EFFECT OF OMEGA 3 FATTY ACIDS AND VITAMIN A ON CARRAGEENAN INDUCED JOINT INFLAMMATION IN FEMALE WISTAR RATS

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BY

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FACULTY OF BASIC MEDICAL SCIENCES,

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Declaration

I declare that the work in this dissertationentitled "Effect of omega 3 fatty acids and vitamin A on carrageenan induced joint inflammation in female wistar rats" has been carried out by me in the Department of Human Physiology, Ahmadu Bello University, Zaria under the supervision of Dr. M.B.Akor-Dewuand Dr. A. Abdulsamad.The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

______________________________ ____________ ____________

Name of Student Signature Date

Certification

Thisdissertationentitled "EFFECT OF OMEGA 3 FATTY ACIDS AND VITAMIN A ON CARRAGEENAN INDUCED JOINT INFLAMMATION IN FEMALE WISTAR RATS" by Rahmatu Ibrahim IDRIS meets the regulations governing the award of degree of Master of Science in Human PhysiologyAhmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

Dr. M.B. Akor-Dewu__________________ _______________

Signature Date

Chairman Supervisory committee

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The Dean, School of Post Graduate Studies. Signature Date

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Dedication

Idedicated this work to my grandmother HajiaUmmaKurthum who has been suffering from rheumatoid arthritis for over 30 years.

Abstract

Joint inflammation is a hallmark of diseases such as arthritis affecting single or multiple joints associated with increased pro-inflammatory markers and free radicals. Efforts are channeled towards discovering therapeutic agents which are more effective, less toxic with prolonged use and more affordable.The aim of this study was to investigate the effect of omega 3 fatty acids and vitamin A supplements on knee circumference, TNF-α, C-reactive protein (CRP), oxidative stress biomarkers: superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and malondialdehyde (MDA) on carrageenan-induced joint inflammation in female Wistar rats.Twenty four female Wistar rats of age 10 weeks weighing between 150-200g divided into six groups of 4 animals each were used for the study.Group 1 (normal control), group 2 (injection control [normal saline]), group 3-6 received 0.02ml of 1% CGN injection 3x/week for 10 weeks but in groups 4-6, after first 2 weeks of CGN injection, oral treatment with 200mg/kg Omg3, 4000IU/kg VA and 200mg/kg Omg3+4000IU/kg VA respectively was given daily with simultaneous injection of the CGN for the remaining 8 weeks. Knee diameter was measured before the first injection (at day 0), at week 2 and thereafter weekly for 8 weeks using veneercalliper. Animals were euthanized using ketamine injection and blood samples of 5 ml were drawn from the heart by cardiac puncture, collectedinplane tubes, allowed to clot and serum was separated by centrifugation. Serum TNF-α, CRP, SOD, GSH, CAT and MDA were determined. There was a significant interaction in knee circumference between time and treatment ($P<0.05$). TNF-α showed a significant decrease ($P<0.05$) in groups 4, 5 and 6 when compared to group 3. CRP level was significantly decreased ($P<0.05$) in group 4 and 6 when compared to group 3. There was a significant increase ($P<0.05$) in Groups 4, 5 and 6 of SOD, GSH and CATwhen compared to group 3. There was a significant decrease ($P<0.05$) in MDA in

groups 4, 5 and 6 when compared to group 3. In conclusion treatment with combination of 200mg/kg omega 3 fatty acidsand 4000IU/kg vitamin A was more effective than vitamin A lone. Combination of omega 3fatty acids and vitamin A did not show a better outcome than Omega 3 fatty acidalone.

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Lists of Abbreviations

CJI chronic Joint Inflammation

VA Vitamin A

Omg 3 Omega 3

Omega 3 FA Omega 3 fatty acids

EPA eicosapentaenioc Acid

DHA Docosahexanoic Acid

CGN Carrageenan

RA Rheumatoid Arthritis

OA Osteoarthritis

TNF-α Tumour Necrosis Factor α

CRP C-reactive Protien

SOD Superoxide Dismutase

CAT Catalase

GSH Glutathione

MDA Malondialdehyde

KnC Knee Circumference

ROS Reactive Oxygen Species

CHAPTER ONE

1.0 INTRODUCTION

1.1 Preamble

Inflammation is a biological reaction to altered tissue homeostasis initiated by several stimuli including chemical and mechanical injuries, autoimmune reactions and other events that invoke the defense against microorganisms (Medzhitov 2008, Dewing *et al.,* 2012). Inflammatory response is associated with a series of cellular and microvascular reactions that prevent tissue damage, however, this reaction may be prolonged in certain conditions leading to painful and chronic diseases requiring pharmacological treatment (Aliyu*et al.,*2017).

Inflammatory reaction occurs in three definitive phases which include an acute phase characterized by a rapid onset and short duration, it manifests withthe recruitment of plasma proteins, fluid, and leukocytes (most notably neutrophils) into the tissue. This recruitment is facilitated by alterations in the local vasculature that lead to vasodilation, increased capillary permeability and increased blood flow (Soehnlein and Lindbon, 2010). It is then followed by a delayed sub-acute phase characterized by infiltration of leucocytes and phagocytic cells and lastly a chronic proliferative phase, which manifests by the presence of lymphocytes and macrophages that results in fibrosis and tissue degeneration (Ashley *et al.*, 2012).

During the inflammatory processes, large amounts of pro-inflammatory mediators such as cytokines, chemokines, prostaglandins (PGs) and nitric oxide (NO) are generated by isoforms of cyclooxygenase (COX-2) and NO synthase (iNOS).These substances contribute to the classic clinical features of inflammation: heat (*calor*), redness (*rubor*), pain (*dolor*), swelling (*tumour*), diminished function and may also produce hyperalgesia or allodynia (Ashley *et al.*, 2012). Inflammation could be triggered by pathogens (bacteria, fungi, viruses and parasites), molecules (such as toxins and drugs), foreign protein in the form of antibodies and physical trauma. Chronic stress condition causes diminished effect of cortisol in regulating inflammatory response and increase production of leukocytes thus increasing risk of inflammatory-related diseases(Ashley *et al.*, 2012).

Chronic inflammation of the joints are known features of arthritic diseases which affect one or more joints with characterised synovitis, cartilage and bone destruction (Roland, 2017). Inflammatory mediators such as leucocytes, macrophages, lymphocytes as well as cytokines, chemokines and eicosanoids play critical roles in the development of chronic joint inflammation (CJI). These mediators are activated by exogenous materials or autologous antigens as in the case of autoimmune diseases like rheumatoid arthritis (RA), while in the case of non-autoimmune arthritis like osteoarthritis (OA), activation of the immune response could be due to obesity, ageing or trauma (Shiel, 2017).

Carrageenans (CGNs) are a family of linear sulphated polysaccharides that are extracted from red edible seaweeds. All carrageenans are high-molecular-weight polysaccharides made up of repeating galactose units and 3,6 anhydrogalactose (3,6-AG), both sulfated and nonsulfated, joined by alternating α-1,3 and β-1,4 glycosidic linkages (Tobacman, 2001). Many investigators use CGN to cause inflammation in the study of how specific drugs could reduce inflammation and in the study of cells and cell products involved in inflammatory response (Uno *et al.*, 2001). Carrageenan activates biologically significant pathways which include activation of reactive oxygen species (ROS), reduction in activity of sulfatase enzymes, and activation of genes involved in carcinogenesis (Tobacman, 2001; Weiner *et al.*, 2007).

Omega 3 fatty acids are polyunsaturated fatty acids (PUFAs). There are threetypes of omega 3 FA involved in human physiology; α-linolenic acid (ALA) (found in plant oils), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) commonly found in marine oils. Common sources of α-linolenic acid include walnut and flaxseed oil, while sources of animal omega 3 FA include fish oils, squid oils and krill oil. All these are important for normal metabolism and are widely used as food supplements (MacLean *et al.*, 2006; Andrew and Bolland, 2014). Due to its wide range of biological actions, public and scientific interest has been directed toward the role of omega 3 FA in health promotion and disease prevention (Car and Webel, 2012). They have been shown to have beneficial effects on some chronic degenerative diseases such as cardiovascular disease, arthritis, diabetes, some autoimmune diseases and cancer (Kaur *et al.*, 2014). Several studies have demonstrated that omega 3 FA can reduce the concentrations of proinflammatory cytokines, chemokines, eicosanoids, and other inflammatory biomarkers (Cockbain *et al.*, 2012).

Vitamin A is recognized as a fundamental element of cellular maturation and cellular differentiation. Retinoic acid, an oxidized form of vitamin A, regulates embryonic development and the differentiation of epithelial and mesenchymal tissues throughout life (Cha *et al.*, 2016). Apart from its protective effect on ROS and modulation of the immune response, vitamin A has essential roles in bone development and maintenance of epithelial tissue (Sowers and LaChance, 1999).

1.2 Statement of Research Problem

Chronic joint inflammations are among the disturbing symptoms of diseases worldwide such as injury or trauma and the most common being osteoarthritis (OA) and rheumatoid arthritis (RA). In Nigeria, the true incidence of RA and OA remains unknown, crude incidence of

RA in a Nigerian urban city (Lagos city) has been estimated to be 12.3% with a female-to-male ratio of 2.4:1 and mean age of 46.9 years (Adeneye *et al.,* 2014) while OA affects about 8 million Nigerians with females at more risk (Dashe, 2014). Survey by the World Health Organization reports that 25% of world population over the age of 65years suffer from pain and disability from OA (Breedveld, 2004) while Sundaram *et al.* (2015) reported that 10-15% of the world are arthritic and the rate expected to double by 2030. In the United States' Centre for Disease Control and Prevention (CDCP), a survey based on data from 2007– 2009 revealed that 22.2% (49.9 million) of adults aged ≥ 18 years had self-reported doctor-diagnosed arthritis, and 9.4% (21.1 million) of those with arthritis had arthritis-attributable-to-activity-limitation and with an increasing aging population this figure is expected to rise (CDCP, 2010; Adeneye *et al.,* 2014).

Although much research has been done, the medical management of chronic joint inflammation is currently inadequate (Scott *et al.,* 2010) with no therapy successfully preventing its progression. Painkillers and anti-inflammatory drugs such as steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) suppress the symptoms of the disease, but they do not stop the progression of joint destruction (Nishimura *et al.*, 2007). Due to their ability to suppress the immune system, these drugs have many side effects such as decreased resistance to infection. Biological agents such as interleukin-1 receptor antagonist (IL-1Ra) have been used and was found to have many significant limitations like systemic side effects, lack of curative response and the need to be regularly re-administered (Scott *et al.*, 2010). This calls for a search for drugs that can diminish the progression of joint inflammation with less side effect with prolong use. So far, several studies have shown that omega 3 FA exert anti-inflammatory activity,with fewer studies reporting effect of vitamin A on joint inflammation; however effect of combination of the two is yet to be ascertained.

1.3 Justification

Efforts are currently channelled towards discovering and developing therapeutic agents which are more effective, less toxic with prolonged use and more affordable. Omega 3 fatty acids and vitamin A have independently been found to have anti-inflammatory and antioxidant activity but the effect of combination of the two is yet to be revealed. As dietary supplements, they are expected to be less harmful or toxic to the body and availability and affordability of their natural sources inform their choice for this study. Based on the fact that over 80% of Nigerian population is reliant partly or wholly on one formor the other of complementary and alternative medicine for the treatment of their ailments (Adeneye *et al.*, 2014) suggests reliable scientific research to back up the use of these supplements.

Literature on the effect of omega 3 fatty acids and vitamin A on joint inflammation is sparse. It is believed that this study will add to the database of related studies and provide useful information that may serve as a lead to the development of novel therapeutic approach for the prevention and treatment of joint inflammation.

1.4 Aim of the Study

The aim of this study is to determine the effect of omega 3 fatty acids, vitamin A and their combination on carrageenan-induced joint inflammation in female Wistar rats.

1.5 Objectives of the Study

Specific objectives of this study include:

1. To determine both individual and combined effect of omega 3 fatty acids and vitamin A in carrageenan-induced joint inflammation on knee joint diameter.
2. Evaluation of the effect of single and coadministration of omega 3 fatty acids and vitamin Ain carrageenan-induced joint inflammation on inflammatory markers: TNF-α and C-reactive protein (CRP).
3. To observe the effect of omega 3 fatty acids, vitamin A and their combination on oxidative stress biomarkers: Superoxide dismutase (SOD), Catalase, Glutathione (GSH) and Malondialdehyde (MDA) in carrageenan-induced joint inflammation in female Wistar rats

1.6 Research Hypothesis

Omega 3 fatty acids and Vitamin A do not affect carrageenan-induced joint inflammation in female Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 JointInflammation

Inflammation, derived from the Latin word '*inflammatio'* meaning to set on fire, is an important process in the biological system which leads to elimination and repair of damaged tissue or neutralization of harmful agents (Ferrero-Miliani *et al.*,2007, Ashley*et al.*,2012). It is a tissue-destroying process associated with recruitment of inflammatory cell, plasma proteins and fluid into the disturbed tissue. This migration is facilitatedby alterations in the local vasculature that lead to vasodilation, increased vascular permeability, growth of new tissue and blood vessels. These processes aid the rapid destruction or isolation of the underlying sourceof the disturbance, remove damaged tissue, and then restore tissue homeostasis (Ashley *et al.,* 2012). As inflammatory activity progresses, chronic inflammation sets in and various cytokines and growth factors are secreted, which result in recruitment of more different forms of immune cells including leukocytes, lymphocytes and fibroblasts. Chronic effect of these inflammatory cells cause persistent tissue damage leading to manifestation of chronic diseases such as cardiovascular diseases, diabetes, arthritis and pulmonary diseases (Aggarwal *et al.,* 2009).

