# ผลของการเสริมกรดไขมันโอเมก้า 3 และเมล็อกซิแคมต่ออุบัติการณ์และการเปลี่ยนแปลงของทูเมอร์เนโครซิสแฟคเตอร์แอลฟ่า และอินเตอร์ลิวคินวันเบต้าในซีรั่มที่สัมพันธ์กับโรคหัวกระดูกสะโพกหลุดในไก่เนื้อ

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# EFFECT OF OMEGA 3 FATTY ACIDS AND MELOXICAM SUPPLEMENTATION ON INCIDENCE AND ALTERATION OF SERUM TNF-$\alpha$ AND IL-1$\beta$ IN RELATION TO FEMORAL HEAD SEPARATION IN BROILER

Mr. Suriya Sooksong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Animal Physiology
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| By | Mr. Suriya Sooksong |
| Field of Study | Animal Physiology |
| Thesis Advisor | Associate Professor Kris Angkanaporn, D.V.M., M.S., Ph.D. |
| Thesis Co-Advisor | Associate Professor Nopadon Pirarat, D.V.M., Ph.D. |

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

................................................Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, D.V.M., Ms.C., Ph.D.)

THESIS COMMITTEE

..................................................................Chairman

(Assistant Professor Anusak Kijtawornrat, D.V.M., Ph.D.)

..................................................................Thesis Advisor

(Associate Professor Kris Angkanaporn, D.V.M., M.S., Ph.D.)

..................................................................Thesis Co-Advisor

(Associate Professor Nopadon Pirarat, D.V.M., Ph.D.)

..................................................................Examiner

(Associate Professor Sutthasinee Poonyachoti, D.V.M., Ms.C., Ph.D.)

..................................................................External Examiner

(Assistant Professor Yuwares Ruangpanit, Ph.D.)

สุริยา สุขส่ง : ผลของการเสริมกรดไขมันโอเมก้า 3 และเมล็อกซิแคมต่ออุบัติการณ์และการเปลี่ยนแปลงของทูเมอร์เนโครซิสแฟคเตอร์แอลฟ่า และอินเตอร์ลิวคินวันเบต้าในซีรั่มที่สัมพันธ์กับโรคหัวกระดูกสะโพกหลุดในไก่เนื้อ (EFFECT OF OMEGA 3 FATTY ACIDS AND MELOXICAM SUPPLEMENTATION ON INCIDENCE AND ALTERATION OF SERUM TNF-α AND IL-1β IN RELATION TO FEMORAL HEAD SEPARATION IN BROILER) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. น.สพ. ดร.กฤษ อังคนาพรกฤษ อังคนาพร, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. น.สพ. ดร.นพดล พิษารัตน์นพดล พิษารัตน์, 66 หน้า.

การเจริญเติบโตอย่างรวดเร็วของไก่เนื้อในปัจจุบัน โดนเฉพาะเนื้อหน้าอก ทำให้เพิ่มแรงกดทับต่อหัวกระดูกสะโพก เหนี่ยวนำให้เกิดหัวกระดูกสะโพกหลุด (FHS) และเกิดข้ออักเสบตามมาได้ จุดประสงค์ของผู้วิจัยในครั้งนี้ ต้องการศึกษาถึงผลของการเสริมกรดไขมันโอเมก้า 3 และเมล็อกซิแคม ต่ออุบัติการณ์และการเปลี่ยนแปลงของทูเมอร์เนโครซิลแฟคเตอร์แอลฟ่า (TNF-α) และอินเตอร์ลิวคินวันเบต้า (IL-1β) ในซีรั่มที่สัมพันธ์กับโรค FHSในไก่เนื้อ ไก่เนื้อสายพันธุ์ Cobb 500 เพศผู้จำนวน 1,152 ตัว ถูกแบ่งออกเป็น 4 กลุ่ม (6ซ้ำ) อย่างละเท่าๆกัน กลุ่มที่ 1 คือกลุ่มควบคุม กลุ่มที่ 2 คือกลุ่มที่เหนี่ยวนำให้เกิดหัวกระดูกสะโพกหลุดด้วยพื้นเอียง กลุ่มที่ 3 เหนี่ยวนำเหมือนกลุ่มที่ 2 แต่มีการให้อาหารเสริม 2% All-G-Rich™ (16% DHA) และกลุ่มสุดท้าย เหนี่ยวนำเหมือนกลุ่มที่ 2 แต่มีการให้เมล็อกซิแคมละลายน้ำ (0.5 มก./กก.) ทำการเก็บเลือดที่อายุ 42 วันเพื่อนำไปตวจหาสารสื่ออักเสบ ไตรกลีเซอไรด์ (TG) และโคเลสเตอรอล (CL) เมื่อไก่อายุ 43 วันจะถูกทำให้ตายอย่างสงบด้วยการรมก๊าซคาร์บอนไดออกไซด์ และทำการให้คะแนนหัวกระดูกสะโพกทุกตัว ผลการทดลองพบว่า กลุ่มที่ 4 มีอุบัติการณ์ของ FHS ลดลง ($p < 0.05$) อย่างมีนัยสำคัญทางสถิติ (42.20±4.20%) เมื่อเปรียบเทียบกับกลุ่มอื่นๆ นอกจากนั้น ด้วยวิธีทางจุลพยาธิวิทยา สามารถตรวจพบการเปลี่ยนแปลงรอยแยก osteochondrotic clefts ในหัวกระดูกสะโพก แม้ว่าลักษณะทางมหกายวิภาคจะดูปกติก็ตาม อย่างไรก็ตามไม่พบการเปลี่ยนแปลงในระดับซีรั่มของ TNF-α IL-1β TG และ CL ในทุกกลุ่มการทดลอง ($p > 0.05$) จากการทดลองครั้งนี้ปริมาณ DHA ที่ใช้ (700 มก.ต่อวัน) ไม่สามารถลดอุบัติการณ์ของ FHS รวมถึงทำให้เกิดการเปลี่ยนแปลงของระดับสารสื่ออักเสบ ในทางตรงกันข้าม เมล็อกซิแคมสามารถลดอุบัติการณ์ของ FHS ลงได้ เป็นเพราะคุณสมบัติของตัวยาเองที่สามารถยับยั้งเอนไซม์ COX-2 ที่บริเวณรอยโรค ทำให้ลดการทำลายกระดูกอ่อนลงได้ จากการทดลองนี้ สรุปได้ว่าการเสริมเมล็อกซิแคมขนาด 0.5 มก./กก. ในน้ำดิ่ม สามารถลดอุบัติการณ์ของ FHS ลงได้ ไม่พบมีการเปลี่ยนแปลงของ TNF-α IL-1β TG และ CL ในซีรั่ม ทั้งในกลุ่ม DHA และเมล็อกซิแคม นอกจากนี้ การเปลี่ยนแปลงทางจุลพยาธิวิทยา สามารถช่วยระบุการเกิดรอยโรคหัวกระดูกสะโพกขั้นเริ่มต้นได้อย่างชัดเจนเพิ่มขึ้นจากลักษณะทางมหกายวิภาค

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SURIYA SOOKSONG: EFFECT OF OMEGA 3 FATTY ACIDS AND MELOXICAM SUPPLEMENTATION ON INCIDENCE AND ALTERATION OF SERUM TNF-α AND IL-1β IN RELATION TO FEMORAL HEAD SEPARATION IN BROILER. ADVISOR: ASSOC. PROF. KRIS ANGKANAPORN, D.V.M., M.S., Ph.D., CO-ADVISOR: ASSOC. PROF. NOPADON PIRARAT, D.V.M., Ph.D., 66 pp.

Femoral Head Separation (FHS) is one of the problems found in fast growing broilers worldwide. This can result in joint inflammation leading to lameness, reduced growth performance and culling. The aim of this study was to examine whether omega 3 fatty acid and meloxicam could reduce the incidence of FHS in relation to changes in serum TNF-α and IL-1β. Total of 1,152 male, Cobb 500 broilers, were divided in to 4 groups of 6 replicate pens as T1 = control, T2 = FHS induction by Speed Bump, T3 = T2 + 2%All-G-Rich™ (16%DHA) in feed and T4 = T2 + meloxicam in tap drinking water (0.5 mg/kg BW). On d 42 of age, blood samples were collected for serum inflammatory cytokines, triglyceride (TG) and cholesterol (CL) analysis. Birds were euthanized using $CO_2$ inhalation, gross and histopathological lesions of femoral head were scored individually at d 43. The results showed that, the incidence of FHS was significantly decreased ($p < 0.05$) in T4 (42.20±4.20%) compared to others. Moreover, an osteochondrotic clefts can be defined by histopathology technique even though gross lesion score is normal. Serum TNF-α, IL-1β, TG and CL were not changed among all groups ($p > 0.05$). From this study, the dosage of DHA (700 mg/day) may be inadequate to reduce the incidence of FHS or alter pro-inflammatory cytokines. In contrast, meloxicam can reduce the incidence of FHS by its activity to inhibit COX-2 enzyme, resulting in decreased degradation of articular cartilage. In conclusion, anti-inflammatory drug meloxicam helped to alleviate the gross appearance of FHS in fast growing broilers. There was no alteration of serum TNF-α, IL-1β, TG and CL in both DHA and meloxicam groups. Furthermore, histopathology finding is able to specify changes in detail of the beginning of FHS lesion.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid (C20:0) |
| AC | Articular cartilage |
| ACZ | Articular cartilage zone |
| ADG | Average daily gain |
| ALA | Alpha linolenic acid (C18:3 n-3) |
| BCO | Bacterial chondronecrosis with osteomyelitis |
| CP | Crude protein |
| CL | Cholesterol |
| cum FI | Cumulative feed intake |
| COX | Cyclooxygenase enzyme |
| d | Day |
| DAMPs | Damage-associated molecular pattern |
| DHA | Docosahexaenoic acid (C22:6n-3) |
| dL | Deciliter(s) |
| EC | Epiphyseal vascular canals |
| EPA | Eicosapentaenoic acid (C20:5n-3) |
| FCR | Feed conversion ratio |
| FHS | Femoral head separation |
| FHT | Femoral head transitional degeneration |
| FHN | Femoral head necrosis |
| g | Gram(s) |
| GE | Gross energy |
| HZ | Hypertrophic zone |
| $I_kB$ | Inhibitor of kappa B |
| IKK | I kappa B kinase enzyme |
| IL-1β | Interleukin 1 beta |
| kg | Kilogram(s) |

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| | |
|---|---|
| LA | Linoleic acid (C18:2n-6) |
| LDL | Low-density lipoprotein |
| LOX | Lipoxygenase enzyme |
| LT | Leukotrienes |
| m | Meter(s) |
| M | Moisture |
| ME | Metabolize energy |
| mg | Milligram(s) |
| ml | Milliliter(s) |
| µl | Microliter(s) |
| µm | Micrometer(s) |
| MSB | Modified speed bump (slope 50%) |
| MVP | Metaphyseal vascular plexus |
| $NF_kB$ | Nuclear factor kappa B |
| NLRs | Intracellular nucleotide binding domain and leucine-rich-repeat-containing receptors |
| NSAIDs | Nonsteroidal anti-inflammation drugs |
| PAMPs | Pathogenic-associated molecular pattern |
| PEV | Penetrating epiphyseal vessels |
| PG | Prostaglandin |
| PPARs | Peroxisome proliferator activated receptors |
| PPZ | Proliferative-prehypertrophic zone |
| T | Treatment |
| TG | Triglyceride |
| TNF-α | Tumor necrosis factor alpha |
| TLRs | Toll-like receptors |
| TX | Thromboxane |

# CHAPTER I

# INTRODUCTION

Over the past decade, chicken breeds have been selected for main purpose of producing more meat in order to meet the increasing demand from the growth of human population. Fast developing breast muscle, high average daily gain (ADG) and low feed conversion ratio (FCR) to meet market weight around 2.5 kilograms (kg) in 6 weeks of new modern broiler can cause deleterious effect on chicken skeletal lead to skeletal disorder such as lameness and stiff gait.

Femoral head separation (FHS), femoral head transitional degeneration (FHT) and femoral head necrosis (FHN) were a group of lesions found in the case of bacterial chondronecrosis with osteomyelitis (BCO). These necropsy lesions can be seen in both obvious lame chickens and normal chickens. High growth rate of breast meat can impose mechanical shear force to femoral head and tibiofibula head. Micro-fracture from mechanical shear force within epiphyseal and physeal cartilage of femoral head can lead to focal ischemia and necrosis. Furthermore, bacteria from environment can enter blood circulation from respiratory tract or gastrointestinal tract and then transfer, colonize and release lytic substances to the lesion that resulting in generalized necrosis and lead to septic arthritis eventually (Wideman and Prisby, 2012). To date, animal welfare and food safety become a major public concern. FHS or BCO can cause detrimental effects to chickens, leading to lameness and resulting in an inability to access to both feed and water. This can create a risk to consumers to get contaminated food with bacteria.

At present, FHS or BCO can be induced by many techniques or models for instances prednisolone injection or raising the chickens on wire floor (speed bump; slope 50%) (Wideman et al., 2012; Wideman and Pevzner, 2012). The mechanism of speed bump inducing FSH or BCO is due to foot instability and then it creates a mechanical shear force on any fulcrum of chicken joint such as femoral head or tibiofibula head (Gilley et al., 2014). Some research works suggested that adding probiotics in chicken diet from day one can reduce the incidence of FHS by reducing

pathogenic bacteria in intestinal tract and can improve gut integrity so that it reduce bacterial load at the local site of femur (Wideman et al., 2012).

Many studies suggested that, in human, omega 3 fatty acids and nonsteroidal anti-inflammation drugs (NSAIDs) can alleviate Rheumatoid arthritis (Shapiro et al., 1996; Watkins et al., 2001b). Proposed mechanisms of action of omega 3 fatty acids were that Alpha linolenic acid (ALA; C18:3 n-3), eicosapentaenoic acid (EPA; C20:5 n-3) and docosahexaenoic acid (DHA; C22:6 n-3) can change composition of cell membrane phospholipids and surface or intracellular fatty acid receptor leading to inflammation reduction. However, there are no evidence of using these omega 3 fatty acids and anti-inflammatory drug, i.e. meloxicam, in which one of its possible mechanism is to alternate pro-inflammatory cytokines such as serum tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) on the incidence of FHS in broiler.

The objectives of this study were to examine the effect of omega 3 fatty acids and meloxicam supplementation on incidence of FHS, alteration of serum TNF-α and IL-1β as well as histopathological changes in relation to FHS.

