ผลของไตรแอมซิโนโลนอะซีโทไนด์ต่อการแสดงออกของยีนที่เกี่ยวข้องกับการตายของเซลล์โดยภาวะเครียดจากออกซิเดชันและการมีชีวิตของเซลล์กระดูกอ่อน

นางสาวมณฑิรา สันติภาพลือชา

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EFFECTS OF TRIAMCINOLONE ACETONIDE ON EXPRESSION OF GENES INVOLVED IN CELL DEATH BY OXIDATIVE STRESS AND CHONDROCYTE VIABILITY

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มณฑิรา สันติภาพลือชา : ผลของไตรแอมซิโนโลนอะซีโทไนด์ต่อการแสดงออกของยีนที่เกี่ยวข้องกับการตายของเซลล์โดยภาวะเครียดจากออกซิเดชันและการมีชีวิตของเซลล์กระดูกอ่อน (EFFECTS OF TRIAMCINOLONE ACETONIDE ON EXPRESSION OF GENESINVOLVED IN CELL DEATH BY OXIDATIVE STRESS AND CHONDROCYTE VIABILITY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. รัชนีกร ธรรมโชติ, 119 หน้า.

ไตรแอมซิโนโลนอะซีโทไนด์ (TA) เป็นยาในกลุ่มกลูโคคอร์ติคอย (GC) ที่นิยมใช้ฉีดข้อโดยตรงเพื่อลดอาการปวดในผู้ป่วยโรคข้อเสื่อม อย่างไรก็ตามสาเหตุการเกิดพิษต่อกระดูกอ่อนจากการใช้ยาในกลุ่มนี้ยังไม่ทราบ แน่ชัด มีทฤษฎีที่กล่าวว่ายาในกลุ่ม GC กระตุ้นให้เซลล์กระดูกอ่อนให้เกิดความเครียดออกซิเดชันและส่งผลให้เกิดการ เปลี่ยนแปลงการแสดงออกของยีนที่เกี่ยวข้องกับเซลล์ตายและการหมุนเวียนภาวะ ธำรงดุลของสารแมทริกซ์ภายนอกเซลล์ ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาว่า TA กระตุ้นเซลล์กระดูกอ่อนให้เกิดความเครียดออกซิเดชัน และเปลี่ยนแปลงการแสดงออกของยีนที่เกี่ยวข้องกับการตายของเซลล์และภาวะธำรงดุลของสารแมทริกซ์ภายนอกเซลล์กระดูกกอ่อนหรือไม่ เซลล์กระดูกอ่อนจากผู้ป่วยโรคข้อเข่าเสื่อม 10 รายถูกบ่มด้วย TA ที่ความเข้มข้น 0, 1, 5 และ 10 มิลลิกรัมต่อมิลลิลิตร ร่วมกับวิตามินซี 100 ไมโครโมลาร์ ในการศึกษาผลของยาต่อการมีชีวิตของเซลล์ จะบ่มเซลล์ 7 และ 14 วัน และในการศึกษาผลของยาต่อความเครียดออกซิเดชันและวิเคราะห์การแสดงออกของยีน เซลล์จะถูกบ่ม 48 ชั่วโมง ผลการทดลองแสดงให้เห็นว่า ระดับการมีชีวิตของเซลล์กระดูกอ่อนลดลงอย่างมีนัยสำคัญเมื่อถูกบ่มด้วย TA ที่ทุกความเข้มข้นที่ทดสอบ ความเครียดจากออกซิเดชันในเซลล์เพิ่มขึ้นอย่างมีนัยสำคัญที่ความเข้มข้นยา 5 มิลลิกรัมต่อมิลลิลิตร นอกจากนี้ วิตามินซีช่วยเพิ่มระดับการมีชีวิตและลดความเครียดออกซิเดชันของเซลล์กระดูกอ่อนที่ TA ความเข้มข้น 5 มิลลิกรัมต่อมิลลิลิตร TA เพิ่มการแสดงออกของยีนที่เกี่ยวข้องกับการหยุดวัฏจักรของเซลล์และการย่อยสารแมทริกซ์ภายนอกเซลล์ ได้แก่ *P21, GDF15, cFos* และ *MMP-3* อย่างมีนัยสำคัญ จากผลการทดลองสามารถสรุปได้ว่า TA ส่งผลให้เกิดพิษต่อเซลล์กระดูกอ่อนโดยกระตุ้นให้เกิดความเครียดออกซิเดชันและเพิ่มการแสดงออกของยีน วิตามินซีเพิ่มการมีชีวิตของเซลล์และลดระดับออกซิเดชันในเซลล์กระดูกอ่อนที่ถูกบ่มด้วย TA ความรู้ที่ได้จากงานวิจัยนี้เป็นข้อมูลสำหรับแพทย์เพื่อใช้เพิ่ม ประสิทธิภาพในการวางแผนการรักษาด้วย TA ในผู้ป่วย OA

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

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MONTHIRA SUNTIPARPLUACHA: EFFECTS OF TRIAMCINOLONE ACETONIDE ON EXPRESSION OF GENESINVOLVED IN CELL DEATH BY OXIDATIVE STRESS AND CHONDROCYTE VIABILITY. ADVISOR: ASST. PROF. RACHANEEKORN TAMMACHOTE, Ph.D., 119 pp.

Triamcinolone acetonide (TA) is a glucocorticoids (GCs) used for pain reduction in patients with osteoarthritic (OA) by intra-articular injection. However, how GCs cause toxicity to cartilage are inconclusive. Hypotheses are that GCs induce oxidative stress in chondrocytes and altering expressions of genes involved in cell death and extracellular matrix homeostasis. Therefore, aims of this study are to determine whether TA induces oxidative stress and altered genes involved in cell death and extracellular matrix homeostasis in chondrocytes. Primary chondrocytes isolated from 10 knee OA patients were treated with TA at 0, 1, 5, and 10 mg/ml, with or without 100 µM of vitamin C. For viability analysis, cells were incubated for 7 and 14 days, and for oxidative status and gene expression analyses, cells were incubated for 48 h. Results showed that viability of TA-treated chondrocytes significantly decreased in all concentrations tested. Oxidative stress significantly increased at 5 mg/ml of TA treatment. Addition of vitamin C significantly increases chondrocyte viability and decrease oxidative stress when treated with 5 mg/ml of TA. TA significantly increased expressions of *P21, GDF15, cFos,* and *MMP-3*. These results suggest that the causes of TA-induced toxicity to chondrocytes by stimulation of oxidative stress and expressions of genes involving cell death and extracellular matrix degradation. Vitamin C improved cell viability and oxidative status of TA-treated chondrocytes. Knowledge gained from this study will provide information for physicians to improve TA treatment plan in OA patients.

Field of Study: Biotechnology Student's Signature .....

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CHAPTER I
INTRODUCTION

Rationales

Osteoarthritis (OA) of the knee is one of the most frequent type of degenerative joint disorders, and is one of the top ten causes of disability in the elderly population (Fransen et al., 2011). General symptoms of knee OA are joint pain, joint stiffness and in some cases, inflammation in and around joint area. There are several pharmaceutical treatments for pain relief in patients with knee OA (Jevsevar, 2013). First, physicians usually use non-steroidal anti-inflammatory drugs (NSAIDs) or anaesthetics, by orally intake or intra-muscularly injection, in patients. However, many OA patients fail to respond to these drugs and systematic adverse effects can occur to the users (Lavelle, Lavelle and Lavelle, 2007). In those cases, steroid drugs (also known as glucocorticoids (GCs) or corticosteroids) are ultimate medicinal treatment administered by intra-articular injection. Synthetic GCs are very potent anti-inflammatory agents and pain reducers. Their structures are similar to cortisone, a natural hormone. Major mechanism of action of GCs is via binding to intracellular GC receptors. The GC-GC receptor complex then move into nucleus and regulate gene expressions by binding to GC-response elements (Barnes, 1998; Rhen and Cidlowski, 2005). This can either enhance expression of target genes such as anti-inflammatory genes *lipocortin1* or decrease expression of target genes by interfering activity of transcription factor such as *NF-κB* (Cruz-Topete and Cidlowski, 2015).

Several GCs can be used for intra-articular injections. Triamcinolone acetonide (TA) is one of the most selected GCs by physicians due to its water insolubility that makes it slowly released to surrounding cells and stays longer in the body than other water-soluble GCs (Douglas, 2012). However, effects of the drug to cartilage and chondrocytes are still in doubt. Guideline for knee OA treatment still has not strongly

recommended to regularly use glucocorticoids for intra-articular injection since balance between advantages and side effects from the drugs is still unclear (Neustadt, 2001; Jevsevar, 2013). Several studies of effects of TA and drugs in the same group have different results concerning toxicity versus benefits of the drug. For example, a study of TA in horses showed that TA altered balance of extracellular matrix turnover of cartilages towards catabolism of cartilages (Celeste et al., 2005). Also, some *in vitro* studies have reported chondrotoxicity of TA either applied alone (Syed et al., 2011) or in combination with anaesthetics (Braun et al., 2012a). However, a study in patients with rheumatoid arthritis suggested that glucocorticoids might help reduce degradation of cartilages (Weitoft et al., 2005).

One of the adverse effects of TA to cartilages is chondrotoxicity, which might be a result of induction of oxidative stress. A study in retinal cell line showed that TA had significant elevation of reactive oxygen species (ROS) levels (Chung et al., 2007). Another *in vitro* study, corticosterone increased protein carbonylation levels, and decreased activities of mitochondrial complex I and superoxide dismutase in PC12 cell line (Tang et al., 2013). ROS produced in cells can react to cellular macromolecules that might lead to cell death. Moreover, ROS can act as signalling molecules which alter expression of genes involved in cell cycle arrest and apoptosis, especially the ones in p53 target group (Han et al., 2008). Also, activator protein-1 (AP-1) transcription factor has been found to be correlated with oxidative stress, and up-regulation of *cFos*, which is a subunit of AP-1, was reported (Kaur, Kaur and Bansal, 2008). Alterations of cartilage homeostasis is also another adverse effect of TA. These alterations might result from up-regulation of expressions of proteolytic enzyme genes or down-regulation of proteolytic enzyme inhibitors (Sondergaard et al., 2010).

Nevertheless, there has been no study regarding effects of TA on viability, oxidative stress, and gene expressions in human primary chondrocytes from OA patients. Also, effects of antioxidants on TA treated chondrocytes have not been analysed. In this

study, primary chondrocytes isolated from OA patients were used which represented the cells under real disease conditions.

Objectives

To examine whether toxicity of TA to chondrocytes and cartilages is by induction of oxidative stress and alteration of genes involved in oxidative-induced cell death and cartilage homeostasis

Expected beneficial outcomes

Knowledge gained from this study will help physicians improve steroid drugs treatment plan in OA patients, also, provides information of genes that involved in TA-induced oxidative chondrotoxicity and cartilage toxicity.

CHAPTER II
LITERATURE REVIEW

1. Osteoarthritis of the knee

Osteoarthritis (OA) is a progressive, degenerative joint disease, which is caused by both biological and mechanical actions. Main characteristic of knee OA is degradation of joint articular cartilage, formation of osteophytes, and narrow joint space which leads to contact of subchondral bones. Other characteristic symptoms of OA are pain and morning stiffness of the joint, enlargement of bones around the affected joint, crepitus (grating sound) during joint movement, and restricted movement of the joint (National Institute of Arthritis and Musculoskeletal and Skin Diseases, 2010). In some cases, inflammation of affected joints might occur. These will lead to deformation of the joints and disability in patients which later affects the patients' quality of life (Felson, 2013).

1.1. Prevalence

OA is the most common type of arthritis and is found in high prevalence in elderly population (≥ 55 years old), and more frequent in women than men (3:1 ratio) (Bunriang Pitsamai, 2012). The most commonly affected articular joints are knee, hip, and hand, from which, knee OA has the highest incidence rate (Sharma, 2001). Some patients do not show any symptoms but evidence of knee OA (thin articular cartilage layer, narrow joint space, and osteophytes) can be seen on the x-ray radiographic film. Symptomatic knee OA has more clinical significance because it can result in disability and reduce the patient's quality of life since knee OA commonly affects both knees rather than one knee. Although, the majority of knee OA patients are in an elderly population, there are increasing numbers of younger patients (40 - 55 years old) (Felson, 2013).

1.2. Causes of knee OA

OA is a multifactorial disease, which means several risk factors can lead to development and progression of the disease. Important risk factors are weight including body mass index (BMI), age, physical activities and injuries, and gender. People with higher weight, BMI, and physical activities have increased risk of knee OA due to access force onto articular cartilage altering the cartilage homeostasis (Sharma, 2001). In older individuals, alteration of metabolic functions and properties of chondrocytes, synoviocytes and other supporting joint components results in the elevated risk. Women have more risk in developing knee OA than men because hormonal influences on metabolism of bone and articular cartilage (Fransen et al., 2011). Primary OA occurs spontaneously without knowing exact causes, which may be from natural degradation process of the cartilage. On the other hand, secondary OA happens from injuries to the joint or underlying joint diseases that lead to abnormal joint components (Robin Poole, 2001).

Those mentioned risk factors can either directly affect a cartilage or systemically affect environment of a joint. When one or more factors affect any joint components, this will tilt the balance of the components' normal functions that eventually result in OA, this can be implied that the joint components themselves act as joint protectors (Felson, 2013). Components of a joint other than cartilage are joint capsule (movement limitation and lubrication of cartilage), muscles, ligaments and tendons (distribution of tension loads by contraction), sensory nerves (signal transduction between brain and muscles), and subchondral bones (distribution of tension loads) (Felson, 2013).

1.3. Structures and functions of articular cartilage

Articular cartilage is a cartilage that covers a capsulated, yet moveable diarthrodial joint (known as synovial joint) (Dewire, 2001). This component distributes the most protection to the joint it covers and, despite its thinness (< 5 mm) allows painless movement and resistance from any distresses. Components of articular cartilage are similar in every synovial joints, and across species. Even though the cartilage is very durable, it has limited ability to repair itself. In addition, its repairing ability decreases with older age, and contributes to its gradual degradation. This process can subsequently lead to joint pain from contact of subchondral bones (Buckwalter, Mankin and Grodzinsky, 2005). Articular cartilage is an avascular organ, which means it does not contain blood vessels, lymphatic vessels, and nerves. It is composed mainly of extracellular matrix and specialised cells called chondrocytes.

There are 4 zones and 3 regions in each zone of articular cartilage (Figure 1) (Sophia Fox, Bedi and Rodeo, 2009). Each zone consists of different composition, orientation, and distribution of ECM and different shapes and activities of chondrocytes, hence, they have slightly different properties. The zones, from deepest to the most superficial, are:

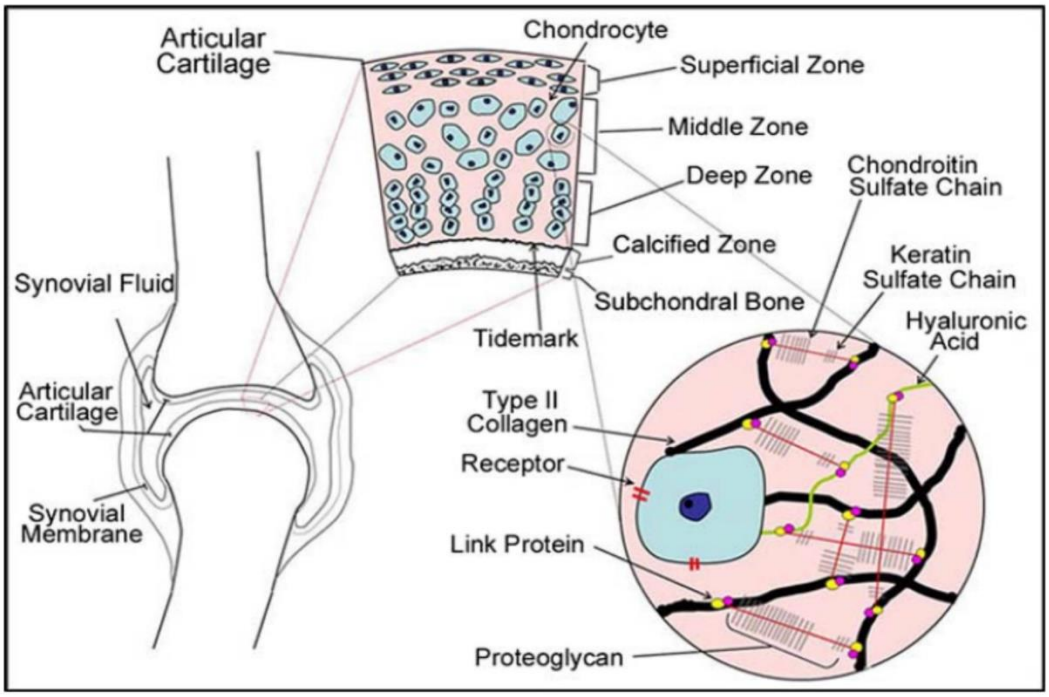

Figure 1 Compositions of articular joint and articular cartilage

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a) *A calcified zone*: This zone connects the cartilage to the bone by attaching deep zone to subchondral bone by thick collagen fibres. The number of chondrocytes and water concentration are low in this zone. The chondrocytes are more hypertropic and less mature than those in other zones. Primary role of the calcified zone is resistance to shear forces (Dewire, 2001; Sophia Fox et al., 2009).

b) *A radial zone* (or a deep zone): The radial zone is separated from the calcified zone by tide mark. This zone contains the largest collagen fibres and the highest concentration of proteoglycans, but lowest concentration of water. The collagen fibres and chondrocytes arrange in vertical orientation to the cartilage plain, in parallel to each other, hence this zone contributes

the highest resistance to compressions (Dewire, 2001; Buckwalter et al., 2005; Sophia Fox et al., 2009).

c) *A transitional zone* (or a middle zone): The transitional zone has the highest volume (40-60%) in articular cartilage. There are 2 types of collagen fibres in this zone, small and large fibres. The large collagen fibres have slant orientation while the small fibres randomly distribute across the cartilage and act as supports for the larger fibres. This zone is the first line of defence to joint impacts. Chondrocytes are in spherical shape and spread sparsely throughout the zone in lower number than in the radial zone. However, they are more active than those in the radial zone (Dewire, 2001; Buckwalter et al., 2005; Sophia Fox et al., 2009).

d) *A tangential zone* (or a superficial zone): The top layer is in contact with synovial fluid. Collagen fibres align in parallel to the surface and their concentrations are at the highest because the main function of tangential zone is to protect the deeper zones from shear, tensile, and compressive forces. Moreover, primary types of collagen are type II and type IX collagens, while in other zones, type II collagen is the major type. There is some type I collagen in this zone. There is a thin collagen fibrils called lamina splendens covers the tangential layer. Chondrocytes in this are in high number and in flatten, fibrocyte-like shape (Dewire, 2001; Buckwalter et al., 2005; Sophia Fox et al., 2009).

1.3.1 Extracellular Matrix (ECM)

The highest component in ECM is water. Collagens and proteoglycans are the highest dry components in ECM. Other molecules such as lipids and

glycoproteins are found in small amount in the ECM (Dewire, 2001; Buckwalter et al., 2005; Sophia Fox et al., 2009).

1.3.2 Water

Eighty percent of total weight of cartilage is water, and more than half is in the matrix. Functions of water, other than associated with and lubricating the matrix protein molecules, water helps carrying nutrients to chondrocytes. Water spreads through the ECM by capillary force from compression onto the matrix. The slow rate of water flowing through cartilage matrix makes the cartilage having ability to endure pressure loads acting on it (Dewire, 2001; Buckwalter et al., 2005; Sophia Fox et al., 2009).

1.3.3 Collagens

Around 60% of macromolecules in ECM are collagens, from which type II collagen is the most abundant (90-95%). Other types of collagens are Type I, IV, V, VI, IX, and XI. These small amount of collagens are important in supporting the type II collagen network. Collagen fibril is triple helix which forms by winding of 3 α-chains via hydrogen bond. The triple helix structure of the collagen maintains stability to the cartilage and resistance to shear and tensile forces (Dewire, 2001; Buckwalter et al., 2005; Sophia Fox et al., 2009).

1.3.4 Proteoglycans

Proteoglycans are the second most abundant marcromolecules in the ECM. They are glycoprotein composed of glycosaminoglycan (GAG) connecting to a protein core with covalent bond. In articular cartilages, 4 types of GAGs, chondroitin sulphates, keratin sulphate, heparin sulphate, and dermatan sulphate, are primarily found, from which chondroitin sulphates are the most prevalence (Harvey, 2011). There are several types of proteoglycans

including aggrecan, decorin, biglycan, and fibromodulin. Proteoglycans are distributed randomly throughout the articular cartilage, but their concentrations are inverted in proportions with amount of collagens in each zone. Main functions of proteoglycans are endurance and distributing the compression loads (Dewire, 2001; Buckwalter et al., 2005; Wescoe et al., 2008; Sophia Fox et al., 2009).

Aggrecan is the primary proteoglycan and the most abundant proteoglycans in the cartilage. It is the largest in size and contains over 100 chains of chondroitin sulphate chains and about 40 chains of keratin sulphate. Characteristic of aggrecan is the ability to non-covalently bind to hyaluronan via link protein and form large aggregates. Aggrecan is located within collagen fibre network. The aggrecan has high negative charge, hence is a strong hydrophilic molecule that can absorb high amount of water compared to their weight. The osmotic properties of aggrecan are important for cartilage's flexibility and ability to resist compressive loads (Dewire, 2001; Buckwalter et al., 2005; Wescoe et al., 2008; Sophia Fox et al., 2009).

Other proteoglycans that do not form aggregates can interact with collagen, such as decorin, biglycan, and fibromodulin. These proteoglycans are small, but their quantities in an articular cartilage are similar to those of aggrecan. (Sophia Fox et al., 2009) Composition of GAGs and types of core protein in these proteoglycans are different from each other, thus they have different functions in the cartilage. For example, dermatan sulphate chains are found in decorin and biglycan, while keratin sulphate chains are found in fibromodulin. Decorin and fibromodulin bind to type II collagen and support fibre network of the cartilage. Biglycan is found around chondrocytes and interacts with type VI collagen, so it helps supporting the cells with surrounding matrix. (Dewire, 2001; Buckwalter et al., 2005; Wescoe et al., 2008).

1.3.5 Chondrocytes

Articular cartilage contains only one type of cells in its structure, which brings about 1-2% of total volume of mature cartilage. Chondrocytes lineage is from mesenchymal stem cells (Goldring, 2007). The chondrocytes are crucial in maintenance and turnover of articular cartilage. They not only produce proteins that built into matrix of the cartilage, but also produce matrix degradation enzymes. Considering there is no vessels in the cartilage for nutrient supply, chondrocytes endure in hypoxic environment and can aerobically and anaerobically break down glycogen. When surrounding environment is changed, chondrocytes will change their activity to maintain integrity of ECM, as a result, if a decreased in the number of cell or altered cells activity will have effects on integrity of the cartilage. (Dewire, 2001; Buckwalter et al., 2005; Wescoe et al., 2008; Sophia Fox et al., 2009)

1.4. Treatments

The main symptom of symptomatic knee OA patients is pain in the affected joint, which results in reduced mobility. The inactivity can later leads to other diseases such as cardiovascular disease and obesity. As a result, aims for treating OA patients are to reduce joint pain in order to increase an ability to move and to improve quality of life of the patients. Normally, patients with mild symptoms would not need pharmaceutical treatments, however, patients with more severe conditions would need both physiotherapy and pharmaceutical treatments (Jevsevar, 2013).

1.4.1 Non-pharmaceutical therapy

At first, physicians use non-pharmaceutical approaches for treatments for OA (Douglas, 2012). For example, information about diet plans, exercise,

or how to avoid using the affected joint would be given to the patients. The best way to relieve pain from the joints is to reduce high compressive forces acting onto the joints like using canes for walking supports or losing weight. Also, physiotherapy is important in increasing strength of muscles that bridge the affected joint to bones. The stronger muscles help supporting the joint, and there is less compression on to the joint (Felson, 2013).

1.4.2 Pharmaceutical treatments

In patients with consistent pain, several drugs can be given by oral intake, and intramuscular or intra-articular injections. Even though intra-articular injection increases risks of introducing infection to the joint, this technique is still widely performed due to low systemic side effects. Furthermore, doses of injected drugs used in intra-articular injection are lower and more accurately introduced compared with those of orally intake medicine (Gerwin, Hops and Lucke, 2006).

1.4.2.1 Analgesic agents

Agents in this category are only used for reducing pains and improving joint function but do not reduce inflammation, if any, in the affected joint.

Pain relievers

First choice of drugs for pain relief in knee OA patients is paracetamol (acetaminophen) because of its low cost and considerably safe outcomes. However, continuous use of the drug and the use in patients with liver problems are not recommended (DeHaan et al., 2007). Opioids such as codeine, tramadol, morphine, and fentanyl (as a patch) are used in patients with moderate to severe joint pain when other

analgesic agents are no longer effective. Used of opioid drugs must be closely monitored by physicians because the risks are dose-related which can lead to the abuse of drug use and death from overdosing (Goodwin, Kraemer and Bajwa, 2009; Ivers, Dhalla and Allan, 2012).

Anaesthetics

Normally, anaesthetics are employed regionally (spinal block) or locally (nerve block) in order to prevent pain in patients undergo joint surgery. Moreover, the drugs can be applied directly into affected joints OA patients either as a treatment or as post-surgery pain relief. Although, an intra-articular injection of anaesthetics has shown to be more effective than systemic application of the drugs, several chondrotoxicity effects have been reported. Frequently used local anaesthetics are bupivacaine and lidocaine, but duration of effect of bupivacaine is 2-fold longer than that of lidocaine (Evans, Kraus and Setton, 2014; Tian and Li, 2016).

