ABSTRACT

Title of Document: BIOLOGICAL EFFICACY, MECHANISMS OF ACTIONS OF SOY-DERIVED PHYTOALEXIN GLYCEOLLINS IN PREVENTION OF CHRONIC DISEASES

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Cardiovascular disease (CVD) is the leading cause of deaths worldwide. Prostate cancer is the most prevalent cancer in U.S. male population. Diet-induced hypercholesterolemia and chronic inflammation promote the development of both CVD and prostate cancer. Glyceollins are a group of soy phytoalexins possessing a variety of biological activities. This research project focused on characterizing glyceollins' bioactivities in alleviating cholesterol dysregulation, prevention of prostate cancer, and regulating gut microbiome.

The first part of the project aimed to evaluate glyceollins' cholesterol-lowering effect in-vivo. Male golden Syrian hamsters were fed high-fat diet with or without glyceollins supplementation for 28 days. Glyceollins supplementation led to a significant reduction of plasma VLDL, hepatic cholesterol esters and total lipid content. Consistent with changes in circulating cholesterol, glyceollins

supplementation also altered expression of the genes related to cholesterol metabolism in the liver.

The second part of the study aimed to evaluate glyceollins' effect in reducing prostate cancer tumor growth in a xenograft model. An initial delayed appearance of tumor was observed in a PC-3 xenograft model. However, no difference in tumor sizes was observed in a LNCaP xenograft model. Extrapolation analysis of tumor measurements indicated that no difference in sizes was expected for both PC-3 and LNCaP tumors. Glyceollins had no effect on the androgen responsive pathway, its proliferation, cell cycle, or on angiogenesis genes in tumor and xenobiotic metabolism, cholesterol transport, and inflammatory cytokine genes in liver. Glyceollins' low bioavailability might have led to the ineffectiveness in reducing tumor growth in-vivo.

The microbiome has emerged as an important and integral part of the human physiology with a significant role in human health and disease. The third part of the study aimed to evaluate the effect of glyceollins on the gut microbiome in mice. Fecal and cecal samples collected from mouse feeding studies were analyzed for microbial population and composition. Glyceollins supplementation did not alter gut bacteria groups in cecal sample examined in this study. Glyceollins significantly affected total Enterobacteriaceae and Ruminococcus population in fecal samples collected at 24 h, indicating the impact and importance of time of collection in interpreting gut microbiome data in fecal analysis.

BIOLOGICAL EFFICACY, MECHANISMS OF ACTIONS OF SOY-DERIVED PHYTOALEXIN GLYCEOLLINS IN PREVENTION OF CHRONIC DISEASES - CHOLESTEROL LOWERING AND PROSTATE CANCER PREVENTATIVE EFFECTS

By

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ABCA	ATP-binding cassette sub-family A member
ABCG	ATP-binding cassette sub-family G member
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACACA	Acetyl-CoA carboxylase alpha
ACAT	Acetyl-Coenzyme A acetyltransferase
ACOX	Acyl-CoA oxidase
AKT	Protein kinase B
AR	Androgen receptor
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CETP	Cholesteryl ester transfer protein
COX-2	Cyclooxygenase 2
CVD	Cardiovascular disease
CYP51	Lanosterol 14 α-demethylase
CYP7A1	Cholesterol 7 alpha-hydroxylase
DPPH	2,2-diphenyl-1-picrylhydrazyl
ER	Estrogen receptor
ERG	ETS-related gene
ETS	E26 transformation-specific
ETV1	ETS variant 1
FAS	Fatty acid synthase
FPP synthase	Farnesyl pyrophosphate synthase
FXR	Farnesoid X receptor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Glutamate-cysteine ligase
GLP-1	Glucagon-like peptide-1
GLUT4	Glucose transporter type 4
GR	Glucocorticoid receptor
GSTP1	Glytathione S-transferase P 1
HDL	High-density lipoprotein
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-CoA reductase
HO1	Heme oxygenase
IDL	Intermediate-density lipoprotein
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Insig	Insulin-induced gene
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPL	Lipoprote lipase

LPS	Lipopolysaccharide
LXRα	Liver X receptor alpha
MAPK	Mitogen-activated protein kinase
MCP-1	Chemokine (C-C motif)ligand 2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PCNA	Proliferating cell nuclear antigen
PECAM	Platelet endothelial cell adhesion molecule
PI3K	Phosphoinositide 3-kinase
PPARα	Peroxisome proliferator-activated receptor alpha
PSA	Prostate-specific antigen
RXR	Retinoid X receptor
SCAP	Sterol regulatory element-binding protein cleavage-activating protein
SCID	Severe combined immunodeficiency
SQS	Squalene synthase
SREBP	Sterol Regulatory Element-Binding Protein
TGF-β	Tumor growth factor-β
TMPRSS2	Transmembrane protease, serine 2
TNF	Tumor necrosis factor
TRAMP	Transgenic adenocarcinoma of the mouse prostate
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein

Introduction

Cardiovascular disease (CVD) has been the leading cause of death worldwide since the 1970s. Prostate cancer is the most prevalent cancer in U.S. male population and accounts for 15% of all malignant cancer incidences. Diet-induced hypercholesterolemia and chronic inflammation promote the development of both CVD and prostate cancer. Despite the prevalence and severity of these chronic conditions, effective cures remain largely unavailable. Hence, prevention presents an important part of overall management strategy for these chronic diseases. Given the role of diet in the development of CVD and prostate cancer, modulation of diet is key in the prevention strategy. Soy is one of the major agricultural commodities in the U. S. and worldwide. Soy and soy-derived foods are rich sources of bioactive phytochemicals and well known for their health promoting effects, include protection against cardiovascular diseases and cancers such as prostate cancer. However, the precise bioactive component(s) as well as the mechanism(s) of action remain largely unresolved. Glyceollins are soy phytoalexins possessing a variety of biological activities, including antibacterial, antifungal, antiproliferative, antiestrogenic, antidiabetic, antioxidant and anti-inflammatory effects.

Hence, the overall goal of this research was to elucidate the bioactive components of soy and the underlay mechanisms of action, and the focus was on the examination of the bioactivities of the novel soy-derived compounds glyceollins. The current research projects characterized glyceollins' bioactivities in alleviating cholesterol dysregulation resulted from consumption of a western-style diet, and glyceollins' effect on prostate cancer prevention. The specific objectives were:

1) To examine the hypothesis that glyceollins can alleviate metabolic and inflammatory disorders resulting from high fat/cholesterol diet.

2) To determine the hypothesis, that glyceollins possess preventative effect against prostate cancer.

3) To investigate the influence of diet and glyceollins supplementation on microbiome in the gastrointestinal tract.

Chapter 1: Literature Review

1.1. Diet and chronic diseases

A chronic disease is a human health condition or disease that is persistent or long-lasting in effects. The World Health Organization (WHO) reported chronic conditions to be by far the leading cause of mortality in the world, representing 36 million deaths in 2008 and over 63% of all deaths (WHO, 2013). Chronic illnesses cause about 60% of deaths in the U. S. in 2005, and CVD, cancer, chronic respiratory diseases, and diabetes were the four top causes of mortality in the general US population (CDC, 2009), among which CVD accounted for 30% of all deaths and cancer cost $263.8 billion in the U. S. in 2010 (AACR, 2012).

Over the past three decades, the role of diet in the prevention and control of morbidity and premature mortality due to chronic diseases, like CVD and cancer, has well been established by the vast population-based epidemiological studies (Kris-Etherton, Eckel, Howard, St Jeor, & Bazzarre, 2001; Lichtenstein, Appel, Brands, Carnethon, Daniels, Franch, et al., 2006). Diet and genetic predisposition are identified to be the most important environmental factors in the development of chronic diseases (Simopoulos, 1990). Humans, as a species, have not changed genetically over the past tens or hundreds millenniums, however, major changes have taken place in food supply and diet compositions (Friend, 1990), thus diet is considered the dominant part in the soaring incidence of CVD and cancer (Freeman & Solomon, 2004; Spence, Jenkins, & Davignon, 2010). The link between dietary intake of cholesterol and elevated circulating

cholesterol level has been well established (Spence, Jenkins, & Davignon, 2010), and hypercholesterolemia is strongly correlated with CVD (Kinosian, Glick, & Garland, 1994). Doll and Peto performed an extensive review in 1981, in which they determined that diet is the largest risk factor of cancer and attributable to approximately 35% of cancer deaths in the U. S. with percentage potentially ranging as low as 10% to as high as 70% (Doll & Peto, 1981). Willett further studied several types of cancers and estimated that 75% (ranging from 20% to 80%) of prostate cancer deaths are avoidable by modification of diet (Willett, 1995).

1.1.1. Hypercholesterolemia

Hypercholesterolemia is a condition that high levels of cholesterol present in the blood caused by the imbalance between cholesterol uptake and excretion (van der Wulp, Verkade, & Groen, 2013). Hypercholesterolemia is strongly associated with CVD, such as atherosclerosis, and can further lead to myocardial infarction, stroke, and peripheral vascular disease. The pathways involved in the development of hypercholesterolemia are regulated via a complex interplay of enzymes, transport proteins, transcription factors and non-coding RNAs (van der Wulp, Verkade, & Groen, 2013).

Current understanding of cholesterol metabolism, absorption, transport, catabolism and excretion, and potential targets in prevention of hypercholesterolemia in the respective stage will also be reviewed.

1.1.1.1. Cholesterol metabolism

Cholesterol is a type of sterol (Fig. 1) and building block for all mammalian cell membranes (Maxfield & Tabas, 2005). It is essential for proper membrane permeability and fluidity. Also, cholesterol serves as a precursor for the biosynthesis of steroid hormones, bile acids, and vitamin D (Rezen, Rozman, Pascussi, & Monostory, 2011).

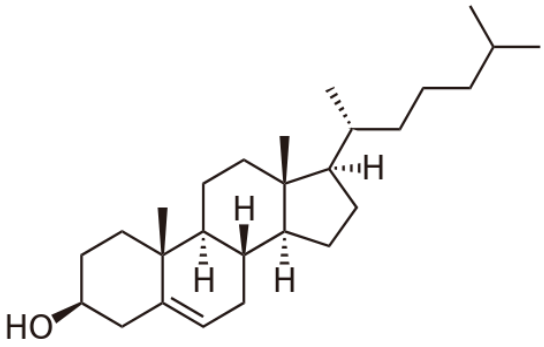

Fig. 1.1. Structure of cholesterol.

Physiologically, cholesterol is mainly de novo synthesized in the liver (Fig18), via a complex 37-step process, which starts with the rate-limiting enzyme 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (Molina, Vazquez, & Gutierrez, 1991). A typical individual has a daily cholesterol synthesis of ~1 g, and a total body content of ~35 g, primarily located within the membranes of cells. Cholesterol can also be derived from the diet. On average, daily dietary intake of cholesterol in the U. S. is 300 - 450 mg (Lecerf & de Lorgeril, 2011).

Normally, the body has the ability to compensate for additional cholesterol consumption by reducing cholesterol synthesis. Therefore, under physiological condition, cholesterol intake in food has little, if any, effect on total body cholesterol content or concentrations of cholesterol in the circulation. This is achieved by excreting the cholesterol via the form of bile into the digestive tract, and about 95% of the excreted

cholesterol is re-absorbed back into circulation. The remainder is lost in the feces. The excretion and re-absorption of bile acids are known as the enterohepatic circulation, which is essential for the digestion and absorption of dietary fats.

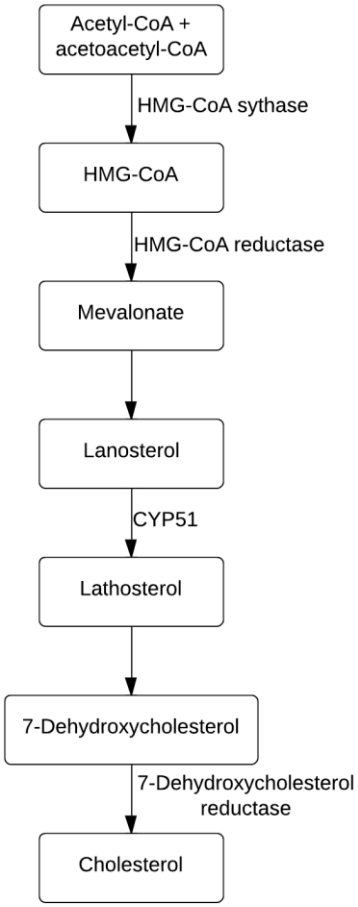

Fig. 1.2. Critical enzymes involved in synthesis of cholesterol.

De novo synthesis of cholesterol is regulated by the cholesterol level that is present in circulation (Gylling, Strandberg, Tilvis, & Miettinen, 1994; Molina, Vazquez, & Gutierrez, 1991). Under normal physiological conditions, higher intake of dietary cholesterol will lead to a decrease in endogenous production, whereas lower intake will induce the increase of de novo synthesis (Lecerf & de Lorgeril, 2011). Upon the accumulation of cholesterol, inhibitory signals of the SREBP family of transcription

factors will be triggered and prevent further synthesis of cholesterol (Brown & Goldstein, 1997). Downstream, a feed-forward pathway will be activated to increase the conversion of cholesterol into bile acids. Additionally, excess cholesterol will be actively removed from peripheral and transported back to the liver for excretion via the reverse cholesterol transport pathway (Lewis & Rader, 2005).

1.1.1.2. Potential targets in cholesterol synthesis in prevention of hypercholesterolemia

Inhibition of HMG-CoA reductase has been proven effective in inhibiting cholesterol synthesis and is widely used in therapeutic treatments (Pedersen, Kjekshus, Berg, Haghfelt, Faergeman, Thorgeirsson, et al., 2004). The reduction of hepatic cholesterol synthesis in liver is compensated for by increase in cholesterol uptake from circulation resulting in lower plasma cholesterol levels (Ma, Gil, Südhof, Bilheimer, Goldstein, & Brown, 1986). Other targets involve the non-rate-limiting steps in cholesterol synthesis (such as CYP51 and squalene synthase) have also been studied, but their effectiveness in reducing cholesterol synthesis has not been proven (Korošec, Ačimovič, Seliškar, Kocjan, Tacer, Rozman, et al., 2008; Wasko, Smits, Shull, Wiemer, & Hohl, 2011).

1.1.1.3. Biomarkers in cholesterol synthesis

Sterol Regulatory Element-Binding Protein (SREBP) 1 and 2 are in charge of sensing of intracellular cholesterol in the endoplasmic reticulum, which is one of the primary mechanisms of cholesterol regulation (Lobaccaro, Repa, Lu, Caira, Henry-Berger, Volle, et al., 2001). There are three isoforms of SREBPs: SREBP-1a, SREBP-1c

and SREBP-2. SREBP-1a is responsible for stimulation of regulatory genes involved in both cholesterol and fatty acid synthesis, SREBP-1c stimulates lipogenesis, and SREBP-2 is responsible for regulation of cholesterol-synthesizing enzymes and LDL receptor in case of cholesterol depletion (Chang, Chang, Ohgami, & Yamauchi, 2006). Non-activated SREBPs are located at the nuclear envelope and endoplasmic reticulum membranes. In the presence of cholesterol, SREBP is bound to sterol regulatory element-binding protein cleavage-activating protein (SCAP) and insulin-induced gene (Insig)-1 to form a complex (Fig. 20). When cholesterol levels drop, Insig-1 dissociates from the SREBP-SCAP complex, and the remaining complex migrates to the Golgi apparatus, where SCAP will activate S1P and S2P, which will then enzymatically cleave SREBP. After that, the cleaved SREBP migrates to the nucleus and acts as a transcription factor to bind to the SRE, and initiates a number of downstream events, among which are the LDL receptor and HMG-CoA reductase. LDL receptor binds to circulating LDL and take the LDL into the cell. When cholesterol levels are high, synthesis can be turned off, and both LDL receptor and HMG-CoA reductase expression are reduced. HMG-CoA reductase contains both a cytosolic domain, which is responsible for its catalytic function, and a membrane domain, which is more susceptible to destruction by the proteasome as cholesterol concentration increases (Guo, Li, Wu, Xie, Zhang, & Cui, 2008; Lagace, Storey, & Ridgway, 2000; Rawson, 2003; Weber, Boll, & Stampfl, 2004).

1.1.1.4. Cholesterol absorption and transport

Transport of cholesterol is an intricate process. Cholesterol is only slightly soluble in water, and free cholesterol concentration is very low in water-based bloodstream.

Cholesterol in the form of free or cholesterol esters and triglycerides are mainly transported in the circulation within lipoproteins. Amphipathic molecules, such as phospholipids, are wrapped on the surface of the lipoprotein particle. Additionally, proteins (e. g. apolipoprotein E, apolipoprotein B100) serve as cell-targeting signaling molecules are also exposed on the surface. Several types of lipoproteins namely, chylomicrons, VLDL, IDL, LDL, and HDL, are classified according to the increasing density. The cholesterol within all these various lipoproteins is identical, with part of cholesterol carried in free form, and some carried as fatty acyl esters. Lipoproteins differ themselves by possessing distinct apolipoproteins so that each lipoprotein can be recognized by specific receptors on cell membranes. In other words, the apolipoproteins on the surface are molecular identifications that determine the start and end points for cholesterol transport. Chylomicrons carry fats from the intestine to muscle and other tissues that need fatty acids for energy or fat production. They are the least dense type of cholesterol transport molecules and contain apolipoprotein B-48, apolipoprotein C, and apolipoprotein E in the shells. Cholesterol that is not used by peripheral tissues remains in chylomicron remnants, which are more cholesterol-rich, will be taken up by the liver from circulation. Liver produces VLDL, which contain excess triacylglycerol and cholesterol. VLDLs contain apolipoprotein B100 and apolipoprotein E in the shells. After released into the bloodstream, triacylglycerol in VLDL are cleaved and absorbed, and the remaining form IDL molecules, which contain an even higher percentage of cholesterol. The IDL molecules can either be metabolized and directly picked up through the LDL receptor on the liver cell surfaces, or continue to lose triacylglycerol in the bloodstream until they become LDL molecules, which contain the highest percentage of cholesterol

within them. LDL molecules are the major carriers of cholesterol in the circulation, and each one contains approximately 1,500 molecules of cholesterol ester. The surface of the LDL molecule contains only one molecule of apolipoprotein B100, which can be recognized by the LDL receptor in peripheral tissues. Upon binding of apolipoprotein B100, LDL will be taken into the cells, and the cholesterol can be used for membrane biosynthesis or esterified and stored within the cell. Synthesis of the LDL receptor is regulated by SREBP, the same regulatory protein controlling de novo synthesis of cholesterol. When a cell has abundant cholesterol, LDL receptor synthesis is blocked so that new cholesterol in the form of LDL molecules cannot be taken up. On the other hand, more LDL receptors will be made if the cell is deficient in cholesterol. HDL particles are responsible for transporting cholesterol back to the liver for excretion or to other tissues that use cholesterol to synthesize hormones in a process known as reverse cholesterol transport. Due to the high prevalence of high circulating cholesterol in the western population, more HDL is usually thought to be “good” for health.

