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PERIPHERAL ANTI-NOCICEPTIVE EFFECT OF OLEANOLIC ACID IN A RAT MODEL OF OSTEOARTHRITIS

by
Israa Mohamad Salman

A thesis
submitted in partial fulfilment of the requirements
for the degree of Master of Science in Neuroscience
to the Department of Anatomy, Cell Biology, and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
December16, 2019

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Israa Mohamad Salman

Approved by:

Dr. Nada B. Lawand, Assistant Professor — Advisor
Department of Neurology and Anatomy, Cell Biology, and Physiological Sciences

Dr. Abdo Jurjus, Professor — Member of Committee
Department of Neurology and Anatomy, Cell Biology, and Physiological Sciences

Dr. Georges Daoud, Assistant Professor — Member of Committee
Department of Anatomy, Cell Biology, and Physiological Sciences

Dr. Firas Kobaissy, Associate Professor — Member of Committee
Department of Biochemistry and Molecular Genetics

Date of thesis defense: December 16, 2019

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ACKNOWLEDGEMENTS

After a year of research experience, I would like to express my appreciation and special thanks to my creative advisor Dr. Nada Lawand for being always there for me, advising me in every step throughout my work. I am grateful for all her support, encouragement, patience and guidance. Dr. Nada Lawand has not only been an ideal advisor but also a great mentor from whom I learned academic work and priceless lifetime lessons. I wish someday I would become as successful, genuine, smart, lovely and a caring person like her.

I also would like to thank my committee members and the Chairperson of our department Dr. Elie Al-Chaer for his precious time, comments and suggestions that enhanced my work. Many thanks to all the faculty members and supervisors in DTS for all their help.

Moreover, I would like to deeply thank my second family Al-Chaer's Lab members for all their help. First of them is Dr. Marc Fakhoury, for all his dedicated help which I appreciate from the bottom of my heart. His great efforts made this work possible. Also, it is important to mention my gratefulness for Ms. Malak Fouani who guided me in every step throughout my experimental work. She was not only a friend but also a sister. Big salute, to our best research assistant Mr. George Merhej who hasn't just been a source of academic help but also a source of great humor which I needed throughout my stressful journey. Finally, I thank the supportive Mr. Wassim Najjar who was the first helper and supporter. Having such a great and supportive family has been one of the most important memories with which I close my M.S chapter.

Last but not least, none of this would have happened without the support and the encouragement of my first family, that is my parents, my precious brother, my sisters and my husband. I would like to express my thanks to my life partner and soulmate for believing in me, being there for me in my good and hard times. I am grateful for all his support in everything I do, for his trust, encouragement and endless love throughout my journey. He is the reason behind my success and I do thank God to have him by my side. I want to thank my mother for all her encouragement, caring, unconditional love and all her prayers. And of course, I would like to thank my father for all his support, encouragement to pursue my learning and higher education, and all his sacrifices that he has afforded for me; I'll forever be grateful to him and hope I was able to make him feel proud of me.

AN ABSTRACT OF THE THESIS OF

Israa Salman for Master of Science
Major: Neuroscience

Title: Peripheral anti-nociceptive effect of oleanolic acid in a rat model of Osteoarthritis

Osteoarthritis, the most common form of joint inflammation, is characterized by severe pain and hypersensitivity to thermal and mechanical stimulation, and by prolonged increase in the synaptic excitability of central nociceptive neurons. Oleanolic acid (OA), a naturally occurring pentacyclic triterpenoid present in food and plants, is thought to play a beneficial role in inflammation owing to its neuroprotective effects. OA modulates several nuclear receptors involved in regulating bile acid through the activation of transcription factors. Converging evidence has pointed towards a role for OA and these receptors in the regulation of inflammation. However, despite significant progress in research, the role of OA in anti-nociception and anti-inflammation is still not fully understood. In this study, we hypothesize that Oleanolic acid exerts an anti-nociceptive and anti-inflammatory effect in a rat model of osteoarthritis. To test this hypothesis, we conducted behavioral and electrophysiological studies to examine the effect of intra-articular OA on the development of nociceptive behaviors, knee joint edema formation, motor incoordination and increased afferent discharges associated with knee joint inflammation.

Materials and Methods: Knee joint Inflammation was induced by injecting a mixture of 3% kaolin and carrageenan (K/C). Sensory and motor tests were performed in rats prior to, and at 4, 8, 24, 48 and 168hrs following the induction of inflammation. The knee joint circumference was measured, at each time point, to monitor the development of joint edema. Rats were divided into two main groups: Pre-treatment and post-treatment. In the pre-treatment groups, two different doses of OA (0.5mg (n=5) and 3mg(n=4)) were administered in the knee joint before induction of inflammation. In the post-treatment group, rats received only one dose (0.5mg g (n=5)) of OA after induction of inflammation. The control groups received a solution of 100% ethanol, as a vehicle substance. Nociceptive behaviors were assessed in all animals using heat hyperalgesia and mechanical allodynia and hyperalgesia tests. In addition, motor coordination was evaluated using the rotarod test. To determine whether OA acts on articular primary afferents, electrophysiological recordings were made from the tibial nerve. OA's effect on sensitized nerves were also tested.

Symptoms of inflammation were manifested in all rats injected with K/C. Inflamed rats receiving OA prior to and after induction of inflammation, showed a decreased joint swelling and a noticeable decrease in pain hypersensitivity in response to noxious and innocuous stimulation as compared to the control groups. On the other hand, recordings from branches of the articular nerve revealed an excitatory effect of OA on sensitized primary afferents. We suggest that the increase in nerve activity leads to activation of central descending inhibitory pathways that reduce afferent input into the spinal cord. Collectively, the results of the present study highlight the potential benefits of OA for the management of pain and inflammation associated with osteoarthritis.

CONTENTS

ILLUSTRATIONS

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CGRP	Calcitonin gene-related peptide
CRF	Corticotropin-releasing factor
COX-2	Cyclooxygenase-2
FTO	Fat mass and obesity-associated
FXR	Farnesoid X receptor
GDF5	Growth differentiation factor 5
GNL3	Nucleostemin-encoding gene
IL-beta	Interleukin 1-β
iNOS	Inducible nitric oxide synthase
K/C	Kaolin/Carrageenan
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
LXR	Lipid X receptor
MAN	Medial articular nerve
mGlut	Metabotropic glutamate
MMP-13	Matrix metallopeptidase 13
NADPH	Nicotinamide adenine dinucleotide phosphate
NFkB	Nuclear factor kappa-light-chain-enhancer
NMDA	N-methyl-D-aspartate
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Oleanolic acid
PAN	Posterior articular nerve

PB	Phosphate buffer
PGE2	Prostaglandin E2
PFA	Paraformaldehyde
PTGS2	Prostaglandin-endoperoxide synthase 2
PXR	Pregnane X receptor
ROS	Reactive oxygen species
SYSA-DOAs	Symptomatic Slow Acting Drugs for Osteoarthritis
TRPV1	Transient Receptor Potential Vanilloid 1
TNF-alpha	Tumor necrosis factor alpha
VIP	Vasoactive intestinal peptide

CHAPTER 1

INTRODUCTION

A. OSTEOARTHRITIS

1. Definition and overview

Osteoarthritis is a common degenerative joint disease and a major cause of pain. It is characterized by a change of homeostatic healthy state into a catabolic state, and by pathological changes in the joints such as thickening of the subchondral bone and continuous loss and destruction of the articular cartilage (Chen et al., 2017). Patients with osteoarthritis also suffer from chronic pain, joints stiffness and instability, narrowing of joint space, and hypertrophy of the joint capsule (Felson, 2006). This disease is classified either as primary osteoarthritis or secondary osteoarthritis. While the former is a more generalized idiopathic form of osteoarthritis with an unknown cause, the latter mostly occurs due to post-traumatic causes, and could also result from congenital, endocrine and metabolic abnormalities (Michael et al., 2010).

Epidemiological studies indicate that osteoarthritis is a large and growing problem to the society affecting more than 25% of the population with an age greater than 18 years old (Chen et al., 2017). In the United States alone, more than 46 million individuals have self-reported doctor-diagnosed arthritis (Helmick et al., 2008) Studies also showed that osteoarthritis is very common among individuals aged 60 years or older (affecting 27 to 44%) (Felson et al., 1987; Dillon et al., 2006), and that its prevalence increases with age throughout the elderly years (Felson et al., 1987). In addition, there appears to be a gender-based difference in the prevalence of osteoarthritis, with women being more affected than men (Felson et al., 1987; Dillon et al., 2006).

2. Causes of osteoarthritis

Although osteoarthritis is classified as an idiopathic disease, there are numerous factors that could lead to its development. Aging is one of such factors. In particular, it has been shown that beyond the age of 56, people are more likely to have osteoarthritis (Felson et al., 1987) mainly due to changes in muscles, tissues and subchondral bone including the cartilage (Chen et al., 2017). In addition to aging, obesity is one of the causes that lead to osteoarthritis (Chen et al., 2017). The increased adipose tissue accumulation observed in individuals with obesity increases the overall load on the joints, thus leading to inflammation and the development of osteoarthritis (Chen et al., 2017). Another cause for osteoarthritis is sports injury of the knee (Chen et al., 2017).

Moreover, sport injury has been associated with increased risk of osteoarthritis; in particular individuals that have experienced some sort of knee injury are four times more likely to develop osteoarthritis, and about 41 to 51% of patients of young ages show symptoms of osteoarthritis in the years following a knee injury (Roos, 2005). Common injuries that occur in such cases are cartilage tissue tear, joint dislocation and ligament strains and tears (Chen et al., 2017). Injury to the anterior cruciate ligament has shown to be a cause for knee osteoarthritis (Hill et al., 2005). Osteoarthritis could also occur due to trauma following an extreme load on the joint, which causes damage in the subchondral bone and rupture of blood vessels in the joint capsule (Lotz and Kraus, 2010)

Last but not least, genetic factors were shown to play an important role in the underlying pathophysiology of osteoarthritis (Spector et al., 1996; Chen et al., 2017). Ex-vivo studies using tissues derived from patients with osteoarthritis and in-vivo studies using animal models of the disease have implicated genes involved in WNT (Sassi et al., 2014), TGF-beta (Serra et al., 1997) and hedgehog (Lin et al., 2009) signaling. In addition, genome-wide association studies have identified several genes associated with osteoarthritis, including the nucleostemin-

encoding gene (GNL3), which is involved in the regulation of cell cycle and differentiation (arc et al., 2012), the fat mass and obesity-associated (FTO) gene, which is involved in regulation of bodyweight (arc et al., 2012), the prostaglandin-endoperoxide synthase 2 (PTGS2), which is responsible for production of inflammatory prostaglandins (Valdes et al., 2008), and the growth differentiation factor 5 (GDF5) gene, which participates in the development, maintenance and repair of bone (Zhang et al., 2015).

3. Clinical symptoms and mode of inflammation

Osteoarthritis is classified as a disease characterized by bone and joint inflammation as a result of articular cartilage loss (Loeser et al., 2012). Inflammation primarily occurs in the joints of the body, most importantly at the level of the knee joint. The knee is made of a synovium joint embedded in the synovial fluid, inside of which there is an articular capsule surrounded by a synovial membrane (Tamer, 2013). The synovium plays a central role not only in the lubrication of the joints, but also in the regulation of inflammation (Tamer, 2013).

