CHANGES IN THE STRUCTURE AND COMPOSITION OF MENISCUS IN HUMAN OSTEOARTHRITIS

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การเปลี่ยนแปลงของโครงสร้างและองค์ประกอบของหมอนรองกระดูกเข่าในภาวะโรคข้อเข่าเสื่อมของมนุษย์

นางสาวปิยฉัตร รุ่งสว่าง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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คำสำคัญ: โรคข้อเข่าเสื่อม/เนื้อเยื่อวิทยา/พยาธิสภาพ/กลูโคซามีน/คอลลาเจน

โรคข้อเสื่อม (OA) เกิดขึ้นเมื่อกระดูกอ่อนที่ปกคลุมพื้นผิวข้อต่อเสื่อมสภาพ ในโรคข้อเข่าเสื่อม จะพบรอยถลอกที่กระดูกอ่อนของข้อเข่า นอกจากนี้ยังสังเกตเห็นการเปลี่ยนแปลงโครงสร้างที่เมนิคัส วัตถุประสงค์หลักของการศึกษาครั้งนี้คือเพื่อศึกษาการเปลี่ยนแปลงเชิงโครงสร้างของเมนิคัสด้านในและด้านนอก จากการศึกษาเนื้อเยื่อวิทยา ผลที่ได้แสดงให้เห็นว่าความเสียหายส่วนใหญ่เกิดขึ้นที่เมนิคัสส่วนหลัง จำนวนเซลล์ของเมนิคัสด้านในมีจำนวนน้อยกว่าด้านนอกโดยเฉพาะบริเวณส่วนนอกและส่วนลึกพบเซลล์ขนาดใหญ่กว่าปกติที่ส่วนหลังของเมนิสคัสด้านใน การเกิดพยาธิสภาพส่งผลให้เกิดการลดลงของโปรติโอไกลแคนในส่วนของเมนิคัสด้านในมากกว่าเมนิคัสด้านนอก เส้นใยคอลลาเจนเพิ่มขึ้นอย่างชัดเจนในส่วนของเมนิคัสด้านในมากกว่าด้านนอก โรคข้อเข่าอักเสบในระยะเริ่มต้นทำให้เกิดวิถีการตายแบบอะพอพโตซิสในเมนิคัสด้านนอกมากกว่าเมนิคัสด้านใน ซึ่งจำนวนอะพอพโตซิสเซลล์ของเมนิคัสด้านในมีน้อยกว่าเมนิคัสด้านนอก อาจจะเป็นเพราะเซลล์ผ่านกระบวนการตายแบบอะพอพโตซิสและได้สลายไปแล้ว แต่อะพอพโตซิสเซลล์ของเมนิคัสด้านนอกกำลังอยู่ในช่วงของการเกิดกระบวนการตายแบบอะพอพโตซิส

ผลการทดสอบ เอ็มทีที แสดงให้เห็นว่าปริมาณของสารที่ใช้ในการรักษาต่อการเพาะเลี้ยงเซลล์คอนโดรไซต์ไม่ก่อให้เกิดความเป็นพิษต่อเซลล์ นอกจากนี้ ผลจากการทดสอบ อีไลซา เทคนิค พบว่ากลูโคซามีนที่ 125 ไมโครกรัม, เมือกหอยทากที่ 50 ไมโครกรัม และคอลลาเจนชนิดที่ 2 ที่ไม่แปลงสภาพจากไก่เนื้อที่ 100 ไมโครกรัม มีความสามารถในการลดปริมาณของอินอส และ ค็อกซ์-2 ที่หลั่งจากเซลล์ที่มีสภาวะอักเสบได้อย่างมีนัยสำคัญ สารเหล่านี้จึงมีศักยภาพในการลดการอักเสบของโรคข้อเข่าเสื่อมได้

สาขาวิชาปรีคลินิก ลายมือชื่อนักศึกษา ปิยฉัตร รุ่งสว่าง

ปีการศึกษา 2564 ลายมือชื่ออาจารย์ที่ปรึกษา ปิยดา เงินสูงเนิน

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Keyword: Osteoarthritis/Inflammatory/Menisci/Immunohistochemistry/Undenatured collagen type II

Osteoarthritis (OA) occurs when the cartilage covering the articular surface wears down. In OA knee, abrasions at the articular cartilage are found. Structural changes are also observed at the menisci. The main objective of the present study was to investigate structural changes in medial and lateral menisci. Using conventional staining, the result showed that damage mostly occurred at the posterior horn of the menisci. The number of cells in the medial meniscus was much less than those in the lateral meniscus, especially in the outer and deep zone. Hypertrophic cells were found in the posterior horn of the medial meniscus, but hypo trophic cells were found in the lateral meniscus. Pathology resulted in a decrease in proteoglycans in the medial meniscus which was greater than those in the lateral meniscus. Collagen fibers markedly increased in the medial meniscus more than those in the lateral meniscus. Early OA caused more cell apoptosis in the lateral meniscus than the medial meniscus. The number of apoptotic cells in the medial meniscus was less than those of the lateral meniscus, possibly because the cells underwent apoptosis and decayed, while the apoptosis cells of the lateral meniscus were during the apoptosis process. The result of the MTT assay showed that every treatment doses to the chondrocyte cells culture were not toxic to the cells. In addition, the result from the ELISA assay showed that glucosamine at 125 µg, snail mucus at 50 µg, and undenatured collagen type II from broiler chicken at 100 µg had the ability to significantly reduce iNOS and COX-2 which were secreted from the inflamed cells (induced by LPS). The substances could have a potential of an inflammatory agent.

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Student's Signature Piyachat Rungsawang
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LIST OF ABBREVIATIONS

µg/ml	=	Microgram per milliliter
µg	=	Microgram
µm	=	Micrometer
μm^2	=	Micro square meter
BSA	=	Bovine serum albumin
°C	=	Degree Celsius
CO_2	=	Carbon dioxide
cm	=	Centimeter
DAB	=	Diaminobenzidine
DI	=	Distilled water
ELISA	=	Enzyme-linked immunosorbent assay
hr	=	Hour
HRP	=	Horseradish peroxidase
H_2SO_4	=	Sulfuric acid
IgG	=	IgG immunoglobulin
KCl	=	Potassium chloride
KH_2PO_4	=	Potassium Dihydrogen Phosphate
M	=	Molar
ml	=	Milliliter
mM	=	Millimolar
mm	=	Millimeter
ng/ml	=	Nanogram per milliliter
min	=	Minute
NaCl	=	Sodium chloride
Na_2CO_3	=	Sodium carbonate
$NaHCO_3$	=	Sodium bicarbonate
Na_2KPO_4	=	Disodium hydrogen phosphate
nm	=	Nanometer

LIST OF ABBREVIATIONS (Continued)

PBS	=	Phosphate buffered saline
pH	=	Potential of hydrogen ion
PMSF	=	Phenylmethylsulphonyl fluoride
SD	=	Standard Definition
TMB	=	3, 3', 5, 5'-Tetramethylbenzidine

CHAPTER I

INTRODUCTION

1.1 Background/Problem

Discharge of meniscus in the knee joint diminished cartilage degeneration and bone refurbishing 50 years ago (Fairbank, 1948). The access was a choice to the basically therapeutic near of this standard work or sports injury. Treatment (when a fissure meniscus is repaired rather than removed) is only possible when the meniscus tissue is still of good quality. For the accident of meniscal tissue, some instead of all meniscectomy is the treatment of alternative to minimize the loss of this main anatomical structure. The knee joint menisci play physiological roles, such as load distribution, joint stabilization, lubrication, and sensory responsibility which led to the thought of displacement of the meniscus when its irreversible damage is occurred (Milachowski et al., 1989). Fifteen years have now passed since the partial meniscectomy and repairment were imported as a treatment in replacement for total meniscectomy (Gillquist et al., 1982; Hamberg et al., 1983) and the publication of the first clinical description of meniscal transplanting was reported 9 years ago (Milachowski et al., 1989).

Osteoarthritis (OA) is a joint disease that mainly effects the cartilage. The cartilage is the slithery tissue that covers the ends of bones in a joint. In OA, the upper layer of cartilage malfunction and deteriorate. This design bones below the cartilage to rub together. The rubbing begets pain, swelling, and loss of joint movement (Messner and Gao, 1998). Over time, the joint may suffer the loss of its common shape. Also, bone spurs may increase on the edges of the joint. Small bone or cartilage can break off and float within the joint area, which causes further pain and injury. Patients with OA often have joint pain and low movement of the joint.

OA is a public health issue. The incidence of OA increases continuously in middle-aged and elderly people. OA is one of the 10 most normal diseases causing a main cause of inefficiency in the elderly (Royal College of Physicians Orthopedic

Thailand, 2554). The World Health Organization (WHO) estimated that 10% of the world population with the age of over 60 years old have OA. The academics contend that the incidence of OA began to occur at 45 years old people (more than 50%). In a group over 65 years old people, degeneration of the knee is often found (Brooks, 2003; Zhang et al., 2010; Richmond et al., 2010). The incidence of the disease increased continuously. In Thailand, in 2553, it was found that more than 6 million people had the degeneration of the knee. OA is a public health problem, commonly found in middle-aged and elderly people, and the incidence of OA raises steadily (Pereira et al., 2011).

The treatment using medicines palliatively with loading on the knee makes gradually repairs joints deteriorating. It is recommended of using a correctly, enough, and compliance treatment. The appropriate methods help alleviate of pain, although the disease cannot be cured. In addition to this approach is the use of drugs and surgery (Arnun, 2014).

1.2 Research objectives

The overall objective of this work was to study the morphological and biochemical changes of the cartilage, compared between horns and zones of the menisci in the same grade of OA.

The specific purposes of this study were as follows:

1.2.1 To measure sizes and numbers of chondrocyte cells in horns and zones of the grade II OA compared between medial and lateral menisci.

1.2.2 To observe the distribution of proteoglycan and collagen in horns and zones of the grade II OA compared between medial and lateral menisci.

1.2.3 To measure the numbers of apoptosis cells in horns and zones of the grade II OA compared between medial and lateral menisci.

1.2.4 To study the effect of snail extract, glucosamine, and undenatured type II collagen in the prevention of inflammation in OA chondrocyte cell culture.

1.3 Research hypotheses

1.3.1 There are differences in degeneration of cartilage in zones and horns of menisci.

1.3.2 There are changes in cellular morphology, density, and biochemistry in zones and horns of menisci.

1.3.3 Snail extract, glucosamine, and undenatured collagen type II plays a role in preventing inflammation in OA chondrocytes.

1.4 Scope and limitations of the study

This study was focused on grade II knee OA. The deterioration of the menisci in each grade of the OA was compared to the control group. The biochemical changes and numbers of apoptotic cells were identified in horns and zones of the menisci. Moreover, effects of snail mucus, glucosamine, and undenatured collagen type II on inflamed chondrocyte cell culture were observed.

CHAPTER II
LITERATURE REVIEW

2.1 Osteoarthritis factors

For main patients, OA is involved in more risk factors that can be grouped into changeable and on- changeable. Non- changeable possibility factors for OA relate to age, sex, ethnicity, genetics, and preceding history of injury or joint trauma. Potential alterable risk factors for OA include overweight, some occupations and sports, joint damage (injury protection), joint deformation, and quadriceps weakness.

2.1.1 Aging

In an elderly population, chondrocytes encounter major changes in degeneration diseases for example OA. Degenerative disc disease affects the chondrocytes phenotype and the total tissue constituent, architecture, and feature, with many important aspects of the mechanisms, relate to cartilage homeostasis, improvement, and degeneration. The apparent pericellular zone of extracellular matrix (ECM) instantly be on all sides of chondrocytes may hold the importance to comprehension of these mechanisms (Davies et al., 1962; Meachim and Stockwell, 1979). Aging and the evolution of cartilage deterioration relate to numerous factors, which either alone or in combination may cause the onset of OA. Many shreds of confirmation indicate that one factor may persuade a number of serial responses and structural modifications, which either affect the cartilage ECM or cell responsibility which makes the tissue weaker to compressive loads or injury (Grogan and D'Lima, 2010). Aging is related to progressively lower cellularity in articular cartilage possibly outcome of cell death over time. Cell death form of apoptosis has been associated with OA (Temple et al., 2007).

2.1.2 Overweight

OA is many regular joint disorders with signs and symptoms usually showing in the hands, knees, hips, back, and neck (National Institute of Arthritis and Musculoskeletal and Skin Diseases, 2014). Clearly, the existence obese increases, the

responsibility placed on the joints, such as the knee, which enhance the force and could it is possible hasten the malfunction of cartilage (Creamer and Hochberg, 1997).

2.1.3 Previous injury

Before the traumatic injury to cartilage, ligaments, and/or meniscus also extend the risk of expanding OA in the affected joint. Post-traumatic arthritis makes up about 12% of all OA events and can result from injuries sustained in automobile or military accidents, falls, or sports (Punzi et al., 2016). People with histories of an in ancient times torn anterior cruciate ligament (ACL) or meniscus are 2.5 times more probably to expand knee OA and 4 times more probably to through an eventual total knee arthroplasty (Vina and Kwoh, 2018; Hunter et al., 2006). During young sportspeople who support an ACL injury, 10-90% will advance to OA within 10-20 years. Moreover, surgical remodeling and rehabilitation do not present to alleviate the risk of developing OA succeeding ACL injury (Padua et al., 2018).

2.1.4 Occupations and sports

The examination has clearly exposed the health advantage of the light to moderate physical activity in taking down OA-related joint pain. While the boundary to which participation in not interacting with recreational sports or again repeatedly activities may increase the possibility of OA has not been fully explained (Driban et al., 2017). There is confirmation that some occupations (e.g., construction, healthcare, farming, law enforcement, first responders, military service) which relate to long-standing, kneeling squatting, lifting, and repetitiveness motion resulting in heavy mechanical stress on a joint, increase the risk of OA and can degrade symptoms. Osteoarthritis and back pain are the extreme common estimates related to disability-causing by the military service, both during times of peacetime and war (Yucesoy et al., 2015; Cameron et al., 2016; Patzkowski et al., 2012). High-impact professional sports (e.g., hockey, soccer, and football), which make repetitive loading or over force, in adding to joint trauma, puts players at the possibility of OA. In a systematic examination, Driban et al. (2017) establish that in addition to elite-level sportsmen (soccer, long-distance running wrestling, and weightlifting), nonelite soccer sportsmen were also at the possibility of developing OA. Appropriate preventative/preventive measures, such as stretching and strengthening exercises, appropriate footwear, and

other helpful devices, along with helpful workplace plans can assist to reduce the onset and development of OA in occupational and sports settings.

2.1.5 Joint position and muscle strength

Knees that are not mechanically alignment appropriately-resulting in either varus (bowlegged) or valgus (knock-kneed) alignments which can accrue possibility of knee OA. In addition, the degenerate the malalignment, the considerable refuse in a physical role likely to be expert by patients. Knee braces or shoe inserts may help with pain and stiffness when these conditions exist (Sharma et al., 2001; Heidari, 2011). Weaker quadriceps strength has been concerned to expand basic disability and pain in patients with knee OA (O'Reilly et al., 1998; Bacon et al., 2018). Muscle build-up exercises with a physical therapist or through a mutuality-based intervention program can help to lower pain and work restrictions.

2.2 Anatomy of the meniscus

Menisci are semilunar in shape. They form fibrocartilagenous morphology which inserts between the femoral condyles and the tibial plateau on the medial and lateral sides of the knee (Messner and Gao, 1998). Menisci function as the centralload-bearingg in the knee joint, including joint balance, shock absorption, and protection of articular cartilage from excessive stress (Rath and Richmond, 2000). Discard menisci reduces contact space in the joint and increases peak stress on articular cartilage load-bearing zones (Paletta et al., 1997). Some or total meniscectomies result in supra-physiological stress on the articular cartilage, which can cause knee degeneration and OA (Mc Dermott and Amis, 2006).

The menisci are in the knee joint and contain both a medial and a lateral intrinsic placed between the corresponding femoral condyle and tibial plateau (Kohn et al., 1995). The part of the meniscus divides into a glossy-white, compound tissue including cells, functional ECM molecules, and region-specific perception and vascularization. Both menisci are critical components of a healthy knee joint (Kohn et al., 1995; Greis et al., 2002). The major protect ligaments are the medial collateral ligament, transverse ligament, and meniscofemoral ligaments, which are located at the anterior and posterior horns (Kusayama et al., 1994). The meniscofemoral ligaments, also known as the Humphrey and Wrisberg ligaments, attach the posterior horn of the

lateral meniscus. Only forty-six percent of people have both ligaments, but 100% of people have at least one (Kusayama et al., 1994). Human medial and lateral menisci have obviously unlike size. Lateral menisci are about 32.4-35.7 mm in length and 26.6-29.3 mm in width. Medial menisci are 40.5-45.5 mm long and 27 mm wide (Shaffer et al., 2000). Though both menisci are roughly wedge-shaped and semi-lunar shaped, the lateral menisci show many varieties in size, shape, thickness, and mobility than the medial meniscus (Clark et al., 1983).

Vascular formation in the meniscus is of high relevance. From prenatal development until shortly after birth, the meniscus is completely vascularized. After, however, vascular formation appears to diminish. At 10 years of age, vascular formation is presented around 10-30% of the meniscus. In adulthood, the meniscus contains blood vessels and nerves only in the peripheral 10-25% of the tissue. Afterward, 2 distinct areas of the meniscus can be prominent: the outer, vascular/neural region (red-red zone), and the inner, avascular/abneural region (white-white zone). These 2 areas are separated by the red-white region. In early growth, all meniscus cells show similar cellular morphology in the label of size and shape with no area variations. Anyway, after development, morphology, and phenotype distinct the cells present, which also vary in terms of number and topographic localization (Figure 2.1).

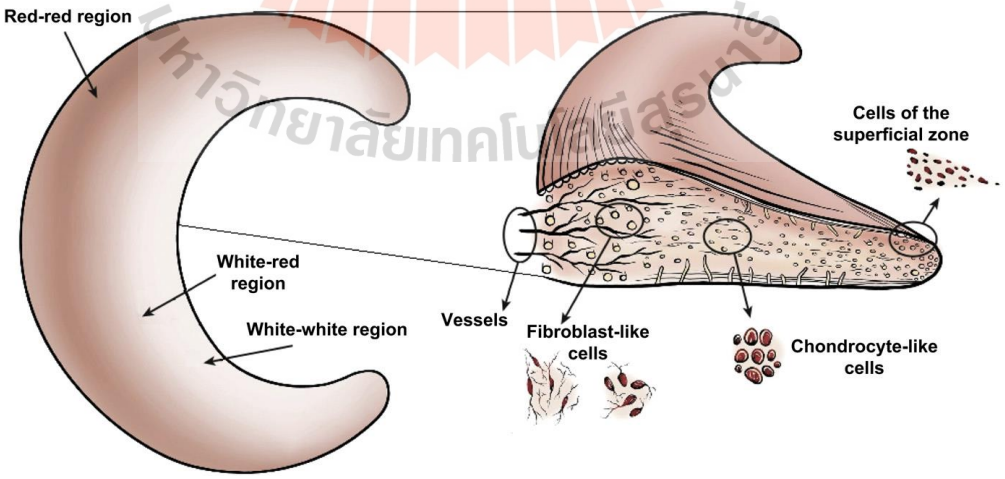

Figure 2.1 Regional variations in vascularization and cell populations of the meniscus.

2.2.1 Medial meniscus

The medial meniscus has a greater anterior-posterior length than width. The fan-shaped interjection of the anterior horn location at the anterior intercondylar fossa of the tibia, with 6-7 mm anterior to the insertion of the anterior cruciate ligament (ACL) (Swenson et al., 1995) (Figure 2.2). In sixty-four percentage of cases, a fibrous strip called the transverse ligament attaches to the anterior horns of the medial and lateral menisci (Johnson et al., 1995). The bigger posterior horn connects to the posterior intercondylar fossa of the tibia and lies posterior to the insertions of the lateral meniscus, but anteromedial to the insertion of the posterior cruciate ligament (PCL) (Moreno, 1995). The total area of tibial entheses of the medial meniscus is larger than that of the lateral meniscus (Swensen and Harner, 1993). In addition, the anterior enthesis of the medial meniscus is 1.3 to 1.7 times larger than that of the posterior enthesis. In rabbits, a larger area of enthesis has been presented to have a higher tensile load to failure. This has not yet been accepted in humans. The medial meniscus is connecting to the joint along its entire periphery. The joint capsule condenses into a distinct band forming the deep medial collateral ligament, which attaches firmly to both the femur and tibia (Swensen and Harner, 1993). The capsular and bony attachments of the medial meniscus constrain its motion, possibly accounting for the higher frequency of injury compared to the lateral meniscus (Moreno, 1995; Swensen and Harner, 1993).

2.2.2 Lateral meniscus

The lateral meniscus has more circular in shape, with about the same width as the anterior-posterior sizes (Figure 2.2). It corresponding covers more tibial surface area than the medial meniscus (Clark and Ogden, 1983). The all-meniscal periphery is loosely connected to the joint, and there is no specification for the lateral collateral ligament. The posterior convexity of the meniscus is indented by the popliteus tendon and accepts an aponeurotic elongation from the popliteus muscle. Its anterior horn inserts anteriorly to the lateral intercondylar predominance of the tibia, but laterally and posteriorly to the ACL connection which is to some extent blends. The posterior horn inserts posteriorly to the lateral intercondylar predominance of the tibia, however anteriorly to the posterior enthesis of the medial meniscus. For fifty percent of cases, anterior fibers enlarge from the posterior

interference ligament to the lateral side of the medial femoral condyle, forming the anterior meniscofemoral ligament, or ligament of Humphrey (Moreno, 1995; Humphrey, 1858; Wan and Felle, 1995). For seventy-six percent of cases, the posterior fibers of the posterior insertion ligament cross obliquely and connect to the intercondylar fossa of the medial femoral condyle, just posterior to the PCL insertion on the tibia. This forms the posterior meniscofemoral ligament or ligament of Weisberg. The absolute function of the meniscofemoral ligaments is debated, but they are thought to upgrade joint congruity by stabilizing the posterior horn of the lateral meniscus for knee flexion and to serve as minor restraints for translation of the tibia (Friederich and Brien, 1990). Insertion region of the lateral meniscus are still controversial. Kohn and Moreno (1995) reported that the anterior enthesis of the lateral meniscus was 1.2 times smaller than that of the posterior enthesis, whereas Johnson et al. (2001) describe that the anterior enthesis was 1.5 times larger.

2.3 Locomotor function of the knee joint

Healthy cartilage authorizes bones to glide over each other. Additionally helps in the absorption of shock of motion. The meniscus is ring-like cartilage that is distributed on 2 sides. The inner medial meniscus and the outers lateral meniscus. The main stabilizing ligaments are the medial collateral ligament, transverse ligament, and meniscofemoral ligaments which connect to the anterior and posterior horns (Figure 2.2). The functional of the cartilage is to a cushion between the bones. It transfers weight and movement when moving. The surface of the cartilage disc attracts each other well (Royal College of Physicians Orthopaedic Thailand, 2554).

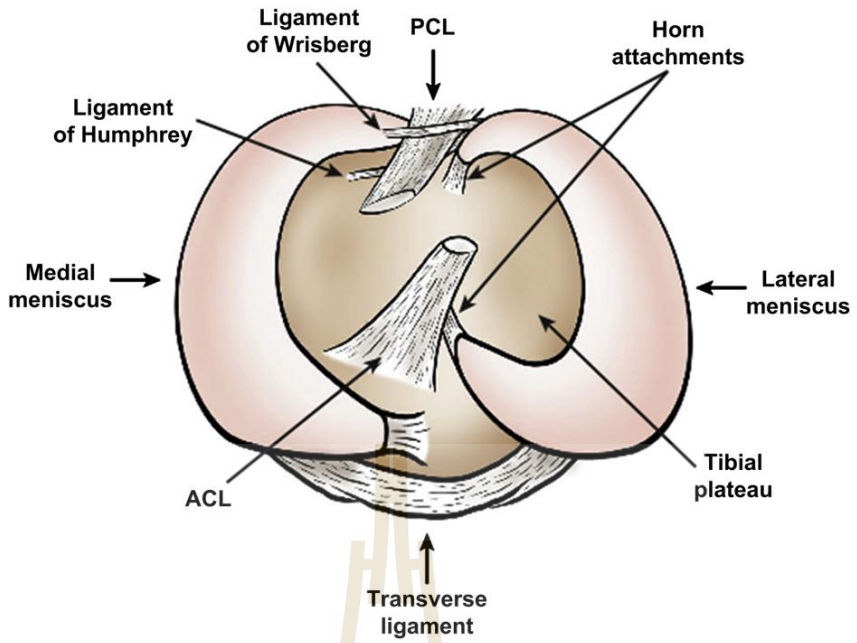

Figure 2.2 Anatomy of the meniscus: superior view of the tibial plateau (Hattam and Smeatham, 2010).

