การเปลี่ยนแปลงของชั้นคอลลาเจนในผิวหนังปกติและการหายของแผลผ่าตัด
ภายหลังการฉีดโบทูลินัมท็อกซิน เอ เข้าชั้นผิวหนังในสุกร

นางสาวปนัดดา ดาราพงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาศัลยศาสตร์ทางสัตวแพทย์ ภาควิชาศัลยศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

CHANGE OF COLLAGEN IN NORMAL SKIN AND HEALING OF SURGICAL WOUND AFTER INTRADERMAL INJECTION OF BOTULINUM TOXIN A IN PORCINE

Miss Panadda Darapong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Surgery
Department of Veterinary Surgery
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2016

Thesis Title	CHANGE OF COLLAGEN IN NORMAL SKIN AND HEALING OF SURGICAL WOUND AFTER INTRADERMAL INJECTION OF BOTULINUM TOXIN A IN PORCINE
By	Miss Panadda Darapong
Field of Study	Veterinary Surgery
Thesis Advisor	Assistant Professor Sumit Durongphongtorn, D.V.M., D.V.Sc., D.T.B.V.S.
Thesis Co-Advisor	Associate Professor Theerayuth Kaewamatawong, D.V.M., Ph.D., D.T.B.V.P.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, D.V.M., M.S., Ph.D., D.T.B.V.P.)

THESIS COMMITTEE

.....Chairman

(Professor Marissak Kalpravidh, D.V.M., M.S., Ph.D., D.T.B.V.S.)

.....Thesis Advisor

(Assistant Professor Sumit Durongphongtorn, D.V.M., D.V.Sc., D.T.B.V.S.)

.....Thesis Co-Advisor

(Associate Professor Theerayuth Kaewamatawong, D.V.M., Ph.D., D.T.B.V.P.)

.....Examiner

(Assistant Professor Chalika Wangdee, D.V.M., M.Sc., Ph.D., D.T.B.V.S.)

.....External Examiner

(Assistant Professor Supranee Jitpean, D.V.M., Ph.D., D.T.B.V.S.)

ปนัดดา ดาราพงษ์ : การเปลี่ยนแปลงของชั้นคอลลาเจนในผิวหนังปกติและการหายของแผลผ่าตัดภายหลังการฉีดโบทูลินัมท็อกซิน เอ เข้าชั้นผิวหนังในสุกร (CHANGE OF COLLAGEN IN NORMAL SKIN AND HEALING OF SURGICAL WOUND AFTER INTRADERMAL INJECTION OF BOTULINUM TOXIN A IN PORCINE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. สุมิตร ดุรงค์พงษ์ธร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร. ธีระยุทธ แก้วอมตวงศ์, 64 หน้า.

งานวิจัยนี้เป็นการศึกษาผลของการฉีด BTX-A เข้าชั้นผิวหนังในผิวหนังสุกรที่ปกติและผิวหนังสุกรที่เหนี่ยวนำให้เกิดบาดแผลผ่าตัดลึกเต็มชั้นผิวหนัง โดยในการทดลองที่หนึ่ง ทำการฉีด BTX-A เป็นกลุ่มทดลอง และน้ำเกลือเป็นกลุ่มควบคุมแบบสุ่มเลือกบริเวณกลางหลังของสุกร ส่วนในการทดลองที่สอง ทำการฉีด BTX-A เป็นกลุ่มทดลอง และน้ำเกลือเป็นกลุ่มควบคุมแบบสุ่มเลือก บริเวณรอบบาดแผลวงกลมลึกเต็มชั้นผิวหนังที่ได้เตรียมไว้บริเวณหลังของสุกรทดลอง ภายหลังหลังการฉีด 24 ชั่วโมง ผลการศึกษาในการทดลองที่หนึ่งพบว่าผิวหนังของทั้งสองกลุ่มการทดลอง มีการอักเสบและมีเลือดออก หลังจากนั้นอาการดังกล่าวค่อยๆลดลงและหายไปใน 72 ชั่วโมงภายหลังการฉีด จากผลทางจุลพยาธิวิทยา ไม่พบความแตกต่างอย่างมีนัยสำคัญของทั้งสองกลุ่มทดลอง ยกเว้นวันที่ 21 ของการทดลองกลุ่ม BTX-A จะพบเซลล์อักเสบมากกว่าในกลุ่มควบคุมอย่างมีนัยสำคัญ โดยเซลล์หลักที่พบจะเป็นเซลล์แมคโครฟาจและลิมโฟไซต์ ในส่วนของจำนวนหลอดเลือดจะพบว่ากลุ่ม BTX-A มีจำนวนหลอดเลือดเพิ่มขึ้นมากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญในวันที่ 3 ของการทดลอง และมีแนวโน้มที่จะขึ้นในวันที่ 7 14 และ 21 ของการทดลอง ส่วนการย้อมพิเศษ Masson trichrome เพื่อดูความเข้มของการติดสีและการจัดเรียงตัวของคอลลาเจนในชั้นหนังแท้ พบว่าความเข้มของการติดสีในกลุ่มควบคุมและกลุ่มทดลองไม่มีความแตกต่างกัน พบจำนวนของนิวเคลียสที่ให้ผลบวกต่อ KI67 ของเซลล์เนื้อเยื่อเกี่ยวพันในชั้นหนังแท้เพิ่มขึ้นอย่างมีนัยสำคัญในวันที่ 3 ของกลุ่ม BTX-A เมื่อเปรียบเทียบกับกลุ่มควบคุม ส่วนในกลุ่มควบคุมจะมีจำนวนนิวเคลียสที่ให้ผลบวกมากกว่ากลุ่ม BTX-A อย่างมีนัยสำคัญในวันที่ 21 ของการทดลอง พบความเข้มของสีที่ให้ผลบวกต่อการย้อม collagen ชนิดที่ 1 ในกลุ่ม BTX-A ในวันที่ 7 มีความเข้มที่มากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ สำหรับผลการทดลองที่สองพบว่าไม่มีความแตกต่างของขนาดแผลในทั้งสองกลุ่มทดลองและกลุ่มควบคุม พบเพียงสะเก็ดปกคลุมแผลและมีขนาดเล็กลงเรื่อยๆ และหายสนิทในวันที่ 28 จากผลทางจุลพยาธิวิทยา พบว่าในกลุ่ม BTX-A มีแนวโน้มในการพบเม็ดเลือดขาวจำนวนน้อยกว่ากลุ่มควบคุมในช่วง 21 วัน และพบจำนวนที่น้อยกว่าอย่างมีนัยสำคัญในวันที่ 3 และ 14 ของการทดลอง ส่วนการเพิ่มจำนวนของหลอดเลือดใหม่ พบว่ามีการเพิ่มจำนวนของหลอดเลือดในกลุ่มทดลองอย่างมีนัยสำคัญทางสถิติในวันที่ 3 ภายหลังการผ่าตัด ส่วนการย้อมพิเศษ Masson trichrome พบว่าแนวโน้มความเข้มของการติดสีในกลุ่ม BTX-A มากกว่ากลุ่มควบคุมตลอดการทดลองและมีความเข้มสีที่มากกว่าอย่างมีนัยสำคัญในวันที่ 21 ของการทดลอง ส่วนการจัดเรียงตัวของคอลลาเจนในกลุ่มทดลองมีความเป็นระเบียบมากกว่ากลุ่มควบคุมอย่างมีนัยสาคัญทางสถิติในวันที่ 14 และ 21 ของการทดลอง จากผลของทั้งสองการทดลองสรุปได้ว่า การฉีด BTX-A เข้าชั้นผิวหนังของสุกรไม่เป็นอันตรายต่อผิวหนังของสุกร โดยในช่วงแรกของการฉีด BTX-A มีส่วนช่วยลดจำนวนเซลล์อักเสบ เพิ่มจำนวนของหลอดเลือดและเซลล์ไฟโบบลาสต์ร่วมกับมีการสร้างคอลลาเจนชนิดที่ 1 เพิ่มขึ้น นอกจากนี้ BTX-A ยังช่วยในเรื่องการหายของบาดแผล จากการลดจำนวนของเซลล์อักเสบ เพิ่มจำนวนของหลอดเลือด เพิ่มจำนวนของคอลลาเจนและทำให้การจัดเรียงตัวของคอลลาเจนดีขึ้นบริเวณแผลผ่าตัด

ภาควิชา ศัลยศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา ศัลยศาสตร์ทางสัตวแพทย์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5775308831 : MAJOR VETERINARY SURGERY
KEYWORDS: BOTULINUM / COLLAGEN / INTRADERMAL INJECTION / PORCINE / SURGICAL WOUND

PANADDA DARAPONG: CHANGE OF COLLAGEN IN NORMAL SKIN AND HEALING OF SURGICAL WOUND AFTER INTRADERMAL INJECTION OF BOTULINUM TOXIN A IN PORCINE. ADVISOR: ASST. PROF. SUMIT DURONGPHONGTORN, D.V.M., D.V.Sc.,D.T.V.B.S., CO-ADVISOR: ASSOC. PROF. THEERAYUTH KAEWAMATAWONG, D.V.M., Ph.D., D.T.B.V.P., 64 pp.

This study was designed to study the effect BTX-A in the normal skin and healing process of full thickness wound after BTX-A intradermal injection in the pig model. In the 1st experiment, both sides of the dorsal region of pig were randomly injected with BTX-A as the treatment group and normal saline as the control group. In the 2nd experiment, round full-thickness wounds were performed at the skin on both sides of the dorsal region of pig and injected with BTX-A around the wound as the treatment group or normal saline as the control group. From the 1St experiment, the skins of both control and BTX-A groups showed inflammation and hemorrhage at the injection site. Severity of the lesions was decreased gradually until 72 hours post injection. For the histological evaluation, there was no difference of the amount of increased inflammatory cells, mainly macrophages and lymphocytes between the BTX-A and control groups. However, 21 days at post injection, in the BTX-A group of the increased inflammatory cells was significantly more than the control group. The increased of blood vessel in the BTX-A group was significantly more than the control group at day 3 and tended to be great at days 7, 14 and 21. For the mason trichrome staining, there was no significant difference of the intensity of positive blue color and arrangement of collagen between the control and BTX-A groups. Immunohistochemically, the number of Ki67 positive nuclei of fibroblasts of the BTX-A group was significantly more than the control group and the BTX-A groups on the 3rd day post-injection day but in reverse on the 21st post-injection days. The color intensity of collagen type I of the BTX-A group was greater than the control group on the 7th post-injection days.

In the 2nd experiment, wound of the BTX-A and control groups had no significant difference of wound size at all time points. For the histological evaluation, the BTX-A groups had significantly less infiltration of inflammatory cells than the control group on the 3th and 14th post-operative day. The increased number of blood vessels of the control group was more than the BTX-A group on the 3rd post-operative day. For the mason trichrome staining, the intensity of positive blue color of collagen in the BTX-A group was significantly greater than the control group on the 21st post-operative days. The BTX-A group had more order arrangement of the collagen than the control group on the 14th and 21st post-operative days. In conclusion, the intradermal injection of the BTX-A is not harmful to the skin in pig model. Injection of BTX-A is advantageous in the early stages after injection. It could reduce inflammation and increases blood vessels, fibroblast proliferation and collagen type I synthesis. Moreover BTX-A could promote wound healing process by reducing inflammatory cells, increasing vessel proliferation and collagen, and inducing order arrangement of the collagen in the wound and adjacent area.

Department: Veterinary Surgery
Field of Study: Veterinary Surgery
Academic Year: 2016

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the Graduate School Thesis Grant 2016 of Chulalongkorn university fund for supporting and giving me a chance to study in this honorable institute. I would like to acknowledge the following individuals who encouraged, inspired, supported and assisted me, and sacrificed themselves to help my pursuit of a higher education. I would like to express my deepest gratitude to my advisor, Assist. Prof. Dr. Sumit Durongphongtorn for his excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research. I would like to thank my kind co-advisors, Assist. Prof. Pasakorn Brikshavana and Assoc. Prof. Dr. Theerayuth Kaewamatawong for guiding my research and helping me to develop my background in surgery and pathology. I would like to thank all teachers in the Department of Surgery, Faculty of Veterinary Science, Chulalongkorn University, especially Prof. Dr. Marissak Kalpravidh for a lot of advices to develop my thesis. I would like to thank Assist. Prof. Dr. Supranee jitpean for her valuable comments. I would like to thank CULAC staffs, who helped me take care the pigs during the research. I would like to thank Mr. Wissanu Prasertsom from Medicare Health Group helping and giving me the suggestions in the laboratory. Many thanks are extended to my beloved friends, who studied together during the master's degree program for helped me in the research. My research would not have been possible without their helps. Importantly, I would like to thank my parents, who always support and encourage me with their best wishes. Finally, I would like to thank my partner, Mr. Kristsayam Kantangen who is always there, cheering me up and standing by me through both the good and bad times.

CONTENTS

Page

Page

Page

Page

List of tables

List of figures

CHARPTER I

INTRODUCTION

1.1 Importance and rationale

Clostridium botulinum, a gram-positive, rod-shaped anaerobic bacterium produces botulinum toxin (BTX or Botox), a neurotoxin substance causing the food related poisoning called botulism. Seven immunologically distinct antigenic subtypes of BTX have been identified: A, B, C1, D, E, F and G (Simpson, 1986). These components have a high affinity for uptake by cholinergic neurons. The therapeutic benefits derived from a local injection of a BTX. BTX-A preparation are based on site-specific for example subcutaneous, intramuscular and the face delivery. Toxin causes show temporary chemo-denervation and the loss or reduction of neuronal activity at the target organ (e.g. muscle, glands) with minimal risks of systemic adverse effects while used in appropriate dose. However, there was a report of the severe systemic adverse reaction such as paralysis and respiratory failure. BTX is a presynaptic neuromuscular blocking agent inducing selective and reversible muscle weakness up to several months when injected intramuscularly in minute quantities (Thomas et al., 2003). In the early 1980s, botulism toxin type A (BTX-A) was first introduced for treatment of disorders as strabismus and blepharospasm (Scott, 1980; Scott et al., 1985). The BTX-A is available for clinical use in many countries. For more than 20 years, the application of BTX-A has been proved to be safe and effective in the treatment of various disorders, including blepharospasm and hyperfunctional lines (Zhibo et al., 2011). The BTX-A is mostly easy to produce in culture and was the first one obtained in a highly purified, stable and crystal line form (Sander et al., 1986). The BTX-A is commonly used in neurologic, ophthalmic, orthopedic and plastic surgery to alleviate muscle contractions and spasm and also used in cosmetic surgery. The indications are blepharospasm, strabismus, cerebral palsy, dystonia, dysphonia, dystonic, hemi facial spasm, essential head tremor, headaches in stress form, spasmodic torticollis, myoclonus, nystagmus, palatal, stuttering, pelvirectal

spasm, tennis elbow and spastic diplegia (Deniz et al., 2014). The BTX-A induces chemodenervation through its action on presynaptic neurons, preventing the release of acetylcholine (ACh) and that leads to functional denervation of striated muscle for 2–6 months after injection, as a result of muscle fiber atrophy subsequent clinical flaccid paralysis (Fagien, 1999). The cosmetic use of BTX-A in treating wrinkles induced by muscle hyperactivity is widespread (Carruthers and Carruthers, 1998; Rohrich et al., 2003).

The BTX-A has recently been recognized only in dermatology. The use of BTX in dermatology includes the treatment of focal hyperhidrosis, hyperfunctional facial lines as well as paralysis of the anal sphincter in the therapy of anal fissures. In addition about wound healing, BTX-A is a potent neuromodulator that temporarily relaxes muscles and improve wound healing (Bansal et al. 2006). BTX-A is used to reduce the tension around the wound by stimulating temporary denervation for the treatment of hypertrophic scar and skin graft contraction. The use of BTX-A by intramuscular injection continues to expand especially in cosmetic fields.

