THE EFFECT OF RICE BRAN OIL IN COMPARISON TO COCONUT OIL ON FAT ACCUMULATION IN 3T3-L1

BY

MISS JIERADA RABALERT

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MEDICAL SCIENCES

FACULTY OF MEDICINE

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was approved as partial fulfillment of the requirements for
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ABSTRACT

Obesity has become a major health problem worldwide. It is associated with the development of type 2 diabetes, coronary heart disease, and hypertension. Rice bran oil (RO) and coconut oil (CO) have been shown to reduce blood cholesterol, lipid profiles and obesity in both human and animal studies. CO is the richest source of medium-chain fatty acids (MCFAs) comprising about 50% of lauric acid (LaA). RO contains mainly oleic acid (OA) and linoleic acid (LA). However, the direct effects of the coconut oil and RO on lipid accumulation and adipogenesis *in vitro* have not been previously investigated. Thus, the objectives of this study were to investigate the anti-obesity effects of RO and CO on lipid accumulation and adipogenesis in 3T3-L1 cells.

The 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of edible oils i.e. RO and CO, and fatty acids i.e. OA, LA, LaA, and PA. The cells were cultured in differentiated media with and without various concentrations of the treatments for 4 and 8 days. After incubation, the cells were measured cell viability by MTS assay, adipogenesis and lipid accumulation by Oil red O staining, and the expressions of PPARγ and C/EBPα mRNA by real time-PCR method. The results demonstrated that OA, LaA, RO and CO treatments at all concentrations did not significantly affect cell viability. In contrast, the cells treated with PA (60 µg/mL) and LA (80, 100 µg/mL) for 8 days significantly reduced cell viability with more than

70% of surviving 3T3-L1 cells. However, PA (80, 100 µg/mL) and LaA (100 µg/mL) significantly reduced cell viability with less than 70% of surviving 3T3-L1 cells. The treatments of OA, LA, PA, LaA, RO, and CO inhibited adipogenesis and lipid accumulation in these cells as determined by decreased Oil red O staining. After 4 days of incubation, OA (20, 40, 60 µg/mL), LA (20, 40, 60 µg/mL), LaA (20 µg/mL), RO (100, 200, 300 µg/mL) significantly decreased lipid accumulation. After 8 days of incubation, OA (20 µg/mL), LA (20, 40 µg/mL), LaA (20, 40 µg/mL), RO (100, 200 µg/mL), CO (100, 200, 300 µg/mL) significantly decreased lipid accumulation. Moreover, the cells treated with LA (20, 40, 60 µg/mL), LaA (20, 40 µg/mL), CO (20, 40 µg/mL) for 8 days had significantly reduced adipocyte size. In gene expression, the treatments with LaA and CO for 4 and 8 days down-regulated PPARγ and C/EBPα while RO and LA treatments for 4 days suppressed only PPARγ. Moreover, OA (20, 40 µg/mL*)* for 8 days decreased the expression levels of *PPARγ* and *C/EBPα*, LA treatments (20, 40 µg/mL) for 4 days and (40 µg/mL) 8 days significantly decreased expression levels of *PPARγ*, and LA treatments (40 µg/mL) for 4 days and (20, 40 µg/mL) for 8 days significantly decreased expression levels of *C/EBPα.*

Therefore, LaA may be a bioactive compound in CO, as well as OA and LA may be bioactive compounds in RO for reducing adipogenesis and lipid accumulation. These properties of the fatty acids in CO and RO may be used to prevent obesity. However, further studies are required to explore more details of molecular mechanisms underlying the anti-obesity effects of CO, RO, and their bioactive compounds.

Keywords: 3T3-L1, Adipogenesis, *PPARγ*, *C/EBPα*, Rice bran oil, Coconut oil, Saturated fatty acids, Unsaturated fatty acids.

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Miss Jierada Rabalert

TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

TABLE OF CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
%	Percent
&	Ampersand
/	Per
<	Less than
=	Equal
>	More than
ATCC	American type culture collection
BMI	Body mass index
C/EBPα	Cytosine-cytosine-adenosine-adenosine-thymidine (CCAAT)/enhancer binding protein-alpha
cDNA	Complementary deoxyribonucleic acid
CO	Coconut oil
CO_2	Carbon dioxide
Conc.	Concentration
DEPC-treated water	Diethylpyrocarbonate-treated water
dH_2O	Distilled water
ddH_2O	Deionized water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
e.g.	For example
et al.	Et alii, and colleagues
etc.	Et cetera, and other things
EtOH	Ethanol
FBS	Fetal bovine serum
g	Gram

LIST OF ABBREVIATIONS (CONTINUED)

Symbols/Abbreviations	Terms
g/mL	Gram per milliliter
h	Hour
HDL-C	High-density lipoprotein-cholesterol
H_2O	Water
i.e.	Id est, that is
L	Liter
LA	Linoleic acid
LaA	Lauric acid
LCT	Long chain triglyceride
m	Meter
M	Molar or mole per liter
mg	Milligram
mg/L	Milligram per liter
mg/mL	Milligram per milliliter
min	Minute
mL	Milliliter
mM	Millimolar
MCFs	Medium chain fatty acids
MLCT	Medium and long chain triglyceride
MTS	3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MUFAs	Monounsaturated fatty acids
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide

LIST OF ABBREVIATIONS (CONTINUED)

Symbols/Abbreviations	Terms
µg/mL	Microgram per milliliter
U/mg protein	Unit per miligram protein
α	Alpha
β	Beta
δ	Delta
γ	Gamma

CHAPTER 1
INTRODUCTION

1.1 Introduction

The prevalence of obesity now affects more than 600 million obese people worldwide. The total number of people worldwide with type 2-diabetes was expected to increase from 171 million in 2000 to 366 million in 2030 (1). It is currently accepted that obesity is associated with increased risks of cardiovascular disease and type 2 diabetes. Obesity is characterized by excess adipose tissue mass that results from both elevated adipocyte number (hyperplasia) and elevated adipocyte size (hypertrophy). Adipogenesis is one mechanism proposed to explain the development of excess adipose tissue mass during obesity (2).

Adipogenesis is tightly regulated by adipogenic transcription factors such as peroxisome proliferator-activated receptor gamma (*PPARγ)* and cytosine-cytosine-adenosine-adenosine-thymidine (CCAAT)/enhancer binding protein-alpha (*C/EBPα*) (3). Both *PPARγ* and *C/EBPα* genes are up-regulated during adipogenesis and highly expressed in the adipose tissue. Therefore, the inhibition of *PPARγ* and *C/EBPα* expression in the adipocytes is considered to be an important protective mechanism for obesity. 3T3-L1 cells are preadipocytes from mice (4). These cells are usually used in a model to study adipose physiology and basic cellular mechanisms related to diabetes, obesity, and related disorders. Thus, 3T3-L1 cell line was chosen for the present study.

Fatty acids have multiple important functions as a component of biological membranes, energy sources, and signal transducer or precursors of lipid mediators and regulating gene expressions in fatty acids transport and metabolism. They are important metabolic factors associated with increased risk, such as plasma cholesterol, insulin resistance, abdominal obesity and triglycerides (5). Fatty acids are a factor in daily intake for maintaining an optimal diet. It can cause illness if improperly consumed. Fatty acids are divided into two major groups: unsaturated (UFA) and saturated (SFA). SFAs are primarily from animal products and vegetable oils such as palm and coconut (tropical oils). However, coconut oil contains high

concentrations of LaA (C12:0) which raise HDL levels, a decrease in LDL cholesterol, improve nutrient absorption and antimicrobial properties.

Monounsaturated fatty acids and polyunsaturated fatty acids improve insulin sensitivity and decrease triglyceride contents (6). Monounsaturated fats primarily come from plants like rice bran oil (RO), olive oil, canola oil, peanut oil and most nuts. Polyunsaturated fat reduces body fat in mice (7). It is found mostly in plant sources - like safflower, sunflower, soybean, corn, and cottonseed. RO reduced plasma LDL-cholesterol, cholesterol, and triglyceride levels when compared with sunflower oil in patients with hyperlipidemia (8). RO mostly contains with oleic acid (OA) and linoleic acid (LA).

All these findings suggest that the consumption of coconut oil, RO, and their bioactive fatty acids may improve lipid metabolism. However, the direct effects of virgin coconut oil (CO), RO and their bioactive fatty acids on adipogenesis and adipocytes lipid accumulation in *vitro* have not been previously elucidated. Thus, in the present study, 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of OA, LA, palmitic acid (PA), LaA, RO, and CO.

1.2 Objectives

The aim of the study was to examine the anti-adipogenic effects of fatty acids and essential oils and the underlying mechanisms using cultured 3T3-L1 adipocytes. The specific objectives of the study were as follow:

1.2.1 To determine the cytotoxicity of fatty acids and edible oils in 3T3-L1 adipocytes.

1.2.2 To determine the effect of fatty acids and edible oils on lipid accumulation as Oil red O in the 3T3-L1 adipocyte.

1.2.3 To determine the effect of fatty acids and edible oils on adipocyte size as Oil red O in the 3T3-L1 adipocyte.

1.2.4 To determine the effect of fatty acids and edible oils on the mRNA expression of nuclear receptor peroxisome proliferator-activated receptor grammar (*PPARγ*) and CCAAT/enhancer binding protein alpha (*C/EBPα*) related to adipogenesis.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Definition of obesity

Obesity is defined as abnormal or extensive fat accumulation which negatively affects health (9). It comes from complex interplays of genetic, psychological, and environmental factors. However, an increase of fat accumulation from energy intake occurs when intake exceeds energy expenditure. The assessment of obesity by measuring body fat can be determined in different ways; skinfold measurement, waist circumference and body mass index which are according to the World Health Organization (10). Body mass index (BMI) is a calculation of weight in relation to height that is commonly used to classify overweight and obesity in adults (11). Overweight is here defined as a BMI of 25-29.9 kg/m^2 and obesity as a BMI $\geq$ 30 kg/m^2. Increasing of BMI is generally associated with insulin resistance that increases risks of heart disease and metabolic disease (12).

2.2 Adipocytes and its role in obesity

It is well-established that adipocyte is a central cell in the homeostatic control of energy metabolism. Adipocytes are the major cellular components of adipose tissue. They are divided into two types: white adipocytes and brown adipocytes. White adipocytes store energy (e.g., triglycerides). White fat is widely distributed and it represents the primary site of fat metabolism and storage, whereas brown adipocytes consume energy (13, 14).

Obesity is a chronic metabolic disorder caused by an imbalance between energy intake and energy expenditure (15), which leads to the pathological growth of adipocytes. Although knowledge and role of genetic factors in obesity is elevating, the fast increase in the prevalence of obesity throughout the world indicates that environmental factors such as high-fat diet, high carbohydrate diet, and physical inactivity are the major contributors to this epidemic (16, 17). Disruption of this

balance can potentially increase fat deposits in adipose tissue, especially abdominal fat accumulation and non-adipose tissue (ectopic fat accumulation) (18). Obesity is strongly associated with the formation of adipocyte and the mass of adipose tissue in the body. The growth of adipose tissue can result from hypertrophy and/or hyperplasia of cells which leads to increase in cell size and fat cell number (18, 19).

The adipose tissues are not only concerned with energy storage but are also endocrine organs that produce and secrete multiple bioactive substances, known as adipokines, that have regulatory activities of metabolic homeostasis and inflammatory response in our bodies such as free fatty acids, lipid metabolisms, immune functions and blood coagulability, and may serve as blood markers of cardiometabolic risk (13, 14).

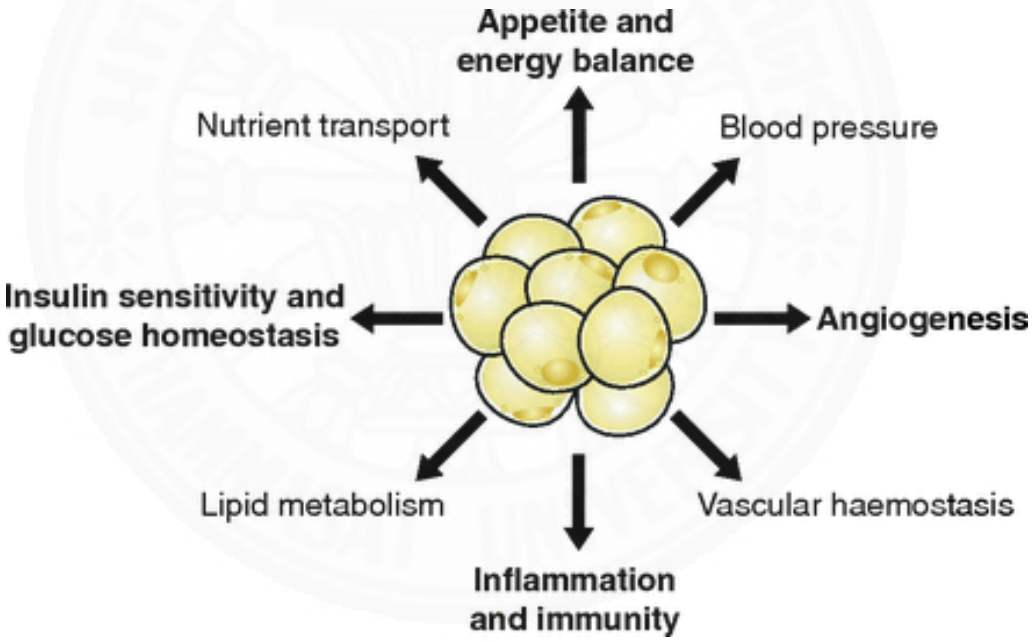


Figure 2.1 Illustration of the major physiological and metabolic processes which adipose tissue is involved in the secretion of adipokines from adipocytes. The interactions may be autocrine, paracrine, or endocrine (20).

The importance of adipose tissue in controlling whole-body metabolism, lack of adipose tissue associated with insulin resistance mice and humans (21-23). Insulin resistance is defined as the decreased ability of tissues to respond to insulin

action. Obesity poses a major risk for serious diet-related chronic diseases, including type 2-diabetes, cardiovascular diseases, hypertension, insulin resistance and stroke (24, 25). Insulin promotes adipocyte triglyceride stores by a number of mechanisms, including differentiation of preadipocytes to adipocytes and, in mature adipocytes, stimulating glucose transport and triglyceride synthesis (26). Accumulation of adipose tissue, as in obesity, is associated with insulin resistance.

Table 2.1 Types of adipocyte (27).

Characteristics	 **White adipocyte**	 **Brown adipocyte**
Origins	Mesenchymal/pericyte origins	Myogenic lineage Trans-differentiation from white adipocyte
Locations	Visceral-epicardial & intermuscular regions	Cervical-supraclavicular-perirenal & paravertebral regions
Structure	Larger cells	Smaller cell
	Unilocular lipid droplet	Multilocular lipid droplets
	Few mitochondrial	Many mitochondria
	Peripheral nucleus	Central nucleus
Functions	Store lipid (TG) & release FA	Dissipate energy
	Produce adipokines	Produce heat
Association with obesity	Abdominal region	Decrease number
	1. Increase size	
	2. Increase number	

2.3 Adipogenesis

Adipogenesis by definition involves the recruitment and differentiation of preadipocytes to mature adipocytes. It has two phases which are determination phase and the termination phase. Determination promotes the transformation of the stem cell to preadipocyte. Adipocyte cannot be illustrious morphologically from its precursor cell and has to lose the potential to differentiate into other cell types. The terminal differentiation phase of differentiation is the preadipocyte transformed into adipocyte cells (28). Adipocyte differentiation and adipogenesis come from in vitro studies of fibroblasts and preadipocytes (29). Adipose tissues, like muscle and bone, are generally regarded as having mesodermal origins. The adipose tissues are believed to arise from the mesenchymal stem cells when to give rise to common early precursors (adipoblast). Mesenchymal stem cells (MSCs) are usually going to differentiate into multiple cell lineages that resemble adipocytes, osteoblasts, chondrocytes, myoblasts, and connective tissues (18). Adipogenesis occurs under the regulation of transcription factors, such as cytosine-cytosine-adenosine-adenosine-thymidine (CCAAT)/ enhancer-binding proteins (*C/EBPs*) and peroxisome proliferator-activated receptor γ (*PPARγ*). These play key roles in the complex transcriptional cascade during adipocyte differentiation (3, 30).

In general, adipogenesis is a well-established multistep process that requires the sequential stimulation of numerous adipogenic transcription factors, including cytosine-cytosine-adenosine-adenosine-thymidine (CCAAT)/ enhancer-binding proteins (*C/EBPs*) and peroxisome proliferator-activated receptors. *C/EBPs*, consisting of *C/EBPβ*, *C/EBPδ*, and *C/EBPα*, are transcription factors that regulate genes involved in adipocyte differentiation. *C/EBPs* are generally classified as pro-adipogenic transcription factors which are not adipocyte-specific. Although *C/EBPs* are not adipocyte-specific, they are expressed before the transcription of most adipocyte-specific genes starts (31).

C/EBPβ and *C/EBPδ* are early expression stage, followed by increasing of *PPARγ* and *C/EBPα*. have been shown to play important roles in regulating adipose tissue development in mice and preadipocyte differentiation in vitro (32). *C/EBPα* is

important in adipogenesis when expressed late. These suggest that increasing in *C/EBPβ* above a certain level induces the expression of *PPARγ*.

PPARγ is a member of the nuclear receptor superfamily of ligand-activated transcription factors. It is necessary for adipocyte differentiation of white and brown adipocytes (33). *PPARγ* is a master regulator of adipogenesis in *vitro* and in *vivo* studies (34). *PPARγ* also appears to be required for maintenance of the terminally differentiated state of the adipocyte. It plays a major role in adipogenesis-associated with lipid homeostasis. And it is necessary and sufficient for adipocyte differentiation (3, 31, 35). The ligand activation of *PPARγ* with *C/EBPα* leads to full adipocyte differentiation (36).

The mature adipocyte is characterized by a large internal fat droplet, which enlarged the cell so that the cytoplasm. The cytoplasm is compacted to a thin layer surrounding the lipid droplet while the nucleus is displaced to the outer edge of the cells, which change into a spherical shape, accumulate lipid droplets and acquire the morphological and biochemical characteristics of the mature white adipocyte.

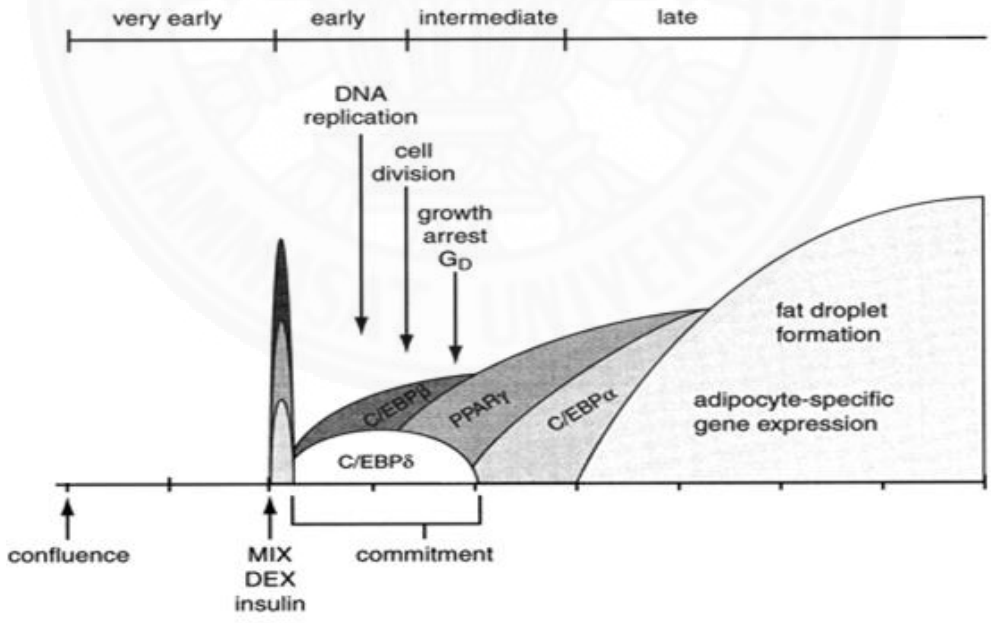


Figure 2.2 Major identified events of preadipocyte differentiation by isobutylmethylxanthine (IBMX or MIX), dexamethasone (DEX) and Insulin (Modified from Avram *et al*, 2007 (37).