2.4 Compositions of cartilage

Meniscal tissue generally contains water, collagens, and proteoglycans. Collagens are dominated at 60-70% of the dry tissue weight. Type I collagen is the highest composition compared to type II, III, V, and VI (McDevitt and Webber, 1990). Throughout life, the development of chondrocytes is associated with the protection and improvement of cartilage. In aging people, chondrocytes present major transformation to degenerative diseases, for example in OA. The degenerate disc disease effect in chondrocytes phenotype and all tissue constituent, architecture, and properties, with many exacting forms of the synchronization mechanism in cartilage homeostasis, remodeling, and degeneration (Foldager et al., 2014).

2.5 Types of cartilage

Cartilage is a type of connective tissue which composes of specific cells known as chondrocytes along with collagen and elastin fibers. The fibers and cells are implant in a firm gel-like matrix moreover in mucopolysaccharides or glycosaminoglycans (GAGs). These long chains of carbohydrates are synthesized from the cells and help in

the structuring of bone and cartilage. GAGs (formerly called mucopolysaccharides) are so present in the fluid that lubricates joints. Cartilage is not as hard and rigid as bone. It is much more flexible and elastic. Cartilage has no blood or lymphatic vessels. Cells within the cartilage accept nutrition from distribution through the matrix. Cartilage has no nerves also, it is insensitive. Cartilage is nearby a fibrous membrane known as the perichondrium. This perichondrium which covers the bone, and articular cartilage has no perichondrium. Thus, regeneration after injury is thus inadequate nutrition because the perichondrium plays a key role in the regeneration of the cartilage. When cartilage calcifies, the chondrocytes die, and the cartilage is replaced by bone-like tissue. Calcium salts which supply structural stability in bones, are not found in the cartilage matrix. Instead, flexible material known as chondroitin is attendance within the cartilage matrix providing flexibility.

There are 3 types of cartilage. Hyaline cartilage has very thin fibers having the same refractive index as the matrix of the cartilage and thus fibers are not visible. Hyaline cartilage is a bluish-white flexible articular cartilage of long bones, sternum, ribs, etc. Fibrocartilage has many white fibers. It is display in the symphysis of the pubis and sternoclavicular joint, etc. It make a glistening white fibers with an opaque shape. Elastic cartilage has many yellow elastic fibers. It is demonstrated in the ear pinna, external auditory meatus, Eustachian tubes, epiglottis, etc. Its color is yellowish, and the shape is opaque (Pritzker et al., 2006).

2.6 Apoptosis in osteoarthritis

General situations interlace chondrocyte apoptosis, for example the terminal differentiation of hypertrophic chondrocytes from the growth, as well as pathological states participate mice and rat or human cartilage degeneration, such as OA, or the ultra-rare alkaptonuria. Histological study expose lacunar emptying and decrease cell intenseness within osteoarthritic cartilage, advise that cell dying could arise during the OA procedure and even associate in OA onset (Adams and Horton, 1998; Heraud et al., 2000; Blanco et al., 1998; Hashimoto et al., 1998; Millucci et al., 2015).

Apoptosis is a seriously controlled system of cell death that is complicated in the progress of homeostasis and aging. The dissolving of apoptosis induces to pathological states, such as cancer, developmental disorders, and diseases. OA, which

is the most common chronic disease in the elderly people, is analyze by the disruption of cartilage, causing significant disorder. Since the cartilage joints based on only cells living, chondrocytes are essential for the preservation of extracellular matrices. Agreement of chondrocyte functions and survival will cause the defeat of the cartilage joints. In late OA, the cartilage becomes hypocellular and is often appear by lacunar flushing, which is considered to be confirmation that chondrocyte mortality is an major feature in OA present cell death arise in the cartilage (Hwang and Kim, 2015).

The pervading of the type II collagen network is remodel, being uniformly pervading all through the normal cartilage layers, but at a reduced level in OA-degenerated region and at an enlarged level in chondrocytes clusters (Speziali et al., 2015). Malfunction of these constituent is managed by a set of aggrecans (e.g., a disintegrin and metalloproteinase with thrombospondin motifs ADAMTS-4 and -5) and collagenases (e.g., matrix metalloproteases, MMP-1, -3, -8, and -13), which are upregulated, moreover in early stages of OA-associated cartilage degradation. Pro-inflammatory cytokines, such as IL-1, TNF-, and IL-6, are correlated with OA event and associate in cartilage degradation through motivation of pathways (e.g., nuclear factor-$_{\kappa}$B_NF-$_{\kappa}$B, toll like receptor TLR) that control MMPs and ADAMTS upregulation (Goldring et al., 2011). Pathomechanisms of OA, reviewed in 2010, the role of proteins, such as HIF-2 (Yang et al., 2010), MMP-13 (Little et al., 2009), ADAMTS5 (Glasson et al., 2005), discoidin domain receptor 2 (DDR2) (Xu et al., 2010), S100A8/9 (Van et al., 2008), and Syndecan-4 (Echtermeyer et al., 2009, which, once knocked down, reduce or even defeat OA property in an OA mouse model (Van, 2011). Overexpression of these proteins, driven by IL-1 and TNF- in OA (Frisch and Francis, 1994) change to a progressive loss of collagen and proteoglycans, distressing cartilage shape and affect to joint stability and function.

Receptor interacting serine/threonine kinase 1 (RIPK1), a protein that control cell death and inflammation, is thoroughly apparent in the ovaries, lungs, liver, intestines, limbs, and 25 other tissues (Yue et al., 2014). RIPK1 control the apoptosis and necroptosis via kinase-dependent and lost of functions, which are important for cell providence and inflammation (Dannappel et al., 2014; Rickard et al., 2014). Necrostatin-1 (Nec-1) is a particular small molecule inhibitor of RIPK1 that particular

inhibits phosphorylation of RIPK1 and RIPK1-mediated necroptosis and apoptosis (Degterev et al., 2005). Necroptosis is executed by RIPK1 and/or RIPK3 (Newton, 2015).

2.7 Inflammation and OA

Usually, OA was discussed as a degenerative disorder effect from the routine wear and tear in the joints, importantly due to mechanical determinant. This concept was based on the observation that chondrocytes, the only cell type present in cartilage were avascular, not innervated, and have no potentiality to regenerate (Sophia et al., 2009). Although, with the furtherance of molecular biology in the past century, there came a paradigm shift in our comprehension of the pathological mechanisms which is the foundation of OA. There a is strong foundation that OA is a multifactorial disease, and the structural changes discover in OA are due to an association of essence among which inflammation has an important role (Robinson et al., 2016). By the early 21 century, synovitis, which arise due to interactivity between damaged tissue and the immune system, was accepted as a critical characteristic of OA and was received as a driver of the pathogenesis of OA (Zhuo et al., 2012). Inflammation in OA is a feature by the participation of the innate immune system mostly and to some scope, an alterable immune system (Haseeb and Haqqi, 2013). Joint inflammation is clearly a reflex in various of the clinical symptoms of OA, such as joint swelling, warmth, and pain (Sellam and Berenbaum, 2010). Previously that ZCCHC6 knockout mice develop less severe OA due to reduced expression of IL-6 (Ansari et al., 2019). Inflammation of the synovium (synovitis) is a common finding in OA and is identify by synovial hypertrophy and insertion of the sub lining tissue with inflammatory cells (Scanzello and Goldring, 2012). Inflammation in OA is submissive and less pronounced as differentiate to RA and differs in terms of the cellular and molecular players complicated (de Lange-Brokaar et al., 2012). The most receive hypothesis to describe the inflammation in OA is that once the reduce cartilage bits encounter the synovium, they are contemplating foreign particles, and there is a preventive inflammatory responsiveness by the synoviocytes (Berenbaum, 2013). The result of cartilage degenerationcausese the motivation of inflammatory indicator pathways inclusive nuclear factor-kappa b (NF-$_K$B), which a central role in the inflammatory reply (Scanzello, 2017). Inflammation in the joint can also be triggered by the stress

response, and obesity-related systemic inflammation might add to the local inflammation (Scanzello, 2017). Cytokines including IL-1β, TNF-α, IL-6, IL-17, IL-18, and IL-21 have been implicated in the pathogenesis of OA and are among the most widely studied intermediate of inflammation. These inflammatory intermediates are destructive for the joint and originate or preserve the cartilage disadvantage and increase the low-grade inflammation, which may also persuade other inflammatory diseases that are affected by systemic low-grade chronic inflammation (Berenbaum, 2013)

iNOS in OA pathogenesis is manufacturing of NO which is irregularly high in OA cartilage as high levels of NO are built by OA chondrocytes (Scher et al., 2007). Besides, high concentrations of nitro tyrosine, a marker of NO-dependent oxidative damage have been present in synovial fluid from OA patients, and extra NO producing in OA cartilage has been explained by immunostaining with anti-nitro tyrosine antibodies (Loeser et al., 2002). In tandem, demonstration of iNOS has been expression by Western blot and immunohistochemical staining in OA cartilage (Vuolteenaho et al., 2001). High levels of nitrite and iNOS mRNA and protein have been present in the synovium of patients with OA (Ersoy et al., 2002; McInnes et al., 1996). Besides, cartilage is an important source of NO in OA joints. Induce expression of iNOS is present in chondrocytes as compared with synovial cells in OA patients (Melchiorri et al., 1998). Chondrocytes isolation from OA cartilage demonstrates more expression of major iNOS in the superficial zone, except for chondrocytes isolated from patients without OA non express iNOS (Amin et al., 1995). Unaccustomed expression of iNOS shows a crucial role in many inflammatory diseases like OA, colitis, asthma, multiple sclerosis, and psoriasis (Kroncke et al., 1998). iNOS generated NO seems to be crucially involved in the path mechanisms of OA, and it support to the OA pathogenesis by regulate ECM homeostasis and cytokines expression, causality oxidative risk, and chondrocyte apoptosis. Over production of NO by iNOS associated cartilage damage by induce MMP activity and downregulating the biosynthesis of aggrecan and collagen (Lepetsos and Papavassiliou, 2016).

Pharmacologic therapy initiates with simple analgesics and studies. In many patients, simple analgesics inadequate handle moderate arthritis pain. Nonsteroidal anti-inflammatory drugs (NSAID) are representative for or added to the analgesic

therapy. While NSAIDs are effective in domination pain in mild to moderate OA, they are related with significant toxicity (most frequently gastrointestinal) and may even cause difficulty resulting in death. Patients who experience the pain participatory with arthritis would therefore advantage from the anti-inflammatory and analgesic activity of agents that are devoid of significant toxicities. Cyclooxygenase-2 (COX-2) inhibitors are being assess in clinical experiment. These agents present to inhibit only the COX-2 isoenzyme, which is assembled largely during inflammation and is responsible for the biosynthesis of prostaglandins and other intermediate of inflammation as well as sensitizers to pain. Because COX-2 inhibitors do not inhibit COX-1 isoenzyme activity at pharmacologic concentrations, they are devoid of many of the toxicities that are typical side effects of NSAIDs. Short-term studies in dental pain, OA, and rheumatoid arthritis establish that the COX-2 inhibitor celecoxib was an effective analgesic but did not cause gastroduodenal erosions. The possible to provide analgesia and anti-inflammatory action in patients with arthritis without the side effects of NSAIDs (Lane, 1997). Besides studies are desire to substantiate this discovery.

2.8 Medicines involving osteoarthritis

Many drugs are known as NSAIDs or painkillers, which control the inflammation and reduce pain caused. However, these drugs do not prevent arthritis. The drug which is used to inject into the knee joint is steroids which can be helpful with severe knee arthritis. The other one is a synovial fluid injection (Royal College of Physicians Orthopedic Thailand, 2554).

2.9 Chondrocytes and OA

Human chondrocytes are acquiring from human menisci cartilage of donors with OA patients. OA is an inflammatory ailment differentiate by expand degradation of cartilage tissue in the joint due to the overproduction of enzymes disgrace the extracellular matrix. Despite the beginning proliferation and activation of chondrocytes, they are not able to successfully repair the degrading cartilage. As an alternative, chondrocytes undergo terminal disparity and in the end apoptosis, leading to mineralization of cartilage in a procedure resembling bone establishing during

enlargement. Therefore, human chondrocytes OA provide a useful model to study modify in chondrocyte biology in reply to the abnormal environment of the OA joint.

Many substances display an anti-apoptotic purpose in rat chondrocytes. Indeed, intra-articular injection of osteoproteger in (i.e., via adenovirus-mediated osteoproteger in vector (Ad-OPG) lower proteoglycan loss and prevents chondrocyte apoptosis in a collagen-induced arthritis rat model) (Feng et al., 2015). In vitro, amiloride (inhibitor of acid-sensing ion channels) supply protection against acid-induced apoptosis in rat chondrocytes through the repairing of mitochondrial membrane potential and Bcl-2 mRNA level (Rong et al., 2012). Remarkably, prolactin (PRL) inhibits apoptosis both in vitro and in vivo (Adan et al., 2013). In vitro, PRL inhibits the apoptosis of rat cultured chondrocytes in response to a mixture of proinflammatory cytokines (TNF-, IL-1, and IFN) by prevention the induction of p53 and reducing the Bax/Bcl-2 ratio through a NO-independent, JAK2/STAT3-dependent pathway. In vivo, induce hyperprolactinemia in rats before or after inducing the supplement model of inflammatory arthritis lower chondrocyte apoptosis whereas proapoptotic effect of cytokines cocktail was supplement in PRL receptor-null mice (Adan et al., 2013).

2.10 Snail mucous and OA

The snail mucus is a substance that covers the entire external surface of the animal. The mucus is secreted from salivary epidermal glands orientated at the foot of the snail (pedal glands). There are studies that state the relationship between mucus and OA. Mucin from jellyfish has been reported to have probable disease-modifying effects on osteoarthritis on rabbit cartilage (Ohta et al., 2009). This action has confidence in to be due to the potentiality of the mucus to recover the viscosity and friction feature of the synovial fluid and increase in self- bundling capacity of the cartilage. The snails mucus can be a potential disease-modifying OA agent, compared to the potential osteoarthritic effect of snail mucus to that of glucosamine in a trial model of knee OA in dogs (Ajadi et al., 2013). The land snail has been used in medicine since ancient times. The mucus from snails has been described to slow down the inflammation of the lungs (Bonnemain, 2005). Besides, lecithin of the land snail has

been used as a evidential descriptor for some cancers, such as those of the breast, stomach, and colon (Dwek et al., 2001).

2.11 Glucosamine and OA

External method management of glucosamine may work in OA is unspecified. It is believed that glucosamine may have a key role in controlling the anabolic processes of cartilage and in the synthesis of synovial fluid. In addition, it may inhibit the degenerative and catabolic process of OA with its anti-inflammatory and even antioxidant efficacy. It is described that glucosamine may affect the cytokine intermediate pathways to control inflammation, cartilage damaged, and immune responses (Chan et al., 2006; Imagawa et al., 2011). *In vitro* experiment on chondrocytes isolation, or cartilage explanation from healthy or OA patients, provision many premises for the present mechanisms concerning about glucosamine supports joint health. Glucosamine enhances the production of cartilage matrix components in chondrocyte culture, such as aggrecan and collagen type II (Lippiello, 2007; Varghese et al., 2007). Glucosamine help increases hyaluronic acid produced in synovium explants. Besides experiments have shown that glucosamine protect collagen degeneration in chondrocytes by inhibiting lipoxidation interaction and protein oxidation (Tiku et al., 2007).

Glucosamine is an amino sugar that acts as a precursor in the biosynthesis of glycosaminoglycan (GAGs) and proteoglycan aggregates (de los Reyes et al., 2000). Glucosamine and snail mucus inhibited the progression of the experiment persuade OA in dogs. There were no differences in plasma concentration of IL-6 and MMP-3 following management of glucosamine or snail mucus (Adetola et al., 2013). Oral glucosamine hydrochloride protective the loss of GAG in joint cartilage degeneration by intra-articular injection of chymopapain (Oegema et al., 2002). Moreover, to their important component in the joint, glucosamine also have anti-inflammatory feature. In vitro studies showed that glucosamine inhibited cartilage catabolic responses and prevention IL-1b-induced excess in nitric oxide synthesis (Häuselmann, 2001; Fenton et al., 2002).

2.12 Undenatured collagen type II and OA

Undenatured collagen type II is a nutritional supplement and product containing glycosylated. Undenatured collagen type II is available for the management of osteoarthritis (Lugo et al., 2013; Lugo et al., 2016; Bagchi et al., 2002). It can be used to enhance joint health, promotes joint flexibility, relieve joint pain, protect OA symptoms (Pabhoo1 and Billa, 2018), and recover the quality of life, and physical and mental health of OA patients. So, it has the potential to enhance a revolutionary functional raw material for lower the symptoms of arthritis, with wide market development opportunities. The possible mechanism of undenatured collagen type II for OA improve may be through oral immune tolerance (Ausar et al., 2001; Lerman et al., 2016). Extremely, undenatured collagen type II can be procedure by antigen-presenting cells and display by a important histocompatibility compound to activate regulatory T cells in Peyer's patches (a group of gut-associated lymphoid tissues). Although, there is no systematic study that which point of interest on the effects of various doses of undenatured collagen type II on inflammatory intermediated and oxidative stress as well as the pathophysiology of synovium and cartilage tissues.

CHAPTER III

RESEARCH METHODOLOGY

3.1 Tissue collection

Menisci samples were collected from Suranaree University Hospital. The medial and lateral menisci from patients who underwent knee replacement were cut into horns and zones as shown in Figure 3.1.

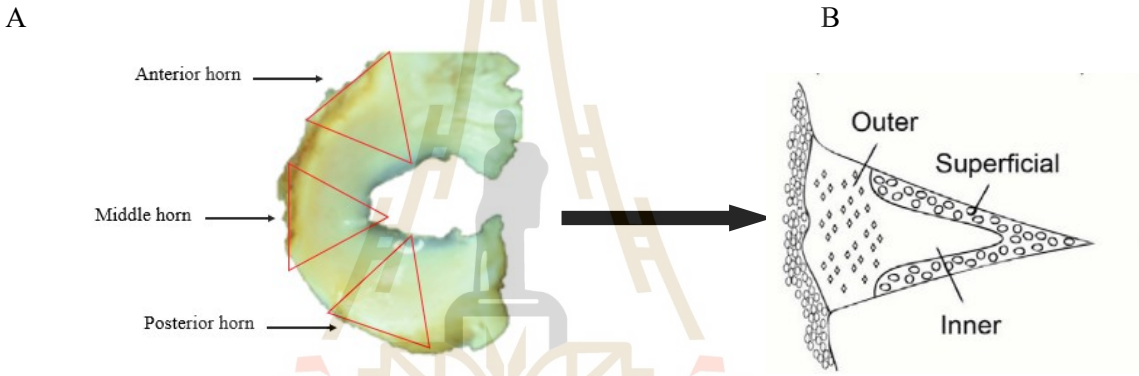

Figure 3.1 Collection of horns (A) and zones (B) of the meniscus (Eleftherios et al., 2011).

3.2 Tissue preparation

The collected menisci were fixed in 4% formalin solution for 24 hr., dehydrated with graded series of ethanol (70%-100%), and finally embedded in paraffin. The tissue blocks were sectioned serially with a rotary microtome (Olympus DP72) at the thickness of 4 μm.

3.3 Morphological study of the menisci under a light microscope

3.3.1 Hematoxylin and eosin staining

The tissues sections were stained with hematoxylin and eosin using the conventional method. H&E is the combination of two histological stains: hematoxylin and eosin. The hematoxylin stains cell nuclei in purplish blue, and eosin stains the

extracellular matrix and cytoplasm in pink (Wissowzky, 1876). Briefly, the sections were immersed in xylene to eliminate paraffin. Subsequently, the sections were rehydrated by immersing in graded series of ethanol (100-70%) and then distilled water. Finally, the sections were dehydrated with graded series of ethanol (70%-100%), cleared with xylene, and mounted per mount. The structure of the cells was determined by hematoxylin and eosin staining.

Stained cells in this experiment were classified into grades of OA using the criteria of Jeanie Kwok and coworkers (2016). The total scores for all criteria (tissue surface structure, cellularity, and matrix staining) ranged from 0 to 25. Tissue surface structure at femoral and tibial sides was scored at scores 0-3 for smooth to severs fibrillations. Cellularity in each zone (outer, inner, and superficial zone) was scored at scores 0 for normal cells, 1 for hypercellularity, 2 for diffused hypocellularity, and 3 for hypocellularity. Matrix staining was scored at score 0 for slight staining for pericellular matrix, 1 for slightly disrupted, 2 for moderately disrupted, and 3 for severely disrupted. These ranges were classified into grades 0 through 4 to present progressive stages of meniscal degeneration; Score 0-5 = Grade 0 OA, 6-10 = Grade I OA, 11-15 = Grade II OA, 16-20 = Grade III OA, 21-25 = Grade IV OA (Figure 4). Blind scoring was performed by four separate individuals and average numbers were reported. Finally, the sizes and numbers of chondrocytes in horns and zones of the grade II OA were compared between medial and lateral menisci. For cell counting and sizing methods, 5 photographs/1 sections were visualized with a total of three sections.

Table 3.1 Criteria, scores, and observations for histological assessment of menisci. The range of possible total scores is 0-25 (edit from Kwok et al., 2016).

Criteria	Score	Histology
Tissue Surface Structure		
Femoral and tibial side, inner rim	0	Smooth
	1	Slight fibrillation or undulating
	2	Moderate fibrillation or undulating
	3	Severe fibrillation or undulating,
		- Disruption or total loss of tissue

Table 3.1 Criteria, scores, and observations for histological assessment of menisci. The range of possible total scores is 0-25 (edit from Kwok et al., 2016) (Continued).

Criteria	Score	Histology
Cellularity		
Outer region	0	Normal distribution of fusiform cells
	1	Hypercellularity
	2	Diffused hypocellularity- Few empty lacuna
	3	Hypocellularity -Empty lacuna, cyst, matrix separation
Inner region	0	Normal distribution of round cells
	1	Hypercellularity
	2	Diffused hypo/acellular zones
	3	Hypocellularity- Empty lacuna, cyst
Superficial zone	0	Normal distribution of round cells
	1	Hypercellularity- Cell clustering
	2	Diffused hypo/acellularity- Cell shrinkage
	3	Hypocellularity
Matrix Staining		
Outer region	0	Normal - slight staining of PCM
	1	Slightly disrupted
	2	Moderately disrupted
	3	Severely disrupted
Inner region	0	Normal - slight staining of ECM
	1	Slightly disrupted
	2	Moderately disrupted
	3	Severely disrupted
Superficial zone	0	Normal - homogenous staining of ECM
	1	Slightly disrupted
	2	Moderately disrupted
	3	Severely disrupted

3.3.2 Alcian blue pH 2.5 staining

Alcian blue staining (Acros Organics) was intended for histological visualization of sulfated and carboxylated acid mucopolysaccharides and sulfated and carboxylate sialomucins (glycoproteins). Glycoprotein is a protein conjugated with the carboxyl group. The sections were deparaffining with xylene and rehydrated by alcohol series, then incubated with alcian blue for 1 hr. The following staining was periodic acid (Acros Organics) and Schiff reagent which indicated type of neutral mucopolysaccharides. The sections were dehydrated with alcohol series, cleared with xylene, and finally mounted per mouth.

3.3.3 Picrosirius red for collagen staining

Picrosirius red staining (Abcam, Cambridge, MA) was used to distinguish between collagen types I and III under polarized light (Nikon, SP-NIK-OPTO2POL). It is one of the best understood histochemical techniques able to selectively highlight collagen networks. Relatively inexpensive, the technique relies on the birefringent properties of collagen molecules. While the picrosirius red stain alone does not selectively bind the collagen network, it becomes more specific than the other common collagen stains when combined with polarized light detection (Laure, 2017). Tissue sections were deparaffinized and hydrated in distilled water. Adequate picrosirius red was applied to the tissue sections and incubated for 60 minutes, and then rinsed quickly in acetic acid. Subsequently, the sections were dehydrated with alcohol series, cleared with xylene, and finally mounted with per mouth. The distributions of proteoglycan and collagen were then compared between horns and zones of medial and lateral menisci in the same grade of OA.

