THE EFFECTS OF CHANNA STRIATUS COMPARED WITH GLUCOSAMINE SULPHATE IN SERUM CARTILAGE AND INFLAMMATORY MARKERS IN KNEE OSTEOARTHRITIS PATIENTS

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by

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LIST OF ABBREVIATIONS AND SYMBOLS

σ	Standard deviation
δ	Detectable difference
4-PL	4-parameter logistic
AA	Arachidonic acid
ACR	American College of Rheumatology
ADL	Activity in Daily Living
ALT	Alanine Transaminase
A-P	Anterior-Posterior
AST	Aspartate Transaminase
BIPED	Burden of disease, Investigative, Prognostic, Efficacy of intervention and Diagnostic
COL2A1	Type II collagen
COMP	Cartilage oligomeric protein
COX-2	Cyclooxygenase-2
cPGES	Cytosolic PGES
CS	Channa striatus
CTX-1	C-terminal cross-linking telopeptide of type I collagen
DHA	Docosahexanoic acid
ELISA	Enzyme-linked immunosorbent
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
GlcN	Glucosamine
GlcN-6-P	Glucosamine-6-phosphate
GlcNAc	N-acetyl-glucosamine
GS	Glucosamine sulphate
HA	Hyaluronic acid
HDL	Higher high density lipoprotein

HED	Human equivalent dose
HRP	Horseradish peroxidase
hsCRP	high sensitive C-reactive protein
IL-16	Interleukin-16
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
IQR	Interquartile range
K-L	Kellgren & Lawrence
KOA	Knee osteoarthritis
KROOS	Knee Injury and OA Outcome Score
LDL	Low density lipoprotein
LFT	Liver function test
LPS	Lipopolysaccharide
LSCS	Post Lower Segment Caesarean Section
LTB4	Leukotrienes B4
LTB5	Leukotrienes B5
MIF	Macrophage inhibitory factor
mg	Miligram
ml	mililitre
ng	nanogram
MMPs	Matrix metalloproteinases
mPGES-1	Microsomal PGES-1
mPGES-2	Microsomal PGES-2
MUFAs	Monounsaturated fatty acids
NAGly	Arachidonoylglycine
NICE	National Institute for Health and Care Excellence
NO	Nitric oxide
NOS2	NO synthase 2
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis

PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E synthase
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PGs	Prostaglandins
PLA_2	Phospholipase A_2
PRP	Platelet rich plasma
PUFAs	Polyunsaturated fatty acids
QOL	Quality of Life
RA	Rheumatoid arthritis
RFT	Renal function test
ROS	Reactive Oxygen Species
SFAs	Saturated fatty acids
SLE	Systemic Lupus Erythematosus
$SPLA_2$-IIA	Type IIA secretory phospholipase A_2
TGF-β	Transforming growth factor beta
TMB	Tetramethylbenzidine
TNF-α	Tissue necrosis factor-α
TWCC	Total white cell counts
TXA_2	Thromboxane A_2
uCTXII	urine collagen type II
UDP-GlcNAc	Uridine diphosphate N-acetyl-glucosamine
VAS	Visual analog scale
WOMAC	Western Ontario and McMaster University Osteoarthritis Index

KESAN EKSTRAK CHANNA STRIATUS BERBANDING DENGAN GLUKOSAMINA SULFAT KE ATAS PENANDA RAWAN DAN PENANDA-PENANDA KERADANGAN DI KALANGAN PESAKIT OSTEOARTRITIS LUTUT (KOA)

ABSTRAK

Osteoarthritis lutut (KOA) adalah sejenis osteoarthritis yang biasa didapati. Rawatan yang sedia ada hanya melegakan kesakitan kepada pesakit osteoarthritis. Kesan sampingan pada rawatan sekarang menggalakkan lebih banyak kajian dijalankan untuk menghasilkan produk semulajadi yang mempunyai kurang kesan sampingan. *Channa striatus* (CS) adalah ikan air tawar yang merupakan remedi semulajadi dikalangan masyarakat setempat yang dapat merawat pelbagai penyakit tanpa bukti sainstifik. Objektif kajian ini adalah untuk menilai dan membandingkan kesan pengambilan ekstrak CS dalam dua dos (1000 mg/hari dan 500 mg/hari) berlainan secara oral ke atas penanda-penanda rawan (COMP) dan keradangan (COMP, COX-2 dan PGE_2) di kalangan pesakit KOA. Seramai seratus empat puluh lapan pesakit mengambil bahagian di dalam kajian rawak, ujian buta dwipihak dan percubaan kawalan plasebo dengan membandingkan kesan dua dos ekstrak CS yang berlainan dengan glukosamina sulfate (GS) berbanding dengan kumpulan plasebo. Sampel darah diambil dalam tempoh masa berlainan (*baseline* dan 6 bulan) dan tahap COMP, COX-2 dan PGE_2 dinilai dengan menggunakan kit yang terdapat di pasaran dan keputusan $p<0.05$ dianggap signifikan secara statistik. Pengurangan tahap COX-2 secara signifikan boleh dilihat pada kedua-dua kumpulan ekstrak pada dos yang berbeza serta kumpulan GS dengan berbanding pada kumpulan plasebo. Pengurangan tahap COMP pada kedua-dua kumpulan dos ekstrak CS memberi

gambaran bahawa ekstrak CS memiliki kesan perlindungan rawan kepada pesakit KOA. Walaubagaimanapun, tiada pengurangan tahap PGE_2 yang signifikan dapat dilihat pada kajian ini. Perbandingan di antara kumpulan CS 1000 mg/hari dan CS 500 mg/hari menunjukkan pengurangan tahap COX-2 yang significant. Kesimpulannya, penggunaan ekstrak CS secara oral mempunyai potensi dalam perawatan pesakit KOA. Namun begitu, kajian lebih lanjut diperlukan untuk memahami tindakan mekanisma CS.