Although the pathogenesis of obesity is complex, the increased adipogenesis is one of the important pathomechanisms for the development of obesity (2). Abnormal adipogenesis is believed to be a consequence of impairment of transcriptional regulation of the key adipogenic transcription factors (38). The previous study has reported that HF feeding induced the expression of *PPARγ* and *C/EBPα* in the adipocytes of rats, thereby increasing the adipocyte differentiation and lipid storage (39). These results support a pathogenic role for the activation of adipogenesis in obesity. Thus, the inhibition of adipogenesis may have the potential to treat obesity and related metabolic diseases as shown in **Figure 2.3**.

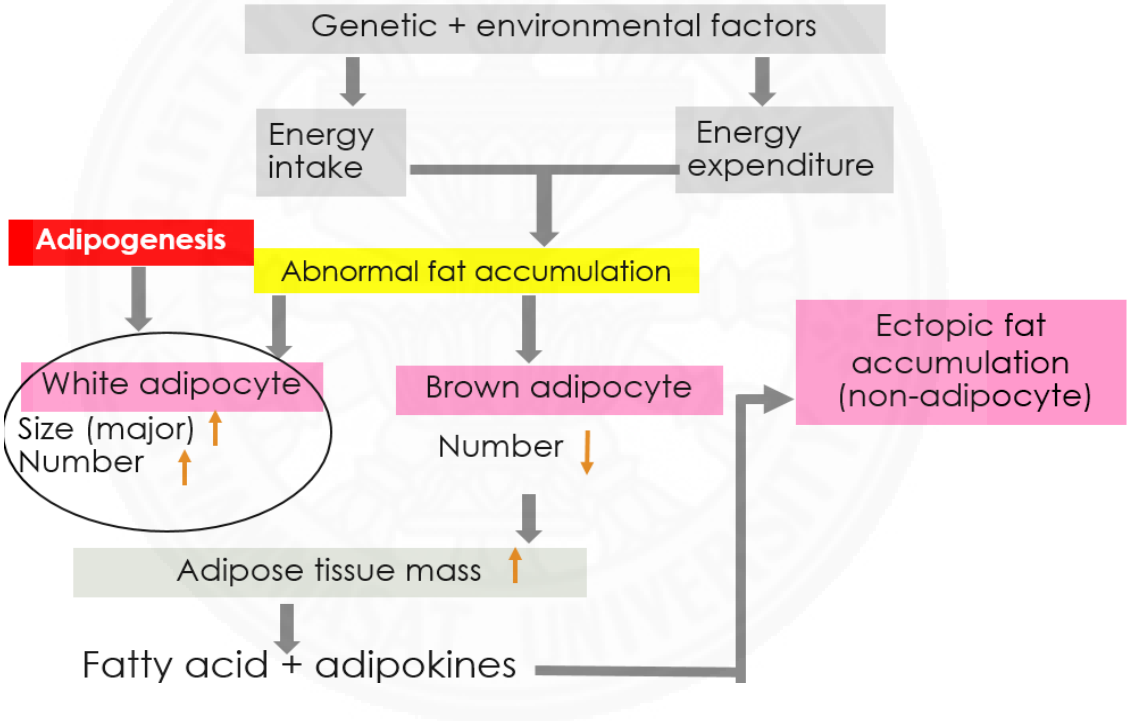


Figure 2.3 Adipocytes and obesity**.**

2.4 The 3T3-L1 Cell Line

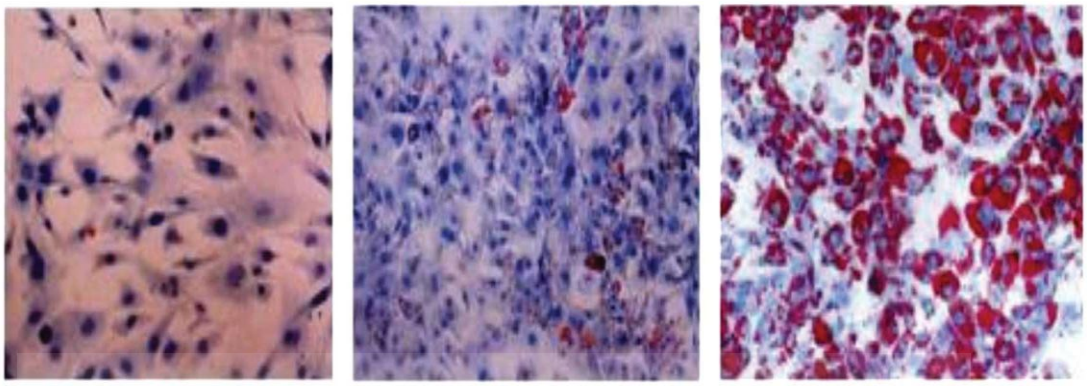
For many years, the cellular and molecular mechanisms of adipocyte differentiation have been extensively studied using preadipocyte culture systems. The 3t3-L1 cell line is the first and most commonly characterized in vitro model established to study adipogenesis. The 3T3-L1 is a preadipose cell line which was

originated from disaggregated 17 to 19 days the 3T3-Swiss albino mouse embryos (40, 41).

The 3T3-L1 cells propagate under normal conditions having a fiblastic phenotype. Several investigations use 3T3-L1 cells because it helps in identifying key molecular markers including transcription factors and various pathways during preadipocyte differentiation (42). The 3T3-L1 cell can be differentiated from fibroblast into complete adipocytes using adipogenesis by inducing media. Adipogenic cocktail refers to a mixture of inducing agents used in cell culture models for adipocytes differentiation. Numerous protocols can be used chemical inducing preadipocyte to adipocyte by hormone cocktail including methylxanthine, dexamethasone and insulin (MDI) (43) in the presence of serum. The role of methylxanthine or isobutylmethylxanthine in adipocyte differentiation at a nuclear level that increases the expression of *C/EBPβ* in early differentiation and induces the expression of *PPARγ* and *C/EBPα*. Isobutylmethylxanthine enhances that increases intracellular levels of cAMP (31, 44), a physiological activator of protein kinase A (45). Dexamethasone is known to induce adipogenesis by the formation of lipid clusters (46, 47). Preadipocytes contain less amount of lipid droplets accumulated, but four days after induction they start to accumulate lipids that grow in size and number over the differentiation time (48).

The morphologically-fibroblast-like preadipocyte undergoes conversion into round adipocytes accumulation fat by an inducer, MDI (3-isobutyl-1-methylxanthine, dexamethasone and insulin). The differentiation in 4 days, adipocyte cells accumulated small lipid droplet in the cytoplasm as shown by Oil red O staining. After incubation for 7 days, more than 90% of the cells showed physiological characteristics of mature adipocytes with a large number of lipid droplets accumulated in the cytoplasm. As shown in **Figure 2.4.**

The 3T3-L1 was performed as described to understand the basic cellular mechanisms associated with diabetes, obesity, and related disorders; therefore, an important physiological process whose function or dysfunction may prevent or promote metabolic disease.



Preadipocytes 4 days post-MDI 7 days post-MDI

Figure 2.4 The differentiation of 3T3-L1 adipocyte. 3T3-L1 preadipocytes were induced to differentiate into mature adipocytes with MDI (3-isobutyl-1-methylxanthine, dexamethasone and insulin). 3T3-L1 cells on various days of differentiation were stained with Oil red O (49).

2.5 Dietary fatty acids and edible oils for health

2.5.1 Fatty acids

Macronutrients from the animal are nutrients needed in large amounts. There are three macronutrients; carbohydrates, proteins, and lipids that are essential for regulation of body function including survival, growth and maintaining health. Lipids are mostly composed of carbon, hydrogen and oxygen. The main types of lipids include monoglycerides, diglycerides, triglycerides, phospholipids, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K) and others. The main biological functions of lipids include storing energy, signaling, and acting as structural components of cell membranes (50). Triacylglycerols are the body's major energy store and also the major form of dietary fat (94%). They are composed of glycerol and three fatty acids. Other forms of dietary fat are cholesterol (1%) and phospholipids (5%).

Non-esterified fatty acids or free fatty acids are circulating in plasma bound to albumin and are released in adipose tissue lipolysis. Triacylglycerol and cholesterol are transported in the plasma associated with various lipoprotein particles.

They are separated by hydrated density; electrophoretic mobility; size; and their relative content of cholesterol, triglycerides, and protein into five main classes: chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL).

Dietary kinds of fats and how different kinds of fats can be used in the food processing industry are associated with metabolic syndrome such as dyslipidemia, hypertension, cardiovascular, type 2 diabetes mellitus. Fats are triglycerides, which are composed of one glycerol and three fatty acid molecules. Glycerol is a polyhydric alcohol containing three carbon atoms, each of which has a hydroxyl (-OH) group as shown in **Figure 2.5**.

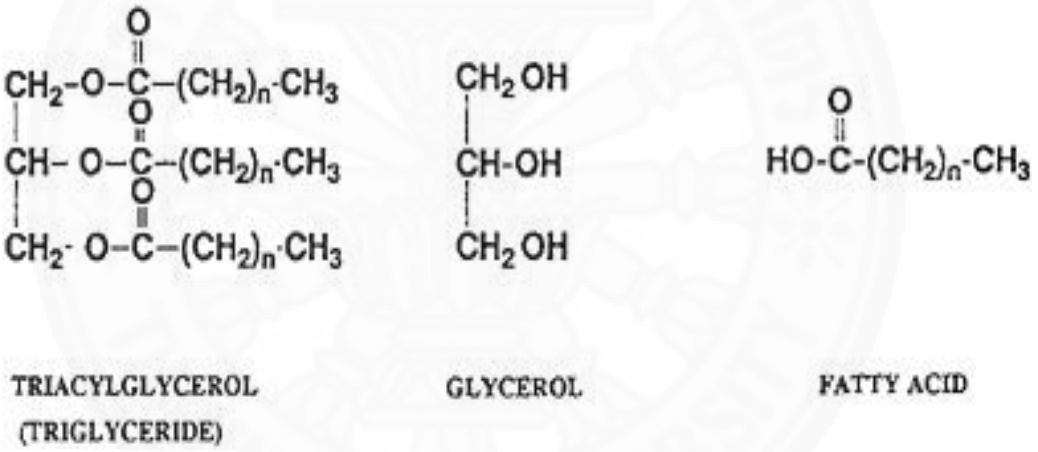


Figure 2.5 Structure of a typical long-chain triacylglycerol fatty acid.
(modified from http://www.nap.edu/read/4563/chapter/24#353)

Fat is an essential component of the normal human diet. Fatty acids are an organic acid usually containing between 4-24 carbon atoms. The fatty acids are divided two classes; SFAs and UFAs depend on a number of double bonds and length (**Table 2.2**). The Saturated fatty acids (SFAs) contain only carbon-carbon single bonds in their chain (C-C) whereas monounsaturated fatty acids (MUFAs) have one (C=C)**,** and polyunsaturated fatty acids (PUFA) contain two or more double bonds.

SFAs are mostly solid at room temperature. The source of fat in pork, lard, lamb, poultry skin, butterfat, and egg yolk are mostly saturated plant fats with a high content of SFA include tropical oils like coconut oil, palm kernel oil, cocoa butter, and palm oil. However, coconut oil is nature's richest source of medium chain fatty acids, particularly LaA (45-52%) as shown in **Table 2.3**. Foods that contain more saturated fat are usually solid at room temperature and are sometimes called "solid" fat.

They are mostly solid at room temperatures such as LaA (C12:0), myristic acid (C14:0) and PA (C16:0). They have importance structural properties and useful source of energy. The research has shown that higher intake of most dietary SFAs is associated with higher levels of blood total cholesterol and low-density lipoprotein (LDL) in humans and animal models (51). The consumption of saturated fat has been shown to increase LDL-C cholesterol and consequently has been associated with increased risk of cardiovascular disease (52, 53). Increasing PUFA consumption as a replacement for SFA reduces coronary heart disease (54).

SFAs, particularly PA, which in the diet and in adipose tissue, negatively correlated with insulin sensitivity has been associated with higher risk of coronary heart disease (55). PA reduce insulin-stimulated glucose transport in muscle from obese (56). Similar, Palm oil (PO) enriched diet, high PA content induces impaired glucose tolerance and increase triacylglycerols, are associated with insulin resistance in mice (57) and mitochondrial dysfunction. More research has shown that trans fatty acids intake increase LDL-cholesterol and decreased HDL-cholesterol (58). However, trans fat was associated with chronic disease than saturated fat (59). Consumption of trans fat and butter was positively associated with an increased risk of metabolic syndrome (60).

The common MUFAs acids are eicosenoic acid 20:1 (n-9), palmitoleic acid (16:1, cis-vaccenic acid (18:1 n−7), Mead acid 20:3 (n-9), erucic acid 22:1 (n-9), Nervonic acid 24:1 (n-9) and OA (18:1 n−9). The source of MUFAs includes olive, avocado, peanut, corn oil whole grain wheat, cereal, sunflower, and rice band. PUFAs containing more than one double bond in the hydrocarbon chain include soybean, corn, and cottonseed oils. Dietary high-MUFA reduce

plasma triglycerides and LDL-cholesterol. MUFAs, as oleic acid, improve lipid profile (61) and prevent mitochondrial dysfunction, insulin resistance and inflammatory signaling in neuronal cells (62). Unsaturated fatty acids improve insulin sensitivity in diabetic patients (63) and impairing cardiovascular parameters (64). Diet rich in MUFAs, from high oleic sunflower oil and olive oil, there was a significant increase in plasma high-density lipoprotein (HDL) cholesterol. Oleic acid and peanut oil high in oleic acid reverse the inhibitory effect of insulin production of the inflammatory cytokine TNF-α both *in vitro* and *in vivo* systems (65).

PUFAs containing more than one double bond in the hydrocarbon chain. PUFAs can be classified in various groups by their chemical structure: methylene-interrupted polyenes conjugated fatty acids and other PUFAs. The omega-3; linolenic acid (18:3 n-3), stearidonic acid (18:4 n-3), eicosapentaenoic acid (20:5 n-3) and/or docosahexaenoic acid **(**22:6 n-3**)**. The omega-6 fatty acids; linoleic acid (18:2 n-6) is the most common polyunsaturated fatty acids, γ-linolenic (18:3 n-6), dihomo-gamma-linolenic (20:3 n-6) and arachidonic acid (20:4 n-6). The source of PUFAs includes sunflower, soybean, walnuts, corn, cottonseed oils, canola oil, sesame seeds, chia seeds and in fatty fish. A diet enriched in PUFAs, in particular of the n-3 family, decreases adipose tissue mass and suppresses the development of obesity in rodents (66). Dietary unsaturated fats and polyunsaturated fats in place of animal fats reduces in total and low-density lipoprotein (LDL) cholesterol that the result was a reduction in the risk of coronary artery disease and heart disease (67, 68). However, saturated fats can be sub-classified into the short chain, medium chain, and long chain fats. MUFAs and PUFAs all long chain fats. Short-chain fatty acids (SCFA) are considered to have a chain length fewer than 6 carbon atoms, medium-chain fatty acids (MCFA) have 6–12 carbons, and long-chain fatty acids (LCFA) generally have 13 more carbon chains.

MCTs are easily absorbed by the body then transported directly to the liver and used for energy. The three MCTs; capric acid, caprylic acid and lauric acid are naturally found in a variety of animal and vegetable fats. The study has found that dietary medium-chain triglyceride (MCT) was shown to better decrease weight, body fat and blood triglyceride to better than long chain triglyceride in Chinese hypertriglyceridemic subjects (69).

Table 2.2 Main fatty acids in the diet
(modified from http://www.efsa.europa.eu/en/efsajournal/pub/1461)

Systematic nomenclature	Common name	Abbreviation
Saturated fatty acids (SFA)		
Butanoic acid	Butyric acid	4:0
Hexanoic acid	Caproic acid	6:0
Octanoic acid	Caprylic acid	8:0
Decanoic acid	Capric acid	10:0
Dodecanoic acid	Lauric acid	12:0
Tetradecanoic acid	Myristic acid	14:0
Pentadecanoic acid	Pentadecilic acid	15:0
Hexadecanoic acid	Palmitic acid	16:0
Heptadecanoic acid	Margaric acid	17:0
Octadecanoic acid	Stearic acid	18:0
Cis-monounsaturated fatty acids (cis-MUFA)		
Hexadecenoic acid	Palmitoleic acid	16:1Δ9c
Octadecenoic acid	Oleic acid	18:1Δ9c (n-9; ω9)
Eicosenoic acid	Gadoleic acid	20:1Δ9c
Trans-monounsaturated fatty acids (trans-MUFA)		
Octadecenoic acid	Elaidic acid	18:1Δ9t (n-9; ω9)
Octadecenoic acid	Trans-vaccenic acid	18:1Δ11t (n-7; ω7)
Polyunsaturated fatty acids (n-6 PUFA)		
Octadecadienoic acid.	Linoleic acid	18:2Δ9c,12c (n-6; ω6)
Eicosatetraenoic acid	Arachidonic acid	20:4Δ5c,8c,11c,14c (n-6; ω6)

Systematic nomenclature	Common name	Abbreviation
n-3 Polyunsaturated fatty acids (n-3 PUFA)		
Octadecatrienoic acid	α-Linolenic	18:3Δ9c,12c,15c (n-3; ω3)
Eicosapentaenoic acid	EPA	20:5Δ5c,8c,11c,14c,17c (n-3; ω3)
Docosapentaenoic acid	DPA	22:5Δ7c,10c,13c,16c,19c (n-3; ω3)
Docosahexaenoic acid	DHA	22:6Δ4c,7c,10c,13c,16c,19c (n-3; ω3)
Octadecadienoic acid		
Octadecadienoic acid	Rumenic acid	18:2Δ9c,11t 18:2Δ10t,12c

2.5.2 Edible oils

Rice bran oil (RO)

RO is the oil extracted from the germ and inner husk of rice milling plant. It is most commonly used in Asian cultures, including Japan and China, primarily because rice is already such a staple food in their cuisines. Rice bran enters preparation process to separate impurities, heated to reduce the moisture and undergoes the hexane solvent extraction process. The solvent will be completely removed from the crude oil before the oil enters refinery plant. The refining process begins by reducing excess free fatty acids; using Alfa-Leval separators, bleaching, deodorizing, dewaxing removed from crude rice bran oil with using proprietary techniques (**Figure 2.6**).

It has a mostly composition with unsaturated fatty acid; OA (43.3%), LA (30%) and SFAs; PA (19.20%) in **Table 2.5**. The major components of RO include γ-oryzanol, tocopherols, phytosterols (β-sitosterol), tocotrienols and triterpene alcohols, can attenuate plasma lipid and lipoprotein profiles of rodents, rabbits and humans (70). They found that rice bran oil reduced blood levels of total cholesterol and triglyceride. Ro diet reduces serum cholesterol, triglycerides and increases HDL-

cholesterol in animal (71). Treatment with RO also significantly reduced the levels of blood total- cholesterol, LDL-cholesterol and triglyceride when compared with sunflower oil in patients with hyperlipidemia (8). Moreover, consumption RO decreased the level of serum total cholesterol and tended to decrease LDL-cholesterol in patients with type 2 diabetes (72).

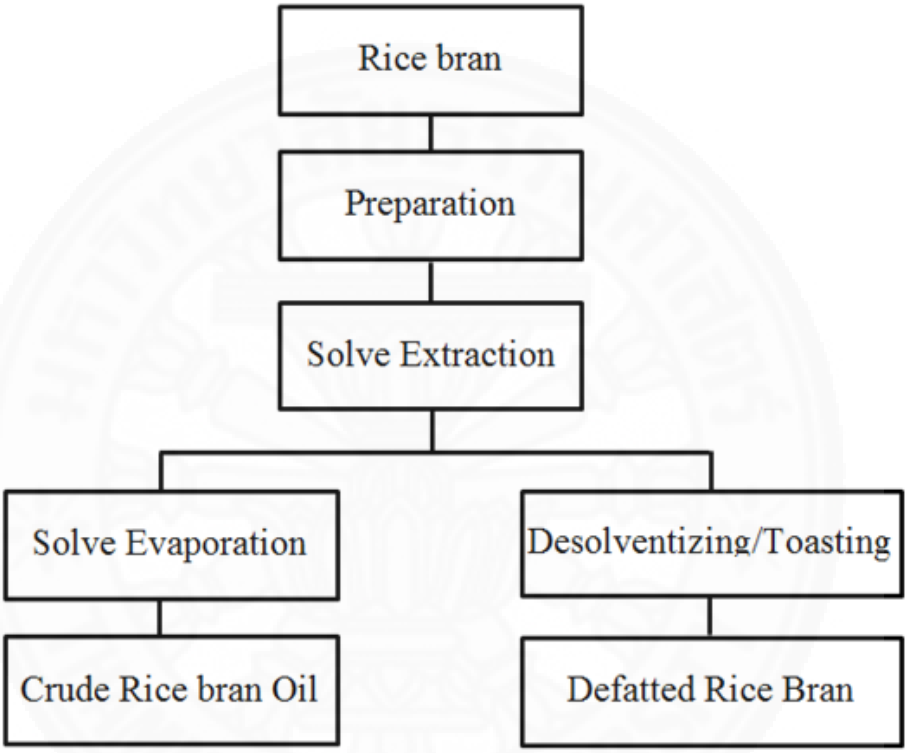


Figure 2.6 RO extraction and physical refining process
(from http://www.surinbranoil.com)

Coconut oil (CO)

CO is increasing especially in South East Asia. It is believed to be a functional food. The oil is rich in medium-chain fatty acids (MCFA), lauric acid (LaA) which is present at approximately 45–53% as shown in **Table 2.5**. LaA can convert into a monoglyceride called monolaurin which is found in breast milk.

