain hydrogen bond. This kind of loosely packed structure, thereby, facilitates the interaction between acrylic acid and chitin to yield ester linkages.

In the second step, the casting solution of chitin–AA was further polymerized by the addition of initiator. The subsequent addition of acrylic acid molecules to the active grafting sites propagated the growing of acrylic side chains on chitin. Meanwhile, both interchain crosslinking and PAA homopolymerization were concurrently occurring. The former resulted in a forming of network structure which strengthened the gels when swelling in water while the latter affected the swelling ability of the gels, which will be discussed later (Tanodekaew *et al.*, 2004).

Novel chitin–PAA hydrogels were synthesized. Chitin was first modified with acrylic acid via an esterification process. The polymerization of acrylic acid monomer then proceeded simultaneously with the film forming process with a use of redox initiator. FTIR results were taken as evidence of ester linkages forming between chitin and acrylic acid. The films possessed a network structure and exhibited an enhancement of water sorption ability. The swelling behavior and gel strength were found to depend upon monomer feed content. Chitin–PAA 1:4 yielded optimal swelling and gel strength. The overall results of the cellular behavior on the chitin–PAA 1:4 film in this present study suggested that the material has a potential for biomedical applications particularly as temporary skin substitute (Tanodekaew *et al.*, 2004).

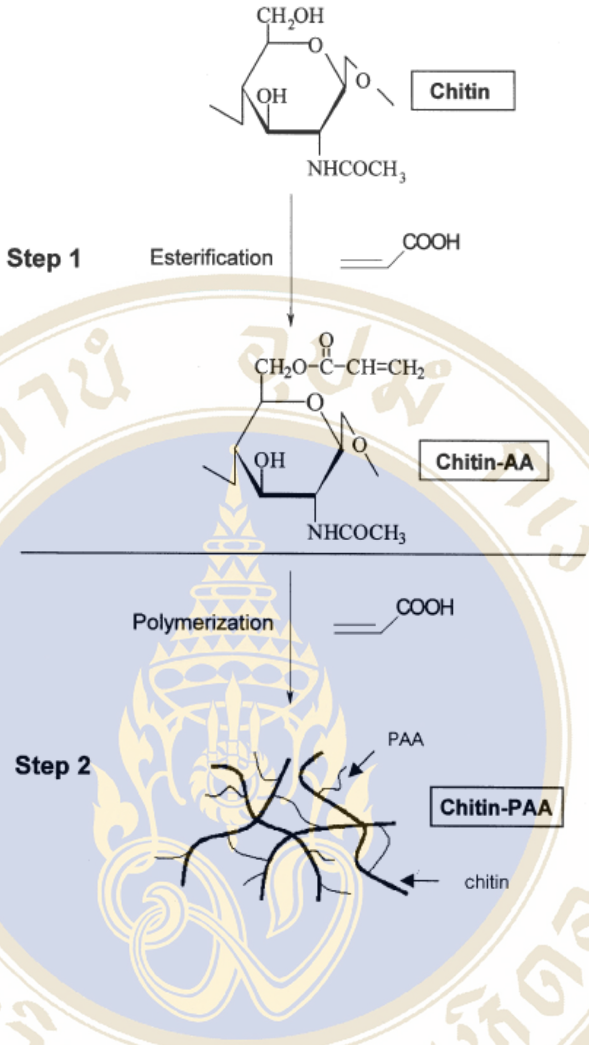


Figure 7 Scheme of the preparation of chitin–PAA hydrogel. (Tanodekaew *et al.*, 2004)

1.11 Chitin–PAA–GTMAC

Chitin–PAA–GTMAC was synthesized by adding carboxylic and quaternary ammonium groups to chitin structure for increase absorption and antibacterial ability by reaction between glycidyl trimethyl ammonium chloride (GTMAC) and chitin–PAA, the derivative synthesized by reacting chitin with acrylic acid. Chitin–PAA–GTMAC has absorbability and antibacterial properties. It not only activates tissue regeneration and wound healing process but also activates the cell to produce interleukin 8 (IL 8) that concerns wound healing and angiogenesis.

1.12 Preparation of chitin–PAA–GTMAC hydrogels

Chitin–PAA–GTMAC was prepared from alteration of chitin–PAA chemical structure with GTMAC to increase antibacterial ability. The 2% with chitin–PAA in water was reacted with quaternary ammonium reagent (glycidyl trimethyl ammonium chloride, GTMAC). Salt solution was used to facilitate a better substitution reaction of quaternary ammonium on to chitin-PAA chain. In addition, Chitin–PAA–GTMAC has lower toxicity than other antibiotic substances for example, povidine-iodine, sodium hypochlorite, Dakin's and silver sulfadiazine etc. These chemicals, although have high quality of antisepsis, at the same time they have high toxicity for fibroblast and keratinocytes that lead to a slow wound healing process.

1.13 Amorphous hydrogel dressings in wound management

Amorphous hydrogels are widely used in practice to debride necrotic and sloughy wounds. The hydrogel dressings fulfill many of the characteristics of an ideal dressing as they provide a moist environment, and non-adherent to the wound, thus can be removed without causing trauma. They are safe to use, free from particulate contaminants and available in the hospital and community settings. Hydrogels are available as flat sheets or amorphous gels. The flat sheets are mainly used in the treatment of burns or scar tissue, whereas the amorphous hydrogels are used in a variety of wound types, e.g. pressure sores, fungating wounds, extravasation injuries etc. It is important to note that all amorphous hydrogels are designed for single-use only. The amorphous hydrogels most commonly used in practice will be examined.

1.14 Properties of amorphous hydrogels

Like many other dressing products available on the market, the amorphous hydrogels look very similar in appearance, but their composition is very different and this should be considered when making a decision as to which dressing should be used. Hydrogels are three-dimensional, cross-linked structures that are formed from hydrophilic polymers. The hydrogel sheets have a stable three-dimensional macro-structure that retain their physical form as fluid is absorbed into the dressing, while the

amorphous hydrogels have no fixed structure and decrease in viscosity as fluid is absorbed into the product (Jones & Milton, 2000). According to Thomas and Andrews (1999), these products are able to donate or absorb fluid depending on the hydration of the wound and the formulation of the dressing product. These products have high water content, which is why they are of value in wound debridement. Autolytic debridement is enhanced, as an increase in the moisture content of necrotic and sloughy tissue in the wound facilitates enzymatic activity.

Amorphous hydrogels consist mainly of water and also contain a small amount of either starch or carboxymethylcellulose polymer, which acts as a gel-forming medium. Many of the amorphous hydrogels also contain 20 per cent propylene glycol, which acts as a humectant and preservative, i.e. it prevents the gel from drying out whilst in use and should also prevent the growth of micro-organisms (Thomas & Andrews, 1999).The amorphous hydrogels although all clear gels, differ in their composition and water content.

Amorphous hydrogels can play a valuable role in the debridement of necrotic and sloughy tissue from a wound, and can be used in a variety of wound types. The dressing products are easy to apply and can be removed without causing pain. The only problem that can occur is maceration if too much hydrogel has been applied to the wound. The appropriate secondary dressing should be used, depending on the amount of exudate. Finally, it is important to remember that these products are single-use only, and any unused hydrogel should be discarded.

Intrasite™ Gel contains water, carboxymethylcellulose polymer and propylene glycol. Intrasite™ Gel is an amorphous hydrogel which promotes rapid but gentle debridement of necrotic tissue, whilst being able to loosen and absorb slough and exudate. It can also be used to provide a moist wound healing environment during the later stages of wound healing. It is non-adherent and does not harm viable tissue or the skin surrounding the wound. This makes Intrasite™ Gel ideal for every stage in the wound healing process (available from Smith & Nephew, 2008).

1.15 Wound Healing process of skin (Romo, 2008)

Wound healing is a natural restorative response to tissue injury. Healing is the interaction of a complex cascade of cellular events that generates resurfacing, reconstitution, and restoration of the tensile strength of injured skin. Healing is a systematic process, traditionally explained in terms of 3 classic phases: inflammation, proliferation, and maturation. A clot forms and inflammatory cells debride injured tissue during the inflammatory phase. Epithelialization, fibroplasia, and angiogenesis occur during the proliferative phase. Meanwhile, granulation tissue forms and the wound begins to contract. Finally, during the maturation phase, collagen forms tight cross-links to other collagen and with protein molecules, increasing the tensile strength of the scar.

For the sake of discussion and understanding, the process of wound healing may be considered a series of separate events. Actuality, the entire process is much more complicated, as cellular events that lead to scar formation occur in tandem. Many aspects of wound healing have yet to be elucidated. Surgeons should have an understanding of the process of wound healing to help produce scars that are cosmetically pleasing and do not impair function.

1.15.1 Inflammatory phase

The early events of wound healing are characterized by the inflammatory phase, a vascular and cellular response to injury. An incision made through a full thickness of skin causes a disruption of the microvasculature and immediate hemorrhage. Following incision of the skin, a 5- to 10-minute period of vasoconstriction ensues, mediated by epinephrine, norepinephrine, prostaglandins, serotonin, and thromboxane. Vasoconstriction causes temporary blanching of the wound and functions to reduce hemorrhage immediately following tissue injury, aid in platelet aggregation, and keep healing factors within the wound.

Endothelial cells retract to expose the subendothelial collagen surfaces; platelets attach to these surfaces. Adhesion to exposed collagen surfaces and to other platelets occurs through adhesive glycoproteins: fibrinogen, fibronectin, thrombospondin, and von Willebrand factor. The aggregation of platelets results in the formation of the primary platelet plug. Aggregation and attachment to exposed

collagen surfaces activates the platelets. Activation enables platelets to degranulate and release chemotactic and growth factors, such as platelet-derived growth factor (PDGF), proteases, and vasoactive agents (e.g. serotonin, histamine).

The coagulation cascade occurs via 2 different pathways. The intrinsic pathway begins with the activation of factor XII (Hageman factor) when blood is exposed to extravascular surfaces. The extrinsic coagulation pathway occurs through the activation of tissue factor found in extravascular cells in the presence of factors VII and VIIa. Both pathways proceed to the activation of thrombin, which converts fibrinogen to fibrin. The fibrin product is essential to wound healing and is the primary component of the wound matrix into which inflammatory cells, platelets, and plasma proteins migrate. Removal of the fibrin matrix impedes wound healing.

In addition to activation of fibrin, thrombin facilitates migration of inflammatory cells to the site of injury by increasing vascular permeability. By this mechanism, factors and cells necessary for healing flow from the intravascular space and into the extravascular space.

The result of platelet aggregation and the coagulation cascade is clot formation. Clot formation is limited in duration and to the site of injury. Clot formation dissipates as its stimuli dissipate. Plasminogen is converted to plasmin, a potent enzyme that aids in cell lysis. Clot formation is limited to the site of injury because uninjured nearby endothelial cells produce prostacyclin, an inhibitor of platelet aggregation. In uninjured adjacent areas, antithrombin III binds thrombin, and protein C binds factors of the coagulation cascade, namely, factors V and VII.

The vasoconstriction period is followed by a more persistent period of vasodilation mediated by histamine, prostaglandins, kinins, and leukotrienes. Vasodilation is responsible for the erythema, edema, and heat observed after tissue injury. Vasodilation is an important means by which the wound can be exposed to increased blood flow, accompanied by the necessary inflammatory cells and factors that fight infection and debride the wound of devitalized tissue. Alterations in pH (secondary to tissue and bacterial degradation), swelling, and tissue hypoxemia at the injury site contribute to the sensation of wound pain.

Following injury, the products of the earliest cellular events activate intricately related inflammatory pathways that modify subsequent events in the

wound-healing process. For example, Hageman factor activates the kinin pathway, which produces bradykinin. Bradykinin stimulates vasodilation and increased vascular permeability. Histamine released from platelets and circulating mast cells increases vascular permeability and indirectly stimulates vasodilation through the production of prostaglandins E_1 and E_2. Prostaglandins cause vasodilation through the activation of the adenylate cyclase pathway via the production of cyclic adenosine monophosphate. Prostaglandins also accumulate at the area of injury through the activation of phospholipases located on injured cell membranes. Phospholipases stimulate the release of arachidonic acid, ultimately leading to the production of prostaglandins, leukotrienes, and other factors.

Hageman factor also activates the classic complement pathway during the inflammatory phase. Inactive proteins of the complement system (i.e. C1-C9) are activated by means of a cascade of reactions. These proteins stimulate important inflammatory events such as chemotaxis, degranulation of mast cells, and cytolysis. C5a and C567 are chemotactic agents for neutrophil migration. C3a, C4a, and C5a cause degranulation of mast cells, leading to release of histamine and increased vascular permeability. The membrane attack complex, C5b6789, is responsible for cytolysis. The cellular aspect of the inflammatory phase occurs within hours of injury. Neutrophils are the predominant cell type for the first 48 hours after injury but do not appear essential to the wound-healing process. Neutrophils cleanse the wound site of bacteria and necrotic matter and release inflammatory mediators and bactericidal oxygen-free radicals. The absence of neutrophils does not prevent healing.

Macrophages are essential to wound healing and perhaps are the most important cells in the early phase of wound healing. Macrophages phagocytose debris and bacteria. Macrophages also secrete collagenases and elastases, which break down injured tissue and release cytokines. In addition, macrophages release PDGF, an important cytokine that stimulates the chemotaxis and proliferation of fibroblasts and smooth muscle cells. Finally, macrophages secrete substances that attract endothelial cells to the wound and stimulate their proliferation to promote angiogenesis. Macrophage-derived growth factors play a pivotal role in new tissue formation, as evidenced by the fact that new tissue formation in macrophage-depleted animal wounds demonstrates defective repair. In studies in which experimental

wounds are rendered monocytopenic, subsequent stages of fibroplasia and granulation tissue formation are impaired and the overall rate of wound healing is delayed.

T-lymphocytes migrate into the wound during the inflammatory phase, approximately 72 hours following injury. T lymphocytes are attracted to the wound by the cellular release of interleukin 1, which also contributes to the regulation of collagenase. Lymphocytes secrete lymphokines such as heparin-binding epidermal growth factor and basic fibroblast growth factor. Lymphocytes also play a role in cellular immunity and antibody production.

1.15.2 Proliferative phase

Formation of granulation tissue is a central event during the proliferative phase. Inflammatory cells, fibroblasts, and neovasculature in a matrix of fibronectin, collagen, glycosaminoglycans, and proteoglycans comprise the granulation tissue. Granulation tissue formation occurs 3-5 days following injury and overlaps with the preceding inflammatory phase. Proliferative phase includes epithelialization, fibroplasia, angiogenesis and contraction.

1.15.2.1 Epithelialization

Epithelialization is the formation of epithelium over a denuded surface. Epithelialization of an incisional wound involves the migration of cells at the wound edges over a distance of less than 1 mm, from one side of the incision to the other. Incisional wounds are epithelialized within 24-48 hours after injury. This epithelial layer provides a seal between the underlying wound and the environment. The process begins within hours of tissue injury. Epidermal cells at the wound edges undergo structural changes, allowing them to detach from their connections to other epidermal cells and to their basement membrane. Intracellular actin microfilaments are formed, allowing the epidermal cells to creep across the wound surface. As the cells migrate, they dissect the wound and separate the overlying eschar from the underlying viable tissue. In superficial wounds (e.g. wounds due to laser resurfacing, dermabrasion, chemical peel treatments) and adnexal structures (e.g. sebaceous glands, hair follicles) contribute to reepithelialization.

Epidermal cells secrete collagenases that break down collagen and plasminogen activator, which stimulates the production of plasmin. Plasmin

promotes clot dissolution along the path of epithelial cell migration. The extracellular wound matrix over which epithelial cells migrate has received increased emphasis in wound-healing research. Migrating epithelial cells interact with a provisional matrix of fibrin cross-linked to fibronectin and collagen. The matrix components may be a source of cell signals to facilitate epithelial cell proliferation and migration. In particular, fibronectin seems to promote keratinocyte adhesion to guide these cells across the wound base.

Wounds in a moist environment demonstrate a faster and more direct course of epithelialization. Occlusive and semiocclusive dressings applied in the first 48 hours after injury may maintain tissue humidity and optimize epithelialization. When epithelialization is complete, the epidermal cell assumes its original form, and new desmosomal linkages to other epidermal cells and hemidesmosomal linkages to the basement membrane are restored.

1.15.2.2 Fibroplasia

The fibroblast is a critical component of granulation tissue. Fibroblasts are responsible for the production of collagen, elastin, fibronectin, glycosaminoglycans, and proteases. Fibroblasts grow in the wound as the number of inflammation cells decrease. The demand for inflammation disappears as the chemotactic factors that call inflammatory cells to the wound are no longer produced and as those already present in the wound are inactivated.

Fibroplasia begins 3-5 days after injury and may last as long as 14 days. Skin fibroblasts and mesenchymal cells differentiate to perform migratory and contractile capabilities. Fibroblasts migrate and proliferate in response to fibronectin, platelet-derived growth factor (PDGF), fibroblast growth factor, transforming growth factor, and C5a. Fibronectin serves as an anchor for the myofibroblast as it migrates within the wound. The synthesis and deposition of collagen is a critical event in the proliferative phase and to wound healing in general.

Collagen consists of 3 polypeptide chains, each twisted into a left-handed helix. Three chains of collagen aggregate by covalent bonds and twist into a right-handed superhelix, forming the basic collagen unit. A striking structural feature of collagen is that every third amino acid is glycine. This repeating structural feature is an absolute requirement for triple-helix formation. Collagen is rich in hydroxylysine

and hydroxyproline moieties, which enable it to form strong cross-links. The hydroxylation of proline and lysine residues depends on the presence of oxygen, vitamin C, ferrous iron, and α-ketoglutarate. Deficiencies of oxygen and vitamin C, in particular, result in underhydroxylated collagen that is less capable of forming strong cross-links and, therefore, are more vulnerable to breakdown.

Collagen is secreted to the extracellular space in the form of procollagen. This form is then cleaved of its terminal segments and called tropocollagen. Tropocollagen can aggregate with other tropocollagen molecules to form collagen filaments. Filaments consist of tropocollagen molecules arrayed in a staggered fashion, joined by intermolecular cross-links. Filaments aggregate to form fibrils. Collagen fibrils, in turn, aggregate to form collagen fibers. Filament, fibril, and fiber formation occur within a matrix gel of glycosaminoglycans, hyaluronic acid, chondroitin sulfate, dermatan sulfate, and heparin sulfate produced by fibroblasts. Intermolecular cross-links within the collagen fiber stabilize it, making it resistant to destruction. Age, tension, pressure, and stress affect the rate of collagen synthesis. Collagen synthesis begins approximately 3 days after injury and may continue at a rapid rate for approximately 2-4 weeks. Collagen synthesis is controlled by the presence of collagenases and other factors that destroy collagen as new collagen is made.

Approximately 80% of the collagen in normal skin is type I collagen; the remaining is mostly type III. In contrast, type III collagen is the primary component of early granulation tissue and is abundant in embryonic tissue. Collagen fibers are deposited in a framework of fibronectin. An essential interaction seems to exist between fibronectin and collagen; experimental wounds depleted of fibronectin demonstrate decreased collagen accumulation. Elastin is also present in the wound in smaller amounts. Elastin is a structural protein with random coils that allow for stretch and recoil properties of the skin.

1.15.2.3 Angiogenesis

A rich blood supply is vital to sustain newly formed tissue and is appreciated in the erythema of a newly formed scar. These blood vessels disappear as they become unnecessary, as does the erythema of the scar. The macrophage is essential to the stimulation of angiogenesis and produces macrophage-derived

angiogenic factor in response to low tissue oxygenation. This factor functions as a chemoattractant for endothelial cells. Basic fibroblast growth factor secreted by the macrophage and vascular endothelial growth factor secreted by the epidermal cell are also important to angiogenesis.

Fibronectin is chemotactic for endothelial cells. Capillaries bud from existing capillaries in response to these growth factors. Endothelial cells coalesce and bind fibrin, which adds support to the vessel wall. Angiogenesis results in greater blood flow to the wound and, consequently, increased perfusion of healing factors. Angiogenesis ceases as the demand for new blood vessels ceases. New blood vessels that become unnecessary disappear by apoptosis.

New blood vessel formation is a complex process that relies on several angiogenic factors such as vascular endothelial growth factor, angiogenin, and angiotropin.

1.15.2.4 Contraction

Wound contraction begins almost concurrently with collagen synthesis. Contraction, defined as the centripetal movement of wound edges that facilitates closure of a wound defect, is maximal 5-15 days after injury. Contraction results in a decrease in wound size, appreciated from end to end along an incision; a 2 cm incision may measure 1.8 cm after contraction. The maximal rate of contraction is 0.75 mm/d and depends on the degree of tissue laxity and shape of the wound. Loose tissues contract more than tissues with poor laxity, and square wounds tend to contract more than circular wounds. Wound contraction depends on the myofibroblast located at the periphery of the wound, its connection to components of the extracellular matrix, and myofibroblast proliferation. Radiation and drugs, which inhibit cell division, have been noted to delay wound contraction. Contraction does not seem to depend on collagen synthesis. Although the role of the peripheral nervous system in wound healing is not well delineated, recent studies have suggested that sympathetic innervation may affect wound contraction and epithelialization through unknown mechanisms.

Contraction must be distinguished from contracture, a pathologic process of excessive contraction that limits motion of the underlying tissues and is typically caused by the application of excessive stress to the wound.

1.15.3 Maturation phase

Collagen and cytokine are required in the maturation process.

1.15.3.1 Collagen

Collagen remodeling during the maturation phase depends on continued collagen synthesis in the presence of collagen destruction. Collagenases and matrix metalloproteinases in the wound assist removal of excess collagen while synthesis of new collagen persists. Tissue inhibitors of metalloproteinases limit these collagenolytic enzymes, so that a balance exists between formation of new collagen and removal of old collagen.

During remodeling, collagen becomes increasingly organized. Fibronectin gradually disappears, and hyaluronic acid and glycosaminoglycans are replaced by proteoglycans. Type III collagen is replaced by type I collagen. Water is resorbed from the scar. These events allow collagen fibers to lie closer together, facilitating collagen cross-linking and ultimately decreasing scar thickness. Intramolecular and intermolecular collagen cross-links result in increased wound bursting strength. Remodeling begins approximately 21 days after injury, when the net collagen content of the wound is stable. Remodeling may continue indefinitely. The tensile strength of a wound is a measurement of its load capacity per unit area. The bursting strength of a wound is the force required to break a wound regardless of its dimension. Bursting strength varies with skin thickness. Peak tensile strength of a wound occurs approximately 60 days after injury. A healed wound only reaches approximately 80% of the tensile strength of unwounded skin.

1.15.3.2 Cytokines

Cytokines have emerged as important mediators of wound healing events. By definition, a cytokine is a protein mediator, released from various cell sources, which binds to cell surface receptors to stimulate a cell response. Cytokines can reach their target cell by endocrine, paracrine, autocrine, or intracrine routes. Some important cytokines are described as follows:

- **Epidermal growth factor (EGF)**

Epidermal growth factor was the first cytokine described and is a potent mitogen for epithelial cells, endothelial cells, and fibroblasts. Epidermal

growth factor stimulates fibronectin synthesis, angiogenesis, fibroplasia, and collagenase activity.

- **Fibroblast growth factor (FGF)**

Fibroblast growth factor is a mitogen for mesenchymal cells and an important stimulus for angiogenesis. Fibroblast growth factor is a mitogen for endothelial cells, fibroblasts, keratinocytes, and myoblasts. This factor also stimulates wound contraction and epithelialization and production of collagen, fibronectin, and proteoglycans.

- **Platelet-derived growth factor (PDGF)**

Platelet-derived growth factor is released from the alpha granules of platelets and is responsible for the stimulation of neutrophils and macrophages and for the production of transforming growth factor-β. PDGF is a mitogen and chemotactic agent for fibroblasts and smooth muscle cells and stimulates angiogenesis, collagen synthesis, and collagenase. Vascular endothelial growth factor is similar to PDGF but does not bind the same receptors.

- **Vascular endothelial growth factor (VEGF)**

Vascular endothelial growth factor is mitogenic for endothelial cells and plays an important role in angiogenesis.

- **Transforming growth factor-β (TGF- β)**

Transforming growth factor-β is released from the alpha granules of platelets and has been shown to regulate its own production in an autocrine manner. This factor is an important stimulant for fibroblast proliferation and the production of proteoglycans, collagen, and fibrin. The factor also promotes accumulation of the extracellular matrix and fibrosis. Transforming growth factor-β has been demonstrated to reduce scarring and to reverse the inhibition of wound healing by glucocorticoids.

- **Tumor necrosis factor-α (TNF-α)**

Tumor necrosis factor-α is produced by macrophages and stimulates angiogenesis and the synthesis of collagen and collagenase. Tumor necrosis factor-a is a mitogen for fibroblasts.

1.16 Proliferating cell nuclear antigen (PCNA)

The proliferating cell nuclear antigen (PCNA) is a 36 kDa molecular weight protein also known as cyclin. The protein has also been identified as the polymerase-associated protein and is synthesized in early G1 and S phases of the cell cycle. In early S phase, PCNA has a very granular distribution and is absent from the nucleoli. At late S phase, PCNA is prominent in the nucleoli. PCNA is a much conserved protein present not only in animal but also in plant cells.

Proliferative cell nuclear antigen (PCNA) is a ring-like protein (Figure 8) that provides the DNA polymerase the processivity of DNA replication and DNA repair (Kelman, 1998). The ring-shaped homotrimeric PCNA encircles and slides along double-stranded DNA, acting as a “sliding clamp” that localizes proteins to DNA (Figure 9). It is required for the coordinated synthesis of both leading and lagging strands at the replication fork during DNA replication. It is found mainly in proliferating cells.