3.3.4 Immunohistochemistry for detection of collagen type I

Immunohistochemistry (IHC) is a method for the detection of antigens in the cells of a tissue segment using the principle of antigen-binding antibodies specifically in biological tissues. Antibody-antigen binding can be visualized in the sample. Enzymes such as Horseradish Peroxidase (HRP) or Alkaline Phosphatase (AP) are often used to catalyze the color reaction. To determine the localization of collagen type I in the meniscus, the method was performed according to a previous study (Gambadella et al., 2010). The paraffin sections were deparaffinized, and rehydrated, followed by an elimination of endogenous peroxidase and picric acid using 1%

hydrogen peroxide and 1% lithium carbonate (Sigma Aldrich), respectively. Free aldehyde was eliminated by 0.1 M glycine (Acros Organics), and the cell membrane was broken by 1% Triton X-100 (Fibertech Co., Ltd, Seoul, Korea). Subsequently, the sections were placed in citrate buffer (pH 6, Acros Organics) and heated in a microwave oven for 10 min, 3 times, to increase binding between the antigen and the antibody. The non-specificity was blocked by emerging the sections in 0.1 M PBS containing 4% bovine serum albumin (BSA, Acros Organics) for 1 hr at room temperature. The sections were then incubated with rabbit anti-collagen type I polyclonal antibody (Abcam, Cambridge, MA), at the dilution of 1:100, and left at 4°C, overnight. The sections were extensively washed with PBS containing 0.1% Tween-20 (Amresco, Solon, OH), twice (10 min, each), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) (1:500) for 1 hr at room temperature. After extensively washing, the antigen-antibody complex was visualized by adding the substrate, 3, 3'-diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA). After stopping the enzyme-substrate reaction, the sections were dehydrated, cleared with xylene, mounted using per mount, and observed under a light microscope. Negative control was performed by omitting the primary antibody.

3.4 Detection of cell apoptosis by TUNEL assay

Tissue sections were deparaffinized in 3 changes of xylene for 5 minutes, each. The sections were then emerged in 2 changes of absolute ethanol for 5 minutes, each, followed by emerging in 95% ethanol and 70% ethanol for 3 minutes, each. The sections were washed with PBS for 5 minutes. To pretreat the tissue, proteinase K (20 µg/mL) was applied to the sections for 15 minutes at room temperature in a coplin jar or directly on the slide (~60 µL/5 cm^2). The sections were then washed twice with dH_2O for 2 minutes, each. Endogenous peroxidase was eliminated with 3.0% hydrogen peroxide in PBS for 5 minutes at room temperature. The sections were rinsed twice with PBS or water, for 5 minutes each. Excess liquid was removed, followed by immediately adding 75 µL/5 cm^2 equilibration buffer directly on the sections and incubated for at least 10 seconds at room temperature. Subsequently, Working Strength TdT Enzyme was applied, followed by gently tap off excess liquid and carefully blotting or aspirating around the sections. Fifty-five µL/5 cm^2 of working

strength TdT enzyme was immediately pipetted onto the sections and incubated in a humidified chamber at 37°C for 1 hour. Then, stop/wash buffer was applied, agitated for 15 seconds, and incubated for 10 minutes at room temperature. An aliquot of the anti-digoxigenin conjugate from the stock vial was prepared for the desired number of sections. The aliquot was stranded in and applied to the section. The sections were then washed at room temperature in 3 changes of PBS for 1 minute, each. Excess liquid was gently tap off and carefully blot or aspirate around the sections. The anti-digoxigenin conjugate was applied to the slides (about 65 µL/5 cm^2 of surface area) and incubated in a humidified chamber for 30 minutes at room temperature. The sections were washed with 4 changes of PBS for 2 minutes per wash at room temperature. The color was developed within a peroxidase substrate (75 µL/5 cm^2) and stained for 3 to 6 minutes at room temperature. In order to determine the optimal staining time, color development was monitored under a microscope. The sections were then washed in 3 changes of dH_2O for 1 minute, each, and incubated in dH_2O for 5 minutes at room temperature. The sections were counterstained in 0.5% (w:v) methyl green for 10 minutes at room temperature and subsequently washed in 3 changes of dH_2O, dipped 10 times each in the first and second washes, followed by 30 seconds without agitation in the third wash. After that, the sections were washed in 3 changes of 100% n-butanol, dipped in the first and second washes, for 10 mins, each, followed by 30 seconds without agitation in the third wash. Finally, the sections were dehydrated with xylene and drained by a gently tap. The sections were not allowed to dry. The numbers of apoptotic cells in horns and zones were compared between medial and lateral menisci in the same grade of OA.

3.5 Treatments of LPS-induced chondrocytes with snail mucus, glucosamine, and undenatured collagen type II

Snail mucus extract (Kungthepchemi Thailand), glucosamin (Phitsanuchemicals Co., LTD, Thailand), and undenatured collagen type II were tested with culturing chondrocytes under a inflammatory condition, which was induced by 1 µg Lipopolysaccharide (LPS) for 2 hr at 37°C with 5% CO_2 prior to the treatment.

3.5.1 Isolation and identification of chondrocytes

Chondrocytes were isolated from the menisci. The menisci were washed in phosphate buffered saline (PBS) 3 times and minced into small pieces, followed by digested with 2% collagenase (Gibco, Thermo Fisher Scientific) overnight at 37°C with 5% CO_2. Thereafter, the cells were collected by centrifugation at 3000 rpm for 5 min. The pellets were suspended in a complete culture medium (CCM) (Gibco, Thermo Fisher Scientific). The freshly isolated chondrocytes were seeded into chambers at 30,000 cells/cm^2 and incubated in CCM for 24 hours at 37°C in a humidified air atmosphere containing CO_2 to allow them to adhere. The adherent isolated cells in a primary culture were identified the characteristics of chondrocytes cells by immunocytochemistry (ICC). 20,000 cells/cm^2 were seeded in a dish and incubated in CCM for 24 hours at 37°C in a humidified air atmosphere containing CO_2. Cells were fixed with cold methanol for 20 min and washed with PBS. Blocking was performed with 1% BSA for 1 hr. Primary antibodies were rabbit anti-collagen I polyclonal antibody (1:100) (Invitrogen, Thermo Fisher, Scientific), rabbit anti-collagen II polyclonal antibody (1:200) (EMD Millipore, Sigma US), mouse anti-aggrecan monoclonal antibody (0.1:200) (Abcam, Cambridge, MA), rabbit anti-Sox 9 monoclonal antibody (1:200) (Abcam, Cambridge, MA). Before application of secondary antibody, cells were washed with PBS. Secondary antibodies were 1:200 goat anti-rabbit IgG (Alexa Fluor® 488), or goat anti-mouse IgG (Alexa Fluor® 647), Nuclei were stained with DAPI (1:500) for 10 min and washed with PBS. Reactivities were observed under a fluorescence microscope (Nikon ECLIPSE Ti), with NIS-Elements D4.00.12 program.

3.5.2 The experimental group of chondrocyte cell culture for therapeutic

Isolated chondrocytes were cultured and treated with snail mucus extract, glucosamine, and undenatured collagen type II prior to induction of inflammation. Chondrocyte cells were incubated with snail mucus at 100, 50, and 25 µg/ml for overnight at 37°C with 5% CO_2. Negative control was performed using cells that were not exposed to mucus (Trapella et al., 2018). Similarly, chondrocyte cells were incubated with glucosamine at concentrations of 500, 250, and 125 µg/ml, while negative control was cells which were not exposed to glucosamine. The treated cells were incubated overnight at 37°C with 5% CO_2. Undenatured collagen type II from chick sternum of Broiler and Korat chicken was used for treatments of

chondrocyte cells at 150, 100, and 50 µg/ml, with an incubation for overnight at 37°C with 5% CO_2. Negative control was performed using cells that were not exposed to collagen. To further characterize the effects of snail mucus, glucosamine, and undenatured collagen type II on chondrocyte cells, the cells were induced with LPS (1 µg) for 2 hr to induce inflammation.

Measurement of the reduction environment (mitochondrial reductase) of cellular mitochondria was performed. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen, Thermo Fisher, Scientific) tetrazolium reduction assay is reduced by mitochondrial reductase. The MTT color changes to the purple of the formazan color. Absorption was measured at 540 nm. This means the number of live cells was counted.

Enzyme-Linked Immunosorbent Assay (ELISA) was used to detect the presence of inflammatory chondrocyte cells. Primary antibodies were rabbit anti-iNOS polyclonal antibody (1:2000) (Invitrogen, Thermo Fisher, Scientific) and rabbit anti - Cox-2 monoclonal antibody (1:500) (Invitrogen, Thermo Fisher, Scientific). The cells were seeded in 96well-plateded (20,000 cells/well) and incubated at 37°C with 5% CO_2, overnight. LPS was added to the cells for 2 hr. Treatment consists of glucosamine (500, 250, and 125 µg/ml), snail mucus (100, 50, and 25 µg/ml), and undenatured collagen type II from chick sternum of Broiler and Korat chicken (150, 100, and 50 µg/ml), and incubated for 24 hr. Cells were then washed with PBS 1X, pH 7.4, lyzed with lysis buffer and resuspended in 4°C buffer at 1 hr. After that cell were coated on an ELISA plate with coating buffer and incubated at 4°C, overnight. After blocking with BSA for 1 hr. at 37°C, primary antibodies (iNOS 1:2000, Cox-2 1:500) were incubated at 4°C for 2 hr. Secondary antibody (goat anti-rabbit IgG-HRP (1:500)) was then applied and incubated for 1 hr. at 37°C. The substrate was added and waited until it began to turn blue. The reaction was stopped with HCL, and color was measured at 450 nm.

CHAPTER IV

RESULTS

4.1 Gross anatomy of menisci

A knee joint contained the menisci, comprised of both medial and lateral menisci situated between the corresponding femoral condyle and tibia plateau (Figure 4.1B). Each meniscus was divided into 3 horns, anterior, middle, and posterior horns (Figure 4.2) In the OA knee, abrasions in the area were found of articular cartilage (Figure 4.1A) fibrillations were also found in the border of menisci (Figure 4.1C).

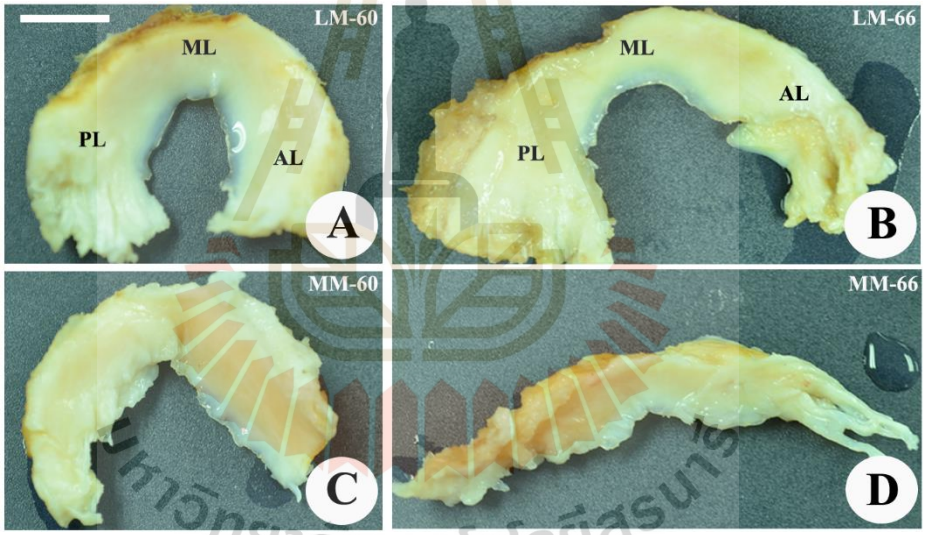

Figure 4.1 A) Lateral meniscus (Age 60); B) Lateral meniscus (Age 66); C) Medial meniscus (Age 60); Medial meniscus (Age 66); AL: Anterior lateral; ML: Middle lateral; PL: posterior lateral; AM: Anterior medial; MM: Middle medial; PM: Posterior medial; Scale bar 1 cm.

The menisci are in the space between the femoral and tibial condyles. They were semi lunar-shaped lamellae, each with anterior and posterior horns, and were triangular in cross-section. The surface of each meniscus was concave superiorly, providing a congruous surface to the femoral condyles, and was flat inferiorly to

accompany the relatively flat tibial plateau. The horns of the medial meniscus were further apart, and the meniscus appears ‘C’ shaped. Though those of the lateral one where the meniscus appeared more ‘O’ shaped. The increasing size of the medial meniscus regrettably leaves a large, declarative area that in turn can be prone to injury.

4.2 Histology of menisci

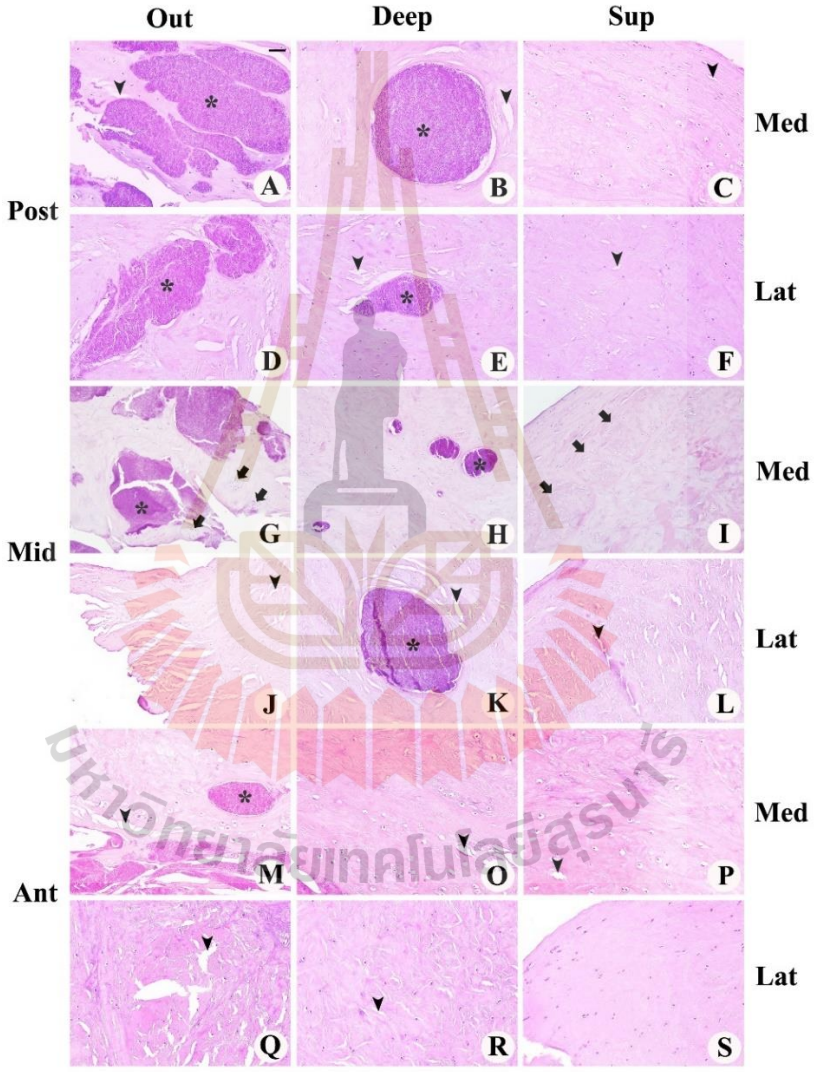

Figure 4.2 Histology of meniscus staining with hematoxylin and eosin. A) Outer zone of the posterior horn; B) Deep zone of the posterior horn; C) Superficial zone of the posterior horn; D) Outer zone of middle meniscus; E) Deep zone of middle meniscus; F) Superficial zone of middle meniscus; G) Outer zone of the anterior horn; H) Deep zone of the anterior horn; I) Superficial zone of the anterior horn; Scale bar 200 μm.

In early stage of OA knee, histological changes were observed in the outer zone of the posterior horn of the medial and lateral menisci. There was an aggregation of small cell clusters called the bone marrow-like region (Figures 4.2A and D (asterisks)). The bone marrow-like region was clearly more abundant in the medial meniscus than in the lateral meniscus. In addition, medial and lateral menisci showed signs of a herniated rupture of the knee bone called fibrocartilage separation, which clearly occurred on the periphery of the medial meniscus. The bone marrow-like region was also identified in the outer zones of posterior horns of medial and lateral menisci. However, they were less in numbers, compared to the outer zone. In addition, they were larger in the medial meniscus than in the lateral meniscus. (Figures 4.2B and E).

A study comparing the pathology of the superficial zone between the medial and lateral menisci revealed that this region did not form a bone marrow-like region as in the outer zone and deep zone, but the presence of fibrocartilage separation was presented. A comparative study of the cells revealed that the number of the hypocellularity was observed in medial and lateral menisci. In addition, hypercellularity was characterized in the medial meniscus (Figure 4.2C).

Histologically, it was found that the outer zone of the medial meniscus showed more pathology than the lateral meniscus, especially at the outer zone. Fibrocartilage separation, and large bone marrow-like regions distributed in the medial meniscus. In contrast, the lateral meniscus showed only the tear. The tissue was not formed the bone marrow-like region. Result from the study comparing the number of cells in the middle part of the medial and the lateral menisci revealed that the outer zone of medial meniscus had a relatively low number of cells (hypocellularity) and the cells tended to cluster together. However, although the tissue of the lateral meniscus showed signs of tearing, the number of cells was normal. The cell distribution was also normal. In deep zones of the medial and lateral menisci, the bone marrow-like region was generally distributed. Sizes of the bone marrow-like regions in the lateral meniscus were larger than those in the medial meniscus. However, the number of bone marrow-like regions was higher at the medial meniscus. The cell number and cell distribution in the deep zone of the medial and lateral meniscui were relatively similar. However, the number of cells was lower than that of normal meniscus cartilage. Cellular changes in the superficial zone and deep zone of the medial meniscus were clearly observed

due to the formation of large clusters. These changes were not observed in lateral meniscus where the cells were distributed widely, similarly to the outer zone. The superficial zones of the medial and lateral menisci did not show the bone marrow-like region like the other parts. The rupture of tissues was clearly seen from the lateral meniscus. However, the superficial zone of the medial meniscus showed a tear (Figures 4.2G-L).

Study in anterior horn, the results showed that the bone marrow-like regions were found in outer zone of medial meniscus (Figure 4.2M (asterisk)). The bone marrow-like region was absent in the lateral meniscus. Only fibrocartilage separation was found. In addition, medial meniscus cells were hypertrophy, with some missing from the lacuna or with pyknotic cells. In a comparative study of cell numbers, the outer zone of the medial and lateral menisci showed a marked reduction in cell proliferation, which could be classified as hypocellularity with fibrocartilage separation. A comparative study of pathology in the deep zone of the medial and lateral menisci revealed that the pathology was different from the posterior horn and middle part because there was no bone marrow-like region in both medial and lateral menisci (Figures 4.2O, R). A comparative study of the cell numbers of the deep zone revealed a significant decrease in the number of cells in the medial and lateral menisci cells (hypo-cellularity). However, the cells in this area showed the same hypertrophy characteristics as in the outer zone. Therefore, it could be concluded that the anterior horn in patients with early-stage knee arthritis, more severity was observed at the deep zone of anterior horn of medial meniscus than the lateral meniscus. A comparative study of pathology in the superficial zone between the medial and lateral menisci revealed that this region did not form a bone marrow-like region, e but showed signs of fibrocartilage separation in the medial meniscus. In addition, cells from the medial meniscus were hypertrophy (Figure 4.2P). The investigators, therefore, concluded that the pathology of the superficial zone of anterior horn of medial meniscus showed more pathology than the lateral meniscus.

4.3 Sizes and numbers of chondrocyte cells in horns and zones of grade II OA compared between medial and lateral menisci

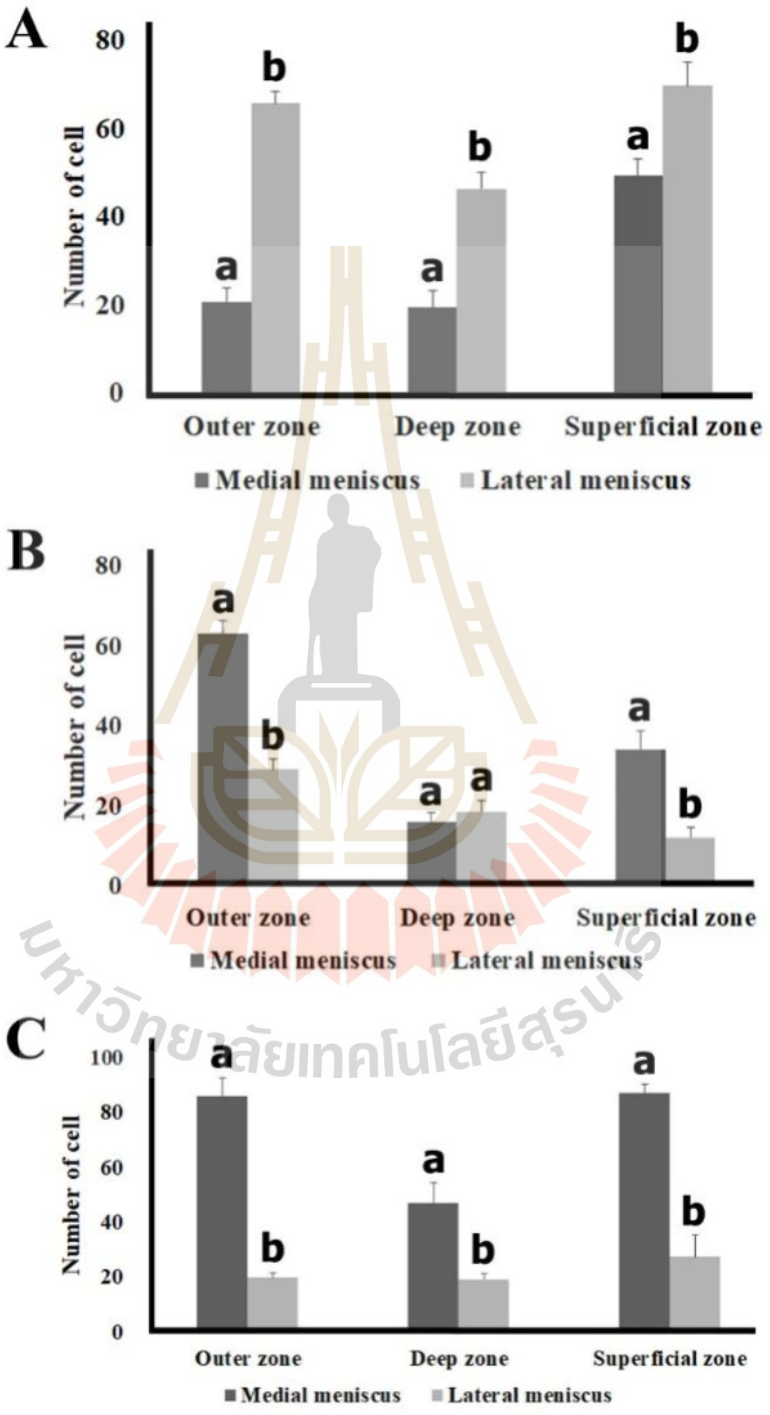

Figure 4.3 Numbers of chondrocyte cells. A) posterior horn; B) middle part; C) anterior horn.

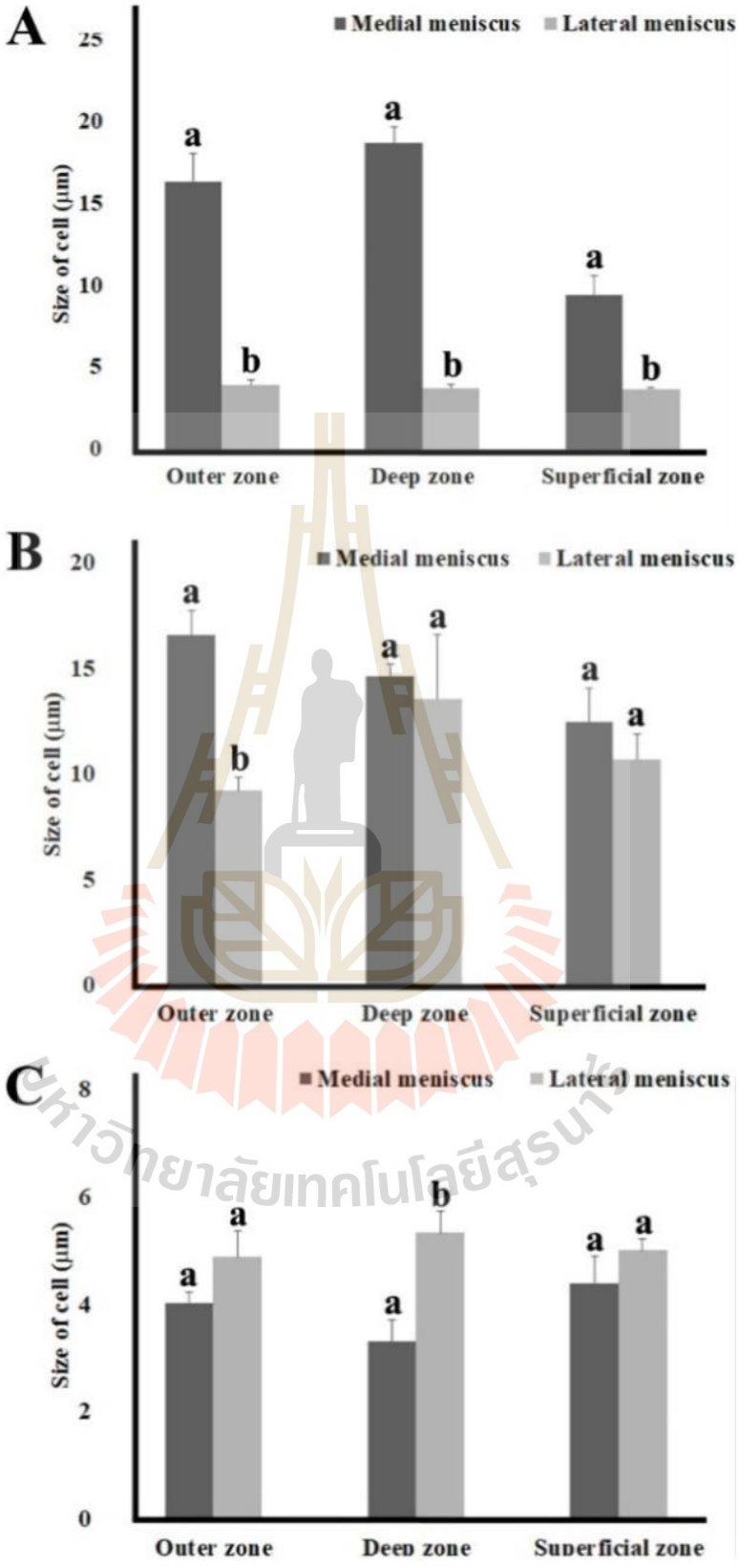

Figure 4.4 Size of chondrocyte cells. A) posterior horn; B) middle part; C) anterior horn.