1.1.1.5. Potential targets in cholesterol absorption and transport in prevention of hypercholesterolemia

Despite the considerable amount of research and effort in inhibiting cholesterol absorption and transport, current treatments are usually nonspecific and require relatively high doses to achieve modest effect in lowering circulating LDL. However, recent findings in plant sterol/stanols, which are rich in cooking oils, wheat cereals, nuts and seeds and structurally similar to cholesterol, may present an alternative approach in controlling cholesterol absorption. Plant sterol can be transported via ABCG5/8 with

comparable efficiency as cholesterol (Wang, Sun, Zhang, Ma, Xu, Belani, et al., 2006), and effectively lower circulating LDL levels in human (Guardamagna, Abello, Baracco, Federici, Bertucci, Mozzi, et al., 2011). Thus, ABCG5/8 may be a better target than LDL in reducing cholesterol absorption and transport.

1.1.1.6. Biomarkers in cholesterol absorption and metabolism

LXR is a member of the nuclear receptor family of transcription factors and is closely related to several other nuclear receptors, such as the PPARs, FXR and RXR. LXR can dimerize with RXR and is an important transcriptional regulator of cholesterol, fatty acid, and glucose homeostasis. LXR can be activated by excessive cholesterol, either dietary or endogenous, and 1) stimulate cholesterol removal from the cell, transportation to the liver and biliary excretion; 2) enhance reverse cholesterol transport; 3) inhibit intestinal cholesterol absorption; and 4) inhibit cholesterol synthesis and uptake by the cells (Beltowski, 2008). Activation of the LXR/RXR heterodimer can lead to an increase of intestinal expression of the energy-dependent transporter protein ABCA1, which is critical in cellular cholesterol efflux and dietary cholesterol absorption. It completely blocks intestinal absorption of cholesterol (McNeish, Aiello, Guyot, Turi, Gabel, Aldinger, et al., 2000; Repa, Turley, Lobaccaro, Medina, Li, Lustig, et al., 2000). In addition to ABCA1, LXR directly regulates another two members of the ABC superfamily, ABCG5 and ABCG8 (Lee & Carr, 2005), which are involved in actively efflux cholesterol and plant sterols back into the intestinal lumen. In addition, ABCG5 and ABCG8 located at the surface of hepatocytes can facilitate efflux of cholesterol and plant sterols into bile (Jakulj, Vissers, Tanck, Hutten, Stellaard, Kastelein, et al., 2010).

Two forms of LXR receptors (α and β) exist. LXRα plays critical a role in the control of cholesterol synthesis (Peet, Turley, Ma, Janowski, Lobaccaro, Hammer, et al., 1998). Knock-down of LXRα can significantly increase the expression of the cholesterol synthesis transcriptional regulator SREBP-2, as well as HMG-CoA reductase and synthase, FPP synthase, and SQS (Alberti, Schuster, Parini, Feltkamp, Diczfalusy, Rudling, et al., 2001; Peet, et al., 1998), suggesting a role of LXRα in down-regulation or suppression of cholesterol synthesis pathways (Millatt, Bocher, Fruchart, & Staels, 2003).

PPARs are a group of nuclear receptor proteins that function as transcription factors, which play essential roles in the regulation of cellular differentiation, development, metabolism (carbohydrate, lipid, protein), and tumorigenesis (van Raalte, Li, Pritchard, & Wasan, 2004).

PPARα plays a major role as a regulator of fatty acid catabolism and targets genes such as FAS, ACACA, and SREBP-1 (Ferre & Foufelle, 2010; Goldstein & Brown, 1997). PPARα is also very important in lipoprotein metabolism, especially in the metabolism of triglyceride-rich lipoproteins (Staels, Dallongeville, Auwerx, Schoonjans, Leitersdorf, & Fruchart, 1998). Activation of PPARα in the liver will shift free fatty acid metabolism from triglyceride synthesis to catabolism, increase the activity of LPL (Heller & Harvengt, 1983), and thus reduce the secretion of VLDL particles from liver (Schoonjans, Staels, & Auwerx, 1996). LPL is the key enzyme in the hydrolysis of triglycerides and also mediates the uptake of atherogenic triglyceride-rich lipoproteins remnants by the liver (Schoonjans, PeinadoOnsurbe, Lefebvre, Heyman, Briggs, Deeb, et al., 1996; Staels, Vudac, Kosykh, Saladin, Fruchart, Dallongeville, et al., 1995). On the other hand, impaired PPAR function leads to increased hepatic triglycerides content,

elevated VLDL production, impaired clearance of triglyceride-rich lipoproteins in the periphery, and increased plasma triglyceride levels (Duval, Muller, & Kersten, 2007).

PPARα activation also up-regulates the synthesis of apo A-I and A-II, two major HDL apolipoproteins in the liver, and promote HDL maturation through the increased hydrolysis of triglyceride-rich lipoproteins. (Bisgaier, Essenburg, Barnett, Auerbach, Haubenwallner, Leff, et al., 1998; Vu-Dac, Chopin-Delannoy, Gervois, Bonnelye, Martin, Fruchart, et al., 1998; Vudac, Schoojans, Kosykh, Dallongeville, Fruchart, Staels, et al., 1995).

The PPAR nuclear receptors appear to play a central role in the regulation of LXRα expression. Activators of both PPARα and PPARγ are shown to induce LXRα expression (Chawla, Boisvert, Lee, Laffitte, Barak, Joseph, et al., 2001; Chinetti, Lestavel, Bocher, Remaley, Neve, Torra, et al., 2001). The binding of the nuclear receptor PPARα to a PPAR response element (PPRE) in the 5' flanking region of LXRα gene can up-regulated expression of LXRα mainly by increasing transcriptional rate (Laffitte, Joseph, Walczak, Pei, Wilpitz, Collins, et al., 2001; Tobin, Steineger, Alberti, Spydevold, Auwerx, Gustafsson, et al., 2000). PPARα up-regulation will induce ABCA1 expression through the nuclear LXRα to promote cholesterol efflux (Chinetti, et al., 2001). PPARα induces the hepatic expression of LXRα, then LXRα up-regulates the expression of CYP7A1, a protein that is key in promoting conversion of cholesterol to bile acids in the liver (Hafner, Rezen, & Rozman, 2011). Once LXRα is activated, LXR-dependent pathways and genes described in the previous section will be activated and lead to down-regulation of cholesterol synthesis pathways.

1.1.1.7. Cholesterol catabolism and excretion

Cholesterol can be converted into a variety of bile acids in the liver (Javitt, 1994), and CYP7A1 is the key enzyme involved in bile acid synthesis (Handschin, Gnerre, Fraser, Martinez-Jimenez, Jover, & Meyer, 2005). Bile acids are usually conjugated with glycine, taurine, glucuronic acid, or sulfate. A mixture of conjugated and nonconjugated bile acids, along with cholesterol, is excreted from the liver into the bile. Approximately 95% of the bile acids are re-absorbed from the intestines, and the remainder is lost in the feces (Wolkoff & Cohen, 2003). The excretion and re-absorption of bile acids form the basis of the enterohepatic circulation, which is essential for the digestion and absorption of dietary fats. Up to 1 g per day of cholesterol enters the colon, which may originate from the diet, bile, and desquamated intestinal cells, and is further metabolized by the colonic bacteria into a nonabsorbable sterol, coprostanol. Free cholesterol, bile acids, and the metabolites are then excreted in the feces.

Cholesterol is susceptible to oxidation and can easily be oxidized into derivatives known as oxysterols. Three different oxidation mechanisms have been identified, which are 1) autoxidation, 2) secondary oxidation to lipid peroxidation, and 3) cholesterol-metabolizing enzyme oxidation (Bosinger, Luf, & Brandl, 1993). Oxysterols can exert inhibitory actions on cholesterol biosynthesis (Kandutsch, Chen, & Heiniger, 1978). Oxysterols are also involved in bile acid biosynthesis, transport of cholesterol, and regulation of gene transcription (Russell, 2000).

1.1.1.8. Diet and hypercholesterolemia

Hypercholesterolemia is typically due to a combination of environmental and genetic factors, with dietary choices being an important part of the environmental factors (Bhatnagar, Soran, & Durrington, 2008). It is well known that diet has an important effect on circulating cholesterol (Howell, McNamara, Tosca, Smith, & Gaines, 1997). Dietary cholesterol can regulate cholesterol synthesis in humans, in which liver is the major organ senses the increase in uptake, and increased intake of dietary cholesterol will suppresses hepatic cholesterol synthesis (Stange & Dietschy, 1985; van der Wulp, Verkade, & Groen, 2013). In recent years, the intestine has come into focus as an important control point in cholesterol homeostasis, and approximately 50% of the non-esterified cholesterol is absorbed in the intestine (Lichtenstein, 1990). Genetic predisposition also plays a major role in hypercholesterolemia, potentially adding to lifestyle factors and multiplying the risk of late complications (Matsushima & Teramoto, 1998).

Consumption of a western-style diet, which is generally high in fat and cholesterol, is associated with an increased level of cholesterol and further leads to a higher level of LDL in the blood. Excessive LDL that is not taken up by the peripheral tissues will remain in the circulation and be oxidized and taken up by macrophages. These macrophages will become engorged and form foam cells. Foam cells are often trapped in the walls of blood vessels and contribute to atherosclerotic plaque formation. These plaques are the main causes of heart attacks, strokes, and other serious medical problems. Thus, the dietary control of cholesterol level may play a critical role in the management of CVD.

1.1.2. Prostate cancer

Prostate cancer is identified as the most prevalent cancer in U.S. population in 2011 and accounts for 15% of all malignant cancer incidences (AACR, 2012). Many factors, including genetics, hormones, race, age, diet, and the environment have been identified to be responsible for the initiation and development of prostate cancer.

Treatment of prostate cancer generally involves surgery, various forms of radiation therapy, cryosurgery, with hormonal therapy and chemotherapy generally reserved for more advanced disease (Macpherson, Ng, Lakhani, Price, Venitz, & Figg, 2002; Tammela, 2012). The age and underlying health of the man, the extent of metastasis, histology and response of the cancer to initial treatment are important in determining the outcome of the disease (Bostwick, Burke, Djakiew, Euling, Ho, Landolph, et al., 2004). However, none of these therapeutic strategies is an effective cure for this disease.

The causes of prostate cancer remain largely unknown (Hsing & Chokkalingam, 2006). In the process of understanding the initiation and progression of prostate cancer, a wide variety of pathways and biomarkers have been identified, which reflect changes in cell morphometry; DNA ploidy; chromosomal gains and losses; cytoplasmic differentiation; cytoskeletal proteins; cell adhesion, proliferation, and apoptosis; growth factors and their receptors; oncogenes and tumor suppressor genes; AR gene mutations; and metastasis suppressor genes (Bostwick, et al., 2004).

1.1.2.1. Role of steroid hormones in prostate cancer

Androgens and estrogens are two groups of steroid hormones involved in the metabolism, inflammation, immune functions, and development of sexual characteristics (Holmes & Shalet, 1996). Aside from androgens' physiological role, androgens also play an important part in prostate cancer progression and metastases (Chan, Stampfer, & Giovannucci, 1998; Huggins & Hodges, 1972). Estrogens are also presented in male, though in lower concentration. More and more evidence supports the role of estrogen in the regulation of prostate cancer, which may act through two different ways: 1) indirect androgens lowering effect, and 2) direct target estrogen receptors presented on prostate cells (Bonkhoff & Berges, 2009). The role of these steroid hormones in prostate cancer is reviewed in the following section.

Androgens are a group of male sex hormones (e. g. testosterone and dihydrotestosterone), which control the differentiation and maturation of male reproductive organs, including the prostate gland. Binding of androgens to the androgen receptor leads to the expression of target genes (Dehm & Tindall, 2006). Androgens may also post-transcriptionally regulate gene expression by modulating the stability of mRNAs (Sheflin, Zou, & Spaulding, 2004). Multiple signaling pathways have been demonstrated to be critical for prostate cancer initiation and progression (De Marzo, DeWeese, Platz, Meeker, Nakayama, Epstein, et al., 2004; Ramsay & Leung, 2009), with the androgen signaling pathway being one of the most prominent (Tindall & Tindall, 2011).

It has long been proposed that androgens promote prostate carcinogenesis (Huggins, 1967; Huggins & Hodges, 1972). A number of epidemiological studies have

supported a link between circulating androgen levels and risk of prostate cancer, although the correlation is not conclusive (Isbarn, Pinthus, Marks, Montorsi, Morales, Morgentaler, et al., 2009; Morgentaler, 2006). A 'saturation' model of androgen action on androgen-dependent growth was suggested (Morgentaler & Traish, 2009), which states that physiological levels of androgen play an important role in both normal and malignant prostate cell proliferation; however, elevated levels of androgens alone do not necessarily lead to uncontrolled cell proliferation. Although epidemiologic data suggest that androgens alone are not sufficient to promote prostate carcinogenesis (Hsing, 2001), abundant biological data make it clear that androgens promote prostate cancer cell proliferation. Animal studies also demonstrated that androgens are very strong tumor promoters for prostate carcinogenesis. Even low doses of testosterone can induce prostate cancer in rodents. When rats are simultaneously treated with estradiol and testosterone, prostate cancer incidence is markedly increased, and even a short course of estrogen treatment results in a high incidence of prostate cancer (Bosland, 2006). Furthermore, ligand-independent activation of androgen receptor (AR) signaling may play a critical role in initiation and progression of prostate cancer (Debes & Tindall, 2004; Dehm & Tindall, 2006).

Androgen is known to regulate the alterations and overexpression of the TMPRSS2 gene and the ETS transcription factor genes in prostate cancer (Lin, Ferguson, White, Wang, Vessella, True, et al., 1999; Tomlins, Rhodes, Perner, Dhanasekaran, Mehra, Sun, et al., 2005; Vaarala, Porvari, Kyllonen, Lukkarinen, & Vihko, 2001). TMPRSS2 is a membrane-bound serine protease mostly limited to prostate luminal epithelial cells (Afar, Vivanco, Hubert, Kuo, Chen, Saffran, et al., 2001; Vaarala, Porvari,

Kellokumpu, Kyllonen, & Vihko, 2001), and ETS transcription factors, namely ERG and ETV1, are involved in multiple processes, including cell proliferation and cancer cell invasion (Hsu, Trojanowska, & Watson, 2004). Current understanding indicates most prostate cancers involve a TMPRSS2 ETS translocation and ERG gene overexpression (Petrovics, Liu, Shaheduzzaman, Furasato, Sun, Chen, et al., 2005; Soller, Elfving, Lundgren, & Panagopols, 2006). Thus, androgen's induction and promotion of expression of ETV1 and ERG are reported to contribute to prostate carcinogenesis.

Androgens also regulate the expression of both IGF-1 through the androgen response elements in the IGF-1 promoter region (Wu, Zhao, Zhao, Pan, Wu, Zhang, et al., 2007) and the expression of IGF-1R through a nongenomic event (Pandini, Mineo, Frasca, Roberts, Marcelli, Vigneri, et al., 2005). Androgens also modulate the IGF-1 signaling pathway through regulation of IGF-binding protein expression. Increased levels of IGF-1 are known to associate with prostate cancer (Kaaks, Lukanova, & Sommersberg, 2000). Increased expression of growth factors and the receptors promotes prostate cell proliferation, migration, and tumor angiogenesis, thereby facilitating prostate carcinogenesis and cancer progression.

EGF is another gene that appears to be subject to androgen regulation. Inhibition of the EGF receptor can completely suppress androgen-induced proliferation of LNCaP cells. Androgens enhance the expression of EGFR, while reduce expression of ERBB2 in LNCaP cells (Pignon, Koopmansch, Nolens, Delacroix, Waltregny, & Winkler, 2009). Furthermore, ligand dependent or ligand-independent activation of AR promotes EGF/EGFR signaling during prostate cancer progression (Dehm, Schmidt, Heemers,

Vessella, & Tindall, 2008; Libertini, Tepper, Rodriguez, Asmuth, Kung, & Mudryj, 2007).

In addition to the regulation of growth factors and respective receptors, androgens also affect the downstream effectors, such as PI3K/AKT. The PI3K/AKT pathway is one of the most frequently altered signaling pathways in a variety of human cancers and plays a critical role in prostate carcinogenesis and its progression (Vivanco & Sawyers, 2002; Yuan & Cantley, 2008). Activation of AKT significantly induces the progression of prostate cancer (Sarker, Reid, Yap, & de Bono, 2009). Proliferation and survival of androgen responsive LNCaP cells depend on the activation of PI3K/AKT, while inhibition of AKT or PI3K significantly attenuates androgen-induced cell proliferation (Sun, Yang, Feldman, Sun, Bhalla, Jove, et al., 2003). Androgen independent prostate cancer cell proliferation is also correlated with increased activity of PI3K/AKT (Murillo, Huang, Schmidt, Smith, & Tindall, 2001).

Estrogens are the primary female sex hormone, however substantial levels of estrogens are presented in the male, although markedly less than androgens. Prostate growth, differentiation and function are primarily controlled by androgens, but estrogens modulate these effects in several ways. Estrogens regulate the development and function of prostate throughout stages of a man's life (Prezioso, Denis, Klocker, Sciarra, Reis, Naber, et al., 2007). Estrogens can directly affect prostate through external hormone or through estradiol produced by local aromatisation of testosterone (Harkonen & Makela, 2004). Indirectly, estrogen regulation can interfere with androgen production.