Local inflammatory response in joint inflammation is mediated by the immune system along with resident non-immune cells (synovial fibroblasts). It starts with an acute inflammatory exudate of neutrophils into the synovial space and followed by chronic inflammation of the synovial tissues, leucocytes phagocytose the antigen-antibody complexes, and release lysosomal enzymes that damage tissues including cartilage (Danquah *et al.*, 2011; Laar *et al.*, 2012). Macrophages are also stimulated, this leads to production of proteases and collagenases that further contribute to the destruction of collagen and bone. The release of multiple factors is also triggered, including pro-inflammatory cytokines, bradykinins, histamines, prostaglandin E2, serotonin, (Laar *et al.,* 2012).

When joint tissues (ligament, tendon, synovium or cartilage) are exposed to injury due to over use, trauma or other inflammation triggering factors, a chain of event in the immune system known as inflammatory signalling cascade is activated (Stills, 2018). It starts with proinflammatory protiens that first detect the damage and cause aggregation of white blood cells to clean up damaged tissues and clear out infection. This is what causes the redness, swelling and pain often seen in injury (Roland, 2017). The next response is that of anti-inflammatory mediators that take over to heal the area once the threat subsides. When this process occur it is referred to as local or acute inflammation and is expected to last for hours or days and the proteins involved in the inflammatory cascade will perform their task and later degrade (Stills, 2018; Baer, 2016). In situations where the symptoms of joint inflammation did not disappear, it indicates that the immune system is unable to turn off itself when it should and this leads to chronic inflammation.

Local injuriesand infections cause the body's immune system to turn on accelerated inflammatory response in the area of injury, while with systemic or chronic inflammation the immune system keeps the inflammatory response seething even when not suffering from the injury (Stills, 2018). This ongoing response has been associated with a range of health conditions and degenerative diseases. Persistent proliferation of the cells of the immune system (cytokines, macrophages mast cells, lymphocytes etc.)in the synovial fluid causes change in the appearance and increase in volume of this fluid and synovitis, resulting in swelling of the synovial membrane and increased joint circumference (Kidds *et al.,* 1996; Howman *et al.,* 2013). Chronic inflammation of the joint causes erosion of bony structure of the joint, it also affects connective tissues around the joint contributing to bone deformity and swelling (Stills, 2018).

Chronic joint inflammation leads to joint derangement associated with degeneration of articular cartilage and synivium, spreading into the bone itself (Shiel, 2017). It occurs in response to local factors like history of trauma, congenital or developmental joint alteration and alteration of subchondaral bone and supporting structures. It could also be due to host factors as in genetic traits and aging, obesity, infection or lifestyle (Breedveld, 2004; Baer, 2016).

Genetic factors contribute to thedevelopment of chronic joint inflammation (CJI), accounting for 50% ormore (Thomas *et al.,* 2017). Evidence of genetic factors in pathogenesis of CJI was supported by a study of 130identical and 120 non-identical female twins, the magnitude of occurrence was estimated to be 39–65% (Breedveld, 2004). Furthermore, evidence of significant genetic component from a classic female twin study showed a higher incidence of radiographic CJI of hands and knees in identical twins than in non-identical twins (Sinkov and Cymet, 2003).Chromosomal aberrations observed in synovial membrane, gene polymorphisms and human leukocyte antigen alleles have been associated with generalized osteoarthritis, while precocious joint inflammation with mild chondrodysplasia have been associated with type II procollagen gene polymorphisms (Sakkas and Platsoucas, 2007).

It has also been reported that being overweight or obese is associated with a higher rate of development and progression of knee joint inflammation. High body mass index results in mechanical stress due to overload of the weight bearing joints (Breedveld, 2004).For every 5-unit increase in body mass index, the risk ofdeveloping chronic knee joint inflammation increases by 35% (Thomas *et al.,* 2017). A report from Breedveld (2004) showed that reduction of 2kg/m^2 would decrease the risk of developing knee joint inflammation by 20–30%. This finding is supported by a study suggesting that mechanical stress in extreme sport activity or heavy physical workload can result in CJI (Lievense *et al.,*2001). Joint inflammation in obesity is also seen in non-weight bearing joint, this could be related to the activities of inflammatory mediators derived from adipose tissue, free fatty acids and ROS. White adipose tissue has been shown to produce adipokines and cytokines (TNF-α, IL-1β and IL-6) thereby increasing the incidence of obesity related joint inflammation (Courties *et al.,* 2015).

Although CJI can be found in almost all age groups, the strongest factor that predict the development of radiographically detectable damage is increasing age (Mathiessen and Conaghan, 2017). It is thought to result in insufficient cartilage repair, hormonal changes and continuous exposure to damaging environmental effects(Breedveld, 2004). Based on European report, a study in Rotterdam in 1997 showed that, out of 1040 participants aged 55–65 years, only 135 (13%) were free of radiographically detectable CJI (Breedveld, 2004).

Exposure to pathogens either bacterial, fungal or viral can cause CJI which has been shown to mostly affect the synovium (Baer, 2016). The infection usually arise from blood born organisms and may also be part of systemic infection, it is associated with intense infiltration of neutrophils with subsequent necrosis of the synovium with formation of granules and scar tissues (Baer, 2016; Shiel 2017). Bacterial products such as heat shock proteins released within the joint are capable of producing rapid cartilage destruction (Lain *et al.,* 2011).

2.2 Mechanism ofJoint Inflammation

Inflammation generally involves a tightly regulated cascade of immunologicalprocesses that are strictly organized by soluble immune signaling molecules called cytokines (Cruvinel *et al.,* 2010). This orchestrated sequence of inflammatory response beginsby recognition ofpathogens, foreign particles or tissue injurythrough detection of pathogen-associated molecular patterns (PAMPs[which are specifically expressed by pathogens and are essential for its survival]) by transmembrane toll-like receptors (TLRs) anddamage-associated molecular patterns (DAMPs[endogenous molecules that signal damage or necrosis and are recognized by the innate immune system]) by intracellular nod-like receptors (NLRs) (Janeway *et al.,* 1999, Ashley *et al.,* 2012). Detecting these signals minimize accidental targeting of host cells and tissues since innate immune system lacks the ability to recognize different forms of pathogens and whether they are harmful to the host (Janeway *et al.,*1999).Upon recognition, TLRs activate common signaling pathways that results in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)such as activation of myeloid differentiation primary response 88 (MyD88)-dependent signal transduction pathway. Activated NF-κB enhance transcription of inflammatory genes(Ashley*et al.,*2012; Haseeb and Haqqi, 2013).NLRs sends signal that activates caspase-1 which leads to conversion of cytokines into active forms, triggering inflammation (Ashley *et al.,* 2012).

Inflammatory response involves the transcription and translation of genesthat stimulate expression of proinflammatory cytokinesand chemokines, when these protiens are released, they stimulaterecruitment of effector cells such as monocytes and neutrophils along with exudate of protein-rich fluid to the site of disturbance (Cruvinel *et al.,* 2010; Soehnlein and Lindbon, 2010). Mast cells and tissue-resident macrophages potentiate this migration by releasing histamine, leukotrienes, and prostaglandins, these substances causevasodilation and increased vascular permeability (Mansouri *et al.,* 2015).Toxic compounds such as ROS, RNS and various proteases are releasedby neutrophils, these compounds are nonspecific and can attack both pathogen and host cell. The resultant effect of thiscascade of reactions is the cardinal signs of local inflammation vis heat, swelling, redness, pain, and loss of function(Janeway *et al.,*1999; Mansouri *et al.,* 2015).

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Figure 2.1: Mechanism of inflammation (Adapted from Ashely *et al*., 2012).

Ag, antigen; DC, dendritic cell; DAMP, damage-associated molecular pattern; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; IKK, inhibitor of kappa B kinase; IFN-γ, interferon-gamma; IL, interleukin; Mφ, macrophage; MHC, major histocompatibility complex; MyD88, myeloid differentiation primary response gene (88); NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLR, nucleotide binding domain and leucine-rich-repeat-containing receptors; PAMP, pathogen-associated molecular pattern; RNS, reactive nitrogen species; ROS, reactive oxygen species; TCR, T-cell receptor; TGF-β, transforming growth factor-beta; Th, T helper cell; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha; Treg, regulatory T cell.

During chronic inflammation, effector functions are further regulated by the adaptive immune systemcausing more lasting damage to cells. This interaction is similar to innate immunity but in this case there is diversity and adaptive capacity to generate new receptors or recognize new molecular patterns other than those already genetically programmed (Janeway *et al.,* 1999; Mansouri *et al.,* 2015). Macrophages, lymphocytes and dendritic cells participate in phagocytosis of antigen through production of more cytokines and antibodies, they also act as antigen presenting cells, presenting antigens bound with molecules of the major histocompatibility complex (MHC)class II receptors to naive T lymphocytes (Th0) causing them to become polarized through stimulation of the TCRs by the antigens (Davis and Pearlman, 1999; Cruvinel *et al.,* 2010).Th0 cells differentiate intosubtypes with varying functions vis Th1 cells (proinflammatory), Th2 cells (anti-inflammatory), Tregs (regulatory) and Th17 cells (proinflammatory). Cytokines that are produced by polarized Th1 and Th2 are mutually inhibitory, those produced by Treg cells dampen both Th1 and Th2 responses while Th17 cells are highly proinflammatory and are regulated by the other Th subtypes (Davis and Pearlman, 1999;Ashley *et al.,* 2012).

The last stage of inflammationoccurs when neutrophils stimulate the conversion of leukotrienes (produced by macrophages and other cells) to lipoxins, initiating termination of inflammation. Moreover, Fas ligand, resolvins, and protectins enhance apoptosis of neutrophilsand macrophages phagocytose apoptotic neutrophils and cellular debris (Ashley, *et al.,*2012).

In addition to the general sequence of inflammatory response, chronic joint inflammation following a mechanical, inflammatory and/or oxidative stress produce an imbalance between production and destruction of cartilage by releasing several cartilage specific auto-antigens which trigger the activation of immune response (Breedveld, 2004; Cruvinel*et al.,* 2010). As the disease progresses, degradation of collagen and proteoglycans in cartilage leads to fibrillation, erosion and cracking in the superficial cartilage layer which could later spread to the deeper layers of cartilage and eventually large clinically observable erosions are formed. (Breedveld 2004; Mathiessen and Conaghan, 2017).

Cells of the immune system such as T-cells, B-cells and macrophages infiltrate the diseased joint; also, cytokines and chemokines, degrading factors such as matrix metalloprotienase (MMP) and PGE_2 are released resulting in further damage to articular cartilage. Macrophage derived cytokines such as TNF-α and IL-1β are the major players in cartilage breakdown. (Haseeb and Haqqi, 2013). Bondeson *el at*. (2006)developed a macrophage deficient synovial cell culture using CD14 conjugated magnetic beads, this result in significantly decreased production of cytokines, IL-1β, TNF-α andIL-6 indicating that macrophages are the source of these cytokines. These cytokines in turn stimulate increased production of MMP which play a key role in cartilage degradation(Breedveld, 2004).

The proteins involved in inflammation as well as immune response and apoptosis are primarily regulated by transcription factor NFκB, it is present in the cytoplasm in an inactive form in association with inhibitory κB (IκB) proteins. Studies have shown the importance of NFκB in cartilage metabolism and progression of joint inflammation (Haseeb and Haqqi, 2013). Abnormal NFκB activation by stimuli such ascytokines, bacterial or viral products or free radicals leads to a cascade of signaling event that result in phosphorylation of IκB by activated IκB kinase (IKKs)that leads to the activation and nuclear translocation of NFκB. NFκB then binds to the DNA elements present in its target genes and facilitate their transcription (Haseeb and Haqqi, 2013; Akkiraju and Nohe, 2015).This triggers the loss of the growth-arrested state of articular chondrocytes, following the production of procatabolic mediators that induce cartilage degradationsuch as aggrecanases and MMPs and the proinflammatory cytokines (IL-1β and TNF-α) that induce them(Akkiraju and Nohe,2015).

As stated, NFκB can be activated by proinflammatory cytokines and a form of positive feedback loop is formed that further upregulate expression ofmore cytokines,along with chemokines, COX2 and angiogenic factors (vascular endothelial growth factor, fibroblast growth factor 2) are also expressed and these protiens leads to synovitis seen in joint inflammation (Akkiraju and Nohe, 2015; Mathiessen and Conaghan, 2017).Additionally, homeostasis of the subchondral bone that involves a balance between bone resorption and bone formation is altered, during normal condition, this balance is mediated by the receptor activator of NFκB and its ligand [(RANK)/RANKL] signaling pathway that activates NF-κB transcription factors controlled by osteoprotegerin (OPG). This homeostasis is disturbed during joint inflammation and the OPG/RANK/RANKL signaling pathway is deregulated (Akkiraju and Nohe, 2015).