There was change in cell number from each zone of the medial and lateral menisci. A comparison of cell numbers from the posterior horn revealed that the mean cell numbers from the outer, deep, and superficial zones of the medial meniscus were 20.8±2.7, 19.4±3.8, 49.4±5.5 cells, respectively. Which were significantly less than the numbers of cells in the outer, deep, and superficial zones of lateral menisci (65.8±3.0, 46.4±3.9, 69.8±3.9 cells respectively) ($p \leq 0.05$). Cell numbers of the outer, deep, and superficial zones of normal tissue were 197.6±14.7, 20.4±1.8, 84.8±2.4 cells, respectively. The deep zone of the lateral meniscus showed hyper-cellularity, because the number of cells was significantly higher than that of normal tissue ($p \leq 0.05$). However, the number of cells in the outer zone of the medial and the lateral menisci was revealed by hypo-cellularity, because the numbers of cells were significantly less than in normal tissue ($p \leq 0.05$).

At the middle part of the medial meniscus, cell numbers from the outer, deep, and superficial zones (63.0±3.5, 16.0±2.4, 34.2±4.7 cells, respectively) were significantly higher than those of the outer and superficial zones of lateral meniscus (29.2±2.7 and 12.3±2.9 cells, respectively ($p \leq 0.05$), except the deep zone (18.6±2.7 cells) which was similar to that of the medial meniscus. The outer and the superficial zones of the medial meniscus revealed hypocellularity.

At the anterior horn of the medial meniscus, cell numbers from the outer, deep, and superficial zones were 85.8±7.0, 47.0±7.4, 87.0±3.5 cells, respectively which were significantly higher than that of the lateral meniscus at outer, deep, and superficial zones (19.8±1.9, 19.2±1.9, 27.4±8.0 cells respectively) ($p \leq 0.05$). The study of the cell numbers indicates that changes in cell numbers can be observed from the medial and lateral menisci and were the most prominent in the outer zone (Figure 4.3).

A comparative study of cell size changes from the medial meniscus and lateral meniscus was performed in the outer, superficial, and deep zones. Comparing cell sizes from the posterior horn revealed that cell sizes from the outer, deep, and superficial zones of the medial meniscus (16.3±1.7, 18.6±1.0, 9.4±1.2 μm, respectively) were larger than those of the outer, deep, and superficial zones of the lateral meniscus (3.9±0.3, 3.7±0.2, 3.6±0.1 μm, respectively) with statistically significant ($p \leq 0.05$). Cell sizes were measured from the outer, and deep zones of normal sample. In the

superficial zone, cell sizes were 5.5±0.5, 7.3±0.3, 5.4±0.3 μm, respectively. Medial meniscus cells were hypertrophy cells, because of their significantly larger size than normal cells, and lateral meniscus cells were hypotrophy, because they were clearly smaller than normal cells.

Comparison of cell sizes from the middle part of the menisci revealed that cell sizes at the outer, deep, and superficial zones of the medial meniscus (16.5±1.1, 14.6±0.5, 12.4±1.6 μm respectively) were larger than those of the outer zone of the lateral meniscus (9.2±0.6 μm) ($p \leq 0.05$) , but the cell size from the deep and superficial zones of the lateral meniscus (14.5±3.0, 10.6±1.2 μm respectively) were similarly to those of the medial meniscus, which was not statistically significantly different ($p \leq 0.05$). In addition, sizes of cells in the 3 zones of the middle part of both medial and lateral menisci were larger than those of the normal cells.

Comparisons of cell sizes from the anterior horn revealed that cell sizes from the medial meniscus at the outer, deep, and superficial zones (4.0±0.2, 3.3±0.4, 4.3±0.5 μm, respectively) were significant differences in cell sizes from the lateral meniscus at the outer, deep, and superficial zones (4.8±0.5, 5.3±0.4, 5.0±0.1 μm respectively, $p \leq 0.05$). Comparisons with normal cells were similar. Therefore, the cell sizes above indicated that changes in cell size were observed from 2 zones, the outer zone, and the deep zone, but no change in size was observed in the superficial zone (Figure 4.4).

4.4 Distribution of proteoglycan and collagen in medial and lateral menisci

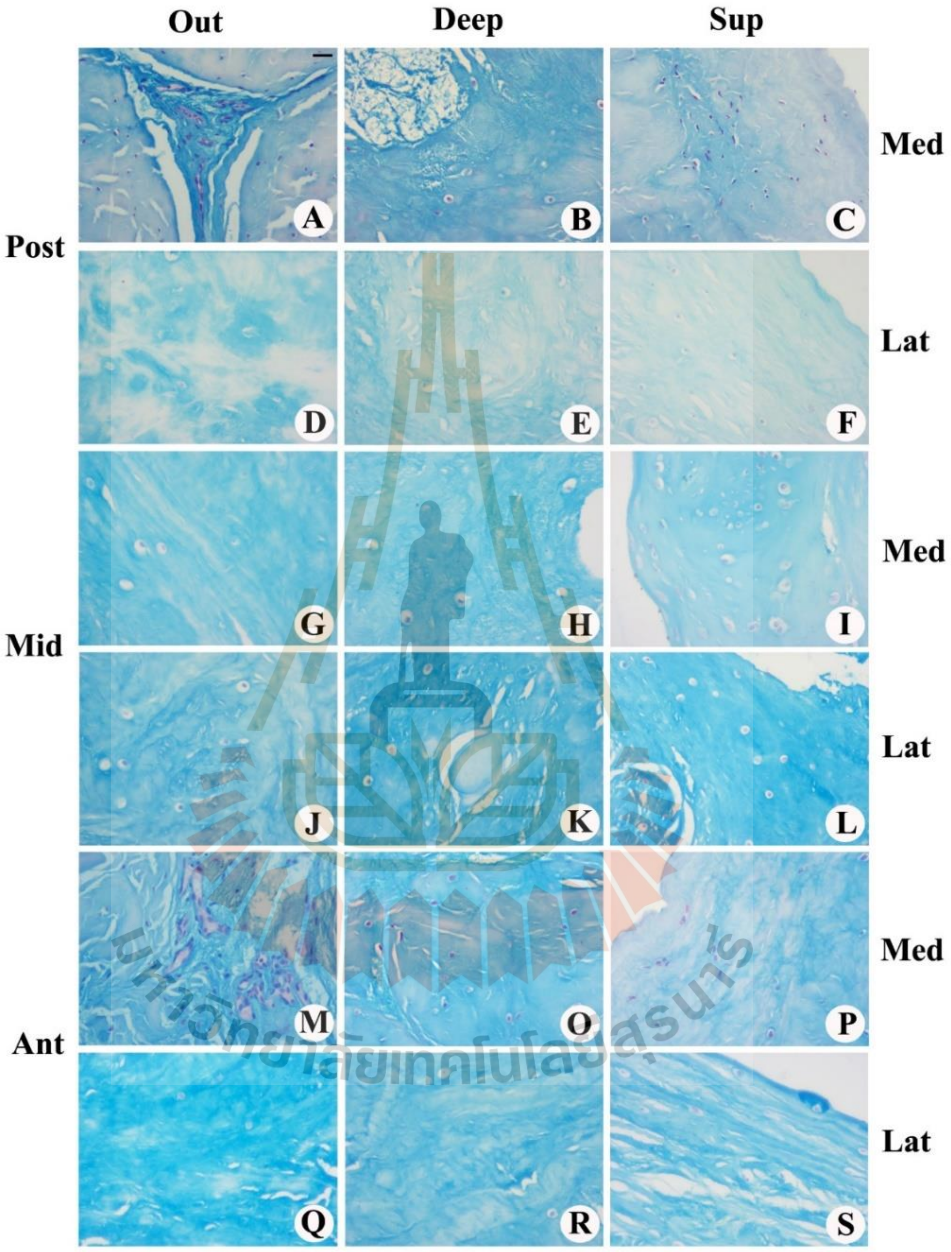

Figure 4.5 Histology of meniscus staining with alcian blue pH 2.5. A) Outer zone of the posterior horn; B) Deep zone of the posterior horn; C) Superficial zone of the posterior horn; D) Outer zone of middle meniscus; E) Deep zone of middle meniscus; F) Superficial zone of middle meniscus; G) Outer zone of the anterior horn; H) Deep zone of the anterior horn; I) Superficial zone of the anterior horn; Scale bar 200 μm.

Table 4.1 Alcian blue staining with meniscus OA.

Horns	Zones	Alcian blue staining		
		Outer	Deep	Superficial
Posterior	Medial	+/++	+/++	+
	Lateral	+	+	+
Middle	Medial	+++	+++	++
	Lateral	+++	+++	+++
Anterior	Medial	++	++	+
	Lateral	+++	++	++

Staining: - no staining, + weak staining, ++ moderate staining, +++ strong staining.

Changes in proteoglycan compared between the medial and lateral menisci are summarized in table 4.1. Study with Alcian blue (pH 2.5) staining, results indicated that the medial meniscus had significantly lower proteoglycan content than the lateral meniscus. In addition, when comparing the changes in the amount of proteoglycan between the medial and the lateral menisci at three horn, it was found that the posterior horn of the lateral meniscus showed the greatest reduction in proteoglycan. However, the comparison between the middle part and the anterior horn of the medial meniscus and lateral meniscus showed low differences in proteoglycan. The amount of proteoglycan from the posterior horn of the medial meniscus decreased the in most at the superficial zone. Changes in proteoglycan and the lateral meniscus are formed in the same way but the staining is more intense than the medial meniscus (Figure 4.5). Pathology affects the proteoglycan content of the outer zone of the meniscus cartilage. In the medial meniscus, alcian blue was stained faintly around the cells. In the lateral meniscus, the surrounding cells were strongly stained with a distinctly dark color. However, the vascular at the periphery of the outer zone of the medial meniscus had an intense alcian blue staining than other regions showing that was more proteoglycan around the blood vessels than in the surrounding area. The study comparing the relationship between amount of proteoglycan and the number of cells revealed that in the area of the meniscus that had hyper-cellularity, amount of proteoglycan was higher than in the area that had hypo-cellularity (Figure 4.5C). In

addition, the study also compared the amount of proteoglycan and the morphology of the cells. The results showed that the peripheral region of the hypertrophy cell had a significantly higher amount of proteoglycan than that of the hypotrophy cells (Figure 4.5D). Comparisons of proteoglycan content between the posterior horn of medial and the lateral menisci at the deep and superficial zones revealed that there was very little difference in proteoglycan content, and the proteoglycan content in the peritoneum was similar to that of the outer zone.

A comparative study of proteoglycan content from the middle part of the medial and lateral menisci revealed that the proteoglycan contents of the outer and the deep zones were relatively similar in that the staining characteristics of Alcian blue were relatively homogeneous (Figures 4.5G-L). However, alcian blue staining from the outer zone of the medial meniscus remained lighter than the lateral meniscus, indicating that the proteoglycan content of the medial meniscus was less than the lateral meniscus. A study comparing the amount of proteoglycan from the superficial zone of the meniscus found that the medial meniscus contained less amount of proteoglycan less than the lateral meniscus. However, a study to compare the cell numbers between the superficial zone of the medial and lateral menisci showed that the medial meniscus had a statistically significantly higher cell number than the lateral meniscus ($p \leq 0.05$). This may be due to the hypertrophy cell trait, but the cell size comparison was not statistically significant ($p \leq 0.05$). As a result, the function of cells in proteoglycan production was different.

Comparison of proteoglycan content from the anterior horn of the medial and lateral menisci revealed that proteoglycan content was similar to the posterior horn, which amount of proteoglycan from the medial meniscus clearly less than the lateral meniscus (Figures 4.5M-S). A peripheral artery had a higher proteoglycan content than other regions, indicating that meniscus pathology effects on proteoglycan. Comparative studies of proteoglycan content from outer and deep zones of the medial and lateral menisci showed that the proteoglycan content was relatively similar. The proteoglycan content of the superficial zone was decreased. The study of the number of cells from superficial zone of the medial and lateral menisci showed that the medial meniscus had significantly more cells than the lateral meniscus ($p \leq 0.05$). Those cells were

hypertrophy. However, there was no statistically significant difference in cell size ($p \leq 0.05$), resulting in unclear differences in the cells for proteoglycan formation.

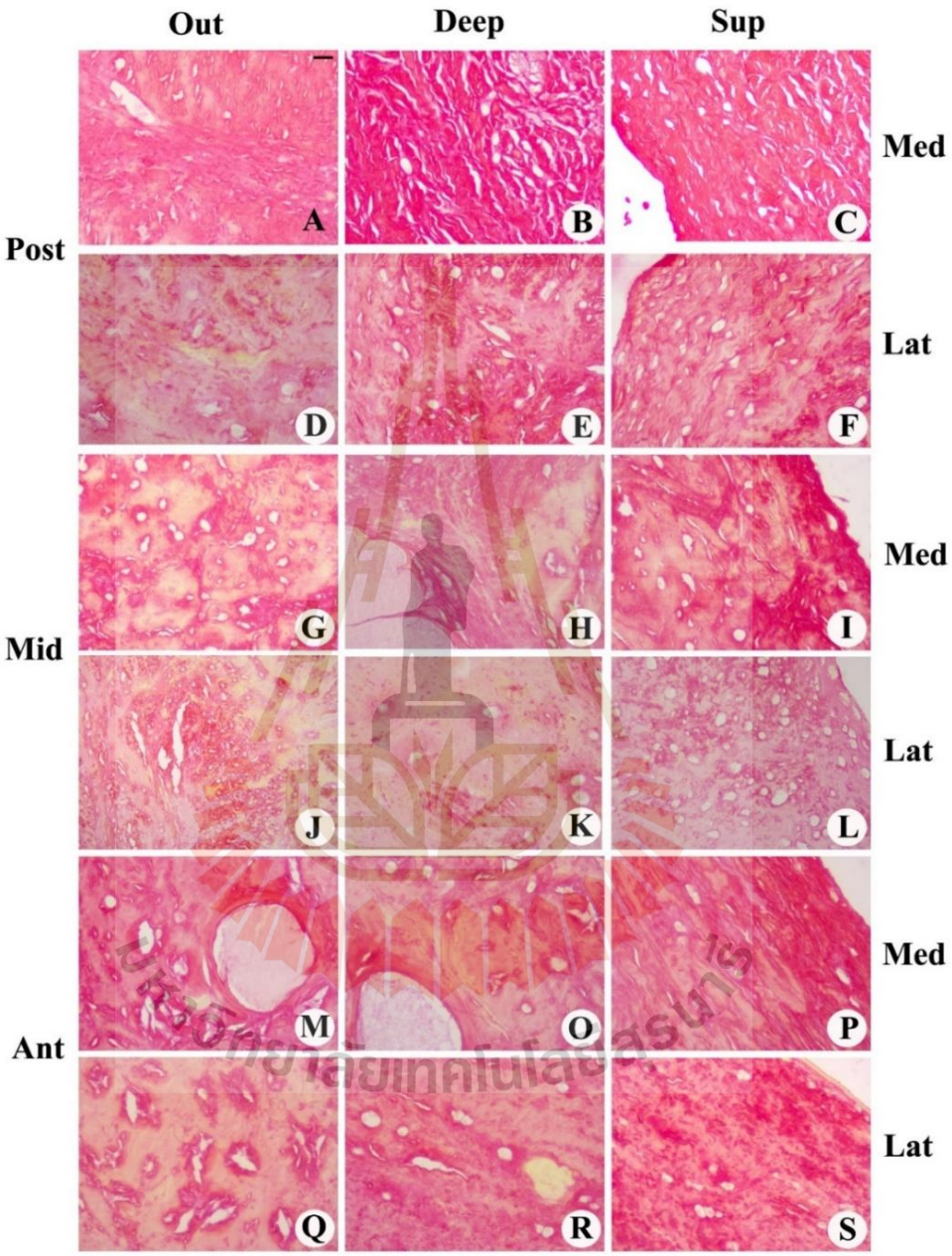

Figure 4.6 Meniscus staining with Picrosirius red. A) Outer zone of the posterior horn; B) Deep zone of the posterior horn; C) Superficial zone of the posterior horn; D) Outer zone of middle meniscus; E) Deep zone of middle meniscus; F) Superficial zone of middle meniscus; G) Outer zone of the anterior horn; H) Deep zone of the anterior horn; I) Superficial zone of the anterior horn; Scale bar 200 μm.

Table 4.2 Picrosirius red staining with meniscus OA (light microscopy).

Horns	Zones	Picrosirius red staining (light microscopy)		
		Outer	Deep	Superficial
Posterior	Medial	++	+++	+++
	Lateral	+	++	+
Middle	Medial	++	++	+++
	Lateral	+	+	+
Anterior	Medial	+	+	++
	Lateral	+	+	+

Staining: - no staining, + weak staining, ++ moderate staining, +++ strong staining.

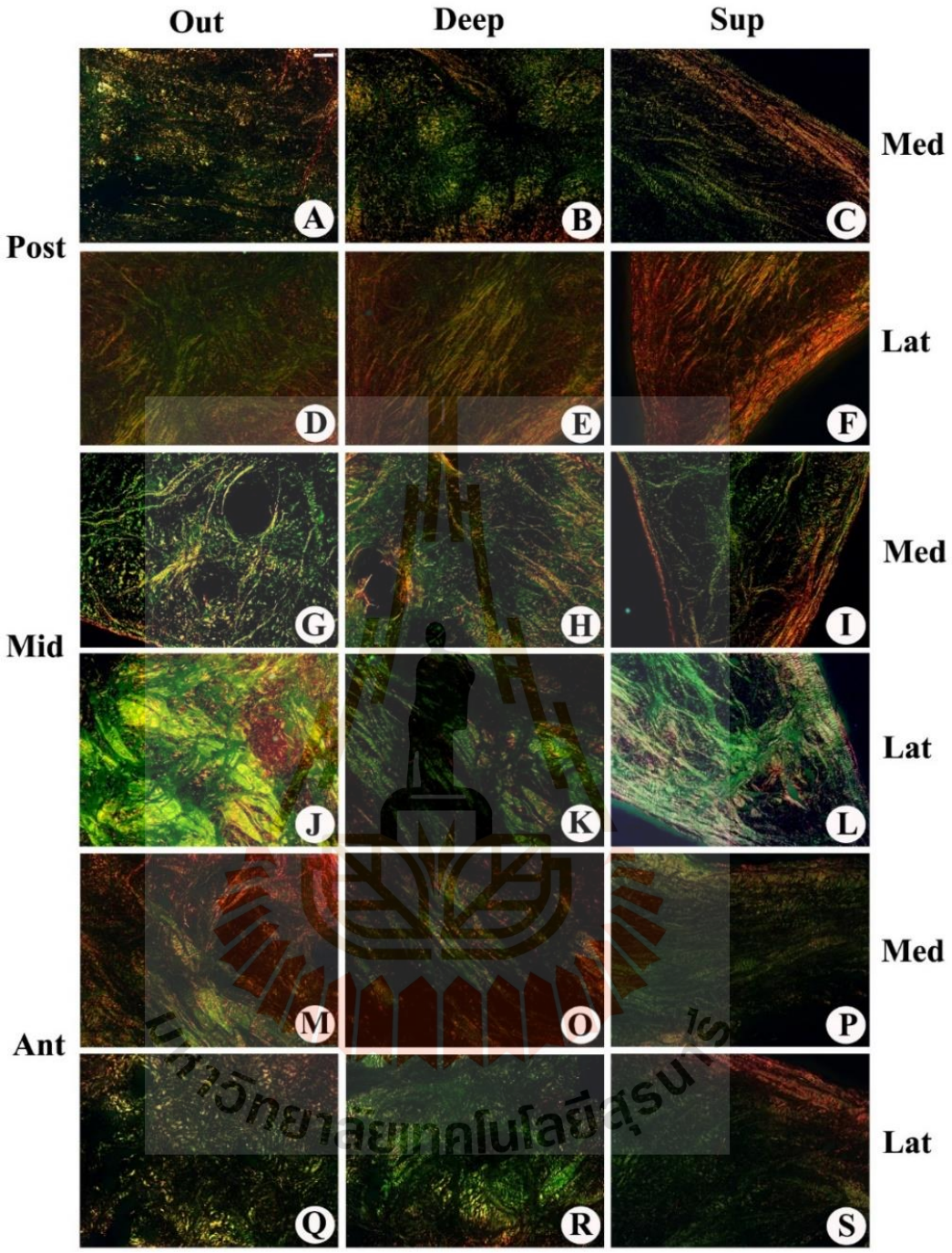

Figure 4.7 Photographs from polarization microscopy showing collagen fibers type I and III in red color. A) Outer zone of the posterior horn; B) Deep zone of the posterior horn; C) Superficial zone of the posterior horn; D) Outer zone of middle meniscus; E) Deep zone of middle meniscus; F) Superficial zone of middle meniscus; G) Outer zone of the anterior horn; H) Deep zone of the anterior horn; I) Superficial zone of the anterior horn; Scale bar 200 μm.

Table 4.3 Picrosirius red staining with meniscus OA (polarization microscopy)

Horns	Zones	Picrosirius red staining (polarization microscopy)		
		Outer	Deep	Superficial
Posterior	Medial	-	-	+
	Lateral	+	+	++
Middle	Medial	-	-	+
	Lateral	-	-	-
Anterior	Medial	+	-	-
	Lateral	-	-	+

Collagen type I: - no staining, + weak staining, ++ moderate staining, +++ strong staining.

Horns	Zones	Picrosirius red staining (polarization microscopy)		
		Outer	Deep	Superficial
Posterior	Medial	++	++	+
	Lateral	+	+	-
Middle	Medial	++	++	+
	Lateral	+++	+	+++
Anterior	Medial	+	+	+
	Lateral	+	+	-

Collagen type III: - no staining, + weak staining, ++ moderate staining, +++ strong staining.

Changes in collagen fibers compared between the medial and the lateral menisci, are summarized in table 4.2-4.3. A study of Picro sirius red staining showed that the medial meniscus was significantly more altered by a decrease in collagen fiber than the lateral meniscus, which was in association with the study of proteoglycan changes. Collagens at posterior horn, middle part, and anterior horn decreased in descending order. A study to compare the changes in the posterior horn collagen fibers between the medial meniscus and the lateral meniscus revealed a moderate decrease in the amount of collagen in the outer zone of the medial meniscus (medium-dark

dye). In contrast, the deep and superficial zones showed a greater reduction in collagen fibers (extremely strong dye) around the tissue rupture pathology showing a staining effect. Picro Sirius Red is darker than the surrounding area, this indicates that the area where the pathology was observed markedly reduced in collagen fibers. In addition, in the outer zone, where the connective tissue from the cartilage capsule was inserted, the Picrosirius red stain was clearly more intense than the cartilage tissue. This indicates that the collagen fiber content of the connective tissue was less than that of the meniscus cartilage. The study of changes in the amount of collagen fiber from the outer, deep, and superficial zones of the lateral meniscus revealed that Picro Sirius Red dye had a lower staining intensity than the medial meniscus (indicating that the amount of collagen fiber from the lateral meniscus was higher than that of the medial meniscus). The staining was significantly darker than the side areas as in the medial meniscus study (Figure 4.6A-F). A detailed study of the types of collagen fibers was performed under Picro Sirius Red polarized light microscopy. Type I collagen was shown in yellow/orange color and type III collagen was shown in green color under polarized light microscopic observation. The results showed that the outer, deep, and superficial zones of the medial meniscus were more type III collagen fibers than type I collagen, but the lateral meniscus contained more type I collagen fibers. The medial meniscus had a greater reduction in collagen fibers than the lateral meniscus, and the collagen fiber types also differed (Figure 4.7A-F).

