การศึกษาฤทธิ์ลดการสะสมและการสลายไขมัน ในเซลล์ไขมันใต้ผิวหนังและเซลล์ไขมันช่องท้อง โดย กรดไลโปอิก กรดไลโปอิกที่ห่อหุ้มด้วยอนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตร และสารคาเฟอีน

นางสาวสุนิสา แก้วแดง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
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Evaluation of lipid accumulation and lipolysis activity in human subcutaneous adipocytes and human visceral adipocytes by α-lipoic acid, α-lipoic acid-incorporated $CaCO_3$ nanoparticles and caffeine.

Miss Sunisa Kaewdang

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
Faculty of Medicine
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Thesis Title	Evaluation of lipid accumulation and lipolysis activity in human subcutaneous adipocytes and human visceral adipocytes by α-lipoic acid, α-lipoic acid-incorporated $CaCO_3$ nanoparticles and caffeine.
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Field of Study	Medical Science
Thesis Advisor	Assistant ProfessorAmornpun Sereemaspun, M.D., Ph.D.
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(Visnu Lohsiriwat, M.D.)

สุนิสา แก้วแดง : การศึกษาฤทธิ์ลดการสะสมและการสลายไขมัน ในเซลล์ไขมันใต้ผิวหนังและเซลล์ไขมันช่องท้อง โดยกรดไลโปอิก กรดไลโปอิกที่ห่อหุ้มด้วยอนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตร และสารคาเฟอีน (Evaluation of lipid accumulation and lipolysis activity in human subcutaneous adipocytes and human visceral adipocytes by α-lipoic acid, α-lipoic acid-incorporated $CaCO_3$ nanoparticles and caffeine.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ.อมรพันธุ์ เสรีมาศพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร.นริศร คงรัตนโชค, 53 หน้า.

ในปัจจุบันได้มีการศึกษาค้นคว้าและวิจัยเกี่ยวกับสารสกัดจากธรรมชาติ ที่มีคุณสมบัติลดการสะสมของไขมันอันเป็นสาเหตุหนึ่งของการเกิดโรคอ้วน อันได้แก่ คาเฟอีนและกรดไลโปอิก เนื่องจากกรดไลโปอิกมีชีวปริมาณการออกฤทธิ์ที่ต่ำ จึงทำให้มีการนำเอาองค์ความรู้ทางด้านนาโนเทคโนโลยีมาประยุกต์ใช้ เพื่อเพิ่มประสิทธิภาพในการลดการสะสมไขมัน แต่อย่างไรก็ตาม ยังไม่มีงานวิจัยใดที่ศึกษาฤทธิ์ลดการสะสมของไขมันโดยใช้ส่วนผสมของกรดไลโปอิกที่ห่อหุ้มด้วยอนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตรและคาเฟอีน ดังนั้นในงานวิจัยนี้จึงมีวัตถุประสงค์ที่จะทำการศึกษาฤทธิ์ลดการสะสมและการสลายไขมัน เพื่อลดกระบวนการอันก่อให้เกิดการพัฒนาไปเป็นโรคอ้วน รวมทั้งได้ทำการศึกษาเปรียบเทียบประสิทธิภาพระหว่างเซลล์ไขมันใต้ผิวหนังและเซลล์ไขมันในช่องท้อง โดยใช้สารที่มีส่วนผสมของกรดไลโปอิกที่ห่อหุ้มด้วยอนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตรและคาเฟอีนที่ได้จากกากกาแฟ จากผลการทดลองพบว่าการผสมกันระหว่างกรดไลโปอิกที่ห่อหุ้มด้วยอนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตรและคาเฟอีนสามารถช่วยลดการสะสมไขมันในเซลล์ไขมันใต้ผิวหนังได้ แต่ไม่สามารถแสดงถึงประสิทธิภาพเสริมฤทธิ์การทำงานของสารทั้งสองชนิดในการลดการสะสมไขมันในเซลล์ไขมันใต้ผิวหนัง และการสลายของไขมัน ทั้งในเซลล์ไขมันใต้ผิวหนังและเซลล์ไขมันในช่องท้อง อีกทั้งจากผลการทดลองยังแสดงให้เห็นว่าเซลล์ไขมันในช่องท้องมีประสิทธิภาพในการสลายไขมันได้ดีกว่าเซลล์ไขมันใต้ผิวหนัง นอกจากนี้ในงานวิจัยยังพบว่า กรดไลโปอิกที่ห่อหุ้มด้วยอนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตร มีความสามารถในการลดการสะสมของไขมันได้ดีกว่าสารคาเฟอีนและกรดไลโปอิกเพียงอย่างเดียว ข้อมูลเหล่านี้แสดงให้เห็นว่า อนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตรสามารถนำมาใช้เป็นทางเลือกหนึ่งในการเพิ่มประสิทธิภาพการทดลองเพื่อลดการสะสมไขมันในเซลล์ไขมันใต้ผิวหนังและเซลล์ไขมันในช่องท้องของมนุษย์

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5874106230 : MAJOR MEDICAL SCIENCE
KEYWORDS: CAFFEINE / ALPHA-LIPOIC ACID / CALCIUM CARBONATE NANOPARTICLES / LIPID ACCUMULATION / LIPOLYSIS

SUNISA KAEWDANG: Evaluation of lipid accumulation and lipolysis activity in human subcutaneous adipocytes and human visceral adipocytes by α-lipoic acid, α-lipoic acid-incorporated $CaCO_3$ nanoparticles and caffeine.. ADVISOR: ASST. PROF.AMORNPUN SEREEMASPUN, M.D., Ph.D., CO-ADVISOR: NARISORN KONGRUTTANACHOK, Ph.D., 53 pp.

Recently, much attention has been paid to natural plant-derived compounds as an alternative strategy for developing anti-obesity agents with minimal detrimental effects. Among various natural compounds, caffeine (1, 3, 7-trimethyl xanthine) and alpha lipoic acid (1, 2-dithiolane-3-pentanoic acid; LA) are the natural agents which show a promising result in decreasing fat deposit. Moreover, we have improved efficiencies of LA by $CaCO_3$ NPs because LA had low bioavailability. In the present study, we investigated the effect of combination between caffeine and LA-$CaCO_3$ NPs in lipid accumulation reduction and lipolysis stimulation in human subcutaneous adipocytes and human visceral adipocytes. Our results showed that intracellular lipid accumulation in human subcutaneous adipocytes treated with the combination (5 mM caffeine and 600 μM LA-$CaCO_3$ NPs) were not significantly different from in the groups treated with LA, LA-$CaCO_3$ NPs, and caffeine while the lipid levels in human visceral adipocytes treated with the combination were lower than those in the groups using caffeine and LA alone. Lipolysis was lower in both human subcutaneous adipocytes and human visceral adipocytes that are treated with the combination than that in the groups treated with each alone. These results indicated that combination between caffeine and LA-$CaCO_3$ NPs were not reduced lipid accumulation in subcutaneous adipocytes but they can reduce lipid accumulation in visceral adipocytes. In term lipolysis, the combination were not stimulated lipolysis in both cells. Moreover, we have established a novel LA-$CaCO_3$ NPs that enhance reduction of lipid accumulation and stimulation of lipolysis in both cells. These results propose that $CaCO_3$ NPs has potential effect for using an alternative method for subcutaneous adipocytes and visceral adipocyte lipid reduction.

Field of Study: Medical Science
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I

Introduction

Obesity is one of the global health problems which links to cardiovascular and metabolic diseases (1). It occurs as a result of imbalance between energy intake and energy expenditure that ultimately leads to the excess energy stored as triacylglycerol (TG) in adipocytes (2). The pathological changes of adipocytes; both adipocyte hyperplasia (increase in cell number) and adipocytes hypertrophy (increase in cell size), result in elevated triglyceride content in plasma and consequently to metabolic dysregulation (3-5). Therefore, it is important to develop effective strategies for obesity prevention and treatment.

Lifestyle change and increase in physical activity have been considered for a long time as the most useful means for weight reduction. Even though these strategies are safe and effective, not so many people succeed in reducing bodyweight by these methods. Nowadays, pharmaceutical drug become a common method for obesity treatment; nonetheless, this intervention is often associated with negative side effects and rebound weight gain when discontinue the medications (6). Recently, much attention has been paid to natural plant-derived compounds as an alternative strategy for developing anti-obesity agents with minimal detrimental effects. There are several natural products such as isolated and crude extracted compounds from plants that show anti-obesity properties. Among various natural compounds, caffeine (1, 3, 7-trimethyxanthine) (7) and alpha lipoic acid (1, 2-dithiolane-3-pentanoic acid; LA) (8) are the natural agents which show promising result in decreasing fat deposit. Caffeine is a xanthine alkaloid of plant found in coffee, tea, chocolate, cola and soft drinks (9). Previous studies showed that caffeine suppresses intracellular lipid accumulation in 3T3-L1 mature adipocytes(10), stimulate lipolysis in adipocytes (11), reduce food intake and increase energy expenditure (12). In similar to caffeine, LA has shown beneficial effects on obesity and diabetes by reducing body weight and adiposity (13). Moreover,

LA was reported to be capable of decreasing food intake in rodents and human.(14, 15)

Although LA and caffeine could be used as potential bioactive compounds for reducing fat deposition, the effectiveness of adiposity reduction by using LA is limited due to poor bioavailability (16, 17). To overcome this issue, a novel drug delivery system is needed for improving cellular targeting. Among many types of delivery system, calcium carbonate nanoparticles ($CaCO_3$ NPs) are one of the effective carriers which were used for delivering drug to targeted area. $CaCO_3$ NPs were applied in various biomedical application due to their longer biodegradation time such as targeted drug delivery, biosensor, and protein encapsulation (18). In addition, the unique characteristic of $CaCO_3$ NPs are that these NPs disintegrate slowly at normal physiological pH while the NPs are broken up as a faster rate and release more drug in acidic pH (19). This NPs can be beneficial for delivering drug to adipocyte due to average intracellular pH in adipocytes is acidic.

Even though caffeine and LA are known to be lipolytic agents, the result of fat reduction by the combination of these drugs has not been well studied. In this study, we combined caffeine and LA-incorporated with $CaCO_3$ NPs and investigated the effect of our synthetic compound on reduce fat reduction and lipolysis stimulation in adipocytes. Primary human adipocytes from both visceral and subcutaneous fat were used in order to compare the difference in fat reduction. We also tested whether the combination of LA and $CaCO_3$ NPs would enhance adiposity reduction. Our approach would determine efficiency of fat lessening in visceral and subcutaneous adipocytes and would address an alternative strategy for reduce fat deposition.