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ABSTRACT

Knee osteoarthritis (KOA) is one of the commonest types of osteoarthritis (OA). Currently, the treatment is to provide the pain relief to OA patients. The concern for the side effects of current treatment encouraged the studies to search for natural products to reduce the side effects. *Channa striatus* (CS) is a freshwater fish that proclaimed as natural remedies to various illnesses by local communities without scientific evidence. Therefore, the objective of this study was to evaluate the effect of oral administration of different doses of CS extract (1000 mg/day and 500 mg/day) on the level of cartilage marker, Cartilage Oligomeric Protein (COMP) and inflammatory markers, Cyclooxgenase-2 (COX-2) and Prostaglandin E_2 (PGE_2) and compared within Glucosamine Sulphate (GS) treatment in KOA patients. One hundred and forty-eight patients were enrolled in this randomized, double-blind, placebo-controlled trial comparing the effect of two doses of oral CS extract (1000 mg/day and 500 mg/day) and glucosamine sulphate (GS) with the placebo group. Blood samples were collected at different-time period (baseline and six months) to assess the level of COMP, COX-2 and PGE_2 using commercially available kits and the result with $p<0.05$ considered as statistically significant. There was a significant reduction of COX-2 level in both doses of the CS extract groups and GS group compared to the placebo group patients. Reduction of COMP level in both doses of CS extract groups might suggest the chondroprotective effect of CS extract on KOA patients. However, there

was no significant difference found in the PGE_2 level. Comparison between CS 1000mg/day and CS 500 mg/day groups with GS group showed a significant reduction in COX-2 level. In conclusion, orally administrated CS extract can serve as potential candidate in the treatment of KOA patients. However, further studies required to understand the mechanism of CS action.

CHAPTER 1

INTRODUCTION

Knee osteoarthritis (KOA) is the leading causes of disability in elderly population globally (Neogi, 2013, French *et al.*, 2016). The leading cause of disability and pain in elderly population is characterized by degradation of articular cartilage. The fundamental reason causing KOA is that the normal mechanism of cartilage production is disrupted and there is no equilibrium between catabolism and anabolism of cartilage production. Erosion of articular cartilage causes pain and disability, hence reduces the quality of life. The pathophysiology of KOA is about the interaction of mechanical, cellular and biochemical processes. Cartilage is composed of water, collagen and proteoglycans. In healthy cartilage, remodeling occurs when there is degradation of cartilage and it is replaced by chondrocytes. Chondrocytes will form a new articular cartilage. However, in KOA, this process is disrupted. The disruption of the remodeling process leads to cartilage damage, joint spaces narrowing and with time subchondral cysts and osteophytes formation.

Biochemically, inflammation had been associated with KOA (Mathiessen and Conaghan, 2017; Haywood *et al*., 2003). It is an element of natural response from host towards injury and infection. In early stage of KOA, synovitis occurs (Benito *et al*., 2005, Atukorala *et al*., 2016). Synovitis is inflammation of synovial lining of the joint. Signs such as swelling of the joint associated with joint pain and stiffness in KOA patients indicate local inflammation (Pelletier *et al*., 2001, Scanzello and Loeser, 2015) and it has been widely acceptable as part of KOA (Myers *et al*., 1990, Ene *et al*., 2015). Synovitis is

representing the production of various inflammatory cytokines (tissue necrosis factor-α [TNF-α], Interleukin-1β [IL-1β], Interleukin-6 [IL-6] and Interleukin-8 [IL-8]), inflammatory biomarkers (prostaglandins [PGs] and cyclooxygenase-2 [COX-2]) (Sokolove and Lepus, 2013, Wojdasiewicz *et al.*, 2014). Over the years, researchers have made tremendous effort in trying to recognize inflammatory biomarkers related to KOA which can be detected even before radiographic changes taking place. The commonly studied inflammatory markers were COX-2 and Prostaglandin E_2 (PGE_2) (Fan *et al*., 2015, Guler *et al*., 2011, Pecchi *et al*., 2012, Zuo *et al*., 2011) and lately some studies were using serum biomarker such as cartilage oligomeric protein (COMP), matrix metalloproteinase-3, type II collagen degradation and interleukin-16 (IL-16) to assess the degree of cartilage destruction in joints (Georgiev *et al*., 2018, Das Gupta *et al*., 2017, Arellano *et al*., 2017).

COMP is a tissue-specific protein that binds to type II collagen fibers network of articular cartilage and it belongs to thrombospondin family. It is synthesized by synovial cells and chondrocytes with the activation of proinflammatory cytokines. Majority of COMP found in the joint is derived from articular cartilage. COMP molecules are important for maintaining the properties and integrity of the collagen network. It contributes to the material properties of biological tissues (Chen *et al*., 2005, Rosenberg *et al*., 1998, Luo *et al*., 2017). It also binds to aggrecan which is the major proteoglycan in articular cartilage. Aggrecan provides a hydrated gel structure that presents the cartilage with load bearing ability by hyaluronan and link protein interaction (Kiani *et al*., 2002, Roughley and Mort, 2014). The breakdown of the cartilage, releases COMP first into synovial fluid followed by into the bloodstream (Wislowska and Jablonska, 2005). The higher level of serum COMP detected indicates the severity of cartilage destruction (Larsson *et al*., 2002).

Another biomarker of interest in KOA is COX-2. COX-2 is multifunctional enzyme that catalyzes conversion of arachidonic acid (AA) to Prostaglandin H_2 (PGH_2) in inflammatory pathway. It is metabolized by Prostaglandin E synthase (PGES) into the end product which is PGE_2. (Martel-Pelletier *et al*., 2003, Wojdasiewicz *et al.*, 2014). The highest levels of COX-2 are found mainly in the brain, vas deferens and renal cortex (Guilak *et al*., 2004). COX-2 expression is increased during inflammation and other pathologic situations. Its expression is highly elevated by tumour promoters, pro-inflammatory agents, growth factors, mitogens and oncogenesis. In normal physiological conditions, COX-2 often found low in blood. However, it plays an important role in articular cartilage disease and it facilitates the inflammatory cytokine-induced metabolic imbalance of cartilage proteoglycans (Maldonado and Nam, 2013).

PGE_2 is the end product of arachidonic acid (AA) which has been studied and associated with KOA. PGE_2 is the most abundant eicosanoid and a potent lipid mediator. There are four principle bioactive PGs generated *in vivo*: PGE_2, Prostacyclin (PGI_2), Prostaglandin D_2 (PGD_2) and Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). PGE_2 is regulated by COX-2 and PGES. It is involved in many physiological conditions such as regulation of immune responses, blood pressure, gastrointestinal integrity and fertility. Secreted PGE_2 acts in an autocrine or paracrine manner through its four G protein coupled receptors which are EP1, EP2, EP3 and EP4. PGE_2 is known as the major prostaglandin synthesized by cartilage in KOA. The amounts of PGE_2 in KOA potential to increase to 50-fold higher than in normal cartilage (Amin *et al*., 1997, Cho *et al*., 2015).