PCNA expression correlates with the proliferation activity of several malignant and non-malignant cell types. Anti-PCNA is relatively specific for SLE; however, it is only found in about 5% of cases. It also can be found in some cases of Sjogren’s syndrome. PCNA labeling index (PLI) is the prognostic value of tumor cell proliferation.

The process of re-epithelialization of a wound in the epidermis comprises the following steps: proliferation of epidermal basal cells, migration of epidermal cells to the wound surface, and cell differentiation. An evaluation of epidermal basal cells proliferation is important process in wound heals, in the wound margin using an immunohistochemical labeling with proliferating cell nuclear antigen (PCNA), as markers of cell proliferation.

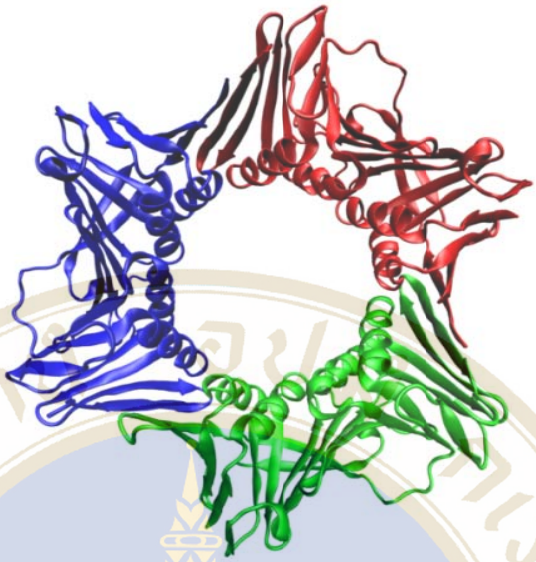


Figure 8 The ring-shaped homotrimeric PCNA (Wikipedia, 2009).

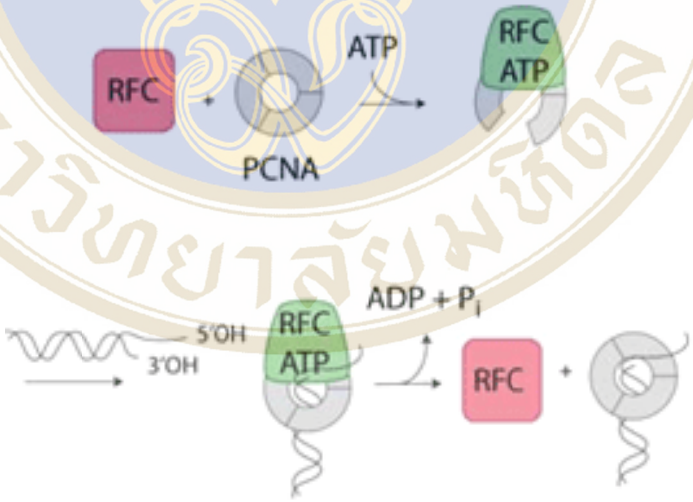


Figure 9 Schematic of the clamp loading cycle. PCNA is the clamp, RFC is the clamp loader. Hydrolysis of ATP releases the loader from the clamp (Bowman, 2004).

CHAPTER II
OBJECTIVES

The present study aims to evaluate wound healing efficiency of chitin–PAA–GTMAC at the ratio of 1:4 and 1:10 as compared with commercial product (Intrasite™ Gel) in the Wistar rats microscopically by routine histological and immunohistochemical technique in three aspects according to the periods of the experiment, namely

1. the appearance and the size of wound area.
2. microscopic study of the wound tissue by hematoxylin and eosin staining.
3. the number of PCNA positive cells which indicates the ability of the healing process of the tissue.

CHAPTER III
LITERATURE REVIEW

In 1995, Flanagan assessed the efficacy of a hydrogel in the treatment of non-viable tissue in a variety of wound types. In her study of 47 patients, she found that the percentage of non-viable tissue in the wounds was reduced by a median value of 75 per cent, although patients with leg ulcers experienced the poorest efficacy results. However, it should be remembered that patients presenting with a leg ulcer frequently have a number of underlying pathology which could influence wound healing.

In 1997, Shutler *et al*. discussed the case study of a patient with a fungating breast lesion, where the use of a hydrogel dressing improved the state of the wound, with reduced pain, and irritation at the wound site. This case study illustrated the value of using an amorphous hydrogel to remove sloughy tissue from fungating wounds and improved the state of the surrounding skin.

In 1998, Bale *et al*. compared the efficacy of two amorphous hydrogels, including Sterigel™ and Intrasite™ Gel in the debridement of necrotic pressure sores in 50 patients. They compared the dressing products in relation to debridement, pain, maceration of the skin and wound odour and found that both hydrogels debrided in similar periods of time, did not cause pain or wound odour during treatment or dressing change, and both caused maceration in some patients.

In 1998, Thomas *et al*. identified how amorphous hydrogels could be beneficial in the management of necrotic meningococcal skin lesions, compared to the traditional practice of allowing the purpural areas to dry out and demarcate prior to surgical debridement. This case study highlighted the various issues in managing this type of wound, and how the use of an amorphous hydrogel limited further tissue damage, facilitated autolysis and promoted wound healing that was cosmetically acceptable to the patient.

In 1999, Cho *et al.* showed the water-soluble chitin as a wound healing accelerator. Its accelerating effect on wound healing in rats was compared with those of chitin and chitosan. The wound treated with water-soluble chitin (WSC) solution was completely re-epithelialized, granulation tissues in the wound were nearly replaced by fibrosis and hair follicles. They were almost healed at 7 days after initial wounding. Also, the WSC-solution-treated skin had the highest tensile strength and the arrangement of collagen fibers in the skin was similar to normal skin. The WSC solution is considered to be a suitable wound-healing agent due to its easy application and high effectiveness.

In 2000, Sukihara *et al*. examined the effects of a silk film on epidermal recovery from full-thickness skin wounds. Full-thickness wounds were prepared on the dorsal wall of CRJ-CD-1 mice. The area of the wounds were dressed with silk film, DuoActive. Histological finding revealed that there was greater collagen regeneration and less inflammation and neutrophil-lymphocyte infiltration of the wounds dressed with silk film than with DuoActive dressing. It was clear that regeneration of the epidermis and dermis of the wound beds covered with silk film was faster than with DuoActive dressing.

In 2000, Trudgian undertook a small study of 10 patients, to investigate the use of Aquaform™. The patients had a variety of wound types containing necrotic or sloughy tissue. It was found that all wounds had improved after 14 days of treatment and that it appeared to stay in place especially on wounds located in difficult areas.

In 2001, Hong *et al*. studied the comparison effect of the Gelatin-hyaluronate sponge with and without antibiotic and epidermal growth factor (EGF). Four types of sponges were applied on the full-thickness dorsal skin defect of Wistar rats. The effect of antibiotic and EGF in gelatin-hyaluronate sponge on wound healing were investigated by light microscopy and image analyzer at postoperative days of 5, 12 and 21. An immunohistochemical technique, employing PC10, a monoclonal antibody against proliferating cell nuclear antigen (PCNA) was applied to wounded tissue sections. The number of PC10-positive cells was very high for the sponge with EGF at postoperative day 5, and then gradually decreased with time. Also it was found that antibiotic restrained the cell proliferation during the migratory phase. The sponge with both antibiotic and EGF showed good wound healing performance on the whole

healing period. The epithelium was regenerated fast with EGF-impregnated sponges at day 5, but each sample had nearly the same length of regenerated epithelium at day 12.

In 2002, Yusof *et al*. presented that chitin films possessing increased flexibility, softness, transparency, and conformability. The chitin films were nontoxic to human skin fibroblast, maintaining 70–80% cell viability. Wound studies showed no sign of allergenicity or the high inflammatory response associated with biodegradable biomaterials. The chitin film displayed accelerated wound healing properties. Based on histological examination, wound sites dressed with the chitin film stabilized and healed faster, and appeared stronger than those dressed with Opsite™ and gauze dressings after 7 days of healing.

In 2004, Sangjun *et al.* studied the use of chitin-based dressing in the treatment of deep partial skin thickness wounds. Chitin modified with acrylic acid (chitin-PAA) was applied as a wound dressing to evaluate its healing effectiveness in Guinea-pigs. The wounds were dressed with chitin-PAA and Cutinova® hydro for a comparison and were inspected every 3 days for up to 18 days. Histological examination performed that the wounds covered with chitin-PAA showed no signs of infection and healed completely within 15 days. A significant improvement in the wound healing was found for chitin PAA. The results demonstrated that chitin-PAA was a good material to be used as a dressing material for wound healing.

In 2006, Roh *et al*. revealed the wound healing effect of silk fibroin with alginate-blended sponge in full thickness skin defect of rat. On the postoperative days of 3, 7, 10 and 14 the residual wound area was calculated and skin wound tissues were biopsied to measure the area of regenerating epithelium and collagen deposition as well as the number of proliferating cell nuclear antigen (PCNA)-immunoreactive cells. Silk fibroin with alginate-blended sponge significantly increased the size of re-epithelialization and the number of PCNA positive cells. These results demonstrated that the wound healing effect of silk fibroin with alginate-blended sponge was the best and this synergic effect was mediated by re-epithelialization via rapid proliferation of epithelial cell.

In 2006, Vidinský *et al.* reported the histological study of the first seven days of skin wound healing in rats. The two paravertebral full thickness skin incisions were performed on the back of male rats. Histological sections from tissue specimens

were stained by hematoxylin and eosin and evaluated during the first seven days after surgery. Histological evaluation revealed that the regeneration of injured epidermis was completed in five days after surgery. The inflammatory phase was recorded during the first three days of healing with the culmination of this phase between day one and day two. The beginning of the proliferative phase was dated to the first day and the peak during day five and day six. The initiation of the maturation and remodeling phase of the healing process was observed on day six after wounding. The wound healing process of rat skin was histologically described during the first seven days. Results of this work could serve as an experimental model for further research using.

CHAPTER IV
MATERIALS AND METHODS

4.1 Materials

4.1.1 Dressings

4.1.1.1 Chitin–PAA–GTMAC at the ratio of 1:4 and 1:10, hydrogel biomaterials, obtained from the National Metal and Materials Technology Center, Thailand. (Figure 10)

4.1.1.2 Commercial product (Intrasite™ Gel). (Figure 11)

Figure 10 Hydrogel biomaterial made of chitin–PAA–GTMAC

Figure 11 Commercial product (Intrasite™ Gel)

4.1.2 Animals

Twenty four female Wistar rats weighing between 250-300 grams and being twelve weeks old were purchased from the National Animal Center of Thailand, Mahidol University, Salaya Campus, Nakornpathom.

4.1.3 Chemicals

1. Alcohol solution (50%, 70%, 80%, 90%, 95% and absolute)
2. Carbon dioxide
3. Ketamine
4. Normal saline
5. Paraplast (VWR International Ltd.)
6. Povidine-Iodine antiseptic solution
7. Xylaxine
8. Xylene solution

Hematoxylin & Eosin staining

1. Distilled water
2. Egg albumin
3. Eosin solution
4. Formalin
5. Glacial acetic acid
6. Hematoxylin (Scharlau Cheme S.A.)
7. Hydrochloric acid
8. Mercuric oxide
9. Potassium alum
10. Resin solution (permount)

Indirect immunoperoxidase method

1. Acetone (Carlo Erba Reagents)
2. Deionized water
3. Disodium hydrogen orthophosphate (Scharlau Cheme S.A.)

4. Ethyl alcohol solution; 50%, 70%, 80%, 90%, 95% and Absolute (J.T.Baker)
5. Glycerol (Ajax Finechem)
6. Hydrogen peroxide solution (Merck KGaA)
7. Phosphate Buffer Saline (PBS)
8. Potassium alum (Scharlau Cheme S.A.)
9. Potassium iodate (Asia Pacific Specialty Chemicals Limited)
10. Primary antibody to Proliferating cell nuclear antigen (PCNA)
11. Rabbit ABC Staining System kit (Santa Cruz Biotechnology, Inc.) consisting of;
    - 1.0 ml of normal blocking serum
    - 250 µg of biotinylated secondary antibody
    - 0.5 ml each of avidin and biotinylated horseradish peroxidase (AB reagents)
    - 1.0 ml of 50x peroxidase substrate
    - 1.0 ml of 50x DAB chromogen
    - 3.0 ml of 10x substrate buffer
12. Sodium dihydrogen orthophosphate (Ajax Finechem)
13. Toluene solution (Fisher Scientific)
14. VECTABOND™ reagent

4.1.4 Tools

1. Analytical weighing apparatus (Precisa, XT 320 M)
2. Auto-mounting mechine (LEICA, CV 5030)
3. Beakers
4. Cylinders
5. Digital pH meter (SUNTEX , Model: SP – 7)
6. Erlenmeyer flask
7. Forceps
8. Funnels
9. Glass stirring rods
10. Gloves

11. Heater (IDAMAG ®, REC – G)
12. Hotplate (IKAMAGE® REC-G)
13. Humidified chamber
14. Incubator
15. Jar
16. Light microscope (Nikon YS100)
17. Light microscope with attached digital camera (Carl Zeiss Vision GmbH, Germany)
18. Masks
19. Microtome (Leica RM 2135)
20. Microscopic clear glass slides 25.4 × 76.2 mm, 1.2 mm thick
21. Mixing bottles for the preparation of working solutions reagent in ABC Staining System Kit
22. Neotape
23. Pipettes
24. Silk (USP 210)
25. Slide boxes
26. Tegaderm™
27. Timers
28. Water bath (MEDEX,KV – 7684)

4.2 Methods

4.2.1 Animal preparation

A total of 24 healthy female Wistar rats were raised in the animal room of the Department of Laboratory Animal, Analysis Division, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. They were kept in individual cages under control-conditioned room, temperature at 24±1 °C, relative humidity at 60±5% with 12 hours light per day and fed on standard diet and clean

water throughout the experimental period. (Guide for the care and use of laboratory animal of National Research Council, 1996)

4.2.2 Deep skin wound preparation

4.2.2.1 The dorsal hair of Wistar rats were shaved by sharp blade (Figure 12) and animals were anaesthetized with Xylaxine 10 mg/kg and Ketamine 75-100 mg/kg. The bare shaved skin was disinfected by Potadine® and 70% ethyl alcohol (Figure 13).

Figure 12 The hairs on the dorsal body wall of the animal were shaved.

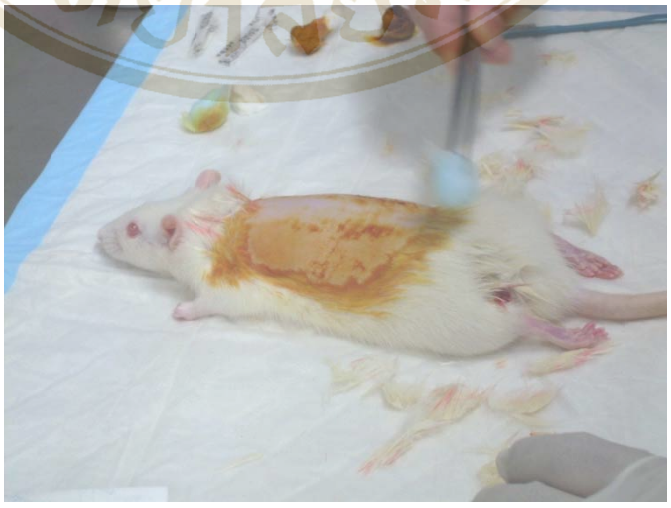


Figure 13 The bare skin area on the dorsal body wall was disinfected with Potadine® and 70% ethyl alcohol.

4.2.2.2 Three full thickness wounds of 1×1 cm in size were prepared by excising the dorsal skin of a rat (Figure 14).

Figure 14 Performing the deep dermatotomies on the dorsal body wall of the animal.

4.2.2.3 The upper left, lower left and upper right wounds were covered with the chitin-PAA-GTMAC 1:4, 1:10 and Intrasite™ Gel respectively (Figure 15).

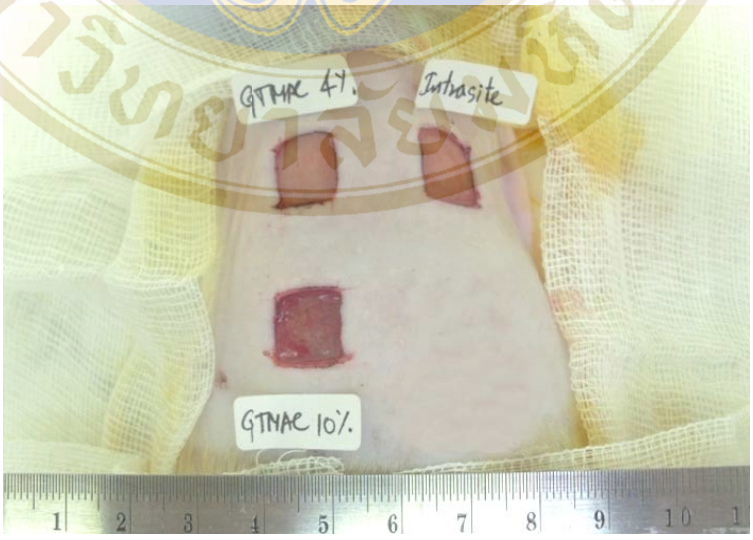


Figure 15 The three wounds were covered with the chitin-PAA-GTMAC 1:4, 1:10 and Intrasite™ Gel.

4.2.2.4 A sterilized elastic band (10×12 cm^2, Tegaderm^{TM}) was employed to wrap all three wounds and followed by the Neotape which was fixed at the corners on the dorsum of a rat with silk, U.S.P.2/0 at the corners (Figure 16).

Figure 16 The wound were wrapped with the Tegaderm^{TM} and followed by the Neotape which was fixed at the corners on the dorsum of a rat with silk, USP.2/0.

4.2.2.5 The animals were kept in individual cages as mentioned in the animal preparation.

4.2.3 Evaluation of deep wound healing

4.2.3.1 Grouping of the animals.

The animals were divided into six groups of four each regarding to the time they were sacrificed. Those were 3, 7, 9, 12, 15 and 18 days after deep wound preparation.

4.2.3.2 Sacrifice of the animals.

Four animals of each group were sacrificed at day 3, 7, 9, 12, 15 and 18 after deep wound preparation by euthanasia with carbon dioxide.

4.2.3.3 Evaluation of the wound size.

Before sacrifice of all animals of each group, the wounds were visually observed and photographed by a digital camera. The areas of the wounds were calculated using a computer program of an image analyzer.

4.2.3.4 Microscopical study of the wound tissue.

The tissue deep into the dermis at the wound area of 2.5 × 2.5 cm^2 strip were removed for conventional light microscopy by hematoxylin and eosin staining and immunohistochemistry method for detection of the PCNA positive cells which are the indicator of the healing process.

4.2.4 Analysis of the data

4.2.4.1 Appearance and the size of the wound surfaces treated with hydrogel biomaterial preparation from chitin–PAA–GTMAC of 1:4, 1:10 and commercial product (Intrasite™ Gel) were observed and analyzed by the computer program.

4.2.4.2 Histological study of the wound tissue dressed with the three dressing was performed by epidermal assessment. Histological preparations were examined for the extent of epidermal growth. The epidermis can easily be distinguished from the dermis on the basis of the standard histological criteria. The developing keratinocytes lying on the basement membrane are revealed in the epidermal layer, whereas the dermis contains connective tissue including mostly collagen fibers and fibroblasts. The degree of epithelialization is determined by the number of the layer of the keratinocytes and the degree of keratinization as well as the pattern of dermal papilla (Figure 17).

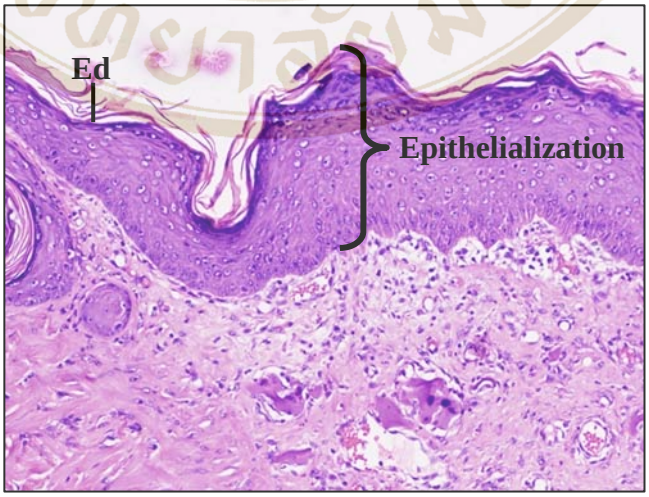


Figure 17 Photomicrograph shows the epithelialization at the edge (Ed) of the wound (Obj x10).

4.2.4.3 Quantitative analysis of PCNA-positive cells was analyzed by determination of the proliferating nuclear antigen (PCNA). The presence of PCNA-positive cells in the wound area of the tissue sections was determined by using the indirect immunoperoxidase staining technique. The PCNA should be localized in the area showing the golden–brown precipitation which located in the nucleus of the PCNA-positive cells. These golden–brown stained areas were examined light microscopically to observe the proliferating cells which were found in both epithelium and granulation tissue of the wounds (Figure 18).

The number of PCNA-positive cells in the epidermis was counted under a light microscope by evaluation of the PCNA labeling cells. The percentage of immunoreactive cells of the wound areas in one section were obtained by random selection of the three fields and counted by using the x100 objective lens.

Data analysis involved the use of SPSS 15.0 (SPSS, Chicago, IL). Data for PCNA-positive cells were expressed as means ± standard deviation of the PCNA indices from each wound dressing category counted in all animals. Statistical analysis was done through Friedman test for k related sample case and ordinal scale nonparametric analysis of variance (ANOVA), with Bonferroni's multiple comparison tests used to evaluate significant differences between groups. Differences with $P<0.05$ were accepted as significant.

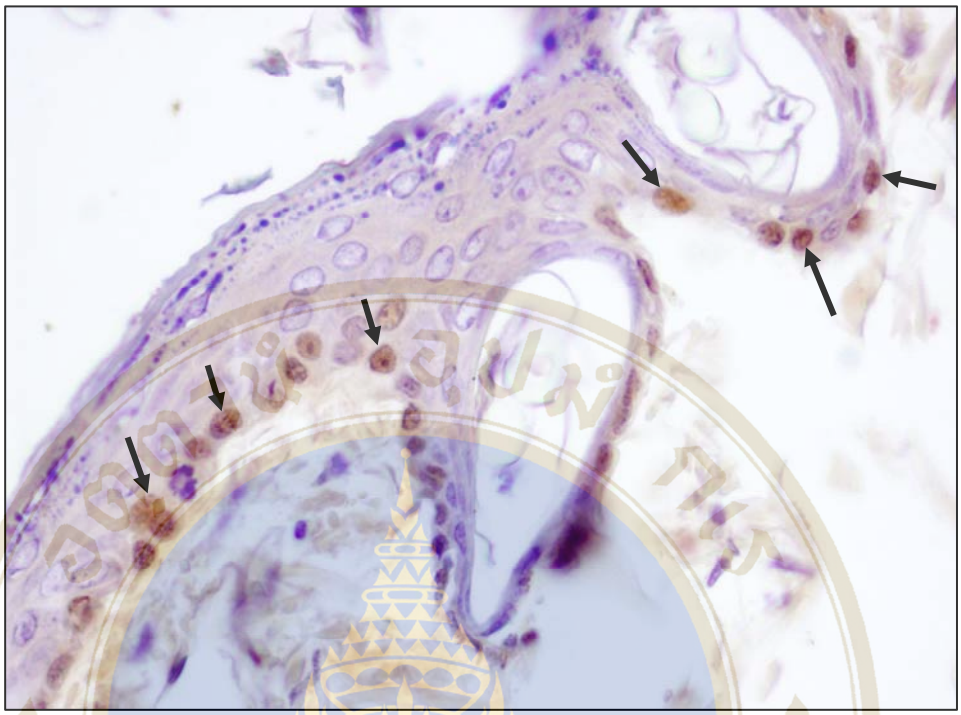


Figure 18 Photomicrograph reveals the PCNA-positive cells which displays the golden – brown coloration in the nucleus at the basal layer of the epithelium and hair follicles (Obj x40).

CHAPTER V
RESULTS

5.1 Appearance and size of the wound surfaces

5.1.1 Appearance of the wound surfaces

The photographs of the wounds of each group were shown in the Figure 19.

On day 0, the day of dermatotomy, three full thickness wounds of 1×1 cm^2 in size were prepared by excising the dorsal skin of a rat.

On day 3, the wound dressed with chitin-PAA-GTMAC 1:4 hydrogel appeared pink with high degree of swelling while those with chitin-PAA-GTMAC 1:10 and Intrasite™ Gel were mild swelling. In addition, those with Intrasite™ Gel were red at the edge of the wound. However, every wound became hyperemia which indicated that all the wounds were in acute inflammatory phase of the wound healing process.

On day 7, each wound dressed with each materials was smaller than those of day 3. The characteristic of the wounds dressed with chitin-PAA-GTMAC 1:4 were moist while those with chitin-PAA-GTMAC 1:10 and Intrasite™ Gel were dry with thin scab covering.

On day 9, all the wounds of different dressings contracted toward the center and became smaller than those of day 7. The wounds dressed with chitin-PAA-GTMAC 1:4 hydrogel showed the most contraction followed by those dressed with chitin-PAA-GTMAC 1:10 hydrogel and Intrasite™ Gel respectively. Besides the center of all wounds appeared red indicating the granulation tissue formation filling the gap of the wounds that make the wounds were more shallow than those of day 7.

On day 12, all wounds dressed with three different materials showed the continuous contraction and the wound areas were obviously smaller than those on day 9.

Especially the wounds dressed with chitin-PAA-GTMAC 1:4 and 1:10 hydrogel looked dry and were covered with the small scabs at the wound center. Meanwhile those dressed with Intrasite™ Gel showed the least contraction and red moist appearance at the center.