Research question

Does the effect of caffeine and LA-CaCO3 NPs combination improve lipid accumulation and lipolysis in human subcutaneous adipocytes and human visceral adipocytes?

Objective

To evaluate the effects of combination between caffeine and LA-$CaCO_3$ NPs in lipid accumulation reduction and lipolysis stimulationin human subcutaneous adipocytes and human visceral adipocytes.

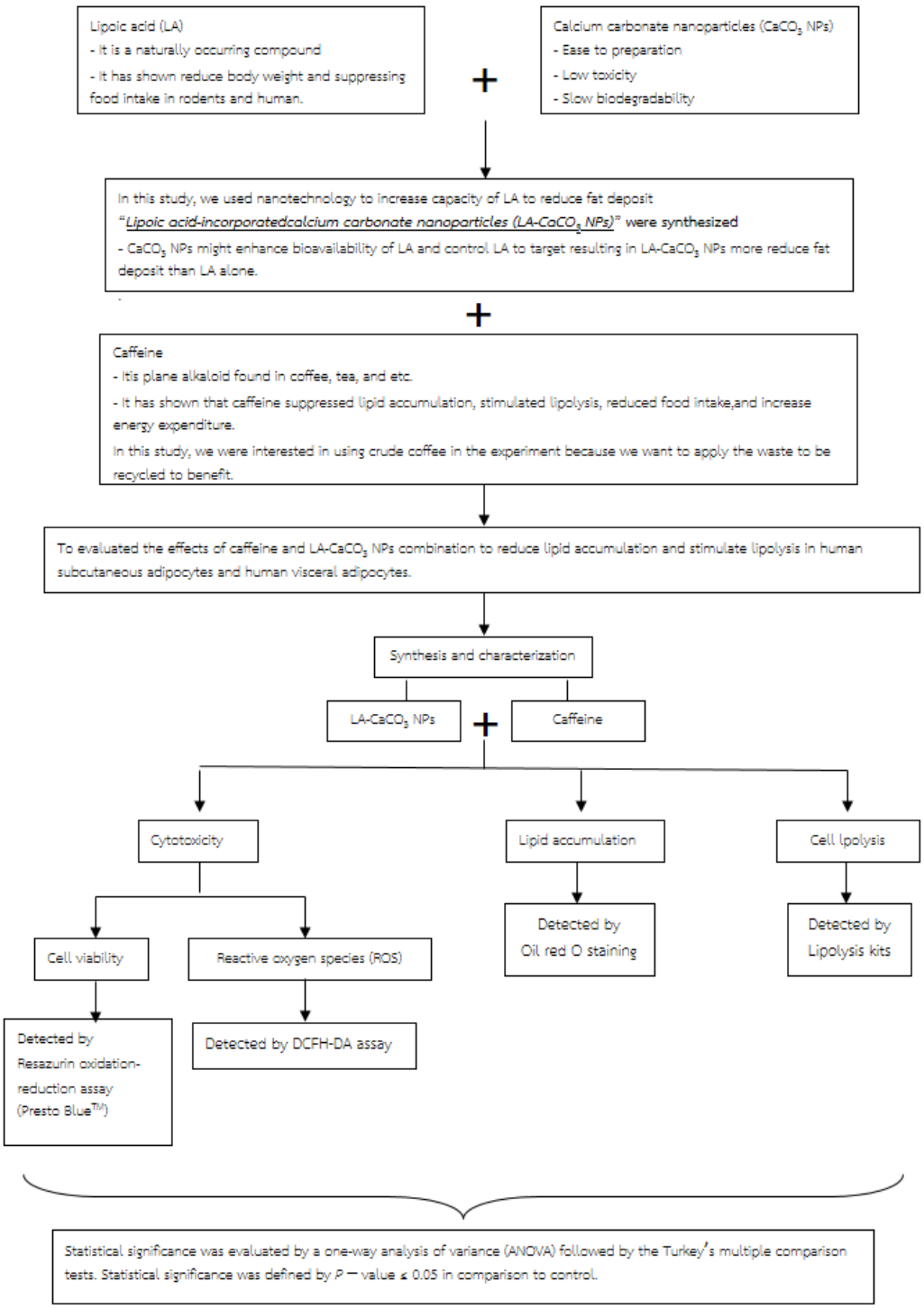
Hypothesis

The combination between caffeine and LA-$CaCO_3$ NPs reduce lipid accumulation and stimulate lipolysis in human subcutaneous adipocytes and human visceral adipocytes.

Expected outcome of this study

The combination between caffeine and LA-$CaCO_3$ NPs has a beneficial impact to increase lipolysis and decrease lipid accumulation in human subcutaneous adipocytes and human visceral adipocytes.

Conceptual framework



CHAPTER II

LITERATURE REVIEW

Obesity

Obesity is a one of global health issues, because it has been associated with metabolic syndrome, type 2 diabetes, heart disease, cancer, nonalcoholic fatty liver disease, sleep apnea, and atherosclerosis (20-23). The consequence of obesity is concerned in term of the modifications in adipose tissue (24). It occurres as a result of imbalance between energy intake and energy expenditure that ultimately leads to the excess energy stored as triacylglycerol (TG) in adipocytes (2). The pathological changes of adipocytes; both adipocyte hyperplasia (increase in cell number) and adipocytes hypertrophy (increase in cell size), result elevated triglyceride content in plasma and consequently metabolic dysregulation (3-5). Therefore, it is important to develop effective strategies for obesity prevention and treatment.

Primary preadipocytes

Human primary preadipocytes represent a highest model for study of adipocyte metabolism pathway and mechanisms controlling adipogenesis (25). The adipogenesis is process of human primary preadipocytes differentiate into mature adipocytes (26). Adipocyte differentiation is a multi-step process concerning a cascade of transcription factors for main protein that gene expression and lead to adipocyte evolution (27). It is well known that the transcription factors, which the essential determinants of adipocyte fate such as peroxisome proliferator-activated receptor gamma (PPAR-**γ**), sterol regulatory element binding protein (SREBP), and CCAAT/enhancer-binding proteins (C/EBPs) (28). Moreover, the PPAR-**γ**2 is highly in adipose tissue and functions to support adipocyte differentiation and triacylglycerols storage (29).

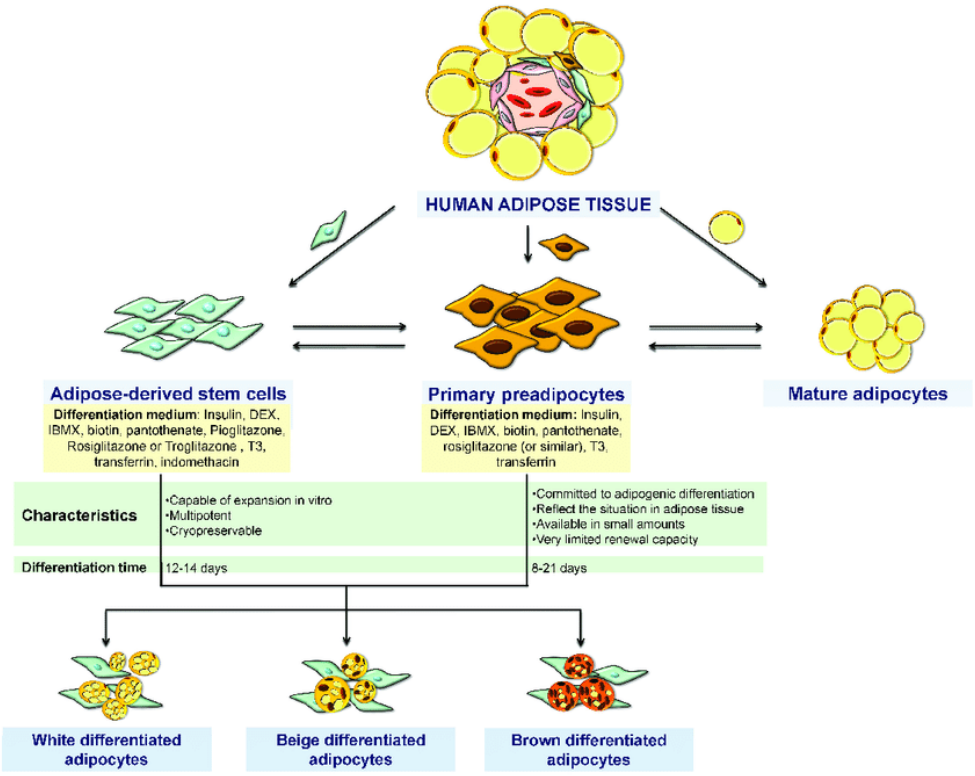


Figure 1 Human models to study the adipogenesis process. DEX, dexamethasone; IBMX, 3-isobutyl-1-methylxanthine; T3, triiodothyronine (27).

Adipose tissue

Adipocytes are derived from mesenchymal stem cells and represent a specific component of adipose tissue. Adipose tissue, or fat is a loose connective tissue. Its ability is maintaining homeostasis by synthesis and storage of triglyceride (30, 31) and production and release of adipokines and cytokines(32). Besides, the distribution of adipose tissue in the body depends on age, gender, sex hormone, and genetics (33). Adipose tissue is commonly characterized into three types such as white adipose tissue, brown adipose tissue, and beige or brite adipose tissue(34). In this study, we focus on white adipose tissue. White adipose tissue functions primarily as a main regulatory centre of a site for fuel storage and energy metabolism. Moreover, it controls other biological processes including blood pressure control, immunity, blood coagulation, and angiogenesis. Moreover, structural properties and functions of white adipose tissue in each area are different. The major types of white adipose tissue depots are visceral adipose tissue and subcutaneous adipose tissue (33).

Visceral adipose tissue

Visceral adipose tissue can be found in depots surrounding internal organs such as abdominal locations are the omental depot, retroperitoneal adipose around the kidney, the mesenteric depot about the intestines, and gonadal (33, 35). The amount of visceral adipose tissue is 5-8% of total fat in women and 5-20% of total fat in men (36). Moreover, visceral adipose accumulation is associated with a trend to hyperinsulinemia, hyperglycemia, hypertriglyceridemia, and increased apolipoproteins B-rich lipoproteins, which are features of the insulin resistance syndrome. Furthermore, higher levels of the visceral fat area had higher tendency TG levels, lower high-density lipoprotein cholesterol values, and plasma cholesterol (31). Thus, the anatomical location of visceral adipose tissue cloud is one potential reason for increased cardio-metabolic risk linked with visceral obesity (37). Recently, studies are interested in a

functional compound of plant source that inhibits adipogenesis and stimulates lipolysis on human visceral adipocyte. For example, the previous report in 2011 established that resveratrol can modulate genes that are associated with lipid metabolism in human visceral adipocytes (38).

