ผลของอนุภาคแคลเซียมซิเทรตบรรจุในแคลเซียมคาร์บอเนตนาโนต่อเซลล์ HK-2 ในสิ่งแวดล้อมที่เป็นกรด

นางสาวทิพากร อุดมสินศิริกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Effect of calcium citrate in calcium carbonate nanoparticles on HK-2 cells in acidic environment

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ทิพากร อุดมสินศิริกุล : ผลของอนุภาคแคลเซียมซิเทรตบรรจุในแคลเซียมคาร์บอเนตนาโนต่อเซลล์ HK-2 ในสิ่งแวดล้อมที่เป็นกรด (Effect of calcium citrate in calcium carbonate nanoparticles on HK-2 cells in acidic environment) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ. ฐสิณัส ดิษยบุตร , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. นพ. อมรพันธุ์ เสรีมาศพันธุ์, ผศ. ดร. โรจน์ฤทธิ์ โรจนธเนศ, 50 หน้า.

ภาวะกรดเกินในเลือดเป็นภาวะสำคัญที่ส่งผลต่อการทำงานของเนื้อเยื่อหลายชนิด ทำให้เกิดการสลายของกระดูกและโปรตีน กระตุ้นการอักเสบ ภาวะเครียดทางออกซิเดชันรวมทั้งการตายของเซลล์ นำมาสู่การเกิดการเสื่อมการทำงานของอวัยวะต่าง ๆ โดยเฉพาะอย่างยิ่งทำให้เกิดภาวะไตเสื่อมเรื้อรัง โซเดียมไบคาร์บอร์เนต (sodium bicarbonate) หรือ สารละลาย Shohl's solution ถูกนำมาใช้เพื่อรักษาอาการกรดเกิน แต่ยาทั้งสองก่อให้เกิดผลข้างเคียงในระบบทางเดินอาหาร เพื่อลดผลข้างเคียงที่เกิดขึ้น ผู้วิจัยจึงได้พัฒนาอนุภาคแคลเซียมคาร์บอเนตนาโนมาใช้ในการขนส่งซิเทรตไปยังเซลล์ไต โดยมีสมมติฐานว่าซิเทรตจะช่วยลดผลข้างเคียงจากสภาวะกรดเกินในเลือด ผู้วิจัยทำการศึกษาคุณลักษณะทางกายภาพเบื้องต้นรวมถึงประสิทธิภาพทางเภสัชวิทยาของอนุภาคแคลเซียมซิเทรตนาโน (Cacit) และอนุภาคแคลเซียมซิเทรตในแคลเซียมคาร์บอเนตนาโน (CCN) ต่อเซลล์ HK-2 ในการเกิดความเป็นพิษต่อเซลล์ ภาวะเครียดทางออกซิเดชัน การสร้างไบคาร์บอเนตและการเปลี่ยนแปลงความเป็นกรดของสิ่งแวดล้อม และการตายของเซลล์จากสภาวะกรด โดยผลการศึกษาพบว่า Cacit และ CCN ที่สังเคราะห์ขึ้นมีขนาดอยู่ในระดับนาโนเมตร ซึ่งอนุภาคมีเส้นผ่านศูนย์กลางประมาณ 50-70 และ 60-80 นาโนเมตร ตามลำดับ โดย Cacit มีค่าประจุที่พื้นผิวเท่ากับ -16.1 mV และ CCN มีค่าเท่ากับ -13.0 mV ซึ่งมีซิเทรตเป็นองค์ประกอบใน Cacit และ CCN ประมาณ 31% และ 25% ตามลำดับ นอกจากนี้ยังพบว่าอนุภาคนาโนทั้งสองชนิดสามารถเข้าสู่เซลล์ HK-2 ได้อย่างอิสระทั้งในสภาวะปกติและสภาวะกรด โดยที่ทั้ง Cacit และ CCN มีความเป็นพิษต่อเซลล์ต่ำที่ระดับความเข้มข้นไม่เกิน 1 mg/mL เมื่อทดสอบดูประสิทธิภาพทางเภสัชวิทยาพบว่า Cacit และ CCN มีคุณสมบัติเป็นสารต้านอนุมูลอิสระซึ่งมีศักยภาพสูงกว่ายามาตรฐานคือโซเดียมซิเทรต (Nacit) แต่ถึงอย่างไรก็ตาม Cacit และ CCN ไม่ได้ส่งผลให้เกิดการเปลี่ยนแปลงความเป็นกรดที่ภายนอกเซลล์ รวมทั้งไม่พบความเปลี่ยนแปลงของปริมาณไบคาร์บอเนตทั้งภายนอกและภายในเซลล์ เราพบว่า Cacit และ CCN ช่วยลดการตายของเซลล์ที่ถูกกระตุ้นจากสภาวะกรดได้เทียบเท่ากับ Nacit จากผลการทดลองทั้งหมดนี้แสดงให้เห็นว่า Cacit และ CCN มีศักยภาพในการใช้เป็นสารเสริมฤทธิ์ในการรักษาร่วมกับยาที่ใช้เป็นมาตรฐานในการรักษาสภาวะกรดเกิน โดยจำเป็นต้องมีการศึกษาเพิ่มเติมเพื่อทดสอบผลกระทบทางเภสัชวิทยาของอนุภาคนาโนทั้งสองในสัตว์ทดลองต่อไป

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: NANOPARTICLES, CALCIUM CITRATE, CALCIUMCARBONATE, ACIDOSIS, CALCIUM CITRATE IN CALCIUM CARBONATE NANOPARTICLES

TIPAGORN UDOMSINSIRIKUL: Effect of calcium citrate in calcium carbonate nanoparticles on HK-2 cells in acidic environment. ADVISOR: ASST. PROF. DR. THASINAS DISSAYABUTR, M.D., CO-ADVISOR: ASST. PROF. DR. AMORNPUN SEREEMASPUN, M.D., ASST. PROF. DR. ROJRIT ROJANATHANES, 50 pp.

Metabolic acidosis exerts several cellular and tissue dysfunctions, such as increases mineral bone and protein degradation, induces inflammation, oxidative stress and cell death. These complications lead to multiple organ failure, including chronic kidney disease. Sodium bicarbonate and Shohl's solution are commonly used to treat metabolic acidosis. However, these agents cause frequent gastrointestinal adverse effects. Hence, we invented the new calcium carbonate nanoparticles to use as a drug delivery system for carrying citrate to renal cell which we hypothesize to reduce adverse effects of metabolic acidosis. We studied the physical characteristics and pharmacological effects of calcium citrate nanoparticle (Cacit) and calcium citrate in calcium carbonate nanoparticles (CCN) on HK-2 cells in acidic environment on the aspect of cytotoxicity, ROS and bicarbonate generation, pH modification and cell death. Our results showed that the Cacit and CCN has spherical molecule with diameter between 50-70 and 60-80 nm, respectively. Cacit has surface charge about -16.1 mV and CCN has -13.0 mV. Citrate content in Cacit and CCN was approximately 31% and 25%, and both nanoparticles was uptake into the cell freely in normal and acidic condition. Cacit and CCN had very low cytotoxicity at the concentration up to 1 mg/ml. Pharmacological characterization revealed that both Cacit and CCN contained antioxidant properties which was appeared to be more potent than standard drug sodium citrate (Nacit). Cacit and CCN treatment did not alkalinize extracellular pH, or extracellular and intracellular bicarbonate concentration. We found that Cacit and CCN treatment ameliorated acidosis-induced cell death similar to Nacit. In conclusion, Cacit and CCN were a potential medication to use as an adjuvant therapy with standard drug in chronic acidosis. Further study is required to elucidate the systemic pharmacological effects of these nanoparticles in animal.

Field of Study: Medical Science
Academic Year: 2017

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

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Chapter I Introduction

Acidosis or acidemia is a common acid-base disorder caused by organ dysfunction. Acidemia is defined by serum pH less than 7.35. Etiology of acidosis is a reduction of serum bicarbonate concentration, high partial pressure of arterial carbon dioxide or accumulation of acidic products. Acidosis is divided by etiology into respiratory and metabolic-caused (1). In this research we focused in chronic metabolic acidosis which is caused by increased acid metabolite production, excessive loss of bicarbonate, and insufficient renal elimination of acids. Acidosis can induce cellular injury, kidney cell damage, osteoporosis, muscle protein degradation, abnormal albumin synthesis and multi-organ dysfunction (2). To treat chronic cidosis, sodium bicarbonate or Shohl's solution are currently used as a standard medication. However, they have frequent gastrointestinal adverse effects such as unpalatability, gastric discomfort and increasing absorption of aluminum. These cause poor patient's compliance and discontinuation of therapy (3). Regarding this, we aimed to develop a novel drug to treat chronic acidosis using nanoparticle envelope, targeting to reduce the gastrointestinal side effects.

In recent years, nanoparticles are introduced in term of nanosized particles which have one dimension less than 100 nm (4). Several nanoparticles have been applied to encapsulate some drugs to improve their efficacy. Nanocarriers have high biocompatibility, modifiable, increase drug stability and carry drug to specific tissue (5). Nanoparticle delivery could be either organic or inorganic compound. Calcium carbonate nanoparticle ($CaCO_3$ NPs) is one of the most common inorganic nanoparticle compounds used in many industries. In medicine, $CaCO_3$ NPs were used as a nanocarrier to delivery certain drugs; such as insulin and betamethasone phosphate (6). $CaCO_3$ NPs have efficacy to control drug releasing. In addition, they have been identified to safety, slow biodegradability and pH sensitivity (7).

Hence in this research, we applied the $CaCO_3$ NPs to combine with properties of citrate for improving acidosis. Citrate is an alkalinizing agent which is taken up and

metabolized to producing bicarbonate (8). In addition, previous study showed that citrate had plays an important role in other cellular processes, such as inflammation (9) and antioxidant activity (10). Citrate may ameliorate renal dysfunction (11). Consequently, we had invented a calcium citrate in calcium carbonate nanoparticle (CCN) which citrate was loaded in $CaCO_3$ NPs. We expected that CCN could deliver citrate and break down in acidic cells, leading to ameliorate metabolic acidosis. In addition, CCN should have lower gastrointestinal adverse reactions than standard medication.

The aims of this research were to study the characterization of CCN and its effects on living cells. We emphasized on the study to evaluate the efficiency of CCN in the generation of bicarbonate, prevention of oxidative stress and cells death in acid condition in human proximal tubular cell line (HK-2 cells). We hypothesized that CCN could be used to treat chronic acidosis and attenuates acidosis-induced cellular injury.

1.1 Research questions

1. What are the basic properties of calcium citrate in calcium carbonate nanoparticles?
2. Can calcium citrate in calcium carbonate nanoparticles increase alkaline generation in acidic condition on HK-2 cells?
3. Can calcium citrate in calcium carbonate nanoparticles reduce oxidative stress and cell death in acidic condition on HK-2 cells?

