ANTI-INFLAMMATORY AND CHONDROPROTECTIVE ROLE OF TART CHERRY POLYPHENOLS IN SW1353 HUMAN CHONDROCYTES

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ABSTRACT

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ANTI-INFLAMMATORY AND CHONDROPROTECTIVE ROLE OF TART CHERRY POLYPHENOLS IN SW1353 HUMAN CHONDROCYTES

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The aim of this study was to investigate the chondroprotective effect of tart cherry polyphenols (TCP) using human chondrocytes. Chondrocytes (SW 1353) were pre-treated with TCP at concentrations of 1.25, 2.5, 5, and 10 µg/mL for 24 h then stimulated with the recombinant interleukin-1β (20 ng/mL) for 24 hours. After an initial increase ($p < 0.05$) in cell proliferation at 1.25 µg/mL TCP, a dose-dependent decrease in cell proliferation was observed with higher doses of TCP. Following a significant decrease in nitrate levels at the 1.25 µg/mL TCP dose, there was a dose-dependent increase in nitrate concentration. The dose-dependent increase in nitrate levels occurred with concomitant decreases in inducible nitric oxide synthase (iNOS) intracellular protein expression at the two highest TCP doses. Reductions in cartilage degradation were observed in the 2.5, 5, and 10 µg/mL TCP doses, as indicated by decreases in glycoprotein-39 (YKL-40) levels. TCP appeared to have no significant effect on total protein expression of the pro-inflammatory transcription factor, necrosis factor kappa B (NF-κB), or the matrix-degrading enzyme, matrix metalloproteinase-3 (MMP-3). In conclusion, these findings suggest that TCP promote cartilage health by modulating pro-inflammatory pathways

implicated in extracellular matrix destruction, as indicated by the effect of TCP doses on nitrite, iNOS, and YKL-40 levels. However, further research is need to elucidate the mechanism by which tart cherry polyphenols elicit these positive effects on cartilage health.

KEYWORDS: Chondroprotective; tart cherry polyphenols, chondrocytes, osteoarthritis

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CHAPTER I

INTRODUCTION

Osteoarthritis (OA) is a degenerative disease involving a progressive deterioration of articular cartilage, resulting in structural and functional impairments in the affected joint organ (Arden & Nevitt, 2006; Hunter, 2011). Among adults residing in the United States, it is the most common cause of disability, and it the fourth leading diagnosis cited for hospitalization (Murphy & Helmick, 2012). The global prevalence of symptomatic OA is estimated to be around 9.6% for men and 18% for women over the age of 60, and four out of every five individuals afflicted, experience functional impairments, which significantly limit the range of motion, mobility, and overall quality of life (Pereira et al., 2011; Woolf & Pfleger, 2003).

Currently, there are no disease-modifying treatments for OA to significantly delay or prevent total joint failure. Late treatment for OA involves highly invasive total joint replacement procedures accounting for approximately 42.2 billion dollars in health costs in the United States, annually. Unfortunately, this estimation accounts only for total joint replacements of the hip and knee, thus making the total costs even higher (Murphy & Helmick, 2012). Indirect costs related to losses in job productivity and increases in the number of disability claims account for an estimated financial loss of 3.4-13.2 billion dollars (Berger, Hartrick, Edelsberg, Sadosky, & Oster, 2011). With aging, the prevalence and incidence of radiographic and symptomatic OA increase drastically

before plateauing off around the age of 70 (Oliveria, Felson, Reed, Cirillo, & Walker, 1995). With a disproportionate segment of the United States population entering senescence, the economic burden of OA is expected to increase significantly.

Prolonged local and systemic inflammation has been implicated in the pathogenesis of many chronic diseases, including OA (Goldring & Otero, 2011; Khansari, Shakiba, & Mahmoudi, 2009; Sokolove & Lepus, 2013). Inflammation can disrupt normal cartilage metabolism through the induction of a hypertrophic catalytic response favoring cartilage turnover in mature articular chondrocytes (Sandell & Aigner, 2001). The pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor (TNFα), are significant mediators in this inflammatory process (Barnes & Karin, 1997; Martel-Pelletier, 2004). Not only is IL-1β an inflammatory agent, it also induces the expression of the transcription factor, necrosis factor kappa-light-chain-enhancer of activated B cells (NF-κB), which regulates critical inflammatory enzymes such as, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Abramson, Attur, Amin, & Clancy, 2001; Barnes & Karin, 1997). NF-κB also regulates the expression of the cartilage degrading enzymes: matrix metalloproteinase-1 (MMP-1), MMP-3, and MMP-13 (Ahmed, Wang, Hafeez, Cheruvu, & Haqqi, 2005).

Bioactive compounds naturally present in fruits and vegetables, such as polyphenols have shown promise in modulating inflammatory and catalytic pathways implicated in the pathogenesis of OA. Polyphenols or phenolic compounds can modulate

aberrant cartilage metabolism observed in OA by (1) suppressing gene expression and downregulating the synthesis of pro-inflammatory cytokines; (2) inhibiting the production of free radicals; (3) inducing expression of anti-inflammatory transcription factors; (4) and inhibiting cytokine-induced activation of apoptotic signaling pathways. (Shen et al., 2012).

Compounds isolated from grapes (resveratrol) and turmeric (diferuloylmethane or more commonly known as curcumin), have exhibited chondroprotective properties via multiple biochemically distinct mechanisms; the inhibition of NF-κB, a powerful proinflammatory transcription factor, the inhibition of cytokine-induced activation of apoptotic signaling pathways (Bcl-2, Bcl-xL, and caspase-3), expression of pro-inflammatory mediators (NO, interleukins, and TNF-α) and enzymes (iNOS, COX-2, MMP-3, MMP-13), and production of cytotoxic reactive oxygen and nitrogen species in chondrocytes (Chowdhury, Salter, Bader, & Lee, 2008; Csaki, Keshishzadeh, Fischer, & Shakibaei, 2008; Csaki, Mobasheri, & Shakibaei, 2009; Lei, Liu, & Liu, 2008; Lei et al., 2012; Liacini et al., 2003; Mathy-Hartert et al., 2009; Schulze-Tanzil, Mobasheri, Sendzik, John & Shakibaei, 2004; Shakibaei, Csaki, Nebrich, & Mobasheri, 2008; Shakibaei, John, Schulze-Tanzil, Lehmann, & Mobasheri, 2007).

Another promising bioactive compound for the treatment of OA is the green tea polyphenol epigallocatechin-3-gallate (EGCG). In a gene study performed by Akhtar and Haqqi (2011), pretreatment of osteoarthritic chondrocytes with EGCG prior to

stimulation with the inflammatory compound IL-1β suppressed the expression of 29 proteins known to be enhanced in pathogenic chondrocytes. These findings indicate EGCG can suppress aberrant inflammatory responses implicated in the pathogenesis of OA by affecting multiple targets. This inhibition of IL-1β induced protein expression has been attributed to EGCG ability to suppress the mitogen-activated protein kinase (MAPK) signaling pathways, c-Jun N-terminal kinases (JNKs), in addition to suppressing NF-κB (Akhtar & Haqqi, 2008; Singh, Ahmed, Malemud, Goldberg, & Haqqi, 2003). MAPKs govern many cellular processes including gene transcription, protein synthesis, differentiation, and regulating cell cycle functions (Kyriakis & Avruch, 2012). Therefore, agents that can target both MAPK signaling pathways and the activation of the pro-inflammatory transcription factor NF-κB may be of great value in treating OA. Tart cherries contain polyphenols in the forms of flavonoids and phenolic acids, which are known for their high antioxidant capacity. All polyphenols possess at least one aromatic ring containing one or more hydroxyl groups; however, variations in number, type, and position of functional groups attached to the aromatic ring(s) dictate their subsequent classification (Ignat, Volf, & Popa, 2011). Phenolic acids differ from flavonoids in the number of aromatic rings the compound contains, as phenolic acids contain a single ring. Flavonoids contained in tart cherry polyphenols can be sub-classified into flavonols (kaempferol, quercetin, and isorhamnetin) and anthocyanins (cyanidin and peonidin), which contain a ketone group and flavanols (catechin and epicatechin) which do not

(Kirakosyan, Seymour, Llanes, Kaufman &, Bolling, 2009). The phenolic acids are present as hydroxycinnamic acids (chlorogenic and p-coumaric acid). The structural diversity of the antioxidants in tart cherry may be exhibit unique chondroprotective properties via multiple mechanisms (Ignat et al., 2011; Kirakosyan et al., 2009)

In a small clinical study performed by Schumacher et al. (2013), the administration of tart cherry juice was shown to improve measures related to joint function and decrease knee pain in individuals with OA. Furthermore, these improvements corresponded to reductions in plasma concentrations of the pro-inflammatory protein, C-reactive protein (CRP). These data indicate that tart cherry consumption may have a role in improving clinical outcomes by modulating proinflammatory pathways via the reduction of systemic inflammation (Altindag et al., 2007; Pearle et al., 2007). In addition to improving symptoms associated with knee OA, consumption of tart cherry juice has also demonstrated the ability to promote recovery from intense exercise, by reducing inflammation and oxidative stress by systemically boosting antioxidant capacity (Bell, McHugh, Stevenson & Howatson, 2014; Howatson et al., 2010).

Similarly, both sweet and tart cherries have demonstrated the ability to decrease oxidative stress and biomarkers of chronic inflammation in healthy adults. Thus, their potential therapeutic effect may be of higher value in individuals with pre-existing inflammatory diseases such as OA (Kelley et al., 2013; Traustadóttir et al., 2009). Given

inflammation often precedes and or is concomitant with cartilage destruction in OA, reducing oxidative stress and other mediators of the inflammatory process are may be vital in preventing irreversible cartilage destruction associated with advanced stage OA. Cartilage health is key to joint health, and the potential chondroprotective role of plant-derived polyphenols need to be investigated as potential alternatives and or ancillaries to current treatment modalities.

Central Hypothesis

Therefore, this research was conducted to determine if tart cherry polyphenols could promote cartilage health through its anti-inflammatory action using a cell culture model.

Specific Aims

Specific Aim One

To determine whether tart cherry polyphenols in a dose-dependent manner decreases the expression of the pro-inflammatory enzyme, iNOS, and its downstream product NO.

Specific Aim Two

To evaluate if tart cherry polyphenols will dose-dependently decrease the expression of YKL-40, a biomarker of cartilage degradation.

Specific Aim Three

To determine if tart cherry polyphenols can dose-dependently decrease protein expression of NF-κB, a gene regulator of pro-inflammatory pathways, and MMP-3, a mediator of cartilage destruction using Western blot analysis.

CHAPTER II

REVIEW OF LITERATURE

Osteoarthritis

Defining Osteoarthritis

Osteoarthritis (OA) is a degenerative disease involving a progressive deterioration of articular cartilage, resulting in functional limitations that can hinder an individual's ability to perform day-to-day activities (Arden & Nevitt, 2006). OA affects each structural component of the joint tissue including the articular cartilage, synovium, and subchondral bone (Arden & Nevitt, 2006). The development, progression, and manifestation of the disease are heterogeneous with multiple factors being implicated in the pathogenesis of the disease (Hunter, 2011). Epidemiological studies of disease prevalence support a pathological model in which both internal and external factors interact to increase susceptibility to OA and can alter the pathological course of the disease (Arden & Nevitt, 2006).

The global prevalence of symptomatic OA in adults over the age of 60 are estimated to be around 9.6% and 18.0% for men and women, respectively, and four out of every five individuals afflicted, experience functional impairments, which significantly limit range of motion and overall mobility (Pereira et al. 2011; Woolf & Pfleger, 2003; World Health Organization, 2018). With aging, the prevalence and incidence of radiographic and symptomatic OA increase drastically before leveling off around the age

of 70 (Oliveria et al., 1995). However, given differences in the criteria used to define OA, this estimation of disease prevalence may be inaccurate. Recent studies using more stringent diagnostic criteria to identify OA have yielded disease prevalence to be approximately 3.8% and 0.76% for knee and hip OA, respectively (Cross et al., 2014). Only symptomatic knee and hip OA with Kellgren-Lawrence grade or two or higher were used to establish OA diagnosis. Therefore, prevalence and disability associated with knee and hip OA may be higher than indicated by this particular study.

In the Framingham OA cohort, 6.8% of individuals over the age of 26 had symptomatic hand OA, and it was approximately 2.4 times more prevalent in women in comparison to men (Murphy & Helmick, 2012). There also has been a significant increase in the prevalence of OA from 2005 to 2015 by 32.9% when correcting for age using the age-standardized rate (ASR). The increase remains significant at an increase of 2.2% in the global prevalence of OA over the past decade (Vos et al., 2016). This increase indicates that the prevalence of OA is increasing independent of the risk of developing OA attributed to normal aging.