Numerous evidence indicates that osteoarthritis is characterized by many changes in the synovium, including fibrosis in the ligaments, remodeling of the subchondral bone and ectopic bone formation **(Figure 1)** (Aspden and Saunders, 2019). Patients with osteoarthritis also have thickened and shortened tissues, reduced flexibility due to capsule and ligaments fibrosis (Lloyd-Roberts, 1953). Pain is another one of the major symptom that an osteoarthritis patient could suffer from (Peat et al., 2001). The sensation of pain observed in patients with osteoarthritis often results from damage of joint structure in the synovium and the loss of the articular cartilage. The mechanism of arthritic pain is not only peripheral but also central since it has been shown that even after joint replacement surgery the patient is still suffering from referred pain sensation (Gwilym et al., 2009; Wylde et al., 2011).

The inflammation observed in osteoarthritis is centrally mediated by glial cells. In an

monoiodoacetate (MIA) osteoarthritic rat model, Ogbonna and colleagues (2015) reported high levels of activated microglia in the spinal cord accompanied by cartilage degradation in the knee joint (Ogbonna et al., 2015). C afferent fibers have also been shown to be an important factor in mediating inflammation (Kelly et al., 2012) by triggering extravasation through antidromic dorsal root stimulation (Couture and Cuello, 1984). On the other hand, sympathetic efferent fibers have shown to play a minor role in joint injury in both adjuvant-induced arthritis and kaolin and carrageenan rat models after sympathectomy (Levine et al., 1985; Sluka et al., 1994).

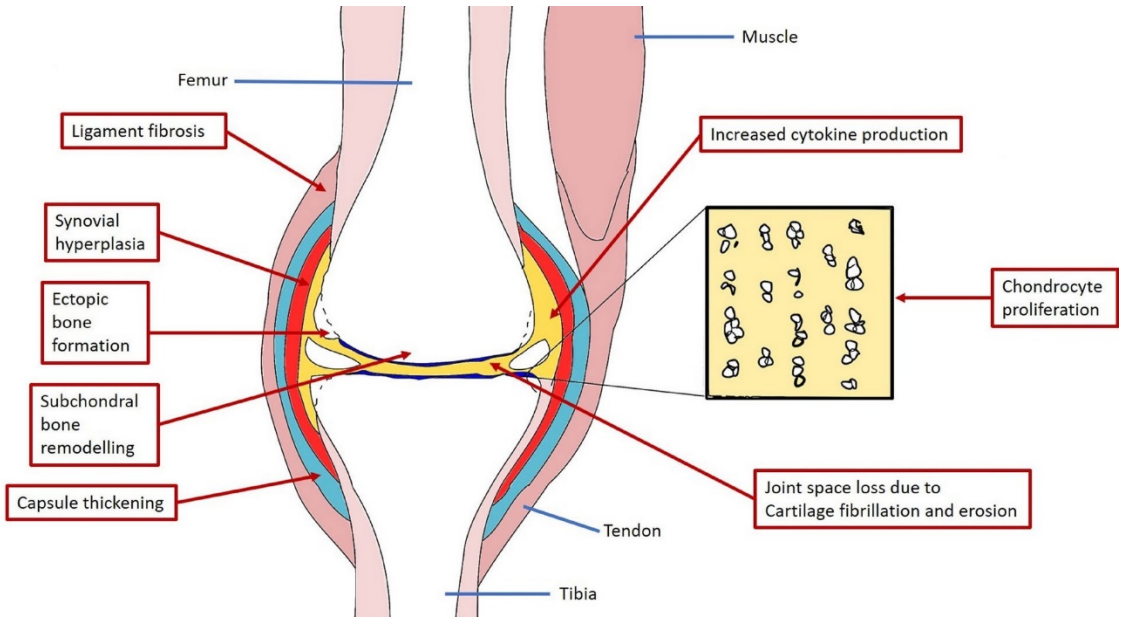


Figure 1: Illustration of a synovial joint indicating some of the tissue changes observed in osteoarthritis. Taken from (Aspden and Saunders, 2019).

4. Neurogenic inflammation in osteoarthritis

The knee is comprised of well-developed menisci, which contain pyramid-shaped ossicles (Hildebrand et al., 1991). The anterior and posterior horns of the rat knee's menisci are attached to the tibial intercondylar area through ligaments (Hildebrand et al., 1991). The posterior horn of the lateral meniscus of the rat knee is also attached to the medial condyle of the femur and

the tibial intercondylar area (Hildebrand et al., 1991). The knee joint is innervated by two sets of primary articular nerves; the posterior articular nerve (PAN) and the medial articular nerve (MAN) (Hildebrand et al., 1991). Between these two nerves, The PAN is the largest and most constant (Hildebrand et al., 1991). The PAN emerges from the tibial nerve as a single trunk from the popliteal fossa. It originates as two branches and passes through the popliteal fat and the joint capsule (Hildebrand et al., 1991). The PAN consists of approximately 400 axons, 80% of which are unmyelinated (Hildebrand et al., 1991). It originates from the tibial nerve and is considered to be the major primary knee joint nerve. On the other hand, the MAN is smaller and more variable relative to the PAN (Hildebrand et al., 1991).

There are several neuropeptides involved in the inflammatory process associated with osteoarthritis, the most important one being substance P, calcitonin gene-related peptide (CGRP), corticotropin-releasing factor (CRF), urocortin and vasoactive intestinal peptide (VIP) (Sutton et al., 2009).

Substance P is a neuropeptide found in the C- fibers of both the peripheral and central nervous system. It plays an important role in joint disease inflammation by interfering with several components of joint inflammation, and by mediating the activation of neutrophils, the de-granulation of mast cells, and the proliferation of fibroblasts (Ferrell and Russell, 1986). Substance P is highly secreted following a traumatic injury (Zacest et al., 2010) and in inflammatory diseases (O'Connor et al., 2004). Secretion of substance P leads to pain sensation in the joints (de Grauw et al., 2006), causes vasodilation (Lam and Ferrell, 1993), and leads to the secretion of Interleukin 1-β (IL-beta), Tumor necrosis factor (TNF)-alpha and IL-6 (Hernanz et al., 2003). In addition to this, secretion of substance P leads to the activation of macrophages, platelets, mast cells, and polymorphonuclear cells (Menkes et al., 1993) and most importantly, causes the synoviocytes to proliferate and express Prostaglandin E2 (PGE2) and collagenase (Lotz et al., 1987).

CGRP is another important neuropeptide involved in the pathogenesis of osteoarthritis (Fernihough et al., 2005). It is predominantly localized in primary afferent neurons that innervate the muscles and the joints (O'Brien et al., 1989; Schwab et al., 1997). In addition, this neuropeptide was shown to play a prominent role in the regulation of inflammation and pain sensation (Benemei et al., 2009). CGRP has also been shown to be involved in peripheral and central sensitization leading to hyperalgesia and pain in arthritis (Zhang et al., 2001; Iyengar et al., 2017). Moreover, in a rat model of acute inflammation, administration of CGRP receptor antagonists close to nociceptive neurons innervating the knee joint was shown to reduce responses to noxious pressure applied to the knee, suggesting that CGRP is involved in the processing of mechanosensory input (Neugebauer et al., 1996).

CRF is a neuropeptide primarily found in the hypothalamus and in sites where inflammation is present (McEvoy et al., 2001; Backstrom and Winberg, 2013). This neuropeptide is produced in the synovium itself and may reach the inflammatory region of the synovium via a neurogenic pathway (McEvoy et al., 2001). CRF also plays an important role in mediating inflammation in the joints by promoting the production of glucocorticoids through its action on the pituitary gland (Seasholtz, 2000).Glucocorticoids in turn inhibit the migration of leukocytes, suppress the release of pro-inflammatory cytokines, and inhibit the production of inflammatory mediators derived from phospholipids (Crofford et al., 1993).

5. Current treatments used

Despite significant progress in research, there is still no cure for osteoarthritis. Most of the current treatments available are only useful in helping patients cope with their symptoms. Common therapeutic strategies include the use of pharmacological interventions to dampen inflammation and related pain, and surgical interventions to repair damage joints (Chen et al., 2017).

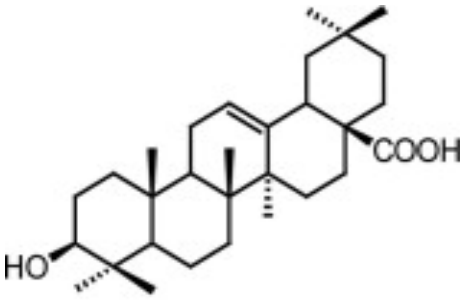
The first attempt in managing the symptoms of osteoarthritis is to perform daily exercise and to follow a healthy life style. With increasing severity of symptoms, the patient can start by taking paracetamol as a pain relief medication. A specific prescribed treatment of Symptomatic Slow Acting Drugs for Osteoarthritis (SYSA-DOAs), such as glucosamine sulfate or chondroitin sulfate, could also help manage the symptoms of osteoarthritis (Reginster et al., 2001; Pavelka et al., 2002; Kahan et al., 2009). Topical nonsteroidal anti-inflammatory drugs (NSAIDS) could also be used for pain relief in osteoarthritis (Zeng et al., 2018). NSAIDs could also be administered orally, though this type of drug administration could lead to deleterious effects in the kidney, stomach and heart if adopted for a long time (Bruyere et al., 2014). When analgesic and anti-inflammatory drugs fail to manage the symptoms of osteoarthritis, intra-articular injections of hyaluronic acid and corticosteroids could be used as treatment option (Ayhan et al., 2014). Cortisol is relatively more robust, though it has a shorter-term efficiency (~ 1 week) than hyaluronic acid (~ 6 months). Patients who did not respond to the above-mentioned treatments could use oral weak opioids or duloxetine (Myers et al., 2014; Wang et al., 2018). These pharmacological agents may have side effects such as morbidity, and may lead to withdrawal in patients (Bruyere et al., 2014). Last but not least, when all pharmacological options failed to manage the symptoms of the patients, a surgical intervention for the knee joint replacement must be envisaged (Bruyere et al., 2014).

B. OLEANOLIC ACID

1. Definition and overview

Oleanolic acid is a pentacyclic triterpenoid acid extracted from numerous types of plants including licorice, hawthorn fruit, basil and brown mustard (Raphael and Kuttan, 2003; Yin et al., 2012; Kao et al., 2014). It can also be extracted from Lufla cylindrical seeds (Kapil and Sharma, 1995) and is found in olive leave extract, olive pomace, mistletoe sprouts, and clove

flowers, among others (Kang et al., 2017b). Interestingly, oleanolic acid can be found in the human diet and in more than 1620 plant species, including food and medicinal plants (Liu, 1995, 2005; Fai and Tao, 2009; Fukushima et al., 2011). As illustrated in Figure2, oleanolic acid is an olean-12-en-28-oic acid substituted by a beta-hydroxy group at position 3, and as such can be interchangeably used with the term *3-beta-Hydroxyolean-12-en-28-oic acid* (Pollier and Goossens, 2012).