In 2015, Elena Pomari and coworkers demonstrated that *Rhodiola rosea* extract can reduce triglyceride incorporation during maturation via down-regulation of PPAR**γ**, the master regulator of adipogenesis and FABP4 on primary human visceral adipocytes (39).

In 2016, Bruno Stefanon and colleagues determined that hydroxytyrosol, a component of olive oil can reduce triglyceride accumulation and stimulate lipolysis in human primary visceral adipocytes during differentiation via down-regulating of gene promoting in adipogenesis and increase gene inhibiting of adipogenesis (40).

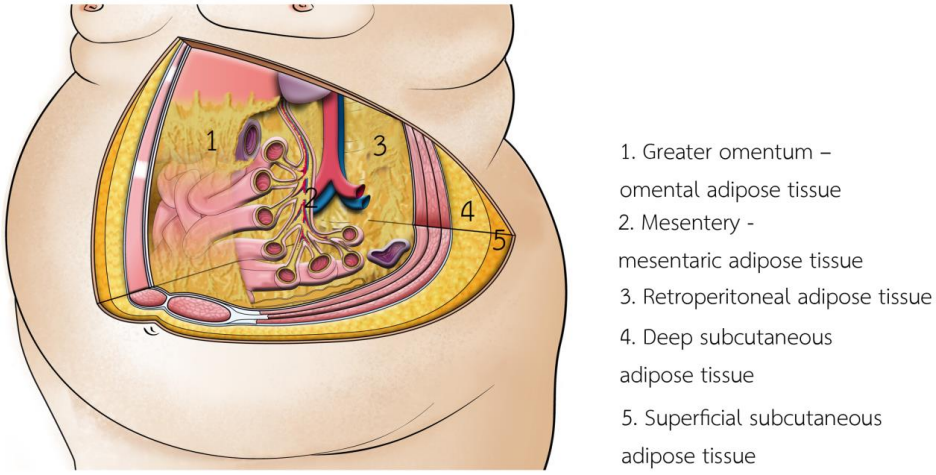


Figure 2 Representation of the anatomical localization of the main abdominal adipose tissue depots (41).

Subcutaneous adipose tissue

The subcutaneous adipose tissue is circulated over the body's surface in the hypodermal layer of the skin. About 80% of all body fat is in the subcutaneous area (31). Furthermore, a thickness of the subcutaneous adipose tissue varies in each area such as a thinner subcutaneous adipose tissue in space of lax skin including eyelids and scrotum and a thicker hypodermis in the hip and buttocks (30). Subcutaneous adipose tissue is important for insulation, thermal regulation, protection form mechanical injuries, and provision or energy (30). Moreover, previous research has compared between visceral adipose tissue and subcutaneous adipose tissue. They found that visceral adipocytes are more metabolically active, more insulin resistant, and more sensitive to lipolysis than subcutaneous adipocytes (31, 33). Moreover, they found that subcutaneous adipose tissue is more athirst in the absorption of circulating free fatty acids and triglyceride than visceral adipose tissue (31). When fat storage ability of subcutaneous adipose tissue is overdone fat is stored outside this tissue in ectopic fat depots which are important contributors, to the obesity-associated inflammation and insulin resistance (5). Recently, studies was interested in the functional compound of plant source that inhibits adipogenesis and stimulates lipolysis on human subcutaneous adipocyte. For example, the previous report in 2009 investigated the effect of white tea extract to influence lipolysis and adipogenesis on human subcutaneous preadipocytes and adipocytes. Their data showed that white tea extract significantly decreased triglyceride absorption during adipogenesis, stimulated lipolytic activity in adipocyte, and inhibited adipogenesis through by decreasing PPARγ, C/EBPα, ADD1/SREBP-1c and C/EBPδ mRNA levels. Thus, they suggested that white tea extract is a natural source that effectively inhibits adipogenesis and activates lipolysis on human subcutaneous preadipocytes and adipocytes (42).

In Marta Fernández-Galilea and co-worker investigated the effect of LA on triglyceride accumulation and lipogenesis in human subcutaneous adipocytes from

overweight/obese subjects. The result showed that LA down-regulated triglyceride content by inhibiting *de novo* lipogenesis and fatty acid esterification via the stimulation of AMPK signaling pathway in human subcutaneous adipocytes from overweight/obese subjects (43).

Nanotechnology

Nanotechnology is related to the development and use of the systems and materials at the nanoscale size (1-100 nm) (44, 45). It has played important role in various fields of science and contributed to the progress in chemistry, physics, engineering, pharmaceutical industries, and medicine (45). In medical, nanotechnology applies to nanomedicine applications such as medical diagnostics (46). Moreover, nanoparticles (NPs) are widely investigated for target and control drug release (47). They can act as vessels for drugs since they are small enough to reach almost every regions of the human organism (45).

Drug delivery system can expand therapeutic efficacy and reduce side effects of therapeutic agents by focusing them at specific target sites in the body. In recent years, researchers have become increasingly interested in calcium carbonate ($CaCO_3$) nanoparticles (NPs) in controlling the release of drug. There are several advantages to use to control drug delivery, biosensing, tissue engineering applications (48) and protein encapsulation in pharmaceutics, for example, their longer biodegradation time. Certainly, $CaCO_3$ NPs are slowly degraded. Thus drugs for longer times after administration (49). Moreover, the pH-sensitive properties of $CaCO_3$ NPs provide a new possibility for targeting and controlling the drug delivery. These $CaCO_3$ NPs decompose slowly at pH 7.4 while they displayed a faster decomposition and release of drug at pH <6.5. Furthermore, $CaCO_3$ NPs have availability, slow biodegradability, low cost, low toxicity, and the most abundant natural materials such as bone and teeth (18).

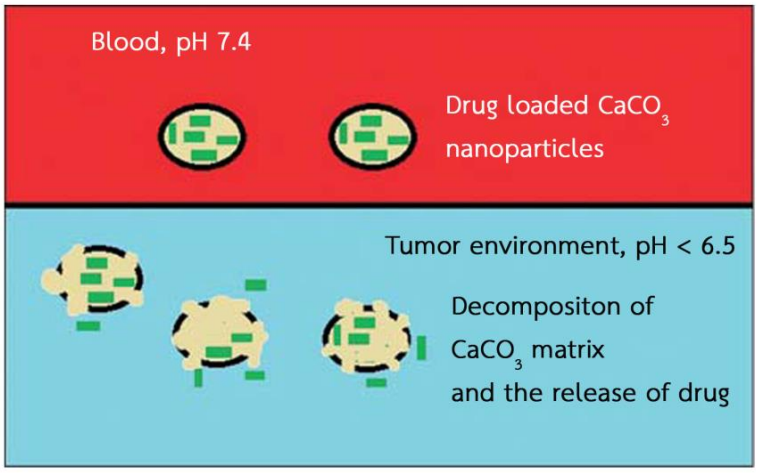


Figure 3 CaCO3 nanoparticles as drug carrier; slow decomposition at normal physiological pH (7.4) and a faster decomposition and release of drug at acidic pH (< 6.5) tumor environment (18).

Recently, there have been many research studies the drug delivery by using $CaCO_3$ NPs as a nanomedicine for medical application. In 2016, Cen Chen and coworkers studied the physicochemical properties, cytotoxicity of $CaCO_3$ NPs and the properties of polyethyleneimine (PEI) -modified $CaCO_3$ NPs as a gene carrier were further investigated *in vitro*. The result showed that prepared PEI-$CaCO_3$ NPs had good biocompatibility and lox cytotoxicity in a certain concentration rang. Moreover, PEI-$CaCO_3$ can transfected pEGFP-C1 gene into epithelial-like cancer cells. And with the expression of GFP-P53 fusion protein, pEGFP-C1-p53-gene-loaded PEI-$CaCO_3$ particles significantly reduced the proliferation of cancer cells. These findings indicate that our PEI-modified $CaCO_3$ NPs are potential to be successfully used as carriers for gene therapy (50).

In 2016, Avik and colleagues determined that $CaCO_3$ NPs with distinct sizes can selectively localize in the extracellular region of tumors and modulate tumor pH in rodents, accompanied by the prevention or reduction of tumor growth. Their data reported that large (> 300 nm) and small (< 20 nm) particles are less effective in increasing the in vivo pH because of limited diffusion and transient retention in the tumor environment, respectively. The ability of $CaCO_3$ NPs to inhibit tumor growth in vivo could serve as a treatment paradigm for long-term tumor static therapy (51).

Lipoic acid

Alpha-lipoic acid (1, 2-dithiolane-3-pentanoic acid; LA) is a strong antioxidant derived from plant and animal tissues (52). It has shown a beneficial effect in chronic inflammatory diseases such as obesity and diabetes by reducing body weight and adiposity in rodents and human. In term of anti-obesity, LA has been shown to increase energy expenditure and reduce food intake (14). Moreover, several studies have also revealed that adipose tissue is a key target in the body weight lowering and insulin-sensitizing actions of LA.

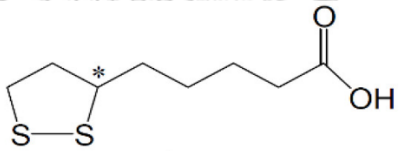


Figure 4 Structure of lipoic acid

Recently, there have been many research studying the effect of LA in adipocytes. In 2014, Marta Fernández-Galilea and others investigated the effect of LA on triglyceride accumulation and lipogenesis in subcutaneous adipocytes and determined the potential mechanisms involved. Their study reported that LA down-regulates key lipogenic enzymes, inhibiting lipogenesis and reducing triglyceride accumulation through the activation of AMPK signaling pathway in human subcutaneous adipocytes (43).

In 2012, Marta Fernández-Galilea and others detected effects of LA on lipolysis in 3T3-L1 adipocytes and the mechanisms involved. Their results showed that LA can stimulate lipolysis in 3T3-L1 adipocytes and suggested that these lipolytic actions of LA are mainly mediated by the phosphorylation of hormone-sensitive lipase through

cAMP-mediated activation of PKA, probably through the inhibition of AdPLA and PGE_2 (14).