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Figure 2.2:Involvement of the synovium in joint pathophysiology (Mathiessen and Conaghan, 2017).

OA, osteoarthritis. IL-1, interleukin-1. TNF, tumour necrosis factor. IL-6, interleukin-6. IL-8, interleukin-8. MMPs, matrix metalloproteinases. ADAMTS, a disintegrin and metalloprotease with thrombospondin. PGE2, Prostaglandin E2. LTB4, leukotriene B4. NO, nitric oxide. NAMPT, nicotinamide phosphoribosyltransferase. IL-4, interleukin-4. IL-13, interleukin-13. IL-12, interleukin-12. IL-1Ra, interleukin-receptor alpha. TiMPs, tissue inhibitors of metalloproteinases. BMPs, bone morphogenic proteins. CXCL13, chemokine (C-X-C motif) ligand 13. CD4+T cell, cluster of differenciation 4+ T cell. V-CAM1, vascular cell adhesion molecule 1. VEGF, vascular endothelial growth factor. EGF, epidermal growth factor.

2.3Diseases Commonly Associated with Joint Inflammation

Arthritis is the most common form of chronic joint inflammation among which are OA, RA, gout and septic arthritis, with the most common being OA and RA. These conditions cause progressive joint destruction through production of cytokines, growth factors and chemokines in cartilage, synovium and bone (Dwivedi, 2014). This results in joint pain caused by abnormal wearing of the cartilage that covers and acts as a cushion inside joints and destruction or decrease of synovial fluid that lubricates those joints. As joint cartilage progressively depletes, painful feelings are experienced upon weight bearing activities like walking and standing (Bonnet and Walsh, 2005). The joint pain of arthritis could be exacerbated by activity and relieved by rest, or initiated from autoimmune reaction in case of autoimmune form of arthritis; in more advanced disease both form of arthritis can be painful at rest and at night(Sinkov and Cymet, 2003). This pain may involve few joints like in case of OA or multiple in case of RA and septic arthritis(Sinkov and Cymet, 2003). Morning joint stiffness are common in CJI disorders and can usually last for 30 minutes or more, as the diseasesprogress, prolonged joint stiffness and joint enlargement (swelling) are prominent; like in case of RA the pain and stiffness may even last for several hours (Hinton *et al.,* 2013). Chronic joint inflammation can affect other organs in the body together with symptoms like malaise and fatigue, poor sleep, weight loss and muscle pains. With advancement of disease, movement become difficult and muscle weakness and loss of flexibility sets in; followed by disability that predisposes patients to risk of obesity, high cholesterol, cardiovascular disease and depression (Gardner, 2016).

Chronic joint inflammation in RA is known to be associated with autoimmunityand has for long being linked with production of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6), while the non-autoimmune type such as OA was first considered non inflammatory but later discovered to be also associated with elevated level of these inflammatory markers in the synovial fluid, synovial membrane, cartilage and subchondral bone (Mathiessen and Conaghan, 2017). These cytokines have catabolic response through their synergistic effects on signaling pathways that increase inflammation and cartilage degradation (Mathiessen and Conaghan, 2017).Ashraf *et al.* (2016) observed an elevated level of TNF-α, synovial inflammation and cartilage degradation in knee joints of rats injected 2% CGN.Analysis has also demonstrated that serum level ofC-reactive protein (CRP) are strongly associated with the presence and progression of OA with positive correlation of evidence of synovitis (Smith *et al.*, 2012). In several experimental studies where rats were induced different models of arthritis within some weeks to months (about 2 months or less) presented a significant increase in these markers. Mohamed *et al.* (2014) reported a significant increase in CRP in the serum of an adjuvant induced arthritis (AIA) rats after 14 days of injection compared to the normal control.

2.4 Factors Associated with Joint Inflammation

2.4.1 Tumour Necrosis Factor-α

It is well established that pro-inflammatory cytokines are involved in the pathogenesis of chronic joint inflammation.Cytokinesare cell signalling molecules in form of peptide, protein and glycoprotein that are involved in intercellular communication in immune responses and movement of cells towards site of inflammation, infection or trauma (Mandel, 2017). They serve as mediators of cellular differentiation, inflammation, immune pathology, andregulation of immune response (Lain *et al.*, 2011).The central role of TNF-α involves the activation of cytokine and chemokine expression, expression of endothelial-cell adhesion molecules, protection of synovial fibroblasts, promotion of angiogenesis, suppression of regulatory T cellsand induction of pain (Lain*et al.*, 2011). Whereas local leukocyte activation and autoantibody production is said to be caused by IL-6, however, it also mediates systemic effects that promote acute-phase responses, anaemia, cognitive dysfunction and lipid-metabolism dysregulation (Hess *et al.*, 2011). It was reported that the fundamental role of these two cytokines has been confirmed by successful therapeutic blockade of their corresponding receptors in patients with chronic joint inflammation (Venkatesha *et al.*, 2014).

A balance between pro-inflammatory (TNF-α, IL-1β and IL-6) and anti-inflammatory (IL-4, IL-10 and IFN-α) cytokines is essential for the development of a well-regulated effector immune response. The overproduction of pro-inflammatory cytokines and/or the deficiency of anti-inflammatory cytokines may lead to immunepathology (Tang *et al.*, 2015; Savio *et al.*, 2015). The pro-inflammatory cytokines IL-1, TNF-α and IL-6 in particular have been identified and intra-articular as well as systemic levels of these cytokines may to some extent reflect disease activity. Interferon-γ (IFN-γ) and IL-17 are also reported to have significant effect on disease process (Choy, 2012).

Through complex signalling pathways, these cytokines activate genes associated with CJI responses, including additional cytokines and MMPs involved in tissue degradation (Clavel *et al.*, 2013).During chronic joint inflammation, elevated level of TNF-α inhibit the synthesis of proteoglycan in cartilage explant, it also stimulate the expression ofMMP-1, MMP3 and MMP13 and stimulate bone resorption (Haseeband Haqqi, 2013). In addition to their articular effects, pro-inflammatory cytokines promote the development of systemic effects, including production of acute-phase proteins (such as CRP), anaemia of chronic disease, cardiovascular disease and osteoporosis and affect the hypothalamic–pituitary–adrenal axis, resulting in fatigue and depression (Choy, 2012). Tumour necrosis factor-α, IL-1β and IL-6 are mainly produced by cells of myeloid origin such as macrophages and dendritic cellsand during inflammatory condition, production of these cytokines are exaggerated (Lain *et al.*,2011; Choy, 2012). It may also acts as a co-stimulator of some of these cells and enhances the pathogen-directed cytotoxicity of monocytes, neutrophils, and eosinophils (Lain *et al.*, 2011). Due to its stimulatory effect on phagocytes, TNF-α plays an important role in destruction of bacteria, yeast, parasites and virus-infected cells (Sipos *et al.*, 2015).

TNF-α is expressed as a trans-membrane protein (mTNF-α) that can be processed into a soluble form (sTNF-α) via proteolytic cleavage by the metalloprotease TNF-α converting enzyme (TACE) (Parameswaran and Patial, 2010). Both soluble and membrane TNF-α bind to two trans-membrane receptor molecules: TNF receptor-1 ($TNFR_1$) referred to as p55/p60 death-domain-containing protein and TNF receptor-2 ($TNFR_2$) also known as p75/p80, whose expression is highly regulated and is typically expressed in the cells of the immune system (Parameswaran and Patial, 2010). While tumour necrosis factor receptor-1 is predominantly associated with inflammation and tissue degeneration, $TNFR_2$ is mainly implicated in tissue regeneration and immune modulation. Moreover, soluble tumour necrosis factor-α and mTNF-α differ in their capability to stimulate signalling via $TNFR_1$ and $TNFR_2$ (Michel *et al.*, 2014). Whereas tumour necrosis factor receptor-1 can be activated by binding of either sTNF-α or mTNF-α, $TNFR_2$ is only fully activated by mTNF-α (Fischer *et al.*, 2015). Accordingly, the pro-inflammatory effects of TNF-α have been associated with signalling of sTNF-α via $TNFR_1$, while signalling of mTNF-α predominantly via $TNFR_2$ is associated with tissue regeneration and immune modulation (Matsuda *et al.*, 2014; Fischer *et al.*, 2015).

It has been reported that $TNFR_1$-deficient mice show reduced development of CGN-induced joint inflammation and in human TNF transgenic (hTNFtg) mice lack of $TNFR_1$ completely protects these animals from arthritis (Boettger *et al.*, 2008; Bluml *et al.*, 2012). Moreover, reintroduction of $TNFR_1$ on mesenchymal cells is sufficient to allow for the development of TNF-dependent joint inflammation. In an hTNFtg mouse model of arthritis, $TNFR_2$-deficient mice develop aggravated arthritis and joint destruction compared with wild-type mice. (Bluml *et al.*, 2012).

Increased level of TNF-α in response to inflammation causes stimulatory effects on endothelial cells, this result in enhanced surface expression of adhesion molecules and an increased permeability of the endothelium. This is of great importance for the penetration of immune cells into the joint tissue which is a central step for the recruitment of immune cells, e.g., neutrophils, lymphocytes, and monocytes (Lee *et al.*, 2013).

Rossol *et al.* (2013) made an observation on the importance of TNF-α and $TNFR_1$ on T-cell migration into synovium using *in vitro*experiments. The results of their study showed that the interaction of TNF-α with$TNFR_1$is important migratory stimulus for CD4+ T cells and that it is TNF-α gradient dependent. Moreover, Wahid *et al.* (2000) tested the capacity of TNF-α to induce cell migration into the synovium using synovial graft implant into severe combined immunodeficient mice (SCID model) subcutaneously. They demonstrated that intragaft injection of TNF-α stimulate the expression of human cell adhesion molecule (CAM) following inhibition for 4 weeks post transplantation, and the upregulation of graft CAM is associated with increased human peripheral blood lymphocytes (PBL) migration into the transplants.

As one of the key cytokine that mediate chronic inflammation, elevated level of TNF-α can influence fibroblast-like synoviocytes (FLS) and macrophages in different ways leading to more proliferation of these cells in prolonged synovial inflammation (McInnes and Schett,2011; Bartok and Firestein, 2010). Activation of macrophages through TNF-α is transient, while the FLS are responsible for prolonged synovial inflammation, a study reported by Lee *et al.* 2013 showed uncontrolled inflammatory response due to ineffective termination of TNFα signalling in FLS. The results they obtained suggested that prolonged and sustained inflammatory responses by FLS in response to synovial TNFα contribute to the persistence of synovial inflammation.

Presently, the clear understanding of the distinct role of TNF-α in activating the macrophages and FLS is limited. With regards to macrophages a study reported that exposure of macrophages to lipopolysaccharides or IL-1β causes the release of TNFα in large quantities and formation of TNF-receptor complex can either cause cell apoptosis or activate the NF-κB/mitogen-activated protein kinase (MAPK) pathway (Shivaprasad *et al.,*2015). This pathway generates several mediators that modulate apoptosis, cell survival and other joint inflammatory processes as earlier mentioned. Similar pathway was described by Lee *et al.* (2013) in attempt to investigate the possible mechanism of FLS activated persistent synovial inflammation. Therefore, at the early stage, they can temporarily respond to TNF-α to initiate inflammation, but since FLS mainly provide a mechanical platform for synovium and usually naive to foreign antigens (Shivaprasad *et al.,*2015) they may lack the haemostatic mechanism to regulate their activation. Although the molecular mechanism behind it is unclear, available reports suggest that activation of FLS by TNFα leads to dysregulation of NF-κB signalling resulting in secretion of inflammatory mediators for longer period of time (Ramanjaneya and Shaslidadharamurthy, 2013; Angiolilli *et al.,* 2016).

2.4.1.1Tumour Necrosis Factor-α and Osteoclastogenesis

With the discovery of osteoclast (OC) in the synovial tissue of patients with CJI, interests have been developed on its fundamental role in the pathogenesis of joint damage. A number of experimental studies have demonstrated that elevated level of TNFα in the inflamed synovium plays a significant role in joint damage, as it increases OC mediated bone resorption (Briot *et al.,* 2017) either by direct OC differentiation, maturation and activation or via expression of RANKL on T-cells as well as synovial fibroblast. Recent studies have shown that TNF-α can magnify the process of osteoclastogenesis in the presence of RANKL as an additional role apart from its pro-inflammatory response (Bartok and Firestein, 2010;Briot *et al.,* 2017). In a study conducted on mice with induced joint inflammation showed that OC activity is strictly related to erosive damage, since RANKL knockout mice had only joint inflammation with absence of erosions. Binder *et al.*(2013) carried out an experiment using *in vitro* studies and human TNF-α (hTNF) transgenic destructive arthritis mouse model and found that for osteoclastogenesis to occur, high level of TNF-α is required than that needed for initiation of inflammation at the synovial joints. This is because he was able to attain a decreased expression of TNF-α using low doses of TNF-α inhibitor which was able to impede osteoclastogenesis but did not inhibit synovial inflammation. Therefore, an increased level of TNF-α inhibitors are required to inhibit both inflammation and bone resorption(Ramanjaneya and Shaslidadharamurthy, 2013; Sarkar *et al.*, 2014).