Up to date, there is still no curative treatment for KOA. Currently KOA is being treated symptomatically with drugs such as nonsteroidal anti-inflammatory drugs

(NSAIDs) and glucosamine (GlcN). Glucosamine provide modest pain relief in patients with KOA. Glucosamine (2-amino-2-deoxy-D-glucose) is a monosaccharide and produced naturally in the body and it is an essential part of proteoglycans, glycosaminoglycans and collagen (Jerosch, 2011). In United States, GlcN is used by five million people annually and consumed as a dietary supplement to promote joint health (Stumpf and Lin, 2006, Kennedy, 2005). Generally, GlcN is absorbed rapidly and in constant state. Under *in vivo* condition, GlcN can be synthesized from glucose and is used to produce glycosaminoglycan chains from which proteoglycans are formed (Huskisson, 2008). In KOA, GlcN was reported to have the ability as chondroprotective and anti-inflammatory agent thus have the possibility to reduce the symptoms in KOA (Lomonte *et al*., 2018; Naraoka *et al*.,2017; Li *et al*., 2018; Veronesi *et al*., 2017; Richy *et al*., 2003).

Alternative therapies used for the treatment of KOA include herbal supplements, acupuncture and electromagnets (Rodriguez-Merchan, 2016; Garland *et al*., 2007). Nowadays, there is an interest towards natural remedies for KOA. *Channa striatus* (CS) also known as Haruan in Malaysia has been considered as a natural remedy for KOA. It has good nutritive value and easily available. It is enriched with many amino acids such as glycine, lysine, aspartic acid, proline, glutamic acid and fatty acids such as palmitic acid, docosahexanoic acid (DHA) and eicosapentaenoic acid (EPA) (Dahlan *et. al*., 2010; Mat Jais *et. al*., 1998; Zakaria *et. al*., 2007; Zuraini *et. al*., 2006). The amino acids and fatty acids contained in CS is known to promote in wound healing in caesarean delivery and improve the wound healing texture (Baie and Sheikh, 2000b, Baie and Sheikh, 2000a, Ab Wahab *et al*., 2015). The fatty acids such as DHA and EPA are capable in reducing the levels of pro-inflammatory mediators (Al-Saffar *et al*., 2011a; Al-Saffar *et al*., 2011b;

Michelle *et al*., 2004). Many studies reported the therapeutic potentials of CS as anti-microbial, anti-depressant, neuro regenerative and anti-inflammatory agent. (Abedi *et. al*., 2012; Mat Jais *et. al*., 1997; Saleem *et. al*., 2011; Wei *et. al*., 2010). It is also popular in traditional medicine to promote wound healing. Given the possible anti-inflammatory property of CS in treating diseases with an inflammatory component, the amelioration of KOA and its mechanism on how it works as an anti-inflammatory agent is still not clear.

This study is done to compare the serum levels of cartilage biomarker (COMP) and inflammatory biomarkers (COX-2 and PGE_2) on KOA patients consuming oral CS and glucosamine sulphate (GS). This study also aims to compare the serum levels of cartilage and inflammatory markers (COMP, COX-2 and PGE_2) at different doses of CS extract (1000 mg/day or 500 mg/day) given to KOA patients. With the understanding of CS acting on KOA could generate further ideas on the use of natural remedies as an alternative in the treatment of KOA.

1.1 Justification of the study

KOA is a major issue globally. Current treatment for KOA is orally administered GlcN and NSAIDs. But many side effects found on treatment with NSAIDs and thus the current focus is by treatment with natural products on KOA (Hammad *et al.*, 2015; Campbell *et al*., 2015; Jo *et al*., 2017). In this study, CS extract chosen as it possesses many anti-inflammatory and many beneficial properties. It is also been shown to have a protective effect on wound healing on post Lower Segment Caesarean Section (LSCS) (Ab Wahab *et al*., 2015). But there is no clinical trial been done on the effect of CS on

KOA patients. GS is used as a positive control in this study since it is a gold standard for the treatment of KOA. Therefore, to our knowledge this is the first study to assess and compare the effectiveness of GlcN and CS extracts on cartilage and inflammatory biomarkers on KOA.

1.2 General objective:

To assess and compare the effect of oral administration of different doses of CS extract versus GS on the level of cartilage and inflammatory biomarkers in KOA patients.

1.3 The Specific Objectives:

a. To assess and compare the levels of inflammatory biomarkers (COX-2 and PGE_2) among CS extract (1000 mg/day and 500 mg/day), GS and placebo administered groups of KOA patients.
b. To assess and compare the level of cartilage biomarker, COMP among CS extract (1000 mg/day and 500 mg/day) and GS and placebo administered groups of KOA patients.
c. To compare the levels of inflammatory biomarkers (COX-2 and PGE_2) and COMP within the CS extract (1000 mg/day and 500 mg/day) administered groups of KOA patients.

1.4 Benefit of the study

Since the side effect and cost of GlcN are increasing, this study will provide scientific evidence on the comparison of cartilage and inflammatory biomarkers level in KOA patients consuming CS extract or GS where CS can have less side effect and cost-effective. Administration of NSAIDs as the first line of KOA treatment is mainly to reduce the inflammation in KOA joint and the properties in CS extract can aid in reducing the inflammation thus slow down the KOA progression.

1.5 Research hypothesis

CS extract provides a significant improvement on articular cartilage of KOA patients with the decreased level of serum COMP, COX-2 and PGE_2 when compared with placebo and GS.