In 2014, Jong Ryeal Hahm and colleagues detected how LA modulates the autophagic process during the adipocyte differentiation. Their data reported that LA significantly attenuated accumulation of lipids in differentiated adipocytes and also the production of several autophagy markers (e.g., LC3-II, AMPK, acidic vacuoles) that has been stimulated at the early stage during adipogenesis (8).

In 2014, Marta Fernández-Galilea and coworkers interested the putative effect of LA on mitochondrial biogenesis and the achievement of brown-like characteristics by human subcutaneous adipocytes from overweight/obese subjects. The data showed that LA can stimulate mitochondrial biogenesis and brown-like remodeling in human subcutaneous adipocytes from overweight/obese subjects (53).

In 2014, Bradley Carrier and others showed that LA can reduce acetyl-CoA carboxylase and fatty acid synthase in hepatocytes (54).

Table 1 The effect of lipoic acid

Model	Dose of LA	Effects of LA	Mechanism described	Author/ year
3T3-L1 adipocytes	100-250 μM	Stimulated lipolysis.	Mediated by the phosphorylation of HSL through cAMP-mediated activation of PKA, probably through the inhibition of AdPLA and PGE 2.	Marta Fernández-Galilea et al., (2012) (14).
C_2C_{12} cells	300 μmol/l	Decreased intracellular triacylglycerol accumulation.	Activated both SIRT1 and AMPK.	Chen et al., (2012) (52).
3T3-L1 preadipocytes	500 μM	Attenuated of adipocyte differentiation and lipid accumulation.	Suppressed activation of AMPK.	Jong Ryeal Hahm et al., (2014) (8).
Human subcutaneous adipocytes	100-250 μM	Reduced of fatty acid esterification and llipogenesis.	Increased activation of AMPK signaling pathways.	Marta Fernández-Galilea et al., (2014) (43).
Hepatocytes	300, 600 μM	Reduced of lipogenesis.	Reduced acetyl-CoA carboxylase and fatty acid synthase	Bradley Carrier et al., (2014) (54)

Caffeine

Caffeine (1, 3, 7-trimethylxanthine) is a naturally occurring alkaloid that is found in coffee, tea, cola, chocolate, and soft drinks. It is one of the main components in coffee and is also used as an additive in more than 60% of available soft drinks. Several studies reported that caffeine has a therapeutic activity and possesses anti-obesity. It increases lipolysis by inhibiting cyclic nucleotide phosphodiesterase. Phosphoodiesterase is an enzyme responsible for degrading cyclic adenosine monophosphate (cAMP). It usually hydrolyzes cAMP to AMP. After consumption of caffeine, the cAMP concentration rises and the sympathetic nervous system (SNS) is activated lead to promotion lipolysis.

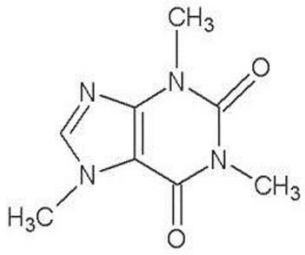


Figure 5 Structure of caffeine (55)

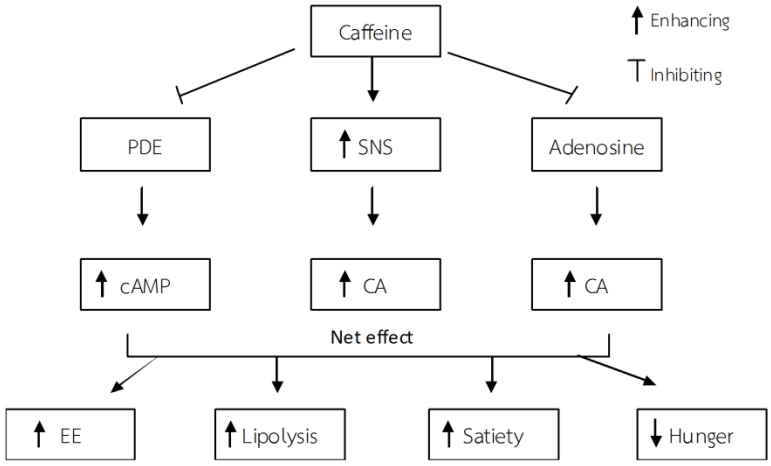


Figure 6 The role of caffeine in thermogenesis and energy intake. PDE, phosphor-diesterase; SNS, sympathetic nervous system; cAMP, cyclic adenosine monophosphate; CA, catecholamines; EE, energy expenditure (12).

There are many previous studies reporting effect of caffeine. In 2013, Shu-Hui Su and colleagues studied the effects of caffeine on adipogenesis using primary rat adipose-derived stem cells (ADSCs) and a mouse bone marrow stromal cell line (M2-10B4) in vitro. Their result reported that caffeine has effectively inhibited adipogenic differentiation of ADSCs and M2-10B4 cells partly through its inhibition of adipogenesis-related factors (7).

In 2016, Hyo Jung Kim and colleagues detected how caffeine affects adipogenesis in 3T3-L1 adipocytes. Their data reported that caffeine is an anti-adipogenic bioactive compound involved in the adjustment of mitotic clonal expansion during adipocyte differentiation through the AKT/GSK3 pathway (56).

In 2017, XIAOJUAN ZHU and others investigated effect of combination therapy with caffeine and catechin in differentiated 3T3-L1 adipocytes. Their result showed that combination therapy with catechins and caffeine inhibited fat deposition by inhibiting the mRNA expression of transcriptional factors and adipogenesis-related enzymes, and enhancing the protein expression of lipolysis-related enzymes in the presence of norepinephrine in 3T3-L1 adipocytes (57).

Table 2 The effect of caffeine

Model	Dose of caffeine	Effects of caffeine	Mechanism described	Author/ year
Primary adipose-derived stem cells and bone marrow stromal cells	0.1-1 mM	Inhibition of adipocyte differentiation.	Down-regulation of PPARγ, C/EBPα, adipocyte lipid binding protein, lipoprotein lipase, leptin, and TNFα.	Shu-Hui Su *et al*., (2013) (7).
3T3-L1 adipocytes	1-5 mM	Inhibition of adipocyte differentiation.	Reduction of AKT and GSK3β phosphorylation lead to decreased levels of C/EBPα and PPARγ.	Hyo Jung Kim *et al*., (2016) (56).
3T3-L1 adipocytes	40 μg/ml catechins, 160 μg/ml caffeine	Inhibition of adipocyte differentiation and stimulation of lipolysis.	Down-regulation of PPARγ, C/EBPα, and FAS	XIAOJUAN ZHU et al., (2017) (57).
Human hepatoma HepG2 cells	0.5-4 mM	Inhibition of lipogenesis and stimulation of lipolysis.	Modulation of AMPK-SREBP signaling pathways.	Hai Yan Quan *et al*., (2013) (58).

CHAPTER III

MATERIALS AND MEDTHODS

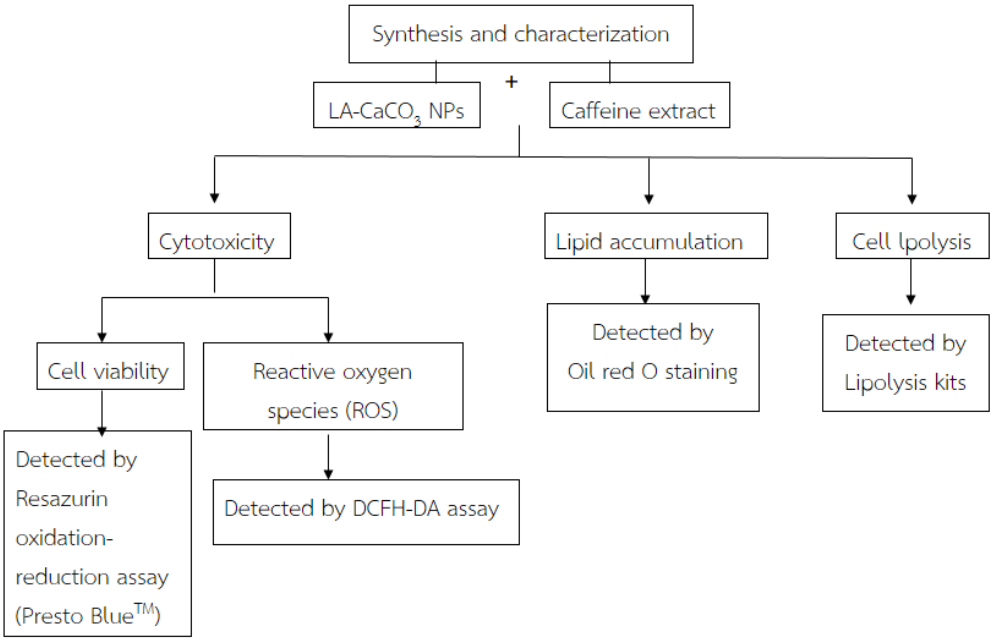


Figure 7 Experimental strategy and step: synthesis and characterization, cytotoxicity, and fat reduction analysis

Isolation of Caffeine from coffee

Crude coffee sample was obtained from Doisaket coffee. Isolation of caffeine from coffee, follow these steps. First, correctly weighed amounts of sieved coffee (about 50 g) were dissolved in 300 ml of distilled water and add 20 g of $NaCO_3$. Secondly, the solution was stirred by using magnetic stirrer and heated gently to remove caffeine easily from the solution for 15 min. Also, the solution was filtered by a glass filter to clear of the particle from solution. Extraction has been done according to the following procedures. The coffee solution prepared above (under coffee sample preparation) was mixed with 8 ml of dichloromethane for the extraction of caffeine from coffee. The extraction of caffeine proceeded 4 times with 5 ml dichloromethane at each round. The caffeine extracted by dichloromethane at each round was stored in beaker. Next, add 0.5 g of anhydrous sodium sulfate to the combined

dichloromethane extracts in the 100 ml flask. The anhydrous sodium sulfate will absorb the small amount of water that is dissolved in the dichloromethane and small amounts of water from the aqueous layer that may have gotten into the flask by accident. Decant the liquid from the flask into a 100 ml beaker. Place one Boiler into the beaker. When the solution in the beaker begins to get dry remove the beaker from the heat and allow it to cool. Then, caffeine to dissolve with distilled water. Finally, the absorbance of the solution was measured by UV/vis spectrophotometer at 273 nm against the corresponding reagent blank.