Oleanolic acid

Figure 2: Chemical structure of oleanolic acid. Taken and adapted from (Pollier and Goossens, 2012).

2. General functions of oleanolic acid

Oleanolic acid has been known to exert multiple beneficial effects. In particular, it has been used for inflammation, cancer, and hepatic protection (Hsu et al., 1997; Jeong, 1999; Giner-Larza et al., 2001; Zhu et al., 2001). Oleanolic acid was also shown to have anti-oxidative actions and protective roles against apoptosis (Tsao and Yin, 2015). This compound exerts anti-oxidative effects mostly by decreasing the level of IL-6 and TNF alpha, and by enhancing and stabilizing mitochondrial stability against oxidative or inflammatory injury of cells (Tsao and Yin, 2015). In addition, oleanolic acid decreases the levels of reactive oxygen species (ROS) and PGE2 and suppresses the expression of nicotinamide adenine dinucleotide

phosphate (NADPH) and p-38, p65, cyclooxygenase-2 (COX-2) and nuclear factor-κB (NFKB) (Tsao and Yin, 2015).

The hepatoprotective effect of oleanolic acid is largely due to its anti-oxidative role, anti-inflammatory action and its action on drug-metabolizing enzymes (Liu, 2005). Oleanolic acid is effective in exerting a protective effect in hepatic injury whether it is an acute injury due to chemicals, a chronic liver fibrosis, or a cirrhosis (Liu, 1995). In addition, this natural compound can be taken orally either alone or in combination with other hepato-protective ingredients (Liu, 2005). On the other hand, the protective effect of oleanolic acid against cancer is largely attributed to its inhibitory action on tumor initiation and proliferation in different tumor development stages (Oguro et al., 1998; Li et al., 2016). This compound was also shown to exert beneficial effects in acute myeloid leukemia by inducing the activation of apoptosis of tumor cells (Konopleva et al., 2004). In addition, oleanolic acid and its derivatives were shown to inhibit angiogenesis, tumor cells metastasis and invasion, and as such, could potentially represent a new class of chemotherapeutics (Ovesna et al., 2004). Last but not least, oleanolic acid was shown to exert antidiabetic and anti-metabolic syndrome actions mostly by attenuating insulin resistance and by maintaining the function and viability of pancreatic beta-cells (Castellano et al., 2013; Li et al., 2015).

3. Oleanolic acid and inflammation

Numerous evidence points to the fact that oleanolic acid exerts an anti-inflammatory action in different inflammatory models of mice and rats (Singh et al., 1992). In a rat model of formaldehyde-induced arthritis, oleanolic acid was shown to inhibit the action of serum transaminase (ALT and AST), which were highly increased in this animal model (Singh et al., 1992). Interestingly, oleanolic acid was shown to have similar actions to NSAIDs in terms of anti-inflammatory effects (Singh et al., 1992). The anti-inflammatory effect of oleanolic acid

is largely attributed to its inhibitory action on the release of High mobility group box protein (HMGB1) and on the production of the pro-inflammatory cytokines including nuclear factor kappa-light-chain-enhancer (NFkB) and TNF alpha (Yang et al., 2012; Lee et al., 2013). Owing to its anti-inflammatory effects, oleanolic acid treatment has shown promising results in a number of diseases and conditions. For instance, oleanolic acid was shown to rejuvenate testicular function by exerting anti-inflammatory actions through the inhibition of nuclear factor kappa B (NFkB) pathway, which in turn inhibits the production of pro-inflammatory cytokines (Zhao et al., 2017). Oleanolic acid could also be used to provide neuroprotection against cerebral damage in case of ischemia (Caltana et al., 2015).

4. Oleanolic acid receptors: mechanism of action

Oleanolic acid acts selectively on the farnesoid X receptor (FXR) (Liu and Wong, 2010). FXR is a nuclear receptor that regulates bile acid synthesis through the activation of transcription factors (Mencarelli et al., 2009). It is predominantly found in the liver and plays important roles in the regulation of the synthesis and storage of triglycerides (Jiao et al., 2015). FXR has four isoforms, namely FXR alpha 1, FXR alpha 2, FXR beta 1, and FXR beta 2 (Zhang et al., 2003). Among these isoforms, FXR beta 2 is the most potent in promoting ligand-induced FXR target gene expression (Zhang et al., 2003). FXR has many functions, ranging from bile acid regulation and glucose homeostasis (Mazuy et al., 2015). FXR also interferes in the lipoprotein and lipid metabolism and plays crucial roles in liver regeneration, cellular proliferation, and inflammation (Mazuy et al., 2015).

Oleanolic acid was also shown to modulate other receptors including the pregnane X receptor (PXR) and the lipid X receptor (LXR) (Lin et al., 2018). PXR is a member of nuclear receptors that play a crucial role in mediating lipogenesis (Lin et al., 2018). PXRs were shown to be involved in bone homeostasis through the regulation of calcium and phosphate homeostasis

(Azuma et al., 2010). LXR is another member of the nuclear receptor family, and is responsible in regulating cholesterol levels and metabolic homeostasis in the liver (Repa et al., 2000). It also plays an important role in synthesizing, storing and transporting lipids (Repa et al., 2000), and in several other processes including lipid metabolism, glucose homeostasis, steroidogenesis, immunity and inflammation (Huang, 2014). In addition, agonist-mediated LXR activation was shown to suppress inducible nitric oxide synthase (iNOS), TNF alpha and COX-2 (Fu et al., 2015). LXR activation was also shown to play a role in inhibiting the pro-inflammatory response induced by microglia and astrocytes (Kim et al., 2006; Zhang et al., 2007), and by decreasing TNF-alpha-induced superoxide and nitrotyrosine production (Spillmann et al., 2014) suggesting that the LXR pathway exerts an anti-inflammatory and anti-apoptotic actions. Numerous evidence indicates that oleanolic acid exerts an inhibitory effect on both PXR and LXR, and by doing so, controls important pathophysiological processes such as lipid homeostasis, immunity and inflammation (Lin et al., 2018).

5. Oleanolic acid as a potential treatment for osteoarthritis

Numerous evidence suggests that oleanolic acid could be used for the treatment of osteoarthritis. In experimental animals, oleanolic acid was shown to inhibit and reverse the degeneration of cartilage in mice by decreasing proteins that are involved in this process and in the dysfunction of joints, including PGE2 and matrix metallopeptidase 13 (MMP-13) (Kang et al., 2017b). MMP-13 plays a potential role in the pathogenesis of osteoarthritis by degrading components of extracellular matrix like proteoglycans (Fosang et al., 1996). Oleanolic acid also prevents the degeneration of articular cartilage by inhibiting the secretion of IL-1beta, which in turn helps suppress the secretion of MMP-13 (Aida et al., 2005; Kang et al., 2017a; Kaneko et al., 2019). Other beneficial effects of oleanolic acid in osteoarthritis include the stimulation of osteoblastic differentiation (Bian et al., 2012), the increase of collagen 2 levels,

the prevention of glucose-induced cell injury, and the decrease of Lactate Dehydrogenase (LDH) level, a marker for cell injury (Kang et al., 2017b). In addition, oleanolic acid was shown to exert beneficial effects in arthritis by specifically suppressing the C3- convertase of the complement pathway; a pathway which influences factors that mediate inflammation through the release of pro-inflammatory cytokines and anaphylotoxins (Kapil and Sharma, 1995). Altogether, these findings suggest that oleanolic acid provides beneficial effects by mitigating the pathological changes associated with osteoarthritis, though more work is needed, specifically from controlled clinical studies to fully evaluate its potential use as a treatment for osteoarthritis.

C. HYPOTHESIS AND SPECIFIC AIMS:

The overarching goal of the present study is to evaluate the use of oleanolic acid in the treatment of pain and inflammation associated with osteoarthritis. We hypothesize that injection of oleanolic acid into the synovial joint can exert anti-nociceptive and anti-inflammatory effects in a rat model of osteoarthritis.

To test our hypothesis, we devised three specific aims:

Aim 1: to investigate whether pre-treatment of the knee joint with oleanolic acid prior to induction of inflammation can prevent the development of nociceptive behaviors, knee joint swelling, and motor dysfunction.

Aim 2: to examine whether Post-treatment of the knee joint with oleanolic acid after induction of inflammation can stop the development of nociceptive behaviors, knee joint swelling, and motor dysfunction.

Aim 3: Assess the direct effect of oleanolic acid on articular nerve afferent before and after induction of inflammation.

CHAPTER 2

MATERIALS AND METHODS

A. Subjects

In this study, adult male Sprague-Dawley rats (n=28) with a weight ranging from 150 g to 300 g were used. All experimental procedures were approved by the institutional animal care and use committee at the American university of Beirut and followed the ethical guidelines for experimental pain on conscious animals. The animals were kept in cages at a controlled room temperature of 25 °C and received water and food ad libitum.

B. Experimental Protocol

Rats were randomly divided into 5 groups as follows: In pre-treatment groups 1 and 2, intra-articular injection of two different doses of oleanolic acid (0.5mg/injection; group 1; n=5) and (3mg/injection group 2; n=4) was performed prior to induction of inflammation. In post-treatment group 3, rats received oleanolic acid in the synovial joint 30 to 60 min post induction of inflammation (n=5). To control for the pre- and post- treatment effects of OA, two groups of rats received an equivalent volume of the the vehicle substance, ethanol.

C. Induction of inflammation

To induce of inflammation, rats were anesthetized with Isoflurane for a short period of time and injected with 0.1ml of 3% kaolin and carrageenan into the synovial cavity of the right knee using a 1 ml syringe.

D. Oleanolic acid injection

Oleanolic acid was dissolved in 100% ethanol and injected in a volume of 0.1ml into the synovial cavity of the right knee 30 min before or 30 min after induction of inflammation.

E. Nociceptive behavioral testing

All rats were tested for nociceptive behaviors prior to and at 4, 8, 24, 48 hrs, and 1 week following the induction of inflammation. Tests were conducted during the light phase of the cycle, and all animals were put in the experimental room for at least 1 hr for accommodation. Three nociceptive behavioral tests were done; the mechanical allodynia test, the mechanical hyperalgesia test, and the heat hyperalgesia test

E.1 Mechanical Allodynia Test

Prior to testing, all rats were placed in a transparent chamber on a metal wire mesh floor and left for 30-min accommodation period. A von Frey Filament with a bending force of 2 g was used to determine the Paw withdrawal responses to a non-noxious stimulus.. The tip of the filament was applied perpendicularly to the medial plantar surface from below the mesh grid until a withdrawal was observed. The filament was applied for five trials (five applications with 2 s per each trial) at approximately 5-min intervals. Baseline values were obtained prior to injection of kaolin and carrageenan into the knee joint. Testing was repeated at 4, 8, 24, 48hrs and 1 week following induction of inflammation. The five trial responses of both hind paws were recorded for each animal and the measurements obtained for each rat were averaged. An increased measure of paw withdrawal frequency indicated the development of mechanical allodynia.

