The Hong Kong Polytechnic University

Department of Applied Biology and

Chemical Technology

Study of Effects of Fructus Ligustri Lucidi on Calcium Balance and Vitamin D Metabolism

DONG XIAO LI

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

May 2009

CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written nor material which has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

_____

(Signature)

Dong Xiao Li

(Name)

Abstract

Osteoporosis is a worldwide public health problem that poses significant economic burden on society as well as on families of patients that suffer reduced functional independence as a result of osteoporosis related fractures. In most frequently affected postmenopausal women, vertebral and hip fractures are often attributed to low estrogen production and secondary hyperparathyroidism as a result of age-related decline in calcium (Ca) intake and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)-mediated Ca absorption. In the present study, the effects of estrogen deficiency on intestinal Ca absorption and renal Ca reabsorption in aged ovariectomized (OVX) female rats fed different levels of dietary Ca for 3 months were characterized. The results indicated that intestinal Ca absorption and renal Ca reabsorption were reduced in response to long-term estrogen deficiency in aged female rats. The blunted Ca absorption during estrogen deficiency in aged female rats was associated with the alterations of expression of epithelial Ca transporting proteins in intestine and kidney. However, $1,25(OH)_2D_3$ dependent Ca transport protein expressions were upregulated in aged normal and OVX female rats fed low Ca diet, suggesting that the blunted Ca transport associated with estrogen deficiency is not due to alteration in $1,25(OH)_2D_3$-mediated Ca absorption.

Previous studies in our laboratories demonstrated that the ethanol extract of Fructus Ligustri Lucidi (Nuzhenzi, FLL), a Chinese Medicinal herb, plays an active role in modulating Ca homeostasis, not only in OVX rats but also in aged

female rats with intact ovaries. In order to identify the active fractions which are responsible for the positive actions of FLL on Ca homeostasis, ethyl acetate soluble (lipophilic) fraction (EAF) and water layer (hydrophilic) fraction (WF) were prepared from the ethanol extract (EE) of FLL and administered to young female rats. The effects of different FLL fractions on Ca balance were evaluated. Our results showed that WF fraction of FLL exerted positive effects on Ca balance in rats fed a diet containing adequate Ca. The positive actions of FLL fractions on Ca balance might relate to their abilities to modulate the expression of epithelial Ca transporting proteins.

Our previous study also showed that the ethanol extract of FLL increased serum $1,25(OH)_2D_3$ levels in aged female rats. Thus, the mechanism by which the ethanol extract of FLL modulates vitamin D_3 metabolism was also investigated. The results indicated that the ethanol extract of FLL could raise the production of serum $1,25(OH)_2D_3$ levels in both aged normal and OVX female rats via the induction of the expression and activity of renal 25-hydroxy-1α-hydroxylase (1-OHase) directly. To identify the active fraction that accounts for the positive effects of FLL on renal 1-OHase, human proximal tubule HKC-8 cells were established as an *in vitro* screening platform. The results indicated that the EA fraction of FLL was the active fraction that contributed to its modulatory action on renal 1-OHase.

In conclusion, the results of the study show that FLL ethanol extract is effective in improving Ca balance in both young and aging animals by inducing

the expression of epithelial Ca transport protein as well as the activity and expression of renal 1-OHase. The hydrophilic fraction of the FLL ethanol extract is responsible for its stimulatory actions on Ca transport and the lipophilic fraction of the FLL ethanol extract is responsible for its stimulatory actions on renal 1-OHase. The study suggests that FLL ethanol extract might be a useful agent for prevention of postmenopausal osteoporosis by improving disturbances in Ca balance associated with estrogen deficiency.

List of Publications

1. Zhang Y, **Dong XL**, Leung PC, Wong MS. Differential mRNA expression profiles in proximal tibia of aged rats in response to ovariectomy and low-Ca diet. *Bone*, 2009, 44:46-52.

2. Zhang Y, **Dong XL**, Leung PC, Che CT, Wong MS. Fructus ligustri lucidi extract improves calcium balance and modulates the calciotropic hormone level and vitamin D-dependent gene expression in aged ovariectomized rats. *Menopause*, 2008, 15(3):558-565

3. **Dong XL**, Wong MS. Regulation of 25-hydroxyvitamin D 1-alpha hydroxylase by Fructus Ligustri Lucidi in vitro. Proceedings of Global Chinese Health (Functional) Food Symposium, Hong Kong, China, August 2009

4. **Dong XL**, Chen WF, Che CT, Wong MS. Effects of different extracts of Fructus Ligustri Lucidi on Ca balance in normal female rats. Proceedings of the 30th American Society of Bone and Mineral Research, Montreal, Canada, September 2008

5. Chen WF, **Dong XL**, Mok SK, Yao XS, Leung PC, Wong MS. Bone anabolic effects of icariin and total flavonoid fraction of Herba Epimedii in ovariectomized mice. Proceedings of the 30th American

Society of Bone and Mineral Research, Montreal, Canada, September 2008

6. **Dong XL**, Zhang Y, Leung PC, Che CT, Wong MS. Study of the effect of Fructus Ligustri Lucidi (Nuzhenzi) on renal and intestinal vitamin D metabolism in aged Rats. Proceedings of 2007 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, HK; August 2007

7. Wan HY, Lai WP, Zhang Y, **Dong XL**, Wong MS. Study of Fructus Lugustri Lucidi (Nvzhenzi) on the regulation of vitamin D in rats with different calcium intake. Proceedings of 2006 Hong Kong-Macau Postgraduate Symposium on Chinese Medicine, HK; August 2006

Acknowledgement

Three years ago, I left Qingdao for my Ph.D study in the Hong Kong Polytechnic University. At that time, I knew that a long and hard journey began. But unexpectly and fortunately, my research work and life went smoothly during this period. And it is due to the generous support and encouragement from many people around me. I want to take this opportunity to express my sincere appreciation and thanks to all of them.

The first and deepest thanks I would like to give to is my chief supervisor, Dr. M.S. Wong. She gave me patient and experienced guidance, invaluable suggestions and discussions during this period of my Ph.D. studies. My every step forward can be directly attributed to her kind guidance. Her enthusiastic attitude to science, her wisdom and her high work efficiency will be the goals for all my life.

I would like to thank Prof. Favus Murray and his team for their invaluable support and guidance in the University of Chicago during my exchange period to the USA. They are not only so helpful for my research work, but also my life there. It's their great helps that make me finish the exchange study smoothly in USA.

I am very grateful to my teammates Ms. Edith Lai, Ms. Summi Lau, Ms. Helen Mok, Ms Wai-Yin Pang and Dr. Zhang Yan for their technique help, generous

support and collaboration. It is a cheerful and enjoyable period to work with these kind and friendly people.

Many thanks are directed to Pro. Chun-Tao Che and Dr. Zhao Ming from the Chinese University of Hong Kong. They offered me great help and provided useful recommendations of the Chinese herb. Their generous support ensured my research going forward.

I also thank the technical and support staff in the Department of Applied Biology and Chemical Technology for their assistance. Thanks give to Mr. C.H. Cheng, Mr. Leung, Miss Sarah Yeung, Miss Mable Yau and Ms. Isabella Chong.

Special thanks are due to my friends Guo-qing Liang, Yuan-shuai Liu, Xiao-hua Li and Lan-Zou. Thanks for their consolation and encouragement during my study.

Finally, I would like to give my sincere and deepest thanks to all of the people who offered me support and help.

Table of Contents

List of Figures and Tables

Abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
1-OHase	25-hydroxyvitamin D1-alpha hydroxylase
24-OHase	25-hydroxyvitamin D-24 hydroxylase
$25(OH)D_3$	25-hydroxyvitamin D_3
μCT	Cone-beam X-ray micro-computed tomography
AJs	Adheres junctions
AP-1	Activator protein 1
BMD	Bone mineral density
BSA	Bovine albumin
BS/BV	Bone surface/Bone volume
BV/TV	Bone volume/Total volume
Ca	Calcium
CaBP	Calbindin protein
CaSR	Calcium sensing receptor
Cr	Creatinine
CNT	Connecting tubule
DBP	Vitamin D-binding protein
DCT	Distal convoluted tubule
DPD	Deoxyopyridinoline
EA	Ethyl acetate
EAF	Ethyl acetate soluble fraction

ECF	Extracellular fluid
EE	Ethanol extract of Fructus Ligustri Lucidi
ER	Estrogen receptor
FDA	Food and drug administration
FE	Femur end
FGF23	Fibroblast growth factor 23
FH	Femur head
FLL	Fructus Ligustri Lucidi
FM	Femur midshaft
HCD	High calcium diet
HKC-8	Human proximal kidney tubule cells
HPLC	High performance liquid chromatography
IEJs	Interendothelial junctions
KHS	Krebs-Henseleit saline
LCD	Low calcium diet
MCD	Medium calcium diet
MDBK	Madin-Darby bovine kidney cells
NAMS	North American Menopause Society
OCN	Osteocalcin
OVX	Ovariectomized
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C

PTCs	Proximal tubular cells
PTH	Parathyroid hormone
SD	Sprague dawley
SEM	Standard error of mean
Sp1	Stimulatory protein
Tb.No	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TCM	Traditional Chinese Medicine
TH	Tibia head
TK	Tissue kallikrein
TM	Tibia midshaft
TRP	Transient receptor potential
TSS	Transcription start site
VDR	Vitamin D receptor
VDRE	Vitamin D-response element
WF	Water layer fraction
WHO	World Health Organization

Chapter 1

Introduction

1.1Osteoporosis

1.1.1 Epidemiology of Osteoporosis

According to World Health Organization (WHO), osteoporosis is a progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. The different variants of this disease are primary osteoporosis, which includes postmenopausal, age-related (senile) and idiopathic osteoporosis, and secondary osteoporosis, which is due to a specific underlying disease or drug therapy (Boonen et al., 2006). Currently affecting more than 10 million people in the United States, it is estimated that osteoporosis will affect approximately 14 million adults over the age of 50 by the year 2020 (Lane, 2006). Aged-related osteoporosis commonly resulted in fractures which are responsible for the greatest proportion of morbidity and mortality from this disease. In Hong Kong, it is reported that 300,000 elderly suffer from osteoporosis and 10 hip fractures occur everyday due to this disease (Book., 1991). Life expectancy is increasing worldwide, and it is estimated that the number of individuals aged 65 years or above will increase from the current figure of 323 million to 1555 million by the year 2050. The social and economic burden of osteoporosis is increasing steadily and this disease has become a major public health problem (Dennison et al., 2006).

1.1.2 Causes of Osteoporosis

Osteoporosis comes from an imbalance between new bone formation and old bone resorption. The body may fail to form enough new bone, or too much old bone may be reabsorbed, or both. Two essential minerals for normal bone formation are calcium (Ca) and phosphate. Throughout youth, the body uses these minerals to produce bones. If Ca intake is not sufficient or if the body does not absorb enough Ca from the diet, bone production and bone tissue may suffer (Bischoff-Ferrari et al., 2008). Ca is the essential element for normal functioning of the heart, brain, and other organs. To keep those critical organs functioning properly, the body may reabsorb Ca from the bones for their use. Thus, the bones may become weaker, brittle and fragile, that can break easily at last. Usually, the loss of bone takes an extended period of years without signs. Often, a person will sustain a fracture before becoming aware that the disease is present. By then, the disease may be in its advanced stages and damage may be serious (Wick, 2009).

A lack of certain hormones, particularly estrogen and vitamin D, is the leading cause of osteoporosis. Women, especially those older than 60 years, are frequently affected by the disease. During menopause, bone loss due to reduced estrogen levels is complicated by the age-related abnormalities of the vitamin D endocrine system (Riggs, 2003). Vitamin D deficiency causes secondary hyperparathyroidism, which leads to increased bone resorption activity. The risk of hip fracture increases with turnover, and recent data suggest that this high turnover state may create large porosities in the cortex of the femur at sites

where hip fractures most often occur. Lowering of bone turnover and reduction of cortical porosity are probably the main explanations for the beneficial effect of vitamin D and Ca supplementation on osteoporotic fractures (Eriksen and Glerup, 2002). Other factors including lack of weight-bearing exercise, and othe r age-related changes in endocrine functions may also lead to bone loss in this elderly group. Other conditions that may lead to osteoporosis include overuse of corticosteroids (Cushing syndrome), thyroid problems, lack of muscle use, bone cancer, certain genetic disorders, use of certain medications, and problems such as inadequate Ca in the diet (Book, 2009).

Vitamin D and Ca supplementation have been shown to reduce the risk of hip and other peripheral fractures by 40% (Dawson-Hughes et al., 1997). Furthermore, Ca and vitamin D supplementation have been adopted to be the basic treatment in association with other specific antiosteoporotic therapies like hormones, biphosphonates and selective estrogen receptor modulators (Eriksen and Glerup, 2002).

1.1.3 Ca and Osteoporosis

The skeleton is the largest Ca reservoir which can be mobilized to compensate for the losses of Ca from the ECF via the kidney, bowel, and skin. The organ prefers to maintain the ionized Ca concentration, in comparison with preserving the integrity of the skeleton. So experimental Ca deficiency in adult does not cause significant hypocalcemia but rather results in PTH-mediated bone resorption (Pettifor and Ross, 1983). In states of chronic Ca deficiency, negative Ca balance and mobilization of the skeleton may continue and lead sooner or later to the condition of osteoporosis, which was manifested as a reduction in the apparent density of bone. This event can be aggravated by estrogen deficiency in postmenopausal females. It has been suggested that estrogen deficiency only mobilizes trabecular bone, whereas Ca deficiency also mobilizes cortical bone, which suggests the severity of the negative Ca balance (Cai Q, 1998) (Figure 1-1-1).

Although it is clear that Ca deficiency causes osteoporosis in animals, but it has not been confirmed in humans. Adults need to be in zero Ca balance to protect their bones. Ca balance attributes to three factors including Ca intake, absorption and excretion, and the last two were shown statistically more important than the Ca intake (Nordin et al., 2004). It was indicated that bioavailability is important when habitual Ca intakes are low, especially during periods of bone growth or loss. But further research is required to quantify the effects of major dietary modulators of Ca balance on bone health and to understand their relationship with genetic and physiological variables

(Fairweather-Tait and Teucher, 2002). Ca malabsorption is a potential cause of negative Ca balance. Ca absorption starts at menopause and falls with age, it is mainly due to a decline in gastrointestinal responsiveness. In established osteoporosis, especially in many patients with vertebral fracture, Ca malabsorption has more severe degree (Morris et al., 1991). But it has been suggested that the malabsorption is a cause rather than a consequence of osteoporosis (Tilyard et al., 1992). The unmistakable rise in urine Ca excretion at menopause has already been referred to and estimated at 0.5-1.0 mmol (20-40 mg) daily, which is compatible with the rate of bone loss after menopause. The rise in urine Ca occurs in both normal and osteoporotic postmenopausal women which is due to a reduction in the tubular reabsorption of Ca and consequent renal Ca “leak” (Nordin et al., 2004). And the strategies of lowering urine Ca excretion reduces bone loss and fracture risk (Nordin et al., 2004). Figure1-1-1 shows the diagrammatic representation of the pathways leading to osteoporosis.

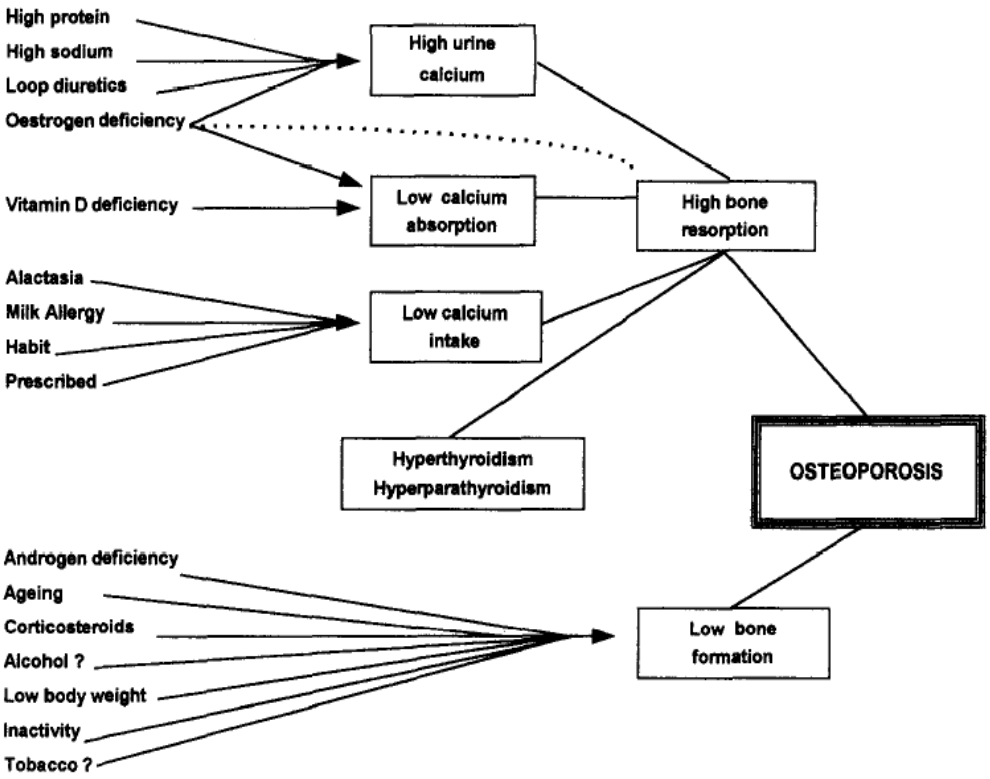


Figure 1-1-1 Diagrammatic representation of the Ca balance pathways leading to osteoporosis

Adapted from (Nordin et al., 2004)

1.1.4 1,25(OH)$_2$D$_3$ and Osteoporosis

1.1.4.1 The effects of aging on vitamin D_3 metabolism

Cutaneous production of vitamin D_3 decreases with advancing age. Dietary intake of vitamin D_3 decreases slightly or remains unchanged with advancing age. Due to differences in dietary supplementation and food preferences, dietary vitamin D_3 varies over a broad range. Intestinal absorption of vitamin D_3 does not appear to change with aging. Despite the states of dietary intake and intestinal absorption, vitamin D_3 status in elderly populations, as judged by serum concentrations of either vitamin D_3 or 25-hydroxyvitamin D_3 (25OHD$_3$), deteriorates with advancing age. The serum concentration of 25OHD$_3$ in the elderly, which is usually referred to a reflection of serum vitamin D_3 levels, is related inversely to age and directly to sun exposure. The age-related decrease in serum 25OHD$_3$, in the absence of disease, is indicative of both a decrease in cutaneous production and reduced dietary intake. In the presence of disease, malabsorption can play a major role. Also, it has been suggested that hepatic hydroxylation at the 25 position of vitamin D_3, in the absence of disease, is not impaired by aging (Dusso et al., 2005).

The decline in serum testosterone with aging may reduce tonic stimulation of the 25-hydroxyvitamin D-1α-hydroxylase (1-OHase; CYP27B1), but if serum 1,25(OH)$_2$D$_3$ remains unchanged indicating that other factors must come into play to compensate for this loss (Gavaia et al., 2006). However, estrogen deficiency leads to a decrease in serum 1,25(OH)$_2$D$_3$ regardless of age. Estrogen

replacement in postmenopausal women can increase both total and free serum $1,25(OH)_2D_3$, suggesting that menopause and the accompanying estrogen deficiency may remove an important trophic factor for the maintenance of serum $1,25(OH)_2D_3$ in aging women (Marcus et al., 1992). Serum Ca has been shown to influence directly 1-OHase activity, but it has not been determined whether the response of serum $1,25(OH)_2D_3$ to direct modulation by Ca changes with age. Phosphorus directly regulates 1-OHase activity, short-term low phosphorus increases serum $1,25(OH)_2D_3$ level in adult rats which is likely to be mediated by a decrease in metabolic clearance via the down-regulation of both renal 24-OHase and VDR expression (Lai et al., 2003).

The serum PTH increases progressively with advancing age in men and women. In men serum PTH increases with aging, but $1,25(OH)_2D_3$ does not change, suggesting that the ability of the kidney to respond to PTH may decrease with advancing age. Tsai et al. reported that the increase in serum $1,25(OH)_2D_3$ induced by infusion of bovine PTH was blunted in elderly postmenopausal women with mild to moderate renal insufficiency when compared to that in healthy young women. Although the increment in serum $1,25(OH)_2D_3$ induced by PTH in the elderly may be delayed relative to that in the young, maximum renal responsiveness to PTH in healthy elderly men was not impaired (Dusso et al., 2005). These studies leave open the question as to how basal serum PTH can be elevated in the elderly without causing an increase in serum $1,25(OH)_2D_3$. Physiological studies have indicated that the age-related decline in serum GH and IGF may reduce tonic stimulation of 1-OHase. In

response, the serum levels of $1,25(OH)_2D_3$ would be predicted to decrease, which would predictably reduce Ca absorption in the intestine, thereby leading to hypocalcemia. PTH would be expected to increase as a result of the hypocalcemia. Accordingly, $1,25(OH)_2D_3$ synthesis would increase through the direct actions of PTH on renal 1-OHase, thus offsetting the normal stimulus provided by GH/IGF (Lieberman et al., 1994).

VDR levels in rat intestine and kidney decrease with advancing age. Similarly, a 23% decrease in the expression of VDR mRNA occurred in aged rats in comparison with adult rats. A comparable decrease in CaBP9k mRNA was also noted. M.A.Hirst and D.Feldman found that the concentration of rat intestinal VDR fell by approximately 25% in chronically ovariectomized, aged rats compared to age-matched controls (Feldman et al., 1979). In an important study of human subjects, Ebeling et al determined VDR levels using an immunoradiometric assay of duodenal biopsy specimens from 35 female volunteers. They found a statistically significant decrease in VDR with age (Dusso et al., 2005).

1.1.4.2 Relationship of $1,25(OH)_2D_3$ with Osteoporosis

Although it is clear that PTH and vitamin D_3 metabolism change with aging, the relationship between these changes and bone loss is not certain. A number of studies have shown that increased bone turnover, bone mineral density or bone loss do not depend on the age-related increase in PTH or decrease in $25OHD_3$ and $1,25(OH)_2D_3$. Indeed, there is no conclusive evidence indicating that bone turnover does increase progressively after menopause or in men. Conversely, some studies have shown a relationship between bone mineral density and the level of $1,25(OH)_2D_3$, PTH and $25OHD_3$. Ooms et al. reported that $25OHD_3$ levels did correlate with hip BMD below the threshold of 30nmol/liter (Ooms et al., 1995). In one study, PTH did relate to rates of bone loss from the forearm in premenopausal women (Lukert et al., 1992). A model to describe the interrelationship between bone loss, PTH, and vitamin D_3 metabolism is shown in Figure1-1-2.

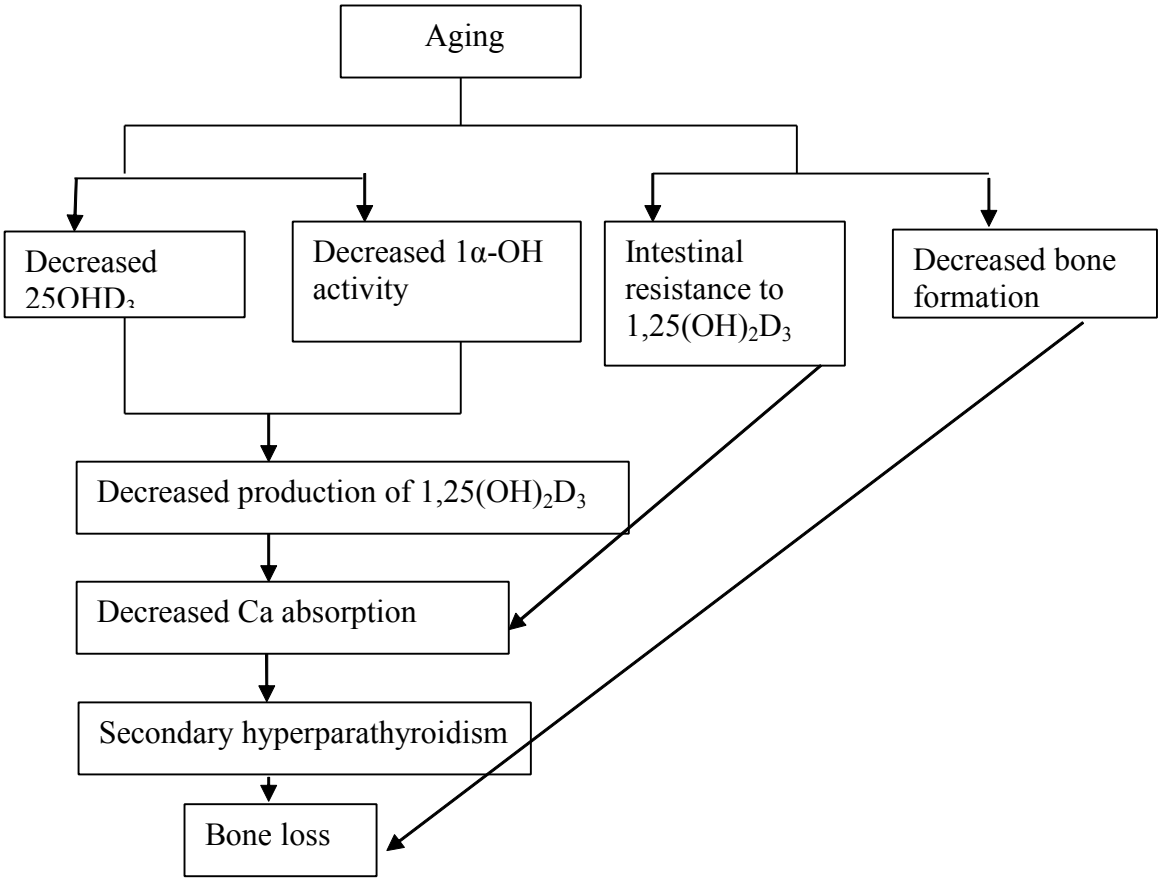


Figure 1-1-2 The interrelationship between bone loss, PTH, and vitamin D_3 metabolism

(Adapted from (Feldman et al., 1997)

Thus, a number of changes in vitamin D_3 and its actions occur with age.

1. A decrease in $25OHD_3$ that probably results from decreased UV light exposure, decreased effect of UV light on the skin sythesis of vitamin D_3, and decreased absorption of vitamin D_3 from the diet.
2. A decrease in $1,25(OH)_2D_3$ after the age of 65 years that is partly a result of the decrease in the substrate, $25OHD_3$, and partly a result of the decrease of renal 1α-OHase activity.
3. A decrease in active Ca absorption that results from the decrease in vitamin D_3 metabolites, intestinal resistance to the action of vitamin D_3, and estrogen deficiency.
4. An increase in PTH secretion.

As stated, Ca homeostasis and vitamin D metabolism are closely related to osteoporosis. It is necessary to make clear how the body maintains Ca homeostasis and vitamin D_3 metabolism.

1.2 Calcium Homeostasis

1.2.1 Body calcium compartments

Ca exists in three quite distinct divisions (or compartments). The first and most obvious compartment is the Ca in the bones and teeth. Ca exists in bones and teeth as inorganic mineral crystals, arranged in an imperfect apatite lattice with variable stoichiometry, and embedded in a dense protein matrix. There is very limited exchange of Ca ions between the bone and the circulating body fluids. So the exchangeable bone Ca moieties occupy only 0.1% of total skeletal Ca. The second biologically critical compartment is intracellular Ca. Here Ca serves as a ubiquitous second messenger, which links signals from outside the cell to the mechanisms constituting the cell's response. Most of the intracellular Ca binds to specialized Ca storage proteins (e.g., sequestrin, parvalbumin, calbindin) and they locate in the storage vesicles and specialized units of the endoplasmic reticulum. This cytosolic Ca compartment is tiny and exchange between extracellular fluid (ECF) and cell stores of Ca is surprisingly slow. Cellular Ca stores are typically unaffected by acute changes in Ca concentrations of ECF. The third and smallest division of body Ca is the Ca present in the circulating blood and the ECF. This compartment contains about 0.4mmol Ca per kg body weight. Ionized Ca concentration in these fluids is about 1.25mmol/L (Feldman et al., 2005).

1.2.2 Mineralization of bone

Mineralization of bone is a passive phenomenon, lagging several days, and even weeks behind the osteoblastic cellular activity that initiates the process. The growing mineral deposits extract Ca and phosphorous from blood. It is much higher during growth, of course, and rises once again after mid life (Chapuy et al., 1996).

1.2.3 Obligatory loss

Obligatory loss consists of a combination of cutaneous loss and the fixed components of urinary and endogenous fecal Ca excretion. Cutaneous losses consist not just of sweat Ca but of the Ca contained in shed skin, hair, and nails. The losses are estimated to be at least 0.4mmol/day and more likely closer to 1.5mmol/day (Rianon et al., 2003). On a normal diet, the endogenous fecal Ca values average within 3mmol/day. Since absorption efficiency in adults is never over 60%, there is an irreducible minimum loss of endogenous Ca through the gut averaging to 2mmol/day. On average, the level of obligatory urinary Ca loss amounts to about 2mmol/day. But it will rise with the increasing excretion of sodium or protein (Heaney and Recker, 1982). So given typical adult diets, the sum of these obligatory losses through skin, gut, and kidney is about 5±1 mmol/day, or about one fifth of the total Ca in the ECF (Feldman et al., 2005).

1.2.4 Ca absorptive input

Ca (Ca^{2+}) absorption occurs in epithelia, including kidney, intestine, placenta, mammary glands, and gills. In mammals, the small intestine and kidney constitute the influx pathways into the extracellular Ca^{2+} pool. Ca^{2+} transport is mediated by a complex array of transport processes that include paracellular and transcellular pathways. The paracellular pathway allows the direct exchange of Ca^{2+} between two compartments, while the transcellular route involves transport across at least two plasma membrane barriers (Hoenderop et al., 2005).

1.2.4.1 Paracellular pathway

Epithelia consist of a continuous layer of individual cells, and the narrow intercellular spaces between the epithelial cells can allow the diffusion of small molecules and ions. The intercellular spaces are called tight junctions. And this route is called the paracellular pathway (Goodenough, 1999). Although this route has not been investigated more than transcellualr pathway, it has been identified to be related to particular inherited diseases, including familial hypomagnesemia (Simon et al., 1999), hypertension (ID, 2003), and autosomal recessive deafness (Wilcox et al., 2001), confirming the importance of paracellular transport.

Tight junctions consist of linear arrays of integral membrane proteins, which include occludin, claudins, and several immunoglobulin superfamily members, such as the junctional adhesion molecule. The claudin family consists

of at least 20 related integral membrane proteins with four transmembrane domains and functions as major structural components of the tight junctional complex (Hoenderop et al., 2005). Tight junctions are crucial for epithelial cell functions. Epithelial cells can rapidly change the structure and permeability of tight junctions through intriguing mechanisms depending on the extracellular and intracellular environment (Rajasekaran et al., 2008). An uncontrolled increase in endothelial permeability underlies pathologies such as atherogenesis, acute lung injury, and metastasis. And the paracellular or junctional permeability is regulated by the integrity of interendothelial junctions (IEJs). Disruption of IEJs is achieved by increasing intracellular Ca^{2+} to stimulate MLC-dependent cell contraction as well as disassembly of junctional proteins comprising tight junctions (TJs) and adheres junctions (AJs) (Vandenbroucke et al., 2008). It has been indicated that growth factors, cytokines, hormones, drugs and nutrients have specific roles in the regulation of tight junction permeability (Laukoetter et al., 2006; Schneeberger and Lynch, 2004). Recent studies revealed that ions and ion transporters are also involved in the regulation of tight junction structure and functions which might function by being physically present at the junctions and modulating a local ionic milieu or changes in the ionic content of the cells and transmit signals to eventually alter tight junction structure and functions. These ion transporters include Na,K-ATPase, Na^+-glucose cotransporters, K^+-ATP channels, chloride channels and non-selective cation channels of the transient receptor potential (TRP) superfamily and so on (Rajasekaran et al., 2008).

1.2.4.2 Transcellular pathway

Transcellular Ca transport is a three-step process, comprises the transfer of luminal Ca into the enterocyte or renal epithelial cell through Ca-selective channels (TRPV5/TRPV6), the translocation of Ca from point of entry to the basolateral membrane by vitamin D_3-dependent Ca binding proteins (Calbindin D9k/Calbindin D28k), and finally active extrusion from the cell into the circulatory system mediated by a high-affinity plasma membrane Ca^{2+}-ATPase (PMCA1b) or Na^{+}/Ca^{2+} exchanger (NCX1) (Hoenderop et al., 2005) (Figure1-2-1).

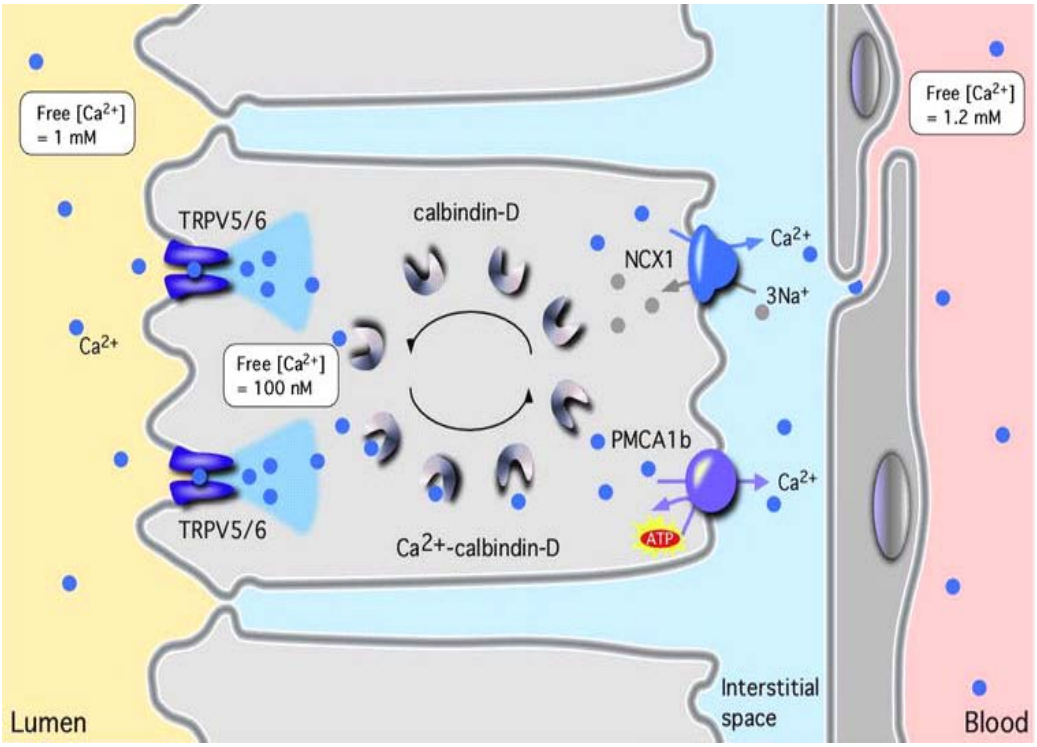


Figure 1-2-1 Transcellular Ca^{2+} transport in kidney and small intestine.

Schematic representation of transcellular Ca^{2+} transport consisting of apical entry of Ca^{2+} through the epithelial Ca^{2+} channels transient receptor potential vanilloid 5 and 6 (TRPV5 and TRPV6), cytosolic diffusion bound to Ca^{2+}-binding proteins (calbindins) and extrusion across the basolateral membrane by a Na^{+}/Ca^{2+} exchanger (NCX1) and/or a plasma membrane Ca^{2+}-ATPase (PMCA1b). Transcellular Ca^{2+} (re)absorption occurs in the distal convoluted and connecting tubules in kidney involving TRPV5 as well as in duodenum involving TR.
Adapted from (Hoenderop et al., 2005)

A. Epithelial Ca^{2+} channels

Ca^{2+} enters the epithelial cell via Ca^{2+}-selective channels at the luminal membrane under the influence of a steep, inwardly directed electrochemical gradient. TRPV5 (previously named ECaC1 or CaT2) and TRPV6 (previously named ECaC2 or CaT1) were discovered to be the epithelial Ca^{2+} channels, which are homologous members of the transient receptor potential (TRP) superfamily. Their expression pattern is quite variable between different species and tissues (den Dekker et al., 2003). TRPV5 is the major isoform in the kidney, while TRPV6 is highly expressed in the intestine. But in humans, they are coexpressed in the kidney and intestine and also in other organs such as pancreas, prostate, mammary, sweat and salivary glands (van Abel et al., 2005a).

The gene encoding TRPV5 consists of 15 exons, which translates into a protein of 729 amino acids in human. This protein contains six putative transmembrane domains and intracellular N and C tail. Functional TRPV5 channels exist as tetramers forming together a single Ca^{2+} selective pore (Hoenderop et al., 1999). TRPV5 plays a critical role in renal Ca^{2+} handling demonstrated by the generation of TRPV5 knockout mice (Hoenderop et al., 2003). In renal epithelial cells, after gene transcription and protein translation, TRPV5 is assembled into functional tetrameric channels. They reach the plasma membrane through the Golgi apparatus and allow the influx of Ca^{2+}. Then TRPV5 enters its recycling process and interaction with Rab11a at last step make it become the active channels again (de Groot et al., 2008) (Figure1-2-2). Factors regulating TRPV5 activity include Klotho, tissue kallikrein, pH, Ca^{2+},

intracellular Mg^{2+}, PIP_2 and WNK4. Klotho is a single-pass transmembrane protein containing a large extracellular domain, which exhibits β-glucuronidase activity upon cleavage. Locally excreted Klotho glycosylated TRPV5 induces the channel accumulation in the plasma membrane of distal convoluted tubule (DCT) and the connecting tubule (CNT) (Chang et al., 2000). In the absence of Klotho, TRPV5 may not be well expressed at the luminal membrane (Tsuruoka et al., 2006). Tissue kallikrein (TK) is a serine protease that is produced as an inactive precursor in the CNT. It was indicated that TK could increase TRPV5 expression in an autocrine/paracrine manner (Hoenderop et al., 2004). TRPV5 activity appeared to be under direct control of the acid-base states. Both intra- and extracellular acidification reduced TRPV5 single-channel activity (Nijenhuis et al., 2006). Intracellular Ca^{2+} levels serve to regulate channel function to maintain optimal Ca^{2+} reabsorption without excessive influx of Ca^{2+} (de Groot et al., 2008). In HEK293 cells, intracellular Mg^{2+} was shown to reversibly inhibit TRPV5 activity during negative membrane potentials. The plasma membrane phospholipids PIP2 significantly reduced TRPV5 sensitivity for Mg^{2+} (Eeleen, 2005). WNKs are a recently discovered family of serine/threonine protein kinases that regulated ion transport. They affected renal Ca^{2+} handling by regulating TRPV5 function (Qin LM, 2002).

The human TRPV6 gene is locacted on chromosome 7q33-q34. TRPV5 is locacted on 7q35, which exhibits approximately 75% identical amino acid to TRPV6 (Peng et al., 2000). TRPV6 usually has posttranslational glycosylation and its molecular weight amounts about 75-100kDa (Bodding et al., 2002).

TRPV6 occurs predominantly in the small intestine and colon of rat (Peng et al., 1999), but in human and murine tissues, no detectable level of TRPV6 was found in the small intestine and colon (Barley et al., 2001). The predominant TRPV6 expression is detected in the placenta and pancreas of human and murine origin (Hirnet et al., 2003). There are many differences in TRPV6 expressions in rat and human. TRPV6 channels have an identical glycosylation site as TRPV5, so they are also influenced by the action of Klotho (Hirnet et al., 2003). It was also demonstrated that Src-dependent tyrosine phosporylation enhanced TRPV6 activity in an overexpression system. And this activation can be prevented by dephosphorylation by the tyrosine phosphatase PTP1B (Sternfeld et al., 2005). Binding of PIP_2 could also activate TRPV6 and furthermore decrease sensitivity of the channel to Mg^{2+}-induced slow inhibition (Eeleen, 2005). One characteristic feature of TRPV6 is the initially rapid and subsequently slower decay of its Ca currents during prolonged stimulation by hyperpolarizing potentials (Bodding et al., 2002).

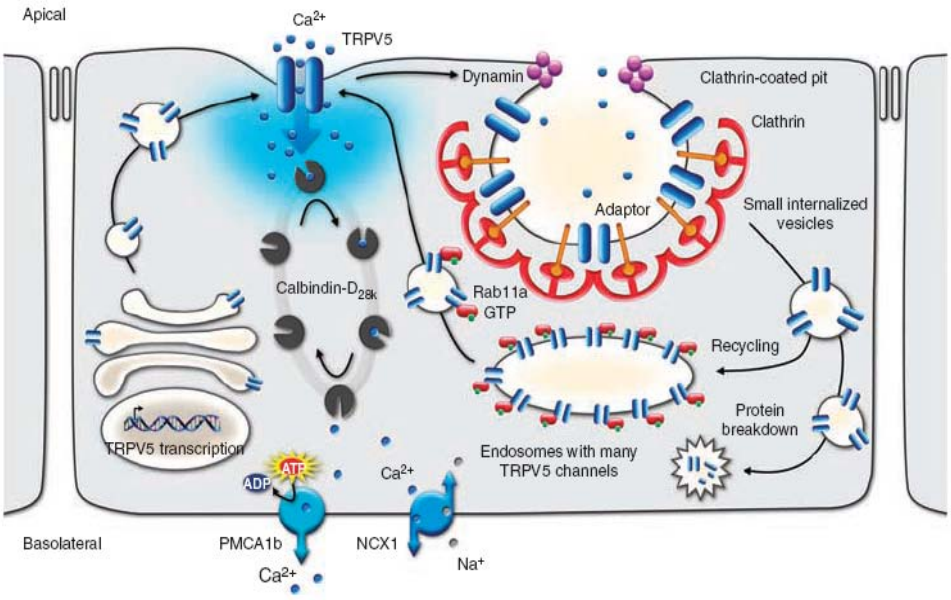


Figure 1-2-2 TRPV5 function and regulation in active Ca^{2+} reabsorption.

Adapted from (de Groot et al., 2008)

B. Cytosolic Diffusion

There are two major subclasses of vitamin D-dependent Ca^{2+}-binding proteins, calbindin-D_{9K} (CaBP9k) and calbindin-D_{28K} (CaBP28k). These cytosolic proteins have been proposed as shuttles that can bind Ca^{2+} and facilitate the Ca^{2+} diffusion between the apical and basolateral surfaces of the cell (Hoenderop et al., 2005). The calbindins, like CaM, belong to a group of intracellular proteins that bind Ca^{2+} with high affinity and undergo structural changes upon binding (Berggard et al., 2002). Each calbindin is encoded by a separate gene, and there is no direct association between the two genes. CaBP28k was first found in the chick duodenum and then CaBP9k was identified and characterized in mammalian duodenum. CaBP28k presents in kidney, small intestine (only birds), pancreas, placenta, bone, and brain, and CaBP9k is present in highest concentrations in small intestine and in kidney (only mouse) (Hoenderop et al., 2005). It has been generally accepted that CaBP9k and CaBP28k facilitate the cytosolic diffusion of Ca^{2+} from the apical influx to the basolateral efflux sites and act as cytosolic Ca^{2+} buffer to maintain low intracellular Ca^{2+} levels during changes in transcellular Ca^{2+} transport (Bindels, 1993). In addition, calbindins may function as Ca^{2+} sensors due to their biochemical properties related to their EF-hand motifs and binding with Ca^{2+} will produce conformational changes in the proteins (Venyaminov et al., 2004). However, the “cytoplasmic ferry” model is cast into doubt by some recent observations. DeLuca and his coworkers found that CaBP9k-null mice were fully capable of absorbing Ca^{2+} from the intestine in response to

$1,25(OH)_2D_3$ and were also able to reproduce normally and had no impaired Ca^{2+} homeostasis, which clearly proves that CaBP9k is not needed for steroid-induced Ca^{2+} absorption (Akhter et al., 2007).

C. Extrusion Mechanisms

The efflux of Ca^{2+} occurs against a considerable electrochemical gradient, and two Ca^{2+} transporters are found in the basolateral membrane of absorptive cells to extrude Ca^{2+}, i.e., a Na^+/Ca^{2+} exchange mechanism (NCX) and a Ca^{2+}-ATPase (PMCA).

Three genes for NCX, designated NCX1, NCX2, and NCX3, have been identified in mammals. They have a homology of ~70% sequence identity. Of the three isoforms, NCX1 is widely distributed among different mammalian tissues, including absorptive epithelial, whereas NCX2 and NCX3 are confined primarily to brain and skeletal muscle. NCX1 has been demonstrated to be primary extrusion mechanism in the distal tubular cells, but its role in the enterocytes may be of minor importance only (Khanal and Nemere, 2008). In fish enterocytes, NCX appears to be the main mechanism by which transcellular Ca^{2+} fluxes are extruded from the cells at the basolateral surface, whereas in mammals PMCA is the predominant extrusion mechanism (Hoenderop et al., 2005). That is to say, in the kidney, basolateral Ca^{2+} extrusion is mainly carried out via NCX1, whereas Na^+/Ca^{2+} exchange seems of minor importance in the small intestine. It has been shown that NCX1 is regulated by PTH which has been shown to stimulate markedly Ca^{2+} reabsorption in the distal part of the

nephron. However, the exact mechanism remains controversial. In addition to PTH, $1,25(OH)_2D_3$ was also shown to regulate the renal expression of NCX1. Studies in vitamin D-deficient knockout models showed an impressive downregulation of NCX1 mRNA that could be normalized by $1,25(OH)_2D_3$ supplementation. In these animal models there was no significant downregulation of PMCA in line with a primary role of NCX in Ca^{2+} extrusion (Hoenderop et al., 2005).

PMCA belongs to the P-type pump family, which is characterized by the formation of a high-energy phosphorylated intermediate during the reaction cycle (Palmgren and Axelsen, 1998). PMCA has two conformational states described to be E1 and E2. In the E1 state, PMCA is assumed to expose a high affinity Ca^{2+} binding site to the cytoplasmic side of the plasma membrane. In the E2 state, PMCA exposes the bound Ca^{2+} to the extracellular face and decreases the affinity of its binding site, liberating it outside of the cell. Then the enzyme will return to the E1 conformation (Di Leva et al., 2008). In mammals, PMCA is the product of four distinct genes (ATP2B1-4), located on human chromosomal loci 12q21-23, 3p25-p26, Xp28 and lq25-q32 (Wang et al., 1994). Four genes encode separate isoforms designated PMCA1-4. All four PMCA isoforms are distinctively expressed in the kidney, and variable abundance of the individual isoforms along the different regions of the nephron has been documented (Strehler and Zacharias, 2001). Of them, PMCA1b is the predominant isoform and abundantly expressed in the small intestine and it is the principal Ca^{2+} extrusion mechanism in intestinal Ca^{2+} absorption (Kip and

Strehler, 2003). In general, there is only limited data available regarding the regulation of PMCA by hormones or signaling mechanisms. Several studies indicated that PMCA is positively regulated by $1,25(OH)_2D_3$ in the intestine to increase Ca^{2+} absorption (Cai et al., 1993). In MDCK cell lysates, it was found to be upregulated by $1,25(OH)_2D_3$ in a time- and dose-dependent manner (Kip and Strehler, 2004). In addition, Prince and colleagues demonstrated a stimulatory effect of estrogen and dihydrotestosterone on PMCA activity measured in isolated vesicles from Madin-Darby bovine kidney cells (MDBK). But it was found that estrogen increased PMCA activity without increasing its protein expression, while $1,25(OH)_2D_3$ upregulated its protein levels (Prince et al., 1995). The traditional PMCA activity activator is calmodulin which interacts with a domain (CaM-BD) located in the C-terminal cytosolic tail of the pump. The binding of calmodulin to the CaM-BD is assumed to displace the latter from the receptor sites, leading to the decrease of K_d for Ca^{2+} from 10-20uM to < 1uM (Di Leva et al., 2008). The activity of PMCA is also influenced by the phospholipids composition in the surrounding plasma membrane. Acidic phospholipids and polyunsaturated fatty acids activate the pump by binding to two distinct regions: one is the CaM-BD itself (Brodin et al., 1992), and the other is located in the first cytosolic loop of the pump (Pinto Fde and Adamo, 2002).

1.2.4.3 Endocrine regulation of epithelial Ca^{2+} transport

A. Regulation by $1,25(OH)_2D_3$

$1,25(OH)_2D_3$ regulates epithelial Ca^{2+} transport through genomic actions involving the classical VDR and nongenomic regulation by a separate membrane receptor (Khanal and Nemere, 2008). The TRPV proteins are primarily regulated by $1,25(OH)_2D_3$, but its effects require the presence of functioning VDR. A vitamin D response element (VDRE) has been detected in TRPV5 (Hoenderop et al., 2005). But TRPV6 gene lacks recognizable VDREs. So it has been suggested that TRPV6 might be controlled by a novel $1,25(OH)_2D_3$-mediated mechanism (Song et al., 2003). TRPV6 expression was $1,25(OH)_2D_3$ dependent in men but not in older women, where expression of both TRPV6 and VDR were reduced (Walters et al., 2006). It has been demonstrated in many early studies that $1,25(OH)_2D_3$ stimulates the expression of calbindin-D9k and calbindin-D28k in humans and many animal models. Functional VDREs have been detected in these mammalian calbindin genes. However, a tight correlation between calbindin expression and Ca^{2+} transport is not always present. Recent studies indicate that CaBP9k is not needed for steroid-induced Ca^{2+} absorption (Akhter et al., 2007). The results indicated that effects of $1,25(OH)_2D_3$ on PMCA might be species, organ, or tissue specific. Both vitamin D and $1,25(OH)_2D_3$ have been found to increase PMCA mRNA and protein levels in the intestine but not in the kidney (van Abel et al., 2005b). The synthesis of PMCA has not been shown to be $1,25(OH)_2D_3$ dependent.