Healthcare Costs

Since 1990, OA has increased from the 19th leading cause of disability attributed to non-communicable diseases to 13th and years lived with disability (YLDs) have increased by 34.8% from 2005 to 2015 (by 3.9% when using age-standardized rate) (Vos et al., 2016). The negative impact OA has on quality of life also appears to be greater in

regions of Eastern Europe, Central, and East Asia (Vos et al., 2016). This disparity may be due to the population's limited access to end-stage OA treatment modalities like total joint replacements, which can greatly reduce disability rates (Vos et al., 2016). For individuals 50 years of age and older, OA remains a perennial top ten cause of disability, globally. The years living with disability (YLDs) attributed to all-cause OA was approximately 2.2% of total global YLDs in 2010 (Cross et al., 2014).

In the United States alone, OA was the primary diagnosis for 921,000 hospital admissions in 2009 with approximately 98% of those admissions undergoing a total joint replacement procedure of the knee or hip (Murphy & Helmick, 2012). The individual hospital costs associated with knee and hip joint replacement procedures were estimated at 28.5 and 13.7 billion dollars in 2007, respectively (Murphy & Helmick, 2012). These costs have more than doubled since 1997 and OA has increased from the 16th to the 4th leading primary diagnosis of discharged patients over that period (Murphy & Helmick, 2012). Of total joint replacement procedures, 6% of knee replacements and 11% of hip replacements required revision, further increasing the economic burden associated with the end-stage treatment of OA (Chen, Gupte, Akhtar, Smith, & Cobb, 2012).

The development and progression of OA are often heterogeneous and has been associated with individual and joint-specific factors. Individual factors include age, gender, genetic susceptibility, diet, weight, fitness, and ethnicity. Joint-specific factors include injury, activity, joint loading, and muscle strength at the level of the joint

(Plotnikoff et al., 2015). Some of these factors can be modified to promote overall health and prevent the development of obesity and or obesity-related comorbidities, such as type II diabetes, hypertension, renal disease, and other diseases of the musculoskeletal system, and decrease the risk of developing symptomatic OA (Berger et al., 2011; Plotnikoff et al., 2015). Although OA is listed as the primary diagnosis for the fourth leading cause of hospitalization, it should be noted that greater than one-third of health care services rendered to employees with OA, had a higher incidence of other health conditions that can negatively impact health. These comorbidities associated with OA may contribute to the significant increase in healthcare care costs assessed to employees with OA when compared employees without OA (Berger et al., 2011; Murphy & Helmick, 2012).

In addition to direct health care costs associated with OA, indirect costs associated with decreased job productivity, increased absenteeism, and increased worker's compensation/short-term disability can be extremely costly. Short-term disability claims are approximately 2.4 times greater in individuals afflicted with OA (Berger et al., 2011). Annually, the job-related costs associated with OA is between $3.4-13.2 billion in the United States and £3.2 billion in the United Kingdom (Berger et al., 2011; Buckwalter, Saltzman, & Brown, 2004). The total economic cost associated with OA in the United States, United Kingdom, France, and the continent of Australia account for 1-2.5% of each respective nation's gross national product (Chen et al., 2012). The economic burden of OA is staggering at the national and global level, and it is only

expected to grow as the disease prevalence rises precipitously as a disproportion segment of the population enters senescence.

Symptomatology

Given the complexity of the pathological model of OA, the onset, manifestation, progression, and prognosis of the disease vary greatly from one individual to another. Despite heterogeneity in disease presentation, OA is characterized by the presence of pain and structural damage to and around the joint and surrounding tissues (Hunter, 2011). Symptoms include pain and stiffness, limited range of motion, weakness or atrophy of surrounding muscles, and in some cases swelling at and adjacent to the afflicted joint. Damage can occur at all levels of the joint including the articular cartilage, subchondral bone, synovium, periarticular ligaments, and muscles (Arden & Nevitt, 2006).

Common pathological features include loss of articular cartilage, abnormal remodeling of the cartilage and subchondral bone, loss of elasticity in surrounding ligaments, and synovial inflammation and distension (Hunter, 2011). Primary OA, also known as idiopathic OA, is the most common form of OA and it is diagnosed when joint degeneration cannot be attributed to an identifiable causative factor (Arden & Nevitt, 2006). It rarely occurs in individuals younger than 40 years of age (Buckwalter et al., 2004).

Secondary OA is less common and can be attributed to a precipitating metabolic, traumatic, or inflammatory event and or an anatomical or genetic defect favoring the development of OA (Arden & Nevitt, 2006). OA can also be classified according to joint involvement or the anatomical site of articulation, and it can be present in single joint (monoarticular), multiple joints (oligoarticular) of the same type, or multiple functionally unique joints (polyarticular) in a condition recognized as generalized OA. Major sites of OA include the hands, spine, hips, and knees with a lesser incidence of OA occurring in the wrist, elbow, shoulder, and ankle joints (Arden & Nevitt, 2006). Studies indicate that primary OA is rare in the lesser affected joint sites. A form of secondary OA, known as post-traumatic OA, is the predominant form of OA at these lesser joint sites. Conversely, primary OA is more common in the chief OA sites (Buckwalter et al., 2004).

Diagnostic Criteria

Diagnostic criteria used to identify OA can be clinical (symptomatic), radiographic, or a combination of clinical and radiographic. Diagnosis of hand OA relies solely on clinical information gathered through individual assessment of patient's medical history and a physical examination. Definitive diagnosis of hip and knee OA often requires additional radiographic evidence to validate clinical measures (Murphy & Helmick, 2012). The criteria developed by the American College of Rheumatology (ACR) is the most commonly used diagnostic for the identification hand, hip, and knee OA. Only clinical measures are used to reliably identify OA specific to the hand. To be

formally diagnosed with hand OA, an individual must experience hand pain, aching or stiffness for most days, have hard tissue enlargement of two or more selected hand joints (bilateral second and third proximal interphalangeal joints, second and third proximal interphalangeal joints, and first carpometacarpal joints), and swelling in two or more of the metacarpal joints. In addition to the aforementioned criteria, the individual must also experience one of the following conditions: hard tissue enlargement of two or more distal interphalangeal joints or deformity of one more of the selected hand joints (Arden & Nevitt, 2006).

Diagnosis of hip OA requires a clinical symptom of hip pain most days occurring with radiographic or laboratory evidence of two of the following three conditions; femoral or acetabular osteophyte formation, hip joint-space narrowing, or increased erythrocyte sedimentation rate (ESR $\leq$ 20 mm/h). Knee OA can be identified using clinical measures exclusively or a combination of clinical and objective lab measurements. Knee pain occurring frequently throughout the week in combination with one following physical abnormalities: frequent crepitus occurring with active knee joint motion or bony enlargement of the knee. In the absence of frequent knee crepitus, an age greater than 38 occurring with frequent pain and bony knee enlarge can be used to identify knee OA (Arden & Nevitt, 2006).

Quantitative values used in combination with clinical criteria to diagnosis knee OA are radiographic evidence of osteophytes at joint margins or abnormal synovial fluid

indicative of OA. Synovial fluid indicative of OA must exhibit one of three following criteria; high clarity, high viscosity, and low white blood cell count (< 2,000 WBC/mm^3) (Altman et al., 1986). Frequent knee pain and radiographic evidence are sufficient to support the diagnosis of knee OA. However, without radiographic evidence, an individual must exhibit knee stiffness equal to or greater than 30 minutes daily, frequent knee crepitus concurrent with laboratory evidence of abnormal synovial fluid, and or be equal to or greater than 40 years of age (Altman et al., 1986; Arden & Nevitt, 2006).

Current Treatment Modalities

In the early stages of OA, non-invasive or minimally-invasive treatment options are preferred and include the use of oral and topical pharmacological agents, intra-articular injections, physical therapy, and custom orthotics (McAlindon et al., 2014). Unfortunately, these treatment strategies are often limited to relieving joint pain and have not demonstrated the ability to delay the progression of the disease or reverse cartilage damage (Shen et al., 2012). More progressive treatments aimed at tissue regeneration have limited utility in the treatment of OA due to the inability to successfully replicate the biomechanical properties of cartilage tissue due to terminal differentiation or dedifferentiation of transplanted cells (Jayasuriya & Chen, 2015). This alteration in cell type and or cell function has been attributed to the abnormalities inherent to the microenvironment within the pathological tissue. Therefore, tissue engineering is not yet

a feasible restorative approach if the pathogenic pathways specific to OA remain active at the time of transplantation (Goldring, 2012).

More traditional approaches use a combination of different therapeutic approaches: pharmaceuticals to manage pain, exercise prescription to strengthen residual muscle weakness and improve joint mobility, management of co-morbidities, and use of assistive devices to correct flawed biomechanics that contribute to structural damage (McAlindon et al., 2014). Unfortunately, prolonged use of the most commonly utilized treatment option such as the use of acetaminophen and nonsteroidal anti-inflammatory drugs (NSAIDs), have been linked to liver damage and gastrointestinal concerns (McAlindon et al., 2014). Given the risks and costs associated with NSAIDs and their limited ability to modify disease course, its use is not a viable long-term treatment option for the management of OA.

The use of pharmacological agents to treat and manage pain related to OA is well established; however, the cost versus benefit ratio must be carefully weighed for each individual to determine if its use is justified. Acetaminophen, duloxetine, NSAIDs, selective COX-2 inhibitors, and capsaicin are common pharmaceuticals considered appropriate for OA management (McAlindon et al., 2014). The therapeutic benefits of the aforementioned agents are generally associated with their ability to reduce pain and inflammation. Stronger analgesics derived from opioids are also prescribed for pain

management; however, patients taking these drugs are three times more likely to experience adverse iatrogenic outcomes (McAlindon et al., 2014).

With the exception of duloxetine, a serotonin-norepinephrine reuptake inhibitor (SNRI), chronic use of all the aforementioned pharmacological agents can have detrimental side effects on various organs throughout the body (McAlindon et al., 2014). Presently, most pharmacological agents are not viable long-term treatment options, as OA is a chronic condition with progressive degeneration. Intra-articular injections of corticosteroids, hyaluronic acid, and platelet-rich plasma (PRP) have shown greater efficacy than oral or topical agents. However, the benefits are only short-term: two to four weeks following the injection for corticosteroids, four to eight weeks for hyaluronic acid injection, and three to 12 months for PRP injections. Greater injection frequency prior to cessation of the PRP therapy was associated with increased duration of reported improvement in pain and joint function parameters (Huang, Wang, Chou, Wang, & Ko, 2017; Hunter, 2011; McAlindon et al., 2014).

Cartilage Biology

Formation

Cartilage tissue arises from multipotent mesenchymal cells. These cells condense and differentiate into three distinct tissues of the musculoskeletal system: bone, muscle, and cartilage (Jayasuriya & Chen, 2015). Differentiation of mesenchymal progenitors will determine the cells subsequent function. Bone and cartilage formation is governed by

similar mediators with chondroprogenitor cells of mesenchymal origin either undergoing terminal differentiation to promote endochondral ossification or differentiation into articular chondrocytes (Pitsillides & Beier, 2011). Prior to cellular condensation of lateral mesenchymal tissue, the tissue is rich in hyaluronan and type I, III, and V collagen (Goldring, 2012; Pitsillides & Beier, 2011). Once the lateral mesoderm plates have migrated and fully condensed they form the structure known as cartilage anlagen, and the pluripotent mesenchymal cells differentiate into chondroprogenitor cells that will give rise to chondrocytic cells (Jayasuriya & Chen, 2015). Anlagen expresses high levels of type IIa collagen, and the extracellular matrix (ECM) becomes hyaluronan-deplete as remodeling occurs (Pitsillides & Beier, 2011). The chondroprogenitor cells proliferate and begin to differentiate into mature chondrocytes in response to pericellular changes in the environment that support permanent cartilage formation (Jayasuriya & Chen 2015).

Mature chondrocytes are responsible for remodeling and maintaining the composition of the extracellular matrix (ECM) within the fully developed cartilage tissue. The ECM composition differs from that of its predecessor, anlagen with type IIb (mature) collagen and the proteoglycan, aggrecan becoming the predominant macromolecules within the fully developed cartilage tissue (Martel-Pelletier, Boileau, Pelletier, & Roughlye, 2008; Pitsillides & Beier, 2011).