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# CHAPTER II
# LITERATURE REVIEW

## 2.1. Bone development

Bone is one of the organ that has its own dynamic function at all time. In poultry, bone serves two major vital functions in the body. It serves as a structural support of skeletal muscle system and serves as calcium and phosphorus reservoir for egg production (Tully, 2002). Development of compact bone in avian species can be divided into two types. First type of bone development is endochondral bone formation which is the development of long bone such as humerus and femur. The process started when chondrocytes that developed from osteogenic progenitor cells, secrete extracellular matrix and accumulate to cartilage. These chondrocytes then became hypertrophy, calcification and changed to calcified cartilage. Further vascular invasion and osteoblast recruitment produced osteoid to woven bone. Subsequently, resorption and reformation lead to lamellar bone. The second type of bone development called intramembranous bone formation which is the development of flat bone. The process started when osteoblasts developed from osteogenic progenitor cells which will then develop further and secrete osteoid. This accumulation of osteoid and its calcification will generate woven bone formation and finally replaced by lamellar (Roughley, 2002).

Basic component of compact bone normally comprises cells (2%) and extracellular matrix (98%). The extracellular matrix is composed of approximately 70% mineral, 20% organic substance and 10% water. Of all the organic matter, 90% is collagen, 9% protein and approximately 1% proteoglycan (Roughley, 2002).

Bone remodeling occurs all the time of life span. Osteoblasts and osteoclasts are the main factor for bone growth and development. Osteoblasts are developed from osteogenic progenitor cells while osteoclasts are developed from pluripotent hematopoietic stem cells. The pluripotent stem cells were transformed to monocyte progenitor cells and evolution to preosteoclasts. Preosteoclasts accumulate and transform to osteoclast which has gigantic and multinucleated nucleus. The border

site of osteoclast has a brush border (ruffled border) attach to the bone surface by integrin protein. After attachment, acid secretion by the action of carbonic anhydrase creates an optimal pH and activating lysosomal enzyme activity which results in bone resorption. After that, osteoblasts on the bone lining surface produce osteoid, calcified and some osteoblasts are embedded in the matrix and differentiate into osteocytes in the clear zone that make bone rigid, increase size and has a suitable shape.

Cartilage is different from bone and it is classified into 3 types: 1) Hyaline cartilage which found in general composition of bone such as articular cartilage (AC), white color and cover of head of bone for reduce movement friction and reabsorb weight, 2) Elastic cartilage such as ear cartilage and epiglottis which were more elastic than others, and 3) fibrocartilage such as annulus fibrous of vertebrae that its most composition was collagen type I and had less ratio of proteoglycan than other cartilage.

Basic component of cartilage comprises cells (5%) and extracellular matrix (95%). The extracellular matrix is composed of approximately 70% water and 30% organic matter. Of all the organic matter, 60% is collagen, 25% proteoglycan and approximately 15% protein (Roughley, 2002).

Bone formation and resorption was controlled by hormones, bone intrinsic factors and cytokines. For hormones, Insulin, growth hormone and estrogen influence bone formation whereas parathyroid hormone, thyroid hormone and glucocorticoids influence bone resorption while calcitonin inhibit bone resorption. For bone intrinsic factor, prostaglandin $E_2$ ($PGE_2$) acts on bone resorption also induces secretion of insulin-like growth factors which related to bone-derived growth factors for balance bone resorption and bone formation that can create bone growth and development. For cytokines and growth factors, insulin-like growth factor I, insulin-like growth factor II, fibroblast growth factors 2 and transforming growth factors induce bone formation whereas IL-1, IL-4, IL-6, IL-11, tumor necrosis factor alpha (TNF-$\alpha$), epidermal growth factors and macrophage colony-stimulating factor induce bone resorption (Watkins et al., 2001b).

## 2.2. Lameness in broiler chickens and Femoral Head Separation (FHS)

Kestin et al. (1992) established lameness score in broiler into 6 levels: score 0 means normal walk and stand, score 1 means slightly lameness but hard to define, score 2 means slightly lameness but the clinical sign disappear when they walk, score 3 means lameness can be observed and has abnormal walk, score 4 means abnormal walk and the chickens spend more time to sit on their hock rather than walk and score 5 means chickens show clinical signs with lameness and cannot walk (Kestin et al., 1992).

Lameness in chicken is occurred by many factors such as an imbalance of calcium and phosphorus in diet or vitamin D deficiency. Moreover, poisoning from sweet pea that contain lathyritic agent β-aminopropionitril which is inhibit lysyl oxidase and prevent mineralization of the bone (Rath et al., 2000). Furthermore, virus such as Reovirus or bacterial chondronecrosis with osteomyelitis (BCO) can cause a septic arthritis as well. Femoral head separation (FHS), femoral head transitional degeneration (FHT) and femoral head necrosis (FHN) are a group of bacterial chondronecrosis with osteomyelitis (BCO). The mechanism of BCO started from a separation between epiphysis and physis (growth plate) zone, leading to blood vessel rupture around osteochondrotic cleft. Consequently, it causes a local ischemia and foci necrosis. Incorporation with bacteremia from respiratory tract or gastrointestinal tract, bacterial colonized can cause emboli at ascending metaphyseal vessels. Bacteria release lytic substances and make necrosis zone expanded into metaphysis zone leading to septic arthritis. Lame chickens prefer to sit rather than walked. Weight of body will load and compress to ischiatic artery (the vessel which supplies nutrient to growth plate and lead to FHN finally (Wideman and Prisby, 2012; Gilley et al., 2014).

## 2.3. Inflammation mechanism

Inflammation is one of normal mechanism to protect body from tissue damage, infection or ischemia. When the cause of inflammation was eliminated, the body will be repaired and stop the process of inflammation. However, if the

inflammation was extended, it would cause a negative effect to the body. The process of inflammation is very complicated and correlated with many systems including cytokines and chemical mediators. There are two detection systems of this process, the first, pathogenic-associated molecular pattern (PAMPs) which is activated by pathogen or virulence factors. Second, damage-associated molecular pattern (DAMPs) which is activated by tissue injuries or foreign particles. When activated either by PAMPs or DAMPs, the major receptors system is initiated by Toll-like receptors (TLRs) or Intracellular nucleotide binding domain and leucine-rich-repeat-containing receptors (NOD-like receptors; NLRs). When TLRs is activated it will send signal to MyD88 and activate I kappa B kinase enzyme (IKK) to phosphorylate inhibitor of kappa B ($I_kB$) lead to nuclear factor kappa B ($NF_kB$) transferred to nucleus and upregulated target inflammatory genes. Consequently, many cytokines will be produced such as Pro-IL-1β, Pro-IL-18, IL-6, IL-8, IL-10, IL-12, IL-15, IFN-gamma and TNF-α. However, when NLRs receptor is activated, it will send signal by activate caspase-1 that will change pro-inflammatory cytokines into active form.

When cytokines are released, the effector cells such as neutrophils, macrophages and dendritic cells will be introduced to damage sites. Neutrophils will release toxic substances such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can destroy pathogens. After macrophages and dendritic cells killed pathogen, its antigen will be presented to MHC class II to Prime naïve T cells (Th0) and then Th0 will be transformed to Th1, Th2, Tregs and Th17. Cytokines from Th1 and Th17 are categorized as inflammatory cytokines. In contrast, cytokines from Th2 are categorized as anti-inflammatory cytokines. However, cytokines from Tregs will control balance between two population of Th1 and Th2. At final stage of inflammation, body will be repaired by changing types of pro-inflammatory mediators from prostaglandins and leukotrienes to lipoxins which will inhibit neutrophils and monocytes accumulation at damage site and allow repairing process to begin (Ashley et al., 2012).

When the process of inflammation occurs, phospholipase will be activated and will transform phospholipid to arachidonic acid (AA). Then cyclooxygenase enzyme (COX) can change AA to two series of PG (i.e. $PGE_2$, $PGD_2$ and $PGI_2$) and

thromboxanes (TX). At the same time, lipoxygenase enzyme (LOX) can change AA to leukotrienes (LT) and lipoxins as well. The metabolite products from COX reaction are $PGE_2$, $PGD_2$ and $PGI_2$ which will create vasodilation effect whereas $TXA_2$ which is also a metabolite product from COX will create vasoconstriction effect. For metabolite products of LOX, 5-hydroxyeicosatetraenoic acid (5-HETE), will be converted to $LTB_4$ (activating neutrophil to release ROS and lysosomal enzyme), $LTC_4$, $LTD_4$, and $LTE_4$ which have vasoconstriction effect. At late stage of inflammation, some $LTE_4$ could interaction with 12-lipoxygenase (12-LO) and create $LXA_4$ and $LXB_4$ to stop inflammation process (Goldring and Gravallese, 2000).

Rheumatoid arthritis is a model for study the inflammation process of bone. There are 3 stages of inflammation occur.

1) Subchondral bone loss; several reports showed that bone had more resorption than formation through osteoclast activation, tartate-resistant acid phosphatase and cathepsin K activation. Moreover, calcitonin receptor mRNA was more expressed in these step. In addition, joint tissue that normally enriched by many cytokines such as IL-1α, IL-1β, TNF-α, macrophage colony-stimulating factor, parathyroid hormone-related peptide, IL-6, IL-11 and IL-17, also showed increased incidence of cytokine production when tissue inflammation and these cytokines could be found in synovial fluid and serum as well (Kutukculer et al., 1998). In consequent, Inflammation cytokines transformed proosteoclasts into mature osteoclasts for bone resorption process (Raisz, 1993).

2) Periarticular osteopenia; lymphocytes and macrophages accumulate in bone marrow adjacent to inflamed joint.

3) Osteoporosis; this is the stage of bone mass reduction. This reduction of bone mass might be the effect of IL-1α. IL-1β, TNF-α and IL-6 from the inflamed joint and expand to other joints (Goldring and Gravallese, 2000). There was a research study showed that Il-1β structural protein in avian similar to IL-1β structure in human (Weining et al., 1998).

Research studies showed that patient with rheumatoid arthritis had hyperlipidemia which might be the effect of TNF-α (Steiner and Urowitz, 2009) by two mechanisms: 1) TNF-α could reduce adipose tissue protein lipase that lead to

reduced elimination rate of triglyceride and finally causes hypertriglyceridemia 2) TNF-α can activate hepatic lipoprotein production resulting in hyperlipidemia and hypocholesteremia (Feingold et al., 1989).

**2.4. Omega 3 fatty acids**

Polyunsaturated fatty acids (PUFA) are divided into two major groups as omega 3 fatty acids and omega 6 fatty acids by its position of double bond in the methyl end. Alpha linolenic acid (ALA) and linoleic acid (LA) are an essential fatty acid in human and mammals because the body cannot synthesize, so the only way to receive these fatty acids is from diet. Omega 6 fatty acids are found in many grains such as corn, wheat and rice but for omega 3 fatty acids are found in green leaves and mostly in deep sea fish such as salmon, tuna and trout (Simopoulos, 1991). Omega 3 fatty acids are a source of energy and composition of cell and important for cell development. Omega 3 fatty acids can be classified as three types: ALA (C18:3 n-3), Eicosapentanoic acid (EPA; C20:5 n-3) and Docosahexaenoic acid (DHA; C22:6 n-3). In human and avian, ALA is found in composition of triglyceride (TG), cholesterol ester and small amount in phospholipid whereas EPA is found in TG, cholesterol ester and phospholipid when DHA is found mostly in phospholipid, cerebral cortex, retina, testis and sperm. In contrast, omega 6 fatty acids (especially LA) are mostly found in phospholipid can be converted to arachidonic acid (AA), which is a substrate of inflammation process. Diet that high in omega 3 fatty acids especially EPA and DHA can replace LA in phospholipid of red blood cells, thrombocytes and hepatocytes. When an inflammation process occur, EPA will be metabolized by COX and LOX into $PGE_3$, $PGI_3$, $TXA_3$ and $LTB_5$ which have a very low inflammation effect compared to metabolite products from AA (Mori and Beilin, 2004).

Omega 3 fatty acids can interact with peroxisome proliferator activated receptors (PPARs), which is an intracellular fatty acid receptor (three isoforms as α, β and γ). PPAR α can be found in hepatocyte, PPAR β can be found in many cells and PPAR γ can be found in adipocytes. Omega 3 fatty acids bind with PPAR γ and lead to inhibit ~~of~~ $NF_kB$ translocation to nucleus, leading to decrease production of

inflammatory cytokines such as IL-1β and TNF-α (Calder, 2012). In addition, omega 3 fatty acids can also bind with G-protein coupled surface receptor (GPR). Both EPA and DHA can bind with GPR120 in adipocytes and macrophages that can maintain $I_kB$ concentration and decreases level of TNF-α and IL-6 resulting in decrease inflammatory cytokine (Oh et al., 2010).

Fish oil is a good source of omega 3 fatty acids which compose of approximately 18% EPA and 12% DHA. Furthermore, adding fish oil in human diet can increase ratio of EPA/DHA in phospholipid and had an anti-inflammation effect (Cleland et al., 2006). Zhao et al. (2004) conducted a research in transgenic mice which express more omega 3 desaturase gene. The data showed that mice in this group had less inflammation when received dextran sulphate-induce colitis with normal level of $PGE_2$ or $LTB_4$. Therefore, the action of EPA and DHA could be its inhibition effect of $NF_kB$, IL-1β and TNF-α.

Omega 3 fatty acids are naturally found in small algae and diatoms such as *Chaetoceros calcitrans*, *Chaetocerosgracilis*, *Skeletonema costatum* and *Thalassiosirapseudonana*. The composition of EPA was around 4.6 – 11.1% of total fatty acid. *Isochrysis sp*. and *Pavlava lutheri* has DHA approximately 8.3 – 9.4% of its total fat (Volkman et al., 1989). In egg industries, omega 3 fatty acids eggs can produced by adding omega 3 fatty acid source from algae (*Schizochytrium limacinum*) in layer feed for 3% (fat 64%, DHA 16%). As a result, yolk from omega 3 fatty acid fed hen had DHA 776 mg/100 g of yolk (Ao et al., 2015). In human who received DHA 1.6 mg/day for 6 weeks, EPA increased from 0.57 to 1.3 g/100 g of fatty acid in serum phospholipid and EPA increased from 0.21 to 0.58 g/100 g of fatty acid in platelet phospholipid. This implied that DHA can be converted to EPA 11.3 – 12.0% in approximately (Conquer and Holub, 1996).