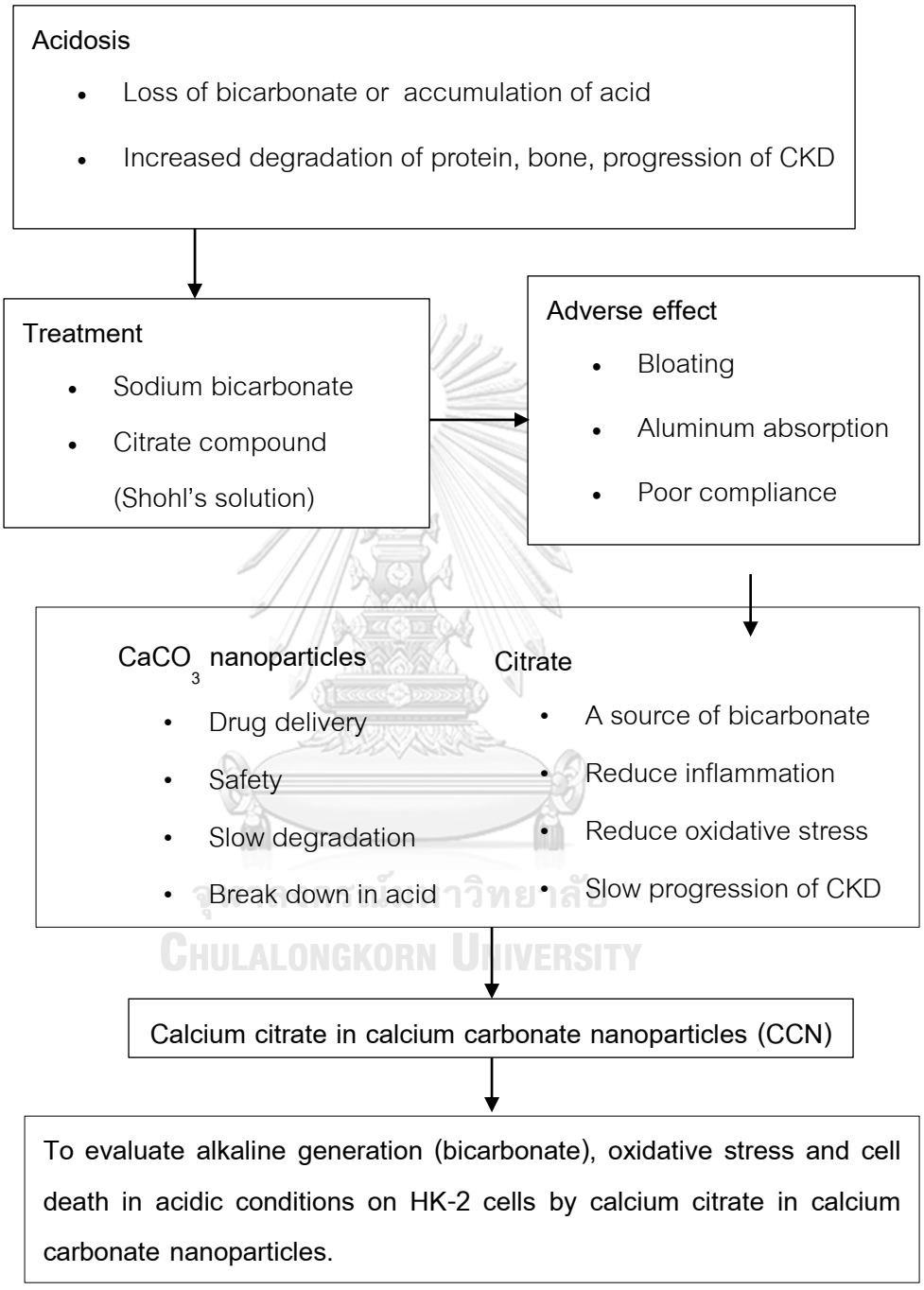
1.2 Research objectives

1. To synthesize calcium citrate in calcium carbonate nanoparticles
2. To validate the basic properties of calcium citrate in calcium carbonate nanoparticles
3. To evaluate alkaline generation, oxidative stress and cell death in acidic conditions on HK-2 cells by calcium citrate in calcium carbonate nanoparticles.

1.3 Research hypotheses

1. Calcium citrate in calcium carbonate nanoparticles can increase alkaline generation in acidic condition on HK-2 cells.
2. Calcium citrate in calcium carbonate nanoparticles can reduces oxidative stress and cell death in acidic condition on HK-2 cells.

1.4 Conceptual frameworks



Keyword: Nanoparticles, Calcium citrate, Calcium carbonate nanoparticles, Acidosis, Calcium citrate in calcium carbonate nanoparticle

Chapter II Literature review

2.1 Chronic acidosis

The body maintains acid-base homeostasis through lungs, kidneys and endogenous buffers. The principle function of lung in acid-base balance is to eliminate carbon dioxide (CO_2) which is accounted for a volatile acid (12). The body detects an increased CO_2 concentration or decreased blood pH via chemoreceptor in medulla oblongata, and responses by increasing the breathing rate for remove CO_2 (1). Likewise, the main function of kidney in acid-base balance is to abolish non-volatile acids (compound of proton). The intercalated cells of distal tubules secrete proton (H^+) into the luminal fluid and proximal tubular cells reclaim urinary bicarbonate (HCO_3^-) back to the bloodstream. When acidosis occurs, the kidney compensates by increased excretion of H^+ and reabsorption of HCO_3^- from the urine as well as generate new HCO_3^- from other biomolecules (13).

Acidosis can be divided into 2 categories according to its cause. Firstly, respiratory acidosis is caused by respiratory failure (14) or diffusion-perfusion defects, resulting in accumulation of CO_2 lead to a low pH in the blood. (15) In case of chronic respiratory acidosis, kidney function adaptably changes occur mainly in the proximal tubular cells leading to increase the reabsorption of HCO_3^- to compensate the low serum pH (16). The common causes of respiratory acidosis are status asthmaticus, upper airway obstruction, high spinal cord injury, primary alveolar hypoventilation or chronic obstructive pulmonary disease. (12) Secondly, metabolic acidosis is occurred when kidney function deteriorates. Therefore, the decreases in kidney function lead to the loss of bicarbonate in urine, retention of non-excretory proton, or diminished ammonium buffer production (3). Metabolic acidosis is a comorbidity found in patients with other organ dysfunction such as kidneys, heart, liver, etc. (17).

Acidosis alters enzyme kinetics, which subsequently disturbs functions of cells, tissues and organelles. Acidosis promotes muscle protein degradation without a change

in muscle protein synthesis (18). In acidosis condition, calcium, sodium and potassium are demineralized from bone in exchange with protons, whereas carbonate is consumed by buffer hydrogen ion. Moreover, acidosis stimulates osteoclasts activity in bone resorption, causing mineral bone loss (19). Acidosis induces macrophage release of interleukins and inflammation, as well as suppresses lymphocyte function and immune response (2). In addition, various proinflammatory cytokines are activated in acidic environment leading to tissue injury and fibrosis (3).

2.2 Role of citrate in chronic acidosis

In the aspect of kidney, previous studies showed that administration of alkalinizing agents to treat acidosis has beneficial effects on kidney protection by slowing progression of kidney impairment, improving glomerulus filtration rate (GFR), decreasing urinary protein excretion, and declining kidney injury markers and endothelin-1 (11, 20). Currently, chronic metabolic acidosis is treated by an alkalinizing agent such as sodium bicarbonate ($NaHCO_3$) and Shohl's solution (compound of sodium citrate and citric acid). Sodium bicarbonate is the most common drug used to treat acidosis, although it forms carbonic acid while contacts with gastric juice and becomes gas. Most carbonic acid turns into carbon dioxide and causes a sensation of fullness and bloating. On the other hand, Shohl's solution is a compound of sodium citrate and citric acid that is metabolized into bicarbonate. However, citrate increases the absorption of aluminum in intestine and risks for aluminum toxicity (3). Additionally, Shohl's solution has very unpalatable taste which deters patient's compliance. Regarding this, a new drug that has efficacy similar to the standard medication, with lower adverse effects should be discovered to promote drug compliance.

Citrate is an intermediate in tricarboxylic acid (TCA) cycle that is present in complexes form with divalent cations. Citrate has two ionic forms: divalent citrate $^{2-}$ and trivalent citrate $^{3-}$. Citrate is freely filtrated at the glomerulus and reabsorbed at the proximal tubule in divalent anion form ($citrate^{2-}$) by luminal sodium dicarboxylate

cotransporter type 1 (NaDC-1). Moreover, rate of citrate uptake depends on urinary pH: lower urine pH raises concentration of divalent citrate, leading to increase citrate reabsorption (21). Most of intracellular citrate is taken up to mitochondria for cellular energy metabolism (22). CO_2 which is the end product of cellular respiration is catalyzed by intracellular carbonic anhydrase type 2 (CAII) to form carbonic acid (H_2CO_3), which subsequently breaks into photon (H^+) and HCO_3^-. HCO_3^- is transported over the basolateral membrane by the Na^+-HCO_3^- cotransporter (NBCe-1) to the blood and H^+ is secreted into urine. (fig.1, 23)

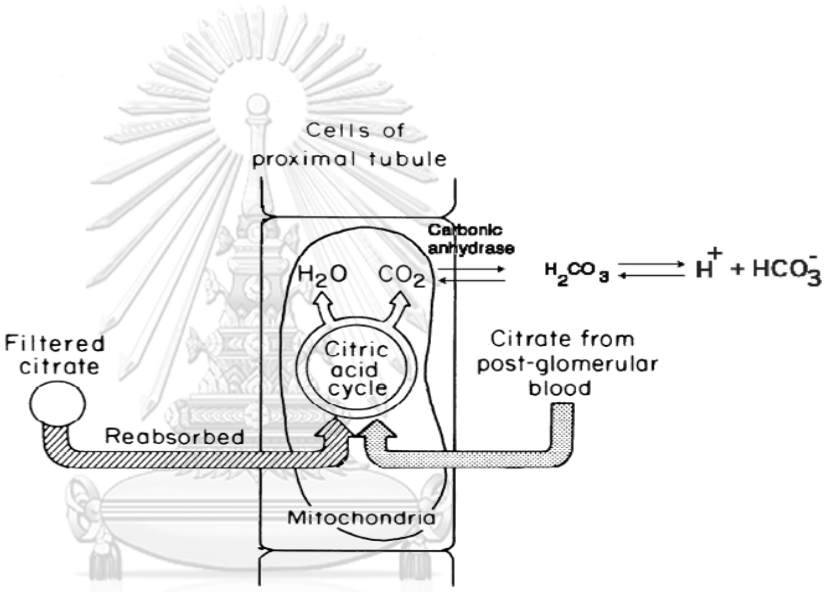


Fig.1 Process of CO_2 binds with H_2O to form H_2CO_3 and ionized to H^+ and HCO_3^-.

Moreover, citrate treatment reduces oxidative stress (9). In previous study, Tosukhowong et al. observed that potassium citrate supplementation in renal stone patient decreased oxidative stress. It reduced urinary lipid peroxidation that is a lipid peroxidation marker and increased vitamin E level (Fig. 2) (24).

	Potassium citrate supplementation		
	Before	**After**	***P***
Plasama			
P-MDA (μM)	3.75±1.2**	2.86±0.97	0.01
Protein thiol (μM)	396±57**	399±32**	NS
Vitamin E (μM)	8.05±3.04***	13.10±3.02**	0.0001

Fig. 2 Levels of plasma malondialdehyde (P-MDA), protein thiol and vitamin E before and after potassium citrate supplementation. (24)

In addition, Tosukhowong et al. used lime powder regimen (LPR) that contained high citrate level to prevent recurrent of kidney stone in patients with stone removal surgery. They found that LPR caused an increase in urine pH and reduction in oxidative stress.

Calcium citrate is a calcium salt that has higher gastrointestinal absorption than calcium phosphate or calcium malate. Previous study showed that calcium citrate improves the epithelial-to-mesenchymal transition (EMT) induced by acidosis in proximal tubular cells (22). EMT is a mechanism that transforms renal tubular cells into mesenchymal cells and then fibroblasts (Fig. 3). These transformed cells produce inflammatory cytokines, adhesion molecules and extracellular matrix that causes tubulointerstitial fibrosis. Decline in renal function in chronic renal failure is caused by loss of nephrons and replacement with fibrotic scar. (25)

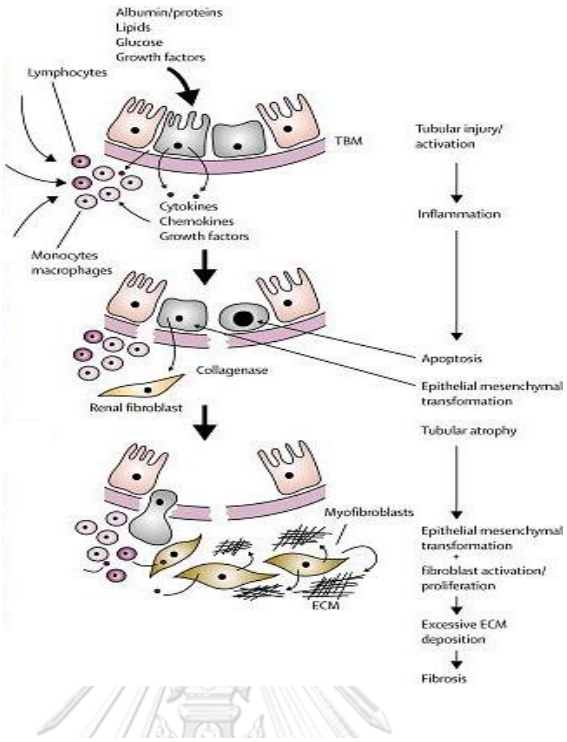


Fig. 3 showed the mechanism of epithelial mesenchymal transition (25)

In addition, calcium citrate ameliorated the progression of kidney injury in rat. (11) At present, calcium citrate is used as a supplement for osteomalacia patients (26). However, calcium promotes the formation of calcium stone in urinary tract when urine pH is high. Regarding the properties of calcium citrate with low solubility, easy to synthesis and high stability, we believed that calcium citrate nanoparticles could be used to alleviate the deleterious effects to tissues in acidic environment.

2.3 Role of nanoparticle in medicine

Nanoparticles (NP) are particles that can be defined in one dimension less than 100 nm (4). The properties of nanoparticles may be changed as their size. Nanoparticles have been applied to drug delivery system because their properties are 1) improved bioavailability 2) increasing half-life for clearance and 3) targeting drug to specific tissue in the body. These properties can improve efficacy and decreasing the several side effect of drug to non-target tissue. Hence, nanoparticles are widely used to

clinical application such as to carrier anticancer, to detect tumor and iron supplement. (27) Calcium carbonate nanoparticle (CCNP) is one of the most common nanoparticles used in medicine. CCNP has low cytotoxicity, slow biodegradability and pH sensitive (decompose slowly at normal pH (7.4) while faster decomposition and release of drug at acidic pH<6.5 (Figure 4).