Upon aging, serum estrogens are known to increase and androgens decrease, which temporally coincide with the increasing incidence of prostate cancer and indicated

a possible causal relationship with each other. However, no conclusive clinical evidence of a strong correlation between elevated serum estrogen or estrogen/androgen ratio and the increased incidence of prostate cancer has been established despite extensive studies (Eaton, Reeves, Appleby, & Key, 1999; Gann, Hennekens, Ma, Longcope, & Stampfer, 1996). However, strong in vitro study evidence shows that excessive or constant exposure to estrogens can promote the development of prostatic alterations, disorders and even malignancies (Ho, 2004). In previous studies, estrogen was found to stimulate DNA synthesis and induce metaplastic epithelial morphology both in human and rat prostate, with synergistic effect observed when combined treatment with androgen (Nevalainen, Harkonen, Valve, Ping, Nurmi, & Martikainen, 1993; Nevalainen, Valve, Makela, Blauer, Tuohimaa, & Harkonen, 1991).

Estrogen regulation has also been considered as one of the hormonal risk factors in association of development of prostate cancer and influences a wide range of genes, including IGF (Bosland, 2000; Henderson & Feigelson, 2000; International Prostate Health Council Study, 2000). Estrogen also regulate the expression of prostate specific genes (Martikainen, Harkonen, Vanhala, Makela, Viljanen, & Suominen, 1987; Nevalainen, Valve, Makela, Blauer, Tuohimaa, & Harkonen, 1991). The presence of estrogen receptors in the prostate suggests that estrogens may act directly on the prostate. In human and rodent prostates, ERβ is the predominant ER subtype, and is expressed in the majority of the epithelial cells, with ERα expressed in a limited amount and sometimes absent.

ERα and ERβ are both expressed in prostate epithelium. ERα and ERβ exhibit similar binding affinity for 17β-estradiol, but their specific roles can be quite distinct,

both complementary and antagonistic (Kuiper, Carlsson, Grandien, Enmark, Haggblad, Nilsson, et al., 1997). In the human prostate, estrogens mediate epithelial elements signaling through ERβ and play a role in repressing cellular proliferation. Absence of the ERβ gene leads to the accumulation of cells. ERβ regulates cellular proliferation by suppressing estrogen-mediated ERα-signaling pathways that promote AR synthesis (Bektic, Berger, Pfeil, Dobler, Bartsch, & Klocker, 2004; Paech, Webb, Kuiper, Nilsson, Gustafsson, Kushner, et al., 1997). In this way, 17β-estradiol can trigger distinct effects depending on the ratio of ERα and ERβ in the cell. Activation of ERβ also induces cell cycle arrest at G2/M phase and cellular apoptosis through p53-independent up-regulation of p21 expression and the down-regulation of cyclin B1 (Hedlund, Johannes, & Miller, 2003). The antiestrogen effect is shown to induce p21 expression and S phase cell cycle arrest in Du145 and PC3 prostate cancer cells (Rohlff, Blagosklonny, Kyle, Kesari, Kim, Zelner, et al., 1998). In vivo studies indicated that antiestrogens effectively inhibit development and progression of experimental and even clinical prostate cancer in a transgenic adenocarcinoma of the mouse prostate (TRAMP) model (Gingrich, Barrios, Morton, Boyce, DeMayo, Finegold, et al., 1996).

1.1.2.2. Androgen responsive pathway markers

Association between androgen and risk of prostate cancer makes androgen responsive genes ideal to assess the effect of bioactive compounds on androgen-dependent pathway and androgen-dependent cancer development.

Prostate-specific antigen (PSA) is a 34 kD, single-chain glycoprotein of 237 amino acids, which is produced almost exclusively by prostatic epithelial cells. PSA is a

serine protease with chymotrypsin-like, trypsin-like, and esterase-like activities. PSA detected in the serum is mainly in the form of a complex with α 1-antichymotrypsin, with a minor fraction of free PSA (Malm & Lilja, 1995). PSA mRNA and protein can be up-regulated by androgen in an androgen receptor-dependent fashion (Payton-Stewart, Schoene, Kim, Burow, Cleveland, Boue, et al., 2009). Serum concentrations of PSA appear to be correlated with circulating level of androgens and activation of androgen receptors. PSA production may be elevated by conditions other than cancer, including prostatitis, prostatic intraepithelial neoplasia, acute urinary retention, and renal failure (Malm & Lilja, 1995). PSA is particularly sensitive and accurate in the detection of residual cancer, recurrent cancer, and cancer progression after treatment, irrespective of the treatment modality. PSA can accurately predict cancer status and detect recurrence several months before detection by any other method (Oesterling, 1991). PSA is also a sensitive and specific immunohistochemical marker for tumors of prostatic origin (Goldstein, 2002). Although PSA has been recommended against clinical testing due to the risk of over-diagnosis and potential harm (USPSTF, 2012), the use of PSA as an indicator in scientific research of prostate cancer is still valid.

NKX3.1 is a putative prostate tumor suppressor that is expressed in a largely prostate-specific and androgen-regulated manner. Human NKX3.1 locates on human chromosome 8p21, a region that experiences a high loss of heterozygosity in human prostate cancer. Loss of NKX3.1 protein expression is a common finding in human prostate carcinomas and prostatic intraepithelial neoplasia (He, Sciavolino, Wing, Augustus, Hudson, Meissner, et al., 1997; Vocke, Pozzatti, Bostwick, Florence, Jennings, Strup, et al., 1996). The NKX3.1 gene knockout mouse develops prostatic intraepithelial

neoplasia, which is the presumed precursor to prostate cancer in humans (Abdulkadir, Magee, Peters, Kaleem, Naughton, Humphrey, et al., 2002; Bhatia-Gaur, Donjacour, Sciavolino, Kim, Desai, Young, et al., 1999). On the contrary, over-expression of NKX3.1 suppresses growth and tumorigenicity of prostate cancer cells in culture (Kim, Bhatia-Gaur, Banach-Petrosky, Desai, Wang, Hayward, et al., 2002), which suggested NKX3.1 to be a tumor suppressor gene. NKX3.1 expression can be regulated by androgens and 17β-estradiol (Korkmaz, Korkmaz, Ragnhildstveit, Kizildag, Pretlow, & Saatcioglu, 2000). No expression of NKX3.1 is detected in androgen-independent PC3 cells, while in androgen-dependent LNCaP cells, NKX3.1 can be upregulated upon androgen stimulation (Korkmaz, Korkmaz, Manola, Xi, Risberg, Danielsen, et al., 2004).

1.1.2.3. Role of angiogenesis in prostate cancer

Angiogenesis is the physiological process of generating new blood vessels from pre-existing vessels (Nicholson & Theodorescu, 2004). Tumor formation and growth need the support of rich supply of blood, and developing tumors are shown to be able to recruit their own blood supply and promote angiogenesis (Fidler, 1995; Gimbrone, Cotran, Folkman, & Leapman, 1972). Tumor cells are known to overexpress angiogenic factors or alter the regulation of endogenous angiogenic factors to establish an imbalance between proangiogenic and antiangiogenic factors in order to induce neovascularization (Folkman & Klagsbrun, 1987; Liotta, Steeg, & Stetlerstevenson, 1991). Angiogenesis also has important implications in cancer progression to distant sites, since tumor cell migration into the circulatory system through surface area of vessels within the tumor (Liotta, Kleinerman, & Saidel, 1976).

During angiogenesis, endothelial cells are signaled to switch from a resting state to rapid growth (Folkman, Watson, Ingber, & Hanahan, 1989). Tumor cells are known to secrete diffusible factors, among which VEGF is the most studied angiogenic growth factor (Senger, Perruzzi, Feder, & Dvorak, 1986). Human tumor biopsies exhibit enhanced expression of VEGF mRNAs by malignant cells and VEGF receptor mRNAs in adjacent endothelial cells, which correlates with microvessel density. On the other hand, normal prostate cells express much less VEGF (Ferrer, Miller, Andrawis, Kurtzman, Albertsen, Laudone, et al., 1998). Blockage of VEGF function can completely suppress the prostate cancer-induced angiogenesis and cease tumor growth at the pre-vascular growth phase (Borgstrom, Bourdon, Hillan, Sriramarao, & Ferrara, 1998).

1.1.2.4. Angiogenesis markers

Angiogenesis plays a role in tumorigenesis and metastasis. Proteins involved in angiogenesis can be used to assess tumor development and effect of bioactive on this pathway as a mechanism.

Vascular endothelial growth factor (VEGF) is a signal protein that stimulates vasculogenesis, which is a vital part in promoting angiogenesis in a wide variety of normal and neoplastic tissues, and is a potent mitogen for endothelial cells. VEGF's normal function is to create new blood vessels during embryonic development, new blood vessels after injury, muscle following exercise, and new vessels to bypass blocked vessels. When VEGF is overexpressed, it can contribute to disease. Cancers need to express VEGF to grow beyond certain size and metastasize (Kollermann & Helpap, 2001). VEGF has been demonstrated in prostate cancer epithelial cells in

immunohistochemical studies (Kollermann & Helpap, 2001; Stewart, Panigrahy, Flynn, & Folkman, 2001), which is significantly reduced after androgen-deprivation therapy (Kwak, Jin, Lee, Park, & Lee, 2002; Latil, Bieche, Pesche, Valeri, Fournier, Cussenot, et al., 2000). Blockage of VEGF inhibits androgen-independent prostate cancer growth in xenograft models due to the induction of endothelial cell apoptosis (Fox, Higgins, Maiese, Drobnjak, Cordon-Cardo, Scher, et al., 2002; Sweeney, Karashima, Kim, Kedar, Mian, Huang, et al., 2002), while exogenous VEGF promotes the growth of xenograft tumors (Gridley, Andres, & Slater, 1997). Androgen-independent prostate cancer showed significantly elevated plasma VEGF concentrations than that of localized prostate cancer, and VEGF levels are correlated inversely with survival (George, Halabi, Shepard, Vogelzang, Hayes, Small, et al., 2001). Current evidence suggests that cancer cells express VEGF for angiogenesis to ensure cancer development, so that circumventing oxygen diffusion will no longer be a rate-limiting step in the growth of prostate cancer. VEGF expression can be mediated and potentiated by cytokines include TNF (Giraudo, Primo, Audero, Gerber, Koolwijk, Soker, et al., 1998), TGF-β (Pertovaara, Kaipainen, Mustonen, Orpana, Ferrara, Saksela, et al., 1994), IGF-1 (Warren, Yuan, Matli, Ferrara, & Donner, 1996), IL-1β (Li, Perrella, Tsai, Yet, Hsieh, Yoshizumi, et al., 1995), and IL-6 (Cohen, Nahari, Cerem, Neufeld, & Levi, 1996), as well as others.

Platelet endothelial cell adhesion molecule (PECAM) is also known as CD31, which is a 130-kD protein belonging to the immunoglobulin superfamily. It is expressed on platelets, monocytes, neutrophils, certain types of T cells and endothelial cell intercellular junctions (Newman, 1997). Engagement of PECAM on the surface of leukocytes activates of integrins and promotes their adhesion and migration (Berman &

Muller, 1995; Reedquist, Ross, Koop, Wolthuis, Zwartkruis, van Kooyk, et al., 2000). Functionally, PECAM is shown to play a role in inflammation, angiogenesis and vascular development (Privratsky, Newman, & Newman, 2010). PECAM takes effect in the adhesion cascade leading to leucocyte–endothelial transmigration during inflammation, which serves as a scaffolding molecule in a number of signaling pathways (Ilan & Madri, 2003). PECAM recruitment and activation will lead to modulation in a number of cellular pathways, including the MAP kinase cascades, which play essential roles in a number of cell processes by regulating transcription or translation, and AKT activation regulating cell survival. Additionally, PECAM has been shown to contain immunoregulatory tyrosine-based inhibitory motifs within its cytoplasmic domain. Phosphorylation of these residues results particularly in inhibition of tyrosine kinase-mediated signaling, proliferation and cellular activation (Newman, 1999). Binding of PECAM and/or soluble PECAM inhibits transendothelial migration of leukocytes and angiogenesis, while PECAM-deficient mice exhibit abnormalities in their inflammatory and angiogenic responses (DeLisser, ChristofidouSolomidou, Strieter, Burdick, Robinson, Wexler, et al., 1997; Duncan, Andrew, Takimoto, Kaufman, Yoshida, Spellberg, et al., 1999; Solowiej, Biswas, Graesser, & Madri, 2003).

1.1.2.5. Role of inflammation in prostate cancer

Chronic inflammation is a risk factor of several forms of human cancer and ~20% of adult cancers are indicated to be related to chronic inflammation (Sfanos & De Marzo, 2012). Several conditions may contribute to the initiation of prostatic inflammation, including infections, dietary components, physical trauma, hormonal changes and urine

reflux (De Marzo, Platz, Sutcliffe, Xu, Gronberg, Drake, et al., 2007). Most inflammation condition in prostate cancer tissues is chronic, with lymphocytes and macrophages involved, however, the factors leading to the chronic inflammation are yet to be identified (Sfanos & De Marzo, 2012).

In prostatic atrophy, inflammatory cell infiltrations are usually observed. It is associated with a high frequency to encompass large regions of the prostate, which are referred to as proliferative inflammatory atrophy. Atrophic epithelial cells can regenerate in response to cellular damage (De Marzo, Marchi, Epstein, & Nelson, 1999). Furthermore, genome alterations, which are associated with prostate cancer, are usually identified in atrophic lesions (De Marzo, et al., 2007; Nelson, De Marzo, & Isaacs, 2003; Putzi & De Marzo, 2000). Previous studies described that 28% instances prostate cancer are closely adjacent to areas with chronic inflammation, indicating the merge of these two conditions (Wang, Bergh, & Damber, 2009b).

Some of the hallmark gene expression changes usually found in prostate cancer have been observed in proliferative inflammatory atrophy. NKX3.1 and p27 are down-regulated in prostate atrophy, which is indicative of initiation of prostate cancer (Bethel, Faith, Li, Guan, Hicks, Lan, et al., 2006; De Marzo, Marchi, Epstein, & Nelson, 1999). p53 expression, together with Ki-67, CK5, COX-2 and GSTP1 expression, also increase in areas of acute inflammation (Wang, Bergh, & Damber, 2009a).

1.1.2.6. Inflammation markers

Cytokines exert cytostatic and immunomodulatory effects on cancer cells, and influence prostate cancer development through regulation of the antitumor immune

response and angiogenesis (McCarron, Edwards, Evans, Gibbs, Dearnaley, Dowe, et al., 2002). Cytokines form a highly entangled system linking cancer and inflammation (Coussens & Werb, 2002; Maeda & Omata, 2008). The involvement of inflammation in prostate cancer discussed in the previous section suggested that the exposure to cytokines may be risk factors in both prostate cancer and inflammation.

TNF-α is strongly expressed in prostate cancer compared with benign prostatic tissue, which makes it a strong risk factor for prostate cancer (Chetcuti, Margan, Mann, Russell, Handelsman, Rogers, et al., 2001; de Miguel, Royuela, Bethencourt, Santamaria, Fraile, & Paniagua, 2000). TNF-α inhibits chemotaxis and proliferation of cancer cell lines (Nakajima, Dellipizzi, Mallouh, & Ferreri, 1995; Ritchie, Spangelo, Krzymowski, Rossiter, Kurth, & Judd, 1997), induces bcl-2-mediated programmed cell death (Kramer, Steiner, Sokol, Handisurya, Klingler, Maier, et al., 2001), activating NF-κB (Gunawardena, Murray, Swope, & Meikle, 2002), and inducing cyclooxygenase-2 expression (Subbarayan, Sabichi, Llansa, Lippman, & Menter, 2001). The effects of TNF-α are mediated by TNF-RI and TNF-RII, both of which are expressed by prostate cancer cell lines (Nakajima, DelliPizzi, Mallouh, & Ferreri, 1996). TNF-α also plays an important role in inflammation by mediating the proliferation and differentiation of immune cells, and development of immune response (Miagkov, Kovalenko, Brown, Didsbury, Cogswell, Stimpson, et al., 1998). TNF-α is one of the major inflammatory mediators secreted by activated macrophage and involved in many crucial events for the initiation of both acute and chronic inflammation, such as regulating the production of several cytokines, up-regulation of adhesion molecule expression, and activation of leukocyte-specific chemotactic cytokines (Simmonds & Foxwell, 2008).

IL-1β can initiate signal transduction by activation of NF-κB and the MAPKs during an inflammatory process (Dinarello, 2004). The activation of NF-κB pathway will in turn induce further expression of a series of cytokines, including IL-1β, TNF-α, IL-6, and promote an inflammatory process. MCP-1 is a potent chemoattractant for monocytes during local immune response and plays an important role in the initiation of atherosclerosis. It has been demonstrated that MCP-1 expression is regulated through NF-κB pathway, and MCP-1 can also be stimulated by TNF-α, IL-1β, and IL-6 (Arefieva, Kukhtina, Antonova, & Krasnikova, 2005; Steube, Meyer, & Drexler, 1999).

1.1.2.7. Cancer proliferation markers

Proliferation is a hallmark of cancer development where cell growth becomes uncontrolled. Proliferation marker can be used to assess efficacies of efficacy of prostate cancer prevention.

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein for DNA polymerase-α involved in DNA repair, replication, post-replication modifications and chromatin assembly, which reaches peak expression during the G1- and G2-phases of the cell cycle and can be used as an index of the proliferative activity of cancers. The PCNA labeling index is indicated to be lowest in benign epithelium and increases progressively in differentiated prostate cancer (Limas & Frizelle, 1994; Nemoto, Kawamura, Miyakawa, Uchida, Hattori, Koiso, et al., 1993). Many studies have shown that the PCNA index is related to cancer stage (Cher, Chew, Rosenau, & Carroll, 1995; Limas & Frizelle, 1994) and a high PCNA labeling index may indicate progression of prostate cancer (Idikio, 1996; Spires, Banks, Davey, Jennings, Wood, & Cibull, 1994), while

patients with a lower PCNA expression survive significantly longer than those with higher ones (Vesalainen, Lipponen, Talja, Alhava, & Syrjanen, 1994).

The Ki-67 antigen is a nuclear protein that is expressed by non-G0 proliferating cells (Cattoretti, Becker, Key, Duchrow, Schluter, Galle, et al., 1992). It was shown that Ki-67 labeling indices correlate with cancer, with higher indices indicating more progressed cancer stage (Bubendorf, Sauter, Moch, Schmid, Gasser, Jordan, et al., 1996; Sadi & Barrack, 1991), which may assist to discriminate between organ-confined and metastatic cancer (Cattoretti, et al., 1992; Cher, Chew, Rosenau, & Carroll, 1995; Harper, Goddard, Wilson, Matanhelia, Conn, Peeling, et al., 1992). Ki-67 expression is also correspond to other factors involved in cancer, such as epidermal growth factor receptor (GlynneJones, Goddard, & Harper, 1996), mutant p53 (Thompson, Mellon, Charlton, Marsh, Robinson, & Neal, 1992), and chromosomal aberrations (Henke, Kruger, Ayhan, Hubner, & Hammerer, 1993).

