GENISTEIN REDUCES PRODUCTION OF

PROINFLAMMATORY MOLECULES IN HUMAN

CHONDROCYTES

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NOMENCLATURE

CON   Control

COX   Cyclooxygenase

EIA   Enzyme immunoassay

ELISA   Enzyme immunosorbent assay

ER   Estrogen receptor

ERT   Estrogen replacement therapy

HRP   Horse radish peroxidase

IL-1β   Interleukin-1 beta

LOX   Lipoxygenase

LPS   Lipopolysaccharide

MMP   Metalloproteinase

NF-κB   Nuclear factor kappa B

NO   Nitric oxide

NSAID   Nonestradiol antiinflammatory drug

OA   Osteoarthritis

PVDF   Polyvinylidene diflouride

RA   Rheumatoid arthritis

SERM   Selective estrogen receptor modulator

SDS   Sodium dodecyl sulfate

TNF-α   Tumor necrosis factor alpha

CHAPTER I

INTRODUCTION

Osteoarthritis (OA) is the most common joint disorder and its etiology is

relatively unknown. After age 45, women experience a more dramatic increase in the

incidence of OA compare to men. The gender difference in prevalence of OA has been

linked to ovarian hormone deficiency. Intraarticular injections of estradiol has been

shown to upregulate both estrogen receptors in condylar cartilage at early stages of OA in

ovariectomized rabbits(1;2). A recent *ex vivo* study (3) indicated that postmenopausal

women with knee OA had elevated estrogen levels in knee synovial fluid without notable

differences in circulating estrogen levels.

Although the etiology of OA is not completely known, it is believed that

production of proinflammatory cytokines in the joints may play a crucial role in the

pathogenesis of OA. Management of OA's symptoms is currently focused on reducing

pain and inflammation through nonestradiol antiinflammatory drugs (NSAIDs) or other

agents. The findings of some recent studies (4;5) have suggested that plant flavonoids

attenuate inflammation and the immune response through their inhibition of important

regulatory enzymes in arachidonic acid metabolism. Flavonoids are powerful inhibitors

of cyclooxygenase-2 (COX-2) activity(5;6) . These antiinflammatory properties of

flavonoids provide the rationale for investigating the role of isoflavones in conditions

such as OA. A recent clinical study by Arjmandi and colleagues (7) indicated thatsoy

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protein supplementation delivering 88 mg isoflavons mainly in the form of genistein, a naturally-occurring selective estrogen receptor modulator (SERM), was effective in alleviating overall OA symptoms and reducing pain medication use. The major isoflavone in soy is genistein, which has been referred to as naturally occurring selective estrogen receptor modulator with resemblance to synthetic SERMs such as tamoxifen, raloxifene, and ipriflavone. Tamoxifen (8), raloxifene (9) and ipriflavone (10) have been shown to have beneficial effect on cartilage metabolism and alleviate OA symptoms. The purpose of the presentstudy was to determine whether genistein inhibits the production of proinflammatory molecules in lipopolysaccharide (LPS)-stimulated chondrocytes.

Hypothesis

The hypothesis of this study was that genistein dose-dependently suppresses the production of LPS-induced proinflammatory molecules in human chondrocytes.

The specific aims of this research were as follows:

1. To investigate the extent to which genistein reduces LPS-induced production of proinflammatory molecule, i.e. COX-2, nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β).

2. To explore whether the antiinflammatory properties of genistein in chondrocytes, in part, is through estrogen receptors. For this purpose ICI-182,780, an estrogen receptor (ER) antagonist, was used.

3. To compare the antiinflammatory properties of genistein against a known selective inhibitor of COX-2.

4. To examine whether genistein restore formation of glycoprotein synthesis in LPS stilmulated chondrocytes by measuring, YKL-40, a marker of cartilage degradation.

CHAPTER II

REVIEW OF LITERATURE

<u>Osteoarthritis</u>

Epidemiology and Prevalence

Osteoarthritis (OA) is the leading cause of disability in the elderly population. In the United States, it afflicts 13% of individuals between the ages of 55 and 64 and 17% of those 65 to 74 years old (11). Currently, 20 million people in the U.S. are affected by OA and this number is predicted to exceed 59 million by the year 2020 (12). Increasing prevalence of OA with age is further aggravated by the lack of treatment to reduce the overall cause of OA. It is the most common joint disorder, both in the U.S. and throughout the world, with relatively unknown etiology. Exhausting a major proportion of health care dollars, OA ranks second in health care problems of the developed world (13). The relative economic impact of musculoskeletal conditions is growing rapidly with the passage of time, having reached 2.5% of the gross national product in 1992. The prevalence of OA is higher in men than in women up to approximately age 45, however, after age 45 the reverse is true (14). The epidemiologic definition of OA encompasses symptoms, disability, and structural changes associated with this disorder. Epidemiological data focus not only on the structural disease but also characterizes the radiographical definition with the presence and absence of symptoms. The most widely used system for grading the severity of the radiograph is known as the Kellgren-

Lawrence grading system (14). This grading defines the degree of osteophyte formation from 0 to 4 but is unclear on the issue of grading the knee with joint space narrowing without the presence of osteophytes.

Incidence of Osteoarthritis

Reports on the percentage of people who have been diagnosed with osteoarthritis based on medical history, examination, or radiographic evaluation confirm a striking increase in the prevalence of OA of the hand, foot, knee, and hip joints with increasing age (15;16). In the Framingham study population, it was found that the percentage of people who have mild, moderate, or severe radiographic changes indicative of osteoarthritis in at least one joint increases progressively from less than 5% in individuals younger than 25 years of age to more than 80% in individuals more than 75 years of age (17;18). More specifically, those with moderate or severe radiographic changes in relation to OA in at least one joint increases dramatically from 5% in individuals younger than 45 years of age to 40% in those 75 years and older (15). Regardless of the relationship between age and OA, the widespread view is that osteoarthritis results from normal wear and tear.

Unlike the joint destruction seen in other rheumatic joint diseases with major systemic inflammatory components such as rheumatoid arthritis (RA), OA consists of a regressive sequence of changes in the cells and matrix that results in the loss of structure and function of articular cartilage accompanied by attempts at cartilage repair and the remodeling of underlying bones (19;20). Because of these repair and remodeling processes, the degeneration of the articular surface in OA is not uniformly progressive, and the rate of degeneration varies among individuals and among joints (21;22). On

occasions, this degeneration may occur rapidly, but in most joints it progresses slowly over many years. However, in some cases, this process may stabilize or even decrease spontaneously with partial restoration of the articular surface and a resulting decrease in symptoms.

<u>Primary and Secondary Osteoarthritis</u>

Primary or idiopathic osteoarthritis is the degeneration of articular cartilage and alterations in single or multiple joints due primarily to aging and wear and tear of the tissue. This results in the loss of structure and function of the articular cartilage causing pain and loss of motion (23). In an increasing number of individuals, OA is classified as secondary on the basis that the joint degeneration is mainly as a result of traumatic joint injury or from developmental, metabolic, and systemic disorders which destroy the articular surface (23). This generally progressive loss of articular cartilage is accompanied by attempted repair of the cartilage, remodeling and sclerosis of the underlying subchondral bone, and in many cases the formation of bone cysts and marginal osteophytes. The diagnosis of OA requires the presence of symptoms and signs that may include; joint pain, restriction of motion, crepitus with motion, joint effusions, and deformity (24). Although OA is most prevalent in the foot, knee, hip, spine, and hand joints (25-27), it can affect any synovial joint as well.