E.2 Mechanical Hyperalgesia Test

Paw withdrawal frequency to application of von Frey filament with a bending force of 15g was recorded. This force has previously been shown to activate mechanoreceptors and nociceptors. Testing started by poking the plantar surface 5 successive times with the 15g filament until the animal elicited a behavioral response. Five trials separated by 5 min time interval were conducted. Baseline values were obtained prior to any treatment. . Testing was repeated at (4, 8, 24, 48 hrs) and 1 week following induction of inflammation. Responses of both paws to mechanical stimulation were recorded for all groups and measurements were averaged for each animal and later analyzed.

E.3 Heat Hyperalgesia Test

The heat hyperalgesia test was assessed by measuring the foot withdrawal latency to radiant heat applied to the plantar surface of the hind paw. Approximately 15 min prior to testing, each rat was placed individually in a clear plastic cage atop an elevated 3-mm thick glass plate for accommodation. The height of the glass plate was adjusted so that heat applied to the plantar surface of the normal foot evoked a withdrawal response after approximately 20 s. Heat stimuli were applied 3 times with a 5-min resting period between trials to avoid conditioning limb withdrawal. The heat stimulus consisted of a heat sensor that generated heat in different intensities. Heat was adjusted at an intensity of 30 (arbitrary units) and directed below the rats hind paw. The withdrawal latency was defined as the elapsed time in seconds from stimulus onset to paw withdrawal. A cut off of 20 s was imposed to avoid tissue damage.

F. Motor Behavioral Test

Motor behavior was assessed using the Rota rod test. The Rota rod test is one of the oldest test used to assess the effects of a drug on animal behavior. It provides a rapid and simple first estimation of whether the inflammatory agent has any effect on neuromuscular coordination. The Rota rod consists of a horizontal rod turning at a constant or increasing speed. Animals placed on the rotating rod try to balance and remain on it to avoid falling into a platform located 30 cm below. Vertical barriers were used to separate the animals from one another. Each rat was tested 5 times, with each trial lasting 300 s and separated by at least a 10 min inter-trial period. Rats were placed on the rod and the apparatus was set at a fixed speed of 5 rpm. The latency to fall off the rotating rod was recorded for each rat at the end of each test.

G. Joint Circumference Measurement

To evaluate the effect of oleanolic acid treatment on the severity of joint inflammation, the knee joint circumference was measured in all rats before and at 4, 8, 24, 48 hrs and 1 week post induction of inflammation. Joint circumference measurements of both knee joints were done using a flexible tape measure wrapped around the center of the knee joint.

H. Electrophysiology

Multiunit recordings of signals from articular afferents were conducted to assess the direct effect of oleanolic acid on sensory articular receptors. Rats were generally anesthetized using ketamine (100 mg/kg) and xylene (10 mg/kg) and the body temperature was maintained at 37 °C . Following isolation of the tibial nerve, the epineurium was removed and a small pool filled with mineral oil was formed around the nerve by retracting the skin flaps. The tibial nerve was cut proximally and fibers were teased apart under a dissecting microscope and placed on a fine silver electrode for single fiber recording. Spontaneous activity and responses to OA injection were recorded. Oleanolic acid was either injected in

the knee joint, near the terminal ends of the nerve or directly on the nerve. The discharges of single fibers were amplified, displayed on a digital oscilloscope and collected via Spike 2 data-acquisition system for analysis.

I. Statistical analysis

All data sets were checked for normality using the Shapiro-Wilkes test with $p<0.05$ accepted as a non-normal distribution. Two normally distributed groups were compared using Student's t-test, while non-normally distributed group were compared using the Mann-Whitney U test. Analysis of differences among groups, and within groups at different time points, was done using one-way ANOVA. All data were expressed as mean ± standard error of the mean (SEM).

CHAPTER 3

RESULTS

A. Effect of oleanolic acid on nociceptive behaviors

Two different treatment regimens were followed in this study. Oleanolic acid was injected into the knee joint at two different doses (0.5mg/injection) and (3mg/injection) prior to induction of inflammation and at one low dose (0.5mg/injection) post induction of inflammation to evaluate its effect on the development of nociceptive behaviors. The analysis of paw withdrawal latency to heat stimulation for animals pre-treated with OA at the high dose, revealed a statistically significant increase in latency at 4, 8, 24, 48hrs and 1 week when compared to baseline and control values ($p<0.05$) (Figure 3). Conversely, rats pre-treated with the low dose of oleanolic acid (0.5mg/injection) did not show any significant change in heat hyperalgesia compared to the control group though a pattern of increasing latency was noted. On the other hand, rats post-treated with the low dose of oleanolic acid (0.5mg/injection) showed a statistically significant decrease in heat hyperalgesia at 4 and 24hrs ($p <0.05$) as demonstrated by an increase in the paw withdrawal latency compared to control and baseline values.

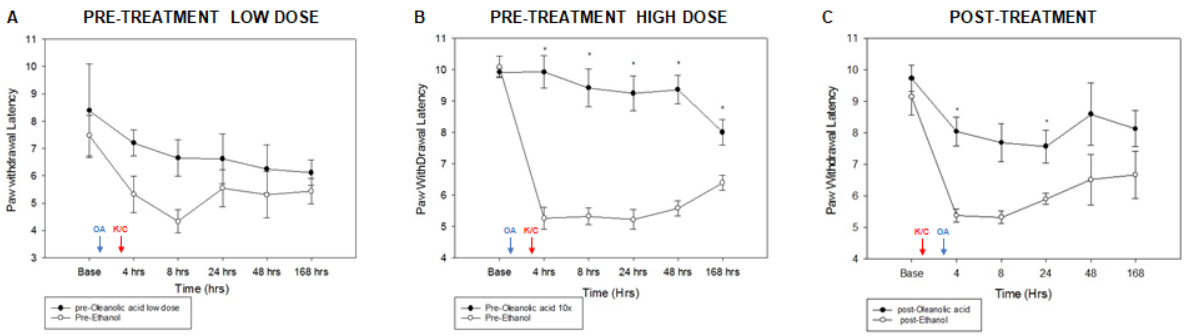


Figure 3: Effect of pre-and post-treatment with oleanolic acid (OA) on heat hyperalgesia. (A) Changes in paw withdrawal latency following oleanolic acid pre-treatment at (0.5mg/injection)

and (B) at (3mg/injection) over a one-week period. (C) Changes in paw withdrawal latency following oleanolic acid post-treatment (0.5mg/injection) over a one-week period. The blue and red arrows indicate the time of injection of OA and K/C, respectively. Data are expressed as mean ± SEM. Statistical significance with the control group is indicated by * for $p < 0.05$.

B. Effect of oleanolic acid pre-treatment and post-treatment on mechanical hyperalgesia

The effect of oleanolic acid on mechanical hyperalgesia was tested prior to and post induction of inflammation by measuring paw withdrawal frequency to a mechanical stimulus.

Compared to control rats, pre-treatment with the high dose of oleanolic acid (3mg/injection) resulted in a decrease in mechanical hyperalgesia at 4hrs post-injection ($p<0.05$). No statistically significant changes were observed at 8, 24, 48hrs and 1-week post-injection ($p>0.05$) although a pattern of decrease at every time point post injection can be noted. On the other hand, rats pre-treated with the low dose of oleanolic acid (0.5mg/injection) showed a similar decrease in mechanical hyperalgesia only at 8hrs and 1-week post-injection ($p <0.05$) as compared to control rats. In the post-treatment group, rats showed a significant decrease in mechanical hyperalgesia at 4, 24, and 48hrs post-injection ($p<0.05$) as evident by a decrease in paw withdrawal frequency to mechanical stimulation.

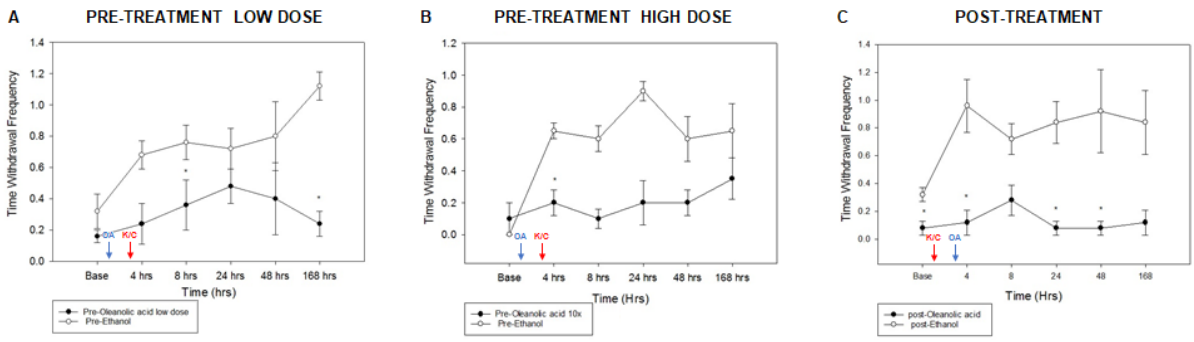


Figure 4: Effect of oleanolic acid on mechanical hyperalgesia. (A) Changes in paw withdrawal frequency following pre-treatment with oleanolic acid (0.5mg) over a one-week period. (B)

Changes in paw withdrawal frequency following Pre-treatment with oleanolic acid (3mg) over a one-week period. (C) Changes in paw withdrawal frequency following oleanolic acid post-treatment (0.5mg/injection) over a one-week period. The blue and red arrows indicate the time of oleanolic acid and K/C injections, respectively. Data are expressed as mean ± SEM. Statistical significance with the control group is indicated by * for $p < 0.05$.

C. Effect of oleanolic acid pre-treatment and post-treatment on mechanical allodynia

When rats received the low and high dose of OA prior to kaolin and carrageenan injection, a decrease in mechanical allodynia was evident at all time points tested. The innocuous stimulus used in this test to activate mechanoreceptors only, did not elicit any increased response in the inflamed paw of rats treated with OA vs. those treated with the vehicle.

Compared to control rats, those pre-treated with the high dose of oleanolic acid (3mg/injection) showed a pronounced decrease in mechanical allodynia at 4hrs and 1week post-injection ($p<0.05$). No statistically significant changes were achieved at 8, 24 and 48hrs post-injection ($p>0.05$). On the other hand, rats pre-treated with the low dose of oleanolic acid (0.5mg/injection) showed a decrease in mechanical allodynia at 8hrs post-injection ($p<0.05$) compared to control rats, but no changes at 4, 24, 48hrs and 1-week post-injection ($p>0.05$). Finally, rats post-treated with the low dose of oleanolic acid (0.5mg/injection) showed a decrease in mechanical allodynia at 8hrs post-injection ($p<0.05$) but no changes at 4hrs, 24hrs, 48hrs and 1-week post-injection ($p>0.05$).