B. Regulation by PTH

The parathyroid glands play a key role in maintaining the extracellular Ca^{2+} concentration through their capacity to sense even minute changes in the level of blood Ca^{2+} from its normal level through the Ca^{2+}-sensing receptor (CaSR). In response to low blood Ca^{2+} levels, PTH is secreted into the circulation and then acts primarily on the kidney and the bone, where it activates the PTH/PTHrP receptor. This receptor directly enhances the tubular Ca^{2+} reabsorption, and it stimulates the activity of 1-OHase and, thereby, increases the $1,25(OH)_2D_3$-dependent absorption of Ca^{2+} from the intestine (Hoenderop et al., 2005). PTH increases transepithelial Ca^{2+} transport via a dual signaling mechanism involving PKA- and PKC-dependent processes (Friedman et al., 1996). The potential mechanisms of PTH action include that membrane insertion of apical Ca^{2+} channels (Bacskai and Friedman, 1990), opening of basolateral chloride channels resulting in cellular hyperpolarization (Friedman and Gesek, 1994), and modulation of PMCA activity (Tsukamoto et al., 1992). It was also reported that PTH affects renal Ca^{2+} handling through regulation of the expression of active renal Ca^{2+} transport proteins, including TRPV5 (Tsukamoto et al., 1992). Recently, many laboratories reported the presence of functional PTH receptors in intestinal epithelial cells which could respond to the hormone with stimulated Ca transport in perfused duodenal loops and enhance Ca uptake in isolated enterocytes (Khanal and Nemere, 2008). The signal transduction pathway activated by PTH binding to its receptor includes PKA-

mediated Ca uptake (Sterling and Nemere, 2007). Further studies need to be done to explore PTH action on intestine.

C. Regulation by Estrogens

Estrogen deficiency results in a negative Ca^{2+} balance and bone loss in postmenopausal women. Estrogen deficiency resulted in bone loss which is associated with a rise in plasma and urinary Ca^{2+} (Hoenderop et al., 2005). There is increasing evidence that estrogen exerts a physiological role in the regulation of renal and intestinal Ca^{2+} (re)absorption. Estrogen receptors (ER) reside in proximal and distal tubules of the kidney and in the duodenum and colon. Estrogen could upregulate the expression of TRPV5 in the kidney and TRPV6 in the intestine in a $1,25(OH)_2D_3$-independent manner (Hoenderop et al., 2002; van Abel et al., 2003). The estrogen effects seem to be mediated solely by ER. An estrogen-responsive element has been found in the promoter sequence of the mouse TRPV6 gene (Gray et al., 1974). Transcriptional activation by the estrogen-liganded estrogen receptor can be mediated through other elements including activator protein 1 (AP-1) binding sites and GC-rich stimulatory protein (Sp1) binding sites which are contained in the human TRPV5 promoter (Hoenderop et al., 2001).

D. Regulation by Dietary Ca^{2+}

The bioavailability of dietary Ca is an important factor in determining the efficiency of intestinal Ca absorption. Diets low in Ca increase intestinal Ca absorption, at least, in part, by increasing $1,25(OH)_2D_3$ production (Hoenderop et al., 2005; Zhang et al., 2007). In mature female rats, it was suggested that OVX induced malabsorption was mediated through PTH and $1,25(OH)_2D_3$ in response to low Ca diet (Zhang et al., 2007). Dietary Ca can act as an additional regulatory mechanism of Ca handling independent of vitamin D (Van Cromphaut et al., 2001). Expression of intestinal TRPV6, CaBP9k, and PMCA1b are normalized by a rescuing Ca diet (Hoenderop et al., 2002). In 1-OHase knockout mice, a high dietary Ca load normalizes plasma Ca concentration and expression of renal TRPV5, CaBP28k, NCX1 and PMCA1b (Hoenderop et al., 2002).

There are still many other endocrine hormones acting on Ca transport across epithelial. Some of the hormones have both positive and negative effects, whereas some others have positive effects only. Varying effects of the same hormone probably depend on the cell system, physiological stage, and hormone concentration (Khanal and Nemere, 2008) (Figure 1-2-3).

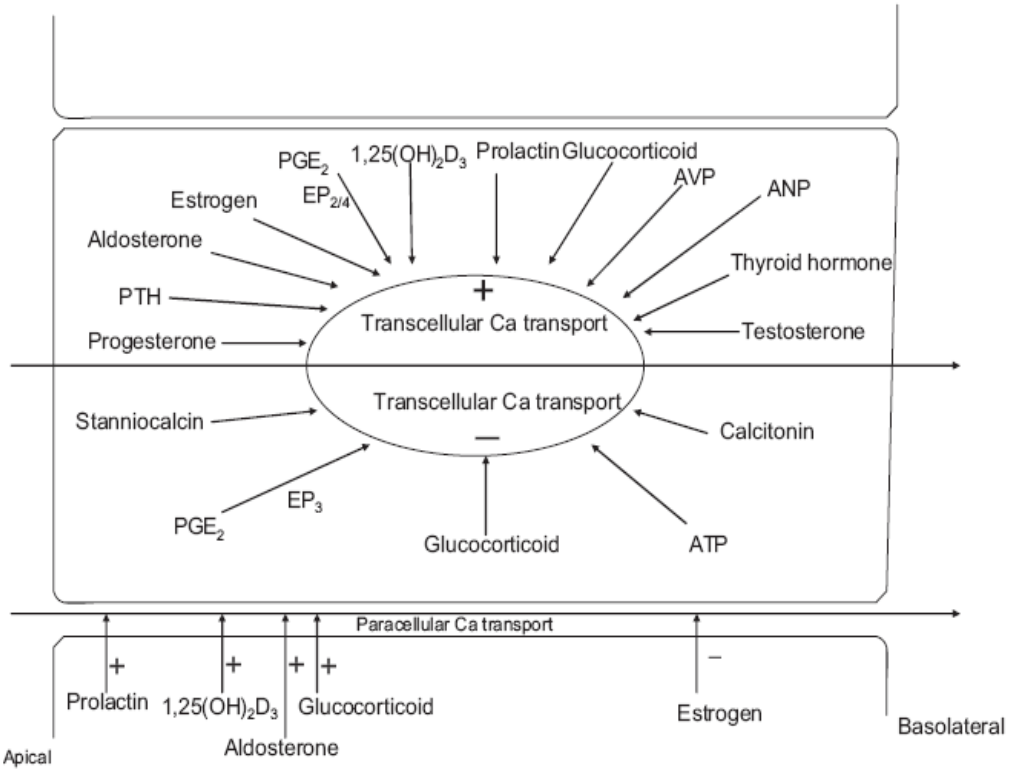


Figure 1-2-3 Schematic model for endocrine control of Ca transport in epithelial cells.

Adapted from (Khanal and Nemere, 2008)

1.3 Vitamin D

1.3.1 Chemistry and Metabolism

Vitamin D was originally identified in the early 1920s as a factor in cod-liver oil that cured rickets, a skeletal disease characterized by undermineralized bones (Orr et al., 1923). The first factor to be identified was designated vitamin D_2 (also known as ergocalciferol), whereas the structure of vitamin D_3 (cholecalciferol) became evident some 4 or 5 years later. Vitamin D_3 is the form of vitamin D that is synthesized by vertebrates, whereas vitamin D_2 is the major naturally occurring form of the vitamin in plants (Norman, 1979).

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is one of the biologically active metabolite of vitamin D_3. Although more than 50 different metabolites of vitamin D_3 have been identified, only $1,25(OH)_2D_3$ is believed to be important for most, if not all, of the biological actions of vitamin D_3 on Ca and bone metabolism. The major portion of vitamin D_3 in the human body, however, comes from photosynthesis in the skin; a process in which UV-B light converts 7-dehydrocholesterol to the unstable previtamin D_3, which isomerizes to vitamin D_3 under the influence of thermal energy (Figure 3-1). Once vitamin D_3 enters the circulation, it is bound to the vitamin D-binding protein (DBP) and transported to the liver where the cytochrome P_{450} –vitamin D-25-hydroxylase introduces an OH on carbon 25 to produce 25-hydroxyviamin D_3 ($25(OH)D_3$). $25(OH)D_3$ enters the circulation and is the major circulating form of vitamin D_3. Measurement of $25(OH)D_3$ is used to determine whether a patient is vitamin D

deficient, vitamin D sufficient or vitamin D intoxicated. $25(OH)D_3$ is biologically inert. It is transported to the kidney where the cytochrome P_{450}–mono-oxygenase, 25(OH)D-1α-hydroxylase (1-OHase; CYP27B1), metabolizes $25(OH)D_3$ to $1,25(OH)_2D_3$ (Feldman et al., 1997) (Figure 1-3-1).

Although the kidney is the major source of circulating $1,25(OH)_2D_3$, there is strong evidence that a variety of tissues and cells, including activated macrophages, osteoblasts, keratinocytes, prostate, colon and breast, express the 1-OHase and have the ability to produce $1,25(OH)_2D_3$ (Wu et al., 2007). In addition, during pregnancy, the placenta produces $1,25(OH)_2D_3$ (Fischer et al., 2007). However, the local production of $1,25(OH)_2D_3$ in tissues not associated with Ca homeostasis may be for the purpose of regulating cell growth and differentiation (Wu et al., 2007). $1,25(OH)_2D_3$ is metabolized in all of its target tissues, as well as in the liver and kidney. It undergoes several hydroxylations in the side-chain by the 25(OH)-24-hydroxylase (24-OHase), causing the cleavage of the side-chain between carbons 23 and 24 and resulting in the biologically inert water soluble acid, calcitroic acid. These metabolites are considered to be biologically inert and are the first step in their biodegradation (Feldman et al., 1997).

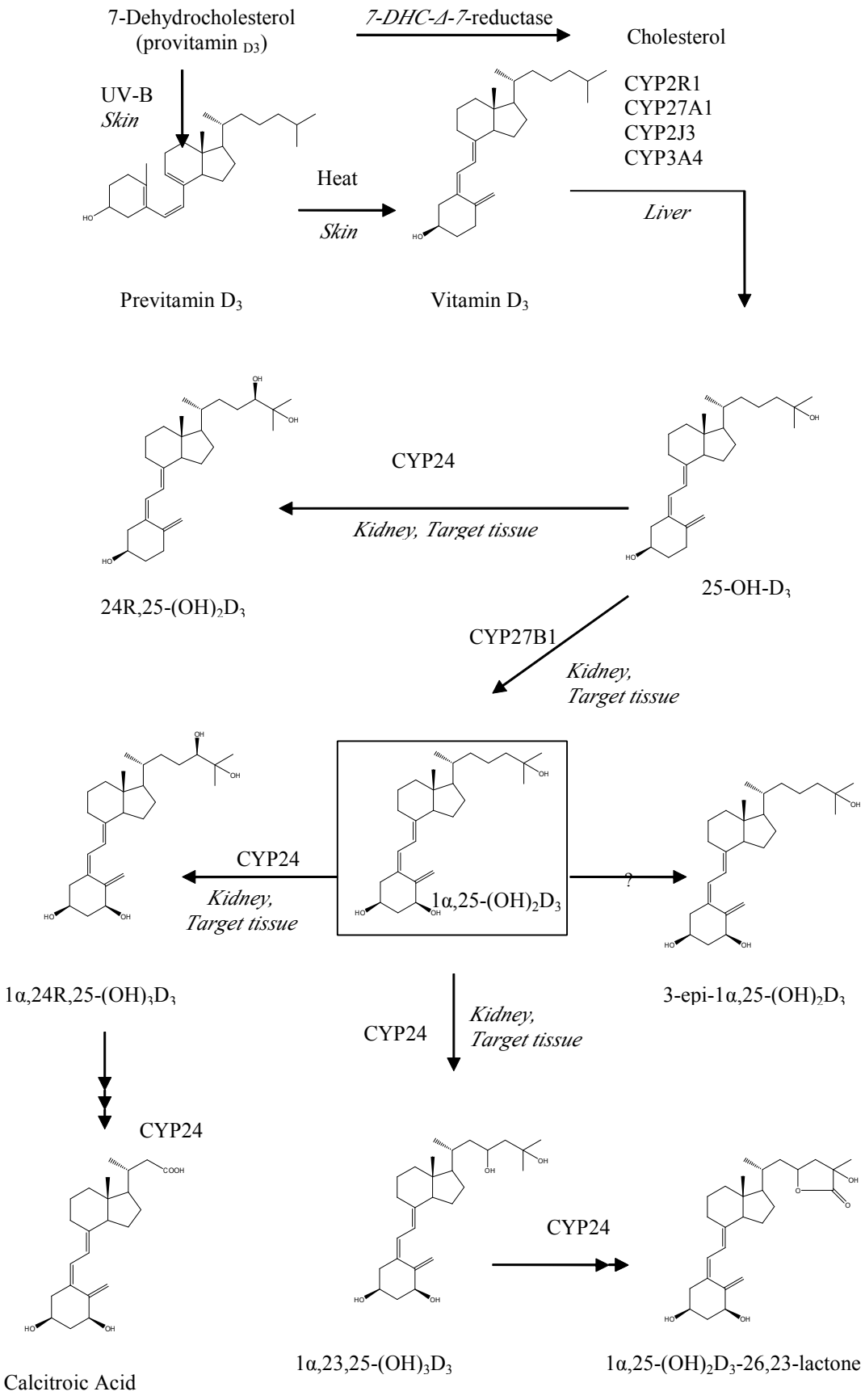


Figure 1-3-1 Pathways for synthesis and metabolism of $1,25(OH)_2D_3$

1.3.2. The 25-hydroxyvitamin D 1α-hydroxylase

$25OHD_3$ is biologically inert. It is transported to the kidney where the cytochrome P_{450}-mono-oxygenase, 25OHD-1α-hydroxylase (1-OHase; CYP27B1), metabolizes $25OHD_3$ to $1,25(OH)_2D_3$. It is now generally accepted that the major contribution to circulating levels of $1,25(OH)_2D_3$ is made by the proximal tubular cells of the renal cortex and that the $1,25(OH)_2D_3$ produced in other cell types most likely serves autocrine or paracrine functions. And the extra renal produced $1,25(OH)_2D_3$ does not respond to the regulatory influences involved in Ca homeostasis, that control the renal enzyme activity (Dusso et al., 2005).

Although the primary sequence of 1-OHase has been deduced from cloned cDNA from several mammalian species, purified preparation of the protein itself is still not routinely obtained. The primary sequence of the cytochrome P450 that catalyzes the 1-OHase of $25OHD_3$, as deduced from its cDNA sequence, reveals that it is structurally related to the mitochondrial sterol side chain hydroxylases and therefore it has been given the systematic name of CYP27B1 (Nelson, 1999).

It has now well been established that the two most important regulators of the activity of the 1-OHase are $1,25(OH)_2D_3$ itself and parathyroid hormone (PTH). But recent research has found that FGF23 is also another important regulator for 1-OHase. In addition, there has been evidence for the involvement

of calcitonin, dietary mineral levels, and hormones of other endocrine systems in the regulation of renal $25OHD_3$ metabolism.

It was recognized that at the time of the localization of the production of $1,25(OH)_2D_3$ in the kidney that, in both avian and mammalian species, vitamin D-deficient animals have higher 1-OHase activity than do vitamin D-replete animals (Fraser and Kodicek, 1970). Within the kidney, both the mRNA and protein product have been observed to be repressed in the presence of $1,25(OH)_2D_3$ in the proximal tubules only (Dusso et al., 2005). However, the suppression of the 1-OHase gene expressed in extra renal tissues by $1,25(OH)_2D_3$ has been described in only a few cell lines derived from extra renal tissues, such as colon-derived cells (Lechner et al., 2007). In the investigation of mechanism of this regression of 1-OHase by $1,25(OH)_2D_3$, Murayama et al proposed that this process occurs via a negative vitamin D response element (nVDRE), located ~500bp upstream from transcription start site (TSS). And this regulation is a cell-type and tissue-specific phenomenon (Murayama et al., 2004). Recent studies by Mikko et al revealed that the responsiveness of the 1-OHase gene to $1,25(OH)_2D_3$ is a cell-type-multiple VDREs that act to recruit and interact with protein super complexes of differing transcriptional abilities (Turunen et al., 2007). They proposed a model that may explain the role of distal and proximal VDREs in the transcriptional regulation of 1-OHase gene in HEK-293 cells (Figure 1-3-2).

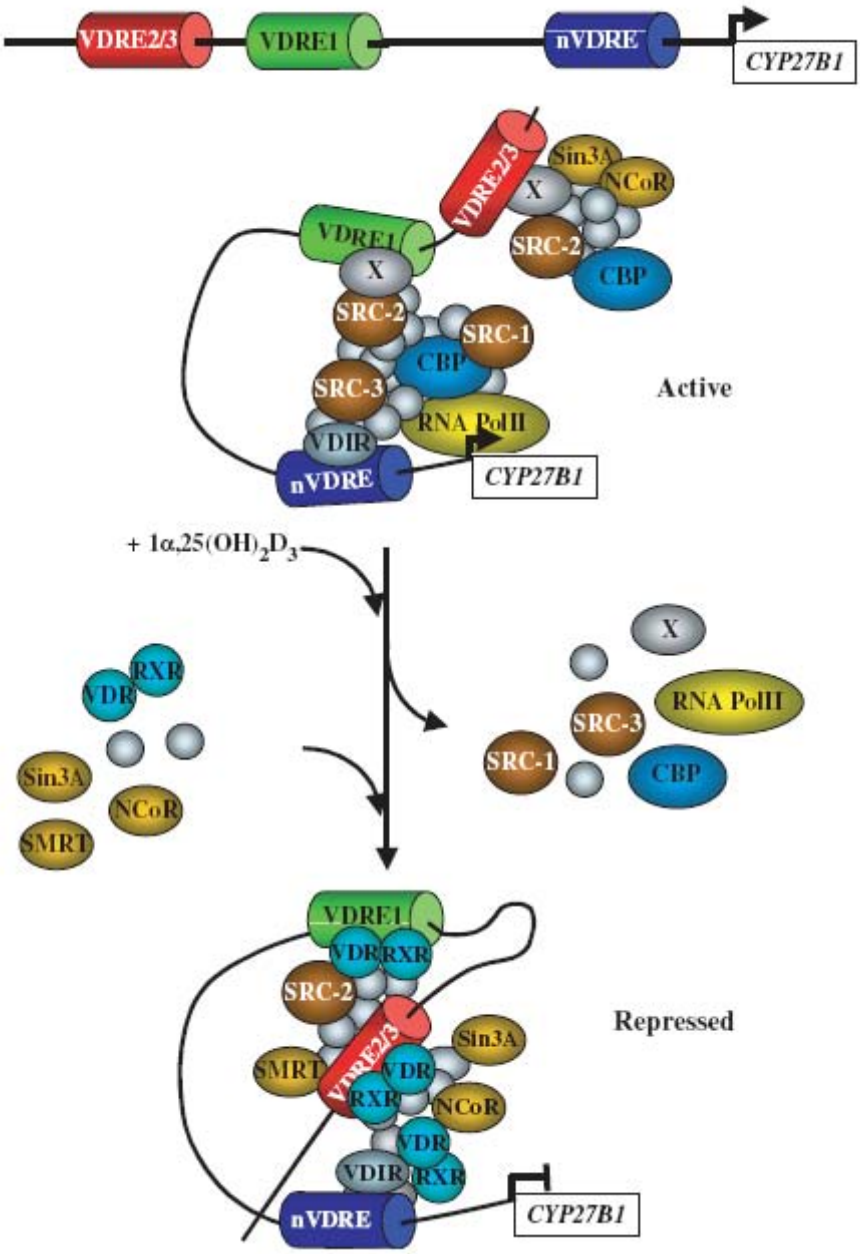


Figure 1-3-2 A model representing the crosstalk of distal and proximal promoter regions during the transcriptional regulation of the CYP27B1 gene.

Adapted from (Turunen et al., 2007)

In whole animal studies, PTH depletion diminishes and PTH repletion increases 1-OHase activity in kidney tissues in both avian and mammalian species (Fraser and Kodicek, 1970). PTH has also been shown to stimulate 1-OHase activity in cultured avian (Rost et al., 1981) and mammalian kidney cells (Kremer and Goltzman, 1982). The stimulatory effect of PTH on 1-OHase activity is mediated at least in part by the cAMP signaling pathway, which can be mimicked by forskolin (Henry, 1985). Protein kinase C (PKC) is also involved in the regulation of renal metabolism of $25OHD_3$ by both the 1-OHase and the 24-OHase (Henry and Luntao, 1989). But the relative physiological importance of the cAMP and PKC signaling pathway in the control of 1-OHase activity has not yet been fully resolved. It has been demonstrated that PTH exerts at least part of its effects on 1-OHase activity at the transcriptional level (Brenza and DeLuca, 2000). During gene array analysis, Claudia et al observed that NR4A2 is markedly up-regulated in a porcine kidney cell line following PTH stimulation (Zierold et al., 2007). NR4A2 (Nurrl) is an orphan nuclear receptor with all of the structural features of a steroid/thyroid hormone receptor, which belongs to a highly conserved subfamily of orphan nuclear receptors (Perlmann and Wallen-Mackenzie, 2004). An unconventional site of action of NR4A2 was proven to be localized to a fragment comprising the sequence from -35/+22 of the 1-OHase promoter at a C/EBP consensus site. And the transcriptional enhancement by NR4A2 on the 1-OHase promoter is inhibited by C/EBPβ which belongs to the CCAAT enhancer binding protein family of transcription factors (Zierold et al., 2007). (Figure 1-3-3)

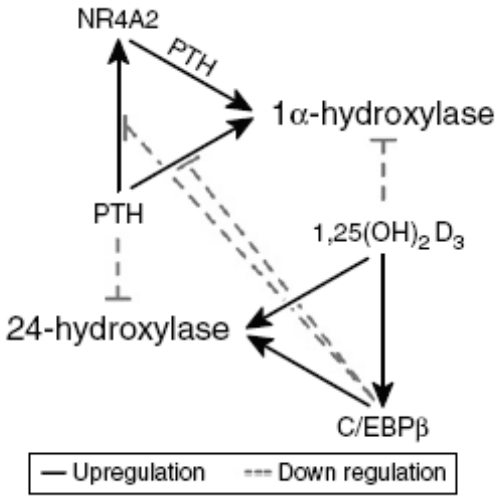


Figure 1-3-3 Diagram of the regulation of the vitamin D hydroxylase by $1,25(OH)_2D_3$, PTH, NR4A2, and cEBP/β.

Fibroblast Growth factor 23 (FGF23) is produced primarily by bone, and in particular by osteoblasts and osteocytes. It has been proven to be a circulating phosphaturic factor that plays a critical role in renal phosphate reabsorption and vitamin D metabolism. Injection of FGF23 *in vivo* causes a reduction in serum $1,25(OH)_2D_3$ followed by hypophosphatemia (Shimada et al., 2004). It was then found that FGF23 decreased expression of 1-OHase and increased 24-OHase in mice and this effect could be confirmed in the kidney cell line as HKC-8 and MCT (Perwad et al., 2007). And FGF-23 directly regulates renal 1-OHase gene expression via activation of the ERK1/2 signaling pathway (Perwad et al., 2007). $1,25(OH)_2D_3$ stimulates the production of FGF23 from bone in as much as FGF23 inhibits $1,25(OH)_2D_3$ production by kidney, this feedback loop like that for PTH secretion maintains a balance in the levels of these important hormones. (Figure1-3-4)

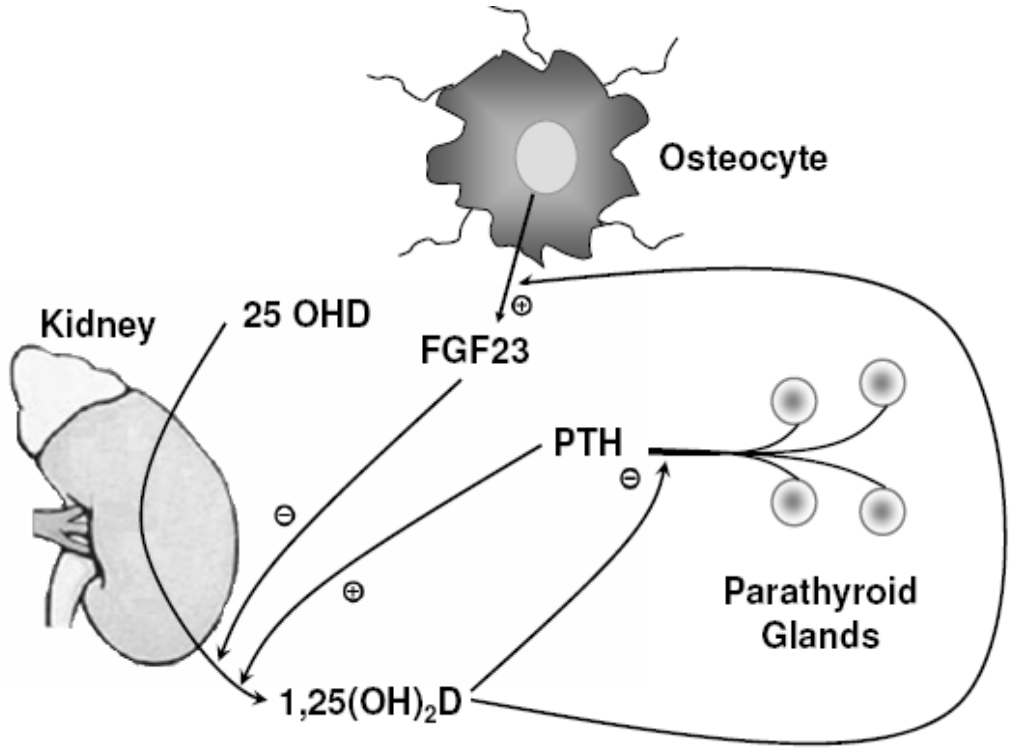


Figure 1-3-4 Interplay of $1,25(OH)_2D_3$, PTH and FGF23 on vitamin D metabolism.

The role of calcitonin in the regulation of renal vitamin D metabolism is controversial. Although there were some *in vivo* reports suggesting that calcitonin injection plays a role in the regulation of vitamin D metabolism (Shinki et al., 1999), experiments in renal cells showed versatile results. A direct stimulatory effect of calcitonin on 1-OHase mRNA levels in the MCT and LLC-PK_1 cells has been reported (Murayama et al., 1999). A recent study reported that in human syncytiotrophoblasts, calcitriol downregulates CYP27B1 expression via a cAMP-dependent signaling pathway, whereas it upregulates 24-OHase gene expression through a VDR-dependent mechanism (Avila et al., 2007).

In human and mammalian animal models, dietary phosphorus is inversely correlated with serum levels of $1,25(OH)_2D_3$ (Portale et al., 1989). It has been demonstrated that the effect of phosphorus is exerted, at least in part, on the transcriptional levels of 1-OHase. And reduced catabolism of $1,25(OH)_2D_3$ may also play a role in the increased serum levels of $1,25(OH)_2D_3$ when dietary phosphorus is low (Zhang et al., 2002).

Steroid hormones of other endocrine systems have been tested for their effects on $1,25(OH)_2D_3$ production *in vivo* or *in vitro*. These include estrogen and the synthetic glucocoticoid dexamethasone. The direct stimulatory effect of estrogen on 1-OHase activity *in vivo* could not be confirmed in cell culture (Baksi and Kenny, 1980). But it was found that estrogen and its analog could up-regulate 1-OHase activity in extra renal tissue culture (Lechner et al., 2006).

Similarly, the effects of glucocoticoid and dexamethasone on 1-OHase activity are variable *in vivo* and *in vitro*. Thus, although interactions between other endocrine systems and the regulation of mineral metabolism by $1,25(OH)_2D_3$ undoubtedly play a role in Ca homeostasis and bone mineral metabolism, they do not apparently take place in a significant way at the level of $1,25(OH)_2D_3$ production in the kidney.

1.3.3 Mechanisms of Action

1.3.3.1 The vitamin D receptor

The vitamin D receptor (VDR) was eventually purified from chicken and porcine intestines in the early 1980s (Evans, 1988). VDR was found to be a 3.3-3.7S protein that bound $1,25(OH)_2D_3$ with high affinity and displayed a pharmacological profile for binding various vitamin D metabolites and analogs that was consistent with their relative biological activities. The VDR has been found in the classic vitamin D target organs, namely, the intestine, bone, kidney, and the parathyroid glands, as well as a host of target tissues not involved in Ca homeostasis, such as skin, muscle, pancreas, reproductive organs, and the hematopoietic, immune and nervous systems (Peng et al., 1999). Table 1-3-1 summarized the VDR distribution and the biological actions of $1,25(OH)_2D_3$ in these target tissues. The range of cultured cell lines defined as targets for $1,25(OH)_2D_3$ were similarly extensive, and now include cells of fibroblastic, osteoblastic, myoblastic, hematopoietic and lymphopoietic origin as well as cells derived from normal kidney, intestine and skin. Furthermore, the biological effects of $1,25(OH)_2D_3$ in tissues and cells extend beyond that of Ca and phosphorus homeostasis to include, among others, a role for $1,25(OH)_2D_3$ in cellular proliferation and differentiation (Feldman et al., 2005).

In some vitamin D target tissues, specific $1,25(OH)_2D_3$-regulated genes have been identified and cloned. Sequencing and promoter analysis of several of these genes has led to the identification of vitamin D responsive elements

(VDREs). The classical VDRE consists of two hexanucleotide direct repeats separated by three intervening base-pairs (DR+3) (Zierold et al., 1995), yet despite the strong homology that exists between the DR+3 VDREs, significant deviations can be observed between elements, as well as between individual half-sites that comprise an element (Koszewski et al., 2000). A list of positive VDREs is shown in Table 1-3-2. These sequences reveal that a "typical" VDRE comprises two hexanucleotide repeats separated by a 3-bp spacer and the general half-site consensus sequence is AGGTCA or GGTTCA, although considerably variability is apparent. Although the sequence of the "spacer" is not generally conserved, these pairs may influence VDR binding (van den Bemd et al., 2002). Traditionally, VDREs are thought to locate relatively close to the transcription start site (TSS) of $1,25(OH)_2D_3$ target genes (Zierold et al., 1995). However, recent studies revealed that the gene promoters may contain multiple response elements that locate not only within proximal promoters but also in more distant regions (Sinkkonen et al., 2005; Vaisanen et al., 2005) and even within coding regions (Healy et al., 2003; Matilainen et al., 2005). On the other hand, a number of negative VDREs (nVDRE) have been described discovered in some genes such as PTH gene (Jaaskelainen et al., 2005), 1-OHase (Turunen et al., 2007), and interleukin-2 gene (Towers and Freedman, 1998). And 1-OHase nVDRE was proved to contain multiple response elements as described before (Turunen et al., 2007).

An important mechanism for the modulation of cellular responsiveness to $1,25(OH)_2D_3$ is mediated by the regulation of receptor abundance. VDR is

regulated by many physiological signals; major differences in VDR regulation exist among species and between various target tissues of the same species; and several different cellular mechanisms are involved in VDR regulation.

Table 1-3-1 $1,25(OH)_2D_3$ actions in classic and nonclassic targets tissues

Tissue	Cell type	Action
Classic		
Intestine	Epithelial	Enhancement of Ca and P absorption
Bone	Osteoblast Osteoclast	Enhancement of bone matrix protein synthesis, bone mineralization, and synthesis of mediators of osteoclastogenis and osteoclastic activity; enhancement of bone resorption
Kidney	Epithelial (proximal and distal)	Inhibition of $1,25(OH)_2D_3$ synthesis and induction of 24-hydroxylase; enhancement of Ca and P reabsorption
Parathyroid gland	Chief	Inhibition of cell growth and PTH synthesis
Nonclassic		
Hematopoietic tissues	Myeloid cell Precursors Colony forming Units	Antiproliferative, prodifferentiating; prodifferentiating
Immune system	Monocyte/ Marcrophages	Enhancement of immune function to control viral and bacterial infectious and tumor growth
	Lymphocyte	Immunosuppression
Skin	Keratinocytes, Fibroblast, Hair follicle, Langerghan cells Melanocytes Smooth muscle	Antiproliferative and prodifferentiating

Muscle	Cell, myoblast Cardiac muscle cell	Antiproliferative, prodifferentiating
Heart	Atrial myocytes	Antiproliferative, prodifferentiating; inhibition of antinatriuretic factor synthesis
Pancreas	ß cells	Enhancement of insulin synthesis and synthesis
Adrenal gland	Medullary cells	Control of catecolamine metabolism
Brain	Hippocampus/ Selected neurons	Neuronal regeneration, enhancement of nerver growth factor and neurotrophin synthesis, control of sphyngomyelin cycle
Cartilage	Chondrocyte	Antiproliferative, prodifferentiating
Female reproductive organs	Myometrial and Endometrial cells	antiproliferative, control of foliculogenesis
Liver	Parenchymal cell (fetal, adult)	Enhancement of liver regeneration; control of glycogen and transferring synthesis
Lung	Fetal pneumocytes Adult pneumocytes	Enhancement of maturation, phospholipids synthesis and surfactant release
Male reproductive organs	Stetoli/ Semminiferus tubule	cell growth Enhancement of Sertoli cell function and spermatogenesis
Pituitary production	Somatomammotroph	Control of T_3-induced growth hormone, prolactin and tyrotrophyn
Thyroid	Follicular cells (C cells)	Inhibition of cell function and calcitonin sythensis

Table 1-3-2 Positive VDREs

Gene	Position	Sequence
Human osteocalcin	-499 to -485	GGGTGAacgGGGGCA
Rat osteocalcin	-460 to –446	GGGTGAatgAGGACA
Mouse osteopontin	-757 to –743	GGTTCAcgaGGTTCA
Mouse calbindin-D_{28K}	-198 to -183	GGGGGAtgtgAGGAGA
Rat calbindin- D_{9K}	-489 to -475	GGGTGTcggAAGCCC
Rat 24-hydroxylase	-151 to -137	AGGTGAgtgAGGGCG
	-239 to -245	CGCACCcgcTGAACC
Avian integrin β3	-770 to -756	GAGGCAgaaGGGAGA
Human p21	-779 to -765	AGGGAGattGGTTCA
	-570 to -556	AGGTGCtccAGGTGC
Synthetic DR3		$PuG^{G}_{T}TCAnnnPuG^{G}_{T}TCA$

1.3.3.2 Homologous regulation of VDR

$1,25(OH)_2D_3$ and other vitamin D metabolites have been shown to up-regulate VDR abundance, whereas many ligands down-regulate their own receptors. This up- regulation was due to an increase in VDR abundance without any change in the affinity for the ligand or in its DNA binding properties. The order of potency among vitamin D metabolites to cause up-regulation was $1,25(OH)_2D_3$= $24,25(OH)_2D_3$ > $1,24,25(OH)_2D_3$ > $25OHD_3$. But this up-regulation requires a functional VDR (Feldman et al., 1997). $1,25(OH)_2D_3$ may or may not increase VDR mRNA abundance, depending on the model system used, and it appears to affect VDR protein turnover by increasing the stability of the VDR protein (Costa et al., 1985). This effect on protein stability may be due to hormone-induced conformational changes that directly affect receptor degradation or that enhances its functional interactions with RXR, DNA, or transcriptional co-regulators and thereby prolong receptor half-life (Feldman et al., 2005). But the mechanism by which $1,25(OH)_2D_3$ regulates renal VDR expression levels may be indirect or posttranscriptional, as no VDREs have been detected in either mouse or human VDR promoter, and no response to $1,25(OH)_2D_3$ has been observed in reporter-gene analysis, suggesting that $1,25(OH)_2D_3$ does not directly activate expression of its own receptor (Byrne et al., 2000).

To prevent ever increasing levels of VDR due to $1,25(OH)_2D_3$ action, the VDR up-regulation must somehow trigger a “turn-off” of the system. This probably occurs by at least two mechanisms. First, $1,25(OH)_2D_3$ induces the

enzyme 24-OHase, which increases the metabolic conversion of the active hormone $1,25(OH)_2D_3$ into inactive metabolites. Second, the actions of $1,25(OH)_2D_3$ increase serum Ca which inhibits PTH production and turns off the renal synthesis of $1,25(OH)_2D_3$ (Dusso et al., 2005).

1.3.3.3 Heterologous regulation of VDR

Heterologous regulation refers to the regulation of VDR abundance by hormones other than $1,25(OH)_2D_3$. VDR can be modulated by numerous physiological stimuli such as dietary composition, steroid hormones and retinoids, growth factors, peptide hormones, and second messenger activators.

The concentrations of Ca and phosphate in the diet are important as indirect modulators of VDR abundance and vitamin D action. In the kidney, Ca is required to act with $1,25(OH)_2D_3$ for VDR production above the basal level, whereas Ca has only a minor effect on intestinal VDR level. Downregulation of renal VDR due to hypocalcemia may be protective measure to block $1,25(OH)_2D_3$-mediated suppression of the 1-OHase and induction of the 24-OHase, which would result in a net increase in serum $1,25(OH)_2D_3$ levels (Beckman and DeLuca, 2002). Recent studies have revealed that extracellular Ca concentration in the physiological range is capable of direct increase of renal proximal tubular VDR expression, and the induction mechanism represents a strategy the body may use to counterbalance effects of PTH on renal vitamin D metabolism (Maiti and Beckman, 2007). Dietary phosphorus restriction up-regulates VDR in a tissue-specific and time-dependent manner (Wong et al.,

1997). A low phosphorus diet resulted in rapid increase in rat intestinal VDR (Meyer et al., 1992), but did not up-regulate kidney or splenic monocyte/macrophage VDR in rat (Hernandez et al., 1996). The mechanisms by which phosphorus may regulate VDR mRNA levels and functional receptor abundance have not yet been elucidated. Increasing evidence showed that lipids regulated VDR levels. An ovariectomy-associated increase in intestinal VDR protein declined after long-term dietary supplementation with essential fatty acids (Leonard et al., 2001). The effects of fatty acids on VDR levels may be indirect, via increased competition by peroxisome proliferators-activated receptor for the VDR heterodimerization partner RXR. Fatty acid's regulation of protein kinase, particularly PKC, may also modulate VDR action and expression levels (Feldman et al., 2005).

Of the steroid hormones that interact with the vitamin D endocrine system, glucocorticoids are of particular interest because of their apparent "anti-vitamin D" effects in several clinical settings. Several investigators have examined the possibility that glucocorticoids modulate $1,25(OH)_2D_3$ actions through the regulation of intestinal VDR, thus inhibiting Ca absorption and contributing to osteoporosis. Thus, there appears to be a pronounced species difference in the response of intestinal VDR to glucocorticoids; an increase occurs in the rat and dog, a decrease occurs in the mouse, and no change is seen in chickens (Manolagas et al., 1979). In addition to VDR regulation in the intestine, a number of *in vitro* studies have investigated the regulation of VDR in bone, the other classic vitamin D target tissue. However, the results were not uniform

(Feldman et al., 1979). The effects of glucocorticoids on VDR might be mediated by direct action of the glucocorticoid receptor on the promoter of the VDR gene and the putative glucocorticoid responsive elements were found to locate in the 5' flanking region of human exon 1C (Byrne et al., 2000).

As steroid hormones that might interact with the vitamin D endocrine system, estrogens are of great interest because of the importance of the syndrome of postmenopausal osteoporosis. The effect of estradiol on VDR is complex and, like glucocorticoids, shows species and organ differences (Feldman et al., 2005). To date, estrogen responsive elements have not been detected in the promoter region of human, mouse, or chicken VDR genes. However, the human exon 1C promoter region was upregulated by E_2 (Byrne et al., 2000). It was demonstrated that E_2 regulates the transcription and expression of VDR *in vivo* in rat colonocytes (Schwartz et al., 2000) and duodenocytes (Liel et al., 1999), and *in vitro* in HT29 human colon cancer cells and MCF7 breast cancer cells (Gilad et al., 2005), by binding to estrogen receptor ß (ERß) and upregulating signal transduction through extracellular signal-regulated kinase (ERK) 1/2 and the activator protein 1 (AP-1) site and Sp-1 site in the VDR promoter (Gilad et al., 2006). Increased VDR expression as a result of E_2 treatment has also been noted in other tissues and cell types, such as the uterus, liver, and human breast cancer cells (Gilad et al., 2006). Estrogen increases VDR expression and bioresponse in rat uterus, duodenal and colon mucosae, and liver; by contrast E_2 decrease VDR expression in the kidneys (Duncan et al., 1991).

PTH is a major Ca regulating hormone which modulates the synthesis of $1,25(OH)_2D_3$. PTH may act on selected vitamin D targets, such as bone and kidney cells, to affect $1,25(OH)_2D_3$ action by regulating VDR abundance. The PTH/PTHrP receptor and VDR are both present in the kidney and osteoblasts. The mechanism of PTH action is mediated through activation of PKA and PKC signal transduction systems, both of which regulate VDR levels in vitamin D target cells (Krishnan et al., 1995). In addition, PTH causes an increase in intracellular Ca levels in target cells, and Ca is another modulator of VDR levels (Bidwell et al., 1991). It was found that PTH and PTHrP could increase VDR content in the preostoblastic cells via cAMP activation (Krishnan et al., 1995), but decrease it in mature osteoblasts (Wong et al., 1997). PTH was also shown to inhibit an increase in intestinal VDR levels after 5-day coinfusion of rats with $1,25(OH)_2D_3$. There was also a partial inhibition of renal VDR levels in that study, accompanied by abolition of CYP24 response to the $1,25(OH)_2D_3$ treatment (Reinhardt and Horst, 1990). And recent studies indicated that PTH-mediated decrease in renal VDR is kidney proximal cell-specific (Bajwa et al., 2005). So the tissue-specific variation in PTH effects on VDR levels presumably relate to the activation of various signaling pathway, depending on the specific target cell type and its state of differentiation.

1.4 Available Medications of Osteoporosis

1.4.1 Approaches used in western medical practice

Numerous pharmacologic and nonpharmacologic interventions may be implemented to slow down or stop bone loss, maintain bone strength, increase bone strength and minimize or eliminate factors that may result in fractures. Nonpharmacologic measures are recommended for the general population at large, while pharmacologic interventions are usually reserved for patients with an increased risk of fractures (Levine, 2006).

At present, Ca supplements are often administered with other agents as a combined therapy for the treatment of osteoporosis. But it seems that Ca has little effect if it is given within the first 5 years of the menopause when bone loss is predominant due to estrogen withdrawal. More recent studies have, however, failed to demonstrate any significant effect of Ca and vitamin D on fracture risk (Keen, 2007).

Hormone replacement therapy (HRT) is an established approach for osteoporosis treatment and prevention. Observational studies have indicated that HRT therapy significantly reduces hip fracture in cohorts of women. However, recent research has shown that HRT is associated with an increase in the risk for postmenopausal women to develop breast, endometrial and ovarian cancers (Davison and Davis, 2003).

Currently, bisphosphonates (alendronate, ibandronate and risedronate), calcitonin, PTH and raloxifene are approved by the US Food and Drug Administration (FDA) for the prevention and/or treatment of osteoporosis. Because they can affect the bone remodeling cycle, they are classified as anti-resorptive medications.

The bisphosphonates reduce bone resorption by inhibiting the activity of osteoclasts and shortening their life span. The North American Menopause Society (NAMS) recommends bisphosphonates as a first line treatment for postmenopausal osteoporotic women. The most common adverse effects associated with these agents are esophageal and gastric irritation, which may result in dysphagia, esphagitis, and esophageal and gastric ulceration (Book, 2006).

Raloxifene is the only approved selective estrogen-receptor modulator, which binds to the estrogen receptor and mimics the activity of estrogen while avoiding some of the adverse effects associated with estrogen. But its application also has side effects, including an increased risk of thromboembolic events and vasomotor symptoms (Ettinger et al., 1999).

Calcitonin is an option for women with osteoporosis who are more than 5 years beyond menopause. It is also used as a second-line therapy for glucocorticoid-induced osteoporosis. Adverse events associated with calcitonin

include nausea, flushing of the face and hands and allergic responses (Book, 2001).

PTH can directly stimulate osteoblasts and increase trabecular bone density and connectivity. The NAMS recommends it to be reserved for patients who are at a high risk of fracture. Its side effects include muscle cramps, nausea, headache, hypercalcemia and dizziness. Moreover, its application may be limited by the daily subcutaneous administration and its high cost (Neer et al., 2001).

1.4.2 Traditional Chinese Medicine

Traditional Chinese Medicine (TCM) has been widely used for thousands of years in China to treat fractures and joint diseases. TCM has been reevaluated by clinicians for the prevention and treatment of postmenopausal osteoporosis as they have fewer adverse effects and are more suitable for long-term use when compared with chemically synthesized medicines (Chen et al., 2005). It was found that Chinese women treated with appropriate TCM had a low morbidity of fracture in climacteric and senescent periods (Nian et al., 2006). TCM theories consider health to be a function of the smooth flow of qi (qi translated as the vital activity of life energy) through a series of pathways or meridians. TCM diagnosis attributes a number of different underlying patterns to menopausal bone loss. A very common pattern is a Kidney qi and yin deficiency pattern, for which TCM can promote and regulate the qi and blood, improve body balance and alleviate menopausal symptoms, activate bone formation and growth (Xu et al., 2005). Thus, many herbs shown to have kidney-tonifying activities have been used in TCM formulas for the treatment of osteoporosis (Zhang et al., 2006).

By 2002, more than 60 kinds of herbs for the prevention and treatment of osteoporosis were reported in China. Some of them have obtained new drug approval, but the majority of them are still under research. The most frequently used traditional Chinese herbs for osteoporosis include Herba Epimedii (Yinyanghuo), Rhizoma Drynariae (Gusuibu), Fructus Cnidii (Shechuangzi),

Radix Dipsaci (Xuduan), Fructus Psoralease (Buguzhi), Fructus Ligustri Lucidi (Nuzhenzi), Radix Achyranthis Bidentatae (Niuxi), Cortex Eucommiae (Duzhong), Radix et Rhizoma Rhei (Dahuang), Radix Astragali (Huangqi) and so on (Wang T, 2006). The formula called Tong Bu Qiang Gu Fang containing Herba Epimedii (Yinyanghuo), Fructus Ligustri Lucidi (Nuzhenzi), Radix et Rhizoma Rhei (Dahuang) and Radix Astragali (Huangqi) has been confirmed to have positive effects for the treatment of osteoporosis (Qin LM, 2002).

1.5 Fructus Ligustri Lucidi

1.5.1 Introduciton

Ligustrum Lucidum, an ornamental shrub or small tree, commonly known as Chinese or Glossy Privet, is a member of the Olive family. Its ripe fruit called Fructus Ligustri Lucidi (Nuzhenzi) has been widely used in Chinese medicine for well over a thousand years. It was first described in the Chinese Materia Medica, Shen Nong Ben Cao Jing (Anonymous, ca. 200 B.C).

The Chinese name of FLL is actually comprised of three words. The first word "Nu" (女) means "female". The second word "Zhen" (贞) means "chaste". The third word "Zi" (子) refers to "fruit". In Chinese, the Fructus Ligustri Lucidi is known as the fruit of a chaste woman. During the Ming Dynasty, Li Shizhen explained in the Bencao Gangmu (Compendium of Materia Medica): "The tree keeps verdant (green) even in cold winter, and seems to have the product of a chasten women. Therefore, it is named as Nuzhen for its chaste quality." Woman, feminine characteristics, winter and quiet properties belong to yin. This herb is actually characterized as being pure yin with extreme quietness. Fructus Ligustri Lucidi is sweet and bitter in taste, cool in nature and manifests its therapeutic actions in the liver and kidney meridians (Liu., 2000). In TCM theory, the liver opens into the eyes while the kidneys open into the ears and manifest in the hair. When there are yin deficiencies in both the liver and kidneys, the eyes and hair lack nourishment resulting in symptoms like blurred

and dark vision, decreased visual acuity and early whitening of the hair. Fructus Ligustri Lucidi can invigorate and slowly nourish both liver and kidney yin making these symptoms generally associated with aging much milder. So it has been proven to benefit liver and kidney, ease the five yin organs, strengthen the lower back and knees, “brighten” the ears and the eyes and “darken” the moustache and hair. Traditionally, it is mainly used to treat tinnitus and hearing loss, to clear vision in the case of blurriness and cataracts; to relieve rheumatic and back pains, reduce heart palpitations and mitigate insomnia (Liang, 1991; Zhang et al., 2006).

Although this herb is seen as cost-effective alternatives by their traditional users, their international acceptance as a major regime would require extensive research using modern science. Actually, increasingly western research is starting to support some of the ethnomedical claims, revealing some very effective biochemical components. Studies have indeed shown that components of Fructus Ligustri Lucidi (FLL) have a powerful effect on respiratory and flu viruses. They also increase blood flow to the heart, reduce palpitations and spasmodic effects and support immune functions especially in tumor patients.

1.5.2 Current extraction and fractionation methods for Fructus Ligustri Lucidi

Living organisms consist of complex mixtures of chemicals, usually held within cellular structures. In order to facilitate testing in bioassays of the components in FLL, it is necessary to separate these chemicals from the cellular structural material (mostly protein, lipid and polysaccharide) and ideally from the larger majority of unrelated substances coexisting in the organism. The initial step in separating apart from the whole can be referred to as extraction, and a number of methods exist for this purpose.

1.5.2.1 Extraction

a. Preparation before extraction

Before extraction, something about the nature of the compound needs to be known so that the approach to take can be determined. The general features of a molecule that are useful to ascertain at this early stage might include: solubility, acid/base properties, charge, stability, and size (Cannell, 1998). Then there are some important points in the process of extraction. First it is important to note the relationship between the method applied and the properties of the substances extracted. A general principle is “like dissolves like”. Thus non-polar solvents will extract out non-polar substances, and polar solvents extract out polar materials. The ionizability of the compounds is another important consideration. The solubility of compounds in a solvent increases with increasing temperature but should note if the compound is unstable at higher temperatures. Secondly, it

also should be noted the properties of the solvents to be used. The boiling point of the solvent will determine the ease with which it can be removed from the extract in order to leave the extracted material. The application of heat to remove a solvent may also have deleterious effects on the substances extracted. Thus a low boiling point solvent may be favoured over a higher boiling solvent with the same or similar polarity. However, the more volatile a solvent is, the more important it becomes to have adequate safe handling procedures in order to protect the environment. It is important to be aware that the solvent itself may react chemically with the compounds to be extracted, resulting in the formation of artifacts. Where large quantities of solvent are to be used, it may be necessary to use the most economical solvent that fulfils the extraction and safety criteria required.

b. The popular extraction methods for Fructus Ligustri Lucidi

The traditional extraction way is solvent extraction, using a liquid solvent at atmospheric pressure, possibly with the application of heat. Recently, some new extraction techniques have been set up, including semi-bionic extraction, supercritical fluid extraction, ultrasound-assisted extraction and macro-porous resin adsorption.