A study at the middle part to compare the changes in the collagen fibers compared between the medial and lateral menisci found that the superficial zone of the medial meniscus showed the greatest reduction in collagen fiber and the outer and deep zones showed a moderate decrease. When comparing the amount of collagen fiber between the medial and lateral menisci, the collagen fiber content of the lateral meniscus was clearly higher than that of the medial meniscus. Collagen fiber content was observed at the periphery of the pathological tissue and the cell periphery. The staining was clearly similar to that of the posterior horn (Figure 4.6G-L). The results showed that the outer, deep, and superficial zones of the medial meniscus contained typed I collagen fibers. Collagen fiber observations of the lateral meniscus were mostly type III. Therefore, the data from the study of collagen fiber found that

the medial meniscus had a greater reduction in collagen fiber than the lateral meniscus, and the types of collagen fibers were also different (Figure 4.7G-L).

A study to compare the changes in the collagen fibers of the anterior horn between the medial meniscus and the lateral meniscus found that the superficial zone of the medial meniscus showed a marked reduction in collagen fibers greater than the outer zone and deep zone. Staining to indicate the amount of collagen fiber from the outer, deep and superficial zones showed that the amount of collagen fiber was similar (the quality of the Picro Sirius Red dye was not different), which was similar to that of the outer and deep zones of the medial the meniscus (Figures 4.6M-S). A detailed study of the types of collagen fibers was performed observations under polarized light microscopy, which revealed that the outer, deep, and superficial zones of the medial meniscus showed prominent type I collagen fibers. Collagen fibers of the lateral meniscus were type III collagen fibers which were evident in the deep zone, but the outer and superficial zones were more likely type I collagen fibers (Figures 4.7M-S).

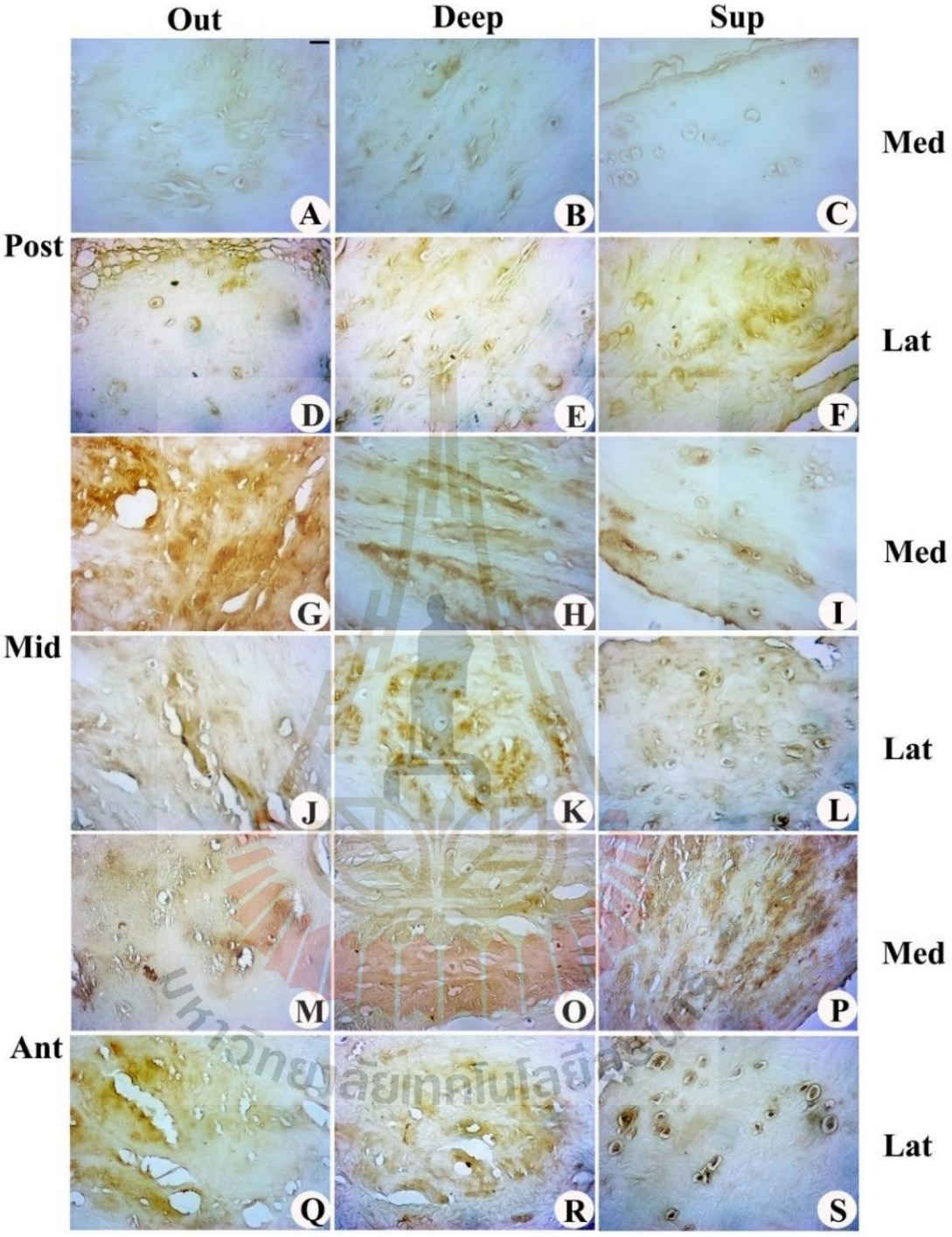

Figure 4.8 Histochemistry staining of type I collagen. A) Outer zone of the posterior horn; B) Deep zone of the posterior horn; C) Superficial zone of the posterior horn; D) Outer zone of middle meniscus; E) Deep zone of middle meniscus; F) Superficial zone of middle meniscus; G) Outer zone of the anterior horn; H) Deep zone of the anterior horn; I) Superficial zone of the anterior horn; Scale bar 200 μm.

Table 4.4 Type I collagen with meniscus OA.

Horns	Zones	Collagen type I		
		Outer	Deep	Superficial
Posterior	Medial	+	+	+
	Lateral	+	+	+
Middle	Medial	+++	++	++
	Lateral	+	++	+
Anterior	Medial	++	++	++
	Lateral	++	++	++

Staining: - no staining, + weak staining, ++ moderate staining, +++ strong staining.

Amounts of type I collagen from IHC staining are summarized in table 4.4. The posterior horn had weak staining, indicating low type I collagen content. Comparing the medial and lateral menisci, there was no difference in the amount of type I collagen in the outer, deep, and superficial zones (Figure 4.8A-F).

From the study of type I collagen in the middle part compared between the medial and lateral menisci, it was found that there were some differences. The outer zone of the medial meniscus has the highest amount of type I collagen (Figure 4.8G-L).

In a study to compare the amount of collagen between medial meniscus and lateral meniscus, the amount of type I collagen in the anterior horn was not different. It was found to have intense staining around the cells and around tissue tears (Figure 4.8M-S).

4.5 Numbers of apoptosis cells in horns and zones, compared between medial and lateral menisci

A comparative study of cell apoptosis from stained TUNEL Assay Kit - HRP-DAB is summarized in Table 4.5. The authors conduct a comparative study of cell apoptosis between the medial meniscus and lateral meniscus from three parts: posterior horn, middle part, and anterior horn.

A study from the TUNEL Assay Kit of the posterior horn showed that the lateral meniscus showed significantly more apoptosis cells than the medial meniscus ($p \leq 0.05$). The results were consistent in outer, deep, and superficial zones. In addition to the greater cell volume from the lateral meniscus, the apparent staining intensity was also greater than that of the medial meniscus (Figure 4.9A-F; 4.10A)

The study comparing the number of apoptosis cells from the middle part showed a difference from the posterior horn because the deep and superficial zones of the medial meniscus contained significantly more apoptosis cells than the lateral meniscus ($p \leq 0.05$). However, the outer zone of the medial meniscus showed significantly more cell apoptosis than the lateral meniscus (Figure 4.9G-L; 4.10B).

Compare the number of apoptosis cells in the anterior horn of the medial meniscus. All zones of the medial meniscus had a statistically significant higher amount of apoptosis cells than the lateral meniscus ($p \leq 0.05$). Staining intensity was clearly greater in the medial meniscus than the lateral meniscus (Figure 4.9M-S; 4.10C).

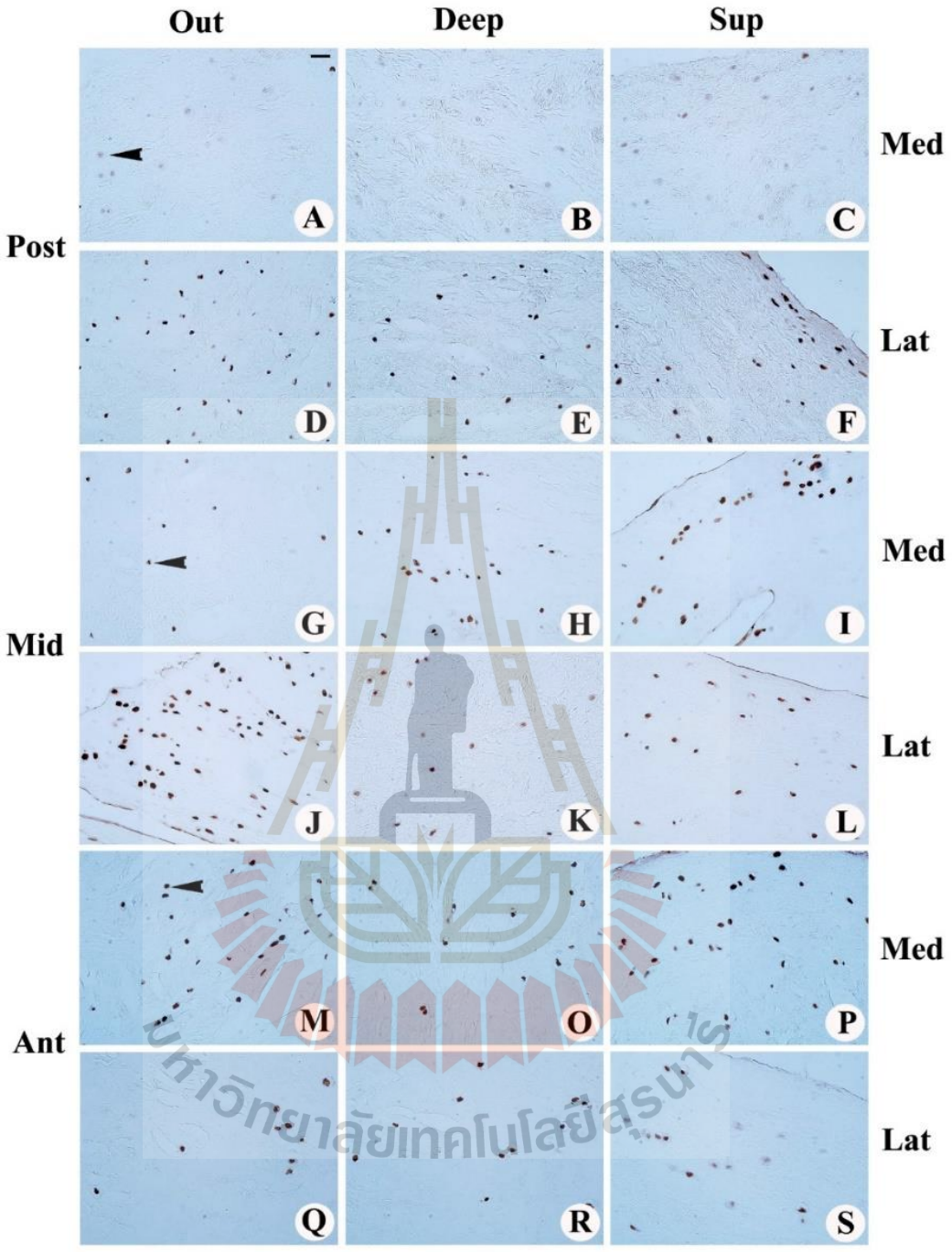

Figure 4.9 Histogram showing apoptosis cells staining with brown color. A) Outer zone of the posterior horn; B) Deep zone of the posterior horn; C) Superficial zone of the posterior horn; D) Outer zone of middle meniscus; E) Deep zone of middle meniscus; F) Superficial zone of middle meniscus; G) Outer zone of the anterior horn; H) Deep zone of the anterior horn; I) Superficial zone of the anterior horn; Scale bar 200 μm.

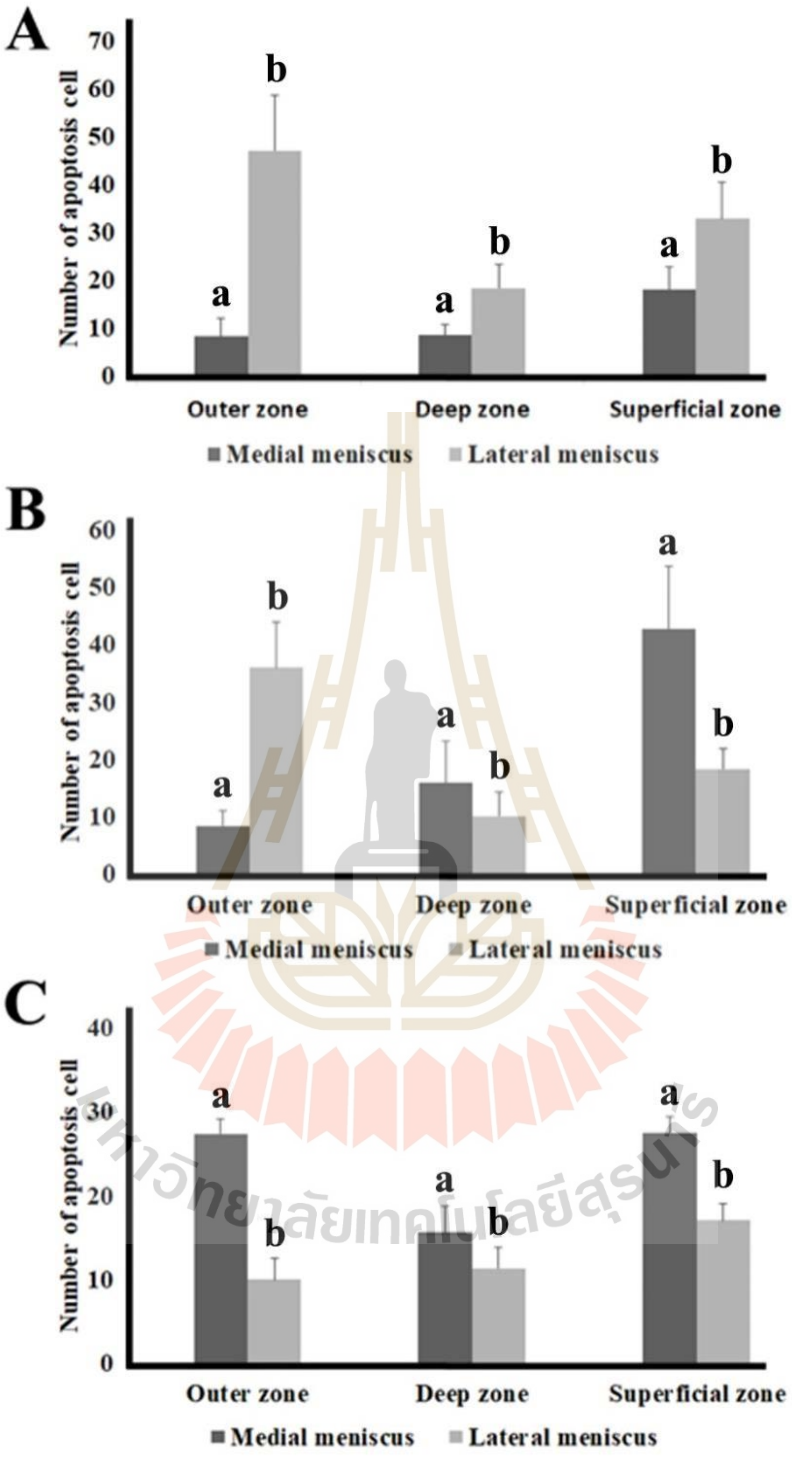

Figure 4.10 The number of apoptosis cells. A) posterior horn; B) middle part; C) anterior horn. Detection of cell apoptosis by TUNEL assay showed apoptosis cells in brow color.

Table 4.5 Comparison of TUNEL assay staining quality between medial meniscus and lateral meniscus.

Horns / Zones		Cell apoptosis by TUNEL assay		
		Outer	Deep	Superficial
Posterior	Medial	+	+	+
	Lateral	+++	+++	+++
Middle	Medial	++/+++	++/+++	++/+++
	Lateral	++/+++	++	++
Anterior	Medial	+++	+++	++/+++
	Lateral	++	+++	+/++

4.6 Effect of snail mucus, glucosamine, and undenatured type II in promote inflammations in OA chondrocyte cell culture

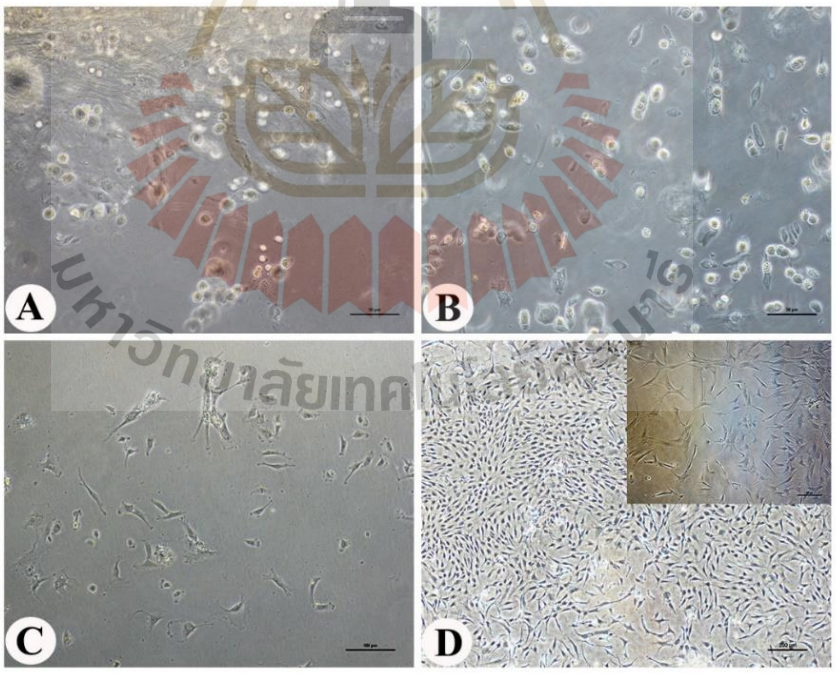

Figure 4.11 Chondrocytes isolation from meniscus tissue. A) After slicing tissue into small pieces; B) After incubated with collagenase, overnight; C) Chondrocyte cell culture for 1 week; D) Chondrocyte cell culture for 2 weeks.

After slicing menisci tissue into small pieces, followed by adding collagenase, the chondrocyte cells began to escape from the tissues, but were not yet adhered to the plate (Figure 4.12A). After overnight incubation, it was found that the tissues were digested, leaving only cells that were still not attached to the plate (Figure 14.2B). After incubation for 1 week, the cells began to adhere to the plate, and the number of chondrocyte cells increased (Figure 14.2C). One week later, cells were found to increase in numbers, and they were all attached to the plate (Figure 4.11D).

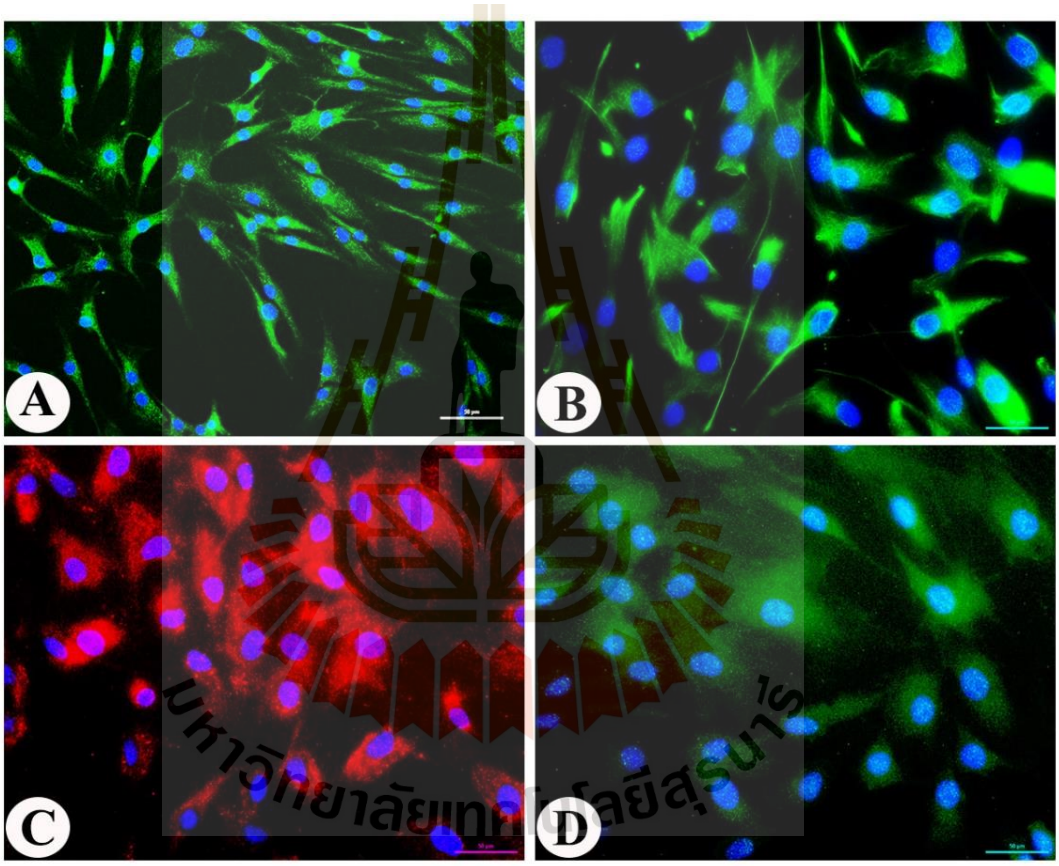

Figure 4.12 Immunocytochemistry (ICC). A) Collagen type I; B) Collagen type II; C) Aggrecan; D) Sox9.

Chondrocyte cells were stained with ICC to confirm their identity. Collagen type I, II, and Sox 9 showed in green color, and blue color indicated nuclei of the chondrocyte cells (Figure 4.12A, B, D), Aggrecan was shown in red color. (Figure 4.12C).

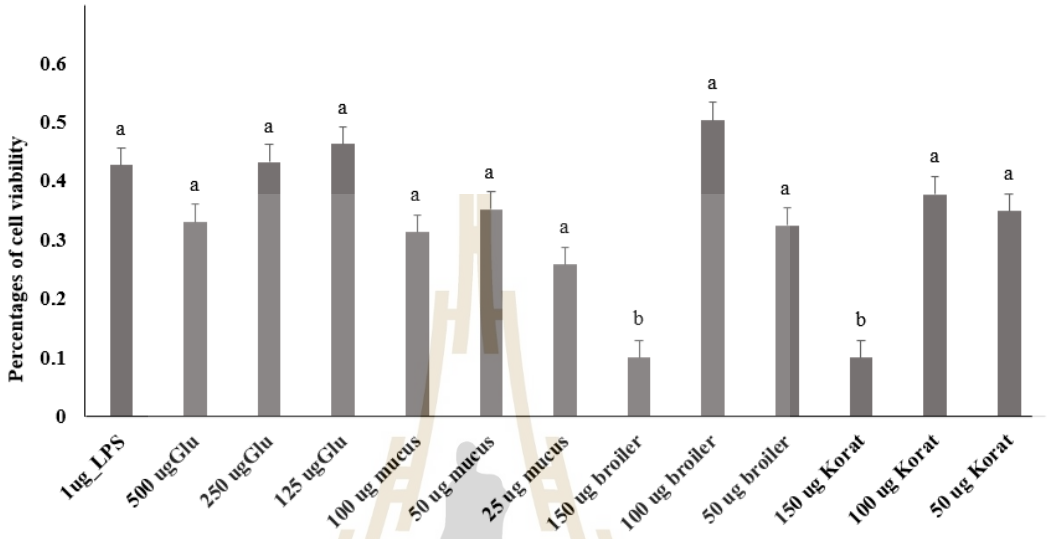

Figure 4.13 Percentages of cell viability by MTT assay.

