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EFFECTS OF CONJUGATED LINOLEIC ACID ON ADIPOGENIC GENES REGULATION IN CHICKENS

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By

SURIYA KUMARI A/P RAMIAH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

December 2015

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DEDICATION

TO MY LATE FATHER, MR.RAMIAH

I Love You Forever

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Doctor of Philosophy

EFFECTS OF CONJUGATED LINOLEIC ACID ON ADIPOGENIC GENES REGULATION IN CHICKENS

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December 2015

Chairman : Associate Professor Goh Yong Meng, PhD
Faculty : Veterinary Medicine

Modern commercial chickens exhibit excessive fat accumulation in the abdominal area. The major goals of the poultry industry are to increase the carcass yield and to reduce carcass fatness. Excessive fat deposition in chicken is detrimental for human consumption. Increasing the leanness of meat will improve the meat quality, and thus makes animals more valuable. Conjugated linoleic acid (CLA) consists of a complex mixture of geometrical (cis and trans) and positional isomers which consist of two major isomers; cis-9, trans-11 CLA and trans-10, cis-12 CLA. Animal studies have reported that dietary intake of CLA changes animal body composition by preventing obesity development. This study was performed with the hypothesis that CLA modified adipose tissue through alteration of the adipocyte genes. Therefore the general objective of current study was to evaluate the effects of CLA on lipid metabolism, cellular morphology and transcription of regulatory genes that were involved in adipogenesis of chickens. Cis-9, trans-11 CLA and trans-10, cis-12 CLA isomers were evaluated individually for their effects on morphological changes, and adipogenic genes expressions on primary adipose tissue isolated from chicken. Adipose tissue isolated from specific pathogen-free (SPF) chicken was cultured in induction media containing Dulbecco's Modified Eagle Medium (DMEM: HEM‟s (50:50); 1.5% bovine serum albumin; 100mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); 2mg/mL collagenease type I, and 1% penicillin/streptomycin. The media were incorporated with two concentrations of both CLA isomers at 1.51% and 2.56% with a CONTROL group (without CLA isomers). After day 7, adipose tissues differentiation was monitored morphologically using ImageJ software, and adipogenic genes expressions were analyzed by real time polymerase chain reaction (PCR). It was observed that the efficacy of cis-9, trans-11 CLA isomer was more pronounced than trans-10, cis-12 CLA isomer in the in vitro study. Adipose tissue morphology data presented in this work revealed that the domination of cis-9, trans-11 isomer CLA effect was observed at higher concentration in the abdominal fat of broiler chickens. This was associated with a lower transcriptional level of peroxisome proliferator-activated receptor gamma (PPAR γ), and adipocyte protein 2 (aP2), together with lesser abdominal adipocyte volume and smaller amount of fat. The acyl-Coenzyme A binding domain containing 5 (ACBD 5), and lipoprotein lipase (LPL) were down-regulated by cis-9, trans-11 CLA isomer. The primary adipose tissue treated with trans-10, cis-12 concentrations had no changes on the adipose cellularity and adipogenic genes. The