Disease-modifying agents

Agents in this category are glucosmine (e.g. hyaluronate), chondroitin sulphate, and diacerein. Since they are components of proteoglycans in cartilage matrix and synovial fluid, aims of using these agents other than pain reduction are to slow disease progression and, hopefully, preserve the cartilage (Uthman, Raynauld and Haraoui, 2003; Kon et al., 2012). They are commonly used via ingestion as supplements. For hyaluronate, direction injection to the affected joint is performed to improve viscoelasticity of synovial fluid and hence increasing resistance to shear strength (Gerwin et al., 2006). However, several studies have showed that benefits of these agents are significant only in patients with

early OA. Also, recommendation for treatment does not support the use of these agents as primary treatment in the patients (Jevsevar, 2013).

1.4.2.2 Anti-inflammatory agents

Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are strongly recommended pharmaceutical agents to be used in OA patients when paracetamol is not effective (Kon et al., 2012; Jevsevar, 2013). NSAIDs are cyclooxygenase inhibitors, usually non-selective to one type of COX. COXs catalyse reaction of prostaglandin production, a pro-inflammatory molecule, so inhibition of COXs activity results in reduction of inflammation (Alvarez-Soria et al., 2008; Kon et al., 2012). NSAIDs can be taken orally, intra-muscular injection, or intra-articular injection, depending on their formula. Aspirin (2-acetoxybenzoic acid) is one of the widely used NSAIDs, even though there are several reports about its gastrointestinal toxicity (Felson, 2013). Ibuprofen and diclofenac have lower toxicity to gastrointestinal tract and are encouraged to be used in OA treatment. Use of two or more NSAIDs in combination is not suggested because of the elevation of side effects but not pain relief effects. Specific COX-2 inhibitors have been reported to be lower toxic but are more expensive (Kon et al., 2012). Topical NSAIDs (e.g. ketorolac) have been developed to avoid systemic effects by local application over or intramuscular or intra-articular injection into the affected joint (Oztuna et al., 2007).

Steroid: Synthetic glucocorticoids

Application of glucocorticoids (GCs) in patients with symptomatic OA is by intra-articular injection, which can be performed as a monotherapy or in parallel with other systemic analgesic agents

(Schumacher and Chen, 2005). Duration of effects is varied from 1 – 4 weeks. GCs for injection can be in solution, emulsion, or suspension forms, depending of their polarity. Examples of GC solutions are disodium hydrogenphosphate form of betamethasone, dexamethasone, and prednisolone. Examples of GC suspensions are acetate form betamethasone, dexamethasone, and prednisolone, and triamcinolone derivatives. All forms of GC contain mannitol or sodium chloride as isotonising agents and some preservation such as benzyl alcohol. Suspended GCs also contain stabilisers such as gelatine to prevent clumping of drug particles which can decrease effectiveness of GCs. GC suspensions significantly stay longer than GC solutions in the injection site, however, GC solutions diffuse through plasma membrane into target cells faster than the suspensions (Gerwin et al., 2006; Douglas, 2012)

1.4.3 Surgical treatment

When the cartilage degradation and joint deformation severely affect the patients' daily life activities and no pharmaceutical treatments are helpful, they may undergo joint replacement surgery. Surfaces of bones and patella (cap) at the joint junction will be prepared for prosthetic joint by removing and trimming the damaged surfaces including articular cartilage. A metal implants and plastic button will be attached onto the resurfaced bones and patella, respectively. Finally, a medical-grade plastic spacer acting as articular cartilage will be placed between the metal implants. There are some opportunities of experiencing complications from the surgery such as infection and blood clot, however, the patients will benefit from significant pain reduction and improved ability to perform daily life activities (American Academy of Orthopedic Surgeons)

2. Glucocorticoids

Glucocorticoids (GCs) or corticosteroids are potent anti-inflammatory agents that have been used to treat arthritis diseases for decades. Their structures and functions are similar to cortisol hormone, which is produced naturally from adrenal cortex. Cortisol is a stress hormone, which is produced in response to stresses such as inflammation (Menkes, 1994; Uthman et al., 2003).

2.1. Mechanisms of action

GCs exert their anti-inflammatory action by binding to glucocorticoid receptor (GR). The GC-GR complexes then alter expressions of genes via several pathways. GC-GR complex action can be carried out by both genomic pathways (Figure 2) and non-genomic pathways (Phuc Le et al., 2005; Rhen and Cidlowski, 2005).

For genomic pathways, GC-GR complexes enter cell nucleus and act as transcription factor by directly binding specific DNA sequences at the promoter region called glucocorticoid responsive element (GRE) and inducing transcription of anti-inflammatory protein lipocortin-1 (Barnes, 1998; Rhen and Cidlowski, 2005). Another genomic action is by interfering with nuclear factor kappa-light chain enhancer of activated B-cells (NF-κB) transcription factor; thus, decreases expressions of NF-κB target genes encoding inflammatory proteins such as cyclooxygenase-2 (Barnes, 1998; Rhen and Cidlowski, 2005). In non-genomic pathways, the complexes induce or block transmission of secondary messengers that eventually result in alteration of expressions of those messengers' target genes (Phuc Le et al., 2005; Rhen and Cidlowski, 2005). For example, GCs activate mitogen-activated protein kinase (MAPK) phosphatase 1 that removes phosphate group from proteins in MAPK pathway and suppresses signal transduction and transcription of MAPK-pathway target genes (Barnes, 1998; Rhen and Cidlowski, 2005).

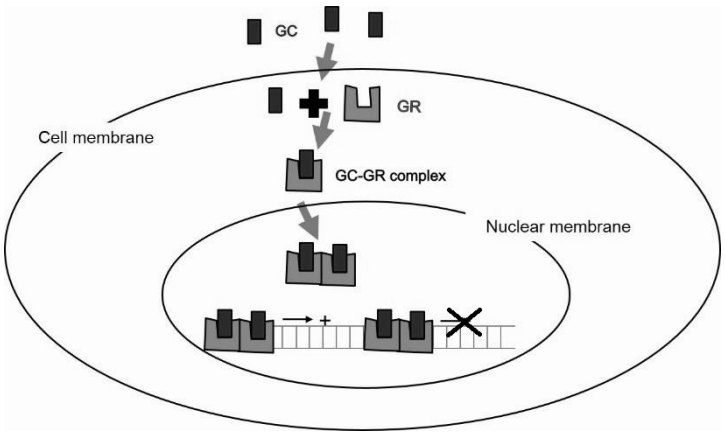


Figure 2 GC mechanism of action – genomic pathway

Drawn by Monthira Suntiparpluacha, 01 September 2013

2.2 Usage

2.2.1 GCs in arthritic diseases

Several GCs can be used for treating OA symptoms, and the preferred route is by intra-articular arthritic joint injection. They are used either to reduce inflammation or to relief pain, especially in those who are allergic or do not respond to NSAIDs (Shapiro et al., 2007; Braun et al., 2012a). Selection of GCs, in most cases, depends on the physician's preference, however, the decision must be made based on potency and duration of effects of GCs (Lavelle et al., 2007). Several GC classes can be used for intra-articular injection such as methylprednisolone, prednisolone, triamcinolone, hydrocortisone, betamethasone, and dexamethasone (Table 1). From these lists, however, the top 3 most commonly used GCs for intra-articular injection by members of American College of Rheumatology are methylprednisolone acetate, triamcinolone acetonide, and triamcinolone hexacetonide (Lavelle et al., 2007). Marked pain reduction effects of intra-articular GC injection have been found to be up to 4 weeks after injection. The effects subside after 8 weeks after injection (Schumacher and Chen, 2005).

2.2.2 GCs in other diseases

GCs are used in several inflammatory diseases. In inflammatory eye diseases, several GCs including TA are used for treatments such as eye drops, subconjuctival or intraocular injection, and pills (Lee et al., 2012; Athanasiadis et al., 2013). Combination of bronchodilator and GC inhalation such as prednisolone can be used to further reduce airway swelling in patients with chronic obstructive pulmonary disease (National Heart Lung and Blood Institute, 2013). GCs are of interest in neoplasm treatments, however, several steroid resistant cases have been reported and mechanism of GC anti-tumour action have not been thoroughly studied (McKay, 2003).

Table 1 Example of GCs used in intra-articular injection

Adapted from (Schumacher and Chen, 2005; Douglas, 2012)

Solubility	Glucocorticoids	Recommended doses (mg/knee)	Potency compared with cortisone (-fold)
Relatively insoluble	Prednisolone acetate	10-25	5
	Triamcinolone acetonide	10-40	5
	Triamcinolone hexacetonide	10-40	5
	Betamethasone sodium phosphate and betamethasone acetate	5-12	25
Slightly soluble	Methylprednisolone acetate	40-80	5
Soluble	Hydrocortisone	50	1
	Dexamethasone sodium phosphate	1.2-4	25

2.3 Triamcinolone acetonide

Triamcinolone acetonide (TA) is one of the most commonly selected GCs for intra-articular injection in patients with knee OA due to its pain relieving efficacy. Known commercial names are Kenalog® and Kanolone-F® (Figure 3) (Douglas, 2012). Chemical structure is shown in Figure 4**,** and its molecular weight is 434.5 (PubChem, 2005). It is white crystalline suspension, and insoluble in water (solubility at 25 °C is 80 mg/L compared with 120 mg/L of methylprednisolone) (Douglas, 2012). Its duration of action is relatively long (14 days) compared with methylprednisolone and dexamethasone (7 days) (Hepper et al., 2009; Dragoo et al., 2012). This longer duration of effects might be from its water insolubility, which lets TA stay longer at the site of injection than water-soluble GC solutions. (Douglas, 2012).

Figure 3 Commercial triamcinolone acetonide for intra-articular injection

Photo taken by Monthira Suntiparpluacha, 01 April 2013

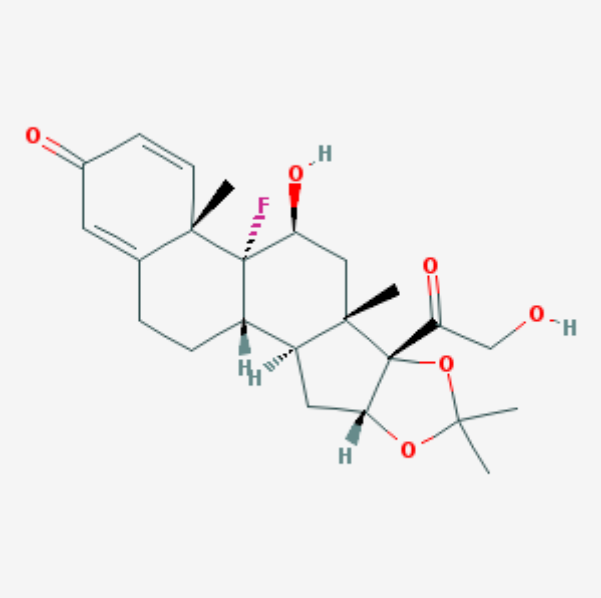


Figure 4 Chemical structure of triamcinolone acetonide

Structure available with identifier CID: 6436 in the PubChem Substance and Compound database (PubChem, 2005)

2.4 Recommendation for usage

Recommendation for treatments of knee OA by American Academy of Orthopaedic Surgeons (American Academy of Orthopedic Surgeons) has stated the inconclusive recommendation for using intra-articular GC injection in patients with symptomatic knee OA. Also, most physicians have suggested that each injection should be at least 3 months apart, as a result, the maximum number of injections per year is 4 injections (Schumacher and Chen, 2005; Jevsevar, 2013).

The inconclusive recommendation is due to conflict evidence between advantages and toxic effects of the use of GCs. Common side effects of intra-articular GC injection is joint flares and risk of joint infection and subcutaneous lipoatrophy (Schumacher and Chen, 2005). The primary concern of intra-articular injection of GCs is joint degradation due to either an increase in cartilage matrix degradation or

decreased the number of chondrocytes. Several reports have showed the toxicity of GCs on articular cartilages and chondrocytes, both *in vitro* and *in vivo.* However, some studies have showed that low dose of GCs for intra-articular injection could decrease degradation of extracellular matrix challenged with inflammatory cytokines (Pelletier and Martel-Pelletier, 1989; Uthman et al., 2003; Garvican et al., 2010; Lu, Evans and Grodzinsky, 2011; Caron et al., 2013).

2.5 Triamcinolone acetonide and chondrotoxicity

As for TA, reports on effects of long-term TA usage have shown worse joint conditions in some users, as a result, studies have been conducted in consideration of determining TA toxicity to cartilages and chondrocytes (Hauser, 2009). Several *in vitro* studies have reported that TA might have chondrotoxicity effects. A study about effects of TA and an anaesthetic bupivacaine on non-OA human chondrocytes and articular plugs by (Syed et al., 2011) showed that clinical doses of TA, and combination of 1:4 TA to bupivacaine decreased both monolayer chondrocyte viability in MTT and plug chondrocyte viability in Live/Dead assays. (Braun et al., 2012a)) demonstrated that 14-day incubation of GCs (betamethasones, methylprednisolones, and TA) enhanced toxicity of anaesthetics to chondrocyte cell line.

3. ECM homeostasis

3.1 Regulation and disruption of ECM homeostasis

Balance between anabolism and catabolism of ECM is regulated by metabolism of chondrocytes. In elders or arthritic patients, the balance is tipped which results in progressive loss of the cartilage because expressions and activities of anabolic proteins are reduced with aging (Goldring and Goldring, 2007). The 3 stages of cartilage degradation are damage and alteration of ECM, decreased synthesis response in chondrocyte, and loss of ECM (Buckwalter et al., 2005). When cartilage is insulted by

mechanical loads, injury, or biochemical changes, disruption or alteration of ECM occurs, such as increased in water content and decreased in concentration and length of aggrecan and glycosaminoglycan (Martel-Pelletier et al., 2008). Matrix swelling increases permeability of the cartilage, hence; cartilage stiffness and strength are decreased (Buckwalter et al., 2005). Chondrocyte responses to ECM damages lead to changes in anabolic and catabolic activities, and cell proliferation. They attempt to repair the damages by increase production of cytokines and proteins involved in matrix synthesis, e.g. bone morphogenetic protein (BMP)-2, BMP-7, transforming growth factor (TGF)-β, insulin-like growth factor (IGF)-1, and cartilage-derived morphogenetic proteins. These proteins can stimulate both ECM synthesis and chondrocyte proliferation. However, cartilage fragments can stimulate production of interleukin (IL)-1 which can further stimulate production of ECM-degrading enzymes. On one hand, these enzymes help clearing the cartilage fragments, allowing better diffusion of anabolic proteins and proliferation of chondrocytes. On the other hand, breakdown of collagens and aggrecan lead to increase water diffusion and swelling of the cartilage. If the chondrocytes are unable to recover ECM homeostasis, progression of cartilage degradation results in OA development (Buckwalter et al., 2005).

3.2 Degradation enzymes and their inhibitors

Chondrocyte responses to stimuli can be either secreting ECM-composing proteins or their proteolytic enzymes. When chondrocytes are stimulated by mechanical pressure, agents, or cytokines like IL-1 and TNF-α, they can produce matrix proteases, inflammatory mediators like cyclooxygenase (COX)-2, and even more inflammatory cytokines (Goldring and Goldring, 2007).

1.1.3 Matrix Metalloproteinase

Important enzymes involved in normal ECM turnover and OA progress are proteases in the matrix metalloproteinase (MMP) family. Enzymes in this

family need zinc for their catalytic activity. There are 26 MMPs discovered so far and they can be classified into 6 groups based on their substrates. MMP classes that are important in cartilage turnover and degradation are collagenases (e.g. MMP-1, MMP-13), stromelysins (e.g. MMP-3, MMP-8), and gelatinases (e.g. MMP-2, MMP-9) (Verma and Hansch, 2007).

MMP-1 (collagenase-1) and MMP-13 (collagenase-3)

The most important collagenases involved in OA cartilage degradation are MMP-1 and MMP-13. They can cleave within the intact triple helix of type II collagen fibrils, while other MMPs with collagenase activity can degrade only the partially digested type II collagens. The cleaved type II collagen fibrils can unwind in body temperature, and later are degraded by MMP-3, MMP-2, and MMP-9 (Goldring and Goldring, 2007). In early OA, the damage of type II collagen by MMP-1 and MMP-13 occurs close to chondrocytes, which later spreads throughout the ECM with the disease progression. Activity of MMP-13 in degrading type II collagen is at least 5-fold higher than activity of MMP-1 (Goldring and Goldring, 2007). Other types of collagen fibrils, which represent 5% of the collagens in a cartilage are more susceptible to MMP-2 and MMP-9 than to MMP-1 and MMP-13 (Gepstein et al., 2002). In a meta-analysis study, elevated protein levels of MMP-1 in synovial fluid were found in OA patients, specifically Asians, compared with healthy controls (Zeng et al., 2015). Also, increased protein levels of MMP-13 in cartilages have been found in OA patients compared with healthy population (Sandell and Aigner, 2001; Goldring and Goldring, 2007).

MMP-3 (stromelysin-1)

In non-inflamed joint, MMP-3 is secreted from chondrocytes and joint synoviocytes. Other than cleaving collagen fibrils, MMP-3 can also degrade other ECM such as proteoglycans, gelatines, laminins, and fibronectins (Nganvongpanit et al., 2009) Another important function of MMP-3 is inducing a full activation of MMP-1 and MMP-13 (Jo et al., 2003). Protein levels of MMP-3 were increased in serum, synovial fluid (Naito et al., 1999; Fernandes, Martel-Pelletier and Pelletier, 2002) and synovial tissue (Chen et al., 2014) of knee OA patients compared with healthy individuals. Moreover, the levels in synovial tissue were significantly different between OA stages I, II and the stage III, as a result, MMP-3 levels might be associated with severity of the disease (Chen et al., 2014).

1.1.4 Disintegrin and metalloproteinase domain with thrombospondin motifs

Several proteins in a disintegrin and metalloproteinase domain (ADAM) and ADAM with thrombospondin motifs (ADAMTS) families possess aggrecanase activity. Similarity in ADAM and ADAMTS proteins are in pro-domain, catalytic domain, disintegrin-like domain and cysteine-rich domain. However, ADAMTS proteins do not contain transmembrane domain, so they are secreted extracellularly. Furthermore, they contain thrombospondin type-1 motifs (TSPs) whose functions are believed to be increasing regulation of ADAMTS activity and anchoring to ECM. To date, 19 ADAMTS have been identified and their functions can be involved in both normal and pathological states (Sandell and Hering, 2001).

ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2)

ADAMTS-4 and ADAMTS-5 are shortest ADAMTS proteins (only 1-2 TSPs) and are important aggrecanases in cartilage due to the highest specificity to aggrecan (Sandell and Aigner, 2001; Song et al., 2007). Although the most important cleavage site on aggrecan for aggrecanases is between Glu373 and Ala 374 within the IGD site (between G1 and G2 domains), ADAMTS-4 and ADAMTS-5 can also effectively cleave at sites within CS-rich regions which are after Glu1545, Glu1714, Glu1819, and Glu1919, generating CS-rich C-terminus fragments necessary for mechanical-endurance properties. Expression regulation is slightly different between these two aggrecanases. For example, in human chondrocyte cell line and cartilage explants, expressions of ADAMTS-4 were upregulated with presence of IL-1 and oncostatin M together, while ADAMTS-5 expression levels were upregulated only with IL-1 (Huang and Wu, 2008). Several studies demonstrated the association between ADAMTS-4 and ADAMTS-5, and OA. They are present in cartilage of OA patients in the aggrecan-loss areas (Song et al., 2007). Moreover, increased serum levels of ADAMTS-4 and ADAMTS-5 in knee OA patients were observed, hence a potential biomarker for OA development and progress (Li et al., 2014). Several studies showed that inhibition of ADAMTS-4 or ADAMTS-5 expression by siRNA in OA cartilage explants or by gene knockout in OA animal models decreased aggrecan degradation. Other than aggrecan, ADAMTS-4 and ADAMTS-5 in cartilage can also use biglycan and decorin as substrates (Huang and Wu, 2008). Although both ADAMTS-4 and ADAMTS-5 are key aggrecanases in OA, activity of ADAMTS-5 catalytic domain is at least 2.5-fold higher than that of ADAMTS-4 and does not decline with age. Mice with knockout ADAMTS-5 were more resistant to surgical-induced OA compared with knockout ADAMTS-4 mice (Verma and Hansch, 2007).

1.1.5 Tissue inhibitors of metalloproteinase

The important MMPs and ADAMTSs inhibitors are Tissue inhibitors of metalloproteinases (TIMPs). There are 4 human TIMPs (1-4) with different affinity to different proteases. Mammalian TIMPs have distinct N-terminal and C-terminal domains, whose conformation is stabilized by 3 disulfide bonds. N-terminal domain folds differently between each TIMPs, hence their different binding affinity and specificity (Brew and Nagase, 2010). Although TIMP-1 and TIMP-2 are the most effective inhibitors to MMPs, TIMP-3 can inhibit both MMPs and ADAMTSs (Huang and Wu, 2008). Balance between expression and activity of proteolytic enzymes and TIMPs is important in regulating cartilage matrix turnover (Gepstein et al., 2002).

TIMP-3

The broad inhibitory effects of TIMP-3 to MMPs and ADAMTSs make it a unique matrix protease inhibitor. TIMP-3 is an effective inhibitor to aggrecanases, especially ADAMTS-4 and ADAMTS-5, so it is an important protein involving progression of arthritis diseases. Because of its role in OA progression, drugs targeting the interaction between TIMP-3 and ADAMTSs may slow cartilage degradation in OA patients. For example, calcium pentosan polysulfate greatly enhances binding of TIMP-3 to ADAMTS-4 and ADAMTS-5 and deceases TIMP-3 reuptake by chondrocytes (Brew and Nagase, 2010). Another special property of TIMP-3 is binding to the ECM via the N-terminal with with heparin sulphate and chondroitin sulphate, hence the fast inhibitory action on MMPs and ADAMTSs (Woessner, 2001; Huang and Wu, 2008).

4. Toxicity effects of glucocorticoids to cartilage and chondrocytes

4.1 Oxidative stress to chondrocytes

How TA causes toxicity to cartilage is still inconclusive, but one possible cause might be from altering oxidative status in chondrocytes. Several *in vitro* and *in vivo* studies have reported the GCs' ability to increase oxidative stress in various types of cells. Dexamethasone-treated human umbilical vein endothelial cells had an increase in levels of ROS and nitric oxide (NO) production at 6 h after treatment, in dose-dependent manner (Iuchi et al., 2003). Moreover, flow cytometry analysis of dexamethasone-treated neonatal rat primary osteoblasts showed a significant increase in intracellular ROS production at 24 h incubation compared with controls (Feng and Tang, 2014). Corticosterone-treated pheochromocytoma-derived cells, PC12, showed that levels of protein carbonyl, representing levels of protein oxidation, were significantly increased in dose-dependent manner (Tang et al., 2013). For TA, a study in rat primary retinal cells reported that levels of reactive oxygen species were significantly elevated in TA-treated cells compared with dexamethasone-treated cells and non-treated controls, even though the cells attempted to recover themselves by increasing production of superoxide dismutase 1 h after TA treatment (Chung et al., 2007). However, a study in porcine retinal pigment epithelial cells reported that TA attenuated effects of hydrogen peroxide by reducing amount of oxidized to total glutathione ratios indicating oxidative stress reduction (Miura and Roider, 2009). In an *in vivo* study, mice were subcutaneously injected with corticosterone. The mice hippocampi were taken for analysis of lipid hydroperoxide (LOOH) levels, protein carbonyl levels, and activities of antioxidant enzymes. The results showed that there were significant increase in levels of LOOH and protein carbonyl, along with reduction in activities of antioxidant enzymes, which indicated an increase in ROS production and oxidative stress (Sato et al., 2010).

Some study has reported beneficial effects of antioxidant in reducing toxicity of GCs. An antioxidant epigallocatechin increased efficacy and decreased toxicity of dexamethasone in arthritic rats. The rats had lower paw swelling, lower inflammatory cytokine production, and lower lipid oxidation compared with dexamethasone-treated and non-treated rats (Roy et al., 2013).

4.2 Expression alterations in genes involved in ECM homeostasis

Several studies have reported that GCs might attenuate effects of pro-inflammatory cytokines in elevating matrix proteases expressions in cartilages both *in vitro* and *in vivo*. However, some studies have shown that GC treatment could further increase expressions of ECM-degrading enzymes and decrease production of cartilage matrix proteins. An *in vitro* study reported that methylprednisolone dose-dependently decreased mRNA level of type II collagen in horse primary chondrocytes (Fubini et al., 2001). Studies in normal bovine primary chondrocytes showed that when the chondrocytes were treated with IL-1α, expressions of MMP-1 and MMP-3 were increased, and addition of dexamethasone and TA decreased both previously elevated MMP-1 and MMP-3 expressions (Sadowski and Steinmeyer, 2001; Sadowski and Steinmeyer, 2002). Studies in horse cartilage explants showed that GCs including TA decreased expressions of *MMP-1*, *MMP-3*, *MMP-13*, and *ADAMTS-4* in the explants stimulated with IL-1 and activated protein C. However, GC treatments did not alter *ADAMTS-5* expressions and increased collagen releases from the cytokine-stimulated explants. GAG release was unchanged although expression and activity of ADAMTS-4 were decreased, indicating an important role of *ADAMTS-5* in GC-treated cartilage degradation (Busschers, Holt and Richardson, 2010). Another study in cartilage explants reported the synergistic effects of dexamethasone with effects of cytokines and mechanical stress on healthy bovine cartilage discs in decreasing expressions of *MMP-3* and increasing expressions of *ADAMTS-5*, however, dexamethasone decreased release of GAG and type II collagen from the stimulated cartilages (Lu et al., 2011).