Monolaurin has a greater antiviral (73), antibacterial (74) and immune response functions (75).

CO that has been refining bleaching and deodorizing (RBD) processes. The RBD process, high heat used to deodorize the coconut oil to remove its distinctive smell and flavor. CO reduced total cholesterol, triglycerides, total and LDL cholesterol, increases HDL cholesterol in serum in experimental animals (76). It also led to decreased lipid deposition in the liver and heart of animals. Consistently, the CO intake has been reported to reduce waist circumference in obese men. This study has also proposed that it is safe to use in humans (77). Moreover, dietary medium and long chain triglyceride (MLCT) was shown better to decrease body fat than long chain triglyceride (LCT) in Chinese hypertriglyceridemic subjects (69). Coconut milk is a natural oil-in-water emulsion extracted from the endosperm of a mature coconut. Coconut milk components a complex biological fluid, fat, carbohydrate, starch and minerals like phosphorus, calcium and potassium as shown in **Table 2.3**.

Table 2.3 Chemical properties of coconut milk

Chemical properties of coconut milk	**Percentage**
Fat	39.77
Water	50.00
Starch	0.09
Protein	2.78
Sugar	2.99
Total solid	10.38
Ash	1.22

The major component of coconut milk is medium-chain triglycerides (MCTs). The consumption of a diet rich in MCTs results in greater loss of adipose tissue compared with long-chain fatty acids (78). Moreover, coconut milk beneficial against metabolic syndrome, which is a major factor in promoting heart disease. Coconut milk is very high in calories. It provides 230 kcal in a 100 ml (gram) portion or 552 kcal/cup, should be consumed in small amounts. Coconut oil has about 862

kcal /100 gram as shown in Table 2.4 (USDA National Nutrient Database for Standard Reference).

Table 2.4 Calories in coconut
(modified from USDA National Nutrient Database for Standard Reference)

Serving size	**Calories per serving**	
	Calories in coconut milk (kilocalories)	**Calories in coconut oil (kilocalories)**
100 g	230	862
1 cup	552 (240 g)	1945 (218 g)
1 tablespoon	34 (15 g)	39 (4.2 g)

Recently, the coconut oil produced by the wet method is known as virgin coconut oil (VCO). VCO is the naturally processed without using heat and without undergoing chemical refining or high heat, may be extracted from fresh meat, coconut milk (79). The cold pressed VCO manufacturer with centrifuge process is able to maintain and preserve all the necessary substances superbly. VCO contained higher total phenolic content, antioxidant compared to refined coconut oil (80, 81). The quality characteristics with CO (Thai pure coconut, Thailand) are clear and transparent like pure water, low viscosity, and no rancidity nor sour-smelling in **Figure 2.7**.

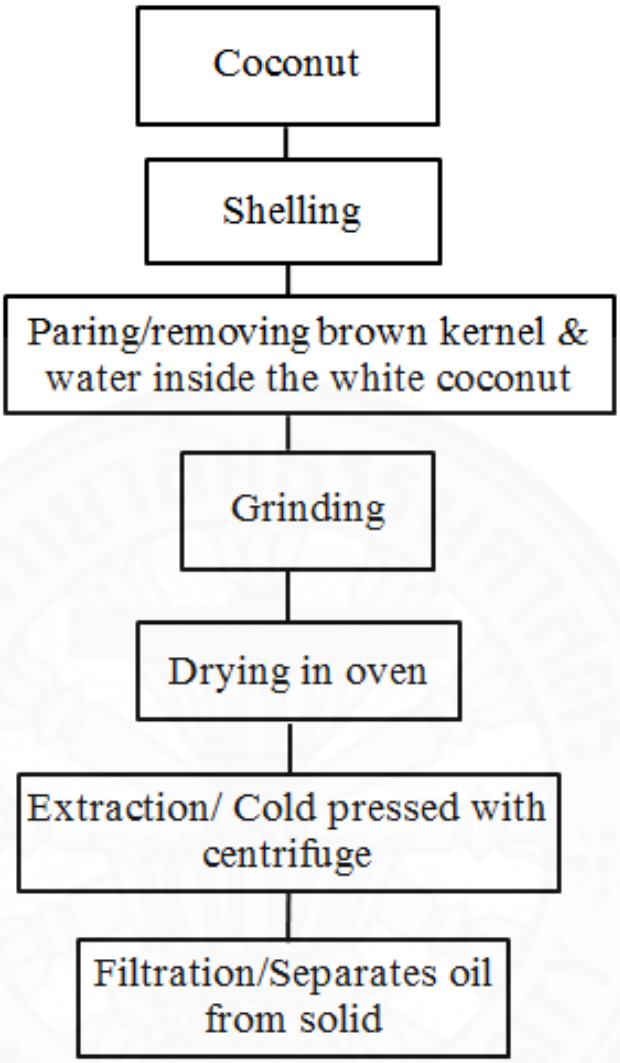


Figure 2.7 Cold pressed CO manufacturer with centrifuge process (from www.coconut-virgin.com/en/home)

Table 2.5 Composition of rice bran oil (table was from Surin bran oil, Thailand) and coconut oil (received from Thai pure coconut, Thailand)

Name of fatty acids	RO Percentage	CO Percentage
Palmitoleic acid (C16:1)	0.2	-
Oleic acid (C18:1, *cisΔ9*)	43.3	-
Linoleic acid (C18:1, cisΔ11)	0.90	0.13
Linoleic acid (C18:2)	30.0	-
Linoleic acid (C18:2 trans)	0.50	0.77
α-Linoleic acid (C18:3)	0.80	-
α-Linoleic acid (C18:3 trans)	0.50	-
Paullinic acid (C20:1, cisΔ11)	0.50	-
Docosapentaenoic acid (C22:5n-3)	0.20	-
Carpoic acid (C6:0)	-	0.41
Octanoic acid (C8:0)	-	6.85
Decanoic acid (C10:0)	-	6.06
Lauric acid (C12:0)	-	49.30
Myristic acid (C14:0)	0.50	-
Palmictic acid (C16:0)	19.20	8.76
Stearic acid (C18:0)	2.00	3.37
Arachidonic acid (C20:0)	0.90	0.07
Lignoceric acid (C24:0)	0.30	-
Moisture and volatile matter	-	0.06

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Chemicals and Reagents

3.1.1 Oil red O staining assay

2-propanol (isopropanol)	(Sigma-Aldrich, USA)
Oil red O	(Sigma-Aldrich, USA)
Formalin (10%)	(International Resin, China)

3.1.2 MTS assay

Cell Titer 96_Aqueous One Solution Cell Proliferation Assay	(Promega, USA)

3.1.3 Fatty acids and edible oils

RO	(Surin bran oil, Thailand)
CO	(Thai pure coconut, Thailand)
OA	(Sigma-Aldrich, USA)
LA	(Sigma-Aldrich, USA)
PA	(Sigma-Aldrich, USA)
LaA	(Promega, USA)

3.1.4 The cell culture

Calf serum (CS)	(Gibco BRL, USA)
Dexamethasone	(Sigma-Aldrich, USA)
Dimethyl sulfoxide, ($(CH_3)_2SO$) (DMSO)	(Sigma-Aldrich, USA)
Dulbecco's modified Eagle's medium (DMEM)	(Gibco BRL, USA)
Fetal bovine serum (FBS)	(Gibco BRL, USA)
Insulin	(Sigma-Aldrich, USA)
Isobutylxanthine (IBMX)	(Sigma-Aldrich, USA)
Sodium Pyruvate	(Gibco BRL, USA)

Penicillin-streptomycin	(Gibco BRL, USA)
Phosphate-buffered saline (PBS)	(Gibco BRL, USA)
Sodium bicarbonate, ($NaHCO_3$)	(Gibco BRL, USA)
Trypan blue stain 0.4%	(Gibco BRL, USA)
Trypsin-EDTA	(Biochrom, Germany)

3.1.5 Determination of target messenger RNA expression

3.1.5.1 RNA isolation

2-propanol (isopropanol)	(Sigma-Aldrich, USA)
Chloroform	(Lab-Scan Analytical Science, Thailand)
Diethylpyrocarbonate (DEPC)-treated water	(Ambion, USA)
Ethanol (EtOH), Analytical grade	(Merck, Germany)
TRIzol reagent	(Invitrogen, USA)

3.1.5.2 Complementary deoxyribonucleic acid synthesis

High Capacity cDNA Reverse transcription Kits	(Applied Biosystems, USA)

3.1.5.3 Quantitative real-time PCR

TaqMan Gene Expression Master Mix	(Applied Biosystems, USA)
TaqMan Gene Expression Assays	(Applied Biosystems, USA)
C/EBP-α	(Assay ID Mm00514283_s1)
PPAR-γ	(Assay ID Mm01184322_m1)
TaqMan® Gene Expression Master Mix	(Applied Biosystems, USA)
TaqMan® Gene Expression Assay	(Applied Biosystems, USA)
GAPDH	(Assay IDMm99999915_g1)

3.2 Instruments

25 cm^2 plastic tissue culture flasks	(Costar Corning, USA)
75 cm^2 plastic tissue culture flasks	(Costar Corning, USA)
96-well plate flat, bottom without lid	(Costar Corning, USA)

Autoclave	(Hirayama, Japan)
Auto pipettes	(Rainin Instrument LLc, USA)
Centrifugation	(Beckham, USA)
Centrifuge tube	(Costar Corning, USA)
CO_2 humidified incubator	(Thermo Scientific, USA)
Disposable pipette	(Costar Corning, USA)
Cryogenic tube 2 mL	(Costar Corning, USA)
Filter paper	(Whatman, USA)
Filter unit (0.22 µm, radio-sterilized)	(Millipore, Ireland)
Freezer	(Sanyo, Japan)
Haemocytometer	(Boeco, Germany)
Hotplate and stirrer	(Sterillin, France)
Inverted microscope	(Nikon, USA)
Inverted microscope	(Zeiss LSM, German)
Laminar air flow	(Boss tech, Thailand)
Liquid nitrogen tank	(Taylor-Wharton, USA)
Magnetic stirrer	(Ika Werke, Germany)
Membrane filters with pore size ratings of 0.22 microns	(Satorius, Germany)
Micropipettes 1-20 µL, 20-200 µL, 100-1,000 µL	(Gilson, USA)
Microcentrifuge machine	(Denville Scientific, USA)
Microcentrifuge machine	(Bertec Enterprise,Taiwan)
Microcentrifuge tube	(Costar Corning, USA)
Microplate reader	(Biotek, USA)
MicroAmp™ Optical 96-Well Reaction Plate	(Applied Biosystems, USA)
MicroAmp™ Optical Adhesive Film	(Applied Biosystems, USA)
Multi-channels pipette	(Costar Corning, USA)
MyCycler™ thermal cycler	(Bio-Rad, USA)
NanoDrop 2000 spectrophotometer	(ThermoScientific, USA)
pH meter	(ThermoScientific, USA)
Paper dish (0.6 cm diameter)	(Costar Corning, USA)

Pipette tips	(Costar Corning, USA)
Pipetteboy	(Integra biosciences, Switzerland)
Spectrophotometer	(Shimadzu, Japan)
Step One Plus™ Real-Time PCR System	(Applied Biosystems, USA)
Syring	(Nipro, Thailand)
Syring filters	(Nipro, Thailand)
Vortex mixer	(Scientific industries,USA)
Water bath	(Memmert, Germany)

3.3 Methods

3.3.1 Evaluation of cell growth using the MTS assay

In this assay, cell viability of 3T3-L1 preadipocytes were assessed by MTS as 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium, inner salt (82, 83). The MTS molecule is bioreduced into a soluble formazan in the cell culture medium by mitochondrial dehydrogenase enzyme of living cells (83).

This conversion is presumably accomplished through NADPH or NADH is produced by dehydrogenase enzymes in metabolically active cells (82).

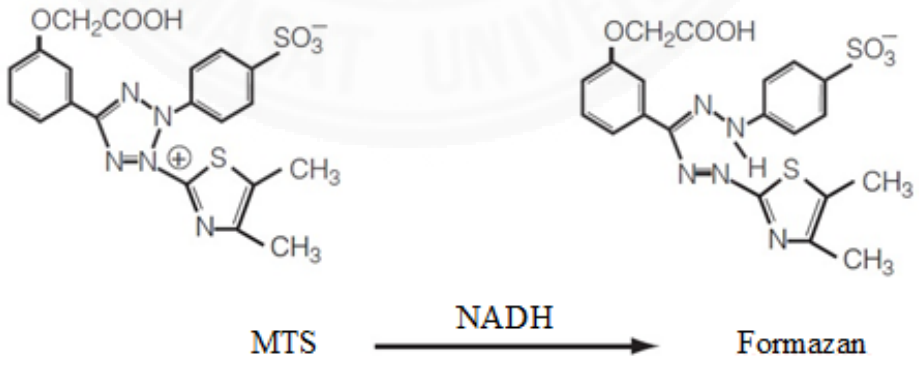


Figure 3.1 Structures of MTS tetrazolium and its formazan product

The 3T3-L1 cells were incubated at a density of 1,000, 2,000, 4,000 and 8,000 cells/well by seeding 100 µL of each density cells into 96–well plates. The preadipocytes were cultured in DMEM supplemented with 10% CS and 1% penicillin-streptomycin in a humidified atmosphere of 5% carbon dioxide at 37 °C. Two days subsequent to reaching confluence, the medium was replaced with DMEM containing 10% FBS and differentiation inducers (1 µg/mL insulin, 0.25 µM dexamethasone, and 0.5 mM IBMX) for inducing cell differentiation. After that, the cells were incubated with maintenance medium as DMEM with 10% FBS supplement and 5 µg/mL of insulin for 2 days. After incubation, the medium was changed with media with 10% FBS/DMEM and replaced every 2 days. The completed differentiation could be achieved between 8 to 10 days.

Evaluation of cell growth was assessed every day for 8 days by MTS assay. The media were replaced every 2 days; the cells were added 20 µL of the freshly prepared MTS solution in each well and then incubated at 37 °C in a humidified atmosphere and 5% carbon dioxide for 2 h. The absorbance of the plate was determined the formazan at 490 nm in a microplate reader. The cells were determined the formazan concentrations which are proportional to the number of live cells. The percentage of cell survival was calculated by the following formula:

$$\% \text{ Cell viability} = \frac{OD_{sample} \times 100}{OD_{control}}$$

Where; OD_{sample} is the absorbance of tested sample. $OD_{control}$ is the absorbance without tested sample. The mean numbers of the cell were calculated and plotted to generate growth curves for 3T3-L1 cells.

A growth curve with various cell density was determined the optimal cell density used in the assays. Cell growth was terminated on one plate per day for eight consecutive days.

3.3.2 The cell culture and differentiation

3T3-L1 preadipocytes obtained from American Type Culture Collection (ATCC, USA). 3T3-L1 is a continuous substrain of 3T3 (Swiss albino) development

through clonal isolation. The 3T3-L1 preadipocytes were incubated in expansion media containing with DMEM supplement with 10% calf serum, 25 mmol/L glucose, 1 mmol/L pyruvate and 4.02 mmol/L L-alanyl-glutamine, 50 U/mL Penicillin and 50 U/mL Streptomycin at 37 °C in humidified atmosphere containing 5% CO_2 for 48 h. The 3T3-L1 preadipocyte differentiation was performed by following the Ericson protocol showed in **Figure 3.1**.

The cells were stimulated with differentiation medium for 2 days (D1-D2) after confluence. The differentiation medium was consisted of 10% FBS, 0.25 µM Dexamethasone, 0.5 mM IBMX, and 1.0 µg/ml Insulin. Then, the differentiation medium was replaced with adipocyte maintenance medium as DMEM with 10% FBS supplement and 5 µg/mL of insulin (D3-D4). After incubation for two days, the medium was changed media with 10% FBS/DMEM (D5-D6). In the incubating process, the medium was replaced every 2 days and incubated until 8 days at 37°C in humidified atmosphere containing 5% carbon dioxide. The complete differentiation could be achieved between 8 to 10 days in **Figure 3.2**.

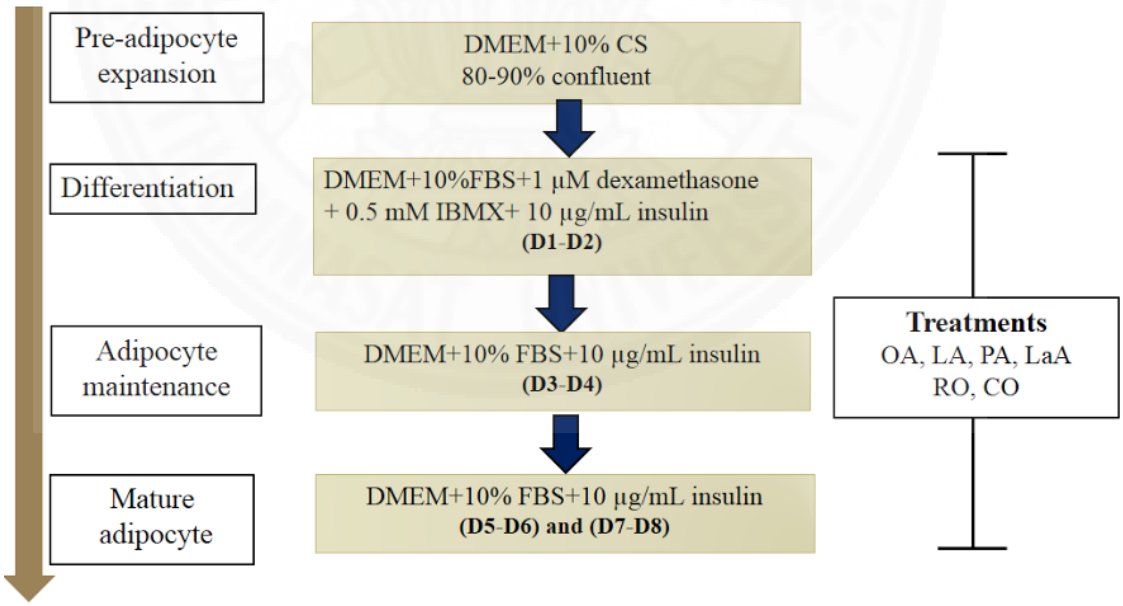


Figure 3.2 Description period of 3T3-L1 cell line maintenance

3.3.3 Preparation of fatty acids and edible oils treatments

3T3-L1 preadipocytes were seeded in 96-well plate at a density of $2.0x10^3$ cells/well. The cells were cultured in expansion medium. The expansion medium consisted of DMEM, 10% calf serum (CS) and 1% penicillin-streptomycin. In the next 48 hours post-confluent 3T3-L1 preadipocytes (day 0), culturing at 37 °C in humidified atmosphere containing 5% carbon dioxide for the expansion of the cells. Two days, the differentiation medium was replaced with DMEM containing 10% FBS and differentiation inducers (1 µg/mL insulin, 0.25 µM dexamethasone and 0.5 mM IBMX) were added to the 3T3-L1 cells to induce differentiation. Cells were treated with fatty acids (OA, LA, PA, LaA at 20, 40, 60, 80 and 100 µg/mL) and 100, 200, 300, 400 and 500 µg/mL rice bran oil (RO), and coconut oil (CO). The media presented with fatty acids, RO, and CO were replaced every 2 days during adipocyte differentiation in 4 and 8 days. The OA, LA, and LaA (20, 40, 60, 80 and 100 µg/mL) were dissolved in 1% DMSO in medium and PA was dissolved with 1mM per 1% bovine serum albumin at 55 °C for 15 min. The CO and RO were dissolved with 1% EtOH and 1% DMSO in medium respectively in differentiation medium at 100, 200, 300, 400, 500 µg/mL. The treatments were tested in triplicates and in the control plates, the medium was changed, but none of the samples was added.

