

การศึกษาผลของกรดไลโปอิกในแคลเซียมคาร์บอเนตระดับนาโนเมตร  
ต่อการเปลี่ยนแปลงและพัฒนาของเซลล์ต้นกำเนิดจากไขมันและผลกระทบต่อหน้าที่ของเซลล์ไขมัน



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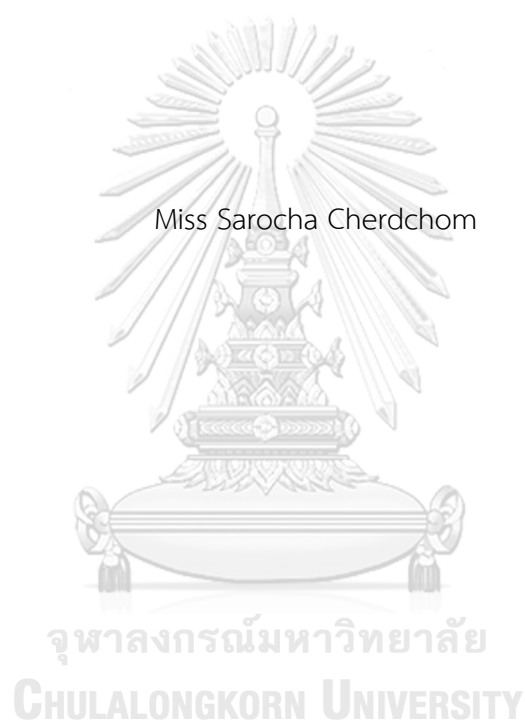
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จุฬาลงกรณ์มหาวิทยาลัย  
**CHULALONGKORN UNIVERSITY**

Lipoic acid-incorporated calcium carbonate nanoparticles  
for controlling of adipose derived stem cells (ADSCs) differentiation  
and adipocyte function

Miss Sarocha Cherdchom



A Dissertation Submitted in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy Program in Medical Science

Faculty of Medicine

Chulalongkorn University

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จุฬาลงกรณ์มหาวิทยาลัย  
**CHULALONGKORN UNIVERSITY**

Thesis Title Lipoic acid-incorporated calcium carbonate nanoparticles for controlling of adipose derived stem cells (ADSCs) differentiation and adipocyte function

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สโรชา เขติโฉม : การศึกษาผลของกรดไลโปอิกในแคลเซียมคาร์บอเนตระดับนาโนเมตรต่อการเปลี่ยนแปลงและพัฒนาของเซลล์ต้นกำเนิดจากไขมันและผลกระทบต่อหน้าที่ของเซลล์ไขมัน (Lipoic acid-incorporated calcium carbonate nanoparticles for controlling of adipose derived stem cells (ADSCs) differentiation and adipocyte function) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ. อมรพันธุ์ เสรีมาศพันธุ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. วิจารณ์ฤทธิ์ วิจารณ์เนศ, ศ. ภาณุ. ดร. พรอนงค์ อร่ามวิทย์, หน้า.

CaCO<sub>3</sub> NPs has received a growing interest due to its potential properties as a safe drug carrier. The LA-CaCO<sub>3</sub> NPs were synthesized by a chemical precipitation method and characterized using transmission electron microscopy, thermogravimetric analysis, and zeta potential measurement. We used LA-CaCO<sub>3</sub> NPs to evaluate their therapeutic effects in lipid accumulation, lipolysis, adipogenesis, and adipokines expression in ADSCs and differentiated adipocytes. The average particle sizes were observed to be 10-20 nanometers. Thermogravimetric analysis results confirmed the percentages of LA in CaCO<sub>3</sub> NPs. The zeta potential showed a negative charge -30.9 mV. Our findings indicated that LA-CaCO<sub>3</sub> NPs were apparently more effective in lipid accumulation and lipolysis than LA alone. Additionally, LA-CaCO<sub>3</sub> NPs were also inhibit adipogenesis in ADSCs as well as adipokine expression. LA-CaCO<sub>3</sub> NPs directly reduced lipid accumulation by enhancing of lipolysis activity. Adipogenesis was also suppressed by LA-CaCO<sub>3</sub> NPs and eventually decreases in adipokine expression. The LA-CaCO<sub>3</sub> NPs with beneficial therapeutic effects could provide a promising approach to target adipose tissue for obesity interventions.

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SAROCHA CHERDCHOM: Lipoiic acid-incorporated calcium carbonate nanoparticles for controlling of adipose derived stem cells (ADSCs) differentiation and adipocyte function. ADVISOR: ASST. PROF. AMORN PUN SEREEMASPUN, M.D., Ph.D, CO-ADVISOR: ASST. PROF. ROJRIT ROJANATHANES, Ph.D., PROF. PORNANONG ARAMWIT, Pharm.D., Ph.D., pp.

ในปัจจุบันได้มีการศึกษาค้นคว้าและวิจัยคุณสมบัติของอนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตร ในการนำมาพัฒนาเพื่อใช้เป็นอนุภาคนาโนสำหรับนำส่งยา (Drug carrier) ที่มีความปลอดภัยและมีประสิทธิภาพ ในงานวิจัยนี้มีวัตถุประสงค์ที่จะทำการพัฒนาการสังเคราะห์อนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตรเพื่อนำส่งกรดไลโปอิกเข้าสู่เซลล์ต้นกำเนิดไขมันและเซลล์ไขมัน จากผลการวิเคราะห์คุณสมบัติทางกายภาพของอนุภาคแคลเซียมคาร์บอเนตที่บรรจุด้วยกรดไลโปอิก พบว่า อนุภาคนาโนที่พัฒนาขึ้นนี้มีคุณลักษณะที่เหมาะสมต่อการนำไปศึกษาประสิทธิภาพในการลดไขมัน การกระตุ้นการสลายไขมัน การเปลี่ยนแปลงและพัฒนาของเซลล์ต้นกำเนิดจากไขมันและผลกระทบต่อหน้าที่ของเซลล์ไขมันได้ ผลการศึกษาในงานวิจัยนี้แสดงให้เห็นว่า อนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตรที่บรรจุด้วยกรดไลโปอิก มีประสิทธิภาพในการลดการสะสมของไขมันจากการกระตุ้นกระบวนการสลายไขมัน อีกทั้งยังพบว่ามีประสิทธิภาพในการยับยั้งการเปลี่ยนแปลงและพัฒนาของเซลล์ต้นกำเนิดจากไขมันส่งผลให้เกิดการยับยั้งการหลั่งไซโตไคน์ของเซลล์ไขมันได้อีกด้วย ด้วยคุณสมบัติของอนุภาคแคลเซียมคาร์บอเนตที่บรรจุด้วยกรดไลโปอิกในการลดการสะสมของไขมันได้อย่างมีประสิทธิภาพนี้ อาจก่อให้เกิดการพัฒนาเพื่อนำเอาอนุภาคแคลเซียมคาร์บอเนตระดับนาโนเมตรที่บรรจุด้วยกรดไลโปอิกไปประยุกต์ใช้สำหรับการลดไขมันในผู้ป่วยโรคอ้วนที่มีการสะสมมากเกินไปได้ในอนาคต

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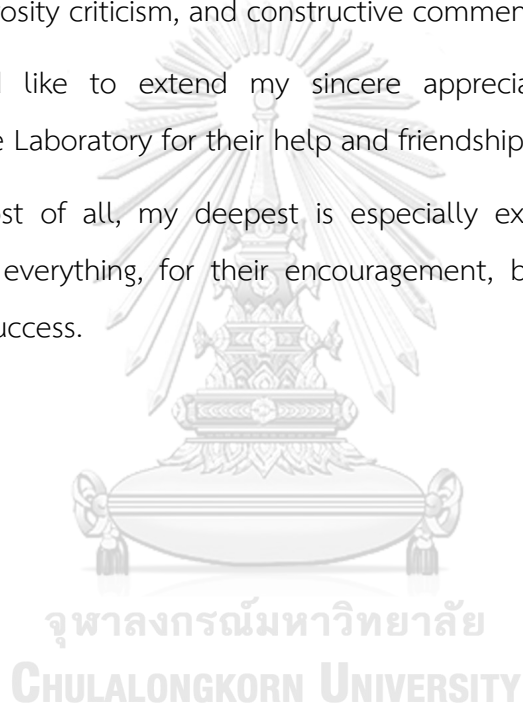
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## CHAPTER I

### BACKGROUND

Obesity is a rising global problem in human. It is an underlying risk factor of many common and severe human diseases, including type 2 diabetes, cardiovascular diseases and hypertension(1, 2). Obesity in human is generally caused by excessive caloric intake accompanied by physical inactivity. Once the caloric intake is more than necessary, the excess fat is accumulated in the form of fatty acids and triglyceride in adipose tissue. Adipose tissue plays a key role in development of obesity and obesity-related disorders. Adipocyte-related cell lineage is originated from stem cells known as adipose-derived stem cells (ADSCs), which differentiated into adipocytes. Three major types of the adipocytes, including white, brown, and beige adipocytes, were found to have distinct functions. The major functions of white adipocyte are energy storage in the form of triglycerides and fatty acid release over the periods of starvation by lipolysis. On the other hand, brown and beige adipocytes have the capacity to use fatty acids and glucose through uncoupled respiration and heat production(3-5). This thermogenesis process in brown and beige adipocytes also appears during prolonged energy restriction, resulting in a reduction of adiposity (6). Furthermore, adipose tissue is known to produce and secrete many bioactive polypeptides known as adipokines including adiponectin, leptin, and resistin. These adipokines play an influential role in energy homeostasis and development of diseases associated with obesity (7). Therefore, modulation of adipogenic differentiation of ADSCs into brown or beige adipocytes and increase of lipolysis attribute as interesting targets for therapeutic intervention in obesity.

The recommended therapy of obesity is non-pharmacological treatment such as physical exercise and lifestyle changes. Nevertheless, these methods for lifestyle modification is often counterproductive and unsuccessful because the success of

exercise in reducing weight loss vary between individuals. The effective pharmacological agents such as orlistat and sibutramine are alternatives for treatment of obesity, but usage of the drugs results in occurrence of some side effects and drug tolerance.(8, 9) Among the alternative medicines for anti-obesity, 1,2-dithiolane-3-pentanoic acid or thioctic acid, also known as  $\alpha$ -lipoic acid (LA), exhibits very potent antioxidant activity (10). It is found in many natural resources and abundant in spinach, cabbage, broccoli, and tomato (11). Interestingly, LA is a compound with beneficial effects on obesity. Previous studies have described functions of LA in anti-obesity by reducing body weight gain in rodents and human,(12, 13) stimulating lipolysis in adipocyte,(14) and inhibiting of adipogenesis in 3T3-L1 cells (15). However, LA is unstable and poor aqueous solubility, leading to low bioavailability (16). Improving a novel drug delivery system is determined to be crucial for enhancing therapeutic efficacy in obesity.

In recent years, there have been widespread interests in the use of nanotechnology as a drug delivery system for enhancing therapeutic efficacy. Among various nanoparticles used as drug carriers,  $\text{CaCO}_3$  nanoparticles ( $\text{CaCO}_3$  NPs) are introduced and it has attracted considerable attention due to a potential drug carrier for their innovative properties such as good bioavailability, low toxicity, and slow biodegradability (17). From a positive viewpoint, the good sustained-release performance and high stability of  $\text{CaCO}_3$  NPs expose new avenues of a proper drug delivery carrier in this study (17).

Our study was conducted to evaluate the properties of  $\text{CaCO}_3$  NPs, including its use as a vehicle for delivery of LA in ADSCs. We developed the LA-incorporated  $\text{CaCO}_3$  NPs (LA- $\text{CaCO}_3$  NPs) using chemical precipitation and evaluated the efficacy of LA- $\text{CaCO}_3$  NPs by lipolytic activity, adipogenesis, and adipokines expression. Here, we show that LA- $\text{CaCO}_3$  NPs exert potent potential benefits for lipid reduction in ADSCs, compared to anti-obesity properties of LA alone.

## CHAPTER II

### LITERATURE REVIEW

#### 1. Obesity

Obesity is a worldwide epidemic. The incidence of obesity (and/or being overweight) is dramatically increasing global in both children and adults. It has been considered public health due to associated with many diseases such as insulin resistance, diabetes, metabolic syndrome, and increased cardiovascular morbidity and mortality (1, 2). Obesity also has serious social and psychological consequences such as low self-esteem and clinical depression and affects all ages and socioeconomic groups (18). Many practical methods used in clinical practice to assess body fat (**Table 1**). Heights and weights were obtained using standardized methods (19). The world health organization (WHO) definition is a person with a BMI of 30 or more is generally considered obese. A person with a BMI equal to or more than 25 is considered overweight. It is defined as a person's weight in kilograms divided by the square of his height in meters ( $\text{kg}/\text{m}^2$ ). A WHO expert committee has proposed the classification of overweight and obesity that applies to both men and women and to all adult age groups (**Table 2**). The behavioral and environmental changes such as poor dietary habits and sedentary lifestyle contribute the development of obesity (2). However, the excess accumulation of lipid within adipose tissue is a fundamental to the development of obesity by alteration of adipose tissue metabolic and endocrine function.



**Table 1** Defining obesity (18).

Method	Definition	Advantages/limitations
<b>BMI</b>	Weight in kilograms divided by square of the height in meters.	BMI correlated strongly with densitometry measurements of fat mass; main limitation is that it does not distinguish fat mass from lean mass.
<b>Waist circumference</b>	Measured (in centimeters) at midpoint between lower border of ribs and upper border of the pelvis.	Waist circumference and waist-to-hip ratio provide measures for assessing upper body fat deposition; neither provide precise estimates of intra-abdominal (visceral) fat.
<b>Skinfold thickness</b>	Measurement of skinfold thickness (in centimeters) with callipers provides a more precise assessment if taken at multiple sites.	Measurements are subject to considerable variation between observers require accurate callipers and do not provide any information on abdominal and intramuscular fat.
<b>Bioimpedance</b>	Based on the principle that lean mass conducts current better than fat mass because it is primarily an electrolyte solution; measurement of resistance to a weak current (impedance) applied across extremities provides an estimate of body fat using an empirically derived equation.	Devices are simple and practical but neither measure fat nor predict biological outcomes more accurately than simpler anthropometric Measurements.

**Table 2** Cut-off points proposed by a WHO expert committee for the classification of overweight (18).

BMI* (kg m <sup>-2</sup> )	Classification	Popular description
<18.5	Underweight	Thin
18.5-24.9	-	'Healthy', 'normal', 'acceptable'
25.0-29.9	class I obesity	Overweight
30.0-39.9	class II obesity	Obesity
≥40.0	class III obesity	Morbid obesity

### 1.1 Adipose tissue

Adipose tissue plays an essential role in regulating energy balance through its metabolic, cellular and endocrine functions. It is an organ that typically functions as the body's energy storage in the form of triglyceride and mobilizing energy in the form of fatty acids. There are homeostatic mechanisms in adipose tissue to balance lipid storage and mobilization because fatty acids and their metabolic products can be toxic in peripheral organs. Lipotoxicity and the accumulation of saturated fat in peripheral tissues are an important step in the development of metabolic syndrome.

Adipose tissue is included of many cell types including the precursor cells of the adipocyte, preadipocytes, adipocytes, vascular endothelial cells, and immune cells. However, adipocytes are abundant in adipose tissue that differentiates from adipocyte progenitors/adipose-derived stem cell (ADSCs) by adipogenesis (**Figure 1**). Adipogenesis, the differentiation of fibroblast-like mesenchymal stem cells (MSCs) into adipocytes, plays an essential role in regulation of whole body energy metabolism. At the cellular level, adipogenesis generally described as a two-step process: a commitment step,

wherein committed adipocyte progenitors (or preadipocytes) are generated from multipotent MSCs, and a differentiation step, wherein pre-adipocytes acquire the features of mature, functional adipocytes (20). The generation of adipocytes by adipogenesis contributes development of obesity (21).

There are three types of adipocytes including white, beige, and brown adipocytes with different phenotype, function, and regulation (**Table 3**). The major adipocytes in the body consist of white adipocytes. It is predominant form found in adults (brown adipose tissue is principally found in neonates) and contribute to metabolic disease in obesity (22). White adipocytes also develop in multiple anatomical sites, and there are known metabolic and endocrine differences between subcutaneous and visceral adipocytes (23). The function of white adipocytes is store excess energy as lipids to regulate energy homeostasis and fatty acid release over periods of starvation by lipolysis. Lipolysis in white adipocytes is a process leading to release of fatty acids and glycerol to the plasma. Additionally, white adipocytes are also an important target for the action of human therapeutics such as the thiazolidinedione (TZD) antidiabetic drugs being the most notable example (24).

In contrast, the function of brown adipocytes is to transfer fatty acids from food and generate heat (thermogenesis), particularly in human neonates. There is abundant mitochondrial content in brown adipocytes because it require a large amount of ATP to maintain processes such as lipolysis,  $\beta$ -oxidation of fatty acids, and fatty acid synthesis. Moreover, brown adipocytes contain a specialized mitochondrial protein called UCP1 to generate heat (25). Cold exposure can stimulate lipolysis, which activates sympathetic nervous system signaling in brown adipocytes. The free fatty acids from lipolysis are used to generate heat using the UCP1 protein. Recent study has shown that brown adipocytes are dispersed throughout the human adipose tissue and are

metabolically active (26). Modulation of brown adipocyte activity has been proposed as a potential strategy in obesity and its associated diseases.

Interestingly, white adipocytes have ability to switch between energy storage and expenditure. White adipocytes phenotype can shift into brown-like adipocytes phenotype in terms of features such as morphology, gene expression pattern, and mitochondrial respiratory activity (27). This process is called the “browning” of white adipocytes. The brown-like adipocytes appearing in white adipocytes are often called “inducible, beige, or brite adipocytes.” The appearance of these inducible brown adipocytes in white adipocytes may also involve trans-differentiation processes of white-to-brown adipocytes (23). The beige adipocytes are derived from precursor cells that are different from classical brown adipocytes and are closer to the white adipocytes lineage (27, 28). These beige adipocytes appear a white adipocyte-like phenotype, such as large lipid droplets and the lack of UCP1 expression, under basal conditions. Nevertheless, in response to certain stimuli, beige adipocytes transform into a brown adipocyte-like phenotype, such as multilocular/small lipid droplets and UCP1 expression (29, 30). The activation of brown/beige adipocytes in humans may contribute to an important strategy for treating obesity.

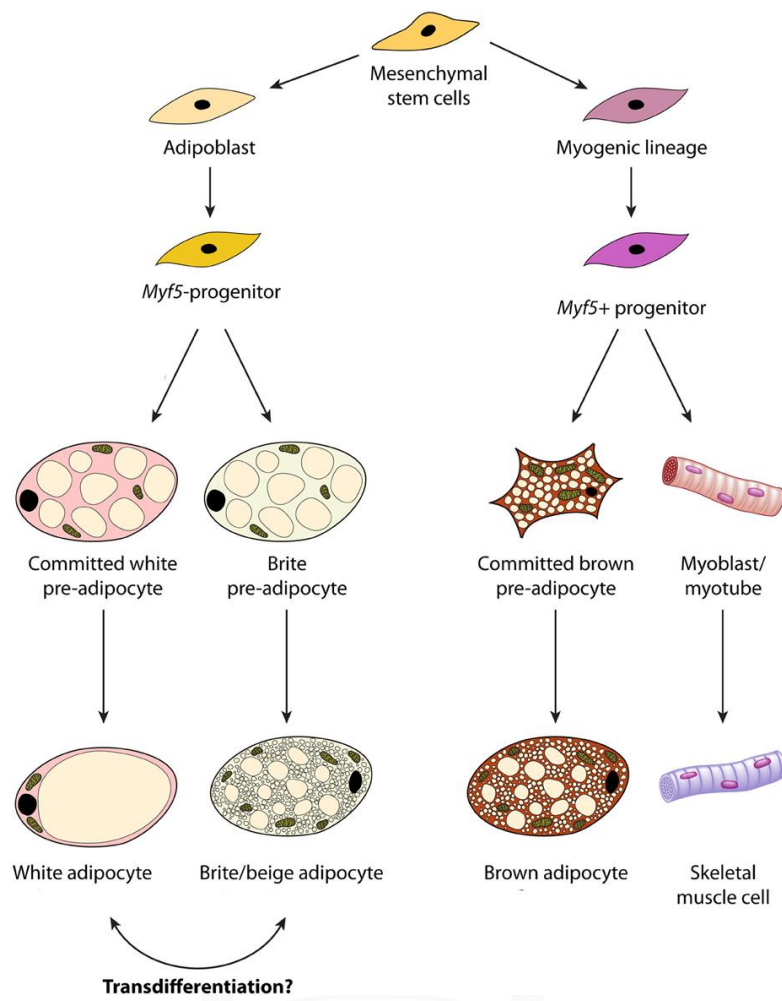


Figure 1 Differentiation into white, beige, or brown adipocytes. Modified from (28)

**Table 3** Differences amongst the three types of adipocytes (31).

	White adipocytes	Beige adipocytes	Brown adipocytes
<b>Location</b>	Inguinal (sWAT), mesenteric, retroperitoneal, perigonadal, omental (vWAT)	Within inguinal WAT, other sWAT?	Interscapular, perirenal, axillary, paravertebral
<b>Morphology</b>	Unilocular/large lipid droplets	Unilocular large/multiple small lipid droplets	Within inguinal WAT, other sWAT?
<b>Function</b>	Storage of energy as triglycerides	Adaptive thermogenesis	Heat production
<b>Mitochondria</b>	(+)	Upon stimulation (++)	(+++)
<b>UCP1</b>	Nearly undetectable	Upon stimulation (++)	(+++)
<b>Obesity</b>	Positive effect	Negative effect	Negative effect

**Abbreviation:** WAT, white adipose tissue; vWAT, visceral white adipose tissue; sWAT: subcutaneous white adipose tissue; UCP1, uncoupling protein 1

### 1.1.1 Specific cell surface marker in adipocyte population

Adipose tissue exists in three forms, white, beige and brown adipocytes, which differ in terms of morphology, function, abundance and regulation. Specific cell surface marker is used to distinguish adipocyte population in adipose tissue. A panel of molecular markers has been identified white, beige, and brown adipocytes (32-34). These include leptin, HOXC8, and HOXC9 for white adipocytes; TBX1 and TMEM26 for beige adipocytes; and UCP-1, CIDEA, and PRDM16 for brown adipocytes. These markers have been used to characterize adipocyte subtypes. Nevertheless, some of these markers such as PRDM16 are also expressed in non-adipose tissues (33). Consequently, specific surface markers would be very helpful in identifying and targeting different adipocyte subpopulations. For this reason, Ussar and colleagues have identified specific cell surface markers for adipocyte population using a combination of *in silico*, *in vitro*, and *in vivo* studies. These results show that ASC-1, PAT2, and P2RX5 are newly identified cell-surface proteins. These proteins located the plasma membrane of adipocytes that can define the heterogeneity and intrinsic differences of adipose tissue depots. ASC-1 is a white adipocyte-specific cell surface protein, with no expression in beige and brown adipocytes. PAT2 are cell surface markers express in beige adipocytes and P2RX5 is specific in brown adipocytes (35).