An *in vivo* study performed in inflammatory-induced arthritis rabbits demonstrated significant increase in *MMP-3* and *MMP-13* expressions and reduction in collagen production compared with healthy controls. After intra-articularly injected with methylprednisolone acetate, expressions of type II collagen were increased and those of MMP-3 and MMP-13 were decreased in the rabbit knee cartilages compared with saline controls (Kydd et al., 2007).

In addition, GCs might cause further cartilage degradation by decreasing levels of TIMP-3, which is an inhibitor of several matrix degrading enzymes. Studies in normal human chondrocytes, and bovine OA chondrocytes and cartilage discs showed that cytokines (TGF-β and TNF-α) increased expressions of *TIMP-3* in both normal and OA chondrocytes and cartilages. Addition of dexamethasone decreased the *TIMP-3* expressions in the chondrocytes to the same levels as controls (Su, Dehnade and Zafarullah, 1996; Lu et al., 2011). However, a study in a different cell type, porcine brain capillary endothelial cells, which were not stimulated with cytokines, reported that mRNA and protein levels of TIMP-3 were up-regulated in response to hydrocortisone treatments (Hartmann et al., 2009).

5. Oxidative stress

Oxidative stress is a state of cells when there is excess in production of prooxidants or deficiency in production of antioxidants (Rahal et al., 2014). An oxidative process occurs normally in cells for energy production. It is important in several biological functions such as digesting foreign antigens, inducing cell apoptosis, etc. In order for cells to function normally, balance between pro- and antioxidants production must be tightly regulated (Noori, 2012).

5.1 Redox reaction

Chemistry involved in occurrence and elimination of oxidative stress in organisms is redox (reduction-oxidation) reaction. Even though the focus is on the oxidation of macromolecules in oxidative stress process, there cannot be oxidation without reduction. Pro-oxidants like reactive oxygen species (ROS) contain at least one unpaired electron which makes them unstable, so they take electrons (oxidation) from nearby molecules (Valko et al., 2007).

Example of lipid oxidation: $RH + OH\cdot + O_2 \longrightarrow ROO\cdot + H_2O$

Oxidation by ROS leads to either damaging organic molecules or production of more dangerous unstable molecules. Anti-oxidation systems prevent further damage of intracellular macromolecules by providing electrons (reducing) to the oxidized molecules and the ROS, hence; redox homeostasis is crucial in maintaining normal function of cellular processes (Valko et al., 2007; Noori, 2012).

Reduction by antioxidant: $ROO\cdot + AH \longrightarrow ROOH + A$

5.2 Reactive oxygen species

Reactive oxygen species (ROS) are oxygen radicals that can be enzymatically (e.g. NADPH oxidase, cyclooxygenase, xanthene oxidase) or non-enzymatically generated. Sources of ROS might be exogenous sources or endogenous sources. Examples of exogenous sources that stimulate ROS production are metal ions (Fe^{3+}, Cu^{2+}) and radiation, and lead to production of hydrogen peroxide (H_2O_2) and hydroxyl radical (OH·) (Kamata and Hirata, 1999; Bender, 2015). Endogenous ROS are mainly from oxidative phosphorylation process in mitochondria due to leakage of electrons at the electron transport chain. This produces superoxide approximately 1.5 mol/day. The leakage of electrons from NADPH cytochrome P450 reductase can also be found at endoplasmic reticulum. In cells such as macrophages, ROS are purposely produced for destroying foreign antigens via pentose phosphate pathway (Kamata and Hirata, 1999; Bender, 2015). O_2^- can also be altered enzymatically to H_2O_2 that can react with metal ions (Fenton reaction) and create a more reactive ROS which is OH·. Moreover, the metal ions can catalyse reaction between O_2^- and H_2O_2 (Haber-Weiss reaction) to create OH· (Kamata and Hirata, 1999; Noori, 2012). The highly reactive OH· is the major molecule that causes damages to macromolecules especially DNA and lipids (Valko et al., 2007).

5.3 Oxidative damage to cellular components

ROS at low concentrations are beneficial to cellular activities, however, excess production of ROS results in deleterious effects to cellular marcromolecules. All components of DNA can react with hydroxyl radical, hence this radical can cause damages both purine and pyrimidine bases, and the sugar backbone of DNA. An example of product of DNA damage is 8-OH-G. If the damages are not repaired, the mutations will accumulate in and, after several of cell divisions, may result in development of malignancy (Bender, 2015). Polyunsaturated fatty acid is highly susceptible to oxidative damage. Oxidation of the unsaturated fatty acid produces

peroxyl radicals (ROO·) that undergo further oxidation process and eventually generate aldehyde products such as malondialdehyde and 4-hydroxy-2-nonenal. These aldehydes can further react with nucleic acids and proteins. Severe lipid oxidation can lead to disruption of plasma membranes. Oxidation of protein molecules occurs at side chains of amino acids especially thiol (SH)-containing amino acids such as cysteine. Oxidation of tyrosine results in generation of dihydroxyphenylalanine and leads to more production of ROS (Valko et al., 2007; Noori, 2012).

5.4 Oxidative stress and association with OA

One mechanism participated in development and progression of OA is oxidative stress since OA and oxidative stress in chondrocytes are increased with aging (Carlo and Loeser, 2003). Blood and synovial fluid of OA patients was found to have increased levels of malondialdehyde, product from lipid oxidation. Moreover, levels of reduced glutathione, vitamin E, vitamin C, and catalase in blood and synovial fluid of OA patients were decreased compared with healthy individuals (Suantawee et al., 2013). Also, several studies have reported that repeated excessive compression, which is one of the OA causes, onto articular cartilages induced oxidative stress to chondrocytes and cell death by impairment of mitochondrial function, and addition of antioxidants e.g. vitamin E and n-acetyl cysteine reduced chondrocyte apoptosis (Beecher et al., 2007; Coleman et al., 2015). Immunohistochemical staining of OA cartilages showed that levels of nitrotyrosine, an evidence of protein oxidation, were higher in the degraded areas of OA cartilage compared with intact areas, indicating the relationship between oxidative stress and disease severity (Yudoh et al., 2005). Several reports showed that an increased production of ROS has also been found in OA chondrocytes (Li, Xie and Wang, 2012). Higher production of ROS would result in the lower number of chondrocytes, which may affect cartilage matrix homeostasis. The correlation between oxidative stress, OA, chondrocyte numbers, and ECM homeostasis is presented in study of Yudoh et al. (2005). The study reported that proteoglycan production in OA

cartilages, telomere length and doubling time of human primary OA chondrocytes treated with 0.1 μM of H_2O_2 were reduced compared with the non-treated and 100 μM of ascorbic acid treated cartilages and chondrocytes (Yudoh et al., 2005). A longitudinal study reported that supplementation with vitamin C might benefit in protection against knee OA (Peregoy and Wilder, 2011). However, vitamin C intake data in the study were self-reported, so the data might not be entirely accurate, unlike a prospective study that dose and duration of vitamin intake can be precisely controlled.

5.5 ROS as signalling molecule – effects in gene expressions

Despite causing cytotoxicity at high concentration, intracellular ROS at moderate concentration act as signalling molecules that influence gene expressions and post-translational modification of several proteins. Two important transcription factors found to be activated by ROS are NF-κB and activator protein-1 (AP-1). They regulate expression of genes involved in oxidative stress response, and several important cellular processes such as cell proliferation and programmed cell death. ROS can activate NF-κB via activation of tyrosine kinase, alteration of Ca^{2+} homeostasis and activation of phosphatases. Thiol anti-oxidant status also impacts activation of NF-κB. Too high or too low levels of oxidized glutathione (GSSG) inhibit binding of NF-κB to DNA. Failure in maintaining Ca^{2+} homeostasis can result in further ROS production by activating degradation of metal-bound proteins, and stimulation of nuclease enzymes, hence, induction of apoptosis (Sen and Packer, 1996; D'Autreaux and Toledano, 2007). Because the AP-1 transcription factors are very responsive to several types of stresses especially oxidative stress, ROS are able to induce AP-1 expression and activity that are important in cellular response to oxidative stress. AP-1 is composed of dimers of Jun-family proteins such as cJun, etc. or Jun- and Fos-family proteins such as cFos, Fra, etc. AP-1 induces or represses gene expression by binding to TPA response element (TRE) on its target genes. The Fos proteins, especially cFos, have shorter live, but highly responsive to stress, than the Jun proteins. As a result, AP-1 members in normal,

non-induced state are dimers of Jun proteins, while under stimulation, AP-1 members are dimers of Jun-Fos proteins and lead to elevating gene transcriptions (D'Autreaux and Toledano, 2007; Schmucker et al., 2012). Other than affecting NF-κB and AP-1-target genes, oxidative stress has been reported to affect expressions of several genes downstream of p53-dependen pathways. A study *in vivo* reported that several altered gene expressions in livers of both SOD-knockout mice and mice injected with Diquat, an oxidative stress-inducing agent, were target genes in the p53-dependent pathways. The genes included *P21, growth differentiation factor-15, DNA-damage-inducible transcript 4, activating transcription factor 3* (Han et al., 2008).

5.6 Antioxidants

5.6.1 Endogenous anti-oxidation molecules

In multicellular organisms, cellular responses to oxidative stress are not as robust as single-cell organisms, but would be rather slow and long response time (D'Autreaux and Toledano, 2007). Mammalian cells contain both enzymatic and non-enzymatic anti-oxidation systems. Major enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), and all of them need coenzymes such as selenium, zinc, copper, and iron for activity. Examples of non-enzymatic antioxidants in mammalian cells are thiols (e.g. glutathione, N-acetyl cysteine), nicotinamide adenine dinucleotide phosphate (NADPH), and ubiquinone (Percival, 1998). SOD is the first enzyme to react to superoxide radicals and dismutase them to O_2 and H_2O_2 that can later be converted to O_2 and water by CAT, GPX, glutathione, and ubiquinone (Percival, 1998; Batinic-Haberle, Reboucas and Spasojevic, 2010). The oxidized glutathione (GSSG) is later converted back to the reduced form by NADPH and glutathione reductase enzyme (Valko et al., 2007; Noori, 2012).

5.6.2 Exogenous antioxidant molecules

Many crucial cellular antioxidants, that are minerals and vitamins, must be obtained from food. Minerals are essential as cofactors of endogenous antioxidant enzymes. Vitamin antioxidants, especially vitamin E and vitamin C, are among the most important vitamin antioxidants in proper functioning of cellular metabolism. Vitamin E, mostly found in α-tocopherol and γ-tocopherol form, is an important antioxidant in protecting lipid oxidation especially of the plasma membrane. After reducing ROS, vitamin E becomes a stable topopheoxyl radical which can be reduced back to tocopherol by other antioxidants such as glutathione and vitamin C (Valko et al., 2007; Noori, 2012). Vitamin C (ascorbic acid, ascorbate) is an important water-soluble antioxidant in recycling of endogenous antioxidants and vitamin E in the cells (Chambial et al., 2013). Flavanoids, a group of non-vitamin phenolic antioxidants (benzopyran derivatives) are found wildly in plants. Examples of flavonoids are beta-carotene in carrots, catechins in black tea, lycopene in tomatoes, and curcumin in turmeric (Hamid, 2010).

CHAPTER III

METHODOLOGY

1. Materials

1.1. Glass and plastic wares

1.1.1. For general use

- 20, 200, and 1000 µl Tips (Extragene, Taichung City, Taiwan)
- 15 and 50 ml Conical tubes (Sarstedt, Numbrecht, Germany)
- 0.2 ml PCR tubes and 1.5 ml Microcentrifuge tubes (Extragene)
- 10 ml Syringe (Nipro, Osaka, Japan)
- 100, 500, and 1000 ml Laboratory bottles (Duran, Mainz, Germany)
- 500 ml Graduated measuring cylinder (Isolab, Wertheim, Germany)

1.1.2. Cell culture associating experiments

- 25 and 75 cm^2 Rectangular canted neck cell culture flasks with vent cap (Corning, NY, USA)
- 6- and 96-well flat bottom microplates with lid (Corning)
- 10 and 25 ml Serological pipettes (Corning)
- Cell Strainer, pore size 70 µm (Corning)
- Syringe filters pore size 0.2 µm (Corning)
- Haemocytometer (Boeco, Hamburg, Germany)
- Vacuum filtration system (Pyrex, Tewksbury, MA, USA)
- Nilon filter membrane, pore size 0.2 µm (Filtrex, Lab-preparation.com (IP: 27.254.81.144), Thailand)
- 27GA Needles (Nipro)

1.1.3 Gene expression experiment

- Microseal® 96-well skirted PCR low-profile plates and adhesive seal (BioRad, Hercules, CA, USA)
- MicroAmp Fast optical 96-well reaction plates and optical adhesive films (Applied Biosystem, Waltham, MA, USA)

1.2 Reagents

1.2.1 Cell culture-associating experiments

- 1×Phosphate buffer saline (PBS), pH 7.4 (Gibco, Billings, MT, USA)
- Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco)
- Foetal bovine serum (Gibco)
- Sodium bicarbonate (Sigma-aldrich, St. Louis, MO, USA)
- Antibiotic-antimycotic containing 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, and 25 µg/ml of Fungizone® (Gibco)
- 0.25% Trypsin-EDTA with phenol-red (Gibco)
- Proteinase (Invitrogen, Carlsbad, CA, USA), dissolved with water
- Collagenase, Type II powder (Gibco), dissolved with water
- 0.4% Trypan Blue stain (Gibco)
- PrestoBlue® cell viability reagent (Invitrogen)
- OxiSelect™ Total glutathione (GSSG/GSH) assay kit (Cell Biolabs, San Diego, CA, USA)
- 4-Vinylpyridine (Sigma)
- Kanolone-F® (Triamcinolone acetonide), 40 mg/ml (L.B.S. Laboratory, Bangkok, Thailand)
- V-C Injection (vitamin C), 250 mg/ml (Vesco Pharmaceutical, Bangkok, Thailand)

1.2.2 Gene expression experiment

- RNeasy® Mini kit (Qiagen, Stanford, VA, USA)
- AccuPower® RT Premix (Bioneer, Seoul, South Korea)
- AccuPower® PCR Premix (Bioneer)
- AccuPower® 2× GreenStar™ qPCR Master Mix (Bioneer)
- 2x QPCR Green Master Mix, LRox (Biotech Rabbit, Henningsdorf, Germany)

1.3 Instruments

- Auto pipettes (Gilson, Middleton, WI, USA)
- Pipetting controller (Corning)
- Universal 32R bench top centrifuge (Hettich, Tuttlingen, Germany))
- Incubator (Thermo Scientific, Walthem, MA, USA)
- Class II laminar flow cabinet (Dwyer, Michigan City, IN, USA)
- Opsys MR™ microplate reader (Dynex Technologies, Chantilly, VA, USA)
- Agilent 8453 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA)
- Submerge-mini agarose gel electrophoresis (Atto, Tokyo, Japan)
- TPersona Thermocycler (Biometra, Göttingen, Germany)
- CFX96™ Real-time PCR Detection System (BioRad)
- StepOnePlus™ Real-time PCR System (Applied Biosystem)

2. Study methods

2.1 Ethical approval

Sample collection and study protocol were approved by the Human Ethics Committee of Faculty of Medicine, Thammasat University (study number MTU-ED-OT-4-099/57). Participants were provided study information sheet and signed in a consent form. Articular cartilages were collected from 10 participants (40-70 years old) who had been diagnosed with radiographic OA of the knee (Kellgren-Lawrence grade 3-4), and underwent knee replacement surgery at Department of Orthopaedic Surgery, Faculty of Medicine, Thammasat University Hospital. The participants must have never been injected intra-articularly with glucocorticoids.

2.2 Chondrocyte Isolation from articular cartilage and cell culture processes

2.2.1 Chondrocyte isolation process

The chondrocyte isolation procedure was adapted from Goldring protocol (Picot, 2005). Articular cartilages were collected in sterile plastic bags immediately after operation and be kept on ice during transportation. All cell culturing steps were performed inside a class II laminar flow cabinet. Cartilages were cut into small size ($5\times5\times1$ mm^3) with sterile surgical knifes and transferred to 50 ml tubes. The cartilage pieces were washed with 5 ml of 1×PBS twice. Then, 5 ml of 0.25% trypsin (Gibco, Billings, MT, USA) was added and incubated at 37 °C for 30 min. After the incubation, trypsin was discarded and the cartilages were washed. Next, 5 ml of filtered sterile 1% proteinase (Invitrogen, Carlsbad, CA, USA) was added and incubated at 37 °C for 30 min. The enzyme was discarded and the cartilages were washed. Then, 5 ml of 1% filtered collagenase (Gibco) was added, and incubated with the cartilage pieces for 6 – 8 hrs in an incubator (37ºC, 5% CO_2). When the incubation was

complete, released chondrocytes were passed through 70 µm Cell Strainers (Corning, NY, USA) into new 50 ml tubes and collected by centrifugation at 1000 ×*g* for 5 min. After that, supernatant was discarded, and the chondrocytes were washed with 1 ml PBS. The cells were collected by centrifugation at 1000 ×*g* for 5 min. Cell pellet was resuspended with complete culture media (DMEM/F12 with 10% FBS and 1% antibiotic-antimycotic (Gibco)) and transferred to 25 cm^2 culture flasks. The chondrocytes were cultured at 37ºC with 5% CO_2 until grow in confluence.

2.2.2 Chondrocyte culture process

Preparation of cell culture media

For 1,000 ml of media, 1 pouch of powdered DMEM/F12 and 1.2 g of $NaHCO_3$ (Sigma-aldrich, St.Louis, MO, USA) were dissolved in 500 ml of sterile water, and media solution was adjusted to pH 7.2-7.4. Then, sterile water was added to reach 1,000 ml, and the media was filtered sterile through nylon membrane using filtration system. Complete media was made by mixing filtered DMEM/F12 with 10% FBS and 1% antibiotic-antimycotic.

Chondrocyte detachment using trypsin (Trypsinisation)

Cell culture media was removed and 5 ml of 1×PBS was added into chondrocyte-containing flasks. After discarding the 1×PBS, 500 µl of 0.25% trypsin was added onto the adhered chondrocytes and incubated at 37 °C until for 5-10 min. After the incubation, 2.5 ml of complete media was added and mixed well. Chondrocyte suspension was transferred to new 1.5 ml microcentrifuge tubes, and centrifuged at 1,000 ×*g* for 5 min to collect the chondrocytes. After discarding supernatant, cell pellets were re-suspended with 1 ml of complete media.

Cell counting using haemocytometer

Ten microliter of the chondrocyte suspension gained after detachment was mixed with the same volume of 0.4% Trypan Blue (Gibco) stain. Then, 10 µl of the suspension was loaded into each end of a haemocytometer. The viable cells were counted under microscope covering all 8 corners of the haemocytometer. The number of cells was determined with the following formula:

$$\text{Number of cells per ml} = 1/8 \times \text{dilution factor} \times 10^{-4}$$

2.3 Chondrocyte confirmation by reverse transcription-polymerase chain reaction (RT-PCR)

2.3.1 RNA extraction

Total RNA was extracted using RNeasy® Mini Kit (Qiagen, Stanford, VA, USA) following the manufacturer's protocol. Chondrocytes were collected as in step 2.2.2 without resuspending with cell culture media. Cell pellets were lysed with 350 µl of Buffer RLT by passing through syringe needle pore size 0.2 µm 10 times. After several steps of purification, RNA was eluted from spin column membrane with 30 µl of nuclease-free water and centrifuged for 1 min at 9,400 ×*g*, and this step was repeated using only 20 µl of water. The RNA concentration was measured using UV/Vis spectrophotometer at 260 nm and 280 nm, and kept at -80 ºC until use.

2.3.2 Reverse transcription

For synthesis of cDNA, AccuPower® RT Premix (Bioneer, Seoul, South Korea) was used and the procedure was adapted from the company's instruction. In brief, 500 ng of total RNA and 100 pmole of Oligo dT primers were added into a clean PCR tube, and nuclease-free water was added to reach 10 µl. RNA and primers mixture was preheated at 70 °C for 5 min, and hold on

ice for another 5 min. During preheating step, 10 µl of water was added to AccuPower® RT Premix PCR tube to dissolve the lyophilised premix. Then the preheated RNA-primer mixture was transferred to the dissolved RT Premix PCR tube and mixed gently. Condition for cDNA synthesis was 42 °C for 1 min and 94 °C for 5 min. cDNA was kept at -20 °C until use.

2.3.3 Polymerase chain reaction and agarose gel electrophoresis for detection of *Type II Collagen* (*COL2A1*)

cDNA from non-treated controls were used for detecting *COL2A1* expression using AccuPower® PCR Premix (Bioneer). In a tube of lyophilised PCR Premix, 10 ng of cDNA was mixed with 300 nM of *COL2A1* primers and nuclease-free water to reach final volume of 20 µl. Condition for PCR was as followed; initial denaturation step at 94 °C for 5 min, amplification step for 35 cycles including denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 40 s, and the last final extension at 72 °C for 5 min. Primers for amplifying *COL2A1* mRNA are as followed; Forward 5'-TGGCCTGAGACAGCATGAC-3' and Reverse 5'-AGTGTTGGGAGCCAGATTGT-3' (Choi et al., 2013). Products from PCR were analysed by 2% of agarose gel electrophoresis separation.

2.4 Determination of effects of TA and vitamin C on chondrocyte viability

2.4.1 TA and vitamin C treatment

Chondrocytes were counted and 15,000 cells were put into each well of a 96-well plate. TA at 0, 2.3, 11.5, and 23 mM (equal 0, 1, 5, and 10 mg/ml) (L.B.S. Laboratory, Bangkok, Thailand) with or without 100 µM of vitamin C (Vesco Pharmaceutical, Bangkok, Thailand) were added into each well, 100 µl/well, in triplicates and incubated for 7 or 14 days. The concentrations of TA used in this study were adjusted from concentrations used clinically in patients.

The adjustment was based on surface area of knee articular cartilage and surface area of a well. Surface area of knee articular cartilage of human is 9000 mm^2 in average. Surface area of a well in 96-well are 32 mm^2, which is about approximately 280 times smaller than the cartilage surface area. Clinical dosage of TA for IA injection of the knee is usually 40 mg (92 mmol), hence dose of TA per well were 0.1 mg (0.23 µmol) (Dragoo et al., 2012).

2.4.2 Determining chondrocyte viability

After the incubation was completed, culture media along with TA were removed. Fresh media was added at 90 µl per well, and 10 µl of PrestoBlue© (Invitrogen, Carisbad, CA, USA) was added into each well. The reagent was incubated for 3 h with the remaining chondrocytes at 37 °C. Colour intensity was measured at 540 nm with an Opsys MR™ Microplate Reader. Cell viability was calculated from their optical density (OD) values following equation (1).

$$\% \text{ Viability} = \left(\frac{\text{OD sample}}{\text{OD control}}\right) \times 100 \qquad (1)$$

2.5 Determination of effects of TA and vitamin C on chondrocyte oxidative stress

2.5.1 TA and vitamin C treatment

Chondrocytes were counted and 10^5 cells were put into each well of a 6-well plate. TA at 0, 2.3, 11.5 mM (0, 1, 5 mg/ml) with or without vitamin C (100 µM) were added into each well, 1 ml/well in replicates and incubated for 48 h. TA concentrations selected were the concentrations that caused chondrotoxicity but did not kill the chondrocytes (results of the 2.4.2 step).

2.5.2 Determining oxidative status

Concentrations of glutathione were measured following protocol of OxiSelect™ Total Glutathione (GSSG/GSH) Assay Kit (Cell Biolabs, San Diego, CA, USA). Chondrocytes were detached by trysinisation and counted. Then, the cells were collected by centrifugation at 100 ×g for 5 min at 4 °C and washed twice with 1 ml of cold 1×PBS. After discarding the PBS, cell pellet was mixed with 300 µl of cold 5% MPA. For protein collection, the cells were lysed by passing ten times through syringe needle size 0.2 µm and centrifuged at 10,000 ×g for 5 min at 4 °C. Supernatant was transferred to a new microcentrifuge tube and store at 4 °C for immediate use or at -80 °C for later use.

On the day of glutathione measurement, standard curve was prepared by double diluting 0.5 µM of glutathione disulphide (GSSG) with 0.5% MPA to reach 0.031 µM of GSSG in duplicates. The extracted samples were diluted 1:10 with 1×Assay Buffer for total glutathione determination. For measuring concentrations of oxidized glutathione (GSSG), 100 µl of diluted samples was mixed with 1 µl of 1 M of 4-vinylpyridine and incubated at room temperature for 1 h. Also, Opsys MR™ microplate reader was set at 405 nm for kinetic assay measurement prior the addition of the kit's reagent. Then, determination of glutathione concentrations was performed in a 96-well plate according to the manufacturer's protocol. Absorbance was recorded immediately every 2 min for 10 min. Average optical densities (OD) of each time points were plotted against incubation time to create linear graphs (y = mx + c). Slope (m) of each sample was used for calculation of glutathione concentrations by comparing to slope of the standard GSSG.