Composition

During growth, chondrocytes can exhibit three functionally distinct phenotypes associated with specific areas of activity: the resting zone, the proliferating or columnar zone, and the hypertrophic zone (Pitsillides & Beier, 2011). Healthy mature articular cartilage is characterized by resting chondrocytes, and this zone does not undergo endochondral ossification. Instead, this zone forms the permanent cartilage located at joint articulations to protect bones against shearing forces. Whereas, the proliferating and hypertrophic zones are localized in and proximal to the growth plate and are involved in bone elongation during growth. Chondrocytes at the point of joint articulations reside in the resting zone and exhibit a stable phenotype. Those designated for bone development undergo terminal differentiation and express a hypertrophic phenotype that will induce apoptosis thereby promoting mineralization of the ECM and subsequent bone development (Goldring, 2012; Pitsillides & Beier, 2011).

Prior to fetal endochondral ossification, growth-plate chondrocytes are dominant within the tissue and exhibit high proliferative, hypertrophic, and apoptotic capabilities. These hypertrophic cells secrete type X collagen in addition to typical ECM components, and this matrix undergoes calcification and is ultimately reabsorbed and replaced by osteoclasts (Goldring, 2012). The articular chondrocytes only proliferate during the fetal chondrogenesis, and their numbers remained relatively fixed after articular cartilage formation ceases. Their primary function is to maintain cartilage tissue to protect the joint

from injury associated with mechanical loading. However, not all mineralized cells undergo apoptosis, and these unmineralized cells may be contained within the growth-plate along with unmineralized growth-plate chondrocytes (Pourmand, Binderman, Doty, Kudryashov, & Boskey, 2007). This zone containing growth-plate chondrocytes reflects a separation between bone and articular cartilage and is characterized by terminally differentiated hypertrophic chondrocytes.

Structure

Cartilage tissue has four functionally distinct zones: the superficial, middle or transitional, radial or deep, and calcified zones. The superficial is composed of one to three layers of chondrocytes, and these cells form a horizontal network of crisscrossing collagen bundles that make up the ECM (Mollenhauer, 2008). The area between the ECM and the superficial layer is the pericellular area. The middle zone contains proteoglycan and collagen fibers arranged into radial bundles and accounts for approximately half of the total cartilage height (Martel-Pelletier et al., 2008). The proteoglycan, aggrecan, is highly concentrated within the deep zone and the fiber bundles are arranged in a manner mirroring that of the middle zone with the only exception being the collagen fibers are thicker. The chondrocytes are positioned perpendicularly to distribute incoming mechanical forces at the joint space thereby protecting the cartilage tissue against shearing forces. The calcified zone contains mineralized cartilage tissue and acts as an anchoring point for cartilage to attach to bone (Mollenhauer, 2008).

Tissue Maintenance/Remodeling

The function of the articular chondrocyte is to build and maintain cartilage over the entirety of the lifespan. Therefore, damage can only be mitigated by replenishing structural components produced by articular chondrocytes, not by synthesizing new chondrocytes. In healthy cartilage, articular and growth-plate chondrocytes govern cartilage metabolism in differing manners (Sandell & Aigner, 2001). Remodeling of articular cartilage occurs via de novo synthesis of structural proteins within the ECM. Healthy articular chondrocytes are referred to as resting chondrocytes, not because they are metabolically inert, but due to their function in maintaining cartilage tissue through the synthesis of structural proteins in the absence of abnormal physiological or pathological stressors (Goldring, 2012).

Cartilage Destruction

In OA, articular chondrocytes appear to revert to the phenotypic expression seen in progenitor cells in an attempt to initiate repair. These articular chondrocytes become hypertrophic, leading to ectopic clustering of chondrocytes in the superficial zone. Unfortunately, this favors long-term cartilage catabolism with degradative and apoptotic pathways being more active than anabolic pathways which regulate growth and maintenance (Martel-Pelletier et al., 2008). This imbalance leads to net loss of articular cartilage despite initial activation of the anabolic response to stimulate repair (Sandell & Aigner, 2001).

Disruptions in inflammatory pathways have also been implicated in cartilage destruction, and it can affect OA development/progression via a multitude of direct and indirect mechanisms (Goldring & Otero, 2011; Martel-Pelletier, 2004). As the upregulation and or activation of proinflammatory pathways have been implicated in cartilage destruction, inflammation may be a key mediator in OA development and its subsequent progression (Goldring & Otero, 2011; Sandell & Aigner, 2001).

Key Factors Associated with Cartilage Destruction

Inflammatory Mediators

The disruption in cartilage metabolism where cartilage catabolism is sustained over synthesis is a defining feature of OA. The dysregulation of catabolic processes has been attributed to localized inflammation in the cartilage tissue (Hunter, 2011). The proinflammatory cytokines, interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα), are major mediators in this inflammatory process (Martel-Pelletier, 2004; Barnes & Karin, 1997). Interleukin-1 beta and TNFα have demonstrated the ability to activate de novo synthesis of the proinflammatory cytokines, interleukin-8 (IL-8) and interleukin-6 (IL-6), and leukocyte inhibitory factor (LIF) in both chondrocytes and synoviocytes (Abramson et al., 2001; Fan, Bau, Yang, & Aigner, 2004; Martel-Pelletier et al., 2008). In addition to inducing the synthesis of these and other proinflammatory molecules that can contribute to the inflammatory process, members of the IL and TNF receptor families can, directly and indirectly, induce mammalian mitogen-activated protein kinase

(MAPK) signaling pathways (Kyriakis & Avruch, 2012). Activation of these pathways can exert diverse and varied physiological changes at the cellular level, many of which can contribute to a metabolic shift seen in osteoarthritic cartilage tissue (Kyriakis & Avruch, 2012; Pitsillides & Beier, 2011). Interleukin-1 beta stimulated chondrocytes exhibited an increase in the protein expression of activated MAPKs from three regulatory pathways: extracellular signaling kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), and p38 MAPK (Cargnello & Roux, 2011; Fan et al., 2004). The p38 and JNK signaling pathways are involved in regulating the cellular response to environmental stress, whereas the ERK signaling pathway is important in the regulation of cellular functions specific to proliferation, differentiation, and apoptosis (Fan et al., 2004; Kyriakis & Avruch, 2012). Interestingly, in a study performed by Fan et al., IL- 1β increased protein expression of the proinflammatory cytokine IL-6, which was significantly blunted upon the addition of an ERK-1/2 inhibitor indicating that ERK pathway is vital in stimulating IL-6 production. Interleukin-6 has also been reported to increase concentration and activity of matrix metalloproteinases (MMPs), thereby promoting cartilage degradation (Fan et al., 2004).

IL-1β mediated activation of MAPKs is similar to that of TNFα mediated activation of MAPKs, as the binding of IL-1β to its respective receptor (IL-1R) recruits a similar yet functionally different adapter protein known as TNF receptor-associated factor-6 (TRAF6). TNF receptor-associated factor-6 engages the ubiquitination of its own

complex thereby activating the upstream MAPK kinase kinase (MAP3K) transforming growth factor beta (TGF-β)-activated kinase-1 (TAK1). This activation is important because TAK1 can also activate inhibitor of nuclear factor kappa-ß kinases (IKKs). IKKs initiate the ubiquitination of the inhibitors of kappa B protein subunit alpha (IkBα) leading to the mobilization and subsequent activation of the nuclear transcription factor kappa beta (NF-kB), which in turn regulates the expression of many proinflammatory and apoptotic genes which have been known to be overexpressed in osteoarthritic tissue (Kryiakis & Avruch, 2012; Martel-Pelletier et al., 2008).

In addition to inducing the activation of NF-kB via induction of IKKs through TAK1, activation of p38 MAPK signaling pathways have been known to increase the stability of messenger RNA (mRNA) used for the synthesis of proinflammatory cytokines such as TNFα, interleukin-3 (IL-3), IL-6, and proinflammatory enzymes such as iNOS and COX-2 (Hitti et al., 2006). Stabilization of mRNA decreases the turnover rate of targeted mRNA thereby increasing the rate of protein translation. This stabilization is one mechanism by which MAPK activation in association with the activation of the transcription factor NF-κB can promote the initiation and propagation of the inflammatory process. As this mechanism is responsible for increasing the transcription of genes with functional end products implicated in cartilage destruction, potential treatments targeting the inhibition of MAPKs signaling pathways should be evaluated (Hitti et al., 2006; Hoernes, Hüttenhofer, & Erlacher, 2016).

The activation of proinflammatory genes by induction NF-kB in association with specific MAPK signaling pathways are responsible for the transcriptional regulation and subsequent production of two key enzymes that are overexpressed in OA, COX-2 and iNOS. Cyclooxygenases are the major rate-limiting enzymes for the production of prostaglandins from arachidonic acid. The COX-2 enzyme produces prostaglandin H_2 that ultimately undergoes isomerization to form the end product prostaglandin E_2 (PGE_2) (Murakami et al., 2000). PGE_2 appears to be a key mediator in the inflammatory process and promotes aberrant cartilage catabolism characteristic of OA (Abramson et al., 2001; Barnes & Karin, 1997). In OA, the activity of the COX-2 isoform is overexpressed resulting in a pathological increase in PGE_2 that occurs independent of COX-1 activity (Bensen et al., 1999).

IL-1β stimulation in OA explants co-cultured with their respective synovial membrane has shown increased COX-2 expression. PGE_2 levels increase concurrently resulting in a significant increase in proteoglycan degradation. Interestingly, the isolated osteoarthritic cartilage explants did not exhibit a significant decrease in proteoglycan in response to IL-1β. The lack of observable change was attributed to a lack of endogenous PGE_2 synthesis occurring within the synovial tissue (Hardy et al., 2002). Furthermore, in the absence of IL-1β stimulation and or in the presence of IL-1β signaling blockade, IL-1β induced PGE_2 production, and proteoglycan destruction was severely blunted. This observation remained even with the addition of exogenous PGE_2, thereby indicating IL-

1β has a modulatory effect on PGE_2 mediated proteoglycan destruction (Hardy et al., 2002).

This effect may be related to IL-1β's ability to promote nitric oxide (NO) production through the induction of iNOS. As NO is a key signaling molecule in cartilage turnover, it is likely that enhanced production of PGE_2 and NO work together to augment cartilage catabolism. PGE_2 levels are increased in osteophyte and fibrocartilaginous tissue, each of which is pathological a feature of OA, resulting from abnormal tissue remodeling (Hardy et al., 2002). This data indicates that PGE_2 may have a role in mediating bone growth and development, whereas NO is associated with cartilage turnover. Extracellular matrix deterioration and abnormal cartilage remodeling precede osteophyte formation, and NO and PGE_2 together appear to promote abnormal cartilage remodeling which in return may promote osteophyte formation in cartilage tissue when present both are present in excess (Hardy et al., 2002).

Nitric Oxide

Increases in cellular NO promote cartilage destruction by increasing the synthesis of cartilage-degrading enzymes and contributing to the production of harmful reactive oxygen species (Abramson et al., 2001; Hunter 2011). Overproduction of NO is believed to occur primarily via the overexpression of the inducible form of nitric oxide synthase (iNOS), and through this non-constitutive enzyme, larger and more sustained quantities of NO are generated in response to a vast array of stimuli. The most potent stimuli are IL-

1β, TNFα, and lipopolysaccharide (LPS). Overproduction of NO in OA also suppresses the synthesis of interleukin-1 receptor antagonist (IL-1Ra) thereby increasing the potential for activation of the proinflammatory IL-1β signaling cascade (Pelletier, Martel-Pelletier, & Abramson, 2001). As previously mentioned, the initiation and subsequent progression of OA is driven by an imbalance between catabolic and anabolic processes with the degradative and apoptotic pathways being favored over the anabolic pathway regulating growth and maintenance. This imbalance leads to the destruction of cartilage and can be greatly influenced by the production of metabolites such as NO.

In addition to inducing proinflammatory pathways implicated in the pathogenesis of OA, the marked increase in NO concentration in chondrocytes have been shown to inhibit the synthesis of two crucial ECM components, type II collagen and proteoglycan (Taskiran et al., 1994). NO also promotes cartilage destruction by increasing the synthesis of cartilage-degrading enzymes and contributes to cellular damage through the generation of free radicals (Clancy, Abramson, Chuck & John, 1998; Hirai et al., 2001; Taskiran et al., 1994). Therefore, NO production exerts a two-fold effect on cartilage destruction by inhibiting the repair of damaged cartilage and by inducing cartilage degradation. Dysregulation of NO production in chondrocytes appears to a key step in the development and progression of OA, thus making it a valuable physiological marker to target for potential intervention.