Solvent extraction involves bringing the material to be extracted into contact with the extraction solvent for a period of time, followed by separation of the solution from the solid debris. The solvent used usually is ethanol. Huang et al has found that the extraction efficiency was highest, when 70% ethanol

was used, and the quantity was 15 times of the herb, and the extraction period was 3 hours twice (Wang et al., 2008).

Semi-bionic extraction is a process which extracts the active components using acid or base water with identified PH continuously. It mimics the transportation process of the drug in the gastrointestinal tract. It has been indicated that extraction efficiency in this method is higher than regular water extraction (Gong et al., 1999).

Supercritical fluid extraction uses supercritical CO_2 which has the high extraction efficiency, without destroying active components and without reductant. Ultrasound-assisted extraction can make good use of the heat, mechanics produced by ultrasound to speed up extraction efficiency and shorten extraction time. Macro-porous resin adsorption can adsorp the active components of the herb selectively.

c. Extraction methods for specific phytochemical groups

The main groups of compounds to be considered are fixed oils, fats and waxes, volatile or essential oils, carotenoids, alkaloids, glycosides, aglycones, phenolic compounds, polysaccharides and proteins. Solvent extraction is the most popular method of extraction and Table 1-5-1 gives a general outline of the solvents that would be appropriate for extraction of these main classes of compounds (PJHaA, 1998).

Table 1-5-1. Types of phytochemicals extracted by different solvents (PJHaA, 1998)

Polarity	Solvent	Chemical class extracted
Low	Light petroleum	Waxes Fats Fixed oils (Volatile oils)
	Hexane	Waxes Fats Fixed oils (Volatile oils)
	Cyclohexane	Waxes Fats Fixed oils (Volatile oils)
	Toluene	Alkaloids Fats Fixed oils (Volatile oils)
	Chloroform	Alkaloids Aglycones Volatile oils
Medium	Dichloromethane	Alkaloids Aglycones Volatile oils
	Diethylether	Alkaloids Aglycones
	Ethylacetate	Alkaloids Aglycones Glycosides
	Acetone	Alkaloids Aglycones Glycosides
	Ethanol	Glycosides
	Methanol	Sugars Amino acids Glycosides
High	Water	Sugars Amino acids Glycosides
	Aqueous acid	Sugars Amino acids Bases
	Aqueous alkali	Sugars Amino acids Acids

1.5.2.2 Fractionation

The components of a mixture, such as an extract from a living organism, can be separated into groups of compounds sharing similar physico-chemical characteristics. This process is called fractionation and can be carried out in various ways. Thus solubility, size, shape, electrical charge and several other features may influence the grouping. The initial fractionation may be based on solubility differences while the second may utilize molecular size. Although in many situations all components of a mixture will need to be tested, in some situations the extract will contain large amounts of unwanted compounds which should be removed. The removal process to yield an extract where the constituents of interest remain are called clean-up procedures. Methods used for achieving fractionation or clean-up are precipitation, solvent-solvent extraction, distillation, dialysis, chromatographic procedures and electrophoresis. It should be noted that chromatography in its various forms is the most common method and is exploited in most fractionation procedures carried out nowadays (ID, 2003; MHaAM, 1985; PJHaA, 1998).

The choice of fractionation method to be used in any particular situation depends on several factors. One is the nature of the substances present in the extract, the other is the immediate fate of the separated fractions and most important one is the safety to health (ID, 2003; PJHaA, 1998).

Chromatographic procedures are the most diverse and the most widely used techniques in the fractionation of extracts. All chromatography relies on

the differential distribution of compounds between two phases, one of which moves relatively to the other. These phases are called the mobile and stationary phase, respectively. The distribution between the two phases, which is an essential feature of chromatography, can be one or more of a range of physico-chemical phenomena. The particular distribution system is very dependent on the type of molecule being separated and the chromatographic materials used (MHaAM, 1985).

1.5.3 Quality control of Fructus Ligustri Lucidi

The main ingredients in FLL include oleanolic acid, ursolic acid, nuzhenide and so on. Oleanolic acid was one of the active components (Tang, 2007). There are many herbs which have been proved to contain oleanolic acid, such as Fructus Forsythiae, Flos Campsis, Herba Plantaginis, Herba Swertiae Mileensis, Patrinia scabiosaefolia Fisch, Russian Olive, Rabdosia Rubesens, Herba Artemisiae Scopariae and so on. FLL could nourish the kidney and strengthen the bone in Chinese medicinal theory. And oleanolic acid was proven to have the function of regulating the immune system, protecting the liver, anti-oxidation, anti-inflammation, anti-cancer, anti-aging and lowering serum glucose (Li, 2002; Tang, 2007). So it is appropritate to appraise the quality of FLL with oleanolic acid. The lowest quantity of oleanolic acid in FLL was reported to be 0.11%, and the highest to be 2.93% (Li, 2002). The pharmacopeia of 2000 year's verson confirmed that FLL with good quality contain at least 0.6% oleanolic acid .

But many factors may influence the content of oleanolic acid and also the quality of FLL. Different production place of FLL contained different quantity of oleanolic acid (Li, 2005a; Shi, 1998) (Table 1-5-3). The researchers also compared the content of oleanolic acid and ursolic acid in FLL in different collection period from August to December of a year. It was usually accepted that the optimum collection season should be at the end of December when the shape of FLL fruit look ripe. But the experimental results indicated that the

content of oleanolic acid and ursolic acid changed with the season and the highest content existed in October, not December (Qin and Gao, 2007). Different extraction methods also influenced the oleanolic acid content in FLL. One research group extracted FLL with water, 95% EtOH, 75% EtOH, acid water and base water. Then after detection of the content of oleanolic acid in different extracts with different solvents, the oleanolic acid extraction efficiency by 95% EtOH was proven to be the highest. Base water appeared to be better than 75% EtOH, and the extracted quantity by acid water was higher than that by only water (Gong et al., 1999). In clinical practice, the effects of this herb were confirmed to be different when the preparation method was different. And experimental results also supported that different herb preparation influenced the content of oleanolic acid in FLL. After mixing FLL with four different preparation solutions including water, yellow wine, vinegar and salt solution, the content of oleanolic acid was determined by thin layer scanning. It was shown that oleanolic acid increased in all products, especially the one prepared by yellow wine and steaming as compared with crude drug (You et al., 2007). And further study indicated that different steaming period by yellow wine determined the extraction efficiency of oleanolic acid from FLL. The optimum steaming period with yellow wine was identified to be 4 hours.

Table 1-5-3. The content of Oleanolic acid from different provinces of China (Shi, 1998)

No.	Region	Content of Oleanolic acid (mg/g)
1	Shanghai	9.17
2	Zhejiang	7.68
3	Jiangsu	10.79
4	Anhui	8.83
5	Hebei	15.16
6	Fujian	13.16

1.5.4 Fingerprint of Fructus Ligustri Lucidi

Xu et al established the HPLC fingerprints of FLL (Xu et al., 2005). In their experiment, HPLC-ELSD analysis was carried out with Lichrospher C_{18} column. The detector was Alltech ELSD 2000. The method was developed by gradient elution with methanol and water as the mobile phase. With oleanolic acid as the marker, sixteen batches of FLL were analyzed with computer-aided similarity evaluation system. The results indicated that HPLC fingerprints of FLL showed 14 characteristic peaks and the similarity of the fingerprints of 10 batches of samples was over 0.90 (Figure 1-5-1). Another group in China set up a new identification and analysis method of FLL. They first got the extracts of FLL using ethanol and chloroform ether as solvent and the extracts were identified by X-ray diffraction Fourier fingerprint spectra and characteristic diffraction peaks were obtained. There were some differences among the spectra of the extracts, but the characteristic diffraction peaks were obvious (Shi, 2007) (Figure 1-5-2).

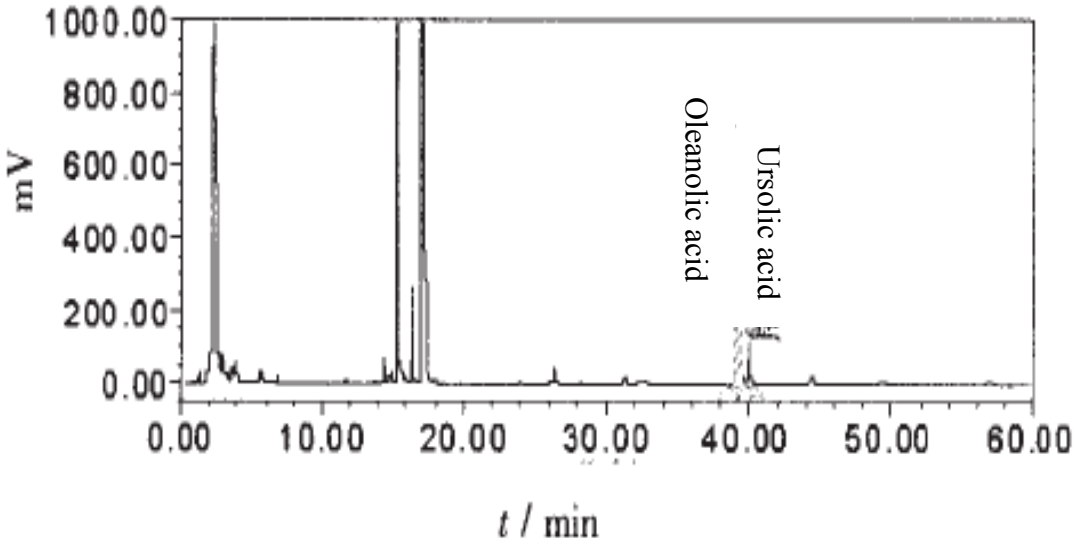


Figure 1-5-1. Fingerpints of Fructus Ligustri Lucidi (Xu et al., 2005)

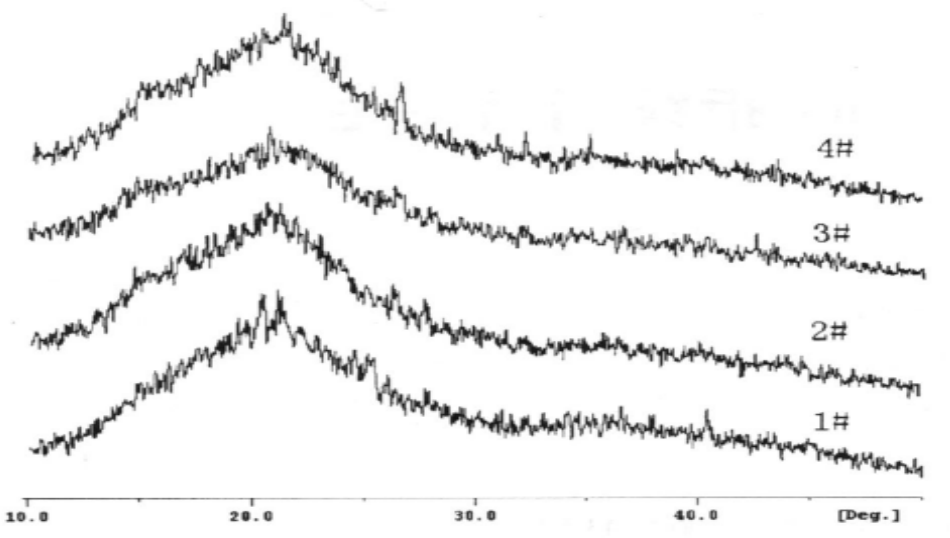


Figure 1-5-2. X-ray Diffraction Fourier Fingerprint spectra (Shi, 2007)

1.5.5 Pharmacological actions of FLL

1.5.5.1 Protective effects on liver

Oleanolic acid in FLL has been shown to have significant protection on acute hepatic injury caused by CCl_4 (Qin and Gao, 2007). It could lessen hepatic denaturation and necrosis and reduce serum glutamate-pyruvate transaminase. It was also indicated to antagonize hepatic toxicity induced by bromobenzene, colchicines and endotoxin (Liu et al., 1995). Rhodioside (3,4-dihydroxyphenethyl-β-D-glucoside) could help to clear free radicals in liver and diminish hepatic injury (Wang, 2004).

1.5.5.2 Effects on immune systems

It was indicated that FLL could regulate specific and non-specific immune systems. Water extract of FLL was reported to be able to improve functions of T cells and increase lymphocyte proliferation (Qiu, 2007). Polysaccharide in FLL was shown to stimulate T cell proliferation in spleen of mice with yin asthenia through directly stimulating mitogen PHA or ConA (Li, 2001). The isolated specneuzhenide and rhodioside from FLL also could strengthen the killing of NK cells suppressed by cyclophosphamide (Cai Q, 1998).

1.5.5.3 Effects of antioxygen and anti-aging

The extract of FLL was proven to ameliorate the state of study and memory of aged mice through improving antioxidase, clearing free radicals, diminishing production of peroxidize lipids (Ding and Xu, 2006). FLL

polysaccharides also showed anti-aging effects by declining MDA levels in liver and kidney and LF in brain by improving activity of SOD and GSH-Px (Zhang, 2006). The iridoids of FLL including oleside dimethyl ester, oleuropein, neuezhenide and lucidumoside were reported to have shown significant anti oxidant action (Neer et al., 2001).

1.5.5.4 Anti tumor effects

Many researches found that FLL extracts could inhibit proliferation of many tumor cells including H22, A548, LLC, LNCap and so on (Shoemaker M., 2005). The involved mechanism was suggested to be possibly relevant to its activity as on reverse transcriptase and DNA polymerase (Kalsuhiko O., 1989). Oleanolic acid could suppress proliferation of human breast cancer cells (MCF27) (Huang MS, 2004) and human lung cancer cells ($PGCL_3$) and induce apoptosis through increasing cells Ca levels.

1.5.5.5 Effects on serum glucose and lipids

Water extracts of FLL could decrease serum glucose induced by adrenaline, alloxan and exogenous sugar (Qin LM, 2002). The compounds of FLL including ligustroside, oleanolic acid also exhibited significant effects on stability of serum glucose (Liu, 2002). FLL extracts was shown to be able to decrease serum cholesterol and triglyceride, so that they could prevent and diminish atherosclerosis of rabbits fed with cholesterol and axungia porci (Sun YW, 1993).

1.5.5.6 Others

FLL extracts could stimulate proliferation of hair follicle in vivo and in vitro by increasing mRNA expression of hepatic growth factor and vascular endothelial growth factor (Fan, 2000). FLL also up-regulated tyrosinase acitivity and expression, so that improved production of melanin (Li, 2005b). And FLL has applied in clinical practice to treat vitiligo.

1.5.5.7 Effects on bone and mineral metabolism

Part A. Effects of FLL on bone turnover and Ca balance in ovariectomized (OVX) rats

In our previous studies, total ethanol extracts of FLL could prevent high bone turnover and Ca loss caused by E_2 deficiency without substantial effects on the uterus in OVX rats. Treatment of OVX rats with FLL ethanol extract for 14 weeks significantly reduced serum osteocalcin (OCN) and urinary deoxypyridinoline (DPD) levels, which were used as bone turnover markers, suggesting that FLL acts on bone as a potent inhibitor of high bone turnover. Treatment with FLL ethanol extract also increased intestinal Ca absorption and Ca retention that were otherwise reduced in OVX rats, indicating that FLL exerts positive effects on Ca balance (Zhang et al., 2006).

Part B. Improvement of Ca balance by FLL in aged female rats

Based on our initial study, we then studied the *in vivo* effects of FLL ethanol extract on Ca homeostasis in OVX and sham-operated female rats fed with diets of different Ca content.

In sham rats, it confirmed the positive effects of FLL on Ca balance which was achieved only when sufficient dietary Ca was provided and could be attributed to its regulation on calciotropic hormones and expression of vitamin D-dependent Ca binding proteins, mainly duodenal CaBP9k and renal CaBP28k (Zhang et al., 2008b).

In OVX rats, the FLL extract was also shown to decrease urinary and fecal Ca loss as well as increased intestinal Ca absorption rate when adequate dietary Ca was available. These results further emphasized the positive function of FLL on Ca homeostasis in aged OVX rats and such actions were not associated with the estrogen levels in rats. In OVX rats, the increased sensitivity to $1,25(OH)_2D_3$ together with the increase in level of serum $1,25(OH)_2D_3$ resulted in the up-regulation of TRPV6 expression and account for the apparent enhanced Ca absorption rate and decreased fecal Ca excretion (Zhang et al., 2008a).

Part C. Improvement of bone properties and enhancement of mineralization by FLL ethanol extract

We also determined the effect of FLL on bone quality under different Ca diets. Our results indicated that a significant improvement of the bone mass by FLL could be found at the diaphysis of tibia and femur in the aged rats fed either LCD or MCD, but not in those fed HCD, suggesting that FLL's effect on the bone mass was also dependent on the level of dietary Ca intake. We also demonstrated that the actions of FLL ethanol extract on bone were site-specific as it could only preserve bone mass and bone strength at diaphysis in the appendicular bones as well as bone mineral density (BMD) in the lumbar spine, but not at the proximal metaphysis and distal metaphysis of the appendicular bones in aged rats. Most importantly, the results from our *in vitro* studies provided additional evidence for the protective effects of FLL on bone as an enhancement of matrix ossification (Zhang et al., 2008c).

Based on previous work done by our group, we still have many potential problems. That is, how the FLL ethanol extract regulated the production of serum $1,25(OH)_2D_3$ and what is the active fractions responsible for the positive effects of FLL ethanol extract on Ca balance. These questions will be answered by my project.

Chapter 2

Objectives and Significance

2.1 Objectives

Osteoporosis is a worldwide public health problem that poses significant economic burden on society as well as on families of patients that suffer reduced functional independence as a result of osteoporosis related fractures. In most frequently affected postmenopausal women, vertebral and hip fractures are often attributed to low estrogen production and secondary hyperparathyroidism as a result of age-related decline in calcium (Ca) intake and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)-mediated Ca absorption. Although it has been shown in humans that estrogens modulate the end organ effect of $1,25(OH)_2D_3$ on intestinal or renal Ca (re)absorption, in aged women, the role and mechanism of ovarian hormones on adaptation to low or high dietary Ca is still unclear. Previous studies in our laboratories demonstrated that the ethanol extract of Fructus Ligustri Lucidi (FLL) plays an active role in modulating Ca homeostasis, not only in OVX rats but also in aged female rats with intact ovaries (Zhang et al., 2008a; Zhang et al., 2008b). However, the active fractions of FLL on Ca homeostasis need to be identified. Our previous study also showed that the ethanol extract of FLL could increase serum $1,25(OH)_2D_3$ levels in aged rats. Thus, the mechanism by which the ethanol extract of FLL modulates vitamin D_3 metabolism will also be investigated.

The specific aims include

Specific aim I:

To determine the effects and possible mechanisms of long-term estrogen deficiency on intestinal Ca absorption and renal Ca reabsorption in aged ovariectomized (OVX) female rats fed different levels of dietary Ca.

Specific aim II:

To establish the *in vivo* screening system for the identification of active fractions of FLL on Ca balance in the young mature female rats.

Specific aim III:

To identify the active fractions in FLL which are responsible for its effects on Ca balance on the established *in vivo* screening system.

Specific aim IV:

To study the mechanism by which the FLL ethanol extract modulates the vitamin D_3 metabolism using *in vivo* and *in vitro* model.

2.2 Significance

It is hoped that this study will provide a mechanistic basis for the development of extracts of Fructus Ligustri Lucidi and its active fractions into a high-quality Chinese Medicine based drug and enhance the international acceptance of Chinese medicine for the prevention and treatment of osteoporosis.

The *in vivo* screening platform for identification of active ingredients for positive Ca balance and *in vitro* screening platform for renal 1-OHase were established. It provided the possibilities to identify the active ingredients of Fructus Ligustri Lucidi and help to develop new drugs for alternative management of osteoporosis.

As traditional therapy agents for Ca and bone metabolism, such as $1,25(OH)_2D_3$, PTH or IGF, proved to be expensive or difficult to obtain, the newly discovered actions of Fructus Ligustri Lucidi on vitamin D metabolism might serve as an alterative low cost oral agent to modulate Ca and bone metabolism.

.

Chapter 3

Estrogen Deficiency Causes Altered Expression of Molecules Critical for Calcium (Re)absorption in the Duodenum and Kidney of Aged Female Rats

3.1 Introduction

Intestinal or renal calcium (Ca) (re)absorption varies according to the age, gender and some physiological or pathological conditions in individuals. Women with postmenopausal osteoporosis often have impaired Ca (re)absorption, which is often characterized by increased renal Ca excretion and reduced intestinal Ca absorption (Heaney et al., 1989). Lower Ca intake in older women will often worsen intestinal and renal Ca (re)absorption which result in negative Ca balance and bone loss (Van Abel et al., 2002). Some *in vivo* experiments in aged female rats indicated that age-related changes in serum parathyroid hormone (PTH), 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$), estrogen level were involved in the regulation of negative Ca (re)absorption (Dusso et al., 2005; Kalu and Orhii, 1999; Morris et al., 1991; O'Loughlin and Morris, 1998). It has been shown in humans that estrogen modulates the end organ effect of $1,25(OH)_2D_3$ on intestinal or renal Ca (re)absorption (O'Loughlin and Morris, 1998). However, in the aged female animals, the role and mechanism of reduced ovarian hormones on adaptation to low or high dietary Ca is still unclear. Ca (re)absorption can be envisaged as a three-step process. The first step indicates the facilitated Ca entry across the apical membrane mediated by an epithelial Ca channel (TRPV5/TRPV6). The second step refers to the cytosolic diffusion of Ca bound to Ca binding proteins such as Cabindin9k (CaBP9k) or Calbindin28k (Ca28k). The last step is the active extrusion of Ca across the basolateral membrane mediated by PMCA1b and NCX1 (Hoenderop et al., 2005). TRPV6 is the major epithelial Ca channels in the intestine, while TRPV5 and TRPV6 coexisted in the kidney, in which, TRPV5 was found to

play a major role. Similarly, CaBP9k was found in the intestine, and CaBP28k existed in the kidney. In fish enterocytes, NCX appears to be the main mechanism by which transcellular Ca^{2+} fluxes are extruded from the cells at the basolateral surface, whereas in mammals PMCA is the predominant extrusion mechanism (Hoenderop et al., 2005). This is a complicated and multi-factor process, which mainly includes the action of estrogen and vitamin D systems.

$1,25(OH)_2D_3$ regulates Ca absorption through acting on the epithelial Ca transporting proteins primarily via a genomic action after binding with its receptor (VDR). The VDRE has been found in TRPV6 (van Abel et al., 2006), CaBP9k (Zierold et al., 1995) and CaBP28k (Gillin et al., 1993). A reduced intestinal VDR level could also cause vitamin D resistance and impair intestinal Ca absorption in mice (Song et al., 2003). PTH seems to act indirectly on intestinal Ca absorption by stimulation of renal 1α-hydroxylase and, thereby, increasing $1,25(OH)_2D_3$-dependent absorption of Ca from the intestine (Ward et al., 2005). In the kidney, PTH receptor has been detected throughout the nephron including DCT and CNT, thus enabling the body to directly control active Ca re-absorption in the kidney via PTH (Hoenderop et al., 2005).

Renal Ca wasting and intestinal Ca mal-absorption caused by estrogen deficiency can be restored by estrogen replacement therapy (Hoenderop et al., 2005; Prince et al., 1991). It was found that estrogen receptors reside in proximal and distal tubules of the kidney and the duodenum and colon (Hoenderop et al., 2005). However, the underlying mechanism by which

estrogen may act on Ca (re)absorption is still poorly understood. In the intestine, estrogen could regulate the expression of TRPV6 in a vitamin D-independent manner, whereas it could not alter the expression of CaBP9k and PMCA1b (Hoenderop et al., 2002; Hoenderop et al., 2005). Estrogen replacement therapy in ovariectomized (OVX) rats revealed an increase in the expression of TRPV5, CaBP28k, NCX1 and PMCA1b in the kidney. It suggests an important role for this hormone in regulating Ca re-absorption (Hoenderop et al., 2002). Renal TRPV5 expression also was upregulated by estrogen in a $1,25(OH)_2D_3$-independent manner and transcriptionally controlled by estrogen (Hoenderop et al., 2002). Estrogen interacts with the action of the calciotropic hormones, regulating the expression of the intestinal and renal Ca transport proteins. Estrogen harbors calciotropic hormone characteristics regulating the expression of TRPV5 and TRPV6 in the kidney and intestine, respectively (Hoenderop et al., 2002).

Deficiency of estrogen at menopause leads to a decrease in intestinal Ca absorption and a corresponding increase in renal Ca excretion (Holloway et al., 2007). But the solution is not as straightforward as simply consuming more Ca because the percentage absorption is inversely related to intake. Thus, increasing Ca intake may be partially negated by a corresponding decrease in the efficiency of Ca absorption in the aged female groups. In order to systemically investigate how the estrogen and vitamin D system interplayed to regulate this process in aged females, aged OVX female rats in our experiment were used to mimic aged women with extremely low level of estrogen. By

contrast, aged sham female rats were used to represent the women with relatively normal estrogen level, as the comparison. This study considered the possible alterations of Ca (re)absorption in OVX rats after long-term estrogen deficiency at different levels of dietary Ca. Moreover, the potential underlying mechanisms in comparison with aged normal female rats were also studied.

3.2 Methods

3.2.1 Animal Study Design

Forty eight eleven-month old aged breeder Sprague-Dawley female rats (270-350g) were purchased from the Experimental Animal Center of Guangdong province (Guangzhou, China). In general, the rats over 1 year are considered to be the aged ones. The rats were 11 month of age at the beginning of our study, and ended at about 14 month of age. So they could also be considered to be the aged rats. The rats were housed in a room which provided alternating 12 h of light and 12 h of darkness with the room temperature at 23 ± 1 °C and humidity 55 ± 5%. Husbandry of the animals was based on the NIH Guide for Care and Use of Laboratory Animals (Council, 2006). Twenty-four rats were sham operated, and the other half were ovariectomized (OVX) to make them estrogen deficient. Following ovariectomy, rats were paired fed with a medium Ca diet (MCD, TD 98005, 0.6% Ca, 0.65% P) for 10 days before the initiation of the treatment regimen. The animals were then randomized to LCD (low calcium diet, TD 05004, 0.1% Ca, 0.65% P), MCD or HCD (high calcium diet, TD 05005, 1.2% Ca, 0.65% P) for 12 weeks. The rats were divided into six groups: sham rats fed LCD (SL, n=8), sham rats fed MCD (SM, n=8), sham rats fed HCD (SH, n=8); OVX rats fed LCD (OL, n=8), OVX rats fed MCD (OM, n=8) and OVX rats fed HCD (OH, n=8). All diets were purchased from Harlan Teklad (Madison, WI, USA). The nutritional composition of different diet was shown in Table 1, which is the same as our previous study (Zhang et al., 2008c). All rats had free access to distilled water, and were fed 15g/day per rat of the respective diet, the minimum average food intake of the rats during the

acclimation period. The experimental protocol was approved by the Animal Ethics Committee of The Hong Kong Polytechnic University.

3.2.2 Sample Collection

In the end of the experiment, the rats were housed individually in a plastic metabolic cage for collection of their 24 hours' urine and feces. After collection of urine and feces samples, the rats were sacrificed, blood was withdrawn from abdominal aorta under light ether anesthesia. Serum was then prepared and stored at -80 °C until biochemical determinations. The duodenum was excised and rapidly rinsed with ice-cold physiological saline. It was slit lengthwise, rinsed again and placed on an ice-chilled glass plate. Duodenal mucosa was separated from the underlying muscle coats with a chilled glass slide. The harvested tissue was placed in an eppendorf tube with 1 ml Trizol reagent and stored at -80 °C for RNA isolation. The two kidneys of each rat were collected and put into liquid nitrogen instantly, and then stored at -80 °C.

3.2.3 Measurement of Ca Balance

Ca concentration in serum and urine were measured by standard colorimetric methods using an ALCYON 300i automatic analyzer (Abbott Laboratories). The level of urine Ca was corrected by the concentration of urinary creatinine (Cr). The Ca absorption rate was calculated from the formula: Ca absorption rate (%) = (intake Ca – fecal Ca)/intake Ca × 100; the Ca net balance was calculated from: Ca net balance (mg) = intake Ca – fecal Ca – urine

Ca. And the amount of Ca in the feces was determined by atomic absorption spectrophotometry (AAnalyst 100 spectrometer; PerkinElmer).

3.2.4 Detection of Calciotropic Hormones

Serum levels of intact parathyroid hormone (PTH 1-84) were determined using rat bioactive intact PTH ELISA assay (Immutopics, Inc., San Clemente, CA). Serum $1,25(OH)_2D_3$ was extracted with two separate extraction columns and measured by competitive enzyme immunoassay (Immundiagnostik AG, Bensheim).

3.2.5 RNA Isolation and Preparation of cDNA

Tissue samples were thawed in Trizol reagent (Invitrogen, Carlsbad, California, USA) and homogenized. RNA extraction was performed according to the Trizol manufacturer's protocol. Reverse transcription reactions were performed using the SuperScript First-Strand Synthesis System (Invitrogen).

3.2.6 Real-time PCR analysis

Total RNA (2ug) was used to generate cDNA in each sample using the Super-Script Ⅱ reverse transcriptase with oligo (dT) 12-18 primers. The RNA, Oligo dT, dNTP were mixed into a PCR tube for amplication on a GeneAmp 9600 PCR system (Perkin Elmer, Foster City, CA, USA). Real-time PCR reactions were performed using SYBR Green PCR Master Mix and an ABI PRISM 7900HT. Thermocycling was done in a final volume of 20ul that contained 0.05ul cDNA and 800 nM of each of the forward and reverse primers.

GAPDH was used as an endogenous control. The primers TRPV5/TRPV6 (Hoenderop et al., 2002; Zhang et al., 2007), CaBP-9k (Hong et al., 2004), CaBP-28k (Wang et al., 2005), PMCA1b (Reinhardt and Horst, 1990), VDR (Zhang et al., 2007) and GAPDH (Zhang et al., 2007) were the same as previously described and were shown in Table 3-2. PCR was performed using the following programme: initial denaturation at 95^{o}C for 10min, 40 cycles of denaturation at 95^{o}C for 1min, primer annealing at 50^{o}C for 1min and polymerization at 72^{o}C for 1min30s. Quantitative values were derived from the threshold cycle number at which the increase in the signal associated with exponential growth of PCR products was first detected. To determine the number of copies of the targeted DNA in the samples, a relative standard curve (concentration-threshold cycle) was generated by the dilution of cDNA from the calibrator (SM group). Data was normalized with GAPDH levels in the samples.

3.2.7 Statistical Analysis

The data from these experiments were reported as mean ± standard error of mean (SEM) for each group. All statistical analyses were performed using PRISM version 4.0 (GraphPad). Analysis of the effects of diet, OVX, and interaction of both factors as grouping variables was performed by two-way analysis of variance (ANOVA). Inter-group differences were analyzed by one-way ANOVA followed by Turkey's multiple comparison test as a post test. Differences in P value of less than 0.05 were considered statistically significant.

Table 3-1 Diet composition

	Low Ca Diet[1] (1 g Ca/kg Diet)	Middle Ca Diet[2] (6 g Ca/kg Diet)	High Ca Diet[3] (12 g Ca/kg Diet)
Casein	110.0	110.0	110.0
Egg White Solids, spray-dried	97.9	97.9	97.9
DL-Methionine	3.0	3.0	3.0
Sucrose	563.5803	551.0903	536.0803
Corn Starch	100.0	100.0	100.0
Corn Oil	50.0	50.0	50.0
Cellulose	20.0	20.0	20.0
Vitamin Mix4	10.0	10.0	10.0
Mineral mixture5	0.042	0.042	0.042

1 Harlan Teklad, Madison, WI. Low Calcium Diet (LCD, TD #05004). This formula is a modification of TD #98005 to reduce calcium to 0.1%.
2 Harlan Teklad, Madison, WI. Control Diet (MCD, TD #98005). This formula is a modification of TD #86464.
3 Harlan Teklad, Madison, WI. High Calcium Diet (HCD, TD #05005). This formula is a modification of TD #98005 to increase calcium to 1.2%.
4 Vitamin mixture from Harlan Teklad (#40060) provided (mg/kg diet): p-aminobenzoic acid, 110.1; ascorbic acid, coated, 1016.6; biotin, 0.44; vitamin B-12, 29.7; calcium pantothenate, 66.1; choline dihydrogen citrate, 3496.9; folic acid, 1.98; inositol, 110.1; menadione, 49.5; niacin, 99.1; pyridoxine HCl, 22.0; riboflavin, 22.0; thiamin HCl, 22.0; dry vitamin A retinyl palmitate 500,000 U/g), 39.65; dry cholecalciferol (500,000 U/g), 4.4; dry vitamin E dl-a-tochopherol acetate (500 U/g), 242.3; cornstarch (diluent), 4666.9.
5 Mineral mixture from Harlan Teklad provided (mg/kg diet): potassium phosphate, monobasic, 24.6; calcium carbonate, 14.74 for MCD, 2.25 for LCD and 29.75 for HCD; potassium chloride, 5.6; sodium bicarbonate, 4.62; magnesium oxide, 3.83; sodium chloride, 3.7; sodium selenite, 0.5; ferric citrate, 0.21; manganous carbonate, 0.123; zinc carbonate, 0.056; chromium potassium sulfate (12H2O), 0.0193; cupric carbonate, 0.011; and potassium iodate, 0.0004.

Table 3-2 Sequences of Primers for Quantitative Real-Time PCR

Gene	Forward primer	Reverse Primer
TRPV5	CTTACGGGTTGAACACCACCA	TTGCAGAACCACAGAGCCTCTA
TRPV6	TCCTGCAAGTCCAAGGAGAA	CACACGCTTTCCCACAATCT
CaBP28k	TCTCTGATCACAGCCTCACA	CTATGTATCCGTTGCCATCC
CaBP9k	AAGAGCATTTTTCAAAAATA	GTCTCAGAATTTGCTTTATT
PMCA1b	GCCATCTTCTGCACAATTGT	TCAGAGTGATGTTTCCAAAC
VDR	CAACCAGTCTTTCACCATG	GCTTCATGCTATTCTCGG

TPPV5/TRPV6, epithelial Ca channel 1 and 2; CaBP28k, calbindin-D28k; CaBP9k, calbindin-D9k; PMCA1b, plasma membrane calcium ATPase; VDR, vitamin D receptor.

3.3 Results

3.3.1 Body weight and Ca and phosphorus levels in serum

There are six groups in our study including sham rats fed with LCD (SL), sham rats fed with MCD (SM), sham rats fed with HCD (SH); OVX rats fed with LCD (OL), OVX rats fed with MCD (OM), OVX rats fed with HCD (OH). As shown in Table 3-3, after 12 weeks' feeding, body weight increased significantly ($P<0.0001$) in all three OVX groups compared with the sham groups, especially in rats fed with MCD and HCD. Two-way ANOVA analysis showed that OVX affected the weight gain of rats during the 12 wk of treatment. The uterine index for each OVX group was much lower than that of the sham group, suggesting that ovariectomy was successful and OVX rats should have minimal estrogen in the circulation (Data not shown).

Two-way ANOVA analysis indicated that both dietary Ca and estrogen deficiency influenced serum Ca levels ($P=0.0003$ and $P<0.0001$, respectively, Table 3-3), though they did not show interaction effect on serum Ca. In both sham and OVX rats, serum Ca levels increased with the increase of dietary Ca ($P<0.05$, SL vs SH; $P<0.05$, OL vs OH; Table 3-3). Statistically significant effects of estrogen deficiency on serum Ca were recorded in those rats fed with LCD and HCD (Table 3-2, $P<0.05$, $P<0.01$). There were no statistically significant differences in serum phosphorus level between sham and OVX group at each dietary Ca level.

Table 3-3 Weight gain, serum Ca, P, $1,25(OH)_2D_3$ and PTH levels in aged female sham and OVX rats fed with LCD, MCD and HCD for 12 weeks[1]

		Weight gain (g)	Serum Ca (mg/dl)	Serum P (mg/dl)	Serum PTH (pg/ml)	Serum $1,25(OH)_2D_3$ (pg/ml)
LCD	SHAM	68 ± 13	9.88 ± 0.33	6.97 ± 0.35	69.75 ± 13.30	39.94 ± 5.78
	OVX	84 ± 11	8.85 ± 0.18*	7.12 ± 0.37	126.7 ± 13.3***	49.14 ± 4.28**
MCD	SHAM	46 ± 19	10.35 ± 0.24	6.46 ± 0.23	52.81 ± 7.70	16.29 ± 2.49
	OVX	85 ± 8*	9.59 ± 0.25	6.68 ± 0.28	55.07 ± 8.6[C']	18.50 ± 1.10[c']
HCD	SHAM	39 ± 6	11.13 ± 0.21[a]	6.06 ± 0.07	47.73 ± 5.68	5.77 ± 3.5 [a]
	OVX	99 ± 15***	9.87 ± 0.21** [a']	6.83 ± 0.24	51.30 ± 6.8[C']	5.02 ± 4.1 [C' d]
			Two-way ANOVA analysis (P value)			
Diet		0.6136	0.0003	0.0594	<0.0001	<0.0001
OVX		<0.0001	<0.0001	0.0545	0.0130	0.0448
Interaction		0.1339	0.5816	0.3465	0.0118	0.0419

Ca, calcium; P, phosphorus; Cr, creatinine; MCD, medium Ca diet; LCD, low Ca diet; HCD, high Ca diet; SHAM, sham-operated; OVX, ovariectomized; ANOVA, analysis of variance; SL, sham rats fed with LCD; SM, sham rats fed with MCD; SH, sham rats fed with HCD; OL, OVX rats fed with LCD; OM, OVX rats fed with MCD; OH, OVX rats fed with HCD.

[1]Values are expressed as mean ± SEM, $n = 8$. *$P < 0.05$, **P<0.01, ***P<0.001 compared to vehicle treated group fed with diet of similar Ca level; [a]P<0.05, [c]P<0.001versus SL; [c']P<0.001 versus OL; [d]P<0.05 versus OM;

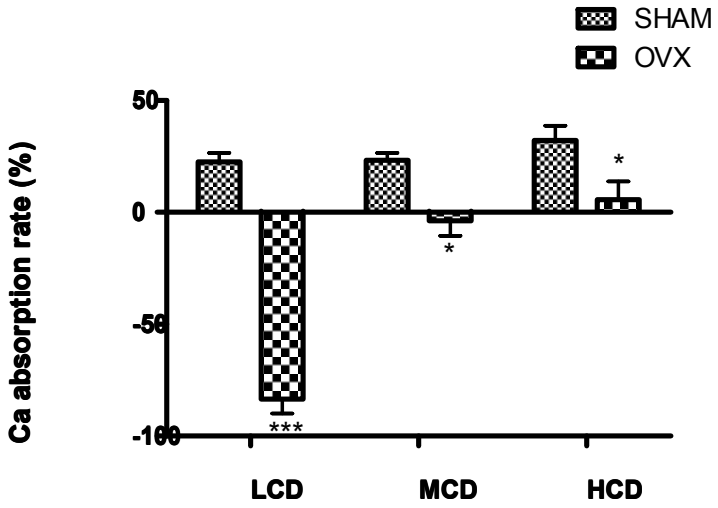
[2] The levels of urinary Ca and P have been corrected by the level of urine Cr;

3.3.2 Ca absorption rate and urinary Ca excretion

OVX significantly reduced the Ca absorption rate at all Ca diet levels, especially at LCD in which OVX resulted in the negative Ca balance (Figure 3-1A, $P<0.05$ and $P<0.001$ sham vs OVX). Two-way ANOVA analysis showed that both a Ca deficient diet and OVX could dramatically reduce the Ca absorption rate (Figure 3-1A, $P<0.0001$). The interaction between the two grouping variables was highly significant for the regulation of Ca absorption of rats (Table 3-4, $P<0.0001$). In sham rats, there were no differences of Ca absorption rate among three dietary groups. But in OVX rats, the Ca deficiency dramatically suppressed the Ca absorption rate (Figure 3-1A, $P<0.001$ OL vs OM or OH). When the OVX rats were fed with MCD and HCD, the Ca balance seemed to be restored from negative to zero or positive (Figure 3-1A). This result suggested that higher dietary Ca could help Ca balance in the aged OVX rats but not the aged normal female rats.

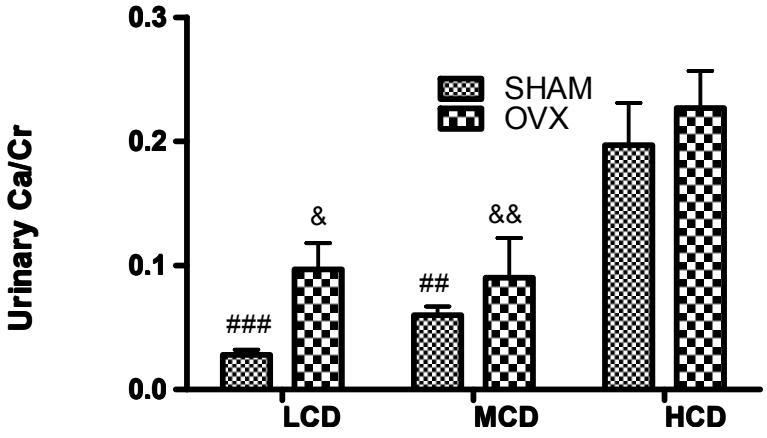
Urinary Ca excretion, expressed relative to creatinine (Cr), could be in part reversely negatively correlated with the Ca re-absorption status in kidney. It was found that urinary Ca/Cr in our study altered with dietary Ca in sham and OVX rats. High dietary Ca significantly elevated urinary Ca excretion compared with low and medium dietary Ca in both sham (Fig1B, $P<0.01$ and $P<0.001$, SL and SM vs SH) and OVX rats (Figure 3-1B, $P<0.05$ and $P<0.01$, OL and OM vs OH). OVX seemed to increase the urinary Ca excretion at each dietary Ca level, though this was not statistically significant (Table 3-4).

A.



n=8

B.



n=8

Figure 3-1. Ca absorption rate (A) and urinary Ca/Cr (B) in each group.

The Ca absorption rate was calculated as "(Ca intake – Fecal Ca excretion) / Ca intake ×100". Data are expressed as mean ± SEM (n=8). *P<0.05, ***P<0.001 vs SHAM group fed a diet with a similar Ca level; ##P<0.01, ###P<0.001 vs SH group; &P<0.05, &&P<0.01, &&&P<0.001 vs OH group. Ca, calcium; LCD, low Ca diet; MCD, medium Ca diet; HCD, high Ca diet.

3.3.3 Calciotropic hormones levels

In order to explore the mechanisms of reduced Ca (re)absorption in OVX rats and its regulation by dietary Ca, the serum calciotropic hormones which are the main regulators for Ca balance were evaluated in our study.

Serum PTH levels were shown to be upregulated by a Ca deficient diet in either sham or OVX rats (Table 3-3, $P<0.001$). In OVX rats, serum PTH levels seemed to be higher elevated under Ca deficient diet than in sham rats ($P<0.05$, Table 3-3). The levels of serum PTH in OVX rats fed with MCD and HCD were restored to the levels in sham rats fed with similar dietary Ca.

Serum $1,25(OH)_2D_3$ levels of rats in all groups seemed to change with serum PTH levels. They decreased with the increase of dietary Ca and also were shown to be elevated higher by LCD in OVX rats than in sham rats ($P<0.01$, Table 3-3). Two-way ANOVA indicated that both low Ca diet and OVX interacted to regulate serum PTH and $1,25(OH)_2D_3$ levels (Table 3-3, $P<0.05$).

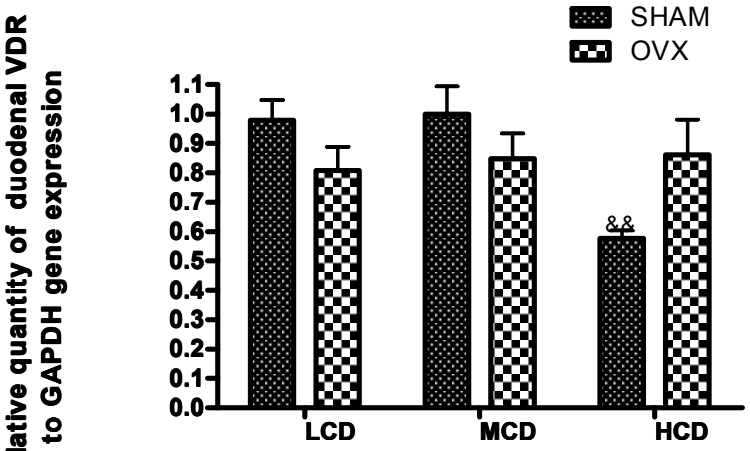
3.3.4 Duodenal and renal VDR gene expression

To further determine whether the alterations of Ca (re)absorption are vitamin D dependent, duodenal and renal VDR mRNA expressions were also evaluated.

The duodenal VDR gene in sham rats was found to be suppressed by HCD, as compared with that in LCD and MCD. No differences of duodenal VDR mRNA among each dietary group in OVX rats were found. Furthermore, it was shown that OVX did not change duodenal VDR gene in comparison with sham rats (Figure 3-2A and Table 3-4).

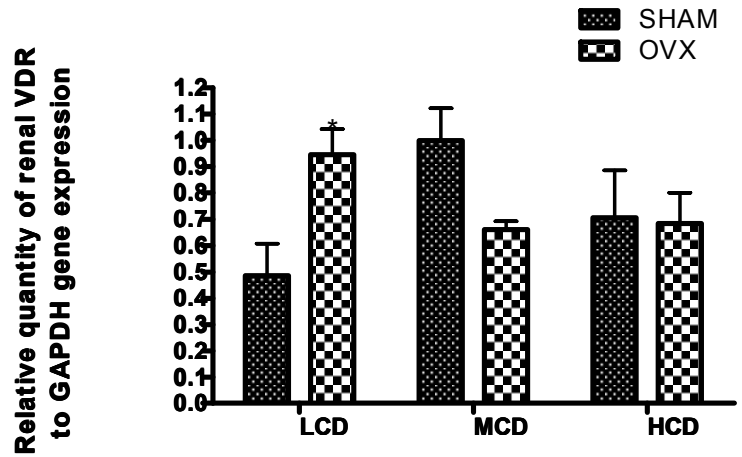
One-way ANOVA analysis in sham or OVX group indicated that dietary Ca did not influence the renal VDR mRNA expression (Table 3-4). But OVX was found to increase renal VDR gene expression in rats fed with LCD ($P<0.05$; Figure 3A).

A.



n=8

B.



n=8

Figure 3-2. Renal (A) and duodenal (B) mRNA expression of VDR in each group.

The expression level is shown as a ratio of target gene/GAPDH. Data are expressed as mean ± SEM (n=8). *P<0.05 vs SHAM group fed with diet with a similar level; &&P<0.01 vs OM group. Ca, calcium; LCD, low Ca diet; MCD, medium Ca diet; HCD, high Ca diet.

3.3.5 Duodenal gene expression for epithelial Ca transporting

To determine whether the decreased Ca absorption rate was associated with abnormal expression of proteins that mediate transcellular Ca absorption through intestine, we performed real-time PCR analysis of the expression of TRPV6, CaBP9k and PMCA1b.

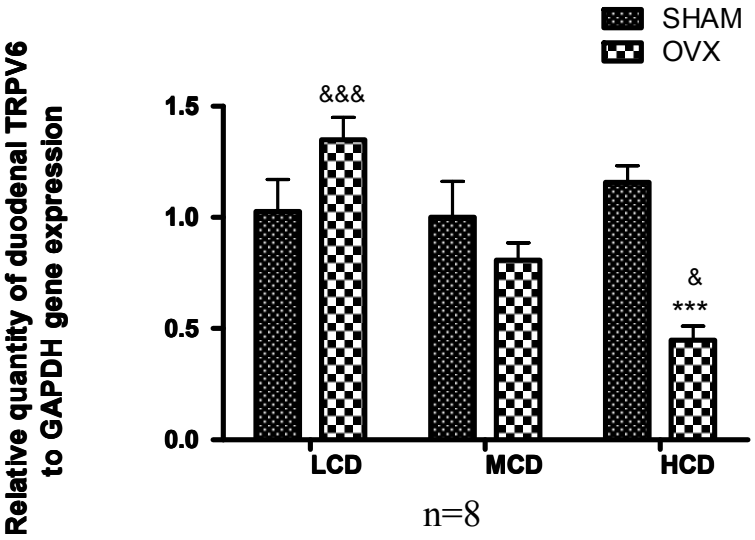
Duodenal TRPV6 gene expression was not affected by different dietary Ca in sham rats and was not altered with the increase of dietary Ca (Figure 3-3A). However, in OVX rats, duodenal TRPV6 mRNA decreased with the increase of dietary Ca ($P<0.001$, OL vs OM; $P<0.05$, OM vs OH; Figure 3-3A). The changing trend in OVX rats was shown to be in line with that of serum $1,25(OH)_2D_3$ levels with the increasing changes of dietary Ca. OVX also had an impact on duodenal TRPV6 gene expression, especially in rats fed HCD in which OVX significantly decreased duodenal TRPV6 mRNA expression ($P<0.001$; Figure 3-3A). The interaction between dietary Ca and OVX was highly significant for the regulation of duodenal TRPV6 mRNA, as indicated by two-way ANOVA analysis. (Table 3-4; $P<0.0001$).

Duodenal CaBP9k gene expression seemed to decrease with the increase of dietary Ca levels in sham or OVX rats. The gene expressions changing trend in both sham and OVX rats was shown to be consistent with that of serum $1,25(OH)_2D_3$ levels with the increase of dietary Ca ($P<0.01$, SH vs SM; $P<0.001$, OL vs OM; $P<0.01$, OH vs OM; Figure 3-3B). Two-way analysis indicated that OVX did not significantly influence its mRNA expression, but

dietary Ca and OVX were shown to interact in regulation of duodenal CaBP9k expression (Table 3-4).