### 2.5. Benefit of omega 3 fatty acids and nonsteroidal anti-inflammatory drugs on arthritis

Fatty acids are essential for bone formation and resorption, it is shown that acidic phospholipids contributed to mineral deposition in growth plate (Li et al.,

1999). Moreover, Watkins et al. (2001b) showed that fatty acids can activate $PGE_2$ and insulin-like growth factor I production in bone tissue and lead to bone formation in chicken and mice by activate osteoblastic activity. Adding omega 3 fatty acids in feed can slow down the mineral bone loss of humerus in ovariectomized mice (Li et al., 1999) and can also increase alkaline phosphatase (serum bone specific) (Watkins et al., 2000). Moreover, in vitro study, AA and IL-1$\alpha$ decreased alkaline phosphatase and osteocalcin in cell line and increase $PGE_2$ when compared to group that received EPA. From this study, they concluded that omega 6 fatty acids could increase $PGE_2$ and lead to bone resorption in spite of bone formation (Watkins et al., 2000). In addition, Seifert and Watkins (1997) showed that linolenic acid (LA) or arachidonic acid (AA) inhibited collagen synthesis whereas omega 3 fatty acids increase collagen synthesis. In rheumatoid arthritis patient, IL-1 was detected in serum that showed inhibitory effect on chondrocyte development and can activate cartilage degradation by increasing $PGE_2$ level. This research demonstrated that omega 3 fatty acid supplementation 3 - 6 mg/day can reduce $PGE_2$ synthesis. Moreover, omega 3 fatty acids can increase insulin-like growth factor I and insulin-like growth factor binding-protein production (the major composition of collagen and proteoglycan of joint) (Watkins et al., 2001b).

Nonsteroidal anti-inflammatory drugs (NSAIDs) modes of action are to decrease COX activities, decrease inflammation metabolites such as PG. There are three isoforms of COX, only two isoforms related to inflammation mechanism and those are COX-1 and COX-2. COX-1 is a constitutive form that can be found in normal condition and it balance cell activity by convert AA to $PGE_2$, $TXA_2$ and $PGI_2$ for vasodilation effect and increase bicarbonate in gastric area. COX-2 is an inducible form that will be increased in the case of tissue damage or inflammation. In chronic inflammation, COX-2 and PG were reportedly increased (Ahmed, 2011).

NSAIDs can be divided into many types as 1) chemical structure; this will be beneficial for avoidance the side effect of drugs that had similar chemical structure, 2) half-life of the drugs, long and short half-life, 3) inhibiting COX activity by IC50 (concentration of drug which can inhibit PG production 50%). This inhibiting COX activity can be divided further into three subcategories as 3.1) non-specific COX

inhibition (classical NSAIDs) which means that drug that had IC50 COX-2:COX-1 >1 such as aspirin, ibuprofen, ketoprofen and piroxicam, 3.2) COX-2 selective inhibitors which mean that drugs that had IC50 COX-2:COX-1 between 0.01 – 1 such as meloxicam, 3.3) COC-2 specific inhibitors which means that drugs that had IC50 COX-2:COX-1 less than 0.01 such as celecoxib and parecoxib.

In avian species, there was a report of using carprofen for lameness treatment in the dosage of 1 mg/kg, subcutaneous route, in broiler that had gait score 2 and 3. The research showed that chickens in the treatment group walked across barrier faster than control group (McGeown et al., 1999). Danbury et al. (2000) added carprofen in broiler feed (4 mg/kg bodyweight or 34.3 mg/kg of feed) for a selective choice feeding in lame and normal chickens for four days. The result showed that normal chickens preferred commercial feed while lame chickens preferred feed with carprofen added and gait score in lame chickens group was improved. Hadipour et al. (2011) tested the toxicity and dosage of diclofenac, carprofen, ketoprofen and meloxicam (0.5, 1, 2, 3 and 4 mg/kg) by drinking water route for 15 days (twice a day for first five days and daily for ten days) in backyard chickens. The result showed that there were no dead chickens at the first five treated days and found dead chickens after day seventh at the dosage of 3 and 4 mg/kg in diclofenac, carprofen, ketoprofen group. There was no dead chicken found in meloxicam group at all dosages and gait score of lame chickens was also improved. In addition, there was a report of using of 5 mg/kg meloxicam and 25 mg/kg carprofen for treated lame chickens which had gait score level 2 and found that gait score and walking ability were improved (Caplen et al., 2013).

### 2.6. The research of FHS

Wideman and Pevzner (2012) reported that FHS can be induced by intramuscular injection of dexamethasone (0.9 – 1.5 mg/kg) skip a day for six times. This research indicated that dexamethasone can induce bacterial chondronecrosis with osteomyelitis (BCO) in femoral head and tibiofibula head and the high dosage of dexamethasone created more necrosis zone in prehypertrophic zone, hypertrophic

zone and metaphysis. In addition, microscopic finding found pyknotic nuclei of chondrocytes in prehypertrophic and hypertrophic zone in BCO chickens. Moreover, there was fat accumulation in metaphyseal blood vessel in calcifying zone of metaphysis. Furthermore, the same research team conducted BCO induction by using speed bump (50% slope slat) from year 2009 to 2011. At the end of experiment speed bump increased the incidence of BCO for 20 – 60% compare with the control. Moreover, the highest incidence of lameness was found between 42 to 56 days of age (Dinev, 2009; Wideman et al., 2012).

Raising chicken on slope slat creates foot instability and increases gravity force to leg fulcrums (proximal head of femur and proximal head of tibiofibula bone). Micro-trauma and collapse of blood vessels which supplies nutrients to these areas may be created, leading to destruction of bone microstructure. Moreover, bacteria in blood circulation from respiratory or gastrointestinal tract can migrate to the local site of the damage area resulting in colonization of bacteria in epiphysis and physis zone. Bacteria may release lytic substances and create necrosis and ultimately lead to BCO. Thorp et al. (1993) collected and identified bacteria type from necrosis area and found that major bacteria species were *Staphylococcus aureus* and *E. coli*. Adding probiotics in broiler feed from first day of age can reduce the incidence of FHS by improve ecosystem of bacteria in gastrointestinal tract (Wideman and Prisby, 2012). Moreover, Wideman et al. (2014) conduct a research which related with broiler breed, sex and bodyweight of broiler related to BCO. The result demonstrated that, only broiler breed that correlated with BCO while sex and body weight were not correlated with the BCO.

Levels of cholesterol (CL), triglyceride (TG) and low-density lipoprotein (LDL) were increased in broilers which were a lesion in growth plate. However, ovotransferrin (protein maker for chronic inflammation) and corticosterone were not difference when compared to control group (Durairaj et al., 2009). In addition, Durairaj et al. (2012) found that supplementing high fat diet (control group contained fat 40 g/kg feed and treatment group contained fat 60 and 80 g/kg feed) in male broiler chickens start from day-old-chick until 37 days can increase body weight gain

and decrease FCR when compared to control group at 28 day of age. However, the incidence of FHS, level of CL, TG and LDL were not different.

# CHAPTER III
# MATERIALS AND METHODS

Experimental animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Faculty of Veterinary Science, Chulalongkorn University (Animal Use Protocol No. 1531055)

## 3.1. Animals and feed

A total of 1,152 male broiler chickens (Cobb 500®) were divided into four groups, each group comprised six replicate pens of 48 chickens (density 10 chickens per square meter). All chickens received *ad* libitum access to water and commercial feed following Cobb 500® standard. In Treatment 1 (T1), the chickens were raised on the floor with rice hull bedding throughout the end of experiment (43 days of age). In addition, Treatment 2 (T2), Treatment 3 (T3) and Treatment 4 (T4), the chickens were raised on rice hull from Day-Old-Chicks (DOC) to 13 days of age and at day 14, a modified speed bump (MSB, slope 50%) were set in these groups across the pen between the feeder and water line in order to force them to walk across the MSB for feed and water until the end of experiment (d 43). Moreover, in T3, the chickens were received 2% omega 3 fatty acid (All-G-Rich™, 16% DHA) in Cobb 500® standard feed starting from DOC to d 43 (Product specification is showed in appendix A, Table VI). During d 32 to d 41, meloxicam (0.5 mg/kg BW) was applied to drinking water in T4 (Table 1).

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**Table 1 Details of each treatment.**

| Treatment | Feed | Floor | Meloxicam |
|---|---|---|---|
| T1 | STD Cobb 500 | Rice hulls | No |
| T2 | STD Cobb 500 | MSB 50%** | No |
| T3 | STD Cobb 500<br>+ All-G-Rich™ 2%* | MSB 50%** | No |
| T4 | STD Cobb 500 | MSB 50%** | Drinking water<br>at d 32 – 41*** |

*All-G-Rich™ 16%DHA: dosage 2% (2 g/feed 100g), supplemented in feed from DOC – 43 day of age

**Modified speed bump (MSB) 50%: W × L × H = 0.64 × 1.50 × 0.16 m.

***Meloxicam: dosage 0.5 mg/kg BW.

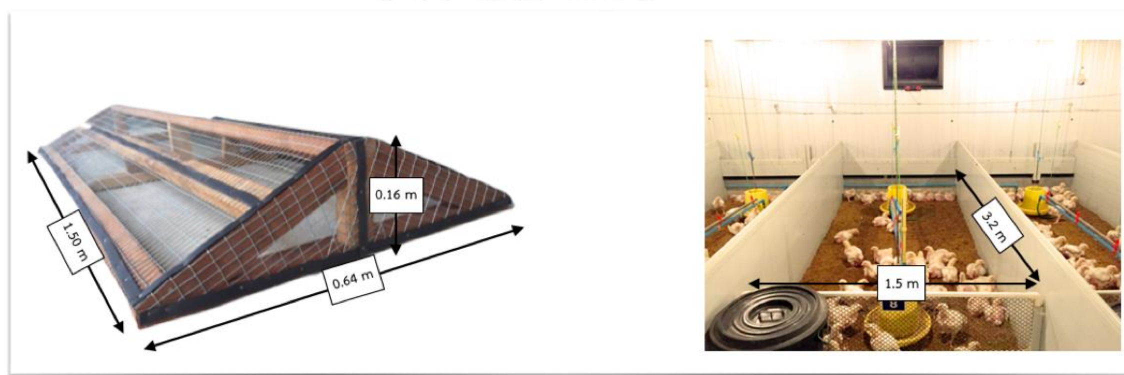



**Figure 1 Modified Speed bump model and pen size.**

Modified Speed bump (MSB) model were applied in T2, T3 and T4 at day 14 of experiment.

### 3.2. Experimental procedure

All chickens were raised in poultry housing with evaporative cooling system and lighting program was followed Cobb 500® standard. Moreover, daily temperature, weekly BW and feed intake (FI) were recorded. At the end of the experiment (d 43), all chickens were euthanized by carbon dioxide inhalation. Both legs of each chicken

were evaluated from the area of femoral head to tibia-fibula. Gross lesion of femoral head was scored individually as normal (score 0) means articular cartilage (AC) remain attached with femoral head, FHS (score 1) means AC detached from femoral head, FHT (score 2) means AC detached from femoral head and found small necrosis zone around growth plate and FHN (score 3) means AC detached from femoral head and found generalized necrosis zone (Wideman and Prisby, 2012) (Figure 2.)

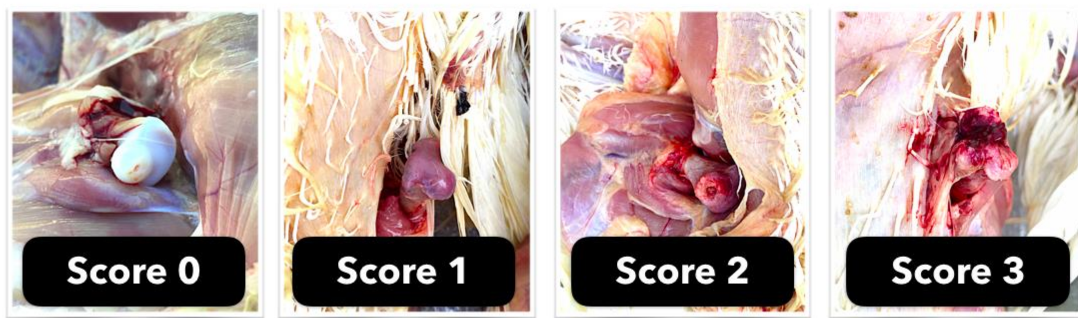



**Figure 2 Gross lesion score of femoral head at 43 day of age.**

Score 0 means normal, Score 1 means FHS, Score 2 means FHT and Score 3 means FHN

Histological changes of femoral head in relation to FHS were further studied, 10 normal femoral head (Score 0) and 10 FHS femoral head (Score 1) from each Treatment were processed to histological examination. In brief, all samples were fixed in Cal-Rite (Deionized water 85 – 86%, Formic acid 9 – 11%, Formaldehyde 3.7% and Methyl alcohol 1.1%) for seven days. After fixation and decalcification process, all femoral head were cut in the same plane (frontal plane through the longitudinal axis) (Figure 3). Then the samples were dehydrating through the series of alcohol-water solution. Afterward, all samples were performed on clearing process which alcohol was replaced by xylol. After clearing step, the samples were embedded in paraffin blocks, 4-μm sections were placed onto two slides, the first slide was stained with hematoxylin and eosin (H&E) and the other slide was stained with 0.04% toluidine blue.

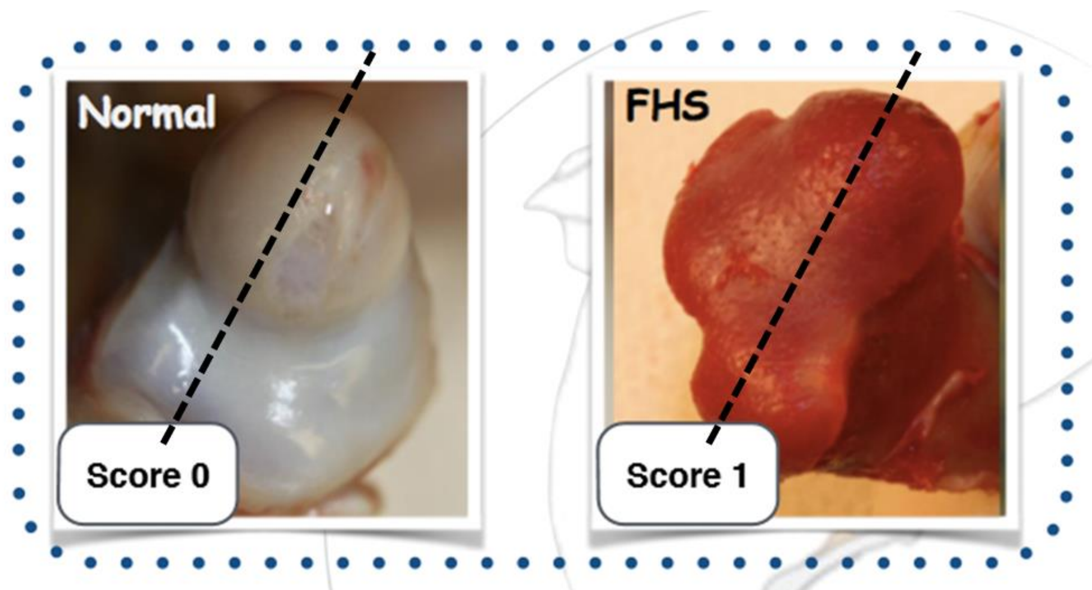



**Figure 3 Femoral head cutting**

From gross lesion score; 10 normal femoral head (Score 0) and 10 FHS femoral head (Score 1) from each Treatment were processed to histological analysis (Dash line is indicated as the cutting direction).