2.4.2 C-reactive Protein

C-reactive protein (CRP) has been recognised as a prototypic marker of inflammation, it is a member of the pentraxin family synthesized in the liver mainly by hepatocytes in response to pro-inflammatory cytokines in particular IL-6 derived from monocytes and macrophages (Molenaar *et al.,* 2001; Rani *et al.,* 2006; Kim *et al.,*2015).It contains five 23-kDa subunits and it can increase by 1,000-fold or more with infection, inflammation and tissue injury. Other cytokines like IL-1 beta, TNF-α and interferon gamma are also relevant to the acute phase response (Chandrashekara, 2014; Kim *et al.,* 2015).

C-reactive protein binds to complement C1q of the immune system and various fragment crystallizable-γ receptors (FcγRs) such as FcγRI (CD64) and FcγRII (CD32) which triggers pro-inflammatory activities by monocytes and macrophages(Bodman-Smith *et al.,*2004; Jones *et al.,* 2015). Fragment crystallisable-γ receptors are a family of membrane receptors found on myeloid cells, B lymphocytes, NK cells and platelets (Heikkila *et al.,* 2007). Signal transducers and activators of transcription later switch on the CRP gene expression, leading to the production of CRP (Heikkila *et al.,* 2007). In inflammation, CRP plays a regulatory role by stimulating endothelial cells to produce intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, E-selectin, and monocyte chemotactic protein (MCP)-1 and inhibits the expression of endothelial nitric oxide synthase (Kim *et al.,* 2015). Moreover, interaction of CRP with FcγRI and FcγRII also enhances the production of proinflammatory cytokines, resulting in strengthening the loop of inflammatory reaction associated with CJI (Bodman-Smith *et al.,* 2004; Kim *et al.,* 2015).

IL-1β and TNF-α are secreted during pro-inflammatory response, this results in the release of the messenger cytokine IL-6 which stimulates the liver to secrete CRP through the activation of Janus kinases (Rani *et al.,* 2006). CRP was taken to be a standby marker of inflammationwithout playing a direct role in the inflammatory process, however, recent studies suggest its direct contribution to inflammation as it is found to stimulate monocyte release of inflammatory cytokines such as IL-1, IL-6 and TNF-α and may also directly act as a pro-inflammatory stimulus to phagocytic cells (Rani *et al.*, 2006). The extra hepatic source of CRP produced in minute concentration include neurons, lymphocytes, monocytes, atherosclerotic plaques and Kupffer cells (Chandrashekara, 2014).An observation made by Jin *et al.*(2015) showed that an increase in CRP level was associated with increase in symptoms of pain and decrease physical function in jointinflammation, furthermore, Pearle *et al.*(2005) demonstrated that OA patient with histologic synovial inflammatory infiltrate had elevated CRP level and synovialIL-6 level compared with OA patients without synovial inflammation.

2.5Oxidative Stress

Oxidative stress have been shown to play important role in joint inflammation, it causes disturbance such as dyslipidaemia, atherosclerosis, connective tissue degradation and joint periarticular deformities (Rahman *et al.*, 2012). Increase in the production of ROS is said to cause damage to lipids, nucleic acids and matrix components. In chronic joint inflammation ROS act as important intracellular signalling molecules in cells of the immune system this helps in amplifying synovial inflammatory-proliferative response. Destruction of cartilage and bones by chronic inflammation can be through oxidative and proteolytic breakdown of collagen and proteoglycans, during this process, neutrophils degranulate and release variety of enzymes and peptides as well as respiratory burst reaction that generate several ROS (Rahman *et al.*, 2012; Smith *et al.*, 2012).

Studies have indicated that the development of joint inflammation is partly related to the excess production of reactive oxygen species and a lowered ability to combat ROS. Recent studies indicated that proinflammatory cytokines such as IL-1β and TNF-α are involved in the formation of toxic peroxynitrite by increasing the activity of nitric oxide synthase (Dwivedi 2014). Imbalance between oxidants and antioxidants due to increased chemical reaction or insufficient antioxidant defence system results in oxidative stress characterized by formation of excess oxidants, antioxidants deficiency or both conditions (Mateen *et al.*, 2016).

An antioxidant is any substance or compound capable of scavenging free radicals or inhibiting the oxidation process in the cell (Quiñonez-Flores, *et al.*, 2016). Aerobic production of the energy needed for the various cell processes occurs through oxidative phosphorylation in which redox reactions in the mitochondria lead to synthesis of adenosine triphosphate (Bracht *et al.,* 2016). During this process,superoxide is converted to hydrogen peroxide (H_2O_2) via the actions of mitochondrial manganese superoxide dismutase (Mn-SOD) and cytosolic Cu/Zn-SOD; subsequently, hydrogen peroxide is eliminated from cells by conversion to water in reactions catalyzed by catalase and glutathione peroxidases (Mattson *et al.,* 2002). However, hydrogen peroxide is an important source of hydroxyl radical (OH^-); a potent inducer of membrane lipid peroxidation. Generation of oxyradicals can also be as a result of calcium influx through the activation of nitric oxide (NO) synthase, this result in NO production which can react with superoxide to produce peroxynitrite (Mattson *et al.,* 2002).

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Figure 2.3: Sources of oxidative stress and molecules damaged by free radical-mediated processes (Mattson *et al.*, 2002).

Arach acid, arachidonic acid; CaM, calmodulin; depol, depolarization; ER, endoplasmic reticulum; GSH, glutathione; GSHPx, glutathione peroxidase; GSHR, glutathione reductase; GSSG, reduced glutathione; LP, lipid peroxidation; PLA2, phospholipase A2; NOS, NO synthase; PS1, presenilin-1; PT, permeability transition pore; SOD1, Cu/Zn-superoxide dismutase; SOD2, manganese superoxide dismutase

During these processes, oxygen serves as the final acceptor of electrons from cytochrome oxidase, producing water as the final product. Partially reduced oxygen metabolites are some of the intermediate products of these reactionstermed reactive oxygen species (García-González *et al.,* 2015).Reactive oxygen species are highly reactive, unstable and short-lived chemical species that have the potential to damage lipids, proteins and DNA in joint tissues (Mateen *et al.*,2016).Among ROS, superoxide radical ($\mathrm{O2}\bullet^{-}$), hydroxyl radical (HO•) and peroxynitrite (ONOO–) contain one or more unpaired electrons in the outermost orbital shell and are called free radicals. Production of free radicals in the body can also be due to leakage in the electron transport chain, inflammatory conditions, drugs, ionizing radiations, chemical toxins etc. (García-González *et al.,* 2015). They remove electrons from other compounds to attain stability and the attacked molecule then loses its electron and becomes a free radical itself, beginning a cascade of chain reaction (Quiñonez-Flores, *et al.,*2016).

Under physiological conditions, ROS participate in multiple processes such as maintenance of the cell redox state, cell signalling, and defence against microorganisms, differentiation, proliferation, growth, apoptosis, cytoskeletal regulation, and phagocytosis (Phaniendra *et al.*,2015). However if the concentrations of ROS are increased beyond normal levels they can damage cellular components, such as the lipids in the cell membranes (Lipid peroxidation) and consequent elevation of degradation products, such as malondialdehyde (MDA), proteins and nucleic acids and structural elements of the extracellular matrix such as proteoglycans and collagen (Quiñonez-Flores, *et al.*, 2016;García-González *et al.*, 2015).The damaging effect of free radicals is counteracted by the action of antioxidants. The non- enzymatic antioxidant defence includes vitamin A, C and E, reduced glutathione (GSH) and antioxidant minerals while enzymatic antioxidant includes superoxide dismutase (SOD), catalase and glutathione related enzymes (glutathione peroxidase (GPx), glutathione reductase (GR), and thioredoxin reductase (Mateen *et al.*, 2016; Quiñonez-Flores, *et al.*, 2016**;** García-González *et al.*, 2015).

Joint inflammation are one of theconditions that induce oxidative stress. A fivefold increase in mitochondrial ROS production in whole blood and monocytes of patients with chronic joint inflammation compared with healthy subjects suggests that oxidative stress is a pathogenic hallmark. (Quiñonez-Flores, *et al.*, 2016). Free radicals are indirectly implicated in joint damage because they also play an important role as secondary messengers in inflammatory and immunological cellular response (Quiñonez-Flores, *et al.*, 2016). When T-cells are exposed to increased oxidative stress they become refractory to several stimuli including those for growth and death and may prolong abnormal immune response. On the other hand, free radicals can degrade directly joint cartilage, attacking its proteoglycan and inhibiting its synthesis (Hassan *et al.*, 2011).

Elevated production of ROS and/or depletion of antioxidants have been observed in a variety of conditions including inflammatory joint diseases. Reactive oxygen speciescatalyse lipid peroxidation and tissue injury, an evidence of oxidative stress activity in CGN model of knee joint inflammation is the study of Mesquita and Costa (2013) reporting an elevated level of antioxidantenzymes following acute exposure. Heidar *et al.*(2014) also reported that rat acute model of knee OA (mono-iodoacetate induced OA) showed an increase in oxidative stress marker super oxide dismutase.Reactive nitrogen species has been reported to participate in the activation of NF-κB, as the formation of peroxynitrite interferes in the redox balance of glutathione. Studies indicate that RNS donors caused NF-κB activation and increased activation of proteolytic systems (Bar-Shai and Reznick, 2006a; 2006b). Oxidative stress can stimulate premature death of chondrocytes and damage of synovial tissue through DNA mutation (Davies *et al.*,2008).

2.6Treatment of Joint Inflammation

Analgesics prescribed for chronic joint inflammation helps in decreasing pain without inhibiting inflammatory responses. Some of these analgesics include Tramadol, Narcotics and Acetaminophen drugs such as Tylenol (Kerkar, 2017).

Chronic joint inflammation and related diseases are commonly treated with NSAIDs as it has been shown to reduce both inflammation and pain. NSAIDs include Ibuprofen and Naproxen. Their intake should be cautioned since they have been reported to cause gastric irritation and are susceptible to stroke and heart attack. The choice of NSAIDs is guided by patient comorbidities and past response to these drugs, it is also guided by dosing frequency; maximum doses are generally needed for effective management. Lower doses may be used if the disease is adequately suppressed with disease modifying anti-inflammatory drugs(DMARDs) (Baer, 2016; Kerkar, 2017).DMARDs are used to depress synovitis, this help in preventing or retarding the development of joint damage and deformity (Baer, 2016).

Treatment of chronic joint inflammation and associated conditions with corticosteroids include cortisone and prednisone which help in suppressing immune system and reducing inflammation. They are either administered orally or injected into the inflamed joint. Corticosteroids in low doses (10mg or less) may also act as an adjunct for the relief of moderate to severe pain in chronic joint inflammation (Ayhan *et al.,* 2014; Kerkar, 2017).

2.7 Omega 3 Fatty Acids and Inflammation

Eicosapentaenoic acid, docosahexaenoic acid and arachidonic acid are essential lipid classes for eicosanoid synthesis. Eicosanoids are among the major inflammation mediators and regulators. They include PGs, leukotrienes (LT), thromboxanes and other oxidized derivatives (Kaur *et al*., 2014; Cockbain *et al*., 2012). They are produced by oxidative pathways of the enzymes cyclooxygenase (COX) and lipoxygenase (LOX). Due to molecular similarity of linoleic and linolenic acid, they compete for the same enzymes to synthesize derivatives with 20 carbon atoms such as arachidonic acid, EPA and eicosatrienoic acid (ETA) (D'Eliseo and Velitto, 2016). A high intake of linoleic acid favours an increased arachidonic acid content in membrane phospholipids, thus increasing the production of prostaglandin E2, thromboxane A2 and leukotriene (LT) B4 through COX and LOX enzymatic pathways (Bistrian, 2003; Lee *et al*., 2012). High production of eicosanoids is related to the occurrence of immune disorders, cardiovascular and inflammatory diseases (Tilley *et al*., 2001). In addition, EPA and DHA are precursors of the lipid mediators resolvins and protectins, which have anti-inflammatory and immunomodulatory characteristic on joints and body system improving blood flow (Car and Webel, 2012; Cockbain *et al.*, 2012; Kaur *et al.*, 2014).

The intake of fatty acids belonging to omega 3 FAfamily such as linolenic acid, EPA or DHA which compete with arachidonic acidfor the same enzymatic pathways competitively inhibits the arachidonic acid oxidation by COX into prostaglandinsand their conversion into LTs via5-LOX pathway, this leads to inhibition of activation of the pro-inflammatory transcription factor NFκB reducing expression of inflammatory genes (Borgesa, *et al.*, 2014). Thus, omega 3 FA act by reducing the formation of eicosanoids with inflammatory characteristics, since it competes with omega-6 fatty acids for the same enzymatic pathway, (Orchard *et al.*, 2014) leading to the inhibition of TNF-α, IL-1, IL-6 synthesis, cartilage-degrading enzymes and reducing the intercellular adhesion molecule-1(ICAM-1) expression (Calder, 2015; Rajaei *et al.*, 2016). Omega 3 FA also provide environment for membrane protein function, maintaining membrane order (fluidity) and disruptionof lipid raft formation (Lee et al., 2012; Rajaei *et al.*, 2016).