3.3.4 MTS cell viability

The cells were performed in 96-well plates. For preadipocyte, the seeding density of $2.0x10^3$ cells/well in preadipocyte expansion medium. After incubated for 48 hr, the medium was replaced with OA, LA, PA, LaA treatments at 20, 40, 60, 80 and 100 µg/mL and 100, 200, 300, 400 and 500 µg/mL RO and CO treatments on day 1 and day 2. The medium was replaced every 2 days and detected at 4 and 8 days, in a humidified atmosphere of 5% CO_2 at 37 °C.

The assay was established by adding 20 µL of MTS reagent to each well and incubated for 2 h, as described previously. The cell viability was expressed as the percentage of control cells, was calculated by the following formula:

$$\% \text{ Cell viability} = \frac{OD_{sample} \times 100}{OD_{control}}$$

Where; OD_{sample} is the absorbance of tested sample. $OD_{control}$ is the absorbance without tested sample. The tested sample will be defined as nontoxic when the percentage of cell survival was higher than 70%.

3.3.5 Oil red O staining

Lipid accumulation in 3T3-L1 adipocytes was determined by Oil red O staining (50, 51). The cells were seeded in 24-well plates at a density of 2×10^4 cells/well. Preadipocytes were subjected to differentiation by the protocol described before in **3.3.2**. Various concentration of concentrations 20, 40 and 60 µg/mL of OA, LA and LaA and 100, 200 and 300µg/mL of CO and RO in the medium were prepared and added to the cultured cells in 4 and 8 days. Oil red O stained was performed on day 4 and 8.

The treated 3T3-L1 cells were determined by Oil red O stained material (OROSM, %). The cells at fourth day and eighth day were washed with PBS and fixed with 10 % formalin (1 mL/well) for 30 minutes. The cells were washed twice with distilled water. After that, the washed cells of each well were stained with Oil red O (1 mL/well) for 2 hours at room temperature. Excessive Oil red O solution was removed. The cells were rinsed twice with DW before allowed to air dry. The stained cells were destained by dissolving with 500 µL isopropyl alcohol per well. Lipid accumulation was measured spectrophotometrically at 520 nm by a microplate reader. Percent of OROSM was compared to control as the cell culture without the sample and calculated according to the following formula:

$$\%\text{OROSM} = \frac{OD_{\text{sample } 520} \times 100}{OD_{\text{control } 520}}$$

Where OD_{sample} is the absorbance of tested sample. $OD_{control}$ is the absorbance without tested sample. The tested sample will be defined as nontoxic when the percentage of cell survival.

3.3.6 Determination of target mRNA expression

3.3.6.1 RNA isolation

Total RNA of 3T3-L1 cells were extracted using TRIzol reagent following the manufacturer's recommendations. A 1 mL of TRIzol reagent was added to cells and subsequently mixed and incubated for 5 min at room temperature. The mixture was mixed with a 200 μL of chloroform, inverted, mix well and then centrifuge at 12000 × g for 15 min at 4 ℃ to get phase separation. The RNA presented in the aqueous phase and collected the aqueous phase into a new microcentrifuge tube. RNA was precipitated with 500 μL of 2-propanol and incubated for 10 minutes at room temperature. The mixture was centrifuged at 12000 × g for 10 min at 4 ℃, and then the supernatant was removed. The RNA pellet was washed with a 1 mL of 75% EtOH and centrifuged at 7500 × g for 5 min at 4 ℃. The EtOH was removed after that the RNA pellet was dried for 10-15 min at room temperature. Dried RNA was dissolved in DEPC-treated water. RNA concentration and purity were determined using the NanoDrop spectrophotometer by absorbance values at 260 nm and 280 nm (A260/A280 ratio) ratio of 1.9-2.1.

3.3.6.2 Complementary deoxyribonucleic acid (cDNA) synthesis

cDNA was synthesized by High Capacity cDNA Reverse Transcription Kits from RNA. The reaction components of cDNA synthesis are showed in **Table 3.1**. cDNA synthesis was performed in 10 μL of total RNA (20 ng/μL) with MultiScribe Reverse Transcriptase in a 10 μL volume of reverse transcription reaction mixture containing reverse transcription buffer (RT buffer), RT random primers, deoxyribonucleotide triphosphates (dNTPs) mix, RNase inhibitor and RNase-free water. Samples were incubated for 10 min at 25 ℃ to anneal the primers to RNA template, followed by cDNA synthesis for 120 min at 37 ℃ and reverse transcriptase de-activation for 5 min at 85 ℃ and 4 ℃ for infinity showed in **Table 3.2**.

Table 3.1 The PCR conditions for cDNA synthesis

Reaction components	Volume/reaction (µl)
	Kit with RNase inhibitor
2X RT master mix:	
1) Nuclease-free H_2O	3.2
2) 10X RT buffer	2.0
3) 10X RT Random Primers	2.0
4) 25X dNTP Mix (100mM)	0.8
5) RNase Inhibitor	1.0
6) MultiScribe™ Reverse Transcriptase	1.0
Total per reaction	**10.0**
cDNA RT reaction:	
1) 2X RT master mix:	10.0
2) RNA sample	10.0
Total per reaction	**20.0**

Table 3.2 Incubating protocol for cDNA synthesis

	Temperature (°C)	**Time (min)**
Step 1 (primer extension)	25	10
Step 2 (cDNA synthesis)	37	120
Step 3 (reaction termination)	85	5
Step 4 (hold)	4	∞

3.3.6.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

Total real-time PCR reaction system was perfumed in Rea-ltime PCR instrument. cDNA was quantitatively amplified using the TaqMan Gene Expression Master Mix and Assay. The reaction components of real-time PCR were showed in **Table 3.3**. Cycling conditions were as follows: an initial set up cycle at 50 °C for 2 min and 95 °C for 10 min. The real-time PCR amplification conditions 40 cycles at 95 °C for 15 and 60 °C for 1 min in **Table 3.4**.

The expression levels of *C/EBPα* and *PPARγ* were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels. The relative gene expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method

Table 3.3 The Reaction components for real-time PCR

Reaction components	Volume/reaction
(1) TaqMan® Gene Expression Master Mix	10 µl
(2) TaqMan® Gene Expression Assays (target gene and reference gene)	1.5 µl
(3) DEPC-treated water	4.5 µl
(4) cDNA	4 µl
Reaction volume/final volume	**20 µl**
Total amount of cDNA	**40 ng**

Table 3.4 Cycling protocol for real-time PCR

Step	**UDG incubation**	**AmpliTaq Gold, UP enzyme activation**	**PCR**	
			Cycle (40 cycles)	
	Hold	**Hold**	**Denature**	**Anneal/extend**
Temperature (ºC)	50	95	95	60
Times	2 min	10 min	15 s	1 min

3.4 Statistical analysis

The data were presented as a mean ± standard error (S.D.) from at least three separate experiments. All statistical analyses were carried out through Statistic Package for the Social Sciences (SPSS) program version 16 for Windows. Differences between control and treatments were determined by independent t-test. The significant difference was considered as $p < 0.05$.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Effect of various concentrations of the cells seeding on cell growth

The 3T3-L1 preadipocytes were seeded in 96–well plate containing 100 µL in expansion medium. DMEM was supplemented with 10% CS and 1% penicillin-streptomycin in a humidified atmosphere of 5% carbon dioxide at 37 °C as described in Method **3.3.1**. The cells were harvested from the culturing plate every day from 1-8. The optical density of cells in each aliquot was determined using MTS assay.

A growth curve with various cell densities was determined for the optimal cell density to be used in the assays. Cell growth was terminated on one plate per day for eight consecutive days. The optical density representing cell growth was plotted against days of culture.

The growth of 3T3-L1 cells at the seeding of 500, 1000, 2000, 4000 and 8000 cells/well was showed in **Figure 4.1**. The values are expressed as the mean from three independent experiments. Normal 3T3-L1 cell growth curve included 4 phases, the lag phase, exponential phase, stationary phase and plateau phase. Cells were cultured in DMEM supplemented with 10% calf bovine serum for 48 h. The results showed that 3T3-L1 cells began to proliferate after 24 h. After 72 h, the number of cells increased and most rapid proliferation occurred in the exponential phase.

Data showed the 1000 and 2000 seeding cells/well gave optimum growth curves. However, the growth curve of 2000 seeding cells/well had slightly higher MTS density which was more convenient for the future studies.

The reduced density of 500 cells/well led to slow growth and a long lag phase. Moreover, the curves of 4000 or 8000 cells/well showed a short lag phase.

On the basis of the proliferation assay, 2000 cells/well are the optimal cell density to use in the experiments.

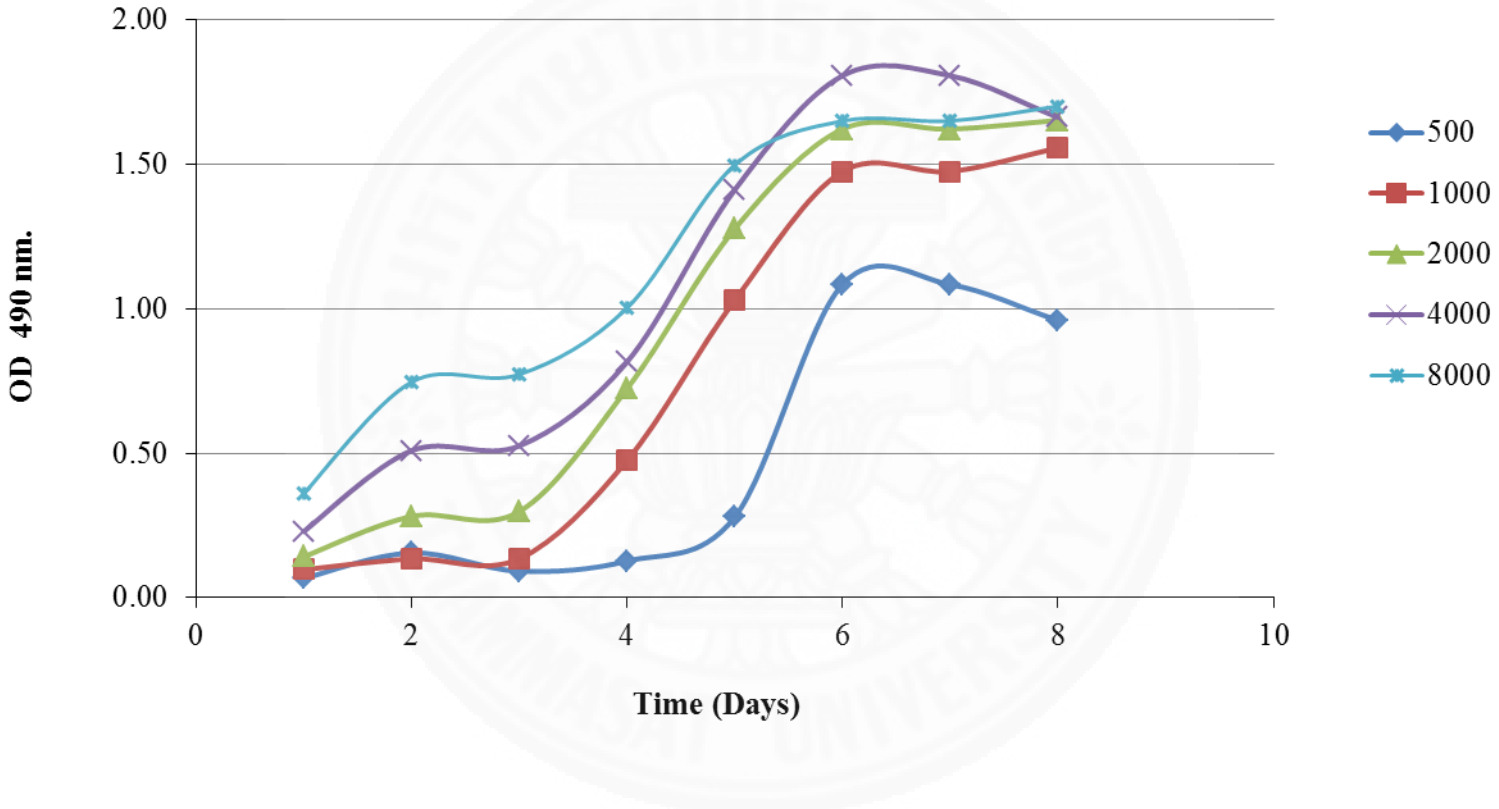


Figure 4.1 Evaluation of cell growth using the MTS assay

4.2 Effect of fatty acids and edible oils on adipocyte cell viability

To determine the effects of OA, LA, PA , LaA treatments at 20, 40, 60, 80 and 100 µg/mL and 100, 200, 300, 400 and 500 µg/mL RO and CO treatments in cell culture media on viability during adipocyte differentiation in 4 and 8 days. From the cell growth studied, the 3T3-L1cells were seeded in 96-well plates at a density of 2000 cells/well. The viability of cell was determined by MTS assay.

The results of cell viability are expressed as the percentage of surviving cells compared to control cells, 0 µg/mL ($p < 0.05$). All treatments were performed in triplicates.

The cell viability results for treatments with fatty acids and edible oils for 4 days and 8 days are shown in **Table 4.1**. The results revealed significantly decreased cell viability at high concentrations of PA (60, 80, 100 µg/mL) and LA (80, 100 µg/mL) when compared to the control ($p< 0.05$). During adipocyte differentiation (4 and 8 days) in the presence of LaA, significantly decreased cell viability was observed when the adipocytes were treated with LaA at 100 µg/mL. However, the OA, LA, PA (except at 80, 100 µg/mL), LaA (except at 100 µg/mL), RO and CO treatments were nontoxic, and the percentage of surviving 3T3-L1 cells was higher than 70%. Moreover, the CO treatment (400 and 500 µg/mL) improved cell viability after 8 days of differentiation. The percentage is shown in **Table 4.1**.

Table 4.1 Effect of fatty acids and edible oils on adipocyte cell viability in 4 and 8 days

Treatments		The measurements (Mean ± S.D., n=3)	
		Cell viability (%) for 4 days	Cell viability (%) for 8 days
OA	20 µg/mL	100.18±12.37	110.05±13.04
	40 µg/mL	103.83±5.83	110.31±18.99
	60 µg/mL	98.75±11.72	100.36±16.94
	80 µg/mL	93.83±11.89	102.29±8.19
	100 µg/mL	87.00±20.57	107.11±1.78
LA	20 µg/mL	100.48±14.44	104.95±3.23
	40 µg/mL	106.89±14.13	102.98±6.70
	60 µg/mL	94.21±16.67	103.75±6.11
	80 µg/mL	95.76±9.43	82.58±7.68*
	100 µg/mL	77.52±15.44	82.08±5.68*
PA	20 µg/mL	90.18±9.07	98.68±9.07
	40 µg/mL	82.32±5.81	97.17±9.75
	60 µg/mL	73.78±7.88*	77.17±9.44 *
	80 µg/mL	71.00±1.41 *	68.44±10.68 *
	100 µg/mL	71.42±7.84 *	64.38±4.76 *
LaA	20 µg/mL	105.16±5.05	88.80±7.79
	40 µg/mL	88.26±5.08	100.74±1.66
	60 µg/mL	109.13±7.95	93.87±4.55
	80 µg/mL	95.26±13.38	91.57±2.38
	100 µg/mL	78.78±4.83	66.64±11.48 *
RO	100 µg/mL	99.93±4.37	97.29±4.37
	200 µg/mL	97.85±4.36	97.90±4.36
	300 µg/mL	96.31±6.13	92.95±6.13
	400 µg/mL	99.77±10.71	92.75±10.71
	500 µg/mL	97.58±9.29	92.71±9.27
CO	100 µg/mL	92.59±3.68	114.59±3.68
	200 µg/mL	103.66±17.01	110.00±17.01
	300 µg/mL	90.59±11.47	107.50±11.47
	400 µg/mL	107.21±7.17	111.22±7.17*
	500 µg/mL	108.02±5.33	106.65±5.33*

*significant compared to control.

4.3 Effect of fatty acids and edible oils on lipid accumulation in 3T3-L1 adipocyte

The intracellular lipid droplet formation was determined by Oil red O staining and observed with an inverted microscope Zeiss LSM. Undifferentiated cells were observed to maintain the initial fibroblast-like shape with non-lipid accumulation on day 1 **(Figure 4.2A)** and 2 days **(Figure 4.2B)**. The pictures clearly show the differentiated cells and control cells on day 4 and 8 **(Figure 4.2C** and **Figure 4.2D**, respectively). The adipocytes and mature adipocytes accumulation of lipid droplets in the cytoplasm were observed on day 4 **(Figure 4.2C)** and 8 **(Figure 4.2D**), respectively. The effects of OA, LA, PA , LaA treatments at 20, 40, and 60 µg/mL and 100, 200, and 300 µg/mL RO and CO treatments on lipid accumulation in cell during adipocyte differentiation in 4 and 8 days as shown in **Figure 4.3-Figure 4.8**.

The Oil red O staining was quantified spectrophotometrically at 520 nm by a microplate reader. The results of the absorbance measurements of Oil red O **(Table 4.2)** showed significantly decreased lipid accumulation after 4 days of differentiation in OA and LA treatments (20, 40, 60 µg/mL), LaA treatment (20µg/mL), RO and CO treatments (except 100 µg/mL) compared with the control ($p < 0.05$). Eight days following the initiation of differentiation, OA treatment (20 µg/mL), LA treatment (20-40 µg/mL), LaA treatment (20, 40 µg/mL), CO treatment, and RO treatment (except 300 µg/mL) resulted in significantly decreased lipid accumulation, when compared with the control cell **(Table 4.2)**. However, the fat accumulation significantly increased by 13.89 % at 60 µg/mL OA treatment in 8 days. Moreover, PA treatment at high concentrations (40, 60 µg/mL) resulted in significantly increased lipid accumulation in 8 days **(Table 4.2)**.

Table 4.2 Effect of fatty acids and edible oils on lipid accumulation (% of control) in

Treatments		The measurements (Mean ± S.D., n=3)	
		Lipid accumulation (%) for 4 days	**Lipid accumulation (%) for 8 days**
OA	20 μg/mL	46.61±6.71 *	75.90±9.00 *
	40 μg/mL	51.87±7.44 *	92.23±10.87
	60 μg/mL	88.10±7.58 *	113.89±3.65*
LA	20 μg/mL	54.95±2.63 *	61.56±2.91 *
	40 μg/mL	71.56±3.75 *	78.58±4.01 *
	60 μg/mL	116.14±2.77 *	107.70±16.19
PA	20 μg/mL	89.96±6.57	97.55±8.71
	40 μg/mL	97.19±9.98	112.09±2.25*
	60 μg/mL	109.66±7.53	123.79±10.20 *
LaA	20 μg/mL	67.53±7.37 *	59.37±5.58 *
	40 μg/mL	85.12±12.69	65.96±5.36 *
	60 μg/mL	93.07±13.09	79.51±10.86
RO	100 μg/mL	78.09±2.34 *	67.67±6.56 *
	200 μg/mL	83.43±3.91 *	69.03±4.89 *
	300 μg/mL	93.83±6.64 *	72.19±13.34
CO	100 μg/mL	93.35±5.94	67.17±7.69 *
	200 μg/mL	112.77±5.03 *	69.27±6.73 *
	300 μg/mL	108.86±1.93 *	74.66±8.69 *

*significant compared to control.