Ussar and colleagues have identified specific cell surface markers for adipocyte population. It arises regarding the physiological role of these markers in adipocyte biology. The specific cell surface markers including ASC-1, PAT2, and P2RX5 are induced upon adipocyte differentiation but nearly absent in mouse and human preadipocytes, suggesting a role in adipocyte formation and/or physiology. Moreover, expression of PAT2 and P2RX5 was lower in BAT of diabetic (*db/db*) mice, which is in line with the “whitening” of brown adipose

tissue. Contrary, whereas *P2RX5* expression was induced in WAT upon cold exposure (also known as “browning” of WAT), *ASC-1* (amino acid transporter) was down-regulated.

PAT2 and P2RX5 are both membrane transporters (amino acid transporter and ion channel, respectively), they may be associated with intracellular signaling pathways involved in activation of brown adipocytes. A main activation route of brown adipocytes is the  $\beta_3$ -adrenergic pathway, resulting in increased levels of intracellular cyclic cAMP, activation of protein kinase A, and subsequent phosphorylation of downstream targets. Whether the  $\beta_3$ -adrenergic pathway and/or other intracellular pathways are coupled to PAT2 and P2RX5 membrane transporters remains to be evaluated.

### 1.1.2 Regulation of lipolysis and thermogenesis in adipocytes

Adipose tissue is richly innervated by sympathetic and parasympathetic fibers. It requires large amounts of ATP to maintain their functions. The main roles of adipose tissue are lipogenesis and lipolysis (36). The lipogenesis is to store excess dietary fat in the form of triglycerides. On the other hand, lipolysis is hydrolysis of triglycerides to fatty acids and glycerol in adipocytes (**Figure 2**). These processes are importance for the control of lipid and carbohydrate metabolism (37).

When nutrient excess, energy uptake by white adipocytes is regulated by insulin’s anabolic role, mediating glucose and lipid uptake, lipogenesis and inhibition of lipolysis. On the other hand, energy deficits caused by energy shortages (for example, fasting) or increased energy expenditure (for example, cold exposure) are regulated by the sympathetic nervous system (SNS) (38, 39). The tone of the sympathetic innervation exerts master control on lipolysis by the release of noradrenaline (also known as norepinephrine) (40).

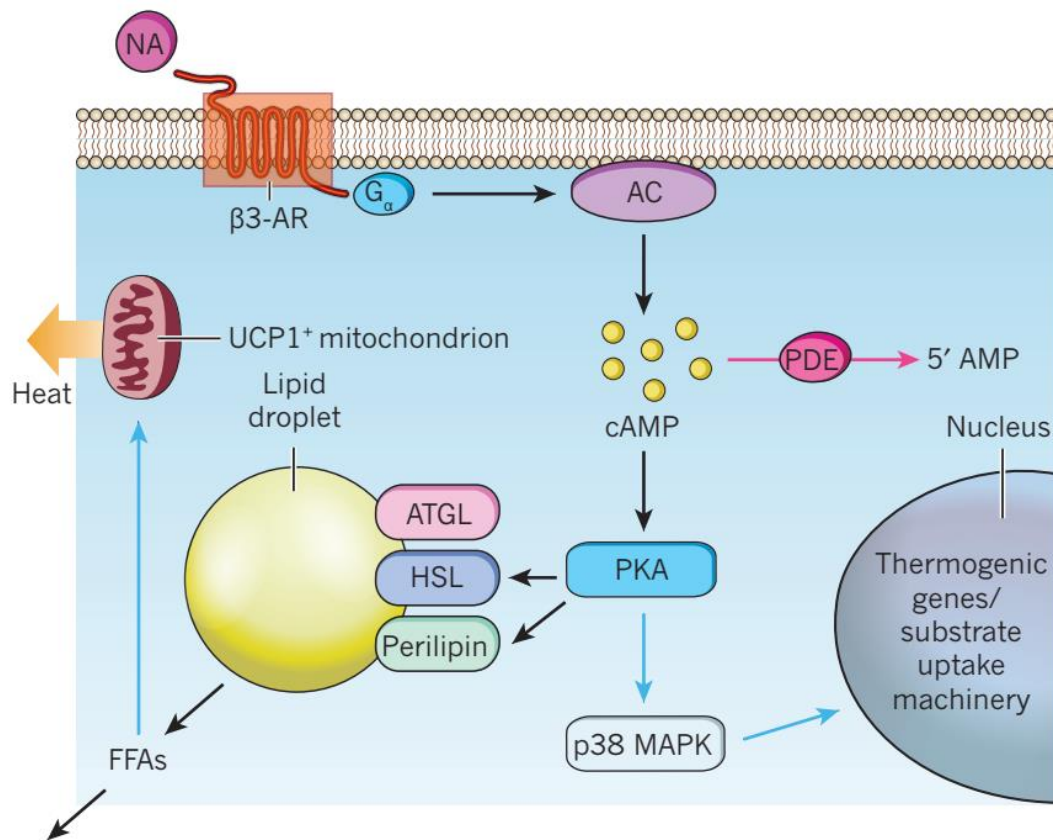


Physiologically, cold exposure stimulates sympathetic nervous system crosstalk between white, beige, and brown adipocytes with respect to nutrient release, partitioning and utilization (41). Upon cold exposure, the neurotransmitter noradrenaline stimulates  $\beta_3$ -adrenergic pathway, resulting in the lipolysis in adipocytes. Increased sympathetic tone to white adipose tissue activates lipolysis resulting in intracellular lipid stores are released to provide nutrients for other organs.

The lipolysis consists of at least three major enzymes including adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoglyceride lipase (MGL). The primary cleavage of triacylglycerol to diacylglycerols is performed by adipose triglyceride lipase (ATGL). Hormone-sensitive lipase (HSL) is the major diglyceride lipase in adipocytes, and monoglyceride lipase (MGL) completes the process by generating glycerol and free fatty acids. The free fatty acids are the main fuel for UCP1 mediated brown adipose tissue thermogenesis (42). Increased sympathetic tone to brown adipose tissue also rapidly induce intracellular lipolysis and eventually lead to free fatty acids release from lipid droplets to locally activate UCP1 and to serve as substrates for oxidation (41). Importantly, the substrate uptake machinery is also upregulated, allowing lipids released by white adipocytes to be taken up by brown adipocytes (42). Sustained SNS activation will also result in increased thermogenic capacity due to increased thermogenic gene expression and, in cases of chronic activation, due to proliferation and differentiation of brown adipocyte precursors (42).

Moreover, white adipocytes also recruit and activate beige adipocytes by increased SNS tone, which might contribute to increased total thermogenic capacity (43, 44). The function of beige adipocytes is an energy burning that has yet to be clarified in rodents. It is difficult to experimentally distinguish the thermogenic contribution of beige adipocytes from that of brown adipocytes. Because there are several factors including cold exposure, which activate and recruit beige adipocytes as well as brown adipocytes.

Additionally, lipolysis has been driven by  $\beta$ -adrenergic pathway in the adipocyte, but other inducers exist and may have physiological relevance. For example, the transcription factor FOXC2 sensitizes adipocytes to adrenergic stimulation by modulating the expression and activity of adrenergic signaling molecules (45-47). Moreover, BMP family is essential for brown adipose tissue development and energy balance (48). BMP8B induces the phosphorylation of downstream adrenergic targets such as hormone-sensitive lipase and p38 MAPK signalling through Smad1, 5 and 8 (49). BMP7 can also drive brown adipogenesis and recruit beige adipocytes by activating p38 MAPK (50). However, there are several alternative mechanisms that activate brown and beige adipocytes independently of the SNS have been studied.



**Figure 2** Regulation of lipolysis and thermogenesis in adipocytes by the sympathetic nervous system (51).

The sympathetic nervous system triggers intracellular signaling events that lead to lipolysis (black arrows). Noradrenaline (NA) released by sympathetic nerve endings binds to  $\beta_3$ -adrenoreceptors ( $\beta_3$ -ARs), which couple to  $G_\alpha$  G-proteins that activate adenylate cyclase (AC), increasing the cellular concentration of cAMP. This activates protein kinase A (PKA), which phosphorylates lipid droplet proteins including hormone-sensitive lipase (HSL) and perilipin, allowing activation of adipose triglyceride lipase (ATGL). The net result is the hydrolysis of triglycerides stored in the lipid droplet to free fatty acids (FFAs), which are released. This response can be attenuated (red arrows) by the activation of phosphodiesterases (PDE), which hydrolyse cAMP. In the case of recruitment and activation of beige adipocytes and the activation of brown adipocytes, this signalling pathway (blue arrows) also leads to increased expression of UCP1 and other thermogenic genes through the p38 MAPK cascade. FFAs released by lipolysis activate existing UCP1 in the mitochondria, and their oxidation produces heat.

## 1.2 Adipose depots

Although the total adipose tissue is important for the development of insulin resistance, it is considered that some fat depots are more linked to risk factors for many diseases. There is different adipose tissue between subcutaneous and visceral adipose tissue. Whole body adipose tissue has generally subdivided in two main components: subcutaneous and visceral adipose tissue.

### 1.2.1 Subcutaneous adipose tissue

Subcutaneous adipose tissue is the most easily identifiable because it is stored directly under the skin. Subcutaneous adipose tissue is less metabolically active than visceral adipose tissue, it may have better short-term and long-term storage capacity. Thus, this depot is important to accumulate triglyceride in periods of excess energy intake and supply the organism with free fatty acids in periods of fasting, starvation, or exercise. Another suggested a role of subcutaneous adipose tissue is to be a buffer during intake of dietary lipids, thus protecting other tissues from lipotoxic effects (37). New smaller adipocytes act as a powerful buffer, which absorb free fatty acid and triglyceride after caloric intake. As adipocytes grow larger, they become dysfunctional capacity. Large adipocytes are insulin resistant, hyper lipolytic and resistant to anti-lipolytic effect of insulin. Subcutaneous adipose tissue contains the small adipocytes, which are more insulin-sensitive and high absorb for free fatty acids and triglyceride, preventing their deposition in non-adipose tissue. On the other hand, visceral adipose tissue contains greater number of large adipocytes.

### 1.2.2 Visceral adipose tissue

Visceral adipose tissue is of great interest. The excess visceral adipocytes accumulation of obesity is associated with many risk factors in obesity-related disorders. It generally has indistinctly included omental, mesenteric as well as extraperitoneal adipose tissue. Mesenteric and omental adipose tissue are drained by the portal vein, a feature which has been at the center of some hypotheses linking visceral adipose tissue accumulation and metabolic disease (52, 53). Anatomical position, molecular biology and physiology in visceral adipose tissue quite different among the subcutaneous adipose tissue. Visceral adipose tissue has been shown to correlate with the development of insulin resistance compared with subcutaneous adipose tissue (54). Additionally, subcutaneous and visceral adipose tissues seem to display depot-specific responsiveness to lipolytic activity. Lipolysis in visceral adipose tissue are more responsive to adrenergic agonist stimulation than subcutaneous adipocytes (55). Lipolysis is markedly increased in visceral adipocytes as compared to subcutaneous adipocytes in obese males, due to  $\beta_3$ -adrenoceptor is higher in visceral adipose tissue (56). In obesity, visceral adipose tissue is more sensitive to weight loss than subcutaneous adipose tissue (57). Furthermore, excess visceral adipocytes accumulation results in altered release of adipokines (7).

### 1.3 Adipokines

Adipose tissue is also recognized as an important endocrine organ, which secretes several peptides and other factors, which are known as adipokines (58). Adipokines have been linked to adipose tissue dysfunction and metabolic complications of obesity.

#### 1.3.1 Adiponectin

Adiponectin, an adipose-tissue derived hormone, are associated with a decreased risk for development of obesity, cardiovascular disease, and type 2 diabetes (59). Adiponectin is mainly secreted by adipocytes and abundantly present in the circulation. There are at least 3 forms in blood circulation including low molecular weight, middle molecular weight, and high molecular weight. The high molecular weight adiponectin is considered the most metabolically active form (60). Adiponectin receptors are expressed in many tissues such as muscle, liver tissue, and mainly adipocytes. The functions of adiponectin have been shown to play important roles in the modulation of glucose and lipid metabolism. It is commonly anti-inflammatory by inflammatory cytokine suppression such as TNF- $\alpha$  (61). Serum levels of adiponectin are reduced in obese and insulin-resistant human subjects and animal models (62-65). Contrary, diet-induced weight loss induces adiponectin synthesis, parallel by an increase in plasma levels of adiponectin. Interestingly, adiponectin is also antidiabetic adipokine that enhances insulin sensitivity (66, 67).

### 1.3.2 Leptin

Leptin, a 16kDa product of obese (Ob) gene, is produced mainly by adipocytes. The functions of leptin regulate food intake, glucose and lipid metabolism and energy expenditure via the central nervous system (68). Biological activities of leptin are mediated through binding to the transmembrane receptor (ObR), a member of the class I cytokine receptor superfamily, that includes six isoforms different in the length of their intracellular tails. The binding of leptin induces the dimerization of receptor molecules and leads to the activation a broad array of multiple intracellular downstream signal pathways (69). Plasma levels of leptin highly correlate with body mass index (BMI). Most obese individuals have an increased food intake despite high circulating leptin levels, which is mentioned to as leptin resistance (70). The leptin levels are decreased while weight loss due to food restriction is associated with a decrease in plasma leptin in mice and humans (71). Additionally, leptin plays a role in controlling the immune system and inflammatory response. It stimulates proliferation of immune cells, by inducing the production of inflammatory cytokines (72, 73). There is strongly association between leptin levels and insulin sensitivity in human (74).

### 1.3.3 Resistin

Resistin is another pro-inflammatory adipokines that derived from both adipocytes and immune cells, including macrophages and monocytes (75). Resistin is a member of the cysteine-rich family of resistin-like molecules (RELMs) that are related to the activation of inflammation. Resistin plays a role in the pathogenesis of obesity and insulin resistance in humans, both of which appear to contribute to the development of type 2 diabetes. Resistin levels are increased in diet-induced obesity as well as in genetic models of obesity and insulin resistance. Administration of anti-resistin antibody improves blood sugar and insulin action in mice with diet-induced obesity (76). Moreover, resistin has been shown to associate Type 2 diabetic patients (77). The mechanisms of resistin in obesity and obesity-related disorders remain unresolved. Interestingly, new targets for pharmacological intervention to decrease the level of resistin will be identified, thereby providing a novel therapeutic strategy for the treatment of obesity.



## 2. Lipoic acid (LA)

Lipoic acid (LA), also known as 1,2-dithiolane-3-pentanoic acid or thioctic acid, has one chiral center and therefore exists in both R- and S-enantiomeric forms (**Figure 3**). The R-LA is essential as a mitochondrial  $\alpha$ -ketoacid dehydrogenases, and thus serves a critical role in mitochondrial energy metabolism (78, 79). LA can be found from food in cabbage, spinach, liver, meat, whole-wheat and yeast of beer. Moreover, it is also endogenously produced by the liver by the lipoic acid synthase (78).

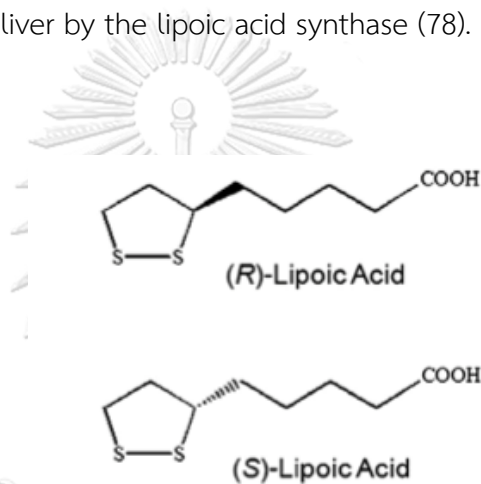


Figure 3 The R and S enantiomers of lipoic acid. Modified from (79)

### Anti-obesity effects LA

Recently, LA has gained considerable because of its diverse biological actions. It is a unique antioxidant because LA has beneficial effects on fuel metabolism. LA exhibits potential to enhance glucose transport into skeletal muscle of lean and insulin-resistant obese animals (80, 81). The effects of LA contribute potential beneficial effects on anti-obesity properties both in rodents and humans without serious side effects (12, 13, 81-83). Supplementation of a high fat diet induced a lower body weight gain and adipose tissue size in both control and high-fat fed rats (12). Oral administration of LA in diabetic mice fed on a high-fat diet and *db/db* mice

dramatically reduced the body weight and visceral fat content by activation of SIRT1 (82). In obese individuals, LA also reduce body weight gain (13). The action of LA decreases hypothalamic AMPK activity, which a fuel sensor in the cell and cause profound weight reduction (84).

During the adipocyte differentiation, lipid droplets are gradually accumulated in the cytoplasm for energy storage. The excessive accumulation of adipocytes by adipogenesis generally causes development of obesity (21). LA directly controls adipogenesis through activation of adipogenic transcription factors, the direct modulation of intracellular degradation process and consequently decrease intracellular lipid accumulation of adipocytes (15, 85). LA has a beneficial property to regulate adipogenesis by induction of beige adipocytes in white adipocytes from overweight/obese subjects. The beige adipose features were characterized by increased mitochondrial biogenesis and the acquirement of beige adipocytes features through the SIRT1/PGC-1 $\alpha$  pathway (86). Nevertheless, it is not known whether the LA is effective in ADSCs.

Adipose tissue is a target for LA therapeutic properties. Activation of lipolysis has been proposed as a promising therapeutic target for the treatment of obesity. LA exerts lipolysis in white adipocytes by significantly increasing the PKA-mediated phosphorylation of hormone-sensitive lipase (14). Dietary LA supplementation increase plasma free glycerol and the rate of free glycerol release from abdominal adipose tissue (87, 88). Increased free fatty acids levels from adipose tissue has been associated with the obesity-related complications (89). Nevertheless, increased lipolysis in adipose tissue does not necessarily increase serum free fatty acids levels. Free fatty acids are taken by other metabolic tissues toward increased free fatty acids utilization and energy expenditure, and therefore

protects against obesity (14, 90). The effect of LA in lipolysis seem to be independent of its antioxidant capacities, other antioxidants such as vitamin C or N-acetylcysteine are not able to induce lipolysis under similar conditions (14). Additionally, LA has potential to prevent or decrease lipid accumulation not only in adipose tissue, but also in other non-adipose (91-94).

The importance of adipokines in the development of obesity and obesity-related diseases has been widely studied. Indeed, adipocytes secrete multiple bioactive peptides, such as adiponectin, leptin and resistin that play key roles in the regulation of energy metabolism and insulin sensitivity (7). Dysregulated adipokine secretion or production from the adipose tissue of obese individuals contributes to the development of obesity-related disorders. LA has potential to regulate adipokines in adipocytes. Adiponectin is an insulin-sensitizing adipokine that regulates glucose and lipid metabolism, and its levels are inversely correlated with fat accumulation in adipose tissue (66, 67). Dietary supplementation with LA can improve circulating adiponectin levels and adiponectin mRNA expression in adipose tissue (95, 96). However, LA induce a dose-dependent inhibition on adiponectin mRNA expression and protein secretion in 3T3-L1 adipocytes (97). Additionally, LA presents a concentration-dependent (1–500  $\mu$ M) inhibition of leptin secretion and gene expression (98). Circulating leptin levels are reduced by LA with the reduction of bodyweight and fat mass (93, 98, 99). Resistin is an adipokine that involved in the development of obesity and insulin resistance (100). There is no report the effect of LA in resistin.

### 3. Nanotechnology

In recent years, there has been widespread interest in the use of nanotechnology as specifically drug delivery system for enhancing therapeutic efficacy. Many substances are under investigation for drug delivery system. Recently, there has been interested in the use of nanoparticles as drug carriers. The reason why nanoparticles are attractive for drug delivery system is based on their important and unique features, such as their surface area is much larger than that of other particles, their quantum properties and their ability to adsorb and carry other compounds. A large (functional) surface of nanoparticles, which can bind, adsorb and carry other compounds such as drugs, probes and proteins (101, 102). Typically, the drug of interest is dissolved, entrapped, adsorbed, attached and/or encapsulated into or onto nanoparticles, which depend on the method of preparation nanoparticles (103). Pharmaceutical nanoparticles can advantageously improve therapeutic efficacy by enhancing the drug bioavailability, serum stability and and/or a reduction in the toxicity of the free drug to non-target organs (101). Various types of nano drug delivery systems, mostly organic and some inorganic ones have been investigated for their ability to serve as carriers in drug delivery. Among various nanoparticles,  $\text{CaCO}_3$  nanoparticles ( $\text{CaCO}_3$  NPs) have been introduced and attracted considerable attention as a relatively safe drug vehicle (104, 105)

$\text{CaCO}_3$  NPs have been interested as drug carrier because of their innovative property such as availability, biocompatibility and low biodegradability. The drug incorporated into  $\text{CaCO}_3$  NPs are stable and released very slowly (106). Moreover,  $\text{CaCO}_3$  NPs appear potential to reduce the cell viability and enhance the apoptosis of the breast cancer cells (107). However, the mechanism of  $\text{CaCO}_3$  NPs should be considered. The engineered  $\text{CaCO}_3$  NPs increase the stability and retention time of the drug (108-111). For a positive viewpoint, especially the good sustained-release performance and high stability provide the new approaches for a proper drug delivery carrier.



**Research questions**

Do LA-CaCO<sub>3</sub> NPs affect the lipolysis in adipocytes and differentiation of ADSCs to adipocytes?