Histological score of femoral head (both normal and FHS) was examined as score 0 – 2 (Figure 4) (Almeida Paz et al., 2009) by pathologist with single blind experiment. Score 0 means complete all normal zone of bone (articular cartilage, proliferative zone and calcifying zone). Score 1 means no articular cartilage zone and found eccentric nuclei in proliferative zone. Score 2 means no articular cartilage zone and cell arrangement was collapsed both in proliferative zone and calcifying zone.

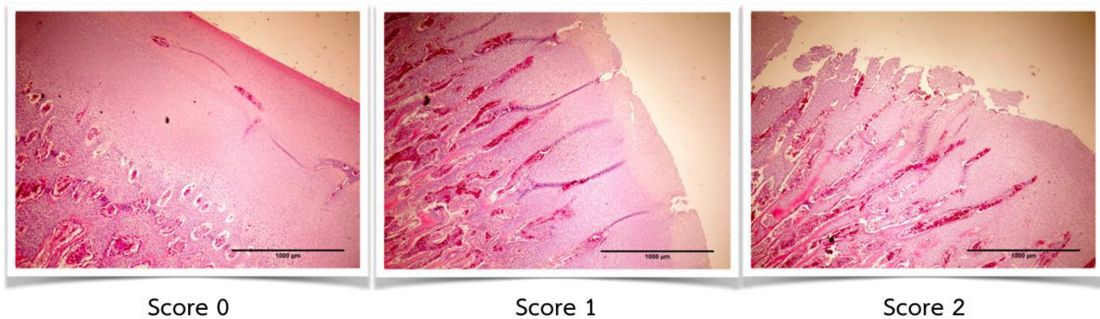



**Figure 4 Histological score of femoral head**

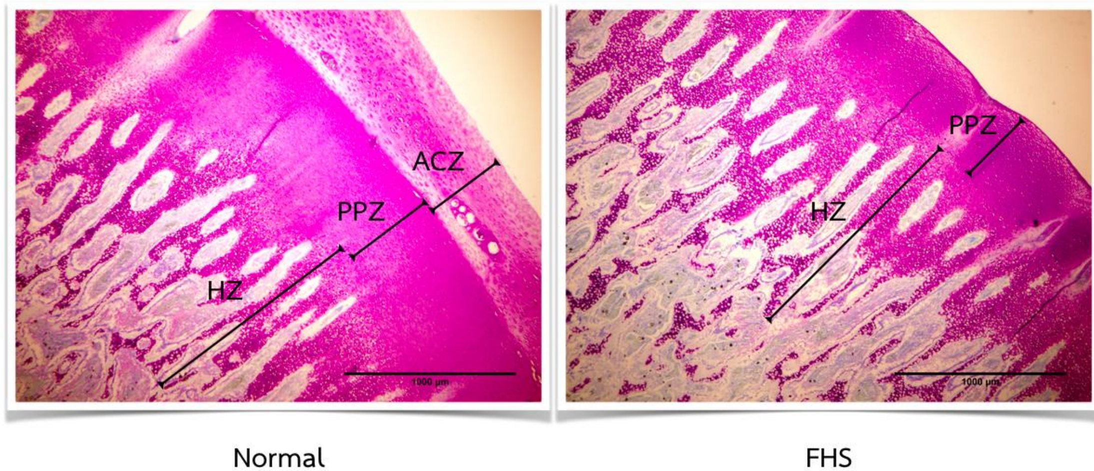
The histological photograph was taken by Olympus microscope (H&E, 40 x magnification)

Score 0 means Complete all normal zone of bone (articular cartilage zone (ACZ), proliferative zone (PZ) and calcifying zone CZ)).

Score 1 means No ACZ and found eccentric nuclei in proliferative zone

Score 2 means No ACZ and cell arrangement was collapsed both in PZ and CZ.

The length of each zone; articular cartilage zone (ACZ), proliferative-prehypertrophic zone (PPZ), hypertrophic zone (HZ), the length of penetrating epiphyseal vessels (PEV), metaphyseal vascular plexus (MVP) were photographed with Olympus microscope at 4 x magnification (objective lens) and 10 x magnification (eye pieces) and measured by ImageJ program. In addition, total number of epiphyseal vascular canals (EC), total number of PEV and total number of MVP were counted (Hedstrom et al., 1986) (Figure 5, 6 and 7).




Normal FHS

**Figure 5 Microphotograph of the proximal femoral head**

The histological photograph was taken by Olympus microscope (Toluidine blue, 40 x magnification). ACZ means articular cartilage zone, PPZ means proliferative prehypertrophic zone, began from the first line of cells of physis due to chondrocytic cells were rounded and HZ means hypertrophic zone, began from chondrocytic lacunae were rounded and lacked of ordering due to the last

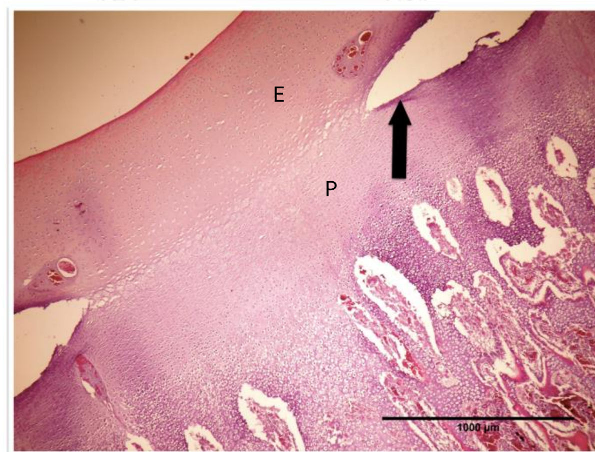



**Figure 6 Microphotograph of osteochondrotic clefts at the boundary between epiphysis and physis.**

The histological photograph was taken by Olympus microscope (H&E, 40 x magnification). The arrow points to the osteochondrotic clefts. E means Epiphysis and P means Physis

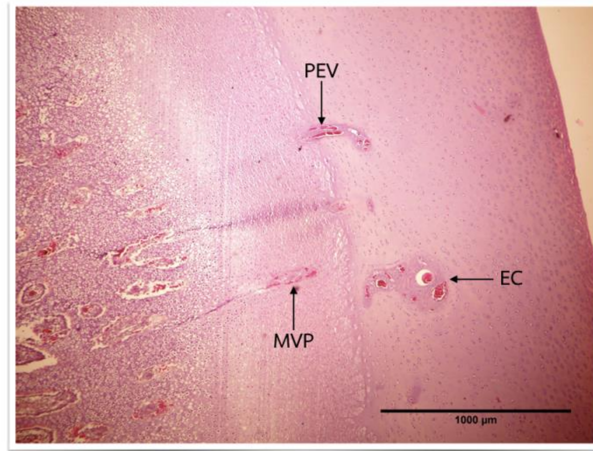



**Figure 7 Microphotograph of blood vessels in proximal femoral head.**

EC means epiphyseal vascular canals, PEV means penetrating epiphyseal vessel and MVP means metaphyseal vascular plexus.

### 3.3. Blood collection and analysis

All chickens were tag-labeled with plastic number tag at the right leg on the day of blood collection (d 42) and 30% of chickens in each pen were venipuncture (1 ml) at ulnar or brachial vein with No. 23 needle (15 chickens per pen or 90 chickens per treatment). Blood samples were kept at the room temperature for two to three hours, centrifuged at 2,500 g, five minutes to separate the serum and then divided the serum into two tubes, stored at -20°C for further analysis. The serum samples which corresponded to gross lesion as normal or FHS were chose for further analysis (10 samples for normal and 10 samples for FHS in each group). Pro-inflammatory cytokines (TNF-αand IL-1β) were analyzed using enzyme linked immunosorbent assays (ELISA) (Novateinbio accelerates research and development for TNF-α and cloud-clone corp. for IL-1β) (Table 2).

TNF-α was performed following assay procedure stated by producer. In brief, all kit components and samples were brought to room temperature before analysis. Seven wells for standard and one well for blank (duplicate for another row of plate) were prepared. A 50 μl of each standard, blank and samples were added into the wells. Furthermore, 100 μl of HRP-conjugate reagent were added to each well, mixed

thoroughly, covered with plastic sealer and incubated for one hour at 37 $^{0}$C. Third, the solution was removed from each well and washed five times with 350 µl of 1x wash solution, the solution was finally removed few minutes afterward. Then 50 µl of chromogen A solution and 50 µl of chromogen B solution were added into each well, covered with the plastic sealer and incubated for 15 minutes at 37 $^{0}$C (protected from light). At the final step, 50 µl of $H_2SO_4$ was added to each well until the color was changed to yellow. The samples were further analyzed with the microplate reader and measured immediately at 450 nm and 620 nm.

IL-1β was performed following assay procedure of the producer. In brief, all kit components and samples were brought to room temperature before analysis. Seven wells for standard and one well for blank (duplicate for another row of plate). Total 100 µl of each standard, blank and samples were added into the wells, covered with plastic sealer and incubated for two hours at 37 $^{0}$C. Additionally, the liquid was removed from each well (do not wash) was removed and 100 µl of detection reagent A was added to each well, covered with plastic sealer and incubated for one hour at 37 $^{0}$C. The solution was then removed from each well and washed three times with 350 µl of 1x wash solution, the solution was finally removed few minutes afterward. Then 100 µl of detection reagent B was added to each well, covered with plastic sealer and incubated for 30 minutes at 37 $^{0}$C. The solution was washed again with 350 µl of 1x wash solution for five times. 90 µl of substrate solution was added to each well, covered with plastic sealer and incubated for 15 – 20 minutes at 37 $^{0}$C (not exceed 30 minutes and protected plate from light), the liquid turn to blue color. For the last step, 50 µl of $H_2SO_4$ was added to each well, the color was changed to yellow and all samples were run using the microplate reader with measurement at 450 nm and 620 nm.

Triglyceride (TG) was analyzed by photometric colorimetric test (TRIGLYCERIDES liquicolor mon; GPO-PAP method) and Cholesterol (CL) was analyzed by enzymatic colorimetric test (CHOLESTEROL liquicolor; CHOD-PAP method) (Table 2).

**Table 2 Amount of serum samples in each treatment.**

| Treatment | Lesion | *n* | | Details |
|---|---|---|---|---|
| T1 | Normal | 10 | samples | CL, TG, TNF-α, IL-1β |
| | FHS | 10 | samples | CL, TG, TNF-α, IL-1β |
| T2 | Normal | 10 | samples | CL, TG, TNF-α, IL-1β |
| | FHS | 10 | samples | CL, TG, TNF-α, IL-1β |
| T3 | Normal | 10 | samples | CL, TG, TNF-α, IL-1β |
| | FHS | 10 | samples | CL, TG, TNF-α, IL-1β |
| T4 | Normal | 10 | samples | CL, TG, TNF-α, IL-1β |
| | FHS | 10 | samples | CL, TG, TNF-α, IL-1β |

*n* = samples, CL = Cholesterol and TG = Triglyceride

### 3.4. Feed analysis

Nutrient compositions of all experimental diets were analyzed for proximate analysis. Percentage of moisture (M) were analyzed by in-house method based on GAFTA (2014) Method 2:1, 2:2, crude protein (CP) were analyzed by in-house method based on GAFTA (2014) Method 4:1, crude fat were analyzed by in-house method based on GAFTA (2014) Method 3:0, crude fiber were analyzed by The Grain and Feed Trade Association (GAFTA), 2014, Method 9:0, gross energy (GE) were analyzed by in-house method technique based on AC500 automatic calorimeter instruction manual, July 2006 and fatty acid profile were analyzed by in-house method based on AOAC (2012) method 996.06 GC/FID (Nutrient composition and feed analysis are showed in appendix A, Table I – IV)

### 3.5. Statistical analysis

The data were expressed as mean ± standard error of the mean, parameter were analyzed by one-way ANOVA using GLM procedure and Duncan's new multiple range tests as post-hoc analysis. Comparison of data involving clinical sign between normal and FHS groups were analyzed using unpaired *t*-test. Differences were considered significant at $p < 0.05$.

# CHAPTER IV

# RESULTS

## 4.1. Broiler performance

The performance of broiler in this study is demonstrated in Table 3. The positive control group (T1, chickens were raised on rice hulls and no modified speed bump; MSB) had the highest BW (3.29±0.02 kg) than other groups ($p < 0.05$) (T2 = 3.22±0.01 kg, T3 = 3.14±0.01 kg and T4 = 3.23±0.02 kg). Moreover, average daily gain (ADG), feed conversion ratio (FCR) and cumulative feed intake (Cum. FI) were also significantly better ($p < 0.05$) when compared to the others. In contrast, chickens in omega 3 group (T3, All-G-Rich™, 16% DHA) had the lowest growth performance especially cum FI, BW and ADG ($p < 0.05$) while FCR was lower than T1 and T2 (MSB) ($p < 0.05$) but not significantly different when compared with T4 (Meloxicam). However, there was no significant difference on depletion (%) among all treatments. In addition, broiler performance (Cum. FI, BW, ADG, FCR and depletion) in each feeding phases (starter, grower, finisher1 and finisher2) were showed in appendix A, Table VIII – XI. The BW of T2 was significantly lower ($p < 0.05$) when compared to T1 (BW at grower phase; T1 = 1,203 g, T2 = 1,170 g, BW at finisher1 phase; T1 = 3.11 kg, T2 = 3.04 kg and BW at finisher2 phase; T1 = 3.29 kg, T2 = 3.22 kg).