1.1.3. Link between cholesterol and prostate cancer

Cholesterol has also been shown to play a role in prostate cancer. It has long been reported that cholesterol and other lipids accumulate in solid tumors (White, 1909). The association between high circulating cholesterol and increased risk of prostate cancer has been reported by a number of cohort studies (Kok, van Roermund, Aben, den Heijer, Swinkels, Kampman, et al., 2011; Magura, Blanchard, Hope, Beal, Schwartz, & Sahmoun, 2008; Mondul, Clipp, Helzlsouer, & Platz, 2010; Platz, Clinton, & Giovannucci, 2008; Shafique, McLoone, Qureshi, Leung, Hart, & Morrison, 2012), furthermore, men with low cholesterol showed a reduced risk of high-grade prostate

cancer (Platz, Till, Goodman, Parnes, Figg, Albanes, et al., 2009). The role of cholesterol in prostate cancer development has also been demonstrated in animal and cell models (Llaverias, Danilo, Wang, Witkiewicz, Daumer, Lisanti, et al., 2010; Xue, Yang, Newmark, & Lipkin, 1997), which suggested that cholesterol level may play a role in progression and metastasis of prostate cancer (Jacobs, Stevens, Newton, & Gapstur, 2012; Kok, et al., 2011; Shafique, McLoone, Qureshi, Leung, Hart, & Morrison, 2012). Epidemiological studies and pre-clinical models have established hypercholesterolemia's role in the progression of prostate cancer, in which cholesterol functions as a mediator of cell proliferation, membrane dynamics, inflammation and steroidogenesis, thus providing multiple avenues for this lipid to contribute to prostate cancer progression (Pelton, Freeman, & Solomon, 2012). Additionally, the regulation of cholesterol-related pathways (such as SREBP-2, HMG-CoA reductase) has been suggested as potential treatment of prostate cancer (Freeman & Solomon, 2004; Krycer, Phan, & Brown, 2012). Hence, dietary modulation of fat/cholesterol may potentially prevent prostate cancer development.

1.1.4. Role of inflammation in prostate cancer

The relationship between inflammation and cancers is well documented. It can trace back to as early as 1863, when Virchow hypothesized that cancers tended to occur at sites of chronic inflammation (Balkwill & Mantovani, 2001). Accumulated epidemiologic studies support that chronic inflammatory diseases are frequently associated with increased risk of cancers (Coussens & Werb, 2002; Philip, Rowley, & Schreiber, 2004). It was indicated that reactive oxygen and nitrogen species generated by

inflammatory cells (Okada, 2002; Reuter, Gupta, Chaturvedi, & Aggarwal, 2010), inflammatory cytokines, chemokines, and enzymes (Coussens & Werb, 2002) all contribute to and facilitate cancer development via multiple signaling pathways (Yang, Hsieh, Ho, & Lin, 2005).

The involvement of inflammation in prostate cancer has also caught a great deal of attention. Inflammatory cell infiltrations are usually observed, associated with a high frequency to encompass large regions of the prostate in proliferative inflammatory atrophy. Previous studies described that 28% prostate cancer instances are closely adjacent to areas with chronic inflammation, indicating the merge of these two conditions (Wang, Bergh, & Damber, 2009b).

1.1.5. The effects of diet on inflammation

The dramatic changes in dietary composition since the industrial revolution and modernized agriculture, and our unchanged physiology, have resulted in a systemic stress induced by simply intake of food (Bosma-den Boer, van Wetten, & Pruimboom, 2012). The stress will spur inflammatory response and lead to long-term persistent inflammation (Kutuk & Basaga, 2003). The western-style diet, in particular, which is high in fat and cholesterol, is known to be the leading cause of overweight and obesity (Dausch, 1992). Obesity can add up to inflammation, due to the increased expression of cytokines coming from the adipocytes (Miranda-Garduno & Reza-Albarran, 2008). Thus, moderation of diet is an important part of reducing chronic inflammation and related conditions.

1.1.6. Microbiome in gastrointestinal tract and health

The word “microbiome” was coined in 2001 to signify "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" (Lederberg & McCray, 2001). Microbial cells (~10^{14} cells) outnumber human cells by about 10 times, which makes it a very large and complex ecosystem between the human body and the residing microorganisms (Hattori & Taylor, 2009; Savage, 1977). Recent advances in DNA sequencing technologies have allowed comprehensive examination of microbial communities without the need to culture the specific microbes in the laboratory.

Although the exact influence and mechanism remains unknown, previous studies have gathered evidence that human microbiome may affect human development, physiology, immunity, and nutrition (Dethlefsen, McFall-Ngai, & Relman, 2007). Introduction of distal gut microbiota into germ-free animals led to a marked increase in adiposity and body fat content within 2 weeks (Backhed, Ding, Wang, Hooper, Koh, Nagy, et al., 2004; Turnbaugh, Ley, Mahowald, Magrini, Mardis, & Gordon, 2006). Recipients of the microbiota from an obese donor animal had a significantly greater increase in adiposity than the ones receiving microbiota from a lean donor, indicating that the gut microbiota from obese animals had an increased and transmissible capacity in promoting adiposity (Turnbaugh, Ley, Mahowald, Magrini, Mardis, & Gordon, 2006). These studies revealed a potential role of the microbiome in the development of obesity and other subsequent chronic conditions.

Considering the overwhelming number and ubiquity of microorganisms, the crosstalk between bacteria and human cells can be playing a central role in the

maintenance of health (Hattori & Taylor, 2009). Previous studies have identified biomarkers associated with inflammation and functional changes in the intestinal microbiome between diseased and healthy human subjects (Karlsson, Tremaroli, Nookaew, Bergstrom, Behre, Fagerberg, et al., 2013), and high-fat diet-induced obesity in animal models appeared to be associated with changes in the gut microbiome and gut inflammation (Albenberg & Wu, 2014; Chen, He, & Huang, 2014). Changes in inflammatory status may lead to other chronic conditions in human.

Long-term and short-term diet patterns can shape the structure and activity of human gut microbiome (David, Maurice, Carmody, Gootenberg, Button, Wolfe, et al., 2014). At early stage of life, breast feeding promotes the colonization of Bifidobacteria (up to 90% of flora) in infant, while, formulated milk harbors more diverse microbiome in infant, including Bacteroides, Clostridium, and Enterobacteriaceae (Martin & Walker, 2008; Martin, Jimenez, Heilig, Fernandez, Marin, Zoetendal, et al., 2009). Another study showed that, people on a plant-based diet have significantly lower levels of Bacteroides spp., Bifidobacterium spp., Escherichia coli, and Enterobacteriaceae spp. than those on an animal-based diet (Zimmer, Lange, Frick, Sauer, Zimmermann, Schwiertz, et al., 2012). Changes in microbiome can be observed within as short as 24 hours shifting from high-fat, low-fiber to low-fat, high-fiber diets (Wu, Chen, Hoffmann, Bittinger, Chen, Keilbaugh, et al., 2011). Additionally, individual differences in the response of the microbial community to dietary change, and in microbial fermentation of dietary substrates in the colon needs to be accounted for in the analysis of human microbiome (Walker, Ince, Duncan, Webster, Holtrop, Ze, et al., 2011). Understanding the interaction

between diet and microbiome may be a critical part in studying diet and the influence on human health.

1.2. Health benefit of soy

Soy (Glycine max) plays an important role in the world's food supply and economy. The United States became the world's leading producer of soy since 1955 and has remained so for over half a century (Liu, 1997). U. S. production of soy in 2011 was 83 million metric tons, which accounted for more than 30% of world production (FAO, 2013). Moreover, soy-food sales increased significantly after a 1999 decision by the U. S. Food and Drug Administration to allow soy-food labels to display health claim that soy protein may reduce risk of heart disease (FDA, 1999). Following sections summarized current knowledge on soy's health promoting effects.

1.2.1. Protection against cardiovascular disease

Soy's cardiovascular protective effects in human subjects have been reviewed and highlighted in a meta-analysis of 38 controlled clinical trials in an effort to reveal the correlation between consumption of soy and lipid levels (Anderson, Johnstone, & Cooknewell, 1995). Analysis showed that the average consumption of 47 g soy protein/day resulted in significant decreases of total cholesterol (9.3%), LDL cholesterol (12.9%) and triglycerides (10.5%). On the other hand, a recent review by the American Heart Association Nutrition Committee found a merely 3% decrease in LDL with soy protein consumption from 8 randomized trials providing approximately 50 g soy protein/day, and no apparent benefit in 14 other trials (Sacks, Lichtenstein, Van Horn,

Harris, Kris-Etherton, Winston, et al., 2006). The responses appeared to be highly dependent on the cholesterol level of the subjects. The meta-analysis performed by Anderson and colleagues included earlier studies with strongly hyperlipidemia (total cholesterol > 250 mg/dL) patients. It was expected that extreme hyperlipidemia would benefit from greater percentage reductions in total and LDL cholesterol, while patients with moderate or minor hyperlipidemia were recruited in the later study, which might result in the lower efficacy observed.

Epidemiological studies have long linked soy consumption to a reduced risk for cardiovascular disease, and soy protein, soy-derived isoflavones, and sterol were to some extent involved in the discussion. (Clerici, Setchell, Pirro, Morelli, Castellani, Giuliano, et al., 2004; Sacks, et al., 2006; Sirtori, Galli, Anderson, Sirtori, & Arnoldi, 2009; Tripathi & Misra, 2005; Xiao, 2008). A recent review identified soy proteins to be partially responsible for the lipid-lowering effects of soy (Sirtori, Galli, Anderson, Sirtori, & Arnoldi, 2009). It has also been suggested that isoflavones may act on vascular tissue to improve circulation (Ghosh, 2009; Ghosh & Scheepens, 2009; Xiao, 2008). However, the active components of soy remain unclear.

One proposed mechanism of soy's cardio-protective effects involves the inhibition of LDL oxidation and reduction of formation of plaque in the arteries. Soy extracts (Astadi, Astuti, Santoso, & Nugraheni, 2009; R. Takahashi, Ohmori, Kiyose, Momiyama, Ohsuzu, & Kondo, 2005) and peptides (Rho, Lee, Il Chung, Kim, & Lee, 2009; Rho, Park, Ahn, Shin, & Lee, 2007) have both been shown to reduce the oxidation of LDL cholesterol in vitro or in vivo. It was also reported that the anthocyanins found in color seed coat of soy displayed potent anti-oxidant properties and was able to inhibit

LDL oxidation (de Pascual-Teresa, Moreno, & Garcia-Viguera, 2010; Kong, Chia, Goh, Chia, & Brouillard, 2003; R. Takahashi, Ohmori, Kiyose, Momiyama, Ohsuzu, & Kondo, 2005). A more recent hypothesis involves the liver's LDL receptors, which may be affected by soy consumption to enhance their uptake of LDL from the serum and consequently lower serum LDL levels (Van Horn, McCoin, Kris-Etherton, Burke, Carson, Champagne, et al., 2008).

1.2.2. Protection against obesity-related metabolic syndrome

A more recent trend of soy research involves examining the potential in mitigating obesity and related complications (Azadbakht & Esmaillzadeh, 2008; Orgaard & Jensen, 2008). The actual efficacy is still under investigation. Mechanistically, the estrogen-like activity of soy isoflavones may be playing a role in regulating adipogenesis by binding to estrogen receptors, thus decreasing lipoprotein lipase activity, and PPAR may also be involved in the regulation (Orgaard & Jensen, 2008). Levels of genistein, a soy isoflavone, ranging from 0.1 to 1.0 μM have been shown to inhibit adipogenesis, while higher concentrations (25-50 μM) enhanced adipogenesis, showing a biphasic effect (Orgaard & Jensen, 2008). However, evidence in the animal model and in-vitro study did not always apply well to human, which made it inconclusive whether consumption of soy will benefit obese population. While, many human clinical experiments did not show any decrease in body weight as expected, but improvements in blood lipids (Orgaard & Jensen, 2008) or improvements in insulin resistance was observed (Bhathena & Velasquez, 2002).

1.2.3. Cancer protective effects of soy

Soy has also exhibited potential in reducing the risk of certain types of cancer (M. Messina, 2006; Nagata, Sonoda, Mori, Miyanaga, Okumura, Goto, et al., 2007; Oba, Nagata, Shimizu, Shimizu, Kametani, Takeyama, et al., 2007; Valachovicova, Slivova, & Sliva, 2004). Among the cancers investigated, breast and prostate cancers are of particular interest due to the sensitivity to sex steroid hormones.

Epidemiological studies among Asian population had long connected the lower breast cancer incident with consumption of soy products, which was usually attributed to soy isoflavones (Trock, Hilakivi-Clarke, & Clarke, 2006; Wu, Yu, Tseng, & Pike, 2008). Alongside the modest protective effects, concern has also been expressed that the estrogenic activity of soy isoflavones may lead to adverse effects on progression and recurrence of breast cancer (Magee & Rowland, 2012). On the other hand, there were some reviews citing a number of human clinical and epidemiological studies indicating no evidence of risk in human (Messina, 2008; Messina & Wu, 2009; Messina & Wood, 2008). Preventative effect of soy against prostate cancer was also indicated and reviewed in previous researches (Messina, 2003), and it is also indicated in clinical trials of the therapeutic effect of prostate cancer (Ahmad, Forman, Sarkar, Hillman, Banerjee, Doerge, et al., 2008; Banerjee, Li, Wang, & Sarkar, 2008). However, other randomized, controlled intervention study did not support that soy consumption can benefit men with prostate cancer, while not excluding the possibility of soy's effect in preventing prostate cancer (Bosland, Kato, Melamed, Taneja, Lepor, Torre, et al., 2001). Despite the amount of effort from experimental and epidemiological studies, soy products' effect in breast and prostate cancer prevention or therapy is largely unresolved.

Mechanisms of cancer prevention by soy can be attributed to isoflavones. Previous studies suggested that multiple mechanisms of action might be responsible for soy's bioactivity. Isoflavones have been shown to affect the cell cycle, apoptosis, differentiation, proliferation, growth, and cell signaling (Banerjee, Li, Wang, & Sarkar, 2008; Messina, Kucuk, & Lampe, 2006; Zhou, Gugger, Tanaka, Guo, Blackburn, & Clinton, 1999). Isoflavones also exhibited antioxidant capacity. They are well-known scavengers for reactive oxygen species, but recent research is suggestive of additional antioxidant activity beyond direct scavenging of radicals. Genistein in particular has been shown to activate transcription factors such as estrogen receptor and stimulate gene expression in breast cancer cells (Banerjee, Li, Wang, & Sarkar, 2008). Genistein can also inhibit androgen responsive gene expression (Lazarevic, Karlsen, & Saatcioglu, 2008). These results indicated that the genistein may have broad ability to impact overall cellular homeostasis through a variety of mechanisms/pathways.

1.2.4. Protection against other chronic diseases

Aside from the diseases mentioned above, soy consumption has also been linked to the effects in other chronic diseases, though not as extensively studied. Diabetes metrics, cognitive function and immune function are among the list of conditions that can potentially benefit from consumption of soy or its components (Bhathena & Velasquez, 2002; Cederroth & Nef, 2009; Lee, Lee, & Sohn, 2005; Ryan-Borchers, Park, Chew, McGuire, Fournier, & Beerman, 2006). As with the diseases discussed above, it remains unclear whether it is isoflavones, proteins, fiber or some other components causing the supposed beneficial effects. The complexity of soy components makes it very hard to

dissect out the exact active component(s) or compound(s) responsible for any particular bioactivity. Compounds may counteract with each other, or show distinct activities resulting from different concentration or ratio. Thus, the study of each individual component is proven to be necessary and important to gather scientific evidence and knowledge to support soy's health promoting effects.

1.2.5. Bioactive components of soy

Isoflavones, tocopherols, carotenoids, and phytosterols are the main bioactive components in soy (Liu, 1997). Isoflavones are a class of polyphenolics found almost exclusively in legumes and most prominently in soybeans. Isoflavones are structurally similar to that of estradiol, which bind weakly to estrogen receptors, eliciting weak estrogenic responses and earning the title of 'phytoestrogen' (Cederroth & Nef, 2009). Genistein, daidzein and glycitein are the predominating isoflavones in soy (Fig. 1.3) (Liu, 1997). Biological activities of soy isoflavones include prevention against atherosclerosis and other CVD, obesity, osteoporosis, and cancer (Clair & Anthony, 2005; Gil-Izquierdo, Penalvo, Gil, Medina, Horcajada, Lafay, et al., 2012; Orgaard & Jensen, 2008; Sarkar & Li, 2003; Taku, Melby, Nishi, Omori, & Kurzer, 2011; Weaver & Cheong, 2005). Due to genistein's estrogenic effect, it was shown that genistein can alter the expression of genes involved in estrogen-mediated pathways and induce proliferative response in breast cancer cells at 1-5 μM, while higher concentration (25 μM) of genistien can induce apoptosis and inhibition of proliferation (Lavigne, Takahashi, Chandramouli, Liu, Perkins, Hursting, et al., 2008). It was also reported that genistein and daidzein affect androgen responsive pathways in prostate cancer cells (Takahashi, Lavigne, Hursting,

Chandramouli, Perkins, Kim, et al., 2006). These findings indicated that the biological activities of soy isoflavones are dose-dependent and involved in multiple pathways.

Tocopherols are members of the Vitamin E family and can act as antioxidants by scavenging peroxyl radicals (Britz, Kremer, & Kenworthy, 2008; Liu, 1997). Carotenoids also possess antioxidant activity, as their highly conjugated structures are extremely vulnerable to oxidation in the presence of light, heat, and oxygen (Lee, Shannon, So, Sleper, Nelson, Lee, et al., 2009). Plant sterols are found in soybean oils mostly in the non-esterified form. Plant sterols have a base structure very similar to that of human cholesterol. The esterified sterols have been shown to reduce serum cholesterol levels when consumed in sufficient quantities (Phillips, Ruggio, Toivo, Swank, & Simpkins, 2002).