On day 15, the sizes of all wounds were smaller and more shallow than those of day 12. The wounds dressed with chitin-PAA-GTMAC 1:4 and 1:10 hydrogel appeared pink with little scabs while the Intrasite™ Gel dressed wounds showed mild red and were covered with scabs at the wound center.

On day 18, three wounds dressed with different materials displayed complete granulation tissue formation and much smaller than those of day 15. Moreover, most part of them were covered with hairs. These indicated that all the wounds became close to normal skin.

Figure 19 Photographs of the wound surfaces show the progression of the wound healing process comparing between the wounds dressed with chitin-PAA-GTMAC 1:4, chitin-PAA-GTMAC 1:10 hydrogel and Intrasite™ Gel.

5.1.2 The remaining wound areas

The remaining wound areas were evaluated at the group of day 3 to day 18 by photographing each wound with a digital camera. The remaining areas of the wounds were shown as the average percentages and calculated by using a computer program of an image analyzer. (Table 2 and Figure 20)

On day 0, the day of three full thickness wounds of 1×1 cm in size were set as 100%.

On day 3, all of the experimental wounds showed higher average percentages of the wound size than those on day 0 but the wound size dressed with chitin-PAA-GTMAC 1:10 and Intrasite™ were lower than those with chitin-PAA-GTMAC 1:4.

On day 7, the size of the wounds dressed with different three experimental materials was clearly smaller than those of day 0 and day 3. However, chitin-PAA-GTMAC 1:4 hydrogel dressed wounds were smallest among others.

On day 9, the size of the wounds dressed with all three dressing was clearly smaller than those of day 7. The average percentage of the wound area dressed with Intrasite™ was the highest. The remaining area of those with chitin-PAA-GTMAC 1:4 demonstrated about 20% less than those with chitin-PAA-GTMAC 1:10 and much smaller than those with Intrasite™ Gel.

On day 12, the average percantage of all wounds dressed with three different experimental materials was obviously smaller than those of day 9. They all showed the average percentage of the remaining wound areas of less than 20. Those with chitin-PAA-GTMAC 1:10 hydrogel were smallest and followed by those with chitin-PAA-GTMAC 1:4 and Intrasite™ Gel respectively.

On day 15, the average percentage of the remaining wound areas dressed with three materials was nearly 0. The chitin-PAA-GTMAC 1:10 hydrogel dressed wounds disappeared and the average percentage of remaining areas become 0.05 while those with chitin-PAA-GTMAC 1:4 and Intrasite™ Gel almost disappeared with the remaining wound areas become 0.94 and 1.86 respectively.

Table 2 The average percentages of remaining wound areas of the chitin-PAA-GTMAC 1:4, chitin-PAA-GTMAC 1:10 hydrogel and Intrasite™ Gel dressed wounds. (* scratched)

Day	Percentages of remaining wound area												Average percentages of remaining wound area		
	Chitin-PAA-GTMAC 1:4				Chitin-PAA-GTMAC 1:10				Intrasite™				Chitin-PAA-GTMAC 1:4	Chitin-PAA-GTMAC 1:10	Intrasite™
3	169.51	90.79	89.03	144.93	114.62	79.77	108.02	110.92	153.33	73.93	94.15	90.55	123.57	103.33	102.99
7	68.76	58.40	101.42	98.11	113.19	99.20	89.98	62.93	81.98	100.00	92.83	56.75	81.67	91.32	82.89
9	22.49	13.10	29.08	2.75	14.87	46.39	85.08	8.94	21.59	63.22	113.16	55.77	16.85	38.82	63.44
12	17.39	5.68	12.82	7.95	12.32	1.37	7.45	2.44	13.60	15.78	9.95	13.91	10.96	5.90	13.31
15	0	0	3.78	0	0	0.22	0	0	4.56	0	2.86	0	0.94	0.05	1.86
18	21.49*	0	0	0	0	0	0	0.85*	0.22	0	2.56	0	0.00	0.00	0.69

On day 18, the last experimental day, three wounds of the groups dressed with chitin-PAA-GTMAC 1:4 and 1:10 disappeared. Another one wound of those two groups still appeared with the percentages of the remaining wound area of 21.49 and 0.85 respectively. Therefore they were excluded in calculating the average percentages of the remaining wound area that made them were 0.00 in the table 2. Whereas those with Intrasite™ Gel also almost disappeared and the average of the wound size was 0.69.

Furthermore, the average percentage of the wound area dressed with chitin-PAA-GTMAC 1:4 and 1:10 of day 9 were significantly decrease as compared with those of day 7 at p-value less than 0.05. Though the wound size went on decreasing from day 3 to day 18 in every experimental groups, only those of day 9 was significantly different from those of day 7 in both groups of wound dressed with chitin-PAA-GTMAC 1:4 and 1:10.

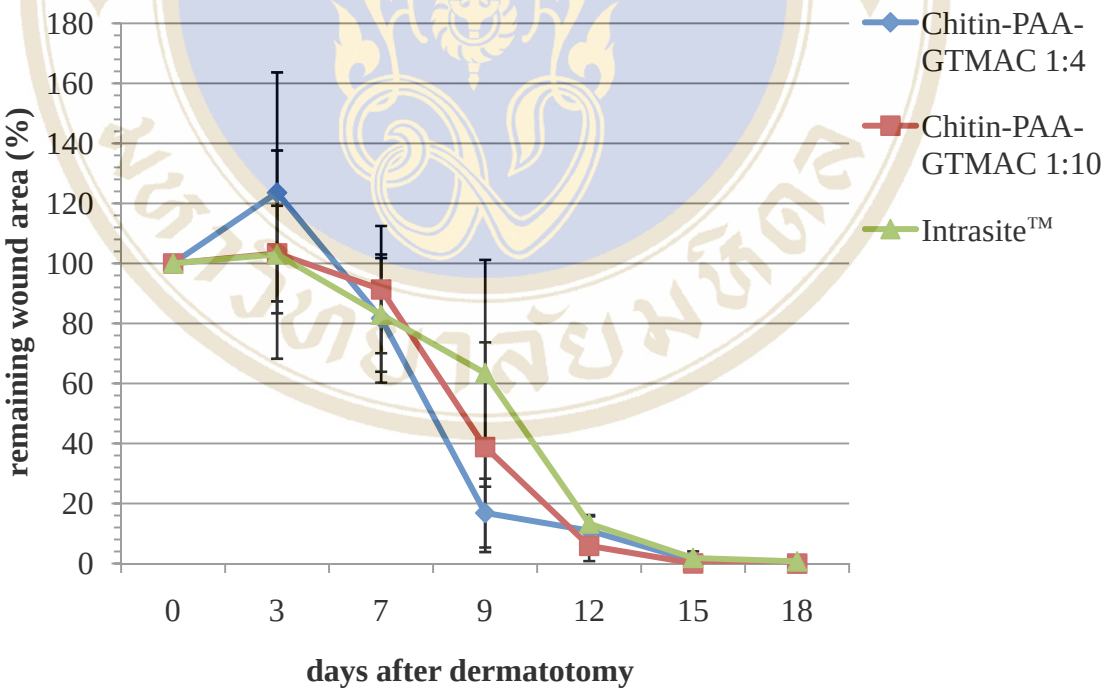


Figure 20 The linear graphs show the comparison of the average percentages of the wounds dressed with chitin-PAA-GTMAC 1:4, chitin-PAA-GTMAC 1:10 hydrogel and Intrasite™ Gel.

5.2 Microscopic study of the wound tissue.

Microscopic study of the wound tissue dressed with the three dressing materials were performed by analyzing the epidermal assessment which examined the extent of epidermal growth.

On day 3, three days after dermatotomy, edges of wounds dressed with chitin-PAA-GTMAC 1:4 and 1:10 showed the developing epidermis beginning to develop toward the central area. No developing epidermis was observed on the wound surfaces dressed with Intrasite™ Gel. Moreover, the many residues of dressing materials and inflammatory cells were observed on the surface of each wound dressed with each dressing material. (Figure 21)

On day 7, the chitin-PAA-GTMAC 1:4 and 1:10 hydrogel-dressed wounds performed new epidermis extending far from the wound edges to the center more than those of day 3. The epidermis showed thick new epithelium containing multilayered keratinocytes. However, the arrangement of new epithelial cell layers was incompletely formed. Moreover, some dermal papillae of the dermis had already developed. The Intrasite™ Gel dressed wounds began to show the epithelialization but they showed less development than those with the former two dressing materials. In addition, the residues of dressing material were observed at the edges and the surfaces of all groups of experimental wounds. Especially, the edges of the wound dressed with Intrasite™ Gel revealed much more material residues scattering from the upper part into the deep subcutaneous layer. (Figure 22)

On day 9, all the wound of each treatment, the migration of the epithelium from the edges are farther than those on day 7. The outermost of the edge areas showed thicker epidermis containing multilayered keratinocytes like in the intact normal skin and the keratin at the external surfaces of the epidermis had been produced already. However, those with Intrasite™, the epidermis had developed less than those with chitin-PAA-GTMAC 1:4 and 1:10. Moreover the dermal papillae were found in all experimental wounds. The hair follicle is observed only at the edge of the wound dressed with chitin-PAA-GTMAC 1:4. The little residues of the dressing material were found in the dermal layer of the wound dressed with chitin-PAA-GTMAC 1:10 while those with Intrasite™ are displayed throughout the section. (Figure 23 and 24)

On day 12, the migration of the new epithelium from the outermost toward the center of the wound was much more than those of day 9. The epithelial cells covering the chitin-PAA-GTMAC 1:4 and 1:10 dressed wounds were complete while those dressed with Intrasite™ Gel revealed the uncovered surface at the central area. The more well developed epidermis completely covering the surfaces of the wounds dressed with the former two dressing materials demonstrated the keratin lining the external surface of the developing epidermis and developing hair follicles at the wound edges. No material residue in dermis is found in the wound dressed with chitin-PAA-GTMAC 1:10, meanwhile those with chitin-PAA-GTMAC 1:4 and Intrasite™ Gel showed the moderate and the large amount of residues respectively. Many inflammatory cells were displayed in those dressed with Intrasite™ Gel. (Figure 25 and 26)

On day 15, the wound dressed with chitin-PAA-GTMAC 1:4 and 1:10 hydrogel revealed the completely developed epidermis covering the wound surfaces and showed more advanced epithelialization than those of day 12. They revealed the thick epidermis containing multilayered keratinocytes with keratin at the external surface like in the normal skin that was the stratified squamous keratinized epithelium. Some dermal papillae, hair follicles and sebaceous glands were observed at the lower part of the well-developed epidermis but the surface of the wounds dressed with chitin-PAA-GTMAC 1:10 hydrogel were rough. Some wound surfaces dressed with Intrasite™ Gel were also completely covered with the epidermis. The epidermis displayed the thick epidermis containing multilayered keratinocytes with keratin at the external surface and some dermal papillae. However, the hair follicle and sebaceous gland were not observed. Anyhow, the center of the wounds dressed with Intrasite™ Gel of some rats had not been covered with the epidermis. (Figure 27 and 28)

On day 18, epidermis had covered the surface of the wounds dressed with three experimental materials completely. The surface of the wounds dressed with chitin-PAA-GTMAC 1:4 and 1:10 were completely covered with well-developed epidermis. It was composed of multilayered keratinocytes with keratin at the external surface like in the normal skin. Many hair follicles, dermal papillae and sebaceous glands had developed in the edge areas. At the same time, the epidermis covering the surface of the wound dressed with Intrasite™ was complete and developed from the

edge areas toward the central area. At the edge areas, the dermal papillae were developed and epidermis was thicker containing multilayered keratinocytes. No developing hair follicle and sebaceous gland were observed at the dermal layer of the wound dressed with Intrasite™ Gel. Little remaining residues of dressing material were still observed in dermal layer of those dressed with chitin-PAA-GTMAC 1:4 and 1:10 while those with Intrasite™ Gel continuously presented many clusters of material residues throughout the experimental period. (Figure 29 and 30)

Figure 21 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A, B and C), chitin-PAA-GTMAC 1:10 (D, E and F) and Intrasite™ Gel (G, H and I) on day 3 after dermatotomy. (H and E staining)

A) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:4 shows the developing epidermis (De) at the edges (Ed) of the wound. The wound surface (Ws) shows inflammatory cells and residues of dressing material (Rd). (X1.25)

B and C) The higher magnification of A. The left (B) and the right (C) edges of the wound are displayed. Few developing epidermis are observed. Besides, the wound edges reveal some remaining residues of the dressing material. (X10)

D) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:10 shows the developing epidermis which begins to migrate toward the center of the wound and the wound surface. The inflammatory cells and some remaining residues of dressing material are displayed. (X1.25)

E and F) The higher magnification of D represented as the left (E) and the right (F) wound edges (Ed) reveal the developing epidermis which begin to develop to the center of the wound and show inflammatory cells and some remaining residues of the dressing material. (X10)

G) The overall surface of the wound dressed with Intrasite™ Gel reveals no developing epidermis. The wound surface shows the inflammatory cells and some residues of the dressing material. (X1.25)

H and I) The higher magnification of G at the left (H) and the right (I) wound edges reveal no developing epidermis and the remaining residues of the dressing material are observed. (X4)

Abbreviation : Dm, dermis; Ic, inflammatory cell

Figure 21

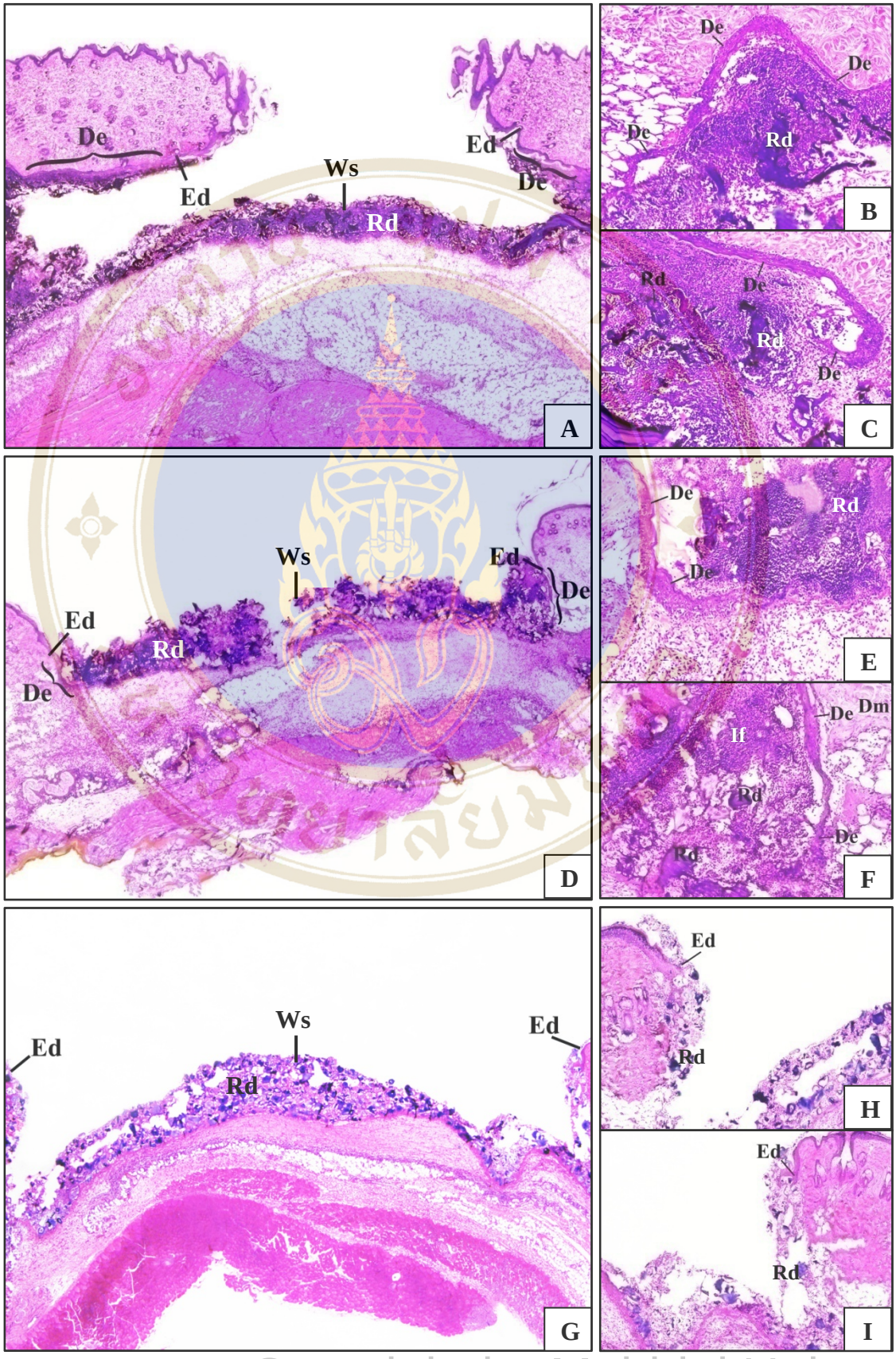


Figure 22 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A, B and C), chitin-PAA-GTMAC 1:10 (D, E and F) and Intrasite™ Gel (G, H and I) on day 7 after dermatotomy. (H and E staining)

A) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:4 performs the migration of the developing epidermis (De) at the edges of the wound (Ed). The wound surface (Ws) performs little number of the inflammatory cells. Some residues (Rd) of the dressing material are observed at the wound edges. (X1.25)

B and C) The higher magnification of A clearly reveal the developing epidermis at left (B) and right (C) edges of the wound. Besides, the right wound edge shows the developing dermal papillae (Dp) which indicates the further step of epidermal development. The remaining material residues are observed at both wound edges. (X10)

D) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:10 shows more developing epidermis from the edges toward the center of the wound. The wound surface reveals the inflammatory cells and material residues (Rd). (X1.25)

E and F) The higher magnification of D show the further step of epidermal development. The left (E) and the right (F) wound edges reveal more epithelial migration from the edge of the wound to the central area and the present of multilayered keratinocytes with keratin (Kr) of epidermis as well as the developing dermal papillae. (X4)

G) The overall surface of the wound dressed with Intrasite™ Gel presents the some developing epidermis. The wound surface shows the inflammatory cells and residual materials. Besides, both wound edges reveal much more material residues scattering from upper part into deep subcutaneous layer.

H and I) The higher magnification of G represent the left (H) and the right (I) wound edges. At the left wound edge shows more migration of the developing epithelium with scab (Sc) from the edge toward the center while the right (I) edge performs the multilayered keratinocytes with keratin, dermal papillae and the residues of material are observed. (X4)

Figure 22

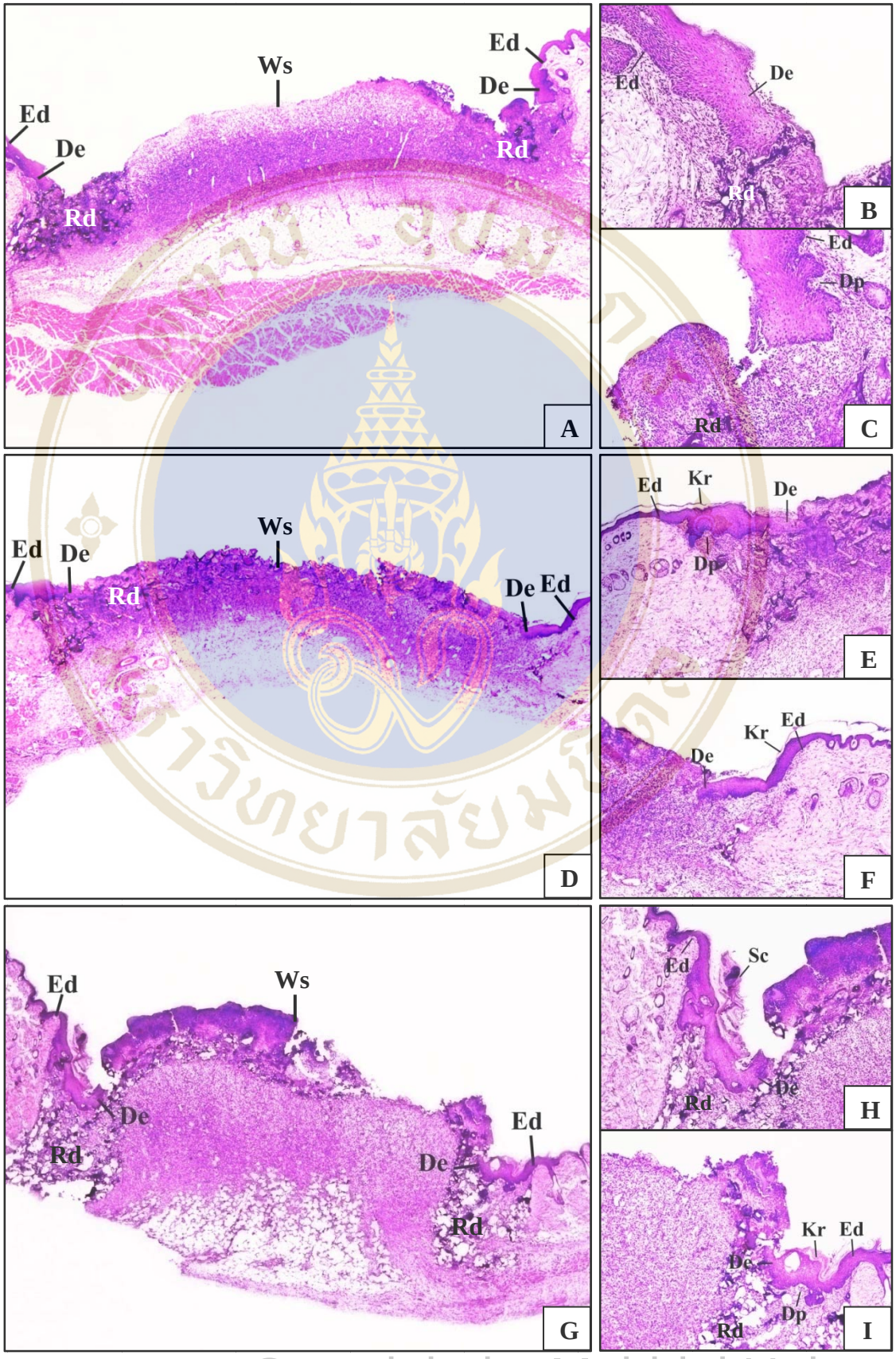


Figure 23 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A, B and C), chitin-PAA-GTMAC 1:10 (D, E and F) and Intrasite™ Gel (G, H and I) on day 9 after dermatotomy. (H and E staining)

A) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:4 is completely covered with the developing epidermis (De). The wound surface (Ws) is smooth with no inflammatory cell and material residue (Rd). (X1.25)

B and C) The higher magnification of A refers to the left (B) and the right (C) wound edges. Both wound edges show completely developed epidermis extending from the edge to the central area. The epidermis contains multilayered keratinocytes. Furthermore, the hair follicle (Hf) and the dermal papillae are observed at the right edge. (X4)

D) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:10 shows the further step of epidermal development with keratin but the central area of the wound surface is uncovered. No inflammatory cell is revealed. Both wound edges show little residues of material (Rd) in dermal layer. (X1.25)

E and F) The higher magnification of D. The epidermis of the left (E) (X10) and the right (F) (X4) wound edges reveal multilayered keratinocytes with keratin (Kr). The dermal papilla is observed at the left wound edge. The remaining residues of the dressing material are observed at the right wound edge.

G) The overall surface of the wound dressed with Intrasite™ Gel represents the multilayered keratinocytes with keratin but the central part of the wound surface is still uncovered. Many inflammatory cells and material residues are displayed throughout the section from wound surface down to the subcutaneous layer (Su). (X1.25)

H and I) The higher magnification of G. The left (H) (X10) and the right (I) (X4) wound edges show the developing epidermis with mild keratinization. Some dermal papillae (Dp) and many material residues are found.