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**Table 3 Broiler performance at 43 days of age.**

| Performance | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|
| Cum. FI (kg/chicken) | $5.44^{a}\pm0.03$ | $5.31^{bc}\pm0.01$ | $5.27^{c}\pm0.03$ | $5.36^{b}\pm0.02$ | <0.01 |
| BW (kg/chicken) | $3.29^{a}\pm0.02$ | $3.22^{b}\pm0.01$ | $3.14^{c}\pm0.01$ | $3.23^{b}\pm0.02$ | <0.01 |
| ADG (g/chicken/day) | $76.44^{a}\pm0.57$ | $74.97^{b}\pm0.28$ | $73.14^{c}\pm0.25$ | $75.03^{b}\pm0.49$ | <0.01 |
| FCR | $1.65^{b}\pm0.01$ | $1.65^{b}\pm0.00$ | $1.68^{a}\pm0.00$ | $1.66^{ab}\pm0.01$ | 0.02 |
| Depletion* (%) | 8.68±1.56 | 5.90±0.64 | 8.75±1.79 | 7.64±1.67 | 0.49 |

[a b c] Means in the same row with unlike superscripts differ significantly ($p < 0.05$)

*Depletion = Dead + Cull

Cum. FI means cumulative feed intake, BW means body weight, ADG means average daily gain and FCR means feed conversion ratio

**4.2. Gross lesion and histopathological score of femoral heads**

At the end of experiment (d 43), all chickens were euthanized, the left and the right femoral head were scored individually (Table 4). T4 (Meloxicam) had the lowest score (0.75±0.06) with significant different ($p < 0.05$) when compared with the other groups while T1, T2 and T3, the score was not significant different (1.18±0.05, 1.08±0.06 and 1.08±0.03 respectively).

Furthermore, the incidence of FHS in T4 was significantly lower ($p < 0.05$) (47.20±4.20 %) than other groups (T1 = 64.75±3.16 %, T2 = 64.33±3.80 % and T3 = 62.06±1.98 %) (Table 4). Chickens in T4 had the highest incidence ($p < 0.05$) of normal chickens without lesion on femoral head (T1 = 32.52±3.27 %, T2 = 34.89±3.54 %, T3 = 36.96±1.59 % and T4 = 52.79±4.20 %). The incidence of FHT was very low in T1 – T3 and was not found in T4 however, T3 had the same incidence (0.98±1.34 %) when compared with T1 and T2. Chickens in T1 had a significantly

higher ($p < 0.05$) incidence of FHT (2.74±0.95 %) when compared to T2 (0.78±1.21 %). On the other hand, the incidence of FHN was not found in all groups.

**Table 4 The incidence of FHS, FHT, FHN and average score of each treatment.**

| Lesion | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|
| Average score | 1.18$^{a}$±0.05 | 1.08$^{a}$±0.06 | 1.08$^{a}$ ±0.03 | 0.75$^{b}$±0.06 | <0.01 |
| Normal (%) | 32.52$^{b}$±3.27 | 34.89$^{b}$±3.54 | 36.96$^{b}$ ±1.59 | 52.79$^{a}$±4.20 | <0.01 |
| FHS (%) | 64.75$^{a}$±3.16 | 64.33$^{a}$±3.80 | 62.06$^{a}$ ±1.98 | 47.20$^{b}$±4.20 | <0.01 |
| FHT (%) | 2.74$^{a}$±0.95 | 0.78$^{b}$±1.21 | 0.98$^{ab}$±1.34 | 0.00$^{b}$±0.00 | 0.03 |
| FHN (%) | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | - |

$^{a\ b\ c}$ Means in the same row with unlike superscripts differ significantly ($p < 0.05$)

FHS = Femoral head separation, FHT = Femoral head transitional degeneration and FHN = Femoral head necrosis

Individual BW of chicken in relation to gross lesion score (normal and FHS) of all treatments were analyzed and showed as profile in Figure 8. The BW of FHS group in all treatment groups tended to be higher than normal group. In addition, an average BW of FHS group was also higher than normal group (T1 FHS = 3,328 g, T1 normal = 3,251 g, T2 FHS = 3,253 g, T2 normal = 3,155 g and T3 FHS = 3,205 g, T3 normal = 3,103 g and T4 FHS = 3,249 g, T4 normal = 3,220 g). Moreover, the average BW of FHS group in T2 and T3 was similar to the average BW of normal group in T1.

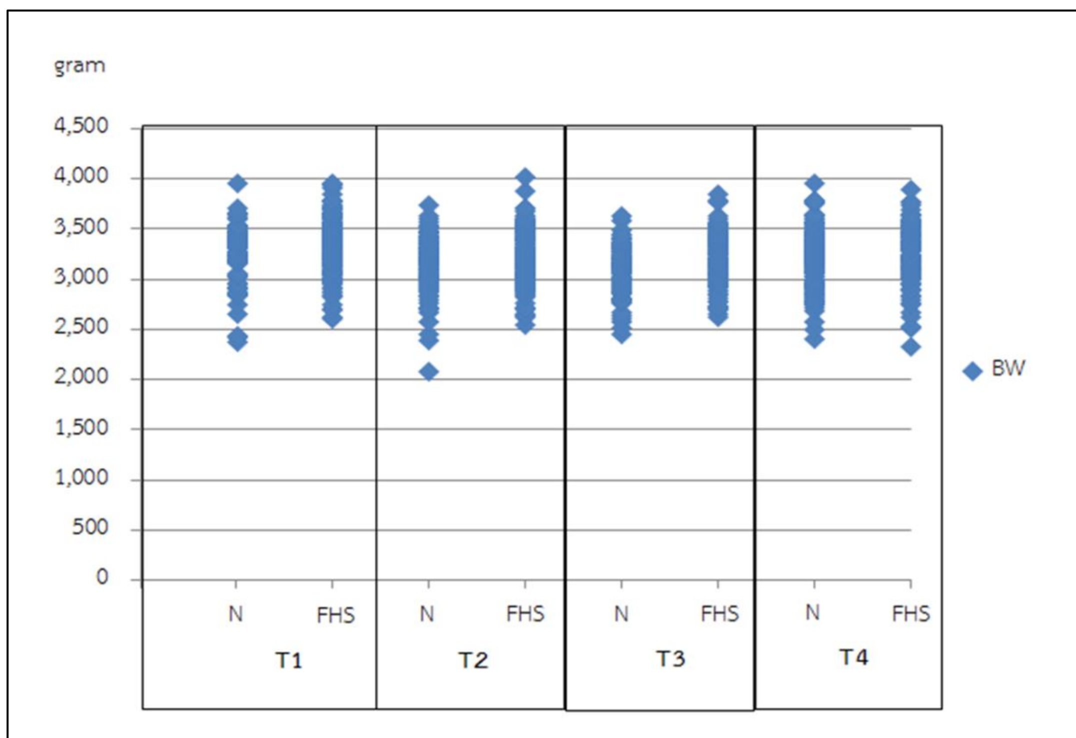


Figure 8 Individual BW related to gross lesion score (normal and FHS).

N = normal, FHS = femoral head separation

Table 5 Average body weight in relation to gross lesion.

| Treatment | Lesion | Average BW (g) | SD | CV |
|---|---|---|---|---|
| T1 | Normal | 3,251 | 290 | 8.93 |
| | FSH | 3,328 | 272 | 8.17 |
| T2 | Normal | 3,155 | 284 | 9.00 |
| | FSH | 3,253 | 256 | 7.87 |
| T3 | Normal | 3,103 | 244 | 7.87 |
| | FSH | 3,205 | 246 | 7.67 |
| T4 | Normal | 3,220 | 272 | 8.45 |
| | FSH | 3,249 | 271 | 8.33 |

Body weight and lesion of normal and FHS were observed at the end of experiment (day 43).

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In order to better define the incidence of FHS and pathological changes in femoral head, the femurs of chicken which scored as normal (score = 0) on both left and right femoral heads and scored as FHS (score = 1) on both left and right femoral heads were fixed in Cal-Rite (10 samples for normal and 10 samples for FHS in each group). Histological score and morphometric measurements data were shown in Table 6 for normal (score = 0) and Table 7 for FHS (score = 1).

The average histological score of normal femoral head was lower than FHS femoral head in all treatments as shown in Table 6 and Table 7. Moreover, when compared between groups with gross lesion scored 0 (normal) (Table 6), there were no significant differences in histological score and all zone length in among treatments. Moreover, the total number of EC, PEV and MVP were not statistically different. The longest length of PEV was T4, followed by T3, T2 and T1 but not statistically different (414±23.7 μm, 406±39.6 μm, 372±31.9 μm and 365±9.2 μm, respectively). In addition, MVP length of T4 was longer than that of T1 with significant different (328±33.7 μm and 242±11.2 μm) ($p < 0.05$) while T2 and T3 were not statistically different (314±12.5 μm and 319±30.2 μm) when compared to T1 and T4.

When compared between groups with gross lesion score 1 (FHS) (Table 7), there were no significant differences in histological score, all zone length and total number of MVP. Furthermore, MVP length were increased in T3 and T4 (388±14.47 μm and 384±23.3 μm) when compared to that of T1 and T2 (294±14.0 μm and 286±25.1 μm) with significant difference ($p < 0.05$).

Moreover, the length of each zone in femoral head was compared. Only the length of PPZ and HZ of T3 from normal group (PPZ = 701±77.8 μm and HZ = 1,711±39 μm) were longer ($p < 0.05$) than FHS group (PPZ = 498±21.7 μm and HZ = 1,525±65 μm) (Figure. 9 and 10).

**Table 6 Morphometric measurements of proximal femoral head of gross lesion score 0 (Normal)**

| Normal* | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|
| Histological score | 0.33±0.17 | 0.17±0.11 | 0.50±0.16 | 0.58±0.15 | 0.22 |
| ACZ length (μm) | 719±66.5 | 741±30.4 | 787±53.5 | 760±74.1 | 0.88 |
| PPZ length (μm) | 534±22.6 | 596±38.6 | 701±77.8 | 601±41.3 | 0.14 |
| HZ length (μm) | 1,468±57 | 1,670±138 | 1,711±39 | 1,541±63 | 0.21 |
| Total length** | 2,722±115 | 3,007±160 | 3,199±88 | 2,902±151 | 0.14 |
| Total number EC | 89.25±9.6 | 113±12.2 | 109±9.3 | 100±8.1 | 0.35 |
| Total number PEV | 2.92±0.64 | 4.33±0.95 | 3.90±0.93 | 4.00±0.43 | 0.58 |
| Total number MVP | 5.42±1.10 | 5.42±1.29 | 6.30±2.02 | 4.58±0.68 | 0.84 |
| PEV length (μm) | 365±9.2 | 372±31.9 | 406±39.6 | 414±23.7 | 0.52 |
| MVP length (μm) | $242^{b}$±11.2 | $314^{ab}$±12.5 | $319^{ab}$±30.2 | $325^{a}$±23.3 | 0.02 |

$^{a\ b\ c}$ Means in the same row with unlike superscripts differ significantly ($p < 0.05$)

* Gross lesion of femoral head score 0

** Calculated from ACZ + PPZ + HZ

ACZ = Articular cartilage zone, PPZ = Proliferative prehypertrophic zone,

HZ = Hypertrophic zone, EC = epiphyseal vascular canals,

PEV = penetrating epiphyseal vessel and MVP = metaphyseal vascular plexus

**Table 7 Morphometric measurements of proximal femoral head of gross lesion score 1 (FHS).**

| FHS* | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|
| Histological score | 1.42±0.20 | 1.30±0.20 | 1.20±0.12 | 1.33±0.17 | 0.86 |
| PPZ length (μm) | 585±43.7 | 513±46.3 | 498±21.7 | 527±30.6 | 0.38 |
| HZ length (μm) | 1,408±143 | 1,506±135 | 1,525±65 | 1,461±73 | 0.88 |
| Total length** | 1,993±170 | 2,018±168 | 2,022±76 | 1,989±98 | 1.00 |
| Total number MVP | 4.00±0.31 | 4.83±1.17 | 4.30±0.34 | 4.00±0.58 | 0.77 |
| MVP length (μm) | $294^{b}$±14.0 | $286^{b}$±25.1 | $388^{a}$±14.5 | $384^{a}$±23.3 | <0.01 |

$^{a\,b\,c}$ Means in the same row with unlike superscripts differ significantly ($p < 0.05$)

* Gross lesion of femoral head score 1

** Calculated from PPZ + HZ

ACZ = Articular cartilage zone, PPZ = Proliferative prehypertrophic zone,

HZ = Hypertrophic zone, EC = epiphyseal vascular canals,

PEV = penetrating epiphyseal vessel and MVP = metaphyseal vascular plexus

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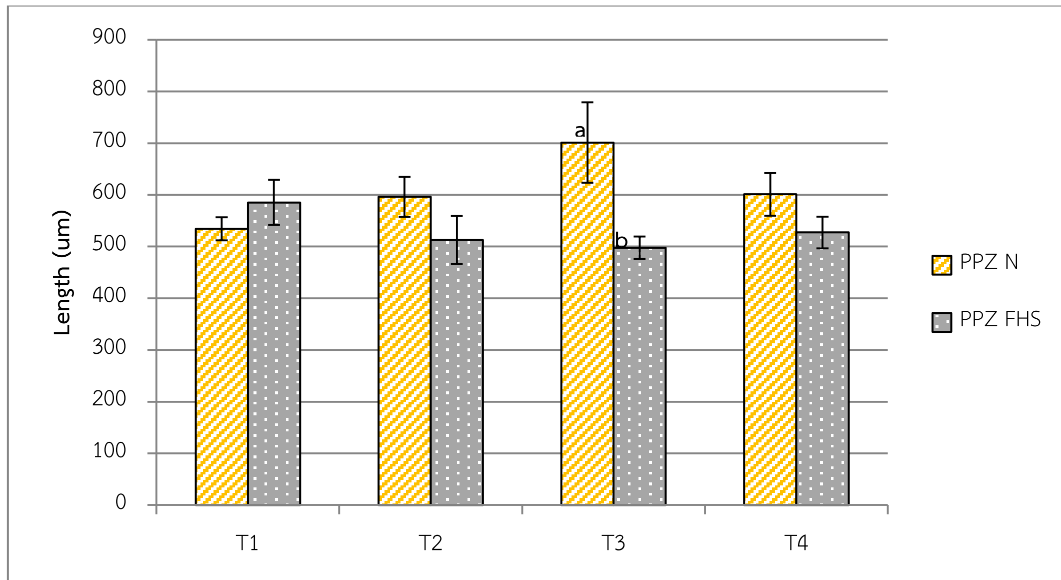