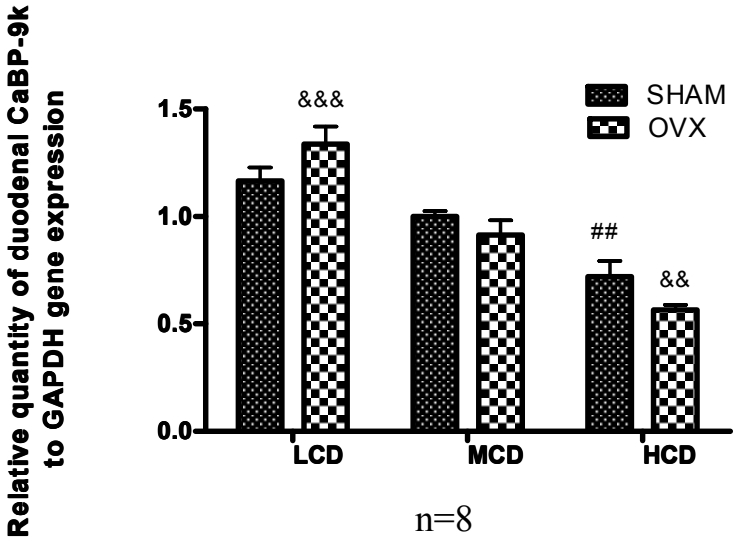
Low Ca diet greatly upregulated duodenal PMCA1b gene expression in sham rats compared with those fed MCD and HCD. Moreover, the lowest mRNA expression level was recorded in sham rats fed MCD (Figure 3-3C). Conversely, the highest duodenal PMCA1b mRNA expression occurred in OVX rats fed MCD, by comparison, LCD did not increase its expression under the estrogen-deficient state (Figure 3-3C). OVX as a grouping variable decreased duodenal PMCA1b gene expression in rats fed LCD and HCD, but increased it in rats fed MCD (Figure 3-3C). It was shown that the changing trend of the duodenal PMCA1b with the increase of dietary Ca levels did not parallel the trend of serum $1,25(OH)_2D_3$ levels in either sham or OVX rats. The interaction between dietary Ca and OVX was highly significant for the regulation of duodenal PMCA1b mRNA, as indicated by two-way ANOVA analysis (Table 3-4).

A.


Relative quantity of duodenal TRPV6 to GAPDH gene expression
SHAM
OVX
1.5
1.0
0.5
0.0
&&&
&
***
LCD
MCD
HCD

n=8

B.


Relative quantity of duodenal CaBP-9k to GAPDH gene expression
SHAM
OVX
1.5
1.0
0.5
0.0
&&&
##
&&
LCD
MCD
HCD

n=8

C.

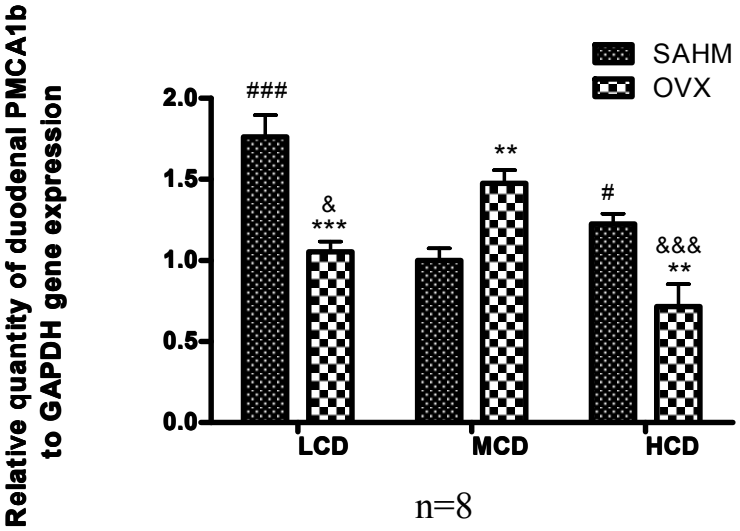


Figure 3-3. Duodenal mRNA expression of TRPV6 (A), CaBP-9k (B), and PMCA1b (C) in each group.

The expression level is shown as a ratio of target gene/GAPDH. Data are expressed as mean ± SEM (n=8). **P<0.01, ***P<0.001 vs SHAM group fed a diet with a similar level; #P<0.05, ##P<0.01, ###P<0.001 vs SM group; &P<0.05, &&P<0.01, &&&P<0.001 vs OM group. Ca, calcium; LCD, low Ca diet; MCD, medium Ca diet; HCD, high Ca diet.

3.3.6 Renal gene expression for epithelial Ca transporting

As stated for duodenal gene expression for epithelial Ca transporting, renal TRPV5, CaBP28k and PMCA1b were also evaluated by real-time PCR, in order to investigate the reasons for hypercalciuria in OVX rats.

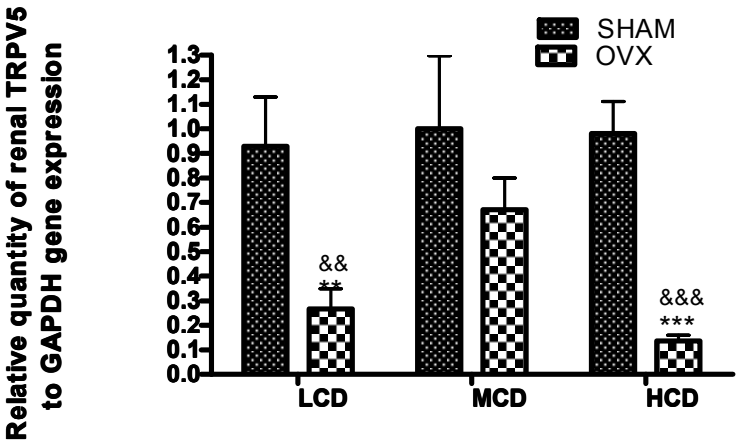
Renal TRPV5 gene expression was not affected by dietary Ca in sham rats in a similar way to that of the duodenal TRPV6 gene (Table 3-4). But in OVX rats, LCD and HCD were shown to greatly suppress renal TRPV5 mRNA expression, as compared with MCD ($P<0.01$, OL vs OM; $P<0.001$, OM vs OH; Figure 3-4A). Another group variable –OVX– suppressed renal TRPV5 gene expression at each dietary Ca level. The statistically significant inhibition was recorded at LCD and HCD ($P<0.01$ and $P<0.001$; Figure 3-4A). In addition, the changing pattern of renal TRPV5 mRNA was not shown to be similar to that of serum $1,25(OH)_2D_3$ levels in either sham or OVX rats.

Dietary Ca intake did not modulate renal CaBP28k mRNA expression in either sham or OVX rats. OVX significantly inhibited the gene expression of renal CaBP28k at each dietary Ca ($P<0.05$, $P<0.001$ and $P<0.001$, respectively; Figure 3-4B). CaBP28k mRNA did not change with the serum $1,25(OH)_2D_3$ levels in the rats.

Renal PMCA1b gene expression alterations in sham and OVX rats were similar to the duodenal PMCA1b gene expression. When the sham rats were fed with MCD, renal PMCA1b declined, compared with rats fed LCD and HCD

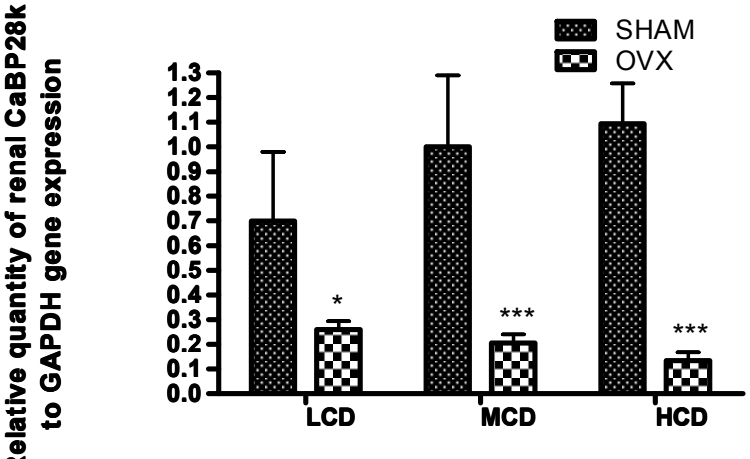
(P<0.05, SL vs SM; P<0.01, SM vs SH; Figure 3-4C). However, when the OVX rats were fed with MCD, renal PMCA1b expressions were found to be higher compared to those in rats fed LCD and HCD (P<0.05, OL vs OM; P<0.01, OM vs OH; Figure 3-4C). OVX could suppress its gene expression in rats fed LCD and HCD, but seemed to upregulate it in rats fed MCD, as compared with sham rats (P<0.001, Figure 3-4C).

A.


Relative quantity of renal TRPV5 to GAPDH gene expression
SHAM
OVX
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
&&
**
&&&
***
LCD
MCD
HCD

n=8

B.


Relative quantity of renal CaBP28k to GAPDH gene expression
SHAM
OVX
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
***
***
LCD
MCD
HCD

n=8

C.

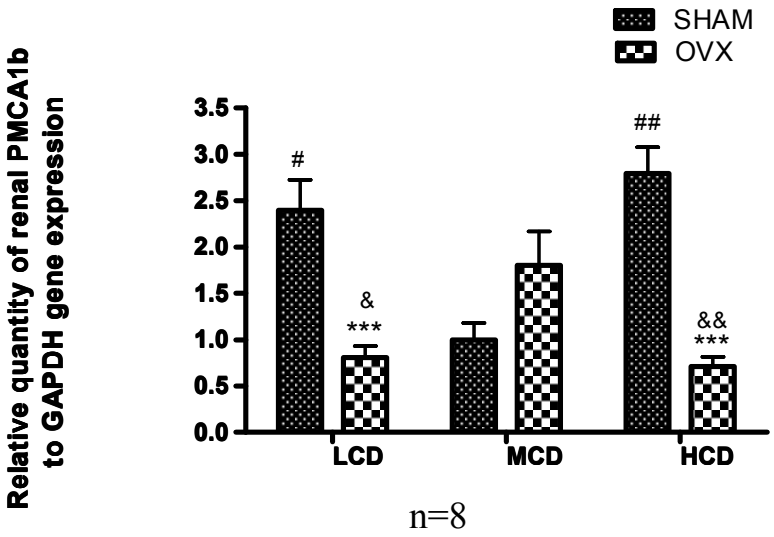


Figure 3-4. Renal mRNA expression of TRPV5 (A), CaBP-28k (B), and PMCA1b (C) in each group.

The expression level is shown as a ratio of target gene/GAPDH. Data are expressed as mean ± SEM (n=8). **P<0.01, ***P<0.001 vs SHAM group fed with diet with a similar level; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs SM group; $^{\&}$P<0.05, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 vs OM group. Ca, calcium; LCD, low Ca diet; MCD, medium Ca diet; HCD, high Ca diet.

Table 3-4. Two-way ANOVA analysis of the effects of Ca diets and OVX on the urinary Ca/Cr ratio, Ca absorption rate and duodenal and renal mRNA expressions involved in epithelial Ca transport, in aged female rats.

A.

	Urine Ca/Cr	Ca absorption rate	Duodenum				Kidney			
			VDR	TRPV6	CaBP9k	PMCA1b	VDR	TRPV5	CaBP28k	PMCA1b
Diet	P<0.0001	P<0.0001	0.0654	0.0025**	P<0.0001	0.0003***	0.5340	0.1451	0.7389	0.3894
OVX	0.06	P<0.0001	0.8644	0.0308*	0.6589	0.0037**	0.7526	P<0.0001	P<0.0001	P<0.0001
Interaction	0.7107	P<0.0001	0.0272*	0.0001***	0.0271*	P<0.0001	0.0156*	0.2273	0.3910	P<0.0001

*P<0.05, **P<0.01, ***P<0.001, statistical analysis by two-way ANOVA

TPPV5/TRPV6, epithelial Ca channel 1 and 2; CaBP28k, calbindin-D28k; CaBP9k, calbindin-D9k;

PMCA1b, plasma membrane calcium ATPase; VDR, vitamin D receptor

3.4 Discussion

It is known that estrogen deficiency induced poor intestinal Ca absorption and increased urinary Ca loss. This is complicated by age-related abnormalities in the vitamin D-endocrine system which result in the development of the Ca disorder known as postmenopausal osteoporosis (Raisz, 2005; Riggs, 2003). Our study has further investigated the process and found that the response of intestinal Ca absorption to different dietary Ca levels was different between the aged female normal and OVX rats. But estrogen deficiency was shown to greatly depress Ca (re)absorption in rats fed a similar Ca diet. This process might be independent of the vitamin D system, and determined by the epithelial Ca transporting proteins.

The present study demonstrated that in aged rats with normal estrogen levels, dietary Ca did not alter intestinal Ca absorption, which seemed to contradict the observations in younger rats (Zhang et al., 2007). It is known that there are two different mechanisms of Ca absorption, passive and active pathways. It is widely believed that higher dietary Ca will result in absorbing more Ca through the paracellular pathway. In the present study, the response of the Ca absorption rate to dietary Ca differences indicated that active pathways might play roles in this process. Serum $1,25(OH)_2D_3$ altered with dietary Ca in aged sham rats, just as in younger

ones in our experiment, which decreased with the increase of dietary Ca. At the same time, duodenal VDR expressions were lower in the rat group fed the higher dietary Ca. That is to say, Ca absorption rate would be expected to decrease with the increase of dietary Ca levels in response to the vitamin D system. But the data from our experiment could not be fully explained by the vitamin D status in the aged normal female rats. The results for epithelial transporting proteins indicated that the changing trend for the three major proteins responsible for active transport also could not explain the changes of Ca absorption rate in the normal rats. So it is suggested that the similar regulation by different dietary Ca on Ca absorption rate might be due to the balanced effects of its passive and active pathways. The reduced capacity of $1,25(OH)_2D_3$ to stimulate Ca absorption in aged rats as reported previously (Armbrecht et al., 1998; Chapuy et al., 1996) could also be one of the reasons for no alterations in response to different Ca diet in the aged normal female rats.

In estrogen-deficient aged rats from our experiments, long-term dietary Ca restriction resulted in a great intestinal Ca mal-absorption. This was showed by the negative Ca absorption rate in OVX rats, especially in those fed with LCD. The result suggested that LCD worsens the intestinal Ca absorption in estrogen deficient

state. This is consistent with many other similar observations on the estrogen or Ca deficiency influencing Ca absorption (Park et al., 2008; Zhang et al., 2008a). An adequate amount of dietary Ca (i.e. MCD and HCD) seemed to partly restore the intestinal Ca absorption in OVX rats. The serum $1,25(OH)_2D_3$ levels in the LCD-fed OVX rats were very high, but they appeared not to impact the intestinal Ca absorption as expected. At the same time, there were higher expressions of three epithelial transporting proteins in the OVX rat group fed with LCD, which also did not help Ca absorption. This indicated that the intestinal Ca in the aged estrogen deficient rats might be absorbed mainly through the paracellular pathway in response to different Ca diet.

Urinary Ca excretion was found to increase with the increase of dietary Ca in both sham and OVX rats. This was reasonable and consistent with the previous studies (Zhang et al., 2008a). The higher Ca diet resulted in higher Ca filtration and higher Ca excretion, which is a natural physiological process. Urinary Ca excretion was shown to be increased in OVX rats at each dietary Ca level, though the increase was not statistically significant. It suggested that renal Ca re-absorption was decreased by OVX. This reduction of Ca (re)absorption also resulted in the decrease of serum Ca levels, especially in OVX rats fed with LCD and HCD. This result

might induce the mineral bone loss just as observed by our previous studies (Zhang et al., 2008c).

We found that OVX further increased circulating PTH levels in aged rats during dietary Ca restriction. This result was in agreement with our previous study, in which OVX worsened secondary hyperparathyroidism in mature rats during 2 weeks of the LCD (Zhang et al., 2007). The increased PTH levels resulted in a proportional increase in serum $1,25(OH)_2D_3$, which suggested a normal vitamin D system in response to PTH in aged females, but seemed to be different from the results gained in our previous study (Zhang et al., 2007). This difference from our previous study might be explained by the different age of rats used and different feeding period. However, the results that serum PTH and $1,25(OH)_2D_3$ levels were reversed by sufficient Ca intake in OVX rats in our study are in agreement with previous reports (Adami et al., 2008; Zhang et al., 2007).

Furthermore, the significant increase of serum $1,25(OH)_2D_3$ in OVX rats fed with LCD did not assist Ca (re)absorption in our experiment. This suggested to us a significant decline of the capacity to respond to $1,25(OH)_2D_3$ in aged rats, especially in the OVX rats without estrogen. At the same time, it was found that duodenal

VDR was not greatly changed by OVX. Moreover, its changing trend could not be enough to explain the changes in intestinal Ca absorption by estrogen deficiency. Renal VDR mRNA was induced by increased endogenous serum $1,25(OH)_2D_3$ in OVX rats fed with LCD. This result was consistent with previous reports (Brown et al., 1995; Healy et al., 2003). It seemed not to explain the increase in urinary Ca excretion in OVX rats. This suggests that $1,25(OH)_2D_3$-independent mechanisms might be involved in the actions of OVX on Ca (re)absorption, especially when the rats are fed with LCD. It might be explained at least in part by the expression of the epithelial transporting proteins alterations in aged female rats with estrogen deficiency and different dietary Ca.

Our results indicated that duodenal PMCA1b gene expression might be responsible for the great decrease in intestinal Ca absorption in OVX rats fed with LCD. PMCA1b gene expression was upregulated by OVX when the rats were fed a normal Ca diet. However, Ca absorption of OVX rats did not return to the similar levels to those of sham rats found in the same dietary Ca group. The underlying mechanism might be the relatively lower expression of TRPV6 and non-reversible PMCA1b activity caused by estrogen deficiency (Wang et al., 2005). But the overload of dietary Ca decreased both duodenum TRPV6 and PMCA1b expression,

so it could not restore Ca absorption in OVX rats to the normal level, as in the sham rats.

Based on our results, the significant decrease of renal proteins responsible for the transcellular Ca reabsorption might result in the increase of urinary Ca excretion. But in the mammalian kidney, 90% Ca reabsorption occurs in the proximal tubule and cortical thick ascending limb by paracellular diffusion (Wang et al., 2005). The current experimental design does not distinguish the renal filtered load of Ca from other factors which could contribute to the urinary Ca excretion. This might explain why the great decrease of epithelial Ca transporting proteins did not result in a significant increase of urinary Ca excretion in the aged OVX rats observed in our experiments.

In the duodenum, mRNA expression of TRPV6 in the aged sham rats was not regulated by dietary Ca, while was altered in the OVX rats. This phenomenon might indicate the fact that estrogen and $1,25(OH)_2D_3$ are two isolated regulators on TRPV6 in duodenum (Hoenderop et al., 2002). The significant decrease of duodenal TRPV6 gene expression in OVX rats fed with HCD might result from the combined actions of estrogen deficiency and HCD induced lower $1,25(OH)_2D_3$ levels. In the

kidney, TRPV5 is thought of as a gatekeeper of epithelial Ca uptake (Hoenderop et al., 2000) and was reported to be upregulated by estradiol treatment (Wang et al., 2005). TRPV5 is primarily involved in the regulation of the Ca transport proteins expression independent of $1,25(OH)_2D_3$. The magnitude of the Ca influx through TRPV5 controls the expression of other Ca transporting proteins (Hoenderop et al., 2002). Our results indicated that renal TRPV5 mRNA seemed not to be influenced by dietary Ca in either sham or OVX rats. Renal TRPV5 gene expression was greatly suppressed by OVX in each dietary Ca group. This study supports the conclusion that estrogen regulation of the gate-keeper TRPV5 in the kidney occurs in a coordinated fashion and can occur independent of vitamin D (Wang et al., 2005).

In rodents, intestinal CaBP9k is regulated at the transcriptional and post-transcriptional levels by $1,25(OH)_2D_3$ (Choi and Jeung, 2008). In our experiments, duodenal CaBP9k expression was altered by dietary Ca but was not influenced by estrogen deficiency. The result suggested that CaBP9k is mainly regulated by $1,25(OH)_2D_3$ but not estrogen in aged female rats. CaBP28k is a vitamin D-dependent protein, but estradiol was also proved to be able to increase its expression in the kidney (Wang et al., 2005). In our experiment, renal CaBP28k mRNA was suppressed by estrogen deficiency, but was not regulated by dietary Ca. In the

TRPV5 knockout mice, renal CaBP28k was found to be downregulated despite elevated levels of $1,25(OH)_2D_3$, which suggested that TRPV5 is primarily involved in the regulation of the Ca transport proteins expression in the kidney independent of $1,25(OH)_2D_3$ (Lambers et al., 2006). This could explain the alterations of renal CaBP28k gene expression in aged normal or OVX rats in our experiment.

Qiang et al have reported that adaptations of chickens to a Ca deficient diet resulted in an increase in duodenal PMCA1b mRNA (Cai et al., 1993). Our results also showed that PMCA1b gene expression could be regulated by LCD, when estrogen level was normal. But the duodenal PMCA1b gene expression in OVX rats seemed not to be induced by LCD. Furthremore, duodenal PMCA1b gene expression was found to be decreased by estrogen deficiency in the rats fed with LCD and HCD. However, under normal Ca diet, OVX seemed to increase it greatly. PMCA1b is usually not regarded as a vitamin D-dependent protein (Choi and Jeung, 2008). In the VDR-knockout mice, it has been found that high or low Ca intake could decrease PMCA1b expression (Van Cromphaut et al., 2001). So, the observation in our experiments might be explained by the simultaneous effects of estrogen deficiency and different dietary Ca. It has been demonstrated that renal PMCA1b, not NCX1, is responsible for extruding Ca in response to in vivo estradiol

hormonal challenge (Wang et al., 2005). In our study, renal PMCA1b expressions were lower in the rats fed with MCD, but not HCD. But in OVX rats, its expressions in those fed with MCD were higher than those fed with LCD and HCD. This result strongly suggests that renal PMCA1b expressions are dependent on different dietary Ca level and they exhibited different response to dietary Ca in estrogen-deficient or non-deficient state. It was also suppressed greatly by OVX in the rats fed with LCD and HCD.

In summary, long-term estrogen deficiency in aged female rats is sufficient to cause mal-absorption of Ca through the intestine and the kidney. In the aged female rats with relatively normal estrogen levels, Ca can be absorbed through the small intestine simultaneously in two ways. However, in the aged estrogen deficient rats, Ca is mainly absorbed through the paracellular pathways. The altered epithelial Ca transporting proteins by estrogen deficiency and different Ca diet is critical for the alterations of Ca (re)absorption in the intestine and the kidney independent of vitamin D. The epithelial Ca transporting proteins were differently regulated in the intestine and the kidney.

Chapter 4

Establishment of *in vivo* Screening System for Active Fractions of Fructus Ligustri Lucidi on Ca Balance in Mature Female Rats

4.1 Introduction

Ca balance is a function of Ca intake, absorption and excretion, with absorption and excretion being the most statistically significant (Nordin et al., 2004). When habitual intakes are low, especially during periods of bone growth or loss, bioavailability is most important. Our previous study showed that the ethanol extract of FLL could improve Ca balance in aged female rats by enhancing Ca absorption through the intestine and the kidney. However the components in FLL which are responsible for its positive effect on Ca balance remain to be elucidated.

As known, *Fructus Ligustri Lucidi* (FLL), the fruit of *Ligustrum lucidum Ait,* is a commonly prescribed herbal material in traditional Chinese Medicine (TCM) to nourish the endocrine and renal system as well as to strengthen bones (Wang et al., 2003). Researchers have isolated and identified more than 100 compounds in *Fructus Ligustri Lucidi*. Furthermore, research has shown that water or ethanol extracts of FLL are useful for regulating the immune system, protecting the liver, lowering serum glucose as well as exerting anti-oxidation, anti-inflammation, anti-cancer, anti-aging functions in human (Ding and Xu, 2006; Huang MS, 2004; Liu et al., 2003; Qiu, 2007). But its effects on Ca balance were firstly reported by our group.

The regulation of Ca absorption is via the intestine and kidney, which is crucial for the maintenance of normal extracellular Ca levels. Both in the intestine and the kidney, Ca absorption occurs in the epithelial cells by two main mechanisms: a transcellular, metabolically driven transport, and a passive non-saturable route, called the paracellular pathway (Bronner, 1998). Transcellular Ca transport constitutes the primary target for regulation by calciotropic hormones, including $1,25(OH)_2D_3$ and parathyroid hormone (PTH), enabling the organism to regulate the extracellular Ca concentration on the body's demand (Hoenderop et al., 2004). Transcellular Ca transport is a three-step process, comprised of the transfer of luminal Ca into the enterocyte or renal epithelial cell through Ca-selective channels (TRPV5/TRPV6), the translocation of Ca from point of entry to the basolateral membrane by vitamin D_3-dependent calcium binding proteins (CaBP9k/CaBP28k), and finally active extrusion from the cell into the circulatory system mediated by a high-affinity plasma membrane Ca^{2+}-ATPase (PMCA1b) or Na^+/Ca^{2+} exchanger (NCX1) (Hoenderop et al., 2005).

$1,25(OH)_2D_3$ regulates epithelial Ca transport through genomic actions involving the classical vitamin D receptor (VDR) and nongenomic regulation by a separate membrane receptor (Khanal and Nemere, 2008). The parathyroid glands play a key role in maintaining the extracellular Ca concentration through their

capacity to sense even minute changes in the level of blood Ca from its normal level through the Ca-sensing receptor (CaSR). In response to low blood Ca^{2+} levels, PTH is secreted into the circulation and then acts primarily on the kidney and bone, where it activates the PTH/PTHrP receptor. This receptor directly enhances the tubular Ca re-absorption, and it stimulates the activity of 25-hydroxy-1α-hydroxylase (1-OHase) and, thereby, increases the $1,25(OH)_2D_3$-dependent absorption of Ca from the intestine (Hoenderop et al., 2005). PTH increases transepithelial Ca transport via a dual signaling mechanism involving PKA- and PKC-dependent processes (Friedman et al., 1996).

In order to identify the active fractions of FLL on Ca balance, the reliable and easily operated screening system is required. In consideration of the difficulties to get aged animals, it was designed to apply FLL ethanol extract to young mature female rats in the present study. Ca balance was detected every week after the start of feeding to the end, in order to determine the earliest time when the herb began to depress urinary Ca and fecal Ca excretion. At the end of experiment, duodenal and renal genes and proteins involved in the epithelial Ca transporting were detected for the mechanisms study.

4.2 Methods

4.2.1 Preparation of Fructus Ligustri Lucidi

Fructus Ligustri Lucidi (FLL) was obtained from Jiangsu province of China. The voucher specimen was appraised in the School of Chinese Medicine, the Chinese University of Hong Kong. The dried (40kg) form of the crude plant was extracted with 70% ethanol two times. The preparation was filtered and concentrated under vacuum to produce a viscous residue at a yield of 36%, by weight of the starting materials. The amount of oleanolic acid in the FLL ethanol extract was quantified as 28.0 mg/g. So the oleanolic acid content in the crude drug should be 1.4%, suggesting that the FLL applied in our study was of high quality according to the Chinese Pharmacopeia (2005). Then the extracta sicca was freeze-dried for 7 days and made into a dried ethanol extract. The dried ethanol extract was stored in a desiccator.

4.2.2 Animal Study Design

Animals were cared for in accordance with the Experimental Animal Center of the Hong Kong Polytechnic University. Sixteen four-month old Sprague-Dawley female rats (220-250g) (Experimental Animal Center of the Hong Kong Chinese University, Hong Kong, China) were used. The rats were housed in a room which

provided alternating 12 h of light and 12 h of darkness with the room temperature at 23 ± 1 °C and humidity 55 ± 5%. Husbandry of the animals was based on the NIH Guide for Care and Use of Laboratory Animals (Council, 2006). After 5 days of acclimation with a medium Ca diet (MCD) (TD 98005, 0.6% Ca), animals were assigned to two groups in random and paired-fed with MCD. The rats in two groups were treated with vehicle (distilled water) and FLL ethanol extract. The control group was fed with distilled water as vehicle. The ethanol extract was fed at a dosage of 700mg/kg/d. The vehicle and FLL ethanol extract were orally fed to the animals through a gastric tube. The total treatment duration was 12 weeks, which was the period used in our previous study that showed improved Ca balance in aged female rats. All rats had free access to water and were fed with MCD at 15g/day per rat, the minimum average food intake of the rats during the acclimation period. The daily drug administration was dependent on the rats' daily weight. The experimental protocol was approved by the Animal Ethics Committee of The Hong Kong Polytechnic University.

4.2.3 Sample Collection

Every week during feeding period, the rats were put into metabolic cages at room temperature to stay for 24 hours, in order to collect their urine and feces for Ca

determination. At the end of the experiment, the rats were killed and the blood, kidney, duodenum were harvested. The blood samples were allowed to clot at room temperature and the serum was separated by centrifuging at 2500 rpm for 20 min. The serum samples were stored at -80°C until analysis. Duodenal mucosa was harvested by excising the duodenum, flushing it with ice-cold physiological saline and placing it on an ice-chilled plate. The duodenum was then incised lengthwise, rinsed again, and the duodenal mucosa was separated from the underlying muscle coats with a chilled glass slide. The harvested tissue was placed in an eppendorf tube with 1 ml Trizol reagent and stored at -80 °C for RNA isolation. The two kidneys of each rat were collected and put into liquid nitrogen instantly, then stored at -80°C.

4.2.4 Biochemical Analysis of Serum, Urine and Feces Samples

The Ca concentrations of both serum and urine samples were measured using standard colorimetric methods with commercial kits (Wako Pure Chemical Industries Ltd., Japan). Urinary creatinine (Cr) was determined using the Jaffe method per the manufacturer's instructions (Wako Pure Chemical Industries Ltd., Japan). The urinary Ca excretion was expressed as the ratio of urinary Ca to Cr level. The amount of Ca in feces was determined by atomic absorption spectrophotometry (PerkinElmer, AAnalyst 100 Spectrometer). The feces was first dried (at 110 °C for

12 h), then incinerated (at 800 °C for 12 h) in a muffle furnace and weighed. Fifty milligrams of fecal ash was then dissolved in 2 mL of 6 M HCl and diluted appropriately with Milli-Q water for atomization. The Ca absorption rate was calculated from the formula: Ca absorption rate (%) = (intake Ca – fecal Ca)/intake Ca × 100; the Ca net balance was calculated from: Ca net balance (mg) = (Ca intake – Ca output) = intake Ca – (fecal Ca + urine Ca).

4.2.5 Detection of Calciotropic Hormones

Serum levels of intact parathyroid hormone (PTH 1-84) were detected using rat bioactive intact PTH ELISA assay (Immutopics, Inc., San Clemente, CA). Serum $1,25(OH)_2D_3$ was extracted with two separate extraction columns and measured by competitive enzyme immunoassay (Immundiagnostik AG, Bensheim).

4.2.6 RNA Isolation and Preparation of cDNA

Tissue samples were thawed in Trizol reagent (Invitrogen, Carlsbad, California, USA) and homogenized. RNA extraction was performed according to the Trizol manufacturer's protocol. Reverse transcription reactions were performed using the SuperScript First-Strand Synthesis System (Invitrogen).

4.2.7 Real-time PCR Analysis

Total RNA (2ug) was used to generate cDNA in each sample using the Super-Script Ⅱ reverse transcriptase with oligo (dT) 12-18 primers. The RNA, Oligo dT, dNTP were mixed into a PCR tube for amplication on a GeneAmp 9600 PCR system (Perkin Elmer, Foster City, CA, USA). Real-time PCR reactions were performed using SYBR Green PCR Master Mix and an ABI PRISM 7900HT. Thermocycling was done in a final volume of 20ul that contained 0.05ul cDNA and 800 nM of each of the forward and reverse primers. 18SrRNA was used as an endogenous control. The primers for TRPV5/TRPV6 (van Abel et al., 2003; Zhang et al., 2007), CaBP-9k (Hong et al., 2004), CaBP-28k (Wang et al., 2005), PMCA1b (Reinhardt and Horst, 1990), VDR (Zhang et al., 2007) and 18SrRNA (Afonina et al., 2006) were the same as previously described. PCR was performed using the following program: initial denaturation at 95^{o}C for 10min, 40 cycles of denaturation at 95^{o}C for 1min, primer annealing at 50^{o}C for 1min and polymerization at 72^{o}C for 1min30s. Quantitative values were derived from the threshold cycle number at which the increase in the signal associated with exponential growth of PCR products was first detected. To determine the number of copies of the targeted DNA in the samples, a relative standard curve (concentration-threshold cycle) was generated by

the dilution of cDNA from the calibrator (Control group). Data was normalized with 18SrRNA levels in the samples.

4.2.8 Western Blot Analysis

Renal proteins were obtained by cell lysis in Nonidet P-40 buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40) supplemented with protease inhibitors (2ug/ml aprotinin, 2ug/ml leupeptin, 1mM PMSF) (Sigma, St. Louis, MO, USA). Duodenal proteins were obtained from Trizol. Protein concentrations were measured by Bradford protein assay (Bio-Rad Laboratory, USA). Equal amounts of cytosolic proteins (50ug) were mixed with the loading dye. After mixing and boiling for 10 min, the samples were separated by SDS-PAGE on appropriate reducing gels at a constant voltage (150V) for 1h until they reached the bottom. Then the proteins were transblotted to PVDF membranes (Immobilin-P, Millipore Corp., Bedford, MA, USA) at a voltage of 100V for 1.5h or 22V overnight. Immuno-blotting was performed after blocking non-specific binding on the membrane with 5% non-fat milk in TTBS for 2h. Then the blots were probed first with the primary antibody for the target enzyme overnight at 4^{o}C, followed by incubation with the correspondent secondary antibody for 1.5-2h. The antigen-

antibody complexes were detected by using an enhanced chemiluminescence reagent and visualized by a Lumi-Imager with the software.

4.2.9 Statistical Analysis

The data from these experiments were reported as mean ± standard error of mean (SEM). All statistical analyses were performed using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by T-test. Differences in P value of less than 0.05 were considered statistically significant.

4.3 Results

4.3.1 Serum chemistries

Serum Ca, P, $1,25(OH)_2D_3$ and PTH concentrations illustrated in Table 4-1, demonstrate that serum Ca and Phosphorus (P) remained consistent after treatment by FLL ethanol extracts in the mature female rats. Serum $1,25(OH)_2D_3$ levels in the FLL-treated group rats were slightly higher than the vehicle treated group, but not statistically significant. There was also an increasing trend in serum PTH levels following FLL ethanol extract treatment, however it was not statistically significant when compared with the vehicle treated group.

Table 4-1 Effects of ethanol extract of FLL on serum Ca, P, $1,25(OH)_2D_3$ and PTH in mature female rats fed a normal Ca diet for 12 weeks[1]

	Serum Ca (mg/dl)	Serum P (mg/dl)	Serum PTH (pg/ml)	Serum $1,25(OH)_2D_3$ (pg/ml)
C	10.19 ± 0.43	5.29 ± 0.18	96.19 ± 14.77	36.49 ± 2.87
FLL	10.24 ± 0.16	5.03 ± 0.19	110.6 ± 23.01	44.19 ± 3.37

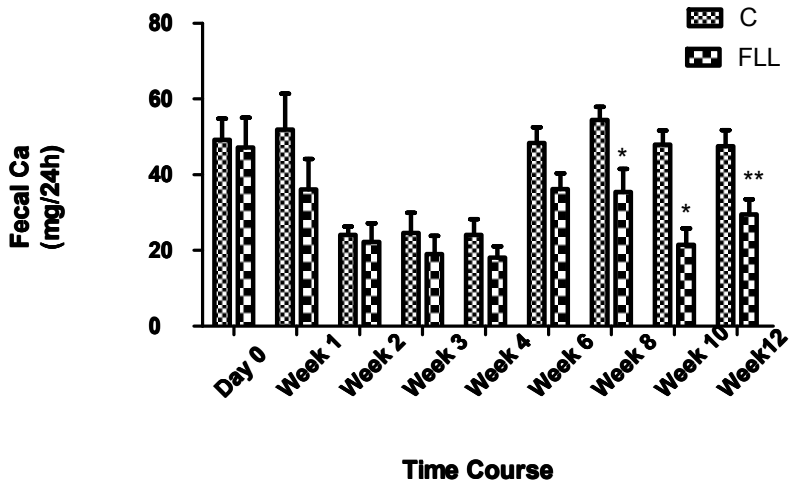
1 Values are expressed as mean ± SEM, n=8.

4.3.2 Ca balance study

It was found that from week 3, FLL ethanol extract began to reduce fecal Ca excretion in comparison with vehicle treatment in the rats, the statistically significant depressive effects occurred on week 8 until week 12. Urinary Ca excretion seemed to be suppressed from week 4, and was shown to be inhibited greatly from week 8 to week 12 (Figure 4-1).

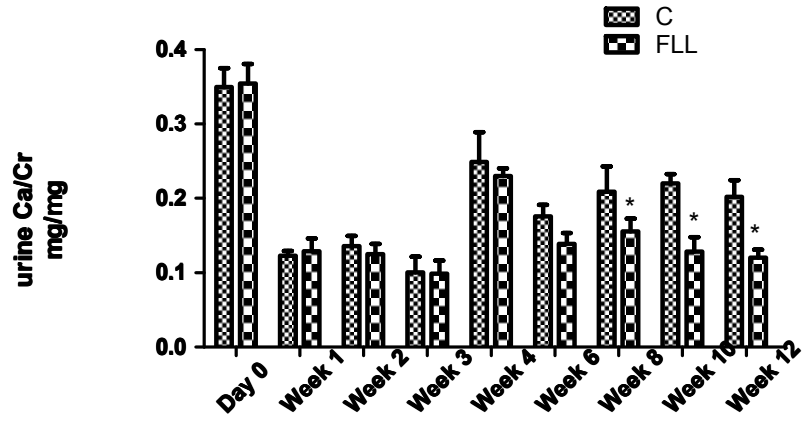
Accordingly, the calculated Ca absorption rate and Ca net balance based on the results of urinary and fecal Ca also showed the similar regulation by FLL ethanol extract. It seemed that Ca absorption rate and Ca net balance were increased by FLL ethanol extract from week 3, though the statistically significant increase was recorded from week 6 onward (Figure 4-2).

A



n=8 in each group

B

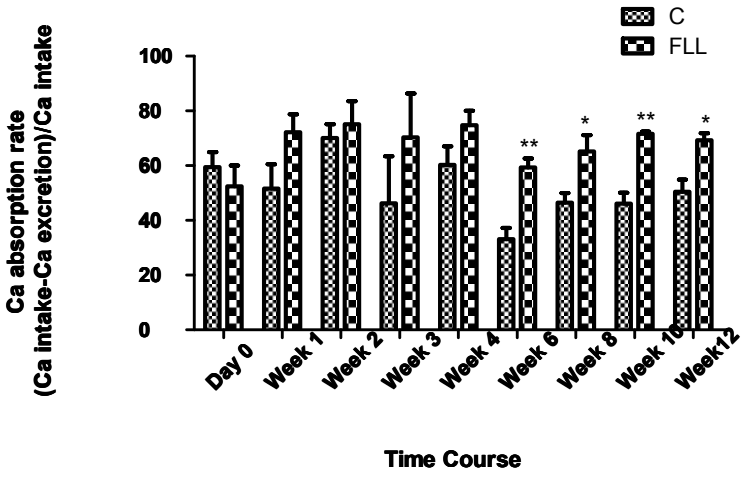


n=8 in each group

Figure 4-1. Effects of FLL ethanol extract on fecal and urinary Ca excretion at every time point.

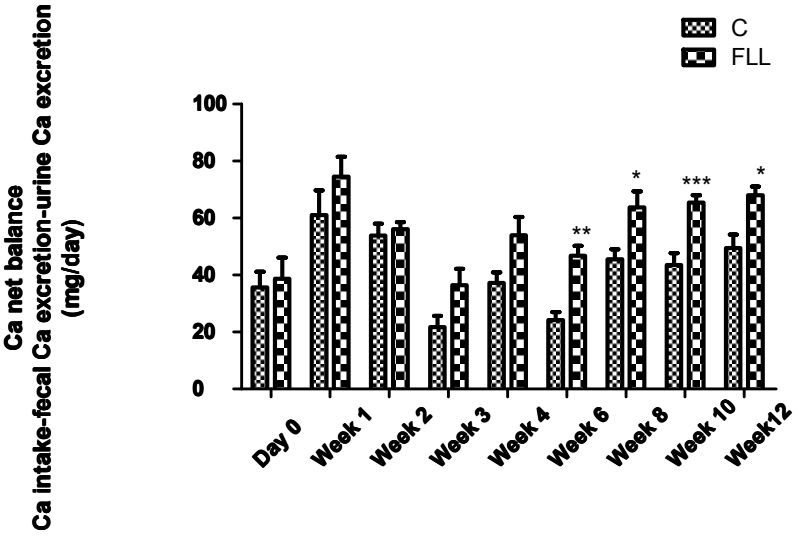
Four month old female rats were assigned to four groups and fed with FLL ethanol extract and vehicle. Every week during the feeding, the rats were put into metabolic cages for the collection of urine and feces. (A) urine Ca/Cr, (B) fecal Ca excretion were shown after being fed with FLL ethanol extract. The level of urine Ca is corrected by the level of urine creatinine (Cr). Data are expressed as mean ± SEM. Results were analyzed by T-test. *P<0.05, **P<0.01 vs Vehicle group were identified to be significant. (n=8)

A.



n=8 in each group

B.



n=8 in each group

Figure 4-2. Effects of FLL ethanol extract on Ca absorption rate and Ca net balance at every time point.

(A) Ca absorption rate is calculated by the formula: (Ca intake – Fecal Ca excretion) / Ca intake ×100. And (B) Ca net balance is calculated by: Ca net balance = Ca intake – Fecal Ca excretion – Urine Ca excretion. Data are expressed as mean ± SEM. Results were analyzed by T-test. *P<0.05, **P<0.01 and ***P<0.001 vs Vehicle group were identified to be significant. (n=8)

4.3.3 Duodenal gene and protein expression for epithelial Ca transporting

As shown in Figure 4-3, mRNA expression of duodenal TRPV6, PMCA1b and VDR showed an increasing trend after FLL ethanol extract treatment, though the increase was not statistically significant. Duodenal TRPV6 and PMCA1b protein expression also had a higher tendency in the FLL ethanol extract treatment group. FLL ethanol extract enhanced the protein expression of duodenal VDR greatly ($P<0.05$; Figure 4-4B). However, it seemed that the expression of CaBP9k which is responsible for transporting Ca in epithelia cells was not upregulated by FLL ethanol extract.

The changing trend of two important proteins involved in the epithelial Ca absorption was shown to be consistent with the result from intestinal Ca absorption after FLL ethanol extract treatment. FLL ethanol extract showed positive up-regulatory effects on Ca absorption through the duodenum at least in part by increasing the mRNA or protein expression which play roles in epithelial Ca transporting process. And the increase of duodenal VDR expression by FLL ethanol extract indicated the possible actions of $1,25(OH)_2D_3$ on induction of TRPV6 and PMCA1b expressions.

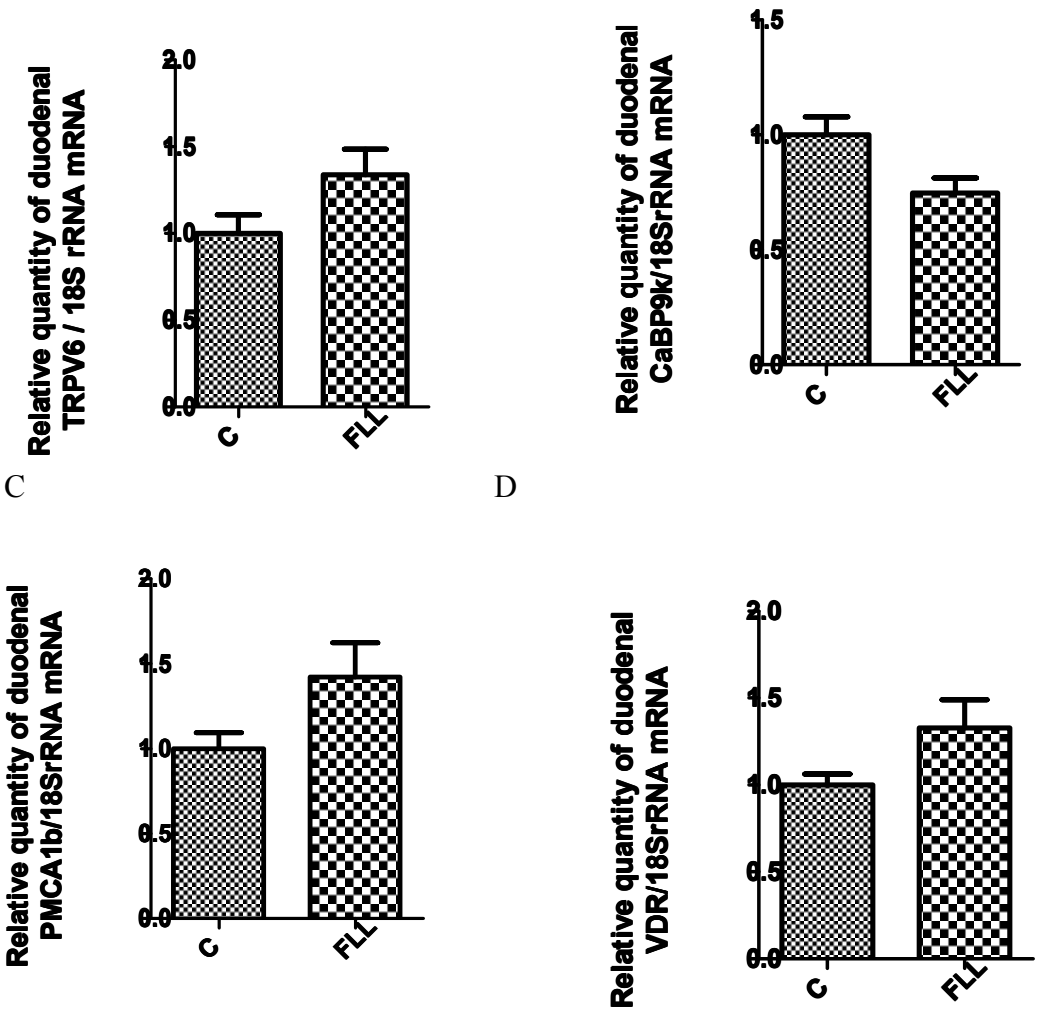


Figure 4-3. Effects of FLL ethanol extract on duodenal mRNA expression of TRPV6, CaBP9k, PMCA1b and VDR.

Four month old female rats were assigned to four groups and fed with FLL ethanol extract and vehicle. On week12, the rats were sacrificed, duodenal mucosa was collected and the duodenal mRNA expressions of TRPV6 (A), CaBP9k (B), PMCA1b (C) and VDR (D) in each group were detected. The expression level is shown as a ratio of target gene/18SrRNA and the ratio in vehicle group is normalized as 1.0. Data are expressed as mean ± SEM (n=8). *P<0.05, **P<0.01 vs Vehicle group.

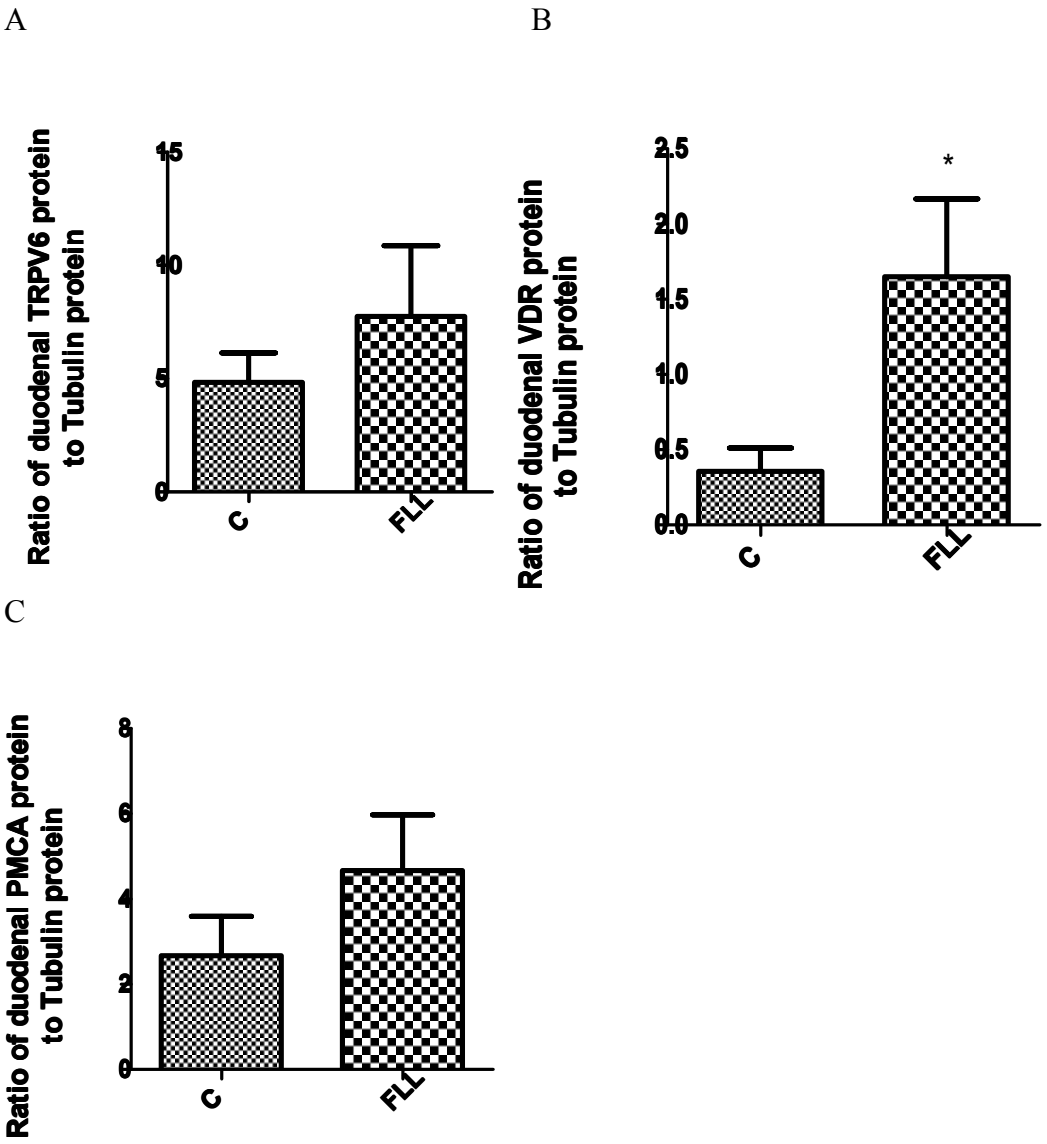


Figure 4-4 Effects of FLL ethanol extract on duodenal protein expression of TRPV6, VDR and PMCA1b.