Matrix Metalloproteinases

In normal adult cartilage, matrix turnover occurs slowly in response to physiological changes within the microenvironment, and it is typically preceded by joint insult/injury (Martel-Pelletier et al., 2008). The initial response to injury results in a marked increase in synthetic activity, however, the initial increase in anabolic activity within chondrocytes is unable to be sustained at a rate that can accommodate tissue repair (Arden & Nevitt, 2006). Remodeling of the damaged tissue becomes outpaced by catabolism resulting in accelerated cartilage loss, a hallmark of OA (Pitsillides & Beier, 2001; Martel-Pelletier et al., 2008). The primary enzymes involved in cartilage degradation belong to a class of enzymes known as MMPs. In osteoarthritic chondrocytes, an elevation in MMP-3 (stromelysin-1), MMP-8 (collagenase-2), and MMP-13 (collagenase-3) have been noted (Sandell & Aigner, 2001). Stromelysin-1 (MMP-3) plays a key role in the destruction of the proteoglycan layer surrounding collagen fibers, and it is upregulated in the early stages of OA (Martel-Pelletier, 2004; Martel-Pelletier et al., 2008). With the limited protection conferred by the hydrolyzed proteoglycan layer, the collagen fibers become more susceptible to the catalytic action of the MMP-1 (interstitial collagenase) and MMP-13 (Vincenti & Brinckerhoff, 2002). In addition to increasing the susceptibility of the extracellular matrix to the catalytic action of other MMPs, MMP-3 is suspected to have a regulatory role in the activation of interstitial collagenase (MMP-1) via the induction of proMMP-1. Interstitial collagenase

(MMP-1) can induce the proteolytic cleavage of multiple collagen fibrils types: I, II, III, VII, and X, however, within cartilage tissue it primarily targets type II collagen with lesser affinity for type X fibrils (Martel-Pelletier et al., 2008). MMP-1 activity is localized primarily within the most superficial zone, whereas the other major collagenase, MMP-13, is localized in the intermediate and deep layers and has an affinity for both type II and IX collagen fibrils, although, the enzyme preferentially targets type II fibrils (Fernandes et al., 1998; Moldovan, Pelletier, Hambor, Cloutier, & Martel-Pelletier, 1997). This marked increase in MMPs concentrations leads to the destruction of the collagen cross-links, further compromising the structural integrity of the ECM.

The crisscrossing type II collagen network contained within the three outer zones: superficial, middle, and radial zones is integral to maintaining the structural integrity of cartilage tissue. As the collagen network is destroyed, the cartilage tissue becomes more susceptible to overhydration and swelling, resulting in the subsequent erosion of the tissue due to the inability of the collagen network to adequately maintain the hydroscopic proteoglycans contained within the tissue (Martel-Pelletier et al., 2008; Sandell & Aigner, 2001). Elevated MMP-1 levels have been associated with the inflammatory process, whereas higher MMP-13 levels are associated with the remodeling phase of cartilage turnover. In OA, the destruction of the fibrous collagen network in cartilage tissue represents an irrevocable step. Hence, the development of therapies targeting the action of collagenases and stromelysins could offer promise in preventing and or delaying the

progression of this condition (Goldring, 2012). An inhibition against the proteoglycanase, MMP-3, could confer protection against the catalytic action of the collagenases, MMP-1 and MMP-13. In addition to contributing to catabolic cartilage turnover, increases in glycoprotein-39 (YKL-40), another potential mediator of pathogenic cartilage remodeling have occurred in concurrence with MMP-1 and MMP-3 (Johansen et al., 1996; Väänänen et al., 2014; Volck et al., 2001). YKL-40 expression is known to be upregulated in the joint tissues and serum of osteoarthritic patients and its concentration has been correlated with advanced cartilage degradation and synovial inflammation (Johansen et al., 1996; Volck, Østergaard, Johansen, Garbarsch, & Price, 1999).

Glycoprotein-39

In addition to increasing in concentration with matrix metalloproteinases (MMP-1 and MMP-3), YKL-40 has also be reported to increase concurrently with the proinflammatory cytokines, IL-6 and IL-17. Interestingly enough, YKL-40 production appears to be promoted by IL-6 and IL-17 in diseased tissue, indicating inflammation may be the precipitating event in the breakdown of the cartilage and the subsequent release of YKL-40 from the cartilage membrane (Väänänen et al., 2014). Thus, YKL-40 may prove to be a valuable tool for assessing both cartilage destruction and inflammation. As inflammation is critical in the progression of OA, reducing inflammatory mediators may be key to preventing cartilage destruction, and some dietary constituents possessing

anti-inflammatory properties have demonstrated the ability to modulate inflammatory pathways.

Dietary and Lifestyle Factors Associated with Osteoarthritis

The end-stage treatment option for OA is a total joint replacement, and it is exorbitant in terms of economic cost and human resources (Berger et al., 2011; Murphy & Helmick, 2012). However, there are proactive strategies that can be employed to prevent and or slow the development of OA. Being aware of risk factors associated with OA; gender (female), age (>40), injury history (joint-specific), vitamin deficiencies (vitamin D and K) and body mass index (BMI > 24.9) and employing strategies to minimize risk profile are preferable to prevent and or delay disease onset and progression (Felson et al., 2000; Hootman, Helmick, & Brady, 2012; Misra et al., 2013; Sanghi et al., 2015; Zhang et al., 2014). Incorporation of physical activity in the form of aerobic and resistance exercise has been known to delay disability, reduce pain, improve overall function, and improve quality of life for those afflicted with OA (Fransen & McConnel, 2009; Kelley, Kelly, Hootman & Jones, 2011).

Even greater benefits were observed when exercise was accompanied by weight loss of five percent or more of total body mass in overweight or obese individuals (Christensen et al., 2017; Messier et al., 2004). A combination of therapies including dietary modification (consuming a diet high in omega-three fatty acids with a macronutrient profile matching that of a typical Mediterranean diet) and the incorporation

of aerobic exercise (mild treadmill exercise for 30 minutes performed day five times a week), has been shown to decrease cartilage degeneration in a joint instability induced OA model. This was attributed to a decrease in IL-1β expression and an increase in lubricin expression that was unable to be replicated by the diet or exercise group alone, indicating that a the chondroprotective effect of diet and exercise is synergistic and requires the incorporation of both treatment modalities to be most effective (Musumeci et al., 2013). Additionally, several foods including olive oil, pineapple, red grapes, salmon, and tart cherry contain bioactive constituents with anti-inflammatory and analgesic properties that can ease symptoms associated with OA by relieving pain and reducing inflammation (Musumeci et al., 2016).

The efficacy of other dietary compounds reported to exhibit "joint protective" properties such as popular dietary supplements like chondroitin, glucosamine, avocado soybean unsaponifiables (ASU), and rosehip have not shown the ability to consistently improve patient outcomes related to pain reduction and joint mobility (McAlindon et al., 2014). However, the reported risks associated with the use of these supplements were small compared to conventional drugs used to manage OA. Thus, further research needs to be conducted to evaluate their potential benefit. Therefore, treatment modalities should focus on the prevention of cartilage destruction and aim to delay OA progression by utilizing novel dietary approaches. Delaying disease progression would greatly reduce economic costs associated with more invasive treatment options (Berger et al., 2011).

Role of Bioactive Constituents in Osteoarthritis

Bioactive compounds naturally present in fruits and vegetables, such as polyphenols have shown promise in modulating inflammatory and apoptotic pathways implicated in the pathogenesis of OA. Polyphenols, or phenolic compounds, can modulate aberrant cartilage metabolism observed in OA by targeting different biological mechanisms; by suppressing gene expression of pro-inflammatory mediators, reducing oxidative stress by inhibiting the production of free radicals and or scavenging free radicals, inducing the expression of anti-inflammatory transcription factors, and or directly inhibiting the expression of pro-inflammatory transcription factors (Shen et al., 2008).

Astragalin, a flavonol derivative of kaempferol and a polyphenol that can be found in whole tart cherry, has demonstrated the ability to suppress pro-inflammatory gene expression directly by suppressing NF-κβ and MAPK activation, and indirectly by increasing activation of the peroxisome proliferator-activated receptor-gamma (PPARλ), an anti-inflammatory antagonist of the proinflammatory transcription factors NF-κβ and activating protein-1 (AP-1) (Ma, Piao, Wang, & Liu, 2014). Rutin, a derivative of the flavonol quercetin, has demonstrated the ability to protect against lipid peroxidation by scavenging the free radical, hydrogen peroxide (H_2O_2), and by increasing anti-inflammatory gene expression via induction of the SIRT1 pathway (Na, Song, Kim, & Kwan, 2016).

Fisetin, a ubiquitous plant flavonoid, isolated from apples, mangoes, grapes, strawberries, peaches, tomatoes, and onions dose-dependently decreased the IL-1β induced production of the pro-inflammatory mediators: NO, PGE_2, IL-6, and TNF-α in OA explants (Zheng et al., 2017). OA explants treated with fisetin, also exhibited a dose-dependent decrease in the protein and gene expression of the pro-inflammatory enzymes iNOS and COX-2 and the cartilage degrading enzymes MMP-3, MMP-13, and a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5), suggesting phenolic compounds can exert a regulatory effect at the gene level. Tart cherries also contain a high quantity of flavonoids along with other polyphenolic compounds; therefore, its use may produce similar outcomes that could be equally beneficial in the treatment of OA.

Additionally, most in vitro studies based on fruit derived phenols have focused on the effect of a single type of polyphenol, not the effects of total phenolic composition of the whole food (Dave et. al, 2008; Lei et. al, 2012; Liu et al., 2010; Shakibaei et al., 2008; Shen et. al, 2012). Resveratrol, a single polyphenol isolated from grapes is the most widely studied bioactive compound, but it only represents the stilbene class of phenolic compounds. Resveratrol has demonstrated the ability to reduce the production of PGE_2, which is of clinical importance because increases in PGE_2 have been shown to disrupt cartilage metabolism (Dave et al., 2008).

However, the potential antioxidant and chondroprotective effects of other polyphenols belonging to classes of flavonoids, phenolic acids, tannins, and lignans are less know. Given that each polyphenol differs in their respective molecular structure, the proposed benefits may occur via different biochemical or physiological mechanisms (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Cherries and grapes both contain anthocyanins, which belong to the flavonoid group. Tart cherries also contain the phenolic derivative, hydroxycinnamic acid. The effect of phenolic acid derivatives such as hydroxycinnamic acid, and or total phenolic composition of whole fruit(s) on OA models are less known. Phenolic acids are reported to have higher antioxidant properties so their mechanism of action may differ significantly from that of grape-derived polyphenols (Fang et al., 2009).

One such phenolic acid, protocatechuic acid (PCA), found in pomegranates has demonstration therapeutic potential in *in vitro* studies. The therapeutic benefit has been attributed to the compounds ability to promote a healthy phenotypic expression in mature articular chondrocytes (Luo et al., 2015). In the condition of OA, articular chondrocytes are exposed to environmental stressors that can cause the cells to dedifferentiate. This process can cause the chondrocytes to undergo a shift phenotypic expression from one favoring repair and maintenance to a catabolic, hypertrophic phenotype expression favoring cartilage erosion and membrane mineralization (Pitsillides & Beier, 2011; Sandell & Aigner, 2001). Protocatechuic acid demonstrated the ability to promote

chondrocyte proliferation, prevent dedifferentiation of articular chondrocytes, and promote the retention of a stable non-hypertrophic phenotype thereby favoring healthy cartilage turnover and repair (Luo et al., 2015).

Healthy matrix turnover was attributed to the ability of PCA to modulate the expression of the chondrogenic transcription factor SRY-related HMG-box-9 (Sox9), as an increase in the synthesis of the ECM components, type II collagen and aggrecan were increased, and a robust increase in glycosaminoglycan synthesis accompanied this. Similar regulation/modulation of phenotypic expression in articular chondrocytes was also observed with the green tea catechin, epigallocatechin-3-gallate (Huang, Liu, Liu, Wu, & Zheng, 2015; Luo et al., 2015). As an extraction of total polyphenols contained within tart cherries would be used as our treatment, it could contain various compounds each with the potential of exhibiting different chondroprotective mechanisms that can be of benefit in inflammatory conditions, such as OA.

Tart Cherry Polyphenols

Tart cherries contain polyphenols in the forms of flavonoids and phenolic acids, which are known for their high antioxidant capacity (Ignat et al., 2011). All polyphenols possess at least one aromatic ring containing one or more hydroxyl groups. However, variations in number, type, and position of functional groups attached to the aromatic ring(s) dictate their subsequent classification. Phenolic acids differ from flavonoids in the number of aromatic rings the compound contains, as phenolic acids contain a single ring

(Kirakosyan et al., 2009). Flavonoids contained in TCP can be sub-classified into flavonols (kaempferol, quercetin, and isorhamnetin) and anthocyanins (cyanidin and peonidin), which contain a ketone group and flavanols (catechin and epicatechin) which do not. The structural diversity of the antioxidants in tart cherry may be exhibit chondroprotective properties via multiple mechanisms (Manach et al., 2004; Ignat et al., 2011).