CHAPTER 2

LITERATURE REVIEW

2.1. Epidemiology of Osteoarthritis (OA)

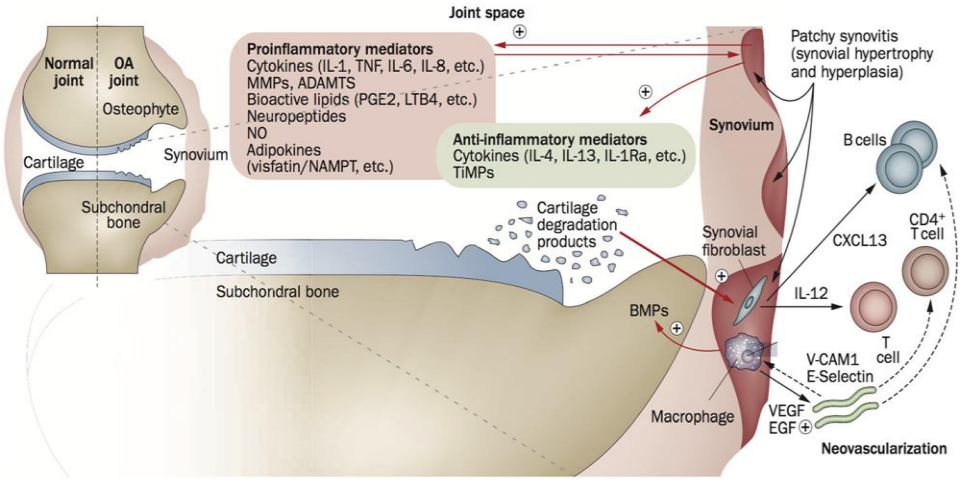
Osteoarthritis (OA) is one of the important causes of chronic disability in old age and is the commonest form of arthritis and joint disorder. OA usually affects the movements on the spine, knee, hip hands and any other smaller joints. Knee Osteoarthritis (KOA) responsible for 83% of OA anxiety. Projection about 10-15% adult age more than 60 years old is affected by OA worldwide. Estimation of more than 20% population will be affected by OA and more than 40 million people will be critically disabled by the year 2050 (Who.int, 2019). In the USA, two-thirds of adults aged between 45 and 65 years old represented two millions cases of symptomatic KOA (Deshpande *et al.*, 2016). Studies indicated that females were more susceptible to KOA (4.5% male and 19% female) in Korea (Park *et al*., 2017) and (5% men and 11.3% female) in Japan (Muraki *et al*., 2010). There is roughly 3.6% of world population (250 million people) is affected by KOA (Vos *et al*., 2012). Prevalence of KOA is accountable to 6% of the global population with estimation of 67 million population in the USA and 100 million people in China by 2020 (Zhang and Jordan, 2008). A retrospective study conducted by Wallace *et al*. dated back to prehistoric until postindustrial era indicated that the prevalence of KOA has doubled since the mid-20th century (Wallace *et al*., 2017).

2.2 Characteristics of Knee Osteoarthritis (KOA)

The prominent characteristics of KOA are the degeneration of articular cartilage and formation of reactive new bones. Stiffness, pain and inflammation are the characteristics of the affected joints. This indirectly restricts joint movements and reduces quality of life to the patients. Further destruction of articular cartilage can cause damage to subchondral bone and leads to subchondral bone hardness which diminish stress absorption ability and integrity of cartilage (Falah *et al*., 2010).

2.3 Pathophysiology of KOA

The pathophysiology of the disease is complex and multifactorial. Generally, KOA joints are characterized by cartilage degeneration and subchondral bone stiffening. Degeneration of cartilage in KOA joints happened when remodeling of cartilage is slower than cartilage degeneration. In some worst condition, erosion of cartilage takes place. Stiffness of subchondral bone reduces the absorption ability of the bone and causing extra stresses on the cartilage (Li and Aspden, 1997).



(Adapted from Jérémie Sellam and Francis Berenbaum, 2010)

Figure 2.1: Pathophysiology of KOA

Everyday activities induced the cyclic loading to produce mechanical stress and deformation of joints. This process makes the chondrocytes at articular cartilage becomes vulnerable to tensile force, fluid flows, osmotic pressure and compressive force (Guilak *et al*., 1999, Bleuel *et al*., 2015). Pathophysiology of KOA started with microfracture, trauma or inflammation by increasing the enzymatic activity and forming "wear" particles (Figure 2.1). The "wear" particles are engulfed by macrophages particularly from synovial cells sited at synovial membrane. The failure to eliminate the overproduction of "wear" particles causes development of inflammatory mediators and decrease in the level of growth factor like transforming growth factor beta (TGF-β)(Park *et al*., 2013, Sokolove and Lepus, 2013). This further trigger the chondrocytes to release various types of degradative enzymes such as collagenases, aggrecanases, stromelysins and gelatinases. The degradative enzymes proceed further to disintegrate collagen and proteoglycan structures in articular cartilage. The fragments are actively taken up by macrophages again and in turn causes the release of pro-inflammatory cytokines such as TNF-α, IL-1 and

IL-6. Cytokine such as IL-1 is known to activate the AA pathway and induces the phospholipase A_2 (PLA_2) which subsequently elevates the cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) level (Gilman *et al*., 1987, Angel *et al*., 1993). The cytokines promptly bind to chondrocytes receptors causing further inhibition of type II collagen production and release of matrix metalloproteinases (MMPs) (Stannus *et al*., 2010). These serials which further worsen the condition of events provoked the disturbance of homeostasis leading to chondrocytes apoptosis and development of KOA.

Basically, there are two types of KOA, primary KOA and secondary KOA. Primary KOA is common in elderly population as the wear and tear of the articular cartilage caused by aging. Secondary KOA is a type of KOA with specific cause, such as injuries, obesity, inactivity, genetics or other diseases. Kellgren & Lawrence (K-L) staging system based on radiological examination is widely used by researchers worldwide. There are divided into four types of grading, Grade I, Grade II, Grade III and Grade IV (Appendix I). Increment in each grades indicating the severity of KOA progression respectively (Kellgren and Lawrence, 1957, Felson *et al*., 2011)

2.4 Risk factors of KOA

Risk factors of KOA can vary from metabolic disorders to physical activities. Zhang and Jordan divided the risk factors of KOA into two types: Systemic risk factors and local risk factors. Systemic risk factors include age, gender and hormones, races or ethnicity, genetics, congenital or development conditions and diet. On the other hand, obesity, injury or surgery, occupations, physical activity or sports, mechanical factors and

alignment are considered as local risk factors (Zhang and Jordan, 2008). Verbeek and colleagues reviewed the studies related to occupational activities leading to KOA. They concluded that longer time exposure of squatting and kneeling at work can expose workers to KOA (Verbeek *et al*., 2017). It has also been identified the role of occupational activity and sporting with the development of KOA (Shelton *et. al.*, 2016).