2.6 Gene expression analysis

Chondrocytes were seeded into 6-well plate at 10^5 cells/well and cultured for 48 h. Then, 3 TA concentrations (0, 2.3, 11.5 mM (0, 1, 5 mg/ml)) were added to each well, 1 ml/well, in replicates and incubated for 48 h. After the incubation, chondrocytes were collected for RNA extraction and reverse transcription as explained in step 2.3. Cycle threshold (Ct) values were used for relative quantification analysis by normalising with expressions of *GAPDH* and comparing with untreated controls. For calculating relative quantification value, equations (2) to (4) were used.

$$\Delta Ct = Ct_{target} - Ct_{GAPDH} \quad (2)$$

$$\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{non\text{-}treated} \quad (3)$$

$$\text{Relative quantification value} = 2^{-\Delta\Delta Ct} \quad (4)$$

Standard curve of each gene was constructed for determining efficiency of the real-time PCR condition for each primer set. cDNA from reverse transcription step was double diluted from starting amount of RNA at 25 ng (1×) to 6.125 ng (1/16×). Ct values were plotted against cDNA concentrations, and then linear equation was created for each gene. The efficiency should be between 95% - 105% to show that the condition is suitable for exponential amplification of the cDNA template. Efficiency percentage was calculated by with equation (5).

$$\% \text{ Efficiency} = (10^{-1/slope} - 1) \times 100 \quad (5)$$

2.6.1 Oxidative stress inducible genes

Genes of interest were *cyclin-dependent kinase 1A* (*P21*), *growth differentiation factor* (*GDF*)-15, and *cFos*. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was a reference gene used for normalisation. Primer sequences for *P21* and *GAPDH* were referred from previous studies (Luo et al.,

2011; Nareerat Rungruang, 2012). Primers for *GDF15* were designed using NCBI Primer-BLAST (Ye et al., 2012) and those for *cFos* were designed using Primer 3 program (Koressaar and Remm, 2007; Untergrasser A, 2012). Real-time PCR was performed on CFX96™ Real-time PCR Detection System (BioRad, Hercules, CA, USA) with AccuPower® 2× GreenStar™ qPCR Master Mix (Bioneer) with some adjustment from the manufacturer's protocol. Gradient PCR and primer-metric were performed in order to determine the optimum annealing temperatures and primer concentrations of each gene. For each reaction, 10 ng of cDNA was added to the reaction mix containing 12.5 µl of AccuPower® 2× GreenStar™ qPCR Master Mix, primers, and water to reach final volume of 25 µl. Condition for real-time PCR was as followed; initial denaturation step at 95 °C for 30 s, amplification step (35 cycles) including steps of denaturation at 95 °C for 5 s, annealing and extension at 58 °C for 10 s, and melting step including steps of holding at 95 °C for 30 s, and melting with temperature rate of 0.1°C/s from 65 °C to 95 °C.

2.6.2 Extracellular matrix homeostasis controlling genes

Genes of interest were *matrix metalloproteinase* (*MMP*)*-1*, *MMP-3*, *MMP-13*, *a disintegrin-like and metalloprotease with thrombospondin type 1 motif* (*ADAMTS*) *-5* and *tissue inhibitor of metalloproteinase* (*TIMP*)*-3*. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used for normalisation. Primers for real-time PCR of *MMP-3, MMP-13,* and *ADAMTS-5* were referred from previously studies (Corps et al., 2008; Nganvongpanit et al., 2009; Kotepui et al., 2012). Primers for *MMP-1* and *TIMP-3* were designed using NCBI Primer-BLAST (Ye et al., 2012) Real-time PCR was performed in StepOnePlus™ Real-time PCR System (Applied Biosystem, Waltham, MA, USA) for study of *MMP-3*, *MMP-13*, and *TIMP-3* expressions using 2x QPCR Green Master Mix, LRox (Biotech Rabbit, Henningsdorf, Germany) with some

adjustment from the manufacturer's protocol. For *MMP-1* and *ADAMTS-5*, real-time PCR was performed in CFX96^{TM} Real-time PCR Detection System (BioRad) also using 2x QPCR Green Master Mix, LRox (Biotech Rabbit). Gradient PCR and primer-metric were performed in order to determine the optimum annealing temperatures and primer concentrations of each gene. For each reaction, 10 ng (for *MMP-3*, *MMP-13*, and *TIMP*-3) or 20 ng (for *MMP-1* and *ADAMTS-5*) of cDNA was added to the reaction mix containing 10 µl of 2x QPCR Green Master Mix, primers, and water to reach 20 µl of total volume. Condition for real-time PCR was as followed; initial denaturation step at 95 °C for 2 min, amplification step (35 cycles) including steps of denaturation at 95 °C for 5 s, annealing and extension at 56 – 62 °C for 15 s, and melting step including steps of holding at 95 °C for 30 s, and melting with temperature rate of 0.1°C/s from 65 °C to 95 °C.

2.7 Statistical analysis

2.7.1 Analysis of results from viability

All statistical analysis was calculated with SPSS v-13.0 (SPSS Inc., Chicago, IL, USA). Differences between days of incubation and effects of vitamin C between each treatments were analysed using T-test statistical analysis. For comparing differences between concentrations of TA, one-way analysis of variance (ANOVA) with Tukey's *post hoc* honest significant difference correlation was used. The differences reached statistical significance when p-value < 0.05.

2.7.2 Analysis of results from oxidative stress determination

Differences between effects of TA concentrations to glutathione levels were analysed with one-way ANOVA. Effects of vitamin C between each

concentrations of TA were analysed with T-test statistics. The differences reached statistical significance when *p*-value < 0.05.

2.7.3 Analysis of results from gene expression study

Differences of relative quantification results between treatments were analysed with one-way ANOVA. The differences reached statistical significance when *p*-value < 0.05.

CHAPTER IV
RESULTS

1. Information of participants in the study

The cartilage samples were collected from 10 patients with knee OA underwent total knee arthroplasty. Participants were consist of 2 males and 8 females with an average age on the day of surgery of 73.33 (range 56-85) years old. Five participants were diagnosed with OA of the right knee, three with OA of the left knee, and one with bilateral knee OA. Information of all participants are shown in Table 2, however, information of one participant (Patient 7) was not available and would be marked as N/A.

Table 2 Participants' information

Participant number	Gender	Age (years old)	Diagnosis
Patient 6	female	71	OA left knee
Patient 7	female	N/A	N/A
Patient 8	female	68	OA right knee
Patient 10	female	65	OA right knee
Patient 11	male	85	OA right knee
Patient 13	female	56	OA right knee
Patient 14	female	85	OA left knee
Patient 15	female	73	OA left knee
Patient 16	male	85	OA bilateral knees
Patient 17	female	72	OA right knee

2. Primary chondrocytes isolated from OA patients

After steps of digestion of articular cartilages, primary chondrocytes were obtained from all ten patients. Under light microscope, the isolated chondrocytes were fibroblast-like spindle shape. Figure 5 shows primary chondrocytes passage 0 that were cultured at 100% confluence.

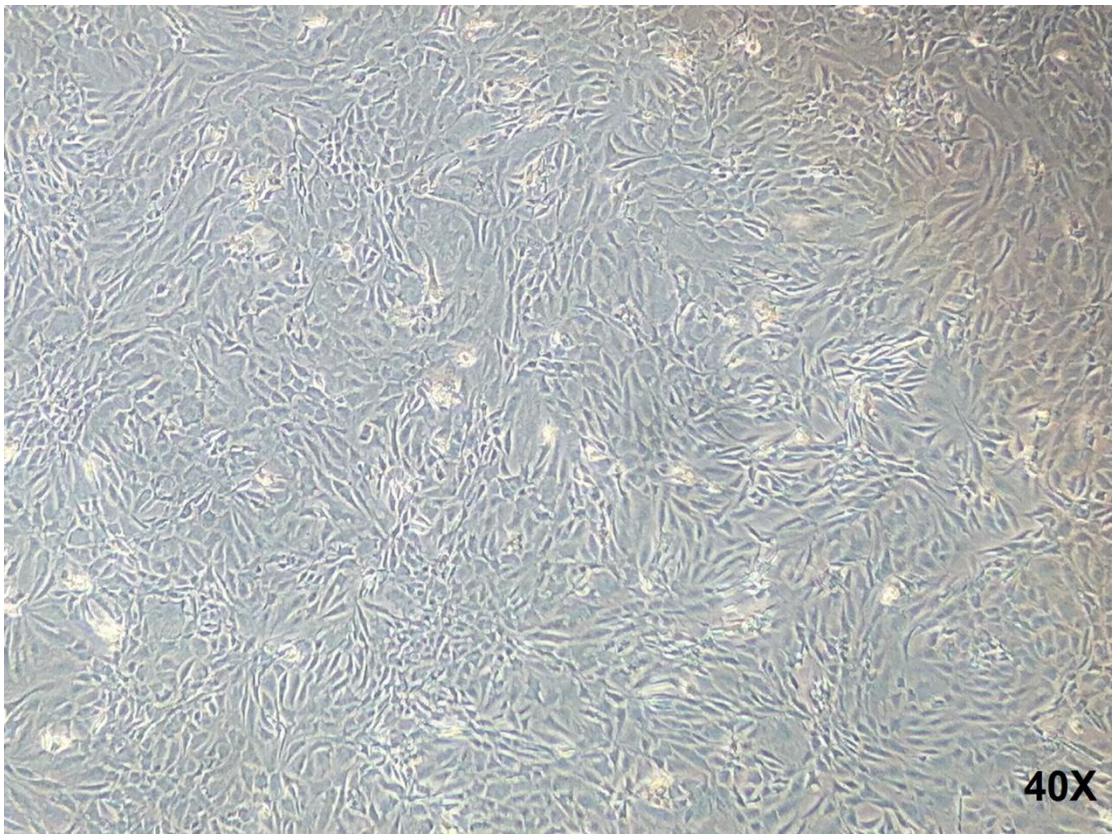


Figure 5 Primary chondrocytes from passage 0 cultured for 7 days

4. Chondrocyte confirmation

Expressions of *COL2A1*, a gene encoding type II collagen, from all 10 chondrocyte samples were evaluated using reverse-transcription polymerase chain reaction and agarose gel electrophoresis techniques. An expected amplified product size of *COL2A1* cDNA is 373 bp. There were amplified genomic DNA products visible on the agarose gel, however, the expected amplified *COL2A1* cDNA (mRNA) PCR product bands showed near a 400 bp marker were detected (Figure 6). The band was confirmed by sequence analysis (Bioneer) and the obtained sequence was compared with nucleotide database using NCBI BLAST® program. Results from BLAST® showed that the sequence had 100% identity with *COL2A1* mRNA (Figure 7).

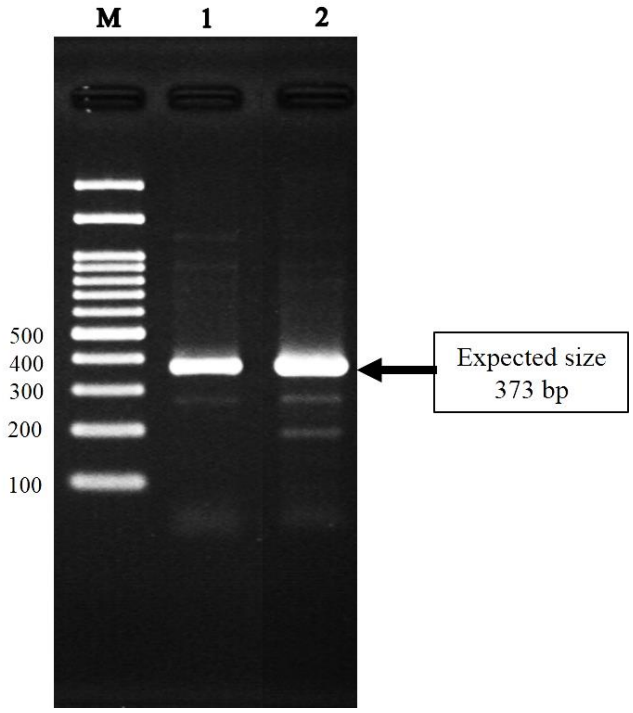
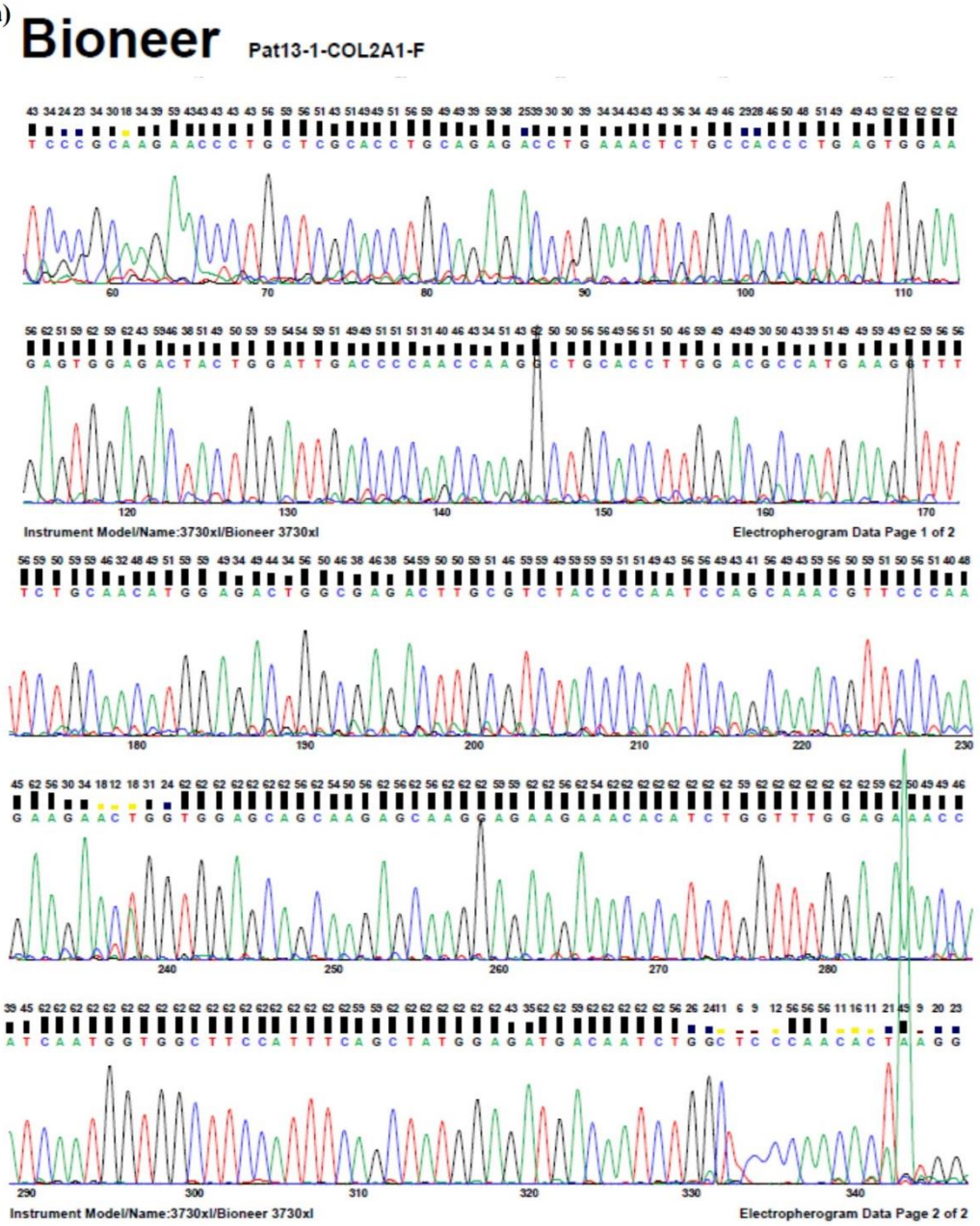


Figure 6 Gel electrophoresis in 2% agarose of products from RT-PCR for detection of COL2A1 mRNA in chondrocytes OA patients (lane 1 and 2). First lane (M) is a 100-bp marker.

a)



b) Sequences

>>

AGTCATGTACTCAGTCCTCACAACCAGATTGAGAGCATCCGCAGCCCCGAGGGCTCCCGCAAGAACCCTGCTCGCACCTG
CAGAGACCTGAAACTCTGCCACCCTGAGTGGAAGAGTGGAGACTACTGGATTGACCCCAACCAAGGCTGCACCTTGGACG
CCATGAAGGTTTTCTGCAACATGGAGACTGGCGAGACTTGCGTCTACCCCAATCCAGCAAACGTTCCCAAGAAGAACTGG
TGGAGCAGCAAGAGCAAGGAGAAGAAACACATCTGGTTTGGAGAAACCATCAATGGTGGCTTCCATTTCAGCTATGGAGA
TGACAATCTGGCTCCCAACACTAAGG

C) Homo sapiens collagen type II alpha 1 (COL2A1), transcript variant 1, mRNA

Sequence ID: ref|NM_001844.4| **Length:** 5087 **Number of Matches:** 1

Range 1: 3970 to 4291 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
581 bits(644)	1e-163	322/322(100%)	0/322(0%)	Plus/Plus

```
Query  1     CAACCAGATTGAGAGCATCCGCAGCCCCGAGGGCTCCCGCAAGAACCCTGCTCGCACCTG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3970  CAACCAGATTGAGAGCATCCGCAGCCCCGAGGGCTCCCGCAAGAACCCTGCTCGCACCTG  4029

Query  61    CAGAGACCTGAAACTCTGCCACCCTGAGTGGAAGAGTGGAGACTACTGGATTGACCCCAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4030  CAGAGACCTGAAACTCTGCCACCCTGAGTGGAAGAGTGGAGACTACTGGATTGACCCCAA  4089

Query  121   CCAAGGCTGCACCTTGGACGCCATGAAGGTTTTCTGCAACATGGAGACTGGCGAGACTTG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4090  CCAAGGCTGCACCTTGGACGCCATGAAGGTTTTCTGCAACATGGAGACTGGCGAGACTTG  4149

Query  181   CGTCTACCCCAATCCAGCAAACGTTCCCAAGAAGAACTGGTGGAGCAGCAAGAGCAAGGA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4150  CGTCTACCCCAATCCAGCAAACGTTCCCAAGAAGAACTGGTGGAGCAGCAAGAGCAAGGA  4209

Query  241   GAAGAAACACATCTGGTTTGGAGAAACCATCAATGGTGGCTTCCATTTCAGCTATGGAGA  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4210  GAAGAAACACATCTGGTTTGGAGAAACCATCAATGGTGGCTTCCATTTCAGCTATGGAGA  4269

Query  301   TGACAATCTGGCTCCCAACACT  322
             |||||||||||||||||||||
Sbjct  4270  TGACAATCTGGCTCCCAACACT  4291
```

Figure 7 Sequencing results

a) electropherogram, **b)** nucleotide sequence, and

c) sequence alignment result from NCBI BLAST® program

6. Effects of TA and vitamin C on chondrocyte viability

The effects of TA on chondrocyte viability were determined using a resazurin-based reagent (PrestoBlue) at day 7 and day 14. Attenuating effects of antioxidant on TA was analysed by addition of 100 µM of vitamin C to the culture medium. Viability percentages were calculated from optical density (OD) by comparing OD from each treatments with the OD from non-treated chondrocytes (0 mM TA) of their matching samples, hence; the viabilities of the controls were considered 100%. The results showed that TA significantly decreased viability of chondrocytes in a dose-dependent manner (Figure 8). The chondrocyte viabilities at 7- and 14-day incubation with TA did not significantly different at all concentrations tested. Compared with controls, significant differences in viability were found at all concentrations tested with *p*-value less than 0.01 (75.4%, 70.1%, and 66.1% at day 7, and 78.3%, 68.6%, and 66.1% at day 14 for 2.3, 11.5, 23 mM TA, respectively). The differences within treatments were significant between 2.3 and 23 mM of TA with *p*-value 0.029 at day 7, and *p*-value 0.037 at day 14. The differences in cell viabilities were not significant between 2.3 and 11.5 mM TA ($p = 0.36$ at day 7 and $p = 0.11$ at day 14), and between 11.5 and 23 mM TA ($p = 0.59$ at day 7 and $p = 0.96$ at day 14).

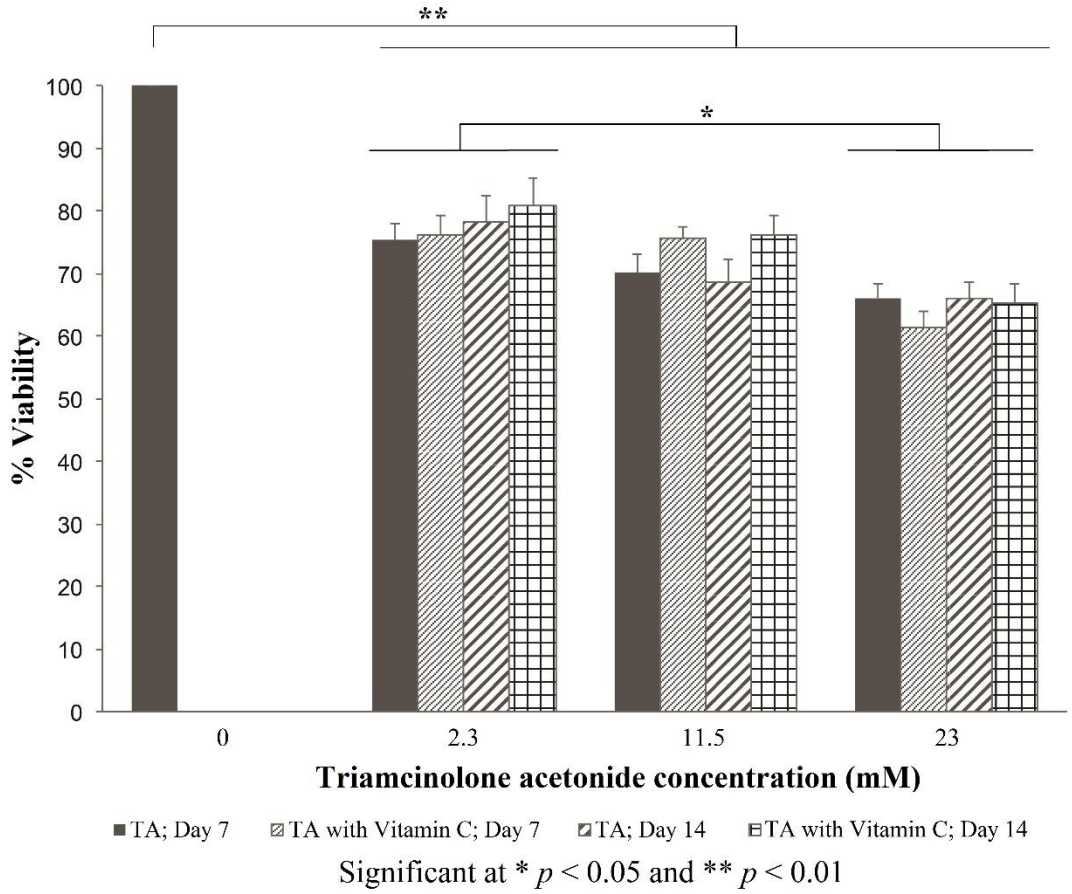


Figure 8 Viability of chondrocytes treated with TA at 0, 2.3, 11.5, and 23 mM (0, 1, 5, and 10 mg/ml) with or without addition of Vitamin C at incubation day 7 and 14

Viability percentage of chondrocytes treated with 2.3, 11.5, and 23 mM (1, 5, and 10 mg/ml) TA with and without 100 µM of vitamin C are shown in Table 3. Addition of vitamin C increased the viabilities of TA-treated chondrocytes, however, the differences between TA-treated and non-treated controls were still significant with *p*-value less than 0.01 (76.3%, 5.6%, and 61.3% at day 7, and 80.8%, 76.1%, and 65.2% at day 14 for 2.3, 11.5, and 23 mM TA, respectively). The significant differences between treatments were also found in vitamin C-treated chondrocytes between 2.3 and 23 mM with *p*-value less than 0.001 at both incubation periods, and between 11.5 and 23 mM TA at day 7 ($p = 0.0003$) but not at day 14 ($p = 0.08$). No statistical difference was found in viabilities between 2.3 and 11.5 mM TA ($p = 0.99$ at day 7 and $p = 1.0$ at day 14) in vitamin C-treated chondrocytes with Moreover, chondrocyte viabilities between vitamin C-treated and non-treated cells were significantly different only at 11.5 mM TA at day 14 with *p*-value 0.035 (from 68.6% in non-treated cells to 76.1% in vitamin C-treated cells) (Table 3). From viability data, the half maximal inhibitory concentration (IC_{50}) of TA could be calculated from graph in Figure 9. The IC_{50} of TA on primary chondrocytes was 58.1 mM.

Table 3 Viability (% viability ± standard error) of chondrocytes treated with 2.3, 11.5, and 23 mM (1, 5, and 10 mg/ml) TA with or without 100 µM vitamin C at 7 days and 14 days after incubation

TA concentration (mM)	% Viability ± Standard Error					
	Day-7			Day-14		
	TA	TA with vitamin C	*p*	TA	TA with vitamin C	*P*
2.3	75.43±2.5	76.27±3.0	0.8	78.30±4.3	80.81±4.4	0.28
11.5	70.10±2.9	75.58±2.03	0.15	68.55±3.7	76.10±3.2	0.035*
23	66.07±2.4	61.34±2.5	0.28	66.05±2.7	65.22±3.15	0.81

*Statistical significance between viabilities of cells treated with and without vitamin C ($p < 0.05$)

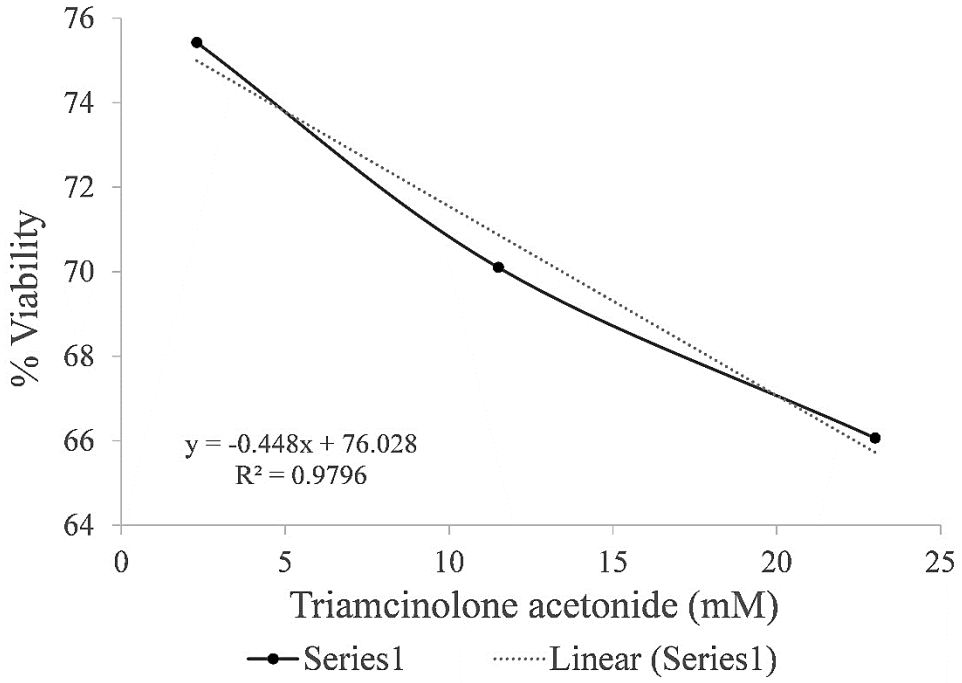


Figure 9 Graph of TA-treated chondrocyte viability at day 7-incubation time used for calculation of IC50

Figure 10 shows morphology of primary chondrocytes before and after treatment of TA at 23 mM (10 mg/ml). The chondrocytes treated with the drug were in lower number compared with the non-treated controls. Moreover, the TA-treated cells appeared to be shrinkage with visible membrane blebbing.