Collagen is the major extracellular protein of the dermis and form about 80% of the extracellular matrix. It is secreted by the skin fibroblasts. Collagen turn over in the dermis is slow. It is controlled by dermal cellular components, particularly fibroblasts, but also inflammatory cells which are able to respond to particular demands such as skin damage and wound healing. In mature individuals, the majority of dermal collagen is formed by type I (87%) and III (10%) which align into relatively large fibrils. Type IV, V and VII are found in basement membranes. Type V collagen represents about 3% of dermal collagen and is found in nearly all connective tissue.

The use of intradermal injection of BTX-A remains a relatively new technique and is an off-label cosmetic application (Shah AR., 2008). The effect of intradermal injection of BTX-A is still controversial**.** Currently, many off-label cosmetic adaptation of BTX-A are under investigated. Some physicians have observed a face-lifting effect due to the increase of collagen synthesis after intradermal injection of BTX-A to the mid and lower face (Alvarez et al., 2005; Seyler et al., 2008). However, the effect of

intradermal injection of BTX-A is still controversial and no experimental trials have been conducted to support this observation.

For these reasons, the present study aimed to preliminarily study the effect of intradermal injection of BTX-A on normal skin and wound healing process in the pig model.

1.2 Objectives of Study

1. The objective of the 1st experiment was to study the effect of skin including inflammatory reaction, number of blood vessels, collagen arrangement, collagen density, intensity of collagen type I and proliferation index of fibroblasts after intradermal injection of BTX-A compared to the controls in the pig model.

2. The objective of the 2nd experiment was to study healing processes of induced full thickness wound after intradermal injection of BTX-A compared to the controls in the pig model.

1.3 Research Frame

This research was designed to study the effect of skin and healing process of the induced full thickness wound after BTX-A injection in the pig model. BTX-A was injected intradermally in normal skin and surgical full thickness wound. Then the visual assessment and histological examination were evaluated on the 3rd, 7th, 14th, 21st and 28th post-operative days.

1.4 Advantages of study

The advantage of this study was the information supporting the veterinary clinical use.

- Knowledge of the effect of BTX-A in skin of the intradermal injection.
- Knowledge of BTX-A on healing of full thickness wound after BTX-A intradermal injection.

CHAPTER II

REVIEW OF LITERATURES

2.1 Botox and history

The term ‘botulism’ is derived from the Latin botulus (sausage) and it linked to the fact, that foodborne botulism was first discovered after eating a sausage. In 1817, Justinus Kerner, a physician and poet, describes the clinical picture of botulism (Villar et al., 1999). Botulism is a potentially fatal, neuropathic illness occurring (Scott et al., 1973) as infant botulism (Scott, 1981), foodborne botulism and in the form of intestinal colonization and wound infection (Villar et al., 1999). Infant botulism is the most common form of botulism, because of the composition of their intestinal flora. If spores of C. botulinum are ingested, they can bind to the gastric mucosa of children, germinate and produce toxin which causes botulism. Symptoms appear 12 - 36 hours after toxin exposure starting with blurred vision, diplopia due to dilates pupils, dry mouth with dysphagia, asthenia, weakness of the upper extremity, diarrhea or constipation, nausea and vomiting as well as abdominal cramps. These symptoms are followed by paralysis and respiratory failure (Villar et al., 1999). The diagnosis of botulism is based on compatible clinical findings, history of exposure to suspect foods or wound infection and supportive ancillary testing to rule out other causes of neurologic dysfunction mimicking botulism, such as stroke, the Guillain-Barre syndrome and myasthenia gravis.

Wound botulism may occur by colonization of a wound by C. botulinum not invading target cells, but producing toxins that act locally or enter the bloodstream, finally damaging internal organs. Wound botulism was mainly described in association with black tar heroin among injecting drug users especially after intramuscular or subcutaneous injection of the heroin (Green and Fahn, 1993). Next to supportive

care, the only therapy available to ameliorate illness and preventing the potential lethal paralysis is trivalent equine botulinum antitoxin (Tacker et al., 1984).

After World War II, Scott et al investigated the pharmacologic weakness of extra ocular muscle after injection BTX-A in primates in 1973. Based on these finding, BTX-A was used in human in the treatment of strabismus (Scott, 1981). Since then, it has been used successfully in a variety of conditions.

2.2 Biochemistry

Botulinum toxins (BTX) are naturally produced during the autolysis of the anaerobic, gram-positive bacterium botulinum, an organism found in soil and water. Seven immunologically distinct antigenic subtypes of botulinum toxin have been identified: A, B, C1, D, E, F and G (Simpson, 1986), the three-dimensional structure of the 150-kD and the 900-kD botulinum neurotoxin serotype A has been determined (Lacy and Stevens, 1999). The BTX molecule consists of a heavy chain (molecular weight 100 kD) and a light chain (molecular weight 50 kD), which are bound together by heat labile disulfide bonds. The heavy chain with its carboxyl terminal is bound to the nerve terminals at the neuromuscular junction and the light chain is the neurotoxic component and internalized by endocytosis. Neither the heavy chain nor the light chain can exert neurotoxicity independently. BTX is bound to a hemagglutinin and forms a dimer reaching a molecular weight of 900 kD (Das Gupta, 1994).

2.3 Mechanism of action

BTX has 3-step model of the mechanism of action: 1. Binding: The heavy chain portion binds to the cell membrane of the motor nerve via an unidentified high-affinity. 2. Internalizing: After binding, the BTX protein molecule passes through the cell membrane of the motor nerve and into its cytoplasm via a process called

endocytosis. It is here that the light chain of the BTX protein molecule is activated. 3. Blocking: Inside the motor nerve, the light chain of the BTX protein molecule cleaves apart a protein that enables vesicles which store the neurotransmitter ACh to attach to the cell membrane. Cleaving protein prevents these vesicles from fusing with the membrane and prevents the release of ACh into the neuromuscular junction. Thus, nerve impulses that control muscle contractions are blocked decreasing muscle activity. Different types of BTX cleave different parts of the protein complex necessary for blocking ACh vesicle. Two types of the toxin are currently available commercially: botulinum toxin type A (BTX-A) and botulinum toxin B (BTX-B).

For BTX-A, the membrane-bound target protein is SNAP-25. Other serotypes of the BTX act on different neuronal proteins, such as syntaxin or vesicle-associated membrane protein (Maisey et al., 1988). Based on evidence, recovery of the chemodenervation from 3 to 6 months is thought to be due to a turnover of presynaptic molecules and nerve sprouting from the nerve terminal forming a new functional synapse (Printer et al., 1991). Not only the BTX inhibits release of ACh, but also shows in adequate quantities inhibition of noradrenaline, dopamine, serotonin, g-amino-butyrate, glycine and peptide methionine-enkephaline (MacKenzie et al., 1982; Coffield and Simpson, 1994). The BTX molecule cannot cross the blood–brain barrier and therefore has no CNS effects (Coffield and Simpson, 1994). In the skeletal muscle, ACh enters the synapse through a calcium-activated release from the presynaptic membrane. Then it binds to the nicotinic receptors on the postsynaptic muscle membrane. These nicotinic receptors allowed transport of sodium and potassium- ions across the postsynaptic cell membrane when activated by ACh. The entry of sodium causes depolarization of the cell membrane and generation of an endplate potential. The endplate potential initiates propagation of an action potential along the cell membrane of the skeletal muscle cell and

ultimately skeletal muscle contraction. In the muscles, BTX-A causes degrees of flaccid paralysis by blocking ACh, required for muscle contraction, from release at the nerve terminal. The activity of sweat glands was regulated by parasympathetic synapses. ACh release can thus be blocked by BTX-A at the postganglionic parasympathetic synapses of sweats glands thus drastically decreasing the production of sweat (Kao et al., 1976). The effect of BTX-A is reversal. In muscle, on day 28, nerve sprouts mediate a partial restoration and on days 62-91, complete recovery can be demonstrated (De Paiva et al., 1999). The duration of action in sudomotor fibers, however, is longer compares to those in motor nerve ending (Naumann et al., 1998).

2.4 The advantages of BTX-A

BTX-A is clinically used in neurologic, ophthalmic, orthopedic and plastic surgery indications such as extreme strabismus, blepharospasm, spasmodic torticollis, dystonia, dysphonia, hemifacial spasm, cerebral palsy, spastic diplegia, essential head tremor, dystonic tic, headaches in stress form, anal fissure, esophageal sphincter stenosis, tennis elbow, palatal myoclonus, stuttering, pelvirectal spasm and nystagmus. It has been used since 1990 in facial cosmetics and had recently been successful used in treatment hyperhidrosis, hypersialorrhea, Frey syndrome, facial asymmetries and platysma bands (Lang, 2004; Ashford and Tumer-Stokes, 2006; Alderson et al., 1991). It was applied to the pectoral muscles after subpectoral implant placement, which induces reversible denervation and better cosmetic result as well as a painless postoperative period.

In the current context, only (BTX-A) has been studied for scar management in the published literature; hence, the current discussion will be limited to this toxin type by selectively and temporarily paralyzing specific muscles, botulinum toxin can treat spastic disorders or fine lines and wrinkles or more relevant to the topic at hand release tension across wounds and scar.

Mechanism of action in wound healing. The effects of BTX-A on wound healing are currently under investigation. Initial studied concentrated on its paralytic effect. Tension exerted on a wound contributes to scar elevation and the formation of hypertrophic or keloid scar. Over time, wound tension can also lead to spread scars. This BTX-A may improve final cosmetic both pre and intraoperatively by decreasing wound tension are reported successful.

At present the BTX-A has recently been recognized only in dermatology. The use of BTX in dermatology includes the treatment of focal hyperhidrosis, hyperfunctional facial lines as well as paralysis of the anal sphincter in the therapy of anal fissures. Currently, many off-label cosmetic adaptation of BTX-A are under evaluation. Some physicians have observed a face-lifting effect due to increased collagen synthesis after intradermal injection of BTX-A to the mid and lower face. It has been claimed that greater collagen synthesis, lower sebum production and smaller facial pore size (Chang et al., 2008; Shah, 2008). As Kurzen and Schallreuter, 2014 reported, the ACh receptor is not only present on neurons, but can also be found on the surface of melanocytes, keratinocytes and other dermal tissues (Kurzen and Schallreuter, 2004). One may reasonably suspect that an effect might be produced on adjacent tissue after BTX-A injection, but what makes this argument even more interesting is that percutaneous needle pricks themselves have been reported create multiple micro bruises in the dermis and to imitate a complex cascade of growth factors that eventually results in collagen production. In 2012, Sang-Ha et al. studied the potential effect of BTX-A on human dermal fibroblast shows interesting effects of BTX-A on collagen production and degradation of human dermal fibroblast in vitro. BTX-A did not stimulate the proliferation or show toxic effect on human dermal fibroblast. Levels of procollagen type I increased significantly in fibroblast growth in the presence of BTX-A. The BTX-A upregulated the expression of type I collagen and decreased the production of some matrix

metalloproteinase in fibroblast that prevent collagen degradation (Sang-HA et al., 2012). In addition about wound healing, the BTX-A is used to reduce the tension around the wound by stimulating temporary denervation for the treatment of hypertrophic scar and skin graft contraction. In 2009, Byung-Joo et al. studied about the effect of BTX-A on a rat surgical wound model, the result showed that the wounds of the Botox-treated group had a larger wound size, less infiltration of inflammatory cells and less fibrosis, a much greater amount of collagen and a lower expression of Transforming growth factor beta 1 (TGF-B1) than did the control group and concluded that Botox might be used to decrease the fibrosis of a surgical wound without damaging the epithelial growth in situations for which decreased fibrosis is necessary, such as for treating laryngeal, tracheal and nasal stenosis (Byung-Joo et al., 2009). In 2013, Deniz et al studied the effect of BTX-A on the wound and skin graft contraction the result showed a decrease in the amount of the contraction. In the histologic examination, it was observed that inflammation and collagen amount was higher, and the arrangement of the collagen was different in the groups receiving BTX-A injection. From the previous researches, the studies were on changes of collagen after injection of BTX-A in small experimental animals and in vitro, but not in large experimental animals such as pigs

2.5 Formulations

Currently, three formations of BTX-A approved by the Food and Drug Administration (FDA) are commercially available in the United States: onabotulinumtoxin A (Botox,allergan, Irvine, CA), incobotulinum A (Xenomin, Merz Pharma GmBH, Frankfurt, Germany) and abobotulinumtoxin A (Dysport, Medicis/Valeant, Scottsdate, AZ). Additional formulations are available around the world but are not legal for use in the United States.

While all formulations contain 150 kD core neurotoxin, the presence and amount of the nontoxic protein component vary. Thus, onabotulinumtoxin A and

abobotulinumtoxin A are synthesized as protein complexes but differ in composition while incobotulinumtoxin A is formulated without any complexing protein. These variations among products have lead researcher to compare, efficacy and potency. In general, there is minimal difference in clinical effect, despite the various compositions of BTX-A. Familiarly and clinical judgment often determine which product is used.

All formulations are dosed by the 'unit' a measure of BTX-A which has been standardized through in vitro mouse assays. However, because of differences in activity, units are required to achieve the same clinical response as 1 unit of onabotulinumtoxin A, whereas incobotulinumtoxin A are typically do dosed similarly Prior to use, all botulinum toxins have to be reconstituted. Although preservative – free 0.9% sodium chloride.

2.6 Toxicity

Toxicity of the toxin is expressed in units. One unit (U) is defined as the lethal dose of toxin required to kill 50% (LD_{50}) of a group of Swiss Webster female mice weighing 18-20 grams in the aggregate after peritoneal injection. The exact lethal dose has not been experimentally defined in humans. Based on these findings from primate studies, the LD50 in monkeys has been determined to be 39U/kg. The human LD_{50} has been estimated as ranging between 2500 and 3000 U for a 70 kg person (40 U/kg). Deaths caused by over dosage with use of BTX-A have not been reported, and it appears clinically that a maximum dose of less than 400 U per treatment session at 3 month intervals is safe in humans (Regan Thomus, 2010).

2.7 Dermis

The dermis

The dermis is the major structural component of the skin. It provides a matrix supporting structures and secretions which maintain and interact with the epidermis

and its adnexae. These include the connective tissue, blood and lymphatic vessels, nerve and receptors and cellular components. It is an important thermoregulatory and sensory structure and also contributes significantly to body water storage.

Connective tissue

The dermal connective tissue matrix consists mainly of collagen and elastic fibers organized in a coherent pattern, principally bundles of collagen bordered by the elastic fibers. The non-fibrous component consists of the proteoglycan ground substance and certain glycoprotein. The superficial dermis is composed of fine irregularly distributed, loose collagen fiber and a network of fine elastin fiber. Deeper in the dermis the collagen is thicker and denser and the fibres tend to run parallel to the skin surface; the elastin fibres are also thicker but less numerous.

2.7.1 Collagen

Collagen is the major extracellular protein of the dermis and form 80% of the extracellular matrix. The fibers provide elasticity and are also involved in cell migration, adhesion and chemotaxis. Collagen is secreted by the skin fibroblasts. The fibers are very resistant to animal proteases but are broken down by collagenases that are secreted chiefly by fibroblasts. The collagenases are natural metalloendoprotease that require calcium as an activator and zinc as the intrinsic metal ion; they are uniquely able to break down the native collagen triple helix.