influences of CLA supplementation on growth performance, fatty acid composition, lipid peroxidation, meat colour and plasma lipids in broiler chicken were investigated in this study. The CLA used in this study was of commercial feed grade (Lutrell® BSAF, SE, Ludwigshafen, Germany). A total of 180 broiler chickens were allocated to 3 dietary treatments (0, 2.5 and 5% CLA), and given a standard broiler starter diet from 1 to 21 days, and finisher diet from 22 to 42 days. Body weight of chickens and feed intake were recorded weekly. After slaughter, the breast meat was aged at 4°C for 0, 3 and 6 days. The fatty acid composition was measured in the breast meat. The dietary CLA supplementation significantly ($P<0.05$) increased the content of CLA in chicken meat. The predominant CLA in meat from birds with supplemented diets was trans-10, cis-9. The proportion of monounsaturated (MUFA) fatty acid in meat decreased significantly ($P<0.05$) with increasing CLA supplementation. Dietary CLA also increased the thiobarbituric acid reactive substances (TBARS) values in breast meat. Conjugated linoleic acid feeding also resulted in the reduction of plasma total cholesterol, low-density protein, and the ratio of high-density protein, particularly among the 5% CLA fed-chickens. The effects of CLA on adipocytes morphology, fatty acid profile and PPARs associated genes were performed in CLA-fed chickens. The adipocyte morphology was analyzed using ImageJ software. It was also observed that the content of cis-9, trans-11 CLA in abdominal fat tissue was higher than that of trans-10, cis-12 CLA. The CLA feeding increased total saturated fatty acid and decreased the MUFA in concentration in broilers compared to the control group. Conjugated linoleic acid fed-chickens have lesser mean abdominal adipocyte volume and a smaller amount of fat because of reduced capacities to store fats. The influence on body composition appears to be dependent on PPAR α (alpha). Current study demonstrated that CLA down-regulated aP2 transcription, which was parallel to PPAR γ transcription in adipose tissue of broiler chickens. Adipocyte protein 2 (aP2) was regulated by PPAR γ as PPAR γ is regarded as „master regulator" of adipocyte differentiation. The upregulation of LPL suggested that this gene might play an independent role in regulation of adipogenesis in chicken model. The upregulation of ACBD 5 seems to be novel; therefore, more studies need to be done on this gene. However, LPL gene was not altered which indicates that the expression of LPL occurs spontaneously at confluence and is independent. Therefore, the current results supported the hypotheses that CLA modified adipose tissue through alteration of the adipocyte genes. It is evident that the CLA down-regulated PPAR γ and aP2, which subsequently resulted in decreased adipocyte size, number and area of abdominal fat cells. The comparatively lower CLA content in poultry species could be mitigated through the use of feed and biotechnology approach to enhance CLA content. In conclusion, the present results proved that CLA feeding is a practical strategy to reduce fat deposition in broiler chicken, while increasing its appeal to the general populace consuming chicken meat.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KESAN ASID LINOLEIK TERKONJUGAT KE ATAS PENGAWALATURAN GEN ADIPOS PADA AYAM

Oleh

SURIYA KUMARI A/P RAMIAH

Disember 2015

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Ayam komersial moden mempunyai lemak berlebihan di bahagian abdomen. Matlamat utama industri ternakan ayam adalah untuk meningkatkan hasil karkas and mengurangkan lemak karkas bagi meningkatkan margin keuntungan penternak. Selain itu, lemak yang berlebihan pada ayam akan memudaratkan kesihatan manusia. Pengurangan lemak daging bukan sahaja meningkatkan kualiti daging, malah ia juga akan menjadikan hasil daging ternakan lebih bernilai. Asid linoleik terkonjugat (CLA) terdiri daripada campuran kompleks isomer, cis dan trans. Terdapat dua isomer utama CLA yakni, cis-9, trans-11 CLA dan trans-10, cis-12 CLA. Kajian haiwan telah menunjukkan bahawa pengambilan CLA mengubah komposisi badan haiwan, dan berkesan untuk mencegah obesiti. Kajian ini telah dilaksanakan dengan hipotesis bahawa induksi gen adipos akibat pendedahan CLA akan memberi kesan kepada pengubahsuaian tisu adipos Oleh itu objektif kajian ini adalah untuk mengkaji kesan CLA pada metabolisme lemak, morfologi selular dan transkripsi gen kawal selia yang terlibat dalam pengawalaturan tisu adipos ayam. Cis-9, trans-11 CLA dan trans-10, cis-12 CLA isomers telah dinilai secara individu untuk menentukan kesan perubahan morfologi dan ekspresi adipos gen, pada kultur primer dari tisu adipos ayam. Tisu adipos yang diasingkan dari sampel ayam dikultur dalam media induksi yang mengandungi Dulbecco's Modified Eagle Medium (DMEM:HEM‟s (50:50); 1.5% albumin serum lembu; 100mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); 2mg/mL collagenease jenis I, dan 1% penicillin/streptomycin. Media tersebut dicampur dengan dua konsentrasi CLA isomer pada 1.51% dan 2.56% dengan kumpulan kawalan (CONTROL) (tanpa CLA isomer). Selepas hari ke-7, pembezaan morfologi tisu adipos dipantau dengan menggunakan perisian ImageJ, dan ekspresi gen adipos dianalisis dengan teknik PCR. Isomer CLA cis-9, trans-11 menunjukkan kesan *in vitro* yang lebih ketara berbanding isomer trans -10, cis 12 CLA. Data morfologi tisu adipos kajian ini menunjukkan bahawa konsentrasi cis-9, trans-11 isomer CLA yang tinggi dikaitkan dengan tahap transkripsi reseptor peridi peroksisom gamma (PPAR γ), dan protein adiposit (aP2) yang rendah, serta jumlah adipos tissue yang minium dan bersaiz kecil. Aktiviti gen ACBD 5 (Acyl-Coenzyme A binding domain containing 5) dan lipoprotein lipase (LPL) nyata susut dengan kahadiran cis-9, trans-11 isomer CLA. Kultur primer tisu adipos ayam yang didedahkan kepada isomer trans-10, cis-12 CLA tidak menunjukkan perubahan dalam pertumbuhan adipos and gen adipos. Pengaruh suplemen CLA ke atas prestasi pertumbuhan, komposisi asid lemak, peroksidasi lipid, warna daging dan lipid plasma turut dikaji. Asid linoleik terkonjugat yang digunakan