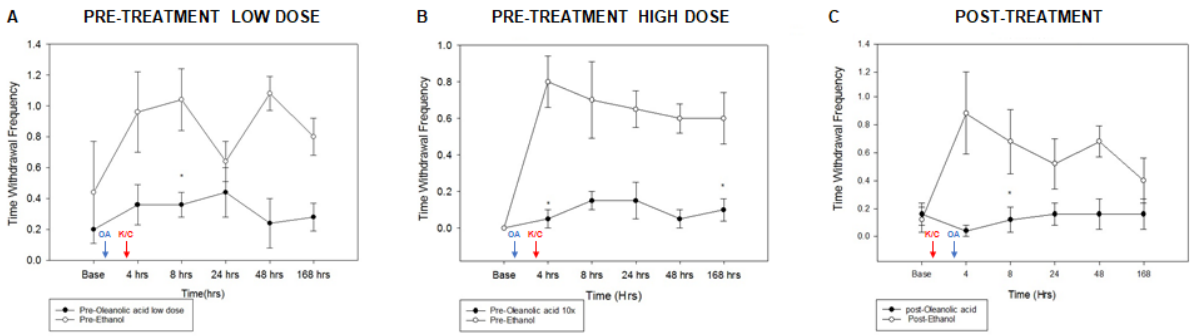


Figure 5: Effect of pre- and post-treatment with oleanolic acid on mechanical allodynia. Paw withdrawal frequency following pre-treatment of joint with 0.5mg (A) and 3mg (B) oleanolic acid and post-treatment with 0.5mg OA (C) over a one-week period. The blue and red arrows indicate the time of oleanolic acid and K/C injections, respectively. Data are expressed as mean ± SEM. Statistically significant data are indicated by * for $p < 0.05$.

D. Effect of oleanolic acid pre-treatment and post-treatment on the rotarod test

The effect of oleanolic acid treatment on motor coordination in inflamed rats was assessed by measuring the duration it took the rat to fall off a horizontal rotating rod. Compared to control rats, pre-treatment with the high dose of oleanolic acid showed an increase in the duration at 4hrs post-injection ($p<0.05$). No statistically significant changes were observed at 8, 24, 48hrs and 1 week post-injection ($p>0.05$). For the pre-treatment group with the low dose of OA, no significant changes were seen when compared to control rats ($p>0.05$). Similarly, post-treatment with the low dose of oleanolic acid had no effect on motor coordination as compared to control rats ($p>0.05$).

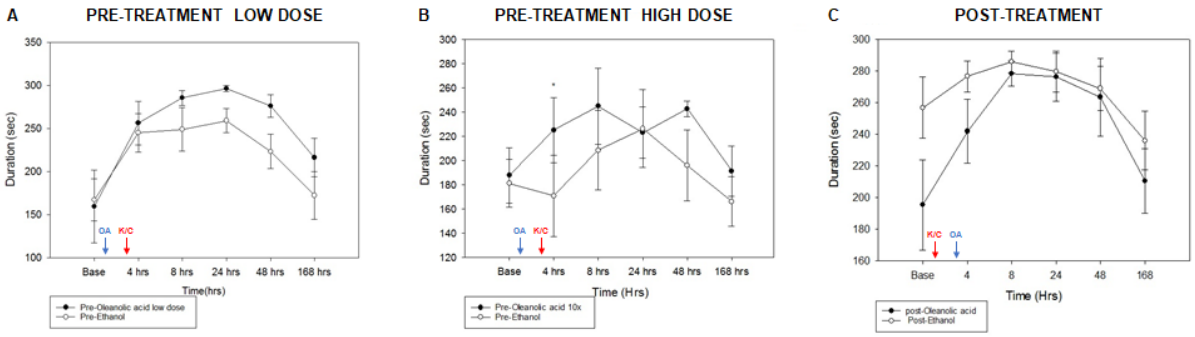


Figure 6: Effect of oleanolic acid pre-and post-treatment on motor activity using Rotarod test. (A) Changes in Duration(s) of the rat's falling from the rotarod wheel following oleanolic acid pre-treatment (0.5mg/injection) over a one-week period. (B) Changes in Duration(s) of the rat's falling from the rotarod wheel following oleanolic acid pre-treatment (3mg/injection) over a one-week period. (C) Changes in Duration(s) of the rat's falling from the rotarod wheel

following oleanolic acid post-treatment (0.5mg/injection) over a one-week period. The blue and red arrows indicate injection of oleanolic acid and K/C, respectively. Data are expressed as mean ± SEM. Statistical significance with the control group is indicated by * for $p < 0.05$.

E. Effect of oleanolic acid pre-treatment and post-treatment on joint circumference

The effect of oleanolic acid treatment on joint circumference was assessed prior to (pre-treatment; low and high dose) or following (post-treatment; low dose only) the induction of inflammation. Compared to control rats, rats pre-treated with the high dose of oleanolic acid (3mg/injection) showed a decrease in joint circumference at 4 and 24hrs post-injection ($p < 0.05$), while no change was noticed at 8, 48hrs and 1-week post-injection ($p > 0.05$). On the other hand, rats pre-treated with the low dose of oleanolic acid showed a decrease in joint circumference at 24hrs post-injection ($p < 0.05$) but no changes at 4, 8, 48hrs and 1-week post-injection ($p > 0.05$). Finally, post-treatment with the low dose of oleanolic acid had no effect on joint circumference when tested at all time points compared to control rats ($p > 0.05$).

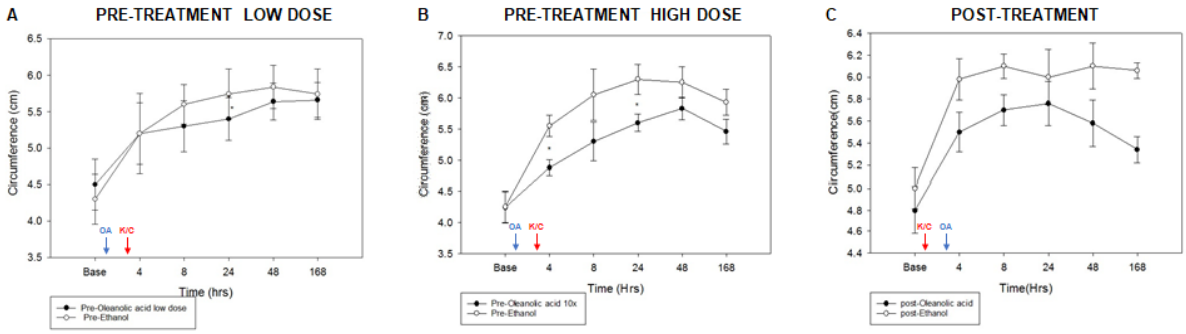


Figure 7: Effect of oleanolic acid pre-and post-treatment on joint circumference measurement. Changes in joint circumference in the oleanolic acid pre-treatment group (0.5mg/injection) (A) and (3mg/injection) (B) over a one-week period. (C) Changes in joint circumference in the post-treatment group (0.5mg/injection). The blue and red arrows indicate the time of oleanolic

acid and K/C injections, respectively. Data are expressed as mean ± SEM. Statistical significance with the control group is indicated by * for $p < 0.05$.

F. Effect of Oleanolic acid on the activity of articular fibers

A total of 12 sensory fibers were isolated using the spike 2 acquisition program and used for the present electrophysiological study. Fibers responding to mechanical stimulation of the skin around the joint or to knee joint flexing were selected. The type of fibers could not be identified since their conduction velocity was not measured. Prior to induction of inflammation, most fibers were silent except for 2 that showed spontaneous activity in a 5-min interval. Some fibers show a short burst-type discharge but became quiescent afterwards. Oleanolic acid injection (3mg/injection) elicited discharges in 3 out of 4 silent fibers, increasing activity by 38.5% over baseline. The average latency for oleanolic acid to evoke discharges in initially quiescent fibers was about 2 sec. This evoked response lasted for only 2 min.

On the other hand, recordings from sensory fibers four hours after induction of inflammation, showed remarkable spontaneous activity with a mean discharge rate of 0.06 spikes/sec. Oleanolic acid administration 4.5 hours after K/C injection, significantly increased the discharge rate by 344% within 10 sec of application ($p<0.05$) (Figure 8). This effect lasted for 10 min, after which it gradually decreased to return to baseline values (Figure 8).

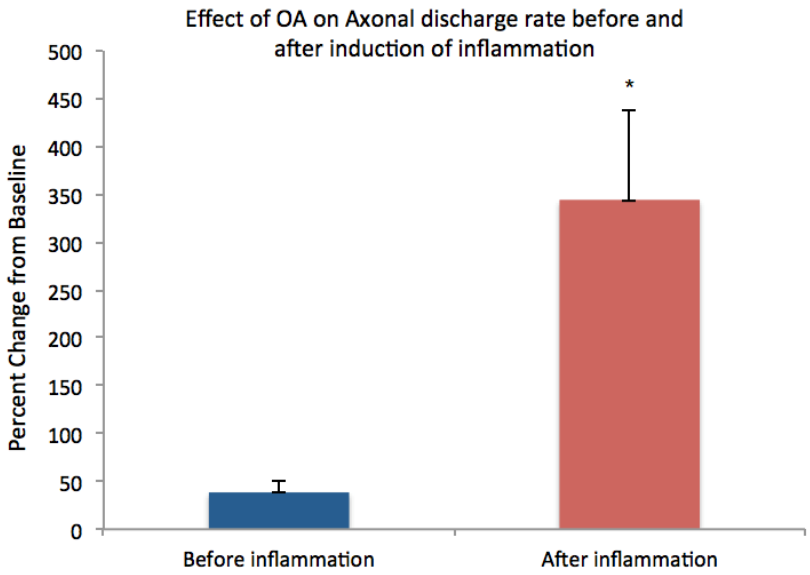


Figure 8: Effect of oleanolic acid on axonal discharge rate of articular fibers. This figure illustrates changes in percentage change from baseline before and after induction of inflammation. Data are expressed as mean + SEM. Statistical significance with the control group is indicated by * for $p < 0.05$.

CHAPTER 4

DISCUSSION

In the present work, a K/C rat model of osteoarthritis was used to investigate the effect of oleanolic acid on osteoarthritis-related symptoms of pain, inflammation, and on motor behavior. Oleanolic acid was applied directly into the knee joint cavity either prior to or post induction of inflammation.

Our results show that pre-treatment with oleanolic acid at the high dose resulted in decreased heat hyperalgesia, mechanical hyperalgesia and mechanical allodynia. Conversely, pre-treatment with oleanolic acid at the low dose resulted in no changes in heat hyperalgesia, but caused a significant decrease in mechanical hyperalgesia and mechanical allodynia. These findings suggest that oleanolic acid exerts a preventive effect on the development of nociceptive behaviors induced by inflammation, particularly mechanical hyperalgesia and allodynia as compared to heat hyperalgesia. In addition, our results show that at some of the tested time points, pre-treatment with oleanolic acid at the high dose resulted in improved motor coordination as assessed by the rotarod test, and that pre-treatment with oleanolic acid at both the low and high dose resulted in decreased joint circumference. Collectively, these findings indicate that oleanolic acid exerts a preventive effect on the development of motor coordination impairment and edema formation caused by the inflammation of the knee joint.

On the other hand, the results of the post-treatment study show that injection of oleanolic acid at a low dose resulted in decreased heat hyperalgesia, and mechanical hyperalgesia and allodynia, suggesting that oleanolic acid exerts a modulatory effect on the activity of nociceptors and mechanoreceptors in the knee joint. Interestingly, post-treatment with oleanolic acid at the low dose failed to significantly affect the performance of rats in the rotarod test, suggesting no changes in their motor coordination. Similarly, no changes in joint

circumference was observed following post-treatment with oleanolic acid at the low dose, indicating that oleanolic acid did not have any effect on the edema caused by the inflammation of the knee joint.