Duodenal protein expressions of TRPV6 (A), VDR (B), PMCA1b (C) in each group. The expression level is shown as a ratio of target protein/β-tubulin (n=8 in each group). Values are means ± SEM. *P<0.05 vs Vehicle group.

4.3.4 Renal gene and protein expressions for epithelial Ca transporting

FLL ethanol extract treatment seemed to elevate renal TRPV6 and CaBP28k mRNA and protein expression, though this was not statistically significant (Figure 4-5A, 4-6A, 4-5B and 4-6B). Different from that in duodenum, Ca transporting protein – CaBP28k but not PMCA1b expression in kidney had an increasing trend in FLL ethanol extract treated group (Figure 4-5C and 4-6C). Renal VDR protein was repressed by FLL ethanol extract, which was differently regulated with that in duodenum ($P<0.05$; Figure 4-6D).

Generally, the regulation pattern of gene and protein expressions for epithelial transporting in kidney was consistent with that of urinary Ca excretion by FLL ethanol extract. So it suggested that decrease of urinary Ca excretion by FLL ethanol extract could partly be explained by the increased active re-absorption in kidney. Due to the suppressive actions of FLL on renal VDR expression, it was unclear if the change of renal proteins for epithelial Ca transporting was attributed to the induction of $1,25(OH)_2D_3$.

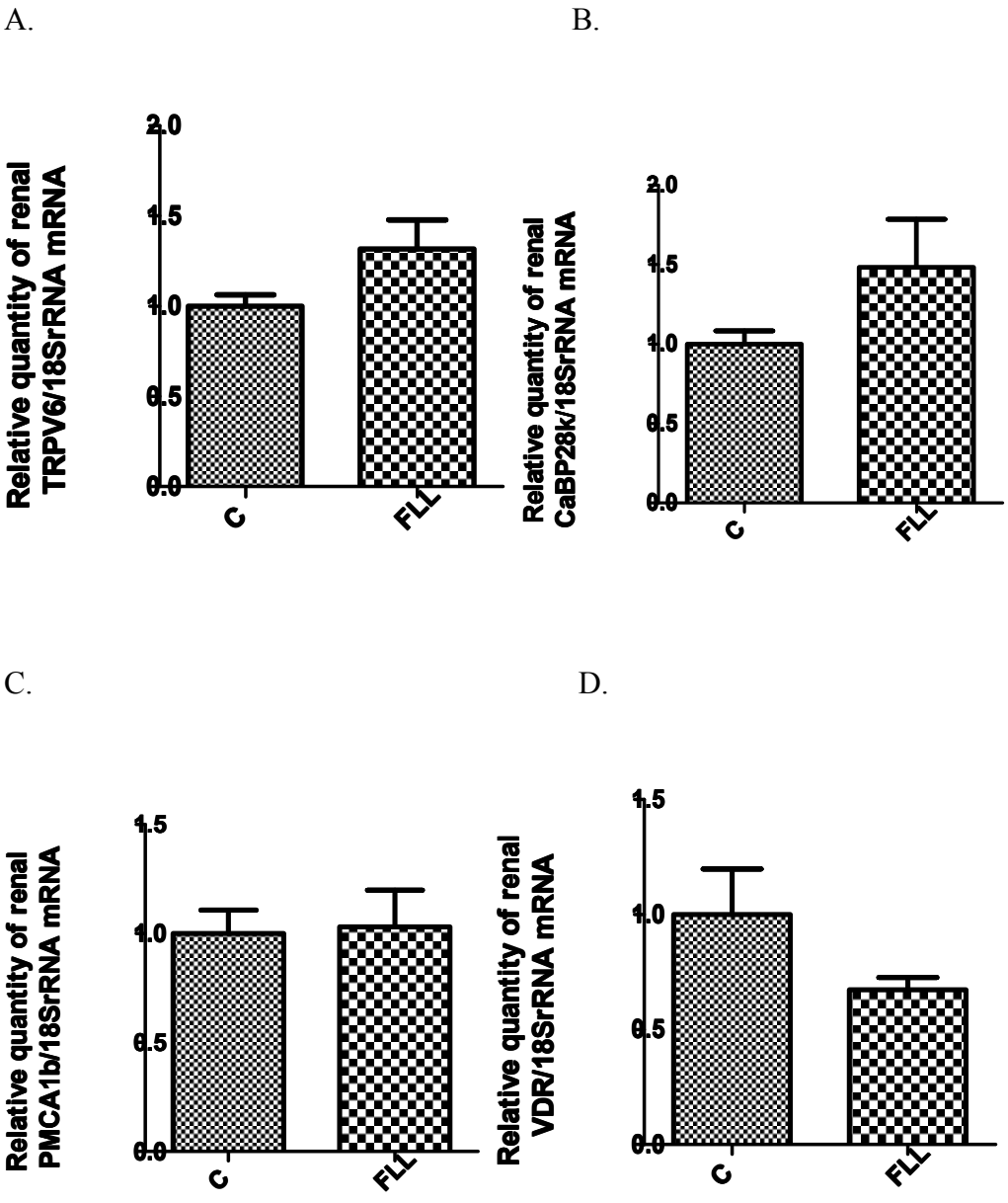


Figure 4-5. Effects of FLL ethanol extract on renal mRNA expression of TRPV6, CaBP28k, PMCA1b and VDR.

Four month old female rats were assigned to four groups and fed with FLL ethanol extract and vehicle. On week12, the rats were sacrificed, duodenal mucosa was collected and the renal mRNA expressions of TRPV6 (A), CaBP28k (B), PMCA1b (C) and VDR (D) in each group were detected. The expression level is shown as a ratio of target gene/18SrRNA and the ratio in vehicle group is normalized as 1.0. Data are expressed as mean ± SEM (n=8).

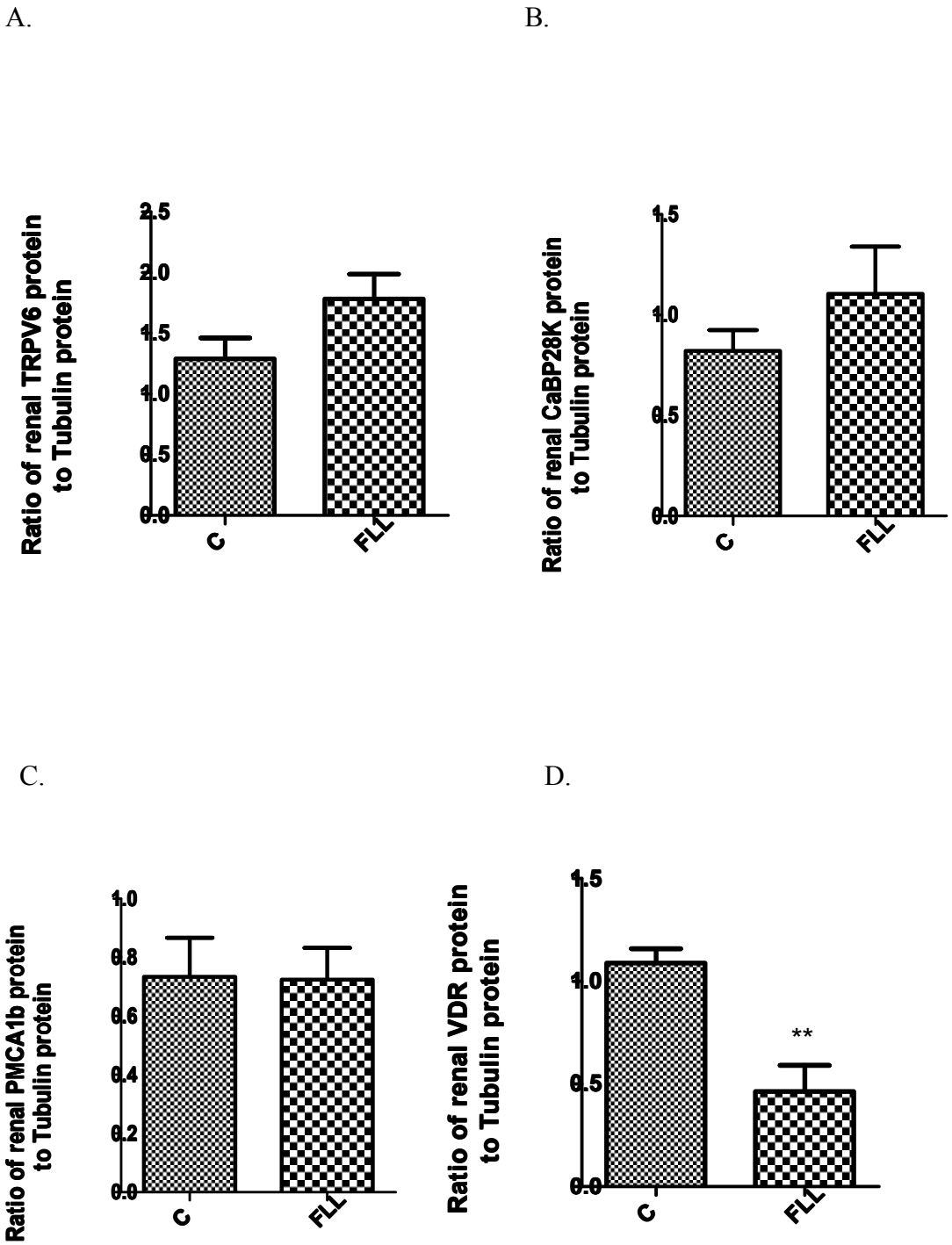


Figure 4-6 Renal protein expressions of TRPV6 (A), CaBP28k (B), PMCA1b (C), VDR (D) in each group.

The expression level is shown as a ratio of target protein/β-tubulin (n=8) in each group). Values are means ± SEM. **P<0.01 vs Vehicle group.

4.4 Discussion

The present study indicated that FLL ethanol extract could also offer positive effects on Ca balance in the young mature female rats. FLL ethanol extract began to depress urinary and fecal Ca excretion from week 3 after the feeding. This effect continued to the end of the experiment. And the genes and proteins involved in epithelial Ca transport were shown to change in a trend with the change of Ca (re)absorption. It suggested that the young mature female rats might be selected to be the *in vivo* screening platform for active fractions of FLL on Ca balance.

In our study, FLL ethanol extract could decrease urinary and fecal Ca excretion and increase the Ca absorption rate through intestine and influence Ca re-absorption through kidney in the young mature rats. This result was consistent with the effect of the herb in the aged female rats. Similarly, FLL ethanol extract could not influence serum Ca and P in the young female rats. It appeared that serum PTH and $1,25(OH)_2D_3$ levels were elevated by FLL treatment, though this was not statistically significant. But the difference of induction degree of serum PTH and $1,25(OH)_2D_3$ levels by FLL ethanol extract in the young and aged female rats might be attributed to the different basal levels between young and aged ones.

The mechanism study indicated that the increased intestinal Ca absorption might be partly explained by the higher expression of intestinal TRPV6 and PMCA1b in the FLL-treated group. It has been proven that TRPV proteins are primarily regulated by $1,25(OH)_2D_3$, but its effects require the presence of functioning VDR. Both vitamin D and $1,25(OH)_2D_3$ have been found to increase PMCA mRNA and protein levels in the intestine but not in the kidney (van Abel et al., 2005b). In our experiment, duodenal VDR mRNA expression was shown to be elevated by FLL ethanol extract treatment. So it suggested that the increase of intestinal TRPV6 and PMCA1b expression might be induced by $1,25(OH)_2D_3$ through VDR, though a direct effect of FLL can not be excluded.

In the kidney, renal TRPV6, CaBP28k gene and protein appeared to be elevated by FLL ethanol extract, though this was not statistically significant. Renal PMCA1b genes were not altered by the herbal treatment. It indicated that the increased TRPV6 and CaBP28k by FLL ethanol extract might contribute to the decrease in urinary Ca excretion. In contrast to the intestine, renal VDR was shown to be inhibited by FLL ethanol extract treatment. This suggested that changes of renal TRPV6 and CaBP28k could not be attributed to vitamin D.

In summary, FLL ethanol extract exerted the positive actions on Ca balance in the young mature female rats as in the aged ones. The changes of epithelial Ca transporting proteins in the intestine and the kidney after FLL ethanol extract treatment could partly explain the effects of FLL on Ca balance. But the regulation of these epithelial proteins in the intestine and kidney were different. The former was induced by vitamin D, while the latter appeared not to be influenced by vitamin D. Based on our results, the younger mature female rats instead of the older animals will be selected to be the initial screening platform for the identification of active fractions in FLL which are responsible for Ca balance.

Chapter 5

Water Layer Fraction is Responsible for the Activity of the Ethanol Extract of Fructus Ligustri Lucidi on Ca balance *in vivo*

5.1 Introduction

Fructus Ligustri Lucidi contained more than 100 compounds including terpenoids, flavonoids, phenethanols, essential oils and others. Of them, terpenoids were the main components in FLL including triterpenes and iridoids. The triterpenes found in FLL include oleanolic acid, ursolic acid, acetyloleanic acid, 2α-hydroxy oleanolic acid (Yin, 1995), D-mannitol (Cheng et al., 2000), α-ursolic acid methylester, 2α,3β,19α-trihydroxylursolic acid (Tormentic acid) (Cheng et al., 2000; O.Masaru, 1977), dammar-24-ene-3β-acetate-20S-ol and dammar-25-ene-3β,20ζ,24ζ-ol (Wu, 1998). Iridoids are typically found in plants as glycosides, most often bound to glucose. The iridoids are produced by plants primarily as a defense against herbivores or against infection by microorganisms. Isolated iridoids in FLL contain nuezhennidic acid (Wilcox et al., 2001), p-hydroxyphenethyl-β-D-glucoside, p-hydroxyphenethyl-α -D-glucoside, sodium oleanolate, specneuzhenide and nuezhengalaside (Monkawa et al., 1997; Shi, 1995), oleoside dimethyl ester, ligustroside, oleuropein, nuezhenide, isonuezhenide, neonuezhenide and lucidumoside (Neer et al., 2001). FLL also contains small quantity of flavonoids, which are identified to be luteolin-7-O-β-D-glucoside, quercitrin (Zhang and Shi, 2004), apigenin and quercetin (Ettinger et al., 1999), cosmosiin, apigenin-7-O-

acetyl-β-D-glucoside,apigenin-7-O-β-D-lutinoside, luteolin, luteolin-7-O-β-D-glucopyranoside and quercetin (Shen et al., 1995).

Research studies have shown that water or ethanol extracts of FLL are useful for regulating the immune system, protecting the liver, lowering serum glucose as well as exerting anti-oxidation, anti-inflammation, anti-cancer and anti-aging functions in humans as decribed above. Our previous study showed that FLL ethanol extract could increase the bone mass of the diaphysis of the tibia and femur in the aged female rats when the rats were fed sufficient dietary Ca. This process was thought to be due to the direct actions on osteoblastic cells by enhancing the mineralization process, leading to a positive Ca balance (Zhang et al., 2008c). At the same time, it was found that the ethanol extract of FLL could improve Ca balance in aged female rats by enhancing Ca absorption (Zhang et al., 2008b). However the components in FLL which are responsible for its positive effect on Ca balance remain to be elucidated.

The purpose of this study was to identify the active fractions of FLL that are responsible for FLL's positive effect on Ca balance, and to further determine the effects of the active fraction on Ca transport in response to differing levels of dietary

Ca. To identify the active fractions in FLL on Ca balance, the two FLL fractions were separated from the ethanol extract. One fraction is EA soluble fraction (EAF) which was extracted by ethyl acetate (EA), the left layer fraction is called water layer fraction (WF). The FLL ethanol extract (EE) with its two separated fractions were administered to female rats. Urine and fecal samples were collected for evaluation of Ca excretion. Ca balance was determined in rats fed varying levels of dietary Ca and treated with different FLL extracts. Our results showed that the water layer fraction of FLL ethanol extract is the active fraction that exerts positive effects on Ca balance when the rats were fed with sufficient dietary Ca.

5.2 Methods

5.2.1 Preparation and Fractionation of Fructus Ligustri Lucidi

Fructus Ligustri Lucidi was obtained from Jiangsu province of China. The preparation of FLL ethanol extract (EE) was described previously (Zhang et al., 2008b).

The ethanol extract of FLL was firstly dried into powder and then dispersed into water. The ethyl acetate was then used to extract the lipophilic fraction, called EA soluble fraction (EAF) in our study, the remaining layer fraction was called water layer fraction (WF). The two separated fractions were also made into power and store in a desiccator.

Oleanolic acid is a commonly used marker for the authentication of FLL according to the Chinese Pharmacopoeia (Pharmacopeia, 2005). The content of oleanolic acid in FLL is often used as one of the criteria to evaluate the quality of this crude drug. In our study, we used oleanolic acid and ursolic acid as standard samples to identify if they existed in the two separated fractions by HPLC detection. The results from HPLC indicated that the two compounds mainly presented in the EA soluble fraction, but not in the water layer fraction (Figure 5-1). At the same

time, the other two compounds—nuzhenide and salidroside were used as standard samples to appraise the water layer fraction (WF). Data from HPLC showed that these two compounds presented in the WF, also constituted the major peaks in this fraction (Figure 5-2).

5.2.2 Animal Study Design

Thirty two four-month old Sprague-Dawley female rats (220-250g) (Experimental Animal Center of the Hong Kong Chinese University, Hong Kong, China) were used. The rats were housed in a room which provided alternating 12 h of light and 12 h of darkness with the room temperature at 23 ± 1 °C and humidity 55 ± 5%. Husbandry of the animals was based on the NIH Guide for Care and Use of Laboratory Animals (Council, 2006). After 5 days of acclimation with a medium Ca diet (MCD) (TD 98005, 0.6% Ca), animals were randomly assigned to four groups and paired-fed. The rats in the four groups were treated with vehicle (C), ethanol extract (EE), EA soluble fraction (EAF) and water layer fraction (WF), respectively. The vehicle group was used as control and fed with distilled water. The dosage of ethanol extract used was 700 mg/kg/d as described in previous studies. While EA and water layer fractions were extracted from FLL ethanol extract, they were fed to the animals at the dosage determined by their extraction ratio. So the

dosage of EA soluble fraction was 126 mg/kg/d, and that of water layer fraction was 574mg/kg/d. All of the FLL extracts were orally fed to the animals through a gastric tube. The duration of treatment was 12 weeks, which was the period used in our previous study that showed improved Ca balance in aged female rats (Zhang et al., 2008b). All rats had free access to water and were fed with MCD at 15g/day per rat, the minimum average food intake of the rats during the acclimation period. The daily drug administration was dependent on the rats' daily weight. The experimental protocol was approved by the Animal Ethics Committee of The Hong Kong Polytechnic University.

To study the effects of water layer fraction in the FLL ethanol extract on Ca balance and bone homeostasis under different levels of dietary Ca, sixty four-month old mature Sprague-Dawley female rats (220-250g) (Experimental Animal Center of the Hong Kong Chinese University, Hong Kong, China) were randomly divided into three groups with differing dietary Ca levels and treated with water layer fraction for 12 weeks. Before treatment, the rats were housed in a room which provided alternating 12 h of light and 12 h of darkness with the room temperature at 23 ± 1 °C and humidity 55 ± 5%. All rats were fed with a medium Ca diet (MCD, TD 98005, 0.6% Ca, 0.65% P) for 10 days before the initiation of the treatment regimen. The

rats were then randomly divided into three groups and fed with diets containing different levels of Ca: low Ca diet (LCD, TD 05004, 0.1% Ca, 0.65% P), MCD and high Ca diet (HCD, TD 05005, 1.2% Ca, 0.65% P). All diets were purchased from Harlan Teklad (Madison, WI, USA). In each dietary Ca group, the rats were sub-divided into two groups of ten rats based on their treatment of either FLL water layer fraction (574 mg/kg/d) or its vehicle (distilled water) for 12 weeks. All rats had free access to distilled water, and were fed 15g/day per rat of the respective diet, the minimum average food intake of the rats during the acclimation period. The body weight of the animals was recorded weekly to adjust the administration of FLL.

5.2.3 Sample Collection

During feeding, the animals were put into metabolic cages every week for urine and feces collection. At the end of the experiment, the rats were killed and the blood, kidney, duodenum were harvested. The blood samples were allowed to clot at room temperature and the serum was separated by centrifuging at 2500 rpm for 20 min. The serum samples were stored at -80°C until analysis. Duodenal mucosa was harvested by excising the duodenum, flushing it with ice-cold physiological saline and placing it on an ice-chilled plate. The duodenum was then incised lengthwise, rinsed again, and the duodenal mucosa was separated from the underlying muscle

coats with a chilled glass slide. The harvested tissue was placed in an eppendorf tube with 1 ml Trizol reagent and stored at -80 °C for RNA isolation. The two kidneys of each rat were collected and put into liquid nitrogen instantly, then stored at -80° C. The tibias and femurs were collected, cleaned of all soft tissues, wrapped together in saline-soaked towels, and stored at -20°C for further analysis.

5.2.4 Biochemical Analysis of Serum, Urine and Feces Samples

The Ca concentrations of both serum and urine samples were measured using standard colorimetric methods with commercial kits (Wako Pure Chemical Industries Ltd., Japan). Urinary creatinine (Cr) was determined using the Jaffe method according to the manufacturer's instructions (Wako Pure Chemical Industries Ltd., Japan). The urinary Ca excretion was expressed as the ratio of urinary Ca to Cr level. The amount of Ca in feces was determined by atomic absorption spectrophotometry (PerkinElmer, AAnalyst 100 Spectrometer). The feces was first dried (at 110 °C for 12 h), then incinerated (at 800 °C for 12 h) in a muffle furnace and weighed. Fifty milligrams of fecal ash was then dissolved in 2 mL of 6 M HCl and diluted appropriately with Milli-Q water for atomization. The Ca absorption rate was calculated from the formula: Ca absorption rate (%) = (intake Ca

– fecal Ca)/intake Ca × 100; the Ca net balance was calculated from: Ca net balance (mg) = (Ca intake – Ca output) = intake Ca – (fecal Ca + urine Ca).

5.2.5 Detection of Calciotropic Hormones

Serum levels of intact parathyroid hormone (PTH 1-84) were measured using rat bioactive intact PTH ELISA assay (Immutopics, Inc., San Clemente, CA). Serum $1,25(OH)_2D_3$ was extracted with two separate extraction columns and measured by competitive enzyme immunoassay (Immundiagnostik AG, Bensheim).

5.2.6 RNA Isolation and Preparation of cDNA

Tissue samples were thawed in Trizol reagent (Invitrogen, Carlsbad, California, USA) and homogenized. RNA extraction was performed according to the Trizol manufacturer's protocol. Reverse transcription reactions were performed using the SuperScript First-Strand Synthesis System (Invitrogen).

5.2.7 Real-time PCR Analysis

Total RNA (2ug) was used to generate cDNA in each sample using the Super-Script Ⅱ reverse transcriptase with oligo (dT) 12-18 primers. The RNA, Oligo dT, dNTP were mixed into a PCR tube for amplication on a GeneAmp 9600 PCR

system (Perkin Elmer, Foster City, CA, USA). Real-time PCR reactions were performed using SYBR Green PCR Master Mix and an ABI PRISM 7900HT. Thermocycling was done in a final volume of 20ul that contained 0.05ul cDNA and 800 nM of each of the forward and reverse primers. 18SrRNA was used as an endogenous control. The primers for TRPV5/TRPV6 (van Abel et al., 2003; Zhang et al., 2007), CaBP-9k (Hong et al., 2004), CaBP-28k (Wang et al., 2005), PMCA1b (Reinhardt and Horst, 1990), VDR (Zhang et al., 2007) and 18SrRNA (Afonina et al., 2006) were the same as previously described. PCR was performed using the following program: initial denaturation at 95^{o}C for 10min, 40 cycles of denaturation at 95^{o}C for 1min, primer annealing at 50^{o}C for 1min and polymerization at 72^{o}C for 1min30s. Quantitative values were derived from the threshold cycle number at which the increase in the signal associated with exponential growth of PCR products was first detected. To determine the number of copies of the targeted DNA in the samples, a relative standard curve (concentration-threshold cycle) was generated by the dilution of cDNA from the calibrator (Control group). Data was normalized with 18SrRNA levels in the samples.

5.2.8 Western Blot Analysis

Renal proteins were obtained by cell lysis in Nonidet P-40 buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40) supplemented with protease inhibitors (2ug/ml aprotinin, 2ug/ml leupeptin, 1mM PMSF) (Sigma, St. Louis, MO, USA). Duodenal proteins were obtained form Trizol. Protein concentrations were measured by Bradford protein assay (Bio-Rad Laboratory, USA). Equal amounts of cytosolic proteins (50ug) were mixed with the loading dye. After mixing and boiling for 10 min, the samples were separated by SDS-PAGE on appropriate reducing gels at a constant voltage (150V) for 1h until they reached the bottom. Then the proteins were transblotted to PVDF membranes (Immobilin-P, Millipore Corp., Bedford, MA, USA) at a voltage of 100V for 1.5h or 22V overnight. Immuno-blotting was performed after blocking non-specific binding on the membrane with 5% non-fat milk in TTBS for 2h. Then the blots were probed first with the primary antibody for the target enzyme overnight at 4^{o}C, followed by incubation with the correspondent secondary antibody for 1.5-2h. The antigen-antibody complexes were detected by using an enhanced chemiluminescence reagent and visualized by a Lumi-Imager with the software.

5.2.9 Analysis of Trabecular Structure using Micro-computed Tomography

Cone-beam X-ray micro-computed tomography (μCT) was used to take μCT images of the left feumur head (FH), femur midshaft (FM), femur end (FE), tibia head (TH) and tibia midshaft (TM) with a tube voltage of 50 KV, tube content of 0.1 mA, slice thickness of 13 μm, and pixel size of 13 μm. The scanned bone contained both cortical and cancellous bone in femur and tibia midshaft located at approximately the middle of femur and tibia. Next, three-dimensional images were set up based on the μCT images and using three-dimensional image analysis software.

5.2.10 Statistical Analysis

The data from these experiments were reported as mean ± standard error of mean (SEM). All statistical analyses were performed using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA followed by Turkey's multiple comparison test as a post test. Differences in P value of less than 0.05 were considered statistically significant.

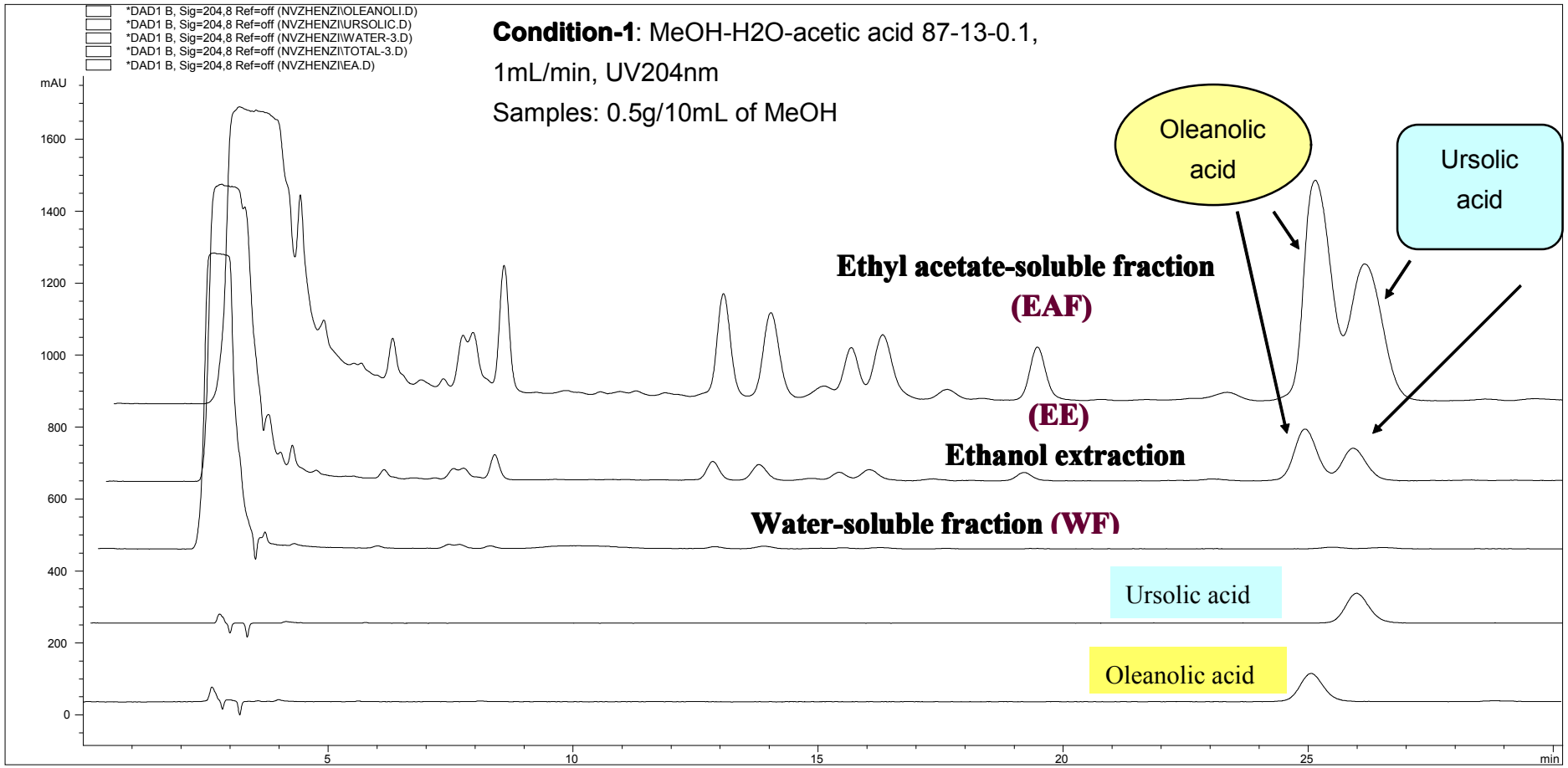


Figure 5-1. Reverse-phase high-performance liquid chromatography (HPLC) for the qualitative analysis of oleanolic acid and ursolic acid in the FLL ethanol extract and its EA soluble fraction with water layer fraction.

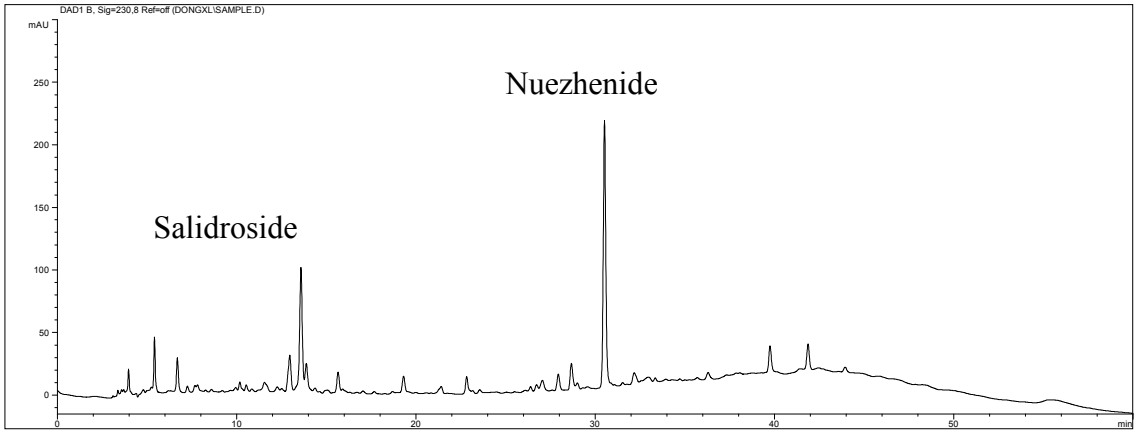


Figure 5-2 HPLC Fingerprinting of Water Layer Fraction in the Ethanol Extract of Fructus Ligustri Lucidi

Conditions: Agilent 1100 series LC/MSD Trap;

Alltech Alltim C18 HPLC column, 4.6 × 250 mm, 5 μm

Mobile phase: 0.1% TFA in water and acetonitrile

ACN% (Time, min): 8%(0), 40%(50), 70%(60)

Detector: DAD; Detector wavelength: 230nm

Flow rate: 0.8 Ml/min; Injection volume: 5 μL

5.3 Results

5.3.1 Serum chemistries

Serum Ca, P and calciotropic hormones are shown in Table 5-1. It was demonstrated that serum Ca and P remained unchanged in rats in response to treatment by different FLL extracts for 12 weeks. Serum $1,25(OH)_2D_3$ levels in rats were found to be slightly higher in the FLL ethanol extract (EE) treated group, but the increase was not statistically significant. There was also a trend towards up-regulation of serum PTH levels following the treatment with FLL ethanol extract (EE) and its water layer fraction (WF), however the increases were not statistically significant.

Table 5-1 Effects of different extracts of FLL on serum Ca, P, $1,25(OH)_2D_3$ and PTH in mature female rats fed normal Ca diet for 12 weeks[1]

	Weight (g)	Serum Ca (mg/dl)	Serum P (mg/dl)	Serum PTH (pg/ml)	Serum $1,25(OH)_2D_3$ (pg/ml)
C	261.16 ± 6.05	10.19 ± 0.43	5.29 ± 0.18	96.19 ± 14.77	36.49 ± 2.87
EE	255.50 ± 7.38	10.24 ± 0.16	5.03 ± 0.19	110.6 ± 23.01	44.19 ± 3.37
EAF	265.56 ± 4.37	9.92 ± 0.19	5.30 ± 0.15	90.71 ± 14.99	33.35 ± 3.78
WF	260.89 ± 7.42	10.08 ± 0.31	4.71 ± 0.15	135.71 ± 14.76	36.25 ± 4.96

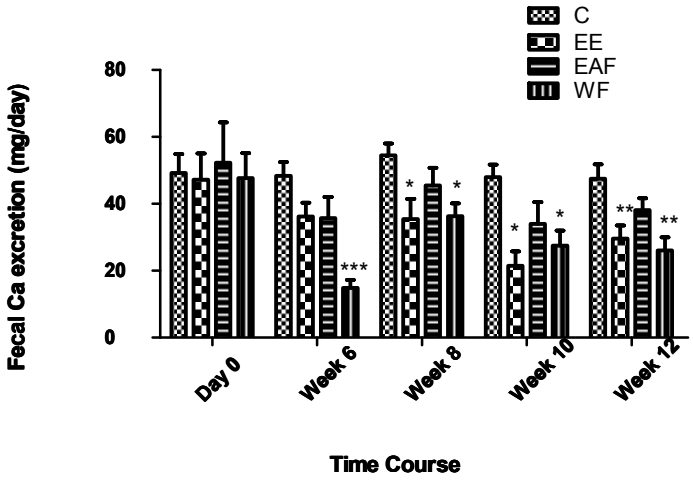
[1] Values are expressed as mean ± SEM, n=8.

5.3.2 Ca balance study

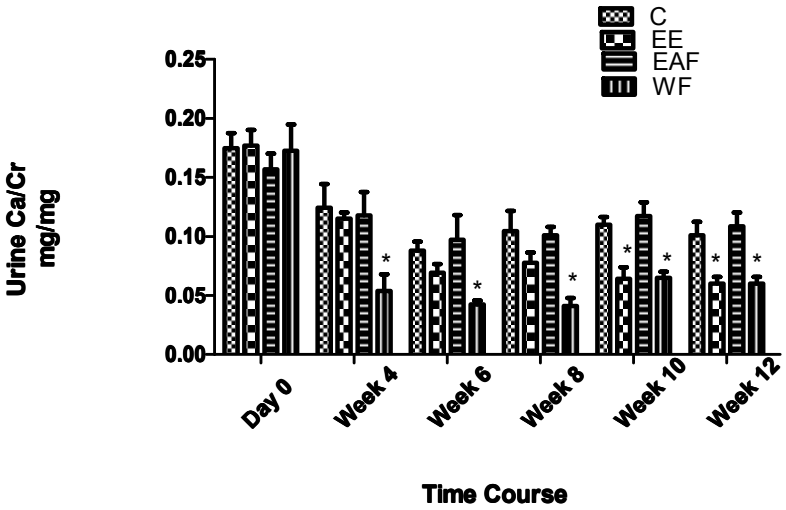
As shown in Figure 5-3, water layer fraction (WF) of the FLL ethanol extract revealed statistically significant repressive effects on fecal Ca excretion from week 6 to week 12 after feeding. The significant inhibitory actions of FLL ethanol extract (EE) on fecal Ca excretion occurred from week 8 to week 12. However, EA soluble fraction did not show the significant inhibition on fecal Ca excretion. Similarly, WF treatment began to decrease urinary Ca excretion compared with the control group from week 4 to week 12. EE treatment inhibited it greatly from week 8 to week 12. The urinary Ca excretion appeared not to be suppressed by EAF treatment.

Accordingly, the calculated Ca absorption rate and Ca net balance based on the results of urinary and fecal Ca also showed the similar regulation by different FLL extracts. FLL ethanol extract (EE) and its water layer fraction (WF) treatment increased Ca absorption rate and Ca net balance from week 6 to week 12 in comparison with vehicle treatment. But its EA soluble fraction (EAF) seemed not to offer the consistent increasing effects on Ca absorption rate and Ca net balance from week 6 to week 12 (Figure 5-4). These results indicate that the water layer fraction (WF) of the FLL ethanol extract might be responsible for the positive effects of FLL ethanol extract on Ca balance by decreasing urinary and fecal Ca excretion.

A.



B. n=8 in each group

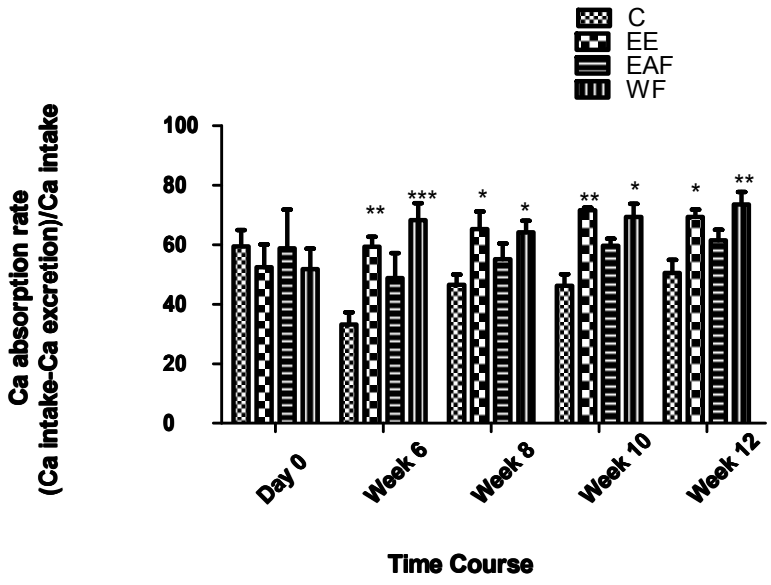


n=8 in each group

Figure 5-3. Effects of different FLL extracts on fecal and urianry Ca excretion at every time point.

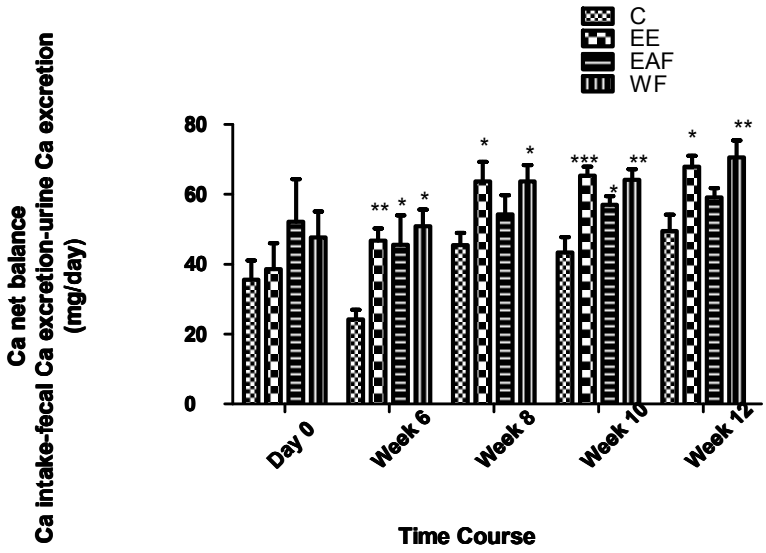
Four month old female rats were assigned to four groups and fed with Ethanol extract (EE), EA soluble fraction (EAF), Water layer fraction (WF) and vehicle (C) respectively. Every week after feeding, the rats were put into metabolic cages for the collection of urine and feces. (A) fecal Ca and (B) urine Ca/Cr excretion were shown after feeding with different FLL extracts. The level of urine Ca is corrected by the level of urine creatinine (Cr). Data are expressed as mean ± SEM. Results were analyzed by one-way ANOVA. *P<0.05, **P<0.01 vs C were identified to be significant. (n=8)

A.



n=8 in each group

B.



n=8 in each group

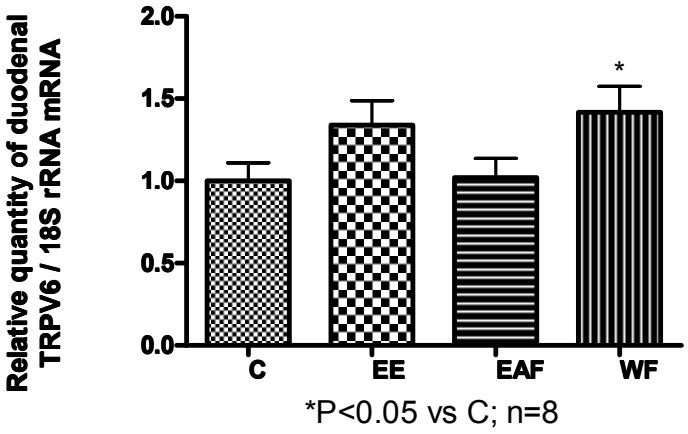
Figure 5-4. Effects of different FLL extracts on Ca absorption rate and Ca net balance at every time point.

(A) Ca absorption rate was calculated by the formula: (Ca intake – Fecal Ca excretion) / Ca intake ×100. And (B) Ca net balance was calculated by: Ca net balance = Ca intake – Fecal Ca excretion – Urine Ca excretion. Data are expressed as mean ± SEM. Results were analyzed by one-way ANOVA. *P<0.05, **P<0.01 vs C were identified to be significant. (n=8)

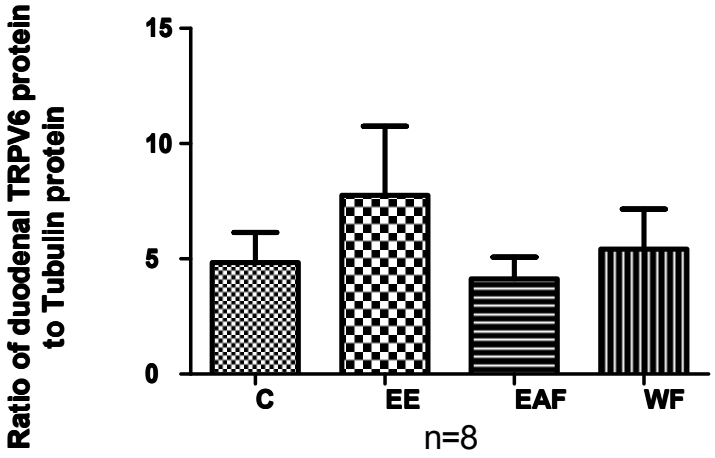
5.3.3 Duodenal gene and protein expression for epithelial Ca transporting

As shown in Figure 5-5A and 5-5B, FLL ethanol extract (EE) and its water layer fractions (WF) enhanced the mRNA and protein expression of duodenal TRPV6 which is responsible for the Ca^{2+} entry. The EA soluble fraction (EAF) lacked the ability to increase the duodenal TRPV6 expression. Similarly, duodenal PMCA1b which acts as pumping Ca^{2+} into circulations was also elevated by FLL ethanol extract (EE) and its water layer fraction (WF), as compared with its EA soluble fraction (EAF) (Figure 5-5C and 5-5D). Unlike the increase in expression of TRPV6 and PMCA1b, CaBP9k which transports Ca^{2+} in epithelial cells was not affected by FLL extracts (Data not shown). Duodenal VDR mRNA and its protein expression regulation by FLL extracts were different. The gene expression of duodenal VDR was elevated by EE and WF, especially by WF treatment ($P<0.05$; Figure 5-5E). However, its protein expression was only enhanced greatly by EE treatment ($P<0.05$; Figure 5-5F).

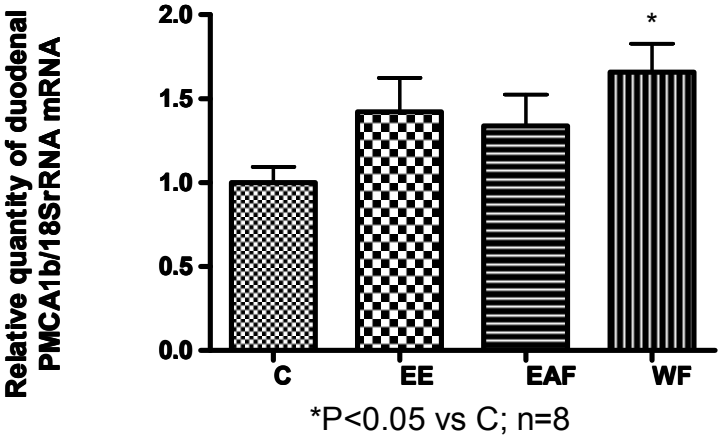
A.


Relative quantity of duodenal TRPV6 / 18S rRNA mRNA
2.0
1.5
1.0
0.5
0.0
*
C
EE
EAF
WF
*P<0.05 vs C; n=8

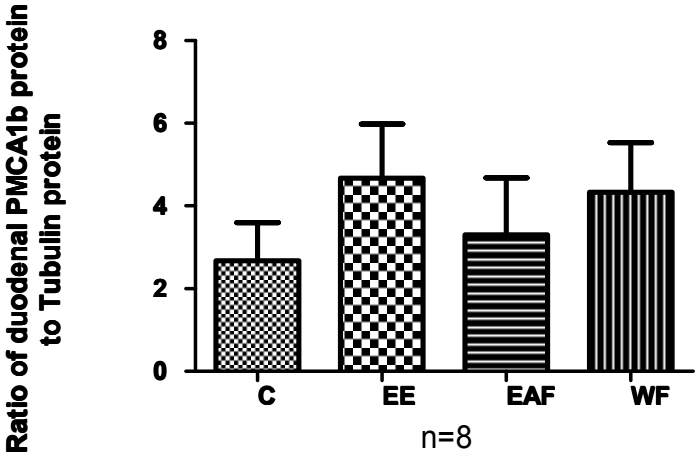
B.


Ratio of duodenal TRPV6 protein to Tubulin protein
15
10
5
0
C
EE
EAF
WF
n=8

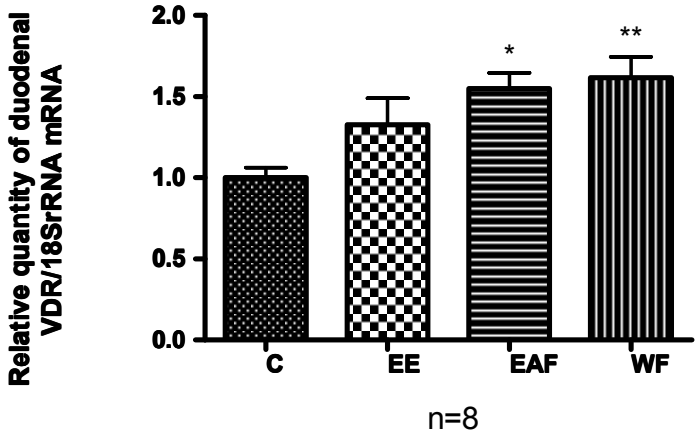
C.


Relative quantity of duodenal PMCA1b/18SrRNA mRNA
2.0
1.5
1.0
0.5
0.0
*
C
EE
EAF
WF
*P<0.05 vs C; n=8

D.


Ratio of duodenal PMCA1b protein to Tubulin protein
8
6
4
2
0
C
EE
EAF
WF
n=8

E.


Relative quantity of duodenal VDR/18SrRNA mRNA
2.0
1.5
1.0
0.5
0.0
*
**
C
EE
EAF
WF
n=8

F.