Abbreviation : Dp, dermal papilla; Ed, edge of wound; Hf, hair follicle; Kr, keratin

Figure 23

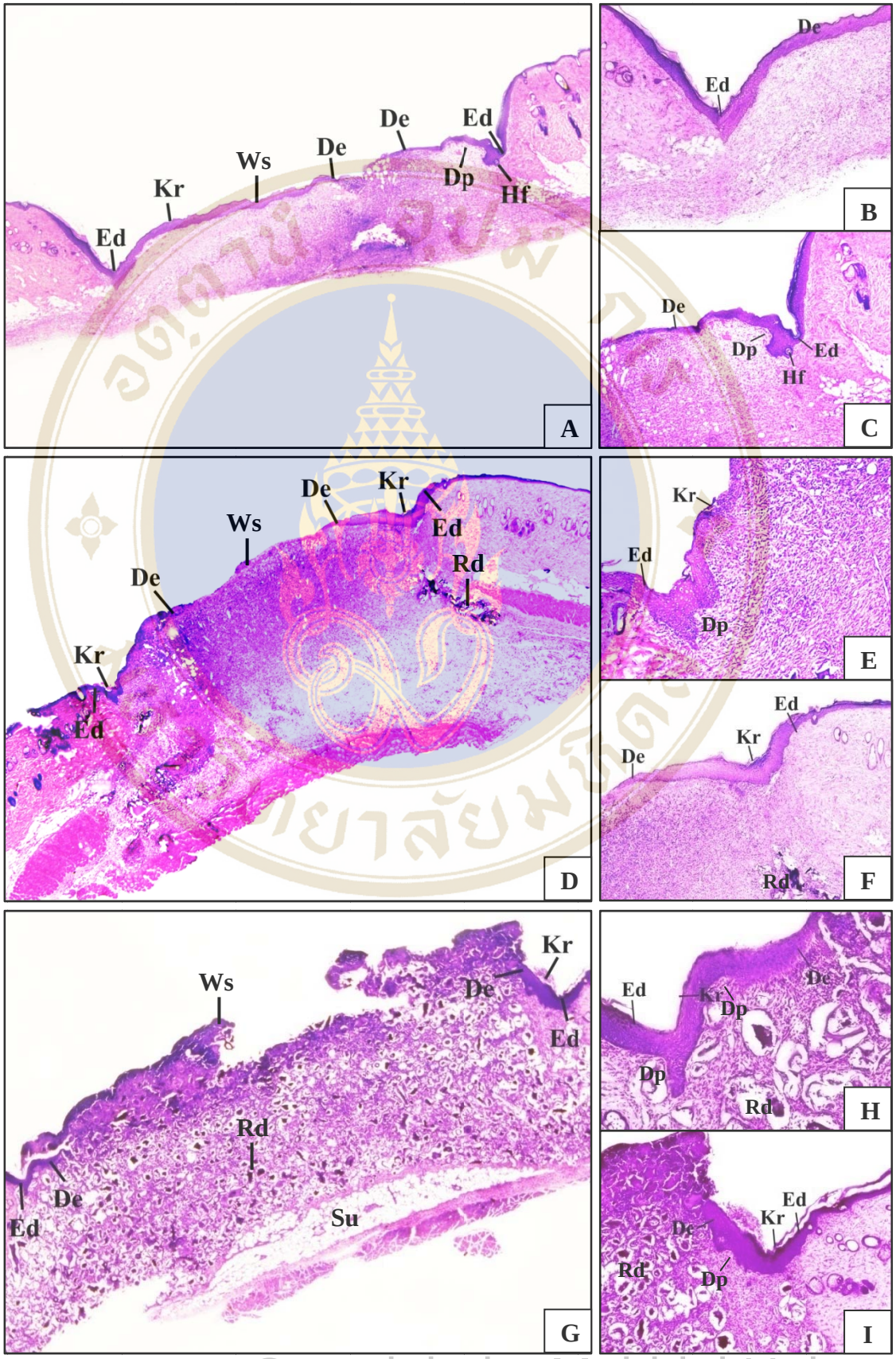


Figure 24 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D) and Intrasite™ Gel (E and F) at the central area of the wound on day 9 after dermatotomy. (H and E staining)

A, C and E The central area of the wound dressed with chitin-PAA-GTMAC 1:4 (A) is completely covered with developing epidermis (De). The epidermis contains the multilayered keratinocytes with keratinization which is close to the normal skin. While the central area of the wound dressed with chitin-PAA-GTMAC 1:10 (C) reveals the developing epidermis containing the multilayered keratinocytes without keratinization. At the same time those dressed with Intrasite™ Gel (E) is still uncovered and performs many inflammatory cells and many clusters of material residues (Rd). (X4)

B, D and F The higher magnification of A, C and E respectively. The epidermis at the central area of the wound dressed with chitin-PAA-GTMAC 1:4 (B) presents the advanced step of development which contains multilayered keratinocytes (Mk) with keratin (Kr) like the normal skin while those dressed with chitin-PAA-GTMAC 1:10 (D) and Intrasite™ Gel (F) are still uncovered. (X10)

Abbreviation : Gt, granulation tissue

Figure 24

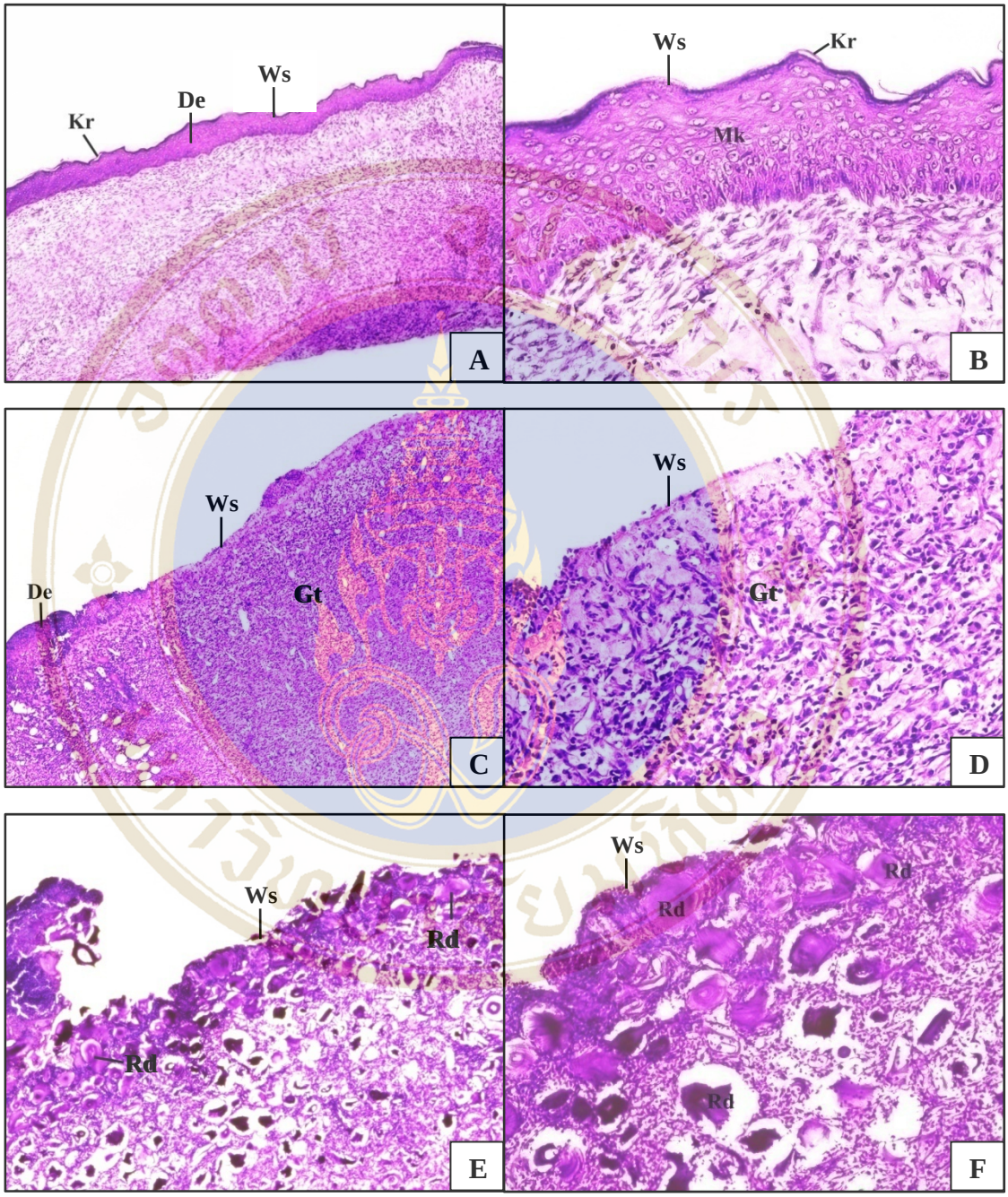


Figure 25 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A, B and C), chitin-PAA-GTMAC 1:10 (D, E and F) and Intrasite™ Gel (G, H and I) on day 12 after dermatotomy. (H and E staining)

A) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:4 is completely covered with the new epidermis (Ne) comprising the multilayered keratinocytes with keratinization. At the edges of wound (Ed), the hair follicles (Hf) are observed. Mild swelling at the center of the wound surface (Ws) is demonstrated. Dermis shows the residues (Rd) of dressing material. (X1.25)

B and C) The higher magnification of A. The left (B) and the right (C) wound edges (Ed) show the developing epidermis (De) containing the multilayered keratinocytes with keratin (Kr) and dermal papillae (Dp). Hair follicle (Hf) is presented at the left wound edge. Moreover, the moderate amount of material residues in the dermis layer is observed. (X4)

D) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:10 reveals the completely formed new epidermis containing the multilayered keratinocytes with keratinization. Especially at the edges of the wound, the epidermis shows the advanced stage of development with keratin (Kr) and obvious dermal papillae. The central area of the wound surface is covered with the scab (Sc). (X1.25)

E and F) The higher magnification of D. The left (E) and the right (F) wound edges show the new epidermis containing the multilayered keratinocytes with keratin and dermal papillae are observed. (X4)

G) The overall surface of the wound dressed with Intrasite™ Gel is incompletely covered with new epidermis. No developing epidermis covers the central area of the wound surface. The developing epidermis found at the wound edges shows the lesser degree of development. Many inflammatory cells and cluster of material residues are closely demonstrated in the dermis. (X1.25)

H and I The higher magnification of G. The left (H) and the right (I) wound edges show the rough pattern of new epidermis with keratin and the dermal papilla is hardly identified. The dermis contains the inflammatory cells and many clusters of material residues. (X10) Gt, granulation tissue

Figure 25

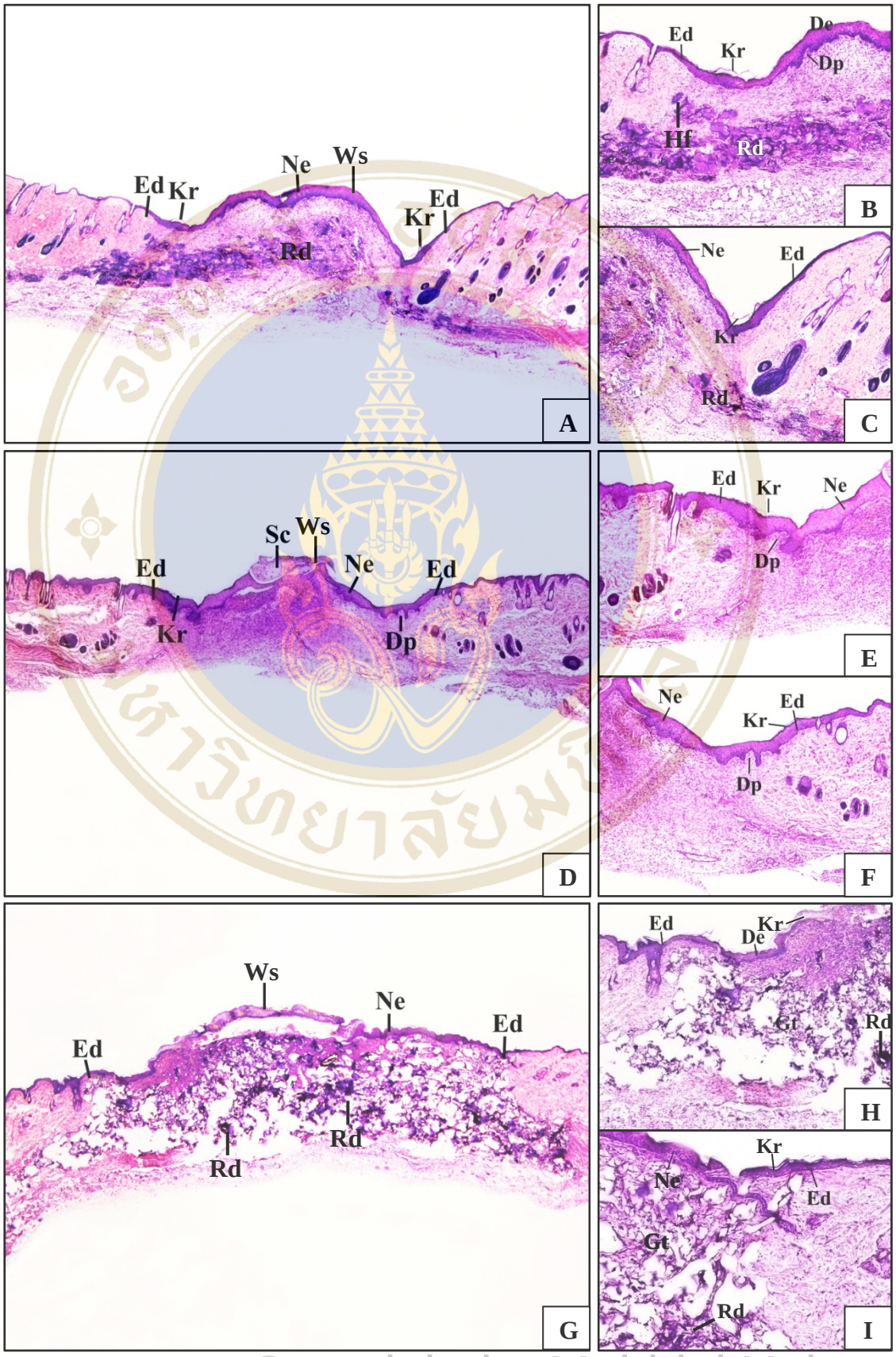


Figure 26 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D) and with Intrasite™ Gel (E and F) dressing materials at the central area of the wound on day 12 after dermatotomy. (H and E staining)

A, C and E The central area of the wounds dressed with chitin-PAA-GTMAC 1:4 (A) chitin-PAA-GTMAC 1:10 (C) and Intrasite™ Gel (E). The central area of the wound surface (Ws) dressed with chitin-PAA-GTMAC 1:4 and 1:10 are completely covered with new epidermis (Ne) while those with the Intrasite™ Gel are still uncovered with developing epidermis. The large scab (Sc) covering the new epidermis at the central area is observed. No material residue in the dermis is found in the wound dressed with chitin-PAA-GTMAC 1:10, meanwhile those with chitin-PAA-GTMAC 1:4 and Intrasite™ Gel shows the moderate and large amount of residues (Rd) respectively. Many inflammatory cells are displayed in those dressed with Intrasite™ Gel. (X4)

B, D and F The higher magnification of A, C and E respectively. The wound surface of the central area of the wound dressed with chitin-PAA-GTMAC 1:4 (B) is completely covered with new epidermis containing the multilayered keratinocytes and dermal papillae (Dp). The moderate amount of material residues appear at the dermal layer. Those dressed with chitin-PAA-GTMAC 1:10 (D) is completely covered with new epidermis containing multilayered keratinocytes with keratin and the scab. No material residue is observed. Those dressed with Intrasite™ Gel (F) is incompletely covered with developing epidermis (De) and the large scab is found covering the uppermost. Moreover, the material residues and inflammatory cells are obviously observed at the wound surface and dermal layer. (X10)

Abbreviation : Gt, granulation tissue; If, Inflammatory cell

Figure 26

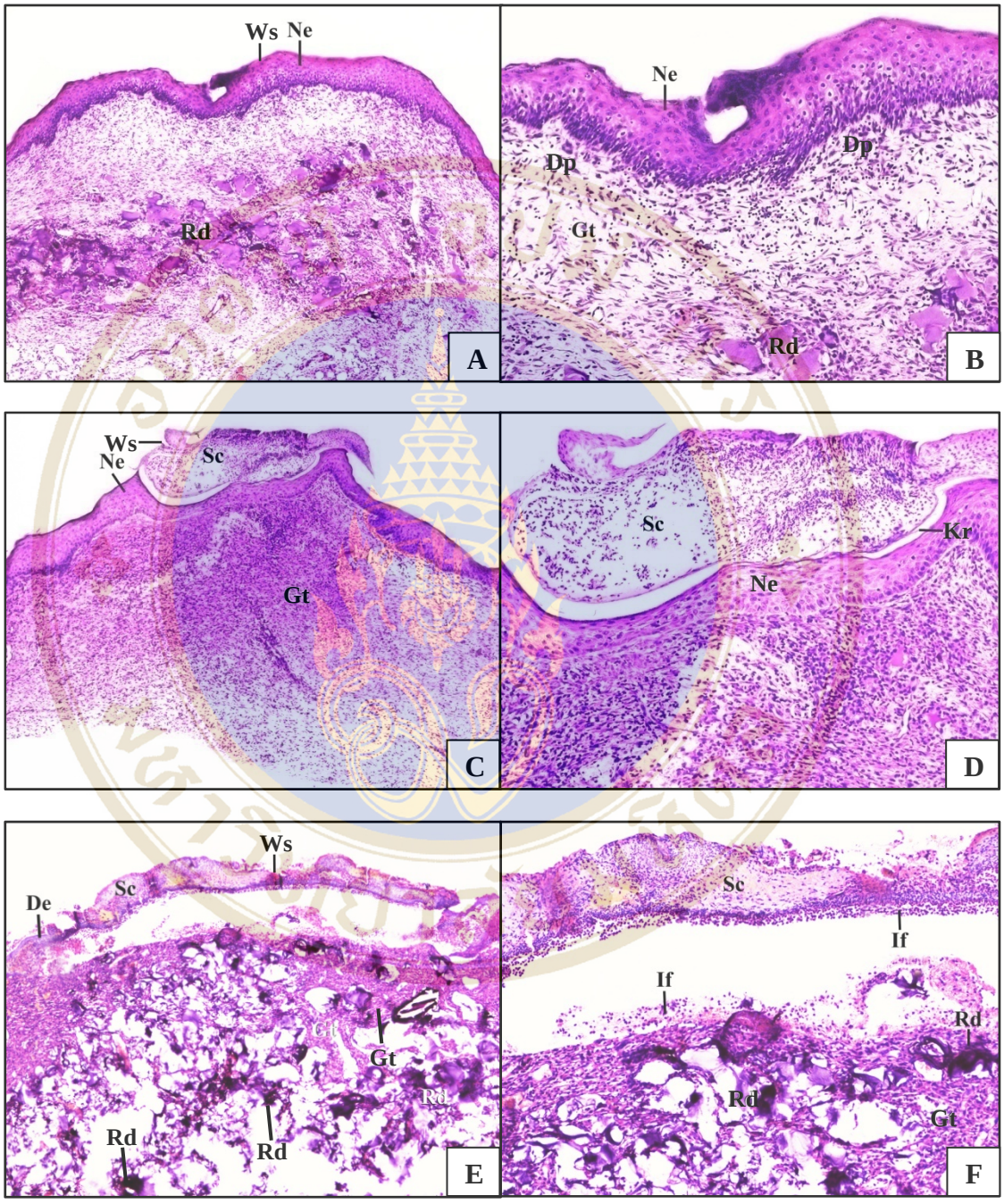


Figure 27 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A, B and C), chitin-PAA-GTMAC 1:10 (D, E and F) and Intrasite™ Gel (G, H and I) on day 15 after dermatotomy. (H and E staining)

A) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:4 is completely covered with new epidermis (Ne) containing the advanced stage of development comprising of multilayered keratinocytes with keratin (Kr) and well developed dermal papillae (Dp) and hair follicles (Hf). The central area beneath the wound surface shows the compact tissue close to the normal skin. (X1.25)

B and C) The higher magnification of A. The left (B) and the right (C) wound edges (Ed) show the developing new epidermis containing the multilayered keratinocytes with keratin. The well developed of dermal papillae and sebaceous gland (Sb) are observed at the left edge. While the hair follicles and dermal papillae are observed at the right edge. (X10)

D) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:10 is completely covered with rough pattern of new epidermis. Keratin, dermal papillae and hair follicles are presented. The dermis at the central area shows the compact tissue close to the normal skin. (X1.25)

E and F) The higher magnification of D. The left (E) and the right (F) wound edges performed the new epidermis with keratin and advanced step of dermal papillae development. The hair follicles and sebaceous glands are presented at both edges. (X10)

G) The overall surface of the wound dressed with Intrasite™ Gel reveals the complete new epidermis with keratin. The little scab (Sc) is presented at the right edge. The dermis shows the dermal papillae and many clusters of material residue (Rd). (X1.25)

H and I) The higher magnification of G. The left (H) and the right (I) wound edges show the complete new epidermis with keratin and the advanced step of developing dermal papillae while many clusters of material residue are observed in the dermis. In addition, the scab is found at the right edge too. (X4)

Abbreviation : Gt, granulation tissue; Ws, wound surface

Figure 27

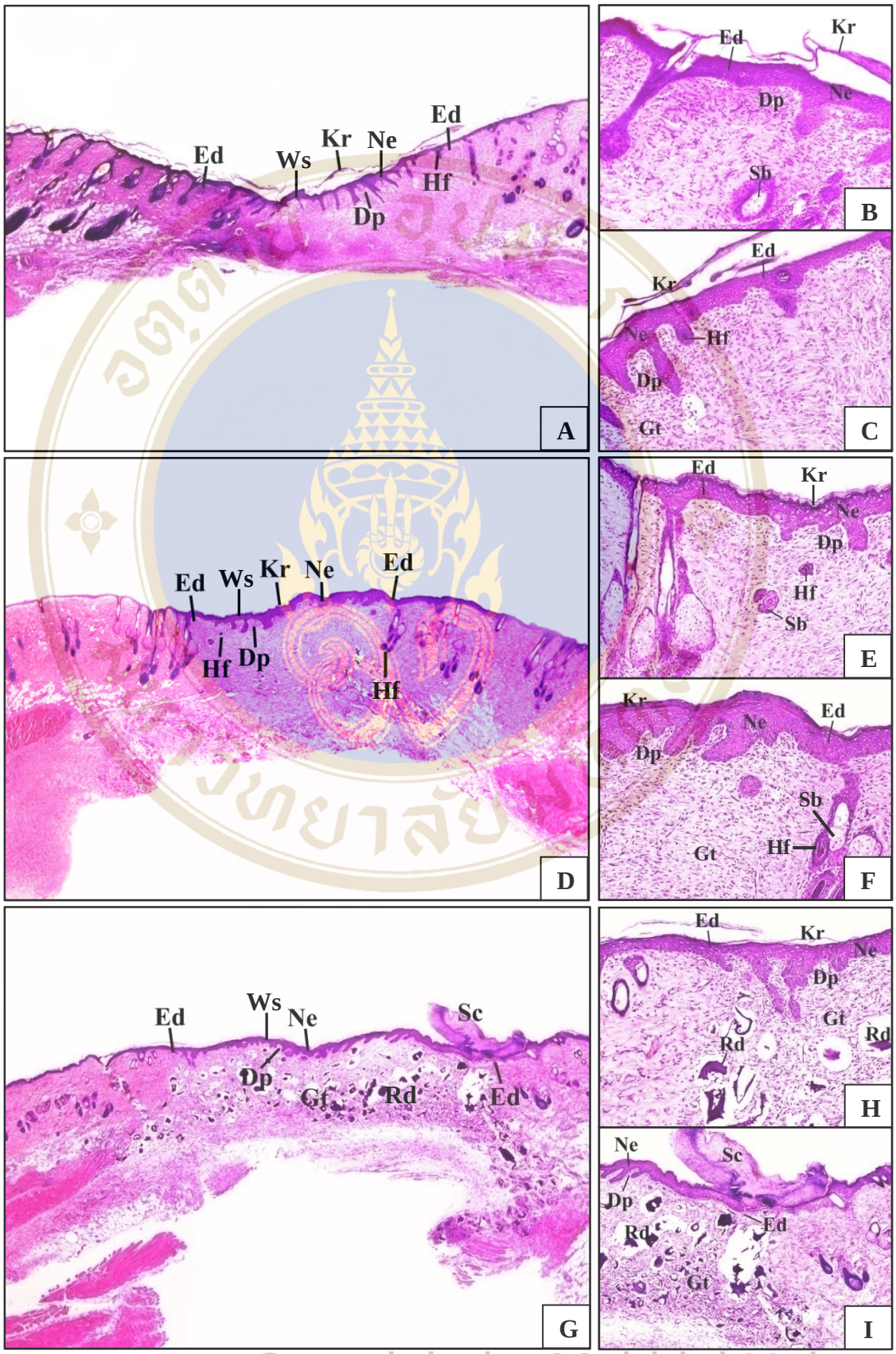


Figure 28 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D) and Intrasite™ Gel (E and F) at the central area of the wound on day 15 after dermatotomy. (H and E staining)

A, C and E The central area of the wound dressed with chitin-PAA-GTMAC 1:4 (A), chitin-PAA-GTMAC 1:10 (C) and Intrasite™ Gel (E) are completely covered with new epidermis (Ne) comprising by the multilayered keratinocytes with keratin (Kr) and advanced step of developing dermal papillae (Dp). In A and C show the hair follicles (Hf) at the edges of the wounds and the tissue beneath the new epidermis of the wounds dressed with all two dressing materials show the compact tissue of dermis. The dermis of the wound dressed with Intrasite™ Gel still appears as loose tissue and contains many material residues. (X4)

B, D and F The higher magnification of A, C and E respectively. The central area of the surface of the wounds dressed with three dressing materials are completely covered with new epidermis containing the multilayered keratinocytes with keratin. The three wound surfaces are compact like the normal skin. Furthermore, the advanced step of developing dermal papillae are observed but the compact tissue of dermis are observed only in B and D while F reveals loose tissue and many material residues. (X10)

Abbreviation : Gt, granulation tissue

Figure 28

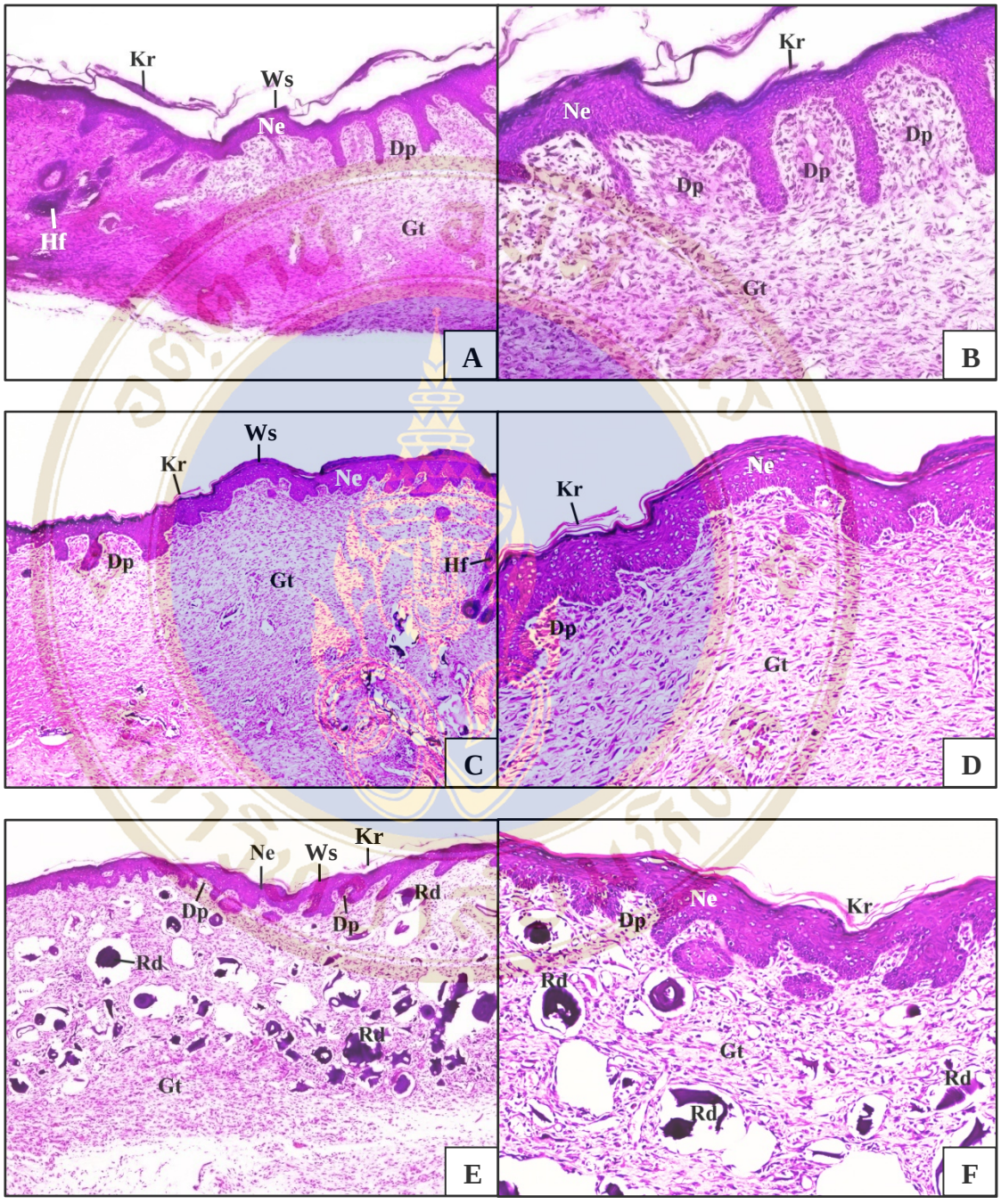


Figure 29 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A, B and C), chitin-PAA-GTMAC 1:10 (D, E and F) and Intrasite™ Gel (G, H and I) on day 18 after dermatotomy. (H and E staining)

A) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:4 reveals the complete smooth new epidermis (Ne) containing the multilayered keratinocytes with keratin (Kr). The compact tissue in the dermis, hair follicles (Hf) and dermal papillae (Dp) are observed like the normal skin. The dermis shows little material residues (Rd). (X1.25)

B and C) The higher magnification of A. The left (B) and the right (C) wound edges (Ed) reveal the completely new epidermis containing the multilayered keratinocytes with keratin. The dermis of both edges show the dermal papillae and hair follicles while sebaceous glands (Sb) are observed at the right edge. (X10)

D) The overall surface of the wound dressed with chitin-PAA-GTMAC 1:10 is completely covered with new epidermis comprising the multilayered keratinocytes with keratin. At the edges of the wound present the hair follicles and dermal papillae. Little material residues are still observed on dermal layer. (X1.25)

E and F) The higher magnification of D. The left (E) (X10) and the right (F) (X4) wound edges represent the complete new epidermis with keratin. The advanced step of developing hair follicles and sebaceous glands are observed at the left edge. However the right edge shows the early developed dermal papillae. The small amount of material residues still appear at the lower part of the dermis.