**Figure 9 Length of PPZ (μm), compared between lesion (normal and FHS) in the same group.**

[a b] Means in the same row with unlike superscripts differ significantly ($p < 0.05$), PPZ N = Proliferative prehypertrophic zone (normal group), PPZ FHS = Proliferative prehypertrophic zone (FHS group)

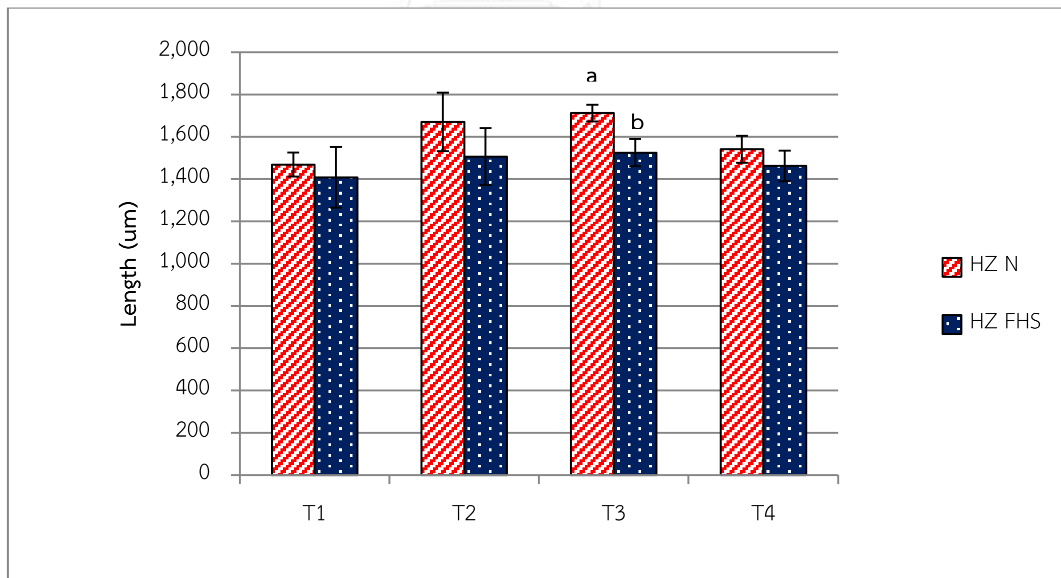


**Figure 10 Length of HZ (μm), compared between lesion (normal and FHS) in the same group.**

[a b c] Means in the same row with unlike superscripts differ significantly ($p < 0.05$), HZ N = Hypertrophic zone (normal group)

HZ FHS = Hypertrophic zone (FHS group)

**4.3. Pro-inflammatory cytokines**

To elucidate the pathological change in femoral heads and mechanism in relation to inflammation, pro-inflammatory cytokines (TNF-α and IL-1β) in serum were analyzed. Blood samples from the same chickens that had femoral head score 0 (normal) and femoral head score 1 (FHS) were selected to analysis using commercial ELISA.

The result of TNF-α and IL-1β is shown in Table 8. When compared among treatment groups (T1 – T4) with the same lesion, both TNF-α and IL-1β levels were not significantly different.

Moreover, the level of TNF-α and IL-1β were compared between the lesion (normal and FHS) in own group (Table 8). The level of TNF-α tended to ($p$ > 0.05) decrease in FHS group in all treatments (T1 = 2.66±0.61 pg/ml, T2 = 3.13±0.82 pg/ml, T3 = 1.95±0.08 pg/ml and T4 = 2.28±0.20 pg/ml) except T2 when compared with normal group (T1 = 3.77±0.64 pg/ml, T2 = 2.69±0.62 pg/ml, T3 = 2.13±0.38 pg/ml and T4 = 2.35±0.32 pg/ml). In contrast, the level of IL-1β tended to increase in FHS group in all treatment (T1 = 1,470±272 pg/ml, T2 = 992±384 pg/ml, T3 = 2,105±434 pg/ml and T4 = 1,220±148 pg/ml) but no significant differences was observed (normal group; T1 = 1,402±305 pg/ml, T2 = 797±215 pg/ml, T3 = 1,420±278 pg/ml and T4 = 1,087±144 pg/ml).

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**Table 8 Serum level of TNF-α and IL-1β at 42 day of age, compared among treatments and between lesion score.**

| Item (pg/ml) | Lesion | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|---|
| TNF-α | Normal | 3.77±0.64 | 2.69±0.62 | 2.13±0.38 | 2.35±0.32 | 0.10 |
| | FHS | 2.66±0.61 | 3.13±0.82 | 1.95±0.08 | 2.28±0.20 | 0.45 |
| | P-value | 0.24 | 0.71 | 0.65 | 0.87 | |
| IL-1β | Normal | 1,402±305 | 797±215 | 1,420±278 | 1,087±144 | 0.24 |
| | FHS | 1,470±272 | 992±384 | 2,105±434 | 1,220±148 | 0.12 |
| | P-value | 0.87 | 0.67 | 0.23 | 0.53 | |

*n* = 10 samples/lesion/treatment

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## 4.4. Serum triglyceride and cholesterol

Level of TG and CL in each treatment is depicted in Table 9. The levels of TG in both normal and FHS groups in each treatment were not significant different. In addition, the levels of CL (normal and FHS) in each treatment were also not different ($p > 0.05$).

Levels of TG and CL between the lesion (normal and FHS) in its own treatment is demonstrated in Table 9. The level of TG tended to increase in FHS group (T1 = 107±7.8 mg/dL, T2 = 104±8.9 mg/dL and T3 = 101±5.9 mg/dL) when compared to normal group (T1 = 84.6±12.3 mg/dL, T2 = 93.4±26.2 mg/dL and T3 = 90.8±16.5 mg/dL) except in T4 (normal = 114±21.3 mg/dL and FHS = 102±9.0 mg/dL), however no significant difference was observed. The level of CL were not different in T2 – T4 but in T1, FHS group had higher level of CL (142±1.6 mg/dL) ($p < 0.05$) with statistical significant when compared to control group (131±4.0 mg/dL).

**Table 9 Serum levels of TG and CL at 42 day of age, compared among treatments and between lesion score.**

| Item (mg/dL) | Lesion | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|---|
| TG | Normal | 84.6±12.3 | 93.4±26.2 | 90.8±16.5 | 114±21.3 | 0.22 |
| | FHS | 107±7.8 | 104±8.9 | 101±5.9 | 102±9.0 | 0.95 |
| | P-value | 0.15 | 0.48 | 0.32 | 0.37 | |
| CL | Normal | 131±4.02 | 143±6.31 | 128±6.83 | 142±4.69 | 0.16 |
| | FHS | 142* ±1.6 | 138±4.6 | 136±5.1 | 146±6.6 | 0.43 |
| | P-value | 0.02 | 0.54 | 0.38 | 0.58 | |

Means in the same column with * differ significantly ($p < 0.05$)

$n$ = 10 samples/lesion/treatment

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# CHAPTER V

# DISSCUSSION

Femoral head separation (FHS) is one of the causes of lameness in broiler production. Separating between physis and epiphysis of femoral head induces cartilage degradation, leads to femoral head transitional degeneration (FHT) and femoral head necrosis (FHN) respectively. In this study, the modified speed bump model (MSB, slope 50%) was used in order to increase the incidence of FHS (Gilley et al., 2014). Moreover, the effects of DHA and meloxicam on histopathological changes and serum TNF-$\alpha$ and IL-1$\beta$ in relation to FHS were discussed as follow.

## 5.1. Modified speed bump (MSB) model on broiler performances and incidence of FHS.

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In this study, FHS was induced by MSB model, started from 14 days of age in T2 – T4, to create foot instability and increase mechanical shear force on femoral head. From the result, the data showed that MSB model in this study cannot increase the incidence of FHS in T2 when compared to T1 (normal rice hull floor). The chickens may prefer to stay on the flat floor than the MSB area. From daily observation, they walked across the MSB only when they eat or drink and spend more time in the rice hull area. Although the slope of MSB in this study was 50% as similar to the original, the size of MSB in relation to the size of pen was different. The area of original speed bump was approximately 30% of the pen size (pen size 3.05 x 3.05 m, speed bump size W x L x H = 0.91 x 3.05 x 0.23 m) (Gilley et al., 2014). In this study, the size of MSB was approximately 20% of the pen size (pen size 1.5 x 3.2 m, MSB size W x L x H = 0.64 x 1.50 x 0.16 m) leaving more flat space than the original one.

Furthermore, MSB in this study did not have a limbo bar to force the chickens to crouch under the bar. The chickens were jumped down from the apex instead of walking down through the instability sloping mesh wire (Gilley et al., 2014).

The higher feed intake and body weight of chickens in control group tended to create more FHS than those raised on MSB. Chickens in T1 had a significantly higher ($p < 0.05$) BW when compared to T2 – T4 due to the higher cumulative feed intake ($p < 0.05$) (Table 3). It was possible that MSB in T2 – T4 might be an obstacle barrier for chickens to reach the feed and water resulting in lower BW. Faster growing broiler tend to show higher incidences of lameness and leg problems when compared to slower growing chickens (Kestin et al., 2001). In addition, reducing growth rate can decrease the incidence of lameness and leg disorder in chickens (Julian, 2005). Moreover, the BW of chickens in FHS group was higher than normal group in T1, T2 and T3 (Figure 8 and Table 5). Wideman et al. (2013) demonstrated that chicken which had a high growth rate and BW leading to increase incidence of FHS or BCO. In contrast, the effect of BW on FHS was not observed in T4 (meloxicam). Meloxicam is able to decrease cartilage degradation (Asano et al., 2006) resulting in alleviation of the effect of BW on FHS in T4. It is demonstrated that the higher BW of chickens was the major predisposing factor that caused FHS lesion resulting in increment of the incidence of FHS in all treatments.

Chickens in T3 had lower BW when compared to T2 and T4. When the omega 3 diet (fish oil 10%) was provided to Weanling male New Zealand White rabbits for 40 days, rabbit in omega 3 group had the lowest cumulative feed intake and BW ($p < 0.05$). Supplementation with a huge quantity of omega 3 fatty acids (fish oil 10 g/100g) and antioxidant (tertiary butyl hydroquinone 0.04%) could reduce energy intake and lower BW in rapid growing rabbit (Judex et al., 2000). In addition, broiler chickens were received full-fat flaxseed (10%, source of omega 3 fatty acid) in feed had a significantly lower ($p < 0.01$) BW approximately 11% and significantly 9% higher ($p < 0.01$) FCR compared to control group (Rahimi et al., 2011). Furthermore, laying hens fed on 1 – 3% of All-G-Rich™ in diets had no difference in hen-day egg production and FCR. However, feed intake (g/hen/day) were slightly 5% decreased ($p > 0.05$) when compared to control diet (Ao et al., 2015). Moreover, increase of omega 3 fatty acid in diet may cause a rancidity in feed resulting in decrease feed intake and leading to low BW (Chae and Lee, 2002). Increasing omega 3 fatty acid in diet can reduce BW and fat deposition in human through post-prandial satiety

mechanism after 120 minutes (Parra et al., 2008). Moreover, omega 3 fatty acid can bind to PPAR α, resulting in increased fatty acid oxidation, reduce BW gain and fat accumulation (Buckley and Howe, 2010).

**5.2. The effect of anti-inflammation on incidence of FHS and pro-inflammatory cytokines level.**

Theoretically, the damaged tissue from mechanical shear force on femoral head can lead to FHS and joint inflammation respectively. The mechanisms that involved in inflammation process were activated by damage-associated molecular patterns (DAMPs), pathogen-associated molecular patterns (PAMPs) and eicosanoid products from phospholipase activity (Goldring and Gravallese, 2000; Ashley et al., 2012). Omega 3 fatty acid (All-G-Rich™, 16% DHA) and NSAIDs (meloxicam, selective COX-2 inhibitor) both had an anti-inflammation properties, were chosen to reduce the FHS in this experiment.

The incidence of FHS was decreased significantly ($p < 0.05$) in meloxicam group (T4) compared to others. It is possible that meloxicam is the selective COX-2 inhibitor which can inhibit directly to COX-2 enzyme, resulting in reduce $PGE_2$ production (Ahmed, 2011). $PGE_2$ is synthesized from AA and this eicosanoid product has a high potency for bone resorption (Watkins et al., 2001b). Moreover, $PGE_2$ can induce osteolytic cytokine such as IL-1, IL-6 and TNF-α (Watkins et al., 2001b). $PGE_2$ can increase osteoclastogenesis by inhibit osteoprotegerin (OPG) secretion (Liu et al., 2006) and upregulate of receptor activator of nuclear factor kβ ligand (RANKL) and macrophage colony stimulating factor (M-CSF) (Li et al., 2000). In general, extracellular matrix of articular cartilage (AC) is composed of two major macromolecules, proteoglycan (aggrecan) and collagen type II (Gendron et al., 2003). Injury chondrocyte, $PGE_2$ and inflammatory cytokines such as IL-1 and TNF-α could induce matrix metalloproteinase (MMP) family. MMP-1 and MMP-13 are importance enzymes in MMP family that can cleave collagen type I and type II in osteoid layer. Degradation of collagen type II and aggrecan, lead to deteriorate of articular cartilage (AC) eventually (Sadowski and Steinmeyer, 2001). Moreover, meloxicam can suppress

the production of MMP in a dose-dependent manner in synovial fibroblasts (Asano et al., 2006). From the reason mentioned above, meloxicam can prevent articular cartilage degradation and resulting in lowest incidence of FHS in this experiment.