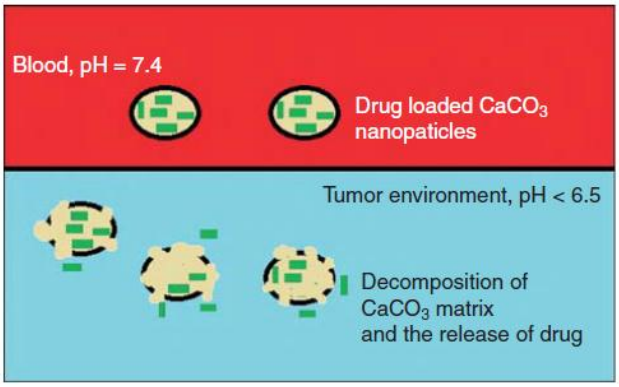


Fig. 4 Shows pH-sensitive drug release profile of $CaCO_3$ nanoparticles (28)

Hence, CaCO3 nanoparticles have been used in drug delivery control, biosensing and protein encapsulation in pharmaceutics. Drugs or bioactive protein loaded in CCNP have high stability. (28) Pervious study has been used CCNP as a drug delivery to carrier insulin, betamethasone phosphate, erythropoietin, validamycin and gentamicin sulfate (6). In the present study, we aimed to synthesize calcium citrate nanoparticle enveloped by CCNP that is efficient in treatment of metabolic acidosis, low cost and lesser adverse effects.

Chapter III Research Methodology

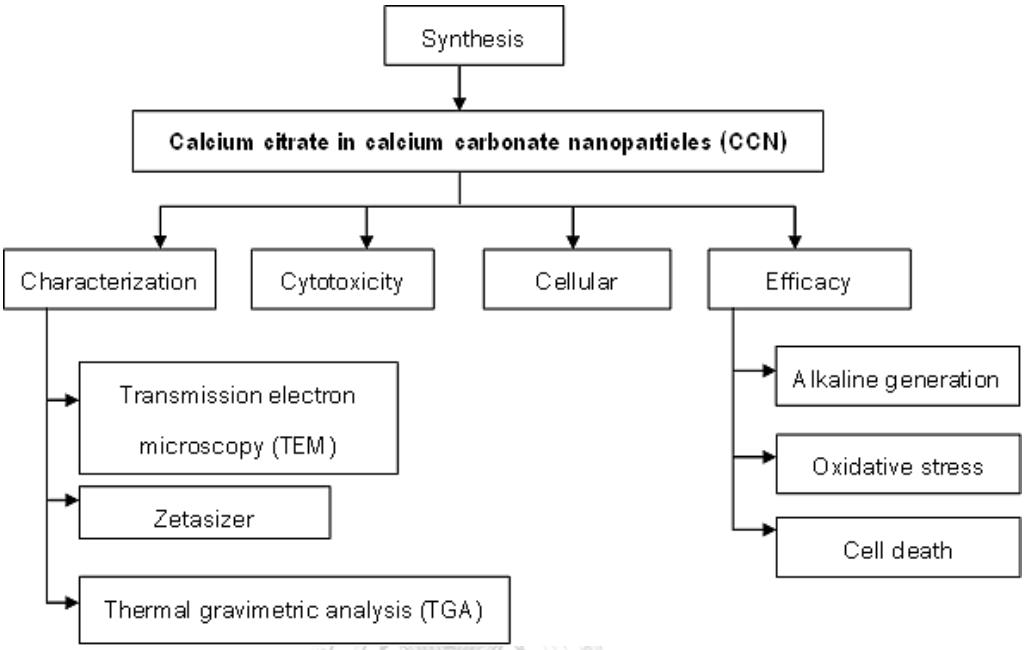


Fig. 5 Experimental strategy and step: synthesis, physical analysis and efficacy of CCN

3.1 Synthesis of calcium citrate in calcium carbonate nanoparticles (CCN)

The method of synthesis was kindly provided by Assistant Professor Dr. Rojrit Rojanathanes (Department of Chemistry, Faculty of Science, Chulalongkorn University). In brief, calcium citrate nanoparticles (Cacit) were prepared by using 0.4 M calcium chloride (MERK, Germany) dissolved in distilled water. Then, a 0.2 M sodium citrate (MERK, Germany) was mixed and vortexed for 5 minutes (Scientific Industries, USA). The solution was shaken for 38 hours on shaking machine (Heidolph, Germany) and then nanoparticles were separated by centrifugation (Sorvall, Germany) at 4500 RPM for 15 minutes. Subsequently, the parts of Cacit were separated to synthesis CCN by coated with 0.5 mM calcium chloride and 0.5 mM sodium carbonate (MERK, Germany)

respectively. The solution was dried at 100 °C and stored in room temperature as a dried form until analysis.

3.2 Particle size and morphology determination

To analyze size and morphology, the sample was prepared by dissolving nanoparticles with distilled water and sonicated for 45 minutes. Then, the diluted sample was dropped on a 200-mesh carbon-coated copper grid for analysis. The diameter and morphology of both nanoparticles were analyzed by transmission electron microscopy (TEM) using a JEM-2010 TEM (JEOL, Japan) at an accelerating voltage of 200 kV. The nanoparticles will be stored in dried powder.

The appropriate nanoparticles size should have at least one dimensional size less than 100 nm which was proven to be taken up to the cells faster than larger dimensional nanoparticles (5). Moreover, shape of nanoparticles should be spherical or oval because it is easily enter to the cells more than rod shape (29).

3.3 Zeta-potential measurement

Zeta potential is the surface charge on nanoparticles which measured electric potential difference between inner electric potential and outer electric potential in aqueous solution. (30) Zeta potential values indicate the stability of colloidal system of the sample, which represents the inhibition of particle aggregation, with the accepted range of $\pm$ 30 mV is required to promote stable dispersion of particles (31). To analyze surface charges, both nanoparticles were analyzed by zeta-sizer (Malvern Instruments, USA). The sample was prepared by diluted nanoparticles with distilled water and sonicated sample for 45 minutes. After that, the diluted sample was injected into the capillary cell of zeta-sizing.

The appropriate zeta potential should be higher than +30 mV or lower than -30 mv, which is sufficient to prevent the aggregation of particles in aqueous form. The

similar charge of each particle can extrude other particles to aggregate which occur by Van der Waals forces (31).

3.4 Composition analysis

Thermogravimetric analysis (TGA) is used to determine the content of sample, representing by the percentage of weight form. On the basis that content of sample will be degraded at different decomposition temperature, this method identified and measured the amount of mass loss of sample when sample were received the temperature at decomposition point. In the present study, we aimed to confirm the existence of citrate and carbonate contents in nanoparticles and measured their content. Approximately 5 mg of CCN and calcium citrate nanoparticles were measured with thermogravimetric analysis using a Thermal Analysis TA Q 50 (TA Instrument, USA). Heating was performed in a nitrogen flow (100 mL/min) using increasing rate of 10 °C/min from 30 to 800 degree Celsius. The decomposition temperature of citrate is about 300-500^{o}C and carbonate is about 600^{o}C

We expected that the content of citrate, which is the active ingredient, should not be less than 20% by weight of total nanoparticles.

3.5 Cell culture condition

HK-2 cells (human proximal tubular cell line) were used in cell culture study of this research. HK-2 cells (ATCC 2290) was provided by Assistant Professor Dr.Chanchai Boonla (Department of Biochemistry, Faculty of Medicine, Chulalongkorn University), were cultured on 75 cm^2 culture flasks and cultured in Dulbecco-Vogot modified eagle medium (DMEM) (Thermo Scientific, USA) supplemented with 10% bovine fetal serum (FBS) (Gibo, USA) and 1% antibiotics (penicillin-streptomycin solution) (Gibo, USA). Cells were maintained in 5% CO_2 and 37 °C regulated incubator (Esco, Singapore).

3.6 Cellular uptake

Fluorescence isothiocyanate (FITC) was use to conjugate CCN as a marker to study the cellular uptake. FITC-conjugated CCN was synthesized by dissolving 0.333 g calcium chloride (MERK, Germany) in distilled water. Then, a 375 µL of FITC and a 0.882 g of sodium citrate (Scientific Industries, USA) were mixed and vortexed for 5 minutes. The solution was shaken for 24 hours on shaking machine (Heidolph, Germany) and then nanoparticles were separated by centrifugation (Sorvall, Germany) at 4500 RPM for 15 minutes. After that the precipitate were coated with 0.5 mM calcium chloride and 0.5 mM sodium carbonate (MERK, Germany) respectively.

HK-2 cells were seeded in 24 well plates and incubated at 5% CO_2 and 37 $^{\circ}C$ for 24 hours. After 70% confluent, culture media was removed and washed with phosphate buffered saline (PBS) (Ameresco, USA). Cells were treated with 1 mg/mL of fluorescence isothiocyanate (FITC) conjugated CCN in DMEM and incubated at the pH 7.4 DMEM and pH 5 DMEM condition for another 24 hours. Then extracellular FITC-conjugated CCN was removed by triple time washes of PBS. To analyses cellular uptake, cells were fixed with 10% formalin and stained with a 4',6-diamidino-2-phenylindole (DAPI). DAPI is a fluorescent staining strongly bind with adenosine-thymine nucleotide, indicating nuclear region of cell. Cellular uptakes were observed by confocal laser scanning microscopy (Carl zeiss, USA) (32).

Cellular uptake of FITC-conjugated CCN could be determined by the overlapping of FITC (green color) and DAPI (blue color) fluorescents detected by confocal microscopy. We anticipated that FITC-conjugated CCN could be uptake into the HK-2 cells in both and acidic condition.

3.7 Cytotoxicity test by Resazurin oxidative-reduction assay

HK-2 cells were seeded in 96 well plates and incubated at 5% CO_2 and 37 $^{\circ}C$ for 24 hours. After 70% confluent, cells were treated with various concentrations of CCN and Cacit at 0, 0.01, 0.1, 0.5, 1 and 5 mg/ml, and incubated for 24 hours. Cells viability was evaluated with Resazurin oxidative-reduction assay. Mitochondria reductase will be reduced resazurin to resofurin that contains high fluorescence intensity. Dead cells cannot change resazurin into resofurin, leading to low fluorescence intensity. A 10 µL PrestoBlue™ reagent (Invitrogen, USA) was added to each well and incubated for additional 30 minutes. Then cells were measured by fluorescence microplate reader (Thermo Scientific, Singapore).

It should be noted that when cell was stressed, the activity of mitochondria reductase may be enhanced, causing increase resofurin conversion (33). In this case, the result of study would be demonstrated as elevated cell survival. In the present study, significantly elevated cell survival than control will be decided as mitochondrial stress or cellular damage.

In the present study, the lowest dose of nanoparticles that induced significant decrease or increase of cell survival (enzyme activity) would be indicated as cytotoxic levels. We will use the maximal dose of nanoparticles that did not induce cytotoxicity for further studies.

3.8 Pharmacologic properties of CCN on HK-2 cells in acidic environment

The HK-2 cells were divided into 5 groups as the following;

A. Control group: DMEM pH 7.4

B. Acidosis group: DMEM pH 5

C. Sodium citrate group: DMEM pH 5 + Sodium citrate (Nacit) 1 mg/ml

D. Calcium citrate nanoparticles group: DMEM pH 5 + Calcium citrate nanoparticles (Cacit) 1 mg/ml

E. CCN group: DMEM pH 5 + CCN 1 mg/ml

Acidosis media (pH 5) was prepared by titration of DMEM with hydrochloric acid (HCl) and measured pH with a pH meter (Denver Instrument, US). We used Nacit as a treatment control based on sodium citrate/citric compound is a standard drug to treat chronic metabolic acidosis. However, citric acid was not used in the present study since it significantly disturbed the extracellular pH. The following experiments were performed at least three times.

3.8.1 Alkalinizing effect

HK-2 cells were seeded in a 6-well plate and incubated at 5% CO_2 and 37 $^{\circ}C$ for 24 hours. After 70% confluent, cells were distributed in 5 experimental conditions: A, B, C, D and E (according to 2.7) for 24 hours. For determination of pH, media was collected and measured for pH by pH meter.

Bicarbonate analyses, cells were seeded in T75 flasks and divide into 4 group pH 7.4 DMEM, 7.4 DMEM with Nacit, 7.4 DMEM with Cacit and 7.4 DMEM with CCN, respectively. After 24 hours of incubation, media and cells were collected. Cells were frozen with liquid nitrogen and thawed at 37$^{\circ}C$ in water bath until ice melting for 4 times. Then, cell pellets and supernatant were separated by centrifugation at 12,000 rpm for 7 minute. Extracellular and intracellular bicarbonate concentration was measured by automated biochemical analyzer (COBRAS2000).

In this study, we expected that the HK-2 cells treated with Nacit, Cacit and CCN should induce higher intracellular and extracellular pH, as well as bicarbonate concentration than control (pH 5 DMEM without treatment).