**Research objectives**

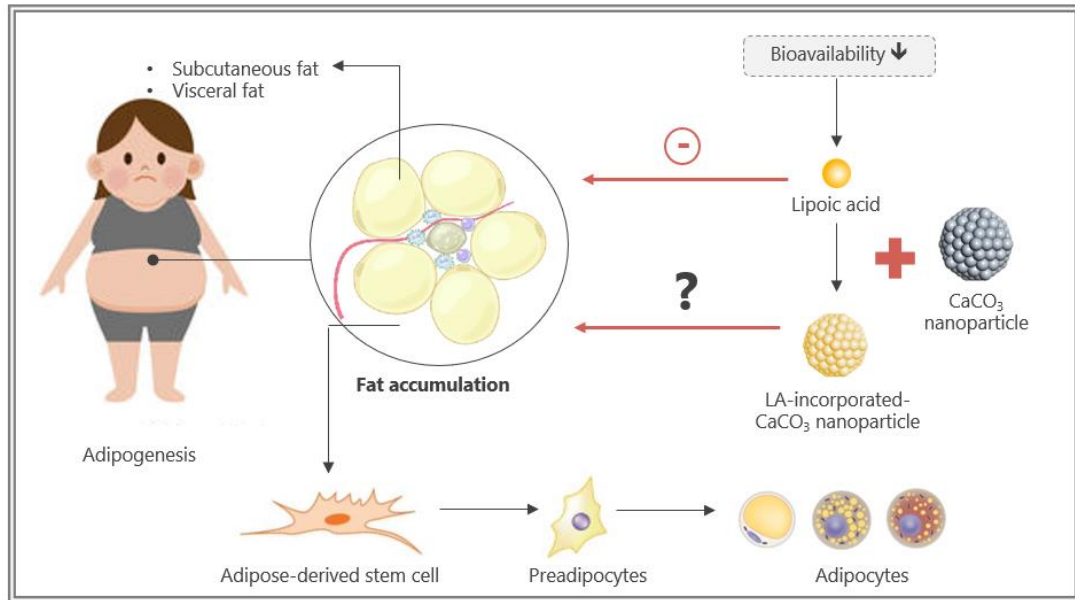
To study the effect of LA-CaCO<sub>3</sub> NPs in lipolysis and differentiation of ADSCs to adipocytes.

**Research hypothesis**

The LA-CaCO<sub>3</sub> NPs stimulate the lipolysis and differentiation of ADSCs to beige/brown adipocytes.



## Conceptual framework



## CHAPTER III

### MATERIALS AND METHODS

#### Part I

##### Preparation of LA-CaCO<sub>3</sub> NPs

The synthesis of LA-CaCO<sub>3</sub> NPs follow the previous paper (106). Chemical precipitation for the preparation of CaCO<sub>3</sub> NPs were used in this study. Briefly, nanoparticles were prepared by the reaction of calcium ions from the aqueous solution from one hand, and carbonate ions from the aqueous solution from another. The aqueous solution of a LA was added into aqueous solutions in the process and then the formation of nanoparticles and entrapment of compounds was performed simultaneously. The LA-CaCO<sub>3</sub> NPs were separated by centrifugation.

##### Characterization of LA-CaCO<sub>3</sub> NPs

###### Thermogravimetric analysis (TGA)

The compositions of the LA-CaCO<sub>3</sub> NPs were determined by thermogravimetric analysis (TGA) using a TGA Q50 instrument with heating from 0 to 850 °C at a rate of 10 °C/min.

###### Zeta potential measurement

For stability analysis of particles, surface charges of NPs were measured by zeta-potential. The zeta potential distribution of nanoparticles was analyzed by a Malvern Zetasizer Nano Series (Malvern Instruments, England).

###### Transmission electron microscopy

Particle size analysis, LA-CaCO<sub>3</sub> NPs were measured by TEM (Hitachi, Japan) at a voltage of 100 kV.



## Adipocytes differentiation

Adipose-derived stem cells (ADSCs) have capacity to differentiate into adipocytes. ADSCs were differentiated using Adipogenesis differentiation kit (GIBCO, USA). Briefly, ADSCs were harvested in 0.25% trypsin/EDTA 3-5 minutes at 37°C. The cells were transferred into a centrifuge tube at 2250 rpm for 5 minutes. ADSCs were seeded into 24 well plates at a density  $2 \times 10^4$  per well, 96 well plates at a density of  $5 \times 10^3$  per well in cell culture medium and incubated for 24 h to allow cell adherence. After that, cells were changed control medium to pre-warmed complete adipogenesis differentiation medium and continue incubation. Cells were changed the complete adipogenesis differentiation medium every 3-4 days. After a specific period of cultivation (14 days), the adipogenic cultures were processed in experiments.

## Cytotoxicity of LA-CaCO<sub>3</sub> NPs in ADSCs

### Cell viability

We evaluate the influence of LA-CaCO<sub>3</sub> NPs on cellular viability with the PrestoBlue™ reagent (Invitrogen, USA). Metabolically active cells can reduce the PrestoBlue reagent, with the colorimetric changes used as an indicator to quantify the viability of cells in culture. Human cell types were used in this experiment including keratinocytes (HaCaT), human dermal fibroblast cells (HDFs), adipose-derived stem cells (ADSCs) and adipocytes. All the cells were seeded in 96 well plates at a density of  $5 \times 10^3$  per well in 45  $\mu$ l of cell culture medium and incubated for 24 h to allow cells adherence. Confluent cells were stimulated with treatment conditions. The plates were incubated for 24, 48 and 72 hours. Untreated cells were used as negative controls. Following incubation, 10  $\mu$ l PrestoBlue solution were added to each well, and then plates were placed back into the incubator for a further 20 min incubation. Fluorescence

was measured using a microplate reader at 560 nm excitation and 590 nm emission (Thermo, Varioskan Flash, England).

A calculation for estimating the percentage of cell viability is:

$$\% \text{ Cell viability} = (\text{Fluorescence treatment} / \text{Fluorescence control}) \times 100$$

### ROS generation

To determine the burst of free radicals and reactive oxygen species (ROS), an intracellular ROS-indicator, 2,7-dichlorofluorescein-diacetate (DCFH-DA) was applied in this experiment. HaCaT, HDFs, ADSCs, and adipocytes were seeded into 96-black well plates at a density of  $5 \times 10^3$  cells per well in 100  $\mu\text{L}$  of culture media and incubated at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$  atmosphere for 24 h. Cells were washed with phosphate-buffered saline and incubated with 0.1  $\mu\text{M}$  of  $\text{H}_2\text{DCF-DA}$  (Invitrogen, USA) at a volume of 100  $\mu\text{L}$ /well for 30 minutes protected from light. After that, cells were washed with phosphate-buffered saline again and stimulated with various conditions.  $\text{H}_2\text{O}_2$  (0.8 mM) was used as positive control. Fluorescence was immediately measured for 1 hour using a microplate reader at excitation and emission wavelengths of 485 and 528 nm (Thermo, Varioskan Flash, England).

A calculation for estimating the percentage of ROS generation is:

$$\% \text{ ROS Generation} = (\text{Fluorescence treatment} / \text{Fluorescence control}) \times 100$$

## Lipid accumulation

### Oil Red O staining

To study the effect of LA-CaCO<sub>3</sub> NPs with the correlation between lipolysis and lipid accumulation in adipocytes. ADSCs were differentiated in 96 well plates. The cells were stimulated with all treatment conditions. Untreated cells were used as negative controls. After 24 hours of exposure to treatments, the cells were stained with Oil Red O to identify lipid droplets. Briefly, media was removed, and cells were fixed in 4% formaldehyde at room temperature for 10 minutes. Cells were washed twice with phosphate-buffered saline and then with 60% isopropanol, before staining with 0.3% Oil Red-O (Sigma, USA) for 20 minutes at room temperature. Cells were washed in running tap water. The images were captured using a phase contrast inverted microscope (Nikon, Eclipse TS 100, Japan). After that, cells were eluted dye with 100% isopropanol. The solutions were transferred into 96 well plates and measured at OD 500 nm using a microplate reader (Thermo, Varioskan Flash, England).

### Lipolysis measurement

Lipolysis was evaluated by measurement the amount of glycerol released to the media. Lipolysis assay was measured using lipolysis assay kit (Abcam, USA). The amounts of glycerol were detected after 0 and 24 hours of treatment following manufacturer's instructions. Briefly, the ADSCs were seeded into 96 well plates and differentiated into adipocytes. The cells were stimulated with treatment conditions for 24 h. Untreated cells were used as negative controls. After that, 50  $\mu$ l reaction mix were added to each well. Plates were incubated at room temperature for 30 minutes protected from light. The amounts of glycerol were measured at OD 570 nm using microplate reader (Thermo, Varioskan Flash, England).

### Real-time PCR

To evaluate the effect of LA-CaCO<sub>3</sub> NPs on ADSCs differentiation into adipocytes. ADSCs were differentiated in 24 well plates in cell culture medium and incubated for 24 h to allow cell adherence. The cells were stimulated with all treatment conditions for 14 days. Adipogenesis was determined after 14 days of growth in the presence or absence of treatments in adipogenesis differentiation medium. The total RNA was isolated from cells using TRIzol reagent (Invitrogen). RNA concentrations and quality were measured by Nanodrop Spectrophotometer (Thermo Scientific, USA). One microgram of total RNA was used for cDNA synthesis with a First strand cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. qPCR was performed with Express SYBR GreenER qPCR Supermix Universal (Invitrogen) using StepOnePlus Real-Time PCR System (ABI Applied Biosystems). The CT (threshold cycle) values of the target genes were normalized to the endogenous GAPDH level (housekeeping gene) and relative to the normalized calibrator. Primer sequences are listed below.

Genes	Primers	Sequence 5'-3'	References
ASC-1	Forward	GTGGCGCTCAAGAAGGAGAT	(35)
	Reverse	CCTTGGGCGAGATGAAGA	
Pat2	Forward	CCTGCCACTGTATGCACATC	(35)
	Reverse	TAGTCCATGCATCACCGTGT	
P2RX5	Forward	TTCACCAACACCTCGGATCT	(35)
	Reverse	CAGGTTGGTGACCACAAAAA	
Adiponectin	Forward	AAGGAGATCCAGGTCTTATTGG	(65)
	Reverse	ACCTTCAGCCCCGGGTAC	
Leptin	Forward	TTTGGCCCTATCTTTTCTATGTCC	(65)
	Reverse	TGGAGGAGACTGACTGCGTG	
Resistin	Forward	GCTGTTGGTGTCTAGCAAGAC	(112)
	Reverse	CATCATCATCATCTCCAG	

### Statistical analysis

The result from triplicates of the sample was expressed as mean  $\pm$  SD. Statistical significance was analyzed by using one-way ANOVA (Analysis of variance), followed by the Tukey's multiple comparison tests. Statistical significance was defined as *the P - value*  $\leq 0.05$  in comparison to control

### Ethical consideration

This study was approved by the Ethic committee of Research affairs, Faculty of medicine, Chulalongkorn University.



## Part II

### Preparation of LA-CaCO<sub>3</sub> NPs

The synthesis of CaCO<sub>3</sub> NPs follow the previous paper (106). Chemical precipitation for the preparation of LA-CaCO<sub>3</sub> NPs were used in this study. Briefly, NPs were prepared by the reaction of calcium ions from the aqueous solution from one hand, and carbonate ions from the aqueous solution from another. The aqueous solution of a LA was added into aqueous solutions in the process and then the formation of nanoparticles and entrapment of LA were performed simultaneously. The LA-CaCO<sub>3</sub> NPs were separated by centrifugation.

### Characterization of LA-CaCO<sub>3</sub> NPs

#### Thermogravimetric analysis (TGA)

The compositions of the LA-CaCO<sub>3</sub> NPs were determined by thermogravimetric analysis (TGA) using a TGA Q50 instrument with heating from 0 to 850 °C at a rate of 10 °C/min.

#### Zeta potential measurement

For stability analysis of particles, surface charges of NPs were measured by zeta-potential. The zeta potential distribution of nanoparticles was analyzed by a Malvern Zetasizer Nano Series (Malvern Instruments, England).

#### Transmission electron microscopy

Particle size analysis, LA-CaCO<sub>3</sub> NPs were measured by TEM (Hitachi, Japan) at a voltage of 100 kV.

## Adipocytes differentiation

Primary human subcutaneous preadipocytes and visceral preadipocytes media were purchased (Lonza, USA), grown and differentiated following the manufacturer's instructions. Briefly, subcutaneous preadipocytes and visceral preadipocytes were grown in preadipocyte growth media, plated into 96 well plates at a density of  $5 \times 10^3$  per well, 24 well plates at a density  $1 \times 10^4$  per well and grown until confluent. Preadipocyte Growth Medium-2 BulletKit™ medium was added every two days until day 14. The intracellular lipid vacuoles begin to appear 4 to 5 days after induction and continue to increase in number and size for 7 to 14 days.

## Cytotoxicity of LA-CaCO<sub>3</sub> NPs in ADSCs

### Cell viability

We evaluate the influence of LA-CaCO<sub>3</sub> NPs on cellular viability in primary human subcutaneous preadipocytes and visceral preadipocytes using the PrestoBlue™ reagent (Invitrogen, USA) Metabolically active cells can reduce the PrestoBlue reagent, with the colorimetric changes used as an indicator to quantify the viability of cells in culture. The primary human subcutaneous preadipocytes and visceral preadipocytes were seeded in 96 well plates at a density of  $5 \times 10^3$  per well in 45  $\mu$ l of cell culture medium and incubated for 24 h to allow cells adherence. Confluent cells were stimulated with all treatment conditions. The plates were incubated for 24, 48, and 72 hours. Untreated cells were used as negative controls. Following incubation, 10  $\mu$ l PrestoBlue solution were added to each well, and then plates were placed back into the incubator for a further 20 min incubation. Fluorescence was measured using a microplate reader at 560 nm excitation and 590 nm emission (Thermo, Varioskan Flash, England).

A calculation for estimating the percentage of cell viability is:

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### ROS generation

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A calculation for estimating the percentage of ROS generation is:

$$\% \text{ ROS Generation} = (\text{Fluorescence treatment} / \text{Fluorescence control}) \times 100$$



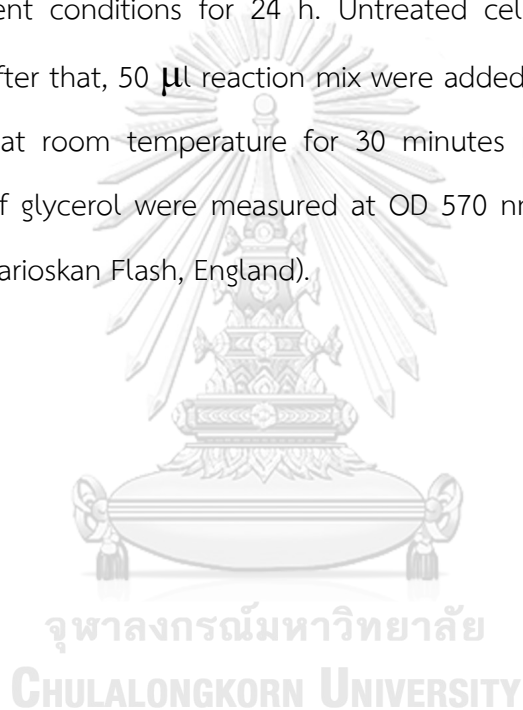
## Lipid accumulation

### Oil Red O staining

To study the effect of LA-CaCO<sub>3</sub> NPs with the correlation between lipolysis and lipid accumulation in primary human subcutaneous preadipocytes and visceral preadipocytes. The primary subcutaneous and visceral preadipocytes were differentiated in 96 well plates. Cells were stimulated with all treatment conditions. Untreated cells were used as negative controls. After 24 hours of exposure, the cells were stained with Oil Red O to identify lipid droplets. Briefly, media was removed, and cells were fixed in 4% formaldehyde at room temperature for 10 minutes. Cells were washed twice with phosphate-buffered saline and then with 60% isopropanol, before staining with 0.3% Oil Red-O (Sigma, USA) for 20 minutes at room temperature. Cells were washed in running tap water. The images were captured using a phase contrast inverted microscope (Nikon, Eclipse TS 100, Japan). After that, cells were eluted dye with 100% isopropanol. The solutions were transferred into 96 well plates and measured at OD 500 nm using a microplate reader (Thermo, Varioskan Flash, England).

### Lipolysis measurement

Lipolysis was evaluated by measurement the amount of glycerol released to the media. Lipolysis assay was measured using lipolysis assay kit (Abcam, USA). The amounts of glycerol were detected after 0 and 24 hours of treatment following manufacturer's instructions. Briefly, primary human subcutaneous preadipocytes and visceral preadipocytes were seeded into 96 well plates and differentiated into adipocytes. The cells were stimulated with all treatment conditions for 24 h. Untreated cells were used as negative controls. After that, 50  $\mu$ l reaction mix were added to each well. Plates were incubated at room temperature for 30 minutes protected from light. The amounts of glycerol were measured at OD 570 nm using microplate reader (Thermo, Varioskan Flash, England).



## Real-time PCR

To evaluate the effect of LA-CaCO<sub>3</sub> NPs on primary human subcutaneous and visceral preadipocytes into adipocytes. The primary subcutaneous and visceral preadipocytes were differentiated in 24 well plates in cell culture medium and incubated for 24 h to allow cell adherence. The cells were stimulated with treatment conditions for 14 days. Adipogenesis was determined after 14 days of growth in the presence or absence of treatments in adipogenesis differentiation medium. The total RNA was isolated from cells using TRIzol reagent (Invitrogen). RNA concentrations and quality were measured by Nanodrop Spectrophotometer (Thermo Scientific, USA). One microgram of total RNA was used for cDNA synthesis with a First strand cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. qPCR was performed with Express SYBR GreenER qPCR Supermix Universal (Invitrogen) using StepOnePlus Real-Time PCR System (ABI Applied Biosystems). The CT (threshold cycle) values of the target genes were normalized to the endogenous GAPDH level (housekeeping gene) and relative to the normalized calibrator. Primer sequences are listed below.

Genes	Primers	Sequence 5'-3'	References
ASC-1	Forward	GTGGCGCTCAAGAAGGAGAT	(35)
	Reverse	CCTTGGGCGAGATGAAGA	
Pat2	Forward	CCTGCCACTGTATGCACATC	(35)
	Reverse	TAGTCCATGCATCACCGTGT	
P2RX5	Forward	TTCACCAACACCTCGGATCT	(35)
	Reverse	CAGGTTGGTGACCACAAAAA	
Adiponectin	Forward	AAGGAGATCCAGGTCTTATTGG	(65)
	Reverse	ACCTTCAGCCCCGGGTAC	
Leptin	Forward	TTTGGCCCTATCTTTTCTATGTCC	(65)
	Reverse	TGGAGGAGACTGACTGCGTG	
Resistin	Forward	GCTGTTGGTGTCTAGCAAGAC	(112)
	Reverse	CATCATCATCATCTCCAG	

### Statistical analysis

The result from triplicates of the sample was expressed as mean  $\pm$  SD. Statistical significance was analyzed by using one-way ANOVA (Analysis of variance), followed by the Tukey's multiple comparison tests. Statistical significance was defined as *the P - value*  $\leq$  0.05 in comparison to control

### Ethical consideration

This study was approved by the Ethic committee of Research affairs, Faculty of medicine, Chulalongkorn University.

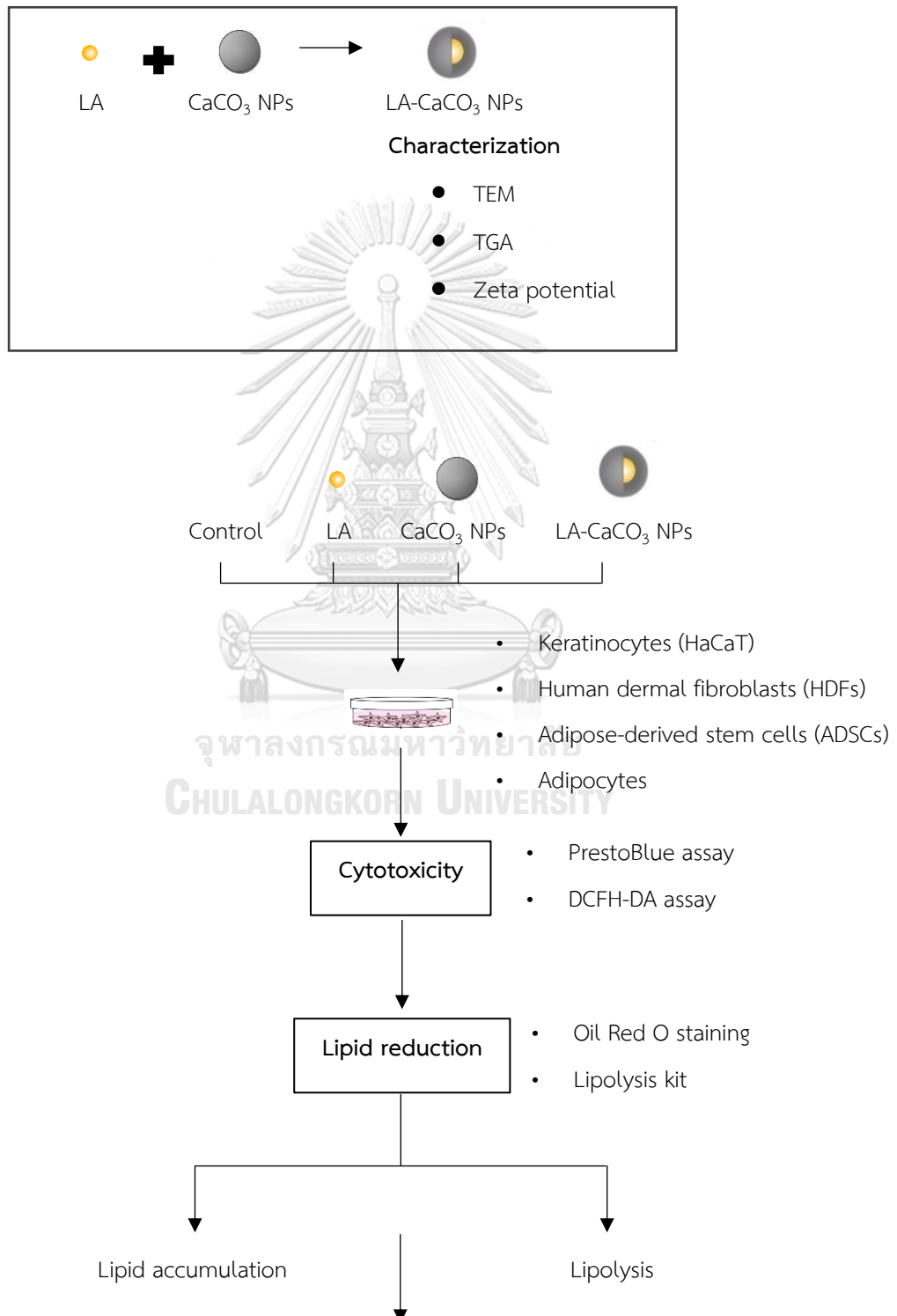


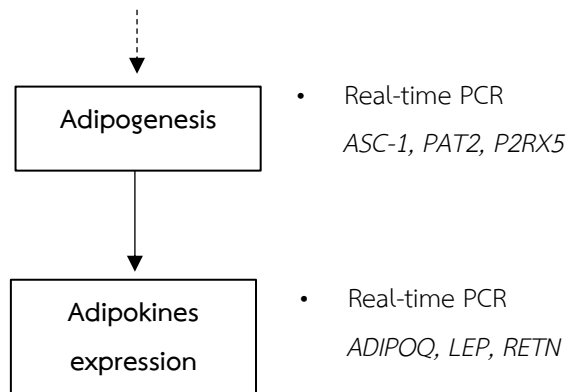
## Research methodology

Overall experimental layout of this study is presented in below.