Until today there are no cure for KOA. The main treatment and intervention offered are to relief pain and provide better quality of life. Many studies began to explore the possibility of KOA related to genetic coding. Recent study managed to identify KOA risk loci and efforts of mapping KOA susceptible loci were granted (Loughlin, 2015). It is important to have the overall mapping of the genetic architecture of KOA currently it is intensively explored by geneticists to prevent and possibly to cure KOA (Zengini *et al*., 2018).

2.5 Assessment of KOA

Assessment of KOA consists of few elements: physical examination, plain radiography and laboratory testing of biochemical markers. Physical examination normally performed when there were complaints of pain at any joints along with patients' demographics and medical history. Plain radiography is an economical and confirmation tool to the integrity of the joints especially the knees. At least two planes anterior-posterior (A-P) and lateral X-ray films needed to assess the infected joints. The results from X-ray were rated with K-L staging system (Kellgren and Lawrence, 1957, Felson *et al*., 2011).

Lab testing of biochemical markers can aid in diagnosis of KOA along with radiography finding. Plasma, serum, urine and synovial fluid are common specimens deployed to detect the biomarkers related to KOA. Burden of disease, investigative, prognostic, efficacy of intervention and diagnostic (BIPED) are the five categories of markers used as the framework for the investigation of KOA biomarker studies (Bauer *et al*., 2006). van Spil *et al*. did a reviewed for 54 types of biomarkers for knee and hip OA by applying BIPED criteria. These included serum COMP, urine collagen type II (uCTXII) and serum hyaluronic acid (HA) among others. The finding was serum COMP considered to have the best performance compared to all biomarkers available in market. It was investigated frequently, widely and had higher scores in most of BIPED categories (van Spil *et al*., 2010). Sudhir Singh *et al.* reported COMP is more sensitive, specific and accurate compared to HA as KOA biomarker (Singh *et al.*, 2015). They concluded COMP is a therapeutic and prognostic indicator of KOA and it provides quantitative commentary about the success fullness of ongoing treatment (Singh *et. al.*, 2014).

Now more researches have ventured into the genetic linkage analysis to investigate the possible genes involved in KOA. Ethnic differences in radiographic KOA and level of biomarkers of KOA offer an interesting insight that genetic factors is one of the factor related to KOA (Clark *et al*., 1999, Holderbaum *et al*., 1999). Fifty percent of OA cases in the hips, hands and knees were genetically related (Spector *et al*., 1996). The discovery of Type II collagen (COL2A1) provides the path for further work on genetic to discover more OA-related genes.

Other biomarkers such as COX-2, PGE_2 and HA are well known for determining the degree of inflammation and destruction in the joints (Willoughby *et al.*, 2000, Lee *et*

al., 2015, Cole *et al*., 2017). C-terminal cross-linking telopeptide of type I collagen (CTX-1) and osteocalcin are important to evaluate the condition of bone metabolism in OA (Bai and Li, 2016, Cantatore *et al*., 2004). There are many more biomarkers undiscovered in our currently available technology. Early detection via laboratory testing is a major challenge to discover a novel biomarker which can detect KOA in the early stage before the disease even starts to progress.

2.6 Cartilage and inflammatory markers associated with KOA

KOA condition is associated with various cytokines which participate in affecting articular cartilage metabolism. IL-1β, TNF-α and IL-6 considered as the important pro-inflammatory cytokines in pathophysiology of KOA. Highlight of these three cytokines can explained the network of pathogenesis of KOA. IL-1β is the essential pro-inflammatory cytokine in the pathophysiology of KOA. It functions as suppressor of aggrecan and type II collagen found commonly at cartilage (Stove *et al*., 2000, Chadjichristos *et al*., 2003). IL-1β can induces production of inflammatory chemokines and cytokines such as IL-6 and IL-8 (Cahill and Rogers, 2008, Chen *et al*., 2005, Eskan *et al*., 2008). Since then, large scale of researches was working on IL-1β as future nominee biochemical marker.

TNF-α is another catabolic biomarker in KOA. TNF-α influenced the formation of cytokines such as IL-6 and IL-8 and is activated by macrophages (Aggarwal *et al*., 2013). TNF-α was first found as pro-inflammatory cytokine back in 1985 where it was known to stimulate PGE_2 and collagenase from isolation of human dermal fibroblasts and synovial

cells (Dayer *et al.*, 1985) . This proposed that TNF-α plays an important part in tissue destruction in inflammatory conditions. Both of IL-1β and TNF-α are recognized to induce lipid peroxidation and reactive oxygen species (ROS) which can lead to degradation of cartilage matrix (Tiku *et al.*, 2000). IL-1β and TNF-α possessed an ability to stimulate production of nitric oxide (NO) which can inhibit proteoglycan synthesis and boost the production of MMP (Tyler, 1985, Dingle *et al*., 1979) .

IL-6 had been identified by several studies as pro-inflammatory cytokine in KOA pathophysiology. IL-6 level was reported high in subjects with radiographic KOA compared to control group (Livshits *et al*., 2009) and found increased in synovial inflammation in KOA (Martadiani *et al*., 2017). It is also capable to activate T-cell, B-cell and liase the engagement of other pro-inflammatory cells to inflammation area (Gabay, 2006).

COMP is the member of the thrombospondin family which is a pentameric glycoprotein. It is synthesized by synovial cells and chondrocytes activated by proinflammatory cytokines (Zivanovic *et al*., 2011). COMP has a part in endochondral ossification, stabilization and accumulation of extracellular matrix through its relationship with matrix components and collagen fibrils (Recklies *et al.*, 1998). Local inflammation of synovial joints caused the COMP to be released initially in the synovial fluid before it can be detected in blood and urine (Kawashiri *et al.*, 2010). High COMP in blood reflects the catabolic events suggesting high turnover degree by the chondrocytes to compensate for the breakdown of cartilage matrix. COX-2 and PGE_2 are two of the inflammatory biomarkers that essential in the inflammation event. Both play an important role in the sequential reactions in the AA pathway. COX-2 enzyme catalyzed the formation of

prostaglandin G_2 (PGG_2) in rate-limiting reaction and finally the production of PGE_2 and the rest of PGs. High level of COX-2 level is accompanied by the induction of inflammatory cytokines. Reduction of COX-2 and PGE_2 level believed to increase proteoglycan synthesis and cartilage damage (Mastbergen *et al*., 2002, Mastbergen *et al.*, 2005) in KOA cartilage. This made COMP (cartilage marker), COX-2 and PGE_2 (inflammatory markers) as the important cartilage and inflammatory biomarkers in the assessment of KOA disease.