Collagen turn over in the dermis is slow. It is controlled by dermal cellular components, particularly fibroblasts, but also inflammatory cells (macrophages, neutrophils, eosinophils, keratinocytes) which are able to respond to particular demands such as skin damage and wound healing. Hydroxyproline, an amino acid that is abundant vital component of collagen, is released during collagen breakdown. Urinary hydroxyproline levels can be used as an indicator of the breakdown in vivo.

In mature individuals, the majority of dermal collagen is formed by type I (87%) and III (10%) which align into relatively large fibrils. Type IV, V and VII are found in

basement membranes. Type V collagen represents about 3% of dermal collagen and is found in nearly all connective tissue.

2.7.2 Elastin fiber

Elastic fibres form a network throughout the dermis and are also present in sheaths on hair follicles and in the walls of the blood and lymphatic vessels. They are composed of two component, elastin and microfibrils. Microfibrillar material in the absence of elastin is called oxytalan. When small amounts of elastin are present it is called elaunin.

Elastin is a covalently cross-linked polypeptide with a very characteristic amino acid composition. Like collagen, it possesses much glycine and also contains hydroxyproline. It is synthesized by fibroblasts and smooth muscle cells. Metabolic turnover is slow but continuous. Degradation is by a variety of elastases including some calcium-dependent metalloenzyme. The microfibrils are composed of type VI collagen and fibrillin

2.7.3 Glycosaminoglycans and proteoglycans

These substances are secreted by fibroblasts. The glycosaminoglycans and proteoglycans form the ground substance, a viscous sol-gel which encompasses and supports other dermal components. The ground substance is composed chiefly of hyaluronic acid and dermatan sulphates. Its degradation and turnover is not well understood but half-lives of 2 to 5 days and 7 to 14 days.

The ground substance appears to be involved in salt and water balance and can bind over 100 times its weight of water. It may also play a part in promoting growth, differentiation and cellular migration.

2.8 Fibroblast

A fibroblast is a type of cell that synthesizes the cellular matrix and collagen the structural framework for animal tissues, and plays a critical role

in wound healing. Fibroblasts are the most common cells of connective tissue in animals.

2.8.1 Information

Fibroblasts and fibrocytes are two states of the same cells, the former being the activated state, the latter the less active state, concerned with maintenance and tissue metabolism. Currently, there is a tendency to call both forms fibroblasts. The suffix "-blast" is used in cellular biology to denote a stem cell or a cell in an activated state of metabolism.

2.8.2 Embryonic origin

Fibroblasts are morphologically heterogeneous with diverse appearances depending on their location and activity. Though morphologically inconspicuous, ectopically transplanted fibroblasts can often retain positional memory of the location and tissue context where they had previously resided, at least over a few generations. This remarkable behavior may lead to discomfort in the rare event that they stagnate there excessively.

2.8.3 Structure and function

The main function of fibroblasts is to maintain the structural integrity of connective tissues by continuously secreting precursors of the extracellular matrix. Fibroblasts secrete the precursors of all the components of the extracellular matrix, primarily the ground substance and a variety of fibers. The composition of the extracellular matrix determines the physical properties of connective tissues.

Like other cells of connective tissue, fibroblasts are derived from primitive mesenchyme. Thus they express the intermediate filament protein vimentin, a feature used as a marker to distinguish their mesodermal origin. However, this test is not specific as epithelial cells cultured in vitro on adherent substratum may also express vimentin after some time.

In certain situations epithelial cells can give rise to fibroblasts, a process called epithelial-mesenchymal transition (EMT). Conversely, fibroblasts in some

situations may give rise to epithelia by undergoing a mesenchymal to epithelial transition (MET) and organizing into a condensed, polarized, laterally connected true epithelial sheet. This process is seen in many developmental situations (e.g. nephron and notochord development), as well as in wound healing and tumor genesis.

2.9 Ki67

Antigen KI-67 also known as Ki-67 or MKI67 is a protein that in humans is encoded by the MKI67 gene (antigen identified by monoclonal antibody Ki-67)

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)), makes it an excellent marker for determining the so-called growth fraction of a given cell population.

2.10 Wound healing

Wound healing is a preferred biologic process that restores tissue continuity after injury. Wound healing begins immediately after injury or incision. The four phases of wound healing are inflammation, debridement, proliferative and remodeling.

Stages of wound healing

2.10.1 Inflammatory phase

Inflammation is a protective tissue response initiated by damage. This phase is a characterized by increase vascular permeability, chemotaxis of circulatory cells, releases of cytokines and growth factors and cell activation (macrophages, neutrophils, lymphocytes and fibroblast). Hemorrhage cleans and fills wounds immediately after injury. Blood vessels constricts for 5 to 10 minutes to limit

hemorrhage, but then dilates and leak fibrinogen and clotting elements into wounds. Vasoconstriction is mediated by catecholamine, serotonin, bradykinin and histamine. The extrinsic coagulation mechanism is activated by thromplastin released from injured cells. Platelets also release potent and growth factors that are necessary in later stages of wound healing. Fibrin and plasma transudates fill wounds and plug lymphatics, localizing inflammation and gluing wound edge together. Fibronectin dimers within the clot become covalently cross-linked to fibrin and to themselves in the presence of activated factor XIII, forming a provisional extracellular matrix. This blood clot formation stabilizes the wound's edges and provides limited wound strength. It also provides an immediate barrier to infection and fluid loss and a substrate for early organization of the wound. Scabs from when the blood clot dries; they protect wound, prevent further hemorrhage and allow healing to progress beneath their surface. Inflammatory mediators cause inflammation that begins immediately after injury and lasts approximately 5 days. White blood cells leaking from blood vessel into wounds imitate the debridement phase.

2.10.2 Debridement phase

An exudate composed of white blood cells, dead tissue and wound fluid forms on wounds during the debridement phase. Chemoattractant stimulate neutrophils and monocytes to appear in wounds (approximately 6 hours and 12 hours after injury, respectively) and initiate debridement. Neutrophils increased number for 2 to 3 days. They prevent infections and debride organism and debris by phagocytosis. Degenerating neutrophils release enzymes and toxic oxygen products that facilitate the breakdown of bacteria, extracellular cells debris and necrotic material and they stimulate monocytes. Monocytes are important for wound healing. Monocytes are major secretory cell synthesizing growth factors that participate in tissue formation and remodeling. Monocytes become macrophages in wounds at 24 to 48 hours. Macrophages secrete collagenases, removing necrotic tissue and foreign

material. They may coalesce and form multinucleated giant cells with phagocytic functions. Macrophages also secrete chemotactic and growth factors. Growth factors can imitate, maintain and coordinate formation of granulation tissue. Chemotactic factors direct macrophages to injured tissue. Macrophages also recruit mesenchymal cells, stimulate angiogenesis and modulate matrix production in wounds. Platelets release growth factors important for fibroblastic activity. Lymphocytes appear later in the debridement phase than neutrophils and macrophages. They secrete soluble factors that may stimulate or inhibit migration and protein synthesis by other cells.

2.10.3 Proliferative phase

The proliferative phase usually begins 3 to 5 days after injury. By this time the phagocytic cells should have cleaned out the wound and disposed of any dead tissue. It is now essential for fibroblasts to migrate into the wound. Fibroblast produce the extracellular material needed for fibrous tissue formation. Fibroblasts are attracted into the wound by growth factors released from macrophages and by chemicals released from damaged matrix. Like phagocytes, fibroblasts are able to actively migrate through but more slowly.

Fibroblasts are necessary for wound healing, they synthesis and secrete collagen and ground substance. Fibroblasts also secrete further growth factors which encourage and control the regeneration of new blood vessels, a process called angiogenesis.

Once in the wound cavity, the fibroblasts secrete collagen strands, these form a three dimensional ‘scaffolding’ through which repair can occur. As soon as 1-2 days after injury, granulation tissue begins to form. Granulation tissue is a combination of fibroblasts, collagen, new capillary loops, new matrix and macrophages. Later is also contains numerous plasma cells. These are derived from B lymphocytes and secrete antibodies. The combination of antibodies and phagocytic cells makes granulation tissue very resistant to infection. Because

circulating blood can be seen through the translucent new tissue, granulation tissue is fragile and bleeds readily because of the new thin walled blood vessels it contains.

Re-epithelialization is also part of the proliferation phase. This refers to the re-growth of epithelial tissue. Viable epidermal cells divide by mitosis and start to migrate over surface of the granulation tissue. Re-epithelialization may develop from the wound edges. Anatomically, epidermis dips down into the hair follicles, into the dermis and even hypodermis. This means there are reverses of epidermal cells in these deeper structures. As results, the epidermis may regenerate from these preserved deep elements. This means that even when the full thickness of the epidermis is loss, full regeneration is still possible.

In wound healing by primary intention re-epithelialization takes place over the granulation tissue but below the scab on the top of the wound, the scab is mostly the residue from the initial blood clot. This scab is very useful as it helps to keep bacteria out of the wound until it can be sealed by the new epithelium. It also protects the new epithelium and granulation tissue from drying out. This is necessary as these new cells and tissues can dry out and so dehydrate. This wound kills the new cells so protect re-epithelialization.

Wound contraction decreases the size of wounds subsequent to fibroblasts, reorganizing collagen in granulation tissue and myofibroblast contraction at the wound edge. Contraction occurs simultaneously with granulation and epithelialization but is independent of epithelialization. Wound contraction involves a complex interaction of cells, extracellular matrix and cytokines. Significant fibroblastic invasion into the wound is necessary for contraction to begin. Centripetal, full-thickness skin edges are pulled inward by contraction and wounds may be noticeably smaller by 5 to 9 days after injury. During wound contraction, the surrounding skin stretches and the wound takes on a stellate appearances.

Contraction progresses at a rate of approximately 0.6 to 0.8 mm/day. Wound contraction stops when wound edges meet, while tension is excessive or when myofibroblasts are inadequate. Wound contraction is limited if skin around wounds is fixed, inelastic or under tension and it is impaired. Contraction can also be impaired by anti-inflammatory steroids, antimicrotubular drugs and local application of smooth muscle relaxants. If wound contraction stops before granulation tissue is covered, epithelialization may continue and cover the wound.

2.10.4 Remodeling phase

Wound strength increase to its maximum level because of changes in the scar during the remodeling phase of wound healing. Wound remodeling begins once collagen has been adequately deposited in wounds about 3 weeks after injury and goes on for years. The cellularity of granulation tissue is reduced as cells die. There is also a reduction in collagen content of the extracellular matrix. Collagen fibers remodel with alteration of their orientation and increased cross-link, which improve wound strength. Fibers orient along lines of stress. Functionally oriented fibers become thicker. Type III collagen gradually decreases and type I collagen increases. Non - functionally oriented collagen fibers are degraded by proteolytic enzymes (matrix metalloproteinases) secreted by macrophages, epithelial cells, endothelial cells and fibroblast within the extracellular matrix. The most rapid gain in wound strength occurs between 7 and 14 days after injury as collagen rapidly accumulates in the wound. Wound gain only about 20% of their final strength in the first 3 weeks after injury. Slower increase in wound strength then occurs but normal tissue strength is never regained in wounds, only 80% original strength may be regained. As the number of capillaries in fibrous tissue declines, the scar becomes paler. Scar also become less cellular, flatten and soften during maturation. Collagen synthesis and lysis occur at the same rate in maturing scars.

2.11 Animal model

The porcine is the suitable model for integument surgery and wound healing study. The porcine skin is commonly used as the skin and wound healing study model in medical experiments, because it has suitable properties such as the mimic physiological function or disease, availability, easy handling, long survival for functioning, fit with commonly used animal housing, and a suitable size for multiple sample. The porcine skin is hairless and has tight attachment between skin and subcutaneous similar to human. In addition, cutaneous blood supply and wound healing characteristics are similar, though the porcine skin is thicker and less vascularity than the human skin (Sullivan et al., 2001).

CHAPTER III

MATERIALS AND METHODS

3.1 Animal

Two healthy female mix breed (Landrace, Large White and Duroc) swine weighing approximately **30** kilograms were used.

3.2 BTX- A preparation

Prior to injection, reconstitute each vacuum-dried vial of 150 kD BTX- A (500 Units vial of DYSPORT® IPSEN UK) with only sterile, preservative –free 0.9% Sodium Chloride Injection, USP, 7 ml. Draw up 7 ml of diluent in appropriate size syringe, and slowly inject the diluent into the vial. Discard the vial if a vacuum does not pull the diluent into the vial. Gently mix BTX-A with the saline by rotating the vial. The resulting concentration will be 70 units/1 ml. Record the date on the space on the label. BTX-A should be administered within 24 hours after reconstitution. During this time period, reconstituted BTX-A should be stored in a refrigerator (2° to 8° C)

3.3 Anesthesia protocol

The pig was withdrawn food and water for 8 hours and 6-12 hours, respectively to prevent aspirate. It was received a complete physical examination and blood work (complete blood counts and blood chemistry) before experimental study. The pig was premeditated with xylazine HCl (2 mg/kg IM), combined with a mixture of tiletamine and zolazepam (Zoletil™, Virbac animal health Inc.) (4 mg/kg IM) and morphine sulphate (0.5 mg/kg IM). General anesthesia was induced with propofol (6-8 mg/kg IV) and maintained with isoflurane 1-3% in oxygen after intubation. Cephalic vein or marginal ear vein was used for the crystalloid fluid (10ml/kg/hr) administration during anesthesia. During recovery, the pig was placed on the soft floor and kept warm with warm air blowing machine. For preventing infection, the pig was received enrofloxacin (Baytril™, Bayer health care,

pharmaceuticals, Germany) 5 mg/kg (PO) pre-operative and post-operatively. To relieved post-operative pain, morphine sulphate 0.5 mg/kg was given (IM) and tramadol HCl (4 mg/kg PO) until the end of the experiment.

3.4 Aseptic protocol

The pig's skin was clipped with hair clipper blade size 40S (0.25mm), scrubbed with 1% chlorhexidine solution and sprayed with 10% povidone iodine and 70% isopropyl alcohol for three times.

3.5 Experiment I: Skin effects after BTX-A ID in normal pig skin

3.5.1 BTX-A injection

Both sides of the dorsal region of the pig was divided into 10 square areas of 3 cm wide and 3 cm long with 5 cm distance apart. Five areas were intradermal injected with 0.05 ml of BTX-A. Other five areas were injected with 0.05 ml of normal saline as control. The observation of effected skins and 6 mm. and full-thickness skin punch biopsies were done at 3, 7, 14, 21 and 28 days after injection (Figure 1, 2).

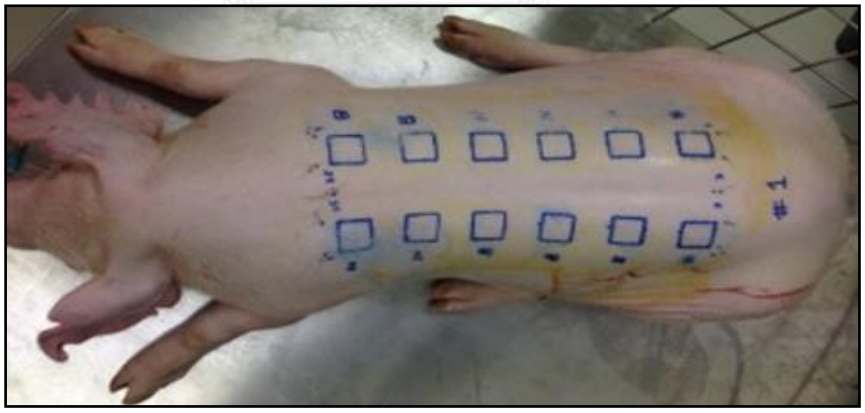


Figure 1. The dorsal region of a pig showing the distinct injected area. There were five areas on each side of the spinal vertebrae.