Osteoarthritis develops most frequently in the absence of a known cause (primary OA) and less often it develops as a result of a joint injury, infection or one of a variety of hereditary, developmental, metabolic and neurologic disorders (secondary OA). The age of onset associated with secondary OA depends on the underlying cause, thus, it may develop in young adults and even children as well as the elderly (28). In contrast, there is

a strong association between the prevalence of primary osteoarthritis and increasing age. The tools to assess the prevalence of osteoarthritis have numerous limitations, including difficulty in defining and establishing the diagnosis and in evaluating more than a few synovial joints in each individual (25-27).

Biology of Articular Cartilage

Composition of Cartilage

Articular cartilage appears histologically to be a simple inert tissue and has a firm slick surface that resists deformation (29). Under the light microscope, the articular cartilage consists primarily of extracellular matrix, with only one type of cell, known as chondrocytes. Cartilage is a non-vascular tissue as it lacks blood vessels, lymphatic vessels, and nerves. In comparison with tissues such as muscle and bone, it has a low level of metabolic activity and appears to be less responsive to changes in loading or to injury (30-32). The morphology of articular cartilage shows that it has a highly intricate and ordered structure and that a variety of complex interactions between the chondrocytes and the protein matrix actively maintain the integrity of the tissue. The articular cartilage is composed of cells, water, and a matrix macromolecular framework, and it is this framework that provides the cartilage its mechanical properties (30;32). Chondrocytes make up about two percent of total volume of cartilage and hence they are considered a small part of the total volume of cartilage (33).

Chondrocytes

Within the articular cartilage, there is only one specialized type of cell known as chondrocyte (29;33). Chondrocytes from different locations of the articular cartilage

differ in size, shape and activity (34). These cells contain organelles necessary for matrix synthesis and in some cases have short cilia extensions, which may play a role in sensing mechanical changes in the matrix. Chondrocytes are surrounded by extracellular matrix and do not form cell-to-cell contacts. These cells in their mature stage are spheroidal in shape and synthesize type II collagen, large aggregating proteoglycan, and specific non-collagenous proteins to form cartilaginous matrix (34;35). Individual chondrocytes are quite active metabolically but the total activity of the cartilage tissue is quite low due to the low cell density (33;35;36).

In relation to the mechano-structural properties of the articular cartilage, chondrocytes appear to remain unchanged in location, appearance, and activity. The type of macromolecules that form the matrix and the concentration of water are important to the structural response of this tissue (37). Maintenance of the articular surface requires turnover of the matrix macromolecules, which involves the continuous replacement of degraded matrix components and alteration in the macromolecular framework of the matrix in response to the use of the joint (37). To achieve these activities, the chondrocytes must sense the changes in the matrix due to degradation as well as changes in macromolecules in relation to the demands placed on the articular surface (38). The cells respond by synthesizing appropriate types and amounts of the macromolecules (30).

Structural Organization of Cartilage

The primary function of the chondrocytes is to synthesize and organize collagens, proteoglycans and the non-collagenous proteins into unique and highly ordered matrix structure to form articular cartilage (30;32). The composition, organization, and mechanical properties of this matrix as well as the chondrocyte morphology and function

vary according to the depth of the articular surface. These changes are identified in layers called zones and there are four zones known as superficial, transitional, middle (radial), and a zone of calcified cartilage (29). The zones differ in respect to their concentration of water, proteoglycan, and collagen including the size of the aggregates. Cells in these zones also differ in size, shape, and orientation relative to the articular surface (29). This also causes a variation in the degree of metabolic activity between the zones. Chondrocytes respond differently to mechanical loading which suggests that the development and maintenance of articular cartilage under normal conditions depends partly on the differentiation of the distinct populations of these cells across the zones (30;32).

Pathogenesis, Clinical Features, and Risk Factors of OA

Osteoarthritis is generally viewed as a degenerative disorder involving cartilage degradation in aging (39;40). Typically, the degenerative changes are accompanied by a local inflammatory component that may accelerate joint destruction (39;41). Hyaline articular cartilage is made up of a matrix of type II collagen fibers and proteoglycans, as well as the chondrocytes that produce the matrix, and water (39;42). In OA, the proteoglycan content of the cartilage is gradually depleted, leading to an initial increase in water content and a loss of compressibility and shock absorption, and culminating in fibrillation and breakdown (42).

The main clinical features of OA are pain, stiffness, loss of function, and ultimately joint deformity. Any of the joints may be affected in isolation or as part of primary generalized OA. The most common joints involved in OA include those of the knee, hands, feet, hips, and the apophyseal joints of the spine. OA is primarily a disorder

of the hyaline articular cartilage that coats the bony surface of all synovial joints and the meniscus (43).

In addition to age, a number of other factors have been suggested as risk factors for the development of OA including: genetics, obesity, joint hypermobility, joint trauma, immobilization, peripheral neuropathy, crystals in joint fluid or cartilage, and repetitive joint overuse (13). The pathological processes and the radiological features vary to some degree across the different joints. Therefore, OA is generally considered to be a heterogeneous disorder and no two joints may be afflicted equally in the same individual. Predominance of generalized OA in women suggests that changes in production and nature of estrogens may be risk factors in the development of OA.

Changes in the Diseased Joint

Morphological Changes

Osteoarthritis involves all of the tissues that form the synovial joint, including articular cartilage, subchondral and metaphyseal bone, synovial tissue and ligaments, joint capsule, and muscles that surround the joint (44). However, the primary changes consist of loss of articular cartilage, remodeling of subchondral bone, and formation of osteophytes (45;46). The earliest histological changes in OA include the superficial zone of the articular cartilage and extend into the transitional zone (47;48). There is decreased staining for proteoglycans in these regions and protrusion of blood vessels from subchondral bone as a result of increased remodeling. Investigators suggest that the stiffening of subchondral bone due to this remodeling causes the degeneration of articular cartilage (47). Alternatively, the loss of articular cartilage could increase the level of mechanical stress on the underlying bone, causing aggressive bone remodeling to occur

(47). However, it is not certain as to what is the correct order of these events. But in most instances, the degeneration of articular cartilage and the remodeling of subchondral bone are both present when symptoms develop and it is this loss of cartilage that leads to the direct loss of joint function.

Stages of Joint Degradation

Many of the mechanisms responsible for the progressive loss of cartilage in degenerative joint disease remain unknown. However, this process can be divided into three overlapping stages which include disruption or alteration of the cartilage matrix, the chondrocyte response to tissue damage, and the decline of the chondrocytic response to restore or maintain tissue leading to loss of articular cartilage (21;49) The first stage is when there is an overall disruption of the matrix framework and an associated increase in the concentration of water (50). The collagen meshwork may also be damaged and the degree of proteoglycan aggregation decreases (21). These changes proceed to the response of the chondrocytes in the second stage by increasing synthesis and decreasing the degradation of the matrix. Rate of degradation is controlled by the balance in activity between both anabolic and catabolic growth factors and cytokines (46).