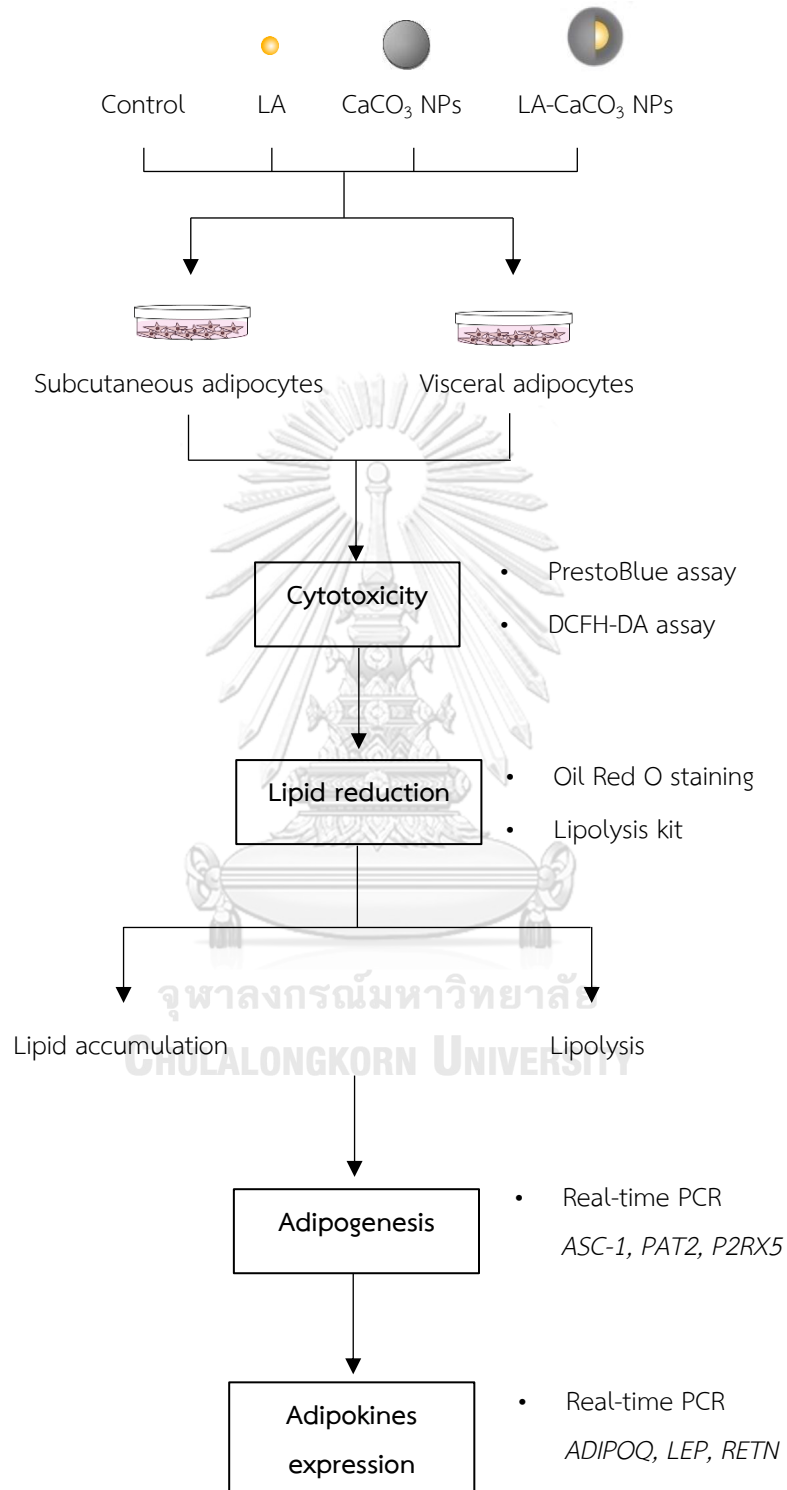
### Part I

#### Synthesis of LA-CaCO<sub>3</sub> NPs





## Part II



## CHAPTER IV

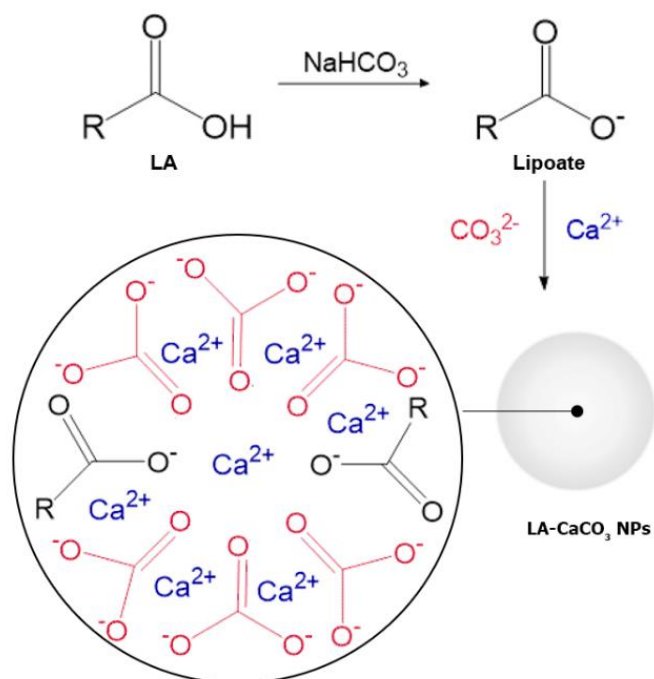
### RESULTS AND DISCUSSION

#### Part I

#### Characterization of LA-CaCO<sub>3</sub> NPs

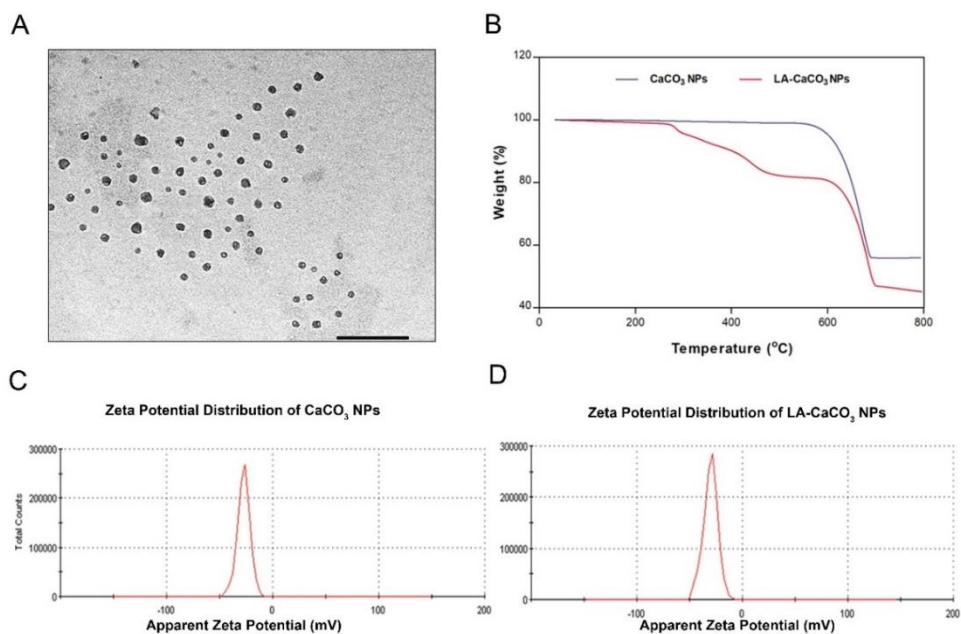
The chemical structure of LA-CaCO<sub>3</sub> NPs were represented in **Figure 4**. It is necessary to characterization material because the toxicity of nanomaterials is closely related to its size, shape and structure. The LA-CaCO<sub>3</sub> NPs were obtained by a multi-stage self-assembled strategy. The TEM image of the LA-CaCO<sub>3</sub> NPs was used to determine shape, size and uniformity of the particles. Transmission electron observations showed that the particles are spherical shaped and monodispersed with an approximate size distribution between 10 and 20 nm (**Figure 5A**). The percentages of LA in CaCO<sub>3</sub> NPs were confirmed by TGA. The concentration of LA in CaCO<sub>3</sub> NPs was approximately 27 percent in CaCO<sub>3</sub> NPs (**Figure 5B**). The zeta potential analysis showed a negative charge of -30.2 and -30.9 mV for the CaCO<sub>3</sub> NPs and LA-CaCO<sub>3</sub> NPs, respectively (**Figure 5C-D**).





**Figure 4** Schematic diagram representing the synthesis process of LA-CaCO<sub>3</sub> NPs.

**Abbreviations:** LA, lipoic acids; NaHCO<sub>3</sub>, sodium bicarbonate; CO<sub>3</sub><sup>2-</sup>, carbonate ion; Ca<sup>2+</sup>, calcium ion; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles.

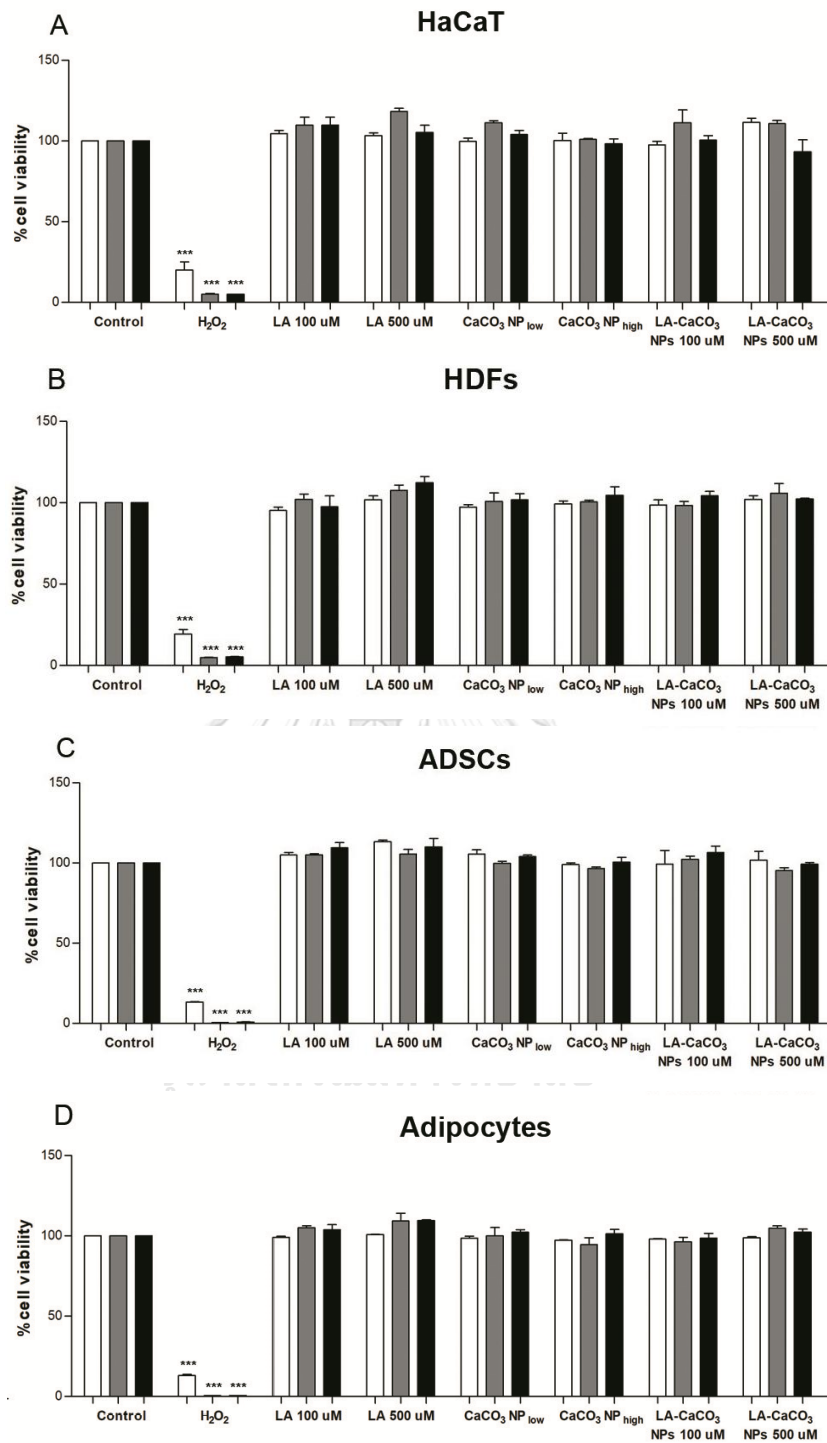


**Figure 5** Characterization of LA-CaCO<sub>3</sub> NPs (A) TEM image of LA-CaCO<sub>3</sub> NPs. (B) TGA thermograms of CaCO<sub>3</sub> NPs and LA-CaCO<sub>3</sub> NPs. (C) Zeta potential of CaCO<sub>3</sub> NPs and (D) LA-CaCO<sub>3</sub> NPs.

**Abbreviations:** CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipolic acids-incorporated calcium carbonate nanoparticles

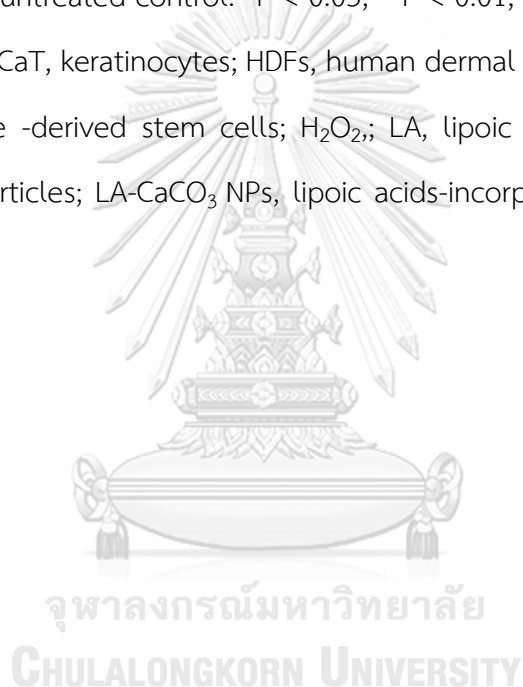
### The effect of LA-CaCO<sub>3</sub> NPs in the cytotoxicity of human cells

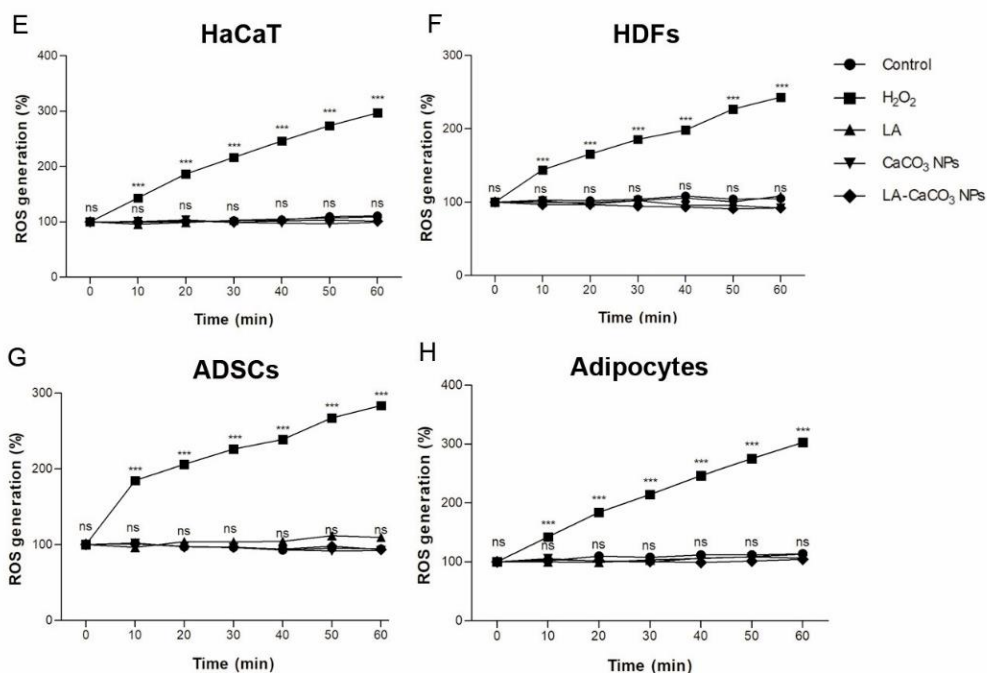
We first examined whether LA-CaCO<sub>3</sub> NPs have an influence on cell viability. After the human cells including HaCaT, HDFs, ADSCs and adipocytes were exposed to NPs for 12, 48 and 72 h, Presto Blue assay was performed to determine the viability of the human cells. (Figure 6A-D) The H<sub>2</sub>O<sub>2</sub>-treated human cells led to the intensified damage of human cells. When compared with LA, CaCO<sub>3</sub> NPs and LA-CaCO<sub>3</sub> NPs, the treated groups were found to be non-toxic in human cells at any incubation time between the treated and control groups. ROS generation are regarded as the developed paradigms to explain the toxic effects of nanomaterials. ROS is a natural byproduct of the normal metabolism in cells. However, excessive ROS can have potentially damaging biological responses resulting in oxidative stress phenomenon. Consequently, we also studied the effect of LA-CaCO<sub>3</sub> NPs with ROS generation. Assessment of ROS generation following 1 h of exposure to NPs showed that intracellular ROS production is indistinguishable between the treated and control groups. (Figure 6E-H).



**Figure 6** Cytotoxicity assessment of LA-CaCO<sub>3</sub> NPs in human cells. (A) HaCaT, (B) HDFs, (C) ADSCs, and (D) adipocytes were cultured in the presence of LA-CaCO<sub>3</sub> NPs following 24, 48 and 72-hour exposure. Cell viability percentage of human cells were estimated by PrestoBlue ® assay. Effect of LA-CaCO<sub>3</sub> NPs on the generation of ROS in human cells. (E) HaCaT (F) HDFs (G) ADSCs and (H) adipocytes were monitoring for 1 hour using DCFH-DA. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001.

**Abbreviations:** HaCaT, keratinocytes; HDFs, human dermal fibroblasts; ADSCs, adipose hydrogen peroxide -derived stem cells; H<sub>2</sub>O<sub>2</sub>; LA, lipoic acids; CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles



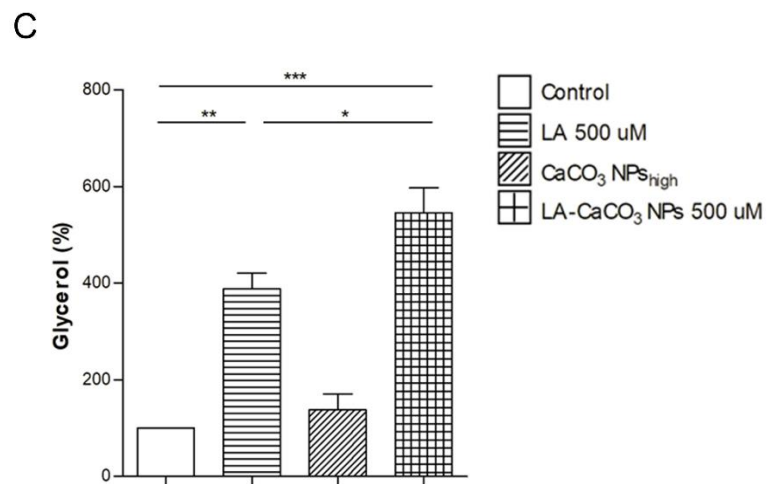
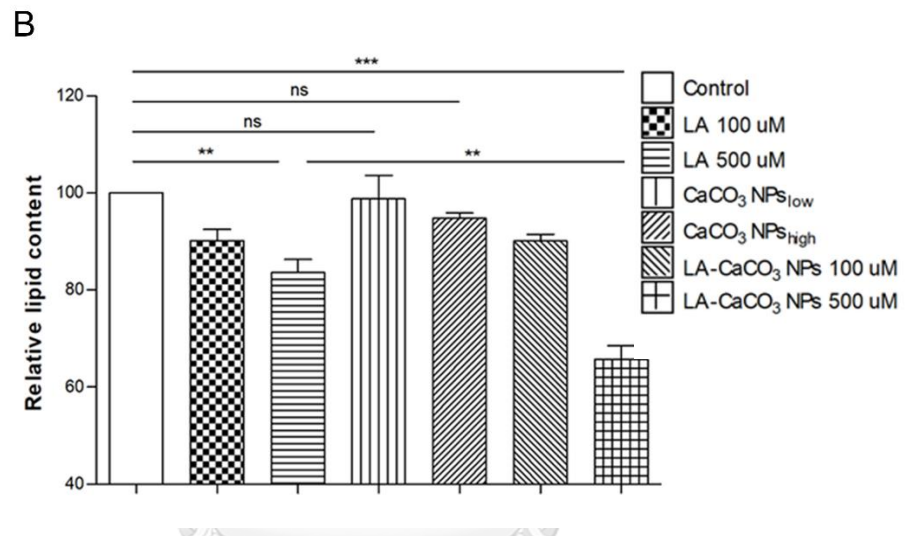
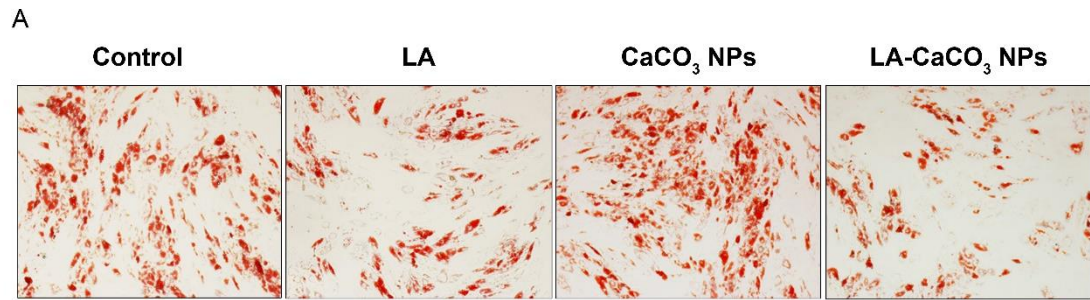


**Figure 6** Cytotoxicity assessment of LA-CaCO<sub>3</sub> NPs in human cells. (A) HaCaT, (B) HDFs, (C) ADSCs, and (D) adipocytes were cultured in the presence of LA-CaCO<sub>3</sub> NPs following 24, 48 and 72-hour exposure. Cell viability percentage of human cells were estimated by PrestoBlue ® assay. Effect of LA-CaCO<sub>3</sub> NPs on the generation of ROS in human cells. (E) HaCaT (F) HDFs (G) ADSCs and (H) adipocytes were monitoring for 1 hour using DCFH-DA. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001.