It has long been established that dietary oily fish and fish oil consumption provide protection against the development of some types of cancer mainly breast, colorectal, and prostatic cancers in humans (Gu *et al.*, 2015). Thereafter, most of the studies conducted either *in vitro* or *in vivo* have demonstrated the protection by omega 3 FA against cancer risk. It induces apoptosis in cancer stem-like cells derived from the colon cancer cell line, (Xiong *et al.*, 2016). In clinical studies, encouraging results concerning the *in vivo* anti-neoplastic activity of omega 3 FA have been obtained, even though they were mainly set-up to investigate cancer prevention and support, rather than cancer therapy,while others argued that an increased omega 3 FA intake could induce some types of cancer (Nabavi*et al.*, 2015; D'Eliseo and Velotti,2016).In other reports it has been shown that the omega 3 FA help in maintenance of structure and function of the membrane, tissue metabolism, and gene regulation. It also reduces low density lipoprotein cholesterol (LDLc), very low density lipoprotein cholesterol (VLDLc), and increasing high density lipoprotein cholesterol (HDLc) concentrations decreasing oxidative and diabetes stress (Lluis *et al.*, 2013).

Omega 3 fatty acids significantly reduce serum lipids and serum sugar in patients with type 2 diabetes through its insulin-stimulating effects by increasing production and secretion of adipocytokines such as adiponectin and leptin and potential prevention of insulin resistance via direct anti- inflammatory effects(Hajianfar *et al.*, 2013; Wu *et al.*,2013). Eissa *et al.* (2015)induced type 2 diabetes in Sprague-Dawley rats by combination of high fat diet and low dose streptozotosin and were treated withomega 3 FA supplement for a period of 4 weeks, they observed that omega 3 FAsignificantly decrease serum and liver fibroblast growth factor 21 (FGF21) an effective regulator of lipid metabolism and improve lipid profile, while its combination with pioglitazone significantly decrease glucose levels and improved insulin resistance.Through modulation of transcription factors, omega 3 FA could also enhance fatty acid oxidation and reduce *de novo* lipogenesis effects which could reduce hepatic fat accumulation and preserve hepatic insulin sensitivity (Wu *et al.*, 2013).

Furthermore, fish oil is said to play a role in preventing reperfusion injury to the heart and brain(Rajaei *et al.*,2016). In an experiment, rats were fed 12% of calories either as corn oil or as fish oil for a month, after induction of myocardial ischemia, among the rats fed fish oil, 76% survived the heart attack and 14% had ventricular tachycardia and fibrillation(Harris*et al.*, 2008; Rajaei *et al.*,2016)and among those fed corn oil, the percentages were 41% and

90%, respectively. Studies on dogs have shown that acute infusion of EPA and DHA can reverse ventricular fibrillation (Harris *et al.*,2008). Study by Li *et al.,* (1996) revealed that several fatty acids, including EPA, DHA, linoleic and linolenic acid could potentiate the action of TNF-α in promoting generation of ROS by human neutrophils. Such effect could result in the activation of compensatory antioxidant enzymes, but the result emphasizes the greater potential for the effect of fatty acid in inflammatory conditions (Dwivedi 2014).

There is increasing evidence thatomega 3 FA play a significant role in neurological development and disease prevention. It exerts neuroprotective action in Parkinson's and Alzheimer's disease (Mirajkar *et al.,* 2011) and scientists found that high doses of omega 3 FAcompletely prevent the neurotoxin-induced decrease of dopamine that ordinarily occurs in such cases (Zivkovic *et al.*,2011). Avramovic *et al.* (2012) experimentedomega 3 FAsupplement effect on brain tissue oxidative stress status in aged Wistar rats (24 month), they obtained a significant decrease inlipid peroxidation and increase in SOD, and they concluded that omega 3 FA supplementation has beneficial effect on brain tissues.

Intake of omega 3 FA mainly from fish oil helps in the protection against stroke caused by build-up of plaque and blood clots in the arteries. A 50% decrease in the risk of stroke was observed when eating at least 2 servings of fish per week (Mirajkar *et al.*,2011).However, eating more than 3 grams of omega 3 FA per day which is equivalent to 3 servings of fish per day may lead to increased risk for haemorrhagic stroke (Mirajkar *et al.*,2011) since report has shown that excess omega 3 FA cause an increase in PGI_2 production (which has an increased vasodilatory and anti-platelet effect), less formation of TXA_2 and platelet activating factor which are both known to activate platelet (Busti, 2015).

2.8 Effectiveness of Vitamin A and Inflammation

Some important pathways through which vitamin A can improve joint inflammation have been postulated, these include protection against oxidative damage, modulation of the inflammatory response, cellular differentiation and biologic actions related to bone and collagen synthesis (Sowers and LaChance, 1999).Vitamin A acts as the first line of defence against free radicals attack and lipid peroxidation, by its ability to stabilize highly reactive free radicalseither independently or as a part of large enzyme system(Sail *et al.,* 2013). Vitamin A is one of the major antioxidants found in diet or dietary supplement products (Sail *et al.,* 2013). It consists of a group of fat-soluble compounds known to be its functional biological metabolites that play an important role in immunity, cellular growth and metabolism, embryogenesis and retinal function (Palczewski, 2010). Gey *et al.* (1993) showed that vitamin A interact with singlet oxygen and can thus prevent the oxidation of polyunsaturated fatty acid by scavenging $O2\bullet^{-}$and reacts directly with peroxy radical(Sail *et al.*,2013).

Vitamin A metabolites can regulate expression of genes that protect the cell from apoptosis and change the cellular redox status, it also activatenuclear retinoic acid receptors and retinoid “X” receptors which can antagonize activator protein (AP)-1-mediated gene expression (Yin *et al.,* 2015; Du *et al.*,2013). In support of this is a report from a study indicating that low consumption of vitamin A may lead to a reduced retinoid X receptor (RXR)-α and β expression in heart tissue which can interfere with the regulation of oxidative stress (Perusek and Maeda, 2013). Study by Fakher *et al.* (2007) showed that vitamin A supplementation leads to a decrease in plasma MDA level in diabetic rats and the same result was seen in the diabetic patients. The serum of OA patients have been shown to have lower levels of a variety of antioxidants, like vitamin E, vitamin C, β-carotene and antioxidant enzymes in comparison with controls (Katti *et al.*,2015). Hagfors *et al.* (2003) also made a similar report in RA patients, this can be associated with either the disease itself or in the course of treatment.

Some studies have reported an inverse relationship between vitamin A status and inflammatory response (Sauer *et al.* 1995; Swamidas *et al.* 1999).However, the mechanism involved is not well understood. Reifen (2002) found that the plasma MDA concentration of the vitamin A deficient(VAD) non-colitic rats was higher than that of the corresponding vitaminA-sufficient and vitamin A-supplemented non-colitic groups by 45% and 78% respectively, this implies that the VAD animals were under oxidative stress. He made a suggestion stating the possibility of oxidativeload in the non-colitic VAD group which may have resulted in lipid peroxidation, activation of NFκB and a subsequent inflammatory cascade (chemotaxis, infiltration of inflammatory cells and collagen deposition in the colon) which was also supported using rat asthma model. The Vitamin A and Pneumonia Working Group(1995) reported that vitamin A supplementation appears to reduce the severity of childhood pneumonia in developing countries. There is evidence that the antioxidant mechanism in vitamin Aexerts an influence in part by inhibiting translocation of the transcription factor NFκB and interrupting the secretion of inflammatory cytokines which have the potential of decreasing inflammation (Reifen,2002).

Vitamin A has essential roles in bone development, the development and maintenance of epithelial tissue, normal reproduction and vision. Its effect on protein synthesis and bone cell differentiation makes it a necessary factor for growth and development of skeletal and soft tissues (Du *et al.,* 2013; Yilmaz*et al.*,2015). Italso plays a role in maintenanceof normal epithelial structures, including the differentiation of basal cells into mucous epithelial cells and its deficiency is associated with the reversal of all the aforementioned beneficial effect (Sowers and LaChance, 1999). A review study reported that insufficient vitamin A consumption is associated with poor bone health (Cha *et al.,* 2016). Vitamin A is also able to repress both MMP activityand vascular endothelial growth factor expression, both of which are correlated with the development of joint inflammation (Yin *et al.,* 2015).

Vitamin A and its derivatives affect many developmental and differentiation pathways in the cell by regulating gene expression through down-regulation ofAP1 transcription and through its free radical scavenging properties (Amann *et al.,* 2011; Yin *et al.,* 2015). These make vitamin A supplementation an attractive therapy for oxidative stress-induced damage in a variety of inflammatory diseases.

However, despite all these beneficial discoveries on vitamin A it should be noted that excess of this vitamin can lead to health problems. In this case, it acts as a prooxidants. Excessive accumulation of vitamin A may cause an imbalance of reactive nitrogen species (RNS) (Allen and Haskell,2002; Harrison, 2012)and subsequently increase the risk of cardiovascular disease. In infants and children, excessive vitamin A consumption may cause leukemia, necrotizing vasculitis, and melancholia (Cha *et al.,* 2016).Histological examination revealed the occurrences of osteolytic osteolyis in human hypervitaminosis A, while in rats, administration of excess vitamin A showed positive correlation between angiogenesis and osteoclast formation as the latter is said to develop and function in close proximity to bone microvasculature and sinosoid(Lind *et al.,* 2017). According to a study by Pasquali *et al.* (2009) conducted in laboratory animals showed that high levels of vitamin A increased thiobarbituric acid reactive species (TBARS) levels and increased the accumulation of lipid peroxides in lung tissue. It can also increase oxidative stress through protein carbonylation. In another investigation, supplementation of vitamin A at clinical levels increased oxidative damage as well as antioxidant enzymes activities in rat liver (Schnorr *et al.*,2011; Cha *et al.*, 2016). Congenital defects has also been reported in excessivevitamin A at similar structures and developmental stages as that found in itsdeficiency(Perusek and Maeda, 2013).

2.9 Carrageenan as a Mediator of Joint Inflammation

Carrageenan is a natural carbohydrate (polysaccharide) obtained from edible red seaweeds. It is a sulfated polygalactan with 15% to40% of ester-sulfate content formed byalternate units of d-galactose and 3,6-anhydro-galactose(3,6-AG) joined by α-1,3 and β-1,4-glycosidic linkage (Tobacman, 2001). Most commonly known forms of CGN are kappa, iota, and lambda. All havesimilar D-galactose backbones (alternating α-1,3 to β-1,4 linkages) but they differ indegree of sulfation, extent of branching,solubility and cation binding (Tobacman,2001).They are large highly flexible molecules that curl forming helical structures. This gives them the ability to form a variety of different gels at room temperature.They are widely used in food industries as thickening and stabilizing agents. It serves as substitute for fat and to thicken non-fat or low fat food and beverages, in toothpaste, cosmetics pesticide and other products (Weiner *et al.*, 2007; Uno *et al.*, 2001). λ-Carrageenan is the least branched and theleast gel forming and is used to induce inflammation (Tobacman, 2001;Zainal-Ariffin*et al.*, 2014). Carrageenan is classified into undegraded (foodgrade) processed with alkali and degraded processed with acid. Degraded CGN is a potent inflammatory agent and is not allowed in food. Animal studies have shown that even the food grade CGN causes gastrointestinal inflammation and higher rate of intestinal lesions, ulcerations and even malignant tumours as it can later be converted to degraded form by gastric acid (Relleve, *et al.*, 2005).

In the past, many investigators used CGN to induce inflammation in laboratory animals to study the effectiveness of anti-inflammatory drugs or to study the cells and cellular products involved in the inflammatory response (Tobacman, 2001; Bhattacharyya *et al.*, 2012). Report states that CGN has been used for decades in animal and cell-based experiments to induce inflammation (Yang *et al.*,2012) andit has been demonstrated to induces chronic inflammatory arthritic changes similar to other established model of arthritis in rats (Hansra *et al.,* 2000, Manole*et al.*,2014).Carrageenan's induction of inflammation is biphasic, the first phase involves release of histamine, bradykinin and serotonin from mast cells, less content of prostaglandin by cyclooxygenase enzyme; this is important in initiation and amplification of inflammatory processes (Coura *et al.,* 2015; Mansouri *et al.,* 2015). The second involves infiltration of neutrophils, increased prostaglandin generation, COX-2 and nitric oxide. This stage is dependent on proinflammatory cytokine production mainly TNF-α, IL-1 and IL-6 by resident cells and neutrophils, alongside with the production of oxygen-derived free radicals such as superoxide anion (O_2-) and hydroxyl radicals(Necas and Bartosikova, 2013; Mansouri *et al.,* 2015).

Studies have demonstrated that CGN induces inflammation throughactivation of ROS mediated pathway that involves heat shock protein 27-inhibitor of nuclear factor kappa-B kinase-inhibitory factor κBα(Hsp27-IKKβ-IκBα) leading to NF-κB stimulation and by activation of innate immunity through the toll-like receptor 4-B cell lymphoma 10 (TLR4-BCL10)-mediated pathway(Bhattacharyya*et al.,* 2011).This causes increased phospho-inhibitor of κB (IκBα), thereby enabling the nuclear translocation of NFκB and the subsequent transcriptional events required for inflammation (Wu *et al.*,2016).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1Materials

3.1.1 Drugs and reagents

Omega-3 fish oil (containing EPA 180 mg and DHA 120 mg) and Vitamin A (10,000 IU [from fish liver oil and retinyl palmitate]) manufactured in USA for Mason Vitamins were used.Carrageenan manufactured in USA by Sigma Aldrich.