In this second stage of the development of OA, the repair mechanisms may increase the synthesis of matrix macromolecules and to a lesser extent cell proliferation which can counter the catabolic effects and stabilize or in some instances restore the tissue (21). This repair response may continue for years and in some individuals reverse the course of osteoarthritis at least temporarily. The failure to stabilize or restore the tissue leads to the third stage in the development of OA (46;50). In the third stage, there is a progressive loss of articular cartilage as well as a notable decline in the anabolic

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response of the chondrocytes (51). This decline can result from mechanical insults and chondrocyte death which no longer can be supported by a stable and functional matrix (21). There is also a down-regulation of the chondrocytic response to anabolic growth factors and cytokines and an increase in synthesis and accumulation of molecules in the matrix such as catabolic cytokines and growth factor-binding proteins which decrease the overall anabolic effect (51). The loss of articular cartilage leads to the symptoms of pain and joint dysfunction associated with OA. This loss occurs most frequently with increasing age, possibly because age related changes in the cartilage matrix and the decrease in the chondrocytic anabolic response compromise the ability of the tissue to maintain and restore itself (12;21;32).

Proposed Role of Estrogen in Osteoarthritis

Epidemiologic studies have indicated that women over the age of 50 are the most vulnerable to joint disorders, especially OA. Sex hormones may play a role in the development of OA in women (52). The prevalence of OA increases in women following menopause and rises faster with age in women then in men (53-55). Tsai and colleagues (8) have suggested that excessive levels of synovial fluid estrogen are responsible for the development of OA in humans. In some animal studies, when estrogen was administered directly to the knee joint, it resulted in an increased frequency and severity of OA (1;2). Furthermore, Tsai and Lui (2) have shown that intraarticular injections of estradiol to ovariectomized rabbits induced an up-regulation of estrogen receptors in condylar cartilage at an early stage of OA and cartilage degeneration and erosion at the late stage. In OA, looser matrix might be synthesized in the medial compartment, allowing

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molecules in the synovial fluid, including estrogens to be taken up by the chondrocytes, and therefore resulting in cell death and cartilage erosion (2). Estrogen may affect articular cartilage by modulating the synthesis of the cartilage matrix and the production of matrix enzymes (56). Estrogen receptors are found in articular cartilage (3;57), but estrogen may also influence cartilage metabolism via cytokines (58). Estrogen has also been suggested to influence the development of OA through its effects on bone metabolism (59;60).

In partially menisectomized rabbits, estrogen accentuates the frequency and severity of OA. Estradiol also modulated articular cartilage metabolism by suppression of DNA and proteoglycan synthesis (56). Furthermore, intraarticular injection of estradiol to the knee joint impaired lactate dehydrogenase resulting in an overall disruption of matrix collagen (56) in cartilage tissue. These observations imply that estrogen can affect chondrocyte metabolism and proliferation, possibly through a receptor-mediated mechanism (56).

There are a number of epidemiological studies that have examined the possible relationship between estrogen replacement therapy (ERT) and risk of OA. Several investigators reported that women who at one time have been on ERT have a lower than expected risk of knee or hip OA (61-65). Prospective cohort investigation of the Framingham study indicated that the use of ERT had a moderate protective effect against worsening of radiographic knee OA among elderly women (66). Although evidence from these studies may suggest that estrogen protects against OA, there are important limitations to be considered. Women on ERT tend to be generally healthier, thinner and more active, as well as better educated than non-users. These characteristics as well

others may benefit the estrogen users and make them less prediposed to have OA than the non-users. Currently there are no published clinical trials on ERT in OA. Longitudinal studies are needed in which estrogen users and non-users can be followed and carefully characterized for OA.

Estrogen may also play an important role in male OA. Higher synovial estradiol and higher estrogen receptor levels in cartilage of individuals with OA compared to non-OA may be primary reasons for the incidence of OA in males as well (8;57). The negative effects of estradiol in synovial fluid may be counterbalanced by endogenous testosterone, resulting in a lower estrogen/testosterone ratio or a lower unopposed, free estradiol to interact with cartilage.

Estrogen Receptors and Selective Estrogen Receptor Modulators (SERM)

Estrogen Receptors

Estrogen has been shown to act genomically by coupling with the estrogen receptor and its coactivators to induce changes in gene expression. 17β-estradiol (E_2) and the estrogen receptors have been shown to form a ligand-estrogen receptor complex (67). The challenge in understanding the mechanisms by which estrogen exerts its effects is due to the existence of its two receptor subtypes, estrogen receptor (ER)-α and ERβ. The two receptors share considerable homology but differ in the C-terminal ligand binding domain and its N-terminal transactivation domain (68). Paech and colleagues (67) have demonstrated that the transcriptional effects elicited by E_2 are determined by whether or not it interacts with ERα or ERβ. The two receptors exhibit different responses to drugs such as tamoxifen and raloxifene which are categorized as either anti-estrogens or SERM. While estrogen induces negative transcription regulation, SERM induce positive

regulatory sequences when interacting with ERβ. This may explain, in part, how SERM are able to induce beneficial estrogenic effects on bone and cardiovascular without dramatically increasing the risk of cancer of the reproductive organs.

Tissue Specific Actions of SERM

The levels of expression for each ER subtype vary from organ to organ, and bind estrogen with varying affinity. The ratio of ERα to ERβ in different vascular beds vary and this may explain the different responses exerted by SERM in various tissues (69). The two well known SERM, tamoxifen and raloxifene, are non-steroidal phenylethylene derivatives that have been shown to bind to ERβ with higher affinity than ERα (70). In some cases, SERM mimic the effects of estrogen, while in other cases, antiestrogenic effects are observed (70). They were first characterized as antagonists/partial agonists on the basis of their binding to ERα. However since then they have been shown to act as full estrogen agonists in bone through their interaction with ERβ. The differential estrogenic effects of SERM vary, as tamoxifen has been shown to have estrogenic actions in the uterus, while raloxifene does not (70). Thus the acronym SERM takes into account the selective modulations of the ER in specific tissues (70). The selective action of both natural and synthetic SERM and their interaction with each of the ER subtype across different tissues needs to be further investigated.

Role of Cytokines and Growth Factors in OA

Function

Cytokines and growth factors are released by cells in response to specific signals

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and influence the function of target cells by exerting a positive or negative effect on gene expression (71). Because they have a relatively short half-life, they usually exert their influence on cells in the local environment. Cytokine production and its effects on target cells are regulated in several ways. Homeostasis is maintained as a balance between a particular cytokine and various naturally occurring molecules that function as cytokine inhibitors (71). The cartilage tissue is maintained by the equilibrium between the effects of catabolic cytokines (e.g. IL-1α and β, TNF-α) which induce the production of specific matrix degrading metalloproteases, and anabolic growth factors such as insulin-like growth factor and transforming growth factor-β which induce the production of building blocks of cartilage such as collagen and proteoglycans (71).

In osteoarthritis, the breakdown of the joint tissue occurs in several phases (72). This depletion suggests an overall failure in the cytokine-controlled matrix homeostasis, with a shift in equilibrium between synthesis and degradation favoring catabolic processes. In the earlier stages of OA, the chondrocytes attempt to repair the cartilage by increasing the synthesis of matrix macromolecules (50). The increased presence of anabolic growth factors presumably activates the chondrocytes to stimulate the matrix synthesis. However, compositional change in the matrix molecules may interfere with this process by reducing their capacity to aggregate properly with hyaluronic acid (73). Over time, the matrix losses its resiliency and fails to withstand the mechanical stress placed on the joints. In the later stages of the disease process, enhanced cartilage degradation far exceeds the ability of the chondrocytes to synthesize new matrix. Furthermore, there is increased presence of catabolic cytokines such as IL-1β and TNF-α that potentiate the expression of matrix metalloproteinases causing proteolysis of the

cartilage matrix (74). These factors can also inhibit cartilage matrix biosynthesis (75).