MTT assay is a way to tell how many cells are alive. Based on the principle of color change (colorimetric assay) from the yellow color of MTT to the purple color of formazan crystals by enzymatic reaction of mitochondrial reductase, which is found only in living cells. For the 1 µg LPS group, cell viability was 0.46±0.02%. After incubated cells with 1 µg LPS for 2 hr, followed by treatment with 10% FBS, cell viability was higher (0.83±0.05%). Cells were incubated with 1 µg LPS for 2 hr and then treated with glucosamine, snail mucus, and undenatured collagen type II, both from broiler and Korat chicken. There was no significant difference in cell viability compared between cells treated with 1 µg LPS, followed by 500, 250, and 125 µg glucosamine (0.31±0.02%, 0.39±0.02%, 0.51±0.01%, respectively), 100, 50, and 25 µg mucus (0.34±0.009%, 0.39±0.01% and 0.27±0.01% respectively), 150, 100, and 50 µg broiler undenatured collagen type II (0.07±0.01, 0.57±0.02, and 0.35±0.01% respectively) and 150, 100 and 50 µg Korat undenatured collagen type II (0.08±0.03, 0.41±0.02, 0.44±0.01% respectively).

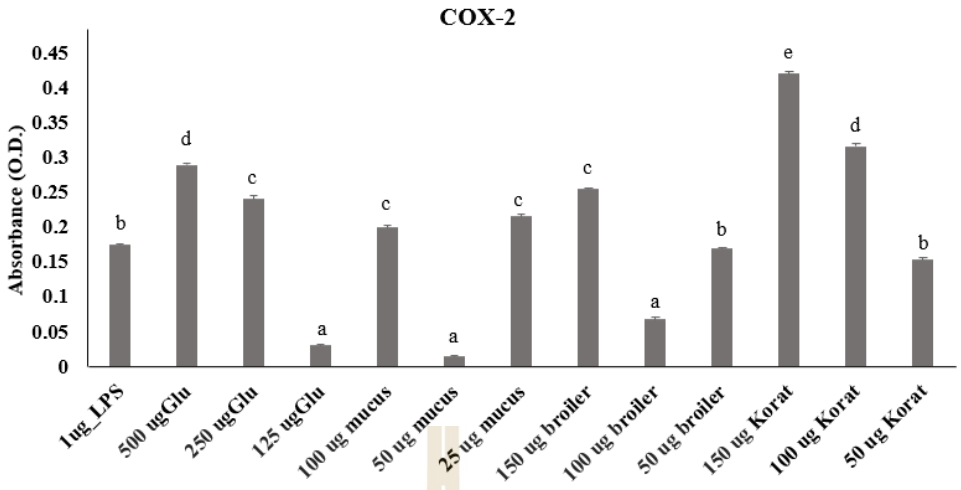

Figure 4.14 ELISA assay of in chondrocyte cell culture for Cox-2.

After incubating the cell with 1 µg LPS for 2 hr and cells were incubated with treatment. The levels of COX-2 were significantly higher in cells treated with 500 µg glucosamine (0.29±0.07), 25 µg mucus (0.216±0.005), 150 µg undenatured type collagen II from Broiler chickens (0.255±0.03), 150 µg undenatured collagen type II from Korat chickens (0.42±0.001). However, levels of COX-2 significantly lower in cells treated with 125 µg glucosamine (0.031±0.06), 50 µg mucus (0.015±0.02), 100 µg undenatured collagen type II from Broiler chickens (0.068±0.02), and 50 µg undenatured collagen type II from Korat chickens (0.153±0.01).

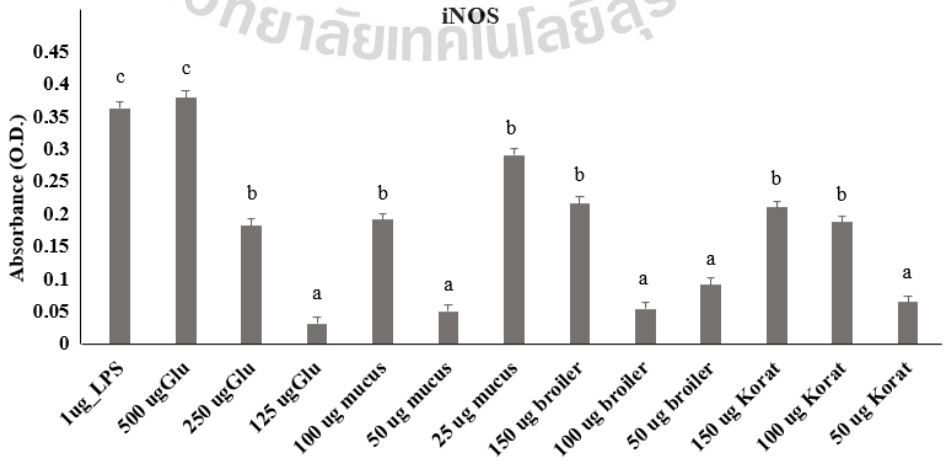

Figure 4.15 ELISA assay of chondrocyte cell culture determines the iNOS.

After incubating the cell with 1 μg LPS for 2 hr and cells were incubated with treatment. The levels of iNOS were significantly higher in cells treated with 500 μg glucosamine (0.379±0.02), 25 μg mucus (0.291±0.07), 150 μg undenatured collagen type II from Broiler chickens (0.216±0.03), 150 μg undenatured type II from Korat chickens (0.21±0.06). In contrast, levels of COX-2 were significantly lower in cells treated with 125 μg glucosamine (0.031±0.03), 50 μg mucus (0.05±0.05), 100 μg undenatured collagen type II from Broiler chickens (0.054±0.04), and 50 μg undenatured collagen type II from Korat chickens (0.64±0.06).

CHAPTER V
DISCUSSION AND CONCLUSIONS

A study of the structure of the knee joint with degenerative characteristics of articular cartilage was compared with that of the knee joint with normal features of articular cartilage. Degeneration of synovial cartilage, erosion of the cartilage covering the tip of the femur is clearly seen where it connects to the patella, and the medial condyle, where it comes into contact with the cartilage. The articular surface of the tibia can also be found in the pathology of eroded cartilage. This pathology is one of the conditions of osteoarthritis of the knee. Preliminary studies have shown that the lateral condyle cartilage of the femur always was less pathology than the medial condyle. From the observations, the articular cartilage of the knee surface is usually glossy, and smooth, but the knee joint with pathology which has less pathology will, look rather rough, not shiny meniscus cartilage/dissected knee from osteoarthritis with normal articular cartilage has a relatively smooth, uniform edge without tearing. However, degenerative meniscus cartilage with articular cartilage pathology is found. The edges are uneven and there are signs of tearing. Therefore, the pathology of articular cartilage also affects the pathology of the menisci cartilage. However, the structure of the lateral condyle from the pathological knee looks normal. However, the menisci cartilage on the lateral condyle has the same pathology as the medial condyle meniscus cartilage. The pathology of the knee joint may be due to the presence of pathology of the meniscus cartilage. The menisci cartilage has a function and role to support and distribute forces. If the pathological structure of the menisci cartilage does not function well, the joint cartilage of the femur and tibia becomes more abrasive, leading to the pathological condition of osteoarthritis of the knee joint. Researchers found that the pathology of damage to the menisci cartilage was proportional to age. The menisci cartilage of older people is more pathologically damaged than younger people. In addition, medial meniscus cartilage is more damaged than lateral meniscus cartilage (Englund et al., 2008). The information may be due to

the bone function of soft mini scaffold on the inside supporting the body's weight more than the outer menisci cartilage, become to more structural damage. The elderly cartilage may have caused deterioration, for example, the loss of water in the menisci cartilage, thus losing flexibility in weight gain and distribution, resulting in more pathologies than younger people.

This research focuses on comparing changes in the structure of the knee disc herniation in patients with knee osteoarthritis. Initially, the study was to compare medial meniscus and lateral meniscus. The study compared the anterior horn, middle part, and posterior horn. The study also detailed subsections including the outer zone, deep zone, and superficial zone. In Knee bones in people with knee osteoarthritis in the early stages, the medial meniscus was clearly more pathological than the lateral meniscus, and these findings indicate that the early stages of knee osteoarthritis The pathology of the medial meniscus was probably preceded by the onset of the lateral meniscus pathology by studying the remodeling of knee herniated discs from knee patients. With osteoarthritis, the medial meniscus in the posterior horn was the most altered (Katsuragawa et al., 2010). Research reports support that the pathology of the medial meniscus can lead to the formation of the knee osteoarthritis because the body weight transfer is unbalanced, the weight is directed downward to the medial rather than the lateral side, and the lateral meniscus is pathological after the pathology of the medial meniscus (Kuma et al., 2013; Bloecker et al., 2013, Li et al., 2019). There are also additional research findings that the development of knee osteoarthritis effect on posterior horn more than the anterior horn (Badlani et al., 2013). A study of changes in meniscus from those at risk of knee osteoarthritis with magnetic resonance imaging (MRI) showed a decrease in the thickness of the posterior horn of the medial meniscus. In addition, the lateral meniscus in those at risk of knee osteoarthritis was observed no change in thickness. Therefore, the medial meniscus of the posterior horn is an important structure to consider in monitoring progress toward the treatment of knee osteoarthritis (Dube et al., 2018). These data are consistent with the results of this study, which found that posterior horn changes in the outer zone and deep zone showed the presence of bone marrow-like regions and diffuse fibrocartilage separation. In general, the pathology of knee osteoarthritis shows damage to the articular cartilage through fibrillation from the superficial zone and, if a repair cannot be effective, results

in loss of articular cartilage. The inner structure is more than the superficial structure (Pauli et al., 2011). The previous research data tends to be in line with the results of this study, changes in the structure of the medial meniscus and lateral meniscus are more pathological from the outer zone, deep zone, and superficial zone. Histological and biochemical studies of the meniscus show the outer 2/3 cells characteristic of fibrocartilage and the inner 1/3 cells characterize the hyaline cartilage (López-Franco and Gómez-Barrena, 2018). The results of this study revealed that the pathogenesis of osteoarthritis results in changes in cell shape and size, with the posterior horn showing the greatest change in cell size, with medial meniscus cells becoming hypertrophy. In addition, the number of chondrocytes in the outer zone and deep zone of the posterior horn from the medial meniscus differed markedly from the lateral meniscus, consistent with research reports on the pathology of knee osteoarthritis, swelling of the cells of the meniscus. Pathology of the meniscus, reduction in cell proliferation, which pathological effects can lead to cellular damage leading to necrosis and/or programmed cell death (apoptosis). In addition, has been reported that pathology to the meniscus is associated with cell apoptosis, and that the percentage of apoptotic cells is also associated with the severity of the pathology, with the percentage increasing as high as 70%. However, approximately 20% of the normal meniscus can develop cell apoptosis (Hashimoto et al., 1999; Lopez-Franco et al., 2016). In addition to meniscus pathology, the occurrence of an injury or tear of the anterior cruciate ligament of the knee joint can contribute to approximately 60 percent of cell apoptosis (Lopez-Franco et al., 2011). This indicates that cell apoptosis can be an indicator of the onset or progression of knee osteoarthritis.

A study to differentiate the types of collagen fibers from picrosirius red staining. The presence of different colors was that type I collagen appeared yellow-red, and type III collagen appeared green (Coelho et al., 2018). There are several types of collagen, including type I, II, III, V, and VI collagen, however, type I collagen is still a major component of the meniscus (Hellio Le Graverand et al., 2001). From a study of changes in collagen fibers of the meniscus with osteoarthritis, reduction in cell numbers, osteoarthritis of meniscus also altered collagen fiber content, the change in collagen fiber content was more pronounced in the medial meniscus than in the lateral meniscus (Pauli et al., 2011). In those with knee osteoarthritis, a decrease in

fibril diameter and an increase in the number of collagen fibers in the medial meniscus, but no changes in the lateral meniscus. Molecular synthesis of collagen fibers revealed an increase in mRNA of type I collagen 52 times, type II collagen 19 times, and type III collagen 400 times, these changes were found in the medial meniscus more than the lateral meniscus (Katsuragawa et al., 2010). In addition, staining to study the transformation of collagen fibers can also indicate cellular changes. The presence of an over-normal stain indicates a transformation from fibroblastic cells to chondrocytic cells, this change appears in the early stages of osteoarthritis.

The meniscus contains quite a variety of extracellular matrix to responsible for maintaining the structure and repairing the damaged. The function of proteoglycan to increase the ability of the meniscus to withstand the compression forces transmitted by body movement (Sophia et al., 2009; Masutani et al., 2020). The results of this study found that the occurrence of knee osteoarthritis in the early stages of medial meniscus show proteoglycan reductions, is clearly greater than that of the lateral meniscus. Pathology in the outer zone and the superficial zone is markedly reduced. From the research report on the changes of extracellular matrix (proteoglycan) from pathology, osteoarthritis has the quantity of the extracellular matrix is reduced (Akkiraju et al., 2015; Roughley et al., 2014). In a study of proteoglycan content in the canine meniscus, 48 h after injury, proteoglycan content was significantly reduced (Nishida et al., 2005), A decrease in the extracellular matrix, in particular proteoglycan, affects the structure and function of the meniscus and leads to the pathology of Osteoarthritis (Lopez-Franco et al., 2016).

The result from immunohistochemistry showed collagen type I staining with brown color. All the menisci tissue was stained with collagen type I. The staining was more intense in the middle area of the anterior horn than the posterior horn. From previous studies, the result of immunohistochemistry, RT-PCR, quantitative real-time PCR, and in situ hybridization revealed that not only collagen type II, but also collagen type I was synthesized by the cells of the diseased cartilage tissue, especially in the later stages of osteoarthritis (Miosge et al., 2004). Similar to the present results, Martin and coworkers (2001) found that some OA patients with higher osteoarthritis scores had higher levels of collagen type 1 mRNA than control patients.

Detection of apoptosis by TUNEL assay has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis. In the present study, the apoptotic cells were found in the superficial zone, especially in the anterior horn more than in another zone. In male Wistar rats, early stages of OA chondrocyte from the superficial zone respond to damage and increased expression of apoptotic cell death (Kouri-Flores et al., 2002; Carlo and Loeser, 2002). Other studies showed that apoptosis occurred in OA cartilage more frequently than in normal cartilage (Kim et al., 2000; Heraud et al., 2000; Blanco et al., 1998).

In the present study, chondrocyte cultures from human OA menisci were identified for, cell identity using collagen type I and II, aggrecan, and Sox9. A previous study detected collagen type I in OA. Most of the cartilage from OA stage I showed a reaction in the territory matrix. A stronger reaction was seen in the interterritorial matrix of OA stage III. Collagen type II was present in all stages of OA. Moreover, they found that OA meniscal cells indicate aggrecan at a significantly higher level than normal meniscal cells (Sun et al., 2010). Upregulation of SOX9 restrained IL-1β-induced inflammatory reply via increasing the level of Smad3 in human chondrocytes and reveal a therapeutic effect on surgically induced OA mice in vivo (Zhang et al., 2017). This discovery present that the knee menisci may be actively complicated in the disease process of OA.

The MTT assay has been used to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. In the present study, the cytotoxicity of the treatment to cells was tested compared to the widely used cell culture medium. The cell viability in treatment groups was decreased compared to the control group. When cells were added with LPS the cell viability was not different compared to the control group. COX-2, the inducible form, is expressed in response to inflammatory and other physiologic stimuli and growth factors. It is involved in the production of prostaglandins that mediate pain and concerns with the inflammatory process. ELISA assay indicated that the level of COX-2 was lesser. The cells were treated with 125 µg of Glucosamine, 100 µg undenatured collagen type II from Broiler chicken, and 150 µg of snail mucus. iNOS is one of the direct consequences of an inflammatory process, ELISA result from iNOS was similar to COX-2. The iNOS level was reduced in cells treated with 125 µg of Glucosamine, 100 µg undenatured collagen

type II from Broiler chicken, and 50 µg of snail mucus. This is of major particular relative to iNOS and COX-2, that the presence of these two inducible proteins shown to be control by the same factors and often occurs under similar conditions in models of acute and chronic inflammation and in several human diseases including OA (Needleman and Manning, 1999). The excessive production of iNOS appeared to elicit cellular cytotoxicity and tissue damage and was thought to contribute to the pathology of several human diseases including OA (Grabowski et al., 1997). Inflammation might be mediated by the chondrocytes once they became damaged due to abnormal wear and tear or trauma. There was some evidence indicating that chondrocytes make specific pro-inflammatory molecules that help to initiate and perpetuate the low inflammation in the OA join (Goldring and Otero, 2011).

Snail extract is reduced knee swellings and glucosamine was found to have preventive actions on OA in humans as well as in rats (Ogata et al., 2018). Glucosamine might have express chondroprotective feature. Inceptive work in vitro showed that glucosamine could moderate certain aspects of the deleterious response of chondrocytes to promotion with IL-1 (Gouze et al., 2001) or lipopolysaccharide (Byron et al., 2003). These aspects included nitric oxide (NO) synthesis (Gouze et al., 200), reduced COX-2 mRNA and protein expression (Shikhman et al., 2001; Largo et al., 2003), and reduced proteoglycan synthesis in articular cartilage (Gouze et al., 2001; Chan et al., 2005; Basslee et al., 1998; Fenton et al., 2002). Restraint of aggrecans based on cleavage of aggrecan was also discover in both rat and bovine cartilage explant cultures when supplemented with glucosamine (Sandy et al., 1998). In addition, NF_KB activation, as well as the nuclear translocation of p50 and p65 proteins, was inhibited in chondrocytes cultured in the existence of glucosamine, propose that glucosamine could inhibited inflammatory. Previous studies have revealed that UC-II reduced lameness after general pain, pain during limb manipulation, and physical exertion in arthritic dogs (D'Altilio et al., 2007). In a cell study, Treg cells specific for type II collagen secreted anti-inflammatory cytokines, which play a chief role in the cells ability to induce oral tolerance (Asnagli et al., 2014). In a cell study with human chondrocytes, the anti-inflammatory action of IL-10 protects against damage from tumor necrosis factor-alpha (TNF-α), a pro-inflammatory mediator elevated in osteoarthritis (Müller et al., 2008).

In summary, the results of the present study revealed that the middle of the anterior horn and posterior horn had a higher number of chondrocytes. The size of chondrocyte cells, apoptosis cells, and extracellular matrix were increased. In addition, biochemical constituents within tissues were studied, such as glycosaminoglycan, collagen fiber, and collagen type I and III. After that, cells were isolated from the menisci tissues to test the inhibition of inflammation, which is detected by iNOS and COX-2.

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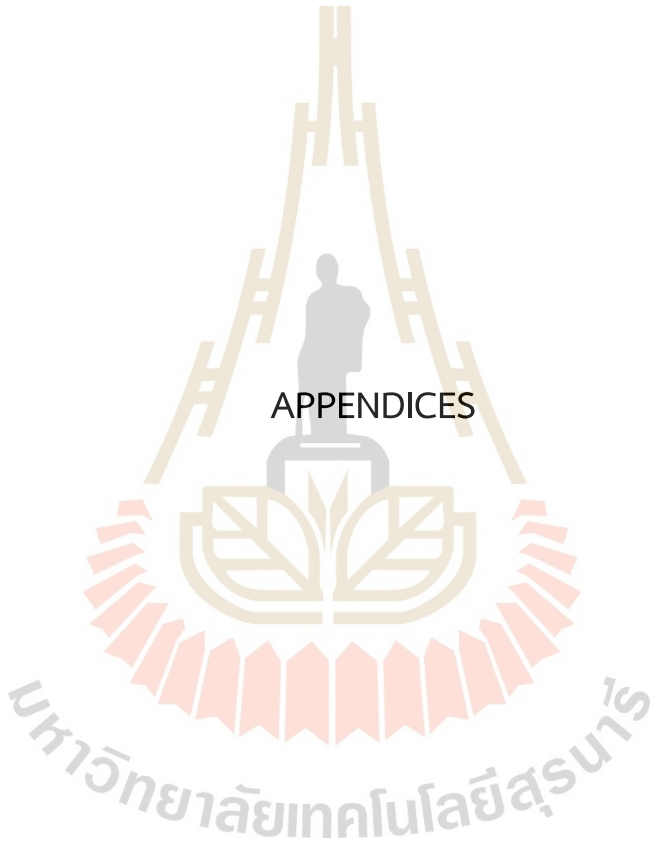
APPENDICES



APPENDIX A

STATISTICAL ANALYSIS

Table A1 Statistic comparison number of outer zone in each horn of menisci.

		Mean			95% Confidence Interval	
(I) zone	(J) zone	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
OPM	OPL	29.80000*	2.59358	.000	21.7808	37.8192
	OMM	20.40000*	2.59358	.000	12.3808	28.4192
	OML	29.00000*	2.59358	.000	20.9808	37.0192
	OAM	28.80000*	2.59358	.000	20.7808	36.8192
	OAL	-16.20000*	2.59358	.000	-24.2192	-8.1808
OPL	OPM	-29.80000*	2.59358	.000	-37.8192	-21.7808
	OMM	-9.40000*	2.59358	.015	-17.4192	-1.3808
	OML	-.80000	2.59358	1.000	-8.8192	7.2192
	OAM	-1.00000	2.59358	.999	-9.0192	7.0192
	OAL	-46.00000*	2.59358	.000	-54.0192	-37.9808
OMM	OPM	-20.40000*	2.59358	.000	-28.4192	-12.3808
	OPL	9.40000*	2.59358	.015	1.3808	17.4192
	OML	8.60000*	2.59358	.031	.5808	16.6192
	OAM	8.40000*	2.59358	.036	.3808	16.4192
	OAL	-36.60000*	2.59358	.000	-44.6192	-28.5808
OML	OPM	-29.00000*	2.59358	.000	-37.0192	-20.9808
	OPL	.80000	2.59358	1.000	-7.2192	8.8192
	OMM	-8.60000*	2.59358	.031	-16.6192	-.5808
	OAM	-.20000	2.59358	1.000	-8.2192	7.8192
	OAL	-45.20000*	2.59358	.000	-53.2192	-37.1808
OAM	OPM	-28.80000*	2.59358	.000	-36.8192	-20.7808
	OPL	1.00000	2.59358	.999	-7.0192	9.0192
	OMM	-8.40000*	2.59358	.036	-16.4192	-.3808
	OML	.20000	2.59358	1.000	-7.8192	8.2192
	OAL	-45.00000*	2.59358	.000	-53.0192	-36.9808
OAL	OPM	16.20000*	2.59358	.000	8.1808	24.2192
	OPL	46.00000*	2.59358	.000	37.9808	54.0192
	OMM	36.60000*	2.59358	.000	28.5808	44.6192
	OML	45.20000*	2.59358	.000	37.1808	53.2192
	OAM	45.00000*	2.59358	.000	36.9808	53.0192

* The mean difference is significant at the 0.05 level.

Table A2 Statistic comparison number of deep zone in each horn of menisci.

		Mean			95% Confidence Interval	
(I) zone	(J) zone	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
DPM	DPL	6.20000*	1.95959	.043	.1411	12.2589
	DMM	6.80000*	1.95959	.022	.7411	12.8589
	DML	-4.40000	1.95959	.255	-10.4589	1.6589
	DAM	6.00000	1.95959	.053	-.0589	12.0589
	DAL	-21.00000*	1.95959	.000	-27.0589	-14.9411
DPL	DPM	-6.20000*	1.95959	.043	-12.2589	-.1411
	DMM	.60000	1.95959	1.000	-5.4589	6.6589
	DML	-10.60000*	1.95959	.000	-16.6589	-4.5411
	DAM	-.20000	1.95959	1.000	-6.2589	5.8589
	DAL	-27.20000*	1.95959	.000	-33.2589	-21.1411
DMM	DPM	-6.80000*	1.95959	.022	-12.8589	-.7411
	DPL	-.60000	1.95959	1.000	-6.6589	5.4589
	DML	-11.20000*	1.95959	.000	-17.2589	-5.1411
	DAM	-.80000	1.95959	.998	-6.8589	5.2589
	DAL	-27.80000*	1.95959	.000	-33.8589	-21.7411
DML	DPM	4.40000	1.95959	.255	-1.6589	10.4589
	DPL	10.60000*	1.95959	.000	4.5411	16.6589
	DMM	11.20000*	1.95959	.000	5.1411	17.2589
	DAM	10.40000*	1.95959	.000	4.3411	16.4589
	DAL	-16.60000*	1.95959	.000	-22.6589	-10.5411
DAM	DPM	-6.00000	1.95959	.053	-12.0589	.0589
	DPL	.20000	1.95959	1.000	-5.8589	6.2589
	DMM	.80000	1.95959	.998	-5.2589	6.8589
	DML	-10.40000*	1.95959	.000	-16.4589	-4.3411
	DAL	-27.00000*	1.95959	.000	-33.0589	-20.9411
DAL	DPM	21.00000*	1.95959	.000	14.9411	27.0589
	DPL	27.20000*	1.95959	.000	21.1411	33.2589
	DMM	27.80000*	1.95959	.000	21.7411	33.8589
	DML	16.60000*	1.95959	.000	10.5411	22.6589
	DAM	27.00000*	1.95959	.000	20.9411	33.0589

* The mean difference is significant at the 0.05 level.