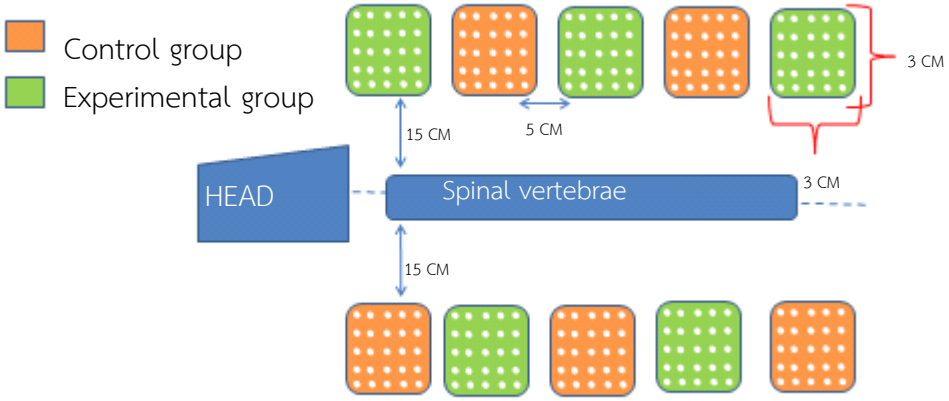


Figure 2. The diagram shows area and location of the control and the experimental groups on the loin area of a pig.

3.5.2 Histopathological evaluations

The pig was sedated and anesthetized according to the anesthetic protocol. The skin from dorsal region of a pig was prepared, cleaned regarding to the aseptic protocol. Full-thickness skin biopsies used a sterile 6 mm disposal skin biopsy punch key were harvested at days 3, 7, 14, 21 and 28 after injection respectively. The skin samples were daily collected 5 samples from each group. The total samples (n) were 50 (Figure 3, 4).

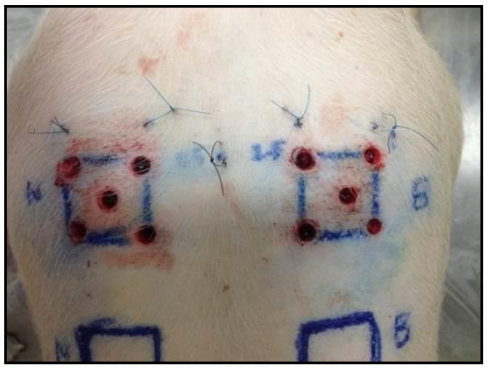


Figure 3. 6 mm skin biopsy which were punched by a sterile 6 mm disposable dermal biopsy punch key at the dorsal region after injection.

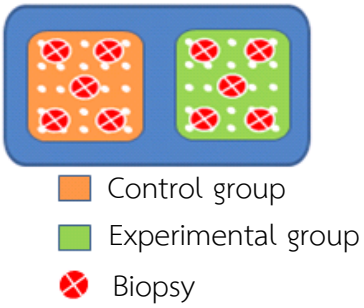


Figure 4. The diagram shows area and location of dermal biopsy punch key at the dorsal region after injection.

The specimens were fixed in formalin, processed and then embedded in paraffin. The paraffin block was cut into 4-6 um thick slices and each slide was stained with hematoxylin-eosin for histologic examination and evaluated the inflammatory response. To evaluate the inflammation response in both groups were counted by a pathologist on high power fields (×400). To evaluate the blood vessel proliferation, we were counted the number of blood vessels on the high power field (×400) and compared the average number of blood vessels in each group. Masson's trichrome was stained for collagen arrangement and density. Immunohistochemistry was stained for positive nucleus fibroblast and collagen type I. Finally the specimens were examined using a light microscope at 40x magnification in blind technique by a pathologist. The inflammatory response, number of blood vessel, collagen arrangement, collagen density, and collagen type I and fibroblast proliferation were assessed.

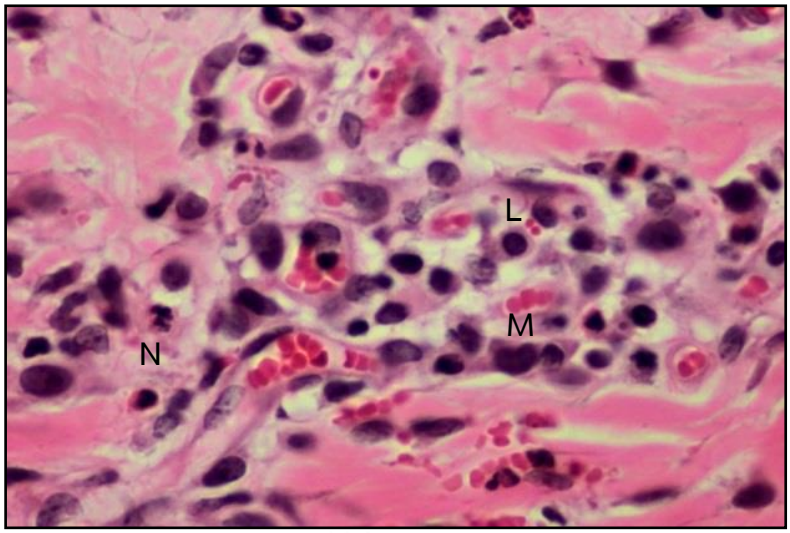


Figure 5. Inflammatory cells in the injected sites that were counted M (Macrophage), N (Neutrophil), and L (Lymphocyte)

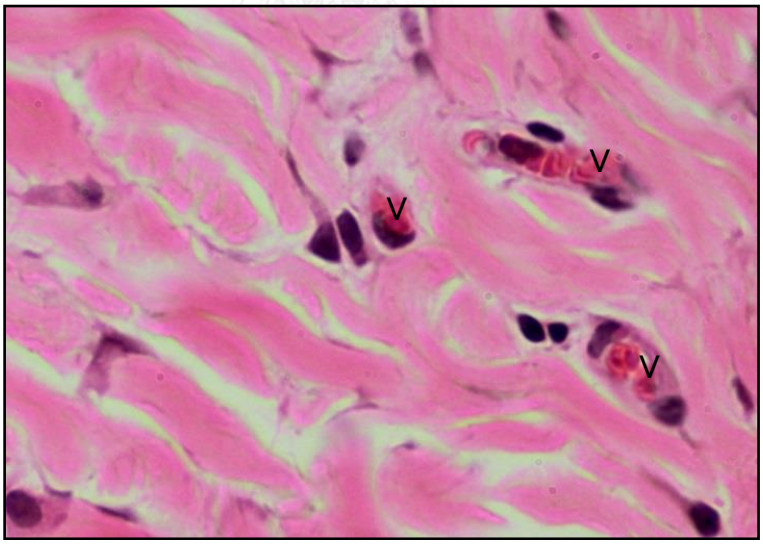


Figure 6. Blood vessels (v) that were counted in the injected sites.

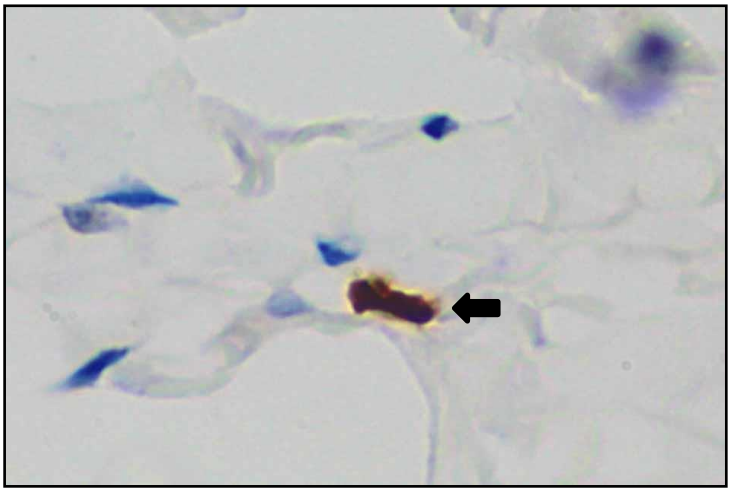


Figure 7. Fibroblast Proliferation that were counted the brown positive staining of fibroblast nucleus (arrow).

The histological observation was designed to estimate the intensity of the inflammation by counting predominant inflammatory cell types. It was quantified by averaging of 50 areas by using 40 x magnifications each group. Predominant cell types were also described, as follows: neutrophils (N), macrophage (M), lymphocytes (L) and eosinophils (E) (Figure 5). The number of vessels was interpreted by and red blood cell within which infiltrated at surrounding tissues (Figure 6). It will be quantified by averaging of 50 areas by using 40 x magnifications each group. The fibroblastic proliferation was quantified by counting a number of positive nucleus fibroblasts which infiltrated at surrounding tissues area. It was quantified by averaging of 50 areas at 40 x magnification in each group. The collagen arrangement and collagen density evaluated by the color intensity and arrangement of the collagen infiltrated in the surrounding tissues, then assessed qualitatively by averaging of 5 areas by using 10 x magnifications in each group. The collagen density grading was described as Grade 0 (absent): No color staining, Grade I (mild): Pale blue staining of the collagen networks, Grade II (moderated): Blue staining of the collagen networks and Grade III (intense): Dark blue staining of the collagen mesh work (Figure 8). The collagen arrangement grading was described as Grade I: unsystematic arrangement:

Grade II moderate connection of the collagen bundles and Grade III: continuously and regularly arrangement of the collagen fibers (Figure 9).

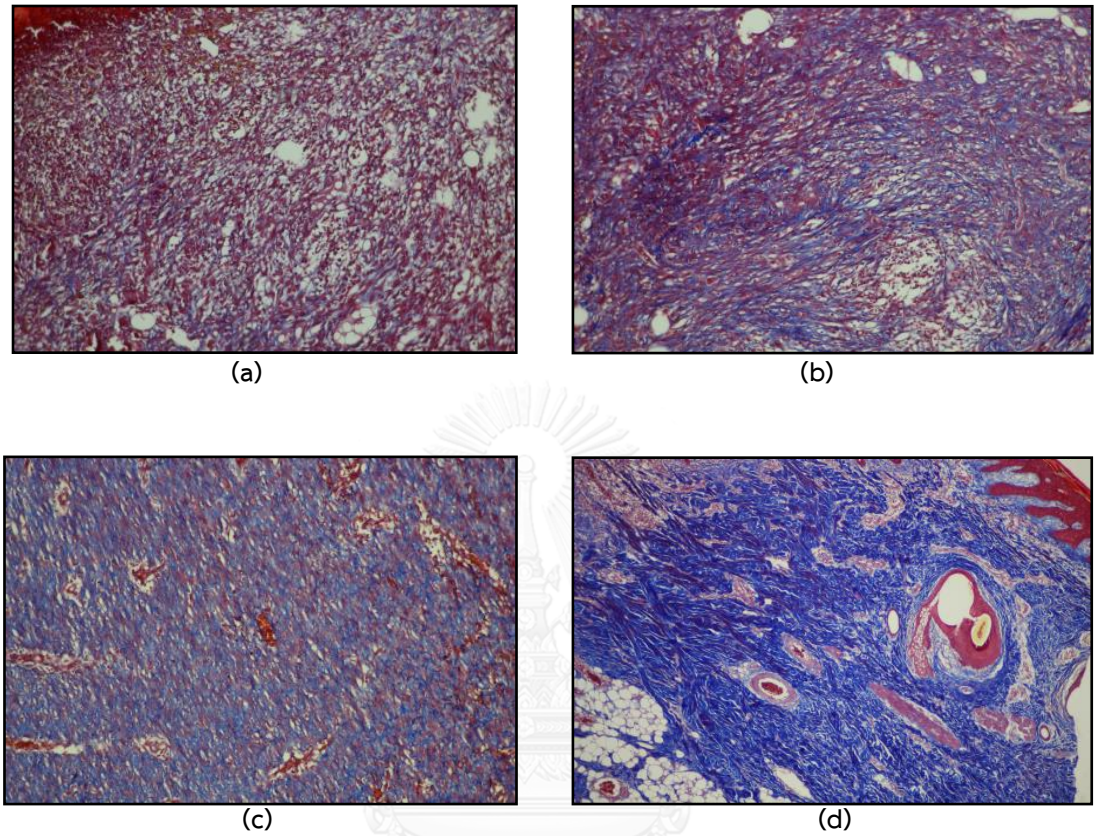
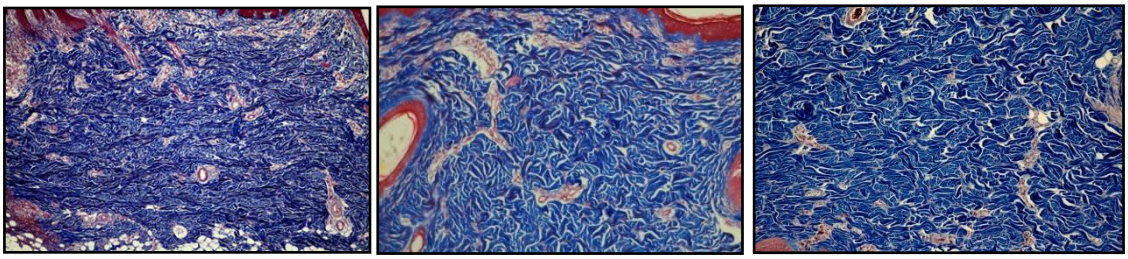


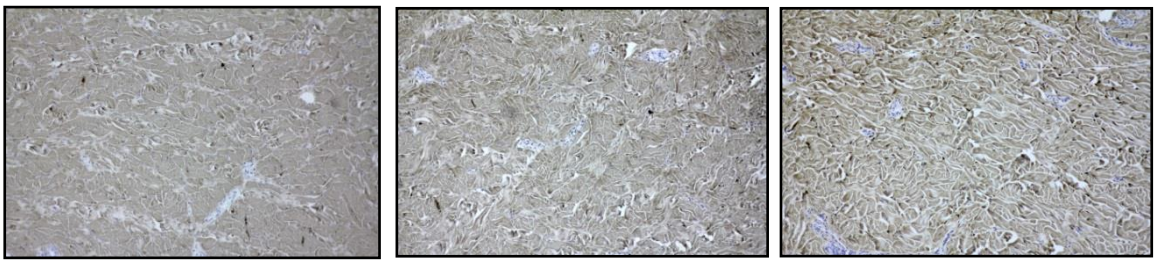
Figure 8. Collagen density grading using color intensity of the collagen fiber; Grade 0 (Absent; No blue color staining) **(a)** Grade I (Mild; Pale blue staining of the collagen networks) **(b)** Grade II (Moderate; blue staining of the collagen networks) **(c)** and Grade III (Intense; Dark blue staining of the collagen networks) **(d),** (10x magnification, Masson's trichrome stain



(a) (b) (c)

Figure 9. Collagen arrangement grading using arrangement of the collagen fiber; Grade I (unsystematic arrangement) **(a)** Grade II (moderate connection of the collagen and regularly arrangement) **(b)** and Grade III (continuously and regularly arrangement of the collagen fibers) **(c),** (10x magnification, Masson's trichrome stain).

The collagen type I evaluated by the color intensity of the collagen infiltrated in the surrounding tissues, Evaluated qualitatively by averaging of 5 areas by using 40 x magnifications in each group. Grade 0 (absent): No color staining, Grade I (mild): Pale brown staining of the collagen networks, Grade II (moderated): Brown staining of the collagen networks and Grade III (intense): Dark brown staining of the collagen networks (Figure 10).



(a) (b) (c)

Figure 10. Collagen Type I grading using color intensity; Grade 0 (Absent, No color staining), Grade I (Mild Pale brown staining of the collagen networks) **(a)** Grade II (Moderated Brown staining of the collagen networks) **(b)** and Grade III (Intense Dark: brown staining of the collagen networks) **(c)** (10x).