In a clinical study performed by Schumacher et al. (2013), consumption of tart juice has been shown to decrease knee pain scores in individuals with OA, and its action may be attributed to the compounds in tart cherry that reduce systemic inflammation. The reduction in inflammation was associated with the ability of tart cherry juice to reduce oxidative stress as indicated by a systemic decrease in C-reactive protein (CRP) and this decrease was attributed to tart cherries high antioxidant capacity (Pearle et al., 2007; Schumacher et al., 2013). In healthy adults, consumption of Bing sweet cherries (280 grams/day) for a month significantly decreased plasma concentrations of several biomarkers associated with chronic inflammatory diseases. Decreases in receptor for extracellular newly identified ligand for the receptor for advanced glycation end products (EN-RAGE), CRP, ferritin, plasminogen activator inhibitor-1(PAI-1), endothelin-1 (ET-1), interleukin-18 (IL-18), and epidermal growth factor (EGF), and an increase in the plasma concentration of IL-1Ra, a receptor agonist that inhibits the activation of the proinflammatory IL-1β signaling cascade were observed. These findings indicate that

regular consumption of cherries can modify one's risk profile for the development of several chronic diseases such as cardiovascular disease, type II diabetes, and metabolic syndrome in healthy adults. This protective effect may extend to other diseases with an inflammatory basis like OA (Kelley et al., 2013).

Consumption of tart cherry juice also attenuated inflammatory and oxidative stress responses following prolonged endurance exercise by decreasing serum levels of IL-6, CRP, and thiobarbituric acid reactive species (TBARS), an indicator of lipid peroxidation, and increasing total antioxidant status post-exercise (Howatson et al., 2010). Despite these promising findings, consumption of tart cherry juice has yet to demonstrate the ability to protect against exercise-induced muscle damage, as indices of muscle damage remained unchanged. However, consumption of tart cherry juice did promote muscle recovery following intense aerobic and resistance-based exercises an effect attributed to the ability of tart cherries to attenuate the secondary muscle damage response by reducing local inflammation and alleviating oxidative stress (Bell, Stevenson, Davison, & Howatson, 2016; Bell, Walshe, Davison, Stevenson, & Howatson, 2015; Bowtell, Sumners, Dyer, Fox, & Mileva, 2011; Howatson et al., 2010; Howatson & van Someren, 2008). Therefore, the antioxidant and anti-inflammatory properties of tart cherry polyphenols may be of particular benefit in chronic inflammatory conditions such as OA. Thus, this study will seek to find if whether the antioxidant and anti-inflammatory

properties of tart cherry polyphenols can promote cartilage health under inflammatory conditions.

CHAPTER III

METHODOLOGY

Extraction and Quantification of Tart Cherry Polyphenols

Freeze-dried tart cherry powder was used for the extraction and lyophilization of tart cherry polyphenols (TCP). Polyphenol extraction began with freeze-dried polyphenol powder (20 g) diluted in 80% ethanol (200 mL) and sonicated for 20 minutes under pulsated nitrogen gas. The solution was then filtered using a vacuum system with a Buchner funnel and Whatman No. 2 filter paper. The filter was then rinsed with 50 mL of 100% methanol to ensure residual was incorporated into the filtrate. Following collection of the filtrate, a rotary evaporator was used at 40°C to remove the solvent. The remaining liquid was then frozen in a shell at -80°C before being attached to the Freeze Dryer (Labconco Inc; Kansas City, MO) for 48-hrs to undergo lyophilization. The lyophilized polyphenol powder was stored in an opaque container and stored at -80°C until it was reconstituted for the quantification of total phenolic concentration and preparation of subsequent treatment doses.

Total phenolic concentration of the lyophilized polyphenol powder was determined spectrophotometrically, by obtaining the optical density (OD) of gallic acid standards generated in Folin-Calteau assay as demonstrated by Kim, Chun, Kim, Moon, & Lee (2003). The tart cherry polyphenols and gallic acid standards were read at 750 nm. The lyophilized tart cherry polyphenols were reconstituted in deionized water for the Folin-Calteau assay. Upon quantification of total polyphenol content contained within the

lyophilized TCP powder, a concentrated stock of TCP was prepared in basal L-15 media at a final concentration of 500 µg/mL. The mother stock was then diluted to create subsequent experimental doses.

Cell Culture Techniques

Human chondrocytes (SW1353) derived from a primary grade II chondrosarcoma, acquired from American Type Culture Collection (ATTCC-HTB-94; Manassa VA), were used for the proposed experiments. These cells were selected to represent an *in vitro* model of cartilage cells in monolayer. The cells were taken out of cryopreservative storage in the Nutrition and Food Science department at Texas Woman's University and cultured. The cells were cultured in complete Leibovitz's L-15 Medium. Complete growth media contained growth serum and antibiotics in the following concentrations 10% (vol/vol) fetal bovine serum (FBS) and 5% (vol/vol) penicillin streptomysin, respectively.

Leibovitz L-15 and FBS were purchased from HyClone Laboratories Incorporated (Logan, UT) through Thermo Fisher Scientific (Waltham, MA). Chondrocytes were incubated at 37°C without CO_2 in a 25 cm^3 flask. After reaching 80% confluency, cells were trypsinized with 1 X Trypsin-EDTA solution obtained from ATCC and sub-cultured in a 75 cm^3 flask. Cells were stored in 95% growth medium and 5% dimethyl sulfoxide (DMSO) obtained from ATCC and cooled to a temperature of 0°C before placing in liquid nitrogen for long-term storage. Basal medium or medium free of growth serum and

antibiotics was used in control and experimental treatments after the initial seeding of the cells to plates.

Cell Viability

The cells were cultured in complete L-15 medium. Upon reaching confluency, the cells were plated in a 96-well plate at a density of $2.5x10^4$ cells per well. After 24 hours, the cells were treated with various doses of TCP obtained from diluting the TCP mother stock to the desired experimental concentrations. Basal media free of TCP was used as the control dosages. After the cells were exposed to the TCP treatment for 24-hours, the cells were stimulated with the proinflammatory cytokine, IL-1β, at a final concentration of 20 ng/mL per a well. The unstimulated control had additional basal media administer to ensure each well contains the same volume of media. The cells were then exposed to the IL-1β for 24 hours before cell viability assay was conducted.

All controls and treatments were incubated for a total of 48 hours with a 24-hour pre-treatment period with tart cherry polyphenols occurring prior to 24-hour stimulation period. Evaluation of cell viability was conducted using the 96-well plate aqueous cell proliferation assay, CellTiter 96®, (Promega; Madison, WI). Upon completion of the 48-hour incubation/stimulation period, the media was removed and the plate was washed with Hank's Balanced Salt Solution (HBSS) twice to remove any residual constituents from the experimental treatment. One hundred microliters of basal media was added back to each well, followed by 20 µL of the working solution (Cell Titer 96®) and incubated

for 3 hours before being read. The plate was read at 490 nm using the using a Biotek Synergy H1 series microplate reader. The assay was repeated until a dose-dependent response was observed and a lethal dose was established. The lethal dose was the TCP concentration at which 50% of the cells were no longer viable, also known as LD 50 ($OD_{dose}/OD_{control}$ x 100 <50). Using the data collected from the Cell Titer assay, the optimal experimental dosages TCP were determined to be at 1.25 2.5, 5, and 10 µg/mL.

Quantification of Nitrite in Cell Media

Cells were cultured in 96-well plates at a density of $2.5x10^4$ cells/well in 100 µL of media. After 24 hours, the cells were treated with TCP at concentrations of 0, 1.25, 2.5, 5 and 10 µg/mL. Twenty-four hours later, the cells were stimulated with IL-1β by adding 10 µL of 220 ng/mL to each well. The final concentration of IL-1β was 20 ng/mL. Two controls were utilized: one unstimulated control and one stimulated control (IL-1β at a final concentration of 20 ng/mL). The unstimulated control was administered 10uL of basal media to ensure uniform volume across the plate (the final volume of each well 110 µL.

Upon completion of the treatment and stimulation period, samples from the two controls and four experimental doses were collected to quantify total nitric oxide content via quantification of its enzymatically reduced counterpart, nitrite. Media samples were then prepped to conduct Measure-iT High Sensitivity Nitrite Assay (Molecular Probes: Eugene, OR). Reference standards containing a known concentration of nitrite were

prepared by diluting 110 µM nitrite standard to the final concentrations of 0, 2.75, 5.50, 11, 22, 33, 44, and 55 µM. The well plate was then loaded with 100 µL of Measure-iT nitrite quantitation reagent before adding 10 µL of each standard and sample in duplicate. The plate containing the nitrite quantitation mixture was then incubated at room temperature for 10 minutes before adding the Measure-iT quantitative developer. After incubation, 5 µL of the developer was added to each well and incubated for another ten minutes at room temperature. Upon completion of the second incubation period, the plate was read at an excitation/emission of 365/450 nm using the Biotek Synergy H1 series microplate reader. The standards were used to generate a linear fit standard curve to determine nitrite concentration based on sample absorbance.

Quantification of Inducible Nitric Oxide Synthase Via In-Cell Enzyme Linked Immunosorbent Assay (ELISA)

Cells were cultured in 96 well plates (2.5x104 cells/well), treated, and stimulated using the same procedure as the previous experiment. Treatment media was removed, and the cells were fixed to the plate using 30% Formalin solution (100 µL/well). The plate was incubated at room temperature for 15 minutes. The Formalin was then removed, and the plate was washed twice for five minutes on a plate shaker using 100 µL of Tris-buffered saline (TBS). All subsequent washes lasted for 5 minutes and occurred on a plate shaker. After the washes, the Permeabilization (100 µL/well) was added and incubated at room temperature for 15 minutes. This was followed by one wash with TBS

and another incubation of 20 minutes after adding the Quenching solution (100 µL/well). Another wash with TBS followed this incubation, followed by blocking the fixed cells with blocking buffer (100µL/well) for 30 minutes at room temperature. After removing the blocking buffer, 50 uL of primary iNOS antibody (1:600 dilution) was added to each well. The primary iNOS antibody was obtained from Cell Signaling (Danvers, MA).

A plate sealer was applied, and the plate was incubated overnight at 4℃. The plate was then washed three times with wash buffer and diluted (1:400) horseradish peroxidase (HRP) conjugate was added (100µL/well) and incubated at room temperature for 30 minutes. After removing excess diluted HRP conjugate, the plate was washed three times with wash buffer. Following the final wash, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (100µL/well) was added and incubated for 15 minutes before stop solution was added (100µL/well). The plate was then read at 450 nm using a Biotek Synergy H1 microplate reader. Optical density was used to observe iNOS expression in relation to the controls. Results were expressed as percent increase and decrease relative to control-IL-1β, and average ODs per each treatment dose in the statistical analysis.

Quantification of Glycoprotein-39(YKL-40) in Cell Media

Samples were collected upon completion of the 48-hour treatment and stimulation period with controls (unstimulated control and stimulated control) and experimental TCP doses (1.25, 2.5, 5, and 10 µg/mL). The concentrations of YKL-40 contained in the media samples were determined using a MicroVue YKL-40 Enzyme Immunoassay

(Quidel; San Diego, CA). Glycoprotein-39 standards, standard controls, and media samples were added to the microwells containing YKL-40 specific antibody. Upon completion of loading, 100 µL of capture solution was added and allowed to incubate for one hour. A series of plate washes (4 washes) with 1X Wash buffer followed this incubation. Following the final wash, 100 µL of enzyme conjugate was added to the plate to incubate one hour. After the second incubation, the plate was washed four more times (using 1X wash buffer) before 100 µL of substrate solution was added to each well. Once the substrate solution was added, the plate was incubated one final time for an hour. The stop solution was added prior to the reading of the plate at 405 nm using the Biotek Synergy H1 microplate reader. A standard was used to generate a linear fit standard curve to determine YKL-40 concentration based on sample absorbance.

Cell Lysate and Total Protein Determination

Cells were plated in six-well plates at a density of 6 x 10^5 cells/well. Treatment doses were added the following day. Twenty-four hours after the administration of treatment doses, the cells were stimulated with IL-1β at a final concentration of 20 ng/mL and incubated at 37℃ another 24 hr. Following the 48-hour treatment and stimulation period, the cells were lysed using RIPA buffer (Cell Signaling). Protease inhibitors (FabGennix Inc.; Frisco, TX) were added to ensure proteins remain intact. For lysate preparation, each well was loaded with 400 µL of radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific; Waltham, MA) and scraped prior to removal. The

residual buffer was then aliquoted into separate microcentrifuge tubes and incubated on ice for five minutes before sonication. Each sample was sonicated for 10 seconds, three times. The samples were then centrifuged for 10 minutes at 12000xg, and the supernatant containing the cell lysate specimens were collected and stored at -80℃.