**Abbreviations:** HaCaT, keratinocytes; HDFs, human dermal fibroblasts; ADSCs, adipose-derived stem cells; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; LA, lipoic acids; CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles

### LA-CaCO<sub>3</sub> NPs effect lipid accumulation and lipolysis in human adipocytes

The effect of LA-CaCO<sub>3</sub> NPs on lipid accumulation was examined by Oil Red O staining. The LA revealed a significant reduction in the lipid droplets in adipocytes. The lipid accumulation was decreased by 9.8% at 100  $\mu\text{M}$  and 16.4% at 500  $\mu\text{M}$  of LA, respectively (**Figure 7A-B**). Interestingly, the LA-CaCO<sub>3</sub> NPs were apparently more effective in lipid accumulation than the LA alone at the same concentration. The result showed that the lipid accumulation was decreased by 9.9% at 100  $\mu\text{M}$  and 34.3% at 500  $\mu\text{M}$  of LA-CaCO<sub>3</sub> NPs. Intracellular lipid accumulation is regulated by lipolysis. Therefore, we hypothesized that LA-CaCO<sub>3</sub> NPs directly reduce lipid accumulation by association with lipolysis. To examine this possibility, we next evaluated lipolytic activity of LA-CaCO<sub>3</sub> NP. The amount of glycerol released into the media was observed in adipocytes treated with LA for 24 h (**Figure 7C**). The LA also exhibited to increase in the amount of glycerol. Nevertheless, the LA-CaCO<sub>3</sub> NPs are more effective in lipolysis than LA-treated group. CaCO<sub>3</sub> NPs were no statistically significant difference in lipid accumulation and lipolysis when compared with the control group. Therefore, these experiments confirmed that LA-CaCO<sub>3</sub> NPs presented anti-adipogenic properties as evidenced by decreasing lipid accumulation and releasing amount of glycerol in adipocytes.





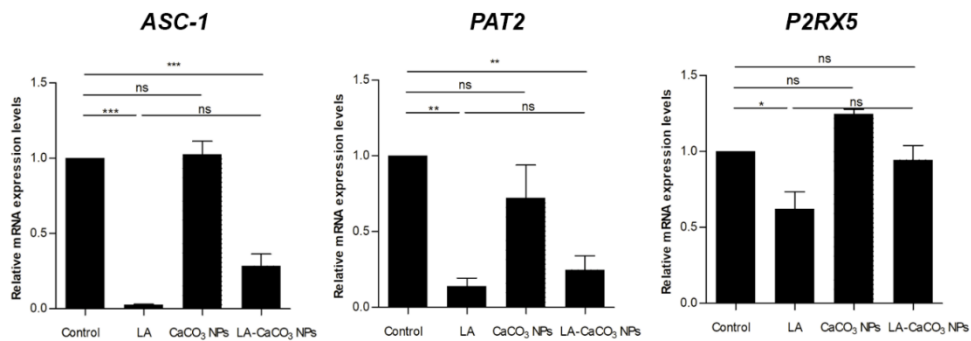
**Figure 7** LA-CaCO<sub>3</sub> NPs stimulates lipid reduction in adipocytes. Adipocytes were treated with LA-CaCO<sub>3</sub> NPs (100–500  $\mu$ M) for 24 hours. (A) Lipid accumulation was assessed by the Oil Red O staining and (B) staining quantification. (C) Lipolysis was measured by the amount of glycerol released into media. The results were represented by a mean $\pm$ SD (n=3). Data was expressed as a percentage of the untreated control. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 between the indicated groups.

**Abbreviations:** LA, lipoic acids; CaCO<sub>3</sub> NPs<sub>+</sub>, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles; ns, not significant.



### The LA-CaCO<sub>3</sub> NPs inhibit adipocyte differentiation

ADSCs can be differentiated to many types of adipocytes with suitable environments. In this study, we examined whether LA-CaCO<sub>3</sub> NPs influence on adipocytes differentiation. With the identification of the adipocytes marker by Ussar *et al.*(35) ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes respectively. To study the expression of ASC-1, PAT2, and P2RX5 in differentiated adipocytes using Real-time PCR. After the ADSCs were exposed with LA, CaCO<sub>3</sub> NPs and LA-CaCO<sub>3</sub> NPs for 14 days. The expression of ASC-1, PAT2 and P2RX5 mRNA expression in the differentiated cells were significantly suppressed in both LA and LA-CaCO<sub>3</sub> NPs (**Figure 8**). Whereas the CaCO<sub>3</sub> NPs did not alter ASC-1, PAT2, and P2RX5 mRNA expression when compared with the control group. All these data suggest that LA and LA-CaCO<sub>3</sub> NPs have a capacity to suppress adipogenesis in ADSCs.

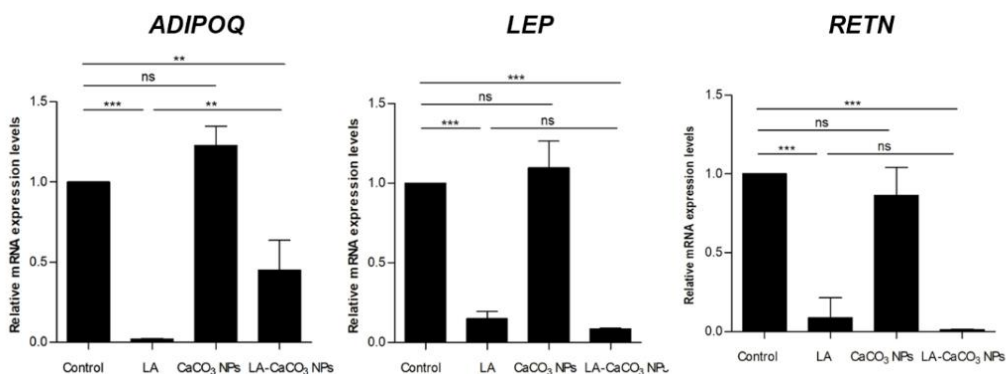


**Figure 8** The LA-CaCO<sub>3</sub> NPs inhibits adipocytes differentiation in ADSCs. The specific markers were measured by real-time PCR in ADSCs. Both LA- and LA-CaCO<sub>3</sub> treated group were suppressed ASC-1, PAT2, and P2RX5 mRNA expression when compared with control groups. GAPDH mRNA expression was used as an internal control. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 between the indicated groups.

**Abbreviations:** ASC-1, neutral amino acid transporter; PAT2, proton assistant amino acid transporter-2; P2RX5, purinergic receptor P2X, ligand-gated ion channel 5; LA, lipoic acids; CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles, ns; not significant.

### The LA-CaCO<sub>3</sub> NPs inhibit adiponectin leptin and resistin mRNA expression

Finally, to gain further insights into the function of adipocytes. Adipose tissue functions as a key endocrine organ by releasing numerous protein, known as adipose-derived secreted factors or adipokines, that have pro-inflammatory (such as leptin and resistin) or anti-inflammatory activities (adiponectin). We measured the adipokines expression in differentiated adipocytes using real-time PCR. Adiponectin leptin and resistin mRNA expression were significantly lower in LA compared with the control group (**Figure 9**). As similarly shown previously, LA-CaCO<sub>3</sub> NPs reduced mRNA expression in adiponectin leptin and resistin. In CaCO<sub>3</sub> NPs, there was no significant difference in adipokines expression when compared with the control group. Consequently, our results further suggest that LA and LA-CaCO<sub>3</sub> NPs inhibit adipokines expression by suppression of adipogenesis.



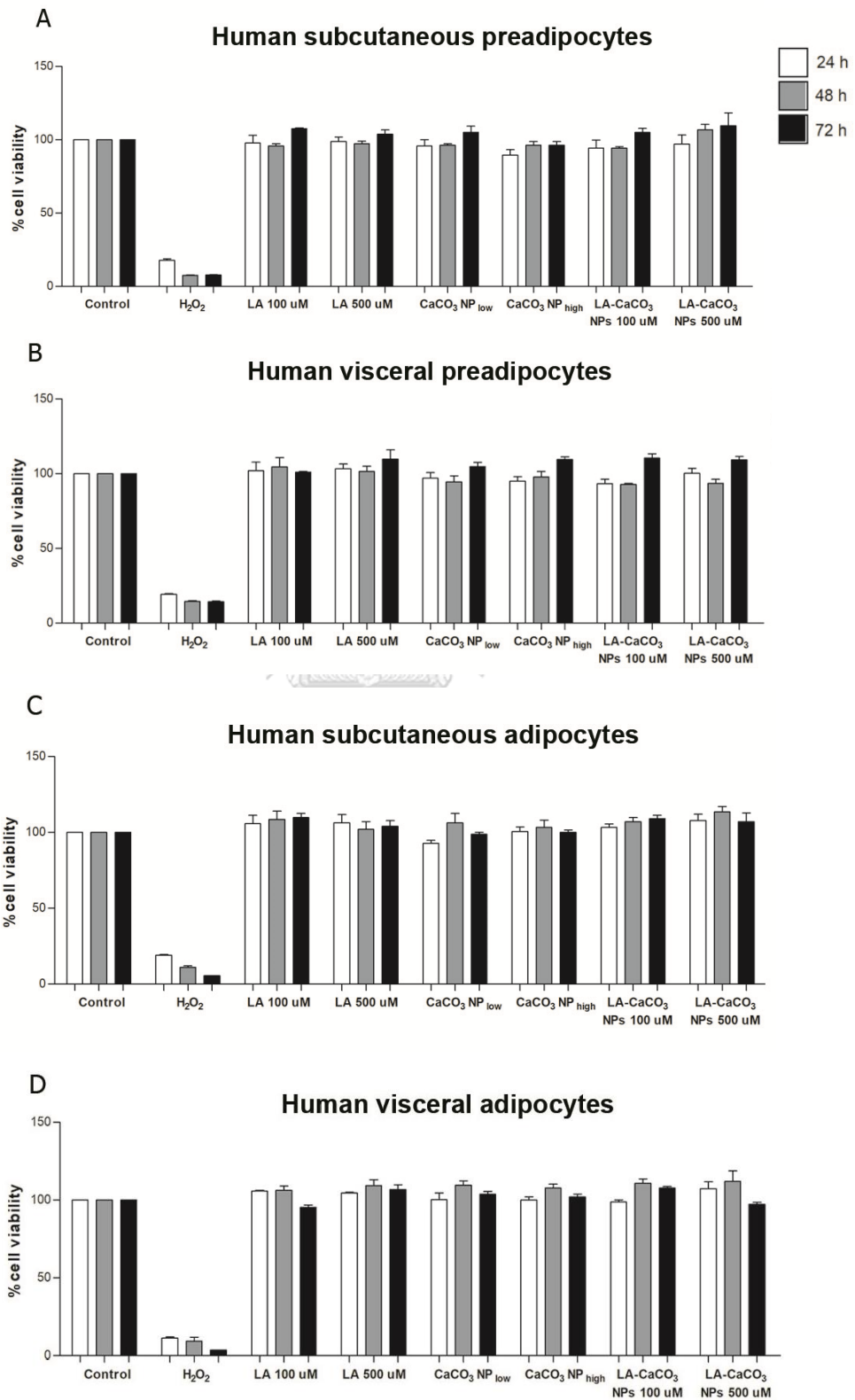
**Figure 9** The LA-CaCO<sub>3</sub> NPs inhibits adipokines expression in ADSCs. ADIPOQ, LEP, and RETN mRNA expression were measured by real-time PCR. Adipokines expression was suppressed in both LA- and LA-CaCO<sub>3</sub> NPs-treated groups when compared with control groups. GAPDH mRNA expression was used as an internal control. \*\*P < 0.01, and \*\*\*P < 0.001 between the indicated groups.

**Abbreviations:** ADIPOQ, adiponectin; LEP, leptin; RETN, resistin; LA, lipoic acids; CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles, ns; not significant.

## Part II

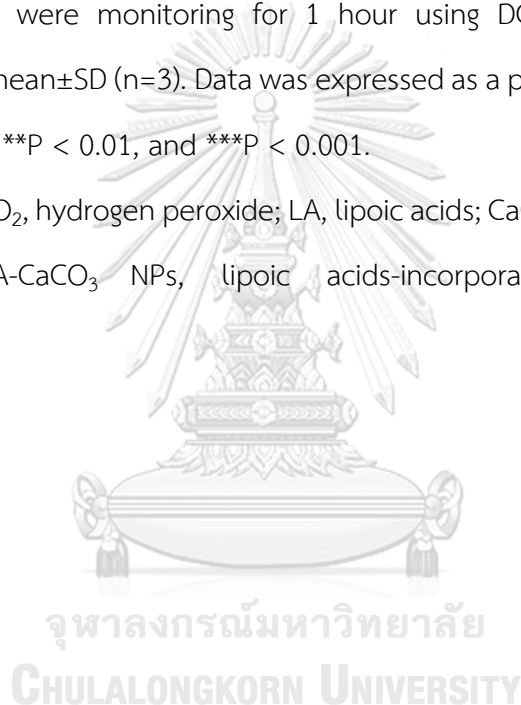
### The cytotoxic effects of LA-CaCO<sub>3</sub> NPs in subcutaneous and visceral adipocytes

In part II, we examined whether LA-CaCO<sub>3</sub> NPs have an influence on cell viability in human subcutaneous and visceral adipocytes. After both cells were exposed to all treatment conditions for 12, 48 and 72 h, Presto Blue assay was performed to determine cell viability. The H<sub>2</sub>O<sub>2</sub>-treated adipocytes were significantly decreased cell viability in subcutaneous and visceral adipocytes. When the cells were exposed to LA, CaCO<sub>3</sub> NPs, and LA-CaCO<sub>3</sub> NPs, we did not observe cytotoxic effects at any incubation time as confirmed by dehydrogenase measurement (**Figure 10A-D**). ROS generation are regarded as the developed paradigms to explain the toxic effects of nanomaterials. ROS is a natural byproduct of the normal metabolism in cells. However, excessive ROS can have potentially damaging biological responses resulting in oxidative stress phenomenon. Consequently, we also examined the effect of LA-CaCO<sub>3</sub> NPs with ROS generation. Assessment of ROS generation following 1 h of exposure to LA-CaCO<sub>3</sub> NPs showed that LA-CaCO<sub>3</sub> NPs had no effect in intracellular ROS production (**Figure 10E-H**).

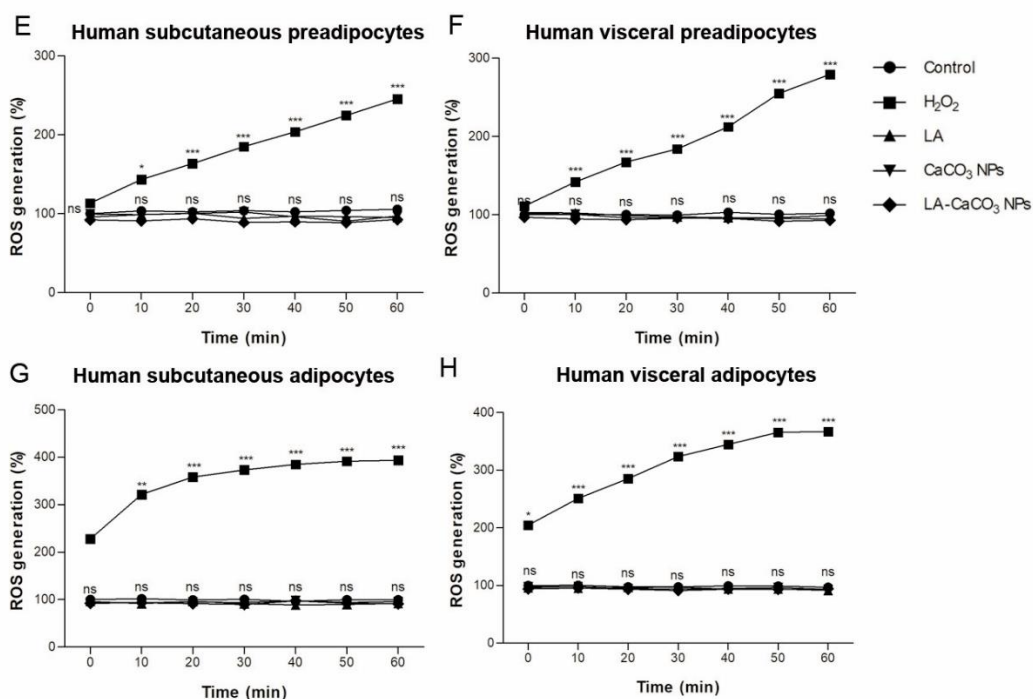


**Figure 10** Cytotoxicity assessment of LA-CaCO<sub>3</sub> NPs in human adipocytes. (A) human subcutaneous preadipocytes, (B) human visceral preadipocytes, (C) human subcutaneous adipocytes, and (D) human visceral adipocyte were cultured in the presence of LA-CaCO<sub>3</sub> NPs following 24, 48 and 72-hour exposure. Cell viability percentage of preadipocytes and adipocytes were estimated by PrestoBlue® assay. Effect of LA-CaCO<sub>3</sub> NPs on the ROS generation. (E) Human subcutaneous preadipocytes, (F) human visceral preadipocytes, (G) human subcutaneous adipocytes, and (H) human visceral adipocyte were monitoring for 1 hour using DCFH-DA. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001.

**Abbreviations:** H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; LA, lipoic acids; CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles





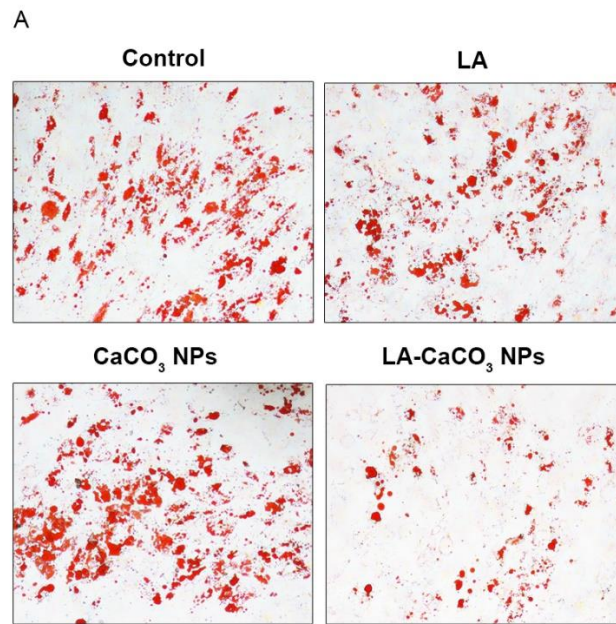


**Figure 10** Cytotoxicity assessment of LA-CaCO<sub>3</sub> NPs in human adipocytes. (A) human subcutaneous preadipocytes, (B) human visceral preadipocytes, (C) human subcutaneous adipocytes, and (D) human visceral adipocyte were cultured in the presence of LA-CaCO<sub>3</sub> NPs following 24, 48 and 72-hour exposure. Cell viability percentage of preadipocytes and adipocytes were estimated by PrestoBlue® assay. Effect of LA-CaCO<sub>3</sub> NPs on the ROS generation. (E) Human subcutaneous preadipocytes, (F) human visceral preadipocytes, (G) human subcutaneous adipocytes, and (H) human visceral adipocyte were monitoring for 1 hour using DCFH-DA. The results were represented by a mean±SD (n=3). Data was expressed as a percentage of the untreated control. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001.

**Abbreviations:** H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; LA, lipoic acids; CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles

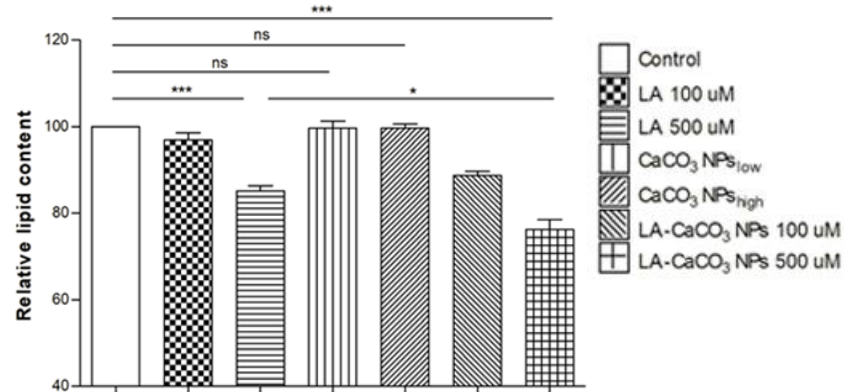
### Effects of LA-CaCO<sub>3</sub> NPs on lipolysis in subcutaneous and visceral adipocytes

To assess the effects of LA-CaCO<sub>3</sub> NPs on lipid reduction, human subcutaneous and visceral adipocytes were cultured in the presence of LA-CaCO<sub>3</sub> NPs. Lipid accumulation was determined by lipid droplets using oil-red O and total glycerol was measured in culture media in 24 h. LA-treated adipocytes decreased lipid droplets in both subcutaneous and visceral adipocytes. In subcutaneous adipocytes, the lipid accumulation was decreased by 3% at 100  $\mu\text{M}$  and 14.8% at 500  $\mu\text{M}$ . In visceral adipocytes, the lipid accumulation was decreased by 3% at 100  $\mu\text{M}$  and 15.8% at 500  $\mu\text{M}$ . Interestingly, the LA-CaCO<sub>3</sub> NPs were more effective in lipid accumulation than the LA alone at the same concentration in both subcutaneous and visceral adipocytes. The lipid accumulation was decreased by 11.3% at 100  $\mu\text{M}$  and 23.7% at 500  $\mu\text{M}$  in subcutaneous adipocytes (**Figure 11A-B**). In visceral adipocytes, the lipid accumulation was decreased by 10.6 % at 100  $\mu\text{M}$  and 25.8 % at 500  $\mu\text{M}$  (**Figure 11D-E**). While, the CaCO<sub>3</sub> NPs treatment did not affect lipid accumulation in both cells. We next evaluated the effects of LA-CaCO<sub>3</sub> NPs on the lipolysis in subcutaneous and visceral adipocytes. The amount of glycerol released into the media was observed in adipocytes. LA-treated adipocytes increased in the amount of glycerol in culture media. However, the LA-CaCO<sub>3</sub> NPs are more effective in lipolysis than LA alone when compared with control group in both cells (**Figure 11C, F**). Therefore, LA-CaCO<sub>3</sub> NPs presented anti-adipogenic properties in both subcutaneous and visceral adipocytes as evidenced by decreased lipid accumulation and amount of glycerol in adipocytes.