3.5.3 Statistical analysis

The variables were intensity of inflammatory cell, blood vessel proliferation collagen arrangement, collagen density, positive nucleus fibroblast and collagen type I.

For each variable on the day 3rd, 7th, 14th, 21st and 28th post-operative day of both control and BTX-A groups were calculated for a central tendency (mean), standard variation (SD) then, compared between the two groups by inferential statistically differences using Wilcoxon rank sum test ($p < 0.05$) by using SPSS for Windows program (version 22.0, IBM, USA).

3.6 Experiment II: Surgically wound model of the experimental animal

3.6.1 Surgical procedures

Pig was sedated and anesthetized according to the anesthetic protocol. The skin from dorsal region of a pig was prepared, cleaned regarding to the aseptic protocol. Ten identical circular areas were prepared on the dorsum of the pig. The diameter of the each wound 2 cm and the distance between the areas were approximately 10 cm. The skin and subcutaneous tissue were then excised, but the deep muscle was preserved at the base of the defect. Five of the wounds were selected at random and it was treated with BTX-A. BTX-A (3.5 U, 0.05 ml) was injected into intradermal away from the wound edges 2 mm. by using 1 ml 27Gx1/2" syringe. As a control, normal saline (0.05 ml) was injected into the other wound (figure 11, 12).

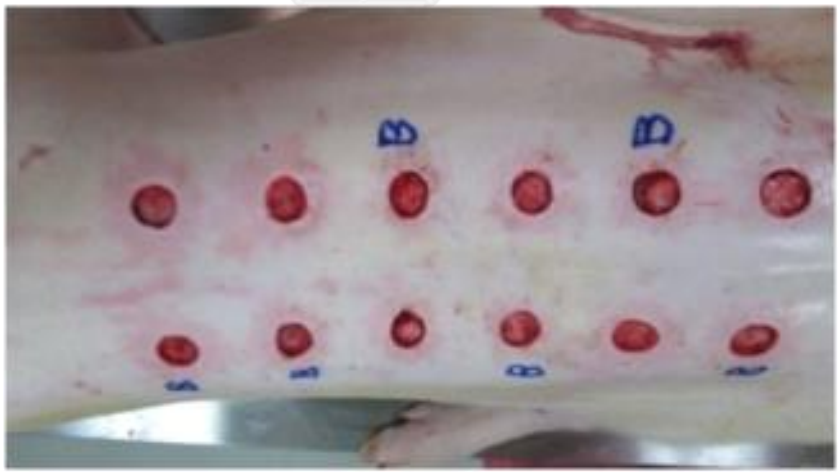


Figure 11. Size and location of the control and the experimental wounds on the loin area of a pig. There were five of 2x2 cm^2 wounds on each side of the spinal vertebrae.

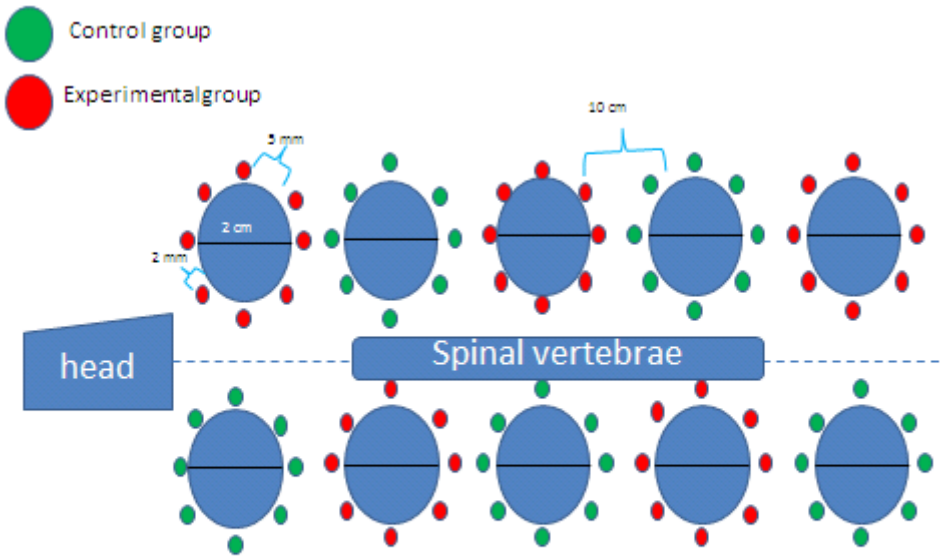


Figure 12. The diagram of Size and location of the control and the experimental wounds on the loin area of a pig. There were five of 2x2 cm2 wounds on each side of the spinal vertebrae.

3.6.2 Assessment of wound size

Visual assessment of the state of the wound healing was done weekly by two veterinarians. They measured the lengths of the major axis and the minor axis of the wound every week with a ruler. The measurement of the area of the wound was calculated by the formula for an ellipse (Kantor and Margolis, 1998).

Area of the wound

= [0.5× the length of the major axis] x [0.5× the length of the minor axis] x [¶]

3.6.3 Histological assessment of wound healing

After surgery, the pig was sedated and anesthetized according to the anesthetic protocol at days 3rd, 7th, 14th, 21st and 28th respectively. Full-thickness skin biopsies 2.5 x 2.5 cm. by using a sterile 15 mm disposal surgical blade were harvested, including the epidermis, dermis and subcutaneous loose tissue with the surrounding normal tissue. The collections were 5 samples from the control group and 5 samples from the experimental group. The total samples (n) were 50 (Figure 13). The biopsy samples were fixed in formalin, processed and then embedded in paraffin. The paraffin blocks were cut into 4-6 μm thick slices and the slides were stained with hematoxylin-eosin for histologic examination. To evaluate the inflammation response in both groups were counted by a pathologist on high power fields (×400). To evaluate the blood vessel proliferation, we were counted the number of blood vessels on the high power field (×400) and we were compared the mean number of new blood vessels in each group. Masson's trichrome was stained for collagen arrangement and collagen density. Finally the specimens were examined using a light microscope at 40x magnification in blind technique by a pathologist. The inflammatory response, number of blood vessel, wound size, collagen arrangement and collagen density were assessed.

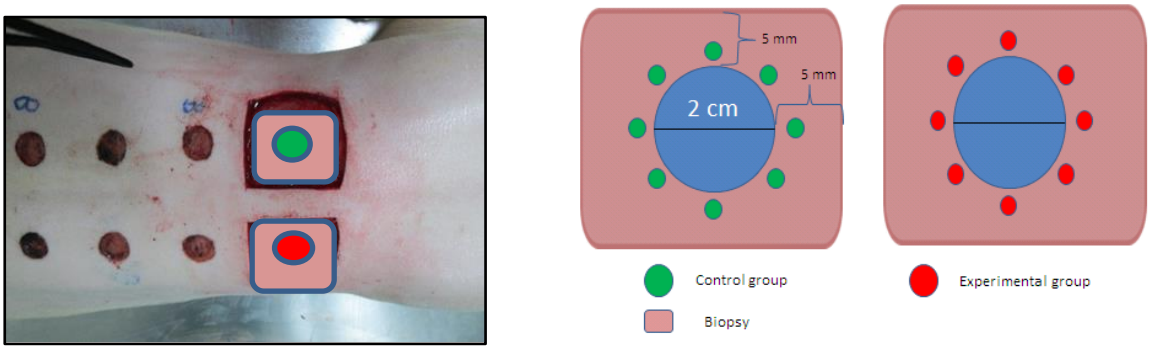


Figure 13. Full-thickness skin biopsies 2.5 x 2.5 cm. by using a sterile 15 mm disposal surgical blade were harvested

Wound Healing of the surgical wound was interpreted by the number of vessels, fibroblast proliferation and intensity of inflammation, collagen arrangement and collagen density. The number of vessels was interpreted by and red blood cell within which infiltrated at surrounding tissues. It will be quantified by averaging of 50 areas by using 40 x magnifications each group. The fibroblastic proliferation was quantified by counting a number of fibroblasts which infiltrated at surrounding tissues area. It was quantified by averaging of 50 areas at 40 x magnification in each group. Finally, the collagen arrangement and collagen density by the color intensity and arrangement of the collagen infiltrated in the surrounding tissues, then assessed qualitatively by averaging of 5 areas by using 40 x magnifications in each group. The collagen density grading was described as Grade 0 (absent): No color staining, Grade I (mild): Pale blue staining of the collagen networks, Grade II (moderated): Blue staining of the collagen networks and Grade III (intense): Dark blue staining of the collagen networks. The collagen arrangement grading was described as Grade I: unsystematic arrangement: Grade II moderate connection of the collagen bundles and Grade III: continuously and regularly arrangement of the collagen fibers

3.6.4 Statically analysis

The variables were wound size, intensity of inflammatory cell, blood vessel proliferation collagen arrangement and collagen density.

For each variable on the day 3rd, 7th, 14th, 21th and 28th post-operative day of both control and BTX-A groups were calculated for a central tendency (mean), standard variation (SD) then, compared between the two groups by inferential statistically differences using Wilcoxon rank sum test ($p < 0.05$) by using SPSS for Windows program (version 22.0, IBM, USA)

CHAPTER IV

RESULT

4.1 Experiment I: Skin effects after BTX-A ID in normal pig skin

4.1.1 Gross appearance

After intradermal injection, skin of both control and BTX-A groups showed redness, hemorrhage and edema immediately. At the 1st day after injection, the skin of both the control and BTX-A groups showed lesser degree of redness, hemorrhage and edema than at Day 0. However both groups showed no remarkable lesion on Day 3 (Figure 14).

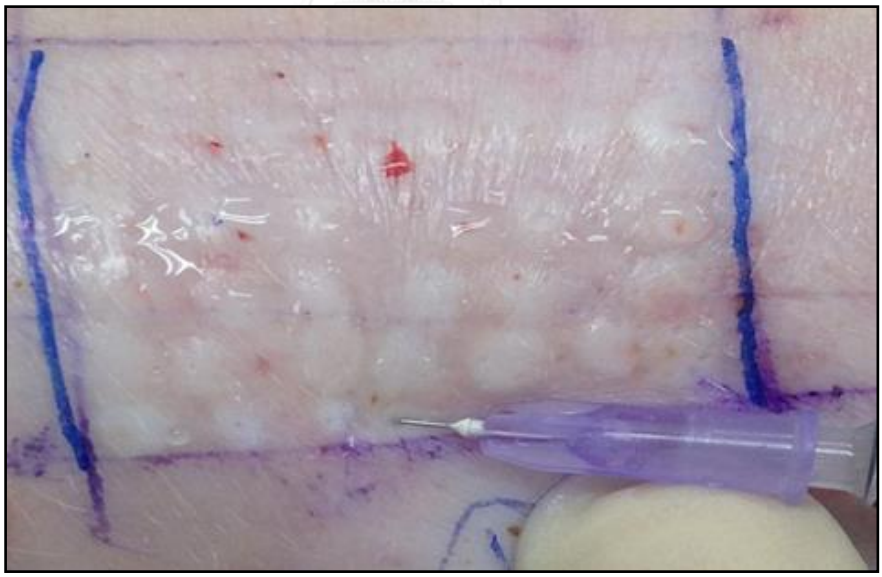


Figure 14 Gross finding of skin after intradermal injection of BTX-A. The skin of both the control and BTX-A groups showed inflammation and hemorrhage at the injection site. The severity of the lesions was decreased gradually until at 72 hours.

4.1.2 Histopathological evaluation

4.1.2.1 The intensity of the inflammation

The control group showed less infiltration of inflammatory cells than the BTX-A group on days 3, 14 and 21. There was statistically significant difference of total inflammatory cells between the control and the BTX-A groups at the 21 post-operative days. ($p<0.05$) (Table 1, Figure 15)

Table 1. The Mean ± standard deviation (SD) and p-value the infiltration of inflammatory cells in the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
3	1.14±1.75	1.16±2.50	0.32
7	2.96±4.96	2.24±4.80	0.26
14	2.64±2.49	3.82±4.03	0.5382
21	7.80±6.50	10.66±5.90	0.01*
28	16.94±11.26	16.30±10.3	0.92

*p-value<0.05

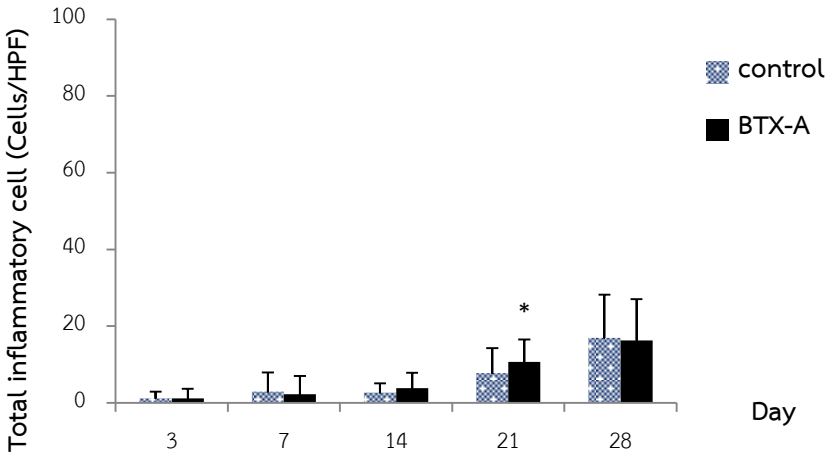


Figure 15. The intensity of inflammatory cell of the BTX-A and control groups There was no statistically significant difference of total inflammatory cells between the BTX-A and control groups. At 21 days post-operative injection, total inflammatory cells of the BTX-A group was significantly more than the control group.

Table 2. The Mean ± standard deviation (SD) and p-value the infiltration of inflammatory cells in control group.

Inflammatory cells in the control group (Mean ± SD)				
Day	Lymphocyte	Macrophage	neutrophil	Eosinophil
3	0.48±0.93	0.52±1.16	0.1±0.3	0.04±0.28
7	0.72±2.05	0.8±0.34*	0.02±0.14	2.14±3.07*
14	1.46±1.83	1.08±1.31*	0.04±0.28	0.06±0.31
21	4.4±4.06*	2.6±2.70*	0.24±1.29	0.56±1.47*
28	9.12±7.46	7.5±5.30	0.24±1.33	0.14±0.61

*p-value<0.05

Table 3. The Mean ± standard deviation (SD) and p-value the infiltration of inflammatory cells in the BTX-A group.

Inflammatory cells in the BTX-A group (Mean ± SD)				
Day	Lymphocyte	Macrophage	Neutrophil	Eosinophil
3	0.64±1.65	0.5±1.23	0.02±0.14	0±0
7	1.48±2.93	0.36±0.83*	0.1±0.58	0.36±1.49*
14	1.84±2.66	1.88±2.16*	0±0	0.1±0.36
21	6.34±4.16*	4.22±2.64*	0.02±0.14	0.08±0.27*
28	8.38±7.47	7.92±4.67	0±0	0±0

*p-value<0.05

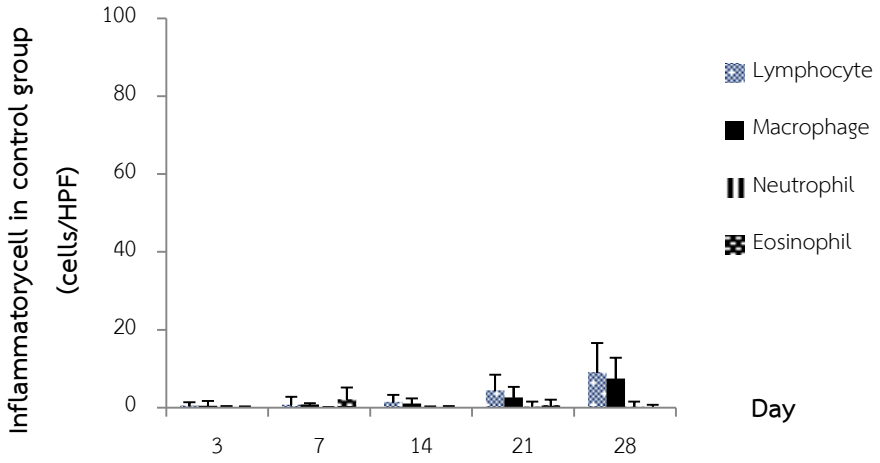


Figure 16. The infiltration of inflammatory cells in the control group.