Protein levels were determined using Thermo Scientific BCA Protein Assay Kit. Cell lysate specimens and protein standards (25µL) were added to 96-well plate. Working solution (200 µL) was then added to each well and mixed on a plate shaker for 30 seconds. The plate was then incubated at 37˚C for 30 minutes. The plate was then read on the microplate reader at 562 nm. The standards containing the known protein concentrations were then used to generate a linear fit standard curve to determine the total protein concentration of cell lysate specimens based on sample absorbance. Due to low protein concentration, the cell lysates were then lyophilized and reconstituted to a concentration of 50 µg protein/10 µL of cell lysate specimen.

Western Blot Analysis

The reconstituted cell lysate specimens were reduced with β-mercaptoethanol (2.5%), and Laemmli buffer (Bio-Rad; Hercules, CA) was added as the coloring agent. The reduced lysate specimens were then heated for 5 minutes at 70°C on a heating block and stored at -80°C. Samples remained in storage until Western blot analysis was performed. Protein samples were loaded and separated on a 10% SDS-polyacrylamide gel

(125 volts) and electroblotted on to a nitrocellulose membrane. Equal protein transfer was confirmed using Bio-Rad Ponceau S staining.

Following staining, the membranes were blocked in 5% milk Phosphate Buffered Saline (PBS) for two hours after which it was washed with PBS three times for five minutes before being incubated with the primary antibodies overnight. NF-κB antibody (Cell Signaling) contained a 1:500 dilution of NF-κB antibody and 1:1000 dilution of the reference protein, β-actin (Cell Signaling). The MMP-3 antibody (Cell Signaling) contained a 1:500 dilution of MMP-3 (Cell Signaling) antibody and 1:1000 dilution of β-actin. Following overnight incubation period, membranes were washed three times with PBS with Tween 20 (PBST) for 10 minutes. Membranes were then incubated with IRDye secondary antibodies (Licor; Lincoln, NE) at a dilution 1:15,000 for 1 hour at room temperature. Three washes with PBST followed this incubation followed by one final wash with PBS (each wash occurred for 10 minutes). Proteins were detected with Enhanced Chemifluorescence Imager by Licor Odyssey® CLx imaging system. The band intensities were quantified by Image Studio™ software and normalized versus β-actin as an internal control for total protein loading. The relative protein expression levels were expressed as the ratio of band intensities to β-actin.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0. Descriptive statistics included means, medians, and standard error, and significance was set at 0.05 ($p < 0.05$).

One-way ANOVA with Tukey's honest significance test was used to identify differences between treatments and control(s) for each outcome. Cell proliferation and viability had a sample size of 10. Quantification of nitrite and glycoprotein-39 (YKL-40) in cell media had a sample size of three and samples were plated in duplicate to ensure consistency. Quantification of inducible nitric oxide synthase via In-cell ELISA had a sample size of seven. All western blot data had a sample size of three. All experiments were performed a minimum of three times.

CHAPTER IV

ANTI-INFLAMMATORY AND CHONDROPROTECTIVE ROLE OF TART CHERRY POLYPHENOLS IN SW 1353 HUMAN CHONDROCYTES

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ABSTRACT

The aim of this study was to investigate the chondroprotective effect of tart cherry polyphenols (TCP) using human chondrocytes. Chondrocytes (SW 1353) were pre-treated with TCP at concentrations of 1.25, 2.5, 5, and 10 µg/mL for 24 hours and then stimulated with the recombinant interleukin-1β (20 ng/mL) for 24 h. After an initial increase ($p < 0.05$) in cell proliferation at 1.25 µg/mL TCP, a dose-dependent decrease in cell proliferation was observed with higher doses of TCP. Following a significant decrease in nitrate levels at the 1.25 µg/mL TCP dose, there was a dose-dependent increase in nitrate concentration. The dose-dependent increase in nitrate levels occurred with concomitant decreases in inducible nitric oxide synthase (iNOS) intracellular protein expression at the two highest TCP doses. A significant decrease in cartilage degradation was observed in the 2.5, 5, and 10 µg/mL TCP doses, as indicated by decreases in glycoprotein-39 (YKL-40) levels. TCP appeared to have no significant effect on total

protein expression of the pro-inflammatory transcription factor, necrosis factor kappa B (NF-κB), or the matrix-degrading enzyme, matrix metalloproteinase-3 (MMP-3). In conclusion, these findings suggest that TCP may promote cartilage health by modulating pro-inflammatory pathways implicated in extracellular matrix destruction, as indicated by the effect of TCP doses on nitrite, iNOS, and YKL-40 levels. However, further research is need to elucidate the mechanism by which TCP elicit these effects on cartilage health.

KEY WORDS: Chondroprotective; tart cherry polyphenols, chondrocytes, osteoarthritis

INTRODUCTION

Arthritis affects approximately 54.4 million Americans (22.7% of the adult population) and of those afflicted 2 in 5 experience disease-related declines in function resulting in the inability to perform day-to-day activities [1]. Over half the physician diagnosed arthritis (30.8 million) is attributable to osteoarthritis (OA), and it is the 13th leading cause of disability worldwide [2,3]. OA is characterized as a degenerative disease affecting all structural components of the joint tissue; articular cartilage, synovium, and subchondral bone [4]. Although, the cause of OA is unknown it is likely due to the complex array of interactions between genomic, cellular, biochemical, and mechanical forces present within the joint organ system [5]. Sustained inflammation is highly implicated in the development of OA, with increases in the presence of pro-inflammatory molecules such as, interleukins (ILs), tumor necrosis factor alpha (TNFα), matrix metalloproteinases (MMPs), and nitric oxide (NO) [6-10]. Glycoprotein-39 (YKL-40), a

biomarker of both inflammation and cartilage degradation has also been found to be elevated in OA, both locally and systemically [11,12].

Acetaminophen, duloxetine, non-steroidal anti-inflammatory drugs (NSAIDs), selective cyclooxygenase-2 (COX-2) inhibitors, and capsaicin are common pharmaceutical agents used in the treatment of OA [13]. However, these treatments are relegated to managing pain symptoms of OA, not for modifying disease course by delaying or preventing total joint failure. Additionally, prolonged use of the most commonly used treatment options such as the use of acetaminophen and NSAIDs has been linked to liver damage and gastrointestinal concerns [13]. Plant-derived bioactive compounds such as polyphenols pose a minimal risk while potentially exerting therapeutic effects beyond acting as an analgesic. Polyphenols have demonstrated the ability to modulate aberrant cartilage metabolism observed in OA by (1) suppressing gene expression and downregulating the synthesis of pro-inflammatory cytokines; (2) inhibiting the production of free radicals; (3) inducing the expression of anti-inflammatory transcription factors and; (4) inhibiting cytokine-induced activation of apoptotic signaling pathways [14-24].

Specifically, polyphenols present in tart cherries have structural diversity and may be able to exhibit unique chondroprotective properties via multiple mechanisms [25,26]. In a small clinical study, the administration of tart cherry juice was shown to improve measures related to joint function and decrease knee pain in individuals with OA [27].

Furthermore, the functional and symptomatic improvements corresponded with decreases in plasma concentrations of the inflammatory biomarker, C-reactive protein (CRP) [27]. These findings indicate that tart cherry consumption may have a role in improving clinical outcomes by modulating pro-inflammatory pathways implicated in chronic inflammatory conditions, such as OA. In addition to improving symptoms associated with knee OA, consumption of tart cherry juice has also been shown to promote recovery from intense exercise, by reducing inflammation and oxidative stress by boosting antioxidant capacity [28-30].

Other plant-derived polyphenols such as, resveratrol and curcumin have consistently exhibited chondroprotective properties by utilizing several distinct molecular mechanisms. The mechanisms include inhibiting the expression of nuclear factor kappa B (NF-κB), a powerful proinflammatory transcription factor; inhibiting cytokine-induced activation of apoptotic signaling pathways (Bcl-2, Bcl-xL, and caspase-3); inhibiting the expression of pro-inflammatory mediators (NO, interleukins, and TNF-α) and enzymes (iNOS, COX-2, MMP-3, and MMP-13) [14,16,17, 20-22]. Based on the available evidence, it is plausible that TCP may exert similar chondroprotective mechanisms. Therefore, the aim of this research study was to investigate the *in vitro* effects of TCP on chondrocytes under inflammatory conditions.

MATERIALS AND METHODS

Extraction and Quantification of Tart Cherry Polyphenols

Freeze-dried tart cherry powder provided by Van Drunen Farms (Momence, IL) was used for extraction of TCP. Initially, 20 g of freeze-dried cherry powder was dissolved in 200 mL of solvent (80% ethanol). The solution was then sonicated for 20 minutes under pulsated nitrogen to minimize oxidation. Following sonication, the solution was filtered through Whatman No. 2 filter paper using a vacuum system. The filter was then rinsed with 50 mL of 100% ethanol that was also incorporated into the filtrate. The solvent was then removed from the filtrate using a rotary evaporator at 62°C, and the residual was lyophilized and stored at -20°C.

Total polyphenol concentration was determined using the Folin-Calteau assay as described by Kim et al. using gallic acid as the standard [31]. Upon quantification of the total phenolic concentration of the TCP powder, a concentrated stock of TCP was prepared in sterile basal Lebovitz L-15 media at a final concentration of 500 µg/mL. The mother stock was diluted to obtain the desired treatment doses for all subsequent experiments.

Cell Culture

Human SW 1353 chondrosarcoma cells were purchased from American Type Culture Collection (ATTCC-HTB-94; Manassa, VA). Cells were cultured in 25 cm^3 flasks in Leibovitz L-15 (Hyclone; Logan, UT) complete growth medium containing 10%

(vol/vol) fetal bovine serum (Hyclone) and 5% (vol/vol) penicillin streptomysin (Thermo Fisher Scientific; Waltham, MA) at 37 °C without CO_2. After reaching 80% confluency, cells were trypsinized with 1X Trypsin-EDTA (ATCC) and sub-cultured into a 75 cm^3 flask for subsequent experiments. For subsequent experiments basal or incomplete Leibovitz L-15, free of FBS and antibiotics was utilized.

Cell Viability

Human SW 1353 chondrosarcoma cells were seeded into a 96-well plate at a density of 2.5 x 10^4 cell/well in 100 µL of basal Leibovitz L-15 media. Cells were pretreated with TCP doses of varying concentrations (1.25, 2.5, 5, 10, 20, and 40 µg/mL) for 24 before being stimulated with IL-1β at a final concentration of 20 ng/mL for an additional 24 hours (total treatment window was 48 hours). Following the 24-hour stimulation with IL-1β, media was removed, and cells were washed with Hank's Balanced Salt Solution (HBSS) (Thermo Fisher Scientific) twice, before the sequential addition of 100 µL basal Leibovitz L-15 media and 20 µL CellTiter 96 Aqueous One Solution (Promega; Madison, WI) to each well. Following a three-hour incubation period (at 37 °C), the absorbance of the plate was read using H1 series microplate reader (Biotek: Winooski, VT) at 490 nm. Cell viability was assessed as the percent increase or decrease in optical density (OD) for each TCP doses relative to the unstimulated control. This data was used to determine a dose-response curve of TCP and for the selection of

the treatment doses for experiments. Tart cherry polyphenols at concentrations of 1.25, 2.5, 5, and 10 µg/mL were used for all subsequent experiments.

Quantification of Nitrite in Cell Media

Cell culture media was collected after the 48-hour treatment and stimulation period as described above. The nitrite concentrations were determined using Measure-iT High Sensitivity Nitrite Assay (Molecular Probes, Eugene, OR) following the manufacturer's instructions. Briefly, the media samples from all TCP treatment doses and nitrite standards were added to a 96-well plate and incubated with Measure-iT quantitation reagent (10 µL) for 10 min at room temperature before the addition of the quantitative developer (5 µL). Following this incubation, the plate was read using the Biotek Synergy H1 series microplate reader at excitation/emission of 365/450 nm. The nitrite concentration was determined using a linear fit standard curve based on sample absorbance.

Quantification of iNOS Expression Via In-Cell ELISA

iNOS expression was evaluated using Colorimetric In-Cell ELISA (Thermo Fisher Scientific). Following treatment and stimulation, the culture media was removed, and the cells were fixed to the plate using 30% Formalin solution, the plate was washed twice with 1X Tris-buffered saline (TBS) before permeabilization solution was added to the plate, and the plate was incubated for 15 minutes. Following this incubation, the plate was washed once with 1X TBS. Quenching solution was added, and the plate was

incubated for 20 minutes. The quenching solution was then removed, and the plate was washed once with TBS before blocking buffer (100 µL) was added to each well and incubated for 20 minutes. The plate was washed once again (with TBS) and blocking buffer was added to incubate for 30 minutes. The blocking solution was removed, and 50 µL of primary iNOS antibody (Cell Signaling; 1:600 dilution) was added to each well and incubated overnight at 4°C. The following day, the plate was then washed 3 times with 1X Wash buffer before adding horseradish peroxidase (HRP) conjugate (1:400 dilution) and incubated at room temperature for 30 minutes. The plate was then washed three times with 1X Wash buffer, and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added (100 µL/well). The reaction was stopped 15 minutes later using the Stop Solution (100 µL/well). The plate was then read at 450 nm using the Biotek Synergy H1 microplate reader. Optical density of the TCP doses was used to quantify iNOS activity relative to the stimulated control cells.