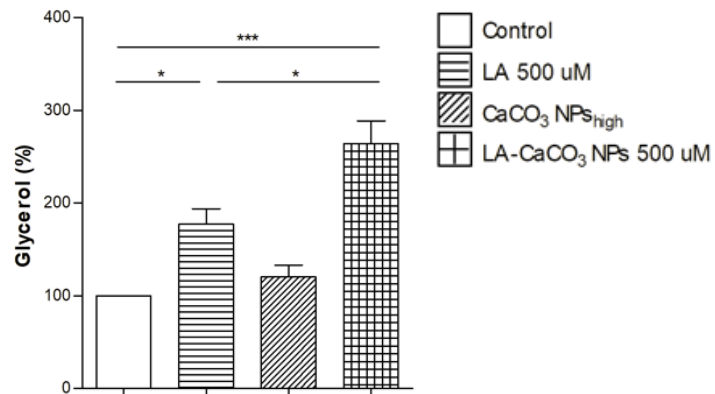


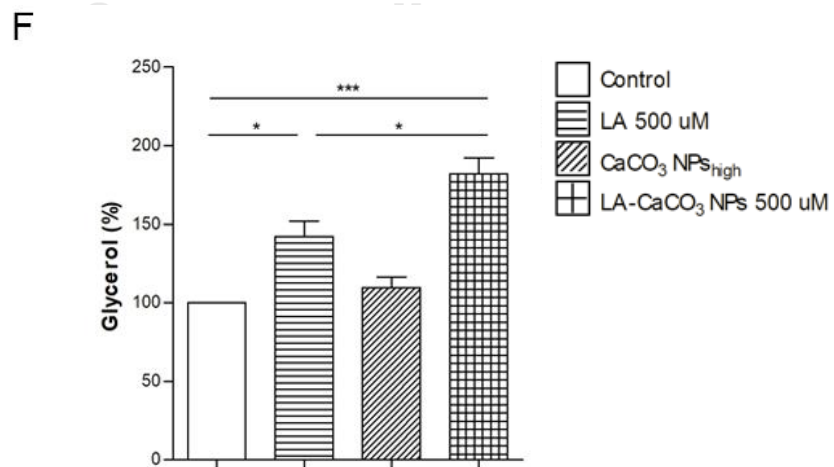
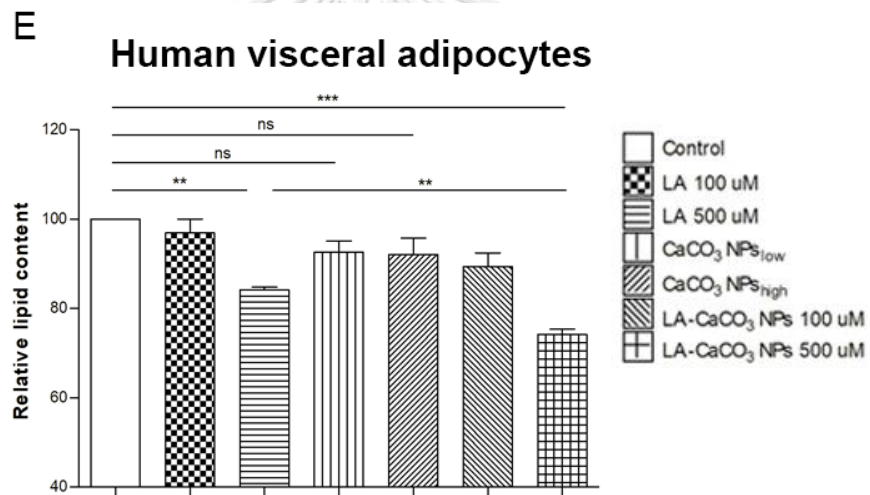
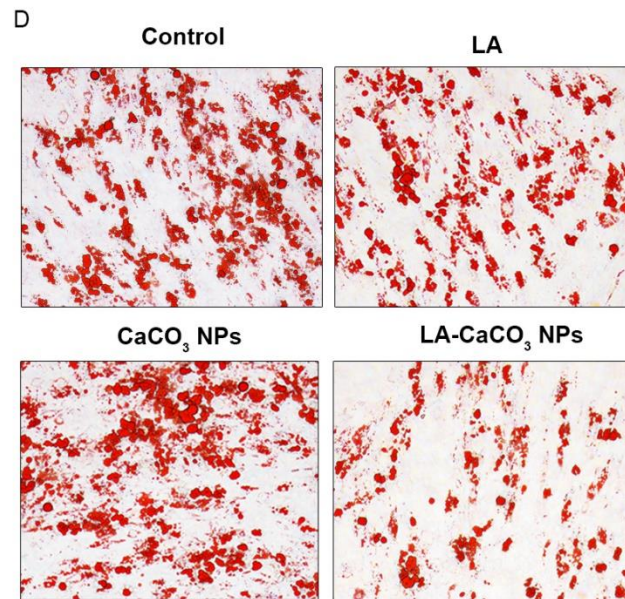
**B**

### Human subcutaneous adipocytes



**C**





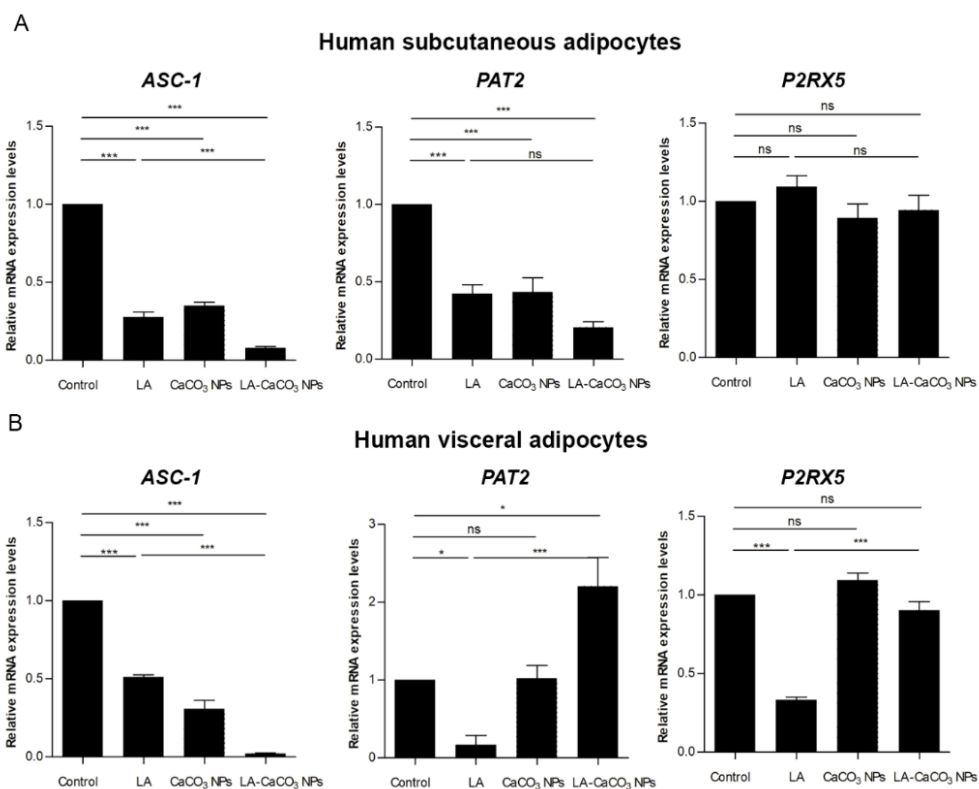
**Figure 11** LA-CaCO<sub>3</sub> NPs decrease lipid accumulation in subcutaneous and visceral adipocytes. Adipocytes were treated with LA-CaCO<sub>3</sub> NPs (100–500  $\mu$ M) for 24 hours. Lipid accumulation was assessed by the Oil Red O staining in (A) subcutaneous and (D) visceral adipocytes treated for 24 hours and (B, E) staining quantification. Lipolysis in (C) subcutaneous and (F) visceral adipocytes were measured by the amount of glycerol released into media. The results were represented by a mean $\pm$ SD (n=3). Data was expressed as a percentage of the untreated control. \*P < 0.05, \*\*P < 0.01, and \*\*\*P < 0.001 between the indicated groups.

**Abbreviations:** LA, lipoic acids; CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles; ns, not significant.



### The effects of LA-CaCO<sub>3</sub> NPs on regulation of differentiation in subcutaneous and visceral adipocytes.

Next, we examined whether LA-CaCO<sub>3</sub> NPs influence on adipocytes differentiation. With the identification of the adipocytes marker by Ussar *et al.* ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes respectively. Human subcutaneous and visceral preadipocytes were treated with differentiating media in the presence or absence of LA, CaCO<sub>3</sub> NPs, and LA-CaCO<sub>3</sub> NPs for 14 days. After treatment exposure, differentiated adipocytes were measured marker expression using real-time PCR. In subcutaneous adipocytes, the results showed that LA-treated groups were inhibited adipogenesis by suppression of *ASC-1* and *PAT2*. Similarly, LA-CaCO<sub>3</sub> NPs treatment significantly reduced the expression of *ASC-1* and *PAT2*. Among the treatment groups, LA-CaCO<sub>3</sub> NPs showed the strongest inhibitory effect on the expression of *ASC-1*. LA, CaCO<sub>3</sub> NPs, and LA-CaCO<sub>3</sub> did not affect the expression of *P2RX5* in subcutaneous preadipocytes (**Figure 12A**). In visceral adipocytes, LA treatment significantly suppressed the expression of *ASC-1*, *PAT2*, and *P2RX5*. However, LA-CaCO<sub>3</sub> NPs-treated groups showed greater *ASC1* suppression compared with LA alone. Surprisingly, LA-CaCO<sub>3</sub> NPs significantly increased *PAT2* expression but did not affect the expression of *P2RX5*. Moreover, we found that CaCO<sub>3</sub> NPs decreased *ACS-1* expression in subcutaneous and visceral adipocytes with *PAT2* expression in subcutaneous adipocytes.



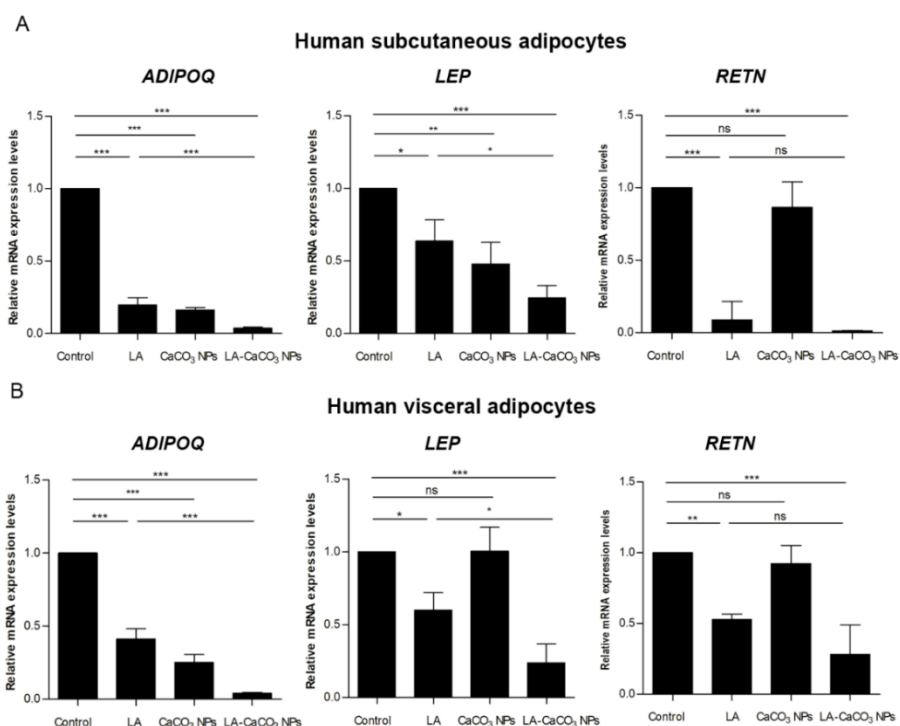
**Figure 12** The LA-CaCO<sub>3</sub> NPs regulate adipocytes differentiation in human subcutaneous and visceral adipocytes. (A) LA-CaCO<sub>3</sub> NP inhibited adipogenesis by suppression of *ASC1* and *PAT2* expression in subcutaneous adipocytes. (B) LA-CaCO<sub>3</sub> NPs-treated groups in visceral adipocytes were induced *PAT2* expression. GAPDH mRNA expression was used as an internal control. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 between the indicated groups.

**Abbreviations:** ASC-1, neutral amino acid transporter; PAT2, proton assistant amino acid transporter-2; P2RX5, purinergic receptor P2X, ligand-gated ion channel 5; LA, lipoic acids; CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles, ns; not significant.

### The effects of LA-CaCO<sub>3</sub> NPs on adipokines expression in subcutaneous and visceral adipocytes.

Finally, to gain further insights into the functions of differentiated adipocytes. Adipose tissue functions as a key endocrine organ by releasing numerous protein, known as adipose-derived secreted factors or adipokines, that have pro-inflammatory (such as leptin and resistin) or anti-inflammatory activities (adiponectin). We measured the adipokines expression in subcutaneous and visceral adipocytes using real-time PCR. *ADIPOQ*, *LEP* and *RETN* mRNA expression were significantly decreased in LA-treated groups compared with the control groups. As similarly shown previously, LA-CaCO<sub>3</sub> NPs reduced mRNA expression in *ADIPOQ*, *LEP* and *RETN* mRNA in subcutaneous and visceral adipocytes. Moreover, we found that CaCO<sub>3</sub> NPs decreased *ADIPOQ* and *LEP* in subcutaneous adipocytes. CaCO<sub>3</sub> NPs also decreased *ADIPOQ* expression in visceral adipocytes. Consequently, our results further suggest that LA and LA-CaCO<sub>3</sub> NPs inhibit adipokines expression by suppression of adipogenesis.





**Figure 13** The LA-CaCO<sub>3</sub> NPs inhibits adipokines expression in adipocytes. *ADIPOQ*, *LEP*, and *RETN* mRNA expression were measured by real-time PCR. Adipokines expression in (A) subcutaneous and (B) visceral adipocytes were suppressed in both LA- and LA-CaCO<sub>3</sub> NPs-treated groups when compared with control groups. GAPDH mRNA expression was used as an internal control. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 between the indicated groups.

**Abbreviations:** ADIPOQ, adiponectin; LEP, leptin; RETN, resistin; LA, lipoic acids; CaCO<sub>3</sub> NPs, calcium carbonate nanoparticles; LA-CaCO<sub>3</sub> NPs, lipoic acids-incorporated calcium carbonate nanoparticles, ns; not significant.

## Discussion

### Part I

In the present study, our findings are worth to present that this is the first report on the potential advantages of LA-CaCO<sub>3</sub> NPs in ADSCs, their physicochemical characterization and their adipocytes functions by comparing with LA alone. Increasing evidence shows that LA has become increasingly candidate agent with therapeutic potential on several diseases such as diabetes and obesity. In agreement with the limited bioavailability of LA, CaCO<sub>3</sub> NPs have been praised for their advantageous drug carrier to improve therapeutic efficacy. Our data demonstrated that LA-CaCO<sub>3</sub> NPs presented a promising treatment for the anti-obesity by reducing lipid accumulation, increasing lipolysis, inhibiting adipogenesis as well as adipokine expression. Cytotoxicity of newly synthesized LA-CaCO<sub>3</sub> NPs had no obvious toxicity in human cells. Notably, LA-CaCO<sub>3</sub> NPs were apparently more effective in lipid accumulation than the LA alone at the same concentration. The significant lipid accumulation of LA-CaCO<sub>3</sub> NPs was attributed to the ability of LA-CaCO<sub>3</sub> NPs to encourage lipolysis in adipocytes, as confirmed by amounts of glycerol. Previous study suggested that a lipolytic action of LA is mainly mediated by the phosphorylation of hormone-sensitive lipase leading to reduction of lipid accumulation in body.(14, 82) Furthermore, CaCO<sub>3</sub> NPs are able to deliver LA, intracellularly at a longer residence time, which results in a sustained therapeutic effect of LA.

Adipogenesis is a differentiation process by stimulation of ADSCs to become terminally differentiated adipocytes. In this study, to our knowledge, this is the first time that the effect of LA was evaluated in differentiation of ADSCs. As reported previously, LA caused inhibition of adipogenesis in 3T3-L1 pre-adipocytes.(15, 85) Here we demonstrate that both LA and LA-CaCO<sub>3</sub> NPs inhibited adipocyte differentiation in ADSCs by suppression of *ASC1*, *PAT2* and *P2RX5* mRNA expression in ADSCs. Moreover, no significant differences were found between LA- and LA-CaCO<sub>3</sub> NPs-treated groups.

The lipid reduction effects have been demonstrated with inhibition of adipogenesis.(113, 114) LA directly modulates the adipocyte differentiation through activation of adipogenic transcription factors, which results in decreased expression of adipocyte-specific genes, consequently contributing to the suppression of adipogenesis. Previous studies reported that LA controls the adipocyte differentiation via the direct modulation of autophagy.(15, 85) However, the precise mechanisms of LA and LA-CaCO<sub>3</sub> NPs in adipogenesis require further investigation, especially in ADSCs. These results, together with the previous findings, suggest that LA-CaCO<sub>3</sub> NPs significantly reduced in accumulation of lipids in adipocytes, whereas they inhibited adipogenesis in ADSCs.

Adipose tissue has been recognized as an endocrine organ that is capable of producing and secreting a variety of proinflammatory and anti-inflammatory factors known as adipokines.(115-117) Next, we investigated whether LA-CaCO<sub>3</sub> NPs regulates adipokines expression including adiponectin (*ADIPOQ*), leptin (*LEP*), and resistin (*RETN*). Adiponectin is mainly secreted by adipocytes. It is an adipokine with insulin-sensitizing property through binding to its receptors AdipoR1 and AdipoR2.(61, 118, 119) The functions of leptin are food intake regulation, glucose and lipid homeostasis, and energy expenditure control via the central nervous system. During food restriction, the leptin levels are decreased while weight loss in mice and humans.(71, 120) Several studies demonstrated that LA treatment up-regulates adiponectin and down-regulates leptin to prevent hyperinsulinemia and insulin resistance.(95, 121) On the contrary, our findings showed a significantly suppressed adiponectin and leptin mRNA expression in both LA- and LA-CaCO<sub>3</sub> NPs-treated groups. These results suggest that LA and LA-CaCO<sub>3</sub> NPs inhibit adipokine expression underlining their inhibitory actions of adipogenesis in differentiated adipocytes. Resistin is an adipocyte hormone that modulates glucose homeostasis. It has been shown to induce pathogenesis of obesity and insulin resistance in humans, both of which appear to contribute to the development of type

2 diabetes.(76, 122, 123) Nevertheless, there have been no reports concerning with the effect of LA in resistin regulation. Our data also showed that LA and LA-CaCO<sub>3</sub> NPs reduced adiponectin leptin as well as resistin mRNA expression. However, we did not find any significant changes between leptin and resistin mRNA expression in LA- and LA-CaCO<sub>3</sub> NPs-treated groups. Adiponectin's mRNA expression was less inhibited by LA-CaCO<sub>3</sub> NPs when compared with LA alone. Indeed, the adiponectin presents protective actions to have a more pronounced anti-inflammatory property than leptin and resistin.(117) We propose that LA-CaCO<sub>3</sub> NPs act in different pathways, which may possibly be ascribed to suppress adiponectin, leptin and resistin mRNA expression. The direct effects of LA and LA-CaCO<sub>3</sub> NPs on lipid accumulation, lipolysis, adipokines expression as well as the potential signaling pathways involved in cultured ADSCs and adipocytes still remains to be further investigated.

Considering the cellular uptake mechanisms of LA-CaCO<sub>3</sub> NPs in ADSCs and differentiated adipocytes, it will be also important to further assess its potential effects of LA-CaCO<sub>3</sub> NPs. Although the CaCO<sub>3</sub> NPs did not alter activities in lipid accumulation, lipolysis, adipogenesis as well as adipokines expression. We herein provide evidence that LA-CaCO<sub>3</sub> NPs present the greater effects in reduction of lipid contents, stimulation of lipolysis, inhibition of adipogenesis and adipokine expression. Different uptake mechanisms enable the consequence of diverse responses. The Na<sup>+</sup>/multivitamin transporter is suggested to transport LA into the cells.(124) The incorporation of LA into CaCO<sub>3</sub> NPs possibly become the different uptake mechanisms by structural alteration of LA. LA-CaCO<sub>3</sub> NPs effectively cross cell membranes by ion channel-mediated endocytosis. The pH-sensitive properties of CaCO<sub>3</sub> NPs provide the dissolution of calcium carbonate in the low acidic medium of endosomal compartments. The CaCO<sub>3</sub> NPs are dissolved in the endosome and destabilize it through osmotic imbalance, and finally deliver the LA into the cytosol.(101, 125-127) Our results indicated that these CaCO<sub>3</sub> NPs were used for safe and efficient delivery of

LA or other drugs. The progressive understanding of the drug release kinetics and transport mechanisms in  $\text{CaCO}_3$  NPs remains deserve further studies for better scientific understanding.



## Part II

To the best of our knowledge, this is the first report comparing the efficacy of LA-CaCO<sub>3</sub> NP properties in both human subcutaneous and visceral adipocytes. Human adipose tissue is a diversified tissue organ with different depots secreting a variety of adipokines. Subcutaneous and visceral adipocytes are two major types of adipocytes in adipose tissue. Moreover, subcutaneous and visceral adipocytes are associated with an adverse metabolic effect (128). Here, we developed the new nanoparticles using CaCO<sub>3</sub> NPs as drug carrier to deliver LA into adipocytes. We examined the effect of LA-CaCO<sub>3</sub> NPs in between subcutaneous and visceral adipocytes. Importantly, this study provides the first report that LA-CaCO<sub>3</sub> NPs exhibit anti-obesity properties by a reduction in lipid accumulation and enhanced lipolysis in both human subcutaneous and visceral adipocytes. Additionally, LA-CaCO<sub>3</sub> NPs effects on adipogenic differentiation throughout adipokines expression in both human subcutaneous and visceral adipocytes.