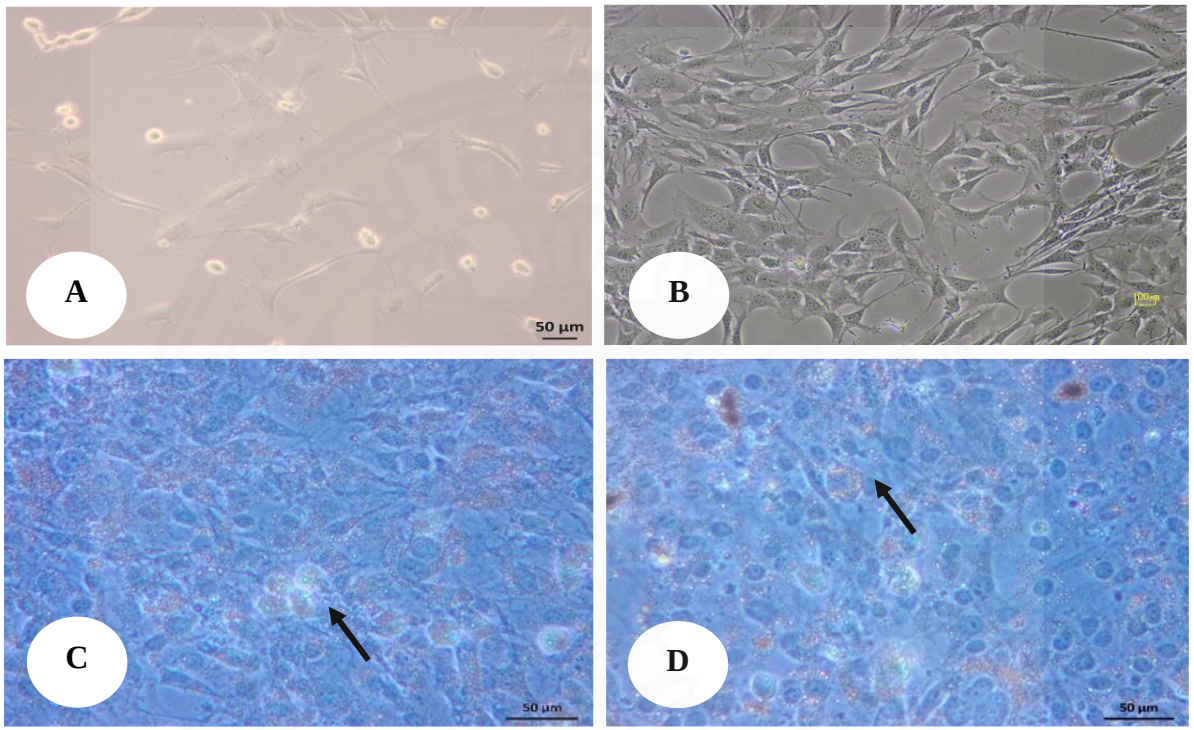


Figure 4.2 Morphological changes of adipocyte cells in differentiation period. Initiated 3T3-L1 cells were not stained before the cells were induced into differentiated cells (A) and 2 days (B). 3T3-L1 cells were induced to differentiate without on treatment. Intracellular lipid droplet formation, as stained with oil red O, was examined with an inverted microscope Zeiss LSM in 4 days (C) and 8 days (D) under ×200 magnification; scale bar = 50 µm.

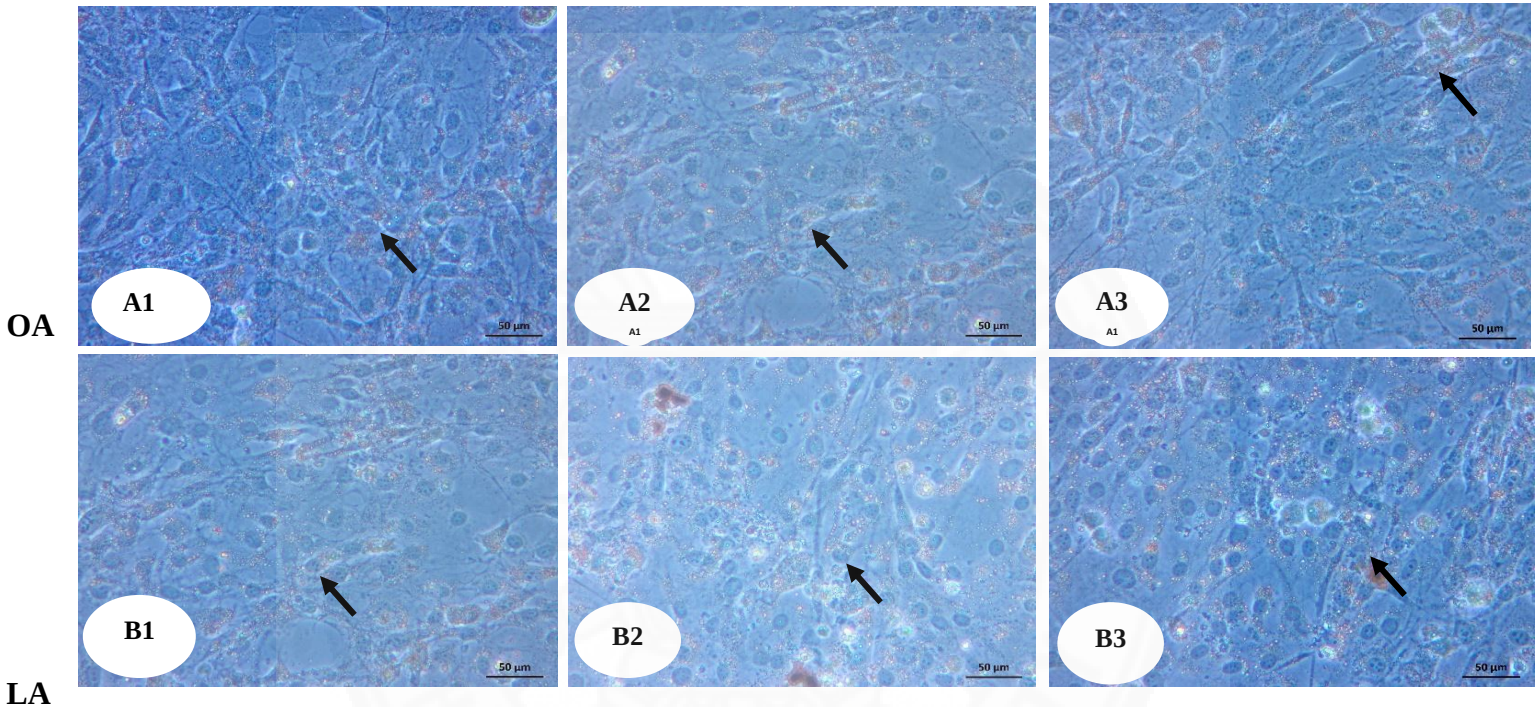


Figure 4.3 Effect of OA and LA treatments on lipid accumulation 3T3-L1 preadipocytes. On day 4 of the treatment with 20, 40 and 60 µg/mL OA (A1-A3), respectively. 20, 40 and 60 µg/mL LA treatment (B1-B3), respectively. Intracellular lipid droplet formation, as stained with oil red O, was examined with an inverted microscope Zeiss LSM under ×200 magnification; scale bar = 50 µm.

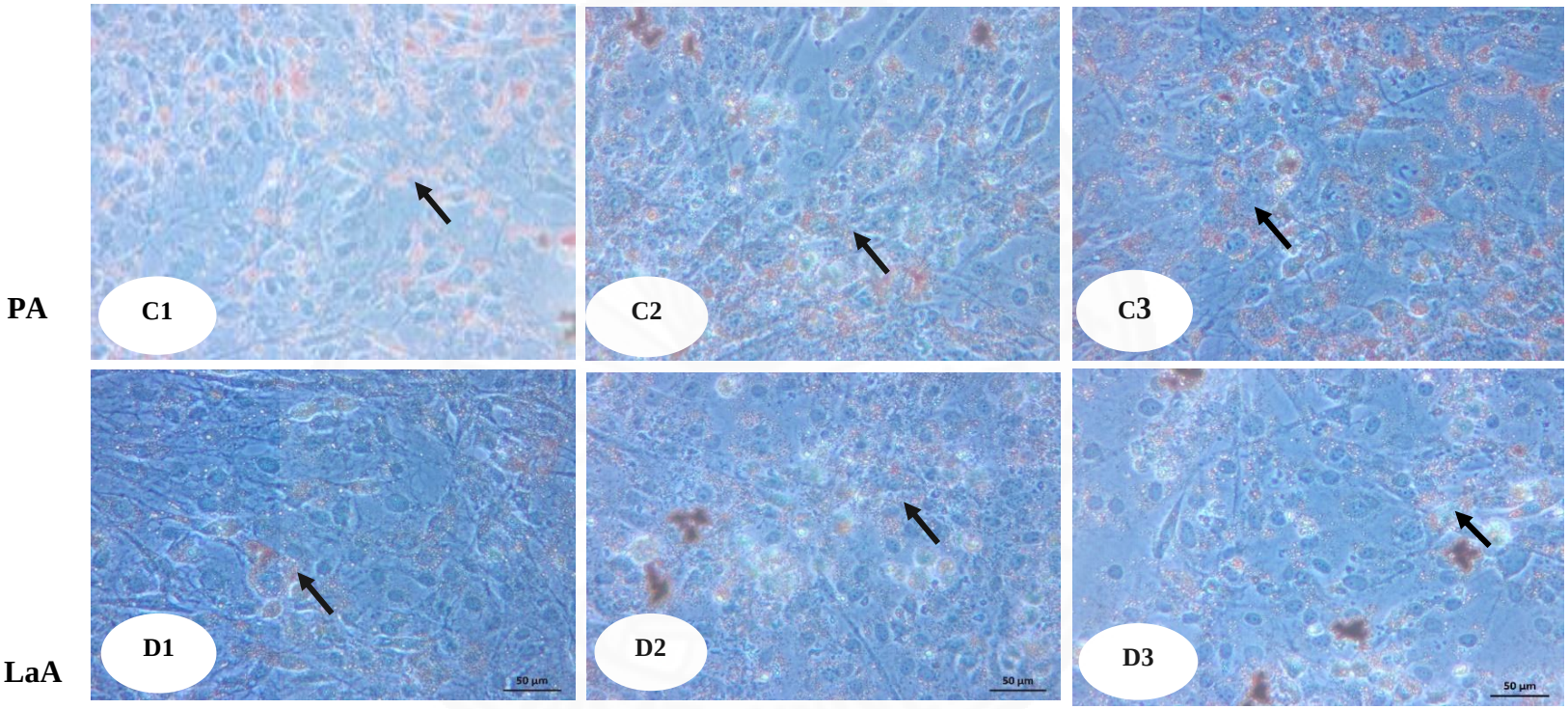


Figure 4.4 Effect of PA and LaA treatments on lipid accumulation of 3T3-L1 preadipocytes. On day 4 of the treatment with 20, 40 and 60 µg/mL PA (C1-C3), respectively. 20, 40 and 60 µg/mL LaA treatment (D1-D3), respectively. Intracellular lipid droplet formation, as stained with oil red O, was examined with an inverted microscope Zeiss LSM under ×200 magnification; scale bar = 50 µm.

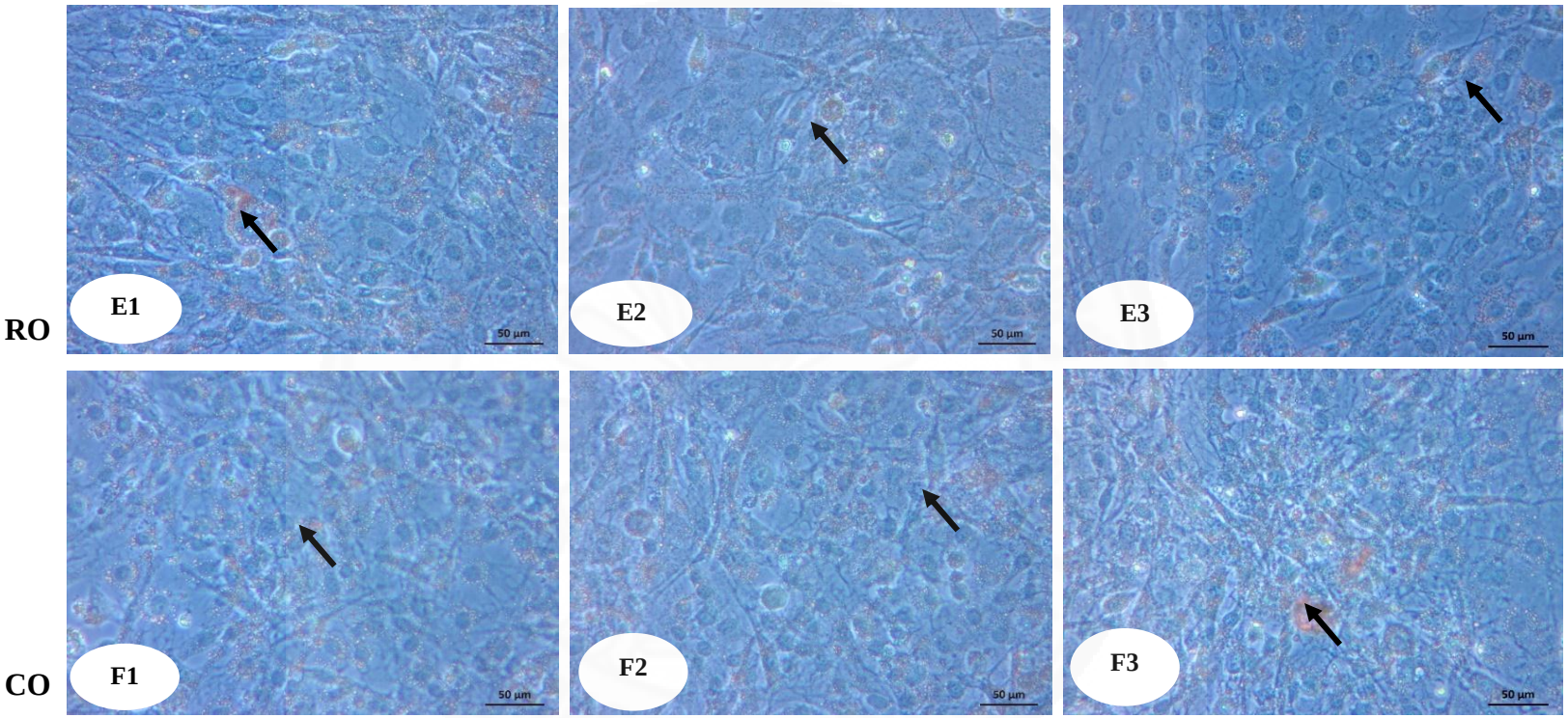


Figure 4.5 Effect of RO and CO treatments on lipid accumulation of 3T3-L1 preadipocytes. On day 4 of the treatment with 100, 200 and 300 µg/mL RO (E1-E3), respectively. 100, 200 and 300 µg/mL CO treatment (F1-F3), respectively. Intracellular lipid droplet formation, as stained with oil red O, was examined with an inverted microscope Zeiss LSM under ×200 magnification; scale bar = 50 µm.

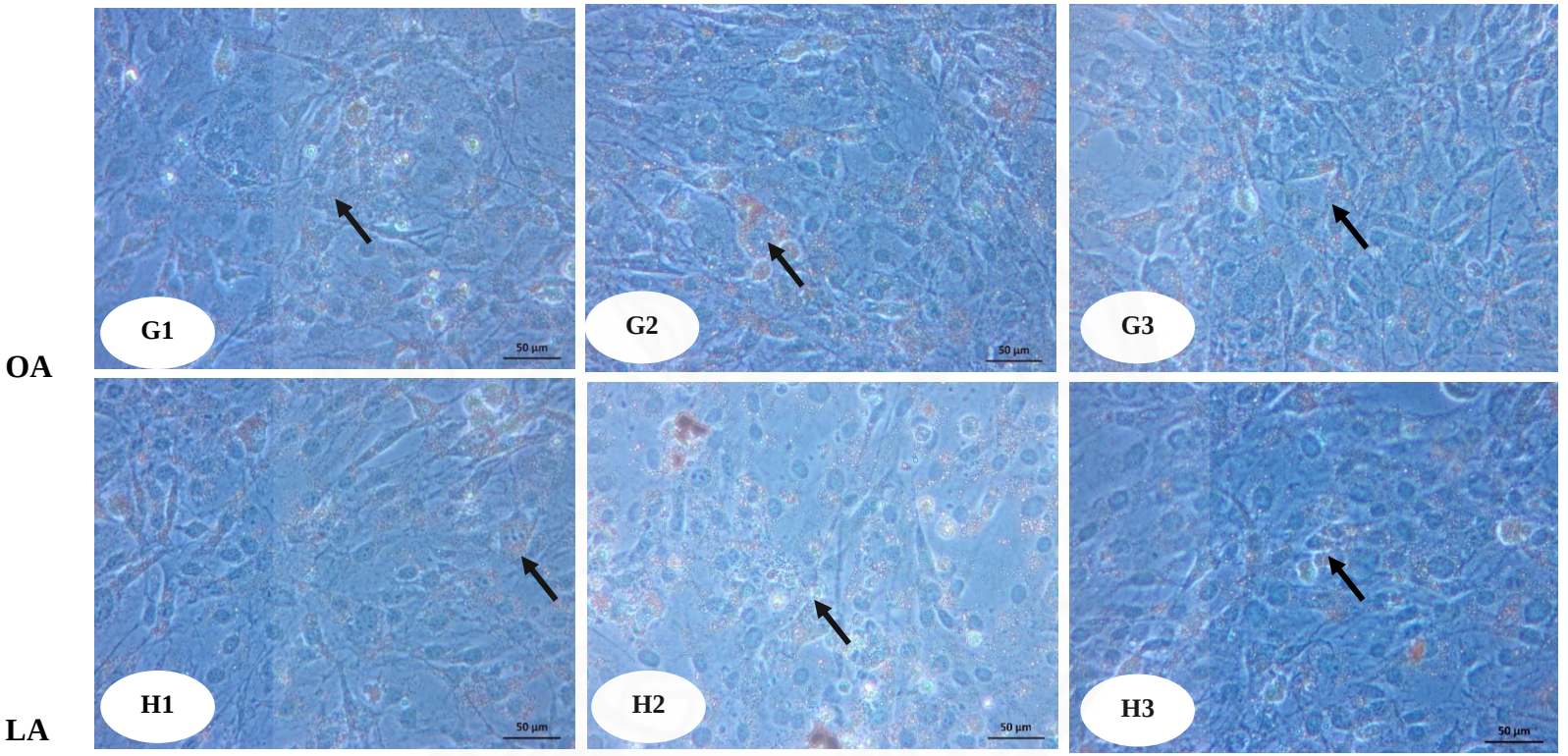


Figure 4.6 Effect of OA and LA treatments on lipid accumulation of 3T3-L1 preadipocytes. On day 4 of the treatment with 20, 40 and 60 µg/mL. OA (G1-G3), respectively. 20, 40 and 60 µg/mL CO treatment (H1-H3), respectively. Intracellular lipid droplet formation, as stained with oil red O, was examined with an inverted microscope Zeiss LSM under ×200 magnification; scale bar = 50 µm.

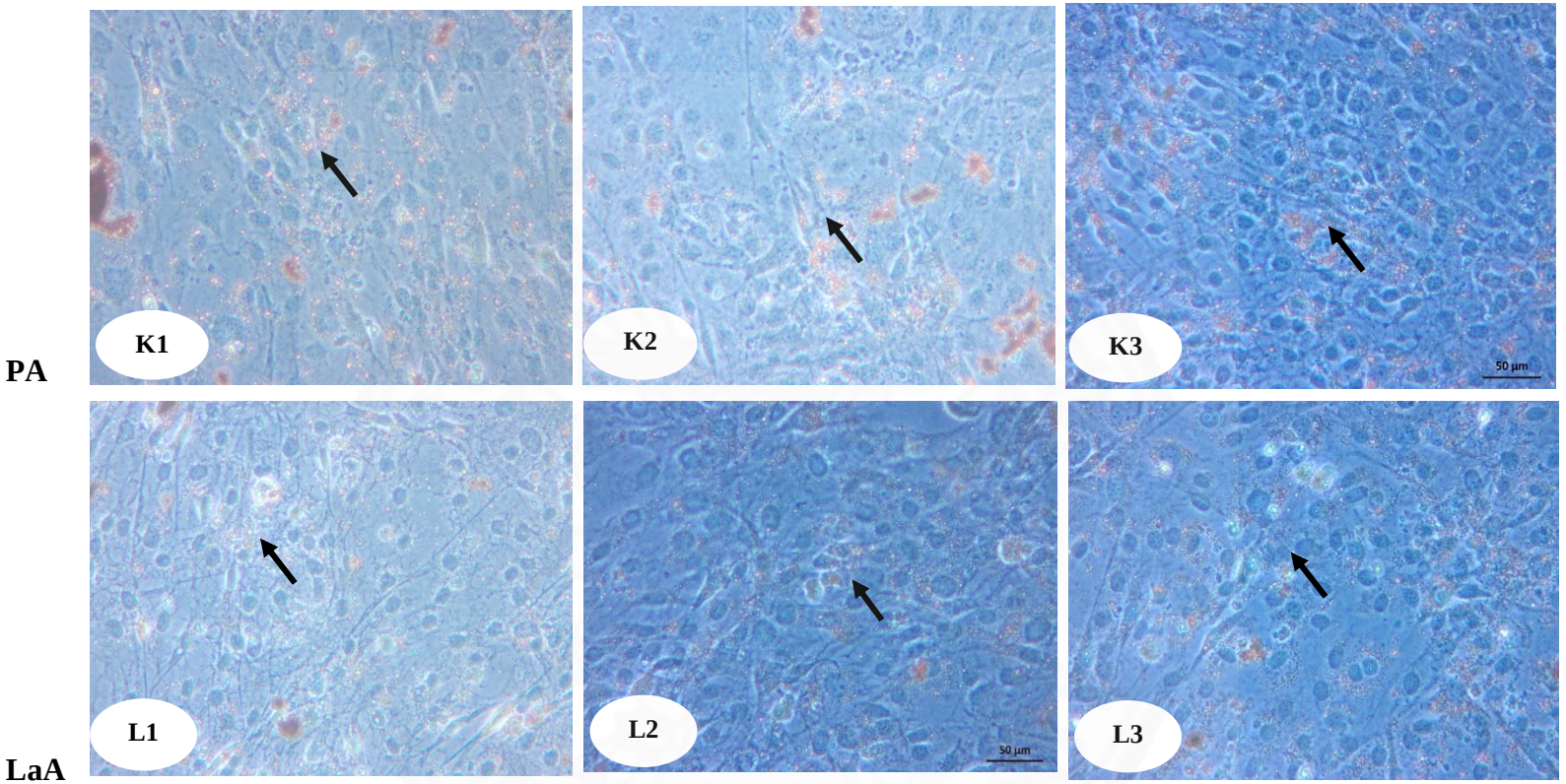


Figure 4.7 Effect of PA and LaA treatments on lipid accumulation of 3T3-L1 preadipocytes. On day 8 of the treatment with 20, 40 and 60 µg/mL OA (K1-K3), respectively. 20, 40 and 60 µg/mL LA treatment (L1-L3), respectively. Intracellular lipid droplet formation, as stained with oil red O, was examined with an inverted microscope Zeiss LSM under ×200 magnification; scale bar = 50 µm.