Evidence is accruing to implicate cytokines in the pathogenesis of joint diseases with major inflammatory and autoimmune components (76). However the mechanisms that initiate and cause cartilage destruction in osteoarthritis remain unclear. The profile for cytokines and growth factors based on quantitative rather than qualitative differences as indicated by fewer cells being recruited for their production in osteoarthritis. This may suggest that other processes are involved in cartilage destruction, and that cytokines are merely indicators of the disease activity. However, the involvement of cytokines and growth factors is a topic of interest in relation to their function in cartilage integrity.

Catabolic Cytokines

Interleukin-1 (IL-1β) was first identified as an inflammatory mediator capable of tissue damage. Its importance in cartilage metabolism appears to be a result of its ability to suppress the synthesis of type II collagen, the predominant form of collagen found in articular cartilage (72). Furthermore, IL-1β is shown to stimulate synthesis of type I collagen, which is predominant in fibroblasts (77;78). The increased presence of IL-1β and the formation of fibroblasts suppresses the ability of chondrocytes to synthesize new proteoglycan, a building block in cartilage formation (79). For cartilage degradation to occur, catabolic cytokines such as IL-1β must act at specific receptors on the target cells (77;79). In osteoarthritis, there is increased presence and sensitivity of IL-1β receptors to interact with IL-1β and trigger the joint destruction processes.

The potency for cartilage breakdown is 10-fold less in the case of tumor necrosis factor (72). Apparently, both IL-1β and TNF-α are produced by the same cells under the

same stimuli. However, in osteoarthritis, the two cytokines are not significantly correlated during active disease(72).

<u>Markers of Cartilage Degradation</u>

A specific marker for cartilage metabolism that is secreted by cultured chondrocytes is human cartilage glycoprotein 39 (YKL-40). The name is derived from its 40 kilodalton molecular weight and the one-letter code for its 3-N-terminal amino acids; tyrosine, lysine, and leucine (80). YKL-40 is synthesized by articular chondrocytes (81-83) and synovial cells (84) in patients with both RA and OA. Histological specimens obtained from patients with OA have shown positive staining for YKL-40 in chondrocytes mainly in the superficial and middle zones of the cartilage whereas it was low or undetectable in normal cartilage samples (83). Elevated levels of both serum and synovial fluid YKL-40 are seen in patients with active RA or severe knee OA in comparison to normal subjects (80). Johansen and colleagues (80;85) demonstrated that YKL-40 was produced in response to removal of chondrocytes from their native extracellular matrix environment related to joint injury and disruption of the cartilage framework. YKL-40 production may be mediated by cytokines and growth factors that have a regulatory effect on chondrocyte function, especially in inflammatory conditions of the joints such as OA.

<u>Current Treatments and Therapies for OA-Medications</u>

Classes of Non-Steroidal Anti-Inflammatory Agents

The choice of effective treatments for individuals with OA is quite a challenge. The consensus recommendation for patients with OA has been to use acetaminophen

(86). Study by Fries and colleagues (87), compared the effects of various doses of acetaminophen with ibuprofen in patient with RA and OA. Overall, their finding indicated that those who took acetaminophen had better tolerability less pain and less gastrointestinal distress in comparison with ibuprofen. Acetaminophen is an excellent analgesic but does not possess any anti-inflammatory activity (86;87). Its mechanism of action is not clearly known but it has been shown to inhibit prostaglandin synthesis at higher doses (88). The adverse effects with the use of acetaminophen have been due to acute overdose which is associated with liver damage. Furthermore, in a case-controlled study, chronic use can lead to kidney failure in some individuals (27).

Celecoxib and Rofecoxib

The new class of non-steroidal anti-inflammatory drugs (NSAIDs) has shown vastly improved function in OA and rheumatoid arthritis patients with similar efficacy to their predecessors but with significantly decreased gastrointestinal and platelet effects (89;90). Americans use these agents on a daily basis and according to the projected statistics by the US Center for Disease Control and Prevention, it is likely that a significant increase in the prevalence of painful and degenerative conditions will likely increase the use of NSAIDs (91). The newer class of NSAIDs selectively inhibit COX-2 more so then COX-1. This makes them more potent anti-inflammatory agents for degenerative joint conditions such as OA without causing detrimental effects on the gastrointestinal tract.

The most commonly used therapeutic compounds for OA in this class are called celecoxib and rofecoxib (92). Both of these compounds are selective COX-2 inhibitors

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and have been shown to be effective in reducing or alleviating OA pain and inflammation associated with rheumatoid arthritis (90;93-96). These studies also presented clear evidence of no endoscopic gastroduodenal damage and no adverse effect on platelet aggregation (89; 90;92-96). However, many of these studies have left the renal effects of these potent COX-2 inhibitors unanswered. Several mechanisms being proposed for these anti-inflammatory compounds include their in vitro inhibition of inducible nitric oxide synthase which subsequently decreases the production of nitrite (49;97). It has also been suggested that NSAIDs, by inhibiting prostaglandin production could also be responsible in reestablishing a more normal cell cycle response which is inhibited by prostaglandins (98-100). Longer-term effects of these agents are not yet fully understood in context of their toxic load on the liver as well as their adverse effects on other tissues and organ systems.

Role of Soy Isoflavones in OA

There is evidence that flavonoids (10), in particular isoflavones, may exert beneficial effects on cartilage metabolism. Soy isoflavones have been shown to exert positive effects on cardiovascular (101)and skeletal health (102). These beneficial effects of isoflavones, in part, may be mediated through their anti-inflammatory properties(6). Studies (6) have suggested that plant flavonoids attenuate inflammation and the immune response through their inhibition of important regulatory enzymes involved in arachidonic acid metabolism. Flavonoids are powerful inhibitors of both lipoxygenase (LOX) and COX-2 activities (6). Lin and colleagues reported that flavonoids are inhibitors of IL-1β, IL-6, TNF-α, and COX-2. They also found that flavonoids downregulated matrix metalloproteinases (MMP), while upregulating MMP inhibitor in

human synovial fibroblasts. Flavonoids may thus inhibit the inflammation response through these modes of action, suggesting that this class of molecules may be effective in conditions such as OA.

Genistein, the prominent soy isoflavone, is structurally similar to tamoxifen and ipriflavone, a synthetic isoflavone(10). Tamoxifen (6) and ipriflavone (10) have both been shown to influence cartilage metabolism and reduce or alleviate the symptoms associated with OA.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

Pure human chondrocytes were purchased from PromoCell Bioscience Alive (Heidelberg, Germany). Chondrocytes were recovered and plated in complete chondrocyte growth medium (PromoCell, Heidelberg, Germany) with 10% fetal calf serum at 37 °C in a 5 % CO_2 humidified incubator in 6 well plates (n=3). Genistein (Sigma, St Louis, MO) and, ICI 182,780 (Tocris, Northpoint, UK) were dissolved in dimethylsulfoxide and added directly to the culture media in various doses of genistein (0, 50, and 100 μM) and 100 μM ICI when cells were 80% confluent. Control cells were treated only with solvent. After one hour, 1 μg/ml LPS (Sigma Diagnostics, St Louis, MO) was added to all treated, except the control (CON) cells. Nitric oxide production in cell culture medium and protein levels of COX-2 and COX-1 in cytosolic fraction were measured.

To compare the effect of genistein to that of NS-398 (Cayman chemical, Ann Arbor, MI, U.S.A.), a COX-2 inhibitor, chondrocytes were plated in 6 well plates (n=4) and treated with different doses of genistein (0, 50 and, 100 μM) and 10 μM NS-398 when cells were 80% confluent. After one hour, 1 μg/ml LPS was added to all the treatment groups, except the control (CON) group. IL-1β and YKL-40 was measured in cell culture medium.