Excessive lipid accumulation due to energy overload causes overweight and obesity. Currently used anti-obesity agents are appetite suppressants (129, 130). However, these drugs often show adverse effects. Due to these undesirable adverse effects of the drugs. Many studies attempt to develop an alternative method for reducing lipid accumulation using natural medicinal agents. Anti-adipogenic activity of LA on adipocytes has been previously well documented. Previous study reveals that the ability of LA to stimulate lipolysis in adipocytes could also contribute to its anti-obesity properties (14). Our results indicate that LA was significantly decreased lipid accumulation and enhanced lipolysis in subcutaneous and visceral adipocytes. Interestingly, the LA-CaCO<sub>3</sub> NPs was also greater than LA in the lipid accumulation. Furthermore, LA-CaCO<sub>3</sub> NPs enhanced lipolytic activity within subcutaneous and visceral adipocytes. Whereas the CaCO<sub>3</sub> NPs treatment did not affect lipid accumulation and lipolytic activity. We postulate that the increased lipid reduction in

subcutaneous and visceral adipocytes is due to CaCO<sub>3</sub> NPs properties including slow biodegradation, allowing for LA accumulation at adipocytes.

Adipogenesis, the new key target of anti-obesity drugs, is the process where mesenchymal cells differentiate into preadipocytes followed by second differentiation into lipid accumulating adipocytes. In this study, subcutaneous and visceral preadipocytes were treated with differentiating media in the presence or absence of all treatment conditions for 14 days. *ASC-1*, *PAT2*, and *P2RX5* are specific marker to identify adipocyte population. Our results indicated that LA treatment significantly decreased *ASC-1*, *PAT2* expression in subcutaneous adipocytes and *ASC-1*, *PAT2*, and *P2RX5* expression in visceral adipocytes. These findings are in accordance with results of previous studies. They suggest that treatment with 500  $\mu\text{M}$  LA acts as a PPAR- $\gamma$  antagonist and inhibits adipogenic differentiation (85). In subcutaneous adipocytes, LA-CaCO<sub>3</sub> NPs exhibited same result in LA treatment. The LA-CaCO<sub>3</sub> NPs inhibited adipogenic differentiation, which was accompanied with lower expression levels of *ASC-1* and *PAT2* genes in subcutaneous adipocytes. Whereas the LA-CaCO<sub>3</sub> NPs suppressed *ASC-1* expression but induced *PAT2* expression in visceral adipocytes. The brown and beige adipocytes have similar thermogenic capacities (34). The mitochondria a central role in metabolism of beige, brown adipocytes (34). In addition, specific function performed by brown adipocytes is converting mitochondrial energy into heat in adaptive thermogenesis. The functions of mitochondria in beige, brown adipocytes relate with metabolic homeostasis and weight control. The ability of LA promotes brown-like remodeling in cultured adipocytes. Previous study reveals that the LA not only increased mitochondrial content, but also mitochondrial function via SIRT1-PGC-1 $\alpha$  signaling pathway (82, 86, 131-133). Regardless of its natural role, increasing the activity of beige adipocytes by LA-CaCO<sub>3</sub> NPs tremendous promise for the treatment of metabolic disease, although the mechanism requires further investigations.

Adipose tissue is essential in energy metabolism. It has endocrine functions and can secrete hundreds of bioactive molecules, many of which are called adipokines. In this study, we explored the effects of LA-CaCO<sub>3</sub> NPs in adipokines regulation. We found that LA significantly inhibited expression of *ADIPOQ*, *LEP*, and *RETN* in subcutaneous and visceral adipocytes. Adiponectin has been focused on the potential beneficial effects of obesity. However, treatment of LA inhibits *ADIPOQ* expression in *In vitro* which contrasts with the stimulation of adiponectin described after *In vivo* supplementation with LA (97). Moreover, many studies report that treatment of adipocytes with LA caused a concentration-dependent inhibition of *LEP* gene (98). Recently, no data are available concerning LA treatment and resistin expression in adipocytes. Resistin has been identified as an adipokine that links obesity to insulin resistance. Resistin serum levels in our obese study participants were significantly higher than in normal-weight controls (134). Our result indicates that *ADIPOQ*, *LEP*, and *RETN* also extensively inhibited by LA-CaCO<sub>3</sub> NPs, while *ASC-1* expression was suppressed by LA and LA-CaCO<sub>3</sub> NPs. It is likely that their inhibitory effect of adipogenesis leading to inhibition of adipokines expression, because adiponectin, leptin, and resistin are mainly produced in white adipocytes.

The LA-CaCO<sub>3</sub> NPs are synthesized by incorporation between LA and CaCO<sub>3</sub> NPs. Our results showed that LA-CaCO<sub>3</sub> NPs treatment play different roles with LA in adipogenesis. LA significantly suppressed adipogenic differentiation in subcutaneous adipocytes. On the other hand, LA-CaCO<sub>3</sub> NPs induced *PAT2* expression in visceral adipocytes. One possibility is that when nanoparticles are combined with LA to create novel properties. Nanomaterials can be designed and synthesized in needed sizes and shapes, and they have unique chemical and physical properties which make them useful as nanocarrier or assistants, excellent signal reporters, transducers, amplifiers or inhibitors (135). Our data indicated the CaCO<sub>3</sub> NPs also inhibited adipogenic differentiation and adipokines expression. We hypothesize that CaCO<sub>3</sub> NPs may involve



adipogenesis and adipokines expression in adipocytes by calcium-releasing activity. Additionally, a biologically active molecules-incorporating nanoparticles are effective than free form (136, 137). Functional LA-incorporating nanostructures might result in a synergism between the LA and  $\text{CaCO}_3$  NPs. Subcutaneous and visceral adipocytes exhibit differential gene expression and function (138). The contrast between cell-type preferences for LA- $\text{CaCO}_3$  NPs is significant and could contribute to different of functional responses. However, we suggest that this mechanism is difference, improved efficiency of adipogenesis in subcutaneous and visceral adipocytes by  $\text{CaCO}_3$  NPs will be considered in the future.



## CHAPTER V

### CONCLUSION

To the best of our knowledge, we successfully synthesized LA-CaCO<sub>3</sub> NPs which exhibited lipid reduction properties in ADSCs, subcutaneous, and visceral adipocytes. The LA-CaCO<sub>3</sub> NPs has efficient activity in reducing lipid accumulation, increasing lipolysis, decreasing both adipogenesis and adipokines expression. Moreover, LA-CaCO<sub>3</sub> NPs regulate adipocytes differentiation to induced *PAT2* expression in visceral adipocytes. Our findings present the new perspective that the delivery of LA to adipose tissue, with the assistance of CaCO<sub>3</sub> NPs exerted a potent therapeutic effect in reducing intercellular lipid deposition. The promising potential of CaCO<sub>3</sub> NPs and LA-CaCO<sub>3</sub> NPs as a stable and effective drug delivery system in targeted adipose tissue for overcoming obesity, metabolic diseases and maintaining a healthy life.

## REFERENCES

1. Cao Y. Angiogenesis modulates adipogenesis and obesity. *J Clin Invest.* 2007;117(9):2362-8.
2. Finkelstein EA, Ruhm CJ, Kosa KM. Economic causes and consequences of obesity. *Annu Rev Public Health.* 2005;26:239-57.
3. Kajimura S, Spiegelman BM, Seale P. Brown and Beige Fat: Physiological Roles beyond Heat Generation. *Cell Metab.* 2015;22(4):546-59.
4. Langin D. Control of fatty acid and glycerol release in adipose tissue lipolysis. *C R Biol.* 2006;329(8):598-607; discussion 53-5.
5. Cypess AM, Weiner LS, Roberts-Toler C, Franquet Elia E, Kessler SH, Kahn PA, et al. Activation of human brown adipose tissue by a beta3-adrenergic receptor agonist. *Cell Metab.* 2015;21(1):33-8.
6. Sasaki N, Uchida E, Niiyama M, Yoshida T, Saito M. Anti-obesity effects of selective agonists to the beta 3-adrenergic receptor in dogs. I. The presence of canine beta 3-adrenergic receptor and in vivo lipomobilization by its agonists. *J Vet Med Sci.* 1998;60(4):459-63.
7. Rabe K, Lehrke M, Parhofer KG, Broedl UC. Adipokines and insulin resistance. *Mol Med.* 2008;14(11-12):741-51.
8. Ioannides-Demos LL, Proietto J, Tonkin AM, McNeil JJ. Safety of drug therapies used for weight loss and treatment of obesity. *Drug Saf.* 2006;29(4):277-302.
9. Padwal RS, Majumdar SR. Drug treatments for obesity: orlistat, sibutramine, and rimonabant. *Lancet.* 2007;369(9555):71-7.

10. Jurisic-Erzen D, Starcevic-Klasan G, Ivanac D, Peharec S, Girotto D, Jerkovic R. The effects of alpha-lipoic acid on diabetic myopathy. *J Endocrinol Invest.* 2018;41(2):203-9.
11. Moini H, Packer L, Saris NE. Antioxidant and prooxidant activities of alpha-lipoic acid and dihydrolipoic acid. *Toxicol Appl Pharmacol.* 2002;182(1):84-90.
12. Prieto-Hontoria PL, Perez-Matute P, Fernandez-Galilea M, Barber A, Martinez JA, Moreno-Aliaga MJ. Lipoic acid prevents body weight gain induced by a high fat diet in rats: effects on intestinal sugar transport. *J Physiol Biochem.* 2009;65(1):43-50.
13. Koh EH, Lee WJ, Lee SA, Kim EH, Cho EH, Jeong E, et al. Effects of alpha-lipoic Acid on body weight in obese subjects. *Am J Med.* 2011;124(1):85 e1-8.
14. Fernandez-Galilea M, Perez-Matute P, Prieto-Hontoria PL, Martinez JA, Moreno-Aliaga MJ. Effects of lipoic acid on lipolysis in 3T3-L1 adipocytes. *J Lipid Res.* 2012;53(11):2296-306.
15. Hahm JR, Noh HS, Ha JH, Roh GS, Kim DR. Alpha-lipoic acid attenuates adipocyte differentiation and lipid accumulation in 3T3-L1 cells via AMPK-dependent autophagy. *Life Sci.* 2014;100(2):125-32.
16. Carlson DA, Smith AR, Fischer SJ, Young KL, Packer L. The plasma pharmacokinetics of R-(+)-lipoic acid administered as sodium R-(+)-lipoate to healthy human subjects. *Altern Med Rev.* 2007;12(4):343-51.
17. Ohgushi H, Okumura M, Yoshikawa T, Inoue K, Senpuku N, Tamai S, et al. Bone formation process in porous calcium carbonate and hydroxyapatite. *J Biomed Mater Res.* 1992;26(7):885-95.
18. Kopelman PG. Obesity as a medical problem. *Nature.* 2000;404(6778):635-43.
19. Jitnarin N, Kosulwat V, Rojroongwasinkul N, Boonpradern A, Haddock CK, Poston WS. Prevalence of overweight and obesity in Thai population: results of the National Thai Food Consumption Survey. *Eat Weight Disord.* 2011;16(4):e242-9.

20. Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell*. 2014;156(1-2):20-44.
21. Nishimura S, Manabe I, Nagasaki M, Hosoya Y, Yamashita H, Fujita H, et al. Adipogenesis in obesity requires close interplay between differentiating adipocytes, stromal cells, and blood vessels. *Diabetes*. 2007;56(6):1517-26.
22. Bartelt A, Heeren J. Adipose tissue browning and metabolic health. *Nat Rev Endocrinol*. 2014;10(1):24-36.
23. Giralt M, Villarroya F. White, brown, beige/brite: different adipose cells for different functions? *Endocrinology*. 2013;154(9):2992-3000.
24. Nolan JJ, Ludvik B, Beerdsen P, Joyce M, Olefsky J. Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N Engl J Med*. 1994;331(18):1188-93.
25. Fedorenko A, Lishko PV, Kirichok Y. Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. *Cell*. 2012;151(2):400-13.
26. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab*. 2007;293(2):E444-52.
27. Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? *Genes Dev*. 2013;27(3):234-50.
28. Obregon MJ. Adipose tissues and thyroid hormones. *Front Physiol*. 2014;5:479.
29. Barbatelli G, Murano I, Madsen L, Hao Q, Jimenez M, Kristiansen K, et al. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. *Am J Physiol Endocrinol Metab*. 2010;298(6):E1244-53.
30. Himms-Hagen J, Melnyk A, Zingaretti MC, Ceresi E, Barbatelli G, Cinti S. Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am J Physiol Cell Physiol*. 2000;279(3):C670-81.

31. Park A, Kim WK, Bae KH. Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells. *World J Stem Cells*. 2014;6(1):33-42.
32. Gesta S, Bluher M, Yamamoto Y, Norris AW, Berndt J, Kralisch S, et al. Evidence for a role of developmental genes in the origin of obesity and body fat distribution. *Proc Natl Acad Sci U S A*. 2006;103(17):6676-81.
33. Lee KY, Russell SJ, Ussar S, Boucher J, Vernochet C, Mori MA, et al. Lessons on conditional gene targeting in mouse adipose tissue. *Diabetes*. 2013;62(3):864-74.
34. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell*. 2012;150(2):366-76.
35. Ussar S, Lee KY, Dankel SN, Boucher J, Haering MF, Kleinridders A, et al. ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes. *Sci Transl Med*. 2014;6(247):247ra103.
36. Bjorndal B, Burri L, Staalesen V, Skorve J, Berge RK. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. *J Obes*. 2011;2011:490650.
37. Frayn KN. Adipose tissue as a buffer for daily lipid flux. *Diabetologia*. 2002;45(9):1201-10.
38. Wilcox G. Insulin and insulin resistance. *Clin Biochem Rev*. 2005;26(2):19-39.
39. Geerling JJ, Boon MR, Kooijman S, Parlevliet ET, Havekes LM, Romijn JA, et al. Sympathetic nervous system control of triglyceride metabolism: novel concepts derived from recent studies. *J Lipid Res*. 2014;55(2):180-9.
40. Vaughan CH, Zarebidaki E, Ehlen JC, Bartness TJ. Analysis and measurement of the sympathetic and sensory innervation of white and brown adipose tissue. *Methods Enzymol*. 2014;537:199-225.
41. Collins S, Surwit RS. The beta-adrenergic receptors and the control of adipose tissue metabolism and thermogenesis. *Recent Prog Horm Res*. 2001;56:309-28.

42. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev.* 2004;84(1):277-359.
43. Loncar D, Afzelius BA, Cannon B. Epididymal white adipose tissue after cold stress in rats. I. Nonmitochondrial changes. *J Ultrastruct Mol Struct Res.* 1988;101(2-3):109-22.
44. Young P, Arch JR, Ashwell M. Brown adipose tissue in the parametrial fat pad of the mouse. *FEBS Lett.* 1984;167(1):10-4.
45. Cederberg A, Gronning LM, Ahren B, Tasken K, Carlsson P, Enerback S. FOXC2 is a winged helix gene that counteracts obesity, hypertriglyceridemia, and diet-induced insulin resistance. *Cell.* 2001;106(5):563-73.
46. Gronning LM, Baillie GS, Cederberg A, Lynch MJ, Houslay MD, Enerback S, et al. Reduced PDE4 expression and activity contributes to enhanced catecholamine-induced cAMP accumulation in adipocytes from FOXC2 transgenic mice. *FEBS Lett.* 2006;580(17):4126-30.
47. Lidell ME, Seifert EL, Westergren R, Heglind M, Gowing A, Sukonina V, et al. The adipocyte-expressed forkhead transcription factor Foxc2 regulates metabolism through altered mitochondrial function. *Diabetes.* 2011;60(2):427-35.
48. Schulz TJ, Tseng YH. Emerging role of bone morphogenetic proteins in adipogenesis and energy metabolism. *Cytokine Growth Factor Rev.* 2009;20(5-6):523-31.
49. Whittle AJ, Carobbio S, Martins L, Slawik M, Hondares E, Vazquez MJ, et al. BMP8B increases brown adipose tissue thermogenesis through both central and peripheral actions. *Cell.* 2012;149(4):871-85.
50. Tseng YH, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM, et al. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. *Nature.* 2008;454(7207):1000-4.

51. Peirce V, Carobbio S, Vidal-Puig A. The different shades of fat. *Nature*. 2014;510(7503):76-83.
52. Bergman RN, Van Citters GW, Mittelman SD, Dea MK, Hamilton-Wessler M, Kim SP, et al. Central role of the adipocyte in the metabolic syndrome. *J Investig Med*. 2001;49(1):119-26.
53. Bjorntorp P. "Portal" adipose tissue as a generator of risk factors for cardiovascular disease and diabetes. *Arteriosclerosis*. 1990;10(4):493-6.
54. Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. *Endocr Rev*. 2000;21(6):697-738.
55. Arner P. Differences in lipolysis between human subcutaneous and omental adipose tissues. *Ann Med*. 1995;27(4):435-8.
56. Hoffstedt J, Arner P, Hellers G, Lonngqvist F. Variation in adrenergic regulation of lipolysis between omental and subcutaneous adipocytes from obese and non-obese men. *J Lipid Res*. 1997;38(4):795-804.
57. Armellini F, Zamboni M, Rigo L, Bergamo-Andreis IA, Robbi R, De Marchi M, et al. Sonography detection of small intra-abdominal fat variations. *Int J Obes*. 1991;15(12):847-52.
58. Arner P, Petrus P, Esteve D, Boulomie A, Naslund E, Thorell A, et al. Screening of potential adipokines identifies S100A4 as a marker of pernicious adipose tissue and insulin resistance. *Int J Obes (Lond)*. 2018.
59. Fruhbeck G, Catalan V, Rodriguez A, Ramirez B, Becerril S, Salvador J, et al. Involvement of the leptin-adiponectin axis in inflammation and oxidative stress in the metabolic syndrome. *Sci Rep*. 2017;7(1):6619.
60. Consitt LA, Saxena G, Schaefer M. Sex-dependent reductions in high molecular weight adiponectin during acute hyperinsulinemia are prevented with endurance training in older females. *Clin Endocrinol (Oxf)*. 2018;88(5):673-82.



61. Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol Metab.* 2002;13(2):84-9.
62. Wang A, Li T, An P, Yan W, Zheng H, Wang B, et al. Exendin-4 Upregulates Adiponectin Level in Adipocytes via Sirt1/Foxo-1 Signaling Pathway. *PLoS One.* 2017;12(1):e0169469.
63. Cho AS, Jeon SM, Kim MJ, Yeo J, Seo KI, Choi MS, et al. Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. *Food Chem Toxicol.* 2010;48(3):937-43.
64. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, et al. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. *J Clin Invest.* 2006;116(1):115-24.
65. Degawa-Yamauchi M, Moss KA, Bovenkerk JE, Shankar SS, Morrison CL, Lelliott CJ, et al. Regulation of adiponectin expression in human adipocytes: effects of adiposity, glucocorticoids, and tumor necrosis factor alpha. *Obes Res.* 2005;13(4):662-9.
66. Aregbesola A, de Mello VDF, Lindstrom J, Voutilainen S, Virtanen JK, Keinanen-Kiukaanniemi S, et al. Serum Adiponectin/Ferritin Ratio in Relation to the Risk of Type 2 diabetes and Insulin Sensitivity. *Diabetes Res Clin Pract.* 2018.
67. Bruun JM, Lihn AS, Verdich C, Pedersen SB, Toubro S, Astrup A, et al. Regulation of adiponectin by adipose tissue-derived cytokines: in vivo and in vitro investigations in humans. *Am J Physiol Endocrinol Metab.* 2003;285(3):E527-33.
68. Ando S, Catalano S. The multifactorial role of leptin in driving the breast cancer microenvironment. *Nat Rev Endocrinol.* 2011;8(5):263-75.
69. Shintani T, Higashi S, Suzuki R, Takeuchi Y, Ikaga R, Yamazaki T, et al. PTPRJ Inhibits Leptin Signaling, and Induction of PTPRJ in the Hypothalamus Is a Cause of the Development of Leptin Resistance. *Sci Rep.* 2017;7(1):11627.

70. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med.* 1996;334(5):292-5.
71. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med.* 1995;1(11):1155-61.
72. Carbone F, La Rocca C, Matarese G. Immunological functions of leptin and adiponectin. *Biochimie.* 2012;94(10):2082-8.
73. Santos-Alvarez J, Goberna R, Sanchez-Margalet V. Human leptin stimulates proliferation and activation of human circulating monocytes. *Cell Immunol.* 1999;194(1):6-11.
74. Flier JS, Maratos-Flier E. Leptin's Physiologic Role: Does the Emperor of Energy Balance Have No Clothes? *Cell Metab.* 2017;26(1):24-6.
75. Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV, et al. Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. *Diabetes.* 2001;50(10):2199-202.
76. Stepan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, et al. The hormone resistin links obesity to diabetes. *Nature.* 2001;409(6818):307-12.
77. Cebeci E, Cakan C, Gursu M, Uzun S, Karadag S, Koldas M, et al. The Main Determinants of Serum Resistin Level in Type 2 Diabetic Patients are Renal Function and Inflammation not Presence of Microvascular Complication, Obesity and Insulin Resistance. *Exp Clin Endocrinol Diabetes.* 2018.
78. Padmalayam I, Hasham S, Saxena U, Pillarisetti S. Lipoic acid synthase (LASy): a novel role in inflammation, mitochondrial function, and insulin resistance. *Diabetes.* 2009;58(3):600-8.