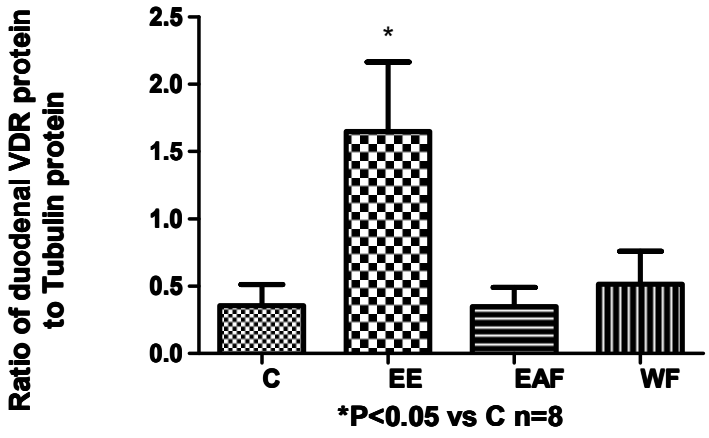

Ratio of duodenal VDR protein to Tubulin protein
2.5
2.0
1.5
1.0
0.5
0.0
*
C
EE
EAF
WF
*P<0.05 vs C n=8

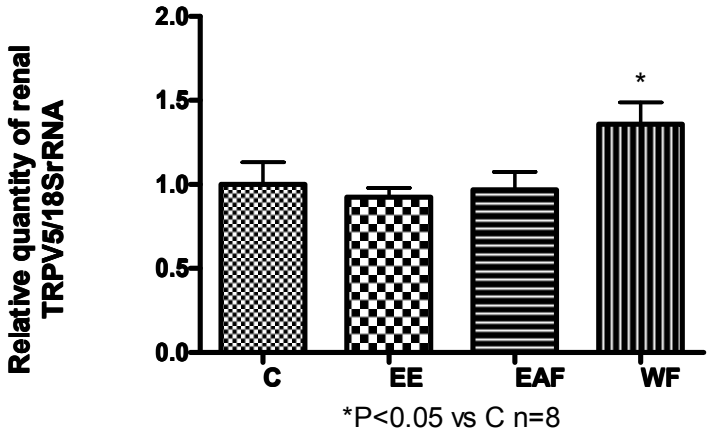
Figure 5-5. Effects of different FLL extracts on duodenal mRNA and protein expression of TRPV6, PMCA1b and VDR.

Four month old female rats were assigned to four groups and fed with Ethanol extract (EE), EA soluble fractions (EAF), Water layer fractions (WF) and vehicle (C) respectively. On week12, the rats were sacrificed, duodenal mucosa was collected and the duodenal mRNA expression of TRPV6 (A), and its protein expression (B); duodenal mRNA expression of PMCA1b (C) and its protein expression (D); duodenal VDR gene (E) and its protein expression (F) in each group were detected. The gene expression level is shown as a ratio of target gene/18SrRNA and the ratio in group C was normalized as 1.0. The protein expression level is shown as a ratio of target protein/Tubulin protein. Data are expressed as mean ± SEM (n=8). *P<0.05, **P<0.01 vs C.

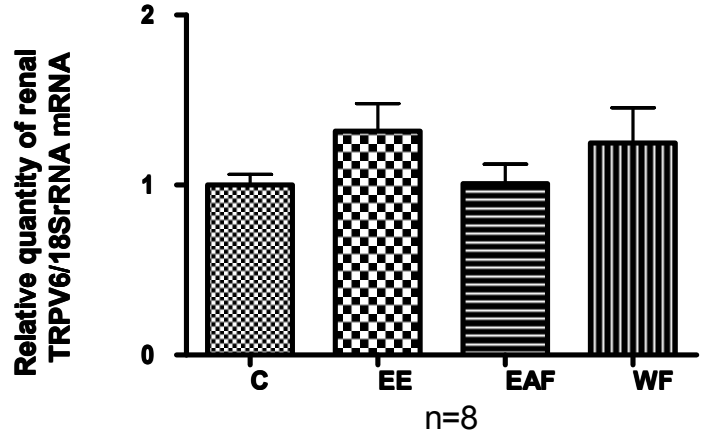
5.3.4 Renal gene and protein expressions for epithelial Ca transporting

Renal TRPV5 mRNA was significantly improved by the water layer fraction (WF) in our study ($P<0.05$; Figure 5-6A). FLL ethanol extract (EE) and its water layer fraction (WF) treatment also seemed to elevate the renal TRPV6 mRNA and protein expression, though this was not statistically significant (Figure 5-6B and 5-6C). Different from that in duodenum, Ca transporting protein – CaBP28k in the kidney appeared to have a tendency to increase after FLL ethanol extract (EE) and its water layer fraction (WF) treatment (Figure 5-6D and 5-6E), but not statistically significant. There was a different trend between renal PMCA1b mRNA and its protein expression after treatment by different FLL extracts. Renal PMCA1b mRNA seemed not to be influenced by different treatments (Figure 5-6F). But the water layer fraction (WF) treatment group seemed to have a higher expression of renal PMCA1b protein in comparison with other treatment groups, though this was not statistically significant (Figure 5-6G). It was shown that all FLL fractions tended to depress renal VDR expressions and renal VDR protein was repressed greatly by FLL ethanol extract (EE) ($P<0.05$; Figure 5-6H).

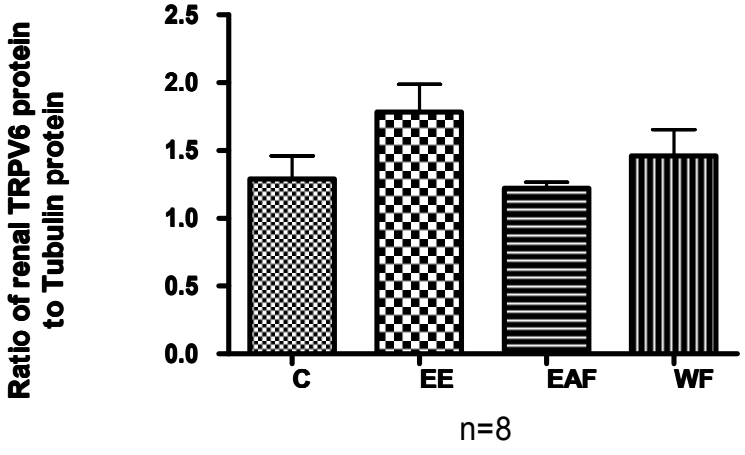
A.


Relative quantity of renal TRPV5/18SrRNA
2.0
1.5
1.0
0.5
0.0
*
C
EE
EAF
WF
*P<0.05 vs C n=8

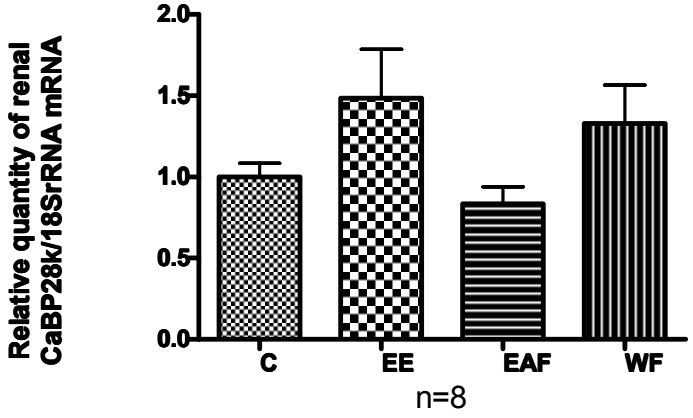
B.


Relative quantity of renal TRPV6/18SrRNA mRNA
2
1
0
C
EE
EAF
WF
n=8

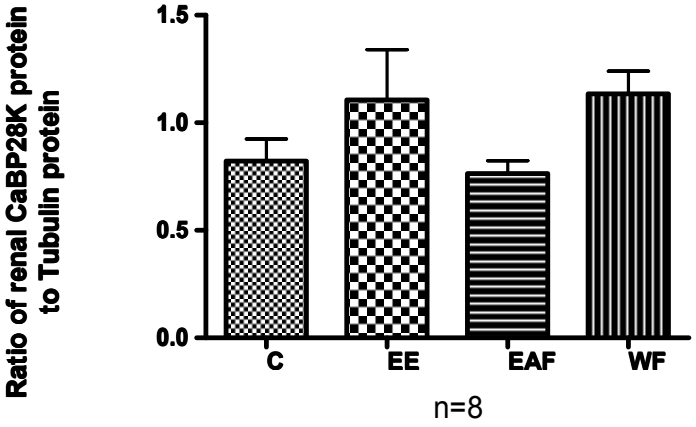
C.


Ratio of renal TRPV6 protein to Tubulin protein
2.5
2.0
1.5
1.0
0.5
0.0
C
EE
EAF
WF
n=8

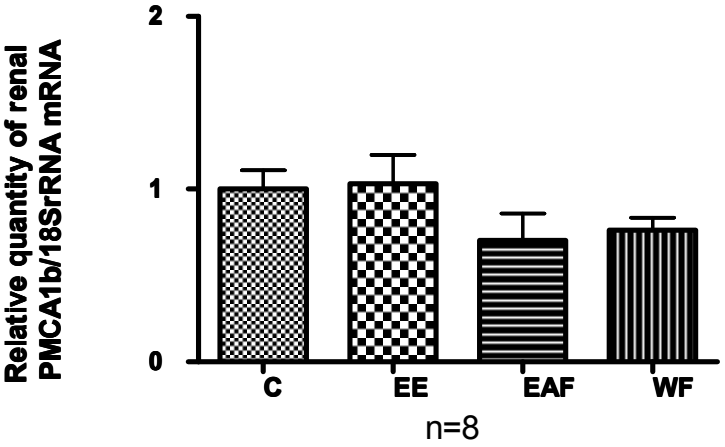
D.


Relative quantity of renal CaBP28k/18SrRNA mRNA
2.0
1.5
1.0
0.5
0.0
C
EE
EAF
WF
n=8

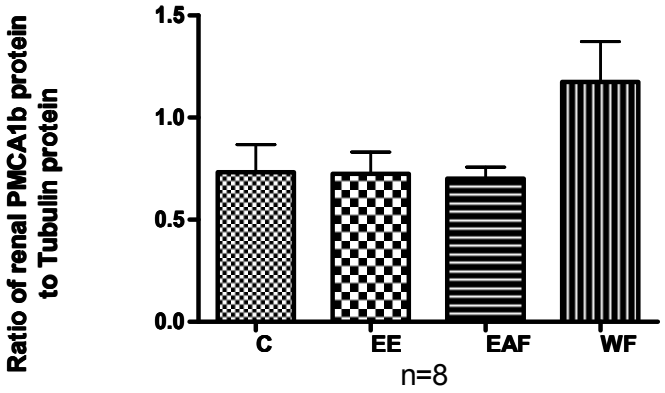
E.


Ratio of renal CaBP28K protein to Tubulin protein
1.5
1.0
0.5
0.0
C
EE
EAF
WF
n=8

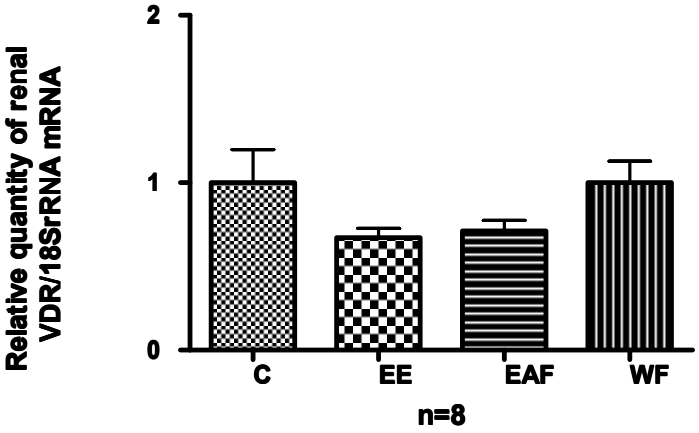
F.


Relative quantity of renal PMCA1b/18SrRNA mRNA
2
1
0
C
EE
EAF
WF
n=8

G.


Ratio of renal PMCA1b protein to Tubulin protein
1.5
1.0
0.5
0.0
C
EE
EAF
WF
n=8

H.


Relative quantity of renal VDR/18SrRNA mRNA
2
1
0
C
EE
EAF
WF
n=8

I.

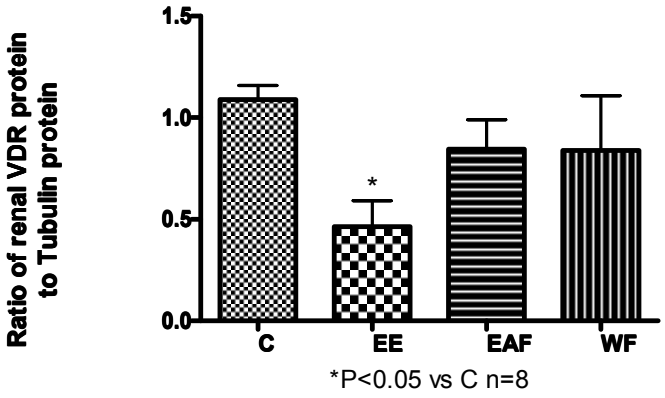

Ratio of renal VDR protein to Tubulin protein
1.5
1.0
0.5
0.0
*
C
EE
EAF
WF
*P<0.05 vs C n=8

Figure 5-6. Effects of different FLL extracts on renal mRNA and protein expression of TRPV5, TRPV6, CaBP28k and PMCA1b.

Four month old female rats were assigned to four groups and fed with FLL ethanol extract (EE), EA soluble fraction (EAF), Water layer fraction (WF) and vehicle (C) respectively. On week12, the rats were sacrificed, rat kidney was collected and the renal mRNA expression of TRPV5 (A), TRPV6 (B) and its protein expression (C); renal mRNA expression of CaBP28k (D) and its protein expression (E); renal PMCA1b mRNA (F) and protein (G) expression; renal VDR gene (H) and protein expression (I) in each group were determined. The gene expression level is shown as a ratio of target gene/18SrRNA and the ratio in group C is normalized as 1.0. The protein expression level is shown as a ratio of target protein/Tubulin protein. Data are expressed as mean ± SEM (n=8). *P<0.05, **P<0.01 vs C.

5.3.5 Effects of water layer fraction in FLL ethanol extract on Ca balance in mature female rats fed with different Ca diet

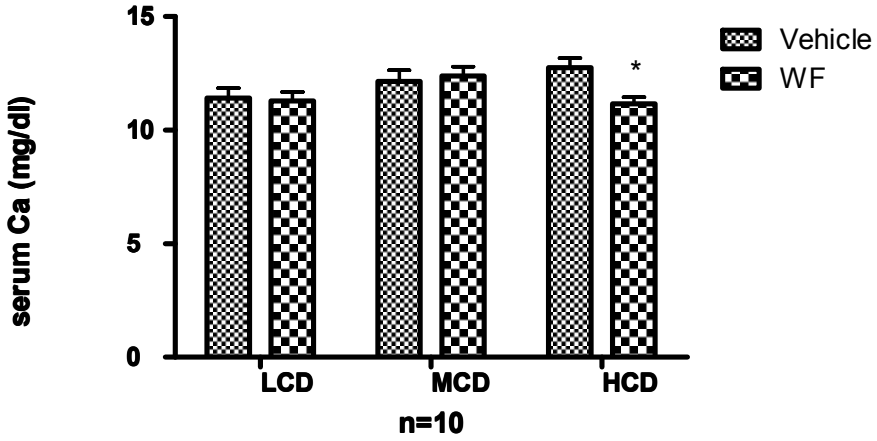
It has been confirmed from the above experiment that water layer fraction (WF) in FLL ethanol extract was the main fraction to promote positive Ca balance in mature female rats. In this study, the actions of water layer fraction (WF) on Ca balance in mature female rats were investigated when the rats were fed with different contents of dietary Ca.

As shown in Figure 5-7A, serum Ca levels in the female rats showed an increase trend with the increasing dietary Ca content in rats treated by the vehicle. FLL water layer fraction (WF) treatment did not significantly change the serum Ca in rats fed with LCD and MCD. However, in the HCD-fed group, serum Ca was shown to be reduced significantly by WF vs vehicle ($P<0.05$; Figure 5-7A). Serum Phosphorus (P) was not altered by dietary Ca and WF treatment (Figure 5-7B).

Urinary Ca excretion and fecal Ca excretion clearly increased with increasing dietary Ca in young female rats, which is a natural physiological process based on previous quantitative studies (Figure 5-8A and 5-8B). In the WF treated groups, urinary and fecal Ca excretion also increased with the increase of dietary Ca, in the

same trend with that in the vehicle treated group. In each dietary group, especially in the MCD and HCD fed group, it was shown that WF treatment significantly suppressed the urinary Ca excretion ($P<0.05$; Figure 5-8A). Similarly, fecal Ca excretion also appeared to be inhibited by WF treatment when the rats were fed with MCD and HCD, though this was not statistically significant (Figure 5-8B). The calculated Ca absorption rate showed a decreased trend with the increasing dietary Ca in both vehicle and FLL water layer fraction (WF) treated groups. However, WF treatment significantly increased the Ca absorption rate in the MCD and HCD fed groups ($P<0.05$; Figure 5-8C). Conversely, Ca net balance increased with the increasing dietary Ca in two treatment groups and it was shown to be enhanced greatly by WF in the rats fed with MCD and HCD ($P<0.05$; Figure 5-8D).

A.



B.

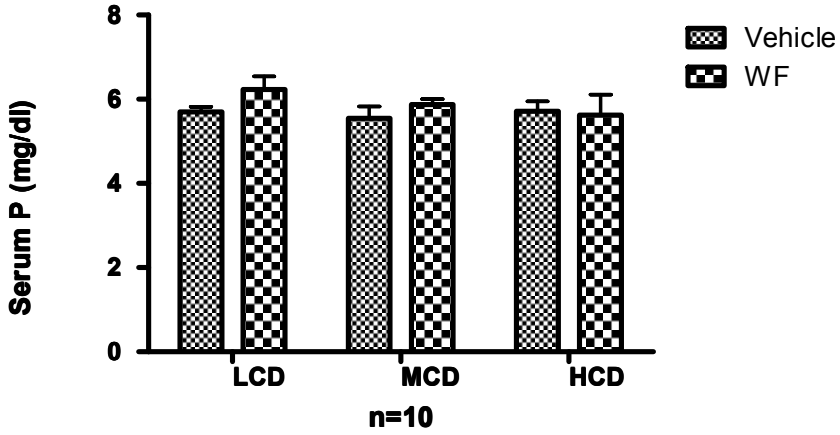
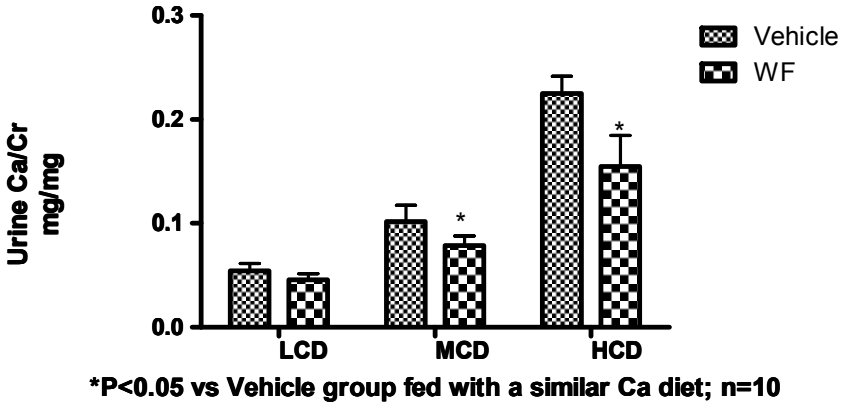


Figure 5-7. Effects of FLL water layer fraction (WF) on serum Ca and P in the mature female rats fed with different levels of dietary Ca.

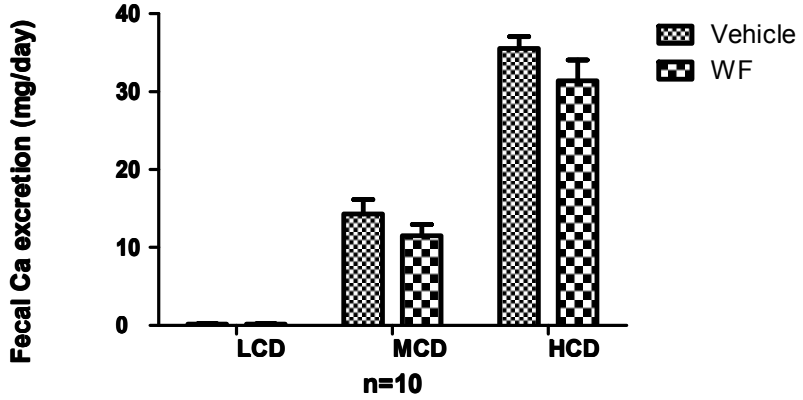
Four month old female Sprague-Dawley rats were treated with the water layer fraction in FLL extract or vehicle on different Ca diet (LCD, low-Ca diet; MCD, medium-Ca diet; HCD, high-Ca diet) for 12 weeks. On week12, the rats were killed, blood and necessary organs were collected. (A) Serum Ca, (B) serum P in each group were detected with commercial kits. Values are means ± SEM. Results were analyzed by two-way ANOVA. *P<0.05 vs vehicle treated group fed with diet of similar Ca level (n=10).

A.

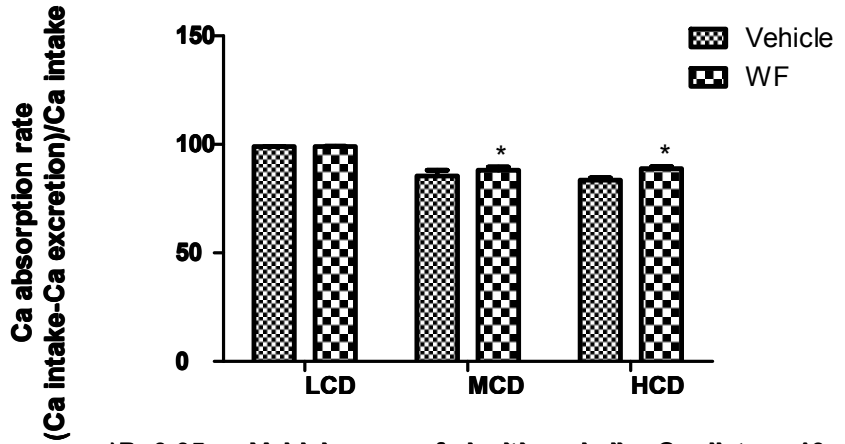


*P<0.05 vs Vehicle group fed with a similar Ca diet; n=10

B.



C.



*P<0.05 vs Vehicle group fed with a similar Ca diet; n=10

D.

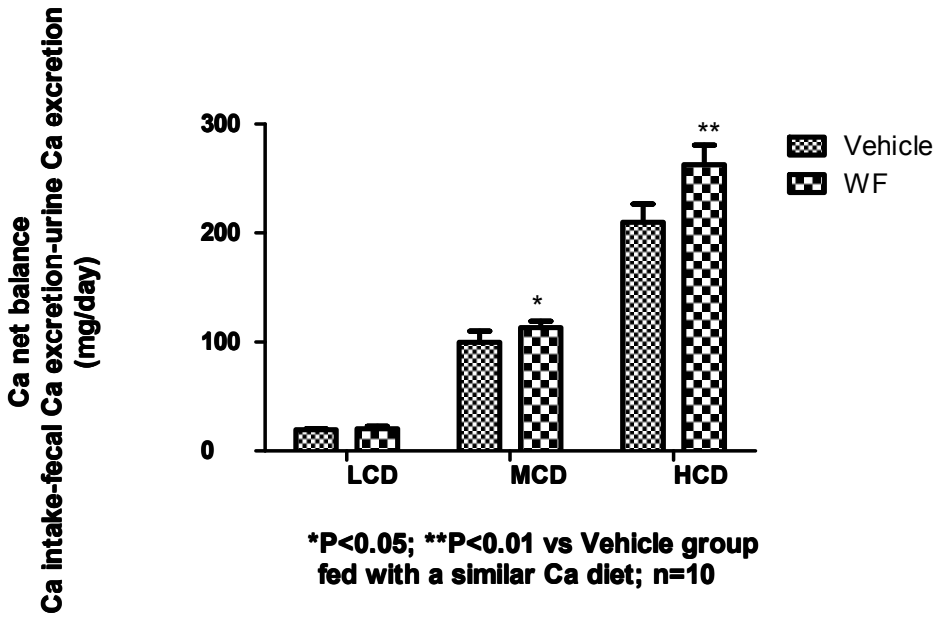


Figure 5-8. Effects of FLL water layer fraction (WF) on urinary and fecal Ca excretion and Ca absorption rate and Ca net balance.

Four month old female Sprague-Dawley rats were treated with water layer fraction in FLL ethanol extract or vehicle on different Ca diet (LCD, low-Ca diet; MCD, medium-Ca diet; HCD, high-Ca diet) for 12 weeks. On week12, the rats were put into metabolic cage for collection of urine and feces. (A) urinary Ca/Cr, (B) fecal Ca excretion in each group were detected. The level of urine Ca is corrected by the level of urine creatinine (Cr). (C) Ca absorption efficiency was calculated by the formula: (Ca intake – Fecal Ca excretion) / Ca intake. And (D) Ca net balance was calculated by: Ca net balance = Ca intake – Fecal Ca excretion – Urine Ca excretion. Data are expressed as mean ± SEM. Results were analyzed by two-way ANOVA. *P<0.05, **P<0.01 vs vehicle treated group fed with diet of similar Ca level (n=10).

5.3.6 Effects of different FLL extracts on bone parameters of different sites in the mature female rats

The different sites of bones from different treatment group were scaned by μCT in our experiment. The bone quality (bone mineral density, bone area, bone volume and bone surface) and bone microarchitecture (trabecular numbers, trabecular thickness and trabecular thickness) were evaluated. It was shown that different FLL extracts did not change significantly the detected bone parameters in comparison with the vehicle (distilled water) as indicated in Table 5-2. This suggested that different FLL extracts showed little influence on bone properties in the mature female rats after 12 weeks' feeding.

Table 5-2 Effects of different extracts of FLL on different bone parameters in mature female rats fed normal Ca diet for 12 weeks

FH	BMD	B.Ar	BV/TV	BS/BV	Tb.No	Tb.Th	Tb.Sp
C	999.907±5.668	1.345±0.089	37.743±1.703	27.914±0.837	5.218±0.127	0.072±0.002	0.121±0.006
EE	997.188±4.440	1.337±0.070	37.575±1.171	27.903±0.437	5.221±0.131	0.072±0.001	0.121±0.005
EAF	982.891±4.513	1.388±0.079	38.170±2.329	27.739±0.808	5.234±0.219	0.073±0.002	0.121±0.010
WF	992.522±5.759	1.337±0.052	36.328±1.249	28.024±0.672	5.056±0.079	0.072±0.002	0.127±0.004
FM	BMD	B.Ar	BV/TV	BS/BV	Tb.No	Tb.Th	Tb.Sp
C	1366.858±0.354	5.740±0.132	61.353±0.794	3.955±0.048	1.213±0.017	0.506±0.006	0.320±0.010
EE	1355.850±3.820	5.733±0.080	59.633±1.618	4.094±0.111	1.216±0.010	0.491±0.013	0.332±0.014
EAF	1354.600±3.055	5.786±0.114	58.516±0.492	4.211±0.066	1.232±0.020	0.476±0.007	0.337±0.07
WF	1354.536±4.489	5.743±0.175	59.394±0.834	4.125±0.107	1.223±0.020	0.486±0.012	0.332±0.007
FE	BMD	B.Ar	BV/TV	BS/BV	Tb.No	Tb.Th	Tb.Sp
C	964.214±4.701	7.195±0.245	36.433±0.985	23.117±0.528	4.189±0.026	0.087±0.002	0.152±0.003
EE	954.150±3.820	7.318±0.161	35.584±0.722	23.815±0.489	4.225±0.051	0.084±0.02	0.153±0.003
EAF	948.868±3.055	7.633±0.262	35.614±1.237	24.255±0.910	4.283±0.050	0.083±0.003	0.151±0.004
WF	956.213±4.489	7.569±0.054	36.574±0.889	23.145±0.606	4.218±0.045	0.087±0.002	0.151±0.003

TH	BMD	B.Ar	BV/TV	BS/BV	Tb.No	Tb.Th	Tb.Sp
C	973.599±4.628	8.567±0.269	38.051±0.967	22.547±0.541	4.266±0.034	0.089±0.002	0.146±0.003
EE	959.193±3.383	8.841±0.229	37.794±1.297	23.753±0.618	4.452±0.067	0.085±0.002	0.140±0.005
EAF	956.723±4.280	9.249±0.247	38.389±1.306	23.653±0.775	4.504±0.068	0.085±0.002	0.137±0.004
WF	963.259±5.289	9.116±0.200	37.701±1.017	23.130±0.654	4.339±0.043	0.085±0.002	0.144±0.007
TM	BMD	B.Ar	BV/TV	BS/BV	Tb.No	Tb.Th	Tb.Sp
C	1344.755±4.665	3.957±0.092	69.080±0.996	4.570±0.076	1.577±0.024	0.439±0.007	0.197±0.008
EE	1357.144±4.983	4.047±0.048	70.479±0.847	4.461±0.064	1.570±0.010	0.449±0.007	0.188±0.005
EAF	1355.478±4.006	4.066±0.063	69.753±1.076	4.485±0.075	1.562±0.011	0.447±0.007	0.194±0.007
WF	1356.184±9.451	3.999±0.108	70.427±1.979	4.393±0.174	1.539±0.017	0.459±0.007	0.192±0.011

[1] Values are expressed as mean ± SEM, $n = 8$.

Sanning site of rats bone:

FH: Femur head; FM: Femur midshaft; FE: Femur end; TH: Tibia head; TM: Tibia Midshaft

Parameters for bone scaning by MicroCT:

BMD: Bone mineral density; B.Ar: Bone area;

BV/TV: Bone volume/Total volume; BS/BV: Bone surface/Bone volume

Tb.No: Trabecular numbers; Tb.Th: Trabecular thickness; Tb.Sp: Trabecular separation

5.4 Discussion

Our study demonstrated that the water layer fraction in FLL ethanol extract was responsible for the positive effects on Ca balance. The increase in intestinal Ca absorption and renal Ca reabsorption could be explained by upregulation of the proteins involved in the epithelial Ca transport process in both the duodenum and kidney. Furthermore, FLL water layer fraction can increase intestinal Ca absorption, decrease urine Ca excretion, increase Ca absorption rate resulting in a positive Ca balance when the rats were provided with sufficient dietary Ca.

In our study, when the rats were fed with MCD, the normal Ca diet, it is obvious that only FLL ethanol extract itself and its water layer fraction could decrease urinary Ca and fecal Ca excretion, increase the Ca absorption rate through intestine and affect Ca net balance at the same time. Combined with the result that no negative effects occurred on bone in our experiment, it could be confirmed that FLL ethanol extract and its fractions help Ca homeostasis through enhancing efficiency in utilizing dietary Ca, without affecting Ca stored in the bone (Zhang et al., 2008b). In the previous study, we also indicated that the increased efficiency of absorbing more Ca from diet was through the induction of serum $1,25(OH)_2D_3$ levels and its dependent CaBPs expressions in the aged female rats. Serum $1,25(OH)_2D_3$ levels seemed to be elevated slightly only by FLL ethanol extract but not its EA or water layer fractions in this study. This difference from the previous

study might be contributed to the use of the female rats of different age which have different basal levels of serum $1,25(OH)_2D_3$ (Feldman et al., 2005).

In our study, the increased Ca absorption through intestine was shown to involve increasing the expression of intestinal TRPV6 and PMCA1b by FLL ethanol extract and its soluble fraction. It has been proven that the TRPV proteins are primarily regulated by $1,25(OH)_2D_3$, but its effects require the presence of functioning VDR. TRPV6 gene lacks recognizable VDREs. So it has been reported that TRPV6 might be controlled by a novel $1,25(OH)_2D_3$-mediated mechanisms (Song et al., 2003). Effects of $1,25(OH)_2D_3$ on PMCA might be species, organ, or tissue specific. Both vitamin D and $1,25(OH)_2D_3$ have been found to increase PMCA mRNA and protein levels in intestine but not in kidney (van Abel et al., 2005b). But the synthesis of PMCA has not been shown to be $1,25(OH)_2D_3$ dependent. Serum $1,25(OH)_2D_3$ levels seemed not to be regulated by different FLL extracts except its slight increase in the FLL ethanol extract treated group. The duodenal VDR proteins were shown to be elevated by FLL ethanol extract and its water layer fraction, which suggests that FLL ethanol extract and its water layer fraction might act on duodenal TRPV6 and PMCA1b through increasing duodenal VDR proteins. However the direct effect of FLL extract on duodenal TRPV6 and PMCA1b can not be excluded.

In the kidney, Ca can re-enter the blood by paracellular as well as transcellular Ca reabsorption and the latter is the main target for the calciotropic hormones (van Abel et al., 2005b). Renal TRPV5 gene expression was increased only by FLL water layer fraction. Renal TRPV6, CaBP28k gene and protein appeared to be elevated by both FLL ethanol extract and its water layer fraction. Renal PMCA1b genes were not changed by FLL treatment, but its protein was significantly increased by treatment with FLL water layer fraction (WF). The overall regulation of the three major proteins responsible for the transcellular Ca transport by FLL extracts also appeared to be in agreement with the result of urinary Ca excretion regulation. The regulation for TRPV6 proteins by FLL ethanol extract and its water layer fraction is similar in the intestine and kidney. However, differences in the regulation of other Ca transport proteins were found in the intestine and kidney. One difference is that renal CaBP28k appeared to be elevated by FLL ethanol extract and its soluble fraction, but no changes for duodenal CaBP9k. Another difference is that renal PMCA1b protein was only increased by FLL water layer fraction, but intestinal PMCA1b was increased by both FLL ethanol extract and its water layer fraction. The last difference is that renal VDR was repressed by the FLL, especially by the ethanol extract, in contrast to the increase in its expression in intestine. Thus, it is unclear how these proteins were regulated by $1,25(OH)_2D_3$ or its receptor in different tissues in response to FLL extracts. We also can not exclude the possibilities of direct regulation by FLL as well as intricate in-vivo processes. Further investigation to elucidate the exact molecular pathways is

warranted.

It is evident from our experiments that the water layer fraction in FLL ethanol extract contained major components for positive Ca balance regulation, by increasing both intestinal and renal Ca reabsorption. The results from the HPLC showed that FLL water layer fraction contained mainly hydrophilic substances with higher molecular weight and polarity. The marker for FLL, including oleanolic acid and ursolic acid was identified to exist in EA soluble fraction, but not in water soluble one. Thus, based on these results, oleanolic acid and ursolic acid may not play important roles in regulating the Ca (re)absorption. In contrast, the two major compounds in FLL water layer fraction, Nuezhenide and Salidroside., may play an active role in promoting Ca (re)absorption and future study on their possible effects on Ca balance is needed.

In our experiments, female rats were fed with three different levels of Ca diet from low to high, and were treated with FLL water layer fraction at the same time. The results showed that FLL water layer fraction can improve Ca (re)absorption to help positive Ca balance when the rats were fed MCD (0.6% Ca) and HCD (1.2% Ca). But when the Ca intake is too low, in LCD (0.1% Ca) group, inductive effects by FLL water layer fraction were not detectable.

In conclusion, FLL water layer fraction was identified to be a major fraction for the positive effects of FLL on positive Ca balance when the rats were fed with sufficient Ca diet. Its actions depend on its regulatory properties of epithelial Ca transporting processes.

Chapter 6

The Ethanol Extract of Fructus Ligustri Lucidi Increased Production of $1,25(OH)_2D_3$ in Aged Female Rats through its Direct Effects on Vitamin D Metabolism

6.1 Introduction

In Chinese clinical practice, *Fructus Ligustri Lucidi* (FLL) is a component in kidney tonifying TCM formulations for treatment of osteoporosis. Our previous study has demonstrated that the ethanol extract of FLL improved calcium (Ca) balance by increasing Ca (re)absorption. The mechanism study indicated that the ability of FLL ethanol extract to increase serum 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) level and vitamin D-dependent Ca binding proteins (CaBPs) expression might play a significant role in its protective effects on Ca balance in aged rats (Zhang et al., 2008b). However, the mechanism by which FLL ethanol extract increases serum $1,25(OH)_2D_3$ levels in aged female rats is still not clear.

$1,25(OH)_2D_3$, one of the biologically active metabolites of vitamin D_3, is believed to be important for most, if not all, of the biological actions of vitamin D on maintaining normal Ca homeostasis and bone metabolism. $1,25(OH)_2D_3$ plays a pivotal role in the regulation of intestinal and renal Ca transport (Dusso et al., 2005; Eeleen, 2005; Feldman et al., 1997; Tissandie et al., 2007). The major portion of vitamin D_3 in the human body, however, comes from photosynthesis in the skin; a process in which UV-B light converts 7-dehydrocholesterol to the unstable previtamin D_3, which isomerizes to vitamin D_3 under the influence of thermal energy. Once vitamin D_3 enters the circulation, it is bound to the vitamin D-binding protein (DBP) and transported to the liver where the cytochrome P_{450} –vitamin D-25-hydroxylase introduces an OH on carbon 25 to produce 25-hydroxyvitamin D_3

($25(OH)D_3$). It is transported to the kidney where the cytochrome P_{450}–mono-oxygenase, 25(OH)D-1α-hydroxylase (1-OHase; CYP27B1), metabolizes $25(OH)D_3$ to $1,25(OH)_2D_3$ (Feldman et al., 1997). Although a variety of tissues and cells express 1-OHase and have the ability to produce $1,25(OH)_2D_3$, the kidney is the major source of circulating $1,25(OH)_2D_3$ in serum (Wu et al., 2007).

The gene that encodes for 1-OHase, which spans approximately 6kb, consists of 9 exons and possesses a 5' untranslated mRNA of approximately 500bp. The latter has also highlighted the homology between CYP1α and its CYP27 counterpart. The cDNA for rat 1-OHase has 82.5% identity to the human cDNA (St-Arnaud et al., 1997). Data using normal human kidneys confirmed the expression of mRNA and protein for 1-OHase in proximal tubules. However, protein and mRNA of 1-OHase were also expressed in distal tubules and in collecting ducts. The other key sites of 1-OHase expression along the nephron were the medullary collecting ducts and papillary epithelium (St-Arnaud et al., 1997). In view of studies with vitamin D deficient animals, it seems likely that production of $1,25(OH)_2D_3$ in the proximal tubules acts in an endocrine fashion to support circulating levels of $1,25(OH)_2D_3$, whereas more distal areas of the nephron fulfil an autocrine or paracrine function (Hewison et al., 2000).

It has now well established that the two most important regulators of the activity of the 1-OHase are $1,25(OH)_2D_3$ itself and PTH. Recent research has found

that Fibroblast growth factor 23 (FGF23) is also another important regulator for 1-OHase. In addition, there is evidence for the involvement of calcitonin, dietary mineral levels, and hormones of other endocrine systems in the regulation of renal $25OHD_3$ metabolism (Dusso et al., 2005).

The present study is aimed at studying the effects of FLL ethanol extract on vitamin D metabolism in aged rats. Renal 1-OHase expression with activity and renal VDR expression were systematically investigated in the aged rats. In order to further confirm if FLL ethanol extract acts on renal 1-OHase directly, its effects on 1-OHase expression and activity in primary cultures of rat proximal tubule cells were also investigated in this study. The results provide evidence to support the hypothesis that FLL ethanol extract could raise the circulating levels of $1,25(OH)_2D_3$ by increasing its biosynthesis directly. Furthermore, human proximal tubule HKC-8 cells were established as an *in vitro* screening platform to identify the active fraction that accounts for the positive effects of FLL on renal 1-OHase. The results indicated that the EA fraction of FLL was the active fraction that contributed to its modulatory action on renal 1-OHase.

6.2 Methods

6.2.1 Preparation of FLL Extract

Fructus Ligustri Lucidi was obtained from Jiangsu province of China. The preparation of FLL ethanol extract (EE) was described previously (Zhang et al., 2008b). Moreover, the two fractions separated from FLL ethanol extract, including EA soluble fraction (EAF) and water layer fraction (WF), were prepared as previously described in chapter 5.

6.2.2 Animal Experiment

Thirty-two ten-month-old retired breeder Sprague-Dawley female rats (280-350g) (Experimental Animal Center of Guangdong province, Guangzhou, China) were used in the study for exploring the effects of FLL ethanol extract on mRNA and protein expression of 1-OHase. After 2 weeks of acclimation with a medium Ca diet (MCD, TD 98005, 0.6% Ca), animals were randomly assigned into two groups. Sixteen rats were bilateral ovariectomized and the remaining sixteen rats were sham-operated and they were fed with MCD. Sham and OVX rats were sub-divided into two groups of eight rats based on treatment with either ethanol extract of FLL at the dosage of 700 mg/kg or its vehicle (distilled water) through gastric tube. The rats were housed in a room which provided alternating 12 h of light and 12 h of darkness with the room temperature at 23 ± 1 °C and humidity 55 ± 5%. Husbandry of the animals was based on the NIH Guide for Care and Use of

Laboratory Animals (Council, 2006). All rats had free access to distilled water, and were fed with MCD at 15g/day per rat of the respective diet, the minimum average food intake of the rats during the acclimation period. The body weight of the animals was recorded weekly to adjust the administration of FLL. The experimental protocol was approved by the Animal Ethics Committee of The Hong Kong Polytechnic University.

In order to further explore the possible effects of FLL ethanol extract on renal 1-OHase enzyme activity, another batch of animals was also used in the experiment. Based on the results from chapter 4 and 5, younger mature female rats were used in this study. Sixteen four-month-old Sprague Dawley female rats were divided into two groups, one group was treated by vehicle and the other with FLL ethanol extract (700mg/kg/d). The treatment period was 4 weeks which was also determined based on the results from chapter 4 and 5. All rats had free access to distilled water and were fed with MCD at 15g/day per rat of the respective diet. FLL administration dose was also adjusted every week by their weights.

6.2.3 Sample Collection of the Aged Rats

After 12 weeks of treatment, blood was withdrawn from abdominal aorta under light ether anesthesia. Serum was then prepared and stored at -80 °C until biochemical determinations. The two kidneys of each rat were collected and put into liquid nitrogen instantly, and then stored at -80 °C. The tibias and femurs were

collected, cleaned of all soft tissues, wrapped together in saline-soaked towels, and stored at -20° C for further analysis.

6.2.4 Isolation and Preparation of Renal Proximal Tubule

Isolation of proximal tubular cells (PTCs) was performed as described by Vinay et al (Vinay et al., 1981). For each experiment, two four-month-old female rats were anesthetized with overdose CO_2 and the abdominal cavity was opened. The kidneys were removed and placed in 30ml ice-cold Krebs-Henseleit saline (KHS) (PH 7.40) previously gassed at 20° C with 95% O_2/5% CO_2 for 30 min. After decapsulation, the kidneys were cut in half and the medulla was carefully dissected out. The kidney cortex was placed in 20 ml of ice-cold KHS and was sliced with a Stadie-Riggs microtome. The slices were pooled in 30 ml of ice-cold KHS, washed 3 times with 30 ml of the solution and re-suspended in 10 ml of KHS containing 0.15 g/100ml of collagenase (Washington Biochemical Corp.) and 0.5 ml of 10% bovine albumin (BSA). The slices in collagenase were then transferred into a 125 ml Erlenmeyer flask. The Erlenmeyer flask was then gassed for 1 min with 95% O_2/5% CO_2, closed with the parafilms, and placed in a 40 rpm shaking bath with temperature control at 37° C to ensure movement of the slices at the bottom of the flask. At the end of the digestion procedure, which lasted about 45 min, approximately 30 ml of ice-cold KHS was added to and the suspension was gently shaken to disperse the fragments of tissue. The whole suspension was

filtered through a tea container to remove the collagen fibers. The tissue suspension was then gently centrifuged (600 rpm) in a 50 ml tube for 1 min. The supernatant was discarded and the tissue rapidly washed by KHS for twice, the last wash was done with half KHS plus half 10% BSA. After the last washing, 10 ml 45% percoll solution (Freshly diluted with water and various salts) was added to mix with the tissue pellet. Mixed percoll solution was then gassed by 95% O_2/5% CO_2 and spun at a speed of 12,000 rpm for 30 min in a Sorvall centrifuge (RC-5) equipped with a fixed-angle rotor head. The tissue was then separated into three distinct bands. The bottom band was generally considered to be mainly the proximal tubule cells.

6.2.5 Measurement of 25-hydroxyvitamin D-1-hydroxylase Activity in the Young Mature Female Rats

After 4 weeks of treatment, rats were anesthetized with overdose CO_2. Both kidneys were harvested for proximal tubule isolation. Aliquots of the proximal tubule band at the bottom were incubated in 1.0 ml KHS containing 5 mmol/L glutamine, 20 mg BSA and crystalline $25OHD_3$ in 0.04 ml 95% ethanol. Each incubation flask contained 60 to 100 ug tubule protein. Incubations were carried out for 20 min at 37 °C in a shaking water bath at 80 oscillations per minute in an atmosphere of 95% O_2/5% CO_2. The reaction was halted by rupturing the cells with 1.0 ml deionized distilled water, immediate icing of the reaction vessel, and tissue homogenization. An aliquot of homogenate was removed for protein determination. The homogenate was extracted once with the two separate extraction columns

(Immundiagnostik AG, Bensheim) to get $1,25(OH)_2D_3$. The concentration of $1,25(OH)_2D_3$ was then determined by competitive enzyme immunoassay (Immundiagnostik AG, Bensheim).

6.2.6 Primary Cell Culture of Renal Proximal Tubules

Cells in the bottom band were extracted out and pelleted by centrifugation at 2500 rpm for 10 min at 4°C and washed with ice-cold KHS twice and once with culture medium (DMEM-F12 1:1 [Invitrogen]). Cells were then seeded on to collagen 1 coated 6-well plates supplemented with DMEM-F12 medium and 10% FBS. Medium was changed every 2 days. For assessment of enzyme activity, mRNA, and protein expression, cells were transferred to defined medium, which consisted of DMEM/F12 containing the following additives: insulin (5 µg/ml), transferrin (5 µg/ml), Na_2SeO_3 (5 ng/ml), T_3 (0.37 nM), epidermal growth factor (2.5 ng/ml), and hydrocortisone (1 nM) (Sigma) for 24 h before enzyme assays or treatments.

6.2.7 25-Hydroxyvitamin D-1α-hydroxylase Assay in Primary Cultures

1α-hydroxylase activity was determined in monolayer cultures of the primary cultures. The assays were performed in the presence of radioactive $25OHD_3$ as the enzyme substrate (20ng of nonradioactive $25OHD_3$ and 0.91uCi of [^{3}H] $25OHD_3$) and DPPD (Sigma, Allentown, PA), an antioxidant and a known inhibitor of free radical-generation $1,25(OH)_2D_3$ (Schwartz et al., 1998). Assays were also

performed in the presence and absence of the cytochrome P450 inhibitor, clotrimazole (20uM; Sigma Chemical Co., St. Louis, MO). After 2h of incubation at 37 °C, cultures were placed on ice, and media was removed. Immediately afterwards, 1ml of methanol was added to extract $25OHD_3$ and $1,25(OH)_2D_3$. After extraction at room temperature for 15 min, the methanol extract was transferred to a glass test tube, and the cells were washed with an additional 0.5ml of methanol. The extract and wash were combined, dried down with a stream of nitrogen, and redissolved in 1ml of acetonitrile, followed by the addition of 1ml of 0.4M K_2HPO_4 (pH 10.0). The mixture was then applied to the two separate extraction columns (Immundiagnostik AG, Bensheim) to get $1,25(OH)_2D_3$. The concentration of $1,25(OH)_2D_3$ was then determined by competitive enzyme immunoassay (Immundiagnostik AG, Bensheim).

6.2.8 Human Proximal Kidney Tubule (HKC-8) Cells Culture

Human proximal kidney tubule cells (HKC-8) cells were maintained in DMEM-Ham's F-12 (DMEM/F12) medium with glutamine (Invitrogen) supplemented with 10% FBS (Invitrogen) and 1% penicillin-streptomycin. Experiments were started when cells were approximately 80%-90% confluent. For assessment of enzyme activity, mRNA, and protein expression, cells were transferred to defined medium, which consisted of DMEM/F12 containing the following additives: insulin (5 μg/ml), transferrin (5 μg/ml), Na_2SeO_3 (5 ng/ml), T_3

(0.37 nM), epidermal growth factor (2.5 ng/ml), and hydrocortisone (1 nM) (Sigma) for 24 h before enzyme assays or treatments.

6.2.9 Detection of Calciotropic Hormones

Serum levels of PTH were detected using rat bioactive intact PTH ELISA assay (Immutopics, Inc., San Clemente, CA). Serum $1,25(OH)_2D_3$ was extracted by using two separated columns offered by the Immundiagnostik Company. The vitamin D was first extracted from the chromabond column by diisopropylether for 4 times. The eluate was dripped on to the silica cartridges. After washing five times with 4/96 Isopropanol/Hexane and 3 times with 6/94 Isopropanol/Hexane, $1,25(OH)_2D_3$ was eluted from the silica cartridge by 25/75 Isopropanol/Hexane. The eluted $1,25(OH)_2D_3$ was combined with its antibody and measured at last by competitive enzyme immunoassay (Immundiagnostik AG, Bensheim).

6.2.10 RNA Isolation and Preparation of cDNA

Tissue samples were thawed in Trizol reagent (Invitrogen, Carlsbad, California, USA) and homogenized. RNA extraction was performed according to the Trizol manufacturer's protocol. Reverse transcription reactions were performed using the SuperScript First-Strand Synthesis System (Invitrogen).

6.2.11 Real-time PCR Analysis

Total RNA (2ug) was used to generate cDNA in each sample using the Super-Script Ⅱ reverse transcriptase with oligo (dT) 12-18 primers. The RNA, Oligo dT, dNTP were mixed into a PCR tube for amplication on a GeneAmp 9600 PCR system (Perkin Elmer, Foster City, CA, USA). Real-time PCR reactions were performed using SYBR Green PCR Master Mix and an ABI PRISM 7900HT. Thermocycling was done in a final volume of 20ul that contained 0.05ul cDNA and 800 nM of each of the forward and reverse primers. GAPDH was used as an endogenous control. The primers for 1-OHase, and GAPDH were the same as previously described (Zhang et al., 2008a; Zhang et al., 2008b; Zhang et al., 2007). PCR was performed using the following program: initial denaturation at 95^{o}C for 3min, 40 cycles of denaturation at 95^{o}C for 15s, primer annealing at 56^{o}C for 20s and polymerization at 72^{o}C for 20s. Quantitative values were derived from the threshold cycle number at which the increase in the signal associated with exponential growth of PCR products was first detected. The relative amount of each PCR product in each sample was calculated by relative quantification — the $2^{-\Delta\Delta C_T}$ method. $\Delta\Delta C_T = (C_{T,Target} - C_{T,\ GAPDH})_{Treatment} - (C_{T,Target} - C_{T,\ GAPDH})_{Control}$ (Livak and Schmittgen, 2001). Finally, a relative gene expression was calculated by assigning the normal control a relative value of 1.0, with all other values relative to the normal control.