Cell Viability Assay

Chondrocytes were plated in 96 wells in density of 15,000 cells/well in phenol red free medium and kept overnight. Cells were treated with different doses of genistein (0, 25, 50, 100 and 200 µM) and one dose of NS-398 (10 µM). After one hour incubation at 37°C in 5% CO_2, chondrocytes were treated with 1 µg/ml LPS for 24 hours. Culture medium was removed and 200 µL culture medium containing 10% resazurin (Sigma, Saint Louis, MO) was added to wells. Cells were incubated at 37°C in 5% CO_2 for 4 hours. The absorbance was measured at reference wavelength of 690 nm and subtracted from the 600 nm measurement. The number of cells was determind as a function of metabolic activity using the dye resazurin according to the manufacture's directions.

Western Immunoblotting

Chondrocytes cultures when reached 80% confluency were washed with phosphate buffer saline (PBS) twice. 300 µL of lysing buffer (100mmol/L HEPES, pH 7.9; 100 mmol/L KCL; 100 mmol/L EDTA, 100 mmol/L DTT, protease inhibitor cocktail and 10% IGEPAL) was added to each plate then plate was incubated on ice and shook at 150 rpm on a rocking platform for 10 min. Cells were then collected and centrifuged at 15000×g for 3 minutes at 4 °C. Supernatant of cytosolic fraction was collected and stored at -80 °C. For protein concentration measurement, 200 µL mixture of bicinchoninic acid and copper (49:1) was added into 5 µL cytosolic fraction using a 96 well-plate. Plate was then covered by an aluminum foil and incubated at room temperature for 20 minutes mildly being shaken using a belly dancer (Stovall Life Science, Greensboro, NS). Absorbance was measured at wavelength of 570 nm. Equal

amounts of protein (20 µg) was brought to volume of 12 µL by adding autoclave water, mixed with 12 µL loading buffer (0.125 mol/LTris, 4 % sodium dodecyl sulfate (SDS) , 20 % glycerol, 10% 2-mercaptoethanol, 0.003% bromophenol blue pH 6.8) and heated at 95-100 °C for 5 minutes. Protein was separated on 8% SDS-polyacrylamide gel for 2 hrs (100v), and transferred to polyvinylidene diflouride (PVDF) membrane (Millipore, Bedford, MA) by using a semi-dry blotting apparatus for one hour (15v). Membrane was blocked with Tris-buffered saline (TBS, 8mmol/L Tris HCL, 16mmol/L Tris-base, 150mmol/LNaCl) containing 5% skim milk for one hour. After washing in TBS, blots were incubated overnight with a 1:200 dilution of COX-2 and COX-1 (Santa Cruz Biotechnology, CA) antibody overnight. This was followed by a 2-hour incubation with 1:2000 dilution of goat polyclonal antibody and rabbit polyclonal antibody (Santa Cruz Biotechnology, CA) in blocking buffer. The protein bands were visualized using an Immun-Star HRP substrate kit (Bio-Rad laboratories, Hercules, CA). Membrane was covered by 2 mL of immuno-star HRP peroxide buffer mixed by 2 mL immuno-star horse radish peroxidase (HRP) enhancer and visualized using VersaDoc Imaging System (Bio-Rad laboratories, Hercules, CA).

Nitric Oxide Assay

Nitric oxide (NO), an important physiological messenger in local inflammation, was measured by Griess reagent system (Promega Co. Madison, WI) in culture medium . This reagent can measure nitrite (NO_2^-), which is one of the two primary, stable and nonvolatile breakdown products of NO. Chondrocytes were plated in 96 wells in density of 15000 cells/well in phenol red free medium and kept overnight at 37ºC incubator

under 95% O_2, 5% CO_2. Cells were treated with different doses of genistein (0, 50 and, 100 µmol/ml medium) and one dose of ICI (100 µmol/ml medium). After one hour incubation at 37°C in 5% CO_2, chondrocytes were treated with 1 µg/ml LPS for 24 hours. Fifty µL of each collected culture supernatant and nitrate standards (0, 0.0935, 0.187, 0.375, 0.75, 1.5 and, 3 µM) were added to 96 well plates. 50 µL medium containing 10% sulfanilamide was added into each well and then incubated for 10 minutes at room temperature away from light. Then 50 µL of 0.1 % N-1-napthylethylenediamine dihydrochloride was added to each well and incubated 10 minutess at room temperature in dark. Absorbance was measured within 30 mins in a plate reader with a filter between 250-550 nm.

Aseessement of IL-1β

Interleukin-1β was measured in culture medium with enzyme-linked immunosorbent assay (ELISA) (PromoKine Bioscience Alive, Germany) which is designed to measure free cytokines in tissue cell culture supernatants. Standards (1000, 500, 125, 31.25, 7.81 and, 0.0 pg/mL) and samples were added to monoclonal antibodies precoated microtiter plates in quantity of 100 µL. Twenty five µl of rabbit anti-human IL-1β polyclonal antibody were added into each well. After 3 hours incubation at room temperature, plate was washed with phosphate buffer saline buffer four times. Fifty µL goat anti-rabbit conjugated alkaline phosphatase was added into wells followed by 45 minutes incubation at room temperature. Plate was washed 4 times with buffer and then 200 µL of color reagent solution was dispensed into each well. The reaction was stopped after 15 minutes incubation at room temperature by adding 50 µL stop solution into each

well. Absorbance was read at 492 nm and values were reported as pg/mL.

YKL-40 EIA Kit

YKL-40, also known as human cartilage glycoprotein 39, a marker of tissue remodeling , produced by chondrcoyets (Hakala BE et al., 1993) was measured by enzyme immunoassay (EIA) kit (Metra, Quidel Co., San Diego, CA) using aliquots of cell culture supernatant. This is a sandwich enzyme immunoassay in a microtiter stripwell format. Twenty µL of cell culture media and standard (0.0, 50, 100, 200 and 300 ng/mL) was added to each streptavidin coated well. The fabricate's fragments of a monoclonal anti-YKL-40 antibody conjugated to biotin binds to striptavidin on the strip and captures YKL-40 in standards and samples. After one hour of incubation at room temperature, the plate was washed with a buffer containing sodium azide (0.05%). 100 µL of polyclonal anti YKL-40 antibody conjugated to alkaline phosphatase was added to each well to bind to the captured YKL-40, followed by one hour incubation at room temperature and washing with buffer containing sodium azide (0.05%). Then 100 µL of a diethanolamine and magnesium chloride solution containing sodium azide (0.05%) and p-nitrophenyl phosphate was added to each well and incubated at room temperature for another hour. Bound enzyme activity was detected with p-nitrophenyl phosphate as substrate and the reaction was stopped with 100 µL 1 mol/L NaOH. The absorbance was read at 405 nm with a microplate reader and values were reported as ng/mL.

Statistical Analysis

Data were analyzed using Student t-test, SAS Version 8.2 (SAS Institute, Cary,

NC) and are presented as mean ± standard error (SE). Significant differences were
determined using alpha level of 0.05.

CHAPTER IV

RESULTS

Cell Viability

Treatment with LPS (1µg/ml), genistein in doses of 0, 25, 50, 100, 200 µM, and NS-398 (10 µM) did not negatively affect the viability of chondrocytes (Figure I). Data are presented as percent of viable cells.