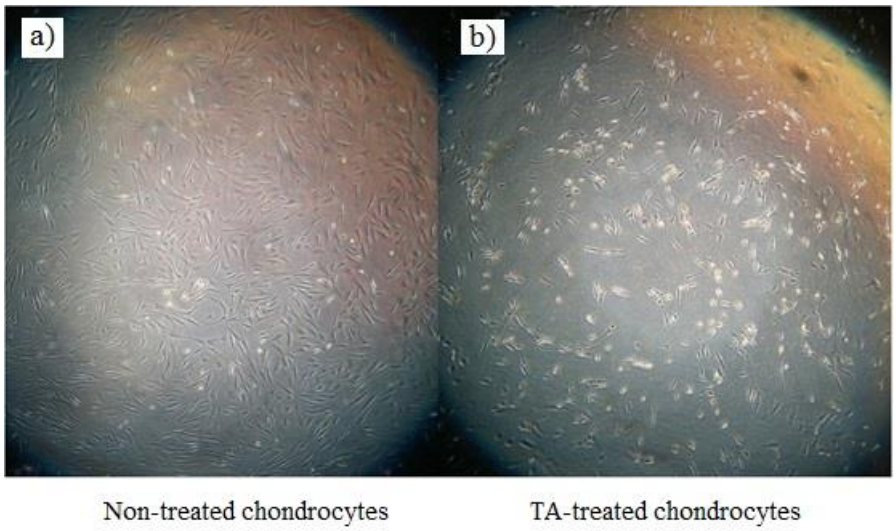


Figure 10 Morphology of chondrocytes **a)** before TA treatment, and **b)** after TA treatment

7. Effects of TA and vitamin C on chondrocyte oxidative stress

The ratio of oxidized glutathione to total glutathione (GSSG:total GSH) was used to assess the oxidative-stress induction effect of TA on chondrocytes. Concentrations of TA and incubation time that could induce stress without killing chondrocytes were based on results from viability assay (0, 2.3, 11.5 mM (0, 1, 5 mg/ml) TA) and preliminary experiment. TA increased the levels of oxidized glutathione (GSSG) to total glutathione (GSH) in chondrocytes in a dose-dependent manner (0.087±0.025, 0.394±0.12, and 0.517±0.135 in cells treated with 0, 2.3, 11.5 mM, respectively) with a significant difference at 11.5 mM TA (p = 0.02) compared with non-treated controls (0 mM TA) (Figure 11), suggesting that TA induced the chondrocytes to undergo

oxidative stress. Even though TA at 2.3 mM increased oxidative stress to chondrocytes, it did not reach statistical significant (p = 0.12).

The values of GSSG:total GSH were reduced following addition of vitamin C to almost the same levels as the non-treated controls (0.089±0.023, 0.129±0.048, and 0.123±0.031 in cells treated with 0, 2.3, 11.5 mM TA, respectively), indicating reduction of oxidative stress by vitamin C. At 11.5 mM TA treatment, the chondrocytes treated with vitamin C had a significant decrease in value of GSSG:total GSH compared with non-treated chondrocytes at p-value 0.025 (from 0.517 in non-vitamin C treatment to 0.123 in vitamin C treatment). Although addition of vitamin C decreased the value of GSSG:total GSH in 2.3 mM TA-treated chondrocytes, the reduction did not reach statistical significant (p = 0.07).

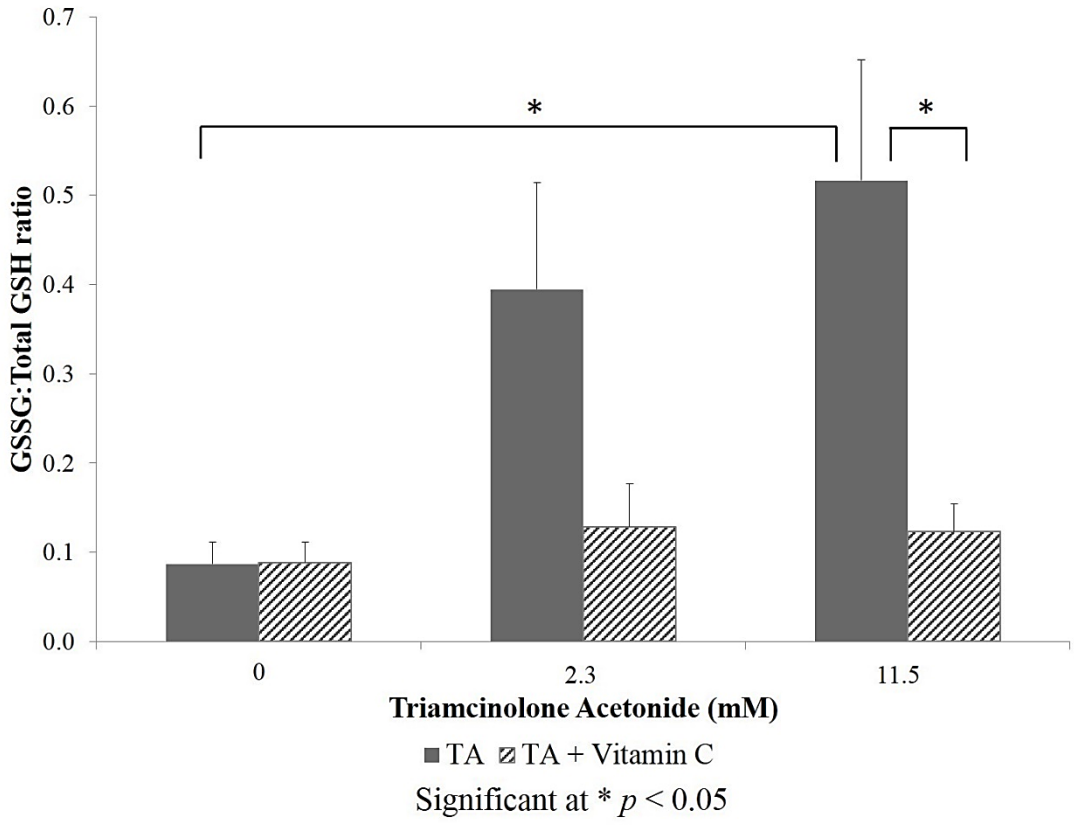


Figure 11 Ratio of oxidized glutathione to total glutathione (with standard error) in chondrocytes treated with 0, 2.3, and 11.5 mM (0, 1, and 5 mg/ml) TA with or without 100 µM vitamin C.

8. Effects of TA on gene expressions by real-time PCR

8.1 Optimisation of real-time PCR condition

Sequences of primers for real-time PCR amplification of *GDF15*, *cFos*, *MMP-1*, and *TIMP-3* were designed and are shown in Table 4. For amplification of *GAPDH*, *P21*, *MMP-3*, *MMP-13*, and *ADAMTS-5*, the primer sequences were referred from previously published studies as stated in methodology chapter, section 2.6 (gene expression analysis) (Corps et al., 2008; Nganvongpanit et al., 2009; Luo et al., 2011; Kotepui et al., 2012; Nareerat Rungruang, 2012).

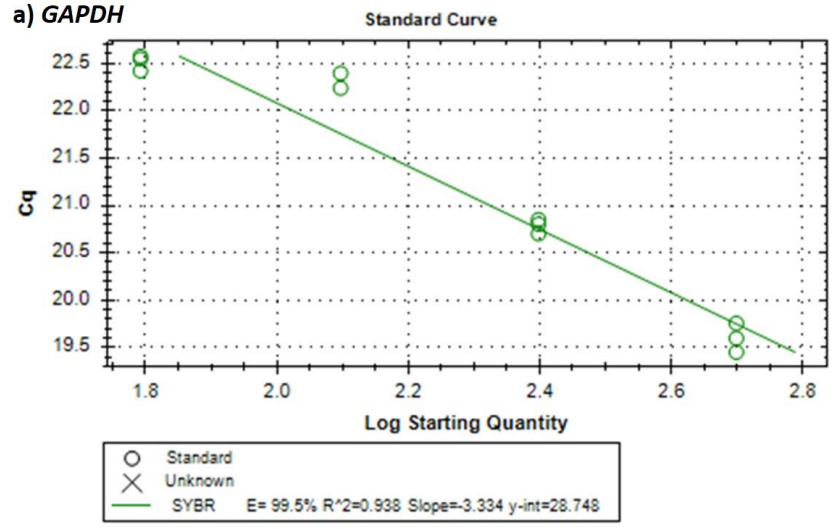
After optimising the primers' concentrations and annealing temperatures, the final concentrations of primers used in real-time PCR were 250 nM for amplification of *P21* and *GAPDH*, and 280 for amplification of *GDF15* and *cFos*. Annealing temperature of the four primer sets was 58 °C. For genes encoding proteolytic enzymes and *TIMP-3*, final concentrations of primers were 220 nM for *MMP-1*, 280 nM for *MMP-13* and *TIMP-3*, and 300 nM for *MMP-3* and *ADAMTS-5*. Annealing temperatures were 56 °C for amplification of *MMP-3* and *TIMP-3*, 59 °C for amplification of *MMP-1* and *ADAMTS-5*, and 62 °C for amplification of *MMP-13* (Table 4).

Standard curves for amplification of each genes were created by performing real-time PCR on 2-fold serial diluted cDNA template in order to calculate real-time PCR efficiencies of each genes. The efficiencies for the amplification of cDNA of all genes studied (Figure 12) were within an acceptable range (95-105% efficiency), and the efficiency percentages were as follow; 99.5% ($R^2 = 0.94$) for *GAPDH*, 98.6% ($R^2 = 0.99$) for *P21*, 102.2% ($R^2 = 0.97$) for *GDF15*, 99.7% ($R^2 = 0.99$) for *cFos*, 99.2% ($R^2 = 0.97$) for *MMP-1*, 98.7% ($R^2 = 0.96$) for *MMP-3*, 101.8% ($R^2 = 0.975$) for *ADAMTS-5*, 95.2% ($R^2 = 0.99$) for *MMP-13*, and 104.1% ($R^2 = 0.99$) for *TIMP-3*.

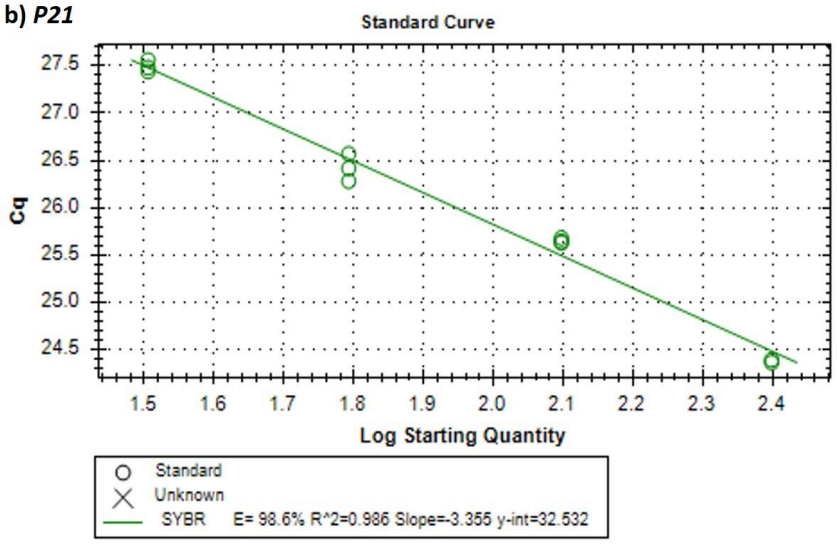
Table 4. Sequences, annealing temperatures and concentrations of primers used in real-time PCR

Gene	Primer sequence (5' → 3')	Product size (bp)	Annealing temperature (°C)	Final concentration (nM)	Source
P21	F- CTGGAGACTCTCAGGGTCGAA R- CCAGGACTGCAGGCTTCC	123	58	250	Luo et al., 2011
GDF15	F- AAACGCTACGAGGACCTGCT R- GTCACGTCCCACGACCTTGA	242	58	280	Designed
cFos	F- ACTTCATTCCCACGGTCACT R- GCAGCCATCTTATTCCTTTCC	277	58	280	Designed
MMP-1	F- GCTGCTTACGAATTTGCCGA R-CATCATACCTCCAGTATTTGTTAGC	217	59	220	Designed
MMP-3	F- CTTTTGGCGCAAATCCCTCAG R- AAAAGAACCCAAATTCTTCAA	404	56	300	Nganvongpanit et al., 2009
MMP-13	F- TCGCGTCATGCCAGCAAATTCCAT R- TTCTTCCCCTACCCCGCACTTCTG	116	62	280	Kotepui et al., 2012
ADAMTS-5	F- AGGAGCACTACGATGCAGCTATC R- CCCAGGGTGTCACATGAATG	73	59	300	Corps *et al.*, 2008
TIMP-3	F- TGACAGGTCGCGTCTATGAT R- GGCAGGTAGTAGCAGGACTT	151	56	280	Designed
GAPDH	F- GAAGGTGAAGGTCGGAGTC R- GAAGATGGTGATGGGATTTC	237	58	250	Nareerat, 2012

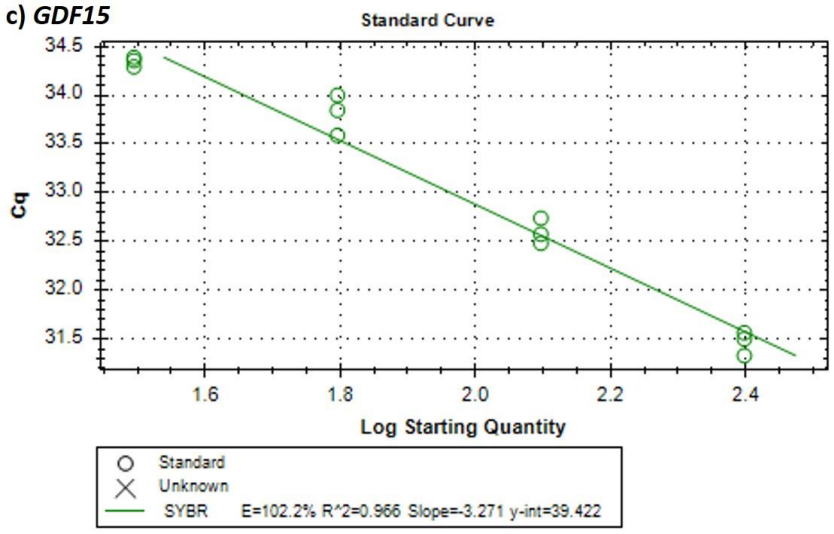
a) *GAPDH*



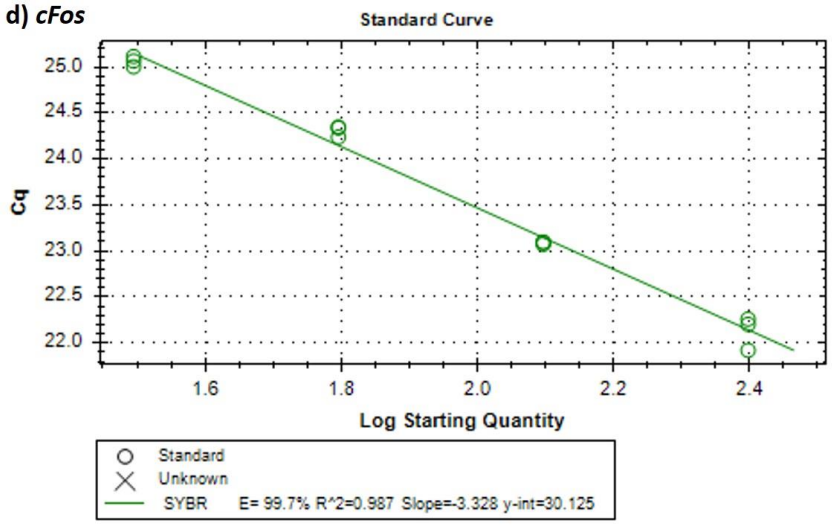
b) *P21*



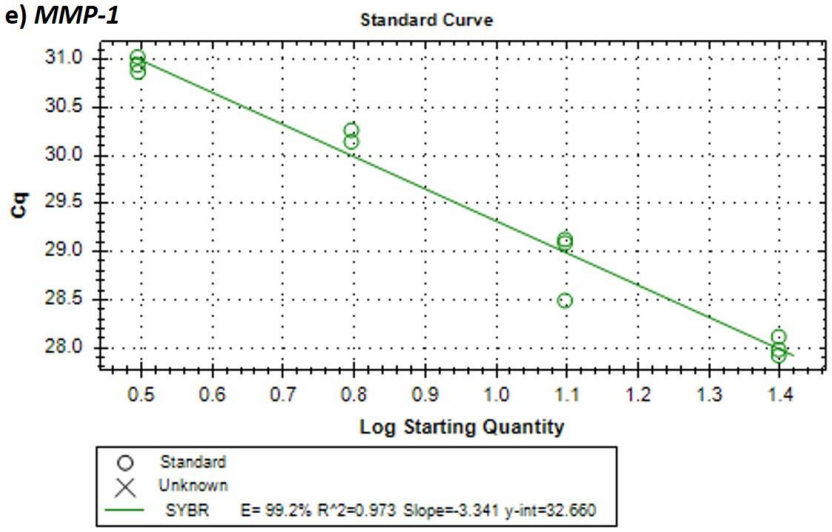
c) *GDF15*



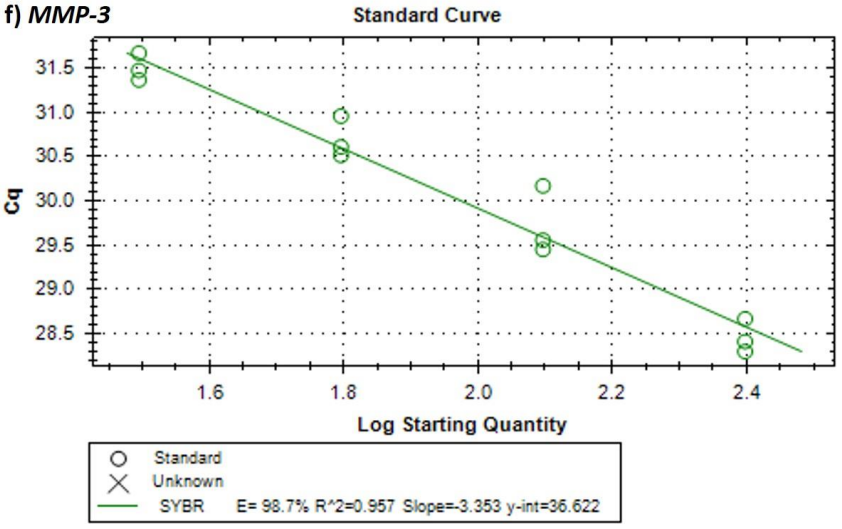
d) *cFos*



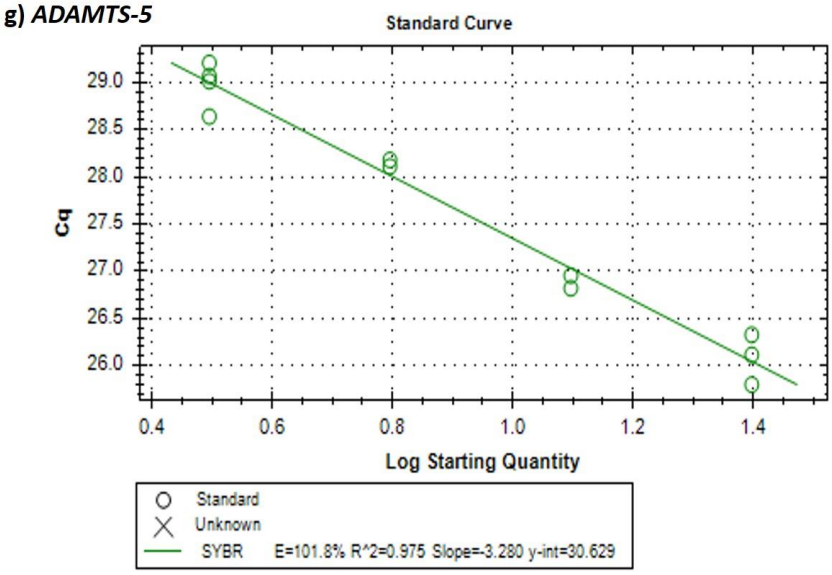
e) *MMP-1*



f) *MMP-3*



g) *ADAMTS-5*



h) *MMP-13* and *TIMP-3*

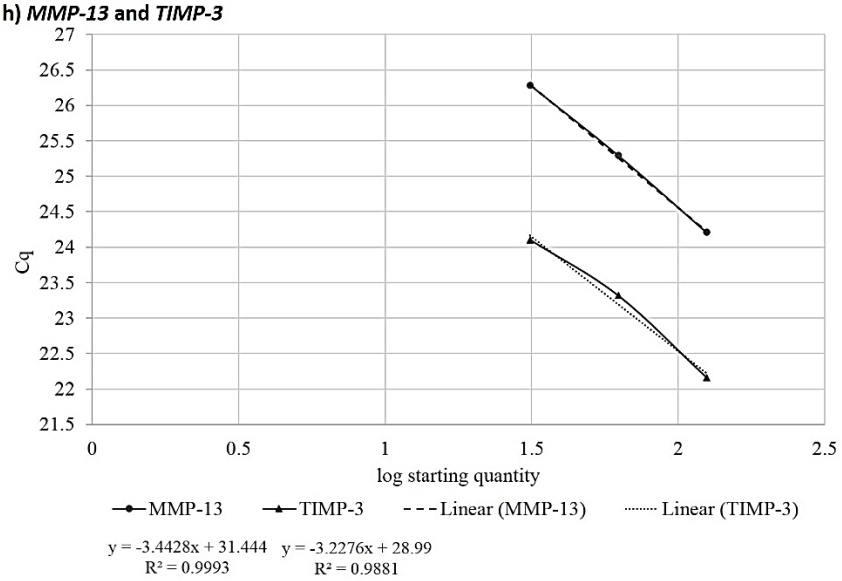


Figure 12 Standard curve from efficiency test conducted with 2-fold serial dilution of cDNA for genes a) *GAPDH*, b) *P21*, c) *GDF15*, d) *cFos*, e) *MMP-1*, f) *MMP-3*, g) *ADAMTS-5*, and h) *MMP-13* and *TIMP-3*

Note: Efficiency curves of *MMP-13* and *TIMP-3* (h) was plotted using StepOnePlus™ Real-Time PCR System (Applied Biosystem), while the rest (a-g) were plotted using CFX96^{TM} Real-time PCR Detection System (BioRad)

8.2 Expressions of genes involved in regulation of the cell cycle (*P21*) and response to oxidative stress (*GDF15*, *cFos*)

Expressions of *P21*, *GDF15*, and *cFos* were normalised with *GAPDH* of their matching treatments and samples, then each normalised Cq value was compared with their non-treated controls (0 mM TA), as a result, the fold change in 0 mM TA was considered unchanged in expression (1-fold). The results reported as fold change in expressions relative to the non-treated controls (Table 5).

Table 5 Fold-change in mRNA expressions of *P21, GDF15,* and *cFos* in chondrocytes treated with 2.3 and 11.5 mM (1 and 5 mg/ml) TA compared with non-treated controls

Genes of interest	Fold ± standard error (*p*-value)	
	2.3 mM TA	11.5 mM TA
P12	5.17±0.8 (< 0.001*)	4.2±0.5 (0.001*)
GDF15	9.97±0.9 (0.001*)	12.97±2.6 (< 0.001*)
cFos	4.96±1.0 (0.029*)	6.65±1.5 (0.002*)

*Statistical significance between fold-change of gene expressions in TA-treated chondrocytes and non-treated controls ($p < 0.05$)

The expressions of all ROS-inducing genes were significantly different between the TA-treated chondrocytes and their non-treated controls that were the expressions of all genes significantly increased after TA treatments in all concentrations tested. Levels of mRNA from *P21* increased 5.2- and 4.2-fold with *p*-value 0.001, those of *cFos* similarly increased 5-, and 6.7-fold with *p*-value less than 0.03, and those of *GDF15* strikingly increased 10-, and 13-fold with *p*-value less than 0.001 in chondrocytes treated with 2.3 and 11.5 mM, respectively (Figure 13). The expressions of *cFos* and *GDF15* were higher in 11.5 mM TA-treated chondrocytes compared with the chondrocytes treated with 2.3 mM, however, the differences did not reached statistical significance. The expressions of *P21* were lower in the cells treated with higher concentration of TA but the reduction was not significant.