Synthesis of lipoic acid loaded $CaCO_3$ nanoparticles

Synthesis LA into $CaCO_3$ NPs by the following method: First, 5 M $CaCl_2$ and 5% lipoic acid were gently mixed for 10 min, and then 1 M Na_2CO_3 was added and stirred gently or vigorously for 10 min. After the addition of 5 ml of distilled water and discarding the large particle of $CaCO_3$ nanoparticles which were precipitated without centrifugation, the suspension was centrifuged (2000 rpm, 5 min) and the supernatant and precipitate were separated.

Dynamic light scattering and zeta-potential measurement

For determining the average size of particles was using Dynamic light scattering (DLS). For analysis of particle surface charges were measured by zeta-potential, and the laser Doppler electrophoresis technique was applied based on the Henry equation. Both techniques were performed on a Malvern Zetasizer Nano Series (Malvern Instruments, England).

Transmission electron microscopy (TEM)

Characterize size of lipoic acid loaded $CaCO_3$ nanoparticles by TEM (Hitachi, Japan) operated at voltage of 100 kV.

Thermogravimetric analysis (TGA)

To determine purity and % concentration of lipoic acid in $CaCO_3$ nanoparticles by TGA (TA, USA)

Cell culture and differentiation

Human subcutaneous preadipocytes and Human visceral preadipocytes were obtained from American Type Culture Collection (ATCC) 10801 University Boulevard Manassas, VA 20110 USA. Cells were cultured in Preadipocyte Growth Medium-2 supplemented with 10% FBS, 2 mM L-glutamine, 30 µg/ml GA-1000 SingleQuots™. Induce the preadipocytes to begin differentiating into adipocytes with the addition of adipocyte differentiation medium to each well for 14 days. The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere

Cell Viability assay

To determine the effect of LA, LA-$CaCO_3$NPs, caffeine extract, and caffeine extract with LA-$CaCO_3$NPs on human subcutaneous preadipocytes, human subcutaneous adipocytes, human visceral preadipocytes, and human visceral adipocytes were using the PrestoBlue™ assay. Cell viability was conducted in 96-well plates at a density of cell $5x10^3$ cells/ well in 90 µl of preadipocyte growth medium-2 and incubated overnight at 37°C in a humidified 5% CO_2 atmosphere for preadipocytes. In term of adipocytes, the cells were add adipocyte differentiation medium and maintained at 37°C in a humidified 5% CO_2 atmosphere for 14 days. The cell were treated with 1 mM H_2O_2 as a positive control, 600 µM LA, 600 µM LA-$CaCO_3$NPs, 5 mM coffee extract, and 5 mM coffee extract with 600 µM LA-$CaCO_3$NPs in 90 µl. Plates were incubated for 24 h and 72 h. Then, 10 µl PrestoBlue™ reagent (Invitrogen, USA) was added to each well, and incubated for 30 min. Finally, the cell viability was measured by microplate reader at 560 and 590 nm (Thermo, Varioskan Flash, England).

ROS generation

To evaluate intracellular ROS levels, we used 2', 7' – dichlorodihydrofluorescein diacetate (DCFH-DA) reagent (Invitrogen, USA). First, the cells were culture under the same conditions of cell viability assay. Subsequently, human subcutaneous preadipocytes, human subcutaneous adipocytes, human visceral preadipocytes, and human visceral adipocytes growing in black 96-well plates were treated with DCFH-DA reagent for 30 minutes in the dark. The cell were treated with 1 mM H_2O_2 as a positive control, 600 µM LA, 600 µM LA-$CaCO_3$NPs, 5 mM coffee extract, and 5 mM coffee extract with 600 µM LA-$CaCO_3$NPs in 100 µl. After nonfluorescent DCFH-DA reagent penetrates into cells, esterases within the cells initiate hydrolysis of DCFH-DA to 2', 7' – dichlorodihydrofluorescein (DCFH). 2', 7'- Dichlorofluorescein (DCF)

produced by oxidation of DCFH in the presence of ROS was immediately detected using a fluorescence microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

Oil Red O *staining*

The lipid accumulation change in human subcutaneous adipocytes and human visceral adipocytes were measured by Oil Red O staining (Sigma, USA). After differentiated of preadipocytes to mature adipocytes and cells were treated with 600 μM LA, 600 μM LA-$CaCO_3$NPs, 5 mM coffee extract, and 5 mM coffee extract with 600 μM LA-$CaCO_3$NPs in 100 μl. The cells were maintained at 37°C in a humidified 5% CO_2 atmosphere for 1 day. After that cells were washed twice with PBS and then fixed with 10% formalin to each well for 30 min at room temperature. Following washing with 60% isopropanol, cells were completely dried before staining with 0.5% Oil Red O for 10 min. Later, the stained cells were washed three times with distilled water, and cells were completely dried before elution Oil Red O by adding 100% isopropanol, incubate about 10 min. Finally, determined by measurement of absorbance at 500 nm, 0.5 sec reading.

Determination of TG level

Grow and differentiate human subcutaneous preadipocytes and human visceral preadipocytes in a 96 –well plate. After differentiation, incubated in the presence of various concentration of 600 μM LA, 600 μM LA-$CaCO_3$NPs, 5 mM coffee extract, and 5 mM coffee extract with 600 μM LA-$CaCO_3$NPs in 100 μl. Media was collected from the cells at time points. The amount of glycerol released was determined using the Lipolysis Assay Kit (abcam). According to the manufacturer's protocol, absorbance was detected at 570 nm and quantified using a spectrophotometric microplate reader.

Statistical analysis

The result from triplicates of the sample was expressed as mean ± SD. Statistical significance was evaluated by a one-way analysis of variance (ANOVA) followed by the Turkey's multiple comparison tests for more than 2-group comparison and Student t-test for 2 independent group comparison. Statistical significance differences were considered when P – value ≤ 0.05.

Ethical consideration

This study was approved by the Ethical committee of Research affairs, Faculty of Medicine, Chulalongkorn University (IRB 106/60).

CHAPTER IV

RESULTS AND DISCUSSION

RESULTS

Caffeine concentration and characterization of Lipoic acid-incorporation $CaCO_3$ nanoparticles (LA-$CaCO_3$ NPs)

In this study, concentration of caffeine used in this experiment was 5 mM, measured by UV-VIS spectrophotometer (see supplement data). In term characterized of LA-$CaCO_3$ NPs, the parameters considered were size, zeta-potential, and concentration of LA. Size of LA-$CaCO_3$ NPs was measured by transmission electron microscope (TEM). The findings showed that the LA-$CaCO_3$ NPs exhibited a spherical shape and the average size was 20 nm (fig. 1B). Regarding, surface charge and stability of LA-$CaCO_3$ NPs represented strongly cationic or anionic, the zeta potential should be less than -30 mV or higher than +30 mV, respectively (ref-zeta potential and measurement). The zeta-potential of LA-$CaCO_3$ NPs showed -40.9 mV (fig. 1C). As for concentration of LA in $CaCO_3$ NPs, we analyzed the percentage of LA using thermo gravimetric analysis (TGA). We considered at residual mass of $CaCO_3$ NPs. The result presented that the concentration of LA was approximately 27% in $CaCO_3$ NPs (fig. 1D).

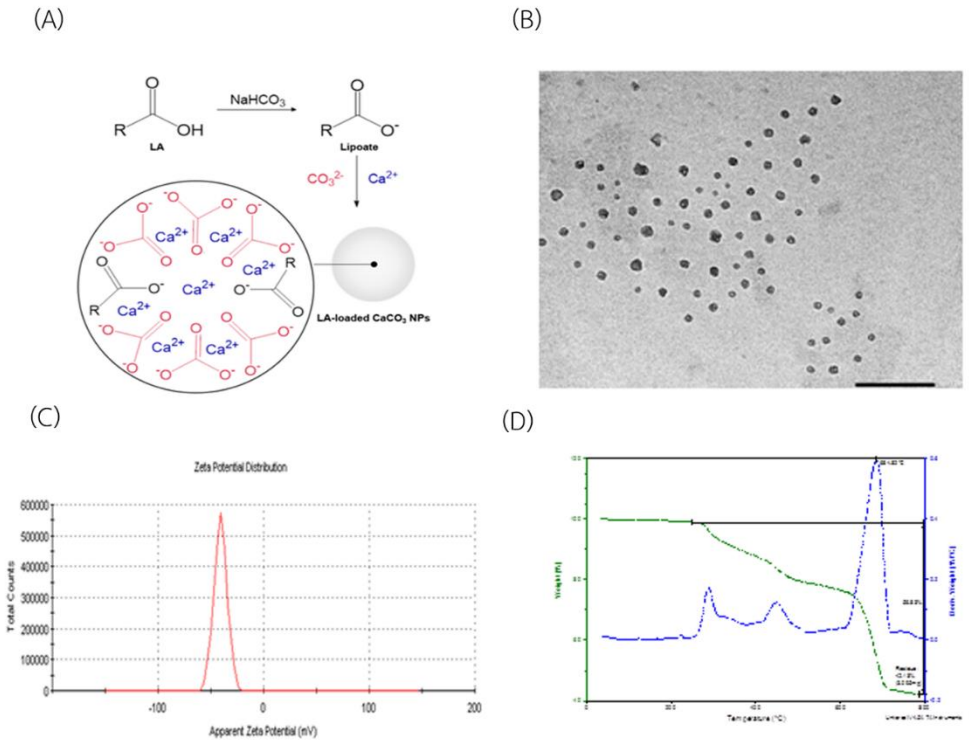


Figure 8 Shows structure of LA-$CaCO_3$ NPs (A), image of LA-$CaCO_3$ NPs by TEM (B), zeta potential of LA-$CaCO_3$ NPs (C), and TGA thermograms of LA-$CaCO_3$ NPs (D).

Cellular toxicity studies

Cell viability assay

After characterizing caffeine and LA-$CaCO_3$ NPs, we determined cell viability and cytotoxicity effect of LA, LA-$CaCO_3$ NPs, caffeine extract, and LA-$CaCO_3$ NPs with caffeine on human subcutaneous preadipocytes, human subcutaneous adipocytes, human visceral preadipocytes, and human visceral adipocytes after incubated for 24 and 72 h. Cell viability was evaluated by the activity of mitochondrial dehydrogenase using a resazurin-based technique. The result presented that treatment of various groups has no toxicity to these cells.