The incidence of FHS in T3 when compared to T2 was lower but not significant different ($p$ > 0.05). In general, fatty acid profiles of AC in chicken comprised oleic acid (C18:1), palmitic acid (C16:0) linoleic acid (C18:2 n-6) stearic acid (C18:0) and arachidonic acid (C20:0; AA) (Dolegowska et al., 2006). AA can be converted to 2 series of PGE (i.e. $PGE_2$) and thromboxane by COX-2 enzyme which can affect degradation of AC (Watkins et al., 2001a; Kruger et al., 2010). Omega 3 fatty acids especially EPA can compete to AA and replace AA composition, thus changing n-3 PUFAs/n-6 PUFAs ratio of cell membrane (Simopoulos, 1991). Increasing the ratio of EPA in cell membrane can reduce inflammation by 3 series of PGE ($PGE_3$) that possess less potency than 2 series of PGE ($PGE_2$) from AA (Kruger et al., 2010; Calder, 2012). In addition, EPA can be metabolized by LOX enzyme hence E series of resolvin are produced. E series of resolvin decrease pro-inflammatory gene expression by binding to chemokine like receptor 1 at cell membrane resulting in decrease inflammation and bone loss (Im, 2012). Furthermore, EPA had a potency to reduce the mRNA levels of MMP-3, MMP-13, COX-2, IL-1$\beta$ and TNF-$\alpha$ than DHA and ALA in bovine chondrocytes (Zainal et al., 2009).

In contrast, DHA is not directly metabolized to eicosanoid products ($PGE_3$ series) as same as EPA. It has been reconverting to EPA and metabolized to 3 series of PG that the inflammation activity was very low (Gorjao et al., 2009; Zainal et al., 2009). In this study, for avoidance a variation of omega 3 fatty acids composition, only DHA (All-G-Rich™, 16% DHA) was used to examine the effect of anti-inflammation on incidence of FHS. When damage occurs between physis and epiphysis of femoral head, 2 series of PG still occur and the incidence of FHS cannot be decreased. In theory, DHA can reconvert to EPA only 11 – 12 % in human (Conquer and Holub, 1996). DHA can protect bone and AC loss by metabolized to D series of resolvin (resolvin D1). Resolvin D1 inhibits neutrophil immigration and reduces inflammation by binding to GPR32 and ALX/FPR2 receptor (Im, 2012).

Furthermore, DHA can directly bind to GPR120, inhibit translocated to nucleus of $NF_kB$ and lead to reduce inflammation eventually (Calder, 2012).

In this study, analysis of histopathological changes of FHS lesion revealed no significant different ($p > 0.05$) among groups. Similar finding was also observed in normal group. Moreover, when compared lesion between histopathological finding of normal femoral head and FHS femoral head. The average histological score from FHS femoral head was markedly higher than normal femoral head (Table 6 and Table 7). All articular cartilage zones of FHS femoral head were separated from physis by definition of FHS (Wideman et al., 2012), resulting in histological score of FHS more than 1. However, femoral heads in normal group which had gross lesion score 0, showed both histopathological score 0 and score 1 (Figure 6). These histopathological findings demonstrated that some chickens developed the FHS lesion with no gross lesion appearance. Furthermore, the length of each zone (articular cartilage zone, proliferative-prehypertrophic zone and hypertrophic zone) and total number of vessels were not different in all treatments except metaphyseal vascular plexus (MVP) length. In normal lesion (gross score = 0), there was a tendency of longer MVP in T3 and T4 than control group. Furthermore, in FHS lesion (gross score = 1), MVP of both T3 and T4 were significantly longer than T1 and T2. In theory, blood vessels which supply nutrient for growth plate are epiphyseal vascular canals and metaphyseal vascular plexus. The expansion of metaphyseal vascular plexus to metaphysis can induce deposition of the bone (Howlett, 1980). In addition, anastomosed of epiphyseal vascular canals and metaphyseal vascular plexus can create penetrating epiphyseal vessels that importance for immature chondrocytes (Howlett et al., 1984). Moreover, Increasing length of MVP has a positive correlation to differentiation in proliferative-prehypertrophic zone (PPZ) chondrocytes into hypertrophic zone (HZ) chondrocytes (Hedstrom et al., 1986). In contrast, inadequate metaphyseal vascular plexus (MVP) can create tibial dyschondroplasia in broiler chickens (Wise and Jennings, 1972). Base on this information, it is postulated that MVP might be important for bone and cartilage formation.

In this experiment, pro-inflammatory cytokines level was not significantly different in all groups. From literature review, IL-1β and TNF-α should be lowest in

T3. DHA reduce the level of IL-1β and TNF-α by binding to GPR120, resulting in inhibit $NF_kB$ translocated to nucleus (Im, 2012). The importance factors for anti-inflammation effects are time and dosage of DHA/EPA. Result from in vitro study showed that 10 μM DHA reduced the level of IL-1β of bovine chondrocytes (Wann et al., 2010). In human and animals study, the range of EPA and DHA for dietary supplement was approximately 3 – 6 g/day which was a beneficial for reducing joint inflammation and arthritis (Cathcart and Gonnerman, 1991; Shapiro et al., 1996; Watkins et al., 2001b; Hurst et al., 2010). Moreover, healthy human who received flaxseed oil in food for 4 weeks can reduced the level IL-1β and TNF-α approximately 30% (Caughey et al., 1996). In this study, the level of both IL-1β and TNF-α in T3 were not significant different ($p > 0.05$) from other groups. It might be possible that the chickens in this experiment received only DHA which was less potency than EPA. Dosage of DHA in this experiment was approximately 700 mg/day that might be inadequate to reduce the level of pro-inflammatory cytokines. The main effect of meloxicam is to inhibit COX-2 enzyme which leads to reduction of the $PGE_2$ level. In addition, there was a positive correlation between $PGE_2$ and proteoglycan released. It is found that the higher concentration of $PGE_2$, the higher of proteoglycan released (Mastbergen et al., 2008). Moreover, meloxicam has indirect effect on the reduction of TNF-α by down regulation of $NF_kB$ binding activity (Safieh-Garabedian et al., 2000). When compared to this experiment, the levels of TNF-α tend to be lower in meloxicam group than control groups but not significant difference ($p > 0.05$).

When the lesion of FHS was compared, serum IL-1β concentrations in FHS groups were higher than normal groups. In contrast, serum TNF-α level was lower than normal group but no significant difference were observed. In general, IL-1β and TNF-α can inhibit proteoglycan synthesis and increase proteoglycan release in human cartilage cells (Mastbergen et al., 2008). IL-1β is important for cartilage degradation while TNF-α acts on inflammatory process (Mohammed et al., 2014). From this experiment, chicks that had FHS lesion did not show the inflammatory cells and bacteria clump accumulated at separating site (between physis and epiphysis) as same as the result of Durairaj et al. (2009). It is postulated that FHS is a

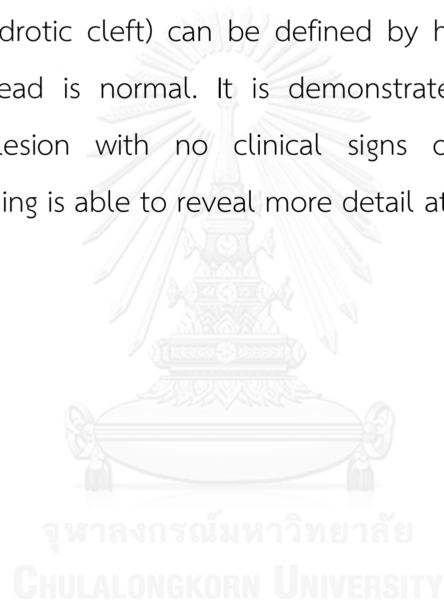
first stage of bacterial chondronecrosis with osteomyelitis (BCO). Only DAMPs pathway is detected while PAMPs pathway still not occur (no bacterial clump at the site of damage). This coincided with the high level of serum IL-1β but low level of serum TNF-α were observed in this study.

**5.3. Change in serum triglyceride and cholesterol level in FHS Chickens.**

In this study, serum triglyceride (TG) and cholesterol (CL) in T3 chicks were not different when compared to others. It is possible that the limitation of DHA dosage used of DHA in this study, thus, cannot decrease the level of serum TG and CL. Moreover, there was no significant difference between levels of serum TG and CL in FHS groups when compared to normal groups. Blood samples and femoral head from FHS, normal and tibial dyschondroplasia chickens were collected, serum lipid such as CL, TG and LDL were observed. The data showed that there was no statistical different among all groups (Durairaj et al., 2009). It is plausible that serum TG and CL may not relate to FHS incidence in this study. Previously, imbalance of lipid metabolism in relation to incidence of FHS was investigated but the results remained unclear. In theory, omega 3 fatty acids can reduce serum TG and CL through many pathways. Omega 3 fatty acids can bind to peroxisome proliferator activated receptor alpha (PPAR α) and form with retinoic-X-receptor, resulting in increased fatty acid oxidation (Calder, 2012). Moreover, omega 3 fatty acids can increase phospholipid synthesis and decrease activity of diacylglycerol acyl transferase or phosphatidic acid phosphor-hydrolase (Harris and Bulchandani, 2006). Furthermore, omega 3 fatty acids can bind to liver X receptor (LXR) which prevents toxicity of CL in cells and can reduce CL absorption in intestine (Davidson, 2006). In human, the serum TG was decreased approximately 20 – 50% from baseline when received 3 – 4 g/day of DHA and EPA more than two weeks (Harris, 1997).

## Conclusions

The main factor that induces FHS in broiler chickens is high growth rate and BW. Modified speed bump used in the current study cannot increase the incidence of FHS. Furthermore, meloxicam 0.5 mg/kg in drinking water for ten days can reduce the incidence of FHS in broiler whereas ALL-G-Rich™ 2% (16% DHA) supplemented in broiler feed for 43 days cannot reduce the incidence of FHS. Moreover, there was no alteration of serum TNF-α, IL-1β, TG and CL in both DHA and meloxicam groups. Histopathological score can differentiate lesion among normal and FHS femoral head as similar to gross lesion score. In addition, small separation between physis and epiphysis (osteochondrotic cleft) can be defined by histopathology although gross lesion of femoral head is normal. It is demonstrated that some chickens may develop the FHS lesion with no clinical signs or gross lesion appearance. Histopathological finding is able to reveal more detail at the beginning of FHS lesion.



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# APPENDICES

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

# APPENDIX A

**Table I. Composition of the feed**

| **Ingredient (%)** | **CB-I-** | | **CB-II-** | | **CB-III-** | | **CB-IV-** | |
|---|---|---|---|---|---|---|---|---|
| | **151** | **152** | **151** | **152** | **151** | **152** | **151** | **152** |
| Corn | 57.32 | 56.86 | 63.67 | 63.30 | 65.28 | 64.82 | 66.32 | 65.97 |
| Full fat soybeans, Bra | 7.10 | 7.10 | 7.80 | 7.80 | 11.20 | 11.20 | 9.90 | 9.90 |
| Soybean meal, Bra | 28.30 | 27.50 | 21.50 | 20.60 | 17.00 | 16.20 | 16.80 | 16.00 |
| Canola | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 |
| All-G- Rich | - | 2.00 | - | 2.00 | - | 2.00 | - | 2.00 |
| Palm oil (crude) | 1.50 | 0.70 | 1.50 | 0.70 | 1.50 | 0.70 | 2.00 | 1.10 |
| MCP | 1.35 | 1.36 | 1.25 | 1.27 | 1.09 | 1.10 | 1.10 | 1.11 |
| Limestone | 1.40 | 1.38 | 1.32 | 1.31 | 1.19 | 1.18 | 1.19 | 1.18 |
| Others | 1.54 | 1.61 | 1.47 | 1.52 | 1.25 | 1.30 | 1.19 | 1.25 |
| **Total** | **100** | **100** | **100** | **100** | **100** | **100** | **100** | **100** |

*Others include: salt, sodium bicarbonate, L-lysine, DL-methionine, L-threonine, choline chloride, feed curb, Vitamin premix, Mineral premix, santoquin 66.7%.*

CB-I = Starter feed, CB-II = Grower feed, CB-III = Finisher1 feed and CB-IV = Finisher2 feed

Code 151 = Standard Cobb 500®, 2012

Code 152 = Standard Cobb 500®, 2012 + All-G-Rich 2% (Reformulated)

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Table II. Calculated values of the feed

| Item | CB-I- | | CB-II- | | CB-III- | | CB-IV- | |
|---|---|---|---|---|---|---|---|---|
| | **151** | **152** | **151** | **152** | **151** | **152** | **151** | **152** |
| ME (kcal/kg) | 3,035 | 3,036 | 3,108 | 3,110 | 3,180 | 3,182 | 3,203 | 3,200 |
| % Crude protein | 21.50 | 21.52 | 19.01 | 18.99 | 17.99 | 18.00 | 17.50 | 17.52 |
| % Crude fat | 5.57 | 5.74 | 5.85 | 6.02 | 6.52 | 6.70 | 6.80 | 6.87 |
| % Crude fiber | 2.71 | 2.69 | 2.61 | 2.59 | 2.63 | 2.61 | 2.60 | 2.59 |
| % Calcium | 0.90 | 0.90 | 0.84 | 0.84 | 0.76 | 0.76 | 0.76 | 0.76 |
| % Total phosphorus | 0.72 | 0.72 | 0.67 | 0.68 | 0.63 | 0.64 | 0.63 | 0.63 |
| % Non phytate phosphorus | 0.45 | 0.45 | 0.42 | 0.42 | 0.38 | 0.38 | 0.38 | 0.38 |
| % Sodium | 0.21 | 0.21 | 0.17 | 0.17 | 0.17 | 0.17 | 0.16 | 0.16 |
| % Chloride | 0.31 | 0.32 | 0.26 | 0.27 | 0.25 | 0.25 | 0.23 | 0.23 |
| % Lysine | 1.32 | 1.33 | 1.19 | 1.19 | 1.05 | 1.05 | 1.00 | 1.00 |
| % Digestible lysine | 1.20 | 1.21 | 1.08 | 1.09 | 0.95 | 0.95 | 0.90 | 0.91 |
| % Methionine | 0.65 | 0.65 | 0.59 | 0.60 | 0.53 | 0.54 | 0.50 | 0.50 |
| % Digestible methionine | 0.62 | 0.63 | 0.56 | 0.57 | 0.51 | 0.51 | 0.47 | 0.48 |
| % Met + Cys | 0.98 | 0.98 | 0.89 | 0.89 | 0.82 | 0.82 | 0.78 | 0.78 |
| % Digestible Met + Cys | 0.90 | 0.90 | 0.82 | 0.82 | 0.75 | 0.75 | 0.71 | 0.71 |
| % Threonine | 0.90 | 0.90 | 0.80 | 0.80 | 0.71 | 0.71 | 0.72 | 0.72 |
| % Digestible threonine | 0.77 | 0.77 | 0.69 | 0.69 | 0.60 | 0.60 | 0.61 | 0.61 |
| % Tryptophan | 0.26 | 0.25 | 0.22 | 0.22 | 0.21 | 0.21 | 0.20 | 0.20 |
| % Digestible tryptophan | 0.23 | 0.22 | 0.19 | 0.19 | 0.18 | 0.18 | 0.18 | 0.17 |
| % Arginine | 1.50 | 1.47 | 1.30 | 1.26 | 1.23 | 1.20 | 1.19 | 1.16 |