3.1.2 Animals

A total of 24 female Wistar rats of age 10 weeks and weighing 150-200g were used for the study. The animals were obtained from Department of Human Physiology Ahmadu Bello University Zaria and housed in cages under standard laboratory conditions and had access to food and water *ad libitum*.

3.2 Methods

3.2.1 Experimental design

A total of 24 female Wistar rats were randomly divided into 6 groups (n=4) and used for the ten weeks study. In the first 2 weeks, animals in groups 3-6 received CGN (0.02ml of 1% CGN) injection at their knee joint 3 times/week to induce/establish inflammation, then injection of CGN 3 times/week was continued in group 3 while groups 4-6 had the injection of CGN3 times/week and oral administration of 200mg/kg omega 3 FA and vitamin A 4000IU/kg daily for the remaining 8 weeks. The experimental groups were as follows:

Group 1:Served as normal control and were given normal feed and water for 10 weeks;

Group 2: Served as injection control and were injected saline solution (0.9% NaCl) in their knee joint 3 times/week for 10 weeks;

Group 3:Served as disease control and were injected 0.02 ml of 1% CGN solution in their knee joint 3 times/weekfor 10 weeks (Manole *et al.,* 2014);

After 2 weeks induction of inflammation:

Group 4: Oral administration of 200mg/kg omega 3 FAdaily(Mesquita *et al.*, 2011) + 0.02 ml of 1% CGN solution injected in their knee joint 3 times/week for 8 weeks;

Group 5: Oral administration of4000IU/kg vitamin A daily(Cha *et al.*, 2016) + 0.02 ml of 1% CGN solution injected in their knee joint 3 times/week for 8 weeks;

Group 6: Oral administration of 200mg/kg omega 3 FA+ 4000IU/kg vitamin A daily + 0.02 ml of 1% CGN solution injected in their knee joint 3 times/week for 8 weeks.

3.2.2Measurement of knee joint inflammation

Joint diameter was measured before first injection (at day 0), second week and thereafter, weekly for 8 weeks.Clinical signs such as swelling, redness, deformity and ankylosis in the knee joints were considered signs of joint inflammation. The development of joint inflammation and its severity was measured by knee swelling in the hind limbs using veneer calliper measurements every 7th day between day 14 and 56 (Balducci *et al.*, 2012; Chen *et al.*, 2013).

3.2.3 Blood collection

At the end of the tenth week of experimental period, the animals were anesthesized using 0.15ml/kg ketamine hydrochloride injection (Kurdi *et al.*, 2014). Blood samples of about 5ml were collected through cardiac puncture. The samplewas placed in plane tubes and serum samples collected after centrifugation at 300rpm. The serum was used for determination of tumour necrosis factor-α (TNF-α), C-reactive protein(CRP), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and Malondialdehyde (MDA) concentration.

3.2.4 Assessment of TNF-α

The assay for TNF-α activity was carried out using TNF-α ELISA kit purchased from Elabscience (Catalog No: E-EL-R0019 96T) USA and was used according to the manufacturer's instruction.

Principle:

This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to rat TNF-α. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Rat TNF-α and avidin-horseradish peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain rat TNF-α, biotinylated detection antibody and avidin-HRP conjugate will appear blue in colour. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the colour turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of rat TNF-α. The concentration of rat TNF-α in the samples can be calculated by comparing the OD of the samples to the standard curve.

Procedure:

All reagents and samples were brought to room temperature then centrifuged before use. All the reagents were mixed thoroughly by gently swirling before pipetting and foaming was avoided.A 100μL of standard blank or sample per well was added; the blank well was added with reference standard and sample diluent. Solutions were added to the bottom of micro ELISA plate well, inside wall touching and foaming was avoided as much as possible. It was gently mixed and the plate was covered with sealer provided and incubated for 90 minutes at 37°C. Liquid of each well was removed, 100μL of biotinylated detection Ab working solution was immediately added to each well, covered with the plate sealer and the plate was gently tapped to ensure thorough mixing and incubated for 1 hour at 37°C. Each well was aspirated and washed, the process was repeated three times by filling with wash buffer (approximately 350μL). Liquid was completely removed at each step. After the last wash, remaining wash buffer was removed by decanting. The plate was inverted and patted against thick clean absorbent paper. HRP Conjugate working solution 100μL was added to each well, covered with plate sealer and incubated for 30 minutes at 37°C. The wash process was repeated for five times. Substrate solution 90μL was added to each well, covered with a new plate sealer and incubated for about 15 minutes at 37°C. The plate was protected from light (the reaction time was shortened or extended according to the actual color change, but not more than 30minutes). When apparent gradient appeared in standard wells, the reaction was terminated. Stop solution 50μL was added to each well, the color turned to yellow immediately. The order to add stop solution was the same as the substrate solution. The optical density of each well was determined at once, using a micro-plate reader set to 450 nm.

3.2.5 Assessment of C-reactive Protein

The assay for CRP was carried out using CRP rapid latex slides (REF 514002: Spectrum bioscience. Germany) and was used according to the manufacturer's instruction.

Principle:

Spectrum CRP latex reagent is a suspension of polystyrene particles sensitized with anti-CRP. When the latex reagent is mixed with a serum containing CRP, visible agglutination occurs. The latex reagent has been produced so that agglutination will take place only when the level of CRP is greater than 6mg/L.

Procedure:

All reagents and specimens were brought to room temperature. Serum to be titrated was serially diluted (1:2, 1:4, 1:8 etc) in 0.9 g/ml saline solution. One drop of positive control was placed on slide. CRP positive control serum was not diluted for comparative or other purposes as no correlation exists between actual titre of the control and titre of unknown sera. Each serum dilution 50µl was placed individually in successive circles on the slide and (50µl) of the positive control into separate circles on the glass slide. CRP latex reagent was shaken gently and one drop (45µl) was added on each circle next to the sample to be tested and control. It was well mixed using a disposable stirrer spreading the mixture over the whole test area and the slide was tilted gently. Agitated for about 2 minutes with hand and the presence or absence of agglutination was observed.

Results and Interpretation:

Presence or absence of agglutination indicate positive (indicating a CRP level of more than 6 mg/L) or negative result respectively. The serum CRP titre can be defined as the highest dilution showing a positive result.

The approximate CRP level (mg/L) present in the sample can be obtained by the following formula:

CRP Titre (mg/L) = Highest dilution with positive reaction xReagent sensitivity (6 mg/lL)

3.2.6Assessment of antioxidant enzymes and lipid peroxidation

3.2.6.1 Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) was determine by the method described by Fridovich (1989).

Principle:

The ability of superoxide dismutase (SOD) to inhibit autooxidation of adrenaline at pH 10.2 formed the basis of this assay.

Procedure:

Carbonate buffer of 0.05M (11.43g of Na_2CO_3 and 4.2g of $NaHCO_3$) were dissolved in distilled water and made up to 1000ml in a volumetric flask. The buffer was adjusted to pH 10.2 and 0.01g of adrenaline was dissolved in 17ml of distilled water, the solution was prepared fresh. Serum of 0.1ml was diluted in 0.9ml of distilled water to make 1:10 dilution of microsome. An aliquant mixture of 0.2ml of the diluted microsome was added to 2.5ml of 0.05M carbonate buffer. The reaction was started with the addition of 0.3ml of 0.3mM Adrenaline. The reference mixture contained 2.5ml of 0.05M carbonate buffer, 0.3ml of

0.3mM Adrenaline and 0.2ml of distilled water. The Absorbance was measured over 30 seconds up to 150 seconds at 480nm.

Calculations: Increase in absorbance per minute = $(A_2 - A_1)/2.5$

% Inhibition = 100 - {(Increase in absorbance for sample/Increase in absorbance of blank) x 100}

1 unit of SOD activity is the quantity of SOD necessary to elicit 50% inhibition of the oxidation of Adrenaline to adenochrome in 1 minute.

3.2.6.2 Catalase (CAT) activity

Catalase (CAT) activity was measured using Abebi's method (1974).

Principle:

Catalase activity is proportional to the rate of H_2O_2 decomposition.

Procedure:

Exactly 100µl of serum was added to a test tube containing 2.80ml of 50mM potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1ml of freshly prepared 30mM H_2O_2 and the decomposition rate of H_2O_2 was measured at 240nm for 5 minute on a spectrophotometer. A molar extinction coefficient (E) of $0.041mM^{-1}$ $-cm^{-1}$ was used to calculate the Catalase activity. Catalase Activity =(Absorbance/ (E. x Protein conc. (mg/ml)

3.2.6.3 Reduced glutathione (GSH) concentration

Reduced glutathione (GSH) was determined by the method of Ellman (1959).

Principle:

Briefly, the reaction of GSH with Ellman's reagent (0.0198% DTNB in 1% sodium citrate) gives rise to a product that can be quantified spectrophotometrically. This reaction is used to measure the reduction of Glutathione disulphide (GSSG) to GSH. The rate of the reaction is proportional to the GSH and GSSG concentration.

Procedure:

1 ml of supernatant (0.5 ml plasma precipitated by 2 ml of 5% TCA) was taken and 0.5 ml of Ellman's reagent and 3 ml of phosphate buffer (pH 8.0) was added. The colour developed was read at 412 nm.

3.2.6.4 Lipid peroxidation

Lipid peroxidation in plasma was estimated colorimetrically by measuring malondialdehyde (MDA) by the method of Albro *et al.* (1986) and Das *et al.* (1990).

Principle:

Malondialdehyde assay is based on the reaction of MDA in the sample with thiobarbituric acid (TBA), forming MDA-TBA2 adduct that absorbs at 532nm.

Procedure:

In brief, 0.1 ml of serum was treated with 2 ml of (1:1:1 ratio) TBA–TCA–HCL reagent (TBA 0.37%, 0.25N HCL and 15% TCA) and placed in water bath for 15 min, cooled and centrifuged and then pink supernatant (TBA- Malonaldehyde complex)was seen after heating at 95^{o}c and was measured at 532nm against reference blank. Malonaldehyde formed was then calculated using the Molar extinction coefficient of 1.56 x 10^{-5} $cm^{-1}M^{-1}$

TBARS Conc. (nmol/mg protein) = {Absorbance of sample / 1.56 x 10^{-5} x protein Conc. (mg/ml)}

2.3 Statistical analysis

The data were expressed as mean ± standard error of mean (SEM) and values were analyzed using mixed analysis of variance (ANOVA) for knee circumference, one-way ANOVA for TNF-α, CRP and oxidative stress biomarkers followed by Tukey's post hoc test. The data were analyzed using SPSS for windows (Version 22). *P*-values of less than 0.05 were considered significant.

CHAPTER FOUR

4.0 RESULT

4.1 Induction of Inflammation

Table 4.1 shows the induction of inflammation following carrageenan injection in group 3 (Omg3) by measuring the levels of knee diameter, TNF-α and CRP. Establishment of inflammation was observed with significant increase in knee diameter (KnD) of carrageenan injected group (CGN) (5.61 ± 0.17, P<0.001) at week 2 when compared to groups 1 (NC) and 2 (NSi) (4.08 ± 0.04 and 4.26 ± 0.10 respectively). Progression of inflammatory response was confirmed by continuous rise in KnC of CGN (7.06 ±0.21, P<0.001)when compared to NC and NSi (4.30 ±0.06 and 4.59 ±0.05 respectively) at week 10. There was a significant increase in TNF-α concentration in CGN (228.81 ± 25.74, P<0.001) when compared to NC and NSi (51.27 ± 4.57 and 61.39 ± 8.45 respectively) and CRP concentration significantly increased (P<0.001) in CGN (2688.00 ± 384.00) when compared to NC and NSi (672.00 ± 96.00 each).

This result showed an elevated inflammatory activity in response to carrageenan injection. The carrageenan injected group showed increase in KnD, TNF-α and CRP all signifying increase in inflammatory activity (P<0.001) when compared to NC and NSi. This indicates an obvious induction as well as continuous flow of inflammation following injection of carrageenan along the time/duration of treatment, thereby confirming inflammation.

Table 4.1: Induction of kneejoint inflammation using carrageenan in Wistar rats

Group	KnD (mm)		TNF-α (mg/L)	CRP (mg/L)
	WK 2	WK 10		
Group 1 (NC)	4.08 ± 0.04	4.30 ±0.06	51.27 ± 4.57	672.00 ± 96.00
Group 2 (NSi)	4.26 ± 0.10	4.59 ±0.05^{c}	61.39 ± 8.45	672.00 ± 96.00
Group 3 (CGN)	5.61 ± 0.17^{ab}	7.06 ±0.21^{abc}	228.81 ± 25.74^{ab}	2688.00 ± 384.00^{ab}

a= significant when compared to NC, b= significant when compared to NSi, c= significant when compared to Wk2. Groups: 1, Normal Control; 2, Normal Saline injection; 3, Carrageenan (0.02ml of 1%).

4.2 Treatment of Inflammation

Table 4.2 shows inflammatory response following administration of omega 3 FA, vitamin A and their combination. Following administration of supplements, Omg3 and Omg3+VAshowed significant decrease in KnD at week 10 (4.51±0.15 and 4.51±0.07, respectively) when compared to week 2 (5.25±0.17 and 5.91±0.16, respectively). Invitamin A group, there was no significant change at week 10 when compared to week 2. There was a significant decrease in KnD at week 10 in Omg3 (4.51 ±0.15), VA (5.20 ±0.18) and Omg3+VA (4.51 ±0.07)when compared toCGN (7.06 ±0.21)with $P<0.001$.