3.8 2 Intracellular reactive oxygen species (ROS) generation

To analyze ROS generation, 2,7-dichlofluorescein-diacetate (DCFH-DA) assay was used to determine ROS production. DCFH-DA passively diffused into cells and then converted to DCFH by intracellular esterase enzymes. These enzymes cleaved diacetate ester (DA) from DCFH-DA. DCFH was non-fluorescent which subsequently oxidized by ROS and turned into highly fluorescent product (34).

HK-2 cells were seeded in a black 96-well plate and incubated at 5% CO_2 and 37 $^{\circ}C$ for 24 hours. After 70% confluent, then 0.1 mM of 2,7-dichlofluorescein-diacetate (DCFH-DA) (Molecular probe, USA) was added and incubated for additional 30 minutes. After 30 minutes, free DCFH-DA was removed by PBS washing for 3 times and then cells were distributed into 5 groups as previously described. Fluorescent intensity at 0 to 60 minutes was measured by microplate reader (485 nm for excitation and 535 nm for emission). (35)

Since acidosis induces ROS generation, we expect to observe high fluorescent intensity in control (pH 5 DMEM), and lower fluorescent intensity in all treated groups, reflecting the suppression of ROS production by Nacit and nanoparticles.

3.8.3 Assessment of cell death

Annexin V and propidium iodides staining was used to analyze cell death. Annexin V staining at the cell membrane indicated early apoptosis stage, which can be visualized by specifically binding with phosphatidylserine and detected with confocal microscope. Late apoptosis stage presented by ruptured plasma membrane, which can be observed by propidium iodides influx into cell and stained DNA. Hence, early apoptosis stage can detect by annexin V and late apoptosis stage can identify by double staining of annexin V and propidium iodides. In addition, necrosis stage was

presented by leaky cells membrane which could be identified by only propidium iodides-bound DNA without annexin V staining (36).

HK-2 cells were seeded in 6 well plates and incubated at 5% CO_2 and 37 $^{\circ}C$ for 24 hours. After 70% confluent, cells were pre-treatment with CCN, Cacit and Nacit for 6 hours in pH 7.4 DMEM. Then cells were distributed in 5 groups as previously described. After 18 hours cells were harvested, washed twice with cold PBS and then resuspened in 100 µL of 1X binding buffer (Thermo Scientific, USA). A 5 µL of annexin V and propidium iodide (Thermo Scientific, USA) was added in each tube and incubated for 15 minutes at room temperature in the dark room. After 15 minutes, cells were added 400 µL of 1X binding buffer and analyzed by flow cytometer (Beckman Coulter, USA). The results would be demonstrated as the percentage of combined cell apoptosis and cell death among each group.

The preliminary results of this study showed that HK-2 cells were well tolerated with pH 5.0 media as very small amount of cells were affected. Accordingly, we performed this study with pH 4.0 DMEM which significantly induced HK-2 cell apoptosis. We anticipated that Nacit and nanoparticles treatment should ameliorate cellular apoptosis and cell death in acidic environment.

3.9 Statistical analysis

GraphPad Prism (California, USA) version 5.0 was used for statistical analysis in the present study. Continuous data was tested by Student t-test for 2 independent groups comparison and ANOVA for more than 2-groups comparison. Significant differences were considered when $P < 0.05$.

3.10 Ethical consideration

This study was approved by the Ethic committee of Research affairs, Faculty of Medicine, Chulalongkorn University (IRB 024/60).

Chapter VI Results

4.1 Characterization of calcium citrate nanoparticles and calcium citrate in calcium carbonate nanoparticles

4.1.1 Physical appearance of calcium citrate nanoparticles and calcium citrate in calcium carbonate nanoparticles

After synthesis, Cacit and CCN were within white aqueous form. Then, both nanoparticles were dried and stored in dry powders. The powders had similar appearance; white color, odorless and tasteless (Figure 6). The powder required to suspend with distilled water before use. The powders were dissolved. In addition, dry powders of Cacit had slightly higher solubility in liquid phase more than CCN which was sonicated before use.

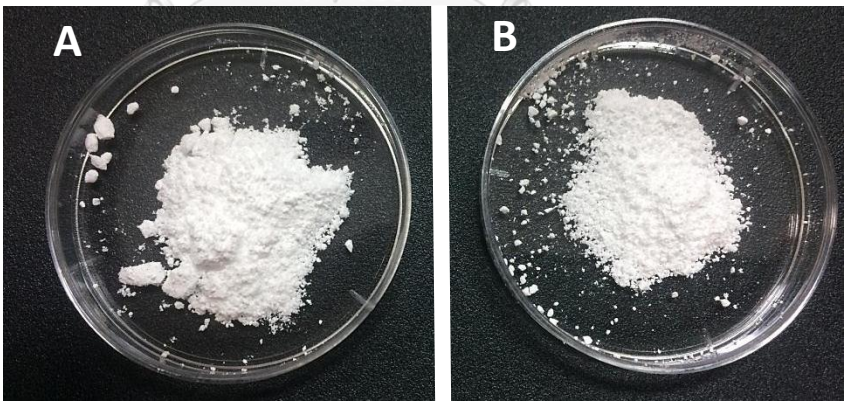


Fig. 6 Dry powders of calcium citrate nanoparticles (A) and calcium citrate in calcium carbonate nanoparticles (B) have white color, odorless and tasteless.

4.1.2 Transmission electron microscopy (TEM) of calcium citrate nanoparticles and calcium citrate in calcium carbonate nanoparticles

TEM showed that Cacit and CCN particles had spherical-shape (Fig. 7A & 7B). The diameter of Cacit was about 50-70 nm, while CCN was slightly larger, approximately 60-80 nm (Fig. 7C & 7D). These results supported our anticipation that the particle size of Cacit and CCN should be less than 100 nm, with spherical shape, which is appropriate to facilitate cell membrane transport.

4.1.3 Zeta potential of calcium citrate nanoparticles and calcium citrate in calcium carbonate nanoparticles

Zeta potential sizer demonstrated that both nanoparticles had negative surface charges. Cacit contained zeta potential of -16.1 mV, but slightly reduced to -13.0 mV when coated with calcium carbonate nanoparticles (Fig. 8 A & 8 B). Nevertheless, the zeta potential was not statistical significance when compared to each other.

The zeta potentials of both Cacit and CCN were lower than our expectation (less than -30 mV or more than +30 mV). This result indicated that Cacit and CCN nanoparticles were susceptible for aggregation in aqueous form.

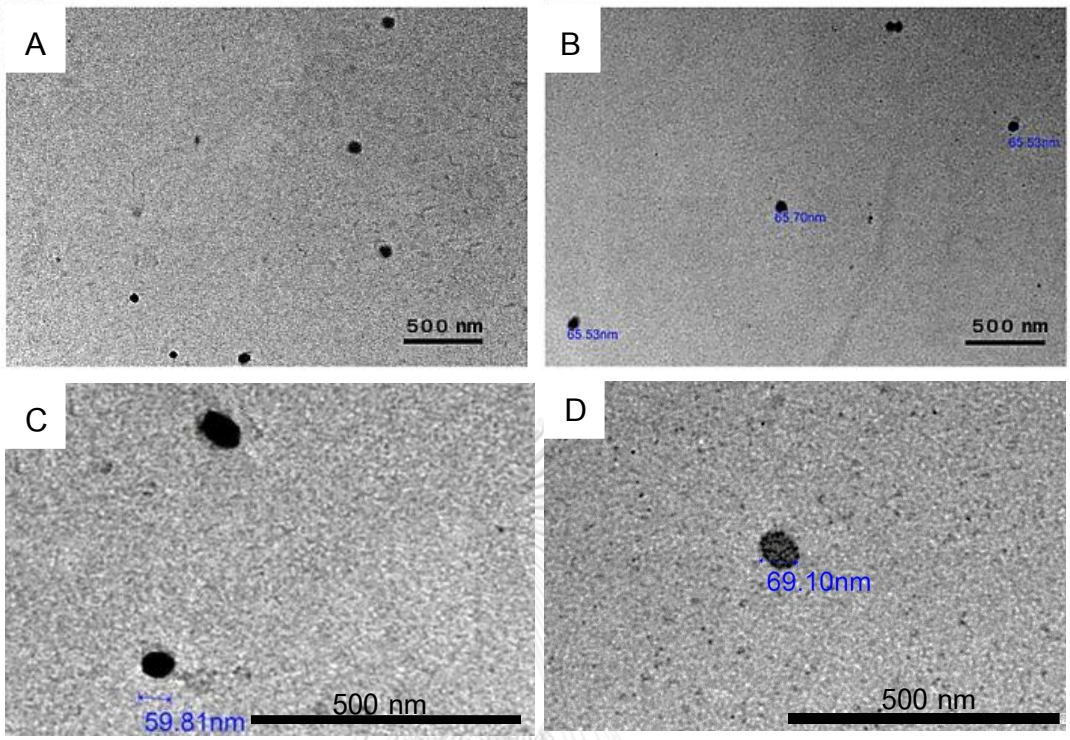


Fig. 7 Nanoparticles characterization. Shape and size of nanoparticles were visualized by TEM. TEM micrograph represented (A) the spherical shape calcium citrate nanoparticles and (B) calcium citrate in calcium carbonate nanoparticles (C) diameter of calcium citrate nanoparticle was about 50-70 nm, and (D) diameter of calcium citrate in calcium carbonate nanoparticle was about 60-80 nm.

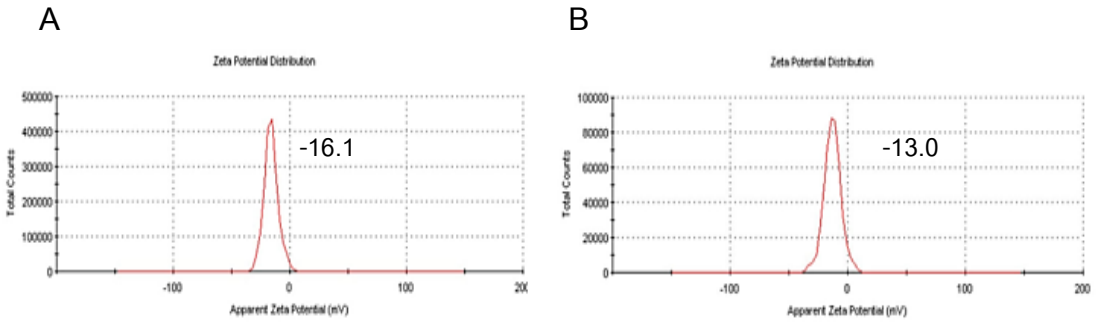


Fig. 8 Nanoparticles characterization by zetaziser. Zeta potential exhibited by micrograph of (A) calcium citrate nanoparticles and (B) calcium citrate in calcium carbonate nanoparticles.

4.1.4 Composition analysis of calcium citrate nanoparticles and calcium citrate in calcium carbonate nanoparticles

TGA curve indicated the composition loss of nanoparticles which decomposed at different temperatures. The decomposed temperature can be used to identify the content and mass loss could be used to calculate the percentage of content. From the curve showed percentage of weight between 25 and 800 degree Celsius indicated by green line in the graph (Fig 9 A & 9 B). The first mass loss in TGA curve was at approximately 80°C to 150°C represented the weight loss of water molecules on the surface of the nanoparticles. The second mass lose ranged from 300°C to 480°C assigned, compatible with the decomposition temperature of citrate ion. The final mass loss was considered to be the decomposition temperature of carbonate which was around 600°C and 700°C. The residual mass was accounted for calcium.

According to the results, Cacit contained water, citrate and carbonate, and the content of citrate was approximately 31% by weight. While CCN contained water, citrate and carbonate as well, but the percentage of citrate dropped to 25% by weight.