Findings of the present study showing that pre- or post-treatment with oleanolic acid decreases nociceptive behaviors in K/C rats are consistent with previous reports indicating that oleanolic acid exerts anti-nociceptive effects in experimental models of pain. In particular, our results corroborate a previous study in mice showing that oral administration of oleanolic acid induces anti-nociceptive effects in various models of pain, including writhing, formalin, substance P and glutamate pain tests (Park et al., 2013). In addition, pre-treatment with oleanolic acid was previously shown to reduce formalin-induced nociceptive behavior in adult zebrafish (Soares et al., 2019) as well as mustard oil-induced nociceptive behaviors in mice (Maia et al., 2006a). Also in line with our findings are studies in rodents showing that a mixture of oleanolic acid and acetylsalicylic acid (Bednarczyk-Cwynar et al., 2016) and a derivative of oleanolic acid (Bednarczyk-Cwynar et al., 2012) induce dose-dependent analgesic effect in a hot plate test. Although the present study did not directly evaluate the mechanisms by which oleanolic acid exerts anti-nociceptive effects, we can speculate that these effects are most likely due to the recruitment of opioid and serotonin systems as pre-treatment with the opioid antagonist, naloxone, or the serotonin receptor antagonist, methysergide, was previously shown to attenuate the anti-nociceptive effects of oleanolic acid in mice (Maia et al., 2006a; Maia et al., 2006b; Park et al., 2013). Another likely mediator of the anti-nociceptive effect of oleanolic acid is the Transient Receptor Potential Vanilloid 1 (TRPV1) receptor, which is expressed by primary afferent sensory neurons of the pain pathway (Soares et al., 2019).

Interestingly, when assessing heat and mechanical hyperalgesia, results of the present study show differential anti-nociceptive effects of oleanolic acid at the low versus high dose. While pre-treatment with oleanolic acid at the high dose was effective in decreasing both heat and

mechanical hyperalgesia, pre-treatment with the low dose was only effective in decreasing mechanical hyperalgesia. These results suggest that heat and mechanical hyperalgesia might be mediated by different mechanisms and might involve molecular players that show different affinity to oleanolic acid. In support of this hypothesis, collective evidence indicates that co-activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and the metabotropic glutamate (mGlut) receptors are both necessary and sufficient for mechanical hyperalgesia, while activation of N-methyl-D-aspartate (NMDA) receptors is necessary for heat hyperalgesia (Meller, 1994). As such, our results could indicate that oleanolic acid has a strong affinity to AMPA and mGlut receptors, which are involved in mechanical hyperalgesia, and a relatively lower affinity to NMDA receptors, which are involved in heat hyperalgesia. However, more work is needed to validate this hypothesis.

Another interesting finding of the present study is that pre-treatment with oleanolic acid at the high, but not the low, dose resulted in improved motor coordination in rats as assessed by the rotarod test, while post-treatment with oleanolic acid at the low dose failed to significantly affect motor coordination. These findings suggest that oleanolic acid show more effectiveness in preventing, rather than treating, the impairment of motor coordination induced by inflammation. However, it is important to note that the lack of effect on motor coordination observed with the post-treatment experiment may be due to the single dose of oleanolic acid used in the present study, as opposed to the pre-treatment experiment where two doses of oleanolic acid were employed. Also, our findings suggest that the anti-nociceptive effects of oleanolic acid— which were observed with both pre- and post-treatment at the low and high dose—are most likely not a consequence of motor abnormality. Lastly, the lack of effect on motor coordination observed with the low dose of oleanolic acid (both pre- and post- treatment) in the present study is in line with previous studies showing that administration of oleanolic acid in experimental models of pain does not alter the locomotor activity in the open field test

(Maia et al., 2006a; Maia et al., 2006b; Soares et al., 2019) nor the motor coordination in the rotarod test (Maia et al., 2006b). However, findings of Maia et al., (2006b) contradict our results showing improved motor coordination following pre-treatment with oleanolic acid at the high dose. Such discrepancy in the results could likely be attributed to differences in the animal model and doses of oleanolic acid used.

In addition, results of the present study showing that pre-treatment with oleanolic acid at both the low and high dose resulted in decreased joint inflammation are in line with previous findings showing that oleanolic acid (Yoo et al., 2017) or analogous compounds (Kang et al., 2008; Bhat et al., 2016; Zhang et al., 2016) exert anti-inflammatory effects. Also consistent with our findings are studies in rodents showing that a mixture of oleanolic acid and acetylsalicylic acid (Bednarczyk-Cwynar et al., 2016) and a derivative of oleanolic acid (Bednarczyk-Cwynar et al., 2012) yielded marked reductions in carrageenan-induced skin inflammation in rats, as demonstrated by the decrease in hind paw thickness. Finally, our results agree with studies in vivo showing that oleanolic acid exerts an anti-inflammatory action in carrageenan and dextran-induced edema in rats (Singh et al., 1992), and with studies in vitro showing that oleanolic acid suppresses lipopolysaccharide (LPS)-mediated pro-inflammatory responses in human endothelial cells, including the production of TNF alpha and the activation of NFKB (Yang et al., 2012; Lee et al., 2013). Conversely, results of the present study showed that post-treatment with oleanolic acid at the low dose failed to decrease inflammation as assessed by changes in joint circumference, suggesting that oleanolic acid is more effective in preventing, rather than, managing, the inflammatory response induced by K/C injection. Last but not least, results of the electrophysiological recordings of the tibial nerve after oleanolic acid treatment suggests that oleanolic acid exert an excitatory effect on articular afferent activity. The increased tibial nerve activity observed in the present study is consistent with a previous study showing that ursolic acid, the isomeric triterpenic acids of

oleanolic acid, promotes the neural regeneration of the sciatic nerve, which innervates the tibial nerve, following injury (Liu et al., 2013). We speculate that the increased excitatory activity of the tibial nerve following oleanolic acid treatment leads to activation of descending inhibitory pathways and thus inhibition of pain processing at the spinal level; effect that could be mediated by alpha adrenoreceptors and glutamate receptors (Wei et al., 2016). This effect may also be mediated by activation of Farnesoid X receptors (FXR); a receptor that predominantly plays a role in the synthesis and storage of triglycerides (Liu and Wong, 2010; Jiao et al., 2015).

In summary, the present work provides clear and robust evidence of the anti-inflammatory and anti-nociceptive effect of oleanolic acid in a K/C model of osteoarthritis and demonstrates that this compound could potentially be used for the prevention and treatment of this disease.

BIBLIOGRAPHY

Aida Y, Maeno M, Suzuki N, Shiratsuchi H, Motohashi M, Matsumura H (2005) The effect of IL-1beta on the expression of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in human chondrocytes. Life Sci 77:3210-3221.

arc OC et al. (2012) Identification of new susceptibility loci for osteoarthritis (arcOGEN): a genome-wide association study. Lancet 380:815-823.

Aspden RM, Saunders FR (2019) Osteoarthritis as an organ disease: from the cradle to the grave. Eur Cell Mater 37:74-87.

Ayhan E, Kesmezacar H, Akgun I (2014) Intraarticular injections (corticosteroid, hyaluronic acid, platelet rich plasma) for the knee osteoarthritis. World J Orthop 5:351-361.

Azuma K, Casey SC, Ito M, Urano T, Horie K, Ouchi Y, Kirchner S, Blumberg B, Inoue S (2010) Pregnane X receptor knockout mice display osteopenia with reduced bone formation and enhanced bone resorption. J Endocrinol. 207(3):257-63.

Backstrom T, Winberg S (2013) Central corticotropin releasing factor and social stress. Front Neurosci 7:117.

Bednarczyk-Cwynar B, Zaprutko L, Marciniak J, Lewandowski G, Szulc M, Kaminska E, Wachowiak N, Mikolajczak PL (2012) The analgesic and anti-inflammatory effect of new oleanolic acid acyloxyimino derivative. Eur J Pharm Sci 47:549-555.

Bednarczyk-Cwynar B, Wachowiak N, Szulc M, Kaminska E, Bogacz A, Bartkowiak-Wieczorek J, Zaprutko L, Mikolajczak PL (2016) Strong and Long-Lasting Antinociceptive and Anti-inflammatory Conjugate of Naturally Occurring Oleanolic Acid and Aspirin. Front Pharmacol 7:202.

Benemei S, Nicoletti P, Capone JG, Geppetti P (2009) CGRP receptors in the control of pain and inflammation. Curr Opin Pharmacol 9:9-14.

Bhat RA, Lingaraju MC, Pathak NN, Kalra J, Kumar D, Kumar D, Tandan SK (2016) Effect of ursolic acid in attenuating chronic constriction injury-induced neuropathic pain in rats. Fundam Clin Pharmacol 30:517-528.

Bian Q, Liu SF, Huang JH, Yang Z, Tang DZ, Zhou Q, Ning Y, Zhao YJ, Lu S, Shen ZY, Wang YJ (2012) Oleanolic acid exerts an osteoprotective effect in ovariectomy-induced osteoporotic rats and stimulates the osteoblastic differentiation of bone mesenchymal stem cells in vitro. Menopause 19:225-233.

Bruyere O, Cooper C, Pelletier JP, Branco J, Luisa Brandi M, Guillemin F, Hochberg MC, Kanis JA, Kvien TK, Martel-Pelletier J, Rizzoli R, Silverman S, Reginster JY (2014) An algorithm recommendation for the management of knee osteoarthritis in Europe and internationally: a report from a task force of the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO). Semin Arthritis Rheum 44:253-263.

Caltana L, Nieto ML, Brusco A (2015) Oleanolic acid: a promising neuroprotective agent for cerebral ischemia. Neural Regen Res 10:540-541.

Castellano JM, Guinda A, Delgado T, Rada M, Cayuela JA (2013) Biochemical basis of the antidiabetic activity of oleanolic acid and related pentacyclic triterpenes. Diabetes 62:1791-1799.

Chen D, Shen J, Zhao W, Wang T, Han L, Hamilton JL, Im HJ (2017) Osteoarthritis: toward a comprehensive understanding of pathological mechanism. Bone Res 5:16044.

Couture R, Cuello AC (1984) Studies on the trigeminal antidromic vasodilatation and plasma extravasation in the rat. J Physiol 346:273-285.

Crofford LJ, Sano H, Karalis K, Friedman TC, Epps HR, Remmers EF, Mathern P, Chrousos GP, Wilder RL (1993) Corticotropin-releasing hormone in synovial fluids and tissues of patients with rheumatoid arthritis and osteoarthritis. J Immunol 151:1587-1596.

Dabby R, Menachem Sadeh, Ilan Goldberg, and Vitaly Finkelshtein (2017) Electrical stimulation of the posterior tibial nerve reduces neuropathic pain in patients with polyneuropathy. J Pain Res. 2017; 10: 2717–2723.