Protein Level of COX-2 and COX-1

LPS significantly increased the protein level of COX -2 in chondrocytes while genistein decreased the protein level of COX-2 by 8.36% in 50 µM dose and significantly $(P< 0.05)$ decreased the COX-2 level protein by 31.8% in 100 µM dose. ICI 182,780 decreased COX-2 protein level but not significantly (Figure II). Genistein treatment had no effect on COX-1 protein level (Figure III).

Nitric Oxide Production

Nitric oxide level in cell culture supernatant tended to increase as a result of LPS treatment (Figure IV). Interestingly, LG50 was more effective in reducing NO production than LG100 (42% vs. 28%) in comparison with LPS-treated control cells. ICI had no effect on NO production.

Interleukin-I Beta Production

IL-1β mean values were numerically lower in genistein-treatment groups by 36.4 % and 48% for both doses of 50 and 100 µmol/ml genistein, respectively in comparison with LPS-treated group. Overall, there were no significant differences among the treatment groups (Figure V). These findings indicate that larger sample sizes may be necessary in order to detect significant differences as a result of treatments which were applied in the present study.

YKL-40 Production

YKL-40, a marker of human cartilage glycoprotein degradation, increased in LPS-treated group. Both doses of genistein were able to suppress its levels in cell supernatant by 18.6 % and 29.3% respectively for LG50 and LG100 compared to LPS-treated group but not significantly. Vix had no effect on YKL-40 production (Figure VI).

CHAPTER V

DISCUSSION

The finding of the present study indicate that genistein, a soy isoflavone, suppress the production of proinflammatory molecules such as COX-2, NO and IL-1β in LPS-induced chondrocytes whileit has no effect on COX-1 production. These findings supporting our earlier study (7) which shows that 40 g soy protein containing 88 mg isoflavone daily for three months improved OA associated symptoms such as range of motion of the knee joint and several factors associated with pain and quality of life. Isoflavones are thought to act similar to estrogen, possibly through estrogen-receptor-mediated events. Soy isoflavones are referred to as natural SERMs (6) because of their conformational ability to bind to estrogen receptor s (ERs), particularly the beta subtypes (68), in a manner similar to other SERMs such as raloxifene. To test this hypothesis we used ICI-182, 780, an estrogen receptor anatagonist, to block the estrogen receptor and inhibit the action of genistein through estrogen receptors. Results showed thatgenistein suppresses the protein level of COX-2, in part, through estrogen receptors. However, several discrete signaling pathways have been implicated in the genesis of COX-2 synthesis that is dependent on the stimulus imposed on cells. Several studies have shown that COX-2 is partly controlled by nuclear factor kappa B (NF-κB)(6). Largo et al. (103) have shown that inhibition of NF-κB activation was related to the down regulation of the expression and synthesis of COX-2. COX is a critical proinflammatory enzyme that

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converts arachidonic acid to prostaglandins. Although prostaglandins have been implicated in the pain and inflammation associated with osteoarthritis (104), they may not fully explain either joint inflammation nor OA symptoms. Nonsteroidal anti-inflammatory drugs (NSAIDs) or COX- inhibitors have been extensively used in the treatment of OA (105;106). It has been suggested that the antiinflammatory action of NSAIDs are due to inhibition of COX-2, a cytokine-induced isoenzyme that mediate pain and inflammation, whereas the unwanted side effects such as the risk of significant injury to the upper gastrointestinal tract and lining of kidneys (107) are due to inhibition of COX-1 (106). Interestingly, in the present study genistein dose-dependently decreased the production of COX-2 protein level while it had no such an effect on COX 1.

Other proinflammatory cytokines such as IL-1β and TNF-α, are believed to cause damage to cartilage by inducing matrix metalloproteinase (MMP) expression in chondrocytes (73;108;109). Both cytokines activate synthesis and release of MMPs which leads to matrix breakdown (46). Elevated levels of IL-1β are also found in OA synovial fluid and gene expression of IL-1β has similarly been reported to be up-regulated in cartilage obtained from patients with knee OA (50).

While the findings of the present study indicate that genistein non-significantly reduces LPS-induced IL-1β in chondrocytes in a dose-dependent manner, these reductions can not be due to cytotoxicity as any of the doses of genistein had no effect on cell viability. Interleukin-1β has been shown to induce production of NO in synovial cells and chondrocytes, which leads to increased vasodilation, permeability, and cartilage resorption in arthritic joints (110). Additionally, NO inhibits proteoglycan synthesis, modulates the activity of metalloproteines, and induce apoptosis in human chondrocytes

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(111). Although in non cell cultural experimental models of inflammation and arthritis NO has been shown to either promote or prevent tissue injury (110), in chondcrocytes NO has been demonstrated to inhibit collagen and proteoglycan synthesis (110) and induce apoptosis (112). Hence, our finding that genistein suppresses the production of NO should viewed as a positive finding.

YKL-40, a marker of cartilage degradation, was numerically increased in LPS-treated chondrocytes, albeit not significantlyin the present study . Elevated serum YKL-40 levels are associated with increased cartilage breakdown (85), which is often triggered by inflammation. Immunohistochemical analysis of articular cartilage biopsy samples from the hip joint of patients with OA have shown positive staining for YKL-40 in chondrocytes. YKL-40 is synthesized by articular chondrocytes (81) of patients with OA or RA. The findings of a study by Volck B. et al. (83) indicated that YKL-40 expression in chondrocytes from normal cartilage was low or not detectable in comparison with patients with OA. In another study by Volck et al., (85) YKL-40 was detected in the inflamed synovial membrane and the number of YKL-40 positive cells were associated with the degree of synovial inflammation.

Athough both doses of 50 and 100 µM genistein numerically decreased the YKL-40 levels in LPS-induced chondrocytes by18.56 % and 29.31%, respectively, in comparison with LPS-treated cells, these values did not reach significance. It can be speculated that if our sample size was larger the differences would have become significant. The notion of anti-inflammatory effect of genistein can be indirectly supported by observations of Volck et al. (85) who injected human arthritic joints with glucocorticoid and noticed remission in joint inflammation followed by a decrease in

serum YKL-40.