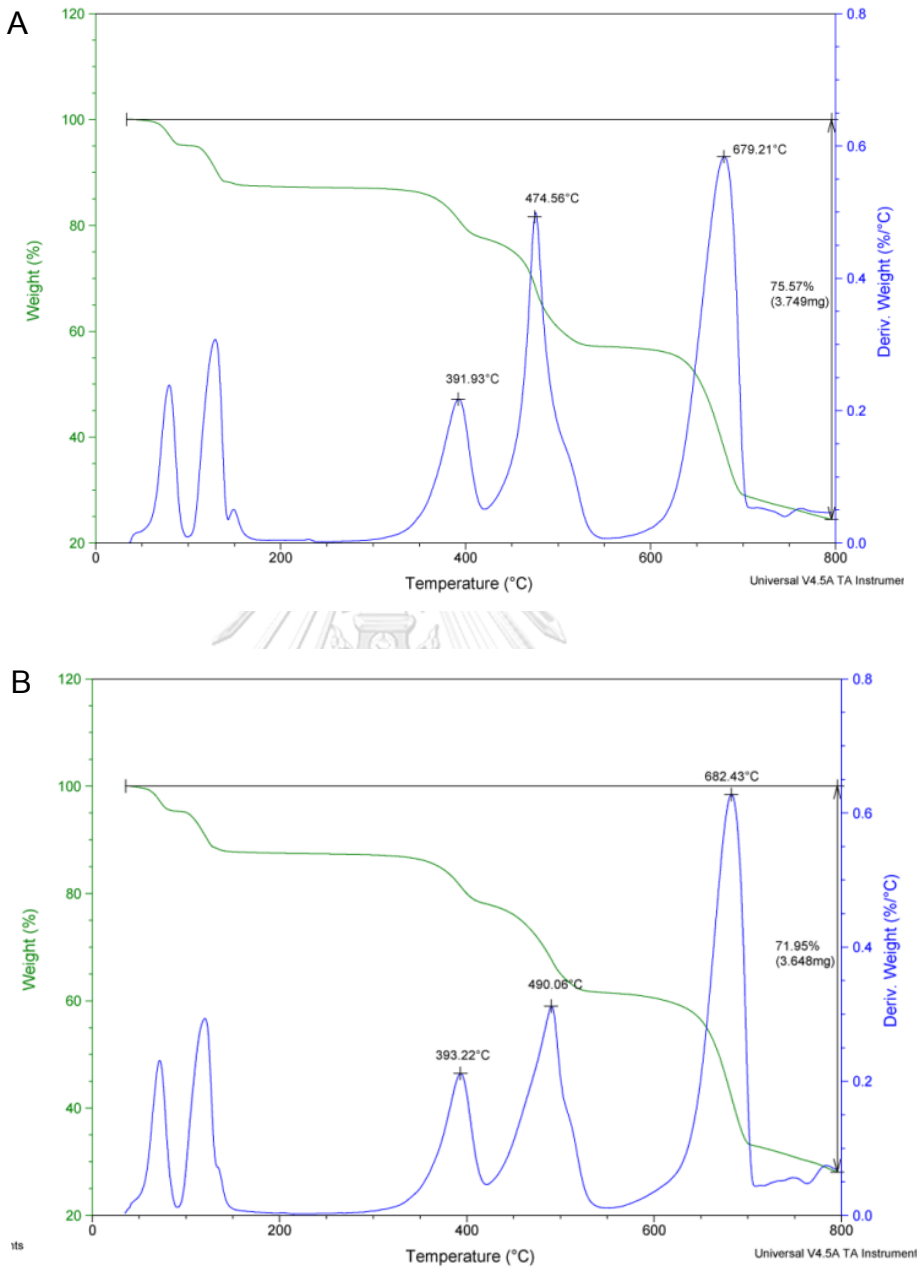


Fig. 9 Analysis of ligand conjugation to calcium carbonate nanoparticles by Thermogravimetric Analysis (TGA). (A) The composition of calcium citrate nanoparticles and (B) the composition of calcium citrate in calcium carbonate nanoparticles.

4.1.5 Cellular uptake of calcium citrate in calcium carbonate nanoparticles

Confocal microscopic images of HK-2 cells treated with FITC conjugated CCN were showed in Fig 10. The images were demonstrated in DAPI channel, FITC channel and merged DAPI and FITC channel. The DAPI channel revealed that only blue fluorescence was detected. There was no auto-fluorescence observed inside the cell. FITC channel showed green fluorescence of FITC conjugated CCN. The combined DAPI and FIT-C channel demonstrated that the green fluorescence was overlaid on blue fluorescence, represented that FITC conjugated citrate was uptake into the cells both in normal and acidic environment. The CCN uptake started since 3 hours after FITC conjugated CCN was incubated and increase over time until 24-hours (Fig 11).

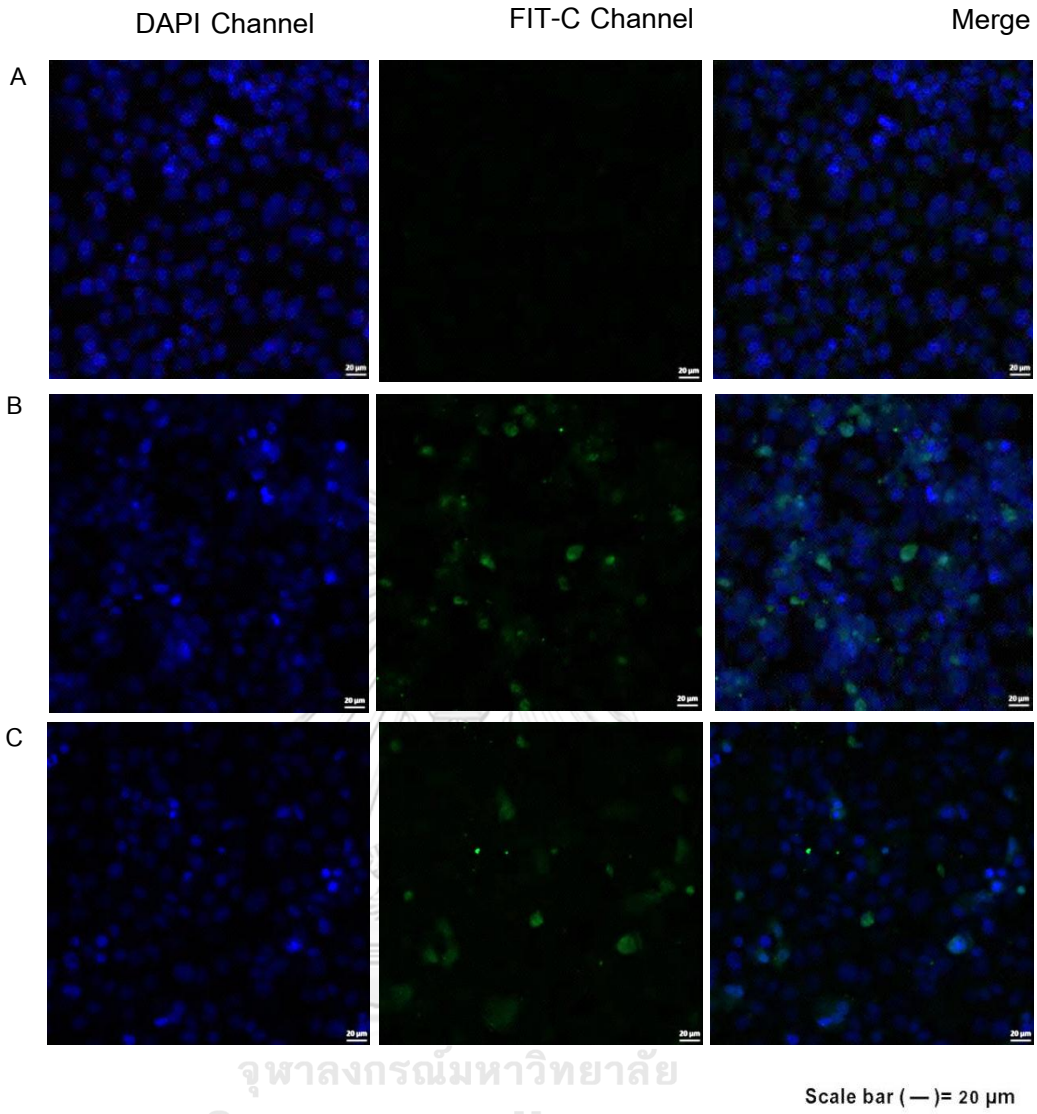


Fig. 10 Confocal images showing the uptake of calcium citrate in calcium carbonate nanoparticles in HK-2 cells. (**A**) HK-2 cells without FITC-conjugated CCN. (**B**) HK-2 cells with FITC-conjugated CCN in pH 7 DMEM and (**C**) HK-2 cells with FITC-conjugated CCN in pH 5 DMEM. Cells were stained with DAPI. Cellular uptake was imaged by DAPI channel (left column), FITC channel (middle column) and combine channel (right column) respectively.

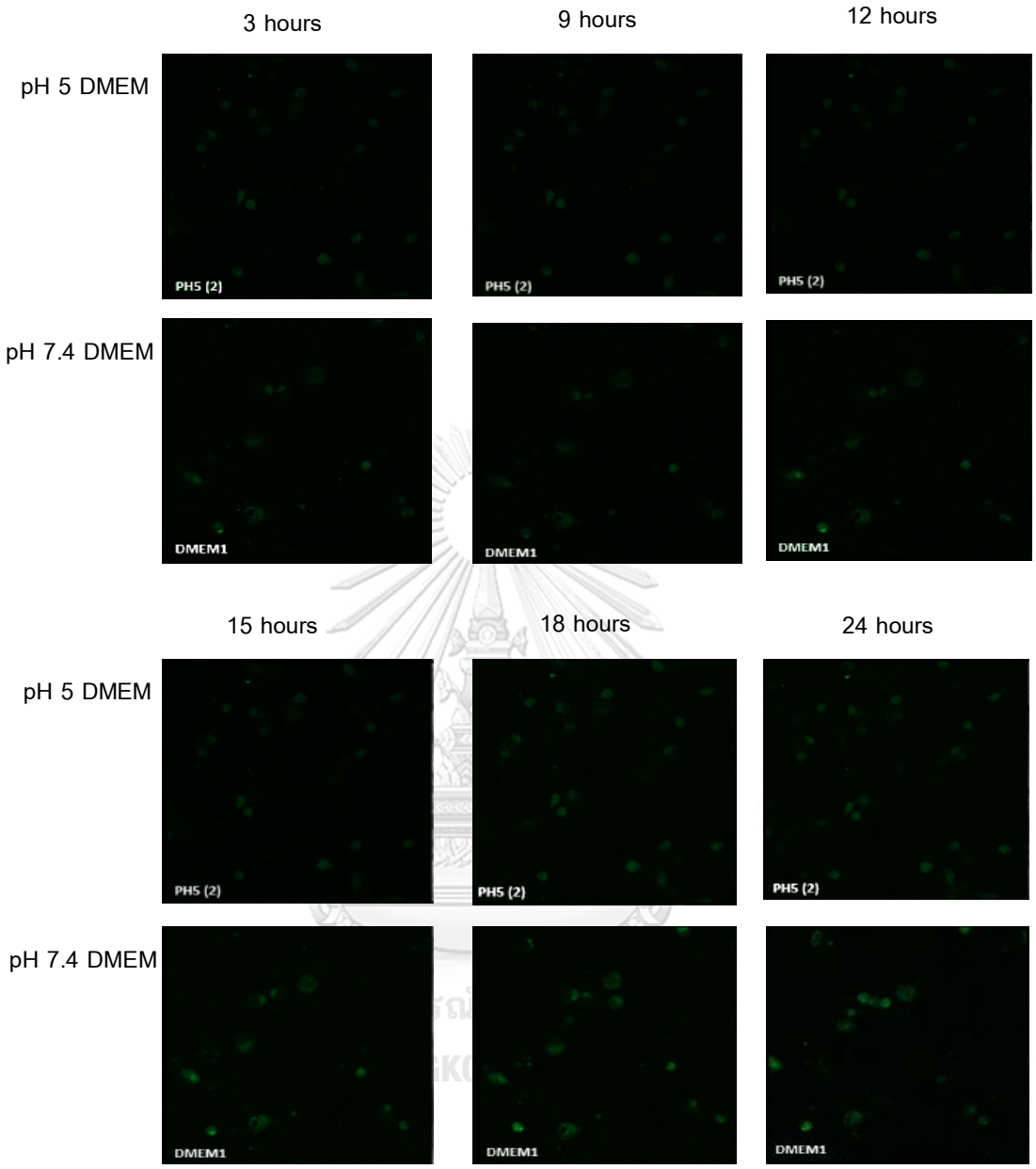


Fig. 11 The cellular uptake of FITC-conjugated calcium citrate in calcium carbonate nanoparticles (CCN) into HK-2 cells detected by confocal microscopy. The retained FITC was increased overtime from 3 to 24 hours in both normal condition (pH 7.4 DMEM) and acidic environment (pH 5.0 DMEM), as represented in increased green fluorescence in FITC-conjugated CCN incubated HK-2 cell culture after PBS washes.

4.2 Pharmacological characteristics of calcium citrate in calcium carbonate nanoparticles

4.2.1 Cytotoxicity of calcium citrate in calcium carbonate nanoparticles

The percentage of cell viability was not changed while HK-2 cell treated with Cacit and CCN with the concentration between 0.01 to 1 mg/ml (Fig 12). This result represented that Cacit and CCN in the concentration 1 mg/ml or less had very low effect on HK-2 cell growth or cell death.