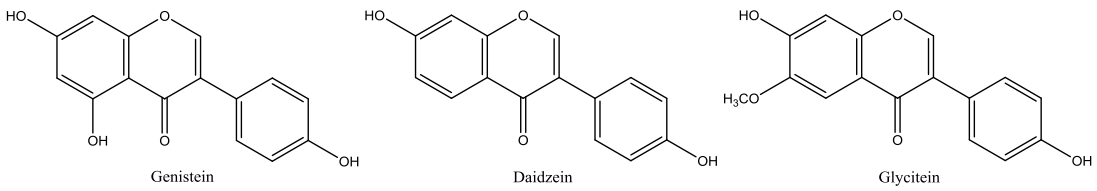

Fig. 1.3. Structure of genistein, daidzein, and glycitein.

Other soy phytochemicals have not been studied as extensively. Recently, a novel group of phytochemicals, glyceollins, which are also soy phytoalexins, was identified for the potential biological activities. The chemical and biological properties of glyceollins are reviewed in the following sections.

1.3. Identification, Isolation of soy phytochmicals glyceollins

Glyceollins are one of the major groups of phytoalexins in soybean. It was first reported in elicited soybean by Zahringer et al. in 1977 (Zahringer, Ebel, Kreuzaler, & Grisebach, 1977). A good number of studies have been conducted to elucidate the biosynthesis of glyceollins in soybean. Banks and Dewick (1983) pointed out that phenylalanine, daidzein, 7,2',4'-trihydroxyisoflavone, 3,9-dihydroxypterocarpan and glycinol are precursors for biosynthesis of glyceollin I, II and III (Banks & Dewick, 1983). In the following decades, plant scientists had shown interest in glyceollins mainly for its antifungal and antibacterial effects in soybean (Olah, Schmitthenner, & Walker, 1982; Parniske, Fischer, Hennecke, & Werner, 1991; Wyss, Boller, & Wiemken, 1991), and a number of fungi were identified to be effective elicitors, e.g. Rhizopus oryzae (Simmons, Vincken, Roidos, Bovee, van Iersel, Verbruggen, et al., 2011), Mucor ramosissimus (Garcez, Martins, Garcez, Marques, Pereira, Oliveira, et al., 2000), Diaporthe Meridionalis (Modolo, Cunha, Braga, & Salgado, 2002), Aspergillus sojae (Kim, Suh, Kim, Park, Joo, & Kim, 2010). It was determined that, glyceollins are in relatively low amount in unstressed soy (1 to 9 µg/g fresh weight of soy, depending on the different parts of soy). While, after induction, glyceollins concentration can go up to 43 to 955 µg/g fresh weight of soy, depending on the different elicitors and parts of soy (Boue, Carter, Ehrlich, & Cleveland, 2000; Degousee, Triantaphylides, & Montillet, 1994; Kraus, Spiteller, Mithofer, & Ebel, 1995). In addition to the effort in isolating glyceollins from elicited soy, chemical synthesis was also studied. However, due to the time-consuming and multiple steps needed in contiguous ring systems preparation, as well as the maintenance of rigorous stereo-control, only glyceollin I has been synthesized

to date with limited preparation capacity (Khupse & Erhardt, 2008; Khupse, Sarver, Trendel, Bearss, Reese, Wiese, et al., 2011; Luniwal, Khupse, Reese, Liu, El-Dakdouki, Malik, et al., 2011; Luniwal, Khupse, Reese, Fang, & Erhardt, 2009).

1.4. Biological activities of soy phytochemical glyceollins

Glyceollins are a group of phytoalexins with antibiotic activity that are synthesized in response to infection. Phytoalexins are inducible chemicals involved in plants' self-defense system (Jeandet, Douillt-Breuil, Bessis, Debord, Sbaghi, & Adrian, 2002), which are low molecular weight, possess anti-microbial activities, and are biosynthesized de novo in response to stress, including microbial attack, heavy metal salts, or UV radiation (Chamberl.Dw & Paxton, 1968; Murch & Paxton, 1980; Paxton, 1971). Aside from the anti-microbial activity, some phytoalexins are also indicated to exhibit chronic disease prevention and health-promoting effects in human (Boue, Cleveland, Carter-Wientjes, Shih, Bhatnagar, McLachlan, et al., 2009).

Glyceollin I, II, and III (Fig. 2) are the most common isomers isolated from soybean (Banks & Dewick, 1983). A number of biological activities were reported by previous studies, including antiproliferation (Lee, Kim, Chun, Park, Kim, Kim, et al., 2010; Payton-Stewart, Khupse, Boue, Elliott, Zimmermann, Skripnikova, et al., 2010; Salvo, Boue, Fonseca, Elliott, Corbitt, Collins-Burow, et al., 2006), antiestrogenic (Burow, Boue, Collins-Burow, Melnik, Duong, Carter-Wientjes, et al., 2001), antibacterial (Weinstein & Albersheim, 1983), antinematode (Huang & Barker, 1991; Veech, 1982), antifungal activities (Lee, Kim, Lee, Jeon, Cui, Lee, et al., 2010; Lozovaya, Lygin, Zernova, Li, Hartman, & Widholm, 2004), insulinotropic (Park, Ahn,

Kim, Lee, Kim, & Kim, 2010) and attenuation of vascular contraction activity in rat (Song, Baek, Jeon, Seo, Kim, Cui, et al., 2010). Hence, accumulating facts suggested that glyceollins possess the potential to be a health promoting phytochemical and deserve further investigation and characterization. Due to the recentness of discovery of glyceollins' health promoting activities and the limited accessibility of this group of compounds, it is noteworthy that most of these reports come from Park and colleagues and Boue and colleagues.

Among the biological activities, glyceollins' insulinotropic, antiestrogenic, antioxidant, anti-inflammation, and antiproliferation effects are of particular relevant to this project and are reviewed in the following sections.

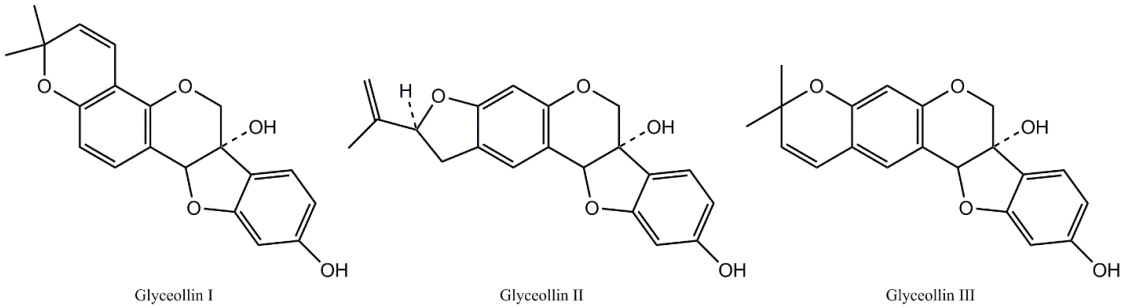

Fig. 1.4. Structure of glyceollins.

1.4.1. Insulinotropic effect of glyceollins

Park and colleagues (Park, Ahn, Kim, Lee, Kim, & Kim, 2010; Park, Kim, Kim, Kim, & Kim, 2012) performed two studies investigating glyceollins' role in improving glucose homeostasis, they concluded that glyceollins act through regulating glucose utilization in adipocytes and modulating β cell function and survival. It was shown that glyceollins could improve insulin-stimulated glucose uptake and decrease triacylglycerol

accumulation in 3T3-L1 mouse adipocytes. 5 μM glyceollins increased basal glucose uptake by 150%. While co-incubation of glyceollins and insulin further stimulated maximal glucose uptake above basal levels than that of either stimulus alone. Mechanistically, glucose transporter GLUT4 mRNA and protein expression significantly increased upon exposure of 5 μM glyceollins for 3 h in 3T3-L1 adipocytes (Boue, Isakova, Burow, Cao, Bhatnagar, Sarver, et al., 2012). In addition, glyceollins slightly improved glucose-stimulated insulin secretion in Min6 pancreatic β cells, and they potentiated insulinotropic actions in dysfunction β cell. This was associated with decreased β cell apoptosis because of the attenuation of endoplasmic reticulum stress. Glyceollins also potentiated GLP-1 secretion to enhance insulinotropic actions in enteroendocrine cells (Park, Ahn, Kim, Lee, Kim, & Kim, 2010). Glyceollins treatment reduced blood glucose levels in diabetic mice and prediabetic rats in oral glucose tolerance testing. The improvement was associated with increased serum insulin levels, hepatic glycogen accumulation and decreased triglyceride storage. It was proposed that glyceollins improved glucose homeostasis partly by enhancing hepatic insulin sensitivity in type 2 diabetic mice (Boue, et al., 2012; Park, Kim, Kim, Kim, & Kim, 2012).

1.4.2. Antiestrogenic and antiproliferative effect of glyceollins

Both estrogenic and antiestrogenic activities have been identified in soy phytochemicals, Daidzein, the precursor of glyceollins, is known to be weakly estrogenic. However, glyceollins have been shown to be antiestrogenic (Burow, et al., 2001; Jiang, Payton-Stewart, Elliott, Driver, Rhodes, Zhang, et al., 2010). Mechanistically, glyceollins exert greater antagonism toward ERα than ERβ in transiently transfected HEK 293

human embryonic kidney cells. It was observed that glyceollins' antiestrogenic effect on ER signaling could lead to a marked suppression of 17β-estradiol-induced proliferation in MCF-7 cells (Burow, et al., 2001). Among the three glyceollin isomers, it was shown that glyceollin I is the most potent antiestrogenic agent. Glyceollin I could effectively inhibit estrogen response element (ERE) transcription and endogenous gene expression in MCF-7 cells (Payton-Stewart, et al., 2010).

The antiestrogenic effect was further tested by Zimmermann et al. in athymic mice model, and resulted in a 53.4% and 73.1% suppression of MCF-7 and BG-1 tumor growth, respectively (Zimmermann, Tilghman, Boue, Salvo, Elliott, Williams, et al., 2010). Trefoil factor 1 and progesterone receptor were reported to be affected by glyceollins treatment, and responsible for their breast cancer protective effect (Wood, Clarkson, Appt, Franke, Boue, Burow, et al., 2006).

Furthermore, glyceollins were also noticed for the effect in suppressing tumorigenesis in triple-negative breast carcinoma MDA-MB-231 (ER-, PgR- and Her2/neu-) cells. Modest suppression of MDA-MB-231 cell tumor growth in vivo was observed upon glyceollins treatment, and a distinct change in microRNA expression profiles and proteomes in MDA-MB-231 was identified to be responsible for glyceollins' effect. This study indicated that, aside from antiestrogenic effect, glyceollins could also exert antitumor activity in triple-negative breast carcinoma cell systems via alteration of microRNA and proteomic expression profiles (Rhodes, Tilghman, Boue, Wang, Khalili, Muir, et al., 2012).

Glyceollins' antiproliferative effect was also studied in LNCaP human prostate cancer cell. It was shown that glyceollins exerted the growth inhibitory effects through

inhibition of G1/S progression and correlated with an up-regulation of CDKN1A and CDKN1B mRNA and protein levels. Furthermore, glyceollins inhibited LNCaP cell growth and cell cycle through a 17-β-estradiol-mediated event instead of an androgen-mediated event. In addition, glyceollin treatments led to down-regulated mRNA levels for androgen responsive genes (Payton-Stewart, et al., 2009).

1.4.3. Antioxidant property of glyceollins

Soy extract is known for its antioxidant capacity, and a number of phytochemicals have been identified, among them genistein and daidzein were the focus of previous studies (Slavin, Cheng, Luther, Kenworthy, & Yu, 2009; Slavin, Kenworthy, & Yu, 2009; Tripathi & Misra, 2005). Structurally, glyceollins (Fig. 2) are similar to genistein and daidzein (Fig. 1). Glyceollins have also been reported for their antioxidant activity. Kim et.al reported that glyceollins possess potent reducing ability, and can inhibit lipid peroxidation, scavenge radicals including singlet oxygen, superoxide anion, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH). In vitro model also indicated glyceollins significantly suppress H_2O_2-induced ROS production in hepa1c1c7 mouse hepatoma cells (Kim, Suh, Kim, Park, Joo, & Kim, 2010).

Additionally, glyceollins were shown to induce NADPH:quinone reductase in a dose-dependent manner in both Hepa1c1c7 mouse hepatoma and BPRc1 cells. Glyceollins also increased the expression of HO1, γ-GCL, and GR by promoting nuclear translocation of the Nrf2. Furthermore, glyceollins could upregulate phosphorylation of AKT and antioxidant response element-mediated reporter gene expression, which

indicated that glyceollins may induce Nrf2-mediated phase 2 enzyme genes through activation of the PI3K signaling pathway (Kim, di Luccio, Kong, & Kim, 2011).

1.4.4. Anti-inflammatory effects of glyceollins

Anti-inflammatory effect of glyceollins was also examined. Glyceollins (0.3 - 3 μg/mL) was able to inhibit NO production and iNOS, IL-6 and COX-2 gene expression induced by lipopolysaccharide (LPS) in RAW264.7 mouse macrophage cells. Mechanistically, glyceollins were shown to suppress the LPS-induced phosphorylation of NF-κB p65 and regulate NF-κB activity (Kim, Sung, & Kim, 2011).

1.5. Animal models for human diseases

Due to ethical concern of using human subjects in scientific research, developing and characterizing animal models that resemble human metabolism are of great importance. Despite the enormous effort and success in human cell model research, only part of the need for studying human metabolism can be met, an intact animal is needed for many areas of study (Suckling & Jackson, 1993). Especially in nutrition and food science area, food is very complex itself with the food content interacting with each other, and food-body interaction is well beyond the analysis capability of cell models. The use of an animal model makes it possible to investigate both a specific endpoint and the big picture of metabolism in a physiological context.

The major concern of employing laboratory animals is to understand the nature of the models in terms of applicability to the particular scientific questions, and also the

potential alternatives. It will be very hard to seek an animal model that is identical to human physiology in every aspect, and researches get increasingly expensive when pursuing animal models genetically closely related to human, e.g. monkey or chimpanzee, not to mention the ethical concern involved. Even though a compromise has to be made, a suitable animal model is key to the success of a scientific research. It is important to identify the structures or pathways related to the research question in the model animals, and characterize and determine the similarity between animal and human counterparts, and also understanding the limitation of the model.

In the field of dietary components' effect in cholesterol metabolism and cancer prevention, the need for animal models is well established and clearly recognized. Such complex systems that involve the digestion and absorption of the food and components, transport between different tissues, plus metabolism and effect on cholesterol profile or cancer development, cannot be studied in depth without the use of animals. The best research strategy is to couple animal models with cell culture systems to gain understanding of both the overall influence and the underlying mechanisms at different levels.

Fortunately, the basic pathways and systems of cholesterol metabolism and cancer development have been studied for decades and well defined, which makes the search of appropriate animal models a targeted endeavor.

Several types of animal models that are widely used, including a wide range of strains of mice, rats, hamsters, rabbits, and other animals. In the following parts, the choices of animal models for each specific aim will be discussed.

1.5.1. Hamster as model for cholesterol metabolisms

Since cholesterol has to be transported via lipoproteins, dysregulation of cholesterol inevitably leads to fluctuation of lipoprotein levels. Plasma lipoproteins and the metabolism are among the most studied attributes in animal models (Suckling & Jackson, 1993). Thus, the comparisons between human and animal plasma lipoprotein profile and metabolism must be made. However, studies indicated that plasma lipoproteins in model animals are significantly different from that of human. In most animal species, HDL is the major plasma lipoprotein, whereas in human LDL is the dominant lipoprotein, despite wide variations between individuals.

Table 1.1. Plasma lipoprotein profiles in model animals.

	HDL (mg/dL)	VLDL (mg/dL)	LDL (mg/dL)
Human *	~ 40	~ 60	~ 100
Swiss mouse	534	41	69
Sprague-Dawley rat	240	107	58
Dog	343	15	26
African green monkey	447	22	196
Hamster	46	20	49

(Chapman, 1986; Quig, Arbeeny, & Zilversmit, 1991)
* Due to the large discrepancy exist in human lipoprotein levels, data represent desirable lipoprotein levels recommended by American Heart Association are used for comparison.

Another major difference between human and model animals is the cholesteryl ester transfer activity associated with the cholesteryl ester transfer protein (CETP). CETP is responsible for promoting the exchange of cholesteryl ester and triglyceride between lipoproteins, which results in the net transfer of cholesteryl ester from HDL to LDL in human. This is one of the reasons that human and hamster have higher plasma LDL

(Chapman, 1986; Quig, Arbeeny, & Zilversmit, 1991). Lipoprotein metabolism in rats is very different. Rat is especially efficient in clearance of chylomicron and VLDL remnants from the circulation, which causes the low LDL levels of the rat. Additionally, CETP is absent in the rat (Oschry & Eisenberg, 1982).

As for cholesterol synthesis, rat shows the highest hepatic cholesterol synthesis rate (100%), with squirrel monkey shows about 40% of this rate, the rabbit 20%, human about 16% and the guinea pig and hamster even less. Similarly, hepatic ACAT activities were shown to be high in the rat and low in the rabbit, human and hamster (Suckling & Stange, 1985).

LDL receptor is the major component in the liver in charge of clearance of plasma LDL. LDL receptor activity is controlled and regulated qualitatively by drug or diet, but the extent to which varies considerably among species. Hamster can substantial lower its hepatic LDL receptor activity in response to dietary saturated fat, which is comparable to that in human. While the rat hepatic LDL receptor is much less sensitive (Spady, Meddings, & Dietschy, 1986).

Hamster is widely accepted as a model for lipoprotein metabolism. It has cholesteryl ester transfer activity in the plasma and has a significant amount of LDL, which is an advantage over other model animals in terms of resemblance to that of human (Chapman, 1986; Jiang, Moulin, Quinet, Goldberg, Yacoub, Agellon, et al., 1991; Quig, Arbeeny, & Zilversmit, 1991). The synthesis of cholesterol and the regulation of the LDL receptor in hamster are sensitive to dietary fat, particularly to saturated fat, in a similar way to human (Fernandez, Wilson, Conde, Vergara-Jimenez, & Nicolosi, 1999; Spady, Meddings, & Dietschy, 1986). Previous study has shown that dietary cholesterol resulted

in decreased hepatic cholesterol synthesis in hamsters (Daumerie, Woollett, & Dietschy, 1992; Woollett, Spady, & Dietschy, 1989). Between the two strains of golden Syrian hamsters, Charles River Laboratories strain (Wilmington, MA) showed a better response to dietary treatment than Bio Breeders strain (Watertown, MA) (Dorfman, Smith, Osgood, & Lichtenstein, 2003).