At week 2, there was establishment of inflammation and commencement of administration/treatment. This marks the baseline of treatment as all the groups showed similar level of inflammatory activity. The significant decrease in KnD presented within the groups treated with omega 3 FA and its coadministration with vitamin A indicatesa high degree of anti-inflammatory activity (suppression/reversion of inflammatory changes) with time/duration of treatment. This effect is assumed to be mostly contributed by omega 3 FA since vitamin A alone could not show a similar outcome, meaning that administering omega 3 FA alone or in combination with vitamin A was more potent than administration of vitamin A alone.

Across weeks vitamin A showed no change. It could be suggested that vitamin A can depress progression of inflammation without necessarily reverting the already formed inflammatory response. This shows a lesser anti-inflammatory activity in VAgroup with time/duration of treatment, this indicates that vitamin A could suppress but not revert inflammatory changes caused by CGN injection.

Significant decrease ($P<0.05$) was obtained in TNF-α concentration of the treatment groups Omg3 (27.13 ± 3.33), VA (123.99 ± 17.92) and Omg3+VA (31.48 ± 1.55) when compared to CGN (228.81 ± 25.74).

Tumour necrosis factor-α concentration significantly decreased ($P<0.05$) in all the treatment groups when compared to CGN group as seen in Table 4.2, this coincides with the decrease in KnD that was observed following treatment.Treatment with vitamin A showed lower anti-inflammatory activity by having a higher TNF-α concentration than that of omega 3 FA alone and in combination, this coincide with the failure of vitamin A to show significant decrease in KnD across experimental periods. This implies that vitamin A could suppress, but not revert inflammatory activity following CGN injection.

There was a significant decrease (P<0.01) in CRP concentration of Omg3 (960.00±192.00) and Omg3+VA (1344.00±192.00) when compared to CGN (2688.00±384.00) but no significant difference in VA (P>0.05). This indicate that Omega 3 FA alone is more effective in lowering inflammatory activity since it has the lowest CRP concentration than its coadministerion with vitamin A. Single administration of vitamin A signifies less effectiveness in decreasing inflammatory response.

Table 4.2: Changes in inflammatory activity in carrageenan-induced joint inflammation in Wistar rats treated with omega 3 FA, vitamin A and their coadministration.

Group	**KnD (mm)**		**TNF-α (mg/L)**	**CRP (mg/L)**
	WK 2	**WK 10**		
Group 3 (CGN)	5.61 ± 0.17	7.06 ±0.21	228.81 ± 25.74	2688.00 ± 384.00
Group 4 (Omg3+CGN)	5.25 ± 0.17	4.51 ± 0.15^{ab}	27.13 ± 3.33^{a}	960.00 ± 192.00^{a}
Group 5 (VA+CGN)	5.12 ± 0.20	5.20 ± 0.18^{a}	123.99 ± 17.92^{a}	1920.00 ± 384.00
Group 6 (Omg3+VA+CGN)	5.91 ± 0.16	4.51 ± 0.07^{ab}	31.48 ± 1.55^{a}	1344.00 ± 192.00^{a}

Significant at P< 0.05. a= when compared to group 3, b= when compared to week 2.Groups: 3, Carrageenan (0.02ml of 1%); 4, Carrageenan+omega 3 (200mg/kg); 5, Carrageenan+Vitamin A (4000 IU/kg); 6, Carrageenan +Omega 3+Vitamin A.

The results of oxidative stress biomarkers following administration of supplements in Table 4.3 shows an increase in SOD and CAT ($P<0.05$) in Omg3 (111.47±3.79, 21.28±0.91 respectively), VA (61.68±4.55, 18.04±1.95 respectively) and Omg3+VA (108.13±1.23, 20.78±2.68respectively) when compared to CGN (16.28±0.85, 8.97 ± 1.02 respectively). In GSH, Omg3 (15.84 ± 1.44), VA (15.53±1.15) and Omg3+VA (19.23±0.95)showed a significant increase ($P<0.05$) when compared to CGN (8.00 ± 0.85). While Omg3 (41.33±5.02), VA (50.76±5.36) and Omg3+VA (38.95±5.77) showed significant ($P<0.05$) decrease in MDA when compared to CGN (82.05 ± 4.37). This proves the ability of omega 3 FA and vitamin A to suppress as well as revert antioxidant activity. It implies that omega 3 FA and vitamin A could activate the antioxidant enzyme system as evident by their increased concentration and could lower lipid peroxidation by a decrease in MDA concentration.

Table 4.3: Oxidative stress biomarkers in carrageenan-induced joint inflammation in rats

Group	SOD (IU/mL)	CAT (IU/mg)	GSH (µg/mL)	MDA (nmols/mg)
Group 1 (NC)	69.94±11.12	16.33±1.03	15.40±1.00	52.34±1.22
Group 2 (NSi)	67.83±6.76	8.42 ± 0.48	11.27±0.99	68.37±11.52
Group 3 (CGN)	16.28±0.85	8.97 ± 1.02	8.00 ± 0.85	82.05 ± 4.37
Group 4 (Omg3+CGN)	111.47±3.79 [a]	21.28±0.91[a]	15.84 ± 1.44[a]	41.33±5.02[a]
Group 5 (VA+CGN)	61.68±4.55[a]	18.04±1.95[a]	15.53±1.15[a]	50.76±5.36[a]
Group 6 (Omg3+VA+CGN)	108.13±1.23 [a]	20.78±2.68[a]	19.23±0.95[a]	38.95±5.77[a]

administered omega 3 FA and vitamin A supplements.

Significant at P< 0.05. a= when compared to group 3.Groups: 1, Normal Control; 2, Normal Saline injection; 3, Carrageenan (0.02ml of 1%); 4, Carrageenan+omega 3 (200mg/kg); 5, Carrageenan+Vitamin A (4000 IU/kg); 6, Carrageenan +Omega 3+Vitamin A.

CHAPTER FIVE

5.0 DISCUSSION

This study examined the influence ofomega 3 fatty acids, vitamin A and their coadministration on TNF-α,CRP and oxidative stress biomarkersfollowing injection of the carrageenan on knee joints of female Wistar rats using the method of Manole *et al.,* 2014.

The induction of inflammation following CGN injection is in line with study of Manole *et al.*(2014) who reported an increase in knee diameter after injection of 0.02ml of 1% carrageenan solution in Wistar rats; while Nobre *et al.* (2013) observed an increase in TNF-α and paw swelling after injection of carrageenan in rats. Assanga *et al.* (2017) reported an increase in rat paw oedema and CRP concentration after injection of (0.1ml/paw) of carrageenan solution.

Carrageenan has been reported to induce inflammation by activating innate immunity through toll like receptor-mediated pathway and through activation of ROS which leads to stimulation of NF-κB, these cause transcription and subsequent release of inflammatory mediators such as histamin and bradykinins from mast cells which are responsible for initiation of inflammation (Bhattacharyya *et al.,* 2012). While progression of inflammation is achieved by neutrophil infiltration, PGs and COX-2 generation through cytokines and ROS production (Necas and Bartosikova, 2013; Mansouri *et al.,* 2015).

Omega 3 fatty acids and its coadministration with vitamin A showed more effectiveness in decreasing KnD than vitamin A alone. This finding support the report of El-Seweidy *et al.* (2016), they stated that omega 3 FA exerted a significant improvement in knee joint of OA rats signifying decreased pathological change in articular surface. This could be through the activity of EPA and DHA which are precursors of the lipid mediators resolvins and protectins that have anti-inflammatory and immunomodulatory effect on joints and body system (Kaur *et al.,* 2014). Omega 3 fatty acid could also reduce inflammation due to the fact that it reduces the formation of eicosanoids with inflammatory characteristics by competing with omega-6 fatty acids for the same enzymatic pathway that leads to the inhibition of TNF-α, IL-1, IL-6 synthesis, cartilage-degrading enzymes and reducing the intercellular adhesion molecule-1 (ICAM-1) expression (Calder, 2015; Rajaei *et al.,* 2016).

Treatment with vitamin A did not show reverting effect but could however decrease KnD when compared to CGN group. This finding supports the work of Nagai *et al.* (1999) who reported that Am80 (a newly synthesized retinol) significantly reduced the development of foot pad swelling and bone damage in collagen induced arthritis. This implies that vitamin A has short-term anti-inflammatory activity which could be through its ability to modulate inflammatory response, cellular differentiation and biologic actions related to bone and collagen synthesis (Sowers and LaChance, 1999).Vitamin A also acts as the first line of defence against free radicals attack and lipid peroxidation, by stabilizing free radicals either independently or as a part of large enzyme system (Sail *et al.,* 2013).

The above observations show that omega 3FA alone and its coadministration with vitamin A have anti-inflammatory activitymore than vitamin A alone. This could be mediated through the supplements' ability to suppress the expression of proinflammatory cytokines which stimulate the production of chemokines, COX2 and angiogenic factors that lead to synovitis as well as MMP_s and PGE_2 that play a key role in cartilage degradation(Bistrian, 2003; Lee *et al.*, 2012). Reactive oxygen species are known to be highly intimidating factors accountable for the progression of chronic joint inflammation or even making it worse by inducing immunological cell activation and signalling cascade and omega 3 FA has the potential to inhibit these reactions. Vitamin A also showed potential for these effects, although at a lesser scale which may probably be improved with a lower dose.

Omega 3 fatty acid showed more effectiveness in lowering inflammatory activity by reverting the concentration of TNF-α to the lowest concentration. The decrease in TNF-α level following omega 3 FA administration is in line with the work of El-Seweidy *et al.* (2017) who reported a significant decrease in TNF-α level following administration of omega 3 FA in murine OA rats. They also stated that omega 3 FA can reduce serum soluble TNF-α receptor p55 and production of pro-inflammatory cytokines induced via the NFκB system. Nobre *et al.* (2013) also reported a decrease in TNF-α along with a decreased paw swelling after treatment with omega 3 FA in carrageenan-induced rat paw oedema. They proposed that omega 3 FAwas able to mediate its effect due to it being an important precursor for resolvins and protectins which are lipid-derived modulators of cell inflammatory processes.These lipid mediators have anti-inflammatory and inflammation resolving capabilities as they inhibit migration of neutrophils from capillaries and limit neutrophil infiltration at sites of inflammation with consequent inhibition of the production of TNF-α (Car and Webel, 2012; Cockbain *et al.*, 2014; Kaur *et al.*, 2014). Omega 3 fatty acids has been shown to reduce the production of arachidonic acid-derived eicosanoids by competing for incorporation into the cell membrane with omega 6, releasing free arachidonic acids by phospholipase (PL)-A2 or by inhibiting the enzyme COX-2 and 5-Lipoxygenase (Orchard *et al.*, 2014).

Vitamin A expressed a significant decrease in TNF-α concentration although not as strong as that of omega 3 FA alone and in combination. This is in contrast with the work of Nagai *et al.,* 1999 who reported that Am80(a synthetic derivative of retinoid) which exhibit specific biological activities of retinoic acid did not inhibit lipopolysaccharide induced TNF-α production in mice.The differences in the results may be due to the fact that in the present study, a different model of inflammation was used, there was a longer duration of treatment, accompanied with continuous feeding on standard nutritive diet. Also, differences in geographical location which could account for varying environmental condition can contribute to the contrasting outcome of these experiments.It could also be proposed that vitamin A was able to decrease TNF-αthrough its ability toinhibit translocation of the transcription factor NFκB and interrupt the secretion of inflammatory cytokines (Reifen, 2002).

The positive outcome observed in coadministration of these supplements could be due to TNF-α reverting effect mainly mediated by omega 3 FA. This implies that omega 3 FA is more effective in decreasing TNF-α concentration than vitamin A in CGN-induced joint inflammation and combination of these supplements did not show a better outcome than omega 3 FA alone.This could be due to potential implication of retinoic acid in cytotoxicity induced NrF2 target genes at some concentrations; NrF2 participate in adaptive cellular defence against retinoid toxicity by enhancing transcription of antioxidant gene (Tan *et al.,* 2008). With chronic administration of vitamin A this mechanism would have been depleted and retinoic acid toxicity outweighed the NrF2 activity. It could also be considered that anti-inflammatory and bone growth supporting effect of vitamin A at the concentration used (4000IU/kg) is improved when co-administered with omega 3 FA rather than vitamin A alone. This could explain the reason why decrease in KnD was more pronounced in Omg3 and Omg3+VA thanVA when compared to CGN, also, when considering response within groups, omega 3 FA alone and in combination with vitamin A had a linear decreasing effect across the weeks of experimental period.