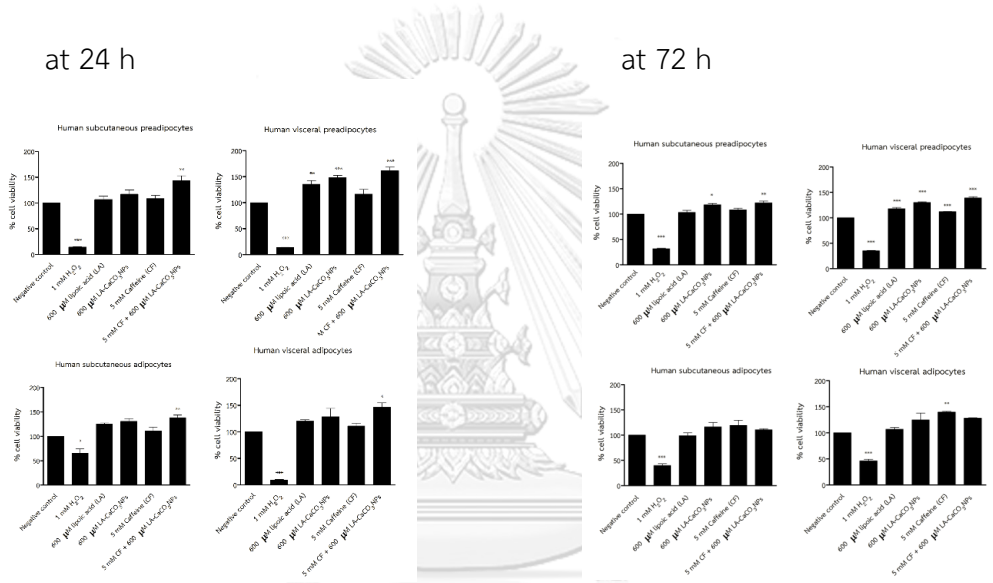


Figure 9 Effect of LA, LA-CaCO3 NPs, caffeine, and LA-CaCO3 NPs combined with caffeine on cell viability. Percentage of cell viability in human subcutaneous preadipocytes, human subcutaneous adipocytes, human visceral preadipocytes, and human visceral adipocytes after incubated with their conditions at 24 h. Data were shown as a percentage of the control group. Values are mean ± SD (n=3). Significance represents the *p<0.05, **p<0.01, and ***p<0.001 versus control group.

ROS generation

To study the cytotoxicity effect of LA, LA-$CaCO_3$ NPs, caffeine, and LA-$CaCO_3$ NPs with caffeine extract on reactive oxygen species generation following treatment in human subcutaneous preadipocytes, human subcutaneous adipocytes, human visceral preadipocytes, and human visceral adipocytes. The evaluation was observed using the DCFH-DA technique from 1 h. Cells were treated with 600 μM LA, 600 μM LA-$CaCO_3$ NPs, 5 mM caffeine, and 600 μM LA-$CaCO_3$ NPs with 5 mM caffeine extract. The results showed that did not displayed the ROS generation in any conditions (figure3) when compared with the control group. Thus, their conditions were used in the subsequent experiment.

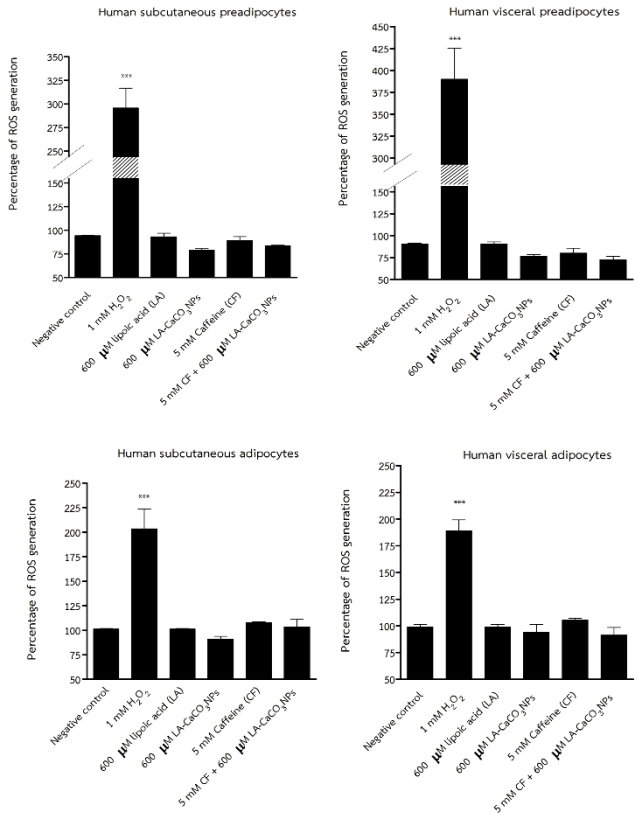


Figure 10 Effect of LA, LA-$CaCO_3$ NPs, caffeine, and LA-$CaCO_3$ NPs combined with caffeine on ROS generation. Percentage of ROS generation in human subcutaneous adipocytes, human subcutaneous adipocytes, human visceral preadipocytes, and human visceral adipocytes after incubated with their conditions at 60 min. ROS generation was detected with DCFH-DA assay and fluorescence intensity were

calculated compared with the control group. Values are mean ± SD (n=3). Significance represents the *p<0.05, **p<0.01, and ***p<0.001 versus control group.

Lipid accumulation

After detecting cell viability and cytotoxicity of these conditions, we found that these conditions have no toxicity. So, we investigated the effect of LA, LA-$CaCO_3$ NPs, caffeine, and LA-$CaCO_3$ NPs with caffeine on lipid accumulation reduction on human subcutaneous adipocytes and human visceral adipocytes. After being treated with individual and combination (LA-$CaCO_3$ NPs with caffeine) in human subcutaneous adipocytes, the results of Oil Red O staining of all conditions in human subcutaneous adipocytes were shown in Fig. 10A and 10B. There were statistically significant decreased lipid accumulations when compared with the control group (untreated). There was no statistically significant difference in lipid accumulation among LA, caffeine, and combination. Also, lipid accumulations in LA-$CaCO_3$NPs group were similar to combination group but lower than in LA group and caffeine group. On the one hand, the results of Oil Red O staining of all conditions in human visceral adipocytes were shown in Fig. 10C and 10D. Lipid accumulations were exhibited significant differences between control group and other groups. Furthermore, the results showed that fat storage was no significant between LA and caffeine group. In addition, there were no significant differences between LA-$CaCO_3$ NPs and combination group, and their lipid accumulation were lower than LA group and caffeine group.

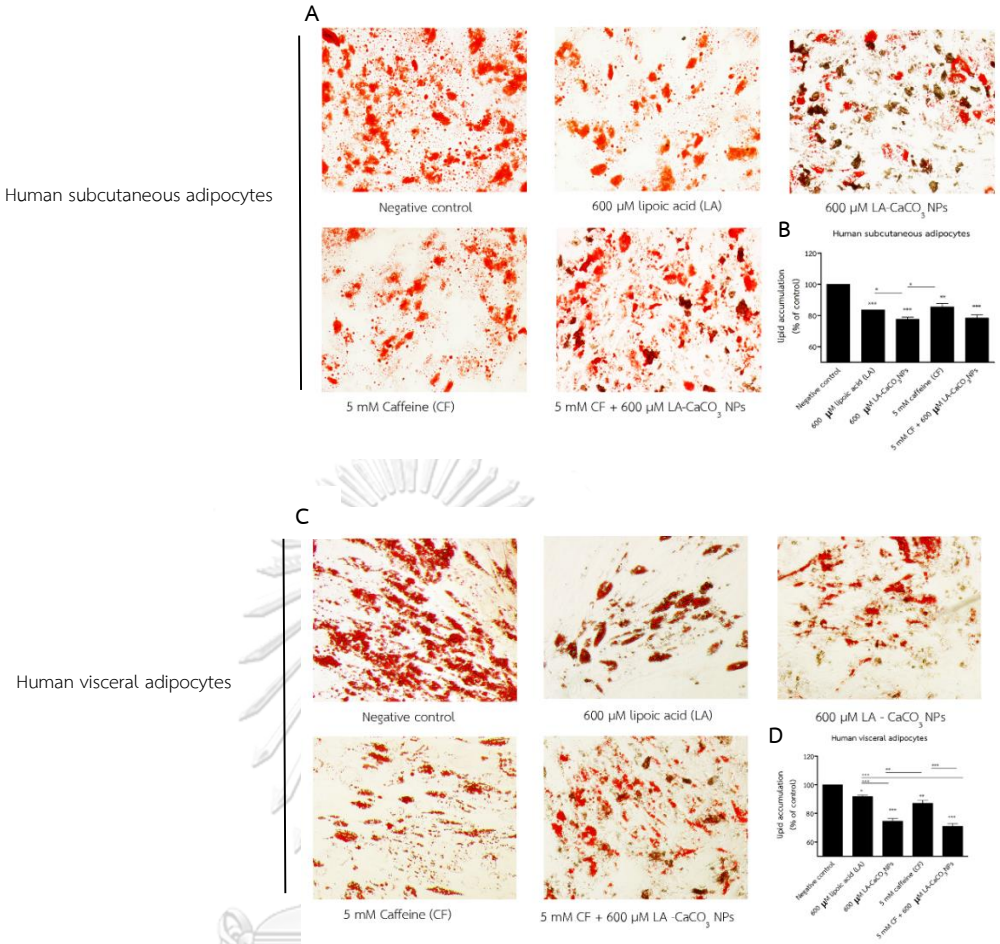


Figure 11 Representative images of Oil Red-O-stained cells captured at 24 h after treated with 600 μM LA, 600 μM LA-$CaCO_3$ NPs, 5 mM caffeine, and 600 μM LA-$CaCO_3$ NPs with 5 mM caffeine on human subcutaneous adipocytes (A) and human visceral adipocytes (B) using inverted microscorpe (magnification, 10X). Our results show that quantitative analysis of lipid accumulation, detected by microplate reader measurement of oil-red-O-stained adipocyte. Values are mean ± SD (n=3). Significance represents the *p<0.05, **p<0.01, and ***p<0.001 versus each group.

Cell lipolysis

In terms of stimulate lipolysis effect of LA, LA-$CaCO_3$ NPs, caffeine, and combination on human subcutaneous adipocytes and human visceral adipocytes. The results of extracellular glycerol contents in human subcutaneous adipocytes were shown in fig. 11A. There were no statistically significant difference in extracellular glycerol contents between a control group and combination group but their glycerol contents were nearly two-fold lower than other groups. Besides, the glycerol levels in caffeine extract group were similar to LA and LA-$CaCO_3$ NPs group. Moreover, the glycerol levels showed in LA-$CaCO_3$ NPs group were higher than LA group. On the one hand, the results of extracellular glycerol contents of all conditions in human visceral adipocytes were shown in Fig. 11B. There were statistically significant increased glycerol level when compared with the control group. In addition, the glycerol levels in LA group were similar to caffeine extract group and combination group but lower than LA-$CaCO_3$ NPs group. Additionally, the glycerol levels in LA-$CaCO_3$ NPs group and caffeine extract group were similar but they were higher than combination group.