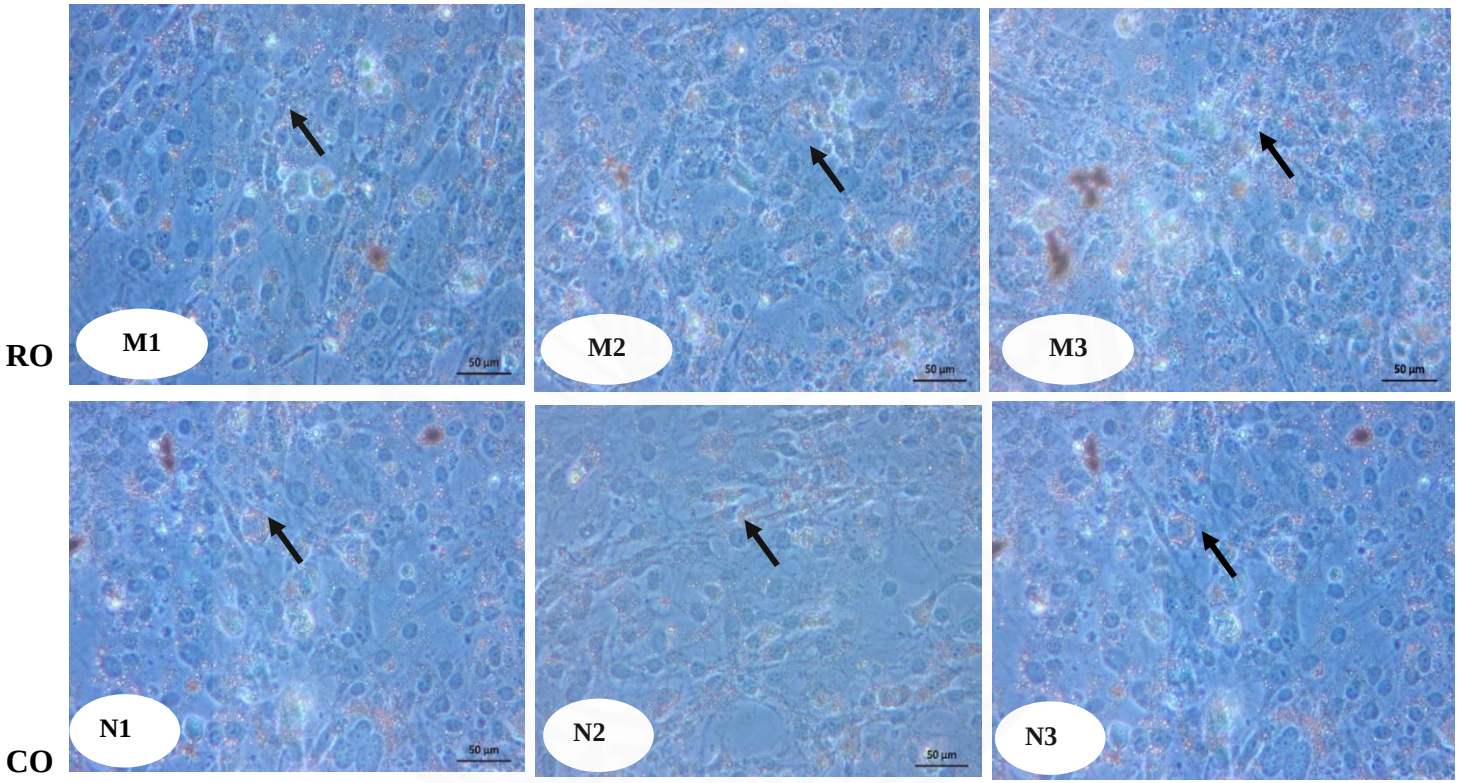


Figure 4.8 Effect of RO and CO treatments on lipid accumulation of 3T3-L1 preadipocytes. On day 8 of the treatment with 100, 200 and 300 µg/mL RO (E1-E3), respectively. 100, 200 and 300 µg/mL CO treatment (F1-F3), respectively. Intracellular lipid droplet formation, as stained with oil red O, was examined with an inverted microscope Zeiss LSM under ×200 magnification; scale bar.

Table 4.3 Effect of fatty acid and edible oils as morphology of adipocyte cells (% of control) in 4 and 8 days

Samples (µg/mL)	Cell number (% of control)			
	4 days		8 days	
	Fibroblast-like cells	Round cells	Fibroblast-like cells	Round cells
Control	23.00 ±2.65	77.00 ±2.65	17.33 ±5.77	82.67 ±5.77
OA20	16.00 ±4.00	84.00 ±4.00	10.67 ±4.04	89.33 ±4.04
OA40	16.33 ±1.53	84.67 ±1.53	9.33 ±4.51	90.67 ±4.51
OA60	16.33 ±3.06	83.67 ±3.06	10.67 ±3.79	89.33 ±3.79
LA20	15.00 ±3.61	85.00 ±3.61	8.33 ±2.52	91.67 ±2.52
LA40	16.00 ±1.73	84.00 ±1.73	9.00 ±4.00	91.00 ±4.00
LA60	16.67 ±3.06	83.33 ±3.06	9.33 ±4.51	90.67 ±4.51
PA20	15.67 ±5.69	84.33 ±5.69	13.00 ±2.65	87.00 ±2.65
PA40	14.33 ±2.52	85.67 ±2.52	14.00 ±3.46	86.00 ±3.46
PA60	11.00 ±2.00	89.00 ±2.00	8.00 ±3.00	92.00 ±3.00
LaA20	28.67 ±4.51	71.33 ±4.51	21.67 ±4.73	78.33 ±4.73
LaA40	24.67 ±11.02	75.33±11.02	15.33 ±2.31	84.67 ±2.31
LaA60	24.33 ±5.13	75.67 ±5.13	16.33 ±2.52	83.67 ±2.52
Ro100	16.00 ±4.36	84.00 ±4.36	15.33 ±2.08	84.67 ±2.08
Ro200	17.33 ±3.06	83.67±3.06	9.67 ±3.79	90.33 ±3.79
Ro300	16.00 ±3.61	84.00 ±3.61	13.67 ±1.53	86.33 ±1.53
CO100	22.00 ±7.55	88.00 ±7.55	21.33 ±5.51	78.67 ±5.51
CO200	25.67 ±6.43	74.33 ±6.43	24.67 ±9.45	75.33 ±9.45
CO300	22.00 ±2.65	78.00 ±2.65	25.00 ±1.73	75.00 ±1.73

4.4 Effect of fatty acids and edible oils on adipocyte size in 3T3-L1 adipocytes

According to the Oil red O staining, adipocyte size in the control group in 4 days (2,826 μm) was lower than that in the control group in 8 days (3,306 μm). Adipocyte sizes of OA, LA, PA, LaA, RO and CO treatments were not different from that of the control group for 4 days as shown in **Table 4.4**. However, treatments with LA (20, 40, 60 μg/mL) and LaA (20, 40 μg/mL), as well as CO at 100 and 200 μg/mL in 8 days significantly reduced adipocyte size when compared with the control group ($p < 0.05$) as shown in **Table 4.4**.

Table 4.4 Effects of fatty acids and edible oils on adipocyte size (μm) in 4 days and 8 days

Treatments		The measurements (Mean ± S.D., n=3)	
		adipocyte size (μm) for 4 days	**adipocyte size (μm) for 8 days**
Control		2826±318.76	3306±266.35
OA	20 μg/mL	2546± 487.22	2525±601.28
	40 μg/mL	3214±971.74	2455±142.39
	60 μg/mL	2641±271.53	2558±187.46
LA	20 μg/mL	2673±152.18	2271±301.38 *
	40 μg/mL	2889±402.46	2517±100.04 *
	60 μg/mL	3448±242.13	2578±96.57 *
PA	20 μg/mL	3057±135.31	2857±151.79
	40 μg/mL	2942±97.21	2884±184.71
	60 μg/mL	2839±106.21	3607±503.87
LaA	20 μg/mL	2588±359.53	2503±61.70 *
	40 μg/mL	2975±204.66	2645±33.72*
	60 μg/mL	2842±307.48	3624±538.24
RO	100 μg/mL	2831±269.72	2631±331.99
	200 μg/mL	2853±411.97	2712±499.92
	300 μg/mL	2674±465.46	3291±75.79
CO	100 μg/mL	3024±347.72	2796±110.76*
	200 μg/mL	2754±261.06	2806±83.70*
	300 μg/mL	3340±720.60	2763±370.60

*significant compared to control.

4.5 Effect of fatty acids and edible oils on mRNA expression of adipogenic transcription factors

The effects of treatments with fatty acids and edible oils for 4 and 8 days on the mRNA expression of adipogenic transcription factors *PPARγ* and *C/EBPα* were detected by real-time PCR as shown in **Figure 4.9** - **Figure 4.12**.

During 4 and 8 days, the mRNA levels of *PPARγ and C/EBPα* were significantly decreased in CO treatment (100, 200 µg/mL) and LaA treatment (20, 40 µg/mL). After differentiation for 4 days, LA (20, 40 µg/mL) and RO *t*reatments (100, and 200 µg/mL) significantly decreased expression of *PPARγ*. Moreover, the mRNA expressions of *PPARγ* and *C/EBPα* in OA, LA (except 40 µg/mL) treatments for 4 and 8 days were significantly decreased when compared to the control ($p < 0.05$) as shown in **Figure 4.9**. However, the mRNA expressions of *C/EBPα* in RO treatment (100, 200 µg/mL) in 4 days was significantly increased when compared to the control ($p < 0.05$) as shown in **Figure 4.10**. In addition, the expression levels of *PPARγ* and *C/EBPα* significantly reduced in LaA and CO treatments in 4 and 8 days as compared with the control except treatment with 200 µg/mL CO for 4 days as shown in **Figure 4.11** and **Figure 4.12**.

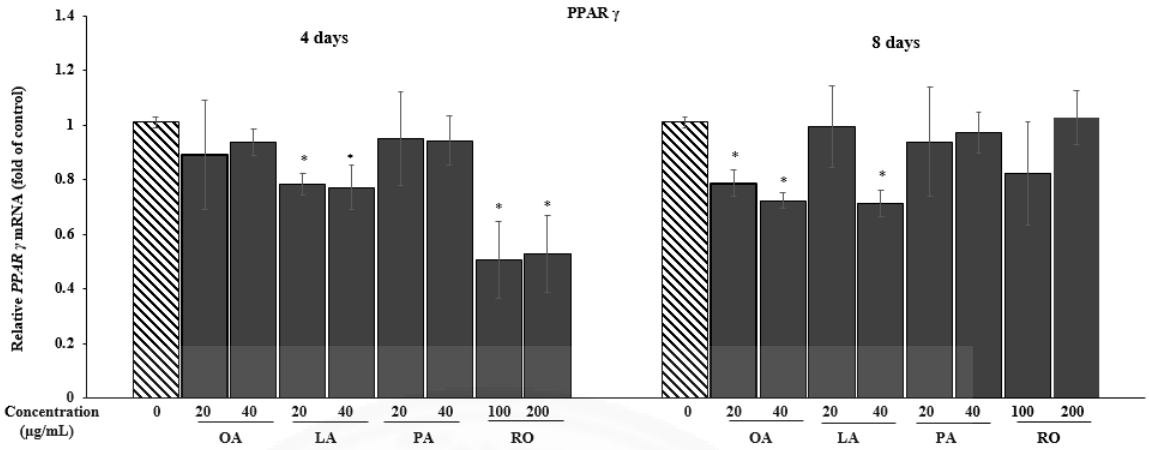


Figure 4.9 Effects of fatty acids and rice bran oil on *PPARγ* gene expression in 4 and 8 days. The values are expressed as mean ± S.D. (n=3). **p*<0.05 compared with control.

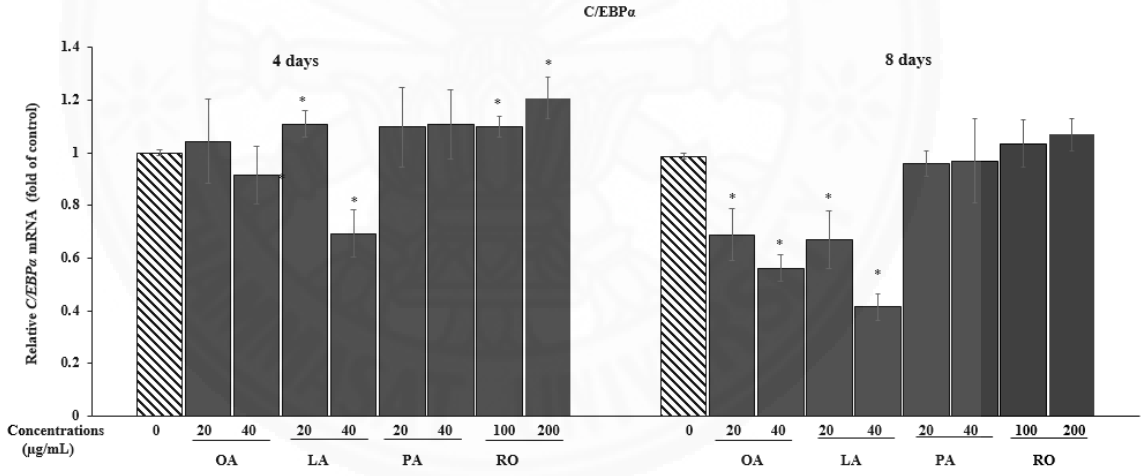


Figure 4.10 Effects of fatty acids and rice bran oil on *C/EBPα* gene expression in 4 and 8 days. The values are expressed as mean ± S.D. (n=3). **p*<0.05 compared with control.

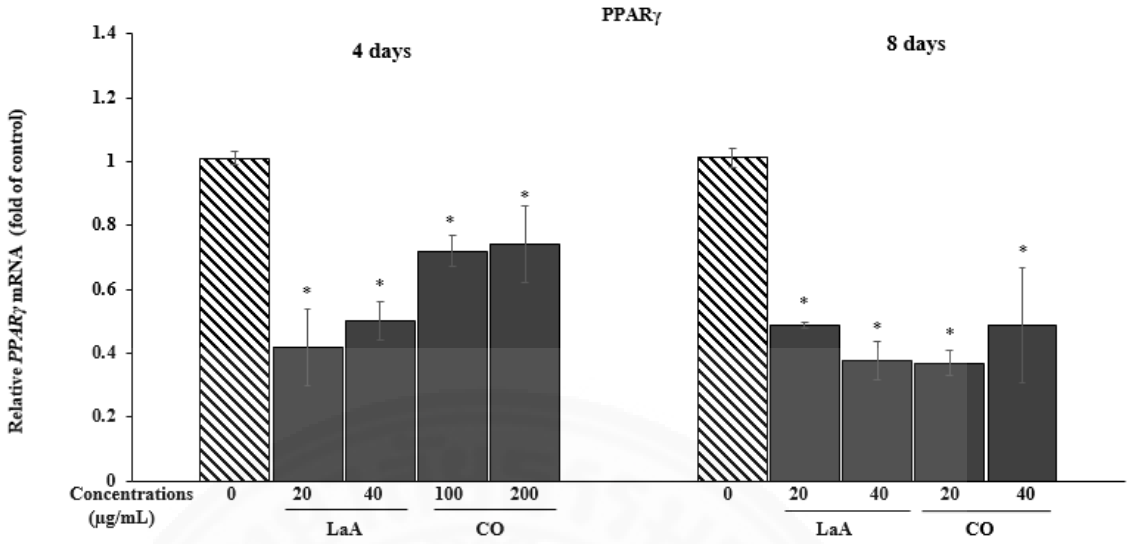


Figure 4.11 Effects of fatty acid and coconut oil on *PPARγ* gene expression in 4 and 8 days. The values are expressed as mean ± S.D. (n=3). **p*<0.05 compared with control.

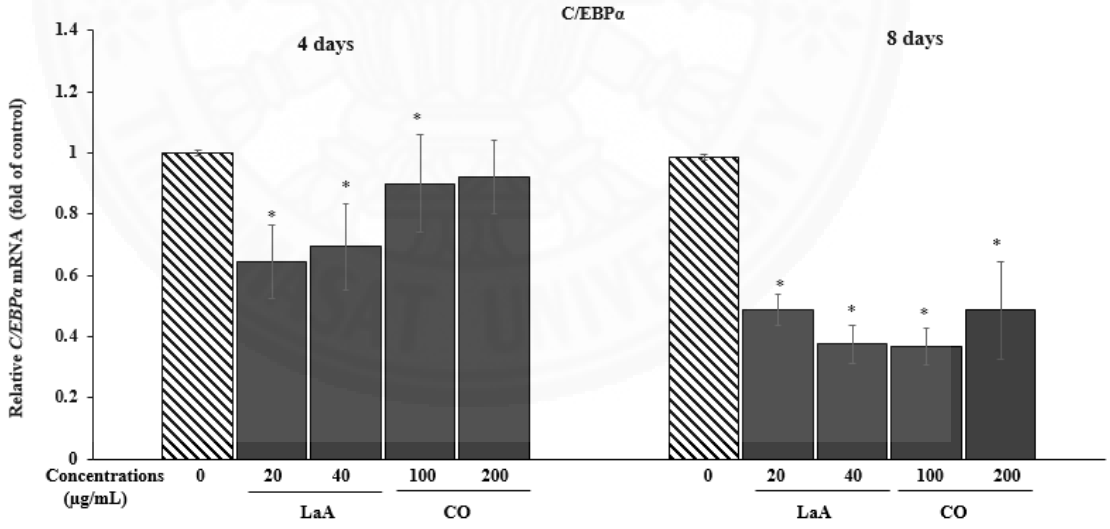


Figure 4.12 Effects of fatty acid and coconut oil on PPARγ gene expression in 4 and 8 days. The values are expressed as mean ± S.D. (n=3). **p*<0.05 compared with control.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

Obesity is a public health problem that has become epidemic worldwide. It is defined as abnormal or excessive fat accumulation, caused by an imbalance between energy intake and expenditure that may impair health. It is the important risk factor for major chronic non-communicable diseases including type 2 diabetes and cardiovascular diseases (84-86). Obesity results from multiple genetic factors and environmental factors such as diet, physical activity and behavior (87). It is characterized by an increase in fat cell number and/or size of fat cell (88). Moreover, adipogenesis is one of the important mechanisms that have been suggested to play a role in the development of obesity.

The abnormal adipogenesis can cause an increase in the excess fat tissue mass via up-regulation of the major adipogenic genes, such as *PPARγ* and *C/EBPα*, leading to an obese state. *PPARγ* and *C/EBPα* can stimulate the expression of various adipocyte-specific genes. Thus, the present study examined the effects of the fatty acids and edible oils on intracellular lipid content, adipocyte size and expression of genes involved in adipogenesis. This study also examined the effect of fatty acids and edible oils on cytotoxicity in 3T3-L1 adipocytes.