David WS, Sadjadi R (2019) Chapter 13 - Clinical neurophysiology of lower extremity focal neuropathies. In Handbook of Clinical Neurology (Eds Levin K.H. and Chauvel, P.) Volume 161, 2019, Pages 207-216

de Grauw JC, van de Lest CH, van Weeren R, Brommer H, Brama PA (2006) Arthrogenic lameness of the fetlock: synovial fluid markers of inflammation and cartilage turnover in relation to clinical joint pain. Equine Vet J 38:305-311.

Dillon CF, Rasch EK, Gu Q, Hirsch R (2006) Prevalence of knee osteoarthritis in the United States: arthritis data from the Third National Health and Nutrition Examination Survey 1991-94. J Rheumatol 33:2271-2279.

Fai YM, Tao CC (2009) A review of presence of oleanolic acid in natural products. Natura Proda Medica 2:77-290.

Felson DT (2006) Clinical practice. Osteoarthritis of the knee. N Engl J Med 354:841-848.

Felson DT, Naimark A, Anderson J, Kazis L, Castelli W, Meenan RF (1987) The prevalence of knee osteoarthritis in the elderly. The Framingham Osteoarthritis Study. Arthritis Rheum 30:914-918.

Fernihough J, Gentry C, Bevan S, Winter J (2005) Regulation of calcitonin gene-related peptide and TRPV1 in a rat model of osteoarthritis. Neurosci Lett 388:75-80.

Ferrell WR, Russell NJ (1986) Extravasation in the knee induced by antidromic stimulation of articular C fibre afferents of the anaesthetized cat. J Physiol 379:407-416.

Fosang AJ, Last K, Knauper V, Murphy G, Neame PJ (1996) Degradation of cartilage aggrecan by collagenase-3 (MMP-13). FEBS Lett 380:17-20.

Fu Y, Hu X, Cao Y, Zhang Z, Zhang N (2015) Saikosaponin a inhibits lipopolysaccharide-oxidative stress and inflammation in Human umbilical vein endothelial cells via preventing TLR4 translocation into lipid rafts. Free Radic Biol Med. 89:777-85.

Fukushima EO, Seki H, Ohyama K, Ono E, Umemoto N, Mizutani M, Saito K, Muranaka T (2011) CYP716A subfamily members are multifunctional oxidases in triterpenoid biosynthesis. Plant Cell Physiol 52:2050-2061.

Giner-Larza EM, Manez S, Recio MC, Giner RM, Prieto JM, Cerda-Nicolas M, Rios JL (2001) Oleanonic acid, a 3-oxotriterpene from Pistacia, inhibits leukotriene synthesis and has anti-inflammatory activity. Eur J Pharmacol 428:137-143.

Gwilym SE, Keltner JR, Warnaby CE, Carr AJ, Chizh B, Chessell I, Tracey I (2009) Psychophysical and functional imaging evidence supporting the presence of central sensitization in a cohort of osteoarthritis patients. Arthritis Rheum 61:1226-1234.

Helmick CG, Felson DT, Lawrence RC, Gabriel S, Hirsch R, Kwoh CK, Liang MH, Kremers HM, Mayes MD, Merkel PA, Pillemer SR, Reveille JD, Stone JH, National Arthritis Data W (2008) Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part I. Arthritis Rheum 58:15-25.

Hernanz A, Medina S, de Miguel E, Martin-Mola E (2003) Effect of calcitonin gene-related peptide, neuropeptide Y, substance P, and vasoactive intestinal peptide on interleukin-1beta, interleukin-6 and tumor necrosis factor-alpha production by peripheral whole blood cells from rheumatoid arthritis and osteoarthritis patients. Regul Pept 115:19-24.

Hildebrandt C, Oqvist G, Brax L et al. (1991) Anatomy of the Rat Knee Joint and Fibre Composition of a Major Articular Nerve. Anat Rec 229:545–555

Hill CL, Seo GS, Gale D, Totterman S, Gale ME, Felson DT (2005) Cruciate ligament integrity in osteoarthritis of the knee. Arthritis Rheum 52:794-799.

Hirasawa Y, Okajimi S, Ohta M, Tokiota T (2000) Nerve distribution to the humna knee joint: anatomical and immunohistochemical study. International Orthopaedics (SICOT) 24:1-4.

Huang C. (2014). Natural modulators of liver X receptors. J Integr Med. 2014 Mar;12(2):76-85.

Hsu HY, Yang JJ, Lin SY, Lin CC (1997) Comparisons of geniposidic acid and geniposide on antitumor and radioprotection after sublethal irradiation. Cancer Lett 113:31-37.

Iyengar S, Ossipov MH, Johnson KW (2017) The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine. Pain 158:543-559.

Jeong HG (1999) Inhibition of cytochrome P450 2E1 expression by oleanolic acid: hepatoprotective effects against carbon tetrachloride-induced hepatic injury. Toxicol Lett 105:215-222.

Jiang W, Wang H, Li YS, Luo W (2016) Role of vasoactive intestinal peptide in osteoarthritis. J Biomed Sci 23:63.

Jiao Y, Lu Y, Li XY (2015) Farnesoid X receptor: a master regulator of hepatic triglyceride and glucose homeostasis. Acta Pharmacol Sin 36:44-50.

Kahan A, Uebelhart D, De Vathaire F, Delmas PD, Reginster JY (2009) Long-term effects of chondroitins 4 and 6 sulfate on knee osteoarthritis: the study on osteoarthritis progression prevention, a two-year, randomized, double-blind, placebo-controlled trial. Arthritis Rheum 60:524-533.

Kaneko Y, Sano M, Seno K, Oogaki Y, Takahashi H, Ohkuchi A, Yokozawa M, Yamauchi K, Iwata H, Kuwayama T, Shirasuna K (2019) Olive Leaf Extract (OleaVita) Suppresses Inflammatory Cytokine Production and NLRP3 Inflammasomes in Human Placenta. Nutrients 11.

Kang DG, Lee HJ, Kim KT, Hwang SC, Lee CJ, Park JS (2017a) Effect of oleanolic acid on the activity, secretion and gene expression of matrix metalloproteinase-3 in articular chondrocytes in vitro and the production of matrix metalloproteinase-3 in vivo. Korean J Physiol Pharmacol 21:197-204.

Kang SY, Yoon SY, Roh DH, Jeon MJ, Seo HS, Uh DK, Kwon YB, Kim HW, Han HJ, Lee HJ, Lee JH (2008) The anti-arthritic effect of ursolic acid on zymosan-induced acute inflammation and adjuvant-induced chronic arthritis models. J Pharm Pharmacol 60:1347-1354.

Kang X, Yang Z, Sheng J, Liu JB, Xie QY, Zheng W, Chen K (2017b) Oleanolic acid prevents cartilage degeneration in diabetic mice via PPARgamma associated mitochondrial stabilization. Biochem Biophys Res Commun 490:834-840.

Kao TC, Wu CH, Yen GC (2014) Bioactivity and potential health benefits of licorice. J Agric Food Chem 62:542-553.

Kapil A, Sharma S (1995) Effect of oleanolic acid on complement in adjuvant- and carrageenan-induced inflammation in rats. J Pharm Pharmacol 47:585-587.

Kelly S, Dunham JP, Murray F, Read S, Donaldson LF, Lawson SN (2012) Spontaneous firing in C-fibers and increased mechanical sensitivity in A-fibers of knee joint-associated mechanoreceptive primary afferent neurones during MIA-induced osteoarthritis in the rat. Osteoarthritis Cartilage 20:305-313.

Kim OS, Lee CS, Joe EH, Jou I (2006) Oxidized low density lipoprotein suppresses lipopolysaccharide-induced inflammatory responses in microglia: oxidative stress acts through control of inflammation. Biochem Biophys Res Commun. 342(1):9-18

Konopleva M, Tsao T, Estrov Z, Lee RM, Wang RY, Jackson CE, McQueen T, Monaco G, Munsell M, Belmont J, Kantarjian H, Sporn MB, Andreeff M (2004) The synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces caspase-

dependent and -independent apoptosis in acute myelogenous leukemia. Cancer Res 64:7927-7935.

Lam FY, Ferrell WR (1993) Acute inflammation in the rat knee joint attenuates sympathetic vasoconstriction but enhances neuropeptide-mediated vasodilatation assessed by laser Doppler perfusion imaging. Neuroscience 52:443-449.

Lee W, Yang EJ, Ku SK, Song KS, Bae JS (2013) Anti-inflammatory effects of oleanolic acid on LPS-induced inflammation in vitro and in vivo. Inflammation 36:94-102.

Levine JD, Moskowitz MA, Basbaum AI (1985) The contribution of neurogenic inflammation in experimental arthritis. J Immunol 135:843s-847s.

Li M, Han Z, Bei W, Rong X, Guo J, Hu X (2015) Oleanolic Acid Attenuates Insulin Resistance via NF-kappaB to Regulate the IRS1-GLUT4 Pathway in HepG2 Cells. Evid Based Complement Alternat Med 2015:643102.

Li X, Song Y, Zhang P, Zhu H, Chen L, Xiao Y, Xing Y (2016) Oleanolic acid inhibits cell survival and proliferation of prostate cancer cells in vitro and in vivo through the PI3K/Akt pathway. Tumour Biol 37:7599-7613.

Lin AC, Seeto BL, Bartoszko JM, Khoury MA, Whetstone H, Ho L, Hsu C, Ali SA, Alman BA (2009) Modulating hedgehog signaling can attenuate the severity of osteoarthritis. Nat Med 15:1421-1425.

Lin YN, Chang HY, Wang CCN, Chu FY, Shen HY, Chen CJ, Lim YP (2018) Oleanolic Acid Inhibits Liver X Receptor Alpha and Pregnane X Receptor to Attenuate Ligand-Induced Lipogenesis. J Agric Food Chem 66:10964-10976.

Liu J (1995) Pharmacology of oleanolic acid and ursolic acid. J Ethnopharmacol 49:57-68.

Liu J (2005) Oleanolic acid and ursolic acid: research perspectives. J Ethnopharmacol 100:92-94.

Liu W, Wong C (2010) Oleanolic acid is a selective farnesoid X receptor modulator. Phytother Res 24:369-373.

Liu B, Liu Y, Yang G, Xu X, Chen J (2013) Ursolic acid induces neural regeneration after sciatic nerve injury. Neural Regen Res 8:2510–2519.

Lloyd-Roberts GC (1953) The role of capsular changes in osteoarthritis of the hip joint. J Bone Joint Surg Br 35-B:627-642.

Loeser RF, Goldring SR, Scanzello CR, Goldring MB (2012) Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum 64:1697-1707.

Lotz M, Carson DA, Vaughan JH (1987) Substance P activation of rheumatoid synoviocytes: neural pathway in pathogenesis of arthritis. Science 235:893-895.

Lotz MK, Kraus VB (2010) New developments in osteoarthritis. Posttraumatic osteoarthritis: pathogenesis and pharmacological treatment options. Arthritis Res Ther 12:211.

Maia JL, Lima-Junior RC, David JP, David JM, Santos FA, Rao VS (2006a) Oleanolic Acid, a pentacyclic triterpene attenuates the mustard oil-induced colonic nociception in mice. Biol Pharm Bull 29:82-85.