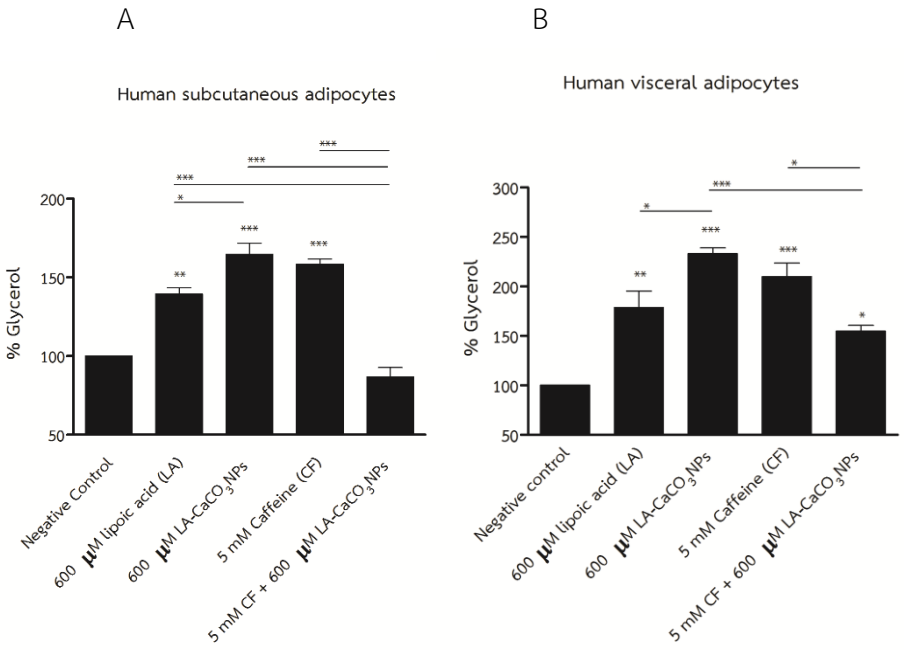


Figure 12 Effect of lipoic acid, lipoic acid loaded $CaCO_3$NPs, caffeine extract, and lipoic acid loaded $CaCO_3$NPs combine caffeine extract on cell lipolysis. Quantification of % glycerol in human subcutaneous adipocytes and human visceral adipocytes after incubated with their conditions at 24 h. Glycerol release was detected by lipolysis assay kit. Data were shown as a percentage of the control group. Values are mean ± SD (n=3). Significance represents the *p<0.05, **p<0.01, and ***p<0.001 versus each group.

Discussion

To our knowledge, this study reported the ability of LA, LA-$CaCO_3$ NPs, caffeine, and combination to reduce fat content in human subcutaneous adipocytes and human visceral adipocytes. Our results indicate that LA-$CaCO_3$ NPs group can reduce lipid accumulation and stimulate lipolysis better than LA group. Furthermore, in both lipid accumulation and lipolysis levels in LA group and in caffeine group were not significantly different. The result of the combination of caffeine and LA-$CaCO_3$ NPs group showed that in term of lipid accumulation the combination were reduce lipid accumulation in visceral adipocytes but did not reduce lipid accumulation in subcutaneous adipocytes. In term of lipolysis, the combination show did not stimulate lipolysis in subcutaneous adipocytes and visceral adipocytes. In addition, we found that human visceral adipocytes more sensitive to stimulated lipolysis than human subcutaneous adipocytes.

The strength of our study is that LA-$CaCO_3$ NPs group can reduce lipid accumulation and stimulate lipolysis batter than LA group. Previous studies showed that abilities of LA were stimulating antioxidant system (59), affecting inflammatory markers (60), and reducing free radicals (61). In term of metabolic processes, LA can improve metabolic disorder, diabetic polyneuropathy, rheumatoid arthritis, neuron degeneration, and body weight (62). Moreover, V. Thirunavukkarasu and colleagues reported that LA can increase High-density lipoprotein cholesterol (HDL-C) and reduce very low-density lipoprotein cholesterol (VLDL-C) and low-density lipoprotein cholesterol (LDL-C) in high fructose–fed insulin-resistant rats (63). Moreover, our data is concordance with previously that LA group can reduce lipid accumulation and activated lipolysis (14, 43). On the other hand, LA only not have high capacity to reduce fat in adipocytes. In this study, we investigate the increased efficacy of lipoic acid by $CaCO_3$ NPs. Our results showed that $CaCO_3$ NPs can increase the performance of LA. This work is in accordance with another study reporting that nanoparticles exhibited significantly improved gene delivery efficiency due to the enhanced cellular uptake and nuclear localization (64).Our results confirmed previous reports that $CaCO_3$ NPs have demonstrated good sustained-release performance and high stability (65). Besides, owing to the slow biodegradability, accessibility, safety, and high stability of $CaCO_3$ NPs, it has been used for controlled drug delivery, biosensing, and encapsulation of different types of drug (18). This suggested that the $CaCO_3$ NPs can be used an

alternative method for subcutaneous adipocytes and visceral adipocyte lipid reduction.

Our results showed that LA and caffeine can significantly affect to reduce lipid accumulation and stimulate lipolysis at concentration 600 μM and 5 mM respectively when compared with control. This is supported by recent studies showed that mechanism of LA to reduce lipid accumulation and stimulate lipolysis. First, in term of lipid accumulation, LA can inhibit lipogenesis in human subcutaneous adipocytes by down-regulating lipogenic enzymes such as fatty acid synthase (FAS), diacylglycerol O-acyltransferase 1, and stearoyl-coenzyme A desaturase 1 and decrease lipid accumulation through the activation of AMP-activated protein kinase (AMPK) signaling pathway (43). Furthermore, LA suppressed FAS protein through the activation of both Sirtuin 1 (SIRT1) and AMPK pathways *in vitro* and *in vivo* (66). Second, in term of lipolysis, LA can induce adipose triglyceride lipase (ATGL) expression, the enzyme is the major triglyceride lipase through AMPK/ transcription factor forkhead box O1 (FOXO1) leading to increase in lipolysis in HepG2 cells (67). LA can increase palmitate β-oxidation and lipolysis via increased AMPK and acetyl-CoA carboxylase (ACC) phosphorylation in C_2C_{12} myotubes (66). Previous studies suggest that LA can activate AMPK pathway lead to lipolysis stimulation in 3T3-L1 cells (14, 68). Regarding, mechanism of caffeine, which could reduce lipid accumulation and promote lipolysis. In term lipid accumulation, a recent study reported that caffeine decreases lipid accumulation through activation of AMPK signaling pathway in HepG2 cells (58). In term lipolysis, previous study reported that caffeine was found to inhibit phosphodiesterase, which is an enzyme responsible for degrading cyclic adenosine monophosphate (cAMP). After phosphodiesterase is inhibited by caffeine, the concentration of cAMP and the sympathetic nervous system (SNS) is activated, resulting in the promotion of lipolysis in adipocytes (11, 12, 69). Our results indicated that caffeine can affect to reduce lipid accumulation and stimulate lipolysis at concentration 5 mM. On the other hand, the result of the combination of 5 mM caffeine with 600 μM LA-$CaCO_3$ NPs suggested that they did not have effect to reduce lipid accumulation in subcutaneous adipocytes and promote lipolysis in subcutaneous adipocytes and visceral adipocytes. This finding might explained from 1) the glomeration between some substances of caffeine and LA-$CaCO_3$ NPs finding they cannot bind the target of lipolysis pathway and fat accumulation pathway. 2) The LA-$CaCO_3$ NPs and caffeine can reduce lipid accumulation via the same target signaling pathway which cause LA-$CaCO_3$ NPs to interfere with caffeine to bind the target. 3) The LA and caffeine has potential to reduce lipid accumulation. However, the limitation of receptor in cells should be consider.

These findings are also in line with those of a previous study by Marta and the other (43), in which LA can reduce lipid accumulation in human subcutaneous adipocyte when compared with control. Conversely, a concentration of LA that use was 100-250 μM while we use 600 μM of LA. The concentration of LA in our study has been used in normal rat liver cells by Bradley Carrier and coworkers (54), who reported that 600 μM of LA can reduce protein abundance of acetyl-CoA carboxylase and a tendency for decrease fatty acid synthase abundance. Moreover, Bradley Carrier and coworkers found that a concentration of LA at 50-300 μM no difference in the mRNA expression to acetyl-CoA carboxylase and fatty acid synthase in rat hepatocytes when compared with control group. This is signifying that the concentration of LA at 600 μM is one of the concentrations that may decrease fat content in human subcutaneous adipocytes and human visceral adipocytes. Therefore, in our experiment, we used 5 mM of caffeine that does not toxic in cells. Also, the higher concentration of caffeine (>1 mM) has been used in *in vitro* by Hyo Jung Kim and the other. They have reported that caffeine had non-cytotoxicity at concentration 5 mM and the concentration of caffeine can decrease lipid accumulation in 3T3-L1 adipocytes (10). Thus, in this study we used 600 μM LA, 5 mM caffeine, and 600 μM LA-$CaCO_3$ NPs to decrease triglyceride content in human adipocytes. The results of individual group showed stimulated lipolysis and fat reduction, but the results of combination group were not additive and synergistic to reduce triglyceride content in human adipocytes. Because we thought that there may have glomeration between some substances of caffeine and LA-$CaCO_3$ NPs and the same target signaling pathway to reduce fat in adipocytes. In addition, our results presented that caffeine, LA-$CaCO_3$ NPs, and the combination can stimulate lipolysis in visceral adipocytes higher than subcutaneous adipocytes. Our finding is in agreement with Shinobu Mori and others, who observed natural products displaying lipolysis-promoting activity in visceral adipocytes, which are more sensitive to hormones than subcutaneous adipocytes (70). Additionally, This is supported by research by previous studies, that reported that lipolysis is high in visceral adipocyte and low in subcutaneous adipocyte (33) due to the visceral adipocyte has less anti-lipolytic action of insulin and more susceptible to the catecholamine-induced lipolysis (31).