G) The overall surface of the wound dressed with Intrasite™ Gel shows mild swelling and is covered by the completely new epidermis with keratin and dermal papillae. A great number of the material residues are still found in the dermis. (X1.25)

H and I) The higher magnification of G. The left (H) and the right (I) wound edges show complete new epidermis with keratin and the dermal papillae. The dermis shows much more clusters of material residues throughout the wound tissue. (X10)

Figure 29

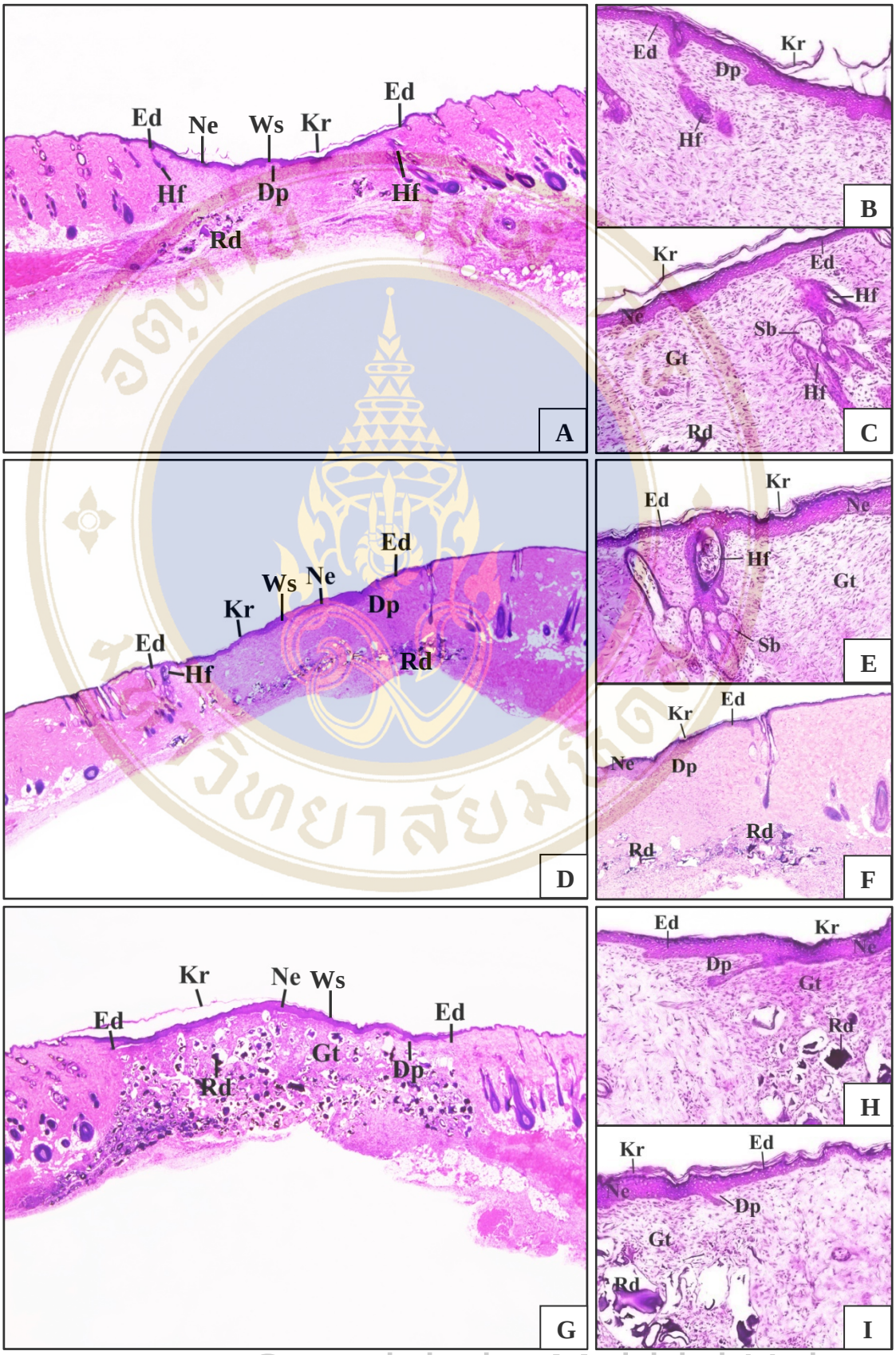


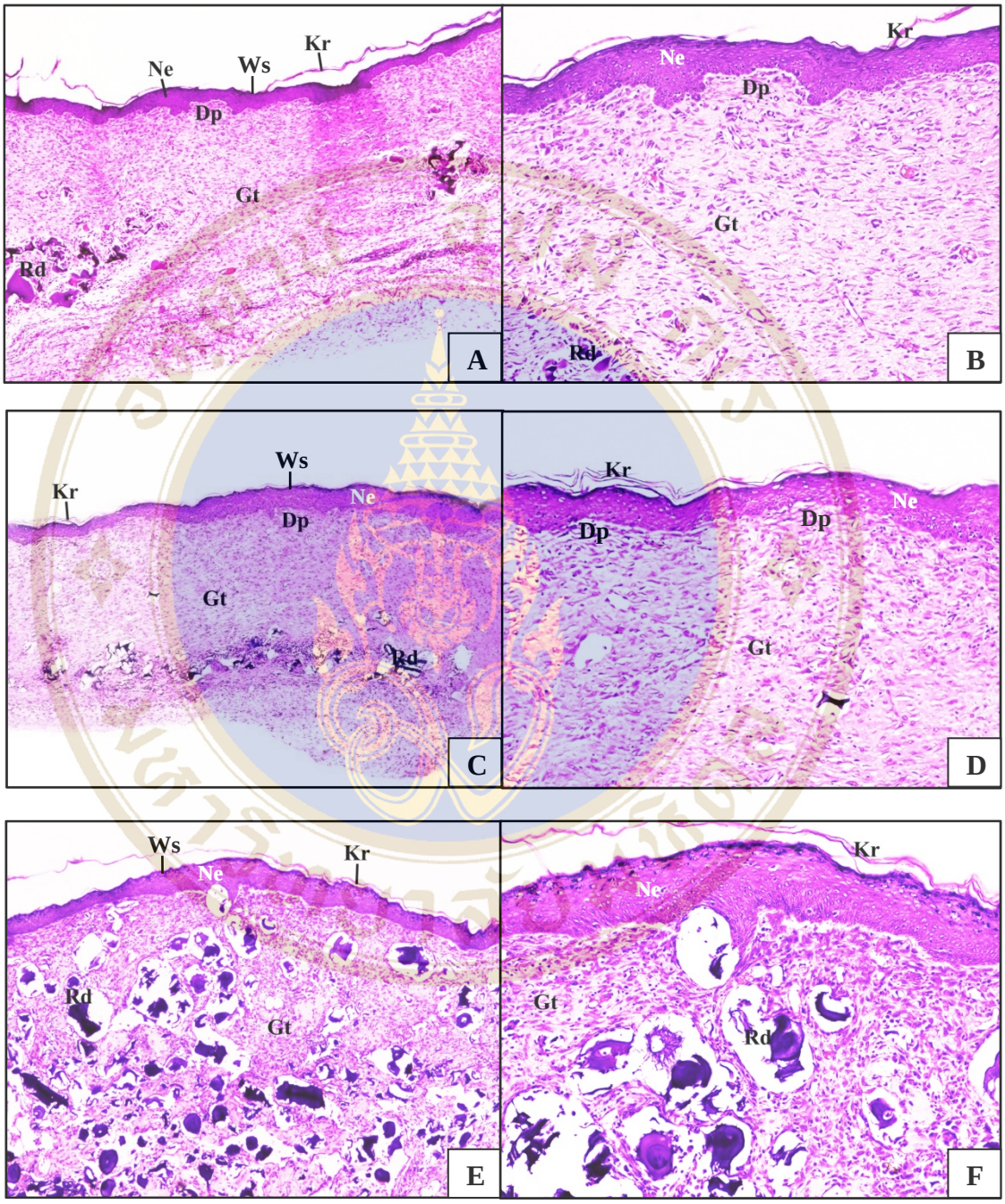
Figure 30 The wound sections dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D) and Intrasite™ Gel (E and F) at the central area of the wound on day 18 after dermatotomy. (H and E staining)

A, C and E The central area of the wound dressed with chitin-PAA-GTMAC 1:4 (A), chitin-PAA-GTMAC 1:10 (C) and Intrasite™ Gel (E) display the wound surface which are completely covered with the multilayered keratinocytes with keratin (Kr). Many clusters of material residues (Rd) are observed in the dermis of the wounds dressed with all experimantal materials but there are more abundant in those with Intrasite™ Gel. (X4)

B, D and F The higher magnification of A, C and E respectively. The surface of the central area of the wound dressed with chitin-PAA-GTMAC 1:4 (B), chitin-PAA-GTMAC 1:10 (D) and Intrasite™ Gel (F) are completely covered with new epidermis (Ne) comprising the multilayered keratinocytes with keratin. The developing dermal papillae (Dp) in the dermis is demonstrated in B and D but not in F. The material residues are observed in all the experimental wounds but it is more abundant in those with Intrasite™ Gel. (X10)

Abbreviation : Ws, wound surface

Figure 30



5.3 Quantitative analysis of PCNA positive cells.

The acquired data was analyzed by determination of the proliferating cell nuclear antigen (PCNA). The PCNA presumed to locate in the nucleus of the cells that exhibited the golden–brown precipitation. The cells that contained the golden-brown stained nucleus were classified as the PCNA positive cells. The PCNA positive cells referred to the proliferating cells in the tissue. These golden–brown stained nuclei were examined light microscopically to observe the proliferating cells of the wound by counting the number of PCNA positive cells. The average percentages of the PCNA positive cells were expressed as means ± standard deviation of the PCNA indices of each wound dressing category counted in all animals and was shown in the Table 3 and Figure 31.

Table 3 The average percentages of PCNA positive cells are expressed as means ± standard deviation of the PCNA indices of each wound dressing category counted in all animals.(n = 60 refer to 3 sections × 5 fields × 4 rats per one group)

Day	Dressing	n	Mean	Std. Deviation
3	chitin-PAA-GTMAC 1:4	60	83.49	9.04
	chitin-PAA-GTMAC 1:10	60	83.44	10.69
	Intrasite™	60	53.63	15.40
	normal	60	41.04	12.99
	Total	240	65.40	22.28
7	chitin-PAA-GTMAC 1:4	60	85.85	8.14
	chitin-PAA-GTMAC 1:10	60	82.67	10.64
	Intrasite™	60	59.04	16.88
	normal	60	27.49	15.33
	Total	240	63.76	26.84
9	chitin-PAA-GTMAC 1:4	60	87.68	7.63
	chitin-PAA-GTMAC 1:10	60	82.20	11.53
	Intrasite™	60	49.67	16.62
	normal	60	32.37	12.00
	Total	240	62.98	26.01
12	chitin-PAA-GTMAC 1:4	60	81.82	9.69
	chitin-PAA-GTMAC 1:10	60	83.22	10.67
	Intrasite™	60	48.66	14.59
	normal	60	27.12	17.86
	Total	240	60.21	27.22
15	chitin-PAA-GTMAC 1:4	60	84.45	9.08
	chitin-PAA-GTMAC 1:10	60	84.84	10.01
	Intrasite™	60	53.63	17.81
	normal	60	30.92	14.27
	Total	240	63.46	26.25
18	chitin-PAA-GTMAC 1:4	60	85.46	8.58
	chitin-PAA-GTMAC 1:10	60	85.77	8.50
	Intrasite™	60	53.47	18.62
	normal	60	31.49	16.22
	Total	240	64.04	26.73

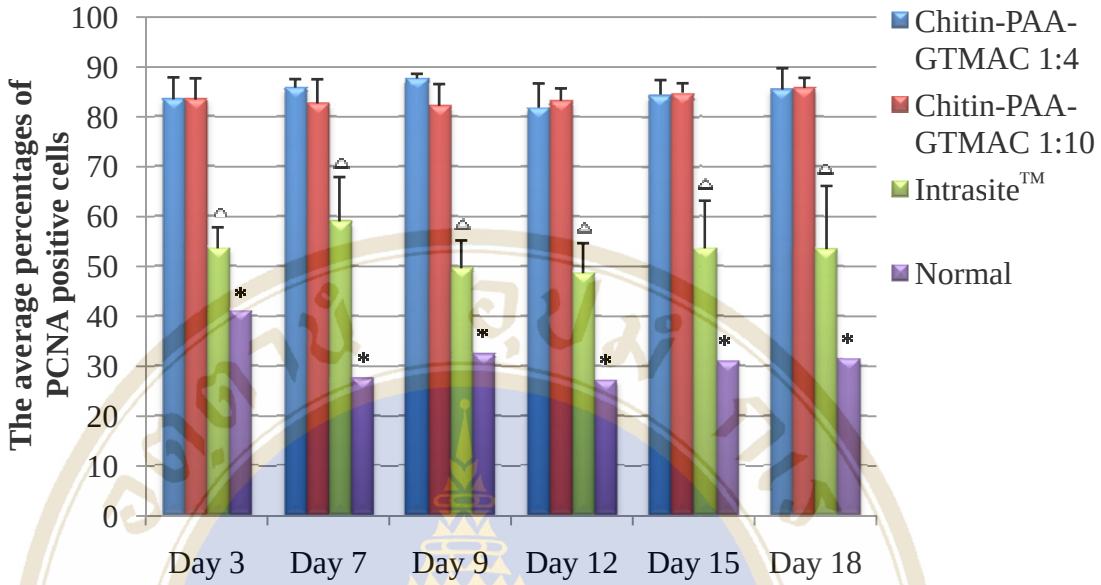


Figure 31 The average percentages of PCNA positive cells of each experimental group comparing between the wounds dressed with chitin-PAA-GTMAC 1:4, chitin-PAA-GTMAC 1:10 hydrogel, Intrasite™ Gel and normal skin.

On day 3, the highest average percentage of PCNA positive cells was expressed in the chitin-PAA-GTMAC 1:4 hydrogel dressed wound group with the amount of 83.49 which was nearly equal to 83.44 of chitin-PAA-GTMAC 1:10. While the Intrasite™ Gel dressed wounds had the average percentage of PCNA positive cells of 53.63 that was significantly less than those of chitin-PAA-GTMAC 1:4 and chitin-PAA-GTMAC 1:10 at p-value less than 0.001. The average percentages of PCNA positive cells in all wounds dressed with three experimental materials were significantly higher than 41.04 of the normal skin at p-value less than 0.001. (Table 3 and Figure 31)

On day 7, the chitin-PAA-GTMAC 1:4 hydrogel dressed wounds showed the highest average percentages of PCNA positive cells, which was 85.85 followed by 82.67 and 59.04 of chitin-PAA-GTMAC 1:10 hydrogel and Intrasite™ Gel respectively. However, the percentage of the wounds covered with Intrasite™ Gel was significantly less than those of chitin-PAA-GTMAC 1:4 and chitin-PAA-GTMAC

1:10 at p-value less than 0.001. Furthermore, all wounds covered with three experimental materials represented the average percentages of PCNA positive cells significantly more than 27.49 of normal skin at p-value less than 0.001. (Table 3 and Figure 31)

On day 9, the wounds dressed with the chitin-PAA-GTMAC 1:4 hydrogel performed the highest average percentages of PCNA positive cells with the amount of 87.68 followed by 82.20 of chitin-PAA-GTMAC 1:10 hydrogel and 49.67 of the Intrasite™ Gel. Moreover, the average percentages of PCNA positive cells of chitin-PAA-GTMAC 1:4 and chitin-PAA-GTMAC 1:10 hydrogel dressed wounds were significantly more than those of Intrasite™ Gel at p-value less than 0.001. In addition, all wounds of each treatment showed the average percentages of PCNA positive cells that were significantly more than 32.37 of normal skin at p-value less than 0.001. (Table 3 and Figure 31)

On day 12, the chitin-PAA-GTMAC 1:10 hydrogel dressed wounds had the highest average percentages of the PCNA positive cells with the amount of 83.22 followed by 81.82 of chitin-PAA-GTMAC 1:4 and 48.66 of Intrasite™ Gel. Furthermore, the percentages those of Intrasite™ Gel were significantly less than those of chitin-PAA-GTMAC 1:10 and chitin-PAA-GTMAC 1:4 hydrogel at p-value less than 0.001. Moreover, the wounds dressed with all three experimental materials showed the average percentages which were significantly more than 27.12 of normal skin at p-value less than 0.001. (Table 3 and Figure 31)

On day 15, the chitin-PAA-GTMAC 1:10 hydrogel dressed wounds show the highest average percentages of PCNA positive cells with the amount of 84.84 which was nearly close to those of chitin-PAA-GTMAC 1:4 hydrogel dressed wounds that was 84.45. Those of the Intrasite™ Gel dressed wounds group was 53.63 which was significant different from those of chitin-PAA-GTMAC 1:10 and chitin-PAA-GTMAC 1:4 hydrogel at p-value less than 0.001. Besides, the average percentages of PCNA positive cells of all experimental wounds was significantly more than 30.92 of normal skin at p-value less than 0.001. (Table 3 and Figure 31)

On day 18, all three dressed wounds had the average percentages of PCNA positive cells that were significantly more than 31.49 of normal skin at p-value less than 0.001. The chitin-PAA-GTMAC 1:10 hydrogel dressed wounds showed the highest average percentages of PCNA positive cells with the amount of 85.77 followed by 85.46 of chitin-PAA-GTMAC 1:4 hydrogel and 53.47 of the Intrasite™ Gel. Those of Intrasite™ Gel was significantly less than those of chitin-PAA-GTMAC 1:10 and chitin-PAA-GTMAC 1:4 hydrogel at p-value less than 0.001. (Table 3 and Figure 31)

Figure 32 The random area of developing epidermis of the wound tissues dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D), Intrasite^{TM} Gel (E and F) dressing materials and normal skin epidermis (G and H) on day 3 after dematotomy. The photomicrographs demonstrate the PCNA positive cells (black arrows) in the epidermis (Ep) which show the golden-brown coloration. These cells are localized either in the stratum basale (Ba) or stratum spinosum (Sp) or both. (Immunoperoxidase staining) (X100)

Abbreviation : Bm, basement membrane; Dm, dermis

Figure 32

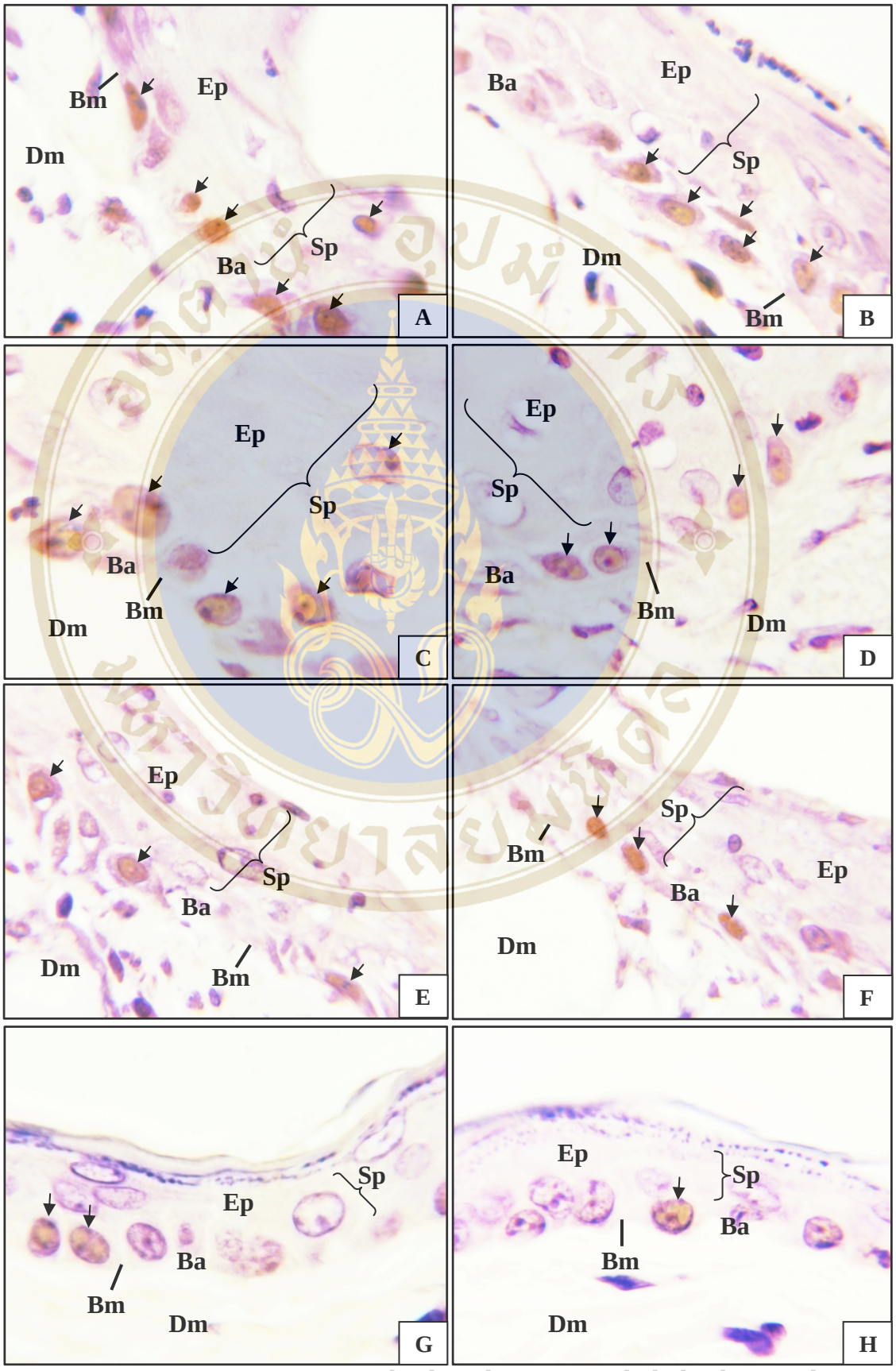


Figure 33 The random area of developing epidermis of the wound tissues dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D), Intrasite™ Gel (E and F) dressing materials and normal skin epidermis (G and H) on day 7 after dematotomy. The photomicrographs demonstrate the PCNA positive cells (black arrows) in the epidermis (Ep) which show the golden-brown coloration. These cells are localized either in the stratum basale (Ba) or stratum spinosum (Sp) or both. (Immunoperoxidase staining) (X100)