dalam kajian ini adalah gred makanan komersial (Lutrell® BSAF, SE, Ludwigshafen, Germany). Sebanyak 180 ekor ayam pedaging diberi makan diet yang mengandungi 3 kumpulan rawatan, terdiri daripada 0, 2.5 and 5% CLA). Ayam tersebut diberikan diet ayam permulaan dari hari 1 hingga 21, dan diet penghabisan dari hari 22 hingga 42. Berat badan ayam dan pengambilan makanan direkodkan setiap minggu. Selepas disembelih, daging dada ayam disimpan pada suhu 4° C untuk 0, 3, dan 6 hari. Komposisi asid lemak daging juga dianalisis. Berat badan dan tahap pengambilan makanan bagi ayam pedaging berkurangan dengan peningkatan tahap CLA. Supplementasi CLA dalam pemakanan meningkatkan kandungan CLA daging ayam dengan signifikan ($P<0.05$). Isomer trans-10, cis-9 CLA merupakan isomer yang utama dalam daging dada ayam. Kadar asid lemak monotaktepu (MUFA) dalam daging berkurangan dengan penambahan CLA dengan signifikan ($P<0.05$). Rawatan dengan CLA juga meningkatkan nilai TBARS reaktif dalam daging. Di samping itu, rawatan CLA juga membawa kepada pengurangan jumlah kolesterol plasma, dan lipoprotein berketumpatan rendah (LDL), serta nisbah lipoprotein berketumpatan tinggi (HDL) kepada LDL, terutamanya pada kumpulan ayam yang menerima rawatan 5% CLA. Kesan CLA ke atas morfologi adipos, profil asid lemak dan reseptor peridi peroksisom (PPARs) juga dicerap pada ayam yang menerima rawatan CLA. Morfologi tisu adipos dianalisa menggunakan perisian ImageJ. Kandungan isomer cis-9, trans-11 CLA didapati lebih tinggi daripada isomer trans-10, cis-12 CLA di dalam lemak abdomen ayam. Supplementasi CLA meningkatkan jumlah kandungan asid lemak tepu, serta mengurangkan tahap MUFA berbanding diet kawalan. Ayam yang diberi CLA mempunyai jumlah sel adipos yang minimum dan saiz lemak yang kecil, disebabkan oleh keupayaannya yang lebih rendah dalam penyimpanan lemak. Komposisi badan ayam pedaging didapati tidak mempunyai kaitan signifikan dengan PPAR α (alpha). Kajian ini menunjukkkan bahawa CLA mengurangkan aktiviti transkripsi aP2 selari dengan aktiviti transkripsi PPAR γ dalam tisu adipos ayam pedaging. Gen protein adiposit (aP2) dikawal oleh PPAR γ dan dianggap sebagai pengawalatur utama bagi pertumbuhan adipos. Peningkatan aktiviti LPL menunjukkan bahawa gen ini tidak memberi sebarang kesan ke atas tumbesaran adipos dalam model ayam. Peningkatan aktiviti transkripsi ACBD 5 merupakan penemuan baharu dalam kajian ini. Oleh itu, lebih banyak kajian lanjutan perlu dilakukan ke atas gen ini. Walau bagaimanapun, tahap ekspresi gen LPL adalah tidak berubah Keputusan ini menyokong hipotesis bahawa CLA mengakibatkan pengubahsuaian tisu adipos melalui kesannya ke atas gen adipos. Adalah didapati juga bahawa CLA mengurangkan aktiviti PPAR γ and aP2. Ini seterusnya mengakibatkan pengurangan pada saiz adipos, bilangan dan liputan sel lemak abdomen. Kesimpulannya, kandungan CLA dalam ayam boleh diperkayakan melalui penggunaan suplemen CLA, serta kaedah bioteknologi. Keputusan kajian ini membuktikan bahawa penggunaan CLA dalam makanan ialah satu strategi praktikal untuk mengurangkan pengumpulan lemak pada ayam pedaging, di samping memberi manfaat kesihatan keapda pengguna.