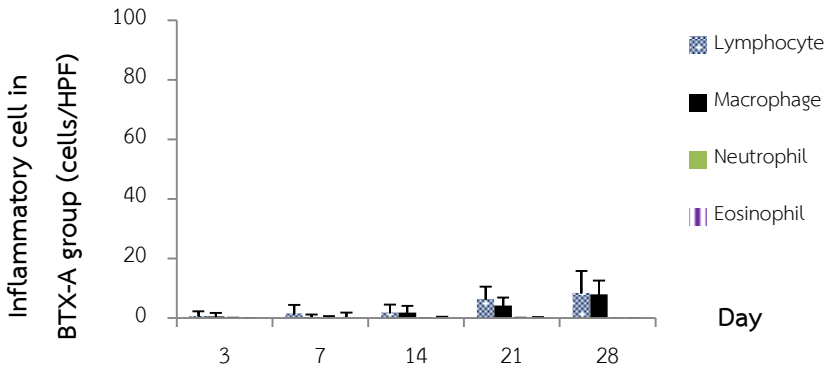


Figure 17. The infiltration of inflammatory cells in the BTX-A group.

4.1.2.2 The number of blood vessel

There was a statistically significant difference of the number of blood vessels between the control and the BTX-A groups on the 3rd post-operative day ($p<0.05$). The average number of blood vessel in the BTX-A group was steadily increased than the control group on the 3rd 7th 14th 21th and 28th post-operative days (Table 4, Figure18).

Table 4. The Mean ± standard deviation (SD) and p-value the infiltration of inflammatory cells in the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
3	1.32±1.90	5.20±5.32	<.0001*
7	3.78±3.15	5.80±4.26	0.52
14	4.02±3.37	6.10±4.94	0.54
21	5.50±4.67	5.82±4.53	0.06
28	6.02±4.10	6.04±3.81	0.88

*p-value<0.05

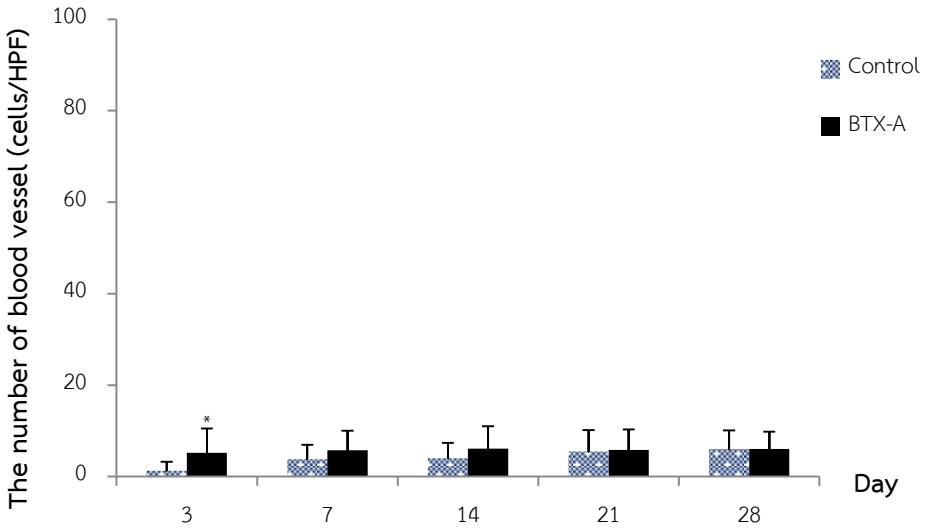


Figure 18. The number of blood vessels in the BTX-A and control groups. The number of blood vessels in the BTX-A group was increased significantly more than the control group at day 3 and likely to at days 7 and 14 after injection.

4.1.3 Masson trichrome staining

4.1.3.1 Collagen density

There were no significant differences of the intensity of collagen density between the control and BTX-A groups at the 3rd 7th 14th 21st and 28th post-operative days (Table 5, Figure 19).

Table 5. The Mean ± standard deviation (SD) and p-value of the intensity of positive nucleus blue color of collagen in the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
3	3± 0	3± 0	
7	2.6±0.55	2.6±0.55	
14	2.8±0.45	2.8±0.45	
21	3± 0	3± 0	
28	3±0	3± 0	0.22

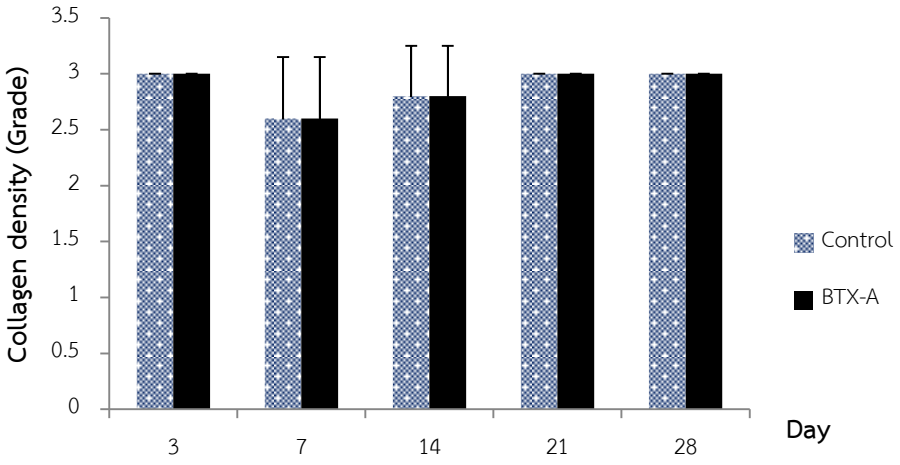


Figure 19. The intensity of positive blue color of collagen in the BTX-A and control groups. There was no significant difference between the control and BTX-A groups at the 3rd, 14th, 21st and 28th post-injection days.

4.1.3.2 Collagen arrangement

There were no statistically significant differences ($p>0.05$) of the collagen arrangement grades between the control and BTX-A groups at the 3rd, 7th, 14th 21st and 28th post-operative days (Table6, Figure 20).

Table 6. The Mean ± standard deviation (SD) and p-value of the intensity of positive blue color of collagen arrangement mesh work in the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
3	3± 0	3± 0	
7	3±0	3±0	
14	2.8±0.45	3±0	
21	3± 0	3± 0	
28	3±0	3± 0	0.43

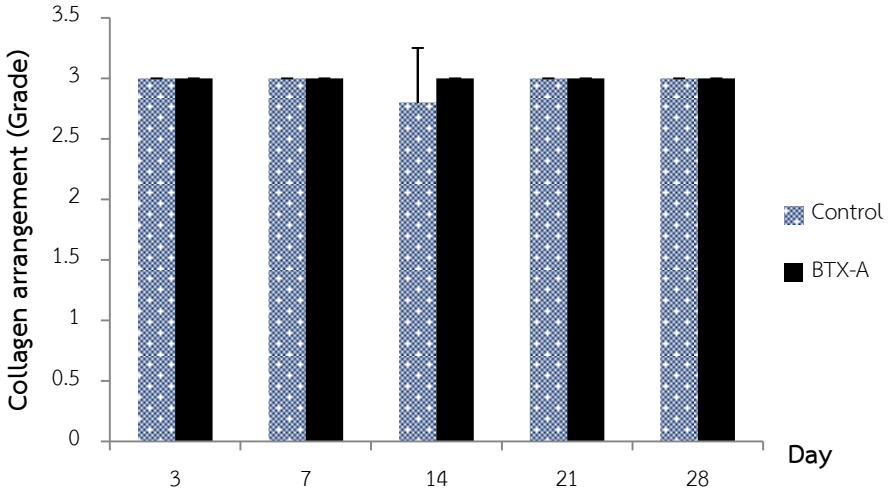


Figure 20. The intensity of positive blue color of collagen arrangement mesh work in the BTX-A and control groups. There was no significant difference of collagen arrangement between the control and BTX-A groups at the 3rd, 14th, 21st and 28th post-injection days.

4.1.4 Immunohistochemically

4.1.4.1 Fibroblasts proliferation

There were statistically significant differences of the number of Ki67 positive nucleus of fibroblasts between the control and the BTX-A groups on the 7th and 21th post-operative days. The average number of Ki69 positive nuclei of fibroblasts was steadily increased in groups at the 3rd, 7th 14th, 21st and 28th post-operative days but the number of Ki67 positive nucleus of fibroblasts BTX-A group was slightly more than the control group at the 7th and 21st post-operative days (Table 7, Figure 21)

Table 7. The Mean ± standard deviation (SD) and p-value of Ki67 positive nuclei of fibroblasts in the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
3	0.7±0.99	0.36±0.66	0.08
7	0.8±0.93	2.96±2.30	<.0001*
14	4.32±2.35	5.62±3.68	0.1352
21	4.9±2.43	3.64±3.09	0.0054*
28	3.2±2.75	3.66±3.72	0.8893

*p-value<0.05

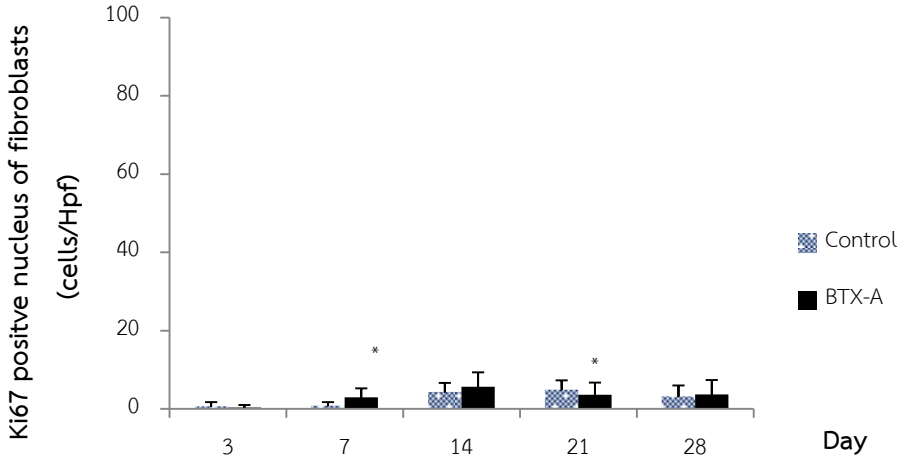


Figure 21. The number of Ki67 positive nucleus of fibroblasts in the BTX-A and control groups. There was statistically significant difference of the number of Ki67 positive in nuclei of fibroblasts between the control and the BTX-A groups at the 7th and 21st post injection days.

4.1.4.2 Collagen type I

There was statistically significant difference of the intensity of collagen type I between the control and the BTX-A groups at the 7th post-operative days ($p<0.05$). However, the intensity of the collagen type I in the BTX-A group was increased more than the control group at the 3rd, 7th, and 14th but decreased more than the control group at the 21th post-operative days (Table 8, Figure 22).

Table 8. The Mean ± standard deviation (SD) and p-value of the intensity of collagen type I in the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
3	2.44±0.58	2.64±0.49	0.2265
7	1.8±0.41	2.20±0.58	0.0085*
14	1.76±0.78	1.92±0.28	0.6222
21	1.72±0.45	1.48±0.59	0.0807
28	2±0.64	2±0.65	1.0000

*p-value<0.05

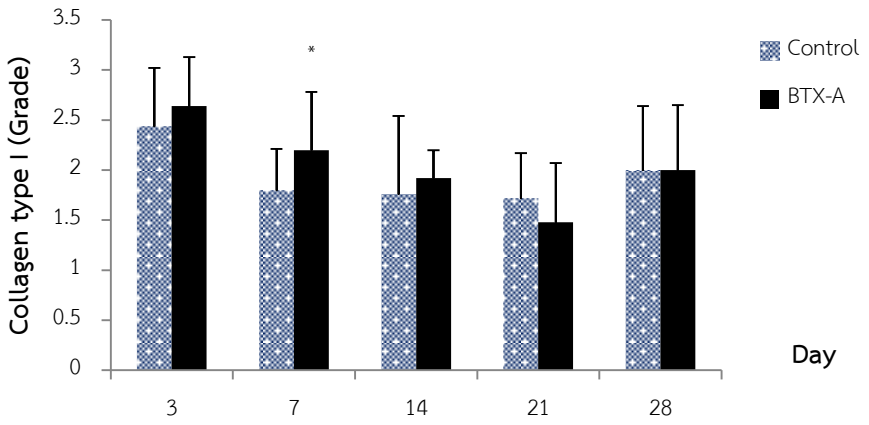


Figure 22. The intensity of collagen type I comparison in the control and the BTX-A groups. There were increase statistically significant differences of the intensity collagen type I between the control and the BTX-A groups at the 7th post-operative days.

4.2 Experiment II: Surgically wound model of the experimental animal

4.2.1 Assessment of the wound size

All wounds were covered with scab at 7 day post-operative. From days 14 to 28, both of the control and BTX-A groups showed progressive decrease in size of the wound scab. There was no significant difference scab and wound size between the BTX-A and control groups (Table 9, Figure 24).

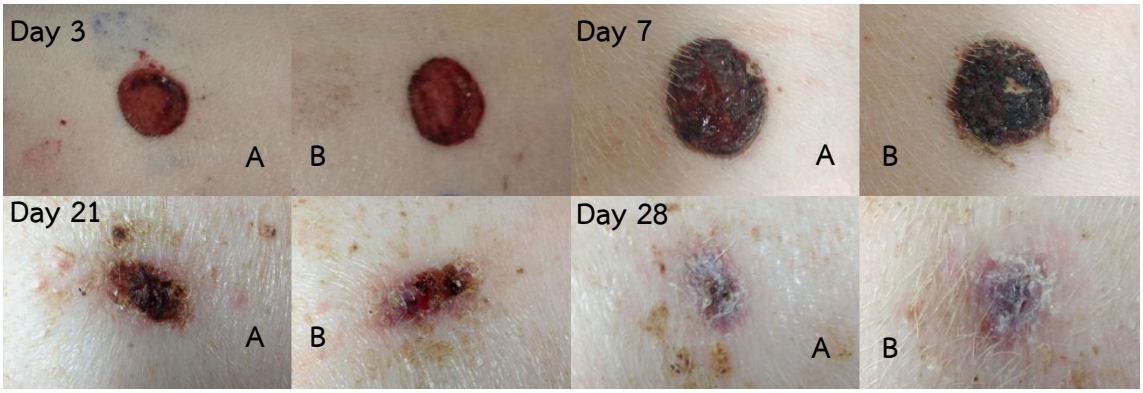


Figure 23. Assessment of the surgical wound. There was no significant difference of the wound size between the control (A, the left wound on each photograph) and BTX-A groups (B, the right wound on each photograph) at the 7th, 14th, 21st and 28th post-operative days.