Abbreviation : Bm, basement membrane; Dm, dermis

Figure 33

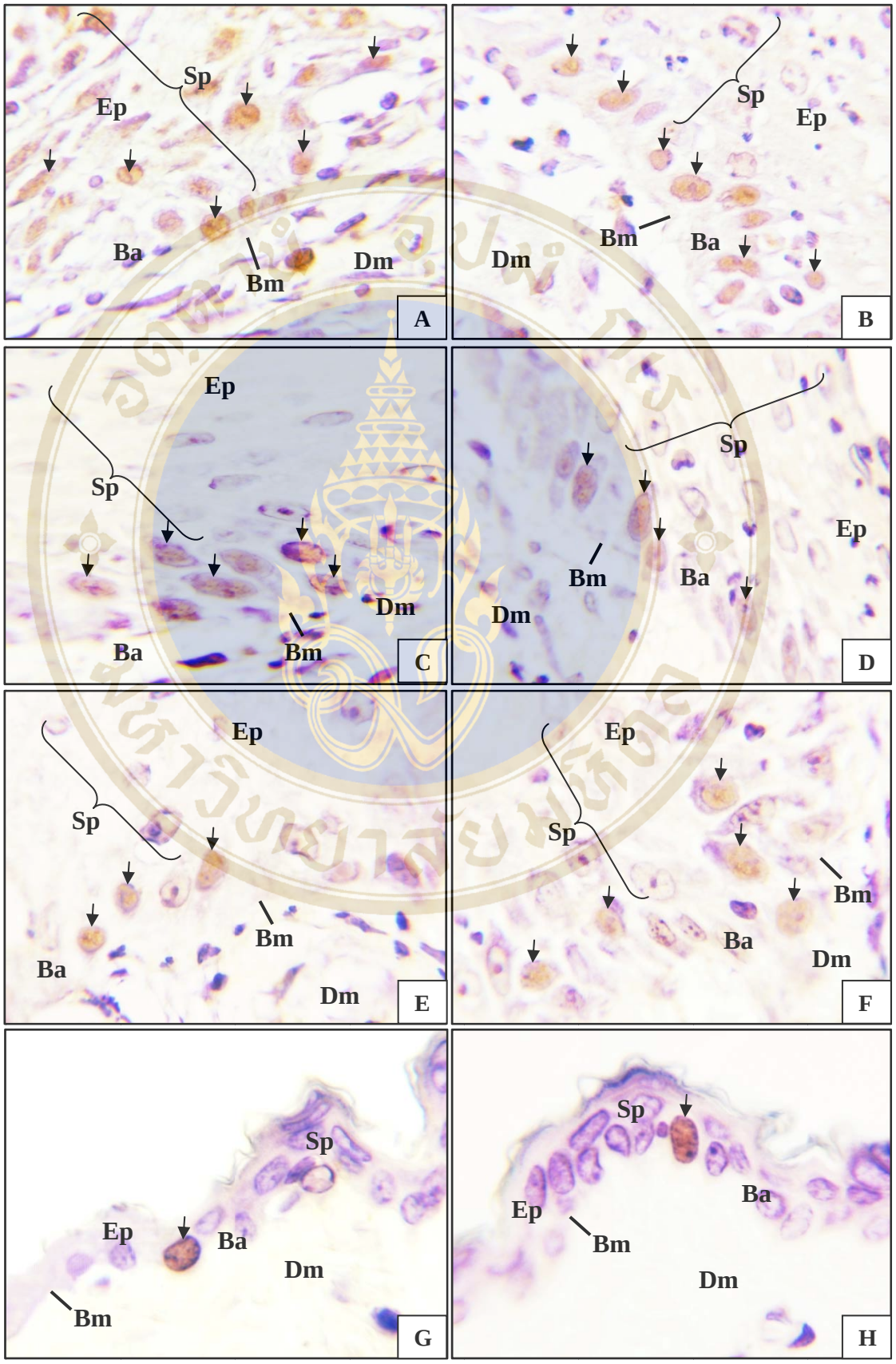


Figure 34 The random area of developing epidermis of the wound tissues dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D), Intrasite™ Gel (E and F) dressing materials and normal skin epidermis (G and H) on day 9 after dematotomy. The photomicrographs demonstrate the PCNA positive cells (black arrows) in the epidermis (Ep) which show the golden-brown coloration. These cells are localized either in the stratum basale (Ba) or stratum spinosum (Sp) or both. (Immunoperoxidase staining) (X100)

Abbreviation : Bm, basement membrane; Dm, dermis

Figure 34

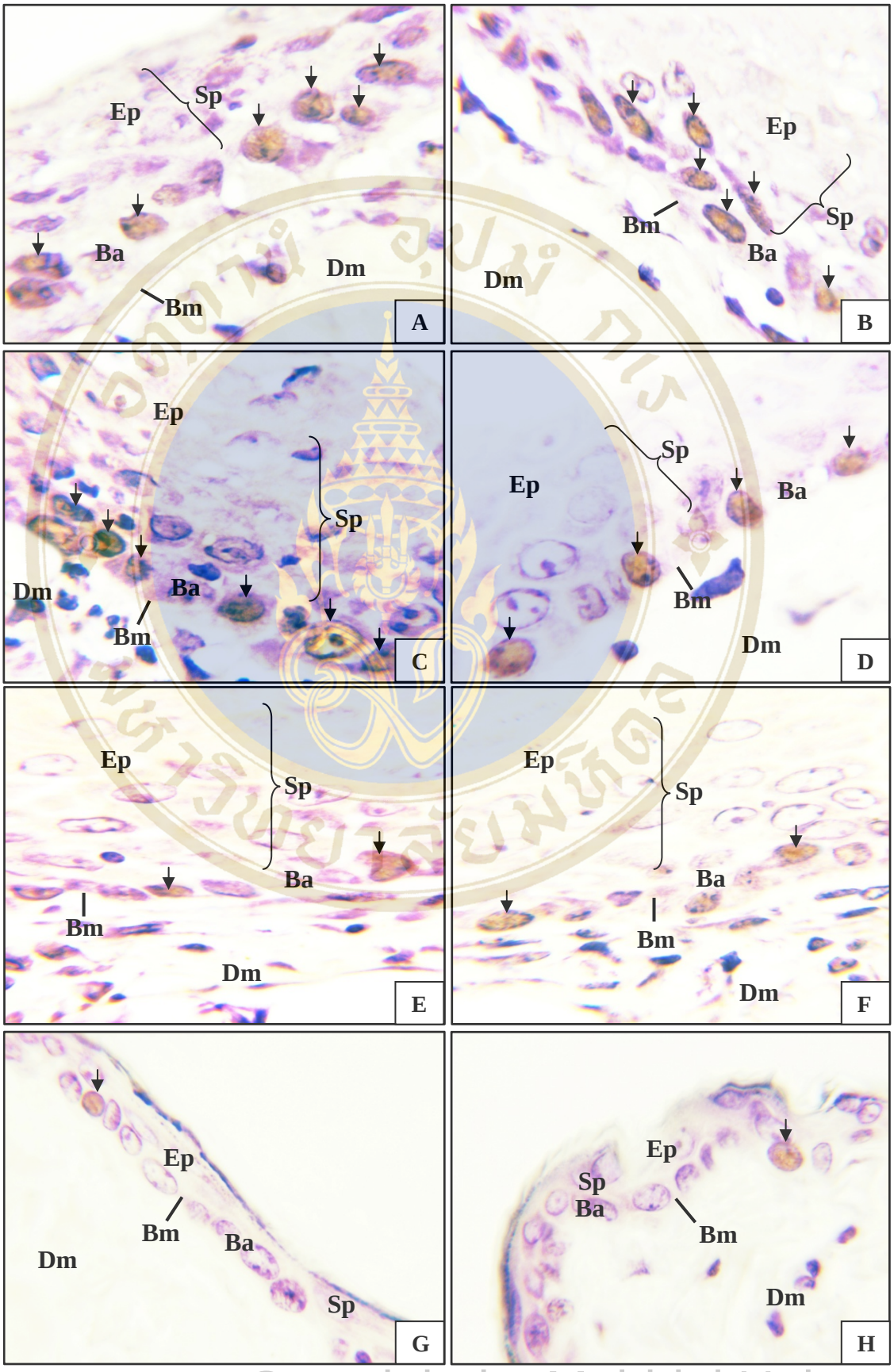


Figure 35 The random area of developing epidermis of the wound tissues dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D), Intrasite™ Gel (E and F) dressing materials and normal skin epidermis (G and H) on day 12 after dematotomy. The photomicrographs demonstrate the PCNA positive cells (black arrows) in the epidermis (Ep) which show the golden-brown coloration. These cells are localized either in the stratum basale (Ba) or stratum spinosum (Sp) or both. (Immunoperoxidase staining) (X100)

Abbreviation : Bm, basement membrane; Dm, dermis

Figure 35

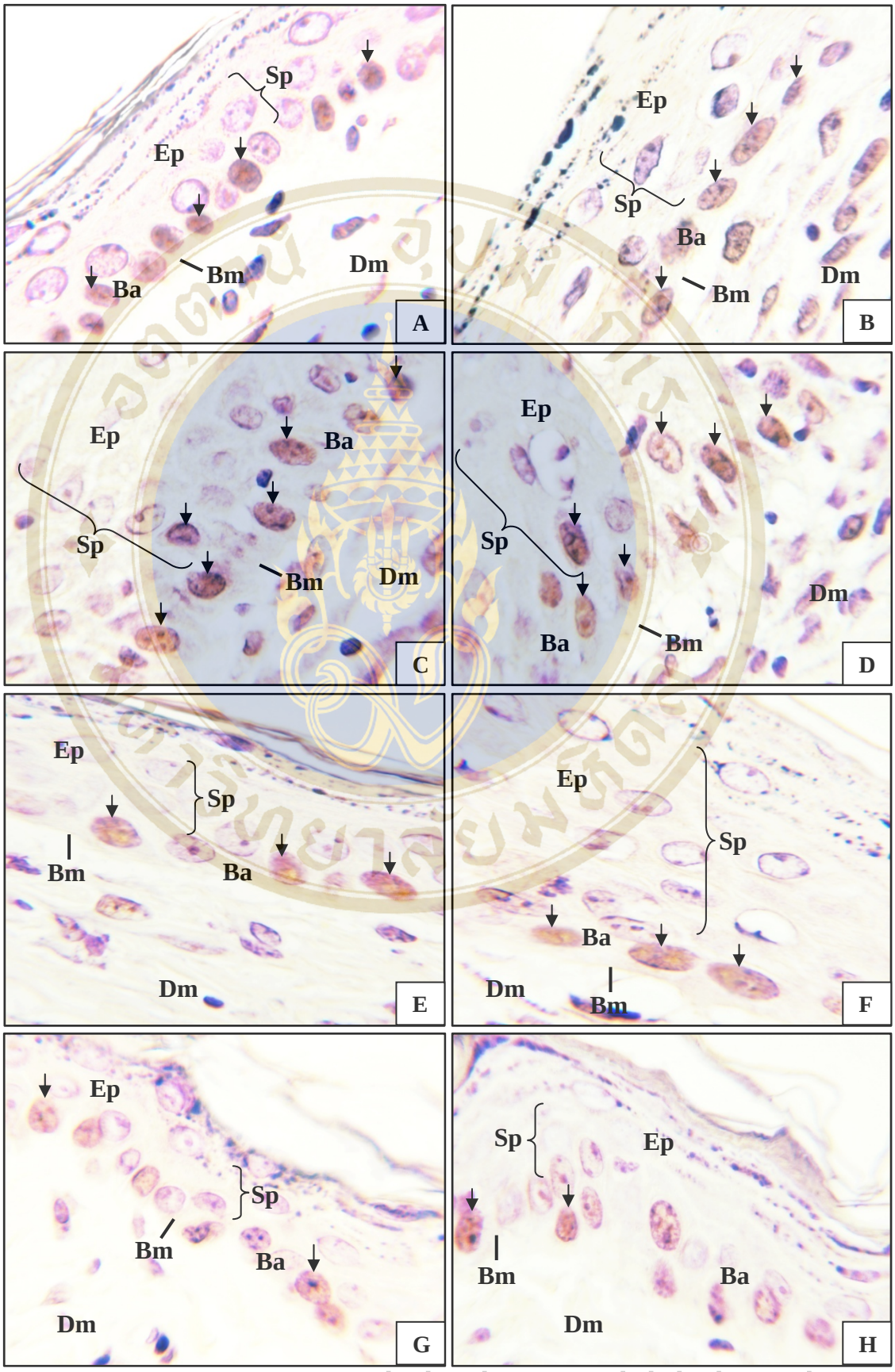


Figure 36 The random area of developing epidermis of the wound tissues dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D), Intrasite™ Gel (E and F) dressing materials and normal skin epidermis (G and H) on day 15 after dematotomy. The photomicrographs demonstrate the PCNA positive cells (black arrows) in the epidermis (Ep) which show the golden-brown coloration. These cells are localized either in the stratum basale (Ba) or stratum spinosum (Sp) or both. (Immunoperoxidase staining) (X100)

Abbreviation : Bm, basement membrane; Dm, dermis

Figure 36

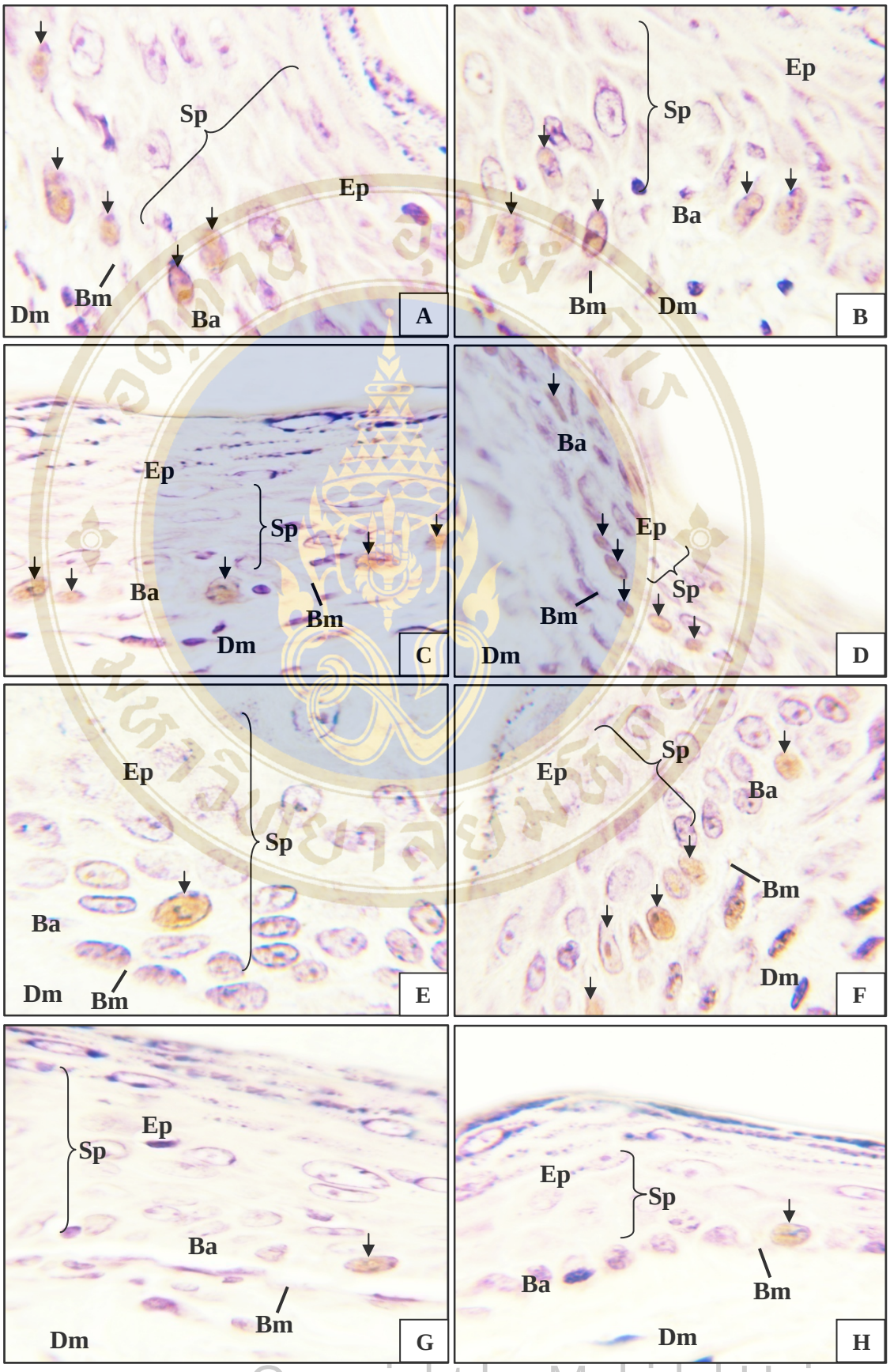


Figure 37 The random area of developing epidermis of the wound tissues dressed with chitin-PAA-GTMAC 1:4 (A and B), chitin-PAA-GTMAC 1:10 (C and D), Intrasite™ Gel (E and F) dressing materials and normal skin epidermis (G and H) on day 18 after dematotomy. The photomicrographs demonstrate the PCNA positive cells (black arrows) in the epidermis (Ep) which show the golden-brown coloration. These cells are localized either in the stratum basale (Ba) or stratum spinosum (Sp) or both. (Immunoperoxidase staining) (X100)

Abbreviation : Bm, basement membrane; Dm, dermis

Figure 37

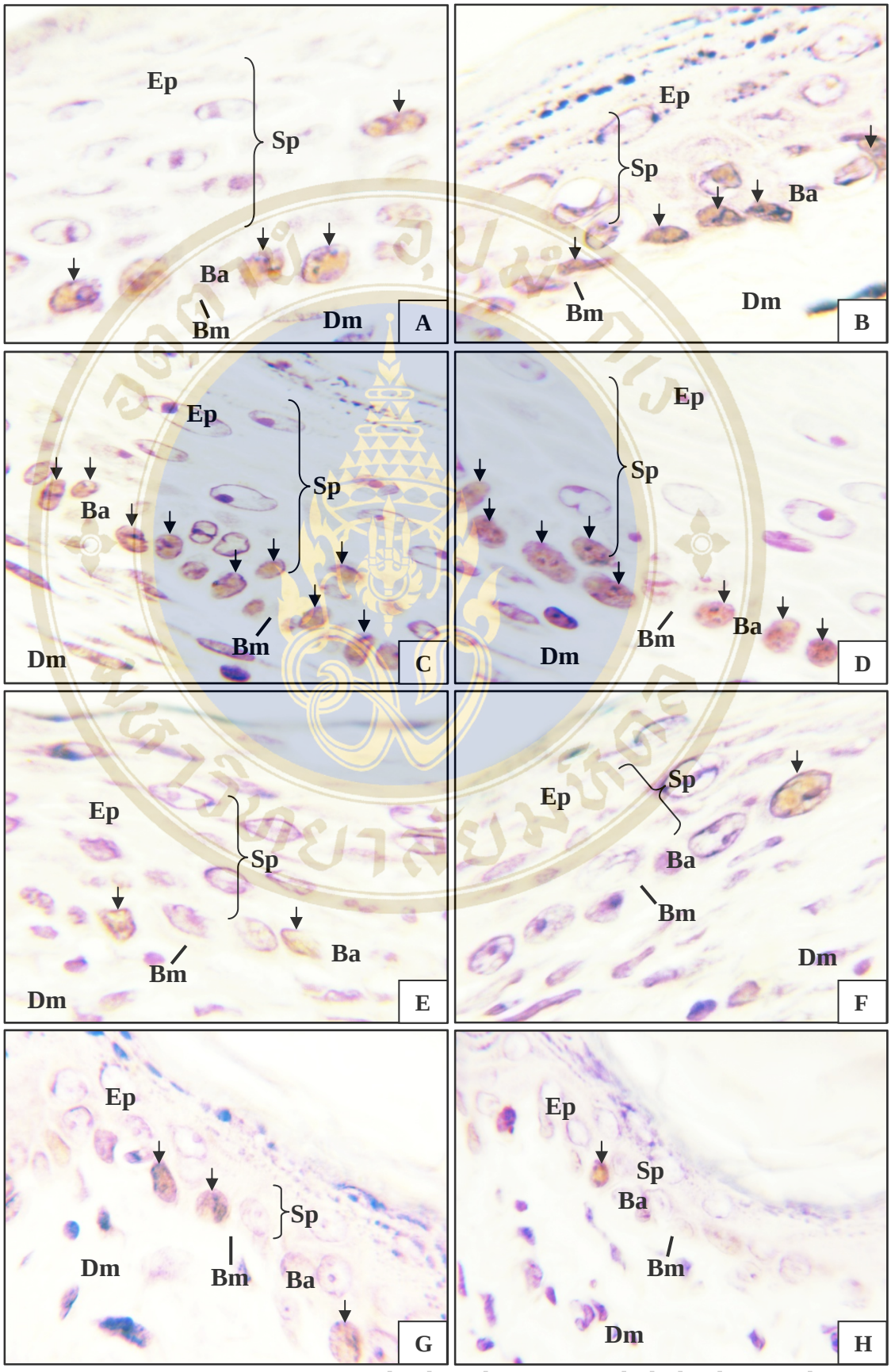
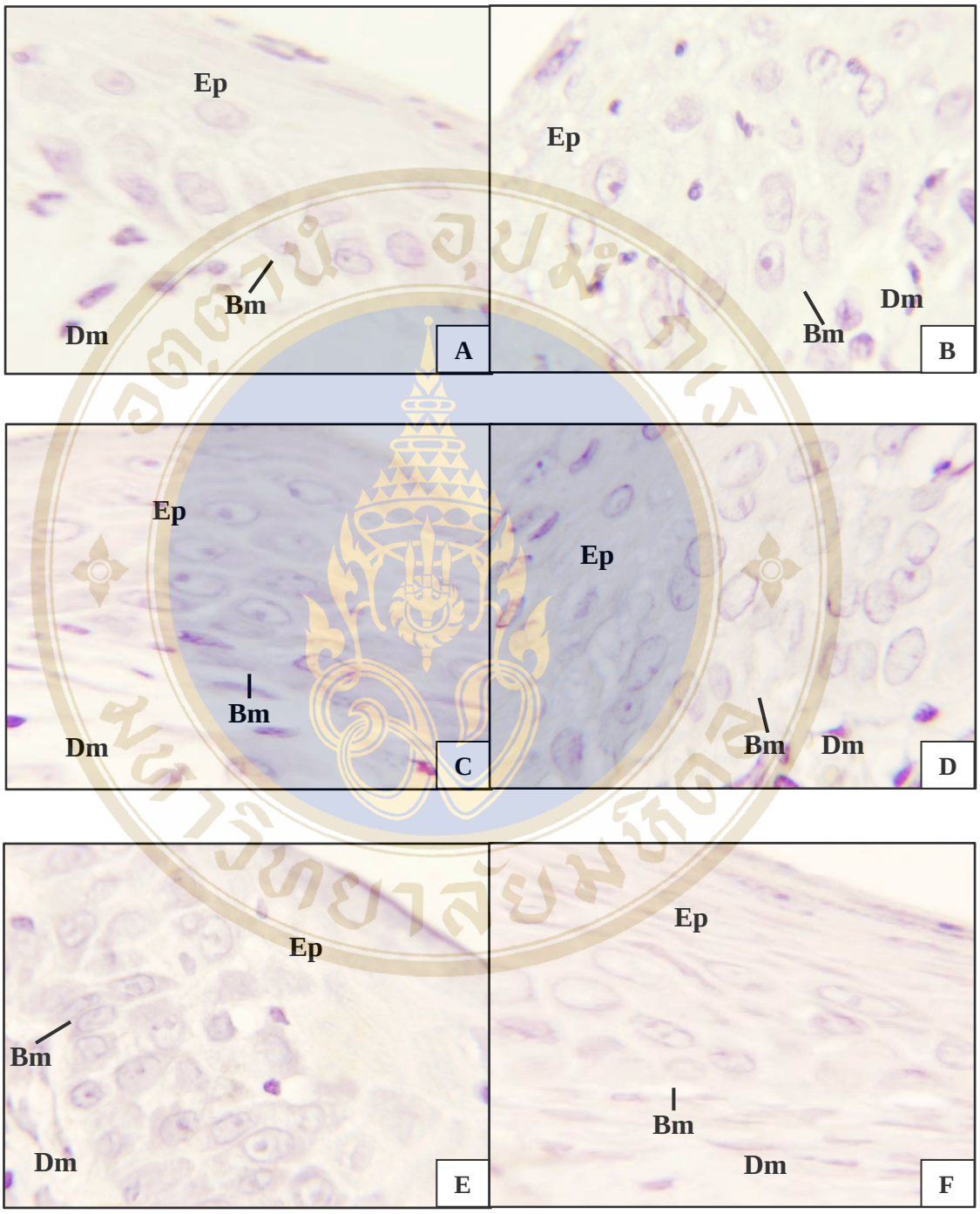


Figure 38 The photomicrographs demonstrate the negative control of the wound tissues in the developing epidermis (Ep) which show no positive golden-brown coloration on day 3 (A), 7 (B), 9 (C), 12 (D), 15 (E) and 18 (F) after dermatotomy respectively. (X100)

Abbreviation : Bm, basement membrane; Dm, dermis

Figure 38



CHAPTER VI
DISCUSSION

Wound healing is one of the most complex biological events after birth (Gillitzer *et al*., 2001). It is a complex process of the replacement of dead tissue by a vital tissue (Rubin *et al.*, 1994). The response of the body to local injury begins very early in the process of inflammation, and result in repair and proliferation. Proliferation is a replacement of injured tissues by parenchymal cells of the same type, sometime leaving no residual trace of the previous injury (Kumar *et al*., 2003). Repair is replacement by connective tissues, which in its permanent state constitutes scar (Menetrey *et al*., 2000). Tissues of mammals consist of three types of cells including permanently dividing-labile cells (e.g. epithelial cells), cells that occasionally go back to the cell cycle-stabile cells (e.g. fibroblast cells) and non-dividing cells (e.g. striated muscle cells) (Kumar *et al*., 2003). The rat skin structure is, in many aspects, similar to human skin. The specific structural characteristics may vary depending on the body region (Marcelo *et al*., 2003). The skin of the dorsum of a normal rat skin is formed by epidermis, dermis and subcutaneous tissue. The epithelial appendages are mostly hair follicles and sebaceous gland. However, no sweat glands were observed in rats (Marcelo *et al*., 2003). Hair follicles can play a role in the process of epithelialization.