1.5.2. Xenograft model for prostate cancer

Xenograft model is an established model in prostate cancer research. Xenograft is established by injecting established human prostate cancer cells into nude mice, usually co-injection with matrigel to increase the success rate (Lim, Liu, Sutkowski, Braun, Lee, & Kozlowski, 1993; Pretlow, Delmoro, Dilley, Spadafora, & Pretlow, 1991). More recently, fragments of primary cell cultures from prostate carcinomas obtained from biopsies, surgery, or intraperitoneal fluid can also be used to initiate xenograft (vanWeerden, deRidder, Verdaasdonk, Romijn, vanderKwast, Schroder, et al., 1996; Wainstein, He, Robinson, Kung, Schwartz, Giaconia, et al., 1994). The success rate in nude mice, which is immunodeficient athymic that lacked T-cell-mediated immunity, was slightly lower than that in SCID mice, which lacked both T-cell-mediated and B-cell-mediated immunity (Lim, Liu, Sutkowski, Braun, Lee, & Kozlowski, 1993; Sato, Gleave, Bruchovsky, Rennie, Beraldi, & Sullivan, 1997). However, the absolute absence of adaptive immunity always poses a concern in terms of physiological relevance. Xenograft models are particularly useful in characterizing the stages of cancer progression, including the emergence of androgen-independence, and assessing the efficacy of cancer preventative treatment (Stearns, Ware, Agus, Chang, Fidler, Fife, et al., 1998). LNCaP

and PC-3 tumor cell xenograft are both well-established xenograft models (Bex, Lummen, Rembrink, Otto, Metz, & Rubben, 1999; Gridley, Andres, & Slater, 1997; Thalmann, Sikes, Wu, Degeorges, Chang, Ozen, et al., 2000; Tymchuk, Barnard, Heber, & Aronson, 2001). Normally, the xenograft can retain the cytogenetic, biologic, and molecular features of the original cancer, even after multiple passages. Also, mouse-derived endothelial cells have been identified between the intravascular space and human tumor cells, suggesting contribution of mouse tissues to the vasculature of the tumor growth (Lehr, Skelly, Buhler, Anderson, Delisser, & Gown, 1997). Thus, xenograft model is suitable for investigating tumor growth, as well as the involvement of angiogenesis and other physiological events.

1.6. Real-time polymerase chain reaction

Polymerase chain reaction (PCR) is a basic molecular biology laboratory technique that amplifies a targeted DNA sequence, and real-time polymerase chain reaction is a PCR technique that simultaneously amplifies and quantifies the targeted DNA. The procedure of real-time PCR follows the general principle of polymerase chain reaction. The key feature of real-time PCR is that the amplification of DNA is detected and quantified at the end of each thermal cycle, comparing to the standard PCR, in which the PCR product is detected at the end of the entire reaction. Common detection methods include: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, such as SYBR Green method, and (2) sequence-specific DNA probes consisting of oligonucleotides, which is designed to hybridize with the complementary sequence in

target DNAs, and a fluorescent reporter, which can be detected only after hybridization, such as Taqmen method.

Real-time PCR is mainly used to provide quantitative measurements of gene transcription. By quantifying the density of fluorescence which is proportional to the concentration of DNA molecules, real-time PCR can be used to determine the genetic expression (such as messenger RNA) level of a particular gene changes over time, such as in the response of tissue and cell cultures to a phytochemical treatment, progression of cell differentiation, or in response to changes in environmental conditions. Real-time PCR can also be used to identify the microorganism by targeting species-specific DNA sequences and quantify the absolute copy number of target DNAs in the sample. The copy number of DNAs can be further calculated to correlate or represent the number of cells or bacteria.

The overarching goal of this research is to advance our understanding of potential health beneficial effect of soy. Based on existing evidence, we hypothesize that components from soy may exert effects on multiple chronic diseases including diet-induced metabolic syndrome and cancers. We focus on examining the effects of the novel soy phytochemicals glyceollins on 1) alleviating metabolic and inflammatory disorders resulting from high fat/cholesterol diet and 2) effect on prostate cancer prevention. The specific aims for this proposal are:

Specific Aim 1: To test the hypothesis that glyceollins can lower diet induced increase in cholesterol levels in plasma and liver. Soy is known to modulate cholesterol level and prevent cardiovascular diseases. However, the active component(s) and the underlying mechanism remain unclear. Existing literature suggested glyceollins as a candidate for soy's cholesterol-lowering effect. This hypothesis will be tested using a hamster model of diet induced metabolic dysregulation. Hamsters fed high-fat diet with or without glyceollins supplementation will be assessed for glyceollins' ability to modulate cholesterol and lipid profiles in plasma and liver. Molecular analysis of lipid/cholesterol metabolisms will be determined to elucidate mechanisms of action.

Specific Aim 2: To test the hypothesis that glyceollins can prevent prostate cancer. Our previous study revealed that glyceollins inhibit androgen responsive LNCaP prostate cancer cell growth in culture through inhibition of androgen-dependent pathways. The effect of glyceollins in vivo is not known. A prostate cancer cell xenograft model will be used to test the effects of glyceollins. The following questions will be

addressed: 1) whether glyceollins differentially affect androgen-dependent and androgen-independent cell-derived tumor, and 2) what is the molecular mechanisms of action of glyceollins. Marker gene expressions in proliferation, angiogenesis, and androgen responsive pathways will be determined to elucidate these questions.

Specific Aim 3: To test the hypothesis that glyceollins supplementation will affect gut microbiome composition or population. Human microbiome is a very large and complex ecosystem, which has been shown to influence human development and health. Recent studies indicated that short and long-term diet can influence the structure and activity of the human microbiome. Glyceollins are a group of phytoalexins, which is synthesized by plants to protect against pathogenic attack. In this study, the influence of low and high-fat and glyceollins supplemented diet on microbiome in animal models will be studied. Certain predominant species of gut bacteria will be selected, and their temporal changes will be measured.

Chapter 2: Lipid and cholesterol-lowering activity of soy-derived glyceollins

Huang, Xie, Boue, Bhatnagar, Yokoyama, Yu, Wang. (2013) Cholesterol-Lowering Activity of Soy-Derived Glyceollins in the Golden Syrian Hamster Model. *Journal of Agricultural and Food Chemistry* 61 (24), 5772-5782.

2.1. Abstract

Hypercholesterolemia is one of the major factors contributing to the risk of cardiovascular disease (CVD), which is the leading cause of death in the developed countries. Consumption of soy foods has been recognized to lower the risk of CVD, and phytochemicals in soy are believed to contribute to the health benefits. Glyceollin is one of the candidate phytochemicals synthesized in stressed soy that may account for many unique biological activities. In this study, the in vivo cholesterol-lowering effect of glyceollins was investigated. Male golden Syrian hamsters were fed diets including 1) 36 kcal% fat diet, 2) 36 kcal% fat diet containing 250 mg/kg diet glyceollins, or 3) chow for 28 days. Hepatic cholesterol esters and free cholesterol, hepatic total lipid content, plasma lipoproteins, fecal bile acid, fecal total cholesterol, and cholesterol metabolism related gene expressions were measured. Glyceollins supplementation led to a significant reduction of plasma VLDL, hepatic cholesterol esters and total lipid content. Consistent with changes in circulating cholesterol, glyceollins supplementation also altered

expression of the genes related to cholesterol metabolism in the liver. In contrast, no change in plasma LDL and HDL, and fecal bile acid or cholesterol content was observed. The cholesterol-lowering effect of glyceollins appeared not to go through the increase of bile excretion. These results supported glyceollins' role as a novel soy-derived cholesterol-lowering phytochemical that may contribute to soy's health effects.

2.2. Introduction

Hypercholesterolemia is one of the major factors contributing to the onset and progression of cardiovascular diseases (CVD), which is the leading cause of death in the adult population of industrialized societies (Ortega, Palencia, & Lopez-Sobaler, 2006). It is estimated that, by 2020, CVD will continue to be the leading cause and account for 37% of all deaths (Thomsen, Hansen, Christiansen, Green, & Berger, 2004). Therefore, it is imperative to develop preventive strategies against this disease. Besides genetic predisposition, increased consumption of high-fat and high cholesterol diets add to the risk of CVD (German, Xu, Walzem, Kinsella, Knuckles, Nakamura, et al., 1996; Lecerf & de Lorgeril, 2008). Mechanistically, previous studies have revealed that diet may modulate oxidative status and chronic inflammation, which may play a pivotal role in atherosclerosis (Hansson, 2009; Kaperonis, Liapis, Kakisis, Dimitroulis, & Papavassiliou, 2006). Dietary intervention would be an economical and efficient preventive measure.

Soy is one of the primary agricultural commodities in the United States (Slavin, Cheng, Luther, Kenworthy, & Yu, 2009). Soy and soy-derived foods are well-known for their health effects and are a rich source of health-promoting bioactive phytochemicals (Friedman & Brandon, 2001). Previous research indicated that soy possesses cholesterol-

lowering effects (Messina & Messina, 2010; Tripathi & Misra, 2005), but the precise mechanism and the active components remain unclear (GatchalianYee, Arimura, Ochiai, Yamada, & Sugano, 1997; Wilson, Nicolosi, Kotyla, & Fleckinger, 2007). Glyceollins (Fig. 2.1), which are synthesized in response to environmental stresses, such as infection, are a family of phytoalexins isolated from soy and possess antibiotic activity. Soy phytoalexins also include bioactive isoflavones such as genistein and daidzein (Fig. 2.1) (Aggarwal, Takada, & Oommen, 2004).

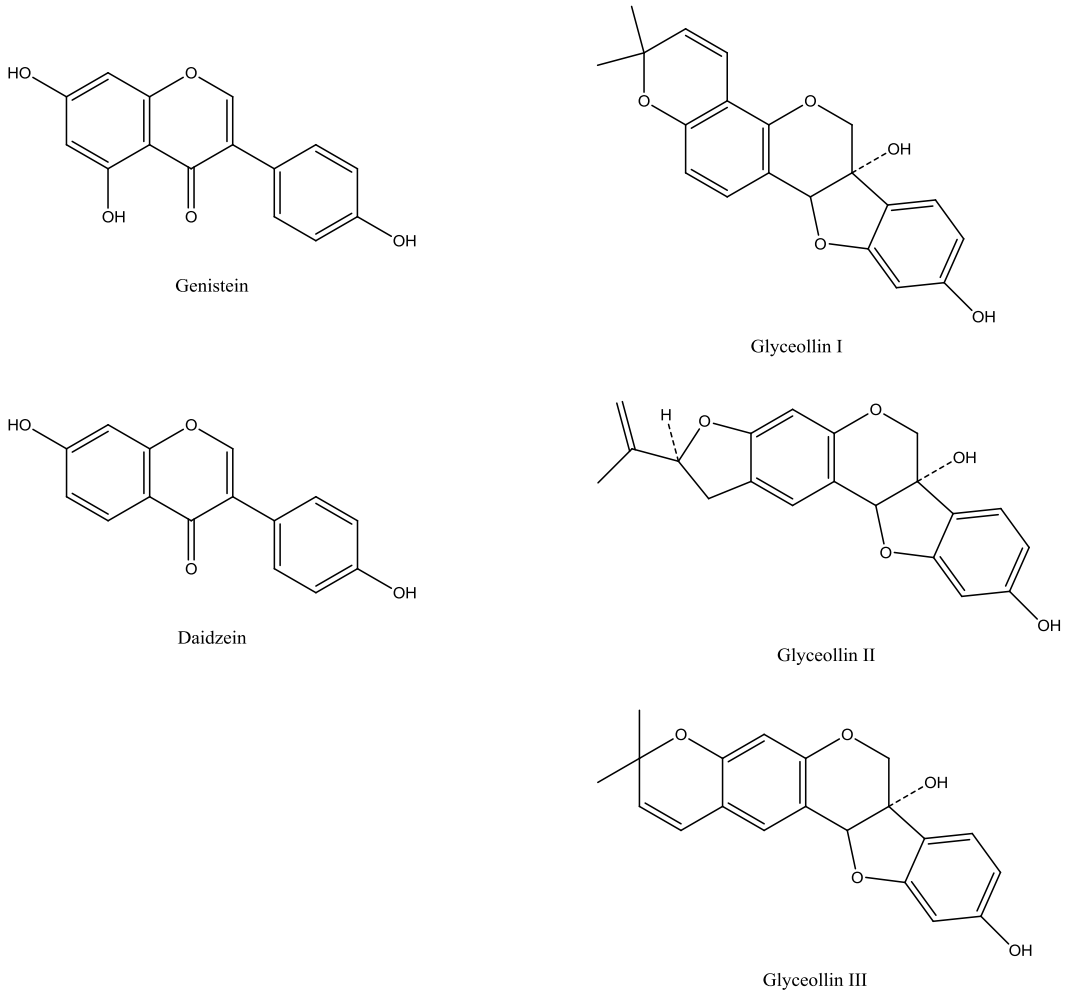

Fig. 2.1. Structures of glyceollins (I, II, and III) and soy phytochemicals genistein, daidzein.

Glyceollins have been reported to exhibit antitumor (Lee, et al., 2010; Payton-Stewart, et al., 2010; Salvo, et al., 2006), antiestrogenic (Burow, et al., 2001), antibacterial (Weinstein & Albersheim, 1983), antinematode (Huang & Barker, 1991; Veech, 1982), antifungal (Lee, et al., 2010; Lozovaya, Lygin, Zernova, Li, Hartman, & Widholm, 2004), antidiabetic (Boue, et al., 2012; Park, Ahn, Kim, Lee, Kim, & Kim, 2010; Park, Kim, Kim, Kim, & Kim, 2012) and vasodilatory effects in rat (Song, et al., 2010). Glyceollins were also reported to possess antioxidant and anti-inflammatory effect (Kim, di Luccio, Kong, & Kim, 2011; Kim, Suh, Kim, Park, Joo, & Kim, 2010; Kim, Sung, & Kim, 2011). The antioxidant and anti-inflammatory properties of glyceollins suggest the potential of glyceollins to prevent, delay or treat cardiovascular condition. Hence, accumulating evidence suggest that glyceollins have the potential to be health promoting phytochemicals and deserve further investigation and characterization. Additionally, the contribution of glyceollins to the cholesterol and lipid lowering-effect of soy has not been reported.

The golden Syrian hamster (Mesocricetus auratus) is widely accepted as a suitable animal model for studying human cholesterol metabolism (Chapman, 1986; Suckling & Jackson, 1993). The lipid profiles and susceptibility to dietary cholesterol of golden Syrian hamster is similar to human (Arbeeny, Meyers, Bergquist, & Gregg, 1992; KrisEtherton & Dietschy, 1997; Suckling & Jackson, 1993). The current study takes advantage of this model to test the hypothesis that glyceollins may exert cholesterol and lipid-lowering effect. Hamsters were fed a high-fat diet or a glyceollins supplemented high-fat diet. Plasma lipid profiles, liver, fecal lipid contents and microbiome, the expression level of cholesterol and lipid metabolism related genes in the liver were

determined to elucidate the cholesterol-lowering activity of glyceollins and the potential mechanisms involved.

2.3. Materials and methods

Animals and diets. Male golden Syrian hamsters (approximately 80 g, LVG strain, Charles River, Wilmington, MA, USA) were given free access to water and rodent chow to acclimatize to the environment for 1 week prior to the experiment. For the experiment, hamsters were fed a high-fat diet (36 kcal% fat diet), a high-fat diet with supplemental glyceollins supplement (36 kcal% fat diet containing 250 mg/kg diet glyceollins.), or chow (8728C Teklad Certified Rodent Diet, Harlan Laboratories, Inc, Frederick, MD, USA). The experimental diets were formulated and purchased from Research Diets, Inc., New Brunswick, NJ, USA. Animals (10 per group) were fed with the respective diet for 4 weeks with water available ad libitum. Food intake was recorded twice a week and body weights were measured weekly. Diets consisted of 18% protein, 45% carbohydrate, and 36% fat on a caloric basis supplemented with 0.14% cholesterol. Diet compositions are listed in Table 2.1. Glyceollins were given as a mixture of 68% glyceollin I, 21% glyceollin II, and 11% glyceollin III. Glyceollins mixture was isolated and purified as described previously (Payton-Stewart, et al., 2009; Virgilo A. Salvo, Boué, Fonseca, Elliott, Corbitt, Collins-Burow, et al., 2006). The animal use and care protocol (Protocol # 10-014) for this study was reviewed and approved by the USDA, ARS, Beltsville Area Animal Care and Use Committee (BAACUC).

Table 2.1. Diet compositions

	High-fat[1]		High-fat+ Glyceollins[1]		Chow[2]	
	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	21.0	18.0	21.0	18.0	24.3	32.0
Carbohydrate	52.0	45.0	52.0	45.0	40.2	54.0
Fat	19.0	36.0	19.0	36.0	4.7	14.0
Others					23.8	
Total		100.0		100.0		100.0
kcal/gm	4.58		4.58		3.00	
Ingredient	gm	kcal	gm	kcal	gm	kcal
Casein	222.0	888.0	222.0	888.0		
DL-Methionine	3.0	12.0	3.0	12.0		
Corn Starch	453.0	1812.0	453.0	1812.0		
Maltodextrin	100.0	400.0	100.0	400.0		
Sucrose						
Cellulose	53.0	0.0	53.0	0.0		
Corn Oil	100.0	900.0	100.0	900.0		
Butter	80.0	720.0	80.0	720.0		
Menhaden Oil	20.0	180.0	20.0	180.0		
Mineral Mix	45.0	40.0	45.0	40.0		
Choline Bitartrate	3.0	0.0	3.0	0.0		
Cholesterol	1.5	0.0	1.5	0.0	0.05	0.0
Glyceollins	0.0	0.0	0.27	0.0	0.0	0.0
Total	1080.5	4952.0	1080.5	4952.0	1000.0	3000.0

[1]High-fat and High-fat+Glyceollins diets were formulated and purchased from Research Diet (New Brunswick, NJ); [2]Chow diet was purchased from Harlan Laboratories (Frederick, MD).