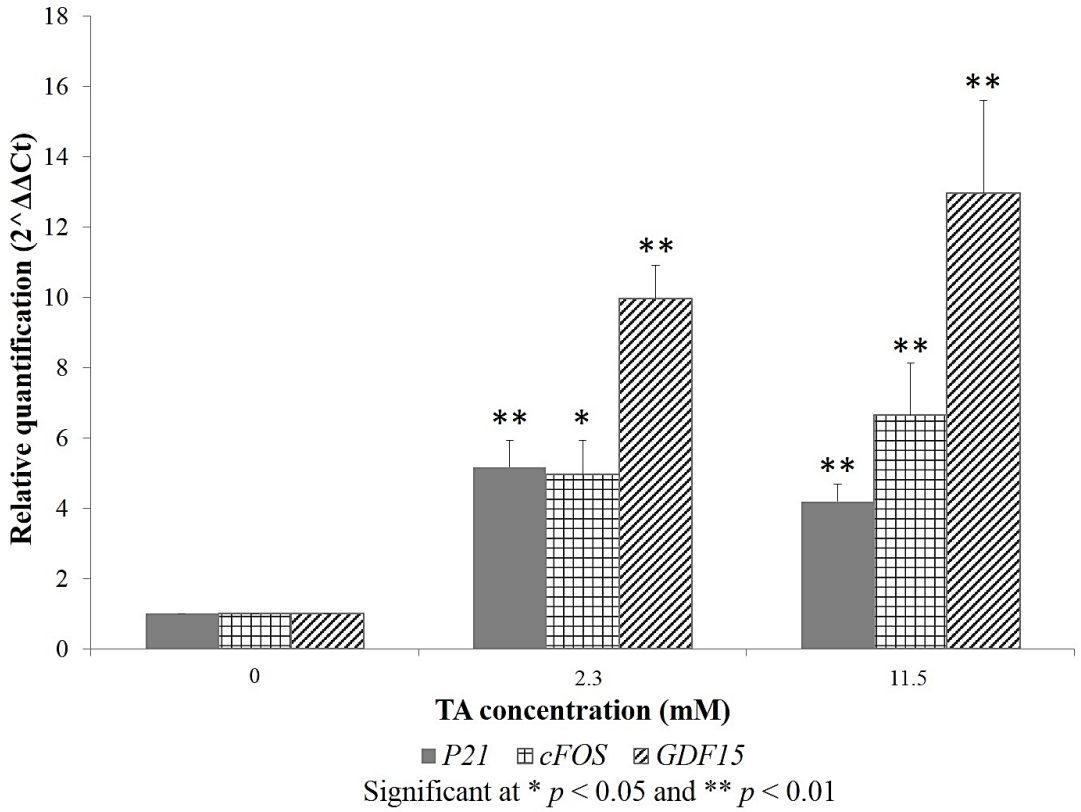


Figure 13 Relative mRNA level of *P21*, *cFos,* and *GDF15* in chondrocytes treated with TA compared with non-treated controls. *GAPDH* was used as an internal control.

8.3 Expressions of genes involved in degradation of extracellular matrix (*MMP-1*, *MMP-3*, *MMP-13*, and *ADAMTS-5*) and their inhibitor (*TIMP-3*)

Expressions of genes ending collagenases (*MMP-1* and *MMP-13*), stromelysin (*MMP-3*), aggrecanase (*ADAMTS-5*), and metalloproteinase inhibitor (*TIMP-3*) were also normalised with *GAPDH*, compared with their non-treated controls, and reported as fold change to the non-treated controls. Results showed that addition of TA increased expressions of proteolytic enzyme genes (*MMP-3*, *MMP-13*, and *ADAMTS-5*) at all concentrations tested (Figure 14) (6.6- and 5.4-fold of *MMP-3*, 2- and 1.9-fold of *MMP-13*, and 2- and 2.3-fold of *ADAMTS-5* for 2.3 and 11.5 mM TA, respectively). Nevertheless, the significant increase was found only in expressions of MMP-3 with *p*-value less than 0.03. Chondrocytes treated with 11.5 mM TA had lower expressions of the proteolytic genes than the cells treated with 2.3 mM TA, but the differences did not reach statistical significant (Table 6).

Table 6 Fold-change in mRNA expressions of *MMP-1*, *MMP-3*, *MMP-13*, *ADAMTS-5*, and *TIMP-3* in chondrocytes treated with 2.3 and 11.5 mM TA compared with non-treated controls

Genes of interest	Fold ± standard error (*p*-value)	
	2.3 mM TA	11.5 mM TA
MMP-1	0.83 ± 0.37 (0.96)	1.63 ± 0.76 (0.63)
MMP-3	6.59±1.64 (0.022*)	5.43±1.09 (0.008*)
MMP-13	2.03 ± 0.86 (0.53)	1.91 ± 0.78 (0.61)
ADAMTS-5	2 ± 0.55 (0.26)	2.29 ± 0.51 (0.87)
TIMP-3	1.37±0.32 (0.6)	1.33±0.39 (0.79)

* Statistical significance between fold-change of gene expressions in TA-treated chondrocytes and non-treated controls ($p < 0.05$)

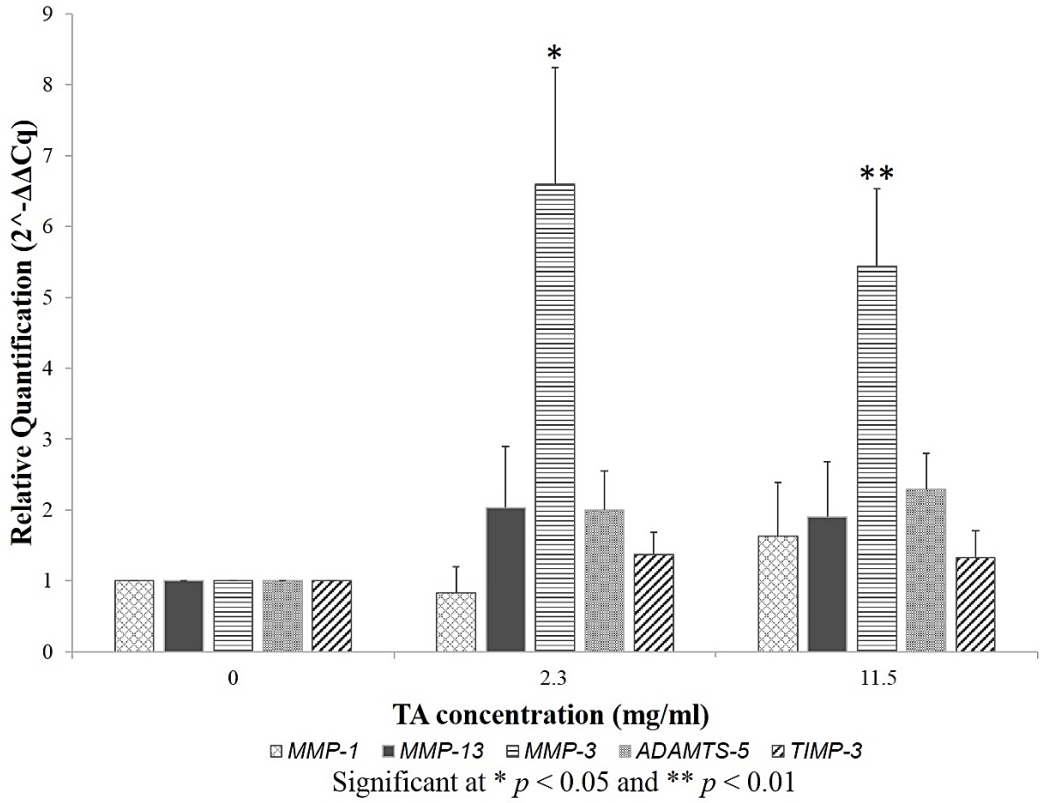


Figure 14 Relative mRNA level of *MMP-1, MMP-13, MMP-3, ADAMTS-5,* and *TIMP-3* in chondrocytes treated with TA compared with non-treated controls. *GAPDH* was used as an internal control.

For *MMP-1*, one outlier (Pat.7) was omitted from analysis; hence, the *MMP-1* graph in Figure 13 showed results from 9 samples. At 2.3 mM TA, expressions of *MMP-1* slightly decreased (0.85-fold) with no statistical significant compared with non-treated controls. However, the *MMP-1* expressions increased after 11.5 mM TA treatment (1.63-fold) but did not reach statistical significant. For *TIMP-3*, gene encoding inhibitors of the proteolytic enzymes, addition of TA did not alter its expressions (1.3-fold in both concentrations of TA compared with the controls).

CHAPTER V
DISCUSSIONS AND CONCLUSIONS

Discussions

TA on oxidative stress induction and cell viability

In spite of its potential adverse effects to a joint, TA is still a widely used GC in treatment for OA patients by intra-articular injection. There are several reports demonstrated a decrease in cartilage integrity and chondrocyte viability following GC treatments. Nevertheless, the cause of cytotoxicity and cartilage degradation are inconclusive. We showed that TA inflicted oxidative stress upon chondrocytes and increased chondrocyte expressions of matrix-degradation enzyme *matrix metalloproteinase-3*.

Normally, proliferation rate of early stage OA chondrocytes is elevated compared with healthy chondrocytes, however, chondrocytes used in this study were to stresses and apoptosis (Goldring and Goldring, 2007). In most studies, chondrocyte cell lines or healthy animals were used for determining the effects of GCs, and they did not represents the disease condition in OA patients. Primary chondrocytes used in this study were from OA patients, as a result, they had pathological conditions similar to the chondrocytes in patients undergoing TA injection. The outcome from this study would more likely represent what might occur in OA chondrocytes in humans.

Results from this study demonstrated that TA decreased chondrocyte viability and increased oxidative stress to chondrocytes in a dose-dependent manner. Our results were correspondent with results from two studies conducted in chondrocyte cell line (Braun et al., 2012a; Dragoo et al., 2012). In both studies, the experimenters determined effects of TA and several GCs commonly used for intra-articular injection at their clinical doses on viability of chondrocytes, and the results showed that GCs increased

a number of chondrocytes death. However, these studies were performed on commercial chondrocytes, and in one study, the GCs were added in combination with anaesthetics. The decrease in viability after TA treatment could be from apoptosis of chondrocytes as seen in the shrinkage and membrane blebbing morphology of the cells. Moreover, there was a review report stating that GCs activated apoptotic machinery of acute lymphoblastic leukemic cell line by increasing expressions of *Bim* (encoding pro-apoptotic protein) and decreasing expressions of anti-apoptotic gene *Bcl-2* (Schmidt et al., 2004). However, further experiments such as flow cytometry, annexin V staining, determination of caspase-3 activity, etc. must be performed in order to confirm whether TA induces chondrocyte apoptosis or necrosis.

Effect of TA in inducing oxidative stress in this study is consistent with results from a study by (Chung et al., 2007), which oxidative stress in primary retinal cells was increased by dexamethasone and TA. Mechanisms of oxidative stress induction to chondrocytes by TA are not known. The mechanisms are believed to be that TA decreased expressions and activities of endogenous antioxidant enzymes since there have been several *in vitro* studies reported these effects of GCs on other types of cells, mostly neurons. Effects of dexamethasone (You et al., 2009; Mutsaers and Tofighi, 2012) and corticosterone (Sato et al., 2010) on rat hippocampal neurons showed that the GCs increased expression of NADPH oxidase, and decreased expressions and activities of superoxide dismutase, catalase, glutathione peroxidase, NADH dehydrogenase (ND)-1, ND-4 enzymes, and cytochrome b leading to increased levels of lipid and protein oxidation. In another study, corticosterone-treated adrenal medulla carcinoma showed that, other than decreasing activity of SOD, the drug also decreased activity of mitochondrial complex I, hence, elevating levels of protein carbonyls (Tang et al., 2013). In an *in vivo* study, pregnant ovine were treated with betamethasone in order to determine effects of the drug on their foetal lambs. The Western blot results showed that the lung vascular epithelia of foetal lambs from the repeatedly GC-injected mothers had increased protein levels of nitric oxide synthase compared with those from

saline-treated mothers. There was no difference between foetal from GC-ingested and single-dose injected mothers (Grover et al., 2000). Taken together, those data could explain the possible mechanisms of TA-induced oxidative stress in chondrocytes in this study.

To study whether antioxidant could attenuate effects of TA on chondrocytes, vitamin C was added. The results showed that addition of vitamin C had significant effects upon treatment with 11.5 mM (5 mg/ml) TA, which were increased chondrocytes viability and decreased oxidative stress. The non-significant results in 2.3 mM (1 mg/ml) TA-treated chondrocytes might be explained by a study from Chothe in 2013 that determined effects of steroid hormones on expressions and regulations of vitamin C transporter, sodium-dependent vitamin C transporter 2 (SVCT2) in primary rabbit intervertebral disc cells (IDCs). Dexamethasone increased *SVCT2* mRNA levels and vitamin C uptake in the rabbit IDCs (Chothe et al., 2013). Thus, the chondrocytes treated with higher concentration of TA might obtain more levels of vitamin C transported into the cells from the higher expression of SVCT2. An importance of SVCT2 in reduction of oxidative stress was reported in a study in 2015 by Sangani et al (2015). The study was performed on primary mouse bone marrow stroma cells (BMCs). Results demonstrated that oxidative stress induced by Sin-1 halted the BMCs in G1 phase and decreased the cell number via induction of autophagy. Addition of vitamin C inhibited the autophagy process and increased the cell viability. However, vitamin C did not rescue the *SVCT2*–knockout BCMs from Sin-1-induced oxidative stress (Sangani et al., 2015).

Since vitamin C is a cofactor of several enzymes such as mono-oxygenase, prolyl and lysyl hydroxylases, it is important in cellular metabolism via synthesis and hydroxylation of carnitine, and amino acids tyrosine, tryptophan, glycine, and proline. Hydroxylation of carnitine is important for energy production by assisting transportation of fatty acid into mitochondria. Therefore, addition of vitamin C might

increase chondrocytes proliferation via pathways other than the non-enzymatic antioxidant role (Chambial et al., 2013).

Results from the present study demonstrated the ability of vitamin C in attenuating effects of TA-induced oxidative stress in chondrocytes, as a result, vitamin C might be potentially used as a supplement in an OA patient by co-injected with TA into the joint. Some *in vivo* studies have suggested formula of vitamin C for local injection. One study examined protecting effects of nutritive mixture solution on surgery-induced OA rabbits. The solution was composed of 10% dextrose solution, 20% amino acids solution important in type II collagen synthesis including proline and glycine, and 5% ascorbic acid. Knee cartilages of the rabbits intra-articularly injected with the nutritive mixture solution compared with the normal saline controls were significantly less severe, which were more chondrocyte number and less swelling, less cleft in the deep zone, more intact cartilage surface (Park et al., 2007). In another study, 15, 50, and 150 mM sodium-L-ascorbate in deoxygenated sodium chloride solution was developed for irrigation during articular anterior cruciate ligament surgery for better post-operation healing. The results showed that, compared with saline controls, rats irrigated with saline-ascorbate mixture had significant lower inflammation at day 1and better integrity of the joint tissues at day 42 after surgery (Fu, 2013). In order to increase chondrocyte viability and antioxidation capacity, vitamin C might be used in combination with fat-soluble antioxidants (e.g. vitamin E, carotenoids) for prevention of ROS reacting with phospholipid in plasma membrane (Buettner, 2004).

TA on expressions of genes involved in cell death

As ROS have been reported to alter expressions of several genes, the role of ROS as signalling molecules and their effects on expression of genes involved in cell cycle control and cell death were evaluated in our study. Results from ROS-induced gene expression analyses showed that all *P21*, *GDF15*, and *cFos* had significantly increased expressions after TA treatments compared with those of controls. *P21*, one

of the p53-target genes, is a member of cyclin-dependent kinase inhibitor (CIP/KIP family), whose role is crucial in cell-cycle checkpoint by binding to cyclin-dependent kinases and inhibiting cell-cycle progression from G1 into S phase, as a result, upregulation of *P21* leads to cell-cycle arrest (Dandrea et al., 2004; Owen, Ahmed and Farquharson, 2009). The increase in expressions of *P21* in the present study are consistent with a study in mouse chondrocyte cell line, which reported that dexamethasone increased expressions of p21 in both mRNA and protein levels at all incubation times (1, 6, and 24 h) (Owen et al., 2009). Considering an oxidative stress-inducing effect of TA on OA chondrocytes in this study, the increased expressions of *P21* in TA-treated chondrocytes could have been consistent with the study mentioned earlier by Sangani (Sangani et al., 2015) that reported an effect of oxidative stress in causing G1 cell-cycle arrest, and the elevated *P21* levels could have been due to stimulation of a p53-dependent pathway in response to DNA damage, which is one of the detrimental consequences of oxidative stress (Dandrea et al., 2004; Valko et al., 2007). However, a study showed that GCs might be able to activate of p21 via other transcription factors independent of the p53 (Yang et al., 2008).

GDF15 is also a p53 target gene. It is a member of transforming growth factor-β/bone morphogenetic factor superfamily and the gene's product is a broad-functional protein regulating various cellular processes including cell proliferation, differentiation, and apoptosis. Generally, expressions of *GDF15* are low in healthy adult tissues but can be rapidly upregulated upon disease conditions (e.g. diabetes, arthrosclerosis), and exposure to stimulants and stresses (e.g. inflammation, and oxidative stress) (Corre, Hebraud and Bourin, 2013; Li et al., 2013). There has been no report on effects of TA on expressions of *GDF15* in chondrocytes, but there have been studies on other types of stresses in induction of *GDF15* in other cell types. Some studies suggested that the primary role of increased expressions of *GDF15* under stress conditions is prevention of apoptosis rather than induction of cell death. Several studies have suggested that GDF15 protects myocardial cells from ischemia-reperfusion injuries. In one study,

nitric oxide (NO)-treated primary rat myocytes, and human myocardial tissues from patients with acute myocardial infarction were determined for expressions of *GDF15*. Also, apoptosis of myocytes in ischemia/reperfusion-induced *GDF15*-deficient mice were studied. The results showed that NO-treated primary myocytes and human myocardial lesions had strongly increased expression of *GDF15* in both mRNA and protein levels, compared with non-treated controls and tissues from healthy individuals. Pre-treatment of the primary myocytes with recombinant GDF15 reduced the number of cell death via PI3K/Akt signalling pathway. After ischemia induction and reperfusion, *GDF15*-deficient mice had larger infarct lesions in the heart compared with wild type mice (Kempf et al., 2006). A study in 2013 by Li et al. demonstrated that high glucose concentration (33 mM) increased apoptosis of HUVECs, and levels of ROS and *GDF15* in human umbilical venous endothelial cells (HUVECs) via p53 pathway. Transfection the HUVECs with *GDF15* siRNA resulted in significant increase number of apoptotic cells and ROS levels after high glucose treatment compared with *GDF15*-expressed HUVECs, which could be suggested that elevation of *GDF15* expression after high glucose treatment could rescue HUVECs from undergoing apoptosis (Li et al., 2013).

However, some previous studies suggested that an increase in *GDF15* expression might not be beneficial. A study in macrophages in arteriosclerotic arteries lesions showed that oxidative stress induction of primary human macrophages *in vitro* by H_2O_2 or oxidized low density lipoprotein (oxLDL) increased expression of *GDF15*, and when immunostaining the arteriosclerotic lesions from patients, the localised macrophages showed co-localisation of GDF15, oxLDL, and apoptotic markers (caspase-3, poly ADP-ribose polymerase (PARP), and apoptosis-inducing factor) with positive correlation by morphometic analyses (Schlittenhardt et al., 2004). Also, a cohort study in patients with chronic kidney disease showed that high serum GDF15 was correlated with high protein-energy wasting and early mortality. The experimenters suggested that GDF15 might have mediated the protein-energy wasting that led to

mortality (Breit et al., 2012). In order to understand a role of *GDF15* in TA-treated chondrocytes in the present study, further study should be performed by knocking-down *GDF15* in chondrocytes.

How an increase in *cFos* expressions in chondrocytes has been in doubt as there has been no report on effects of TA on expressions of *cFos* in chondrocytes. However, there have been studies on oxidative stress-induced expression of *cFos* in several types of cells. *cFos* encodes cFos protein, a member of a Fos family protein, which can dimerise with proteins from a Jun family to form an important stress-response AP-1 transcription factor (Milde-Langosch, 2005). The roles of *cFos* induction in response to oxidative stress have been reported to be either protective or deleterious in several cell types. Effects of ultraviolet (UV)-C and H_2O_2 on primary mouse embryonic fibroblasts (MEFs) showed increase in levels of cFos, JunD, and cleaved PARP (target protein of caspase-3) starting 1 h after exposure. Overexpressions of both proteins in MEFs led to a significant decrease in levels of cleaved PARP, indicating a decrease in cell death UV-C or H_2O_2. Moreover, the study also demonstrated that mechanism of cFos and JunD expression and activation were through extracellular signal-regulated kinases 1 and 2 (ERK1/2) since an inhibition of ERK1/2 activation decreased the expression and activity of both proteins and increased levels of cleaved PARP after the UV-C exposure (Zhou et al., 2007). A study in human retinal pigment epithelial cell line ARPE19 showed that UV-C exposure triggered AP-1 activity via phosphorylation of cJun and cFos by JNK and p38 kinases. Inhibition of JNK and p38 activities lowered the level of phosphorylated cJun and cFos in the ARPE19 cells at 48h after UV-C exposure, and resulted in the increase in number of viable cells, indicating the destructive role of cJun and cFos after UV-C-induced stress (Roduit and Schorderet, 2008).

The increase in expressions of *GDF15* and *cFos* observed in our study might have had destructive effects upon chondrocytes because of the significant decrease in chondrocyte viability.

TA on expressions of genes involved in cartilage homeostasis

Given that the cause of OA is from an altered homeostasis in production and degradation of the cartilages' ECM, understanding the consequences of intra-articular GC treatment on expressions of matrix-degrading enzymes and their inhibitors is necessary for treatment plans in OA patients. As a result, further investigation on the effects of TA on expressions of collagenases (*MMP-1, MMP-13*), stromelysin (*MMP-3*), aggrecanase (*ADAMTS-5*), and a broad inhibitor of metalloprotinases and aggrecanases *TIMP-3* was performed on the primary chondrocytes. The significant increase was found in expressions of only the *MMP-3* gene in the TA-treated chondrocytes compared with non-treated controls. For *MMP-1,* sample 'Pat.7' was discarded from analysis because of the suspiciously high values (52-fold for 2.3 mM TA and 15-fold for 11.5 mM TA treatments), which could be from the biological variation of this patient from other patients.

The increase in *MMP-3* expressions by TA in this study is consistent with an increase in MMP-3 protein levels in an *in vivo* study. The study was carried out in rats to determine mechanisms of GCs (TA and prednisolone) in lessening tendon function. The rats were injected with GCs or normal saline at Achilles tendon calcaneus junction. One week after injection, the tendons from GCs-injected rats had increased expressions of MMP-3 and number of apoptotic tenocytes that led to decreased levels of collagen fibres and reduced strength and elasticity of the tendons (Muto et al., 2014). Altogether, the TA-increased expressions of *MMP-3* in this study might result in destructive effect on articular cartilage integrity that can lead to further cartilage destruction in OA patients.

Results from several studies demonstrated the effects of GCs in attenuating the cytokines' effects on expressions of several metalloproteinase genes. An *in vitro* study by Garvican *et al.* (2010) determined effects of pro-inflammatory cytokines (interleukin (IL)-1β and activated protein C (APC)) and clinical doses of GCs (Methylprednisolone acetate and TA) on equine articular cartilage explants. Treating the cartilage explants with GCs without stimulating them with cytokines did not affect degradation of proteoglycans and collagens, and expressions of *MMP-1*, *MMP-3*, *MMP-13*, and *TIMP-3* compared with non-treated controls, which are consistent with unaltered expressions of these genes by TA in the present study. The effects of GCs, however, were seen in the cytokine-treated cartilage explants that GCs decreased expressions of the previously upregulated *MMP-1*, *MMP-3, and MMP-13* to the levels of non-treated controls (Garvican et al., 2010). Another study on equine cartilage also reported that GCs (TA and dexamethasone) decreased the expressions of *MMP-3* and *MMP-13* that previously upregulated by IL-1β. However, expressions of *ADAMTS-5* remained high after GC treatments, and the drugs were also unable to reduce proteoglycan degradation, suggesting the different mechanisms of GC-controling expressions between *MMPs* and *ADAMTS-5* (Busschers et al., 2010). Other studies on GC-attenuating cytokine effects (Su et al., 1996; Lu et al., 2011) also showed the decrease in expressions of *MMP-1*, *MMP-13*, and *MMP-3* after dexamethasone treatment in cytokine-stimulated bovine cartilage explants. However, those studies reported that GC also decreased expressions of the enzyme inhibitor *TIMP-3* and increased expressions of *ADAMTS-5* in the cytokine-induced cartilages. Results from the present study showed no altered expressions of *MMP-1*, *MMP-13*, *TIMP-3*, and *ADAMTS-5* after TA treatment. Nevertheless, the previous studies mentioned above performed on cytokines-stimulated cartilages from healthy animals to mimic an inflammatory state, thus, results from those studies might not represent the exact effects of GCs when used in non-inflamed joints. In this study, the primary chondrocytes were taken from OA patients and were not stimulated by pro-inflammatory cytokines in order to reflect the patients' pathological

states. As a result, effects of TA on gene expressions in this study were not consistent with the previous reports mentioned above.

Though this study's results suggested that TA treatment could have adverse effect to cartilage due to induction of *MMP-3* expressions, the use of GCs in OA patients with evident of joint inflammation or in patients with rheumatoid arthritis (RA) might be beneficial. A clinical study in RA patients reported that serum levels of cartilage turnover marker, cartilage oligomeric matrix protein significantly decreased, with lowest concentration at 48h, after intra-articular injection of THA, a TA derivative (Weitoft et al., 2005).