Maia JL, Lima-Junior RC, Melo CM, David JP, David JM, Campos AR, Santos FA, Rao VS (2006b) Oleanolic acid, a pentacyclic triterpene attenuates capsaicin-induced nociception in mice: possible mechanisms. Pharmacol Res 54:282-286.

Mazuy C, Helleboid A, Staels B, Lefebvre P (2015) Nuclear bile acid signaling through the farnesoid X receptor. Cell Mol Life Sci 72:1631-1650.

McEvoy AN, Bresnihan B, FitzGerald O, Murphy EP (2001) Corticotropin-releasing hormone signaling in synovial tissue from patients with early inflammatory arthritis is mediated by the type 1 alpha corticotropin-releasing hormone receptor. Arthritis Rheum 44:1761-1767.

Meller ST (1994) Thermal and mechanical hyperalgesia: A distinct role for different excitatory amino acid receptors and signal transduction pathways? APS Journal 3:215-231.

Mencarelli A, Renga B, Distrutti E, Fiorucci S (2009) Antiatherosclerotic effect of farnesoid X receptor. Am J Physiol Heart Circ Physiol 296:H272-281.

Menkes A, Mazel S, Redmond RA, Koffler K, Libanati CR, Gundberg CM, Zizic TM, Hagberg JM, Pratley RE, Hurley BF (1993) Strength training increases regional bone mineral density and bone remodeling in middle-aged and older men. J Appl Physiol (1985) 74:2478-2484.

Michael JW, Schluter-Brust KU, Eysel P (2010) The epidemiology, etiology, diagnosis, and treatment of osteoarthritis of the knee. Dtsch Arztebl Int 107:152-162.

Myers J, Wielage RC, Han B, Price K, Gahn J, Paget MA, Happich M (2014) The efficacy of duloxetine, non-steroidal anti-inflammatory drugs, and opioids in osteoarthritis: a systematic literature review and meta-analysis. BMC Musculoskelet Disord 15:76.

O'Brien C, Woolf CJ, Fitzgerald M, Lindsay RM, Molander C (1989) Differences in the chemical expression of rat primary afferent neurons which innervate skin, muscle or joint. Neuroscience 32:493-502.

O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F (2004) The role of substance P in inflammatory disease. J Cell Physiol 201:167-180.

Ogbonna AC, Clark AK, Malcangio M (2015) Development of monosodium acetate-induced osteoarthritis and inflammatory pain in ageing mice. Age (Dordr) 37:9792.

Oguro T, Liu J, Klaassen CD, Yoshida T (1998) Inhibitory effect of oleanolic acid on 12-O-tetradecanoylphorbol-13-acetate-induced gene expression in mouse skin. Toxicol Sci 45:88-93.

Ovesna Z, Vachalkova A, Horvathova K, Tothova D (2004) Pentacyclic triterpenoic acids: new chemoprotective compounds. Minireview. Neoplasma 51:327-333.

Park SH, Sim YB, Kang YJ, Kim SS, Kim CH, Kim SJ, Suh HW (2013) Mechanisms involved in the antinociceptive effects of orally administered oleanolic acid in the mouse. Arch Pharm Res 36:905-911.

Pavelka K, Gatterova J, Olejarova M, Machacek S, Giacovelli G, Rovati LC (2002) Glucosamine sulfate use and delay of progression of knee osteoarthritis: a 3-year, randomized, placebo-controlled, double-blind study. Arch Intern Med 162:2113-2123.

Peat G, McCarney R, Croft P (2001) Knee pain and osteoarthritis in older adults: a review of community burden and current use of primary health care. Ann Rheum Dis 60:91-97.

Pollier J, Goossens A (2012) Oleanolic acid. Phytochemistry 77:10-15.

Preston D, Shapiro B (2013) Electromyography and Neuromuscular Disorders (Third Edition). Elsevier

Raphael TJ, Kuttan G (2003) Effect of naturally occurring triterpenoids glycyrrhizic acid, ursolic acid, oleanolic acid and nomilin on the immune system. Phytomedicine 10:483-489.

Reginster JY, Deroisy R, Rovati LC, Lee RL, Lejeune E, Bruyere O, Giacovelli G, Henrotin Y, Dacre JE, Gossett C (2001) Long-term effects of glucosamine sulphate on osteoarthritis progression: a randomised, placebo-controlled clinical trial. Lancet 357:251-256.

Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev 14:2819-2830.

Roos EM (2005) Joint injury causes knee osteoarthritis in young adults. Curr Opin Rheumatol 17:195-200.

Sassi N, Laadhar L, Allouche M, Achek A, Kallel-Sellami M, Makni S, Sellami S (2014) WNT signaling and chondrocytes: from cell fate determination to osteoarthritis physiopathology. J Recept Signal Transduct Res 34:73-80.

Schwab W, Bilgicyildirim A, Funk RH (1997) Microtopography of the autonomic nerves in the rat knee: a fluorescence microscopic study. Anat Rec 247:109-118.

Seasholtz A (2000) Regulation of adrenocorticotropic hormone secretion: lessons from mice deficient in corticotropin-releasing hormone. J Clin Invest 105:1187-1188.

Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, Moses HL (1997) Expression of a truncated, kinase-defective TGF-beta type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J Cell Biol 139:541-552.

Singh GB, Singh S, Bani S, Gupta BD, Banerjee SK (1992) Anti-inflammatory activity of oleanolic acid in rats and mice. J Pharm Pharmacol 44:456-458.

Sluka KA, Lawand NB, Westlund KN (1994) Joint inflammation is reduced by dorsal rhizotomy and not by sympathectomy or spinal cord transection. Ann Rheum Dis. 53(5):309-14.

Soares ICR, Santos S, Coelho RF, Alves YA, Vieira-Neto AE, Tavares KCS, Magalhaes FEA, Campos AR (2019) Oleanolic acid promotes orofacial antinociception in adult zebrafish (Danio rerio) through TRPV1 receptors. Chem Biol Interact 299:37-43.

Spector TD, Cicuttini F, Baker J, Loughlin J, Hart D (1996) Genetic influences on osteoarthritis in women: a twin study. BMJ 312:940-943.

Spillmann F, Van Linthout S, Miteva K, Lorenz M, Stangl V, Schultheiss HP, Tschöpe C. (2014) LXR agonism improves TNF-α-induced endothelial dysfunction in the absence of its cholesterol-modulating effects. Atherosclerosis. 232(1):1-9.

Sutton S, Clutterbuck A, Harris P, Gent T, Freeman S, Foster N, Barrett-Jolley R, Mobasheri A (2009) The contribution of the synovium, synovial derived inflammatory cytokines and neuropeptides to the pathogenesis of osteoarthritis. Vet J 179:10-24.

Tamer TM (2013) Hyaluronan and synovial joint: function, distribution and healing. Interdiscip Toxicol 6:111-125.

Tsao SM, Yin MC (2015) Antioxidative and antiinflammatory activities of asiatic acid, glycyrrhizic acid, and oleanolic acid in human bronchial epithelial cells. J Agric Food Chem 63:3196-3204.

Valdes AM et al. (2008) Genome-wide association scan identifies a prostaglandin-endoperoxide synthase 2 variant involved in risk of knee osteoarthritis. Am J Hum Genet 82:1231-1240.

Wang J, Wang Y, Zhang H, Lu M, Gao W, Yin L, Yin Z (2018) Comparative efficacy and safety of oral or transdermal opioids in the treatment of knee or hip osteoarthritis: a systematic review and Bayesian network meta-analysis protocol. BMJ Open 8:e022142.

Wei L, Zhu YM, Zhang YX, Liang F, Jia H, Qu CL, Wang J, Tang JS, Lu SM, Huo FQ, Yan CX (2016) Activation of α1 adrenoceptors in ventrolateral orbital cortex attenuates allodynia induced by spared nerve injury in rats. Neurochem Int. 99:85-93.

Wylde V, Hewlett S, Learmonth ID, Dieppe P (2011) Persistent pain after joint replacement: prevalence, sensory qualities, and postoperative determinants. Pain 152:566-572.

Yang EJ, Lee W, Ku SK, Song KS, Bae JS (2012) Anti-inflammatory activities of oleanolic acid on HMGB1 activated HUVECs. Food Chem Toxicol 50:1288-1294.

Yin MC, Lin MC, Mong MC, Lin CY (2012) Bioavailability, distribution, and antioxidative effects of selected triterpenes in mice. J Agric Food Chem 60:7697-7701.

Yoo SR, Jeong SJ, Lee NR, Shin HK, Seo CS (2017) Quantification Analysis and In Vitro Anti-Inflammatory Effects of 20-Hydroxyecdysone, Momordin Ic, and Oleanolic Acid from the Fructus of Kochia scoparia. Pharmacogn Mag 13:339-344.

Zacest AC, Vink R, Manavis J, Sarvestani GT, Blumbergs PC (2010) Substance P immunoreactivity increases following human traumatic brain injury. Acta Neurochir Suppl 106:211-216.

Zeng C, Wei J, Persson MSM, Sarmanova A, Doherty M, Xie D, Wang Y, Li X, Li J, Long H, Lei G, Zhang W (2018) Relative efficacy and safety of topical non-steroidal anti-inflammatory drugs for osteoarthritis: a systematic review and network meta-analysis of randomised controlled trials and observational studies. Br J Sports Med 52:642-650.

Zhang L, Hoff AO, Wimalawansa SJ, Cote GJ, Gagel RF, Westlund KN (2001) Arthritic calcitonin/alpha calcitonin gene-related peptide knockout mice have reduced nociceptive hypersensitivity. Pain 89:265-273.

Zhang R, Yao J, Xu P, Ji B, Luck JV, Chin B, Lu S, Kelsoe JR, Ma J (2015) A comprehensive meta-analysis of association between genetic variants of GDF5 and osteoarthritis of the knee, hip and hand. Inflamm Res 64:405-414.

Zhang Y, Kast-Woelbern HR, Edwards PA (2003) Natural structural variants of the nuclear receptor farnesoid X receptor affect transcriptional activation. J Biol Chem 278:104-110.

Zhang-Gandhi C, Drew PD (2007) Liver X receptor and retinoid X receptor agonists inhibit inflammatory responses of microglia and astrocytes. J Neuroimmunol. 183(1-2):50-9.

Zhang Y, Song C, Li H, Hou J, Li D (2016) Ursolic acid prevents augmented peripheral inflammation and inflammatory hyperalgesia in high-fat diet-induced obese rats by restoring downregulated spinal PPARalpha. Mol Med Rep 13:5309-5316.

Zhao H, Liu J, Song L, Liu Z, Han G, Yuan D, Wang T, Dun Y, Zhou Z, Liu Z, Wang Y, Zhang C (2017) Oleanolic acid rejuvenates testicular function through attenuating germ cell DNA damage and apoptosis via deactivation of NF-kappaB, p53 and p38 signalling pathways. J Pharm Pharmacol 69:295-304.

Zhu YM, Shen JK, Wang HK, Cosentino LM, Lee KH (2001) Synthesis and anti-HIV activity of oleanolic acid derivatives. Bioorg Med Chem Lett 11:3115-3118.