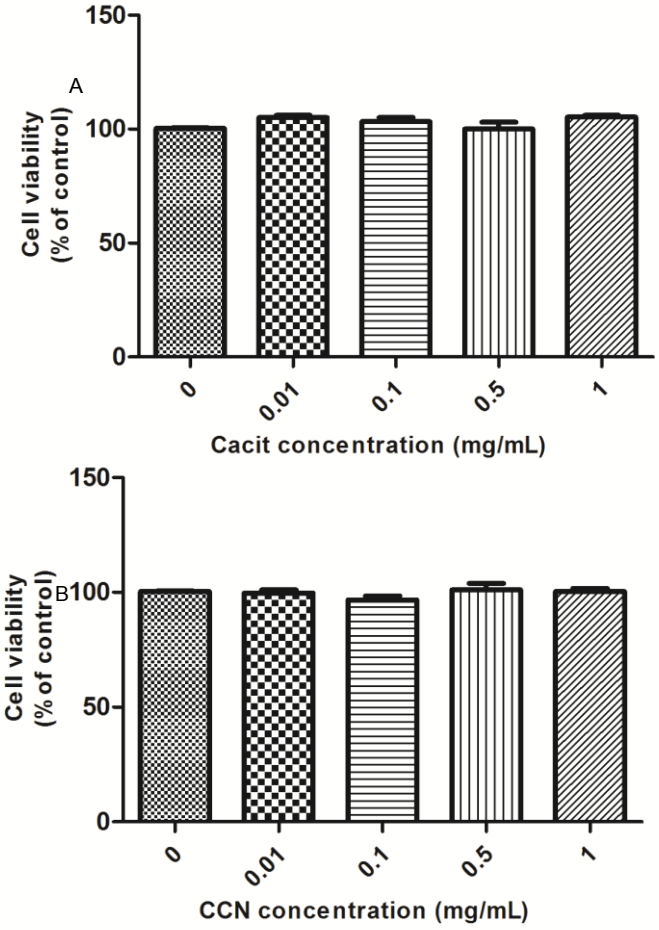


Fig. 12 The cytotoxicity of nanoparticles on HK-2 cell compared with negative control using Resazurin test. (A) Calcium citrate nanoparticles with the concentration between 0.01 to 1 mg/ml; (B) Calcium citrate in calcium carbonate nanoparticles with the

4.2.2 Generation of ROS by calcium citrate and citrate calcium citrate in calcium carbonate nanoparticles in acidic condition

Regarding to cell viability analysis, the concentration of 1 mg/ml CCN and Cacit were chosen for the following studies. Sodium citrate (Nacit) was used in the present study representing standard treatment of acidosis. Intracellular ROS generation was investigated in the CCN-treated HK-2 cells at acidic condition (Fig. 13). The result showed that in acidic condition (pH 5.0 DMEM), the ROS was significantly increased when compared with control (pH 7.0 DMEM). When cells in acidic condition were treated with either CCN or Cacit, the ROS was significantly decreased in relative to the acidic condition. Moreover, the Nacit-treated cells insignificant decreased ROS when compared with acidic condition. This data indicated that CCN and Cacit can reduce ROS generation in acid condition greater than Nacit treatment.

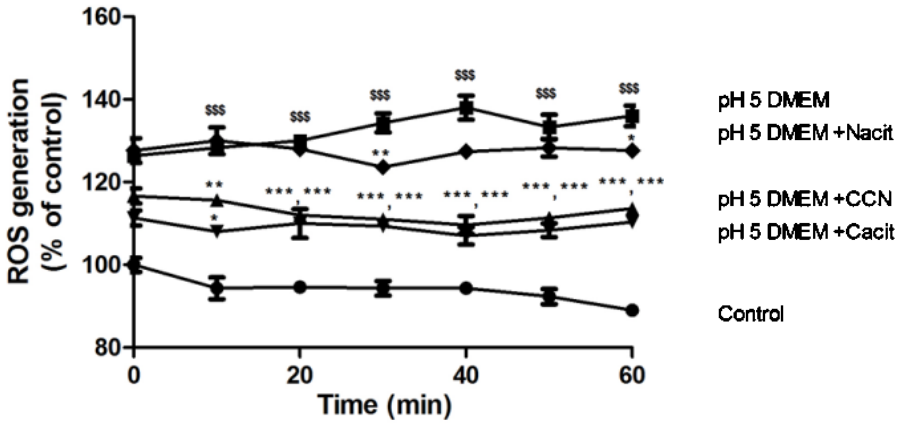


Fig. 13 ROS generation of HK-2 cells in acidic environment. The level of ROS was markedly increased in acidic condition. Treatment of HK-2 cell in acidic condition with calcium citrate in calcium carbonate nanoparticles (CCN) or calcium citrate nanoparticles (Cacit) significantly lower ROD generation, but sodium citrate (Nacit) treatment cannot reduce ROS generation. *$P < 0.05$, **$p < 0.01$ and ***$P < 0.001$ vs. pH 5 DMEM. $^{\$\$\$}P < 0.001$ vs. control

4.2.3 Alkaline generation of calcium citrate and citrate calcium citrate in calcium carbonate nanoparticles in acidic condition

The result showed that when HK-2 cells were incubated in acidic condition, extracellular pH raised to 6.69 (Fig.11 A). However, extracellular pH in acidic condition was significantly lowered when compared with control. When the cells were treated with CCN, Cacit and Nacit, extracellular pH was markedly increased, however, the increased pH was lower than acidic control with no treatment. In the part of bicarbonate analysis, the result showed that treatment of CCN, Cacit and Nacit had no effect on intracellular and extracellular bicarbonate concentration of HK-2 cells in both treatment and control group (Fig. 11 B). This data suggested that citrate treatment may increase cellular proton excretion, resulting in reduced extracellular alkalinization comparing with normal cells in acidic condition without treatment.

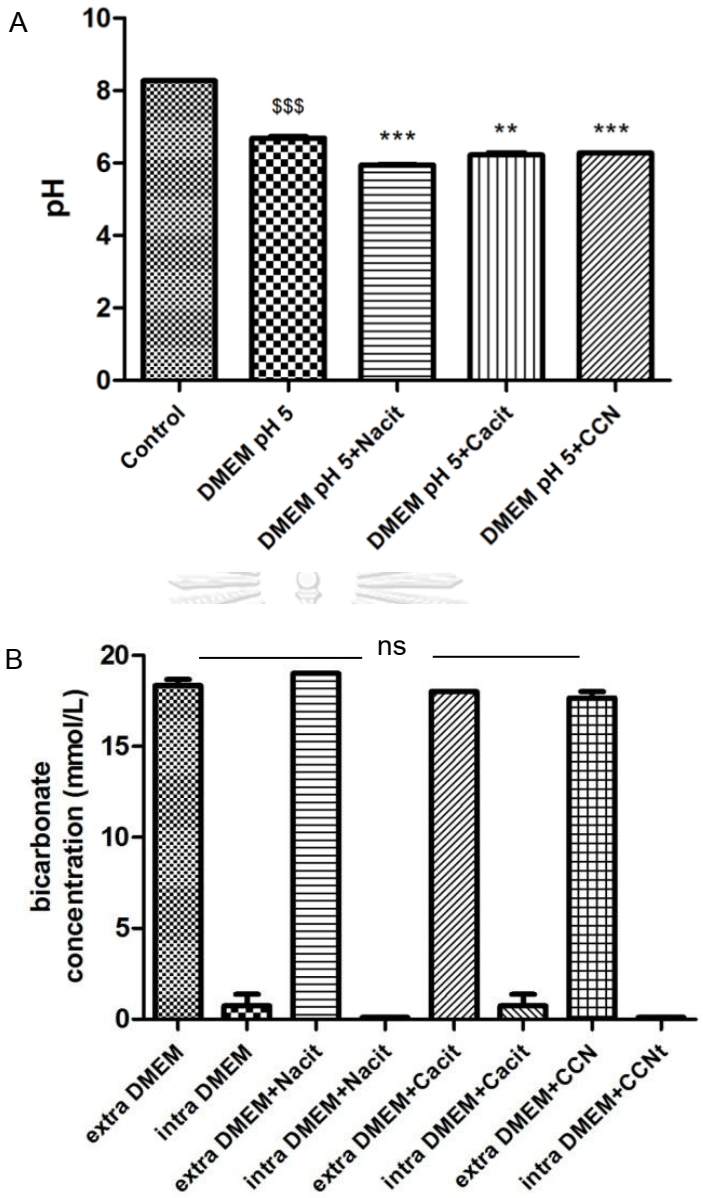


Fig. 14 Alkaline generation of HK-2 cells in acid condition. (A) Effect of calcium carbonate nanoparticles (CCN), calcium citrate nanoparticles (Cacit) and sodium citrate (Nacit) on extracellular pH of HK-2 cells after 24-hour incubation in acid condition. (B) Effect of calcium carbonate nanoparticles (CCN), calcium citrate nanoparticles (Cacit) and sodium citrate (Nacit) on bicarbonate concentration in extracellular and intracellular of HK-2 cells after 24-hour incubation in normal condition (pH 7.4). ***$P < 0.001$ vs. DMEM pH 5. $^{\$\$\$}P < 0.001$ vs. control

4.2.4 Impact of calcium citrate in calcium carbonate nanoparticles on cell death induced by acidic condition

To determine the effect of CCN on acid-induced cell death, cells were incubated in acidic condition for 18 hours. The results showed that there was undetectable HK-2 cell death in acidic condition after 18 hours of experiment when compared with control (Fig. 15 B). This might be due to HK-2 cells tolerate pH 5 to 8 environment which normally found in urine. To solve this problem, pH of DMEM was reduced to pH 4. We found that cell death was significantly increased in pH 4.0 acidic condition in relative to control (Fig. 16 B). The average of percentage on cell death stages (early apoptosis, late apoptosis and necrosis) were increased in acidic condition as showing in Table 1. The total percentage of cell death/apoptosis was 65.3 % for untreated group. We found that acidic-induced cell death/apoptosis was significant alleviated by the treatment of CCN and CaCit (Fig. 12 D). The average percentages of cell death/apoptosis were reduced to 14.32% by CCN and 12.38% by Cacit treatment, respectively. Nacit, as a standard treatment for acidosis, could reduce cell acidosis-induced cell death/apoptosis as well, but no significant difference obtained when compared with Cacit and CCN treatment (Fig. 16 C and 16 F).

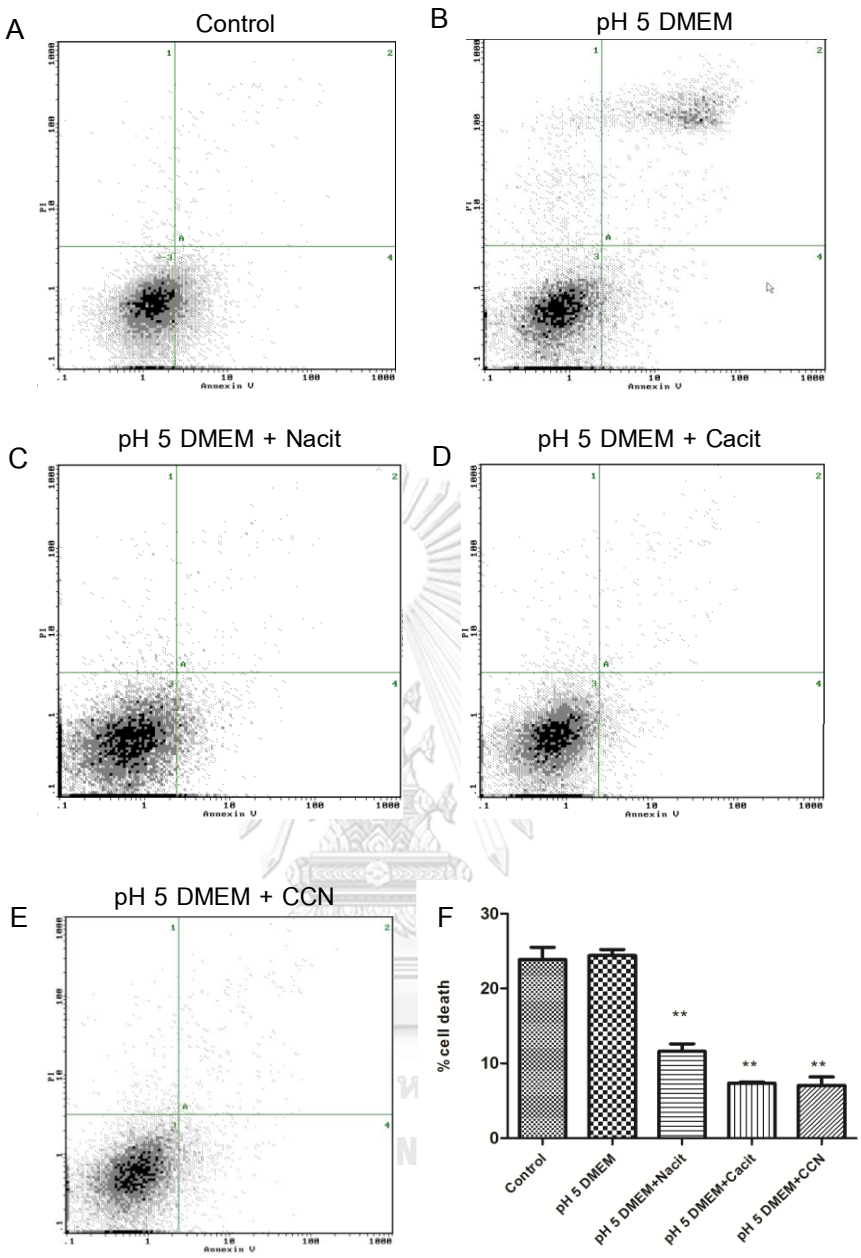


Fig. 15 Flow cytometry analysis of cell death in pH 5 determined by annexin V–FITC/PI staining in HK-2 cells. Representative plots are shown in (A) control; (B) HK-2 cell in acidic environment; (C) HK-2 cell in acidic environment treated with 1mg/mL sodiuim citrate (NaCit); (D) HK-2 cell in acidic environment treated with calcium citrate nanoparticle (Calcit); (E) HK-2 cell in acidic environment treated with calcium citrate in calcium carbonate nanoparticles (CCN); (F) quantification percentage of living cells compared with dead cells. $^{**}P < 0.01$ vs. pH 5 DMEM.