Mechanism of how TA increased *MMP-3* expressions in this study has not been resolved. Several protein kinase pathways have been reported to mediate expressions of *MMP* genes (Reuben and Cheung, 2006). One of the pathways might be via p38 MAPK pathway. One study showed that induction of total MMP activity and expressions by cytokines (oncostatin M and tumour necrosis factor) were mediated via p38 and P44/42 MAPK pathways and addition of either p38 or P44/42 inhibitors decreased activity and expressions of MMPs. However, inhibiting only P44/42 reduced activity and expression of aggrecanases. (Sondergaard et al., 2010). Moreover, an *in vivo* study showed that rats injected with SB203580, a selective inhibitor of p38, after undergoing anterior cruciate ligament surgery presented better cartilage integrity and lower expressions of MMP-3 and MMP-13 proteins compared with saline controls (Chen et al., 2007). Also, as TA increased oxidative stress in this study and one of the pathways regulated by oxidative stress-mediated gene expressions is also p38 MAPK pathway (Ho et al., 2006; Pocrnich et al., 2009), the increase in *MMP-3* expressions by TA in this study might be mediated via the p38 MAPK.

One limitation of this study was that the primary chondrocytes were not embedded in three-dimensional extracellular matrix as they would be in the body, but they were instead cultured in monolayer. In order to minimise chondrocyte phenotypic

changes, passages of chondrocytes used in all experiments were less than three passages, since there have been reports demonstrated that chondrocytes cultured for less than 21 days and at passage 2 still maintain expressions of the chondrocyte-specific proteins (Schnabel et al., 2002; Ma et al., 2013). Moreover, to determine whether the increase in mRNA expressions actually has an effect on chondrocytes, analysis of protein expressions by Western blot analysis can be performed in future studies.

Conclusions

Results from the present study demonstrated that toxicity of TA to chondrocytes was by induction of oxidative stress that later resulted in an increase in expressions of genes controlling cell cycle progression (*P21*), and genes involved in oxidative stress-induced cell death (*GDF15* and *cFos*). Addition of vitamin C decreased oxidative stress and increased chondrocyte viability at low concentrations (1 mg/ml and 5 mg/ml TA), even though the effects were significant in 5 mg/ml TA-treated chondrocytes. Thus, employing vitamin C in patients undergoing TA treatment (or possibly other type of GCs) might be helpful in rescuing cell viability owing to its anti-oxidant activity. An *in vivo* study can be performed in order to confirm the benefits of vitamin C co-injection with TA in OA patients.

Besides its ability to induce oxidative stress that led to chondrotoxicity, effects of TA from this study showed to be increased expressions of stromelysin *MMP-3*, which is an enzyme capable of degrading both collagens and proteoglycans in the cartilage. Consequently, using TA in patients with knee OA might promote degradation of the patient's cartilage. Still, further study in MMP-3 protein expression and activity in TA-treated cartilage explants with no prior cytokine treatments can be performed to confirm the TA-toxicity to OA cartilage.

Knowledge from this study might be useful for physicians when selecting TA as pharmaceutical agent for intra-articular injection in OA patients. Antioxidants such as vitamin C supplementation or co-injection with TA might be useful and be included in treatment plans for the OA patients.

REFERENCES

Alvarez-Soria, M.A., et al. 2008. Long-term NSAID treatment directly decreases COX-2 and mPGES-1 production in the articular cartilage of patients with osteoarthritis. Osteoarthritis and Cartilage 16: 1484-1493.

American Academy of Orthopedic Surgeons (AAOS). 2015. Total knee replacement. (Pamphlet)

Athanasiadis, Y., Tsatsos, M., Sharma, A. and Hossain, P. 2013. Subconjunctival triamcinolone acetonide in the management of ocular inflammatory disease. Journal of Ocular Pharmacology and Theraputics 29: 516-522.

Barnes, P.J. 1998. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clinical Science 94: 557-572.

Batinic-Haberle, I., Reboucas, J.S. and Spasojevic, I. 2010. Superoxide dismutase mimics: chemistry, pharmacology, and therapeutic potential. Antioxidants and Redox Signaling 13: 877-918.

Beecher, B.R., Martin, J.A., Pedersen, D.R., Heiner, A.D. and Buckwalter, J.A. 2007. Antioxidants block cyclic loading induced chondrocyte death. Iowa Orthopaedic Journal 27: 1-8.

Bender, D.A. 2015. Free radicals and antioxidant nutrients. In V.W. Rodwell, Bender, D.A., Botham, K.M., Kennelly, P.J., Weil, P.A. (ed.). Harper's Illustrated Biochemistry. pp.564-569. New York, USA: McGrew Hill Education.

Braun, H.J., et al. 2012. The effect of local anesthetic and corticosteroid combinations on chondrocyte viability. Knee Surgery, Sports Traumatology, Arthroscopy 20: 1689-1695.

Breit, S.N., et al. 2012. Macrophage inhibitory cytokine-1 (MIC-1/GDF15) and mortality in end-stage renal disease. Nephrology Dialysis Transplantation 27: 70-75.

Brew, K. and Nagase, H. 2010. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochemica et Biophysica Acta 1803: 55-71.

Buckwalter, J.A., Mankin, H.J. and Grodzinsky, A.J. 2005. Articular cartilage and osteoarthritis. Instructional Course Lectures 54: 465-480.

Buettner, G.R., and Schafer, F.Q. 2004. Ascorbate as an antioxidant. In H. Asard, May, J.M., and Smirnoff, N. (ed.). Vitamin C: Functions and biochemistry in animals and plants. pp.173 - 185. Oxon, UK: Garland Science/BIOS Scientific Publishers.

Bunriang Pitsamai, M.T., Nirat Imamee, Suparb Aree-ue. 2012. Factors related to self-management behaviors for persons with osteoarthritis. Journal of Public Health 42: 54-67.

Busschers, E., Holt, J.P. and Richardson, D.W. 2010. Effects of glucocorticoids and interleukin-1 beta on expression and activity of aggrecanases in equine chondrocytes. American Journal of Veterinary Research 71: 176-185.

Carlo, M.D.J. and Loeser, R.F. 2003. Increased oxidative stress with aging reduces chondrocyte survival: correlation with intracellular glutathione levels. Arthritis and Rheumatism 48: 3419-3430.

Caron, J.P., Gandy, J.C., Schmidt, M., Hauptman, J.G. and Sordillo, L.M. 2013. Influence of corticosteroids on interleukin-1beta-stimulated equine chondrocyte gene expression. Veterinary Surgery 42: 231-237.

Celeste, C., Ionescu, M., Robin Poole, A. and Laverty, S. 2005. Repeated intraarticular injections of triamcinolone acetonide alter cartilage matrix metabolism measured by biomarkers in synovial fluid. Journal of Orthopaedic Research 23: 602-610.

Chambial, S., Dwivedi, S., Shukla, K.K., John, P.J. and Sharma, P. 2013. Vitamin C in disease prevention and cure: an overview. Indian Journal of Clinical Biochemistry 28: 314-328.

Chen, J.J., Huang, J., Du, W.X. and Tong, P.J. 2014. Expression and significance of MMP3 in synovium of knee joint at different stage in osteoarthritis patients. Asian Pacific Journal of Tropical Medicine 7: 297-300.

Chen, W.D., Jiang, Q., Chen, D.Y., Xu, H. and Zhang, Y.F. 2007. Effects of intra-articular injection of p38 mitogen-activated protein kinase inhibitor on matrix metalloproteinase in articular cartilage of a rat model of osteoarthritis. Zhongguo Yi Xue Ke Xue Yuan Xue Bao 29: 777-781.

Choi, S., Cho, T.J., Kwon, S.K., Lee, G. and Cho, J. 2013. Chondrogenesis of periodontal ligament stem cells by transforming growth factor-beta3 and bone morphogenetic protein-6 in a normal healthy impacted third molar. International Journal of Oral Science 5: 7-13.

Chothe, P.P., et al. 2013. Sodium-coupled vitamin C transporter (SVCT2): expression, function, and regulation in intervertebral disc cells. The Spine Journal 13: 549-557.

Chung, H., Hwang, J.J., Koh, J.Y., Kim, J.G. and Yoon, Y.H. 2007. Triamcinolone acetonide-mediated oxidative injury in retinal cell culture: comparison with dexamethasone. Investigative Ophthalmology and Visual Science 48: 5742-5749.

Coleman, M.C., Ramakrishnan, P.S., Brouillette, M.J. and Martin, J.A. 2015. Injurious loading of articular cartilage compromises chondrocyte respiratory function. Arthritis and Rheumatology1-35.

Corps, A.N., et al. 2008. The regulation of aggrecanase ADAMTS-4 expression in human Achilles tendon and tendon-derived cells. Matrix Biology 27: 393-401.

Corre, J., Hebraud, B. and Bourin, P. 2013. Concise review: growth differentiation factor 15 in pathology: a clinical role? Stem Cells Translational Medicine 2: 946-952.

Cruz-Topete, D. and Cidlowski, J.A. 2015. One hormone, two actions: anti- and pro-inflammatory effects of glucocorticoids. Neuroimmunomodulation 22: 20-32.

D'Autreaux, B. and Toledano, M.B. 2007. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nature Review Molecular Cell Biology 8: 813-824.

Dandrea, T., et al. 2004. The transcriptosomal response of human A549 lung cells to a hydrogen peroxide-generating system: relationship to DNA damage, cell cycle arrest, and caspase activation. Free Radical Biology and Medicine 36: 881-896.

DeHaan, M.N., Guzman, J., Bayley, M.T. and Bell, M.J. 2007. Knee osteoarthritis clinical practice guidelines - how are we doing? The Journal of Rheumatology 34: 2099-2105.

Dewire, P., Einhorn T.A. 2001. The joint as an organ. In D.S. Howell, Altaman, R.D., Buckwalter, J.A., Goldberg, V.M. (ed.). Osteoarthritis: diagnosis and medical/surgical management. pp.49-66. Philadelphia: W.B.Saunders.

Douglas, R.J. 2012. Corticosteroid injection into the osteoarthritic knee: drug selection, dose, and injection frequency. International Journal of Clinical Practice 66: 699-704.

Dragoo, J.L., Danial, C.M., Braun, H.J., Pouliot, M.A. and Kim, H.J. 2012. The chondrotoxicity of single-dose corticosteroids. Knee Surgery, Sports Traumatology, Arthroscopy 20: 1809-1814.

Evans, C.H., Kraus, V.B. and Setton, L.A. 2014. Progress in intra-articular therapy. Nature Review Rheumatology 10: 11-22.

Felson, D.T. 2013. Osteoarthritis. In A.S. Fauci, Langford, C.A. (ed.). Harrison's Rheumatology. pp.232 - 243. USA: McGrew Hill Education.

Feng, Y.L. and Tang, X.L. 2014. Effect of glucocorticoid-induced oxidative stress on the expression of Cbfa1. Chemico-Biological Interaction 207: 26-31.

Fernandes, J.C., Martel-Pelletier, J. and Pelletier, J.P. 2002. The role of cytokines in osteoarthritis pathophysiology. Biorheology 39: 237-246.

Fransen, M., et al. 2011. The epidemiology of osteoarthritis in Asia. International Journal of Rheumatic Diseases 14: 113-121.

Fu, S.C., Cheng, W.H., Cheuk, Y.C., Mok, T.Y., Rolf, C., Yung, S.H., Chan, K.M. . 2013. Develpment of vitamin C irrigation saline to promote graft healing in anterior cruciate ligament reconstruction. Journal of Orthopaedic Translation 1: 67-77.

Fubini, S.L., Todhunter, R.J., Burton-Wurster, N., Vernier-Singer, M. and MacLeod, J.N. 2001. Corticosteroids alter the differentiated phenotype of articular chondrocytes. Journal of Orthopaedic Research 19: 688-695.

Garvican, E.R., Vaughan-Thomas, A., Redmond, C., Gabriel, N. and Clegg, P.D. 2010. MMP-mediated collagen breakdown induced by activated protein C in equine cartilage is reduced by corticosteroids. Journal of Orthopaedic Research 28: 370-378.

Gepstein, A., Shapiro, S., Arbel, G., Lahat, N. and Livne, E. 2002. Expression of matrix metalloproteinases in articular cartilage of temporomandibular and knee joints of mice during growth, maturation, and aging. Arthritis and Rheumatism 46: 3240-3250.

Gerwin, N., Hops, C. and Lucke, A. 2006. Intraarticular drug delivery in osteoarthritis. Advanced Drug Delivery Reviews 58: 226-242.

Goldring, M.B. 2007. Update on the chondrocyte lineage and implications for cell therapy in osteoarthritis. In L. Sharman, Berenbaum, F. (ed.). Osteoarthritis: A companion to rheumatology. pp.53 - 66. Philadelphia, PA, USA: MOSBY Elsevier.

Goldring, M.B. and Goldring, S.R. 2007. Osteoarthritis. Journal of Cellular Physiology 213: 626-634.

Goodwin, J.L., Kraemer, J.J. and Bajwa, Z.H. 2009. The use of opioids in the treatment of osteoarthritis: when, why, and how? Current Rheumatology Reports 11: 5-14.

Grover, T.R., Ackerman, K.G., Le Cras, T.D., Jobe, A.H. and Abman, S.H. 2000. Repetitive prenatal glucocorticoids increase lung endothelial nitric oxide synthase expression in ovine fetuses delivered at term. Pediatric Research 48: 75-83.

Hamid, A.A., Aiyelaagbe, O.O., Usman, L.A., Ameen, O.M., Lawal, A. 2010. Antioxidants: its medicinal and pharmacological applications. African Journal of Pure an Applied Chemistry 4: 142-151.

Han, E.S., et al. 2008. The in vivo gene expression signature of oxidative stress. Physiological Genomics 34: 112-126.

Hartmann, C., et al. 2009. TIMP-3: a novel target for glucocorticoid signaling at the blood-brain barrier. Biochemical and Biophysical Research Communications 390: 182-186.

Harvey, R., and Ferrier, D. 2011. Glycosaminoglycans, proteoglycans, and glcoproteins. In R.A. Harvey (ed.). Lippincott's illustrated reviews: Biochemistry. pp.157-163. Baltimore, MD, USA: Lippincott Williams & Wilkins.

Hauser, R.A. 2009. The deterioration of articular cartilage in osteoarthritis by corticosteroid injections. Journal of Prolotherapy 2: 107-123.

Hepper, C.T., et al. 2009. The efficacy and duration of intra-articular corticosteroid injection for knee osteoarthritis: a systematic review of level I studies. The Journal of the American Academy of Orthopaedic Surgeons 17: 638-646.

Ho, T.C., et al. 2006. Activation of mitogen-activated protein kinases is essential for hydrogen peroxide -induced apoptosis in retinal pigment epithelial cells. Apoptosis 11: 1899-1908.

Huang, K. and Wu, L.D. 2008. Aggrecanase and aggrecan degradation in osteoarthritis: a review. The Journal of International Medical Research 36: 1149-1160.

Iuchi, T., et al. 2003. Glucocorticoid excess induces superoxide production in vascular endothelial cells and elicits vascular endothelial dysfunction. Circulation Research 92: 81-87.

Ivers, N., Dhalla, I.A. and Allan, G.M. 2012. Opioids for osteoarthritis pain: benefits and risks. Canadian Family Physician 58: e708.

Jevsevar, D.S., Brown, G.A., Jones, D.L., et al. . 2013. Treatment of osteoarthritis of the knee: based guideline - 2nd editiond. Illinois.

Jo, H., et al. 2003. The in vitro effects of dehydroepiandrosterone on human osteoarthritic chondrocytes. Osteoarthritis and Cartilage 11: 585-594.

Kamata, H. and Hirata, H. 1999. Redox regulation of cellular signalling. Cellular Signalling 11: 1-14.

Kaur, P., Kaur, G. and Bansal, M.P. 2008. Upregulation of AP1 by tertiary butyl hydroperoxide induced oxidative stress and subsequent effect on spermatogenesis in mice testis. Molecular and Cellular Biochemistry 308: 177-181.

Kempf, T., et al. 2006. The transforming growth factor-beta superfamily member growth-differentiation factor-15 protects the heart from ischemia/reperfusion injury. Circulation Research 98: 351-360.

Kon, E., et al. 2012. Non-surgical management of early knee osteoarthritis. Knee Surgery, Sports Traumatology, Arthroscopy 20: 436-449.

Koressaar, T. and Remm, M. 2007. Enhancements and modifications of primer design program Primer3. Bioinformatics 23: 1289-1291.

Kotepui, M., Thawornkuno, C., Chavalitshewinkoon-Petmitr, P., Punyarit, P. and Petmitr, S. 2012. Quantitative real-time RT-PCR of ITGA7, SVEP1, TNS1, LPHN3, SEMA3G, KLB and MMP13 mRNA expression in breast cancer. Asian Pacific Journal of Cancer Prevention 13: 5879-5882.

Kydd, A.S., Reno, C.R., Tsao, H.W. and Hart, D.A. 2007. Early inflammatory arthritis in the rabbit: the influence of intraarticular and systemic corticosteroids on mRNA levels in connective tissues of the knee. The Journal of Rheumatology 34: 130-139.

Lavelle, W., Lavelle, E.D. and Lavelle, L. 2007. Intra-articular injections. Anesthesiology Clinics 25: 853-862.

Lee, J.M., Lee, H., Park, M. and Baek, S. 2012. Subconjunctival injection of triamcinolone for the treatment of upper lid retraction associated with thyroid eye disease. The Journal of Craniofacial Surgery 23: 1755-1758.

Li, D., Xie, G. and Wang, W. 2012. Reactive oxygen species: the 2-edged sword of osteoarthritis. The American Journal of The Medical Sciences 344: 486-490.

Li, J., et al. 2013. Adaptive induction of growth differentiation factor 15 attenuates endothelial cell apoptosis in response to high glucose stimulus. PLoS One 8: e65549.

Li, W., Du, C., Wang, H. and Zhang, C. 2014. Increased serum ADAMTS-4 in knee osteoarthritis: a potential indicator for the diagnosis of osteoarthritis in early stages. Genetics and Molecular Research 13: 9642-9649.

Lu, Y.C., Evans, C.H. and Grodzinsky, A.J. 2011. Effects of short-term glucocorticoid treatment on changes in cartilage matrix degradation and chondrocyte gene expression induced by mechanical injury and inflammatory cytokines. Arthritis Research & Therapy 13: R142.

Luo, Y., et al. 2011. Autophagy regulates ROS-induced cellular senescence via p21 in a p38 MAPK alpha dependent manner. Experimental Gerontology 46: 860-867.

Ma, B., et al. 2013. Gene expression profiling of dedifferentiated human articular chondrocytes in monolayer culture. Osteoarthritis and Cartilage 21: 599-603.

Martel-Pelletier, J., Boileau, C., Pelletier, J.P. and Roughley, P.J. 2008. Cartilage in normal and osteoarthritis conditions. Best Practice and Research Clinical Rheumatology 22: 351-384.

McKay, L.I., Cidlowski, J.A. 2003. Corticosteroids in the treatment of neoplasms. Holland-Frei cancer medicine. D.W. Kufe, Pollock, R.E., Weichselbaum, R.R., Bast, R.C.Jr., Gansler, T.S., Holland, J.F., Frei, I.E. Hamilton (online), BC Decker.

Menkes, C.J. 1994. Intraarticular treatment of osteoarthritis and guidelines to its assessment. The Journal of Rheumatology Supplement 41: 74-76.

Milde-Langosch, K. 2005. The Fos family of transcription factors and their role in tumourigenesis. European Journal of Cancer 41: 2449-2461.

Miura, Y. and Roider, J. 2009. Triamcinolone acetonide prevents oxidative stress-induced tight junction disruption of retinal pigment epithelial cells. Graefe's Archive for Clinical and Experimental Ophthalmology 247: 641-649.

Muto, T., et al. 2014. Temporary inductions of matrix metalloprotease-3 (MMP-3) expression and cell apoptosis are associated with tendon degeneration or rupture after corticosteroid injection. Journal of Orthopaedic Research 32: 1297-1304.

Mutsaers, H.A. and Tofighi, R. 2012. Dexamethasone enhances oxidative stress-induced cell death in murine neural stem cells. Neurotoxicity Research 22: 127-137.

Naito, K., et al. 1999. Measurement of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in patients with knee osteoarthritis: comparison with generalized osteoarthritis. Rheumatology 38: 510-515.

Nareerat Rungruang. 2012. Effects of the anti-inflammatory drug, ketorolac, on chondrocyte apoptosis and expression of repair genes. Master's Thesis, Botany Department, Faculty of Science, Chulalongkorn University.

National Heart Lung and Blood Institute, N. 2013, 31 July 2013. How is COPD treated? Retrieved 3 January 2016, 2016, from http://www.nhlbi.nih.gov/health/health-topics/topics/copd/treatment.

National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS). 2010. What Is Osteoarthritis? National Institutes of Health. (Pamphlet)

Neustadt, D.H. 2001. Intra-articular therapy. In D.S. Howell, Altaman, R.D., Buckwalter, J.A., Goldberg, V.M. (ed.). Osteoarthritis: diagnosis and medical/surgical management. pp.284-301. Philadelphia, USA: W.B.Saunders.

Nganvongpanit, K., et al. 2009. In vitro suppression of the MMP-3 gene in normal and cytokine-treated human chondrosarcoma using small interfering RNA. Journal of Orthopaedic Surgery and Research 4: 45.

Noori, S. 2012. An overview of oxidative stress and antioxidant defensive system. Open Access Scientific Reports.

Owen, H.C., Ahmed, S.F. and Farquharson, C. 2009. Chondrocyte p21(WAF1/CIP1) expression is increased by dexamethasone but does not contribute to dexamethasone-induced growth retardation in vivo. Calcified Tissue International 85: 326-334.

Oztuna, V., Eskandari, M., Bugdayci, R. and Kuyurtar, F. 2007. Intra-articular injection of tenoxicam in osteoarthritic knee joints with effusion. Orthopedics 30: 1039-1042.

Park, Y.S., et al. 2007. Intra-articular injection of a nutritive mixture solution protects articular cartilage from osteoarthritic progression induced by anterior cruciate ligament transection in mature rabbits: a randomized controlled trial. Arthritis Research and Therapy 9: R8.

Pelletier, J.P. and Martel-Pelletier, J. 1989. Protective effects of corticosteroids on cartilage lesions and osteophyte formation in the Pond-Nuki dog model of osteoarthritis. Arthritis and Rheumatism 32: 181-193.

Percival, M. 1998. Antioxidants. Clinical Nutrition Insites 10: 1-4.

Peregoy, J. and Wilder, F.V. 2011. The effects of vitamin C supplementation on incident and progressive knee osteoarthritis: a longitudinal study. Public Health Nutrition 14: 709-715.

Phuc Le, P., et al. 2005. Glucocorticoid receptor-dependent gene regulatory networks. PLoS Genetics 1: e16.

Picot, J., Jones, G.E. 2005. Human cell culture protocols.2nd. New Jersey: Human Press.

Pocrnich, C.E., et al. 2009. p38 mitogen-activated protein kinase protects human retinal pigment epithelial cells exposed to oxidative stress. Canadian Journal of Ophthalmology 44: 431-436.

PubChem. 2005. Triamcinolone acetonide; CID=6436 [Online]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/6436

Rahal, A., et al. 2014. Oxidative stress, prooxidants, and antioxidants: the interplay. BioMed Research International 2014: 761264.

Reuben, P.M. and Cheung, H.S. 2006. Regulation of matrix metalloproteinase (MMP) gene expression by protein kinases. Frontiers in Bioscience 11: 1199-1215.

Rhen, T. and Cidlowski, J.A. 2005. Antiinflammatory action of glucocorticoids-new mechanisms for old drugs. The New England Journal of Medicine 353: 1711-1723.

Robin Poole, A.a.H., D.S. 2001. Etiopathogenesis of osteoarthritis. In D.S. Howell, Altaman, R.D., Buckwalter, J.A., Goldberg, V.M. (ed.). Osteoarthritis: diagnosis and medical/surgical management. pp.29 - 42. Philadelphia, USA: W.B. Saunder.

Roduit, R. and Schorderet, D.F. 2008. MAP kinase pathways in UV-induced apoptosis of retinal pigment epithelium ARPE19 cells. Apoptosis 13: 343-353.

Roy, S., Sannigrahi, S., Ghosh, B., Pusp, P. and Roy, T. 2013. Combination therapy of dexamethasone with epigallocatechin enhances tibiotarsal bone articulation and modulates oxidative status correlates with cartilage cytokines expression in the early phase of experimental arthritis. European Journal of Pharmacology 698: 444-454.

Sadowski, T. and Steinmeyer, J. 2001. Effects of non-steroidal antiinflammatory drugs and dexamethasone on the activity and expression of matrix metalloproteinase-1, matrix metalloproteinase-3 and tissue inhibitor of metalloproteinases-1 by bovine articular chondrocytes. Osteoarthritis and Cartilage 9: 407-415.

Sadowski, T. and Steinmeyer, J. 2002. Effects of polysulfated glycosaminoglycan and triamcinolone acetonid on the production of proteinases and their inhibitors by IL-1alpha treated articular chondrocytes. Biochemical Pharmacology 64: 217-227.

Sandell, L.J. and Aigner, T. 2001. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. Arthritis Research 3: 107-113.

Sangani, R., et al. 2015. The crucial role of vitamin C and its transporter (SVCT2) in bone marrow stromal cell autophagy and apoptosis. Stem Cell Research 15: 312-321.

Sato, H., Takahashi, T., Sumitani, K., Takatsu, H. and Urano, S. 2010. Glucocorticoid Generates ROS to Induce Oxidative Injury in the Hippocampus, Leading to Impairment of Cognitive Function of Rats. Journal of Clinical Biochemistry and Nutrition 47: 224-232.