Table A3 Statistic comparison number of superficial zone in each horn of menisci.

		Mean			95% Confidence Interval	
(I) zone	(J) zone	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
SPM	SPL	19.80000*	4.33897	.002	6.3842	33.2158
	SMM	33.00000*	4.33897	.000	19.5842	46.4158
	SML	-67.60000*	4.33897	.000	-81.0158	-54.1842
	SAM	-4.20000	4.33897	.924	-17.6158	9.2158
	SAL	-24.60000*	4.33897	.000	-38.0158	-11.1842
SPL	SPM	-19.80000*	4.33897	.002	-33.2158	-6.3842
	SMM	13.20000	4.33897	.056	-.2158	26.6158
	SML	-87.40000*	4.33897	.000	-100.8158	-73.9842
	SAM	-24.00000*	4.33897	.000	-37.4158	-10.5842
	SAL	-44.40000*	4.33897	.000	-57.8158	-30.9842
SMM	SPM	-33.00000*	4.33897	.000	-46.4158	-19.5842
	SPL	-13.20000	4.33897	.056	-26.6158	.2158
	SML	-100.60000*	4.33897	.000	-114.0158	-87.1842
	SAM	-37.20000*	4.33897	.000	-50.6158	-23.7842
	SAL	-57.60000*	4.33897	.000	-71.0158	-44.1842
SML	SPM	67.60000*	4.33897	.000	54.1842	81.0158
	SPL	87.40000*	4.33897	.000	73.9842	100.8158
	SMM	100.60000*	4.33897	.000	87.1842	114.0158
	SAM	63.40000*	4.33897	.000	49.9842	76.8158
	SAL	43.00000*	4.33897	.000	29.5842	56.4158
SAM	SPM	4.20000	4.33897	.924	-9.2158	17.6158
	SPL	24.00000*	4.33897	.000	10.5842	37.4158
	SMM	37.20000*	4.33897	.000	23.7842	50.6158
	SML	-63.40000*	4.33897	.000	-76.8158	-49.9842
	SAL	-20.40000*	4.33897	.001	-33.8158	-6.9842
SAL	SPM	24.60000*	4.33897	.000	11.1842	38.0158
	SPL	44.40000*	4.33897	.000	30.9842	57.8158
	SMM	57.60000*	4.33897	.000	44.1842	71.0158
	SML	-43.00000*	4.33897	.000	-56.4158	-29.5842
	SAM	20.40000*	4.33897	.001	6.9842	33.8158

* The mean difference is significant at the 0.05 level.

Table A4 Statistic comparison size of outer zone in each horn of menisci.

		Mean			95% Confidence Interval	
(I) zone	(J) zone	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
OPM	OPL	4.65600*	.69344	.000	2.5119	6.8001
	OMM	.31600	.69344	.997	-1.8281	2.4601
	OML	-1.89600	.69344	.105	-4.0401	.2481
	OAM	-6.83400*	.69344	.000	-8.9781	-4.6899
	OAL	5.59400*	.69344	.000	3.4499	7.7381
OPL	OPM	-4.65600*	.69344	.000	-6.8001	-2.5119
	OMM	-4.34000*	.69344	.000	-6.4841	-2.1959
	OML	-6.55200*	.69344	.000	-8.6961	-4.4079
	OAM	-11.49000*	.69344	.000	-13.6341	-9.3459
	OAL	.93800	.69344	.753	-1.2061	3.0821
OMM	OPM	-.31600	.69344	.997	-2.4601	1.8281
	OPL	4.34000*	.69344	.000	2.1959	6.4841
	OML	-2.21200*	.69344	.040	-4.3561	-.0679
	OAM	-7.15000*	.69344	.000	-9.2941	-5.0059
	OAL	5.27800*	.69344	.000	3.1339	7.4221
OML	OPM	1.89600	.69344	.105	-.2481	4.0401
	OPL	6.55200*	.69344	.000	4.4079	8.6961
	OMM	2.21200*	.69344	.040	.0679	4.3561
	OAM	-4.93800*	.69344	.000	-7.0821	-2.7939
	OAL	7.49000*	.69344	.000	5.3459	9.6341
OAM	OPM	6.83400*	.69344	.000	4.6899	8.9781
	OPL	11.49000*	.69344	.000	9.3459	13.6341
	OMM	7.15000*	.69344	.000	5.0059	9.2941
	OML	4.93800*	.69344	.000	2.7939	7.0821
	OAL	12.42800*	.69344	.000	10.2839	14.5721
OAL	OPM	-5.59400*	.69344	.000	-7.7381	-3.4499
	OPL	-.93800	.69344	.753	-3.0821	1.2061
	OMM	-5.27800*	.69344	.000	-7.4221	-3.1339
	OML	-7.49000*	.69344	.000	-9.6341	-5.3459
	OAM	-12.42800*	.69344	.000	-14.5721	-10.2839

*. The mean difference is significant at the 0.05 level.

Table A5 Statistic comparison size of deep zone in each horn of menisci.

		Mean			95% Confidence Interval	
(I) zone	(J) zone	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
DPM	DPL	5.46800*	.93572	.000	2.5748	8.3612
	DMM	-3.79840*	.93572	.005	-6.6916	-.9052
	DML	-8.67680*	.93572	.000	-11.5700	-5.7836
	DAM	-7.89780*	.93572	.000	-10.7910	-5.0046
	DAL	7.08400*	.93572	.000	4.1908	9.9772
DPL	DPM	-5.46800*	.93572	.000	-8.3612	-2.5748
	DMM	-9.26640*	.93572	.000	-12.1596	-6.3732
	DML	-14.14480*	.93572	.000	-17.0380	-11.2516
	DAM	-13.36580*	.93572	.000	-16.2590	-10.4726
	DAL	1.61600	.93572	.528	-1.2772	4.5092
DMM	DPM	3.79840*	.93572	.005	.9052	6.6916
	DPL	9.26640*	.93572	.000	6.3732	12.1596
	DML	-4.87840*	.93572	.000	-7.7716	-1.9852
	DAM	-4.09940*	.93572	.002	-6.9926	-1.2062
	DAL	10.88240*	.93572	.000	7.9892	13.7756
DML	DPM	8.67680*	.93572	.000	5.7836	11.5700
	DPL	14.14480*	.93572	.000	11.2516	17.0380
	DMM	4.87840*	.93572	.000	1.9852	7.7716
	DAM	.77900	.93572	.958	-2.1142	3.6722
	DAL	15.76080*	.93572	.000	12.8676	18.6540
DAM	DPM	7.89780*	.93572	.000	5.0046	10.7910
	DPL	13.36580*	.93572	.000	10.4726	16.2590
	DMM	4.09940*	.93572	.002	1.2062	6.9926
	DML	-.77900	.93572	.958	-3.6722	2.1142
	DAL	14.98180*	.93572	.000	12.0886	17.8750
DAL	DPM	-7.08400*	.93572	.000	-9.9772	-4.1908
	DPL	-1.61600	.93572	.528	-4.5092	1.2772
	DMM	-10.88240*	.93572	.000	-13.7756	-7.9892
	DML	-15.76080*	.93572	.000	-18.6540	-12.8676
	DAM	-14.98180*	.93572	.000	-17.8750	-12.0886

* The mean difference is significant at the 0.05 level.

Table A6 Statistic comparison size of superficial zone in each horn of menisci.

		Mean			95% Confidence Interval	
(I) zone	(J) zone	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
SPM	SPL	8.22500*	.99569	.000	5.1464	11.3036
	SMM	2.55340	.99569	.145	-.5252	5.6320
	SML	-4.65840*	.99569	.001	-7.7370	-1.5798
	SAM	3.83200*	.99569	.009	.7534	6.9106
	SAL	9.59580*	.99569	.000	6.5172	12.6744
SPL	SPM	-8.22500*	.99569	.000	-11.3036	-5.1464
	SMM	-5.67160*	.99569	.000	-8.7502	-2.5930
	SML	-12.88340*	.99569	.000	-15.9620	-9.8048
	SAM	-4.39300*	.99569	.002	-7.4716	-1.3144
	SAL	1.37080	.99569	.740	-1.7078	4.4494
SMM	SPM	-2.55340	.99569	.145	-5.6320	.5252
	SPL	5.67160*	.99569	.000	2.5930	8.7502
	SML	-7.21180*	.99569	.000	-10.2904	-4.1332
	SAM	1.27860	.99569	.791	-1.8000	4.3572
	SAL	7.04240*	.99569	.000	3.9638	10.1210
SML	SPM	4.65840*	.99569	.001	1.5798	7.7370
	SPL	12.88340*	.99569	.000	9.8048	15.9620
	SMM	7.21180*	.99569	.000	4.1332	10.2904
	SAM	8.49040*	.99569	.000	5.4118	11.5690
	SAL	14.25420*	.99569	.000	11.1756	17.3328
SAM	SPM	-3.83200*	.99569	.009	-6.9106	-.7534
	SPL	4.39300*	.99569	.002	1.3144	7.4716
	SMM	-1.27860	.99569	.791	-4.3572	1.8000
	SML	-8.49040*	.99569	.000	-11.5690	-5.4118
	SAL	5.76380*	.99569	.000	2.6852	8.8424
SAL	SPM	-9.59580*	.99569	.000	-12.6744	-6.5172
	SPL	-1.37080	.99569	.740	-4.4494	1.7078
	SMM	-7.04240*	.99569	.000	-10.1210	-3.9638
	SML	-14.25420*	.99569	.000	-17.3328	-11.1756
	SAM	-5.76380*	.99569	.000	-8.8424	-2.6852

* The mean difference is significant at the 0.05 level.

Table A7 Statistic comparison apoptosis cells of outer zone in each horn of menisci.

		Mean			95% Confidence Interval	
(I) zone	(J) zone	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
OPM	OPL	5.80000	2.18378	.101	-.6519	12.2519
	OMM	1.30000	2.18378	.991	-5.1519	7.7519
	OML	3.50000	2.18378	.600	-2.9519	9.9519
	OAM	6.80000*	2.18378	.033	.3481	13.2519
	OAL	.60000	2.18378	1.000	-5.8519	7.0519
OPL	OPM	-5.80000	2.18378	.101	-12.2519	.6519
	OMM	-4.50000	2.18378	.323	-10.9519	1.9519
	OML	-2.30000	2.18378	.897	-8.7519	4.1519
	OAM	1.00000	2.18378	.997	-5.4519	7.4519
	OAL	-5.20000	2.18378	.181	-11.6519	1.2519
OMM	OPM	-1.30000	2.18378	.991	-7.7519	5.1519
	OPL	4.50000	2.18378	.323	-1.9519	10.9519
	OML	2.20000	2.18378	.913	-4.2519	8.6519
	OAM	5.50000	2.18378	.137	-.9519	11.9519
	OAL	-.70000	2.18378	1.000	-7.1519	5.7519
OML	OPM	-3.50000	2.18378	.600	-9.9519	2.9519
	OPL	2.30000	2.18378	.897	-4.1519	8.7519
	OMM	-2.20000	2.18378	.913	-8.6519	4.2519
	OAM	3.30000	2.18378	.659	-3.1519	9.7519
	OAL	-2.90000	2.18378	.768	-9.3519	3.5519
OAM	OPM	-6.80000*	2.18378	.033	-13.2519	-.3481
	OPL	-1.00000	2.18378	.997	-7.4519	5.4519
	OMM	-5.50000	2.18378	.137	-11.9519	.9519
	OML	-3.30000	2.18378	.659	-9.7519	3.1519
	OAL	-6.20000	2.18378	.066	-12.6519	.2519
OAL	OPM	-.60000	2.18378	1.000	-7.0519	5.8519
	OPL	5.20000	2.18378	.181	-1.2519	11.6519
	OMM	.70000	2.18378	1.000	-5.7519	7.1519
	OML	2.90000	2.18378	.768	-3.5519	9.3519
	OAM	6.20000	2.18378	.066	-.2519	12.6519

* The mean difference is significant at the 0.05 level.

Table A8 Statistic comparison apoptosis cells of deep zone in each horn of menisci.

		Mean			95% Confidence Interval	
(I) zone	(J) zone	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
DPM	DPL	6.10000*	1.77545	.014	.8545	11.3455
	DMM	7.40000*	1.77545	.001	2.1545	12.6455
	DML	7.40000*	1.77545	.001	2.1545	12.6455
	DAM	-.50000	1.77545	1.000	-5.7455	4.7455
	DAL	2.90000	1.77545	.581	-2.3455	8.1455
DPL	DPM	-6.10000*	1.77545	.014	-11.3455	-.8545
	DMM	1.30000	1.77545	.977	-3.9455	6.5455
	DML	1.30000	1.77545	.977	-3.9455	6.5455
	DAM	-6.60000*	1.77545	.006	-11.8455	-1.3545
	DAL	-3.20000	1.77545	.473	-8.4455	2.0455
DMM	DPM	-7.40000*	1.77545	.001	-12.6455	-2.1545
	DPL	-1.30000	1.77545	.977	-6.5455	3.9455
	DML	.00000	1.77545	1.000	-5.2455	5.2455
	DAM	-7.90000*	1.77545	.001	-13.1455	-2.6545
	DAL	-4.50000	1.77545	.132	-9.7455	.7455
DML	DPM	-7.40000*	1.77545	.001	-12.6455	-2.1545
	DPL	-1.30000	1.77545	.977	-6.5455	3.9455
	DMM	.00000	1.77545	1.000	-5.2455	5.2455
	DAM	-7.90000*	1.77545	.001	-13.1455	-2.6545
	DAL	-4.50000	1.77545	.132	-9.7455	.7455
DAM	DPM	.50000	1.77545	1.000	-4.7455	5.7455
	DPL	6.60000*	1.77545	.006	1.3545	11.8455
	DMM	7.90000*	1.77545	.001	2.6545	13.1455
	DML	7.90000*	1.77545	.001	2.6545	13.1455
	DAL	3.40000	1.77545	.404	-1.8455	8.6455
DAL	DPM	-2.90000	1.77545	.581	-8.1455	2.3455
	DPL	3.20000	1.77545	.473	-2.0455	8.4455
	DMM	4.50000	1.77545	.132	-.7455	9.7455
	DML	4.50000	1.77545	.132	-.7455	9.7455
	DAM	-3.40000	1.77545	.404	-8.6455	1.8455

* The mean difference is significant at the 0.05 level.

Table A9 Statistic comparison apoptosis cells of superficial zone in each horn of menisci.

		Mean			95% Confidence Interval	
(I) zone	(J) zone	Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
SPM	SPL	.20000	1.88788	1.000	-5.3777	5.7777
	SMM	-1.10000	1.88788	.992	-6.6777	4.4777
	SML	5.20000	1.88788	.081	-.3777	10.7777
	SAM	-.70000	1.88788	.999	-6.2777	4.8777
	SAL	-28.70000*	1.88788	.000	-34.2777	-23.1223
SPL	SPM	-.20000	1.88788	1.000	-5.7777	5.3777
	SMM	-1.30000	1.88788	.982	-6.8777	4.2777
	SML	5.00000	1.88788	.103	-.5777	10.5777
	SAM	-.90000	1.88788	.997	-6.4777	4.6777
	SAL	-28.90000*	1.88788	.000	-34.4777	-23.3223
SMM	SPM	1.10000	1.88788	.992	-4.4777	6.6777
	SPL	1.30000	1.88788	.982	-4.2777	6.8777
	SML	6.30000*	1.88788	.018	.7223	11.8777
	SAM	.40000	1.88788	1.000	-5.1777	5.9777
	SAL	-27.60000*	1.88788	.000	-33.1777	-22.0223
SML	SPM	-5.20000	1.88788	.081	-10.7777	.3777
	SPL	-5.00000	1.88788	.103	-10.5777	.5777
	SMM	-6.30000*	1.88788	.018	-11.8777	-.7223
	SAM	-5.90000*	1.88788	.032	-11.4777	-.3223
	SAL	-33.90000*	1.88788	.000	-39.4777	-28.3223
SAM	SPM	.70000	1.88788	.999	-4.8777	6.2777
	SPL	.90000	1.88788	.997	-4.6777	6.4777
	SMM	-.40000	1.88788	1.000	-5.9777	5.1777
	SML	5.90000*	1.88788	.032	.3223	11.4777
	SAL	-28.00000*	1.88788	.000	-33.5777	-22.4223
SAL	SPM	28.70000*	1.88788	.000	23.1223	34.2777
	SPL	28.90000*	1.88788	.000	23.3223	34.4777
	SMM	27.60000*	1.88788	.000	22.0223	33.1777
	SML	33.90000*	1.88788	.000	28.3223	39.4777
	SAM	28.00000*	1.88788	.000	22.4223	33.5777

* The mean difference is significant at the 0.05 level.

Table A10 Statistic comparison iNOS in each treatment group after cells inflammation.

	(J)	Mean	Std.		95% Confidence Interval	
(I) treatment	treatment	Difference (I-J)	Error	Sig.	Lower Bound	Upper Bound
10%	LPS	-.33833*	.01912	.000	-.4088	-.2679
	LPS+10%	-.00433	.01912	1.000	-.0748	.0661
	500Glu	-.33333*	.01912	.000	-.4038	-.2629
	250Glu	-.14667*	.01912	.000	-.2171	-.0762
	125Glu	.01067	.01912	1.000	-.0598	.0811
	100Mucus	-.15967*	.01912	.000	-.2301	-.0892
	50Mucus	-.00267	.01912	1.000	-.0731	.0678
	25Mucus	-.25633*	.01912	.000	-.3268	-.1859
	150Broiler	-.15800*	.01912	.000	-.2285	-.0875
	100Broiler	-.01067	.01912	1.000	-.0811	.0598
	50Broiler	-.05267	.01912	.322	-.1231	.0178
	150Korat	-.18600*	.01912	.000	-.2565	-.1155
	100Korat	-.14833*	.01912	.000	-.2188	-.0779
	50Korat	-.02033	.01912	.999	-.0908	.0501
LPS	10%	.33833*	.01912	.000	.2679	.4088
	LPS+10%	.33400*	.01912	.000	.2635	.4045
	500Glu	.00500	.01912	1.000	-.0655	.0755
	250Glu	.19167*	.01912	.000	.1212	.2621
	125Glu	.34900*	.01912	.000	.2785	.4195
	100Mucus	.17867*	.01912	.000	.1082	.2491
	50Mucus	.33567*	.01912	.000	.2652	.4061
	25Mucus	.08200*	.01912	.011	.0115	.1525
	150Broiler	.18033*	.01912	.000	.1099	.2508
	100Broiler	.32767*	.01912	.000	.2572	.3981
	50Broiler	.28567*	.01912	.000	.2152	.3561
	150Korat	.15233*	.01912	.000	.0819	.2228
	100Korat	.19000*	.01912	.000	.1195	.2605
	50Korat	.31800*	.01912	.000	.2475	.3885

* The mean difference is significant at the 0.05 level.

Table A10 Statistic comparison iNOS in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
LPS+10%	10%	.00433	.01912	1.000	-.0661	.0748
	LPS	-.33400*	.01912	.000	-.4045	-.2635
	500Glu	-.32900*	.01912	.000	-.3995	-.2585
	250Glu	-.14233*	.01912	.000	-.2128	-.0719
	125Glu	.01500	.01912	1.000	-.0555	.0855
	100Mucus	-.15533*	.01912	.000	-.2258	-.0849
	50Mucus	.00167	.01912	1.000	-.0688	.0721
	25Mucus	-.25200*	.01912	.000	-.3225	-.1815
	150Broiler	-.15367*	.01912	.000	-.2241	-.0832
	100Broiler	-.00633	.01912	1.000	-.0768	.0641
	50Broiler	-.04833	.01912	.452	-.1188	.0221
	150Korat	-.18167*	.01912	.000	-.2521	-.1112
	100Korat	-.14400*	.01912	.000	-.2145	-.0735
	50Korat	-.01600	.01912	1.000	-.0865	.0545
500Glu	10%	.33333*	.01912	.000	.2629	.4038
	LPS	-.00500	.01912	1.000	-.0755	.0655
	LPS+10%	.32900*	.01912	.000	.2585	.3995
	250Glu	.18667*	.01912	.000	.1162	.2571
	125Glu	.34400*	.01912	.000	.2735	.4145
	100Mucus	.17367*	.01912	.000	.1032	.2441
	50Mucus	.33067*	.01912	.000	.2602	.4011
	25Mucus	.07700*	.01912	.022	.0065	.1475
	150Broiler	.17533*	.01912	.000	.1049	.2458
	100Broiler	.32267*	.01912	.000	.2522	.3931
	50Broiler	.28067*	.01912	.000	.2102	.3511
	150Korat	.14733*	.01912	.000	.0769	.2178
	100Korat	.18500*	.01912	.000	.1145	.2555
	50Korat	.31300*	.01912	.000	.2425	.3835

* The mean difference is significant at the 0.05 level.

Table A10 Statistic comparison iNOS in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
250Glu	10%	.14667*	.01912	.000	.0762	.2171
	LPS	-.19167*	.01912	.000	-.2621	-.1212
	LPS+10%	.14233*	.01912	.000	.0719	.2128
	500Glu	-.18667*	.01912	.000	-.2571	-.1162
	125Glu	.15733*	.01912	.000	.0869	.2278
	100Mucus	-.01300	.01912	1.000	-.0835	.0575
	50Mucus	.14400*	.01912	.000	.0735	.2145
	25Mucus	-.10967*	.01912	.000	-.1801	-.0392
	150Broiler	-.01133	.01912	1.000	-.0818	.0591
	100Broiler	.13600*	.01912	.000	.0655	.2065
	50Broiler	.09400*	.01912	.002	.0235	.1645
	150Korat	-.03933	.01912	.751	-.1098	.0311
	100Korat	-.00167	.01912	1.000	-.0721	.0688
	50Korat	.12633*	.01912	.000	.0559	.1968
125Glu	10%	-.01067	.01912	1.000	-.0811	.0598
	LPS	-.34900*	.01912	.000	-.4195	-.2785
	LPS+10%	-.01500	.01912	1.000	-.0855	.0555
	500Glu	-.34400*	.01912	.000	-.4145	-.2735
	250Glu	-.15733*	.01912	.000	-.2278	-.0869
	100Mucus	-.17033*	.01912	.000	-.2408	-.0999
	50Mucus	-.01333	.01912	1.000	-.0838	.0571
	25Mucus	-.26700*	.01912	.000	-.3375	-.1965
	150Broiler	-.16867*	.01912	.000	-.2391	-.0982
	100Broiler	-.02133	.01912	.998	-.0918	.0491
	50Broiler	-.06333	.01912	.114	-.1338	.0071
	150Korat	-.19667*	.01912	.000	-.2671	-.1262
	100Korat	-.15900*	.01912	.000	-.2295	-.0885
	50Korat	-.03100	.01912	.940	-.1015	.0395

* The mean difference is significant at the 0.05 level.

Table A10 Statistic comparison iNOS in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
100Mucus	10%	.15967*	.01912	.000	.0892	.2301
	LPS	-.17867*	.01912	.000	-.2491	-.1082
	LPS+10%	.15533*	.01912	.000	.0849	.2258
	500Glu	-.17367*	.01912	.000	-.2441	-.1032
	250Glu	.01300	.01912	1.000	-.0575	.0835
	125Glu	.17033*	.01912	.000	.0999	.2408
	50Mucus	.15700*	.01912	.000	.0865	.2275
	25Mucus	-.09667*	.01912	.002	-.1671	-.0262
	150Broiler	.00167	.01912	1.000	-.0688	.0721
	100Broiler	.14900*	.01912	.000	.0785	.2195
	50Broiler	.10700*	.01912	.000	.0365	.1775
	150Korat	-.02633	.01912	.983	-.0968	.0441
	100Korat	.01133	.01912	1.000	-.0591	.0818
	50Korat	.13933*	.01912	.000	.0689	.2098
50Mucus	10%	.00267	.01912	1.000	-.0678	.0731
	LPS	-.33567*	.01912	.000	-.4061	-.2652
	LPS+10%	-.00167	.01912	1.000	-.0721	.0688
	500Glu	-.33067*	.01912	.000	-.4011	-.2602
	250Glu	-.14400*	.01912	.000	-.2145	-.0735
	125Glu	.01333	.01912	1.000	-.0571	.0838
	100Mucus	-.15700*	.01912	.000	-.2275	-.0865
	25Mucus	-.25367*	.01912	.000	-.3241	-.1832
	150Broiler	-.15533*	.01912	.000	-.2258	-.0849
	100Broiler	-.00800	.01912	1.000	-.0785	.0625
	50Broiler	-.05000	.01912	.399	-.1205	.0205
	150Korat	-.18333*	.01912	.000	-.2538	-.1129
	100Korat	-.14567*	.01912	.000	-.2161	-.0752
	50Korat	-.01767	.01912	1.000	-.0881	.0528

* The mean difference is significant at the 0.05 level.