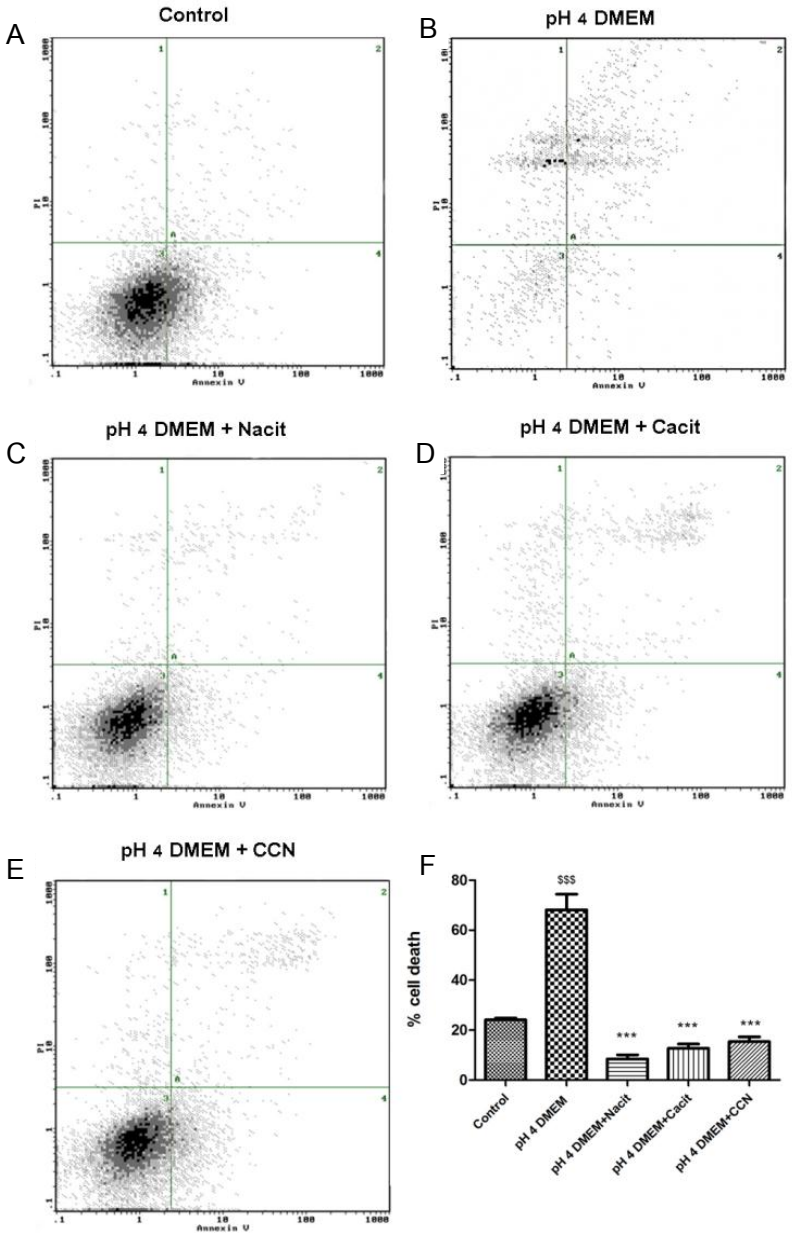


Fig. 16 Flow cytometry analysis of cell death in pH 4 determined by annexin V–FITC/PI staining in HK-2 cells. Representative plots are shown in (A) control; (B) HK-2 cell in acidic environment; (C) HK-2 cell in acidic environment treated with 100 mg% sodiuim citrate (NaCit); (D) HK-2 cell in acidic environment treated with calcium citrate nanoparticle (Calcit); (E) HK-2 cell in acidic environment treated with calcium citrate in calcium carbonate nanoparticles (CCN); (F) quantification percentage of living cells compared with dead cells. ***$P < 0.001$ vs. pH 4 DMEM. $$$ $P < 0.001$ vs. control

Table 1 Percentage of living cells and cell death stages of HK-2 cells in pH 5 DMEM (A) and pH 4 DMEM (B)

Cell type	Control	pH 5 DMEM	pH 5 DMEM+Nacit	pH 5 DMEM+Cacit	pH 5 DMEM+CCN
Live cells	76.11±1.64	75.64±0.81	88.40±1.02	92.66±0.14	92.57±1.56
Early apoptosis	21.55±1.27	6.54±0.09	8.86±0.87	4.45±0.10	4.34±1.25
Late apoptosis	1.50±0.34	13.94±1.43	1.27±0.02	1.37±0.11	1.30±0.42
Necrosis	0.85±0.04	4.34±0.08	1.48±0.14	1.54±0.16	1.79±0.11

Cell type	Control	pH 4 DMEM	pH 4 DMEM+Nacit	pH 4 DMEM+Cacit	pH 4 DMEM+CCN
Live cells	75.69±1.04	34.70±7.85	91.92±2.24	87.62±2.48	85.68±2.37
Early apoptosis	21.85±0.79	13.50±4.56	5.55±1.69	8.74±2.07	8.77±1.30
Late apoptosis	1.57±0.21	35.30±5.17	1.42±0.34	2.35±0.60	3.51±0.64
Necrosis	0.90±0.05	16.50±7.82	1.11±0.32	1.56±0.12	2.04±0.49

Data presented as mean ± SE

Chapter V Discussion

In the present study, we successfully developed the new calcium carbonate, calcium citrate and calcium citrate in calcium carbonate nanoparticles. Cacit and CCN were stored in white, dry powder that was odorless, tasteless and had good water solubility. Physical characteristics of Cacit was; spherical shape, approximate diameter is 50-70 nm, with zeta potential of -16.1 mV, which represented the chance of particle aggregation is kept in aqueous form. The size and shape of Cacit were appropriate for transmembrane transport. Cacit contained 31% of citrate by weight and cellular uptake study revealed that Cacit can enter the HK-2 cell in both normal and acidic condition.

Likewise, calcium citrate nanoparticle coated with calcium carbonate nanoparticle or CCN has spherical shape with a diameter around 60-80 nm, slight larger than Cacit. Zeta potential of CCN was -12.0 mV, slightly more neutral than Cacit. Citrate content in CCN was about 25% by weight and cellular uptake was observed in normal and acidic condition, similar to Cacit.

The pharmacologic properties of Cacit and CCN were performed by comparing with blank and sodium citrate (Nacit) treatment. We found that both Cacit and CCN contained very low cytotoxicity to HK-2 cells at the concentration between 0.01 to 1 mg/ml. Treatment of either Cacit or CCN to the HK-2 cell in acidic condition (DMEM pH 4.0 to 5.0) were able to suppress ROS production and cell death caused by acidosis. The ROS scavenging property of Cacit and CCN appears to be more potent than Nacit, while anti-apoptotic capacity was similarly. Our study revealed that both Cacit and CCN had no effect on environmental acidic neutralization or bicarbonate regeneration.

In the present study, we expected the nanoparticle diameter should be less than 100 nm, since a previous study reported that nanoparticles sized less than 100 nm could enter to the cell freely and was not eliminated by circulation phagocytic cells (5). The zeta potential is an important property to determine the surface charge and stability of the nanoparticles. A high value of zeta potential is high stability, as their charge inhibits aggregation and increases stability (37). In our study, CCN and Cacit had good

water solubility, however, their zeta potential values were lower than our expectation. According this, we decided to store the nanoparticles in solid, dry powder form to prevent the aggregation.

TGA study revealed that both Cacit and CCN contained citrate, as we observe the curve of mass losses at the decomposition of citrate and carbonate, respectively (38). It should be noted that calcium citrate was decomposed by high temperature caused calcium citrate to turn into calcium carbonate, and subsequently calcium oxide. This phenomenon explained the loss mass curve of carbonate in Cacit which originally did not contain carbonate. The content of citrate in CCN was lower than Cacit, which was anticipated since CCN was Cacit which had been coated by calcium carbonate nanoparticle. Regarding this, we could expect that in the following experiment, Cacit should exert more potent pharmacologic effect than CCN at the same concentration, because the content of active ingredient (citrate) was lower. Although we anticipate that the potency of both nanoparticles should not largely different because the different content was small, and CCN should have benefit in targeting acidic cells.

Metabolic acidosis causes degenerative change and progression of several organ dysfunction in human. The mechanism was mediated by the processes of inflammation, oxidation, and abnormal healing. A previous study reported that acidosis reduced cellular total glutathione and protein thiol content and increase in glutathione peroxidase activity (39). Treatment acidosis with citrate promoted NADPH generation by suppression of phosphofructokinase activity, which lead to shifting glucose-6-phosphate into pentose phosphate pathway to produce isocitrate required for isocitrate dehydrogenase to convert NADP to NADPH (10). NADPH converts oxidized glutathione to reduced glutathione which is an important free radical scavenging molecule in human body. This should be the mechanism for citrate to alleviate ROS generation observed in the present study. In addition, citrate is a main ingredient in regular drugs used to increased plasma pH in acidosis patients, such as sodium citrate/citric acid or Shohl's solution used to treat chronic acidosis in chronic renal disease, and potassium citrate compounds or potassium citrate/citric acid used to alkalinize urine in urolithiasis.

Our study demonstrated that CaCit and CCN could be used to alleviate chronic acidosis complication, since these nanoparticles scavenged ROS and alleviated cell death. The disadvantage of these nanoparticles was that they could not neutralize or alkaline acidosis. However, we hypothesized that the alkalinizing property of nanoparticles could be more clearly demonstrated in animal model, since hepatocyte is the main cell utilizing citrate to produce bicarbonate.

Although Cacit and CCN could not neutralize acidosis, the antioxidant and anti-apoptotic properties were convinced that Cacit and CCN could be used as the adjuvant medication with standard drug to treat acidosis and its complication. Otherwise, these nanoparticles might be used as the main drug to treat any disease associated with intracellular acidosis but normal or high plasma pH, such as cancer cells. Further study is essential to validate the efficacy of these nanoparticles in animal.

The major limitation of this study was that we could not demonstrate the molecular mechanism of CCN to rescue ROS overproduction, cell injury and apoptosis. The only evidence we found was that these benefits were independent with pH change. The other limitation was that we could not clearly demonstrate the intracellular bicarbonate change since the method has high limit of detection (LOD). Another technique that has lower LOD (1 mmol/L) should be used for further analysis. Finally, we could not verify that most of CCN breaks down in intracellular or extracellular compartments, because extracellular fluid should be acidic in acidosis condition. However, calcium carbonate nanoparticles ordinarily break down intracellularly with the pH of 5.5 to 6.5, which is not observed in plasma of any acidosis patients. For pharmacokinetics and pharmacodynamics studies of CCN must be done on animals.

In summary, our research team invented the Cacit and CCN that has very low cytotoxicity, antioxidant and anti-apoptotic properties. Cacit and CCN can be stored in dry powder form. Cacit or CCN administration did not modify acid-base status in cell culture model. These nanoparticles had potential to use as an adjuvant therapy with standard medication in chronic acidosis and disease associated with intracellular acidosis.

Chapter VI Conclusion

In conclusion, we have developed new calcium citrate nanoparticles (Cacit) and calcium citrate in calcium carbonate nanoparticle (CCN) to treat chronic acidosis. Cacit and CCN has spherical molecule with diameter between 50-70 and 60-80 nm, respectively. Cacit and CCN contain slightly negative zeta potential charge of -16.1 mV and -13.0 mV and can be stored stably in dried powder form. CCN was uptake by cell in normal and acidosis conditions. In addition, Cacit and CCN are water soluble and have very low cytotoxicity at the level 1 mg/mL or lower. Treatment cell with acidic environment with Cacit and CCN alleviated free radical generation and cellular apoptosis without significant change of extracellular pH.