Table 9. Mean ± standard deviation (SD) and p-value of the surgical wounds between the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
0	3.14±0	3.14±0	1
3	3.14±0	3.08±0.14	1
7	3.04±0.26	3.04±0.26	0.88
14	1.16±0.46	1.34±0.51	0.4
21	0.76±0.20	0.83±0.42	0.89
28	0.59±0.06	0.51±0.17	0.46

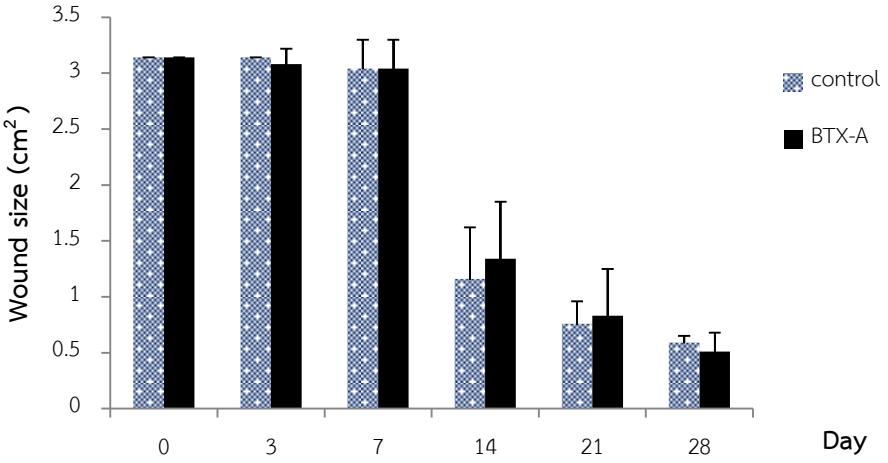


Figure 24. Change of wound size during healing period. There were no significant difference of formation scab and wound size between the BTX-A and control groups at all time points.

4.2.2 Histopathological evaluation.

4.2.2.1 The intensity of the inflammation

The BTX-A group had less infiltration of the inflammatory cells than the control group at days 3th. There was statistically significant difference of infiltration of the inflammatory cells between the control and the BTX-A groups of the 3rd and 14th post-operative day ($p<0.05$) (Table 10 Figure 25).

Table 10. Mean ± standard deviation (SD) of the infiltration of the inflammatory cells in the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
3	16.12±16.61	7.16±6.28	<.0001*
7	15.7±11.56	14.62±11.18	0.5436
14	17.2±10.14	10.96±6.25	0.0012*
21	11.92±7.11	10.1±6.73	0.2377
28	10.2±5.98	10.6±8.19	0.9311

*p-value<0.05

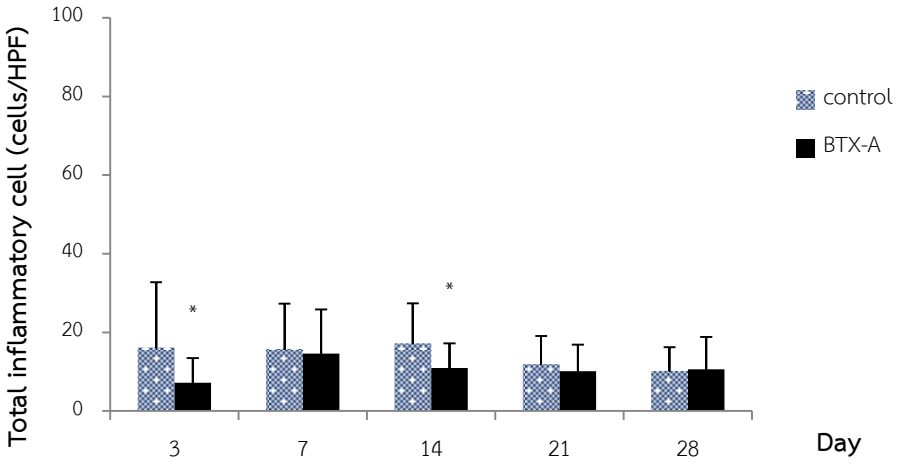


Figure 25. The infiltration of the inflammatory cells in the BTX-A and control groups. The BTX-A group had less infiltration of the inflammatory cells than the control group at day 21 and there was statistically significant between the two groups at the 3th and 14th post-operative days.

Table 11. Mean ± standard deviation (SD) and p-value of the infiltration of the inflammatory cells in the control group.

Day	Lymphocyte	Macrophage	neutrophil	Eosinophil
3	6.6±9.64*	7.32±5.41*	2.06±5.21*	0.06±0.42
7	5.34±5.33*	9.74±9.27	0.22±0.79*	0.4±1.43
14	8.74±6.34*	7±4.28	1.12±3.88*	0.34±1.88
21	3.86±3.08	7.48±5.73*	0±0	0.58±1.44
28	3.96±4.50	5.92±4.40	0±0	0.32±0.77

*p-value<0.05

Table 12. Mean ± standard deviation (SD) and p-value of the infiltration of the inflammatory cells in the BTX-Al group.

Day	Lymphocyte	Macrophage	Neutrophil	Eosinophil
3	3.5±4.03*	3.34±4.08*	0.28±1.60*	0.04±0.28
7	8.26±6.89*	6.3±6.10	0±0*	0.06±0.31
14	4.66±3.05*	6.02±4.41	0.02±0.14*	0.26±0.83
21	4.26±3.99	5.28±5.22*	0±0	0.56±1.93
28	3.3±5.28	6.14±5.33	0±0	0.84±1.54

*p-value<0.05

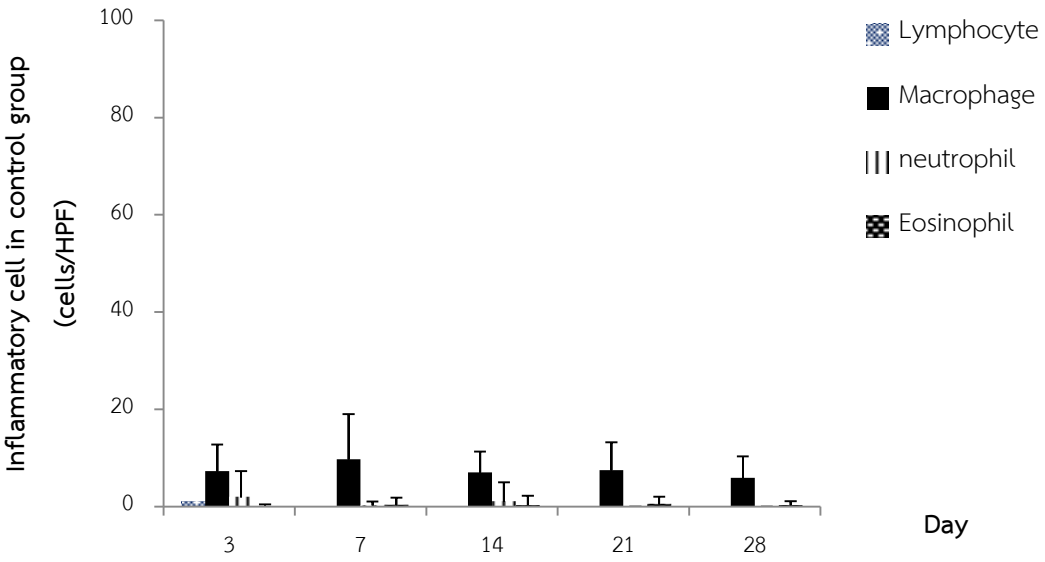


Figure 26. The infiltration of the inflammatory cells in the control group.

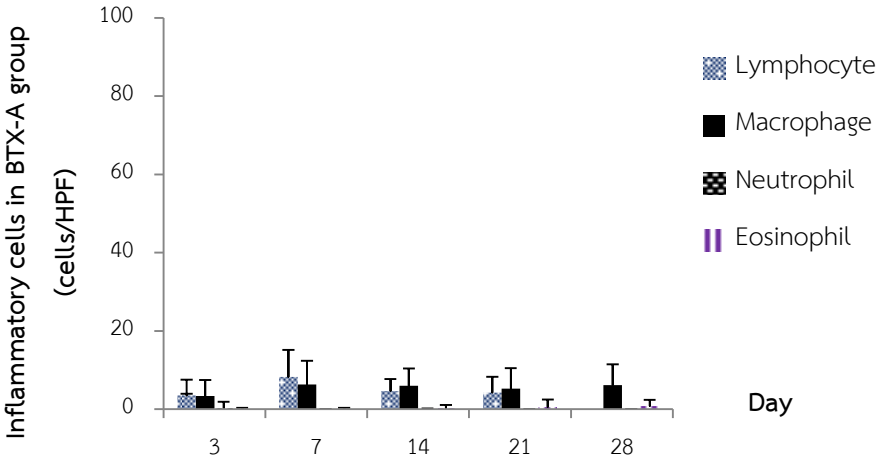


Figure 27. The infiltration of the inflammatory cells in the BTX-A group.

4.2.2.2 The number of blood vessel

There was statistically significant difference of the number of blood vessels between the control and the BTX-A groups at the 3rd post-operative day ($p<0.05$).

The average numbers of blood vessels in the BTX-A group at the 3rd, 7th and 14th post-operative days were more than control group, but there was significant at the 3rd post - operatives (Table 13, Figure 28).

Table 13. Mean ± standard deviation (SD) and p-value of the number of blood vessels in the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
3	4.32±4.1	5.98±3.48	0.0113*
7	5.68±4.20	6.68±4.4	0.1727
14	7.14±5.72	7.28±5.38	0.7688
21	5.96±3.48	6.62±3.94	0.1167
28	5.02±2.37	5.18±4.64	0.1057

*p-value<0.05

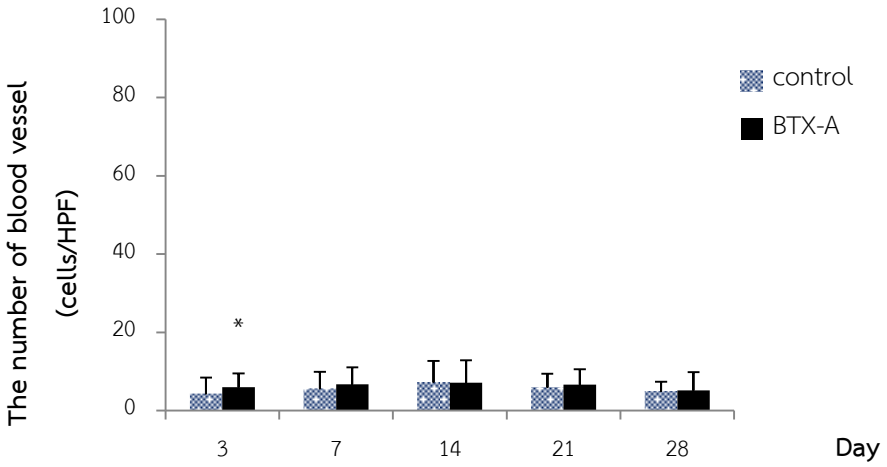


Figure 28. The number of blood vessels in the BTX-A and control groups. There was statistically significant difference of the number of blood vessels between the control and the BTX-A groups at the 3rd post-operative days.

4.2.3 Masson trichrome staining

4.2.3.1 Collagen density

There was statistically significant difference of the intensity of collagen density between the control and BTX-A groups at the 21st post-operative days ($p<0.05$).

The intensity of collagen in BTX-A group tended to be darker than in the control group. This means that the BTX-A group had amount of collagen greater than the control group (Table 14, Figure 29).

Table 14. Mean ± standard deviation (SD) and p-value of the intensity of positive blue color of collagen in the BTX-A and control groups.

Day	Control Mean ± SD	BTX-A Mean ± SD	p-value
3	0± 0	0± 0	1
7	0.8±0.84	1.±1	0.82
14	1.4± 0.45	1.8±0.45	0.27
21	1.4±0.55	2± 0	0.02*
28	1.8±0.45	2.2± 0.45	0.23

*p-value<0.05

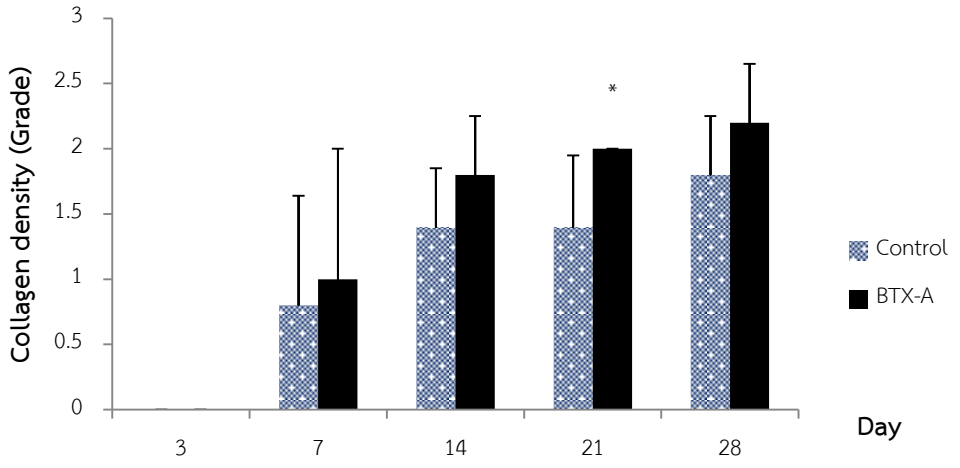


Figure 29. The intensity of positive blue color of collagen in the BTX-A and control groups. The increased intensity of positive blue color of collagen in BTX-A group tended to be more than control group at the 7th, 14th, 21st and 28th post-operative days, but was significant only at the 21th post-operative days.

4.2.3.2 Collagen arrangement

There were statistically significant differences of the arrangement of the collagen between the control and BTX-A groups at the 14th and 21st post-operative days ($p<0.05$) (Table 15, Figure 30).

The BTX-A group had the arrangement of the collagen more orderly than the control group.

Table 15. Mean ± standard deviation (SD) and p-value of the intensity of positive blue color of collagen arrangement mesh work in the BTX-A and control groups.

Day	Control Mean ± SD	Botox Mean ± SD	p-value
3	1±0	1±0	1
7	1±0	1.4±0.55	0.1770
14	1±0	2±00	0.0027*
21	1± 0	2± 0	0.004*
28	2±0	2± 0	1

*p-value<0.05

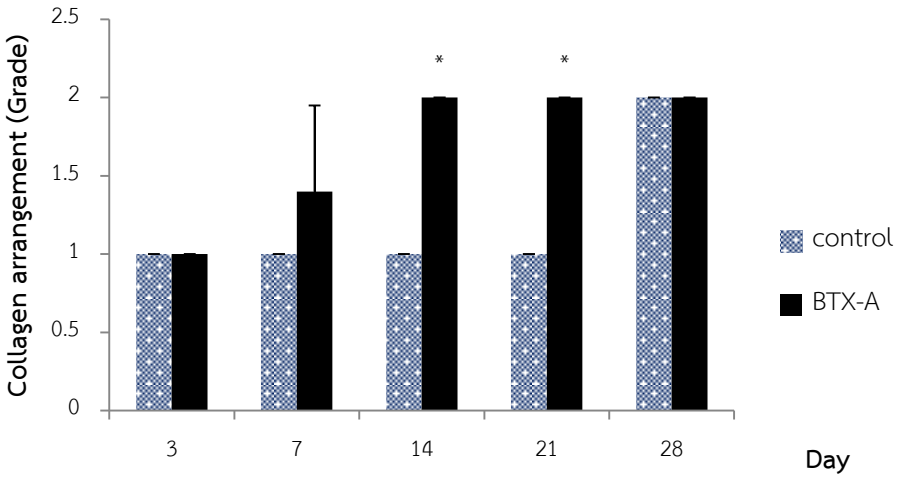


Figure 30. The intensity of positive blue color of collagen arrangement mesh work in the BTX-A and control groups. The intensity of positive blue color of collagen mesh work in the BTX-A group showed more orderly arrangement of the collagen than the control group at the 3rd, 14th and 21st post-operative days.