In summary, the results of the present study show that genistein selectively decreases the production of LPS- induced COX-2 protein level in chondrocytes without affecting COX-1. If the results are shown to be reproducible, genistein can be of particular interest to individuals who suffer from chronic inflammatory conditions such OA. As discussed earlier, there are no pharmaceutical agents that selectively can inhibit COX-2 production without having serious side effects. Therefore, Genistein decreased LPS-induced NO production in chondrocytes though not in a dose-dependent manner. Genistein also numerically decreased IL-1β and YKL-40.
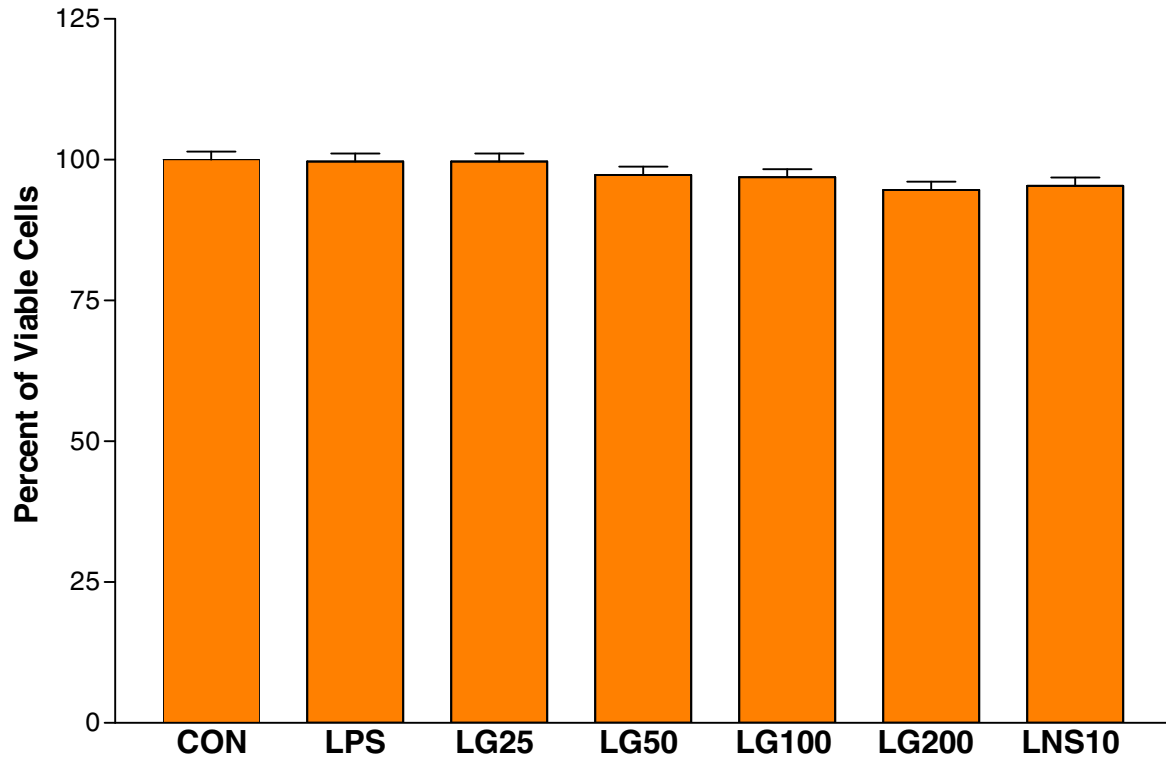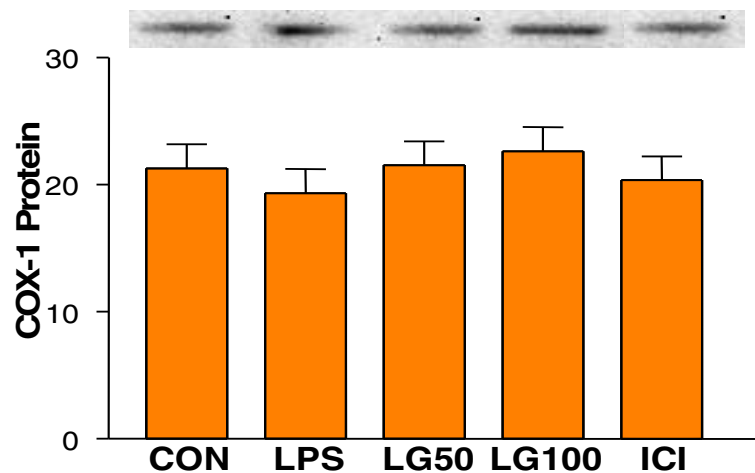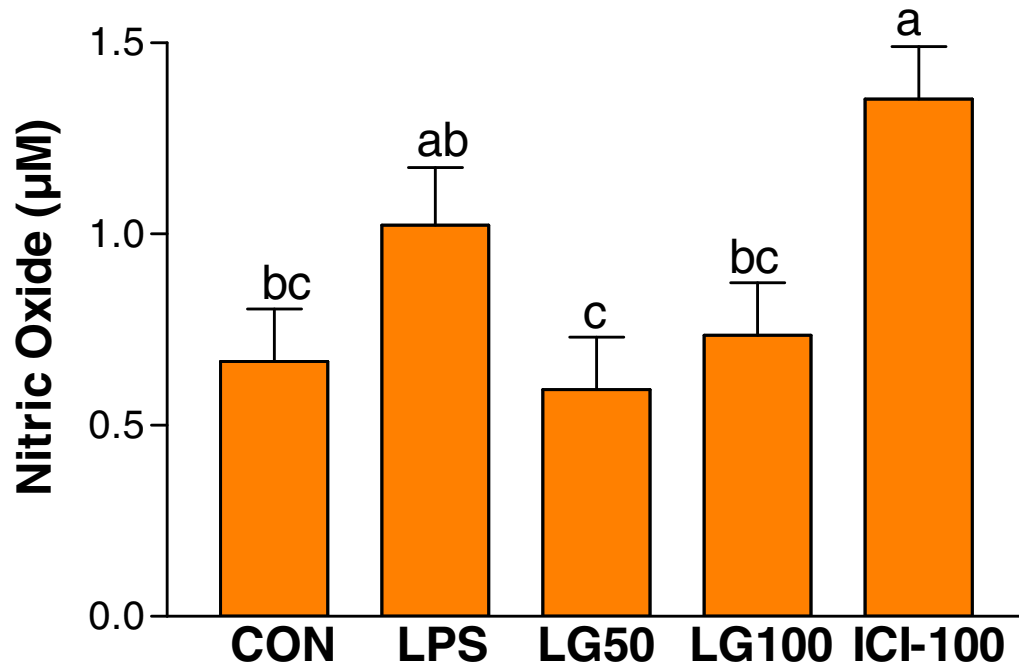
Figure 1. Represents cell viablelity which was measured via resazurin method. CON, control without added lipopolysaccharide; LPS, 1µg/mL; LG25, 25 µM genistein; LG50, 50 µM genistein; LG100, 100 µM genistein; LG200, 200 µM genistein; and LNS10, 10 µM Viox. All treatment groups were treated with 1µg/mL LPS. Bars represent mean ± SE, n = 4 per treatment group.
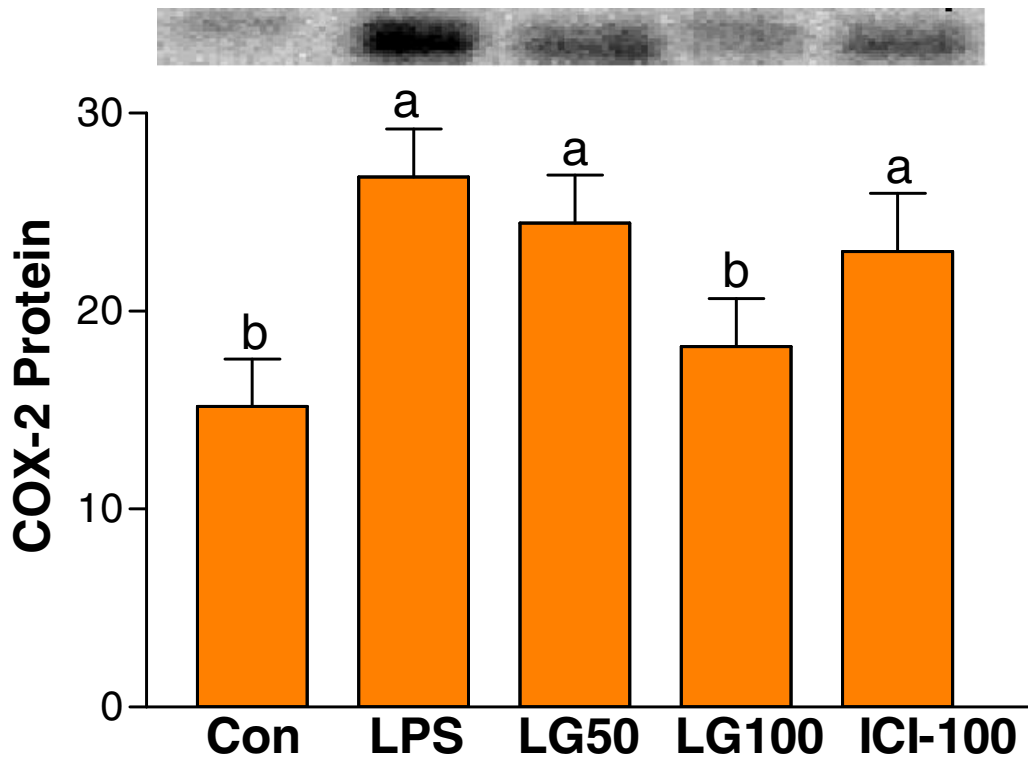
Figure 2. Represent protein level of COX-2 in cytosolic fraction of chondrocytes which was measured via westernblot method. CON, control without added lipopolysaccharide; LPS, 1µg/mL; LG25, 25 µM genistein; LG50, 50 µM genistein; LG100, 100 µM genistein; LG200, 200 µM genistein; and ICI-100, 100 µM. All treatment groups were treated with 1µg/mL LPS. Bars represent mean ± SE, n = 3 per treatment group. Bars with different letters are significantly different (*P< 0.05*).