Plasma, tissue and fecal sample collection. Hamsters were subject to 12 h fasting prior to sacrifice and anesthetized with CO_2. Blood was collected by cardiac puncture with syringes previously rinsed with potassium EDTA solution (15% w/v), and plasma was separated after centrifugation at 1500 rpm for 30 min at 4 °C. Livers and adipose tissues were collected, and one part of the tissue immediately frozen in liquid nitrogen for analysis, the other part was preserved in RNA Stabilization Solution was

purchased from Ambion (Austin, TX, USA) and kept at -80 °C. At the end of the fourth week, 48 h fecal samples were collected from the bottom of the cage and kept at -80 °C.

Plasma lipoprotein analysis. Plasma lipoprotein cholesterol concentrations were determined by size exclusion chromatography as previously described (German, et al., 1996). Briefly, an Agilent 1100 chromatograph was employed with a postcolumn derivatization reactor, consisting of a mixing coil (1615-50 Bodman, Aston, PA, USA) in a temperature-controlled water jacket (Aura Industrials, Staten, NY, USA). A Hewlett-Packard (Agilent, Palo Alto, CA, USA) HPLC pump 79851-A was used to deliver cholesterol reagent (Roche Diagnostics, Indianapolis, IN, USA) at a flow rate of 0.2 mL/min. Bovine cholesterol lipoprotein standards (Sigma Aldrich, St. Louis, MO, USA) were used to calibrate the signal on the basis of peak areas. 15 µL of plasma was injected via an Agilent 1100 auto sampler onto a Superose 6HR HPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). The lipoproteins were eluted with a pH 7.0 buffer solution containing 0.15 M sodium chloride and 0.02% sodium azide at a flow rate of 0.5 mL/min. Plasma lipoprotein concentration was calculated based on a standard curve.

Liquid chromatography-mass spectrometry analysis of plasma concentration of glyceollins. LC-ESI-MS and LC-ESI-MS/MS analyses were conducted on an Agilent 1100 series LC system (Agilent, Santa Clara, CA) coupled to a TSQ Vantage™ Triple Quadrupole Mass Spectrometer (Thermo Scientific, Middletown, VA). Separation was performed on an Agilent Eclipse XDB C18 column (4.6 × 150 mm ID, 5 µm). Ten microliters was injected onto the column held at 25 °C. The binary mobile phase consisted of mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid). The gradient was 0-4 min 30% A to 2% A and hold

at 2% A for 10 min; 14-16 min 2% A to 30% A and hold at 30% A for 7 min; the flow rate was 0.200 mL/min. The UV absorbance detector was set at 285 nm. For positive ion LC-ESI-MS and LC-ESI-MS/MS analyses, electrospray parameters were set at the following: sheath gas pressure of 60 psi, ionspray voltage of 3500 V, auxiliary gas pressure of 15 psi, collision gas pressure of 1.5 mTorr, capillary temperature of 268 °C. Declustering potential and collision energy were maintained at -12 V and 16 eV, respectively. Parent and product mass scan were performed at 339.000 and 229.042.

Hepatic lipid extraction. Livers were excised and immediately frozen in liquid nitrogen, and then stored at –80 ºC prior to analysis. The extraction method was modified from Folch method (Ametaj, Bobe, Lu, Young, & Beitz, 2003; Folch, Lees, & Sloane Stanley, 1957). Approximately 0.15 g of frozen liver was minced and transferred into a test tube. 6 mL of chloroform/methanol (2:1, v/v) was then added, followed by a 2 min homogenization and 30 s of sonication at 30% power level. Samples were then incubated with shaking for 2 h on a platform shaker. After incubation, 2 mL of double distilled water was added. Samples were then centrifuged for 20 min at 500 g. After centrifugation, the bottom layer was carefully aspirated into a new test tube and incubated overnight. It was then filtered through a 0.22 µm filter and dried by stream of nitrogen. The dried lipid was weighed and redissolved in isopropanol with 10% Triton X-100 and used for triglyceride and cholesterol analysis as described below.

Triglyceride, total cholesterol and free cholesterol in the liver. Hepatic triglyceride, total cholesterol and free cholesterol were enzymatically determined using commercial kits (Triglyceride-SL, Genzyme Diagnostics PEI Inc., PE, Canada;

Cholesterol E and Free Cholesterol E, Wako Chemicals, Richmond, VA, USA) following manufacturer's protocols.

Fecal bile acids and cholesterol extraction. Fecal bile acids and cholesterol were extracted using a modified protocol (Allen, Bristow, & Yu, 2004). Fecal samples were collected during 48 h period on days 26-28 after initiation of the experiment. The samples were lyophilized, pulverized using pestle and mortar, and weighed. A dried fecal sample (0.10 g) was hydrolyzed in 1.0 mL of 2 M KOH at 50 °C for 5 h. The cooled mixture was then extracted with two 6-mL portions of diethyl ether to remove nonsaponifiable components. Subsequently, 1 mL of 20% sodium chloride followed by 0.2 mL of 12 M hydrochloric acid was added to the remaining mixture. The acidified mixture was extracted with two 6-mL portions of diethyl ether, and the pooled ether extracts were evaporated by nitrogen and redissolved in 0.5 mL of ethanol. The samples were used for fecal bile acid and cholesterol determination as follows.

Fecal bile acids and cholesterol analysis. Fecal bile acid content was enzymatically determined by 3α-hydroxysteroid dehydrogenase (3α-HSD) (Liu, Wang, Yao, Gao, & Yu, 2010). β-nicotinamide adenine dinucleotide hydrate (NAD), nitroblue tetrazolium chloride (NBT), diaphorase, 3α-HSD, and cholic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). NAD, NBT, diaphorase, and 3α-HSD were prepared in 0.01 M phosphate buffer at pH 7.0. The reaction mixture included 40 μL of sample or standard with 4 μL of Triton X-100, 50 μL of NAD (2.5 mM), 50 μL of NBT (0.61 mM), 50 μL of diaphorase (625 U/L), 50 μL of 3α-HSD (625 U/L). The mixture was incubated for 60 min at ambient temperature, after which 40 μL of phosphoric acid (1.33 M) was added to stop the reaction. The absorbance of each reaction mixture was

measured at 530 nm. Cholic acid in ethanol was used to generate a standard curve, and the amount of fecal bile acid obtained was determined using the standard curve. Cholesterol was determined by the same assay as for liver described above.

Total RNA isolation, cDNA synthesis and gene expression analysis from liver and adipose tissue. To determine the gene expression changes, liver and adipose preserved in RNALater were cut into 0.1 to 0.2 g pieces and homogenized using a Precellys 24 (Bertin Technologies, Villeurbanne, France). RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) were used for total RNA isolation for liver and adipose, respectively. StrataScript First Strand complementary DNA Synthesis kit from Stratagene (Santa Clara, CA, USA) was used to reverse transcribe complementary DNA. Real-time PCR was performed on an Applied Biosystems 7900HT Sequence Detection System using Fast SYBR Green Master Mix by Applied Biosystems (Carlsbad, CA, USA). Primers used in this study are listed in Table 2.2. Relative mRNA expression levels were calculated using the delta Ct method (J. S. Yuan, Reed, Chen, & Stewart, 2006). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as the house-keeping gene for calculations.

Table 2.2. Sequences of Real-time PCR primers

Genes	Direction	Sequence (5'-3')
SYBR Green primers		
GAPDH	Forward	GAACATCATCCCTGCATCCA
	Reverse	CCAGTGAGCTTCCCGTTCA
HMGCoAR	Forward	CGAAGGGTTTGCAGTGATAAAGGA
	Reverse	GCCATAGTCACATGAAGCTTCTGTA
LDLR	Forward	TGAGGAACATCAACAGCATAAAC
	Reverse	ATCCTCCAGGCTGACCATCTGT
LXRα	Forward	ATTGCCATCAGCATCTTCTCT
	Reverse	GCATCCGTGGGAACATCAGT
PPARα	Forward	CTCCACCTGCAGAGCAACCA
	Reverse	CGTCAGACTCGGTCTTCTTGAT
ABCG5	Forward	TGATTGGCAGCTATAATTTTGGG
	Reverse	GTTGGGCTGCGATGGAAA
ABCG8	Forward	TGCTGGCCATCATAGGGAG
	Reverse	TCCTGATTTCATCTTGCCACC
CYP7A1	Forward	GGTAGTGTGCTGTTGTATATGGGTTA
	Reverse	ACAGCCCAGGTATGGAATCAAC
CYP51	Forward	GAGAGAAGTTTGCCTATGTGCC
	Reverse	TGTAACGGATTACTGGGTTTTCT
SREBP	Forward	GCGGACGCAGTCTGGG
	Reverse	ATGAGCTGGAGCATGTCTTCAAA
FA Synthase	Forward	AGCCCCTCAAGTGCACAGTG
	Reverse	TGCCAATGTGTTTTCCCTGA
ACOX	Forward	TTACATGCCTTTGTTGTCCCTATC
	Reverse	CGGTAATTGTCCATCTTCAGGTA
IL-1β	Forward	GGTTGAATCTATACCTGTCCTGTGTG
	Reverse	TTTTCCATCTTCTTCTTTGGGTATT
IL-6	Forward	AGACAAAGCCAGAGTCATT
	Reverse	TCGGTATGCTAAGGCACAG
TNF-α	Forward	AACGGCATGTCTCTCAA
	Reverse	AGTCGGTCACCTTTCT
TGF-β	Forward	ACGGAGAAGAACTGCT
	Reverse	ACGTAGTACACGATGGG
IFN	Forward	GGCCATCCAGAGGAGCATAG
	Reverse	CCATGCTGCTGTTGAAGAAGTTAG
TaqMan primers		
GAPDH	Forward	GAACATCATCCCTGCATCCA
	Reverse	CCAGTGAGCTTCCCGTTCA
	Probe	CTTGCCCACAGCCTTGGCAGC
LPL	Forward	TTTAACTACCCCCTGGACAATGTC
	Reverse	ACCTTCTTGTTGGTCAGACTTCCT
	Probe	AGCCTTGGAGCCCACGCTGCT

Statistical analysis. All end point assays for each sample were conducted in triplicate, and the average was used for group analysis, data for each treatment group were presented as mean ± standard error. Significance level of differences in means was detected using one-way ANOVA and Tukey's test. Statistics analysis was performed using IBM SPSS Statistics 19.0 (2010, IBM Corporation, Armonk, NY, USA) or Graphpad Prism 6 (2012, Graphpad Software, La Jolla, CA, USA). Statistical significance was defined at $p \leq 0.05$.

2.4. Results

Body Weight and Food Intake. There was no difference in body weight or body weight gain between any of the diet treatments during the experimental period (Table 2.3). Food intake was significantly higher in the group fed with chow diet, but caloric intake was higher in the high-fat diet, and high-fat diet supplemented with glyceollins groups due to their higher fat content (Table 2.3).

Table 2.3. Effect of different diets on body weight and food intake

	High-fat	Glyceollins	Chow
body weight (g)	95.83 ± 5.30^{a}	95.91 ± 4.04^{a}	96.87 ± 3.94^{a}
body weight gain (g)	17.48 ± 4.98^{a}	18.95 ± 6.54^{a}	20.83 ± 3.97^{a}
food intake (g/day)	5.82 ± 0.43^{a}	5.89 ± 0.60^{a}	7.59 ± 0.31^{b}
calorie intake (kcal/day)	26.67 ± 1.97^{b}	26.97 ± 2.76^{b}	22.76 ± 0.93^{a}

Body weight and food intake was presented as mean ± SEM (n = 10). Significance level of differences in means was detected using one-way ANOVA and Tukey's test. Numbers marked with different letter are significantly different from each other at $p \leq 0.05$.

Plasma Lipoprotein Cholesterol Content. Compared to the chow diet, consumption of the high-fat diet significantly elevated very low-density lipoprotein

(VLDL) (366%), low-density lipoprotein (LDL) (482%), and total lipoprotein levels (51%) in hamster plasma (Fig. 2.2). Animals on high-fat diet supplemented with glyceollins showed significantly lower (30% less) VLDL than animals on the high-fat diet. We also found a trend in reduction of LDL by 19% and total lipoprotein level by 9% in the animals fed with glyceollins, however reductions in LDL and total lipoprotein level did not reach statistical significance (Fig. 2.2). There was no difference in high-density lipoprotein (HDL) level between the animals on different diets.

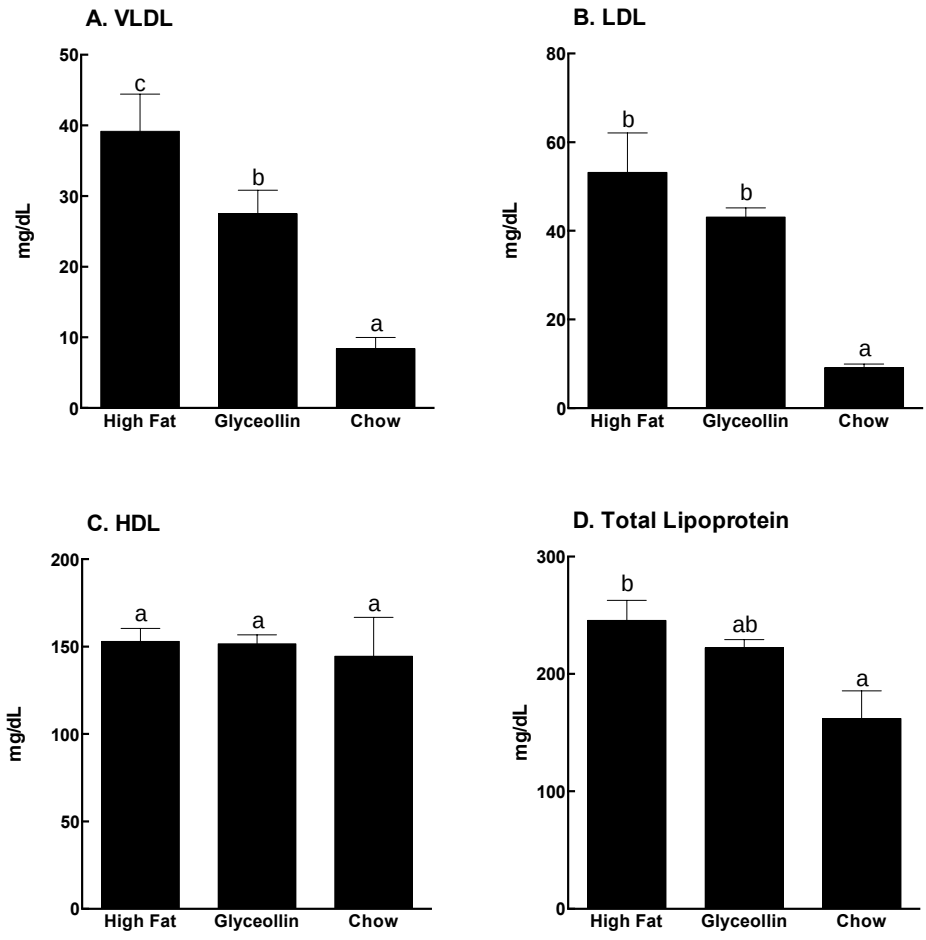

Fig. 2.2. Effects of glyceollins supplement on plasma lipoprotein cholesterol level. Hamster plasma from different diet groups was harvested and Plasma lipoprotein cholesterol concentrations were determined by size exclusion chromatography as

described in Material and Methods. A) Very Low-Density Lipoprotein (VLDL), B) Low-Density Lipoprotein (LDL), C) High-Density Lipoprotein (HDL), and D) Total Lipoprotein. Each column represents the mean ± SEM (n = 10). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

Cholesterol and Lipid Content of Liver. In animals fed the high-fat diet, hepatic cholesterol esters and free cholesterol increased 759% and 27%, respectively, compared to that of animals on the chow diet, and cumulatively resulted in 266% increase in total cholesterol (Fig. 2.3). Glyceollins supplementation in the high-fat diet reduced hepatic cholesterol esters and free cholesterol by 20% and 14% (Fig. 2.3B, 2.3C). Overall, the total lipid content in liver of high-fat diet animals was 82% higher than those on chow diet (Fig. 2.3E). Total lipid in the liver of glyceollins supplemented animals were 18% lower than high-fat diet fed animals. There were no differences in hepatic triglyceride level between the animals from different diet groups (Fig. 2.3D). Consistent with lower lipid level in glyceollins treated animals, we also observed that the livers of the glyceollins fed animals appeared dark red, smooth and more similar to that of chow fed animal, whereas livers in high-fat diet animals were pale and spotted (data not shown).

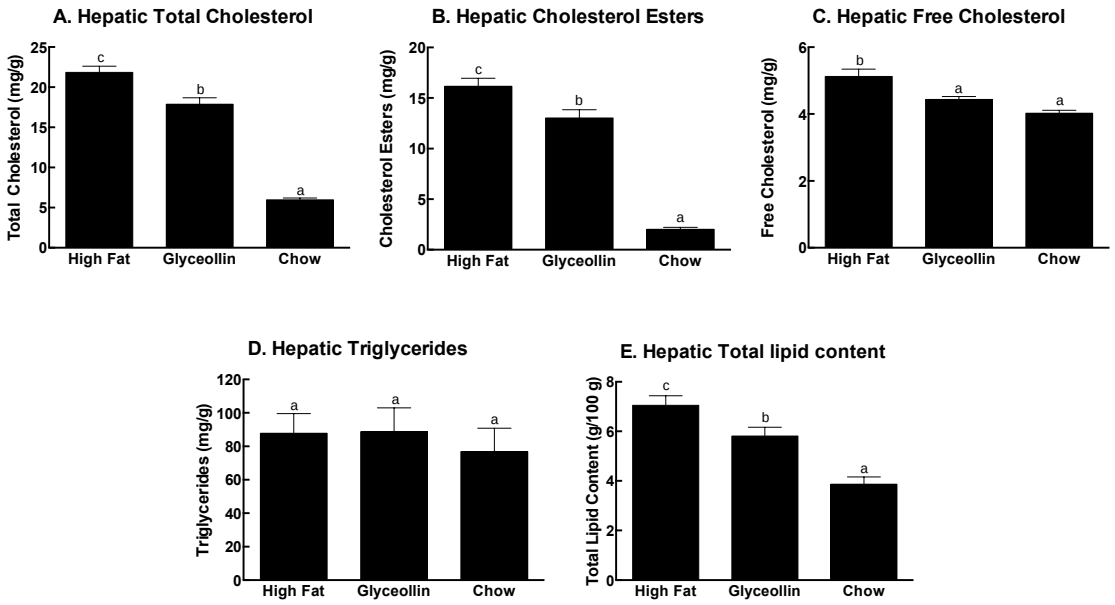

Fig. 2.3. Effects of glyceollins on hepatic triglyceride and cholesterol level. Livers were harvested from animals on different diets and hepatic lipid extracted and enzymatically determined as described in Materials and Methods. A) Hepatic Total Cholesterol, B) Hepatic Cholesterol esters, C) Hepatic Free Cholesterol, D) Hepatic Triglycerides, and E) Hepatic Total Lipid Content. Each column represents the mean ± SEM (n = 10). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

Bile Acid and Cholesterol Content of Feces. There was no significant difference between the diet groups for fecal bile acid content (Fig. 2.4). Compared to animals on the chow diet, higher fecal total cholesterol (170%) levels were detected in high-fat diet fed animals, but there was no difference between animals fed with or without glyceollins.