Quantification of Glycoprotein-39 (YKL-40) in Cell Media

Following the same treatment protocol as described before, culture media was collected upon completion of the treatment and stimulation period for the determination of glycoprotein-39 (YKL-40) concentration. Quidel's MicroVue YKL-40 Enzyme Immunoassay was used in accordance with the manufacturer's instructions. Briefly, YKL-40 standards and samples were added to the microwells coated with YKL-40 specific antibodies. Capture solution was added to each well (100 µL) and allowed to

incubate for one hour, followed by a series of plate washes with1X wash buffer. Following the final wash, 100 µL of enzyme conjugate was added to each well to incubate for one hour. The plate was then washed four more times before the substrate solution (100 µL) was added to the plate. Once the substrate solution was added, the plate was incubated one final time for one hour. The stop solution was added prior to the reading of the plate at 405 nm using the microplate reader. The standards were used to generate a linear fit standard curve to determine YKL-40 concentrations of the TCP doses and controls.

Cell Lysate and Total Protein Determination

Cells were seeded in 6-well plates at a density of 6x 10^5 cell/well in 2 mL of basal Leibovitz L-15 media. Treatment doses and time were the same as the previous experiments. Following the treatment and stimulation period, cells were lysed using (400 µL) radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling). Protease inhibitors (FabGennix; Frisco, TX) were added to preserve protein integrity, and the wells were scraped. The residual was then collected and aliquoted into separate micro centrifuge tubes and incubated on ice for five minutes. Samples were then sonicated three times for 10-second intervals before undergoing centrifugation at 1200xg for 10 minutes. The supernatant was collected, and the lysate specimens were stored at -80°C. Protein concentration was determined using BCA Protein Assay Kit (Thermo Scientific). Due to

low protein concentration, the cell lysates were lyophilized and reconstituted to a concentration of 50 µg protein/10 µL of cell lysate specimen.

Western Blot Analysis of NF-κB and MMP-3

The reconstituted cell lysate specimens were reduced with β-mercaptoethanol (2.5%), and Laemmli (Bio-Rad) was added as a coloring agent. The specimens were then heated for 5 minutes at 70°C on a heating block and stored at -80°C. All specimens remained in storage until Western blot analysis was performed. Protein samples were loaded and separated on a 10% SDS-polyacrylamide gel (125 volts) and electroblotted onto a nitrocellulose membrane. Protein transfer was confirmed using Bio-Rad Ponceau S staining. Membranes were then blocked for two hours in 5% Milk Phosphate Buffered Saline (PBS) before being separately incubated with 1:500 dilution of the primary antibodies; NF-κB (Cell Signaling) or MMP-3 (Cell Signaling). A 1:1000 dilution of β-actin (Cell Signaling) was added with each separate primary antibody and served as an internal control to correct for differences in sample loading.

Following overnight incubation, the membranes were washed thoroughly with PBS with Tween-20 (PBST) and then incubated with a 1:15,000 dilution of the secondary antibody, IRDye (Licor) for one hour at room temperature in low light condition. This incubation was followed by a series of washes with PBST and a final wash with PBS. Proteins were then detected using the Enhanced Chemifluorescence Imager by Licor

Odyssey® CLx imaging system, and band intensities were quantified by Image StudioTM software and normalized versus β-actin.

Statistical Analysis

Cell viability and iNOS expression assays were performed twice at sample sizes of at least seven ($n \geq 7$) per treatment dose. Samples collected for the nitrite and YKL-40 determinations were plated in duplicate to ensure consistency within each sample collected and had a sample size of three (n=3). Statistical analysis was performed using SPSS 16.0. Descriptive statistics include means, medians, and standard error (SE) of each continuous variable. Significance was set at 0.05 ($p<0.05$). One-way ANOVA was used to determine differences between treatment doses of TCP and control(s) for each outcome. Tukey's honest significance test was used to identify significance.

RESULTS

Cell Viability

Cell viability was assessed utilizing a CellTiter 96 Aqueous One Assay and was used to identify a dose-dependent response for our TCP doses and to establish the lethal dose (the TCP concentration at which fifty percent of cells were no longer viable). The dose-dependent effect of TCP on cell viability is shown in Figure 1. With IL-1β (20 ng/mL) stimulation, there was a significant decrease ($p<0.001$) in cell viability when compared to the unstimulated control. The lowest dose of TCP (1.25 µg/mL) in the presence of IL-1β elicited a significant increase ($p<0.001$) in cell viability (an increase of

21.5%) in comparison to the stimulated control. In the stimulated chondrocytes, pretreatment with TCP at 1.25 µg/mL restored cell viability to levels similar to that of the unstimulated control. At TCP doses exceeding 1.25 µg/mL, a significant decrease in cell viability was observed.

Effect of TCP on Nitrite Concentrations and iNOS Expression

The lowest dose of TCP (1.25 µg/mL) resulted in a decline in nitrite concentrations. In comparison to IL-1β stimulated cells, all subsequent doses (2.5, 5, and 10 µg/mL) resulted in significantly higher nitrite concentrations. There was no significant difference in iNOS expression between the stimulated and unstimulated control. However, there was a significant decrease in iNOS expression at the 2.5 and 10 µg/mL TCP doses with an 18% and 16% decrease respectively, in comparison to the stimulated control (Figure 2A). Decreases in iNOS expressions did not correspond to reduction in nitrite concentrations.

Effect of TCP on Glycoprotein-39 (YKL-40) Concentrations in Cell Media

As expected, the concentration of YKL-40, a marker of cartilage degradation increased significantly in the IL-1β stimulated cells. Figure 3 depicts a dose-dependent decrease in YKL-40 concentrations with TCP (2.5, 5, and 10 µg/mL) treated cells.

Effect of TCP on NF-κB andMMP-3 Using Western Blot Analysis

Figure 4A shows the effect of tart cherry polyphenols on NF-κB expression in IL-1β stimulated chondrocytes. There was a two-fold increase in the protein expression of

NF-κB in the highest TCP dose (10 10 µg/mL) relative to the stimulated control; however, it did not achieve a level of significance. The protein expression of NF-κB was not affected by the doses of TCP (1.25, 2.5, 5, and 10 µg/mL). The protein expression densities for NF-κB were corrected by the housekeeping (β-actin) densities (Figure 4A) across all TCP doses and stimulated control cells.

Protein expression of MMP-3 in TCP treated cells relative to the stimulated control is shown in Figure 4C. There was an increase in MMP-3 expression at 1.25 µg/mL TCP dose, which was subsequently lower at higher doses of TCP. However, there was no significant difference between any of the doses of TCP in comparison to IL-1β stimulated control.

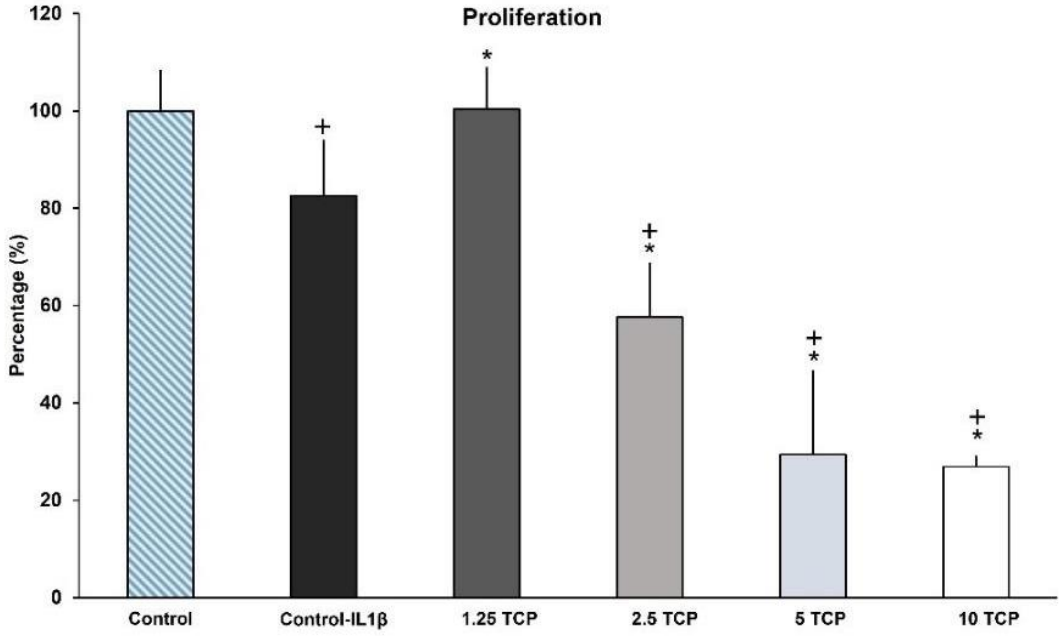

Figure 1. Dose-dependent effect of tart cherry polyphenols of cell proliferation with IL-1β stimulation. Analyzed by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (n=10). * denotes statistical significance ($p<0.05$) compared to Control. + denotes statistical significance ($p< 0.05$) compared to Control-IL1β.

Nitrite and Inducible Nitric Oxide Synthase Quantification

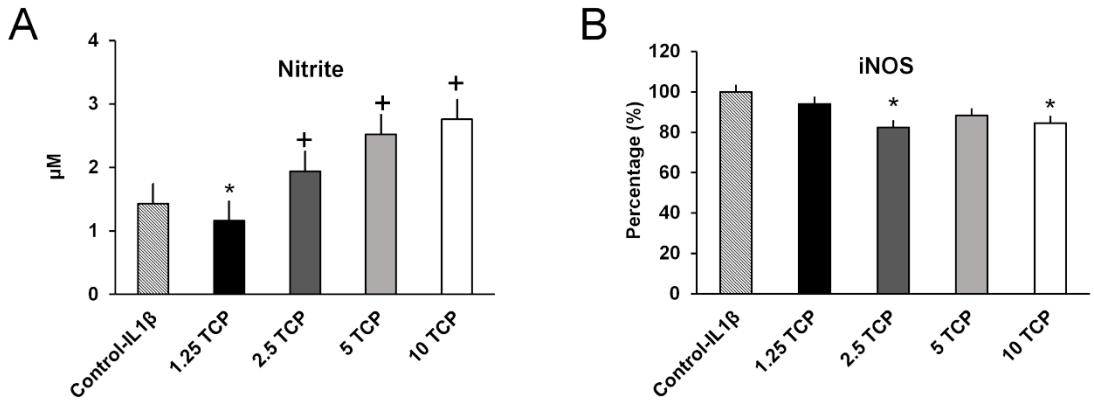

Figure 2 A. Effect of tart cherry polyphenols (TCP) on nitrite concentrations in cell media. Evaluated by Measure-iT High Sensitivity Nitrite Assay after the completion of the 48-hour treatment and stimulation period (n=3). B. Effect of TCP on iNOS expression. Evaluated by In-Cell colorimetric Elisa after 48-hour treatment and stimulation period (n=7). * denotes significant ($p<0.05$) decrease compared to Control-IL-1β. + denotes significant ($p<0.05$) increase compared to Control-IL-1β

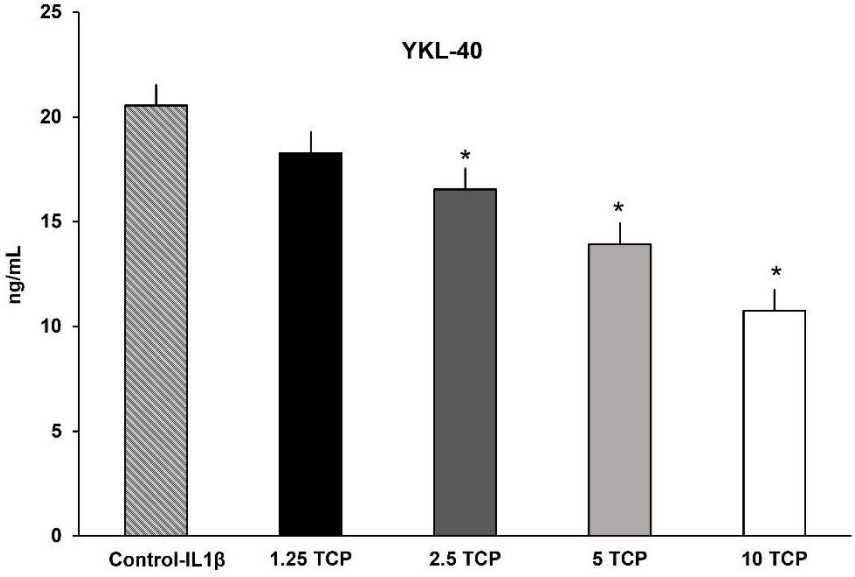

Figure 3. Effect of tart cherry polyphenols on YKL-40 concentrations in cell media. Evaluated by MicroVue YKL-40 Immunoassay. Cell media was collected after the

completion of the 48-hous treatment and stimulation period (n=3). * denotes significant ($p<0.05$) decrease compared to Control-IL-1β.