2.6.1 Cartilage Oligomeric Protein (COMP)

COMP is a non-collagenous protein found abundantly in KOA cartilage. COMP was identified as a useful biomarker in distinguishing between normal and diseased individuals. It is also used to monitor the prognosis of KOA (Arellano *et al.*, 2017, Kluzek *et al*., 2015, Georgiev *et al*., 2018, El-Arman *et al*., 2010). In the longest observational study by Kluzek *et al.*, they observed and concluded their twenty years duration study with high serum COMP is associated with increased risk of developing radiographic KOA (Kluzek *et al*., 2015). This conclusion was similar with a study done by Blumenfeld *et al.* in 2013. However, other studies failed to find the relationship between serum COMP with radiographical grading (Verma and Dalal, 2013, Das Gupta *et al*., 2017). A study done by Gupta *et al.* on multi-ethnic Malaysian population with subjects varies from K-L grade 2 to grade 4 showed gender is a factor of consideration in evaluating the level of serum and synovial fluid COMP (Das Gupta *et al.*, 2017). Another study showed that higher level of serum COMP was reported in males (52%) compared to females (Verma and Dalal, 2013).

In Johnston County OA Project, a large-scale community study participated by 3187 African American or Caucasian individuals age more than forty-five years old reported level of serum COMP of Caucasian men were higher than Caucasian women (Jordan *et al.*, 2003). Singh *et al.* showed the male subjects had slightly higher level of serum COMP, but it is not statistically significant.

Positive finding regarding association of serum COMP and OA were demonstrated in animal studies. Animal study in China showed an increase of serum COMP level in the OA animal group and COMP level increased as OA advance (Bai and Li, 2016). This provided the evident that serum COMP could be considered as a biomarker in detecting and monitoring amelioration of cartilage loss. Measurement of serum COMP and joint fluids COMP were utilized as a specimen to determine the level of COMP during cartilage self-repair. As OA progress, there will be constant breakdown and self-repair by the cartilage. An animal study indicated that COMP level decreases as cartilage in repairing process (Chu *et al*., 2015). High COMP level also detected in several diseases other than KOA. Systemic Lupus Erythematosus (SLE) is an autoimmune disease in which own antibodies attack the joints, mucous membranes, lungs and sometimes the heart. A study comparing the patients with SLE with arthritis and SLE without arthritis indicated the SLE with arthritis had a high level of COMP in serum compared to SLE without arthritis (Fawzy *et al.*, 2011). Sakthiswary *et al.* and Liu *et al.* conducted the researches of COMP level on rheumatoid arthritis (RA). Both came to a strong conclusion that elevated serum COMP in the disease suggested that COMP is a suitable test candidate in RA diagnosis (Sakthiswary *et al*., 2017, Liu *et al*., 2016). The changes of COMP level can serve as early diagnosis of KOA but must be presented with clinical observations.

2.6.2 Cyclooxygenase-2 (COX-2)

COX-2, a 72-kd protein, is the essential enzyme in AA cascade. In physiological condition, COX-2 is expressed at low level (Funk, 2001). It is bounded within inner layer at phospholipid bilayer of nuclear envelope and endoplasmic reticulum (ER) (Spencer *et al.*, 1998, Morita *et al.*, 1995). It is not constitutively expressed as COX-1 but it can be induced tremendously by number of cytokines such as TNF-α, IL-1 and inflammatory stimuli (Crofford *et al.*, 1994). COX-2 was released from many different types of cells such as endothelial cells, fibroblasts synovial cells, macrophages and monocytes (Wu *et al.*, 2013). The induction of COX-2 by pro-inflammatory cytokines further converted AA which originated from cell membrane to form prostaglandin H_2 (PGH_2) and subsequent reactions from various PG syntheses metabolized to form multiple types of PGs and thromboxanes (Vane, 1971).

COX-2 gene coordinated through 5' (transcriptional and 3' [post-transcriptional]) upon activation by IL-1β signal (Faour *et al.*, 2001). The availability of binding sites for transcriptional factors utterly increase the gene transcription directly after activated by external stimuli (Yamamoto *et al.*, 1995). The COX-2 protein level in synovial fibroblast cultures echoed the COX-2 mRNA in a study by Faour *et al.* and confirmed the explicit chemistry between message translation and expression (Faour *et al.*, 2001). Several studies reported induction of COX-2 expression in human synovial fibroblast influenced by lipopolysaccharide (LPS), IL-1β, macrophage inhibitory factor (MIF) and TNF-α (Crofford *et al.*, 1994, Hulkower *et al.*, 1994, Roshak *et al.*, 1996, Sampey *et al.*, 2001). Study in China reported OA and RA subjects had high COX-2 level in supra patellar bursa

fluid compared to control group (Yang *et. al*., 2016). mRNA levels of COX-2 in synovial cells were elevated in OA and RA patients however COX-2 mRNA level in OA was much higher compared to patients with RA (Fan *et al*., 2015). The increment of COX-2 and the subsequent increased of PGE_2 level is believed to cause the deterioration of proteoglycan synthesis within OA chondrocytes in concentration dependent manner (Hardy *et al.*, 2002). COX-2 also promotes inflammatory cytokines causing an imbalance in homeostasis of cartilage proteoglycan synthesis and further worsen the OA condition (Lee *et al*., 2015).

2.6.3 Prostaglandin E_2 (PGE_2)

PGE_2 is a prostanoid and lipid mediator of inflammation and pain. It is not stored but immediately metabolized. PGE_2 is part of proactive PGs which is involved in the enzymatic metabolism conversion of AA to PGG_2 and later converted to PGH_2. PGH_2 is tightly regulated by PG synthases and formed five primary prostanoids: prostaglandin D_2 (PGD_2), PGE_2, prostaglandin I_2 (PGI_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane A_2 (TXA_2) (Figure 2.2).