CHAPTER V

CONCLUSION, DISCUSSION, COMMENT

Conclusion

Intradermal injection of BTX-A into normal skin of the pig model was not harmful. In the early stage of the first experiment the number of blood vessel, Ki67 positive nucleus of fibroblasts and the collagen type I in the BTX-A group was congruously increased more than the control group.

In the second experiment, wound of the BTX-A group had less infiltration of inflammatory cells than the control group at the 21st post-operative days. In the early stage of operation, the number of blood vessels in the BTX-A group was increased more than the control group. The intensity of collagen in the BTX-A group tended to increase more than the control group. The BTX-A group had more orderly arrangement of the collagen than the control group. In conclusion, the BTX-A intradermal injection into the surgical wound of the pig model induced less inflammation at the early stage after the injection and could promote wound healing as evidence of more vessel proliferation, intensity of collagen and more orderly arrangement of the collagen in the wound when compared to the control.

Discussion

BTX-A Intradermal injection was not harmful to the pig as the evidence of no significant allergic reaction and systemic adverse reaction after the injection, similar to the intradermal injection of BTX-A into the mid and lower face in human (Alvarez et al., 2005; Seyler et al., 2008). Intramuscular injection is the common route of the injection of BTX-A having minimal risk of systemic adverse effects when used the appropriate dose. However, there was a report of the severe systemic adverse reaction such as paralysis and respiratory failure. Therefore, the intradermal may be an alternative route of the intramuscular injection of BTX-A.

Histopathologically, BTX-A induced less inflammation at early stage after injection. We could not find any previous study that reported inflammatory

mechanism of BTX-A intradermal injection. The infiltration of inflammatory cells at the BTX-A intradermal injection is different from the intramuscular injection. It might be due to release of several cytokines causing decreased response and short period of inflammation. BTX-A could increase vessel proliferation, which could have been due to increased angiogenesis. Deniz et al, 2014 reported that BTX-A could increase neovascularization in the wound and skin graft contraction.

From Masson trichrome staining, The BTX-A group had increased collagen density. To explain the reason behind BTX-A effects on bundle of collagen fiber, previous study found that BTX-A could increase collagen maturation and accelerate maturation on wound contraction and skin flap in rats (Deniz et al, 2014). Study on the effect of BTX-A in a rat surgical wound, explained the difference of the degree of collagen maturation was associated with the increased collagen maturation by decreasing inflammatory response and shortening of the inflammatory phase in the surgical wound of Botox group (Byung-Joo et al, 2009). In the present study, the BTX-A group showed improve arrangement of collagen. The study on the effect of BTX-A on collagen deposition in hypertrophic scars in a rabbit ear model had shown that the orderly arrangement of collagen fibers more was resulted from the action on the biological behavior of fibroblasts (Zhibo and Guofan, 2012). Some scholars found that BTXA could inhibit the growth of hypertrophic scars and improve the appearance of hypertrophic scars in clinical experiments (Xiao et al. 2010). Byung-Joo et al, 2009 reported that BTX-A might be used to decrease the fibrosis of surgical wounds such as treating laryngeal, tracheal and nasal stenosis.

We are interested in the effect of BTX-A on dermal fibroblasts because the fibroblast are the main cellular component in dermis that secretes collagen. From the immunohistochemical staining, BTX-A could stimulate fibroblast proliferation and stimulated the expression of type I collagen. Fibroblast proliferative could improve wound healing by producing the matrix proteins hyaluronic, fibronectin proteoglycan, and type I and type III procollagen. Collagens are the important component in all phase of wound healing. Synthesized by fibroblasts, they impart integrity and strength to all tissues and play a key role, especially in the proliferative and remodeling phases. Unwounded dermis contains 80% type I and 25% type III

collagen, whereas wound granulation tissue expression 40% type III collagen (Robson MC et. al, 2001.). In this study, BTX-A could increase fibroblast proliferation and collagen type I. Deniz et al 2014 reported the effect BTX-A on the wound and skin graft in rats was the increase number of fibroblasts during wound recovery. The study on the effect of BTX-A on human dermal fibroblasts showed that BTX-A could stimulate the expression of type I collagen. Type I collagen is the most collagen component of dermis (Sang-Ha et. al, 2012). Therefore, BTX-A can help wound healing.

Comment

The majority of skin and unwounded dermis contains collagen type I and type III. Further studies are needed to determine other types of collagen. The present study, studied only the collagen type I. Considering that there have limited studies on difference type of collagen. Therefore the antibodies for other collagen types are not available.

REFERENCES

Alderson K, Holds J and Anderson R 1991. Botulinum-induced alteration of nerve-muscle interactions in the human orbicularis oculi following treatment for blepharospasm. Neurology. 41(11): 1800–1805.

Alvarez C, Trewell S, Keenan S and Beauchamp R 2005. Treatment of idiopathic clubfoot utilizing botulinum A toxin: a new method and its short-term outcomes. J Pediatr Orthop. 25: 229-235.

Ashford S and Tumer-Stokes L 2006. Goal attainment for spasticity management using botulinum toxin. Physiother Res Int. 11: 24-34.

Byung-Joo L, Jae-Hwi J, Soo-geun W, Jin-Choon L, Eui-Kyun G and Hwal-Woong K 2009. Effect of Botulinum Toxin Type A on a rat surgical wound model. CEO. 2: 20-27.

Carruthers A and Carruthers J 1998. Clinical indications and injection technique for the cosmetic use of botulinum A exotoxin. Dermato Surg. 48: 1189-1994.

Chang S, Tsai H, Chen W and Lee W 2008. The wrinkles soothing effect on the middle and lower face by intradermal injection of botulinum toxin type A. Int J Dermatol. 47: 1287-1294.

Coffield J and Simpson L 1994. The site and mechanism of action of botulinum neurotoxin. In: Jankovic J, Hallet M, editors. Therapy with botulinum toxin. New York: Marcel Dekker. 3-13.

De Paiva A, Meunier F, Molgo J, Auki K and Dolly J 1999. Functional repair of motor endplates after botulinum neurotoxin Type A poisoning: Biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci USA. 96: 3200-3205.

Deniz K, Saime I, Selman O and Nazan S 2014. The effects of botulinum toxia A on the wound and skin graft contraction. J Craniofac Surg. 25: 1908-1911.

Fagien 1999. Botox treatment of dynamic and hyperkinetic facial lines and furrows:adjuvant use in facial aestgetic surgery. Plast Reconstr Surg. 701-703.

Green P and Fahn S 1993. Development of antibodies to botulinum toxin Type A in patients with torticollis treated treated with injections of botulinum toxin Type A; in Das Gupta BR (ed) Botulinum and Tetanus Neurotoxin: Neurotransmission and Biomedical Aspects. New York, Plenum Press. 651-654.

Kantor J and Margolis D 1998 Efficacy and prognostic value of simple wound measurements. Arch Dermatol. 12 (134): 1571-1574.

Kao I, Drachman D and Price D 1976. Botulinum toxins: Mechanism of presynaptic blockade. Science. 193: 1256-1258.

Kurzen H and Schallreuter K 2004. Novel a spaects in cutaneous biology of acethylcholine synthesis and acetylcholine receptors. Exp Dermatol. 13 (Suppl 4): 27-30.

Lacy D and Stevens R 1999. Sequence homology and structure analysis of the costridial neurotoxins. J MoL Biol. 1091-1104.

Lang A 2004. History and uses of Botox (botulinum toxin type A). Lippincotts Case manag. 9: 109-112.

MacKenzie I, Burnstock G and Dolly J 1982. The effects of purified botulinum neurotoxin type A on cholinergic, adrenergic and nonadrenergic, atropine-resistant autonomic neuromuscular transmission. Neuro science. 7 (4): 997–1006.

Maisey E, Wadsworth J, Poulain B, Shone C, Melling J, Gibbs P, Tauc L and Dollt J 1988. Involvement of the constituent chains of botulinum neurotoxins A and B in the blockade of neurotransmitter release. Eur J Biochem. 177: 683-691.

Naumann M, Bergmam I, Hofmann U, Hamm H and Reiners K 1998. Botulinum toxin for focal hyperhidrosis: Technical considerations and improvements in application (letter). Br J Dermatol. 139: 1123-1124.

Printer M, Vanden NS, Muccio D and Wallace N 1991. Axotomy-like changes in cat motorneuron electrical properties elicited by botulinum toxin depend on the complete elimination of neuromuscular transmission. J Neurosci 11: 657-666.

Regan Thomus J 2010. Botulinum exotoxin A injections. In: Advanced theraphy in facial plastic & reconstructive surgery. Jason O. Malley and Carole caro Wonsiewicz (eds). China: PMPH-USA. 591.

Rohrich R, Janis J, Fagien S and Stuzin J 2003. Botulinum toxin: expanding role in medicine. Plast Reconstr Surg. 15-35.

Sander DB, Massey W and Buckley EG 1986. Botulinum toxin for blepharospasm: Single-fiber. EMG studies. 36: 545.

Sang-HA O, Young L, Young-joon S, Jeung -Hoon L, Jung D Y, Ho Y C and Byung C C 2012. The potential effect of Botulinum toxin Type A on human dermal fibroblast : As in vitro study. Dermatol Surg. 38: 1689-1694.

Scott AB 1980. Botulinum toxin injection into extraocular muscle as an alternative to strabismus surgery. Opthamol. 1044-1049.

Scott AB 1981. Botulinum toxin injection of eye muscle to correct strabism. Trans Am Ophthamol Soc. 79: 734-770.

Scott AB, Kennedy R and Stbbs H 1985. Botulinum A toxin injection as a treatment for blepharospasm. Arch Ophthamol. 347-350.

Seyler T, Smith B, Marker D and Ma J 2008. Botulinum neurotoxin as a therapeutic modality in orthopedic surgery : more than twenty years of experience. J Bone g AMJoint Sur. 90 (Suppl4): 133 - 145.

Shah A 2008. Use of intradermal botulinum toxin to reduce sebum produntion and facial pore size. J drugs Dermatol. 7: 847-850.

Simpson L 1986. Molecular pharmacology of botulinum toxin and tetanus toxin. Annu Rev Pharmacol Toxicol. 26: 427-453.

Thomas L, Andre R and Brigitte S 2003. BotulinumToxin as a New Therapy Option for Voiding Disorders: Current State of the Art European. Urology. 44: 165–174.

Villar R, Shapiro R, Busto S, Riva-Posse C, Verdejo G, Farace M, Rosetti F, San Juan J, Julia C, Beeher J, Maslanka S and Swerdlow D 1999. Outbreak of type A botulism and developmemt of a botulism surveillance and antitoxin release system in argentina. JAMA. 1334-1340.

Zhibo X, Miaobo Z, Ying l and Lihong R 2011. Botulinum toxin type A inhibits connective tissue growth factor expression in fibroblast derived from hypertrophic scar. Aesth Plast Surg. 35: 802-807.

APPENDIX

Haematoxylin and Eosin (H&E) Staining

Slides were placed in staining jar and deparaffinized by submerging into three series of absolute xylene for 4 minutes followed by 100%, 100%, 95%, 90%, and 70% of ethanol for 4 minutes of each percentage. Next, slides were washed in running tap water for 2 minutes. Then, slides were submerged into Harris Hematoxylin (Sigma-Aldrich, Germany) for 2 minutes and then washed in running tap water for 2 minutes. The slides then were submerged into 1% acid alcohol for 3 dips to decolorize it and washed in running tap water for 2 minutes. Next, slides then were submerged into 2% potassium acetate for 3 minutes and again washed in running tap water for 2 minutes. After that, slides were submerged into Eosin for 2 minutes followed by washing in running tap water for 2 minutes. Stained slides were dried for 24 hours at 38°C. Before observation, Slides were dipped into absolute xylene for 1 minute and finally mounted with cover slip using DPX Mounting.

Modified Masson's trichrome staining

Method Was modified from Kiernan (2008), Skin tissue slides were placed in staining jar and deparafinished by submerging into three series of absolute xylene for 4 minutes each follow by 100%, 95%, 90%, 80% and 70% of ethanol for 4 minutes in each percentages. The slides then were submerged in warmed Bouin's solution at 60° C for 45 minutes. Next, the slides were washed in running tap water until yellow color in samples disappeared. To differentiate nuclei, slides then were immerged in modified Wiegert's haematotoxin for 8 minutes, after that washed in running water for 2 minutes. In order to stain cytoplasm and erythrocytes, slides were submerged in anionic dyes, acid fuschin (C.I. 42590, Merck, Germany) for 5 minutes; Then again slides were washed in running tap water for 2 minutes. Next, slides were treated with phosphomolybidic acid solution for another 10 minutes as a mordant and immediately slides were submerged into methyl blue (C.I. 42780, Merck, Germany) solution for 5 minutes in order to stain fibroblast and collagen. After that, slides were

washed in running water for 2 minutes. and lastly treated with 1% acetic acid solution for 1 minutes. Slides then were dehydrated into a series of alcohol of 70%, 80%, 95% and 100% for 1 minutes each percentage. Before observation, slides were dipped into absolute xylene for 1 minute and finally mounted with cover slip using DPX Mounting.

Immunohistochemical

Collagen type I

Immunohistochemical analysis performs on formalin-fixed, the paraffin-embedded materials by using a primary antibody for collagen type I Briefly the sections were deparaffinized in xylene, rehydrated, washed in distilled water, immersed in phosphate buffered saline (pH6) and then microwave for 5 min. The sections were treated with 3 % H_2O_2 solution to blocked the endogenous peroxidase activity, and then they were washed in phosphate buffered saline ; they were subsequently subjected to incubation with the primary antibody(Rabbit polyclonal Anti-Collagen I antibody) overnight 4°C. Detection of the immunoreactive staining was obtained by Envision with using polymer DAKO. The sections were subjected to a color reaction with diaminobenzidine tetrahydrochloride (DAB) and they were counterstained with hematoxylin. Collagen type I showed cyto-plasmic staining as a brown color.

KI67

Immunohistochemical analysis was performed on the Leica Microsystems Bond max System (Leica Microsystems, Bannockburn, IL). Slides were incubated for 60 minutes at 60°C and treated with Bond Dewax Solution (Leica Microsystems). Epitope retrieval was performed by incubating the slides in Bond Epitope Retrieval Solution 2 (Leica Microsystems) for 30 minutes at100°C. Immunohistochemical analysis was performed using the Bond Polymer Refine Detection kit (Leica Microsystems), a 3-step indirect immunoperoxidase technique. Briefly, primary antibody was applied for 40 minutes at room temperature followed by 3 consecutive rinses with Bond Wash Solution (Leica Microsystems). Peroxide block (3% hydrogen peroxide) was then applied for 5 minutes and rinsed 3 times with Bond Wash

Solution. Post Primary Polymer (Leica Microsystems) was applied for 8 minutes before rinsing 3 times with Bond Wash Solution. Polymer Poly-HRP IgG (Leica Microsystems) was applied for 8 minutes and rinsed 3 times with Bond Wash Solution and once with deionized water before the diaminobenzidine chromogen was applied for 4 minutes followed by 3 deionized water rinses. Slides were counterstained with hematoxylin for 5 minutes. KI 67 showed nucleus staining as a brown color.

VITA

Panutda Darapong was born in January, 19 1984 in Chiang Rai, Thailand. She is the middle one of Darapong's family. In April 2008, she graduated bachelor degree in Veterinary Science from Faculty of Veterinary, Chiang Mai University, and then she worked as a veterinarian at Suvarnachad Animal Hospital, Bangkok, Thailand. In 2014, she began to study in the master degree of veterinary surgery, Faculty of Veterinary Science, Chulalongkorn University. Currently, she is studying accompany to work as a part-time veterinarian at Suvarnachad Animal Hospital, Bangkok, Thailand.