The MTS assay indicated that treatment of differentiated 3T3-L1 adipocytes with PA at concentrations of 60, 80, 100 µg/mL and 100 µg/mL LaA decreased the cell viability in **Figure 4.2** and **Figure 4.3**. The previous observation has shown that PA induced apoptosis in both mouse 3T3-L1 and rat primary preadipocytes grown in a normal medium containing serum (89). Moreover, PA induces apoptotic cell death by ER stress (90). In this study, treatments with OA, LA, RO and CO had no effect on adipocyte viability during adipocyte maturation. However, cell death markers were not evaluated in this study.

The 3t3-l1 cell line is well characterized and widely used as a model for adiogenesis, metabolism and obesity research. Adipogenesis is the differentiation from fibroblast-like preadipocyte into mature adipocyte. Fibroblast-like preadipocytes

were differentiated using the hormone cocktail containing insulin, methylisobutylxanthine, dexamethasone in culture medium to start storing triglycerides in lipid droplets (3, 48, 91, 92). The adipocyte characteristics of 3T3-L1 cells were an accumulation of numerous lipid droplets with various sizes in their cytoplasm.

CO, the oil extracted from the fresh coconut meat, is the richest nature source of medium chain triglycerides (MCTs) such as lauric acid (46-48%) (93), which is more easily digested than long chain fatty acids (94). Anti-obesity property of CO has been claimed for various health effects. CO has also been shown to have anti-inflammatory, analgesic, and antipyretic properties (95).

To support this notion, the present study demonstrated that treatment of post-confluent 3T3-L1 preadipocytes with CO significantly reduced lipid accumulation, as indicated by decreased Oil red O staining. CO treatment for 8 days reduced the lipid accumulation. Consistently, CO treatment markedly decreased adipocyte size (**Figure 4.9)**. Treatment of LaA at low doses for 4 and 8 days significantly decreased lipid accumulation. To investigate the underlying mechanism of anti-adipogenic effect of CO and LaA during adipocyte differentiation, the expressions of *PPARγ* and *C/EBPα* genes were examined. In the present study, treatments with CO and LaA for 4 and 8 days significantly suppressed *PPARγ* and *C/EBPα* mRNA levels in differentiated and mature adipocytes. These improvements are likely to be associated with the reduction of lipid accumulation in adipocytes, leading to a decrease in adipocyte size.

RO contains several nutrients and phytochemicals such as MUFAs, PUFAs, α-tocopheral, phytosterol, teperine alcohols and tocotrienols (96). Many studies have shown that RO consumption can reduce blood cholesterol, improve plasma lipid profiles, and increase high-density lipoprotein cholesterol (HDL-C) in mammals and hypocholesterolemia in the rats (96, 97). RO significantly reduced total plasma cholesterol concentrations in hypercholesterolemic hamsters (98). Moreover, RO reduced plasma LDL-cholesterol and triglyceride levels compared to sunflower oil in patients with hyperlipidemia (8). RO contains a monounsaturated and polyunsaturated fatty acids such as oleic acid (OA) and linoleic acid (LA).

Consistently, the post-confluent preadipocytes treated with OA and LA significantly reduced lipid accumulation as shown in **Figure 4.8** and **Figure 4.9**.

In this experiment, 4 days incubation with RO significantly decreased the mRNA levels of *PPARγ* and *C/EBPα* in differentiated adipocytes. However, 8-day incubation of RO had no effect on *PPARγ* and *C/EBPα* expression during maturation period. The current findings suggest that of decreased lipid accumulation in adipocytes by RO treatment is not a direct effect of regulation of these adipogenic genes. However, treatments with OA and LA significantly inhibited mRNA levels of *PPARγ* and *C/EBPα* in adipocytes.

In summary, the findings of this study revealed that CO treatment decreased the lipid accumulation in adipocytes, probably via down-regulating the expression of adipogenic genes (*PPARγ* and *C/EBPα*). Consistently, RO treatment caused a significant reduction in the lipid accumulation in adipocytes. However, the effect of RO treatment on lipid accumulation in adipocytes was not dependent on gene expression of *PPARγ* and *C/EBPα*. In this regard, *FoxC2* and *SREBP-1c* play a major role in maintaining metabolic homeostasis and lipogenesis in the adipocytes, (99, 100) suggesting that RO treatment may decrease adipocyte lipid accumulation via the function of these two genes.

Although CO has benefit to health due to its MCFs, the optimal amount of CO should be consumed. The MCFs provide a ready source of energy and may be useful in baby foods or diet therapy for patients with abnormal digestion and absorption. Moreover, RO appears to have healthy effects on cholesterol and blood sugar. RO dietary is the most effective approach for diabetics.

LaA may be bioactive compounds in CO, and OA and LA may be bioactive compounds in RO. These fatty acids inhibited adipogenesis and lipid accumulation in adipocytes. These properties of fatty acids in CO and RO may help prevent obesity. However, further studies are required to explore more details of mechanisms underlying the effects of CO, RO and their bioactive compounds against obesity.

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APPENDICES

APPENDIX A

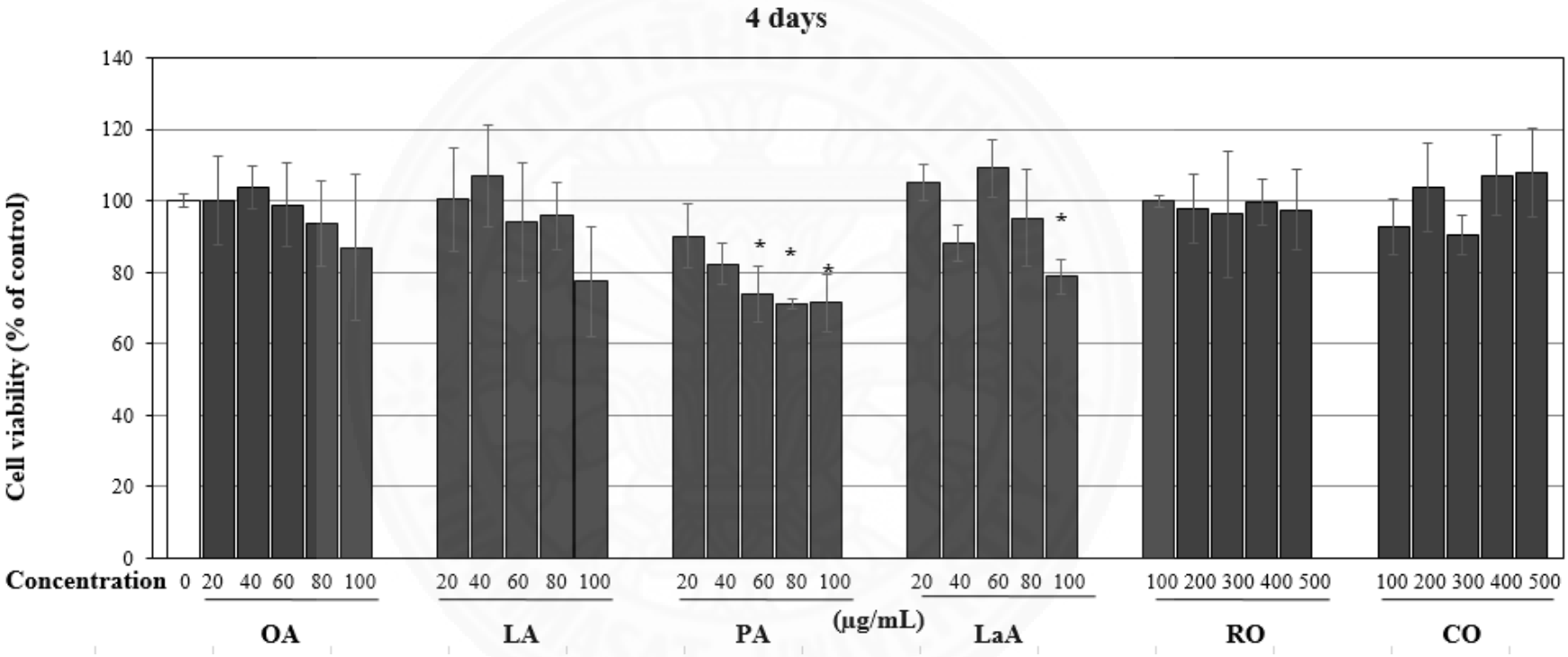

Figure 4-1 Effect of fatty acids and edible oil on adipocyte cell viability in 4 days. The values are expressed as mean ± S.D., (n=3). *p<0.05 compared with control.

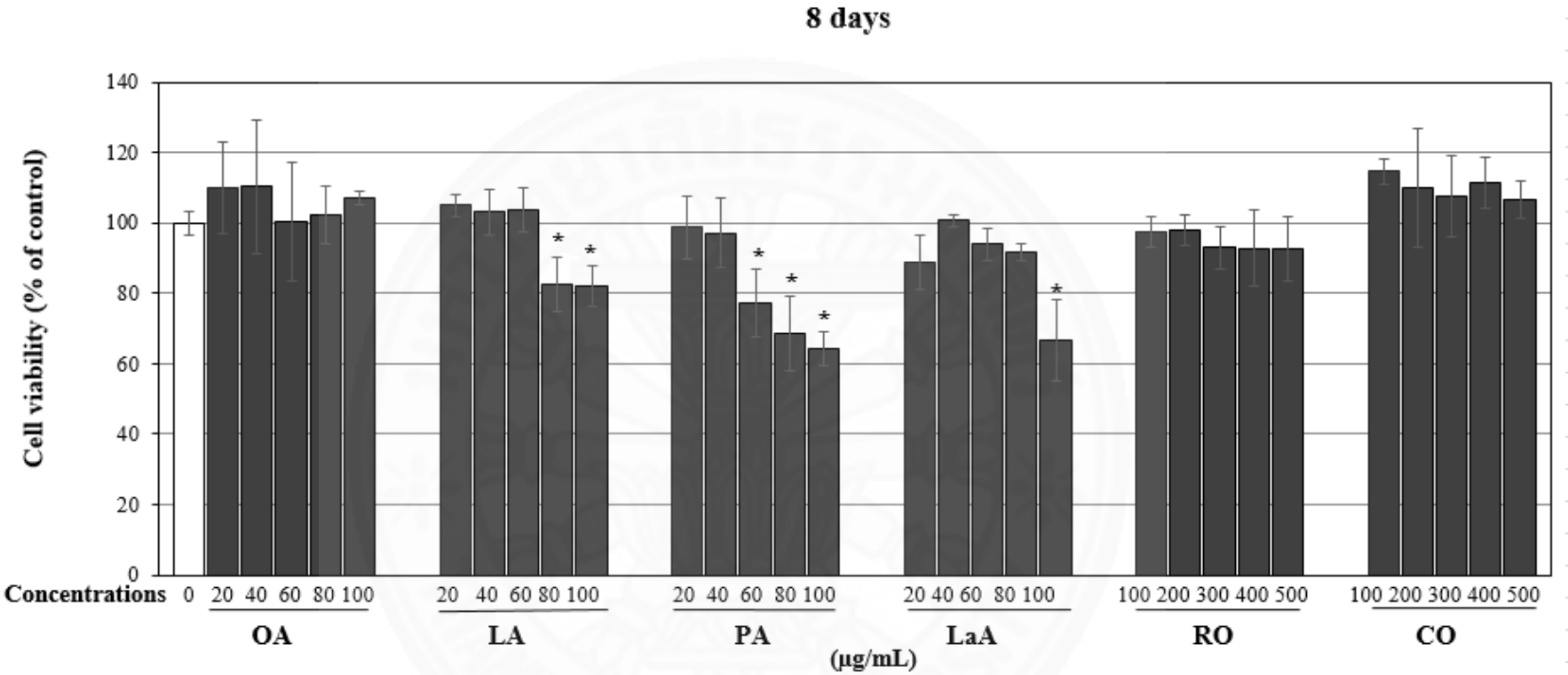

Figure A-2 Effect of fatty acids and edible oil on adipocyte cell viability in 8 days**.** The values are expressedbas mean ± S.D., (n=3). **p*<0.05 compared with control.

APPENDIX B

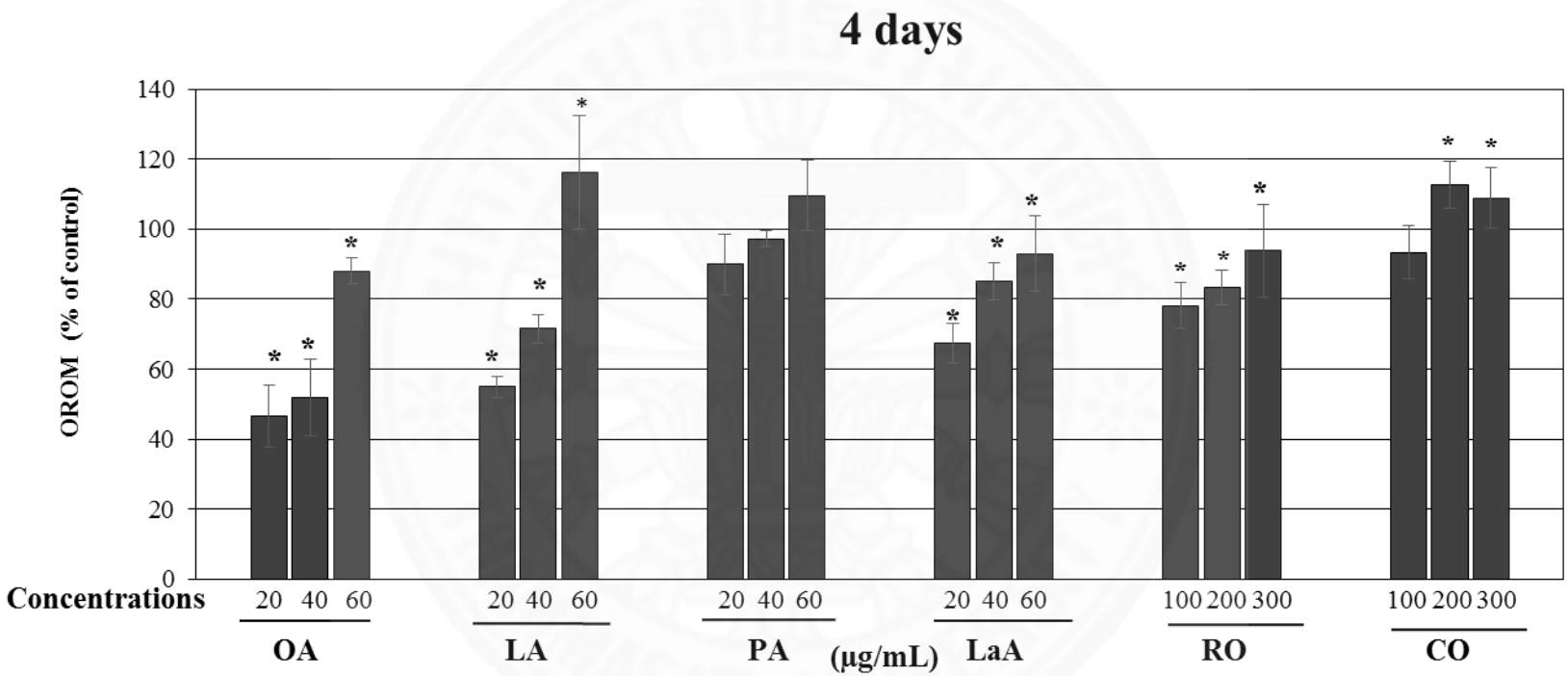

Figure B-1 Effects of fatty acids and edible oils on lipid accumulation (% of control) in 4 days. The values are expressed as mean ± S.D., (n=3). *p<0.05 compared with control.

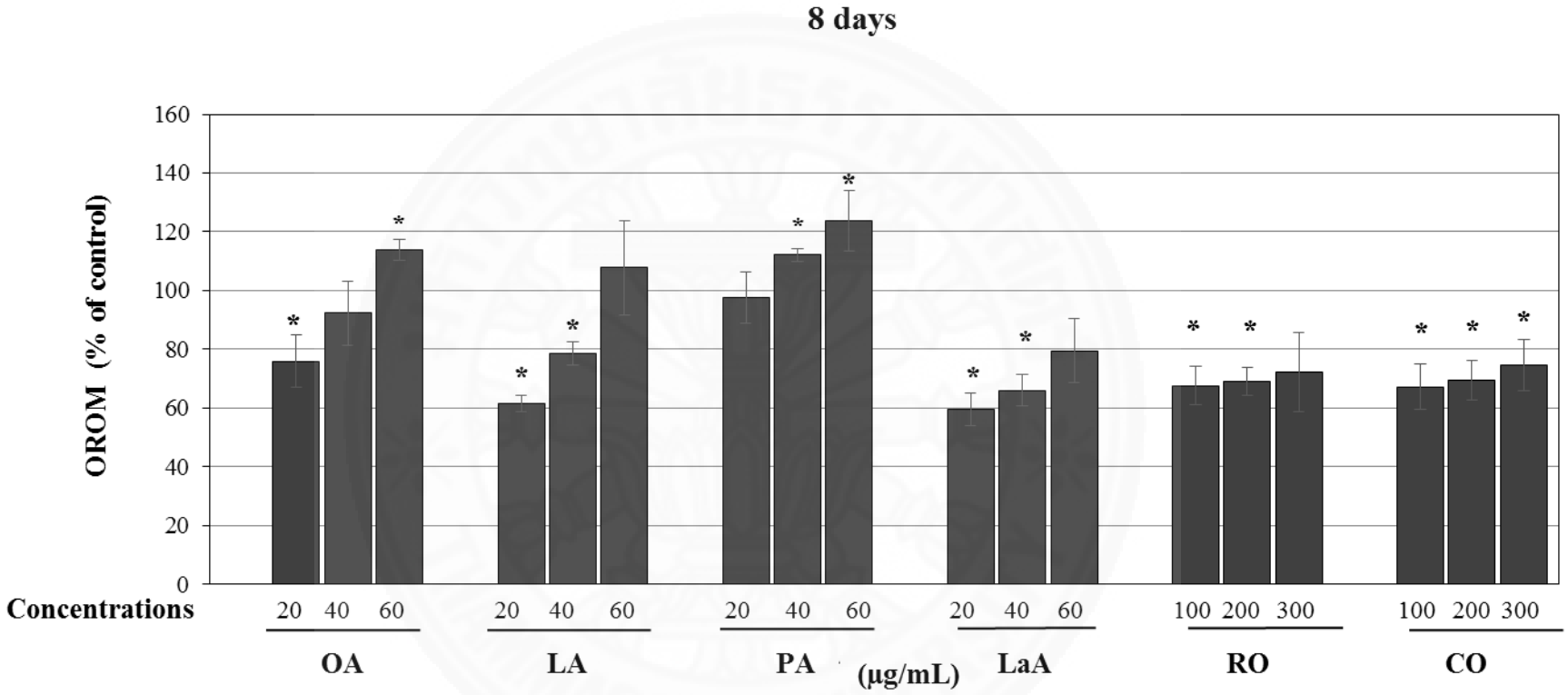

Figure B-2 Effects of fatty acids and edible oils on lipid accumulation (% of control) in 8 days. The values are expressed as mean ± S.D., (n=3). *p<0.05 compared with control.