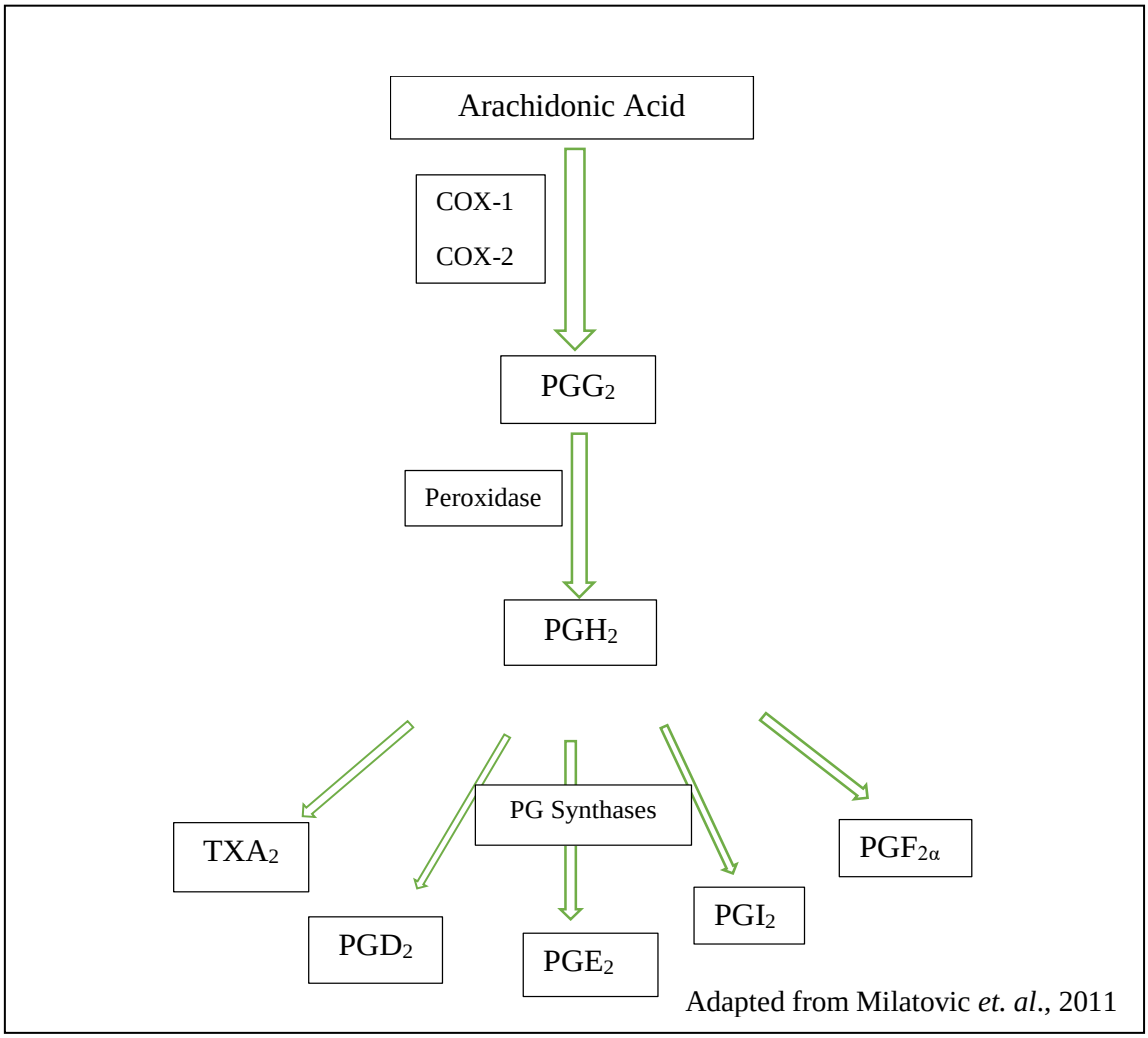


Figure 2.2: Arachidonic acid Pathway

There are three types of PGE_2 synthases: microsomal PGES-1 (mPGES-1), microsomal PGES-2 (mPGES-2) and cytosolic PGES (cPGES). All PGE_2 synthases had their specific function to regulate formation of PGE_2 concentration (Park *et al*., 2006). PGE_2 is well reported with the involvement in multiple normal physiological function such as fertility, blood pressure, gastrointestinal integrity and immune response (Legler *et al*., 2010). In pathophysiological aspect, PGE_2 is also known to be associated with inflammation, pain and cancer (Nakanishi and Rosenberg, 2013, Hsu *et al*., 2017). Cytokines such as IL-1, TNF-α, NO synthase 2 (NOS2), MMP-1 and MMP-3 are some of the inflammatory cytokines and catabolic factors that downregulate the expression and synthesized by PGE_2 (Blanco *et al*., 1995, Haas *et al.*, 1990, Takayama *et al.*, 1990, Fushimi *et al*., 2007, Noguchi *et al*., 2005). Avascular joint cartilages were due to the effects of PGE_2 which are by autocrine and paracrine fashion (Guilak *et al*., 2004). These effects were dose-dependent (Funk, 2001, Harris *et al*., 2002, Sugimoto and Narumiya, 2007).

2.7 Treatment for KOA

Basic treatment of KOA can divided into four categories: Non-pharmacologic, pharmacologic, complementary and alternative and lastly surgical (Sinusas, 2012). The fundamental understanding for KOA treatment is to begin with non-invasive type. Physical exercise is the commonest non-pharmacologic treatment of KOA patients. However, it is recommended for patients suffering with mild OA (Castrogiovanni *et al.*, 2016). Aquatic exercise is relatively a new regime of exercise to treat knee and hip OA

(Rewald *et al.*, 2016, Bressel *et al*., 2014, Guerreiro *et al*., 2014). However, it has short-term and small effects on OA patients.