CB-I = Starter feed, CB-II = Grower feed, CB-III = Finisher1 feed and CB-IV = Finisher2 feed

Code 151 = Standard Cobb 500®, 2012

Code 152 = Standard Cobb 500®, 2012 + All-G-Rich 2% (Reformulated)

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Table III. Analyzed values of the feed

| Item | CB-I- | | CB-II- | | CB-III- | | CB-IV- | |
|---|---|---|---|---|---|---|---|---|
| | **151** | **152** | **151** | **152** | **151** | **152** | **151** | **152** |
| GE (kcal/kg) | 4,054 | 4,085 | 4,069 | 4,110 | 4,123 | 4,146 | 4,148 | 4,177 |
| % Crude protein | 21.900 | 21.330 | 19.400 | 18.560 | 18.400 | 17.590 | 17.700 | 16.870 |
| % Crude fat | 4.810 | 5.930 | 5.710 | 6.580 | 6.840 | 7.280 | 7.060 | 7.580 |
| % Crude fiber | 1.720 | 2.670 | 2.060 | 2.580 | 1.970 | 2.730 | 2.070 | 2.530 |
| % Calcium | 0.940 | 0.990 | 0.910 | 0.850 | 0.720 | 0.830 | 0.790 | 0.780 |
| % Total phosphorus | 0.600 | 0.600 | 0.620 | 0.600 | 0.560 | 0.560 | 0.530 | 0.530 |

CB-I = Starter feed, CB-II = Grower feed, CB-III = Finisher1 feed and

CB-IV = Finisher2 feed

Code 151 = Standard Cobb 500®, 2012

Code 152 = Standard Cobb 500®, 2012 + All-G-Rich 2% (Reformulated)

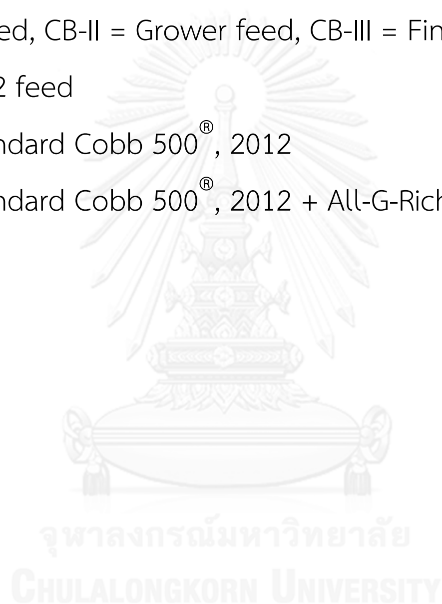

Table IV. Fatty acid profile analyzed of the feed

| Item | CB-I- | | CB-II- | | CB-III- | | CB-IV- | |
|---|---|---|---|---|---|---|---|---|
| | 151 | 152 | 151 | 152 | 151 | 152 | 151 | 152 |
| %Butyric acid (C4:0) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Caporic acid (C6:0) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Caprylic acid (C8:0) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Capric acid (C10:0) | 0.000 | 0.000 | 0.000 | 0.000 | 0.011 | 0.000 | 0.010 | 0.000 |
| %Lauric acid (C12:0) | 0.015 | 0.040 | 0.019 | 0.040 | 0.023 | 0.020 | 0.025 | 0.030 |
| %Myristic acid (C14:0) | 0.010 | 0.070 | 0.013 | 0.070 | 0.017 | 0.060 | 0.019 | 0.070 |
| %Palmitic acid (C16:0) | 1.060 | 1.470 | 1.350 | 1.550 | 1.460 | 1.610 | 1.680 | 1.820 |
| %Palmitoleic acid (C16:1) | 0.000 | 0.000 | 0.000 | 0.010 | 0.000 | 0.010 | 0.000 | 0.010 |
| %Stearic acid (C18:0) | 0.056 | 0.180 | 0.089 | 0.190 | 0.118 | 0.220 | 0.135 | 0.240 |
| %Oleic acid (C18:1) | 1.640 | 1.590 | 1.990 | 1.800 | 2.200 | 1.960 | 2.220 | 2.090 |
| %Linoleic acid (C18:2 n-6) | 2.030 | 2.140 | 2.210 | 2.310 | 2.780 | 2.680 | 2.780 | 2.800 |
| %Alpha linolenic acid (C18:3 n-3) | 0.065 | 0.160 | 0.112 | 0.160 | 0.122 | 0.200 | 0.111 | 0.200 |
| %Stearidonic acid (C18:4 n-3) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Arachidic acid (C20:0) | 0.025 | 0.020 | 0.031 | 0.030 | 0.027 | 0.030 | 0.024 | 0.030 |
| %Eicosenoic acid (C20:1) | 0.000 | 0.010 | 0.000 | 0.010 | 0.000 | 0.020 | 0.000 | 0.020 |
| %Eicosadienoic acid (C20:2 n-6) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Eicosatrienoic acid (C20:3 n-3) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

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Table IV. Fatty acid profile analyzed of the feed (continue)

| Item | CB-I- | | CB-II- | | CB-III- | | CB-IV- | |
|---|---|---|---|---|---|---|---|---|
| | 151 | 152 | 151 | 152 | 151 | 152 | 151 | 152 |
| %Eicosatetraenoic acid (C20:4 n-6) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Eicosapentaenoic acid (C20:5 n-3) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Erucic acid (C22:1) | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 | 0.010 | 0.000 | 0.010 |
| %Docosatrienoic acid (C22:3 n-3) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Docosatetraenoic acid (C22:4 n-6) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Docosapentaenoic acid (C22:5 n-3) | 0.000 | 0.050 | 0.000 | 0.070 | 0.000 | 0.070 | 0.000 | 0.060 |
| %Docosahexaenoic acid (C22:6 n-3) | 0.000 | 0.260 | 0.000 | 0.280 | 0.000 | 0.290 | 0.000 | 0.310 |
| %Nervonic acid (C24:1) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| %Sum n-3 | 0.065 | 0.470 | 0.112 | 0.510 | 0.122 | 0.560 | 0.111 | 0.570 |
| %Sum n-6 | 2.030 | 2.140 | 2.210 | 2.310 | 2.780 | 2.680 | 2.780 | 2.800 |

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CB-I = Starter feed, CB-II = Grower feed, CB-III = Finisher1 feed and CB-IV = Finisher2 feed

Code 151 = Standard Cobb 500®, 2012

Code 152 = Standard Cobb 500®, 2012 + All-G-Rich 2% (Reformulated)

Table V. Analyzed values of All-G-Rich™

| Item | All-G-Rich™ |
|---|---|
| % Crude protein | 13.400 |
| % Crude fat | 62.700 |
| % Crude fiber | 0.912 |
| %Butyric acid (C4:0) | 0.000 |
| %Caporic acid (C6:0) | 0.000 |
| %Caprylic acid (C8:0) | 0.000 |
| %Capric acid (C10:0) | 0.000 |
| %Lauric acid (C12:0) | 0.041 |
| %Myristic acid (C14:0) | 2.960 |
| %Palmitic acid (C16:0) | 36.400 |
| %Palmitoleic acid (C16:1) | 0.000 |
| %Stearic acid (C18:0) | 0.206 |
| %Oleic acid (C18:1) | 0.000 |
| %Linoleic acid (C18:2 n-6) | 0.000 |
| %Alpha linolenic acid (C18:3 n-3) | 0.000 |
| %Stearidonic acid (C18:4 n-3) | 0.000 |
| %Arachidic acid (C20:0) | 0.000 |
| %Eicosenoic acid (C20:1) | 0.000 |
| %Eicosadienoic acid (C20:2 n-6) | 0.000 |
| %Eicosatrienoic acid (C20:3 n-3) | 0.000 |
| %Eicosatetraenoic acid (C20:4 n-6) | 0.000 |
| %Eicosapentaenoic acid (C20:5 n-3) | 0.000 |
| %Docosatrienoic acid (C22:3 n-3) | 0.000 |
| %Docosatetraenoic acid (C22:4 n-6) | 0.000 |
| %Docosapentaenoic acid (C22:5 n-3) | 0.000 |
| %Docosahexaenoic acid (C22:6 n-3) | 21.900 |
| %Nervonic acid (C24:1) | 0.000 |
| %Sum n-3 | 21.900 |

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Table VI. Product specification (All-G-Rich™)

| Label information | Details |
|---|---|
| Fat | Minimum 50% |
| DHA | Minimum 14% |
| Appearance | Brown powder |
| Aroma and taste | Characteristic |
| Solubility | Cloudy solution |

Table VII. Calculated of omega 3 in feed per chicken

| Omega 3 fatty acids | T1 | T2 | T3 | T4 |
|---|---|---|---|---|
| Cum. n-3 (g/43day) | 6.58 | 6.38 | 30.17 | 6.48 |
| Cum. n-3 (mg/day) | 153 | 148 | 702 | 151 |

Table VIII. Performance during starter phase (1 – 10 days of age)

| Treatment | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|
| Cum. FI (g) | $289^{ab}$ | $283^{b}$ | $287^{ab}$ | $293^{a}$ | 0.01 |
| BW at DOC (g) | 46.09 | 46.55 | 46.44 | 46.51 | 0.38 |
| BW at 10 days (g) | $289^{ab}$ | $284^{b}$ | $293^{a}$ | $292^{a}$ | <0.01 |
| ADG (g/b/d) | $28.89^{ab}$ | $28.43^{b}$ | $29.29^{a}$ | $29.24^{a}$ | <0.01 |
| FCR | 1.00 | 1.00 | 0.98 | 1.00 | 0.16 |
| Depletion (%) | 1.39 | 0.00 | 0.42 | 0.69 | 0.22 |

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Table IX. Performance during grower phase (11 – 22 days of age)

| Treatment | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|
| Cum. FI (kg) | $1.28^{a}$ | $1.24^{b}$ | $1.27^{a}$ | $1,27^{a}$ | <0.01 |
| Initial BW at 10 days (g) | $289^{ab}$ | $284^{b}$ | $293^{a}$ | $292^{a}$ | <0.01 |
| BW at 22 days (g) | $1,203^{a}$ | $1,170^{b}$ | $1,206^{a}$ | $1,198^{a}$ | <0.01 |
| BW gain (g) | $914^{a}$ | $885^{b}$ | $913^{a}$ | $906^{a}$ | <0.01 |
| ADG (g/b/d) | $76.21^{a}$ | $73.77^{b}$ | $76.07^{a}$ | $75.50^{a}$ | <0.01 |
| FCR | 1.40 | 1.40 | 1.39 | 1.40 | 0.45 |
| Depletion (%) | 1.04 | 1.39 | 0.42 | 1.06 | 0.83 |

Table X. Performance during finisher1 phase (23 – 40 days of age)

| Treatment | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|
| Cum. FI adj. (kg) | 3.32[a] | 3.26[b] | 3.20[c] | 3.27[ab] | <0.01 |
| Initial BW at 22 days (kg) | 1.20[a] | 1.17[b] | 1.21[a] | 1.20[a] | <0.01 |
| BW at 40 days (kg) | 3.11[a] | 3.04[b] | 2.98[c] | 3.10[a] | <0.01 |
| BW gain (kg) | 1.91[a] | 1.87[a] | 1.77[b] | 1.90[a] | <0.01 |
| ADG (g/b/d) | 106[a] | 104[a] | 98[b] | 106[a] | <0.01 |
| FCR | 1.74[b] | 1.74[b] | 1.80[a] | 1.72[b] | <0.01 |
| Depletion (%) | 5.71 | 3.83 | 7.56 | 5.30 | 0.32 |

Table XI. Performance during finisher2 phase (41 – 43 days of age)

| Treatment | T1 | T2 | T3 | T4 | P-value |
|---|---|---|---|---|---|
| Cum. FI adj. (kg) | 0.52 | 0.51 | 0.49 | 0.49 | 0.65 |
| Initial BW at 40 days (kg) | 3.11[a] | 3.04[b] | 2.98[c] | 3.10[a] | <0.01 |
| BW at 43 days (kg) | 3.29[a] | 3.22[b] | 3.14[c] | 3.23[b] | <0.01 |
| BW gain (g) | 172[a] | 184[a] | 168[a] | 124[b] | <0.01 |
| ADG (g/b/d) | 57.48[a] | 61.19[a] | 55.87[a] | 41.35[b] | <0.01 |
| FCR | 3.11[b] | 2.79[b] | 3.00[b] | 4.19[a] | 0.01 |
| Depletion (%) | 0.77 | 0.73 | 0.43 | 0.74 | 0.96 |

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APPENDIX B

Figure I. Feed Research and Innovation Center

Figure II. Feed Research and Innovation Center: Pen allocation

| Pen | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 | T3 | T4 | T2 | T3 | T1 | T2 | T4 | T1 | T4 | T2 | T3 |
| | T2 | T4 | T1 | T3 | T4 | T2 | T3 | T1 | T3 | T2 | T4 | T1 |
| Pen | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |

Figure III. Pen allocation chart

Figure IV. 7 days of age

Figure V. 14 days of age: the first date of MSB was settled (1)

Figure VI. 14 days of age: the first date of MSB was settled (2)

Figure VII. 20 days of age

Figure VIII. 31 days of age

Figure IX. 32 days of age: Meloxicam was applied in drinking water

Figure X. 36 days of age

Figure XI. 37 days of age

Figure XII. 40 days of age

Figure XIII. 43 days of age: FHS was scored individually (1)

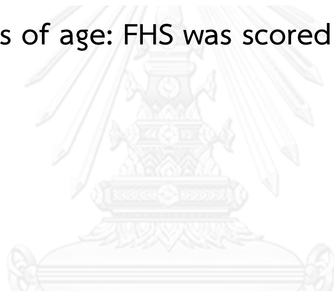

Figure XIV. 43 days of age: FHS was scored individually (2)

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# VITA

Mister Suriya Sooksong was born on August 6th, 1982 in Yasothorn, Thailand. He graduated from the Faculty of Veterinary Science, Chulalongkorn University, Thailand in 2006. He has been working for CPF CO., LTD. since he graduated. After that he enrolled the Master degree of Science in the Department of Veterinary Physiology, Faculty of Veterinary Science, Chulalongkorn University since academic year 2015.