When observing the findings of CRP concentration in Table 4.2, it could be seen that omega 3 FA and its coadministration with vitamin A had a significant decrease compared to CGN control while VA did not show significant decrease. This confirms omega 3 FA ability to suppress inflammatory response which is also supported with the decrease in KnD as well as a fall in TNF-α concentration that was earlier mentioned since vitamin A alone could not give a significant outcome. Omega 3 fatty acids could have mediated its effect through Protectin D1 (PD1), which is a bioactive product of DHA generated from 17S-hydroperoxy DHA (a metabolic intermediate). It potently regulates critical events related to inflammation and its resolution which involves inhibition of polymorphonuclear cells (PMN) infiltration, T-cell migration and decreased TNF-α level. PD1 has also been shown to decrease COX-2 mRNA expression and block NFκB activation (Spite *et al.,* 2014; Gao, 2017). This activities could have decrease the production of pro-inflammatory cytokines which would subsequently decrease the hepatic production of CRP. The inflammation lowering effect of omega 3 FA could also be through its ability to inhibit the activities of δ-6 desaturase, δ-5 desaturase and cyclooxygenase, all of which are involved in fatty acid regulation that influences pro- and anti-inflammatory mediators. It could also be through modifying the activity of transcription factors, such as peroxisome-proliferator-activated receptors and NFκB. Peroxisome proliferator-activated receptors can interfere with the activation of nuclear factor κB by inhibiting signalling molecules and therefore impeding the production of pro-inflammatory cytokines (Muka *et al.,* 2015; El-Seweidy *et al.*, 2017).

The C-reactive protein concentration observed after treatment with vitamin A could be the reason why the KnD of vitamin A group at week 10 could not show significant decrease when compared to week 2, signifying higher inflammatory activity than omega 3 FA alone and in combination. However, treatment with vitamin A showed significant decrease in KnD when compared to CGN control; this at least shows a degree of anti-inflammatory activity which supports its lower CRP counts compared to CGN control. The finding that VAcould not show significant decrease in CRP is in contrast with that of Cha *et al.* (2016) who reported that 4000IU/kg of vitamin A supplementation might be considered as adequate level that should show favourable effect in rats than 20000IU. In this study however, vitamin A at 4000IU/kg showed a weak response to decrease CRP concentration; this could probably be due to difference in geographical as well as laboratory conditions or model of inflammation used. Apart from that, report have shown that vitamin A could be toxic to cells even at levels considered safe affecting cell survival and function.

The outcomes observed in antioxidant effect of omega 3 FA, vitamin A and their combination also explain the decrease in KnD that was observed when these groups were compared to CGN group and within themselves (Omg3 and Omg3+VA) across various weeks of experiment. The finding of this study is in agreement with that of Lluis *et al.,* 2013 who reported that diet of a ratio 1:1 of EPA/DHA significantly activated oxidative stress enzyme SOD and GPx in erythrocytes, also Iraz *et al.* (2005) reported a significant increase in CAT and decrease in MDA after supplementation with omega 3 FA in rats erythrocytes indicating that omega 3 FA was able to prevent lipid peroxidation and safeguard cellular oxidative injury. Omega 3 fatty acidshave been reported to have the ability to incorporate into the cell membrane and become available for conversion into bioactive oxidized derivatives to promote inflammation (Ahmad and Bassima, 1990). The oxidized omega 3 FA can react directly with keap1, a negative regulator of NrF_2, initiating its dissociation with Cullin3, this induces NrF_2-directed gene expression that regulate cytoprotective responses to endogenous and exogenous stress caused by ROS (Gao *et al.,* 2007).

Although vitamin A alone could not significantly decrease KnD within the group itself as was earlier seen, significant decrease seen between it and CGN group support its present ability to decrease oxidative stress activity. This finding supports that of Cha *et al.*(2016) who reported that vitamin A showed significant improvement in antioxidant system in rats where they obtained an increase in the level of SOD, GSH and MDA following their deregulation in vitamin A deficient rats. With regards to the observed effect of vitamin A, it has been reported that retinol reacts by lipoperoxyl radicals in addition to the cyclohexenyl ring. This can support the radical scavenging activity of all-transretinol as a chain-breaking antioxidant (Kamiloglu *et al.,* 2006).

Results obtained from antioxidantbiomarkers in this study showed more antioxidant activity in omega 3 FA alone and in combination than vitamin A only when compared to CGN group. At molecular level, mechanisms proposed include modulation of peroxisome proliferator-activated receptor (PPAR)-γ, synthesis of inflammatory lipid mediators such as resolvins and protectins, and novel G-protein coupled receptors that mediate potent anti-inflammatory actions. It can also be that high DHA diet counteracts reduced sirtuin 1 (SIRT1) that is associated directly with p65 subunit and deacetylates lys310 residue, both critical for NFκB transcriptional activity (Herrera *et al.,* 2015).

Joint inflammation is said to be associated with increased production of ROS (Dwivedi, 2014). Pro-inflammatory cytokines such as IL- 1β and TNF-α are involved in the formation of toxic peroxynitrite by stimulating the activity of nitric oxide synthase. These ROS participate in the process of joint destruction as secondary messengers or directly attack the joint cartilage causing damage to proteoglycans and suppressing their synthesis (Quiñonez-Flores, *et al.,* 2016).

The measure of inflammatory activity through analysis of various parameters such as the KnD, inflammatory markers TNF-α and CRP, antioxidant enzymes SOD, CAT and GSH and marker of lipid peroxidation MDA have been shown to be regulated at various level in response to administration of omega 3 FA and vitamin A either alone or in combination. From the result, these supplements show a promising outcome in reverting and suppressing CGN-induced joint inflammation in Wistar rats; but omega 3 FA has a greater effect on KnD and the other parameters investigated better than vitamin A and in combination.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

In this study, carrageenan was used to induce chronic joint inflammation in female Wistar rats with the aim of investigating the extent to which omega 3 FA and vitamin A supplements (at doses 200mg/kg and 4000IU/kg), respectively, alone or in combination could effectively suppress or revert inflammation.

Changes in knee diameter were observed in all the treatment groups after two weeks of CGN injection. Animals that received CGN only showed increase in KnD throughout the experimental period, treatment groups showed a linear pattern of decreasing KnD with omega 3 FA and its combination with vitamin A having lower value which signify higher anti-inflammatory responsethan vitamin A alone.Chronic joint inflammation is associated with elevated level of pro-inflammatory cytokines released by macrophages, lymphocytes, dendritic cells and other antigen-presenting cells that are stimulated by inflammatory mediators such pathogen, obesity or trauma. These immune cells infiltrate the diseased joint and degrading factors such as matrix metalloprotienase (MMP) and PGE_2 are released resulting in further damage to articular cartilage. Elevated TNF-α level also triggers production of other inflammatory cytokines such as IL-6, IL-1β and IL-17, along with chemokines, COX2 and angiogenic factors. These inflammatory activities causes articular derangement like synovitis and cartilage degradation.

Serum TNF-α and CRP concentration were elevated in CGN group, all the treatment groups had significant decrease in TNF-α concentration; while significant decrease in CRP was seen in omega 3 FA alone and in combination with vitamin A without a significant decrease in vitamin A group.Pro-inflammatory cytokines most especially TNF-α form a positive feedback loop that further up-regulate expression of more macrophages, lymphocytes and dendritic cells.

Serum antioxidant enzymes (SOD, CAT and GSH) along with lipid peroxidation marker (MDA) were used to assess oxidative status in chronic joint inflammation. The result showed a disturbed antioxidant enzyme system by a decrease in SOD, CAT, and GSH, followed by an increase in MDA in CGN control following chronic inflammation; the antioxidant enzymes concentrations were increased and MDA was decreased in omega 3 FA and its combination with vitamin A more than vitamin A alone.

6.2 Conclusion

Omega 3 fatty acids in this study have been found to be more effective than vitamin A alone and in combination, it showed more effectiveness in suppressing CGN-induced joint inflammation by decreasing KnD, TNF-α, CRP, an increase in antioxidant enzymes SOD and CAT than vitamin A alone and its coadministration. Combination of omega 3 FA showed significant decrease in KnC, TNF-α, CRP, MDA and GSH more than vitamin A alone. Therefore, omega 3 fatty acids, its combination and vitamin A alone suppressed carrageenan induced joint inflammation with the most effective being omega 3 FA.

6.3 Recommendations

Based on the outcome of the study, it is recommended that:

1. Studies should be carried out using different doses of these supplements which could help in finding the concentration at which vitamin A could be more effective in decreasing joint inflammation.
2. Coadministration of vitamin A with omega 3 fatty acids have proven more effective in decreasing inflammation than vitamin A alone, this could serve as a guide in figuring out ways to improve effectiveness that relate to vitamin A supplements.
3. Omega 3 fatty acids have shown most appreciable outcome in decreasing joint inflammation, this also serve as a guide to the use of this supplement in managing related ailments and in healthy condition to serve as preventive measure.
4. The effectiveness of the individual components of omega 3 fatty acids (EPA and DHA) should be tested in joint inflammation, targeting their modulatory effect on peroxyzome proliferator-activated receptors (PPAR)-γ and G-protein coupled receptors.

6.4 Contribution to Knowledge

1. Treatment with 200mg/kgomega 3 fatty acids alone and in combination with 4000IU/kgvitamin A decreased knee swelling (5.25±0.17, 4.51±0.15 and 5.91±0.16,

4.51±0.07) along the period of experiment [F (45, 8.156) =22.260, P<0.001]. Omega 3 fatty acids, vitamin A (5.12±0.20, 5.20±0.18) and their combinationshowed a significant decrease in knee diameter when compared to CGN group (5.61±0.17, 7.06±0.21)in CGN-induced joint inflammation.

2. Omega 3 fatty acids and vitamin A showed a significant decrease in serum TNF-α27.13 ± 3.33 (mg/L) and 123.99 ± 17.92 (mg/L) respectively and in combination 31.48 ± 1.55 (mg/L) when compared to CGN control 228.81 ± 25.74 (mg/L) in CGN-induced joint inflammation.
3. Omega 3 fatty acid at 200mg/kg had a significant change in CRP level (960.00 ± 192.00 mg/L) and combination of the supplements showed a significant decrease (1344.00 ± 192.00 mg/L)when compared to CGN group (2688.00 ± 384.00 mg/L) in CGN-induced joint inflammation.
4. Single and combined administration of omega 3 fatty acids and vitamin A at doses 200mg/kg and 4000IU/kg respectively had a significant increase in SOD, CAT and GSH and a significant decrease in MDA in CGN-induced joint inflammation.

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APPENDIX

This table shows knee circumference of carrageenan induced joint inflammation in wistar ratstreated with omega-3 fatty acids and vitamin A.

Knee Circumference (mm)

Group	KnC W0	KnC W2	KnC W3	KnC W4	KnC W5	KnC W6	KnC W7	KnC W8	KnC W9	KnC W10
Group 1	4.03 ± 0.03	4.08 ± 0.04	4.10 ± 0.04	4.14 ± 0.05	4.17 ± 0.06	4.20 ± 0.07	4.22 ± 0.06	4.26 ± 0.06	4.28 ± 0.06	4.30 ±0.06
Group 2	3.94 ± 0.03	4.26 ± 0.10	4.23 ± 0.08^{kl}	4.25 ± 0.05^{klb}	4.23 ± 0.02^{klb}	4.30 ± 0.06^{klb}	4.35 ± 0.02^{cgkb}	4.21 ± 0.20^{lb}	4.51 ± 0.02^{cdib}	4.59±0.05 cdib
Group 3	3.93 ± 0.10	5.61 ± 0.17^{ca}	5.79 ± 0.16^{dela}	5.97 ± 0.16^{dela}	6.14 ± 0.14^{defla}	6.31 ± 0.14^{defka}	6.55 ± 0.17^{defga}	6.53 ± 0.22^{defka}	6.90 ± 0.20^{dfghia}	7.06±0.21 dghijka
Group 4	4.00 ± 0.06	5.25 ± 0.17^{ca}	5.35 ± 0.25^{kl}	5.27 ± 0.28^{ekl}	5.11 ± 0.28^{eflb}	4.94 ± 0.23^{defgb}	4.87 ± 0.21^{defgb}	4.77 ± 0.19^{defgb}	4.66 ± 0.17^{dghib}	4.51± 0.15^{dhikb}
Group 5	4.06 ± 0.06	5.12 ± 0.20^{ca}	5.14 ± 0.22	5.13 ± 0.21^{b}	5.06 ± 0.18^{b}	5.02 ± 0.15^{b}	5.08 ± 0.18^{b}	5.07 ± 0.16^{b}	5.14 ± 0.16^{b}	5.20±0.18^{b}
Group 6	4.12 ± 0.06	5.91 ± 0.16^{ca}	5.80 ± 0.17^{jkl}	5.58 ± 0.17^{del}	5.41 ± 0.19^{defl}	5.22 ± 0.19^{defgb}	5.09 ± 0.18^{defgb}	4.65 ± 0.27^{dfghb}	4.65 ± 0.09^{dfghb}	4.51± 0.07^{dhikb}

a= significant when compared to NC, b= significant when compared to CGN, c= significant when compared to Wk0, d= significant when compared to Wk2, e= significant when compared to Wk3, f= significant when compared to WK4, g= significant when compared to WK5, h= significant when compared to WK6, i= significant when compared to Wk7, j= significant when compared to Wk8, k= significant when compared to WK9, l= significant when compared to WK10.

Groups: 1 Normal Control, 2 Normal Saline injection, 3 Carrageenan (0.02ml of 1%), 4 Carrageenan+omega 3 (200mg/kg), 5 Carrageenan+Vitamin A (4000 IU/kg), 6 Carrageenan +Omega 3+Vitamin A,Wk (week).