REFERENCES

1. Kiessling SG, Goebel J, Somers MJG. Pediatric Nephrology in the ICU. 1st ed. Heidelberg: Springer Berlin Heidelberg; 2009.
2. Kraut JA, Madias NE. Metabolic acidosis: pathophysiology, diagnosis and management. Nature reviews nephrology. 2010;6(5):274-85.
3. Kraut JA, Madias NE. Metabolic Acidosis of CKD: An Update. American journal of kidney diseases. The official journal of the National Kidney Foundation. 2016;67(2):
   307-17.
4. Khan I, Saeed K, Khan I. Nanoparticles: Properties, applications and toxicities. Arabian Journal of Chemistry. 2017 May 10. [Epub ahead of print]
5. Bahrami B, Hojjat-Farsangi M, Mohammadi H, Anvari E, Ghalamfarsa G, Yousefi M, et al. Nanoparticles and targeted drug delivery in cancer therapy. Immunology Letters. 2017;190:64-83.
6. Maleki Dizaj S, Lotfipour F, Barzegar-Jalali M, Zarrintan MH, Adibkia K. Ciprofloxacin HCl-loaded calcium carbonate nanoparticles: preparation, solid state characterization, and evaluation of antimicrobial effect against Staphylococcus aureus. Artif Cells Nanomed Biotechnol. 2016;45(3):535-43.
7. Ueno Y, Futagawa H, Takagi Y, Ueno A, Mizushima Y. Drug-incorporating calcium carbonate nanoparticles for a new delivery system. Journal of controlled release : official journal of the Controlled Release Society. 2005;103(1):93-8.
8. Kim J-A, Choi H-J, Kwon Y-K, Ryu DH, Kwon T-H, Hwang G-S. 1H NMR-Based Metabolite Profiling of Plasma in a Rat Model of Chronic Kidney Disease. PLOS ONE. 2014;9(1):e85445.
9. Iacobazzi V, Infantino V. Citrate-new functions for an old metabolite. Biological chemistry. 2014;395(4):387-99.

10. Liu J, Litt L, Segal MR, Kelly MJS, Pelton JG, Kim M. Metabolomics of Oxidative Stress in Recent Studies of Endogenous and Exogenously Administered Intermediate Metabolites. International Journal of Molecular Sciences. 2011;12(10): 6469-6501.
11. Gadola L, Noboa O, Marquez MN, Rodriguez MJ, Nin N, Boggia J, et al. Calcium citrate ameliorates the progression of chronic renal injury. Kidney International. 2004;65(4):1224-30.
12. Ayers P, Warrington L. Diagnosis and treatment of simple acid-base disorders. Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition. 2008;23(2):122-7.
13. Koeppen BM. The kidney and acid-base regulation. Advances in physiology education. 2009;33(4):275-81
14. Epstein SK, Singh N. Respiratory acidosis. Respiratory care. 2001;46(4):366-83.
15. Gattinoni L, Taccone P, Carlesso E. Respiratory acidosis: is the correction with bicarbonate worth?. Minerva anestesiologica. 2006;72(6):551-7.
16. Ramadoss J, Stewart RH, Cudd TA. Acute Renal Response to Rapid Onset Respiratory Acidosis. Canadian journal of physiology and pharmacology. 2011;89(3):227-31.
17. Lim S. Metabolic acidosis. Acta medica Indonesiana. 2007;39(3):145-50.
18. Kraut JA, Kurtz I. Metabolic Acidosis of CKD: Diagnosis, Clinical Characteristics, and Treatment. American Journal of Kidney Diseases. 2005;45(6):978-93.
19. Krieger NS, Frick KK, Bushinsky DA. Mechanism of acid-induced bone resorption. Current opinion in nephrology and hypertension. 2004;13(4):423-36.
20. Phisitkul S, Khanna A, Simoni A, Broglio K, Rajab MH, Wesson DE. Amelioration of metabolic acidosis in patients with low GFR reduced kidney endothelin production and kidney injury, and better preserved GFR. Kidney International. 2010;77(7):617-623.
21. Unwin RJ, Capasso G, Shirley DG. An overview of divalent cation and citrate handling by the kidney. Nephron Physiology. 2004;98(2):15-20.

22. Cabalgante MJR, Gadola L, Luzardo L, Márquez M, Boggia J, Boim MA. Calcium citrate improves the epithelial-to-mesenchymal transition induced by acidosis in proximal tubular cells. Jornal Brasileiro de Nefrologia. 2012;34(4):343-8.
23. Both T, Zietse R, Hoorn EJ, van Hagen PM, Dalm VA, van Laar JA, et al. Everything you need to know about distal renal tubular acidosis in autoimmune disease. Rheumatology international. 2014;34(8):1037-45.
24. Tungsanga K, Sriboonlue P, Futrakul P, Yachantha C, Tosukhowong P. Renal tubular cell damage and oxidative stress in renal stone patients and the effect of potassium citrate treatment. Urological research. 2005;33(1):65-9.
25. Meguid El Nahas A, Bello AK. Chronic kidney disease: the global challenge. Lancet. 2005;365(9456):331-40.
26. Choi EY, Kim HJ, Han JS. Anti-inflammatory effects of calcium citrate in RAW 264.7cells via suppression of NF-kappaB activation. Environmental toxicology and pharmacology. 2015;39(1):27-34.
27. Mudshinge SR, Deore AB, Patil S, Bhalgat CM. Nanoparticles: Emerging carriers for drug delivery. Saudi pharmaceutical journal: the official publication of the Saudi Pharmaceutical Society. 2011;19(3):129-41.
28. Maleki Dizaj S, Barzegar-Jalali M, Zarrintan MH, Adibkia K, Lotfipour F. Calcium carbonate nanoparticles as cancer drug delivery system. Expert opinion on drug delivery. 2015;12(10):1649-60.
29. Adjei IM, Sharma B, Labhasetwar V. Nanoparticles: cellular uptake and cytotoxicity. Advances in experimental medicine and biology. 2014;811:73-91.
30. Kaszuba M, Corbett J, Watson FM, Jones A. High-concentration zeta potential measurements using light-scattering techniques. Philosophical transactions Series A, Mathematical, physical, and engineering sciences. 2010;368(1927):4439-51.
31. J¨²nior JAA, Baldo JoB. The Behavior of Zeta Potential of Silica Suspensions. New Journal of Glass and Ceramics. 2014;4:29-37.

32. Kuo JH, Lin CW. Cellular uptake on N- and C-termini conjugated FITC of Rath cell penetrating peptides and its consequences for gene-expression profiling in U-937 human macrophages and HeLa cervical cancer cells. Journal of drug targeting. 2013;21(9):801-8.
33. Rampersad SN. Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays. Sensors (Basel, Switzerland). 2012;12(9):12347-60.
34. Chen X, Zhong Z, Xu Z, Chen L, Wang Y. 2',7'-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy. Free radical research. 2010;44(6):587-604.
35. Chariyavilaskul P, Poungpairoj P, Chaisawadi S, Boonla C, Dissayabutra T, Prapunwattana P, et al. In vitro anti-lithogenic activity of lime powder regimen (LPR) and the effect of LPR on urinary risk factors for kidney stone formation in healthy volunteers. Urolithiasis. 2015;43(2):125-34.
36. Rieger AM, Nelson KL, Konowalchuk JD, Barreda DR. Modified Annexin V/Propidium Iodide Apoptosis Assay For Accurate Assessment of Cell Death. Journal of Visualized Experiments. 2011; 50:2597-4.
37. Guo X, Zheng H, Guo Y, Wang Y, Anderson GJ, Ci Y, et al. Nasal delivery of nanoliposome-encapsulated ferric ammonium citrate can increase the iron content of rat brain. Journal of nanobiotechnology. 2017;15(1):42.
38. Herdtweck E, Kornprobst T, Sieber R, Straver L, Plank J. Crystal Structure, Synthesis, and Properties of tri-Calcium di-Citrate tetra-Hydrate [Ca3(C6H5O7)2(H2O)2]·2H2O. Zeitschrift für anorganische und allgemeine Chemie. 2011;637(6):655-9.
39. Rustom R, Wang B, McArdle F, Shalamanova L, Alexander J, McArdle A, et al. Oxidative Stress in a Novel Model of Chronic Acidosis in LLC-PK1 Cells. Nephron Experimental Nephrology. 2003;95(1):13-23.

APPENDIX

List of abbreviations

CAII	Carbonic anhydrase type 2
Cacit	Calcium citrate nanoparticles
CCN	Calcium citrate in calcium carbonate nanoparticles
CCNP	Calcium carbonate nanoparticles
CO_2	Carbon dioxide
DCFH-DA	2,7-dichlofluorescein-diacetate
DMEM	Dulbecco-Vogot modified eagle medium
EMT	Epithelial-to-mesenchymal transition
FBS	Fetal bovine serum
FITC	Fluorescence isothiocyanate
GFR	Glomerulus filtration rate
H^+	Proton
HCO_3	Bicarbonate
H_2CO_3	Carbonic acid
HK-2	Proximal tubule epithelia
LLC-PK1	Renal tubular epithelial porcine
LPR	Lime powder regimen
Nacit	Sodium citrate
NBCe-1	Sodium-bicarbonate cotransporter
NaDC-1	Sodium dicarboxylate cotransporter type 1
NADP	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
NP	Nanoparticles
PBS	phosphate buffered saline
P-MDA	plasma malondialdehyde
ROS	Reactive Oxygen Species

TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis

Equipment and chemicals

Autoclave	HVE-25, Ireland
Autopipette 10, 100 and 1000 µL	Biorad, USA
Pipette 2, 5, 10 and 25 mL	Thermo Scientific, USA
Centrifuge and Centrifuge tubes	Hettich Centrifuge, USA
Class II biohazard safety cabinet	Esco Micro, Singapore
CO_2 Incubator	Esco Micro, Singapore
Light microscope	Nikon, Japan
Microplate reader	Thermo Scientific, USA
Microtube 1.5 and 5 mL	Nest Biotechnology, China
pH-meter	METTLER TOLEDO, USA
Sonicator	P.Intertrade Equipment Co.,Ltd
Tissue culture flasks 25 cm^2	Nest Biotechnology, China
Tissue culture flasks 75 cm^2	Nest Biotechnology, China
Tissue culture flasks 6,96-well	Corning Incoperated, USA
Vortex mixer	Scientific Industries, USA
Transmission electron microscopy (TEM)	Hitachi, Japan
Zetasizer Nano Series	Malvern Instrument, England
Thermal gravimetric analysis (TGA)	TA Instrument, USA
Flow cytometer	Becton Dickenson, USA
Calcium chloride	MERK, Germany
Sodium citrate	MERK, Germany
Sodium carbonate	MERK, Germany
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Scientific, USA
Fetal bovine serum	Gibco, USA
Phosphate buffer saline (PBS)	Ameresco, USA

Penicillin-Streptomycin solution	MERK, Germany
Trypsin/EDTA	SIGMA, Germany
Fluorescein isothiocyanate (FITC)	SIGMA, Germany
Annexin-V and Propidium Iodide	Molecular probe, USA
Prestro Blue	Thermo Scientific, USA
2,7,-dichlorofluorescein diacetate	Molecular probe, USA

Chemical preparations

1) Dulbecco's Modified Eagle Medium (DMEM)

DMEM	13.4	g
Sodium bicarbonate	3.7	g

1) Mix all of chemical component and dissolve in DI water 800 mL
2) Adjust pH to 7.4 with HCl and add 200 mL of DI water
3) Filtrate by 0.22 μM filter as a stock media
4) Add FBS 100 mL and antibiotic 10 mL to media

2) Dulbecco's Modified Eagle Medium (DMEM) pH 5

DMEM	3.35	g

5) Mix all of chemical component and dissolve in DI water 150 mL
6) Adjust pH to 5.0 with HCl and add 100 mL of DI water
7) Filtrate by 0.22 μM filter as a stock media
8) Add antibiotic 2.5 mL to media

3) Phosphate buffer saline (PBS)

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

1) Mix all of chemical component and dissolve in DI water 1000 mL
2) Adjust pH to 7.4 with HCl
3) Sterile by autoclave and filtrate by 0.22 μM filter as a stock media

Fixation cells protocol

Materials

1) Phosphate buffer saline (PBS)
2) Formalin 4%
3) DAPI

Method

1) Remove media and wash with PBS 2 mL for 12 well plate
2) Add formalin 4% which dilute from 100% concentration 300 µL
3) Incubate 5 min and wash with PBS 2 mL 2 time
4) Add DAPI 200 µL and incubate 10 min
5) Wash with PBS 3 mL 3 time
6) Add PBS 1 mL for protect cell dry

Freeze-thaw lysis cells protocol

Materials

1) Liquid nitrogen
2) Water bath 37 ^{o}C
3) Vortex
4) Phosphate buffer saline (PBS)

Method

1) Cells were resuspend with PBS 1 mL
2) Freeze cells with liquid nitrogen until ice crystal form
3) Thaw cells in water bath until ice crystal disappear
4) Disperse cells with vortex
5) Repeat this cycle freeze and thaw 5 time
6) Centrifuge at 12000 rpm 7 min and separate supernatant

VITA

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