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^{o}C	degrees centigrade
ACBD 5	acyl-Coenzyme A binding domain containing 5
acetyl-CoA	acetyl-coenzyme
ADG	average daily gain
ANOVA	analysis of variance
aP2	adipocyte protein 2
BSA	bovine serum albumin
BW	body weight
cal	calorie
CCAAT	enhancer binding protein alpha C/EBP-α
CD-36/FAT	fatty acid translocase
DISC	death-inducing signaling complex
DM	dry matter
DMEM	dulbecco's nodified eagle medium
FAME	fatty acids methyl esters
FAR	fatty acid ratio
FAS	fatty acid synthase
FBS	foetal bovine serum
FFA	free fatty acid
g	gram
GLUT4	glucose transporter 4
GPDH	glycerol-3-phosphate dehydrogenase
HDL	high-density lipoprotein
kcal	kilo calories
Kg	kilogram
L	liter
LA	linoleic acid
LDL	low-density lipoprotein
LPL	lipoprotein lipase
MDA	malondialdehyde
ME	metabolizable energy

mg	milligram
min	minute
mL	milliliter
mL/min	milliliters per minute
mmol/L	millimoles per liter
MUFA	monounsaturated fatty acid
n-6: n-3 ratio	total n-6 PUFA to total n-3 PUFA ratio
PBS	phosphaste buffer saline
PCR	polymerase chain reaction
PPAR	peroxisome proliferator-activator receptors
PUFA	polyunsaturated fatty acids
PUFA: SFA ratio	total PUFA to total SFA ratio
SCD	stearoyl-CoA desaturase
SEM	standard error of mean
SFA	saturated fatty acid
SPF	specific pathogen-free
TAG	triglycerides
TBARS	thiobarbituric acid reactive substances
TC	total cholesterol
UFA	unsaturated fatty acids
UFS: SFA ratio	total UFA to total SFA ratio
VLDL	very low-density lipoprotein

CHAPTER 1

GENERAL INTRODUCTION

According to Food and Agriculture Organization (FAO) data, the human population benefits significantly from poultry meat and eggs (Marangoni et al., 2015). From a nutritional point of view, poultry meat contains major source of protein (18-25%) and significant amounts of monounsaturated fatty acids (only a third of total fat is made up of saturated fatty acids (Stangierski and Lesnierowski, 2015). Malaysia is the third largest poultry producer in Asia Pacific (MIDA, 2014). This industry has been self-sufficient since 1984. In Malaysia, poultry meat output are estimated to be in the region of RM7, 603.77 million in year 2014 (FLFAM, 2015). The broilers are the most efficient major food animal surpassed both beef and pork meat.

The high growth rate of the modern broiler chicken has resulted in a tremendous increase in nutrient requirement for muscle protein synthesis. Excess fat is a major economic loss to the broiler industry as it not only reduces the carcass yield but also rejection from of the meat by consumers (Sahraei, 2012). The accumulation of fat in modern broiler chicken strains is about 13–14.5% fat of their body weight (Tumova and Teimouri, 2010). This situation most commonly occurs with broiler chickens that consume feed *ad libitum*.

In poultry, the major site of "*de novo*" fat synthesis is the liver (Cornejo *et al.*, 2007). The fat will be transported via low-density lipoproteins or chylomicrons for storage in adipose tissues as triglycerides (Hermier, 1997). In general, the abdominal fat deposition reduced via decreasing the size and/or number of abdominal adipose cells by inhibiting the absorption of dietary fat and fatty acid synthesis, and/or promoting fatty acid β-oxidation (Fouad and El-Senousey, 2014). The abdominal fat pad can be used as a parameter to estimate the total body fat content as it is linked directly to total body fat content in avian species (Fouad and El-Senousey, 2014). The broiler industry needs new method to reduce the fatness in broilers, which would economically benefit producers, and lead to improved health and welfare of chickens.

It has been well documented that nutrition has a significant effect on fat deposition in poultry (Fouad and El-Senousey, 2014). The deposition of fat and fatty acids in animal tissues has been ascribed to a complex regulation network of lipogenic genes (Costa *et al.*, 2013). It gave a negative impact for consumers who are conscious of dietary fat intake. The increased amount of adipose tissue mass may be due to increase preadipocytes, as well as through adipogenesis and/or increased deposition of cytoplasmic triglyceride per cell (Soukas *et al*., 2001). Adipogenesis is regulated by adipogenesis-related candidate genes such as peroxisome proliferator-activator receptors (PPARs), C/EBP-α (CCAAT/enhancer binding protein alpha), aP2 (adipocyte protein 2), and lipoprotein lipase (LPL) (Yaqiong *et al*., 2014). Therefore, it is

important to know the regulation of adipose tissue deposition and metabolism in chicken.