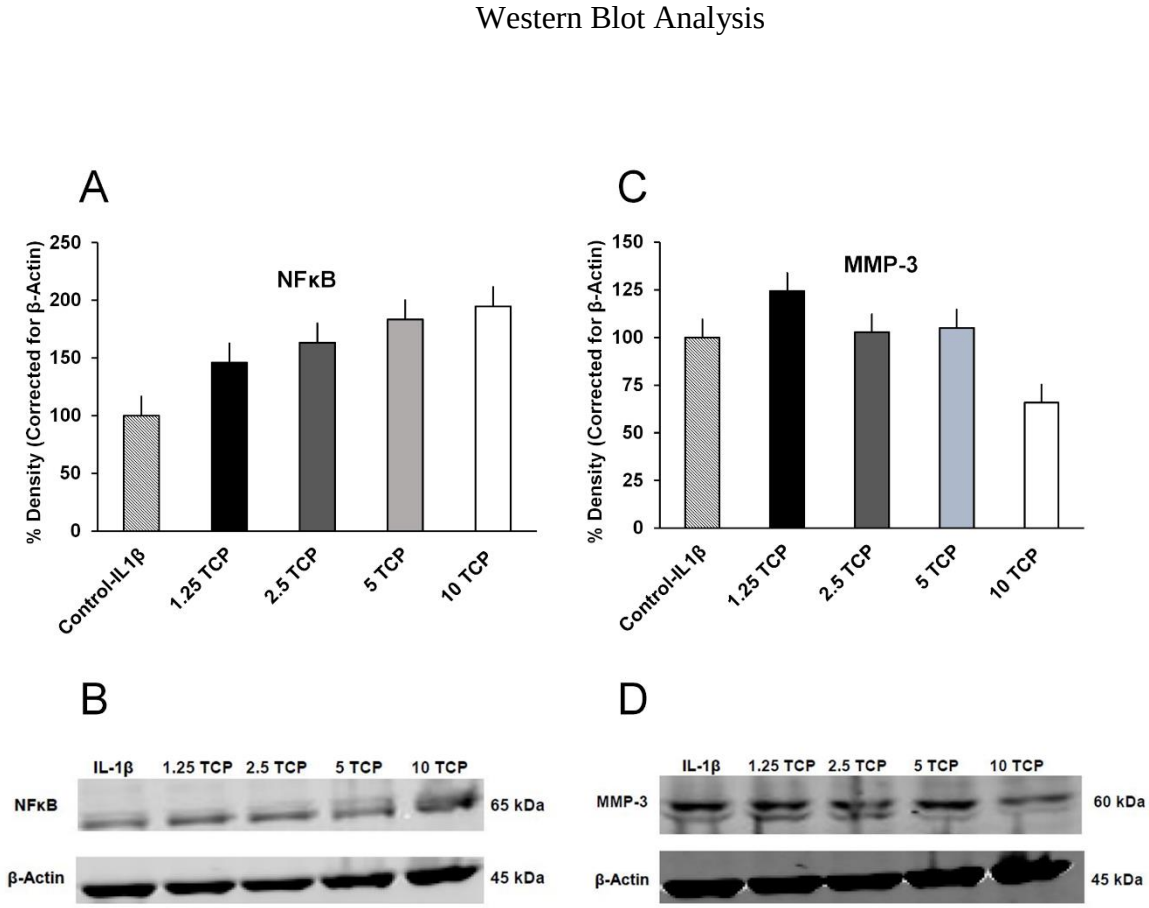

Figure 4 A. Effect of tart cherry polyphenols (TCP) on NF-κB protein expression. B. Representative immunoblot of NF-κB C. Effect of TCP on MMP-3 protein expression. D Representative immunoblot for MMP-3. All Western blot analysis was conducted on cell lysates collected after 48-hour treatment and stimulation with tart cherry polyphenols and IL-1β, respectively. Each Western blot was performed in triplicate. Protein densities of NF-κB and MMP-3 were expressed as ratios relative to β-actin to correct for any potential differences in the loading of the samples.

DISCUSSION

The anti-inflammatory and chondroprotective role of tart cherry polyphenols (TCP) was examined using human chondrocytes. Chondrocytes stimulated with IL-1β had a significant increase in nitrite, a reduced metabolite of nitric oxide (NO) concentration in comparison to the unstimulated control. These findings are consistent with other studies evaluating the effect of IL-1β on NO synthesis in chondrocytes [7, 32,33]. The lowest dose (1.25 μg/mL) elicited a significant decrease in NO production; however, at higher doses a dose-dependent increase in NO production was observed. These findings differ from previous studies in which a dose-dependent decrease in NO production was attributed to a concurrent decrease in iNOS expression with the administration of fruit-derived polyphenols, fisetin and resveratrol [18,34]. Our findings indicate that the decrease in NO production at low doses of TCP occurred independent of the modulation of iNOS protein expression. Within the context of our study, TCP at low doses exhibits a capacity for scavenging NO and or scavenging upstream reactive oxygen species (ROS) implicated in the production of NO [35]. The steep increase in NO production as observed in our study may have been responsible for decreased proliferation and cell viability at the higher doses of TCP. Aberrant NO production is implicated in triggering an apoptotic signaling cascade through the induction of the p38 MAPK pathway [36].

In vivo studies have shown that inhibition of mechanistic target of rapamycin (mTOR), a master regulator of cellular growth, proliferation, and survival, decreases the severity of experimental OA [37,38]. Thus, the induction of apoptosis in prehypertrophic, hypertrophic, and dedifferentiated articular chondrocytes may be of therapeutic benefit in instances of abnormal cartilage metabolism. The benefit of inducing cell death in cells exhibiting hyperplasia, a crucial feature involved in the pathogenesis of rheumatoid arthritis, has been documented in synoviocytes [39,40]. However, the potential benefits of inducing apoptosis in abnormal chondrocytes have not yet been investigated.

Unfortunately, in our study, we did not utilize methods to differentiate between differences in the phenotypic expression of our cells. Cell culture studies that allow for such distinction in cell type and or function typically rely on the expression of cartilage-specific genes, like aggrecan and type II collagen, or the chondrogenic transcription factor, Sox9 [41]. Future studies should incorporate gene expression to evaluate if TCP may be of value in the regulating phenotypic expression of articular chondrocytes, which is known to be dysregulated in OA.

The biomarker YKL-40 is strongly indicative of cartilage destruction, and it has been found to be elevated locally in both synovial and cartilage tissue in individuals with OA [12,42]. Our findings depicted a significant decrease in concentrations of YKL-40 by 19.5%, 32.3%, and 52.3%, respectively as the doses of TCP increased. These findings are similar to those reported by Hooshmand et al., who found a decrease in YKL-40

concentrations in lipopolysaccharide (LPS) stimulated chondrocytes pretreated with the isoflavone, genistein. Genistein is a flavonoid abundant in soybeans and has demonstrated the ability to modulate arachidonic acid metabolism by suppressing the expression of key regulatory enzymes lipoxygenase-2 (LOX-2) and COX-2 [24,43]. However, Hoosmand et al. findings with genistein at various doses did not achieve statistical significance. Despite various studies evaluating the efficacy of YKL-40 as a biomarker for cartilage destruction, it has not been used extensively to assess cartilage degeneration in response to different treatment modalities [44-46]. Despite this limitation, YKL-40 is still an important biomarker, as is can be used to assess cartilage-specific degradative changes.

There appeared to be no significant effect of TCP on the protein expressions of NF-κB or MMP-3. These findings were inconsistent with other *in vitro* studies using curcumin or resveratrol, in which the protein and or gene expression of NF-κB and MMP-3 were significantly decreased [16,21,22]. A dose-dependent increase in NF-κB expression was observed in our study; however, the increase in protein expression was not considered statistically significant. These findings conflict with those of Singh et al. and Ahmed et al. both of whom found significant decreases in IL-1β induced NF-κB expression in treated OA explants. The former used epigallocatechin-3-gallate isolated from green tea, and the latter used an anthocyanin-rich pomegranate fruit extract from their respective polyphenol treatments. Both studies found a decrease in NF-κB binding

activity and protein expression [47,48]. However, Ahmed et al. attributed this decrease in NF-κB expression to the inhibition kappa B protein subunit alpha (IkBα); Whereas, Singh et al. did not see any effect on IkBα activation with epigallocatechin-3-gallate. These findings suggest that there may be different mechanisms by which structurally unique polyphenols modulate the activation of the NF-κB pathway. A decrease in the expression and activation of the NF-κB pathway is of therapeutic interest because it regulates the transcription of proinflammatory (iNOS, COX-2) and catabolic (MMP-1, MMP-3, MMP-13, aggrecanase-1, and aggrecanase-2) enzymes implicated in the pathogenesis of OA [16, 48,49]. Although a non-significant increase in the protein expression of NF-κB was observed in our study, it does not necessarily translate to the transcription of genes regulated by NF-κB. As NF-κB requires phosphorylation of its transactivation subunit, p65, to become fully activated [23,50]. It is possible that TCP could still modulate the activation of the NF-κB pathway by inhibiting the activation of p65, not by decreasing the total protein expression of NF-κB. Conversely, if a decrease in the activation of the NF-κB pathway occurred, a decrease in some of its transcriptional end products such as MMP-3 could occur. Such is the case in our study where MMP-3 levels decreased with the TCP doses. Thus in the future, incorporating western blot analysis of active (phosphorylated) and inactive (dephosphorylated) p65 in relation to total protein expression of NF-κB could be of value in evaluating potential therapeutic mechanism.

A non-significant, dose-dependent decrease in the protein expression of MMP-3 was observed in our study. Decreases in proteoglycan and type II collagen content are early pathological changes associated with cartilage destruction. These decreases result from an increase in the expression of MMPs [51-53]. The collagenase, MMP-13, is associated with extensive and irreversible damage to the collagen network; however, this destruction is typically preceded and or accompanied by extensive proteoglycan loss [54]. Given the lack of the MMP-13's specificity for proteoglycans, it is likely that MMP-3 plays an indirect yet significant role in the destruction of the collagen network since MMP-3 degrades the protective proteoglycans surrounding type II collagen fibrils [55]. As type II collagen destruction represents an irrevocable step in cartilage degeneration, any slight decrease in MMP-3 expression may prove to be clinically relevant despite only seeing a modest decrease in MMP-3 protein expression as observed in our study with TCP.

The microenvironment of cell culture is not reflective of the pathological differences inherent in diseased tissue and therefore; it presents a limitation in regards to clinical implications of our findings. Explants collected from OA tissue have a greater propensity to express pro-inflammatory genes in response to stimulation with IL-1β due to the development and subsequent expression of the pathogenic phenotype associated with the disease state [56,57]. Therefore, stimulation with IL-1β in the cell model we utilized may have been insufficient to increase the expression and or activation of

proinflammatory pathways implicated in the pathogenesis of OA. This assertion may be supported by the lack of difference detected in the protein expression of NF-κB or MMP-3 between our unstimulated and IL-1β stimulated cells (data not shown). In lieu of using OA explants, perhaps future studies could replicate this series of experiments with the current cell culture using additional inflammatory mediators such as LPS or TNF-α. This approach may result in a more robust activation of the inflammatory pathways associated with OA and enable us to see a more significant treatment effect.

In conclusion, TCP had modest benefits on cartilage metabolism, as indicated by lower YKL-40 concentrations. At lower doses, TCP appears to improve cell viability by acting as an antioxidant as indicated by the decrease in nitrite concentration independent of inducing changes in the expression of iNOS. Tart cherry polyphenols appeared to have no effect on protein expression of the proinflammatory gene regulator, NF-κB, and had marginal effect on MMP-3. In summary, our *in vitro* findings suggest that the chondroprotective effect of TCP was potentiated through its antioxidant role and not so much as its impact on the inflammatory pathway.

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