6.2.12 Western Blot Analysis

Renal proteins were obtained by cell lysis in Nonidet P-40 buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40) supplemented with protease inhibitors (2ug/ml aprotinin, 2ug/ml leupeptin, 1mM PMSF) (Sigma, St. Louis, MO, USA). Protein concentrations were measured by Bradford protein assay (Bio-Rad Laboratory, USA). Equal amounts of cytosolic proteins (50ug) were mixed with the loading dye. After mixing and boiling for 10 min, the samples were separated by SDS-PAGE on appropriate reducing gels at a constant voltage (150V) for 1h until they reached the bottom. Then the proteins were transblotted to PVDF membranes (Immobilin-P, Millipore Corp., Bedford, MA, USA) at a voltage of 100V for 1.5h or 22V overnight. Immuno-blotting was performed after blocking non-specific binding on the membrane with 5% non-fat milk in TTBS for 2h. Then the blots were probed first with the primary antibody for the target enzyme overnight at 4^{o}C, followed by incubation with the correspondent secondary antibody for 1.5-2h. The antigen-antibody complexes were detected by using an enhanced chemiluminescence reagent and visualized by a Lumi-Imager with the software.

6.2.13 Statistical Analysis

The data from these experiments were reported as mean ± standard error of mean (SEM) for each group. All statistical analyses were performed using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by unpaired T-Test. Differences in P value of less than 0.05 were considered statistically significant.

6.3 Results

6.3.1 Body weight, serum Ca, P level and serum calciotropic hormones levels in aged normal and OVX female rats

The body weight of the two groups in the aged normal female rats increased slowly during the experimental period. The body weight in two groups of OVX rats increased greater than that in normal female rats. Uterine index for each OVX group was much lower than that of the sham group, suggesting that ovariectomy was successful and OVX rats should have minimal estrogen in the circulation (Data not shown).

It was shown that FLL ethanol extract treatment did not alter the weight gain in normal or OVX rats. In the aged sham rats, serum Ca and P values remained constant after FLL ethanol extract treatment. In comparison, OVX rats without FLL ethanol extract treatment appeared to have slightly lower serum Ca level than the vehicle treated normal ones ($P<0.05$, Table 6-1). Moreover, FLL ethanol extract treatment significantly raised serum Ca in OVX rats (vs vehicle treatment, $P<0.05$, Table 6-1). It was shown clearly that FLL ethanol extract treatment significantly increased serum $1,25(OH)_2D_3$ levels by about 2.25 fold in the aged normal female rats and 1.28 fold in the OVX rats when compared with vehicle treatment controls ($P<0.01$, $P<0.05$, Table 6-1).

Table 6-1 Effects of ethanol extract of FLL on serum Ca, P, 1,25(OH)$_2$D$_3$ and PTH in aged normal and OVX female rats fed with MCD for 12 weeks[1]

		Weight gain (g)	Serum Ca (mg/dl)	Serum P (mg/dl)	Serum 1,25(OH)$_2$D$_3$ (pg/ml)
	Vehicle	46 ± 9	10.34 ± 0.13	6.46 ± 0.23	16.3 ± 2.5
Sham	FLL	38 ± 10	10.48 ± 0.09	6.22 ± 0.33	36.8 ± 5.8**
	Vehicle	85 ± 8$^{\#\#}$	9.58 ± 0.17	6.68 ± 0.28	18.5 ± 1.1
OVX	FLL	83 ± 9$^{\#\#}$	11.02 ± 0.34*	6.49 ± 0.19	23.7 ± 2.2*

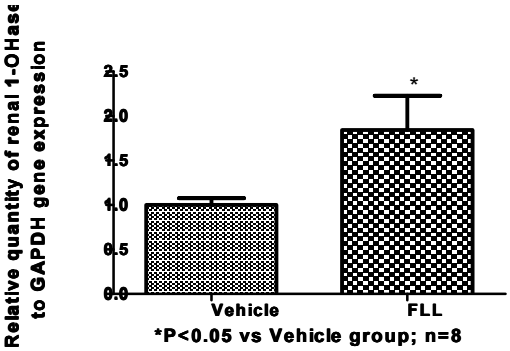
[1] Values are expressed as mean ± SEM, n = 8. *P<0.05, **P<0.01 compared to vehicle treated group. $^{\#\#}$P<0.01 compared with sham group.

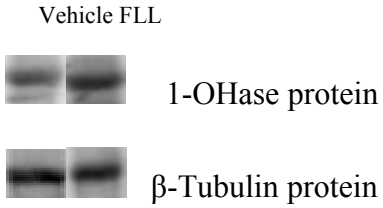
6.3.2 Renal 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) and vitamin D receptor (VDR) mRNA with protein expression in aged normal female rats

To determine if FLL ethanol extract increased serum $1,25(OH)_2D_3$ levels by acting on the biosynthetic enzyme in the aged normal female rats, renal 1-OHase expression was studied. Renal 1-OHase mRNA expression increased 84.3% by FLL ethanol extract treatment for 12 weeks compared with vehicle treatment ($P<0.05$, Figure 6-1A). Accordingly, renal 1-OHase protein expression was also elevated (by 28%) after treatment, though it was not as highly increased as its mRNA expression ($P<0.05$, Figure 6-1B).

To determine if FLL ethanol extract treatment altered the responsiveness of kidney to $1,25(OH)_2D_3$, renal expression of VDR was studied. FLL ethanol extract treatment significantly increased renal VDR mRNA expression by 60.8% ($P<0.05$, Figure 6-2A). Renal VDR protein was also increased in FLL-treated aged rats, and the magnitude of increase is similar to that of its mRNA expression (by 63.8%; $P<0.05$, Figure 6-2B).

A.





B.

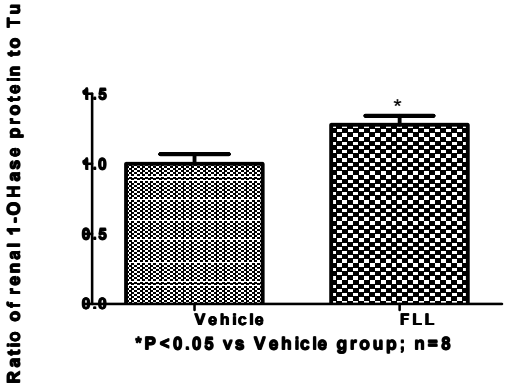
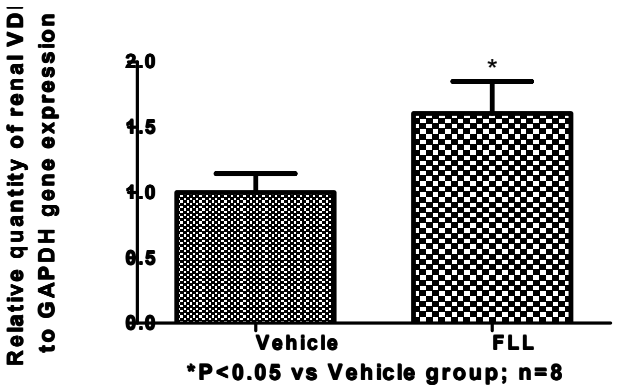


Figure 6-1 Renal mRNA and protein expression of 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) in the aged normal female rats.

Ten-month-old aged female Sprague-Dawley rats were treated with FLL ethanol extract or vehicle on medium Ca diet (MCD) for 12 weeks. Expression level of mRNA is shown as a ratio of relative quantity ($2^{-\Delta\Delta C_T}$) of each group to vehicle group (n=8 in each group). Protein expression level is shown as a ratio of target protein/β-tubulin (n=8 in each group). Values are means ± SEM. *P<0.05 vs vehicle treated group.

A.



B.

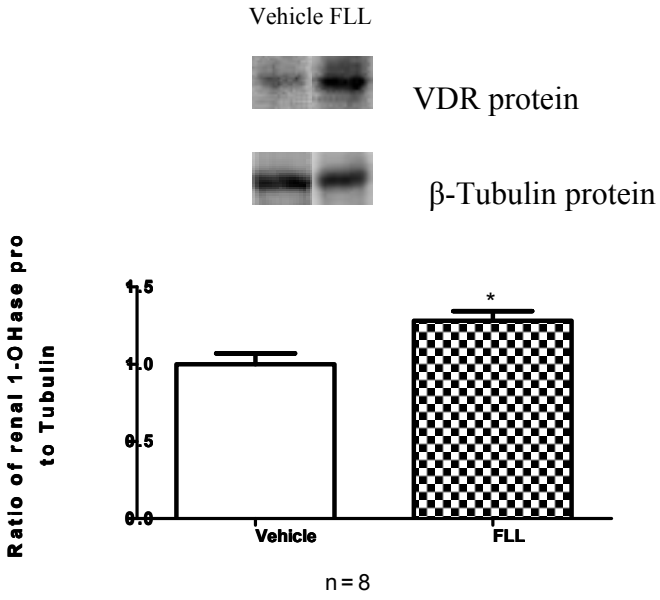


Figure 6-2 Renal mRNA (A) and protein (B) expression of vitamin D receptor (VDR) in the aged normal female rats.

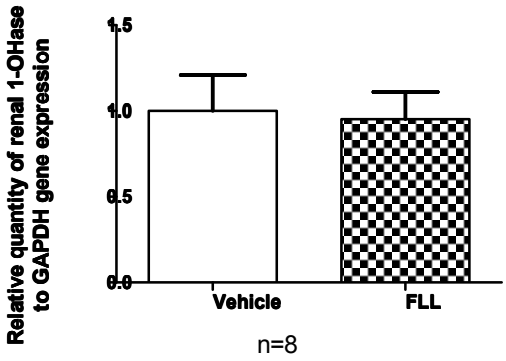
Ten-month-old aged female Sprague-Dawley rats were treated with FLL or vehicle on medium Ca diet (MCD) for 12 weeks. Expression level of mRNA is shown as a ratio of relative quantity ($2^{-\Delta\Delta C_T}$) of each group to vehicle group (n=8 in each group). Protein expression level is shown as a ratio of target protein/β-Tubulin (n=8 in each group). Values are means ± SEM. *P<0.05 vs vehicle treated group.

6.3.3 Renal 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) and vitamin D receptor (VDR) mRNA with protein expression in aged OVX rats

In aged OVX rats, renal 1-OHase mRNA expression did not change after 12 weeks' treatment with FLL ethanol extract in comparison with vehicle treatment. However, it was shown that the protein expression of 1-OHase increased significantly (by 55%) after FLL ethanol extract treatment ($P<0.05$, Figure 6-3B). This indicated that FLL ethanol extract could increase the production of $1,25(OH)_2D_3$ by inducing the expression of renal 1-OHase protein in both normal aged female rats and OVX rats. Furthermore, it indicated that the effects of FLL ethanol extract on 1-OHase might not be affected by estrogen status *in vivo*.

It was found neither renal VDR mRNA nor its protein expression in OVX rats were influenced by FLL ethanol extract treatment. In other words, renal VDR could not be induced in response to the increase of serum $1,25(OH)_2D_3$ under the estrogen deficient state of the aged female rats (Figure 6-4).

A.



B.

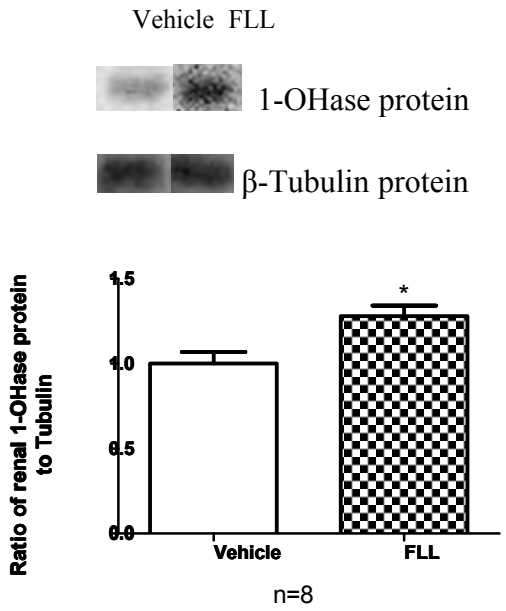
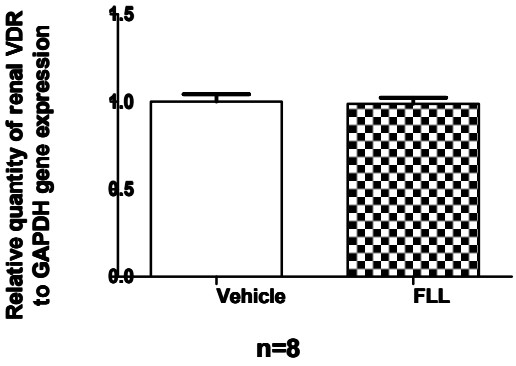


Figure 6-3 Renal mRNA and protein expression of 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) in aged OVX rats.

Ten-month-old aged OVX Sprague-Dawley rats were treated with FLL or vehicle on medium Ca diet (MCD) for 12 weeks. Expression level of mRNA is shown as a ratio of relative quantity ($2^{-\Delta\Delta C_T}$) of each group to it of vehicle group (n=8 in each group). Protein expression level is shown as a ratio of target protein/β-tubulin (n=8 in each group). Values are means ± SEM. *P<0.05 vs vehicle treated group.

A.



B.

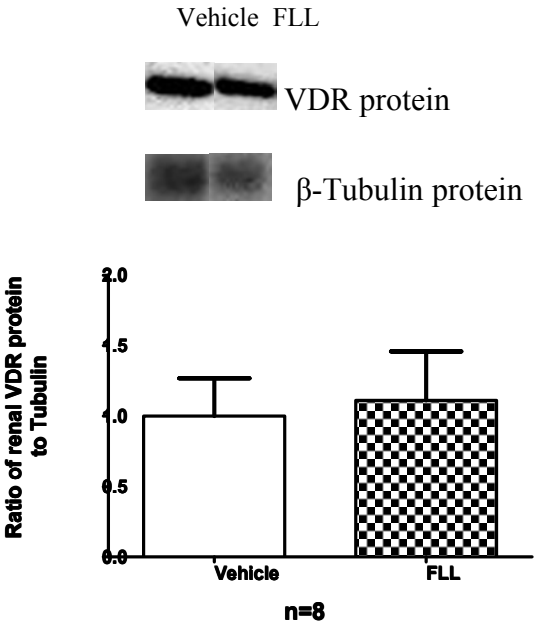


Figure 6-4 Renal mRNA and protein expression of vitamin D receptor (VDR) in aged OVX rats.

Ten-month-old aged OVX Sprague-Dawley rats were treated with FLL or vehicle on medium Ca diet (MCD) for 12 weeks. Expression level of mRNA is shown as a ratio of relative quantity ($2^{-\Delta\Delta C_T}$) of each group to it of vehicle group (n=8 in each group). Protein expression level is shown as a ratio of target protein/β-tubulin (n=8 in each group). Values are means ± SEM.

6.3.4 Effects of FLL ethanol extract on the production of serum $1,25(OH)_2D_3$ and 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) activity in renal proximal tubules of mature female rats

To determine if FLL ethanol extract altered the activity of renal 1-OHase, four month old rats were treated by FLL ethanol extract for four weeks, serum $1,25(OH)_2D_3$ levels with renal 1-OHase activity were detected at the end of experiment. It was shown that serum $1,25(OH)_2D_3$ levels were elevated by 79% ($P<0.05$, Figure 6-5A) in the FLL-fed rats. The renal 1-OHase activity was expressed as the production of $1,25(OH)_2D_3$ per mg proximal tubule protein. The results showed that renal 1-OHase activity was also increased by FLL ethanol extract treatment ($P<0.05$, Figure 6-5B).

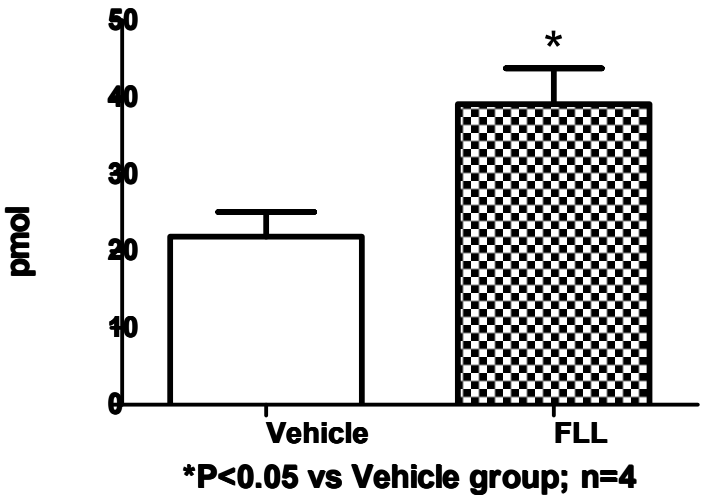


Figure 6-5A Effects of ethanol extract of FLL on serum $1,25(OH)_2D_3$ levels in mature normal female rats.

Four-month-old mature normal female Sprague-Dawley rats were treated with FLL or vehicle on medium Ca diet (MCD) for 4 weeks. Blood was drawn and serum was detected the levels of serum $1,25(OH)_2D_3$ by the commercial ELISA kit. Values are means ± SEM. *P<0.05 vs vehicle treated group (n=4).

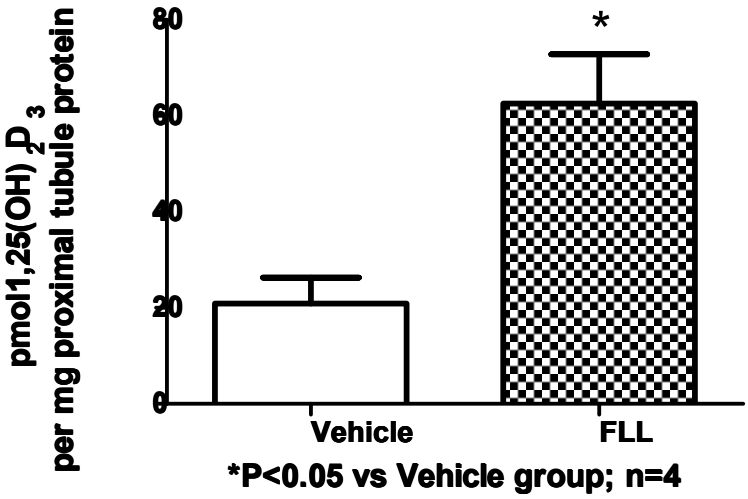


Figure 6-5B Effects of FLL ethanol extract on 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) activity in kidney proximal tubules of mature normal female rats.

Four-month-old mature normal female Sprague-Dawley rats were treated with FLL ethanol extract or vehicle on medium Ca diet (MCD) for 4 weeks. Rat kidney proximal tubules were prepared and the renal 1-OHase activity was detected rapidly after sacrifice of the animals. Data are expressed as the ratio of the production of $1,25(OH)_2D_3$ to the proximal tubule proteins based on the same quantity of substrate $25(OH)D_3$. Values are means ± SEM. *P<0.05 vs vehicle treated group (n=4).

6.3.5 Effects of FLL ethanol extract on 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) mRNA and protein expression in primary cultures of rat proximal tubule cells

In order to determine if FLL ethanol extract directly acted on renal 1-OHase expression, its effects on 1-OHase expressions in primary cultures of rat proximal tubule cells were systemically investigated. The kidney cells taken from rat proximal tubules were plated on dishes for about 4 days and incubated with different concentrations of FLL ethanol extract after pre-treatment with serum free medium containing supplements. After 48 hours' incubation, it was shown that 10ug/ml and 100ug/ml FLL ethanol extract treatment significantly increased 1-OHase mRNA expression in the primary culturing cells of rat proximal tubules ($P<0.01$, Figure 6-6A). Its protein expression in the cells also seemed to increase upon incubation with 10ug/ml and 100ug/ml FLL ethanol extract for 48 h, though the increase was not statistically significant (Figure 6-6B).

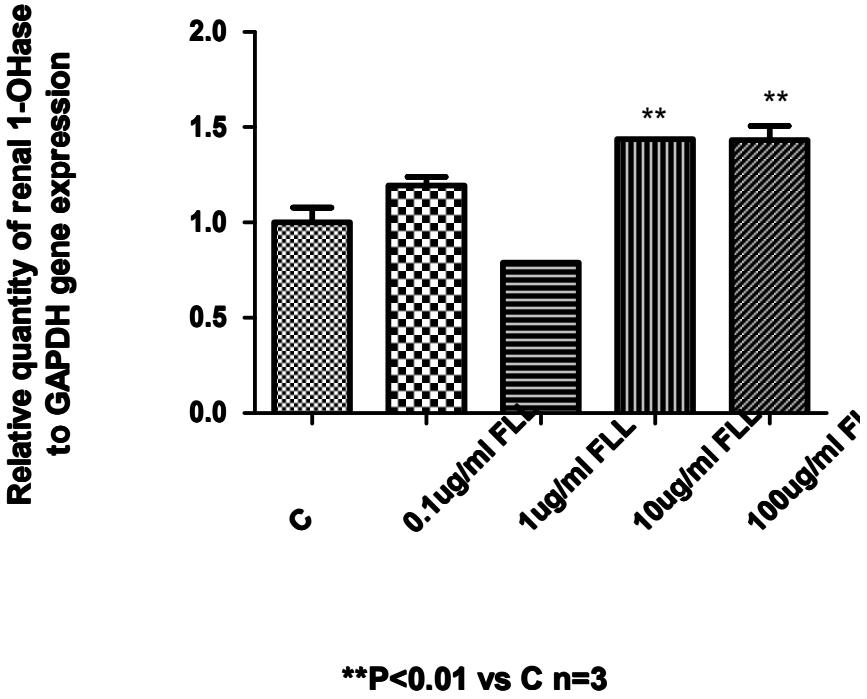


Figure 6-6A Effects of different concentration of FLL ethanol extract on mRNA expression of 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) in primary cultures of rat proximal tubule cells.

Four-month-old mature normal female rats were sacrificed and their kidneys were harvested for preparation of proximal tubule cells. The cells were then plated on 6-well plates. Upon culturing for 4 days, the cells were cultured in serum-free medium with supplements for 24 hours, followed by incubation with different concentrations of FLL ethanol extract. After incubation for 48 hours, mRNA expression levels were detected by real time PCR. The expression level is shown as a ratio of target gene/GAPDH and the ratio in group C is normalized as 1.0. Values are means ± SEM. The experiment was repeated for three times (n=3). **P<0.01 vs group C.

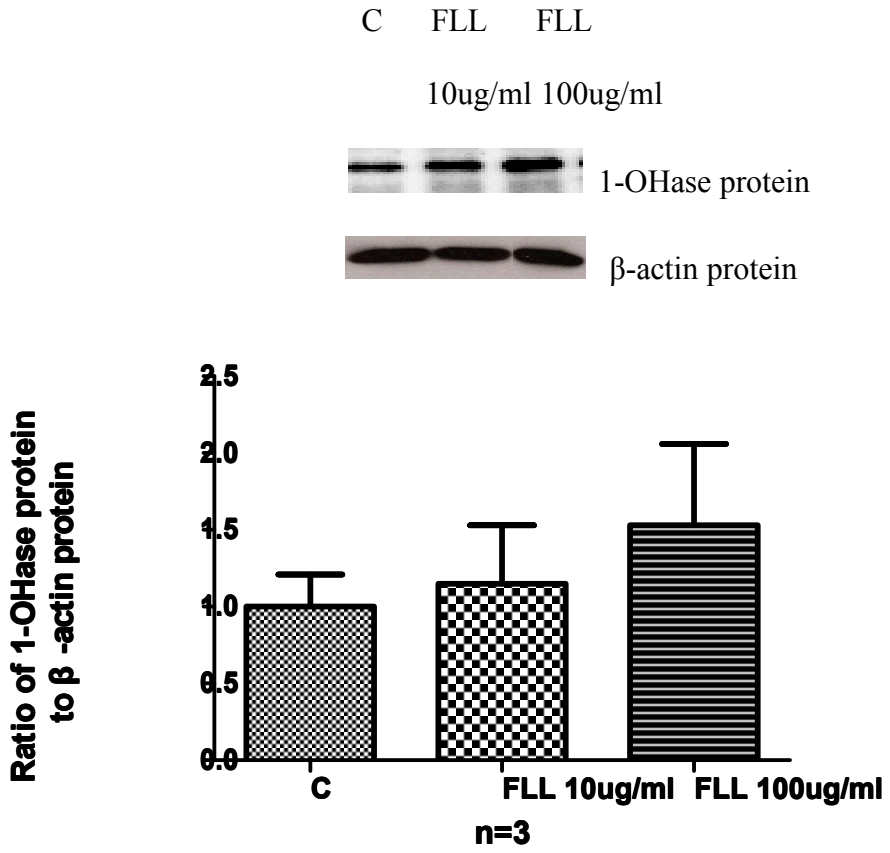


Figure 6-6B Effects of FLL ethanol extract on protein expression of 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) in primary cultures of rat proximal tubule cells.

Four-month-old mature normal female rats were sacrificed and their kidneys were taken out to prepare the proximal tubule cells. The cells were then plated on 6-well plates. Upon culturing for 4 days, the cells were cultured in serum-free medium with supplements for 24 hours, followed by incubation with different concentrations of FLL ethanol extract. After incubation for 48 hours, protein expression levels were detected by western blot. Protein expression level is shown as a ratio of target protein/β-actin. The experiment was repeated for three times (n=3). Values are means ± SEM.

6.3.6 Effects of FLL ethanol extract on 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) activity in primary cultures of rat proximal tubule cells

To determine the possible direct effects of FLL ethanol extract on 1-OHase activity, the 1-OHase activity in primary cultures of rat proximal tubule cells were investigated. Upon incubation with 10ug/ml and 100ug/ml FLL ethanol extract for 48 hours, the ability of 1-OHase to produce $1,25(OH)_2D_3$ was unchanged in the extract treatment group compared with the control group (Data not shown). However, when the cells were incubated with the extract for 72 hours, 10ug/ml and 100ug/ml of FLL ethanol extract ($P<0.05$, Figure 6-7) significantly increased the 1-OHase activity, especially the concentration of 10ug/ml of FLL ethanol extract ($P<0.01$, Figure 6-7).

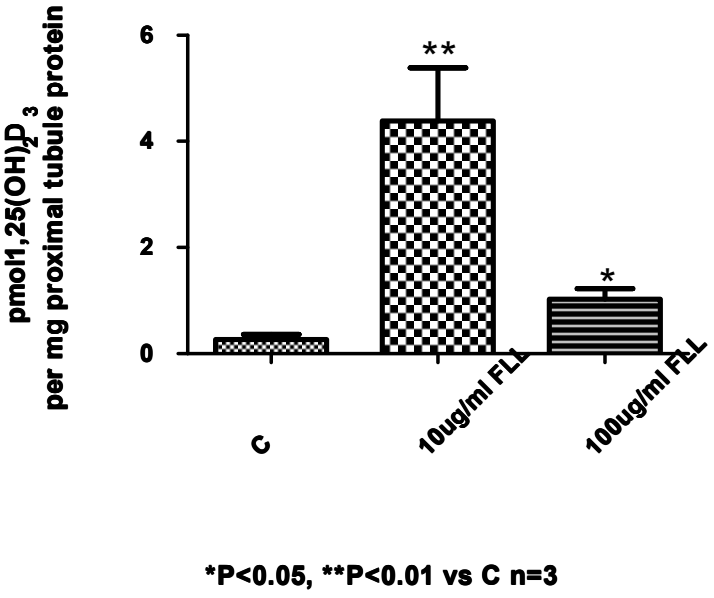


Figure 6-7 Effects of FLL ethanol extract on 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) activity in primary cultures of rat proximal tubule cells.

Four-month-old mature normal female rats were sacrificed and their kidneys were harvested for preparation of proximal tubule cells. The cells were then plated on 6-well plates. Upon culturing for 4 days, the cells were cultured in serum-free medium with supplements for 24 hours, followed by incubation with different concentrations of FLL ethanol extract. After incubation for 72 hours, the substrate $25(OH)D_3$ was added into each well and the activity of 1-OHase was detected accordingly. Data are expressed as the ratio of the production of $1,25(OH)_2D_3$ to the proximal tubule proteins based on the same quantity of substrate $25(OH)D_3$. Values are means ± SEM. The experiment was repeatly for three times (n=3). *P<0.05, **P<0.01 vs group C.

6.3.7 Effects of FLL ethanol extract on 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) mRNA expression in human proximal kidney tubule (HKC-8) cells

To identify the active fractions in the FLL ethanol extract responsible for its actions on 1-OHase, the screening platform was established in HKC-8 cells. The HKC-8 cells were treated with FLL ethanol extract for 48 hours in the same culture conditions as the primary cultures of rat proximal tubules. The results indicated that 0.1ug/ml and 10ug/ml of FLL ethanol extract significantly increased 1-OHase mRNA expression. Furthermore, the responses of 1-OHase in HKC-8 cells to different concentration of FLL ethanol extract were similar to that found in primary proximal tubule cells. This suggested that HKC-8 cells could be selected as the screening cells to identify the active fractions of FLL ethanol extract on 1-OHase mRNA expression.

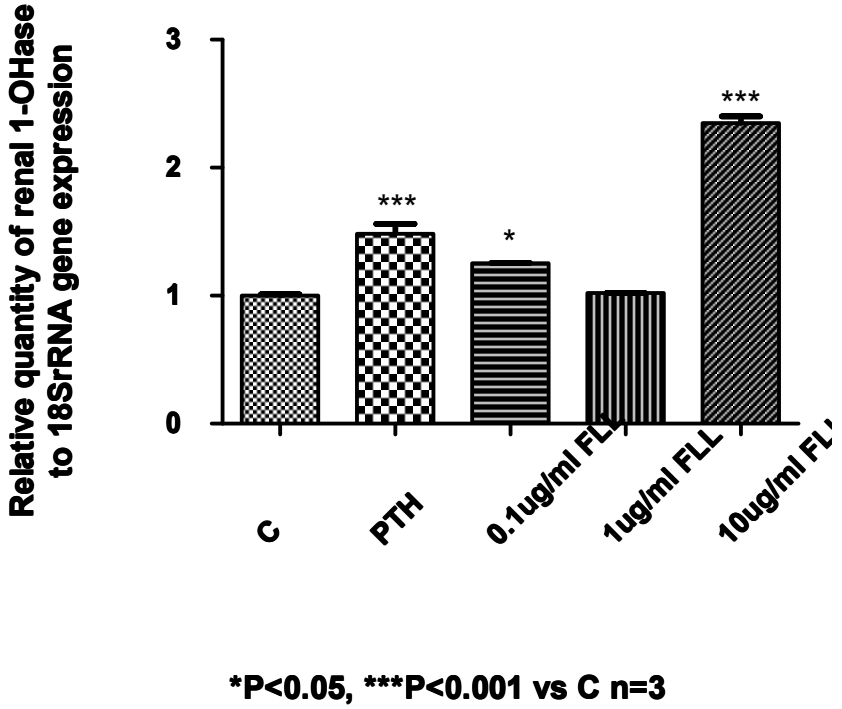


Figure 6-8 Effects of different concentration of FLL ethanol extract on mRNA expression of 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) in HKC-8 cells.

HKC-8 cells were maintained in DMEM/F12 medium supplemented with 10% FBS. Upon 80%-90% confluency, cells were transferred to defined medium containing supplements for 24 h before treatment. Then the cells were treated with different concentrations of FLL ethanol extract and its vehicle and 10^{-7}M PTH as positive control for 48 hours. After 48 hours' incubation, mRNA expression levels were detected by real time PCR. The expression level is shown as a ratio of target gene/18SrRNA and the ratio in group C is normalized as 1.0. Values are means ± SEM. The experiment was repeated for three times (n=3). *P<0.05, ***P<0.001 vs group C.

6.3.8 Effects of FLL ethanol extract and its separated fractions on mRNA expression of 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) in HKC-8 cells

FLL ethanol extract (EE) and its separated fractions, including water layer fraction (WF) and EA soluble fraction (EAF), were incubated with HKC-8 cells for 48 hours for determination of the active fraction that regulates the expression of 1-OHase mRNA. The result showed that FLL ethanol extract and its EA soluble fraction significantly increased 1-OHase mRNA expression ($P<0.05$, $P<0.01$; Figure 9). However, water layer fraction significantly suppressed 1-OHase gene expression ($P<0.05$, Figure 6-9). This suggested that EA soluble fraction from the FLL ethanol extract contained the active ingredients which upregulated 1-OHase gene.

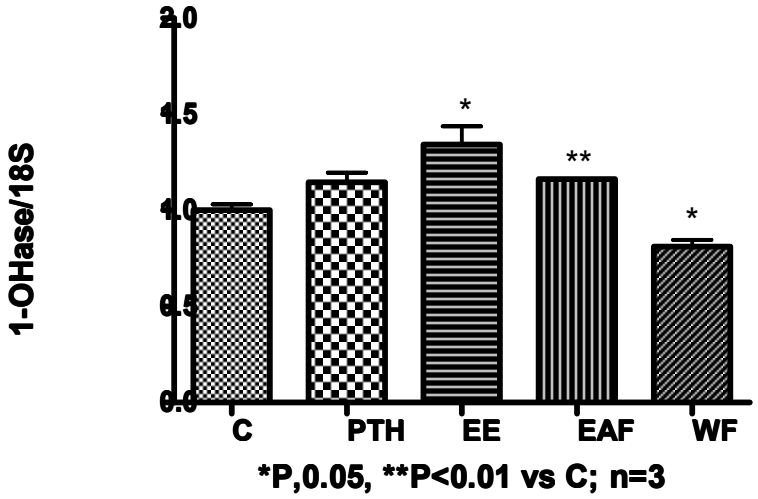


Figure 6-9 Effects of different FLL extracts on mRNA expression of 25-hydroxyvitamin D 1-alpha hydroxylase (1-OHase) in HKC-8 cells.

HKC-8 cells were maintained in DMEM/F12 medium supplemented with 10% FBS. Upon 80%-90% confluency, cells were transferred to defined medium containing supplements for 24 h before treatment. Then the cells were treated with FLL ethanol extract (EE) and its two separated fractions (WF and EAF) and its vehicle and 10^{-7}M PTH as positive control for 48 hours. After 48 hours' incubation, mRNA expression levels were detected by real time PCR. The expression level is shown as a ratio of target gene/18SrRNA and the ratio in group C is normalized as 1.0. Values are means ± SEM. The experiment was repeated for three times (n=3). *P<0.05, ***P<0.001 vs group C.

6.4 Discussion

The present study demonstrates that the FLL ethanol extract could modulate vitamin D metabolism by altering the expression of the enzymes for the biosynthesis of $1,25(OH)_2D_3$, resulting in an increase in serum $1,25(OH)_2D_3$ concentration. Furthermore, our study indicates that the effects of FLL on the biosynthetic enzymes of $1,25(OH)_2D_3$ is direct, and does not require the action of other hormones.

In our experiments, the basal levels of serum $1,25(OH)_2D_3$ in the normal aged female rats treated with vehicle are low, in comparison with that in the normal younger ones (Zhang et al., 2007). Our results also clearly indicate that FLL ethanol extract treatment significantly increased serum $1,25(OH)_2D_3$ levels in the aged female and OVX rats after 12 weeks feeding. Serum Ca and Pi levels in the aged normal females remained constant after FLL ethanol extract treatment. Serum Ca levels decreased in the vehicle treated OVX rats compared with that in the vehicle treated sham rats. However, FLL ethanol extract seemed to help restore serum Ca to the relatively normal levels in the sham rats treated by vehicle. The induction of serum $1,25(OH)_2D_3$ levels by FLL ethanol extract in OVX rats might in part explain the benefit of FLL ethanol extract on the increase of serum Ca levels. The increase of serum $1,25(OH)_2D_3$ levels appeared not to be able to regulate serum Ca in the aged normal female rats. The results suggested that the responses

of Ca (re)absorption to $1,25(OH)_2D_3$ under different estrogen level in the aged female rats were different, as indicated in the previous chapter of the thesis.

To determine if FLL ethanol extract could increase the biosynthesis of 1-OHase, renal expressions of 1-OHase mRNA and protein in aged rats were studied. It was indicated in our study that the FLL ethanol extract treated group had a higher mRNA and protein expression level of renal 1-OHase in the normal aged female rats. In contrast, only protein expression was found to be elevated by FLL ethanol extract treatment in the aged OVX rats, while its mRNA expression were not affected. Regardless of the estrogen status, our results indicated that increase of serum $1,25(OH)_2D_3$ levels by FLL ethanol extract can be explained by the elevations of renal 1-OHase protein expression in aged rats.

Our results clearly showed that FLL ethanol extract significantly induced renal VDR mRNA and protein expression in aged normal female rats. $1,25(OH)_2D_3$ (Brown et al., 1995; Feldman et al., 1997; Healy et al., 2005; Healy et al., 2003) is known to induce renal VDR expression. A substantial number of *in vitro* studies have demonstrated that $1,25(OH)_2D_3$ up-regulates VDR at least partially through the activation of its gene expression or the stabilization of the receptor protein (Healy et al., 2005). Both exogenous and endogenous $1,25(OH)_2D_3$ (as increased by dietary Ca deficiency) can elevate renal VDR expression *in vivo* (Brown et al., 1995; Healy et al., 2003). Thus, an increase in VDR levels in the FLL ethanol

extract treated aged normal rats might be mediated by the induction of circulating $1,25(OH)_2D_3$ levels. However, renal VDR expression in aged OVX rats was not induced by the increased levels of serum $1,25(OH)_2D_3$. This might be attributed to the different expression patterns affected by estrogen deficiency, just as indicated by our findings in the chapter 3.

In order to further confirm the positive effects of FLL ethanol extract on renal 1-OHase, younger normal female rats were fed with FLL ethanol extract for a relatively short period to determine its effect on renal 1-OHase activity. The results showed that serum $1,25(OH)_2D_3$ levels in the younger female rats were elevated by FLL ethanol extract treatment. At the same time, the activity of renal 1-OHase was increased after the four weeks' feeding. Thus, FLL ethanol extract could raise the production of serum $1,25(OH)_2D_3$ levels through increasing the expression together with the activity of its biosynthetic enzymes. The results also indicated that this effect is not dependent on the age and estrogen levels of the female rats.

As mentioned, the activity of the 1-OHase is influenced by many regulators, such as $1,25(OH)_2D_3$ itself, PTH and FGF23 *in vivo*. Thus, the positive results of FLL ethanol extract on renal 1-OHase could be due to the direct action of this herb or through other hormone regulators observed *in vivo*. In order to differentiate the two possibilities, the effects of FLL ethanol extract in primary cultures of rat proximal tubules were studied. It was found that 1-OHase mRNA and protein

expression in the proximal tubule cells were increased after incubation with FLL ethanol extract for 48 hours. The activity of 1-OHase in the cells was elevated by FLL ethanol extract after incubation for 72 hours. Although the involvement of other hormones in the induction of renal 1-OHase by FLL ethanol extract *in vivo* could not be excluded, our results indicated that FLL ethanol extract had the ability to regulate renal 1-OHase expression and activity, directly. The elevation of 1-OHase expression might account for the increase in activity of this enzyme, because activity increased at the time after its protein was upregulated. 100ug/ml and 10ug/ml of FLL ethanol extract similarly increased 1-OHase mRNA and protein expression in the cells. However, 10ug/ml FLL ethanol extract had a greater induction effects on the activity of 1-OHase in comparison with the concentration of 100ug/ml. Thus, it is still possible that the FLL ethanol extract could amplify activity of 1-OHase through direct combination of some components in FLL with the enzyme pockets.

The similar 1-OHase regulation pattern elicited by FLL ethanol extract in the primary of rat proximal tubules cells and the HKC-8 cells indicated that HKC-8 cells could function as an in vitro screening system for the identification fractions active on 1-OHase. Our results showed that EA soluble fraction might be the active fraction responsible for the enhancing actions of FLL ethanol extract on 1-OHase expression. However, the water layer fraction suppressed 1-OHase expression in HKC-8 cells, suggesting it is not the active fraction in FLL ethanol extract.

Our previous study reported that FLL increased Ca balance and intestinal Ca absorption in aged rats (Zhang et al., 2008a). The present study shows that FLL increased serum $1,25(OH)_2D_3$ levels possibly by inducing renal 1-OHase expressions as well as its activity directly. This effect is not dependent on the age and estrogen levels of female rats. EA soluble fraction was the active fraction acting on 1-OHase expression. Our results also demonstrate that the responses of renal VDR expression to serum $1,25(OH)_2D_3$ level were increased in the aged normal female rats, but not in the aged OVX rats.

Chapter 7

Discussion and Conclusion

7.1 Discussion

Previous studies in our laboratories demonstrated that the ethanol extract of Fructus Ligustri Lucidi (FLL) plays an active role in modulating Ca homeostasis, not only in OVX rats but also in aged female rats with intact ovaries (Zhang et al., 2008a; Zhang et al., 2008b). In the present study, the effects of long-term estrogen deficiency on the Ca (re)absorption in the aged female rats were first investigated. The ethyl acetate soluble (lipophilic) extract (EAF) and water soluble (hydrophilic) extract (WF) were prepared from the ethanol extract (EF) of FLL and administered to young female rats to identify the active fractions which are responsible for the positive actions of FLL on Ca homeostasis. Our previous study also showed that the EF extract of FLL could increase serum $1,25(OH)_2D_3$ levels in aged rats. Thus, the mechanism by which the EF extract of FLL modulates vitamin D_3 metabolism was also investigated.

7.1.1 Effects of estrogen deficiency on Ca (re)absorption in the aged female rats

Deficiency of estrogen at menopause leads to a decrease in intestinal Ca absorption and a corresponding increase in renal Ca excretion (Holloway et al., 2007). However, the solution is not as straightforward as simply consuming more Ca, because the percentage absorption is inversely related to intake. Thus, increasing Ca intake may be partially negated by a corresponding decrease in the

efficiency of Ca absorption in the aged female groups. In order to systemically investigate how the estrogen and vitamin D system interplayed to regulate this process in aged females, aged OVX female rats in our experiment were used to mimic aged women with extremely low level of estrogen. By contrast, aged sham female rats were used to represent the women with relatively normal estrogen level, as the comparison. This study considered the possible alterations of Ca (re)absorption in OVX rats after long-term estrogen deficiency at different levels of dietary Ca. Moreover, the potential underlying mechanisms in comparison with aged normal female rats were also studied.

In the aged female rats with relatively normal estrogen levels, dietary Ca did not alter intestinal Ca absorption, although serum $1,25(OH)_2D_3$ levels, together with duodenal VDR and the epithelial Ca transporting proteins altered with dietary Ca. This suggested that intestinal Ca absorption could not be solely explained by paracellular or transcellular pathway and Ca might be absorbed through the small intestine simultaneously in the two ways. However, in the aged estrogen deficient rats, an adequate amount of dietary Ca seemed to restore the intestinal Ca absorption. While, the changing trend could not be consistent with the changes of serum $1,25(OH)_2D_3$ levels and duodenal VDR with other epithelial Ca transporting proteins. This indicated that serum Ca might be mainly absorbed through the paracellular pathways in the OVX rats. Long-term estrogen deficiency in aged female rats resulted in mal-absorption of Ca through the intestine under different

levels of dietary Ca. However, the alterations of serum $1,25(OH)_2D_3$ levels and duodenal VDR expression did not explain the changes of great decrease of intestinal Ca absorption in the aged OVX rats compared with that in the aged sham rats. It was found that altered epithelial Ca transporting proteins, especially the duodenal PMCA1b expression, by estrogen deficiency and different Ca diet is critical for the alterations of Ca (re)absorption in the intestine independent of vitamin D.

In kidney, urinary Ca excretion increased with the increase of dietary Ca in both aged sham and OVX rats. This suggested that higher Ca diet resulted in higher Ca filtration and excretion, which should be a natural physiological process. Estrogen deficiency seemed to increase urinary Ca excretion at each dietary Ca level, though the increase was not statistically significant. Serum $1,25(OH)_2D_3$ levels and duodenal VDR expression did not change in the same trend as the urinary Ca excretion. However, the three epithelial Ca transporting proteins in kidney were inhibited greatly under different dietary Ca levels in the OVX rats compared with those in the sham rats. The alteration of epithelial Ca transporting proteins is also critical for Ca reabsorption alterations in the kidney independent of vitamin D. Furthermore, they were differently regulated in the intestine and the kidney.

7.1.2 Screening for the active fractions of FLL ethanol extract on Ca balance

In order to identify the active fractions of FLL on Ca balance, the reliable and easily operated screening system is required. In consideration of the difficulties to get aged animals, it was designed to apply FLL ethanol extract to young mature female rats in the present study. Ca balance was detected every week after the start of feeding to the end, for the purpose of determination of the earliest time when the herb began to repress urinary Ca and fecal Ca excretion. At the end of experiment, duodenal and renal genes and proteins involved in the epithelial Ca transporting were detected for the mechanisms study. Our results indicated that FLL ethanol extract exerted the positive actions on Ca balance in the young mature female rats as in the aged ones. The changes of epithelial Ca transporting proteins in the intestine and the kidney after FLL ethanol extract treatment could partly explain the effects of FLL on Ca balance. Based on our results, the younger mature female rats will be selected to be the initial screening platform for the active fractions which are responsible for Ca balance in place of the aged ones. Moreover, our results also indicated that the regulation of these epithelial proteins was different in the intestine which seemed to be induced by vitamin D and in the kidney which appeared not to be influenced by vitamin D.

Based on the screening platform, two FLL extracts, the EA soluble lipophilic fraction (EAF), as well as the hydrophilic water layer fraction (WF) were separated

from FLL ethanol extract and administered to the mature female rats. Urine and fecal samples were collected for evaluation of Ca excretion. Duodenum and kidney were also collected at the end of experiment for mechanism study. The results showed that the water layer fraction (WF) is the active fraction that exerts positive effects on Ca balance. The increased intestinal Ca absorption by WF could be explained at least in part by the increase of duodenal TRPV6 and PMCA1b expression. In order to further determine the actions of WF on Ca balance, the mature female rats were fed with different levels of dietary Ca. The results indicated that WF exerts positive effects on Ca balance when the rats were fed a sufficient dietary Ca.

7.1.3 Effects and identification of active fractions of FLL ethanol extract on vitamin D metabolism

Our previous study has demonstrated that the actions of FLL ethanol extract on Ca balance were due to the ability of FLL ethanol extract to increase serum $1,25(OH)_2D_3$ levels and vitamin D-dependent Ca binding proteins expression (Zhang et al., 2008b). The present study is aimed at studying the mechanism of FLL ethanol extract on vitamin D metabolism in aged rats. Although a variety of tissues and cells express 1-OHase and have the ability to produce $1,25(OH)_2D_3$, the kidney is the major source of circulating $1,25(OH)_2D_3$ in serum (Wu et al., 2007). In the present study, renal 1-OHase and VDR expression were systematically

investigated in the aged rats, including the aged normal and OVX rats. At the same time, renal 1-OHase activity was detected in the mature female rats. In order to further confirm that FLL ethanol extract acts on renal 1-OHase directly, its effects on 1-OHase expression and activity in primary cultures of rat proximal tubule cells were also investigated. The results showed that FLL raised the circulating levels of $1,25(OH)_2D_3$ in both sham and OVX aged female rats by increasing the protein expression of renal 1-OHase. This effect is not relying on the age and estrogen levels of female rats. Renal 1-OHase activity was also improved by FLL ethanol extract treatment. The *in vitro* study indicated that FLL ethanol extract directly acted on renal 1-OHase expression and activity. Furthermore, to identify the active fraction that accounts for the positive effects of FLL on renal 1-OHase, human proximal tubule HKC-8 cells were established as an *in vitro* screening platform. The EA fraction of FLL (EAF) was confirmed to the active fraction that contributed to its modulatory action on renal 1-OHase in the screening system. Our results also demonstrate that the responses of renal VDR expression to serum $1,25(OH)_2D_3$ level were increased in the aged normal female rats, but not in the aged OVX rats. This might be explained by the regulation of estrogen deficiency on renal VDR in our findings.

7.2 Conclusion

In conclusion, long-term estrogen deficiency in aged female rats is sufficient to cause mal-absorption of Ca through the intestine and the kidney. In the aged female rats with relatively normal estrogen levels, Ca can be absorbed through the small intestine simultaneously in two ways. However, in the aged estrogen deficient rats, Ca is mainly absorbed through the paracellular pathways. The altered epithelial Ca transporting proteins, especially the PMCA1b, by estrogen deficiency and different Ca diet is critical for the alterations of Ca (re)absorption in the intestine and the kidney independent of vitamin D. The epithelial Ca transporting proteins were differently regulated in the intestine and the kidney.

FLL ethanol extract exerted the positive actions on Ca balance in the young mature female rats as in the aged ones. The younger mature female rats will be selected to be the initial screening platform for the active fractions which are responsible for Ca balance in place of the aged ones. Based on this screening system, FLL water layer fraction was identified to be a major fraction for the positive effects of FLL on Ca balance when the rats were fed a sufficient Ca diet. Its actions might relate to its regulatory properties of epithelial Ca transporting processes.

FLL increased serum $1,25(OH)_2D_3$ levels possibly by inducing renal 1-OHase expressions as well as its activity directly. This effect is not relying on the age and estrogen levels of female rats. EA soluble fraction might be the active fraction acting on 1-OHase expression.

References

Pharmacopoeia Comission of the Ministry of Public Health. Chinese Pharmacopoeia, Part I, 2005 edition, Chemical Industrial Press, Beijing, China.

Adami, S., Viapiana, O., Gatti, D., Idolazzi, L., and Rossini, M. (2008). Relationship between serum parathyroid hormone, vitamin D sufficiency, age, and calcium intake. Bone *42*, 267-270.

Afonina, I. A., Mills, A., Sanders, S., Kulchenko, A., Dempcy, R., Lokhov, S., Vermeulen, N. M., and Mahoney, W. (2006). Improved biplex quantitative real-time polymerase chain reaction with modified primers for gene expression analysis. Oligonucleotides *16*, 395-403.

Akhter, S., Kutuzova, G. D., Christakos, S., and DeLuca, H. F. (2007). Calbindin D9k is not required for 1,25-dihydroxyvitamin D3-mediated Ca2+ absorption in small intestine. Arch Biochem Biophys *460*, 227-232.