Peroxisome proliferator activated receptors (PPAR) is a ligand-dependent transcription factor and a member of the nuclear receptor super family (Wang *et al.*, 2008b). Conjugated linoleic acid (CLA) serves as ligand activator for PPAR isoforms able to regulate cellular metabolism, differentiation and in part of carbohydrate homeostasis (Peters *et al*., 2001). Royan *et al.* (2011a); Wang *et al.* (2008b); Sato *et al.* (2009) suggested that, PPAR γ (gamma), is strongly associated with abdominal fat deposition. In avian species, however, its mechanism has yet to be extensively investigated, as the PPAR may mediate their effects through a different signaling path. In view of the far-reaching effects of conjugated linoleic acid, this study will attempt to elucidate the PPAR expression and its influence on lipid mobilization, adipogenesis and fat deposition in chickens.

In recent years, focus by researchers on health-promoting properties in poultry meat by fatty acid has been increasing (Yari et al., 2015). Modification of these attributes in meat with bioactive substances such as CLA, vitamins and antioxidants, and a balanced n-6 to n-3 PUFA is a simpler and much more commonly applied method than the genetic approach (Stangierski and Lesnierowski, 2015). Polyunsaturated fatty acids (PUFA), particularly the conjugated linoleic acid is increasingly recognized for its beneficial health impact (Park et al., 1997; West 1998; Pariza et al., 2001). Conjugated linoleic acid refers to a group of geometric and positional dienoic isomers of linoleic acid [C18:2(n-6)]. The cis-9,trans-11 CLA isomer is the principal dietary form of conjugated linoleic acid, comprises approximately 90% found primarily in beef and dairy products and the 10,12 isomer comprises the remaining 10% (Carvalho et al., 2010). Collectively, animal studies demonstrated dietary intake of a crude mixture of conjugated linoleic acid isomers prevents the development of obesity (Ostrowska et al., 1999). It is possible to theorize that conjugated linoleic acid could be used as a metabolic modifier in the poultry industry as there are many studies by Zhang et al. (2009a); Zhou (2008); Ramiah et al. (2014a), evidenced that conjugated linoleic acid is a new tool that can be used to reduce fatness in modern broiler chickens.

Most animals or human subjects have consumed a mixture of the two main CLA isomers: cis-9, trans-11 CLA and trans-10, cis-12 CLA (Larsen et al., 2003). Feitoza *et al* (2009) reported that trans-10, cis-12 CLA is the main isomer responsible for alterations in lipid profile and body composition. Nonetheless, 90% CLA in dairy products compromise of cis-9, trans-11 CLA (Feitoza *et al*., 2009). The body fat-lowering effect of CLA has also been reported inconsistent humans and less significant in animals (Riserus et al., 2001; Blankson et al., 2000). Physiological alterations induced by conjugated linoleic acid in relation to the gene expression depend on the animal species (Azain et al., 2000). Conjugated linoleic acid intake has been associated with numerous health benefits and inclusion of conjugated linoleic acid into animal feed improves the meat quality and provides nutritional values for human consumption. Therefore, the general objective of this study is to investigate effects of conjugated linoleic acid on the fat deposition in broiler chickens with the following hypothesis and objectives:

Hypothesis Statements

1. The effects of conjugated linoleic acid isomers on the morphological changes in adipose tissue and adipogenic gene expression on primary adipose tissue mainly related to trans-10, cis-12 CLA isomer.
2. Incorporation of conjugated linoleic acid will have an effect on the growth performance, fatty acid profile of meat and meat quality of broiler chicken.
3. Incorporation of conjugated linoleic acid will have an effect on adipose tissue cellularity and the regulation of adipogenic gene transcription.

Based on these hypotheses, the **SPECIFIC OBJECTIVES** of the present study were:

1. To assess the effects of conjugated linoleic acid isomers on the morphological changes in adipose tissue, and adipogenic genes expression on primary adipose tissue.
2. To investigate the effects of conjugated linoleic acid on the growth performance, fatty acid composition of breast muscle, lipid peroxidation, meat colour, and plasma lipids in broiler chicken.
3. To determine the possible effects of conjugated linoleic acid on the morphology of adipose tissue, the expression of PPARs and associated genes in broiler chickens.

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