The wound healing process involves considerable complex factors. Consequently a detailed evaluation of curative nature of healing material in an inflicted skin wound may require a wide range of observations including gross and microscopic examinations as well as biochemical and pharmacological analyses (Kýlýc *et al*., 2002). However, an experimental design including macroscopic evaluation (appearance and size of the wounds), histological observation (epidermal assessment) and quantitative evaluation of the proliferating cell nuclear antigen (PCNA) positive cells of wound healing process were adopted in this study. According to this current study, the effects of chitin-PAA-GTMAC 1:4, 1:10 and Intrasite^{TM} Gel on the healing of the full-thickness wound were examined in Wistar rats. As indicated,

chitin-PAA-GTMAC was developed as a new biological dressing material from chitin that carboxylic group and quaternary ammonium were added to chitin structure for increased absorption and antibacterial ability to promote wound healing. Intrasite™ Gel is a clear amorphous hydrogel containing a modified carboxymethyl cellulose polymer, propylene glycol and water. It is a commercial dressing product for cavity wounds, extravasation injuries, venous ulcers and decubitus ulcers (Smith & nephew FZE, 2008).

The result of macroscopic study was achieved by investigating the appearance of the wound surfaces. On day 3 after dermatotomy, the three wounds dressed with each experimental materials became hyperemia. These indicated that all the wounds were in the acute inflammatory phase of the wound healing process. The wound dressed with chitin-PAA-GTMAC 1:4 hydrogel appeared pink with great swelling while those with chitin-PAA-GTMAC 1:10 and Intrasite™ Gel were mild swelling as well as those with Intrasite™ Gel were red at the edges of the wound. On day 7, 9, 12, 15 and 18 after dermatotomy, the healing process in the wounds of all groups progressed to proliferative phase of wound healing process. The wounds showed the increase in contraction which leaded to the decrease of the wound size and deepness. The wounds dressed with chitin-PAA-GTMAC 1:4 and 1:10 healed faster than those dressed with Intrasite™ Gel.

Full-thickness cutaneous wounds heal by two independent processes: contraction and epithelialization. Contraction reduces the size of a wound by centripetal movement of the dermis and epidermis that border the defect (Lee *et al*., 1986, 1988; Swaim and Lee, 1987; Swaim and Henderson, 1997). Wound contraction is a major component of second intention wound healing and the center for contraction is granulation tissue. The proportion of wound that heals by contraction varies depending on the properties of the surrounding skin (Swaim *et al.*, 2001).

In the present study, scab appeared on day 7 to day 15 after dermatotomy. It has been indicated that the healing of the wound can be promoted under a moist wound condition (Atiyeh *et al*., 2002). Therefore the scabs found in any wounds studied should provide the moist condition.

In the study of the remaining wounds areas, the areas of the wound were calculated using a computer program of an image analyzer which was the same as the

measurement of residual wound area of Roh *et al*., 2006. On day 3 after dermatotomy, the wounds of three experimental material groups showed the highest average percentages of the wounds areas when compared to those of the other experimental days, besides they were more than those of day 0 after dermatotomy which was presumed to be 100 at start. This indicated that all the wounds were in acute inflammatory phase of the wound healing process corresponding to the results of the macroscopic observation that revealed the hyperemia. Especially, the wounds dressed with chitin-PAA-GTMAC 1:4 had the highest average percentages of the remaining wound areas and followed by the progressive decrease in size in the wounds of the later groups. Until on day 7 and day 9 their wound size became smallest compared to those of chitin-PAA-GTMAC 1:10 and Intrasite™ Gel. These signified that the wounds dressed with chitin-PAA-GTMAC 1:4 had the highest acceleration ability to healing of the wound during the first three experimental days (day 3, 7 and 9 after dermatotomy). While in the last three experimental days (day 12, 15 and 18 after dermatotomy) all the wounds of each group continued decreasing in size until they were almost disappeared.

On day 12 after dermatotomy, the size of three wounds were obviously smaller than those of the corresponding wounds on day 9. All three groups revealed the average percentages of remaining wound area which were less than 20 and became nearly 0 on day 15 after dermatotomy. At the last three experimental days, the results showed the highest acceleration ability to healing in the wound dressed with chitin-PAA-GTMAC 1:10 as showed a greater progressive decrease in size than other groups.

According to the results of the remaining wound area on day 18 after dermatotomy, the wounds area of one rat in the groups dressed with chitin-PAA-GTMAC 1:4 and 1:10 showed a larger remaining wound area than those of day 15 after dermatotomy instead of closing to 0. Those errors occurred by the way that the rats scratched themselves at the wound areas making them larger. Consequently, they were excluded from the raw data and the average percentages of remaining wound areas of both groups were 0.00.

All the experimental days, the wounds in Intrasite™ Gel dressing group showed the least progressive decrease in the wound size. This result indicated that Intrasite™ Gel had least acceleration ability to healing process of the wound.

Concerning the microscopic observation, the epithelialization of wound skin is an essential and important process of wound healing. Successful reconstitution of disrupted epithelial covering is not only for cosmetic purposes, but also for functional restoration of skin (Mast and Cohen, 2000). Epithelialization starts hours soon after skin injury. The process is composed of proliferation of the epithelial cells and migrations of these cells to cover the edge of the wound. Consequently, this experiment was designed to evaluate the efficiency of the chitin-PAA-GTMAC 1:4 and 1:10 hydrogel in wound healing compared with those of the commercial product (Intrasite™ Gel). Accordingly, histological study for evaluation of the progressive steps of the epithelialization by mean of hematoxylin and eosin staining and immunoperoxidase staining for evaluation of the PCNA positive cells of developing epidermis from day 3 to day 18 after dermatotomy were performed. Furthermore, it had been reported that the proliferating cell nuclear antigen expression was closely correlated with the migration epithelial cells during the wound healing process (Hall *et al*., 1990; Hergett *et al*., 1991; Kim *et al*., 1997).

The results of the present study expressed that the wounds dressed with chitin-PAA-GTMAC 1:4 and 1:10 showed epithelialization faster than those dressed with Intrasite™ Gel from day 3 to day 18 after dermatotomy. Upon the different aspects of the epithelialization such as epithelial cell growth and keratinization as well as dermal papillae and hair follicle formation on the first three experimental days, the wounds dressed with chitin-PAA-GTMAC 1:4 showed the highest rate and quality of the mentioned aspects followed by those with chitin-PAA-GTMAC 1:10 while those with Intrasite™ Gel were the least. Moreover, on the last three experimental days, chitin-PAA-GTMAC revealed the promoting effect in wound healing efficiency more than those of Intrasite™ Gel. Especially, the wounds dressed with chitin-PAA-GTMAC 1:4 had shown the complete new epithelium with keratin, dermal papillae and hair follicles since day 9 while those with chitin-PAA-GTMAC 1:10 on day 15 after dermatotomy. Furthermore, those with chitin-PAA-GTMAC 1:4 and 1:10 had developed the sebaceous glands since day 15 after dermatotomy. Although the

complete new epithelium with keratin and dermal papillae had been observed in the wounds dressed with Intrasite™ Gel on day 15 already. The hair follicles and sebaceous gland were still disappeared on day 18, the last experimental day. This may arise from the dermis of Intrasite™ Gel dressed wounds were filled with the residues of dressing materials that made the wound tissues weak, resulting in loose and late skin derivative formation.

The results of microscopic study demonstrated the remaining residues of all three experimental materials in all experimental wound areas. On day 3 after dermatotomy, these all three experimental materials of the corresponding wounds showed much more material residues on both the wound surfaces and the edge area. It could be explained that each wound was in the first stage of treatment with each experimental dressing material which had not been entirely absorbed. Not only large quantity amount of material residues were observed, but also the inflammatory cells were revealed around the residues. On day 7, the amount of residues of chitin-PAA-GTMAC 1:4 and 1:10 decreased but those of Intrasite™ Gel, large amount of material residues still remained with loosely arranged tissue beneath the wound surface at the edge areas. On day 9, 12, 15 and 18 after dermatotomy, the amount of residues of chitin-PAA-GTMAC 1:4 and 1:10 and inflammatory cells were progressively decreased until almost disappeared at the same time that the tissues beneath the wound surface were compact and strong. These indicated that chitin–PAA–GTMAC had compatibility and low toxicity for applying to the full-thickness skin wound.

At the same time, the wounds dressed with Intrasite™ Gel were continuously showed many clusters of material residues scattering throughout the wound tissue till the last experimental day. Therefore the Intrasite™ Gel might have the incompatibility with the wound tissue and the residues scattering throughout the wound tissue leaded to the inflammatory cell infiltration and still remained as performed as ever. Although, the external appearance of the wounds dressed with Intrasite™ Gel displayed the progressed healing with the disappear area of the wound which completely covered with new epithelium on day 18 after dermatotomy, the wound tissues beneath the complete new epithelium were developed later than those of other experimental wounds. In all cases, the wounds dressed with Intrasite™ Gel presented the prolonged proliferative phase and the remodeling or maturation phase of

the healing process was delayed. These indicated that Intrasite™ Gel had toxic qualification for apply to the full-thickness skin wound.

The results of histological study by mean of hematoxylin and eosin staining demonstrated the potent antibacterial effect of chitin-PAA-GTMAC that might also play a role in enhancing wound healing and thus promote epithelialization corresponding to the result of the macroscopic observation and the remaining wound area. These observations also corresponded to the previous studies demonstrating the biomedical applications of chitin which had an enhancing effect on epithelialization in the wound healing process (Oshima *et al.*, 1987; Cho *et al.*, 1999; Yusof *et al.*, 2003; Han, 2005).

Furthermore, the efficiency of chitin-PAA-GTMAC dressing material on the epithelialization or growth of keratinocytes was confirmed by utilizing immunoperoxidase staining for PCNA expression in the cells of hypertrophic epithelium. On all the experimental days, the number of PCNA-positive cells in chitin-PAA-GTMAC 1:4 and 1:10 hydrogel dressing groups were significantly more than those of Intrasite™ and normal skin at p-value less than 0.001. Anyhow, on the first three experimental days, the average percentage of the PCNA-positive cells was found highest in the group of chitin-PAA-GTMAC 1:4 while on the last three experimental days those with chitin-PAA-GTMAC 1:10 displayed the highest percentage. However, no difference between those of chitin-PAAGTMAC 1:4 and 1:10 was observed. These results corresponded to the results of the remaining wound areas and histological study by mean of hematoxylin and eosin staining as compared in wound healing efficiency aspects, including accelerative wound healing ability and promotive effect on epithelialization. All acquired results suggested that the wound healing efficiency of chitin-PAA-GTMAC was mediated by the acceleration of epithelialization process via rapid proliferation of epithelial cells. It was demonstrated that the proliferation of epithelial cells could promote epithelialization, which accelerated wound repair process (Lansdown *et al*., 2000).

The results of all aspects of the present study demonstrated that the wound healing efficiency of chitin PAA-GTMAC hydrogel was better than those of Intrasite™ Gel during the whole wound healing period studied. The healing effect was mediated by rapid reduction of the wound size, epithelialization and granulation tissue

formation. Especially, the wound healing efficiency of chitin PAA-GTMAC involved in the acceleration of epithelialization. Based on these findings, chitin-PAA-GTMAC hydrogel exhibited suitable properties of ideal wound dressing such as: (1) increased wound healing rate by showing rapid reduction of the wound size, (2) promoted and accelerated of epithelialization via rapid proliferation of epithelial cells with better pattern of epidermal development and a great number of PCNA positive cells under the routine histological and immunohistochemistry technique respectively. The number of PCNA positive cells was the indicator of the keratinization ability of the wound dressed with each dressing material, (3) promoted and accelerated skin derivatives via rapid formation of hair follicles and sebaceous glands and (4) lower cytotoxicity according to the amount of residues of chitin-PAA-GTMAC 1:4 and 1:10 as well as the inflammatory cells progressively decreased until almost disappeared at the same time that the wound tissues were compact and strong. These indicated that chitin–PAA–GTMAC had compatibility and low toxicity for applying to the full-thickness skin wound.

Chitin-PAA-GTMAC hydrogel is the biomedical application of chitin which is one of the most abundant organic materials in nature. It can be easily prepared from crustaceous shells and developed as a new biological dressing material by adding the carboxylic group and quaternary ammonium to chitin structure in order to improve the absorption and antibacterial ability to promote the wound healing.

Therefore, for this reason, the chitin-PAA-GTMAC hydrogel has biocompatibility and biodegradability which is suitable and useful for wound treatment. Although, the results of the present study could not specify which possessed the highest wound healing efficiency between the two ratios (1:4 and 1:10) but it was clear that the chitin-PAA-GTMAC promoted wound healing process and showed less cytotoxicity than the commercial product (Intrasite™ Gel).

Finally, chitin-PAA-GTMAC will be a choice for manufacturing the new bioactive wound dressing material in Thailand. Since it has potential to substitute the commercial product, Intrasite™ Gel, that must be imported. Moreover, the Intrasite™ Gel is more expensive than hydrogel biomaterials developed for this study. This can help to decrease the expense of the patients and trade deficit in the near future.

CHAPTER VII
CONCLUSION

The study was to evaluate the wound healing efficiency of chitin–PAA–GTMAC hydrogel compared with the commercial product (Intrasite™ Gel) in Wistar rats microscopically by routine histological and immunohistochemical technique. Twenty four female Wistar rats weighing between 250-300 grams and being twelve weeks old were divided into six groups of four each regarding to the time they were sacrificed. Those were 3, 7, 9, 12, 15 and 18 days after deep wound preparation. Three full thickness wounds were prepared by excising the dorsal skin of a rat. Each wound was covered individually with the chitin-PAA-GTMAC 1:4, 1:10 and Intrasite™ Gel. Before sacrifice of all animals of each group, the wounds were visually observed and photographed by a digital camera. The areas of the wounds were calculated using a computer program of an image analysis. After sacrifice, the tissue strip deep into the dermis at the wound area were removed for conventional light microscopy by hematoxylin and eosin staining for the assessment of the epithelialization as well as immunoperoxidase staining method for detection of the PCNA positive cells which were the indicator of the healing process.

All results of the study demonstrated that the wound healing efficiency of chitin PAA-GTMAC hydrogel was better than of Intrasite^{TM} Gel during the whole experimental period. These included the macroscopic study achieved by the appearance of the wound surface and the analyzer of the remaining wound area as well as the microscopic study for the assessment of epithelialization and quantitative analysis of PCNA positive cells of developing epidermis. The appearance and the size of the wound surfaces dressed with each three dressing materials in all experimental days revealed the progressive healing process. The size of the wound areas diminished progressively until disappeared. On day 3 after dermatotomy, all wounds dressed with each experimental material became hyperemia of the inflammatory phase. The wound dressed with chitin-PAA-GTMAC 1:4 hydrogel appeared pink with great swelling

while those with other were mild swelling. In addition, those with Intrasite™ Gel appeared red at the edge areas.

On day 7, 9, 12, 15 and 18 after dermatotomy, the healing process of all groups progressed to proliferative phase of wound healing process which showed the increase in contraction leading to the decrease of the wound size and deepness. However, the wounds dressed with chitin-PAA-GTMAC 1:4 and 1:10 healed faster than those dressed with Intrasite™ Gel. Especially, on day 15 after dermatotomy, the wound dressed with chitin-PAA-GTMAC 1:4 were completely covered with skin and those with chitin-PAA-GTMAC 1:10 were almost complete while those with Intrasite™ Gel still appeared.

Upon the average percentages of remaining wound areas, the chitin-PAA-GTMAC 1:4 dressed wound showed the highest average percentages of remaining wound area on day 3 compared to those of the other experimental materials and other experimental days. Later, they progressively decrease in size until on day 7 and day 9 the wound size became smallest compared to those of chitin-PAA-GTMAC 1:10 and Intrasite™ Gel. While the last three experimental days (day 12, 15 and 18 after dermatotomy) all wounds of each group continued decreasing in size until they were almost disappeared. The average percentages of remaining wound areas of the chitin-PAA-GTMAC 1:4 and 1:10 dressed wounds became close to zero and disappeared on day 15 whereas those of Intrasite™ Gel almost disappeared and the average percentage of remaining wound area was 1.86.

The microscopic study was performed by hematoxylin and eosin staining for the assessment of epithelialization. The wounds dressed with chitin-PAA-GTMAC 1:4 and 1:10 showed the epithelialization faster than those of Intrasite™ Gel since day 3 to day 18 after dermatotomy. The epithelialization was early performed on day 3 after dermatotomy from the edges of the wound surface of those dressed with chitin-PAA-GTMAC 1:4 and 1:10 while those with Intrasite™ Gel on day 7. The wound dressed with chitin-PAA-GTMAC 1:4 showed the complete new epithelium with keratin, dermal papillae and hair follicle as soon as on day 9 after dermatotomy while those with chitin-PAA-GTMAC 1:10 showed complete characteristics on day 15 after dermatotomy. Moreover, those with chitin-PAA-GTMAC 1:4 and 1:10 showed sebaceous glands on day 15 after dermatotomy. Though, the complete new epithelium

with keratin and dermal papillae had been observed in the wounds dressed with Intrasite™ Gel on day 15 after dermatotomy already but on day 18, the last experimental day, the hair follicles and sebaceous gland still disappeared.

Furthermore, the results of the microscopic study demonstrated the residues of all three experimental materials in each corresponding wound. On day 3 after dermatotomy, all three experimental materials showed large amount of material residues on both the wound surfaces and the edges area. Not only the material residues, but also the inflammatory cells were found surrounding the residues. On day 7, 9, 12, 15 and 18 after dermatotomy, the amount of residues of chitin-PAA-GTMAC 1:4 and 1:10 and inflammatory cells progressively decreased until almost disappeared at the same time the wound tissue were compact and strong while those of Intrasite™ Gel still showed large amount of clusters of material residues and inflammatory cells scattering throughout the wound tissue until the last experimental day. Although, the external appearance of the wounds dressed with Intrasite™ Gel displayed progressive healing process, disappearance and complete covering with new epithelium on day 18 after dermatotomy, the wound tissues beneath the complete new epithelium developed slower than those of chitin-PAA-GTMAC 1:4 and 1:10.

Moreover, the microscopic study was performed by immunoperoxidase staining technique for the quantitative analysis of PCNA positive cells. On all the experimental days, the number of PCNA-positive cells in chitin-PAA-GTMAC 1:4 and 1:10 hydrogel dressing groups was significantly greater than those of Intrasite™ and normal skin at p-value less than 0.001. Anyhow, on the first three experimental days, the average percentage of the PCNA-positive cells was highest in the group of chitin-PAA-GTMAC 1:4 while on the last three experimental days those with chitin-PAA-GTMAC 1:10 displayed the highest percentage. However, no significant difference between those of chitin-PAAGTMAC 1:4 and 1:10 was observed.

The results of all aspects of the present study suggested that the wound healing efficiency of chitin PAA-GTMAC hydrogel was better than those of Intrasite™ Gel. It had biocompatibility, biodegradability and promoted wound healing process as well as less cytotoxicity than the commercial product, Intrasite™ Gel. Therefore it was suitable and useful for wound treatment. In the near future, chitin-PAA-GTMAC will be a choice for manufacturing the new bioactive wound dressing material in Thailand.

REFERENCES

1. Alibaba. Fresh_shrimp [Online]. 2006 [cited 2009 Feb 18]; Available from: URL: http://www.goldenhover.en.alibaba.com
2. Atiyeh BS, Ioannovich J, Al-Amm CA, El-Musa KA. Management of acute and chronic open wounds: the importance of moist environment in optimal wound healing. Curr Pharm Biotechnol 2002; 3(3): 179-195.
3. Bale S, Banks V, Haglestein S, Harding K. A comparison of two amorphous hydrogels in the debridement of pressure sores. Journal of Wound Care 1998; 7(2): 65-68.
4. Bancroft JD, Stevens A. Theory and Practice of Histological Techniques. 4th ed, New York: Churchill Livingstone Inc.1996; 36: 102-729.
5. Beaulieu C. How to extract chitin from the crustacean? [Online]. 2008 [cited 2009 Feb 17]; Available from: URL: http://www.plasticstrends.net
6. Blackwell J, Gardner KH, Kolpak FJ, Minke R, Classey WB. Refinement of cellulose and chitin structures.ACS Symp Ser 1980; 14: 315-34.
7. Bowman GD, O'Donnell M, Kuriyan J. Structural analysis of a eukaryotic sliding DNA clamp–clamp loader complex. Nature 2004; 429: 724.
8. Buf. Squid [Online]. 2006 Mar 6 [cited 2009 Feb 17]; Available from: URL:http://www.khonbaakpae.com
9. Campbell NA. Biology. 4th ed. New Work: Benjamin Cummings 1996; 69.
10. Chen XG, Wang Z, Liu WS, Park HJ. The effect of carboxymethyl-chitosan on proliferation and collagen secretion of normal and keloid skin fibroblasts. Biomaterials 2002; 23: 4609-14
11. Chitin: A Natural Product for the 21st Century [Online].2008 [cited 2008 Dec 2]; Available from: URL:http://www.virilityhealth.com/fat-complex-report.php
12. Cho YW, Cho YN, Chung SH, Yoo G, Ko SW. Water-soluble chitin as a wound healing accelerate. Biomaterials 1990; 20: 2139-45.

13. Dada.Insect [Online]. 2006 Apr 26 [cited 2008 Sep 11]; Available from: URL:http://www.bloggang.com
14. Deng CM, Lan-Zhen He, Ming Zhao, Dan Yang and Yi Liu. Biological properties of the chitosan-gelatin sponge wound dressing. Carbohydrate polymers 2007; 69(3): 583-589.
15. Flanagan M. The efficacy of a hydrogel in the treatment of wounds with non viable tissue. Journal of Wound Care 1995; 4(6): 264-7.
16. Gillitzer R, Goebeler M. Chemokines in cutaneous wound healing. J Leukoc Biol 2001:69: 513-521
17. Goosen MFA. Applications of chitin, chitosan. Pennsylvania: Technomic Publishing; 1997.
18. Hall PA, Levison DA, Woods AL, Yu CC, Kellock DB, Watkins JA, et al. Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. J Pathol 1990; 162: 285-294.
19. Hempel I, Hempel G. Field observations on the developmental ascent of larval Euphausia superba (Crustacea). Polar Biol 1986; 6: 121-6.
20. Hergott GJ, Kalnins VI. Expression of proliferating cell nuclear antigen in migrating retinal pigment epithelial cells during wound healing in organ culture. Exp Cell Res 1991; 195: 307-314.
21. Hong SR, Lee SJ, Shim JW, Choi YS, Lee YM, Song KW, et al. Study on gelatin containing artificial skin IV: a comparative study on the effect of antibiotic and EGF on cell proliferation during epidermal healing. Biomaterials 2001; 22: 2777-2783.
22. Jang MK, Kong BG, Jeong YI, Lee CH, Nah JW. Physicochemical characterization of α-chitin, β-chitin and γ-chitin separated from natural resources. J Polym Chem 2004; 42: 3423-32
23. Jones V, Milton T. When and how to use hydrogels. NT Plus 2000; 96(23): 3-4.
24. Kelman Z, Hurwitz J. Protein-PCNA interactions: a DNA-scanning mechanism? Trends Biol. Sci 1998; 23: 236–8.

25. Kim JS, McKinnis VS, Adams K, White SR. Proliferation and repair of guinea pig tracheal epithelium after neuropeptide depletion and injury in vivo. Am. J. Physiol. 273 Lung Cell.Mol. Physiol 1997; 17: L1235–L1241.

26. Kumar V, Cotran RZ, Robbins SL. Basic Pathology. 7th Ed. Saunders, Philadelphia, London, Toronto, Montreal, Sydney, Tokyo; 2003. p. 873.

27. Kurita K. Chitin and Chitosan: Functional biopolymers from marine crustaceans. Marine Biotech 2006; 8: 203-26.

28. Kurita K. Controlled functionalization of the polysaccharide chitin. Prog Polym Sci 2001; 26(9): 1921–71.

29. Kurita K, Tomita K, Tada T, Shigeru I, Nishimura SI, Shimoda K. Squidchitin as potential alternative chitin source: deacetylation behavior and characteristic properties. J Polym Sci Polym Chem 1993; 31: 485–91.

30. Kýlýc S, Timurkan N, Ünsaldý S, Günay C, Ýstek Ö and Yýlmaz B. Comparison of the effect of some wound healing material on full thickness skin wound in rabbits. Turk: J Anim Sci 2002; 26: 263-272.

31. Lansdown AB. Calcium: a potential central regulator in wound healing in the skin. Wound Repair Regen 2002; 10(5): 271-85.

32. Lee AH, Swaim SF, McGuire JA, Hughes KS. Effects of chlorhexidine diacetate, povidone iodine, and polyhydroxydine on wound healing in dogs. J Am Anim Hosp Assoc 1986; 24: 77–84.