Armbrecht, H. J., Hodam, T. L., Boltz, M. A., Partridge, N. C., Brown, A. J., and Kumar, V. B. (1998). Induction of the vitamin D 24-hydroxylase (CYP24) by 1,25-dihydroxyvitamin D3 is regulated by parathyroid hormone in UMR106 osteoblastic cells. Endocrinology *139*, 3375-3381.

Avila, E., Diaz, L., Barrera, D., Halhali, A., Mendez, I., Gonzalez, L., Zuegel, U., Steinmeyer, A., and Larrea, F. (2007). Regulation of Vitamin D hydroxylases gene expression by 1,25-dihydroxyvitamin D3 and cyclic AMP in cultured human syncytiotrophoblasts. J Steroid Biochem Mol Biol *103*, 90-96.

Bacskai, B. J., and Friedman, P. A. (1990). Activation of latent Ca2+ channels in renal epithelial cells by parathyroid hormone. Nature *347*, 388-391.

Bajwa, A., Horst, R. L., and Beckman, M. J. (2005). Gene profiling the effects of calcium deficiency versus 1,25-dihydroxyvitamin D induced hypercalcemia in rat kidney cortex. Arch Biochem Biophys *438*, 182-194.

Baksi, S. N., and Kenny, A. D. (1980). Estradiol-induced stimulation of 25-hydroxyvitamin D3-1-hydroxylase in vitamin D-deficient Japanese quail. Pharmacology *20*, 298-303.

Barley, N. F., Howard, A., O'Callaghan, D., Legon, S., and Walters, J. R. (2001). Epithelial calcium transporter expression in human duodenum. Am J Physiol Gastrointest Liver Physiol *280*, G285-290.

Beckman, M. J., and DeLuca, H. F. (2002). Regulation of renal vitamin D receptor is an important determinant of 1alpha,25-dihydroxyvitamin D(3) levels in vivo. Arch Biochem Biophys *401*, 44-52.

Berggard, T., Miron, S., Onnerfjord, P., Thulin, E., Akerfeldt, K. S., Enghild, J. J., Akke, M., and Linse, S. (2002). Calbindin D28k exhibits properties characteristic of a Ca2+ sensor. J Biol Chem *277*, 16662-16672.

Bidwell, J. P., Carter, W. B., Fryer, M. J., and Heath, H., 3rd (1991). Parathyroid hormone (PTH)-induced intracellular Ca2+ signalling in naive and PTH-desensitized osteoblast-like cells (ROS 17/2.8): pharmacological characterization and evidence for synchronous oscillation of intracellular Ca2+. Endocrinology *129*, 2993-3000.

Bindels, R. J. (1993). Calcium handling by the mammalian kidney. J Exp Biol *184*, 89-104.

Bischoff-Ferrari, H. A., Kiel, D. P., Dawson-Hughes, B., Orav, J. E., Li, R., Spiegelman, D., Dietrich, T., and Willett, W. C. (2008). Dietary Calcium and Serum 25-hydroxyvitamin D Status in Relation to Bone Mineral Density Among U.S. Adults. J Bone Miner Res *29*, 29.

Bodding, M., Wissenbach, U., and Flockerzi, V. (2002). The recombinant human TRPV6 channel functions as Ca2+ sensor in human embryonic kidney and rat basophilic leukemia cells. J Biol Chem *277*, 36656-36664.

Book (2001). Recommendations for the prevention and treatment of glucocorticoid-induced osteoporosis: 2001 update. American College of Rheumatology Ad Hoc Committee on Glucocorticoid-Induced Osteoporosis. Arthritis Rheum *44*, 1496-1503.

Book (2006). Management of osteoporosis in postmenopausal women: 2006 position statement of The North American Menopause Society. Menopause *13*, 340-367; quiz 368-349.

Book (2009). Correlations between bone mass density and osteoporosis risk factors in postmenopausal women. Ter Arkh *81*, 76-79.

Book. (1991). Summary findings of the 1991 population census. Hong Kong Mon Dig Stat, 107-118.

Boonen, S., Vanderschueren, D., Haentjens, P., and Lips, P. (2006). Calcium and vitamin D in the prevention and treatment of osteoporosis - a clinical update. J Intern Med *259*, 539-552.

Brenza, H. L., and DeLuca, H. F. (2000). Regulation of 25-hydroxyvitamin D3 1alpha-hydroxylase gene expression by parathyroid hormone and 1,25-dihydroxyvitamin D3. Arch Biochem Biophys *381*, 143-152.

Brodin, P., Falchetto, R., Vorherr, T., and Carafoli, E. (1992). Identification of two domains which mediate the binding of activating phospholipids to the plasma-membrane Ca2+ pump. Eur J Biochem *204*, 939-946.

Bronner, F. (1998). Calcium absorption--a paradigm for mineral absorption. J Nutr *128*, 917-920.

Brown, A. J., Zhong, M., Finch, J., Ritter, C., and Slatopolsky, E. (1995). The roles of calcium and 1,25-dihydroxyvitamin D3 in the regulation of vitamin D receptor expression by rat parathyroid glands. Endocrinology *136*, 1419-1425.

Byrne, I. M., Flanagan, L., Tenniswood, M. P., and Welsh, J. (2000). Identification of a hormone-responsive promoter immediately upstream of exon 1c in the human vitamin D receptor gene. Endocrinology *141*, 2829-2836.

Cai, Q., Chandler, J. S., Wasserman, R. H., Kumar, R., and Penniston, J. T. (1993). Vitamin D and adaptation to dietary calcium and phosphate deficiencies increase

intestinal plasma membrane calcium pump gene expression. Proc Natl Acad Sci U S A *90*, 1345-1349.

Cai Q, S., L.D., Shen, Q. (1998). Effect of p-hydroxyphenethyl-D-glucoside on natural killer cell in mice in vitro. . Chinese Journal of Pharmacology and Toxicology *12*, 207-209.

Cannell, R. J. P. (1998). Natural Products Isolation. Methods in Biotechnology, Human Press.

Chang, W., Chen, T. H., Pratt, S., and Shoback, D. (2000). Amino acids in the second and third intracellular loops of the parathyroid Ca2+-sensing receptor mediate efficient coupling to phospholipase C. J Biol Chem *275*, 19955-19963.

Chapuy, M. C., Schott, A. M., Garnero, P., Hans, D., Delmas, P. D., and Meunier, P. J. (1996). Healthy elderly French women living at home have secondary hyperparathyroidism and high bone turnover in winter. EPIDOS Study Group. J Clin Endocrinol Metab *81*, 1129-1133.

Chen, H., Emura, S., Isono, H., and Shoumura, S. (2005). Effects of traditional Chinese medicine on bone loss in SAMP6: a murine model for senile osteoporosis. Biol Pharm Bull *28*, 865-869.

Cheng, X. F., He, M. F., Zhang, Y., and Meng, Z. M. (2000). Studies on Constituents of Fructus Ligustri lucidi. JOURNAL OF CHINA PHARMACEUTICAL UNIVERSITY *31*, 169-170.

Choi, K. C., and Jeung, E. B. (2008). Molecular mechanism of regulation of the calcium-binding protein calbindin-D(9k), and its physiological role(s) in mammals: a review of current research. J Cell Mol Med *12*, 409-420.

Costa, E. M., Hirst, M. A., and Feldman, D. (1985). Regulation of 1,25-dihydroxyvitamin D3 receptors by vitamin D analogs in cultured mammalian cells. Endocrinology *117*, 2203-2210.

Council, N. R. (2006). Guide for the care and the use of laboratory animals. National Academy Press, Washington DC.

Davison, S., and Davis, S. R. (2003). Hormone replacement therapy: current controversies. Clin Endocrinol (Oxf) *58*, 249-261.

Dawson-Hughes, B., Harris, S. S., Krall, E. A., and Dallal, G. E. (1997). Effect of calcium and vitamin D supplementation on bone density in men and women 65 years of age or older. N Engl J Med *337*, 670-676.

de Groot, T., Bindels, R. J., and Hoenderop, J. G. (2008). TRPV5: an ingeniously controlled calcium channel. Kidney Int *74*, 1241-1246.

den Dekker, E., Hoenderop, J. G., Nilius, B., and Bindels, R. J. (2003). The epithelial calcium channels, TRPV5 & TRPV6: from identification towards regulation. Cell Calcium *33*, 497-507.

Dennison, E., Mohamed, M. A., and Cooper, C. (2006). Epidemiology of osteoporosis. Rheum Dis Clin North Am *32*, 617-629.

Di Leva, F., Domi, T., Fedrizzi, L., Lim, D., and Carafoli, E. (2008). The plasma membrane Ca2+ ATPase of animal cells: structure, function and regulation. Arch Biochem Biophys *476*, 65-74.

Ding, Y. Q., and Xu, C. H. (2006). EFFECT OF LIGUSTRUM IUCIDUM ON LEARNING AND MEMORY IN D-GALACTOSE-INDUCED SENILE MICE. Journal of Preventive Medicine of Chinese People's Liberation Army *24*, 247-249.

Duncan, W. E., Glass, A. R., and Wray, H. L. (1991). Estrogen regulation of the nuclear 1,25-dihydroxyvitamin D3 receptor in rat liver and kidney. Endocrinology *129*, 2318-2324.

Dusso, A. S., Brown, A. J., and Slatopolsky, E. (2005). Vitamin D. Am J Physiol Renal Physiol *289*, F8-28.

Eeleen, G. (2005). Molecular modes of action of 1α, 25-dihydroxyvitamin D3 and analogs. Leuven University Press, Belgium.

Eriksen, E. F., and Glerup, H. (2002). Vitamin D deficiency and aging: implications for general health and osteoporosis. Biogerontology *3*, 73-77.

Ettinger, B., Black, D. M., Mitlak, B. H., Knickerbocker, R. K., Nickelsen, T., Genant, H. K., Christiansen, C., Delmas, P. D., Zanchetta, J. R., Stakkestad, J.*, et al.* (1999). Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. Jama *282*, 637-645.

Evans, R. M. (1988). The steroid and thyroid hormone receptor superfamily. Science *240*, 889-895.

Fairweather-Tait, S. J., and Teucher, B. (2002). Calcium bioavailability in relation to bone health. Int J Vitam Nutr Res *72*, 13-18.

Fan, W. X., Zhu, W.Y. (2000). Expression of Growth Factor mRNA in Hair Follicles Cultured with Some Chinese Herbs. Chinese Journal of Dermatology *33*, 229-231.

Feldman, D., Glorieux, F. H., and Pike, J. W. (1997). Vitamin D. Academic Press, USA.

Feldman, D., McCain, T. A., Hirst, M. A., Chen, T. L., and Colston, K. W. (1979). Characterization of a cytoplasmic receptor-like binder for 1 alpha, 25-dihydroxycholecalciferol in rat intestinal mucosa. J Biol Chem *254*, 10378-10384.

Feldman, D., Pike, J. W., and Glorieux, F. H. (2005). Vitamin D. Elsevier Academic Press *2*.

Fischer, D., Schroer, A., Ludders, D., Cordes, T., Bucker, B., Reichrath, J., and Friedrich, M. (2007). Metabolism of vitamin D3 in the placental tissue of normal and preeclampsia complicated pregnancies and premature births. Clin Exp Obstet Gynecol *34*, 80-84.

Fraser, D. R., and Kodicek, E. (1970). Unique biosynthesis by kidney of a biological active vitamin D metabolite. Nature *228*, 764-766.

Friedman, P. A., Coutermarsh, B. A., Kennedy, S. M., and Gesek, F. A. (1996). Parathyroid hormone stimulation of calcium transport is mediated by dual signaling mechanisms involving protein kinase A and protein kinase C. Endocrinology *137*, 13-20.

Friedman, P. A., and Gesek, F. A. (1994). Hormone-responsive Ca2+ entry in distal convoluted tubules. J Am Soc Nephrol *4*, 1396-1404.

Gavaia, P. J., Simes, D. C., Ortiz-Delgado, J. B., Viegas, C. S., Pinto, J. P., Kelsh, R. N., Sarasquete, M. C., and Cancela, M. L. (2006). Osteocalcin and matrix Gla protein in zebrafish (Danio rerio) and Senegal sole (Solea senegalensis): comparative gene and protein expression during larval development through adulthood. Gene Expr Patterns *6*, 637-652.

Gilad, L. A., Bresler, T., Gnainsky, J., Smirnoff, P., and Schwartz, B. (2005). Regulation of vitamin D receptor expression via estrogen-induced activation of the ERK 1/2 signaling pathway in colon and breast cancer cells. J Endocrinol *185*, 577-592.

Gilad, L. A., Tirosh, O., and Schwartz, B. (2006). Phytoestrogens regulate transcription and translation of vitamin D receptor in colon cancer cells. J Endocrinol *191*, 387-398.

Gillin, A. G., Star, R. A., and Sands, J. M. (1993). Osmolarity-stimulated urea transport in rat terminal IMCD: role of intracellular calcium. Am J Physiol *265*, F272-277.

Gong, M., Jia, F., and Wang, M. (1999). Influence of different extract methods on Oleanolic acid production. Beingjing Chinese Medicine *18*, 53.

Goodenough, D. A. (1999). Plugging the leaks. Proc Natl Acad Sci U S A *96*, 319-321.

Gray, R. W., Weber, H. P., Dominguez, J. H., and Lemann, J., Jr. (1974). The metabolism of vitamin D3 and 25-hydroxyvitamin D3 in normal and anephric humans. J Clin Endocrinol Metab *39*, 1045-1056.

Healy, K. D., Frahm, M. A., and DeLuca, H. F. (2005). 1,25-Dihydroxyvitamin D3 up-regulates the renal vitamin D receptor through indirect gene activation and receptor stabilization. Arch Biochem Biophys *433*, 466-473.

Healy, K. D., Zella, J. B., Prahl, J. M., and DeLuca, H. F. (2003). Regulation of the murine renal vitamin D receptor by 1,25-dihydroxyvitamin D3 and calcium. Proc Natl Acad Sci U S A *100*, 9733-9737.

Heaney, R. P., and Recker, R. R. (1982). Effects of nitrogen, phosphorus, and caffeine on calcium balance in women. J Lab Clin Med *99*, 46-55.

Heaney, R. P., Recker, R. R., Stegman, M. R., and Moy, A. J. (1989). Calcium absorption in women: relationships to calcium intake, estrogen status, and age. J Bone Miner Res *4*, 469-475.

Henry, H. L. (1985). Parathyroid hormone modulation of 25-hydroxyvitamin D3 metabolism by cultured chick kidney cells is mimicked and enhanced by forskolin. Endocrinology *116*, 503-510.

Henry, H. L., and Luntao, E. M. (1989). Interactions between intracellular signals involved in the regulation of 25-hydroxyvitamin D3 metabolism. Endocrinology *124*, 2228-2234.

Hernandez, A., Concepcion, M. T., Rodriguez, M., Salido, E., and Torres, A. (1996). High phosphorus diet increases preproPTH mRNA independent of calcium and calcitriol in normal rats. Kidney Int *50*, 1872-1878.

Hewison, M., Zehnder, D., Bland, R., and Stewart, P. M. (2000). 1alpha-Hydroxylase and the action of vitamin D. J Mol Endocrinol *25*, 141-148.

Hirnet, D., Olausson, J., Fecher-Trost, C., Bodding, M., Nastainczyk, W., Wissenbach, U., Flockerzi, V., and Freichel, M. (2003). The TRPV6 gene, cDNA and protein. Cell Calcium *33*, 509-518.

Hoenderop, J. G., Chon, H., Gkika, D., Bluyssen, H. A., Holstege, F. C., St-Arnaud, R., Braam, B., and Bindels, R. J. (2004). Regulation of gene expression by dietary Ca2+ in kidneys of 25-hydroxyvitamin D3-1 alpha-hydroxylase knockout mice. Kidney Int *65*, 531-539.

Hoenderop, J. G., Dardenne, O., Van Abel, M., Van Der Kemp, A. W., Van Os, C. H., St -Arnaud, R., and Bindels, R. J. (2002). Modulation of renal Ca2+ transport protein genes by dietary Ca2+ and 1,25-dihydroxyvitamin D3 in 25-hydroxyvitamin D3-1alpha-hydroxylase knockout mice. Faseb J *16*, 1398-1406.

Hoenderop, J. G., Muller, D., Suzuki, M., van Os, C. H., and Bindels, R. J. (2000). Epithelial calcium channel: gate-keeper of active calcium reabsorption. Curr Opin Nephrol Hypertens *9*, 335-340.

Hoenderop, J. G., Muller, D., Van Der Kemp, A. W., Hartog, A., Suzuki, M., Ishibashi, K., Imai, M., Sweep, F., Willems, P. H., Van Os, C. H., and Bindels, R. J. (2001). Calcitriol controls the epithelial calcium channel in kidney. J Am Soc Nephrol *12*, 1342-1349.

Hoenderop, J. G., Nilius, B., and Bindels, R. J. (2005). Calcium absorption across epithelia. Physiol Rev *85*, 373-422.

Hoenderop, J. G., van der Kemp, A. W., Hartog, A., van de Graaf, S. F., van Os, C. H., Willems, P. H., and Bindels, R. J. (1999). Molecular identification of the apical Ca2+ channel in 1, 25-dihydroxyvitamin D3-responsive epithelia. J Biol Chem *274*, 8375-8378.

Hoenderop, J. G., van Leeuwen, J. P., van der Eerden, B. C., Kersten, F. F., van der Kemp, A. W., Merillat, A. M., Waarsing, J. H., Rossier, B. C., Vallon, V., Hummler, E., and Bindels, R. J. (2003). Renal Ca2+ wasting, hyperabsorption, and reduced bone thickness in mice lacking TRPV5. J Clin Invest *112*, 1906-1914.

Holloway, L., Moynihan, S., Abrams, S. A., Kent, K., Hsu, A. R., and Friedlander, A. L. (2007). Effects of oligofructose-enriched inulin on intestinal absorption of calcium and magnesium and bone turnover markers in postmenopausal women. Br J Nutr *97*, 365-372.

Hong, E. J., Choi, K. C., Jung, Y. W., Leung, P. C., and Jeung, E. B. (2004). Transfer of maternally injected endocrine disruptors through breast milk during lactation induces neonatal Calbindin-D9k in the rat model. Reprod Toxicol *18*, 661-668.

Huang MS, H., W., Wu, Q.N., et al. (2004). Study on oleanolic acid-induced apoptosis and related to intracellular Ca~(2+) level in human breast carcinoma cells. China Journal of Modern Medicine *14*, 58-63.

ID, W. (2003). Handbook of analytical separations. AstraZeneca Macclesfield, UK.

Jaaskelainen, T., Huhtakangas, J., and Maenpaa, P. H. (2005). Negative regulation of human parathyroid hormone gene promoter by vitamin D3 through nuclear factor Y. Biochem Biophys Res Commun *328*, 831-837.

Kalsuhiko O., H. N., Meng Z., et al. (1989). Differential inhibitory effects of various herb extracts on the activities of reverse transcriptase and various Dexxyribonucleic Acid polymerase. Chem Pharm Bull *37*, 1810-1812.

Kalu, D. N., and Orhii, P. B. (1999). Calcium absorption and bone loss in ovariectomized rats fed varying levels of dietary calcium. Calcif Tissue Int *65*, 73-77.

Keen, R. (2007). Osteoporosis: strategies for prevention and management. Best Pract Res Clin Rheumatol *21*, 109-122.

Khanal, R. C., and Nemere, I. (2008). Regulation of intestinal calcium transport. Annu Rev Nutr *28*, 179-196.

Kip, S. N., and Strehler, E. E. (2003). Characterization of PMCA isoforms and their contribution to transcellular Ca2+ flux in MDCK cells. Am J Physiol Renal Physiol *284*, F122-132.

Kip, S. N., and Strehler, E. E. (2004). Vitamin D3 upregulates plasma membrane Ca2+-ATPase expression and potentiates apico-basal Ca2+ flux in MDCK cells. Am J Physiol Renal Physiol *286*, F363-369.

Koszewski, N. J., Malluche, H. H., and Russell, J. (2000). Vitamin D receptor interactions with positive and negative DNA response elements: an interference footprint comparison. J Steroid Biochem Mol Biol *72*, 125-132.

Kremer, R., and Goltzman, D. (1982). Parathyroid hormone stimulates mammalian renal 25-hydroxyvitamin D3-1 alpha-hydroxylase in vitro. Endocrinology *110*, 294-296.

Krishnan, A. V., Cramer, S. D., Bringhurst, F. R., and Feldman, D. (1995). Regulation of 1,25-dihydroxyvitamin D3 receptors by parathyroid hormone in osteoblastic cells: role of second messenger pathways. Endocrinology *136*, 705-712.

Lai, W. P., Chau, T. S., Cheung, P. Y., Chen, W. F., Lo, S. C., Favus, M. J., and Wong, M. S. (2003). Adaptive responses of 25-hydroxyvitamin D3 1-alpha

hydroxylase expression to dietary phosphate restriction in young and adult rats. Biochim Biophys Acta *1639*, 34-42.

Lambers, T. T., Bindels, R. J., and Hoenderop, J. G. (2006). Coordinated control of renal Ca2+ handling. Kidney Int *69*, 650-654.

Lane, N. E. (2006). Epidemiology, etiology, and diagnosis of osteoporosis. Am J Obstet Gynecol *194*, S3-11.

Laukoetter, M. G., Bruewer, M., and Nusrat, A. (2006). Regulation of the intestinal epithelial barrier by the apical junctional complex. Curr Opin Gastroenterol *22*, 85-89.

Lechner, D., Bajna, E., Adlercreutz, H., and Cross, H. S. (2006). Genistein and 17beta-estradiol, but not equol, regulate vitamin D synthesis in human colon and breast cancer cells. Anticancer Res *26*, 2597-2603.

Lechner, D., Kallay, E., and Cross, H. S. (2007). 1alpha,25-dihydroxyvitamin D3 downregulates CYP27B1 and induces CYP24A1 in colon cells. Mol Cell Endocrinol *263*, 55-64.

Leonard, F., Haag, M., and Kruger, M. C. (2001). Modulation of intestinal vitamin D receptor availability and calcium ATPase activity by essential fatty acids. Prostaglandins Leukot Essent Fatty Acids *64*, 147-150.

Levine, J. P. (2006). Pharmacologic and nonpharmacologic management of osteoporosis. Clin Cornerstone *8*, 40-53.

Li, A. J. (2002). Summary in studying assay methods of oleanolic acid in Fructus Ligustri Lucidi and preparations. Chinese Medicine Standardization *3*, 3-5.

Li, L., Ding, A.W., Meng, L. (2001). The study of Ligustrum Lucidum polysaccharrides on immunoregulation effect in mice. Chinese Medicinal Pharmacology and Clinic application *17*, 11-12.

Li, T., Hao, W.C., Xu, C.G., Ma, P.F. (2005a). HPLC-ELSD analysis of the content of oleanolic acid and ursolic acid from different regions. Chinese Medcinal Materials *28*, 391-393.

Li, Y. W., Xu, A.E., Wei, X.D., et al. (2005b). Effects of Glossy Privet Fruit on the Proliferation, Melanin Compound and C-kit Gene Expression in Normal Human Melanocytes in Vitro. Chinese Journal of Dermatovenerology of Integrated Iraditional and Western Mdeicine *4*, 150-152.

Liang, S. M. (1991). Tonic Methods. Zhong Yao Fang Ji Xue, Guangdong, Guangdong Science and Technology Press.

Lieberman, S. A., Holloway, L., Marcus, R., and Hoffman, A. R. (1994). Interactions of growth hormone and parathyroid hormone in renal phosphate, calcium, and calcitriol metabolism and bone remodeling in postmenopausal women. J Bone Miner Res *9*, 1723-1728.

Liel, Y., Shany, S., Smirnoff, P., and Schwartz, B. (1999). Estrogen increases 1,25-dihydroxyvitamin D receptors expression and bioresponse in the rat duodenal mucosa. Endocrinology *140*, 280-285.

Liu, H., Shi, Y., Wang, D., Yang, G., Yu, A., and Zhang, H. (2003). MECC determination of oleanolic acid and ursolic acid isomers in Ligustrum lucidum Ait. J Pharm Biomed Anal *32*, 479-485.

Liu, J., Liu, Y., Parkinson, A., and Klaassen, C. D. (1995). Effect of oleanolic acid on hepatic toxicant-activating and detoxifying systems in mice. J Pharmacol Exp Ther *275*, 768-774.

Liu, Z. B., Zhang, X.P., Hu, G. (2002). Dynamic study of oleanolic acid on glycogen content in alloxan-induced hyperglycemia rats. Journal of Medicine and Pharmacy of Chinese Minorites *8*, 29-30.

Liu., D. (2000). Chinese Medicine. Shanghai Scientific and Technical Publishers.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402-408.

Lukert, B., Higgins, J., and Stoskopf, M. (1992). Menopausal bone loss is partially regulated by dietary intake of vitamin D. Calcif Tissue Int *51*, 173-179.

Maiti, A., and Beckman, M. J. (2007). Extracellular calcium is a direct effecter of VDR levels in proximal tubule epithelial cells that counter-balances effects of PTH on renal Vitamin D metabolism. J Steroid Biochem Mol Biol *103*, 504-508.

Manolagas, S. C., Anderson, D. C., and Lumb, G. A. (1979). Glucocorticoids regulate the concentration of 1,25-dihydroxycholecalciferol receptors in bone. Nature *277*, 314-315.

Marcus, R., Villa, M. L., Cheema, M., Cheema, C., Newhall, K., and Holloway, L. (1992). Effects of conjugated estrogen on the calcitriol response to parathyroid hormone in postmenopausal women. J Clin Endocrinol Metab *74*, 413-418.

Matilainen, M., Malinen, M., Saavalainen, K., and Carlberg, C. (2005). Regulation of multiple insulin-like growth factor binding protein genes by 1alpha,25-dihydroxyvitamin D3. Nucleic Acids Res *33*, 5521-5532.

Meyer, J., Fullmer, C. S., Wasserman, R. H., Komm, B. S., and Haussler, M. R. (1992). Dietary restriction of calcium, phosphorus, and vitamin D elicits differential regulation of the mRNAs for avian intestinal calbindin-D28k and the 1,25-dihydroxyvitamin D3 receptor. J Bone Miner Res *7*, 441-448.

MHaAM, K. H. (1985). Preparative Chromatography Techniques. Application in Natural Product Isolation.

Monkawa, T., Yoshida, T., Wakino, S., Shinki, T., Anazawa, H., Deluca, H. F., Suda, T., Hayashi, M., and Saruta, T. (1997). Molecular cloning of cDNA and genomic DNA for human 25-hydroxyvitamin D3 1 alpha-hydroxylase. Biochem Biophys Res Commun *239*, 527-533.

Morris, H. A., Need, A. G., Horowitz, M., O'Loughlin, P. D., and Nordin, B. E. (1991). Calcium absorption in normal and osteoporotic postmenopausal women. Calcif Tissue Int *49*, 240-243.

Murayama, A., Kim, M. S., Yanagisawa, J., Takeyama, K., and Kato, S. (2004). Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator switching. Embo J *23*, 1598-1608.

Murayama, A., Takeyama, K., Kitanaka, S., Kodera, Y., Kawaguchi, Y., Hosoya, T., and Kato, S. (1999). Positive and negative regulations of the renal 25-hydroxyvitamin D3 1alpha-hydroxylase gene by parathyroid hormone, calcitonin, and 1alpha,25(OH)2D3 in intact animals. Endocrinology *140*, 2224-2231.

Neer, R. M., Arnaud, C. D., Zanchetta, J. R., Prince, R., Gaich, G. A., Reginster, J. Y., Hodsman, A. B., Eriksen, E. F., Ish-Shalom, S., Genant, H. K*., et al.* (2001). Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. N Engl J Med *344*, 1434-1441.

Nelson, D. R. (1999). Cytochrome P450 and the individuality of species. Arch Biochem Biophys *369*, 1-10.

Nian, H., Qin, L. P., Zhang, Q. Y., Zheng, H. C., Yu, Y., and Huang, B. K. (2006). Antiosteoporotic activity of Er-Xian Decoction, a traditional Chinese herbal formula, in ovariectomized rats. J Ethnopharmacol *108*, 96-102.

Nijenhuis, T., Renkema, K. Y., Hoenderop, J. G., and Bindels, R. J. (2006). Acid-base status determines the renal expression of Ca2+ and Mg2+ transport proteins. J Am Soc Nephrol *17*, 617-626.

Nordin, B. E., Need, A. G., Morris, H. A., O'Loughlin, P. D., and Horowitz, M. (2004). Effect of age on calcium absorption in postmenopausal women. Am J Clin Nutr *80*, 998-1002.

Norman, A. W. (1979). Vitamin D metabolism and calcium absorption. Am J Med *67*, 989-998.

O'Loughlin, P. D., and Morris, H. A. (1998). Oestrogen deficiency impairs intestinal calcium absorption in the rat. J Physiol *511 (Pt 1)*, 313-322.

O.Masaru, G. A. C., Norman R.Farnsworth (1977). Potential anticancer agents. J Nat Prod *40*, 157.

Ooms, M. E., Lips, P., Roos, J. C., van der Vijgh, W. J., Popp-Snijders, C., Bezemer, P. D., and Bouter, L. M. (1995). Vitamin D status and sex hormone binding globulin: determinants of bone turnover and bone mineral density in elderly women. J Bone Miner Res *10*, 1177-1184.

Orr, W. J., Holt, L. E., Wilkin, L., and F.H., B. (1923). The calcium and phosphorus metabolism in rickets, with special reference to ultraviolet rat therapy. Am J Dis Children *26*, 362-372.

Palmgren, M. G., and Axelsen, K. B. (1998). Evolution of P-type ATPases. Biochim Biophys Acta *1365*, 37-45.

Park, J. H., Omi, N., Nosaka, T., Kitajima, A., and Ezawa, I. (2008). Estrogen deficiency and low-calcium diet increased bone loss and urinary calcium excretion but did not alter arterial stiffness in young female rats. J Bone Miner Metab *26*, 218-225.

Peng, J. B., Chen, X. Z., Berger, U. V., Vassilev, P. M., Tsukaguchi, H., Brown, E. M., and Hediger, M. A. (1999). Molecular cloning and characterization of a channel-like transporter mediating intestinal calcium absorption. J Biol Chem *274*, 22739-22746.

Peng, J. B., Chen, X. Z., Berger, U. V., Weremowicz, S., Morton, C. C., Vassilev, P. M., Brown, E. M., and Hediger, M. A. (2000). Human calcium transport protein CaT1. Biochem Biophys Res Commun *278*, 326-332.

Perlmann, T., and Wallen-Mackenzie, A. (2004). Nurr1, an orphan nuclear receptor with essential functions in developing dopamine cells. Cell Tissue Res *318*, 45-52.

Perwad, F., Zhang, M. Y., Tenenhouse, H. S., and Portale, A. A. (2007). Fibroblast growth factor 23 impairs phosphorus and vitamin D metabolism in vivo and suppresses 25-hydroxyvitamin D-1alpha-hydroxylase expression in vitro. Am J Physiol Renal Physiol *293*, F1577-1583.

Pettifor, J. M., and Ross, F. P. (1983). Low dietary calcium intake and its role in the pathogenesis of rickets. S Afr Med J *63*, 179.

Pharmacopeia (2005). Pharmacopoeia of the PR China, p31.

Pinto Fde, T., and Adamo, H. P. (2002). Deletions in the acidic lipid-binding region of the plasma membrane Ca2+ pump. A mutant with high affinity for Ca2+ resembling the acidic lipid-activated enzyme. J Biol Chem *277*, 12784-12789.

PJHaA, R. (1998). Laboratory Handbook for the Fractionation of Natural Extracts. . Chapman and Hall.

Portale, A. A., Halloran, B. P., and Morris, R. C., Jr. (1989). Physiologic regulation of the serum concentration of 1,25-dihydroxyvitamin D by phosphorus in normal men. J Clin Invest *83*, 1494-1499.

Prince, R. L., Dick, I., Devine, A., Price, R. I., Gutteridge, D. H., Kerr, D., Criddle, A., Garcia-Webb, P., and St John, A. (1995). The effects of menopause and age on calcitropic hormones: a cross-sectional study of 655 healthy women aged 35 to 90. J Bone Miner Res *10*, 835-842.

Prince, R. L., Smith, M., Dick, I. M., Price, R. I., Webb, P. G., Henderson, N. K., and Harris, M. M. (1991). Prevention of postmenopausal osteoporosis. A comparative study of exercise, calcium supplementation, and hormone-replacement therapy. N Engl J Med *325*, 1189-1195.

Qin, H. L., and Gao, Y. (2007). Advances for constituents and pharmacological study of fructus ligusti lucidi. Taditional Chinese Drug Research and Clinical Pharmacology *18*, 84-85.

Qin LM, X. Y., Zou LZ, Hao RF, Niu FL, Jiang LD (2002). The influence of four single herbs on proliferation of osteoblast-like cells in vitro: Study on the components of Chinese Formula--Tong Bu Qiang Gu Fang. Chinese Journal of Experimental Traditional Medical Formulae *8*, 18-21.

Qiu, R. L. (2007). Study process on chemistry and pharmacology of Fructus Ligustri Lucidi. . Journal of Chinese Medicinal Materials *30*.

Raisz, L. G. (2005). Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest *115*, 3318-3325.

Rajasekaran, S. A., Beyenbach, K. W., and Rajasekaran, A. K. (2008). Interactions of tight junctions with membrane channels and transporters. Biochim Biophys Acta *1778*, 757-769.

Reinhardt, T. A., and Horst, R. L. (1990). Parathyroid hormone down-regulates 1,25-dihydroxyvitamin D receptors (VDR) and VDR messenger ribonucleic acid in vitro and blocks homologous up-regulation of VDR in vivo. Endocrinology *127*, 942-948.

Rianon, N., Feeback, D., Wood, R., Driscoll, T., Shackelford, L., and LeBlanc, A. (2003). Monitoring sweat calcium using skin patches. Calcif Tissue Int *72*, 694-697.

Riggs, B. L. (2003). Role of the vitamin D-endocrine system in the pathophysiology of postmenopausal osteoporosis. J Cell Biochem *88*, 209-215.

Rost, C. R., Bikle, D. D., and Kaplan, R. A. (1981). In vitro stimulation of 25-hydroxycholecalciferol 1 alpha-hydroxylation by parathyroid hormone in chick kidney slices: evidence for a role for adenosine 3',5'-monophosphate. Endocrinology *108*, 1002-1006.

Schneeberger, E. E., and Lynch, R. D. (2004). The tight junction: a multifunctional complex. Am J Physiol Cell Physiol *286*, C1213-1228.

Schwartz, B., Smirnoff, P., Shany, S., and Liel, Y. (2000). Estrogen controls expression and bioresponse of 1,25-dihydroxyvitamin D receptors in the rat colon. Mol Cell Biochem *203*, 87-93.

Schwartz, G. G., Whitlatch, L. W., Chen, T. C., Lokeshwar, B. L., and Holick, M. F. (1998). Human prostate cells synthesize 1,25-dihydroxyvitamin D3 from 25-hydroxyvitamin D3. Cancer Epidemiol Biomarkers Prev *7*, 391-395.

Shen, V., Birchman, R., Xu, R., Lindsay, R., and Dempster, D. W. (1995). Short-term changes in histomorphometric and biochemical turnover markers and bone mineral density in estrogen-and/or dietary calcium-deficient rats. Bone *16*, 149-156.

Shi, L. F., Cai, Q., Wu, G.T., Yang, S.T. (1998). Reverse HPLC analysis of water-soluble active ingrediets and oleanolica acid in Fructus Ligustri Lucidi from different regions. Journal of Chinese Medicinal Materials *23*, 77-79.

Shi, L. F., Wang, P., Chen, H.S., et al. (1995). Studies on water-soluble constituents from the fruits of Ligustrum Lucidum Ait. Acta Parmaceutics Sinica *30*, 935-938.

Shi, Q. B., Sheng, Z.H. (2007). Identification of Ligustrum Lucidum Ait. by X-ray diffraction fourier pattern method. Journal of Chinese Medicinal Materials *30*, 643-645.

Shimada, T., Hasegawa, H., Yamazaki, Y., Muto, T., Hino, R., Takeuchi, Y., Fujita, T., Nakahara, K., Fukumoto, S., and Yamashita, T. (2004). FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. J Bone Miner Res *19*, 429-435.

Shinki, T., Ueno, Y., DeLuca, H. F., and Suda, T. (1999). Calcitonin is a major regulator for the expression of renal 25-hydroxyvitamin D3-1alpha-hydroxylase gene in normocalcemic rats. Proc Natl Acad Sci U S A *96*, 8253-8258.

Shoemaker M., H. B., Dairkee SH., et al. (2005). In vitro anticancer activity of twelve Chinese medicinal herbs. Phytotherapy Research *19*, 649-651.

Simon, D. B., Lu, Y., Choate, K. A., Velazquez, H., Al-Sabban, E., Praga, M., Casari, G., Bettinelli, A., Colussi, G., Rodriguez-Soriano, J*., et al.* (1999). Paracellin-1, a renal tight junction protein required for paracellular Mg2+ resorption. Science *285*, 103-106.

Sinkkonen, L., Malinen, M., Saavalainen, K., Vaisanen, S., and Carlberg, C. (2005). Regulation of the human cyclin C gene via multiple vitamin D3-responsive regions in its promoter. Nucleic Acids Res *33*, 2440-2451.

Song, Y., Peng, X., Porta, A., Takanaga, H., Peng, J. B., Hediger, M. A., Fleet, J. C., and Christakos, S. (2003). Calcium transporter 1 and epithelial calcium channel messenger ribonucleic acid are differentially regulated by 1,25 dihydroxyvitamin D3 in the intestine and kidney of mice. Endocrinology *144*, 3885-3894.

St-Arnaud, R., Messerlian, S., Moir, J. M., Omdahl, J. L., and Glorieux, F. H. (1997). The 25-hydroxyvitamin D 1-alpha-hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. J Bone Miner Res *12*, 1552-1559.

Sterling, T. M., and Nemere, I. (2007). Calcium uptake and membrane trafficking in response to PTH or 25(OH)D3 in polarized intestinal epithelial cells. Steroids *72*, 151-157.

Sternfeld, L., Krause, E., Schmid, A., Anderie, I., Latas, A., Al-Shaldi, H., Kohl, A., Evers, K., Hofer, H. W., and Schulz, I. (2005). Tyrosine phosphatase PTP1B interacts with TRPV6 in vivo and plays a role in TRPV6-mediated calcium influx in HEK293 cells. Cell Signal *17*, 951-960.

Strehler, E. E., and Zacharias, D. A. (2001). Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. Physiol Rev *81*, 21-50.

Sun YW, Z., Y.J. (1993). Experimental study on therapy of Fructus Ligustri Lucidi on hyperlipemia. . Journal of Traditional Chinese Medicine *34*, 493-494.

Tang, H. C., Zhang, D.J., Zhao, L. (2007). Advances in the research of Oleanolic acid. Food science and technology *9*, 16-18.

Tilyard, M. W., Spears, G. F., Thomson, J., and Dovey, S. (1992). Treatment of postmenopausal osteoporosis with calcitriol or calcium. N Engl J Med *326*, 357-362.

Tissandie, E., Gueguen, Y., Lobaccaro, J. M., Grandcolas, L., Voisin, P., Aigueperse, J., Gourmelon, P., and Souidi, M. (2007). In vivo effects of chronic contamination with depleted uranium on vitamin D3 metabolism in rat. Biochim Biophys Acta *1770*, 266-272.

Towers, T. L., and Freedman, L. P. (1998). Granulocyte-macrophage colony-stimulating factor gene transcription is directly repressed by the vitamin D3 receptor. Implications for allosteric influences on nuclear receptor structure and function by a DNA element. J Biol Chem *273*, 10338-10348.

Tsukamoto, Y., Saka, S., and Saitoh, M. (1992). Parathyroid hormone stimulates ATP-dependent calcium pump activity by a different mode in proximal and distal tubules of the rat. Biochim Biophys Acta *1103*, 163-171.

Tsuruoka, S., Nishiki, K., Ioka, T., Ando, H., Saito, Y., Kurabayashi, M., Nagai, R., and Fujimura, A. (2006). Defect in parathyroid-hormone-induced luminal calcium absorption in connecting tubules of Klotho mice. Nephrol Dial Transplant *21*, 2762-2767.

Turunen, M. M., Dunlop, T. W., Carlberg, C., and Vaisanen, S. (2007). Selective use of multiple vitamin D response elements underlies the 1 alpha,25-dihydroxyvitamin D3-mediated negative regulation of the human CYP27B1 gene. Nucleic Acids Res *35*, 2734-2747.

Vaisanen, S., Dunlop, T. W., Sinkkonen, L., Frank, C., and Carlberg, C. (2005). Spatio-temporal activation of chromatin on the human CYP24 gene promoter in the presence of 1alpha,25-Dihydroxyvitamin D3. J Mol Biol *350*, 65-77.

van Abel, M., Hoenderop, J. G., and Bindels, R. J. (2005a). The epithelial calcium channels TRPV5 and TRPV6: regulation and implications for disease. Naunyn Schmiedebergs Arch Pharmacol *371*, 295-306.

Van Abel, M., Hoenderop, J. G., Dardenne, O., St Arnaud, R., Van Os, C. H., Van Leeuwen, H. J., and Bindels, R. J. (2002). 1,25-dihydroxyvitamin D(3)-independent stimulatory effect of estrogen on the expression of ECaC1 in the kidney. J Am Soc Nephrol *13*, 2102-2109.

van Abel, M., Hoenderop, J. G., van der Kemp, A. W., Friedlaender, M. M., van Leeuwen, J. P., and Bindels, R. J. (2005b). Coordinated control of renal Ca(2+) transport proteins by parathyroid hormone. Kidney Int *68*, 1708-1721.

van Abel, M., Hoenderop, J. G., van der Kemp, A. W., van Leeuwen, J. P., and Bindels, R. J. (2003). Regulation of the epithelial Ca2+ channels in small intestine as studied by quantitative mRNA detection. Am J Physiol Gastrointest Liver Physiol *285*, G78-85.

van Abel, M., Huybers, S., Hoenderop, J. G., van der Kemp, A. W., van Leeuwen, J. P., and Bindels, R. J. (2006). Age-dependent alterations in Ca2+ homeostasis: role of TRPV5 and TRPV6. Am J Physiol Renal Physiol *291*, F1177-1183.

Van Cromphaut, S. J., Dewerchin, M., Hoenderop, J. G., Stockmans, I., Van Herck, E., Kato, S., Bindels, R. J., Collen, D., Carmeliet, P., Bouillon, R., and Carmeliet, G. (2001). Duodenal calcium absorption in vitamin D receptor-knockout mice: functional and molecular aspects. Proc Natl Acad Sci U S A *98*, 13324-13329.

van den Bemd, G. J., Jhamai, M., Staal, A., van Wijnen, A. J., Lian, J. B., Stein, G. S., Pols, H. A., and van Leeuwen, J. P. (2002). A central dinucleotide within vitamin D response elements modulates DNA binding and transactivation by the

vitamin D receptor in cellular response to natural and synthetic ligands. J Biol Chem *277*, 14539-14546.

Vandenbroucke, E., Mehta, D., Minshall, R., and Malik, A. B. (2008). Regulation of endothelial junctional permeability. Ann N Y Acad Sci *1123*, 134-145.

Venyaminov, S. Y., Klimtchuk, E. S., Bajzer, Z., and Craig, T. A. (2004). Changes in structure and stability of calbindin-D(28K) upon calcium binding. Anal Biochem *334*, 97-105.

Vinay, P., Gougoux, A., and Lemieux, G. (1981). Isolation of a pure suspension of rat proximal tubules. Am J Physiol *241*, F403-411.

Walters, J. R., Balesaria, S., Chavele, K. M., Taylor, V., Berry, J. L., Khair, U., Barley, N. F., van Heel, D. A., Field, J., Hayat, J. O*., et al.* (2006). Calcium channel TRPV6 expression in human duodenum: different relationships to the vitamin D system and aging in men and women. J Bone Miner Res *21*, 1770-1777.

Wang, M. G., Yi, H., Hilfiker, H., Carafoli, E., Strehler, E. E., and McBride, O. W. (1994). Localization of two genes encoding plasma membrane Ca2+ ATPases isoforms 2 (ATP2B2) and 3 (ATP2B3) to human chromosomes 3p26-->p25 and Xq28, respectively. Cytogenet Cell Genet *67*, 41-45.

Wang, P., Tian, L., Shan, Y., Wang, J., and Wang, Z. X. (2008). Review on extraction techniques and determination methods of oleanolic acid in Fructus Ligustri Lucidi. Chemical Industry Times *22*, 51-54.

Wang, T., Zeng, Y., and Xu, M. (2003). Studies on quality control of Gushuling capsule. Trad Chin Drug Res & Clin Pharm *14*, 403-405.

Wang T, Z. J., Yang MS, Xiao PG (2006). Progress in anti-osteoporosis activity of traditional Chinese medicine and medicinal plants. CHINA JOURNAL OF CHINESE MATERIA MEDICA *31*, 718-722.

Wang, T. T., Tavera-Mendoza, L. E., Laperriere, D., Libby, E., MacLeod, N. B., Nagai, Y., Bourdeau, V., Konstorum, A., Lallemant, B., Zhang, R*., et al.* (2005). Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. Mol Endocrinol *19*, 2685-2695.

Wang, X. D., Liu, Y.G., Su, W.W. (2004). Protection of salidroside on experimental hepatic injury in mice. Journal of Chinese Medicinal Materials *27*, 198-199.

Ward, D. T., Maldonado-Perez, D., Hollins, L., and Riccardi, D. (2005). Aminoglycosides induce acute cell signaling and chronic cell death in renal cells that express the calcium-sensing receptor. J Am Soc Nephrol *16*, 1236-1244.

Wick, J. Y. (2009). Spontaneous fracture: multiple causes. Consult Pharm *24*, 100-102, 105-108, 110-102.

Wilcox, E. R., Burton, Q. L., Naz, S., Riazuddin, S., Smith, T. N., Ploplis, B., Belyantseva, I., Ben-Yosef, T., Liburd, N. A., Morell, R. J.*, et al.* (2001). Mutations in the gene encoding tight junction claudin-14 cause autosomal recessive deafness DFNB29. Cell *104*, 165-172.

Wong, M. S., Sriussadaporn, S., Tembe, V. A., and Favus, M. J. (1997). Insulin-like growth factor I increases renal 1,25(OH)2D3 biosynthesis during low-P diet in adult rats. Am J Physiol *272*, F698-703.

Wu, L. J., Xiang, T., Hou, B.L., et al. (1998). CHEMICAL CONSTITUENTS FROM FRUITS OF LIGUSTRUM LUCIDUM. Acta Botanica Sinica *40*, 83-87.

Wu, S., Ren, S., Nguyen, L., Adams, J. S., and Hewison, M. (2007). Splice variants of the CYP27b1 gene and the regulation of 1,25-dihydroxyvitamin D3 production. Endocrinology *148*, 3410-3418.

Xu, H., Lawson, D., Kras, A., and Ryan, D. (2005). The use of preventive strategies for bone loss. Am J Chin Med *33*, 299-306.

Yin, S., Tang, X.Z., Wu, L.J., Wang, S.X., et al (1995). Study of Chemical components of Fructus Ligustri Lucidi. Journal of Shenyang Pharmaceutical University *12*, 125-126.

You, H. L., Yan, Y. P., Gao, Y. Z., and Li, C. H. (2007). Influences of different preparation methods on the content of oleanolic acid in Fructus Ligustri Lucidi. Journal of Chinese Medicine *9*, 24-25.

Zhang, M. Y., Wang, X., Wang, J. T., Compagnone, N. A., Mellon, S. H., Olson, J. L., Tenenhouse, H. S., Miller, W. L., and Portale, A. A. (2002). Dietary phosphorus transcriptionally regulates 25-hydroxyvitamin D-1alpha-hydroxylase gene expression in the proximal renal tubule. Endocrinology *143*, 587-595.

Zhang, X. H., and Shi, L. F. (2004). Studies on chemical constituents of Fructus Ligustri lucidi. Academic Journal of Second Military Medical University *25*, 333-334.

Zhang, Y., Dong, X. L., Leung, P. C., Che, C. T., and Wong, M. S. (2008a). Fructus ligustri lucidi extract improves calcium balance and modulates the

calciotropic hormone level and vitamin D-dependent gene expression in aged ovariectomized rats. Menopause *6*, 6.

Zhang, Y., Lai, W. P., Leung, P. C., Che, C. T., and Wong, M. S. (2008b). Improvement of Ca balance by Fructus Ligustri Lucidi extract in aged female rats. Osteoporos Int *19*, 235-242.

Zhang, Y., Lai, W. P., Leung, P. C., Wu, C. F., Yao, X. S., and Wong, M. S. (2006). Effects of Fructus Ligustri Lucidi extract on bone turnover and calcium balance in ovariectomized rats. Biol Pharm Bull *29*, 291-296.

Zhang, Y., Lai, W. P., Wu, C. F., Favus, M. J., Leung, P. C., and Wong, M. S. (2007). Ovariectomy worsens secondary hyperparathyroidism in mature rats during low-Ca diet. Am J Physiol Endocrinol Metab *292*, E723-731.

Zhang, Y., Leung, P. C., Che, C. T., Chow, H. K., Wu, C. F., and Wong, M. S. (2008c). Improvement of bone properties and enhancement of mineralization by ethanol extract of Fructus Ligustri Lucidi. Br J Nutr *99*, 494-502.

Zhang, Z. M., Ge, B., Xu, A.X, et al. (2006). Antisenile effect of polysaccharides from Fructus ligustri lucidi. Chinese Journal of Pharmacology and Toxicology *20*, 108-111.

Zierold, C., Darwish, H. M., and DeLuca, H. F. (1995). Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. J Biol Chem *270*, 1675-1678.

Zierold, C., Nehring, J. A., and DeLuca, H. F. (2007). Nuclear receptor 4A2 and C/EBPbeta regulate the parathyroid hormone-mediated transcriptional regulation of the 25-hydroxyvitamin D3-1alpha-hydroxylase. Arch Biochem Biophys *460*, 233-239.