Our findings demonstrated that the LA, LA-$CaCO_3$ NPs, caffeine, and combination exhibited significant when compared with control. Nevertheless,

limitations of the present study should be mentioned. First, in this study, we don't have proven mechanism of their conditions to decrease lipid accumulation and promote lipolysis in adipocytes. Additional studies within this area should consider the mechanism of their conditions to reduce lipid accumulation and activate lipolysis in adipocytes. Second, we don't have proven release of caffeine and LA-$CaCO_3$ NPs into the cells. Therefore, further experimental researcher is required to clarify the conformation of caffeine and LA-$CaCO_3$ NPs including proven drug release by confocal laser scanning microscope. Third, we don't have established purified caffeine. Consequently, it is recommended that further studies should be conducted purified of caffeine to more effectively determine by using high-performance liquid chromatography (HPLC).

CHAPTER IV

Conclusion

The purpose of this study is to determine the effects of caffeine and LA-$CaCO_3$ NPs combination to reduce lipid accumulation and stimulate lipolysis in human subcutaneous adipocytes and human visceral adipocytes. The main points of discussion are as follows. First, the LA-$CaCO_3$ NPs group enhance lipid accumulation reduction and lipolysis stimulation in subcutaneous adipocytes and visceral adipocytes. Second, we propose that LA-$CaCO_3$ NPs increase bioavailability in subcutaneous adipocytes and visceral adipocytes. Third, the combination of LA-$CaCO_3$ NP to caffeine suggest that they were reduced lipid accumulation in visceral adipocytes but not reduce lipid accumulation in subcutaneous adipocytes. Moreover, the combination showed that they did not stimulate lipolysis in visceral adipocytes and subcutaneous adipocytes. Finally, this $CaCO_3$ NPs has potential effect for using an alternative method for subcutaneous adipocytes and visceral adipocyte lipid reduction.

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APPENDIX

LIST OF ABBREVIATIONS

AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
$CaCO_3$	Calcium carbonate
cAMP	Cyclic adenosine monophosphate
CE	Caffeine extract
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
H_2DCF-DA	2', 7' –dichlofluorescein-deacetate
HPLC	High-performance liquid chromatography
LA	Lipoic acid
LA-$CaCO_3$NPs	Lipoic acid-incorporation $CaCO_3$ nanoparticles
NPs	nanoparticles
OD	Optical density
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SNS	Sympathetic nervous system
TEM	Transmission electron microscopy
TGA	Thermogravimatric analysis
UV-VIS	Ultra violet -Visible

EQUIPMENT

1. Autoclave	Hirayama, Japan
2. Autopipette 10, 100 amd 1000 µL	Biorad, USA
3. Class II biohazard safety cabinet	Esco Micro, Singapore
4. CO_2 incubator	Esco, Singapore
5. Cell culture flask	SPL, Korea
6. Centrifuge tube 1.5 mL	Corning, USA
7. Centrifuge tube 15 mL	Corning, USA
8. Centrifuge tube 50 mL	Corning, USA
9. Filter Tip	Corning, USA
10. Flow cytometer	Becton Dickenson, USA
11. Fluorescent microscope	Nikon, Japan
12. Hot plate	Becthai,Thailand
13. Labaratory balance	Denver instrument, Germany
14. Microcentrifuge	Hettich, Germany
15. Varioskan Flash microplate reader	Thermo, England
16. Oven	Contherm, New Zealand
17. Parafilm	Bemis, USA
18. Phase contrast inverted microscope	Nikon, Japan
19. pH meter	Denver instrument, Germany
20. Shaker incubator	Heidolph, Germany
21. Sonicator	P.Intertrade Equipment Co.,Ltd
22. Thermal gravimetric analysis (TGA)	TA Instrument, USA
23. Tissue culture flasks 25 cm^2	Nest Biotechnology, China
24. Tissue culture flasks 75 cm^2	Nest Biotechnology, China
25. Tissue culture flasks 24, 96 well plate	Corning, USA

26. Transmission electron microscopy	Hitachi, Japan
27. Upright Ultra-Low Temperature Freezers	Thermo, England
28. Vortex mixer	Scientific industries, USA
29. Water bath	Memmert, Germany
30. Zetasizer Nano Series	Malvern Instrument, England

Chemical

1.	Alpha-lipoic acid	Sigma, USA
2.	Calcium chloride	Merck, Germany
3.	Dulbecco' s Modified Eagle' s Medium	Sigma, USA
4.	Dichloromethane	Merck, Germany
5.	Fetal Bovine Serum	Gibco, USA
6.	Formaldehyde	Merck, Germany
7.	H_2DCFDA	Invivogen, USA
8.	Isopropanol	Merck, Germany
9.	Lipolysis assay kit	Abcam, USA
10.	Oil Red O	Sigma, USA
11.	Penicillin/ Streptomycin	Gibco, USA
12.	Phosphate buffer saline (PBS)	Ameresco, USA
13.	PrestoBlue™ Cell viability Reagent	Invitrogen, USA
14.	Sodium carbonate	Merck, Germany
15.	Sodium sulphate anhydrous	Mallinckrodt®, US
16.	Trypsin/EDTA	SIGMA, Germany

CHEMICAL PREPARATIONS

1. Phosphate buffer saline

KCl	0.2	g
KH_2PO_4	0.2	g
NaCl	8.0	g
Na_2HPO_4	1.15	g

Mix all of chemical component and add DI water to 1,000 mL, then adjust pH to 7.4 with HCl

2. Dulbecco' s Modified Eagle' s Medium (DMEM)
   1) Dissolve 13.4 g of DMEM with 800 mL DI water
   2) Add 3.7 g of Na_2HCO_3
   3) Adjust pH to 7.2 with HCl
   4) Add DI water to 1,000 mL
   5) Filtrate by 0.2 μM filter and keep as a stock medium
   6) For working medium preparation, add 100 mL of heat Fetal Bovine Serum, 5 mL of antibiotic (Pen-Strep), and 1 mL of normocin into 900 mL of stock medium.

Cell Viability Assay Protocol (PrestoBlue™, Invitrogen, USA, Catalog number A13261)

1. Cell culture

Materials

1. 96-well plate
2. Cell Culture Media
3. Micropipetters
4. CO_2 incubator

Method

1. Cells are seed in 96-well plates at a density of 1-10 x 10^3 cells/well in 45 μL.

2. Incubate at 37 °C and 5% CO_2 for 12 h.

2. Cell viability assay

Materials

1. Unknown sample for toxicity test
2. H_2O_2 (positive control)

Method

1. Add 45 µL of culture medium for negative control.
2. Add 45 µL of H_2O_2 for positive control.
3. Treat with 45 µL of unknown samples.
4. Incubate at 37 °C and 5% CO_2 for 24 or 48 h.
5. Add 10 µL PrestoBlue™ reagents and incubate for 30 min.
6. Measure fluorescent product by using a microplate reader at 560 and 590 nm.

Reference

- Product Information Sheet : PrestoBlue ™ Cell Viability Reagent Protocol from Invitrogen™

Reactive Oxygen Species (ROS) Generation Protocol (H_2DCFDA, Invitrogen, USA, Catalog number D399)

1. Cell culture

Materials

1. 96-black well plate
2. Cell Culture Media
3. Micropipetters
4. CO_2 incubator

Method

1. Cell are seed in 96-black well plate at a density of 1-10 x 10^3 cells/well in 100 µL

2. Incubate at 37 $^{\circ}$C and 5% CO_2 for 12 h.

2. DCFH-DA assay

Materials

1. Unknown sample for ROS generation test
2. H_2O_2 (positive control)
3. Phosphate Buffer Saline (PBS)

Method

1. Wash the cells 2 times with PBS
2. Add 100 µL of 0.1 M H_2DCFDA
3. Incubate at 37 $^{\circ}$C and 5% CO_2 for 30 min
4. Wash 2 times with PBS
5. Add 100 µL of culture medium for control
6. Add 100 µL of 0.5% H_2O_2 for positive control
7. Treat with 100 µL of unknown samples.
8. Measure fluorescence excitation and emission at 485 and 528 respectively by using microplate reader

Reference

- Product Information Sheet : H_2DCFDA from Invitrogen™

Supplement

Characterization of Caffeine in crude extract from coffee

Table 3 Standard absorbance value

Concentration (mg/ml)	Absorbance
0.02	0.1614
0.04	0.3064
0.06	0.4767
0.08	0.6734

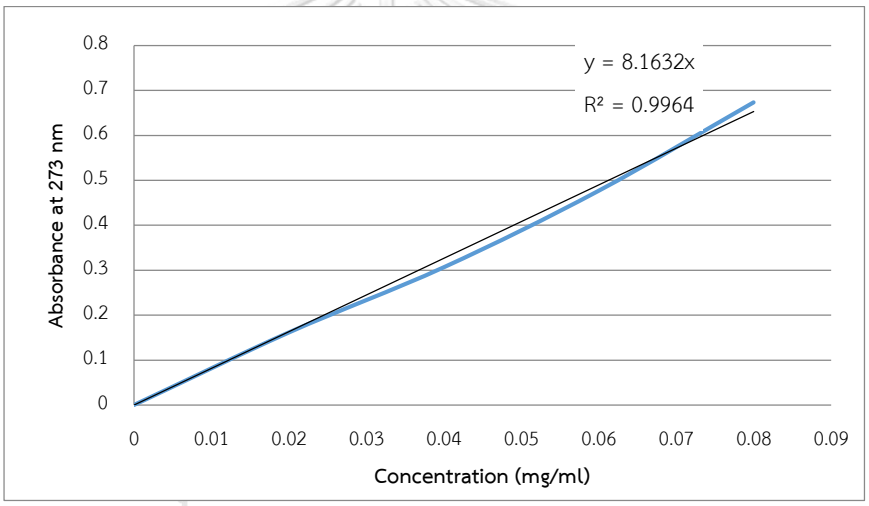

Figure 13 Show chart concentration of caffeine

$$y = 8.1632x$$

$$0.268 = 8.1632x$$

$$x = 0.032$$

Thus, the concentration of caffeine extract compared to caffeine standard at 0.02 mg/ml was 0.032 mg/ml.

VITA

Sunisa Kaewdang was born in Bangkok, Thailand on 3 February 1993. She graduated with the Bachelor Degree of Biotechnology from Faculty of Applied science, King Mongkut's University of Technology North Bangkok in 2015. She enrolled in Master Degree of Medical Science, Faculty of Medicine at Chulalongkorn University in 2015.