Figure 3. Represent protein level of COX-1 in cytosolic fraction of chondrocytes which was measured via westernblot method. CON, control without added lipopolysaccharide; LPS, 1µg/mL; LG25, 25 µM genistein; LG50, 50 µM genistein; LG100, 100 µM genistein; LG200, 200 µM genistein; and ICI-100, 100 µM. All treatment groups were treated with 1µg/mL LPS. Bars represent mean ± SE, n = 3 per treatment group.

Figure 4. Represents NO level in culture supernatant which was measured via griess reagent. CON, control without added lipopolysaccharide; LPS, 1µg/mL; LG25, 25 µM genistein; LG50, 50 µM genistein; LG100, 100 µM genistein; LG200, 200 µM genistein; and LNS10, 10 µM Viox. All treatment groups were treated with 1µg/mL LPS. Bars represent mean ± SE, n = 3 per treatment group. Bars with different letters are significantly different (*P< 0.05*).
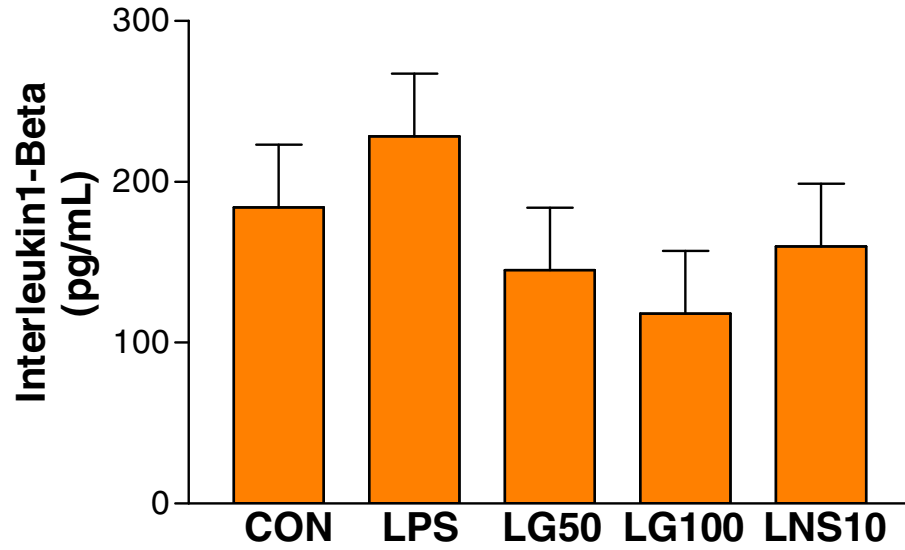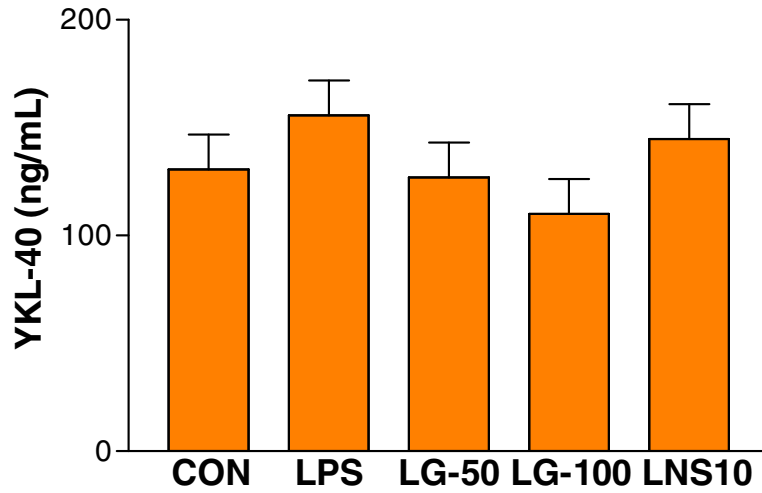
Figure 5. Represents IL-1β level in culture supernatant which was measured via ELISA kit. CON, control without added lipopolysaccharide; LPS, 1µg/mL; LG25, 25 µM genistein; LG50, 50 µM genistein; LG100, 100 µM genistein; LG200, 200 µM genistein; and LNS10, 10 µM Viox. All treatment groups were treated with 1µg/mL LPS. Bars represent mean ± SE, n = 4 per treatment group.

Figure 6. Represents YKL-40 level in culture supernatant which was measured via EIA kit. CON, control without added lipopolysaccharide; LPS, 1µg/mL; LG25, 25 µM genistein; LG50, 50 µM genistein; LG100, 100 µM genistein; LG200, 200 µM genistein; and LNS10, 10 µM Viox. All treatment groups were treated with 1µg/mL LPS. Bars represent mean ± SE, n = 4 per treatment group.

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VITA

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Previously, we reported that cartilage is an estrogen receptor (ER) positive tissue and that mRNA levels of ERß increase in postmenopausal women with osteoarthritis. Based on our findings and those of other investigators, we hypothesized that local rather than circulating estrogen levels negatively affect chondrocyte metabolism and that selective estrogen receptor modulators (SERM) augment cartilage health. To test the latter part of our hypothesis, we explored the role of genistein, a naturally occurring SERM with high affinity to bind ERß, in inhibiting the lipopolysaccharide (LPS)-stimulated cyclooxygenase (COX)-2 but not COX-1 in human chondrocytes (HCH). Cells (PromoCell, Germany) were treated with three levels of genistein (0, 50, and 100 µM). After one hour, the genistein-treated cells were stimulated by one µg/mL LPS for six hours. Cells were then harvested and the cytosolic fraction was isolated for assessing COX-1 and COX-2 protein levels using Western blot technique. Nitric oxide (NO), interleukin-I Beta (IL-1β), and YKL-40 productions were also measured in cell culture supernatants. NO, and IL-1β were measured as markers of inflammation and YKL-40 was assessed as a marker of cartilage catabolism. Interestingly, LG50 was more effective in reducing NO production than LG100 (42% vs. 28%) in comparison with LPS-treatred control cells. Genistein had no significant effect on either YKL-40 or IL-1β levels. Our data indicate that the LPS-stimulated increases in COX-2 protein level and NO in supernatant are reduced by pretreatment of genistein, whereas COX-1 protein level is not affected by genistein.

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