By far, NSAIDs therapy are the first line treatment. NSAIDs are more effective to reduce the inflammation and swelling caused by OA (da Costa *et al.*, 2017, Geba *et al*., 2002) . Recently, intra articular injection become a popular therapy among OA patients. Experiments by intra articular on patients using platelet rich plasma (PRP), mesenchymal stem cells or glucosamine/chondroitin combination provided strong evidence that intra articular injection is a good option with low side effects to reduce pain and disability (Hammad *et al.*, 2015; Campbell *et al*., 2015; Jo *et al*., 2017). However, intra articular injection of PRP only provided symptomatic relief up to 12 months (Campbell *et al.*, 2015) . Complementary medicine like acupuncture is widely used and believed to improves the effect of medical treatment. Manheimer *et al*. and Manyanga *et al.* drew a conclusion and agreed that acupuncture is an effective therapy in reducing pain, enhance flexibility and quality of life (Manheimer *et al*., 2010, Manyanga *et al*., 2014) . Nonetheless, United Kingdom National Institute for Health and Care Excellence (NICE) was against the practice of acupuncture on KOA because lack of secured evidence related to the practice (Birch *et al*., 2017) .

Glucosamine (2-amino-2-deoxy-D-glucose) (GlcN) and chondroitin supplementation are another popular remedy consumed as part of treatment for KOA patients worldwide. GlcN is a monosaccharide derivative of glucose. It is found consolidated into glucosaminoglycans, collagen and proteoglycan (Bassleer *et. al*., 1998). These are the essential components of articular cartilage. Proteoglycans are formed from glycosaminoglycan chain which is produced by glucosamine. Proteoglycans complexes

can create the pressure within cartilage, attract water and cope with mechanical loading (Huskisson, 2008). The proteoglycans are constantly undergoing breakdown and resynthesis with subjected to regular metabolic turnover. Hexosamine pathway is part of glycolytic pathway (Figure 2.3). Glucosamine directly entered the cells into glucose transport system and phosphorylated to glucosamine-6-phosphate (GlcN-6-P). N-acetyl-glucosamine (GlcNAc) is formed before the pathway reach the end-product, uridine diphosphate N-acetyl-glucosamine (UDP-GlcNAc). GlcNAc retains a spectrum of anti-inflammatory activities and inhibit IL-6, NO and COX-2 production *in vitro* (Shikhman *et al*., 2001). An *in vitro* study trying to elucidate the mechanism lied behind the effectiveness of GS in OA. They discovered that GS capable of reducing PLA_2 and collagenase activity. GS acted on decreasing the cellular activity of PLA_2 and directly slow down the AA pathway (Piperno *et al.*, 2000). Reduction of enzyme PLA_2 might cause the reduced synthesis of AA or n-3 fatty acids (which is substrate for COX-2) and indirectly reduced the COX-2 activity. This study confirmed the rational of consuming GS as part of KOA treatment in context of reducing COX-2 levels. However, many studies reported both the supplementations had side effects such as abdominal pain, heartburn, diarrhea, epigastric pain, nausea and flatulence are the known side effects of these supplementations (Tapadinhas *et al*., 1982, Huskisson, 2008). Even some studies reported administration of GlcN can induce mild dysfunction in β-cell secretion and exaggerate asthmatic condition (Monauni *et al*., 2000, Tallia and Cardone, 2002). A multicenter, randomized, double-blind, placebo-controlled study in Spain disclosed that there were no different found in Western Ontario and McMaster University Osteoarthritis Index (WOMAC), score and visual analog scale (VAS) between the intervention group and placebo (Roman-Blas *et al*., 2016). The same result was obtained by Rindone *et al*. in his

study to determine the capability of GlcN in reducing pain on ninety-eight KOA patients (Rindone *et al*., 2000).

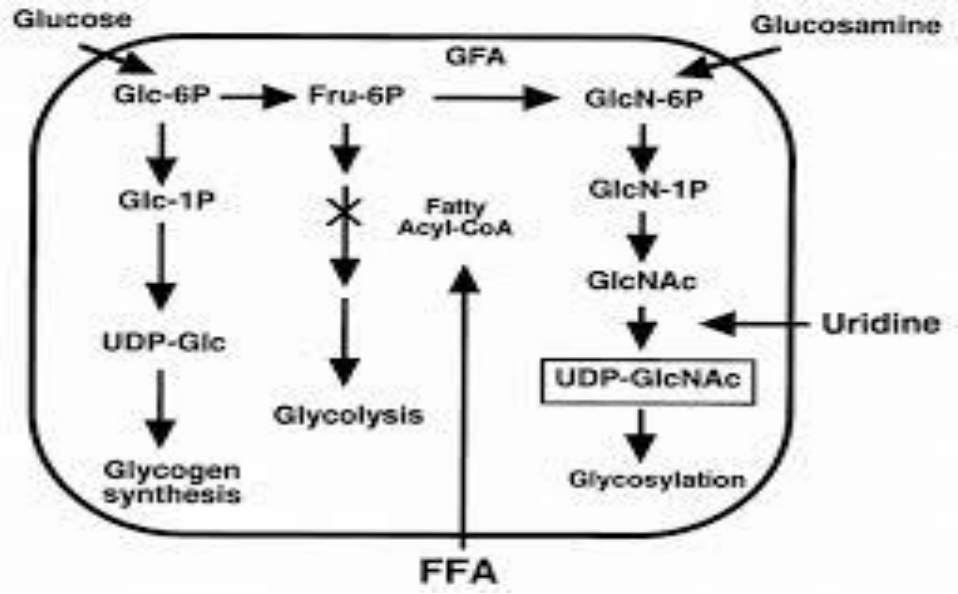


Figure 2.3: Hexosamine Pathway

Glc-6P, Glucose-6-phosphate; Glc-1P,Glucose-1-phosphate; UDP-Glc, Uridine Diphosphate glucose; Fru-6P, Fructose-6 phosphate; GFA, glutamine:fructose-6-P amidotransferase; GlcN-6-P, Glucosamine-6-phosphate; GlcN-1-P, Glucosamine-1-phosphate; GlcNA, N-Acetyl-glucosamine; UDP-GlcNAc, Uridine Diphosphate N-Acetyl- glucosamine; FFA, Free Fatty Acids (Adapted from (Hawkins *et al.*, 1997))

Interventions such as diet with nutrition, occupational related KOA and physiotherapy can be helpful in offering pain relief to KOA patients. Obese and overweight KOA patients were recommended to enforce a weight-loss plan which include suitable exercise regime to cater to their mobility ability. Supplements such as long-chain n-3 fatty acids may reduce pain and improve functions or controlled diet and integrating rich intake of vitamin K may aid to slow down OA progression (Thomas *et al*., 2018).