79. Shay KP, Moreau RF, Smith EJ, Smith AR, Hagen TM. Alpha-lipoic acid as a dietary supplement: molecular mechanisms and therapeutic potential. *Biochim Biophys Acta*. 2009;1790(10):1149-60.
80. Jacob S, Streeper RS, Fogt DL, Hokama JY, Tritschler HJ, Dietze GJ, et al. The antioxidant alpha-lipoic acid enhances insulin-stimulated glucose metabolism in insulin-resistant rat skeletal muscle. *Diabetes*. 1996;45(8):1024-9.
81. Song KH, Lee WJ, Koh JM, Kim HS, Youn JY, Park HS, et al. alpha-Lipoic acid prevents diabetes mellitus in diabetes-prone obese rats. *Biochem Biophys Res Commun*. 2005;326(1):197-202.
82. Chen WL, Kang CH, Wang SG, Lee HM. alpha-Lipoic acid regulates lipid metabolism through induction of sirtuin 1 (SIRT1) and activation of AMP-activated protein kinase. *Diabetologia*. 2012;55(6):1824-35.
83. Mohammadi V, Khorvash F, Feizi A, Askari G. The effect of alpha-lipoic acid supplementation on anthropometric indices and food intake in patients who experienced stroke: A randomized, double-blind, placebo-controlled clinical trial. *J Res Med Sci*. 2017;22:98.
84. Kim MS, Park JY, Namkoong C, Jang PG, Ryu JW, Song HS, et al. Anti-obesity effects of alpha-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. *Nat Med*. 2004;10(7):727-33.
85. Cho KJ, Moon HE, Moini H, Packer L, Yoon DY, Chung AS. Alpha-lipoic acid inhibits adipocyte differentiation by regulating pro-adipogenic transcription factors via mitogen-activated protein kinase pathways. *J Biol Chem*. 2003;278(37):34823-33.
86. Fernandez-Galilea M, Perez-Matute P, Prieto-Hontoria PL, Houssier M, Burrell MA, Langin D, et al. alpha-Lipoic acid treatment increases mitochondrial biogenesis and promotes beige adipose features in subcutaneous adipocytes from overweight/obese subjects. *Biochim Biophys Acta*. 2015;1851(3):273-81.

87. Hamano Y. Effects of dietary lipoic acid on plasma lipid, in vivo insulin sensitivity, metabolic response to corticosterone and in vitro lipolysis in broiler chickens. *Br J Nutr.* 2006;95(6):1094-101.
88. Hamano Y. Influence of lipoic acid on lipid metabolism and beta-adrenergic response to intravenous or oral administration of clenbuterol in broiler chickens. *Reprod Nutr Dev.* 2002;42(4):307-16.
89. Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, et al. Mechanism of free fatty acid-induced insulin resistance in humans. *J Clin Invest.* 1996;97(12):2859-65.
90. Zhang Y, Han P, Wu N, He B, Lu Y, Li S, et al. Amelioration of lipid abnormalities by alpha-lipoic acid through antioxidative and anti-inflammatory effects. *Obesity (Silver Spring).* 2011;19(8):1647-53.
91. Lee WJ, Song KH, Koh EH, Won JC, Kim HS, Park HS, et al. Alpha-lipoic acid increases insulin sensitivity by activating AMPK in skeletal muscle. *Biochem Biophys Res Commun.* 2005;332(3):885-91.
92. Park KG, Min AK, Koh EH, Kim HS, Kim MO, Park HS, et al. Alpha-lipoic acid decreases hepatic lipogenesis through adenosine monophosphate-activated protein kinase (AMPK)-dependent and AMPK-independent pathways. *Hepatology.* 2008;48(5):1477-86.
93. Huong DT, Ide T. Dietary lipoic acid-dependent changes in the activity and mRNA levels of hepatic lipogenic enzymes in rats. *Br J Nutr.* 2008;100(1):79-87.
94. Valdecantos MP, Perez-Matute P, Gonzalez-Muniesa P, Prieto-Hontoria PL, Moreno-Aliaga MJ, Martinez JA. Lipoic acid administration prevents nonalcoholic steatosis linked to long-term high-fat feeding by modulating mitochondrial function. *J Nutr Biochem.* 2012;23(12):1676-84.

95. Prieto-Hontoria PL, Perez-Matute P, Fernandez-Galilea M, Alfredo Martinez J, Moreno-Aliaga MJ. Effects of lipoic acid on AMPK and adiponectin in adipose tissue of low- and high-fat-fed rats. *Eur J Nutr.* 2013;52(2):779-87.
96. Vidovic B, Milovanovic S, Stefanovic A, Kotur-Stevuljevic J, Takic M, Debeljak-Martacic J, et al. Effects of Alpha-Lipoic Acid Supplementation on Plasma Adiponectin Levels and Some Metabolic Risk Factors in Patients with Schizophrenia. *J Med Food.* 2017;20(1):79-85.
97. Prieto-Hontoria PL, Fernandez-Galilea M, Perez-Matute P, Martinez JA, Moreno-Aliaga MJ. Lipoic acid inhibits adiponectin production in 3T3-L1 adipocytes. *J Physiol Biochem.* 2013;69(3):595-600.
98. Prieto-Hontoria PL, Perez-Matute P, Fernandez-Galilea M, Martinez JA, Moreno-Aliaga MJ. Lipoic acid inhibits leptin secretion and Sp1 activity in adipocytes. *Mol Nutr Food Res.* 2011;55(7):1059-69.
99. Cheng PY, Lee YM, Yen MH, Peng JC, Lam KK. Reciprocal effects of alpha-lipoic acid on adenosine monophosphate-activated protein kinase activity in obesity induced by ovariectomy in rats. *Menopause.* 2011;18(9):1010-7.
100. Nieva-Vazquez A, Perez-Fuentes R, Torres-Rasgado E, Lopez-Lopez JG, Romero JR. Serum resistin levels are associated with adiposity and insulin sensitivity in obese Hispanic subjects. *Metab Syndr Relat Disord.* 2014;12(2):143-8.
101. De Jong WH, Borm PJ. Drug delivery and nanoparticles: applications and hazards. *Int J Nanomedicine.* 2008;3(2):133-49.
102. Wilczewska AZ, Niemirowicz K, Markiewicz KH, Car H. Nanoparticles as drug delivery systems. *Pharmacol Rep.* 2012;64(5):1020-37.
103. Barratt GM. Therapeutic applications of colloidal drug carriers. *Pharm Sci Technolo Today.* 2000;3(5):163-71.

104. Biradar S, Ravichandran P, Gopikrishnan R, Goornavar V, Hall JC, Ramesh V, et al. Calcium carbonate nanoparticles: synthesis, characterization and biocompatibility. *J Nanosci Nanotechnol*. 2011;11(8):6868-74.
105. Kamba AS, Ismail M, Ibrahim TA, Zakaria ZA. Biocompatibility of bio based calcium carbonate nanocrystals aragonite polymorph on NIH 3T3 fibroblast cell line. *Afr J Tradit Complement Altern Med*. 2014;11(4):31-8.
106. Ueno Y, Futagawa H, Takagi Y, Ueno A, Mizushima Y. Drug-incorporating calcium carbonate nanoparticles for a new delivery system. *J Control Release*. 2005;103(1):93-8.
107. Jabbari N, Zarei L, Esmaeili Govarchin Galeh H, Mansori Motlagh B. Assessment of synergistic effect of combining hyperthermia with irradiation and calcium carbonate nanoparticles on proliferation of human breast adenocarcinoma cell line (MCF-7 cells). *Artif Cells Nanomed Biotechnol*. 2018:1-9.
108. Zhao P, Li M, Wang Y, Chen Y, He C, Zhang X, et al. Enhancing anti-tumor efficiency in hepatocellular carcinoma through the autophagy inhibition by miR-375/sorafenib in lipid-coated calcium carbonate nanoparticles. *Acta Biomater*. 2018;72:248-55.
109. He XW, Liu T, Xiao Y, Feng YL, Cheng DJ, Tingting G, et al. Vascular endothelial growth factor-C siRNA delivered via calcium carbonate nanoparticle effectively inhibits lymphangiogenesis and growth of colorectal cancer in vivo. *Cancer Biother Radiopharm*. 2009;24(2):249-59.
110. Hammadi NI, Abba Y, Hezmee MNM, Razak ISA, Jaji AZ, Isa T, et al. Formulation of a Sustained Release Docetaxel Loaded Cockle Shell-Derived Calcium Carbonate Nanoparticles against Breast Cancer. *Pharm Res*. 2017;34(6):1193-203.
111. Hammadi NI, Abba Y, Hezmee MNM, Razak ISA, Kura AU, Zakaria ZAB. Evaluation of in vitro efficacy of docetaxel-loaded calcium carbonate aragonite nanoparticles

(DTX-CaCO<sub>3</sub>NP) on 4T1 mouse breast cancer cell line. *In Vitro Cell Dev Biol Anim.* 2017;53(10):896-907.

112. Sadashiv, Tiwari S, Paul BN, Kumar S, Chandra A, Dhananjai S, et al. Over expression of resistin in adipose tissue of the obese induces insulin resistance. *World J Diabetes.* 2012;3(7):135-41.

113. Li Y, Rong Y, Bao L, Nie B, Ren G, Zheng C, et al. Suppression of adipocyte differentiation and lipid accumulation by stearidonic acid (SDA) in 3T3-L1 cells. *Lipids Health Dis.* 2017;16(1):181.

114. Ji E, Jung MY, Park JH, Kim S, Seo CR, Park KW, et al. Inhibition of adipogenesis in 3T3-L1 cells and suppression of abdominal fat accumulation in high-fat diet-feeding C57BL/6J mice after downregulation of hyaluronic acid. *Int J Obes (Lond).* 2014;38(8):1035-43.

115. Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur Cytokine Netw.* 2006;17(1):4-12.

116. Fantuzzi G. Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol.* 2005;115(5):911-9; quiz 20.

117. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol.* 2011;11(2):85-97.

118. Yamauchi T, Kadowaki T. Adiponectin receptor as a key player in healthy longevity and obesity-related diseases. *Cell Metab.* 2013;17(2):185-96.

119. Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, et al. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature.* 2003;423(6941):762-9.

120. Yadav A, Kataria MA, Saini V, Yadav A. Role of leptin and adiponectin in insulin resistance. *Clin Chim Acta.* 2013;417:80-4.

121. Kandeil MA, Amin KA, Hassanin KA, Ali KM, Mohammed ET. Role of lipoic acid on insulin resistance and leptin in experimentally diabetic rats. *J Diabetes Complications*. 2011;25(1):31-8.
122. Fehmann HC, Heyn J. Plasma resistin levels in patients with type 1 and type 2 diabetes mellitus and in healthy controls. *Horm Metab Res*. 2002;34(11-12):671-3.
123. Gharibeh MY, Al Tawallbeh GM, Abboud MM, Radaideh A, Alhader AA, Khabour OF. Correlation of plasma resistin with obesity and insulin resistance in type 2 diabetic patients. *Diabetes Metab*. 2010;36(6 Pt 1):443-9.
124. Zehnpfennig B, Wiriyasermkul P, Carlson DA, Quick M. Interaction of alpha-Lipoic Acid with the Human Na<sup>+</sup>/Multivitamin Transporter (hSMVT). *J Biol Chem*. 2015;290(26):16372-82.
125. Cohen H, Levy RJ, Gao J, Fishbein I, Kousaev V, Sosnowski S, et al. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. *Gene Ther*. 2000;7(22):1896-905.
126. Bisht S, Bhakta G, Mitra S, Maitra A. pDNA loaded calcium phosphate nanoparticles: highly efficient non-viral vector for gene delivery. *Int J Pharm*. 2005;288(1):157-68.
127. Palmqvist NGM, Nedelec JM, Seisenbaeva GA, Kessler VG. Controlling nucleation and growth of nano-CaCO<sub>3</sub> via CO<sub>2</sub> sequestration by a calcium alkoxide solution to produce nanocomposites for drug delivery applications. *Acta Biomater*. 2017;57:426-34.
128. Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu CY, et al. Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. *Circulation*. 2007;116(1):39-48.
129. Luque CA, Rey JA. Sibutramine: a serotonin-norepinephrine reuptake-inhibitor for the treatment of obesity. *Ann Pharmacother*. 1999;33(9):968-78.



130. Bray GA, Greenway FL. Pharmacological treatment of the overweight patient. *Pharmacol Rev.* 2007;59(2):151-84.
131. Shen W, Hao J, Feng Z, Tian C, Chen W, Packer L, et al. Lipoamide or lipoic acid stimulates mitochondrial biogenesis in 3T3-L1 adipocytes via the endothelial NO synthase-cGMP-protein kinase G signalling pathway. *Br J Pharmacol.* 2011;162(5):1213-24.
132. Valdecantos MP, Perez-Matute P, Gonzalez-Muniesa P, Prieto-Hontoria PL, Moreno-Aliaga MJ, Martinez JA. Lipoic acid improves mitochondrial function in nonalcoholic steatosis through the stimulation of sirtuin 1 and sirtuin 3. *Obesity (Silver Spring).* 2012;20(10):1974-83.
133. Shen W, Liu K, Tian C, Yang L, Li X, Ren J, et al. R-alpha-lipoic acid and acetyl-L-carnitine complementarily promote mitochondrial biogenesis in murine 3T3-L1 adipocytes. *Diabetologia.* 2008;51(1):165-74.
134. Jonas MI, Kurylowicz A, Bartoszewicz Z, Lisik W, Jonas M, Domienik-Karłowicz J, et al. Adiponectin/resistin interplay in serum and in adipose tissue of obese and normal-weight individuals. *Diabetol Metab Syndr.* 2017;9:95.
135. Liang H, Zhang XB, Lv Y, Gong L, Wang R, Zhu X, et al. Functional DNA-containing nanomaterials: cellular applications in biosensing, imaging, and targeted therapy. *Acc Chem Res.* 2014;47(6):1891-901.
136. Wong HL, Bendayan R, Rauth AM, Xue HY, Babakhanian K, Wu XY. A mechanistic study of enhanced doxorubicin uptake and retention in multidrug resistant breast cancer cells using a polymer-lipid hybrid nanoparticle system. *J Pharmacol Exp Ther.* 2006;317(3):1372-81.
137. Lam FC, Morton SW, Wyckoff J, Vu Han TL, Hwang MK, Maffa A, et al. Enhanced efficacy of combined temozolomide and bromodomain inhibitor therapy for gliomas using targeted nanoparticles. *Nat Commun.* 2018;9(1):1991.

138. Gerhard GS, Styer AM, Strodel WE, Roesch SL, Yavorek A, Carey DJ, et al. Gene expression profiling in subcutaneous, visceral and epigastric adipose tissues of patients with extreme obesity. *Int J Obes (Lond)*. 2014;38(3):371-8.





Appendix

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## LIST OF ABBREVIATIONS

AC	Adenylate cyclase
ADIPOQ	Adiponectin
$\mu\text{L}$	Microliter
$\mu\text{M}$	Micromolar
ADSCs	Adipose-derived stem cells
ANOVA	Analysis of variance
ASC-1	Neutral amino acid transporter
ATGL	Adipose triglyceride lipase
BMI	Body mass index
BMP	Bone morphogenic protein
$\text{Ca}^{2+}$	Calcium ion
$\text{CaCO}_3$	Calcium carbonate
$\text{CaCO}_3$ NPs	Calcium carbonate nanoparticles
cDNA	Complementary DNA
$\text{CO}_2$	Carbon dioxide
$\text{CO}_3^{2-}$	Carbonate ion
$C_T$	Threshold cycle
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal bovine serum
FFAs	Free fatty acids
G	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
$\text{H}_2\text{DCFDA}$	2,7-dichlorofluorescein-diacetate
$\text{H}_2\text{O}_2$	Hydrogen peroxide
HaCaT	Keratinocytes

HDFs	Human dermal fibroblasts
HSL	Hormone-sensitive lipase
kV	Kilovolt
LA	Lipoic acid
LA-CaCO <sub>3</sub> NPs	Lipoic acids-incorporated calcium carbonate nanoparticles
LEP	Leptin
MGL	Monoglyceride lipase
mM	Millimolar
NA	Noradrenaline
NaHCO <sub>3</sub>	Sodium bicarbonate
nm	Nanometer
NPs	Nanoparticles
ns	Not significant
OD	Optical Density
P2RX5	Purinergic receptor P2X
PAT2	Proton assistant amino acid transporter-2
PDE	Phosphodiesterases
RELMs	Resistin-like molecules
RETN	Resistin
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard deviation
SNS	Sympathetic nervous system
sWAT	Subcutaneous white adipose tissue
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis

TNF- $\alpha$	Tumor necrosis factor-alpha
TZD	Thiazolidinedione
UCP1	Uncoupling proteins 1
vWAT	Visceral white adipose tissue
WAT	White adipose tissue
WHO	World health organization



## EQUIPMENTS AND CHEMICALS

### Equipment

1. 24-well plate (Corning, USA)
2. 96-well plate (Corning, USA)
3. Autoclave (Hirayama, Japan)
4. Autopipette 10  $\mu$ L (Biorad, USA )
5. Autopipette 100  $\mu$ L (Biorad, USA )
6. Autopipette 1000  $\mu$ L(Biorad, USA )
7. Autopipette 200  $\mu$ L(Biorad, USA )
8. Biohazard Laminar Flow (Gibco, USA)
9. Cell culture flask (SPL, Korea)
10. Centrifuge tube 1.5 mL (Corning, USA)
11. Centrifuge tube 15 mL (Corning, USA)
12. Centrifuge tube 5 mL (Corning, USA)
13. Centrifuge tube 50 mL (Corning, USA)
14. Class II biohazard safety cabinet (Esco Micro, Singapore)
15. CO<sub>2</sub> incubator (Esco, Singapore)
16. CO<sub>2</sub> Incubator (Esco Micro, Singapore)
17. Filter Tip (Corning, USA)
18. Laboratory balance (Denver instrument, Germany)
19. Malvern Zetasizer Nano Series (Malvern Instrument, England)
20. Microcentrifuge (Hettich, Germany)
21. Oven (Contherm, New Zealand)
22. Parafilm (Bemis, USA)
23. pH meter (Denver instrument, Germany)
24. Phase contrast inverted microscope (Nikon, Japan)
25. Shaker incubator (Heidolph, Germany)

26. Sonicator (P.Intertrade Equipment Co.,Ltd)
27. StepOnePlus Real-Time PCR System (ABI Applied Biosystems, USA)
28. Thermal gravimetric analysis (TGA) (TA Instrument, USA)
29. Transmission Electron Microscope (Hitachi, Japan)
30. Upright Ultra-Low Temperature Freezers (Thermo, England)
31. Varioskan Flash microplate reader (Thermo, England)
32. Veriti 96 Well Thermal Cycler (ABI Applied Biosystems, USA)
33. Vortex mixer (Scientific industries, USA)
34. Water bath (Mettler, Germany)
35. Zetasizer Nano Series (Malvern Instrument, England)





## Chemicals

1. Calcium chloride (MERK, Germany)
2. Chloroform (MERK, Germany)
3. Dulbecco's Modified Eagle's Medium (Sigma, USA)
4. Ethanol (MERK, Germany)
5. Fetal Bovine Serum (Gibco, USA)
6. First strand cDNA synthesis kit (Roche®) (Thermoscientific, USA)
7. H2DCFDA (Invitrogen, USA)
8. Hydrochloric acid (MERK, Germany)
9. Isopropanol (MERK, Germany)
10. Lipoic acids (Sigma, USA)
11. Lipolysis kit (Abcam, USA)
12. Oil Red O (Sigma, USA)
13. Paraformaldehyde (Sigma, USA)
14. Penicillin/Streptomycin (Gibco, USA)
15. PrestoBlue™ Cell viability Reagent (Invitrogen, USA)
16. Sodium bicarbonate (MERK, Germany)
17. SYBR GreenER qPCR Supermix Universal (Invitrogen, USA)
18. TRIzol reagent (Invitrogen, USA)
19. Ultrapure distilled water (Invitrogen, USA)

## Chemical preparation

### 1. Phosphate buffer saline

KCl	0.2	g
KH <sub>2</sub> PO <sub>4</sub>	0.2	g
NaCl	8.0	g
Na <sub>2</sub> HPO <sub>4</sub>	1.15	g

Mix all 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<sub>2</sub>HCO<sub>3</sub>
- 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 Fetal Bovine Serum, 10 mL of antibiotic (Pen-Strep) into 900 mL of stock medium.

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

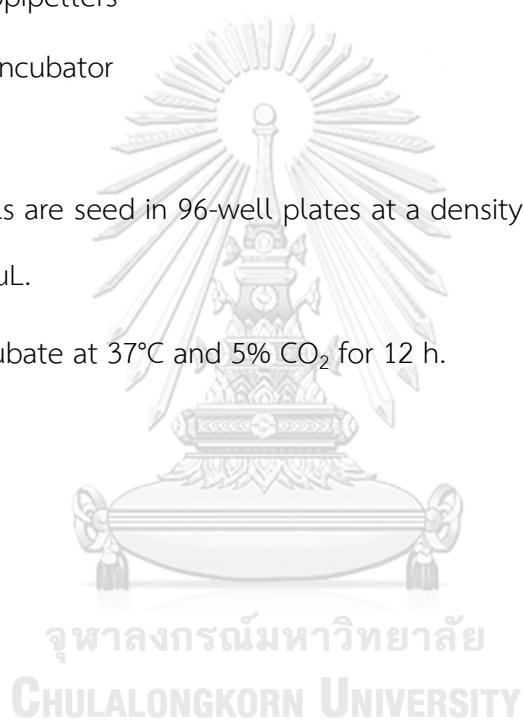
### 1. Cell culture

#### Materials

1. 96-well plate
2. Cell Culture Media
3. Micropipettors
4. CO<sub>2</sub> incubator

#### Method

1. Cells are seed in 96-well plates at a density of  $1-10 \times 10^3$  cells/well in 45  $\mu$ L.
2. Incubate at 37°C and 5% CO<sub>2</sub> for 12 h.



## 2. Cell viability assay

### Materials

1. Unknown sample for toxicity test
2. H<sub>2</sub>O<sub>2</sub> (positive control)

### Method

1. Add 45 µL of culture medium for negative control.
2. Add 45 µL of H<sub>2</sub>O<sub>2</sub> for positive control.
3. Treat with 45 µL of unknown samples.
4. Incubate at 37°C and 5% CO<sub>2</sub> for 24, 48, or 72 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<sub>2</sub>DCFDA, Invitrogen, USA, Catalog number D399)

### 1. Cell culture

#### Materials

1. 96-black well plate
2. Cell Culture Media
3. Micropipettors
4. CO<sub>2</sub> incubator

#### Method

1. Cells are seed in 96-black well plate at a density of  $1-10 \times 10^3$  cells/well in 100  $\mu$ L.
2. Incubate at 37°C and 5% CO<sub>2</sub> for 12 h.

## 2. DCFH-DA assay

### Materials

1. Unknown sample for ROS generation test
2. H<sub>2</sub>O<sub>2</sub> (positive control)
3. Phosphate Buffer Saline (PBS)

### Method

1. Wash the cells 2 times with PBS
2. Add 100 µL of 0.1M H<sub>2</sub>DCFDA
3. Incubate at 37°C and 5% CO<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>DCFDA from Invitrogen™

## VITA

Miss Sarocha Cherdchom was born in Bangkok, Thailand on April 2, 1991. She was graduated with Bachelor's Degree in Biology from faculty of Science, Srinakharinwirot University in 2013. She was granted the 100th Anniversary Chulalongkorn University Fund for Doctoral Scholarship from Graduate School, Chulalongkorn University to enroll in Doctor of Philosophy Program in Medical Sciences in 2014.





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