Table A10 Statistic comparison iNOS in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
25Mucus	10%	.25633*	.01912	.000	.1859	.3268
	LPS	-.08200*	.01912	.011	-.1525	-.0115
	LPS+10%	.25200*	.01912	.000	.1815	.3225
	500Glu	-.07700*	.01912	.022	-.1475	-.0065
	250Glu	.10967*	.01912	.000	.0392	.1801
	125Glu	.26700*	.01912	.000	.1965	.3375
	100Mucus	.09667*	.01912	.002	.0262	.1671
	50Mucus	.25367*	.01912	.000	.1832	.3241
	150Broiler	.09833*	.01912	.001	.0279	.1688
	100Broiler	.24567*	.01912	.000	.1752	.3161
	50Broiler	.20367*	.01912	.000	.1332	.2741
	150Korat	.07033	.01912	.051	-.0001	.1408
	100Korat	.10800*	.01912	.000	.0375	.1785
	50Korat	.23600*	.01912	.000	.1655	.3065
150Broiler	10%	.15800*	.01912	.000	.0875	.2285
	LPS	-.18033*	.01912	.000	-.2508	-.1099
	LPS+10%	.15367*	.01912	.000	.0832	.2241
	500Glu	-.17533*	.01912	.000	-.2458	-.1049
	250Glu	.01133	.01912	1.000	-.0591	.0818
	125Glu	.16867*	.01912	.000	.0982	.2391
	100Mucus	-.00167	.01912	1.000	-.0721	.0688
	50Mucus	.15533*	.01912	.000	.0849	.2258
	25Mucus	-.09833*	.01912	.001	-.1688	-.0279
	100Broiler	.14733*	.01912	.000	.0769	.2178
	50Broiler	.10533*	.01912	.000	.0349	.1758
	150Korat	-.02800	.01912	.972	-.0985	.0425
	100Korat	.00967	.01912	1.000	-.0608	.0801
	50Korat	.13767*	.01912	.000	.0672	.2081

* The mean difference is significant at the 0.05 level.

Table A10 Statistic comparison iNOS in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
100Broiler	10%	.01067	.01912	1.000	-.0598	.0811
	LPS	-.32767*	.01912	.000	-.3981	-.2572
	LPS+10%	.00633	.01912	1.000	-.0641	.0768
	500Glu	-.32267*	.01912	.000	-.3931	-.2522
	250Glu	-.13600*	.01912	.000	-.2065	-.0655
	125Glu	.02133	.01912	.998	-.0491	.0918
	100Mucus	-.14900*	.01912	.000	-.2195	-.0785
	50Mucus	.00800	.01912	1.000	-.0625	.0785
	25Mucus	-.24567*	.01912	.000	-.3161	-.1752
	150Broiler	-.14733*	.01912	.000	-.2178	-.0769
	50Broiler	-.04200	.01912	.665	-.1125	.0285
	150Korat	-.17533*	.01912	.000	-.2458	-.1049
	100Korat	-.13767*	.01912	.000	-.2081	-.0672
	50Korat	-.00967	.01912	1.000	-.0801	.0608
50Broiler	10%	.05267	.01912	.322	-.0178	.1231
	LPS	-.28567*	.01912	.000	-.3561	-.2152
	LPS+10%	.04833	.01912	.452	-.0221	.1188
	500Glu	-.28067*	.01912	.000	-.3511	-.2102
	250Glu	-.09400*	.01912	.002	-.1645	-.0235
	125Glu	.06333	.01912	.114	-.0071	.1338
	100Mucus	-.10700*	.01912	.000	-.1775	-.0365
	50Mucus	.05000	.01912	.399	-.0205	.1205
	25Mucus	-.20367*	.01912	.000	-.2741	-.1332
	150Broiler	-.10533*	.01912	.000	-.1758	-.0349
	100Broiler	.04200	.01912	.665	-.0285	.1125
	150Korat	-.13333*	.01912	.000	-.2038	-.0629
	100Korat	-.09567*	.01912	.002	-.1661	-.0252
	50Korat	.03233	.01912	.920	-.0381	.1028

* The mean difference is significant at the 0.05 level.

Table A10 Statistic comparison iNOS in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
150Korat	10%	.18600*	.01912	.000	.1155	.2565
	LPS	-.15233*	.01912	.000	-.2228	-.0819
	LPS+10%	.18167*	.01912	.000	.1112	.2521
	500Glu	-.14733*	.01912	.000	-.2178	-.0769
	250Glu	.03933	.01912	.751	-.0311	.1098
	125Glu	.19667*	.01912	.000	.1262	.2671
	100Mucus	.02633	.01912	.983	-.0441	.0968
	50Mucus	.18333*	.01912	.000	.1129	.2538
	25Mucus	-.07033	.01912	.051	-.1408	.0001
	150Broiler	.02800	.01912	.972	-.0425	.0985
	100Broiler	.17533*	.01912	.000	.1049	.2458
	50Broiler	.13333*	.01912	.000	.0629	.2038
	100Korat	.03767	.01912	.799	-.0328	.1081
	50Korat	.16567*	.01912	.000	.0952	.2361
100Korat	10%	.14833*	.01912	.000	.0779	.2188
	LPS	-.19000*	.01912	.000	-.2605	-.1195
	LPS+10%	.14400*	.01912	.000	.0735	.2145
	500Glu	-.18500*	.01912	.000	-.2555	-.1145
	250Glu	.00167	.01912	1.000	-.0688	.0721
	125Glu	.15900*	.01912	.000	.0885	.2295
	100Mucus	-.01133	.01912	1.000	-.0818	.0591
	50Mucus	.14567*	.01912	.000	.0752	.2161
	25Mucus	-.10800*	.01912	.000	-.1785	-.0375
	150Broiler	-.00967	.01912	1.000	-.0801	.0608
	100Broiler	.13767*	.01912	.000	.0672	.2081
	50Broiler	.09567*	.01912	.002	.0252	.1661
	150Korat	-.03767	.01912	.799	-.1081	.0328
	50Korat	.12800*	.01912	.000	.0575	.1985

* The mean difference is significant at the 0.05 level.

Table A10 Statistic comparison iNOS in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
50Korat	10%	.02033	.01912	.999	-.0501	.0908
	LPS	-.31800*	.01912	.000	-.3885	-.2475
	LPS+10%	.01600	.01912	1.000	-.0545	.0865
	500Glu	-.31300*	.01912	.000	-.3835	-.2425
	250Glu	-.12633*	.01912	.000	-.1968	-.0559
	125Glu	.03100	.01912	.940	-.0395	.1015
	100Mucus	-.13933*	.01912	.000	-.2098	-.0689
	50Mucus	.01767	.01912	1.000	-.0528	.0881
	25Mucus	-.23600*	.01912	.000	-.3065	-.1655
	150Broiler	-.13767*	.01912	.000	-.2081	-.0672
	100Broiler	.00967	.01912	1.000	-.0608	.0801
	50Broiler	-.03233	.01912	.920	-.1028	.0381
	150Korat	-.16567*	.01912	.000	-.2361	-.0952
	100Korat	-.12800*	.01912	.000	-.1985	-.0575

* The mean difference is significant at the 0.05 level.

Table A11 Statistic comparison COX-2 in each treatment group after cells inflammation.

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
10%	LPS	-.17467*	.02978	.000	-.2844	-.0649
	LPS+10%	.00067	.02978	1.000	-.1091	.1104
	500Glu	-.26633*	.02978	.000	-.3761	-.1566
	250Glu	-.20633*	.02978	.000	-.3161	-.0966
	125Glu	-.01667	.02978	1.000	-.1264	.0931
	100Mucus	-.20133*	.02978	.000	-.3111	-.0916
	50Mucus	-.07133	.02978	.536	-.1811	.0384
	25Mucus	-.26500*	.02978	.000	-.3747	-.1553
	150Broiler	-.24800*	.02978	.000	-.3577	-.1383
	100Broiler	-.06567	.02978	.660	-.1754	.0441
	50Broiler	-.15933*	.02978	.001	-.2691	-.0496
	150Korat	-.44333*	.02978	.000	-.5531	-.3336
	100Korat	-.30800*	.02978	.000	-.4177	-.1983
	50Korat	-.14400*	.02978	.003	-.2537	-.0343
LPS	10%	.17467*	.02978	.000	.0649	.2844
	LPS+10%	.17533*	.02978	.000	.0656	.2851
	500Glu	-.09167	.02978	.182	-.2014	.0181
	250Glu	-.03167	.02978	.999	-.1414	.0781
	125Glu	.15800*	.02978	.001	.0483	.2677
	100Mucus	-.02667	.02978	1.000	-.1364	.0831
	50Mucus	.10333	.02978	.081	-.0064	.2131
	25Mucus	-.09033	.02978	.198	-.2001	.0194
	150Broiler	-.07333	.02978	.493	-.1831	.0364
	100Broiler	.10900	.02978	.053	-.0007	.2187
	50Broiler	.01533	.02978	1.000	-.0944	.1251
	150Korat	-.26867*	.02978	.000	-.3784	-.1589
	100Korat	-.13333*	.02978	.007	-.2431	-.0236
	50Korat	.03067	.02978	.999	-.0791	.1404

* The mean difference is significant at the 0.05 level.

Table A11 Statistic comparison COX-2 in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
LPS+10%	10%	-.00067	.02978	1.000	-.1104	.1091
	LPS	-.17533*	.02978	.000	-.2851	-.0656
	500Glu	-.26700*	.02978	.000	-.3767	-.1573
	250Glu	-.20700*	.02978	.000	-.3167	-.0973
	125Glu	-.01733	.02978	1.000	-.1271	.0924
	100Mucus	-.20200*	.02978	.000	-.3117	-.0923
	50Mucus	-.07200	.02978	.521	-.1817	.0377
	25Mucus	-.26567*	.02978	.000	-.3754	-.1559
	150Broiler	-.24867*	.02978	.000	-.3584	-.1389
	100Broiler	-.06633	.02978	.645	-.1761	.0434
	50Broiler	-.16000*	.02978	.001	-.2697	-.0503
	150Korat	-.44400*	.02978	.000	-.5537	-.3343
	100Korat	-.30867*	.02978	.000	-.4184	-.1989
	50Korat	-.14467*	.02978	.003	-.2544	-.0349
500Glu	10%	.26633*	.02978	.000	.1566	.3761
	LPS	.09167	.02978	.182	-.0181	.2014
	LPS+10%	.26700*	.02978	.000	.1573	.3767
	250Glu	.06000	.02978	.775	-.0497	.1697
	125Glu	.24967*	.02978	.000	.1399	.3594
	100Mucus	.06500	.02978	.674	-.0447	.1747
	50Mucus	.19500*	.02978	.000	.0853	.3047
	25Mucus	.00133	.02978	1.000	-.1084	.1111
	150Broiler	.01833	.02978	1.000	-.0914	.1281
	100Broiler	.20067*	.02978	.000	.0909	.3104
	50Broiler	.10700	.02978	.062	-.0027	.2167
	150Korat	-.17700*	.02978	.000	-.2867	-.0673
	100Korat	-.04167	.02978	.981	-.1514	.0681
	50Korat	.12233*	.02978	.018	.0126	.2321

* The mean difference is significant at the 0.05 level.

Table A11 Statistic comparison COX-2 in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
250Glu	10%	.20633*	.02978	.000	.0966	.3161
	LPS	.03167	.02978	.999	-.0781	.1414
	LPS+10%	.20700*	.02978	.000	.0973	.3167
	500Glu	-.06000	.02978	.775	-.1697	.0497
	125Glu	.18967*	.02978	.000	.0799	.2994
	100Mucus	.00500	.02978	1.000	-.1047	.1147
	50Mucus	.13500*	.02978	.006	.0253	.2447
	25Mucus	-.05867	.02978	.800	-.1684	.0511
	150Broiler	-.04167	.02978	.981	-.1514	.0681
	100Broiler	.14067*	.02978	.004	.0309	.2504
	50Broiler	.04700	.02978	.951	-.0627	.1567
	150Korat	-.23700*	.02978	.000	-.3467	-.1273
	100Korat	-.10167	.02978	.092	-.2114	.0081
	50Korat	.06233	.02978	.729	-.0474	.1721
125Glu	10%	.01667	.02978	1.000	-.0931	.1264
	LPS	-.15800*	.02978	.001	-.2677	-.0483
	LPS+10%	.01733	.02978	1.000	-.0924	.1271
	500Glu	-.24967*	.02978	.000	-.3594	-.1399
	250Glu	-.18967*	.02978	.000	-.2994	-.0799
	100Mucus	-.18467*	.02978	.000	-.2944	-.0749
	50Mucus	-.05467	.02978	.865	-.1644	.0551
	25Mucus	-.24833*	.02978	.000	-.3581	-.1386
	150Broiler	-.23133*	.02978	.000	-.3411	-.1216
	100Broiler	-.04900	.02978	.934	-.1587	.0607
	50Broiler	-.14267*	.02978	.003	-.2524	-.0329
	150Korat	-.42667*	.02978	.000	-.5364	-.3169
	100Korat	-.29133*	.02978	.000	-.4011	-.1816
	50Korat	-.12733*	.02978	.012	-.2371	-.0176

* The mean difference is significant at the 0.05 level.

Table A11 Statistic comparison COX-2 in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
100Mucus	10%	.20133*	.02978	.000	.0916	.3111
	LPS	.02667	.02978	1.000	-.0831	.1364
	LPS+10%	.20200*	.02978	.000	.0923	.3117
	500Glu	-.06500	.02978	.674	-.1747	.0447
	250Glu	-.00500	.02978	1.000	-.1147	.1047
	125Glu	.18467*	.02978	.000	.0749	.2944
	50Mucus	.13000*	.02978	.009	.0203	.2397
	25Mucus	-.06367	.02978	.702	-.1734	.0461
	150Broiler	-.04667	.02978	.953	-.1564	.0631
	100Broiler	.13567*	.02978	.006	.0259	.2454
	50Broiler	.04200	.02978	.980	-.0677	.1517
	150Korat	-.24200*	.02978	.000	-.3517	-.1323
	100Korat	-.10667	.02978	.063	-.2164	.0031
	50Korat	.05733	.02978	.823	-.0524	.1671
50Mucus	10%	.07133	.02978	.536	-.0384	.1811
	LPS	-.10333	.02978	.081	-.2131	.0064
	LPS+10%	.07200	.02978	.521	-.0377	.1817
	500Glu	-.19500*	.02978	.000	-.3047	-.0853
	250Glu	-.13500*	.02978	.006	-.2447	-.0253
	125Glu	.05467	.02978	.865	-.0551	.1644
	100Mucus	-.13000*	.02978	.009	-.2397	-.0203
	25Mucus	-.19367*	.02978	.000	-.3034	-.0839
	150Broiler	-.17667*	.02978	.000	-.2864	-.0669
	100Broiler	.00567	.02978	1.000	-.1041	.1154
	50Broiler	-.08800	.02978	.229	-.1977	.0217
	150Korat	-.37200*	.02978	.000	-.4817	-.2623
	100Korat	-.23667*	.02978	.000	-.3464	-.1269
	50Korat	-.07267	.02978	.507	-.1824	.0371

* The mean difference is significant at the 0.05 level.

Table A11 Statistic comparison COX-2 in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
25Mucus	10%	.26500*	.02978	.000	.1553	.3747
	LPS	.09033	.02978	.198	-.0194	.2001
	LPS+10%	.26567*	.02978	.000	.1559	.3754
	500Glu	-.00133	.02978	1.000	-.1111	.1084
	250Glu	.05867	.02978	.800	-.0511	.1684
	125Glu	.24833*	.02978	.000	.1386	.3581
	100Mucus	.06367	.02978	.702	-.0461	.1734
	50Mucus	.19367*	.02978	.000	.0839	.3034
	150Broiler	.01700	.02978	1.000	-.0927	.1267
	100Broiler	.19933*	.02978	.000	.0896	.3091
	50Broiler	.10567	.02978	.068	-.0041	.2154
	150Korat	-.17833*	.02978	.000	-.2881	-.0686
	100Korat	-.04300	.02978	.975	-.1527	.0667
	50Korat	.12100*	.02978	.020	.0113	.2307
150Broiler	10%	.24800*	.02978	.000	.1383	.3577
	LPS	.07333	.02978	.493	-.0364	.1831
	LPS+10%	.24867*	.02978	.000	.1389	.3584
	500Glu	-.01833	.02978	1.000	-.1281	.0914
	250Glu	.04167	.02978	.981	-.0681	.1514
	125Glu	.23133*	.02978	.000	.1216	.3411
	100Mucus	.04667	.02978	.953	-.0631	.1564
	50Mucus	.17667*	.02978	.000	.0669	.2864
	25Mucus	-.01700	.02978	1.000	-.1267	.0927
	100Broiler	.18233*	.02978	.000	.0726	.2921
	50Broiler	.08867	.02978	.219	-.0211	.1984
	150Korat	-.19533*	.02978	.000	-.3051	-.0856
	100Korat	-.06000	.02978	.775	-.1697	.0497
	50Korat	.10400	.02978	.077	-.0057	.2137

* The mean difference is significant at the 0.05 level.

Table A11 Statistic comparison COX-2 in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
100Broiler	10%	.06567	.02978	.660	-.0441	.1754
	LPS	-.10900	.02978	.053	-.2187	.0007
	LPS+10%	.06633	.02978	.645	-.0434	.1761
	500Glu	-.20067*	.02978	.000	-.3104	-.0909
	250Glu	-.14067*	.02978	.004	-.2504	-.0309
	125Glu	.04900	.02978	.934	-.0607	.1587
	100Mucus	-.13567*	.02978	.006	-.2454	-.0259
	50Mucus	-.00567	.02978	1.000	-.1154	.1041
	25Mucus	-.19933*	.02978	.000	-.3091	-.0896
	150Broiler	-.18233*	.02978	.000	-.2921	-.0726
	50Broiler	-.09367	.02978	.160	-.2034	.0161
	150Korat	-.37767*	.02978	.000	-.4874	-.2679
	100Korat	-.24233*	.02978	.000	-.3521	-.1326
	50Korat	-.07833	.02978	.390	-.1881	.0314
50Broiler	10%	.15933*	.02978	.001	.0496	.2691
	LPS	-.01533	.02978	1.000	-.1251	.0944
	LPS+10%	.16000*	.02978	.001	.0503	.2697
	500Glu	-.10700	.02978	.062	-.2167	.0027
	250Glu	-.04700	.02978	.951	-.1567	.0627
	125Glu	.14267*	.02978	.003	.0329	.2524
	100Mucus	-.04200	.02978	.980	-.1517	.0677
	50Mucus	.08800	.02978	.229	-.0217	.1977
	25Mucus	-.10567	.02978	.068	-.2154	.0041
	150Broiler	-.08867	.02978	.219	-.1984	.0211
	100Broiler	.09367	.02978	.160	-.0161	.2034
	150Korat	-.28400*	.02978	.000	-.3937	-.1743
	100Korat	-.14867*	.02978	.002	-.2584	-.0389
	50Korat	.01533	.02978	1.000	-.0944	.1251

* The mean difference is significant at the 0.05 level.

Table A11 Statistic comparison COX-2 in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
150Korat	10%	.44333*	.02978	.000	.3336	.5531
	LPS	.26867*	.02978	.000	.1589	.3784
	LPS+10%	.44400*	.02978	.000	.3343	.5537
	500Glu	.17700*	.02978	.000	.0673	.2867
	250Glu	.23700*	.02978	.000	.1273	.3467
	125Glu	.42667*	.02978	.000	.3169	.5364
	100Mucus	.24200*	.02978	.000	.1323	.3517
	50Mucus	.37200*	.02978	.000	.2623	.4817
	25Mucus	.17833*	.02978	.000	.0686	.2881
	150Broiler	.19533*	.02978	.000	.0856	.3051
	100Broiler	.37767*	.02978	.000	.2679	.4874
	50Broiler	.28400*	.02978	.000	.1743	.3937
	100Korat	.13533*	.02978	.006	.0256	.2451
	50Korat	.29933*	.02978	.000	.1896	.4091
100Korat	10%	.30800*	.02978	.000	.1983	.4177
	LPS	.13333*	.02978	.007	.0236	.2431
	LPS+10%	.30867*	.02978	.000	.1989	.4184
	500Glu	.04167	.02978	.981	-.0681	.1514
	250Glu	.10167	.02978	.092	-.0081	.2114
	125Glu	.29133*	.02978	.000	.1816	.4011
	100Mucus	.10667	.02978	.063	-.0031	.2164
	50Mucus	.23667*	.02978	.000	.1269	.3464
	25Mucus	.04300	.02978	.975	-.0667	.1527
	150Broiler	.06000	.02978	.775	-.0497	.1697
	100Broiler	.24233*	.02978	.000	.1326	.3521
	50Broiler	.14867*	.02978	.002	.0389	.2584
	150Korat	-.13533*	.02978	.006	-.2451	-.0256
	50Korat	.16400*	.02978	.000	.0543	.2737

* The mean difference is significant at the 0.05 level.

Table A11 Statistic comparison COX-2 in each treatment group after cells inflammation (Continued).

(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
50Korat	10%	.14400*	.02978	.003	.0343	.2537
	LPS	-.03067	.02978	.999	-.1404	.0791
	LPS+10%	.14467*	.02978	.003	.0349	.2544
	500Glu	-.12233*	.02978	.018	-.2321	-.0126
	250Glu	-.06233	.02978	.729	-.1721	.0474
	125Glu	.12733*	.02978	.012	.0176	.2371
	100Mucus	-.05733	.02978	.823	-.1671	.0524
	50Mucus	.07267	.02978	.507	-.0371	.1824
	25Mucus	-.12100*	.02978	.020	-.2307	-.0113
	150Broiler	-.10400	.02978	.077	-.2137	.0057
	100Broiler	.07833	.02978	.390	-.0314	.1881
	50Broiler	-.01533	.02978	1.000	-.1251	.0944
	150Korat	-.29933*	.02978	.000	-.4091	-.1896
	100Korat	-.16400*	.02978	.000	-.2737	-.0543

* The mean difference is significant at the 0.05 level.

APPENDIX B

INFORMATION FOR ANALYSIS

The output of the sample size calculation from n4studies. For estimating the infinite population proportion.

$$n = \frac{z^2_{1-\frac{\propto}{2}}\, p(1-p)}{d^2}$$

Proportion (p) = 0.95, Error (d) = 0.1

Alpha (α) = 0.05 Z (0.975) = 1.959964

Sample size (n) = 19 ˜20

APPENDIX C

THE PREPARATIONS OF REAGENTS

Phosphate Buffer saline pH 7.4

Chemicals

Sodium chloride (NaCl)	8 g
Potassium chloride (KCl)	0.2 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.44 g
Potassium dihydrogen Phosphate (KH_2PO_4)	0.24 g
Distilled water	1 L

Preparation

Add chemical compounds one by one into 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Add distilled water to a total volume of 1 liter. Sterilization by autoclaving (20 min, 121°C, liquid cycle). Store at room temperature.

Coating Buffer (carbonate buffer), 0.05 M pH 9.6

Chemicals

15 mM Sodium carbonate (Na_2CO_3)	1.59 g
35 mM Sodium bicarbonate ($NaHCO_3$)	2.93 g
Distilled water	1 L

Preparation

The total chemical compounds were mixed in distilled water. pH was adjusted to 9.6. Sterilization by autoclaving (20 min, 121°C, liquid cycle).

Lysis Buffer

Chemicals

10 mM TRIS hydrochloride (Tris-HCl) pH 7.2	0.16 g
150 mM Sodium chloride (NaCl)	0.87 g
1 mM Ethylenediaminetetraacetic acid (EDTA)	0.04 g
0.5% Triton X-100	25 µl
1 mM Phenylmethylsulfonyl fluoride (PMSF)	50 µl
Distilled water	100 ml

Preparation

Prepare stock solution by adding Tris-HCl, NaCl, and EDTA into 80 ml distilled water. Adjust pH to 7.2 and then add distilled water to a total volume of 100 ml. Sterilization by autoclaving (20 min, 121°C, liquid cycle program). Prepare working solution by adding PMSF and Triton X-100 into 4.945 ml stock solution.

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