33. Lee AH, Swaim SF, Yang ST, Wilken LO, Miller DP, Wilt GR, Hughes KS. The effects of petrolatum, polyethylene glycol, nitrofurazone and hydroactive dressing on open wound healing. J Am Anim Hosp 1988; 22(6): 443-451.

34. Lloyd LL, Kennedy JF, Methacanon P, Paterson M, Knill CJ. Carbohydrate polymers as wound management aids. Carbohydrate Polymers 1998; 37: 315–22.

35. Marcelo BT, Kemli RB, Cristiano N, Comesu MC. Progressive effects of diabetes mellitus on the skin epithelium of the rat. Int J Morphol 2003; 21**:** 143-48.

36. Mast BA, Cohen K. Normal wound healing. In: B.M. Achauer E, Eriksson B, Guyuron JJ, Coleman III RC, Russell, Vander Kolk CA. (eds.) Plastic Surgery - Indications, Operationsand Outcomes. Mosby, Inc., St. Louis; 2000. p. 37–52.

37. Menetrey J, Kasemkijwattana C, Day CS, Bosch P, Vogt M, Fu FH, Moreland MS, Huard J. Growth factors improve muscle healing in vivo. J Bone Joint Surg Br 2000; 82: 131-139.

38. Ncfisheries. Oster shell [Online]. 2007 [cited 2009 Jan 13]; Available from: URL:http://www.ncfisheries.net/shellfish/recycle1.htm

39. Ohshima Y, Nishino K, Yonekura Y, Kishimoto S, Wakabayash S. Clinical application of Chitin non-woven fabric as wound dressing. Eur J Plast Surg 1987; 10: 66-69.

40. Pudner R. Amorphous hydrogel dressings in wound management [Online]. 2008 [cited 2009 Feb 17]; Available from: URl:http://www.jcn.co.uk/journal. asp

41. Roh Dh, Kang SY, Kim JY, Kwon YB, Kweon HY, Lee KG, et al. Wound healing effect of silk fibroin/alginate-blended sponge in full thickness skin defect of rat. J mater Sci: Mater Med 2006; 17: 547-52.

42. Rinaudo M, Chitin and chitosan: Properties and applications, Prog Polym Sci 2006; 31(7): 603–32.

43. Romo T. Wound healing, skin [Online]. 2008 Feb 18 [cited 2009 Feb 17]; Available from: URL: http://emedicine.medscape.com/article/884594overview

44. Sangjun N, Pilakasiri K, Muensongnoen J, Parichatikanond P, Tanodekaew S. The use of chitin-based dressing in the treatment of deep partial skin thickness wounds: animal study. Chitin Sci 2004; 2: 258-260.

45. School of Anatomy and Human Biology – The University of Western Australia. Blue – Histology – Integumentary System [Online]. 2006 [cite 2009 Aug 10]; Available from: URL:http://www.lab.anhb.uwa.edu.au/mb140/CorePages/Integumentary/Integum.htm

46. Shutler S, Jones M, Thomas S. Management of a fungating breast wound. Journal of Wound Care 1997; 6(5): 213-4.

47. Smith & nephew FZE. Intrasite™ Gel. Trademark of Smith & nephew [Online]. 2008 [cited 2009 Feb 17]; Available from: URL:http://wound.smith-nephew.com/no/Product.asp?NodeId=555

48. Snowden JM. Wound contraction. A quantitative interpretation. Aust J Exp Biol Med Sci 1981; 59: 203–217.

49. Sukihara A, Sugiura K, Morita H, Ninagawa T, Tubouchi K, Tube R, et al. Promotive effects of a silk film on epidermal recovery from full-thickness skin wounds. EBM 2000; 225: 58-64.

50. Surawattanawan P. Chitin-chitosan [Online]. 2003 [cited 2008 Oct 25]; Available from: URL:http://www.gpo.or.th/rdi/html/chitin.html

51. Swaim SF, Henderson RA. Wound dressing materials and topical medication. In: Swaim SF, Henderson RA (eds). Small animal wound management. Lea and Febiger, Philadelphia; 1997. p. 34–52.

52. Swaim SF, Hinkle SH, Bradley DM. Wound contraction: basic and clinical factors. Compend Contin Educ Pract Vet 2001; 23: 20–24.

53. Swaim SF, Lee AH. Topical wound medications: a review. J Am Vet Med Assoc 1987; 190: 1588–1593.

54. Tanodekaew S, Prasitsilp M, Swasdison S, Thavornyutikarn B, Pothsree T, Pateepasen R. Preparation of acrylic grafted chitin for wound dressing application. Biomaterials 2004; 25: 1453–1460.

55. Thomas S, Andrews A. The effect of hydrogel dressings on maggot development. Journal of Wound Care 1999; 8(2): 75-77.

56. Thomas S, Humphreys J, Fear-Price M. The role of moist wound healing in the management of meningococcal skin lesions. Journal of Wound Care 1998; 7(10): 503-7

57. Trudgian J. Investigating the use of Aquaform Hydrogel in wound management. British Journal of Nursing 2000; 9(14): 943-8.

58. Usami Y, Okamoto Y, Takayama T, Shigemasa Y, Minami S. Chitin and chitosan stimulate canine polymorphonuclear cells to release leukotriene B_4 and prostaglandin E_2. J Biomed Mater Res 1998; 42: 517–22.

59. Vidinský B, Gal P, Toporcer T, Longauer F, Lenhardt L, Bobrov N, et al. Histological Study of the First Seven Days of Skin Wound Healing in Rats. Acta Vet. Brno 2006; 75: 197-202.

60. Wikipedia. The free encyclopedia: Chemistry, physical properties and biological function of chitin [Online]. 2008 [cited 2009 Feb 17]; Available from: URL: http://en.wikipedia.org/wiki/File:Chitin_fixed.png

61. Wikipedia. The free encyclopedia: Etymology of chitin [Online]. 2008 [cited 2009Feb 17]; Available from: URL:http://en.wikipedia.org/wiki/File:Chitin_fixed.png

62. Wikipedia. The free encyclopedia: Structure of the chitin molecule [Online]. 2008 [cited 2009Feb 17]; Available from: URL:http://en.wikipedia.org/wiki/File:Chitin_fixed.png

63. Wikipedia. The free encyclopedia: The assembled human DNA clamp, a trimer of the protein PCNA. [Online]. 2009 [cited 2009 Feb 17]; Available from: URL: http://en.wikipedia.org/wiki/File:1axc_tricolor.png

64. Wikipedia. The free encyclopedia: Uses of chitin [Online]. 2008 [cited 2009 Feb 17]; Available from: URL :http://en.wikipedia.org/wiki/File:Chitin_fixed.png

65. Wittaya-areekul S, Prahsarn C. Development and in vitro evaluation of Chitosan -Polysaccharides Composite Wound Dressings, Inter J of Pharm 2006; 139(1-2): 123-8.

66. Wpopp. Blue crab [Online]. 2004 Dec 1 [cited 2009 Feb 17]; Available from: URL:http://www.answers.com/topic/tatoo-food-and-drink

67. Wunneesuthi. Mushroom [Online]. 2006 Aug 11 [cited 2009 Jan 12]; Available from: URL:http://www.shutterfreaks.com/galery2/album225/mushroom

68. Yusof NLBM, Wee A, Lim LY, Khor E. Flexible chitin film as potential wound-dressing materials: wound model studies. Biomed Mater Res: 2003; 66(A): 224-233.

APPENDICES

APPENDIX A
CONVENTIONAL PARAFFIN TECHNIQUE FOR LIGHT MICROSCOPY

1. Fixation

The removed pieces of tissue were fixed in 4% neutral buffered formaldehyde (Appendix B) for 48 hours at room temperature.

2. Washing

All of tissues were washed with neutral buffer three times for 20 minutes each.

3. Processing

Dehydration

This process was the first step of the processing which aqueous tissue fluid had been removed from the tissues by graded series of ethanol. The tissues were immersed first in 80% ethanol then progressed through 90%, 95% and finally to 100% ethanol. (Diagram 1) In this way all the aqueous tissue fluid were removed with little disruption to the tissue due to diffusion currents.

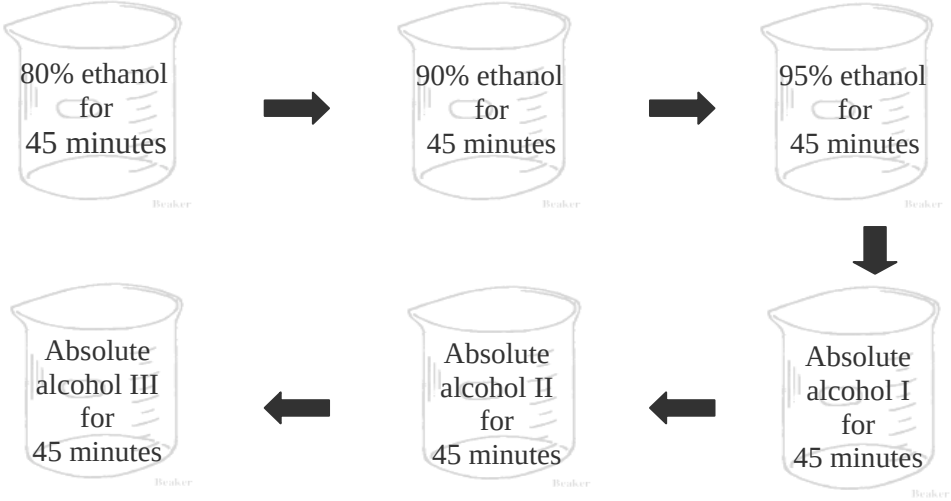


Diagram 1 Dehydration processes

Clearing

The pieces of tissue were now filled with dehydrant, absolute ethanol. This dehydrant were removed from the tissue by clearing agent, xylene.The xylene is appropriate for routine histology schedules. Immersion in xylene must not be prolonged. After clearing the tissue became translucent. (Diagram 2)

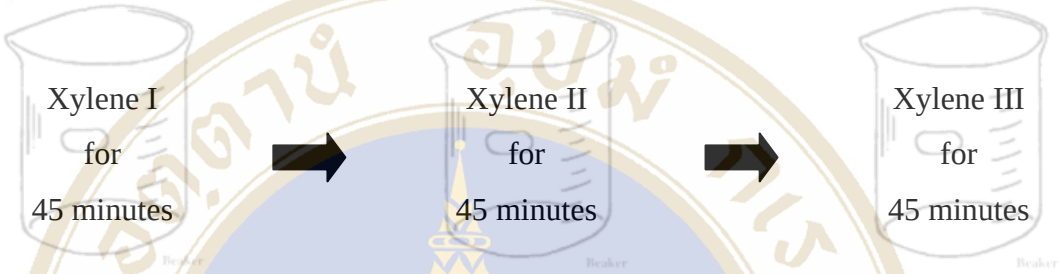


Diagram 2 Clearing processes

Infiltration

The cleared tissues were placed in the melting paraplast in order to replace the clearing agent with the embedding medium. For this study paraplast had been use as the embedding medium. (Diagram 3)

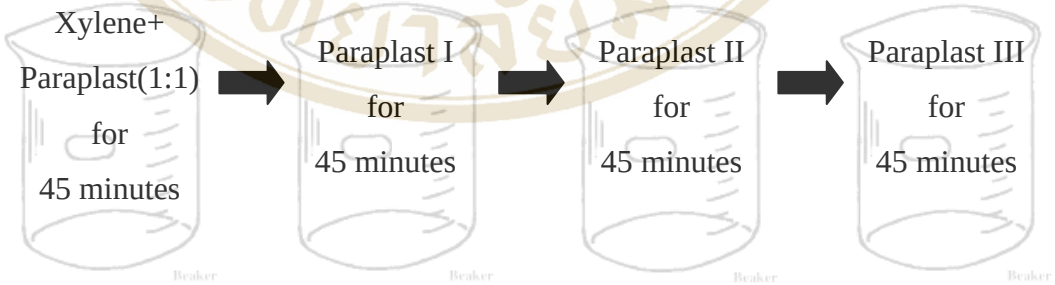


Diagram 3 Infiltration processes

4. Embedding

The infiltrated tissues were placed in the melting paraplast which occupied in the base of the embedding mold. Other melting paraplast was added immediately over the embedded tissue until the mold was full. Then, the mold was placed on the cool plate and after the paraplast had solidified, the embedded tissue block was removed and ready for sectioning.

5. Sectioning with microtome

The embedded tissue blocks were then cut to a thickness of 5 µm with a rotary microtome. (Figure 39) The tissue sections were placed on the glass slides which were coated and then incubated at 60° C for 45 minutes.

Figure 39 Microtome (Leica RM 2135)

6. Hematoxylin and Eosin staining

In order to examine the section, hematoxylin and eosin staining is needed. (Appendix C and Diagram 4)

Diagram 4 Hematoxylin and Eosin staining

APPENDIX B

4% NEUTRAL BUFFERED FORMALDEHYDE (BANCROFT, 1996)

Composition:

40% formaldehyde	100	ml.
Distilled water	90	ml.
Sodium dihydrogen phosphate monohydrate or $NaH_2PO_4 \cdot H_2O$	4	gm.
Disodium hydrogen phosphate anhydrous	6.5	gm.

4% Neutral buffered formaldehyde was prepared, it will be immediately to use.

APPENDIX C
HEMATOXYLIN AND EOSIN STAINING SOLUTION

1. Harris's hematoxylin solution (Bancroft *et al*,1996)

Hematoxylin	2.5	gm.
Absolute ethanol	25	ml.
Ammonium aluminum sulfate	50	gm.
Distilled water	500	ml.
Mercuric oxide	1.25	gm.
Glacial acetic acid	20	ml.

The hematoxylin is dissolved in the absolute alcohol, and is then added to the ammonium aluminum sulfate, which has previously been dissolved in the warm distilled water in a 2-liter flask. The mixture is rapidly brought to boil and the mercuric oxide is then slowly and carefully added. Rapidly cool the mixture by plunging the flask into cold water or into a sink containing chipped ice. When the solution is cold, the acetic acid is added, and the stain ready for immediate use. The glacial acetic acid is optional but its inclusion gives more precise and selective staining of nuclei.

2. Eosin Y Solution (Bancroft *et al*,1996)

<u>Eosin Y Stock Solution (1%):</u>		
Eosin Y	1	gm.
Distilled water	20	ml.
95% Ethanol	80	ml.

Mix to dissolve and store at room temperature.

Eosin Y Working Solution (0.25%):

Eosin Y stock solution	1	part
80% Ethanol	1	part
Glacial acetic acid (concentrated)	1% (of working solution)	

Mix well and store at room temperature.

3. Acid Alcohol Solution (1%)

Hydrochloric acid	0.5	ml.
70% ethanol	50	ml.

Mix well.

4. Ammonia Water Solution (0.2%)

Ammonium hydroxide (concentrated)	2	ml.
Distilled water	1000	ml.

Mix well.

APPENDIX D
IMMUNOPEROXIDASE STAINING TECHNIQUE

1. Sectioning with microtome

The embedded tissue blocks were then cut to a thickness of 5 µm with a rotary microtome. (Figure 39) The tissue sections were placed on the glass slides which were coated with VECTABOND™ reagent and then dried at room temperature.

2. Coating glass slides with Vectabond™ reagent

VECTABOND™ reagents are frequently used to strengthen the bonding of glass fiber to the tissue sections, to promote the adhesion of tissue section to glass substrates.

Coating glass slides protocol

1. Wash slides thoroughly in detergent and rinse in water.
2. Immerse slides in acetone for 5 minutes.
3. Remove slides from acetone.
4. Immerse slides in a freshly prepared 2% solution for VECTABOND™ reagents in acetone for 5 minutes.
5. Remove slides and drain.
6. Eliminate excess reagent by gently dipping them several times over 30 seconds in deionized or distilled water (do not create bubbles).
7. Gentle agitation or tapping of the rack before allowing to dry to decrease water droplets and their resulting spots.
8. Air dry slides thoroughly at room temperature or at 37 °C.
9. Slides can be used immediately after drying, or stored in a box at room temperature.

3. Immunoperoxidase staining procedure (Diagram 5)

All steps are carried out at room temperature in a humidified chamber. Staining dishes or coplin jars may also be used. Apply sufficient volumes of dropping reagents (approximately 50 ml) to cover the sections completely; avoid drying of specimens between each step.

- Preparation of the paraplast-embedded tissue sections for deparaffinizing by immerging in the solutions as follows:
  - Xylene for 5 minutes. (3 times)
  - Absolute alcohol for 10 minutes. (2 times)
  - 95% ethanol for 10 minutes. (2 times)
  - Wash in deionized H_2O for 1 minutes

- After deparaffinizing, the tissue slides were stained by dropping the reagents (Appendix E) as following procedure:
  - 14 minutes in 3% hydrogen peroxide diluted in deionized H_2O to quench endogenous peroxidase activity.
  - Washed in PBS twice for 5 minutes each.
  - Incubated the tissue sections for one hour in 1.5% blocking serum in PBS (Mixing bottle 1).
  - Incubated the sections with primary antibody (dilution; 1:100) for 30 minutes at room temperature in the humidified chamber.
  - Washed the tissue sections with three changes of PBS for 5 minutes each.
  - Incubated the sections for 30 minutes with biotinylated secondary antibody as prepared in mixing bottle 2
  - Washed with three changes of PBS for 5 minutes each.
  - Incubated for 30 minutes with AB enzyme reagent (AB mixing bottle).
  - Washed with three changes of PBS for 5 minutes each.
  - Incubated in 1–3 drops of peroxidase substrate (substrate mixing bottle) for 4 minutes to develop golden–brown precipitation.

- Washed in deionized H_2O for 5 minutes.
- Counterstained in Carazzi's haemotoxylin solution for 1 dip.
- Immediately washed with several changes of distilled H_2O.

- The stained tissue sections were dehydrated by immerging in the solution as follows:
  - Immerged in 95% ethanol twice for 10 seconds each.
  - Immerged in absolute alcohol twice for 10 seconds each.
  - Immerged in xylene three times for 10 seconds each.
- Wiped off excess xylene.
- Immediately added 1 – 2 drops of permanent mounting medium (Resin solution) and covered with a glass coverslip.
- Observed the immunoperoxidase staining tissue sections by the light microscope.
- The stained tissues were photographed by the digital camera attaching to the light microscopy.

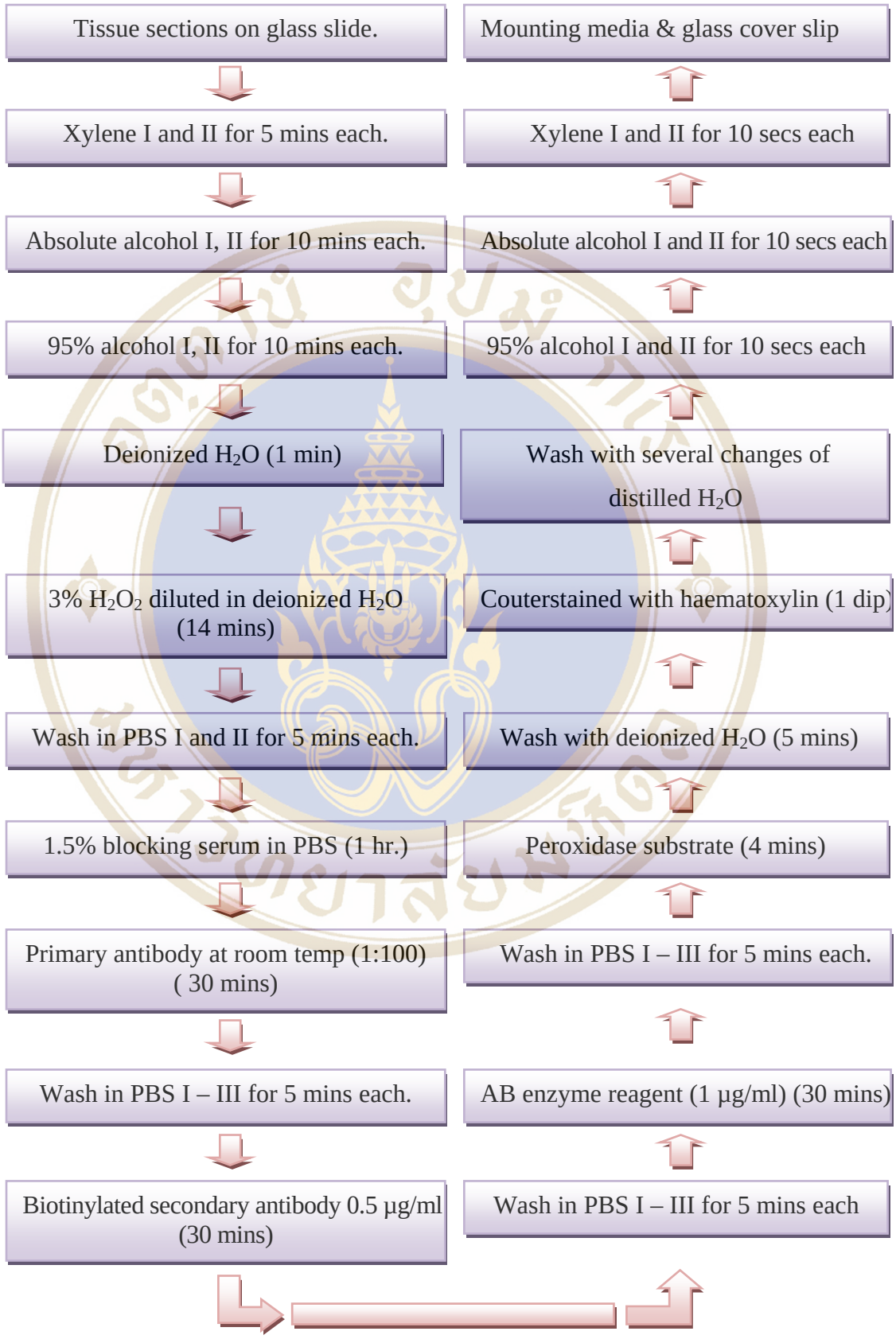


Diagram 5 Immunoperoxidase staining procedure

APPENDIX E
IMMUNOPEROXIDASE STAINING SOLUTION

1. Phosphate Buffer Saline solution; PBS pH 7.2 (Bancroft, 1996)

Preparation of stock solutions

Stock A: 3.12 gm of 0.2 M sodium dihydrogen orthophosphate (mw 156) dissolved in 100 cm^3 of distilled water.

Stock B: 2.83 gm of 0.2 M disodium hydrogen orthophosphate (mw 142) dissolved in 100 cm^3 of distilled water.

Phosphate Buffer Saline pH 7.2 is a mixture between stock A and B. For preparing the PBS 100 cm^3, the 14.0 cm^3 of stock A is mixed with 36.0 cm^3 of stock B, and then make up to 100 cm^3 with distilled water. Finally, the digital pH meter is used to check the pH of the solution and pH 7.2 is adjusted by 0.2 M of sodium dihydrogen orthophosphate in distilled water and 0.2 M disodium hydrogen orthophosphate in distilled water.

2. Preparation of ABC Staining System working solutions (Figure 40)

Use only freshly prepared buffers. Prepare all working solutions in the mixing bottles provided. After preparation, insert the drop dispenser top (supplied in inverted position) into the cap in correct orientation. Place the entire unit on the bottle and twist the cap. The drop dispenser will snap into place. To remove the drop dispenser for refilling, press laterally with thumb until the top snaps off. To prevent evaporation, secure the caps on bottles when not in use. After completion of the staining procedure, working solutions should be discarded and mixing bottles washed with distilled H_2O.

- Blocking serum: In mixing bottle 1 (Blue cap)

  Mix;

  - 75 µl of normal blocking serum stock
  - 5 ml of PBS

- Biotinylated secondary antibody: In mixing bottle 2 (Green cap)

  Mix;

  - 75 µl of normal blocking serum stock
  - 5 ml of PBS
  - 25 µl of biotinylated secondary antibody stock

- AB enzyme reagent: In AB mixing bottle (Purple cap)

  Mix;

  - 50 µl of reagent B (biotinylated HRP)
  - 2.5 ml of PBS

  Mix and let stand for approximately 30 minutes.

- Peroxidase substrate: In substrate mixing bottle (Yellow cap)

  Mix;

  - 1.6 ml of distilled H_2O
  - 5 drops of 10x substrate buffer
  - 1 drop of 50x DAB chromogen
  - 1 drop of 50x peroxidase substrate

Figure 40 Rabbit ABC staining system kit: sc-2018 (Santa Cruz Biotechnology, Inc.) (Goat)

3. Carazzi's haematoxylin solution (Bancroft, 1996)

This is an alum haematoxylin which is chemically ripened by using potassium iodate.

Stock A:	Composition		
	- Haematoxylin	5.0	gm.
	- Glycerol	100	ml.
Stock B:	Composition		
	- Potassium alum	25.0	gm.
	- Distilled water	400	ml.

Carazzi's haematoxylin solution is a mixture between stock A and B as the haematoxylin-alum-glycerol solution. And then, add potassium iodate for 0.1 gm into the haematoxylin-alum-glycerol solution. The staining solution is mixed well and is ready to use. Care might be taken in preparing the haematoxylin solution to avoid overoxidation by not using heat to dissolve the reagents. Finally, filter before use.

BIOGRAPHY

NAME	Miss Patamaporn Molee
DATE OF BIRTH	11 September 1984
PLACE OF BIRTH	Phetchaburi, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 2006 Bachelor of Science (Radiological Technology) Mahidol University, 2009 Master of Science (Anatomy)
RESEARCH GRANTS	Siriraj graduate thesis scholarship, 2008
HOME ADDRESS	15 Moo 7 Banlad, Banlad, Phetchaburi, Thailand 76150 Tel. 087-677-1695 E-mail : junee_pm@hotmail.com
PUBLICATION / PRESENTATION	32nd AAT Annual Conference, Proceedings of the Anatomy Association of Thailand, 29 April-1 May, 2009