APPENDIX C

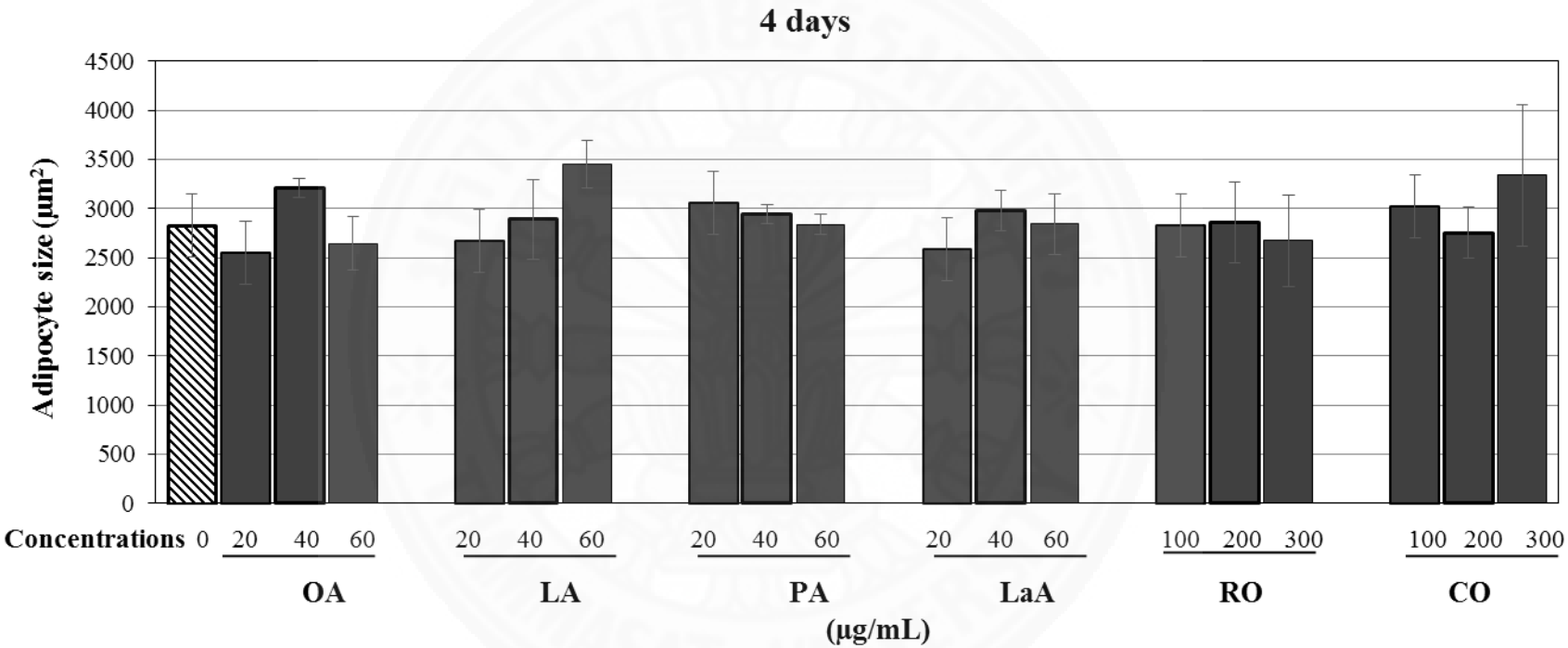

Figure C-1 Effects of fatty acids and edible oils on adipocyte size (μm) in 4 days. The values are expressed as mean ± S.D., (n=3). *p<0.05 compared with control

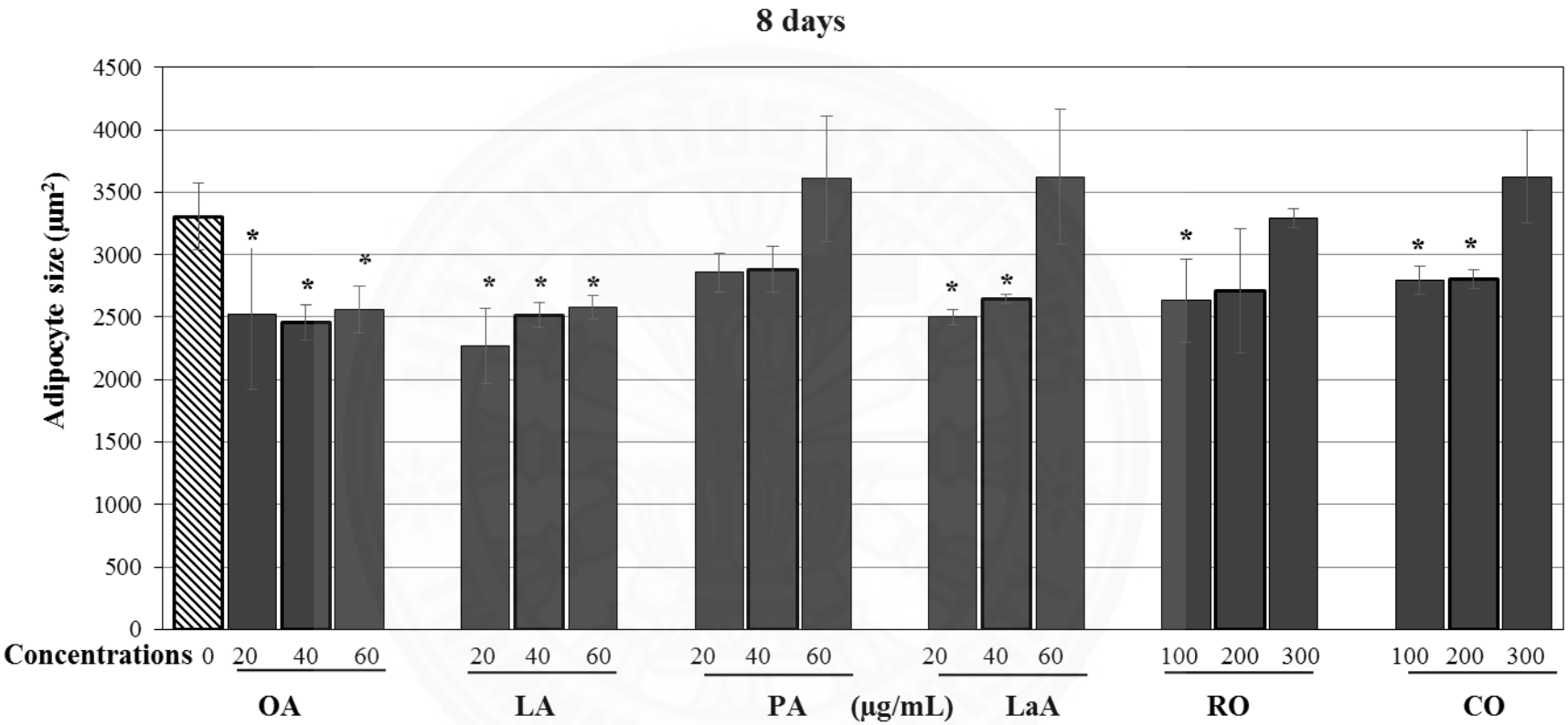

Figure C-2 Effects of fatty acids and edible oils on adipocyte size (µm) in 8 days. The values are expressed as mean ± S.D., (n=3). *p<0.05 compared with control.

APPENDIX D

RESULTS OF EXPERIMENTS

Table D-1 Effects of fatty acids and edible oils on adipocyte cells for 4 days

Treatments		The measurements (Mean ± S.D., n=3)					
		Cell viability (%)	Lipid accumulations (%)	Rounded cells (%)	Cell sizes (µm)	PPARγ expression (fold)	C/EBPα expression (fold)
Control		-	-	77.00 ±2.65	2826±318.76	1.01±0.02	1±0.01
OA	20 µg/mL	100.18±12.37	46.61±6.71 *	84.00 ±4.00	2546±487.22	0.89±0.02	1.04±0.05
	40 µg/mL	103.83±5.83	51.87±7.44 *	84.67 ±1.53	3214±971.74	0.94±0.1	0.92±0.11
	60 µg/mL	98.75±11.72	88.10±7.58 *	83.67 ±3.06	2641±271.53	-	-
	80 µg/mL	93.83±11.89	-	-	-	-	-
	100 µg/mL	87.00±20.57	-	-	-	-	-
LA	20 µg/mL	100.48±14.44	54.95±2.63 *	85.00 ±3.61	2673±152.18	0.78±0.04*	1.11±0.05*
	40 µg/mL	106.89±14.13	71.56±3.75 *	84.00 ±1.73	2889±402.46	0.77±0.08*	0.69±0.09*
	60 µg/mL	94.21±16.67	116.14±2.77 *	83.33 ±3.06	3448±242.13	-	-
	80 µg/mL	95.76±9.43	-	-	-	-	-
	100 µg/mL	77.52±15.44	-	-	-	-	-
PA	20 µg/mL	90.18±9.07	89.96±6.57	84.33 ±5.69	3057±135.31	0.95±0.17	1.10±0.15
	40 µg/mL	82.32±5.81	97.19±9.98	85.67 ±2.52	2942±97.21	0.94±0.16	1.11±0.13
	60 µg/mL	73.78±7.88*	109.66±7.53	89.00 ±2.00	2839±106.21	-	-
	80 µg/mL	71.00±1.41 *	-	-	-	-	-
	100 µg/mL	71.42±7.84 *	-	-	-	-	-
LaA	20 µg/mL	105.16±5.05	67.53±7.37 *	71.33 ±4.51	2588±359.53	0.42±0.12*	0.65±0.12
	40 µg/mL	88.26±5.08	85.12±12.69 *	75.33±11.02	2975±204.66	0.50±0.06*	0.69±0.03
	60 µg/mL	109.13±7.95	93.07±13.09*	75.67 ±5.13	2842±307.48	-	-
	80 µg/mL	95.26±13.38	-	-	-	-	-
	100 µg/mL	78.78±4.83	-	-	-	-	-
RO	100 µg/mL	99.93±4.37	78.09±2.34 *	84.00 ±4.36	2831±269.72	0.51±0.14*	1.10±0.04*
	200 µg/mL	97.85±4.36	83.43±3.91 *	83.67±3.06	2853±411.97	0.53±0.14*	1.21±0.08*
	300 µg/mL	96.31±6.13	93.83±6.64*	84.00 ±3.61	2674±465.46	-	-
	400 µg/mL	99.77±10.71	-	-	-	-	-
	500 µg/mL	97.58±9.29	-	-	-	-	-
CO	100 µg/mL	92.59±3.68	93.35±5.94	88.00 ±7.55	3024±347.72	0.72±0.05*	0.90±0.16*
	200 µg/mL	103.66±17.01	112.77±5.03*	74.33 ±6.43	2754±261.06	0.74±0.12*	0.92±0.12
	300 µg/mL	90.59±11.47	108.86±1.93*	78.00 ±2.65	3340±720.60	-	-
	400 µg/mL	107.21±7.17	-	-	-	-	-
	500 µg/mL	108.02±5.33	-	-	-	-	-

Table D-2 Effects of fatty acids and edible oils on adipocyte cells for 8 days.

Treatments		The measurements (Mean ± S.D., n=3)					
		Cell viability (%)	Lipid accumulations (%)	Rounded cells (%)	Cell sizes (μm)	PPARγ expression (fold)	C/EBPα expression (fold)
Control		100	-	82.67 ±5.77	3306±266.35	1.01±0.03	0.99±0.01
OA	20 μg/mL	110.05±13.04	75.90±9.00 *	89.33 ±4.04	2525±601.28	0.79±0.05*	0.96±0.16*
	40 μg/mL	110.31±18.99	92.23±10.87	90.67 ±4.51	2455±142.39	0.72±0.03*	0.56 ±0.05*
	60 μg/mL	100.36±16.94	113.89±3.65*	89.33 ±3.79	2558±187.46	-	-
	80 μg/mL	102.29±8.19	-	-	-	-	-
	100 μg/mL	107.11±1.78	-	-	-	-	-
LA	20 μg/mL	104.95±3.23	61.56±2.91 *	91.67 ±2.52	2271±301.38*	0.99±0.15	0.67±0.11*
	40 μg/mL	102.98±6.70	78.58±4.01 *	91.00 ±4.00	2517±100.04*	0.71±0.05*	0.41±0.23*
	60 μg/mL	103.75±6.11	107.70±16.19	90.67 ±4.51	2578±96.57*	-	-
	80 μg/mL	82.58±7.68*	-	-	-	-	-
	100 μg/mL	82.08±5.68*	-	-	-	-	-
PA	20 μg/mL	98.68±9.07	97.55±8.71	87.00±2.65	2857±151.79	0.94±0.2	0.96±1.92
	40 μg/mL	97.17±9.75	112.09±2.25*	86.00±3.46	2884±184.71	0.97±0.16	0.97±1.63
	60 μg/mL	77.17±9.44 *	123.79±10.20 *	92.00±3.00	3607±503.87	-	-
	80 μg/mL	68.44±10.68 *	-	-	-	-	-
	100 μg/mL	64.38±4.76 *	-	-	-	-	-
LaA	20 μg/mL	88.80±7.79	59.37±5.58 *	78.33 ±4.73	2503±61.70*	0.38±0.01*	0.49±0.05*
	40 μg/mL	100.74±1.66	65.96±5.36 *	84.67 ±2.31	2645±33.72*	0.55±0.06*	0.37±0.05*
	60 μg/mL	93.87±4.55	79.51±10.86	83.67 ±2.52	3624±538.24	-	-
	80 μg/mL	91.57±2.38	-	-	-	-	-
	100 μg/mL	66.64±11.48 *	-	-	-	-	-
RO	100μg/mL	97.29±4.37	67.67±6.56 *	84.67 ±2.08	2631±331.99	0.82±0.19	1.04±0.09
	200 μg/mL	97.90±4.36	69.03±4.89 *	90.33 ±3.79	2712±499.92	1.03±0.10	1.07±0.06
	300 μg/mL	92.95±6.13	72.19±13.34	86.33 ±1.53	3291±75.79	-	-
	400 μg/mL	92.75±10.71	-	-	-	-	-
	500 μg/mL	92.71±9.27	-	-	-	-	-
CO	100 μg/mL	114.59±3.68	67.17±7.69 *	78.67 ±5.51	2796±110.76*	0.54±0.04*	0.37±0.06*
	200 μg/mL	110.00±17.01	69.27±6.73 *	75.33 ±9.45	2806±83.70*	0.62±0.49*	0.49±0.16*
	300 μg/mL	107.50±11.47	74.66±8.69 *	75.00 ±1.73	2763±370.60	-	-
	400 μg/mL	111.22±7.17*	-	-	-	-	-
	500 μg/mL	106.65±5.33*	-	-	-	-	-

APPENDIX E

REAGENTS FOR LABORATORY EXPERIMENTS

10X Phosphate buffered saline (PBS)

NaCl	38.25 g
Na_2HPO	44.97 g
KH2PO4	2.04 g
Distilled water	450 mL

Adjust pH 7.4 and volume to 500 mL and store at 4 ºC.

1X Phosphate buffered saline (PBS)

10X PBS	50 mL
Distrilled water	450 mL

The solution was stored at at 4 ºC.

DMEM + 10% CS (500 mL)

CS	50 mL
Sodium pyruvate	5 mL
1X penicillin/streptomycin	5 mL
DMEM solution	440 mL

DMEM + 10% FBS (500 mL)

FBS	50 mL
Sodium pyruvate	5 mL
1X penicillin/streptomycin	5 mL
DMEM solution	440 mL

IBMX solution

Dissolve IBMX in a solution made of 0.5N KOH to a final concentration of 0.0115 g/mL.

Insulin stock solution

167 µM (1 mg/mL) in 0.02 M HCL filter sterilized through 0.22 mm filter and store at -20 ºC for long term, 4 ºC for short term.

Dexamethasone stock solution

Freezer stock: 10 mM of Dexamethasone in 100 % ethanol
(store at -20 ºC)
Working stock: Dilute freezer stock to 1mM in PBS
Filter sterilize and store at 4 ºC

MDI induction media (10 mL)

10% FBS/DMEM	
IBMX	1:100

Insulin media (10 mL)

10% FBS/DMEM	
Insulin	1:1000

Oil red O stock (100 mL)

Oil red O	0.5 g
2-Propanol	100 mL

The solution was mixed and store at 4 ºC

Oil red O working solution (10 mL)

Oil red O stock	6 mL
Disstrill water	4 mL

The solution was filtrated with Whatman filter paper

APPENDIX F

Coconut milk nutritional information

Nutrient	Unit	1Value per 100 g	1 cup = 240.0g
Proximates			
Water	g	67.62	162.29
Energy	kcal	230	552
Energy	kJ	962	2309
Protein	g	2.29	5.5
Total lipid (fat)	g	23.84	57.22
Ash	g	0.72	1.73
Carbohydrate, by difference	g	5.54	13.3
Fiber, total dietary	g	2.2	5.3
Sugars, total	g	3.34	8.02
Minerals			
Calcium, Ca	mg	16	38
Iron, Fe	mg	1.64	3.94
Magnesium, Mg	mg	37	89
Phosphorus, P	mg	100	240
Potassium, K	mg	263	631
Sodium, Na	mg	15	36
Zinc, Zn	mg	0.67	1.61
Copper, Cu	mg	0.266	0.638
Manganese, Mn	mg	0.916	2.198
Selenium, Se	mg	6.2	14.9
Vitamins			
Vitamin C, total ascorbic acid	mg	2.8	6.7
Thiamin	mg	0.026	0.062
Riboflavin	mg	0	0
Niacin	mg	0.76	1.824
Pantothenic acid	mg	0.183	0.439
Vitamin B-6	mg	0.033	0.079
Folate, total	mg	16	38

Nutrient	Unit	1Value per 100 g	1 cup = 240.0g
Folic acid	mg	0	0
Folate, food	mg	16	38
Folate, DFE	mg	16	38
Choline, total	mg	8.5	20.4
Vitamin B-12	mg	0	0
Vitamin B-12, added	mg	0	0
Vitamin A, RAE	mg	0	0
Retinol	mg	0	0
Carotene, beta	mg	0	0
Carotene, alpha	mg	0	0
Cryptoxanthin, beta	mg	0	0
Vitamin A, IU	IU	0	0
Lycopene	mg	0	0
Lutein + zeaxanthin	mg	0	0
Vitamin E (alpha-tocopherol)	mg	0.15	0.36
Vitamin E, added	mg	0	0
Vitamin D (D2 + D3)	mg	0	0
Vitamin D	IU	0	0
Vitamin K (phylloquinone)	mg	0.1	0.2
Lipids			
Fatty acids, total saturated	g	21.14	50.736
4:00	g	0	0
6:00	g	0.136	0.326
8:00	g	1.67	4.008
10:00	g	1.327	3.185
12:00	g	10.576	25.382
14:00	g	4.176	10.022
16:00	g	2.021	4.85
18:00	g	1.234	2.962
Fatty acids, total monounsaturated	g	1.014	2.434
16:1 undifferentiated	g	0	0

Nutrient		Unit	1Value per 100 g	1 cup = 240.0g
18:1 undifferentiated		g	1.014	2.434
	20:01	g	0	0
22:1 undifferentiated		g	0	0
Fatty acids, total polyunsaturated		g	0.261	0.626
18:2 undifferentiated		g	0.261	0.626
18:3 undifferentiated		g	0	0
	18:04	g	0	0
20:4 undifferentiated		g	0	0
20:5 n-3 (EPA)		g	0	0
22:5 n-3 (DPA)		g	0	0
22:6 n-3 (DHA)		g	0	0
Cholesterol		mg	0	0
Phytosterols		mg	1	2
Amino Acids				
Tryptophan		g	0.027	0.065
Threonine		g	0.083	0.199
Isoleucine		g	0.09	0.216
Leucine		g	0.17	0.408
Lysine		g	0.101	0.242
Methionine		g	0.043	0.103
Cystine		g	0.045	0.108
Phenylalanine		g	0.116	0.278
Tyrosine		g	0.071	0.17
Valine		g	0.139	0.334
Arginine		g	0.376	0.902
Histidine		g	0.053	0.127
Alanine		g	0.117	0.281
Aspartic acid		g	0.224	0.538
Glutamic acid		g	0.524	1.258
Glycine		g	0.108	0.259
Proline		g	0.095	0.228

Nutrient	Unit	1Value per 100 g	1 cup = 240.0g
Serine	g	0.118	0.283
Other			
Alcohol, ethyl	g	0	0
Caffeine	mg	0	0
Theobromine	mg	0	0
Other			
Caffeine	mg	0	0

Coconut milk nutritional information

Nutrient	Unit	1Value per 100 g	1 cup = 218.0 g
Proximates			
Water	g	0.03	0.07
Energy	kcal	862	1945
Protein	g	0	0
Total lipid (fat)	g	99.06	215.95
Carbohydrate, by difference	g	0	0
Fiber, total dietary	g	0	0
Sugars, total	g	0	0
Minerals			
Calcium, Ca	mg	1	2
Iron, Fe	mg	0.05	0.11
Magnesium, Mg	mg	0	0
Phosphorus, P	mg	0	0
Potassium, K	mg	0	0
Sodium, Na	mg	0	0
Zinc, Zn	mg	0.02	0.04
Vitamins			
Vitamin C, total ascorbic acid	mg	0	0
Thiamin	mg	0	0
Riboflavin	mg	0	0
Niacin	mg	0	0
Vitamin B-6	mg	0	0
Folate, DFE	mg	0	0
Vitamin B-12	mg	0	0
Vitamin A, RAE	mg	0	0
Vitamin A, IU	IU	0	0
Vitamin E (alpha-tocopherol)	mg	0.11	0.24
Vitamin D (D2 + D3)	mg	0	0

Nutrient	Unit	1Value per 100 g	1 cup = 218.0 g
Vitamin D	IU	0	0
Vitamin K (phylloquinone)	mg	0.6	1.3
Lipids			
Fatty acids, total saturated	g	82.475	179.796
Fatty acids, total monounsaturated	g	6.332	13.804
Fatty acids, total polyunsaturated	g	1.702	3.71
Fatty acids, total trans	g	0.028	0.061

BIOGRAPHY

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