Schlittenhardt, D., et al. 2004. Involvement of growth differentiation factor-15/macrophage inhibitory cytokine-1 (GDF-15/MIC-1) in oxLDL-induced apoptosis of human macrophages in vitro and in arteriosclerotic lesions. Cell and Tissue Research 318: 325-333.

Schmidt, S., et al. 2004. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ 11 Suppl 1: S45-55.

Schmucker, A.C., Wright, J.B., Cole, M.D. and Brinckerhoff, C.E. 2012. Distal interleukin-1beta (IL-1beta) response element of human matrix metalloproteinase-13 (MMP-13) binds activator protein 1 (AP-1) transcription factors and regulates gene expression. The Journal of Biological Chemistry 287: 1189-1197.

Schnabel, M., et al. 2002. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis and Cartilage 10: 62-70.

Schumacher, H.R. and Chen, L.X. 2005. Injectable corticosteroids in treatment of arthritis of the knee. The American Journal of Medicine 118: 1208-1214.

Sen, C.K. and Packer, L. 1996. Antioxidant and redox regulation of gene transcription. The FASEB Journal 10: 709-720.

Shapiro, P.S., et al. 2007. The effect of local corticosteroid or ketorolac exposure on histologic and biomechanical properties of rabbit tendon and cartilage. Hand 2: 165-172.

Sharma, L. 2001. Epidemiology of osteoarthritis. In D.S. Howell, Altaman, R.D., Buckwalter, J.A., Goldberg, V.M. (ed.). Osteoarthritis: diagnosis and medical/surgical management. pp.3-21. Philadelphia, USA: W.B. Saunder.

Sondergaard, B.C., et al. 2010. MAPKs are essential upstream signaling pathways in proteolytic cartilage degradation--divergence in pathways leading to aggrecanase and MMP-mediated articular cartilage degradation. Osteoarthritis and Cartilage 18: 279-288.

Song, R.H., et al. 2007. Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. Arthritis and Rheumatism 56: 575-585.

Sophia Fox, A.J., Bedi, A. and Rodeo, S.A. 2009. The basic science of articular cartilage: structure, composition, and function. Sports Health 1: 461-468.

Su, S., Dehnade, F. and Zafarullah, M. 1996. Regulation of tissue inhibitor of metalloproteinases-3 gene expression by transforming growth factor-beta and dexamethasone in bovine and human articular chondrocytes. DNA and Cell Biology 15: 1039-1048.

Suantawee, T., et al. 2013. Oxidative stress, vitamin e, and antioxidant capacity in knee osteoarthritis. Journal of Clinical and Diagnostic Research 7: 1855-1859.

Syed, H.M., Green, L., Bianski, B., Jobe, C.M. and Wongworawat, M.D. 2011. Bupivacaine and triamcinolone may be toxic to human chondrocytes: a pilot study. Clinical Orthopaedics and Related Research 469: 2941-2947.

Tang, V.M., Young, A.H., Tan, H., Beasley, C. and Wang, J.F. 2013. Glucocorticoids increase protein carbonylation and mitochondrial dysfunction. Hormone and Metabolic Research 45: 709-715.

Tian, J. and Li, Y. 2016. Comparative effects of vitamin C on the effects of local anesthetics ropivacaine, bupivacaine, and lidocaine on human chondrocytes. Brazilian Journal of Anesthesiology 66: 29-36.

Untergrasser A, C.I., Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3 - new capabilities and interfaces. Nucleic Acids Research 40: e115.

Uthman, I., Raynauld, J.P. and Haraoui, B. 2003. Intra-articular therapy in oteoarthritis. Postgraduate Medical Journal 79: 449-453.

Valko, M., et al. 2007. Free radicals and antioxidants in normal physiological functions and human disease. International Journal of Biochemistry and Cell Biology 39: 44-84.

Verma, R.P. and Hansch, C. 2007. Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. Bioorganic and Medical Chemistry 15: 2223-2268.

Weitoft, T., Larsson, A., Saxne, T. and Ronnblom, L. 2005. Changes of cartilage and bone markers after intra-articular glucocorticoid treatment with and without postinjection rest in patients with rheumatoid arthritis. Annals of Rheumatic Diseases 64: 1750-1753.

Wescoe, K.E., Schugar, R.C., Chu, C.R. and Deasy, B.M. 2008. The Role of the Biochemical and Biophysical Environment in Chondrogenic Stem Cell Differentiation Assays and Cartilage Tissue Engineering. Cell Biochemistry and Biophysics 52: 85-102.

Woessner, J.F., Jr. 2001. That impish TIMP: the tissue inhibitor of metalloproteinases-3. Journal of Clinical Investigation 108: 799-800.

Yang, J.-Q., et al. 2008. Cell Density and Serum Exposure Modify the Function of the Glucocorticoid Receptor C/EBP Complex. American Journal of Respiratory Cell and Molecular Biology 38: 414-422.

Ye, J., et al. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 13: 134.

You, J.M., et al. 2009. Mechanism of glucocorticoid-induced oxidative stress in rat hippocampal slice cultures. Cananadian Journal of Physiology and Pharmacology 87: 440-447.

Yudoh, K., et al. 2005. Potential involvement of oxidative stress in cartilage senescence and development of osteoarthritis: oxidative stress induces chondrocyte telomere instability and downregulation of chondrocyte function. Arthritis Research and Therapy 7: R380-391.

Zeng, G.Q., Chen, A.B., Li, W., Song, J.H. and Gao, C.Y. 2015. High MMP-1, MMP-2, and MMP-9 protein levels in osteoarthritis. Genetics and Molecular Research 14: 14811-14822.

Zhou, H., et al. 2007. Role of c-Fos/JunD in protecting stress-induced cell death. Cell Proliferation 40: 431-444.

APPENDIX

Approval of study protocol from The Human Research Ethics Committee of Thammasat University

Human Research Ethics Committee of Thammasat University No.1 (Faculty of Medicine)

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Number of COA	**135/2557**
Title of Project	Effects of Triamcinolone acetonide on cytotoxicity and oxidative stress of chondrocytes.
Project No	MTU-EC-OT-4-099/57
Principal Investigator	Assistant Professor Rachaneekorn Thammachote, (MD.)
	Assosiate Professor Nattapol Thammachote, (MD.)
	Monthira Santiparpluacha
Study Center	Faculty of Science
Responsible Department	Botany, Faculty of Science
	Chulalongkorn University, Bangkok , Thailand 10330
	Tel. 02-218-5484 , 089-694-3235

Document Reviewed

1. Protocol Revised No 1: dated July 25, 2014.
2. Information Sheet Revised No 1: dated July 25, 2014.
3. Consent Form Revised No 1: dated July 25, 2014.

This document is a record of review and approval / acceptance of a clinical study protocol. The Human Research Ethics Committee of Thammasat University No.1 (Faculty of Medicine) has approved the above study and the following documents for use in the study at the Ethics Committee meeting on July 15, 2014. (9/2014)

Approval period 3 years.

Progress report deadline: September 11, 2014

Signed: .....

(Associate Professor Thipaporn Tharavanij)

Secretary of the Human Research Ethics Committee of Thammasat University No.I (Faculty of Medicine)

Signed:.....

(Associate Professor Waipoj Chanvimalueng)

Chairman of the Human Research Ethics Committee of Thammasat University No.I (Faculty of Medicine)

Date of Approval: **September 12, 2014**

Date of Expire : **September 11, 2017**

คณะอนุกรรมการจริยธรรมการวิจัยในคน มหาวิทยาลัยธรรมศาสตร์ ชุดที่ 1 (คณะแพทยศาสตร์)

.....

หนังสือรับรองเลขที่ **135/2557**

โครงการวิจัยเรื่อง ผลของยาไตรแอมซิโนโลน อะซีโทไนด์ต่อการเป็นพิษต่อเซลล์กระดูกอ่อน และการเกิดความเครียดจากปฏิกิริยาออกซิเดชั่น

รหัสโครงการวิจัย MTU-EC-OT-4-099/57

ผู้วิจัย ผู้ช่วยศาสตราจารย์ ดร. รัชนีกร ธรรมโชติ
รองศาสตราจารย์ นายแพทย์ ณัฐพล ธรรมโชติ
นาวสาวมณฑิรา สันติภาพลือชา

หน่วยงานที่รับผิดชอบ สาขาพันธุศาสตร์ ภาควิชาพฤกษศาสตร์
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
โทร. 02-218-5484 , 089-694-3235

เอกสารที่รับรอง

1. โครงร่างการวิจัย ฉบับแก้ไขครั้งที่ 1 วันที่ 25 กรกฎาคม 2557
2. เอกสารชี้แจงผู้เข้าร่วมการวิจัย ฉบับแก้ไขครั้งที่ 1 วันที่ 25 กรกฎาคม 2557
3. หนังสือแสดงเจตนายินยอม ฉบับแก้ไขครั้งที่ 1 วันที่ 25 กรกฎาคม 2557

คณะอนุกรรมการจริยธรรมการวิจัยในคน มหาวิทยาลัยธรรมศาสตร์ ชุดที่ 1 (คณะแพทยศาสตร์) ได้พิจารณาอนุมัติด้านจริยธรรมการทำวิจัยในคนให้ดำเนินการวิจัยข้างต้นได้ ตามมติที่ประชุมครั้งที่ 9/2557 วันที่ 15 กรกฎาคม 2557

ระยะเวลาที่อนุมัติ 3 ปี

กำหนดส่งรายงานความก้าวหน้า 1 ปี : วันที่ 11 กันยายน 2558

ลงชื่อ.....
(รองศาสตราจารย์ นายแพทย์ไวพจน์ จันทร์วิเมลือง)
ประธานคณะอนุกรรมการฯ

ลงชื่อ.....
(รองศาสตราจารย์ แพทย์หญิง ทิพาพร ธาระวานิช)
อนุกรรมการและเลขานุการ

อนุมัติ ณ **วันที่ 12 กันยายน 2557**

หมดอายุ **วันที่ 11 กันยายน 2560**

Viability assay

Optical density; OD and viability percentages of chondrocytes from all patients treated with 2.3, 11,5, and 23 mM (1, 5, and 10 mg/ml) TA without vitamin C addition at incubation day 7

Day 7	0 mM	2.3 mM	11.5 mM	23 mM
	OD (% viability)	OD (% viability)	OD (% viability)	OD (% viability)
Patient 6	0.249 (100)	0.173 (69.52)	0.169 (67.65)	0.150 (60.29)
Patient 7	0.180 (100)	0.142 (79.07)	0.138 (76.85)	0.142 (78.70)
Patient 8	0.229 (100)	0.169 (73.94)	0.152 (66.52)	0.152 (66.52)
Patient 10	0.187 (100)	0.128 (68.51)	0.137 (73.31)	0.130 (69.57)
Patient 11	0.235 (100)	0.179 (76.20)	0.140 (59.49)	0.141 (59.77)
Patient 13	0.257 (100)	0.154 (59.97)	0.143 (55.70)	0.142 (55.57)
Patient 14	0.220 (100)	0.183 (83.46)	0.189 (85.89)	0.144 (65.71)
Patient 15	0.217 (100)	0.166 (76.23)	0.141 (65.03)	0.125 (57.67)
Patient 16	0.193 (100)	0.165 (85.17)	0.136 (70.52)	0.144 (74.31)
Patient 17	0.204 (100)	0.168 (82.19)	0.163 (80.07)	0.148 (72.55)

Optical density; OD and viability percentages of chondrocytes from all patients treated with 2.3, 11,5, and 23 mM (1, 5, and 10 mg/ml) TA without vitamin C addition at incubation day 14

Day 14	0 mM	2.3 mM	11.5 mM	23 mM
	OD (% viability)	OD (% viability)	OD (% viability)	OD (% viability)
Patient 6	0.178 (100)	0.119 (67.04)	0.114 (63.86)	0.107 (60.11)
Patient 7	0.225 (100)	0.129 (57.40)	0.121 (53.85)	0.127 (56.36)
Patient 8	0.172 (100)	0.149 (86.82)	0.146 (84.88)	0.140 (81.20)
Patient 10	0.177 (100)	0.112 (62.97)	0.110 (62.03)	0.110 (62.03)
Patient 11	0.194 (100)	0.155 (79.76)	0.126 (64.67)	0.126 (64.67)
Patient 13	0.233 (100)	0.177 (76.22)	0.131 (56.16)	0.137 (58.74)
Patient 14	0.224 (100)	0.174 (77.65)	0.161 (71.98)	0.135 (60.51)
Patient 15	0.209 (100)	0.171 (81.82)	0.136 (64.91)	0.139 (66.67)
Patient 16	0.205 (100)	0.187 (91.06)	0.148 (72.03)	0.146 (71.38)
Patient 17	0.212 (100)	0.216 (102.3)	0.193 (91.10)	0.167 (78.80)

Optical density; OD and viability percentages of chondrocytes from all patients treated with 2.3, 11,5, and 23 mM (1, 5, and 10 mg/ml) TA with vitamin C addition at incubation day 7

Day 7	0 mM	2.3 mM	11.5 mM	23 mM
With vitamin C	OD (% viability)	OD (% viability)	OD (% viability)	OD (% viability)
Patient 6	0.219 (100)	0.173 (79.00)	0.174 (79.5)	0.131 (59.82)
Patient 7	0.236 (100)	0.139 (58.82)	0.155 (65.44)	0.121 (51.20)
Patient 8	0.239 (100)	0.183 (76.54)	0.176 (73.60)	0.169 (70.67)
Patient 10	0.248 (100)	0.167 (67.52)	0.161 (65.10)	0.143 (57.70)
Patient 11	0.227 (100)	0.166 (73.44)	0.167 (73.88)	0.138 (60.78)
Patient 13	0.231 (100)	0.162 (77.38)	0.162 (77.38)	0.148 (72.68)
Patient 14	0.318 (100)	0.246 (77.46)	0.254 (79.87)	0.218 (68.56)
Patient 15	0.256 (100)	0.186 (72.93)	0.191 (74.89)	0.134 (52.32)
Patient 16	0.248 (100)	0.219 (88.31)	0.206 (83.20)	0.130 (52.55)
Patient 17	0.204 (100)	0.186 (91.34)	0.169 (83.01)	0.137 (67.16)

Optical density; OD and viability percentages (in brackets) of chondrocytes from all patients treated with 2.3, 11,5, and 23 mM (1, 5, and 10 mg/ml) TA with vitamin C addition at incubation day 14

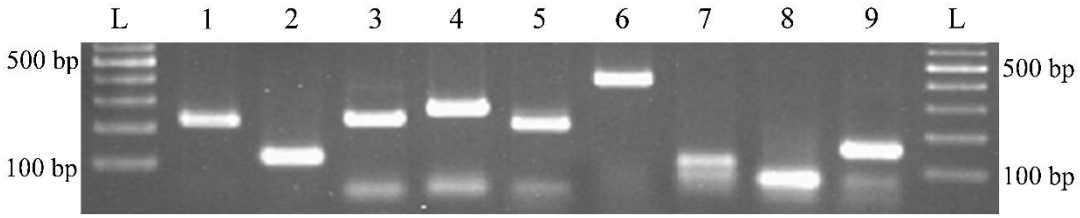
Day 14	0 mM	2.3 mM	11.5 mM	23 mM
With vitamin C	OD (% viability)	OD (% viability)	OD (% viability)	OD (% viability)
Patient 6	0.210 (100)	0.140 (66.40)	0.140 (66.40)	0.137 (65.13)
Patient 7	0.203 (100)	0.130 (63.93)	0.145 (71.48)	0.118 (58.20)
Patient 8	0.256 (100)	0.219 (85.66)	0.201 (78.49)	0.181 (70.93)
Patient 10	0.244 (100)	0.163 (67.08)	0.156 (64.07)	0.140 (57.49)
Patient 11	0.297 (100)	0.264 (88.93)	0.251 (84.54)	0.146 (49.24)
Patient 13	0.185 (100)	0.153 (82.70)	0.133 (71.89)	0.118 (63.78)
Patient 14	0.306 (100)	0.251 (82.22)	0.230 (75.25)	0.223 (73.06)
Patient 15	0.236 (100)	0.173 (73.32)	0.158 (66.95)	0.145 (61.43)
Patient 16	0.241 (100)	0.200 (82.99)	0.182 (75.38)	0.144 (59.75)
Patient 17	0.212 (100)	0.233 (110.01)	0.208 (98.50)	0.175 (82.74)

Total glutathione assay

Values of oxidized glutathione (GSSG) to total glutathione (GSH) in chondrocytes from all patients treated with 2.3 and 11.5 mM (1 and 5 mg/ml) TA with and without vitamin C addition for 48 h

	GSSG:total GSH					
	0 mM		2.3 mM		11.5 mM	
	No Vitamin C	100 µM Vitamin C	No Vitamin C	100 µM Vitamin C	No Vitamin C	100 µM Vitamin C
Patient 6	0.043	0.092	0.261	0.039	0.455	0.085
Patient 7	0.014	0.015	0.206	0.010	0.500	0.068
Patient 8	0.069	0.069	0.027	0.206	0.125	0.011
Patient 10	0.220	0.027	0.810	0.065	1.238	0.068
Patient 11	0.013	0.007	0.064	0.495	0.375	0.364
Patient 13	0.230	0.200	0.733	0.015	1.159	0.089
Patient 14	0.078	0.205	1.115	0.040	0.867	0.081
Patient 15	0.110	0.061	0.531	0.030	0.122	0.139
Patient 16	0.017	0.140	0.089	0.155	0.182	0.140
Patient 17	0.072	0.069	0.108	0.232	0.144	0.186

Gene expression analysis



Gel electrophoresis of real-time PCR products

Lane L = 100 bp ladder

Lane 1 = *GAPDH* expected amplicon size 237 bp

Lane 2 = *P21* expected amplicon size 123 bp

Lane 3 = *GDF15* expected amplicon size 242 bp

Lane 4 = *cFos* expected amplicon size 277 bp

Lane 5 = *MMP-1* expected amplicon size 217 bp

Lane 6 = *MMP-3* expected amplicon size 404 bp

Lane 7 = *MMP-13* expected amplicon size 116 bp

Lane 8 = *ADAMTS-5* expected amplicon size 73 bp

Lane 9 = *TIMP-3* expected amplicon size 151 bp

Fold change in expressions with standard deviation of *P21*, *GDF15*, and *cFos* in chondrocytes from all patients treated with 2.3 and 11.5 mM (1 and 5 mg/ml) TA for 48 h

	Fold change in expression ± standard deviation of Cq triplicates								
	P21			*GDF15*			*cFos*		
	0 mM	2.3 mM	11.5 mM	0 mM	2.3 mM	11.5 mM	0 mM	2.3 mM	11.5 mM
Pat.6	1±0.26	5.1337±0.3	5.8159±0.34	1±0.013	6.0629±0.41	6.3643±0.06	1±±0.76	8.9383±0.09	2.8679±0.19
Pat.7	1±0.02	6.4531±0.01	5.063±0.12	1±0.24	9.063±0.17	8.6338±0.39	1±0.21	9.3827±0.24	8.0556±0.09
Pat.8	1±0.20	3.5064±0.19	4.0278±0.19	1±0.15	9.9866±0.03	20.393±0.34	1±0.16	3.7842±0.08	2.5668±0.08
Pat. 10	1±0.01	0.1731±0.11	1.5052±0.02	1±0.1	9.7136±0.14	15.671±0.1	1±0.07	2.4453±0.05	7.8354±0.14
Pat. 11	1±0.06	9.2535±0.32	6.498±0.04	1±0.14	12.641±0.91	14.222±0.34	1±0.34	4.3469±0.04	17.388±0.01
Pat. 13	1±0.13	6.4531±0.13	3.6301±0.14	1±0.09	5.9794±0.25	3.7063±0.38	1±0.12	1.1329±0.06	3.6301±0.04
Pat. 14	1±0.19	5.2054±0.18	3.3403±0.13	1±0.20	7.8899±0.11	6.5887±0.21	1±0.12	0.6329±0.12	2.7321±0.09
Pat. 15	1±0.16	3.8105±0.15	3.0314±0.07	1±0.10	10.411±0.27	11.876±0.42	1±0.10	6.7272±0.14	5.063±0.12
Pat. 16	1±0.02	4.6589±0.10	3.2266±0.03	1±0.38	13.269±0.12	10.267±0.23	1±0.03	7.3615±0.01	11.236±0.15
Pat. 17	1±0.11	7.0128±0.11	5.8159±0.1	1±0.1	14.723±0.34	32±0.12	1±0.06	4.8568±0.09	5.0982±0.11

Fold change in expressions with standard deviation of *MMP-1*, *MMP-3*, and *MMP-13* in chondrocytes from all patients treated with 2.3 and 11.5 mM (1 and 5 mg/ml) TA for 48 h

	Fold change in expression ± standard deviation of Cq triplicates								
	MMP-1			*MMP-3*			*MMP-13*		
	0 mM	2.3 mM	11.5 mM	0 mM	2.3 mM	11.5 mM	0 mM	2.3 mM	11.5 mM
Pat.6	1±0.11	1.613±0.38	0.448±0.07	1±0.056	4.896±0.38	2.631±0.15	1±0.13	0.879±0.25	1.876±0.24
Pat.7	1±0.02	51.804±0.20	14.571±0.30	1±0.06	5.167±0.18	3.681±0.18	1±0.29	9.335±0.26	8.674±0.11
Pat.8	1±0.10	0.525±0.05	0.224±0.14	1±0.14	1.999±0.29	1.596±0.21	1±0.26	1.529±0.07	0.992±1.1
Pat. 10	1±0.16	0.067±0.09	0.233±0.19	1±0.07	0.988±0.82	0.943±5.6	1±0.07	0.488±0.48	1.244±0.75
Pat. 11	1±0.04	0.363±0.06	0.399±0.04	1±0.15	4.095±0.46	5.913±0.23	1±0.07	1.344±0.11	1.557±0.20
Pat. 13	1±0.26	0.979±0.31	2.204±0.32	1±0.03	18.725±0.30	8.8±0.17	1±0.24	3.391±0.10	0.93±0.21
Pat. 14	1±0.24	3.482±0.16	7.260±0.11	1±0.71	7.017±0.37	9.556±0.06	1±0.04	0.703±0.06	0.435±0.48
Pat. 15	1±0.31	0.023±0.28	0.173±0.08	1±0.04	9.696±0.20	5.333±0.11	1±0.11	0.964±0.15	1.001±0.38
Pat. 16	1±0.36	0.203±0.40	2.428±0.50	1±1.2	3.307±0.04	11.15±0.13	1±0.10	0.108±0.34	0.106±0.17
Pat. 17	1±0.02	0.212±0.02	1.292±0.05	1±0.18	10.034±0.27	4.734±0.35	1±0.10	1.582±0.17	2.252±0.25

Fold change in expressions with standard deviation of *ADAMTS-5* and *TIMP-3* in chondrocytes from all patients treated with 2.3 and 11.5 mM (1 and 5 mg/ml) TA for 48 h

	Fold change in expression ± standard deviation of Cq triplicates					
	ADAMTS-5			*TIMP-3*		
	0 mM	2.3 mM	11.5 mM	0 mM	2.3 mM	11.5 mM
Pat.6	1±0.04	1.347±0.12	4.377±0.24	1±0.13	0.076±0.12	0.083±0.16
Pat.7	1±0.27	0.940±0.21	0.859±0.44	1±0.04	1.811±0.41	0.603±0.30
Pat.8	1±0.02	1.306±0.01	1.735±0.01	1±0.41	1.34±0.53	1.849±0.16
Pat.10	1±0.06	1.580±0.51	0.859±0.43	1±0.15	1.798±0.21	2.002±0.22
Pat.11	1±0.08	6.612±0.20	5.296±0.22	1±0.30	1.828±0.24	1.274±0.07
Pat.13	1±0.25	1.266±0.31	1.079±0.23	1±0.54	0.601±0.37	0.303±0.55
Pat.14	1±0.03	0.940±0.14	1.197±0.14	1±0.87	0.283±0.79	0.126±0.83
Pat.15	1±0.11	1.753±0.05	3.387±0.08	1±0.12	3.451±0.02	2.938±0.07
Pat.16	1±0.12	3.160±0.17	2.969±0.50	1±0.23	0.565±0.26	0.539±0.15
Pat.17	1±0.10	1.102±0.01	1.173±0.13	1±0.13	1.934±0.05	3.542±0.24

VITA

Miss Monthira Suntiparpluacha was born on the 13th of September 1984 in Songkla province, Thailand. She moved to Bangkok at the age of six and has now been living there since. She graduated from Department of Food Technology, Science Faculty for her Bachelor's degree from Chulalongkorn University in 2007. She received her Master's Degree with Distinction in Biotechnology from The Royal Melbourne Institute of Technology, Melbourne, Australia in 2009. After the graduation, she worked as an assistant researcher for Assoc. Prof. Tanawan Kummalue and Prof. Pornchai O-chareonrat at Siriraj Hospital. In 2012, she pursued her Ph.D. study in Program in Biotechnology, Science Faculty, Chulalongkorn University.

Some of her publications

1.Kummalue T, Suntiparpluacha M, Jiratchariyakul W. Antiproliferative activity of combination of Thai herbal remedy and chemotherapeutic agents on human cancer cell lines. Journal of Medicinal Plants Research. 2012 Jan; 6:200-205.

2.Kummalue T, Suntiparpluacha M, Tongkao K, Chuangsuwanich T, Pongpruttipan T, O-charoenrat P. CK19 detection by multiplex real-time RT-PCR correlates with presence of angiolymphatic and perineural invasion in sentinel lymph nodes of Thai breast cancer patients. Journal of Clinical and Experimental Pathology. 2012; doi:10.4172/2161-0681.1000122.