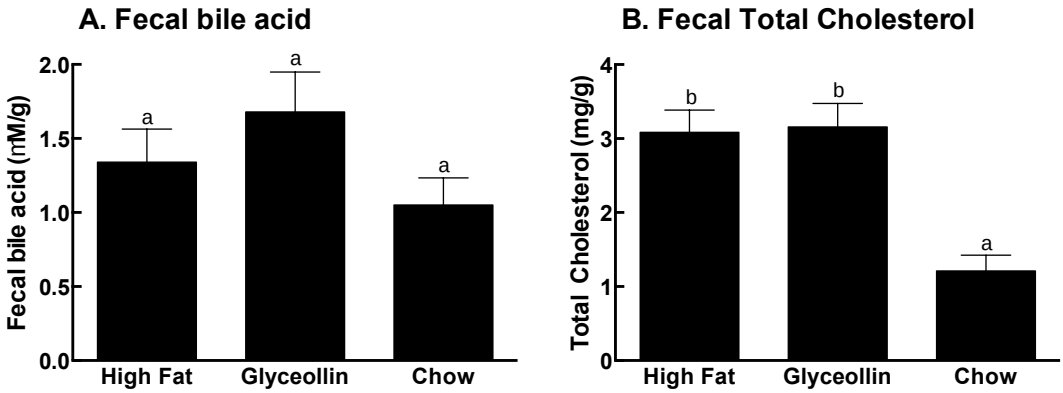

Fig. 2.4. Effects of glyceollins on fecal bile acid and cholesterol level. Two-day fecal samples were collected for animals on different diets and fecal bile acid and total cholesterol was extracted and determined as described in Materials and Methods. A) Fecal Bile Acid, and B) Fecal Total Cholesterol. CA equiv stands for cholic acid equivalents. Each column represents the mean ± SEM (n = 10). Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

Relative Expression of Genes Related to Cholesterol and Bile Acid Metabolism. Expression of hepatic genes related to cholesterol, bile acid, and fatty acid metabolism were determined to elucidate potential mechanisms of action. There was no difference in the expression of the mRNA levels of LDL receptor, which binds to LDL particles in circulation, between animals fed with different diets (Fig. 2.5A). Compared to the chow diet, the high-fat diet significantly decreased the hepatic mRNA level of the HMG-CoA reductase (32%) (Fig. 2.5B), which is the rate-limiting enzyme of the mevalonate pathway involved in cholesterol synthesis. Animals fed the glyceollins supplemented diet showed a similar decrease in hepatic HMG-CoA reductase as animals fed the high-fat diet. Another rate limiting enzyme in cholesterol synthesis, CYP51, which converts lanosterol to cholesterol, was down-regulated ~80% in high-fat diets with or without glyceollins supplemented as compared to chow diet (Fig. 2.5C). Liver X receptor (LXR) α and peroxisome proliferator activated receptor (PPAR) α, transcription

factors regulating fatty acid β-oxidation, did not exhibit differences between the diets (Figs. 2.5D, 2.5E).

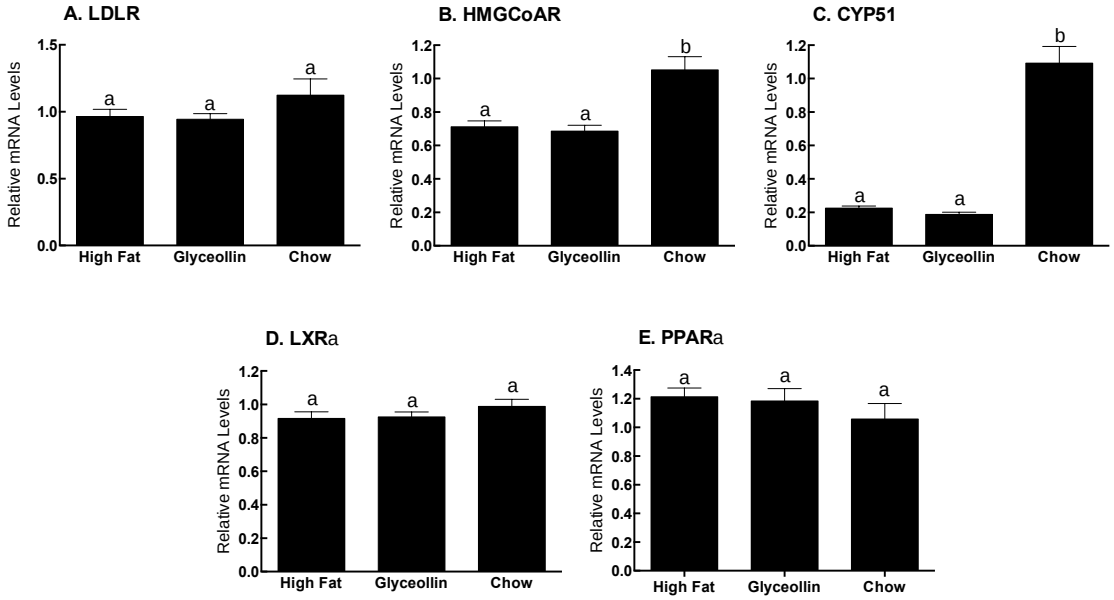

Fig. 2.5. Effects of glyceollins on liver LDL receptors, cholesterol synthesizing enzymes, and cholesterol metabolizing transcription factors mRNA levels. Livers were harvested from animals on different diets, total mRNA extracted and mRNA level determined using Real Time PCR as described in Materials and Methods A) liver LDL receptor, B) HMGCoAR, C) CYP51, D) LXRα and E) PPARα gene. Results express as relative expression levels (mean ± SEM, n = 10) to chow diet. Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

ABCG5 and ABCG8 function as half-transporters to limit intestinal absorption and promote biliary excretion of sterols. High-fat diet significantly elevated both ABCG5 and ABCG8 mRNA expression by 309% and 128%, respectively (Figs. 2.6A, 2.6B). Animals fed glyceollins supplemented diet expressed significantly lower levels of ABCG5 (29%), and ABCG8 (25%) mRNA as compared to the high-fat diet (Figs. 2.6A, 2.6B). CYP7A1 encoding the enzyme for the initial rate-limiting step of bile acid synthesis, was increased by 63% in glyceollins supplemented diet, though did not reach

statistical significance (Fig. 2.6C), while high-fat diet and chow diet exhibited similar expression level of CYP7A1.

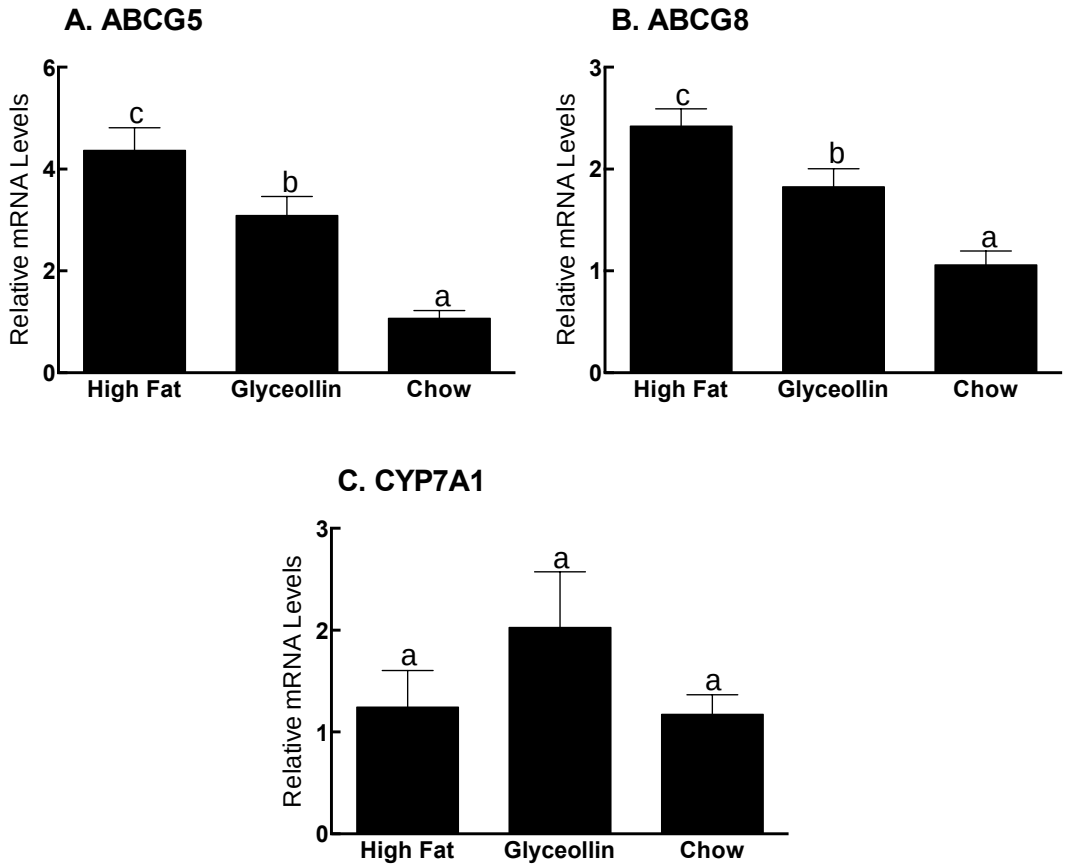

Fig. 2.6. Effects of glyceollins on liver cholesterol transporting and catabolizing enzymes mRNA levels. Livers were harvested from animals on different diets, total mRNA extracted and mRNA level determined using Real Time PCR as described in Materials and Methods A) ABCG5, B) ABCG8, and C) CYP7A1. Results were expressed as relative expression levels (mean ± SEM, n = 10) to chow diet. Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

The mRNA levels of fatty acid synthase (FAS) and acyl-CoA oxidase (ACOX), genes encoding the rate-limiting enzymes in peroxisomal β-oxidation, were not affected by the different diets (Figs. 2.7A, 2.7B). Sterol Regulatory Element-Binding Protein (SREBP)-1c is a transcription factor that binds to the sterol regulatory element DNA

sequence upstream of genes for fatty acid biosynthesis. Animals on the high-fat diet, with or without glyceollin, had higher hepatic SREBP-1c expression (~170%) than animals on chow diet (Fig. 2.7C).

The LPL gene encodes lipoprotein lipase, which has the dual functions of triglyceride hydrolase and ligand bridging factor for receptor-mediated lipoprotein uptake. In this study, LPL expression level in liver was elevated in the high-fat diet by 158%, while glyceollins supplementation reduced liver LPL by 27% (Fig. 2.7D). On the other hand, adipose LPL mRNA levels were significantly lower (35%) in animals that consumed glyceollins as compared to animals on the high-fat or chow diets (Fig. 2.7E).

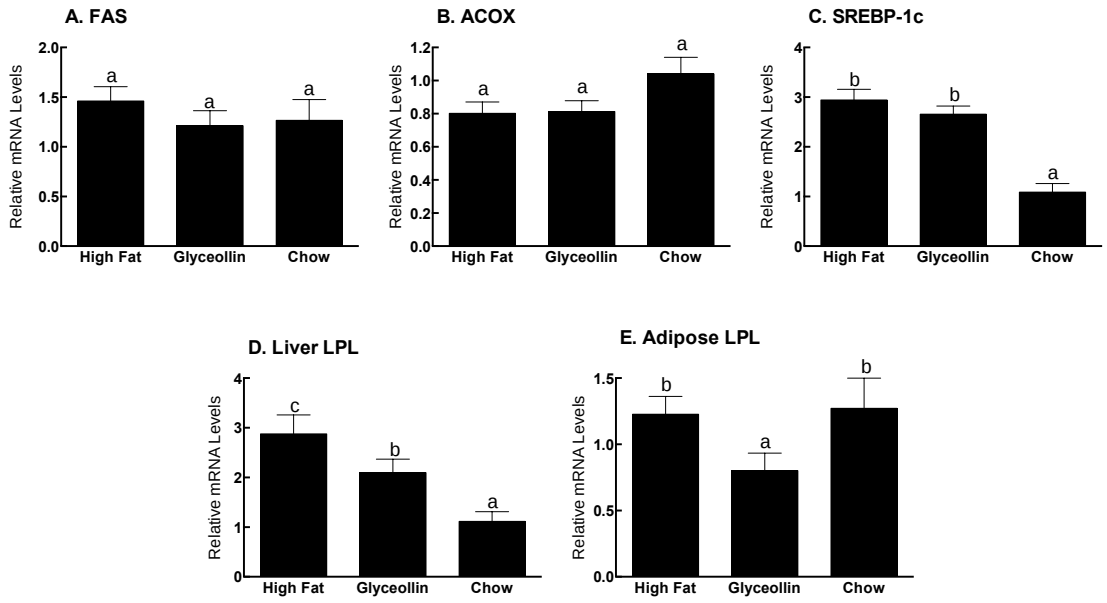

Fig. 2.7. Effects of glyceollins on lipid metabolizing genes in liver and adipose tissue. Livers and adipose tissues were harvested from animals on different diets, total mRNA extracted and mRNA level determined using Real Time PCR as described in Materials and Methods. A) FA synthase, B) ACOX, C) SREBP, D) Liver LPL, and E) Adipose LPL. Results were expressed as relative expression levels (mean ± SEM, n = 10) to chow diet. Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

Glyceollins Reduced Inflammation Cytokine Expressions in the Liver. In liver, we observed inhibition of high-fat diet induced increase in IL-6 and TGF-β in the animals supplemented with glyceollins (Fig. 2.8). TGF-β in particular, as described above, is an important regulator of hepatic stellate cells and liver fibrosis (20). These results thus support an anti-inflammatory effect of glyceollin in liver. In our short term feeding model, although changes in cholesterol/lipid occurred, we did not observe differences in inflammatory markers, such as IL-1β, IL-6, or IFNγ, within the diet groups in the adipose tissue. These results suggest longer feed period may be necessary to observe an effect by glyceollins on inflammation parameter in adipose.

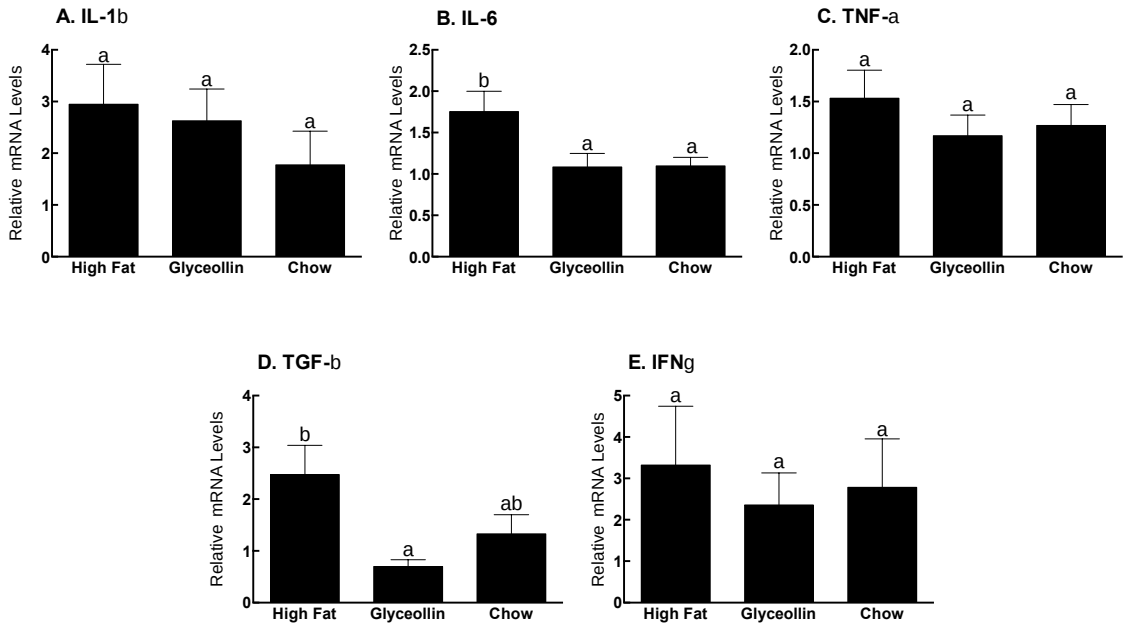

Fig. 2.8. Effects of glyceollins on inflammatory cytokine genes in liver. Livers were harvested from animals on different diets, total mRNA extracted and mRNA level determined using Real Time PCR as described in Materials and Methods. A) IL-1β, B) IL-6, C) TNF-α, D) TGF-β, and E) IFNγ. Results were expressed as relative expression levels (mean ± SEM, n = 10) to chow diet. Columns marked with different letters are significantly different from each other at $p \leq 0.05$.

Fecal Microbiome in Hamster. In the 4-week feeding study in hamster model, Bifidobacteria was significantly reduced by high-fat diet (87% comparing to chow diet group) and a significant increase in Lactobacillus (140%) was observed (Fig. 2.9A). High-fat diet also induced Ruminococcus (940%), Bacteroidetes (814%), and Firmicutes (1021%) comparing to chow diet group, however, due to the huge individual differences within and across groups, none of these changes achieved statistical significance (Fig. 2.1A). In animals fed high-fat diet supplemented with glyceollins, a trend of increase was observed in Akkermansia, Ruminococcus, Bacteroidetes, and Firmicutes, however, no significant change was observed comparing to the high-fat diet group (Fig. 2.9B).

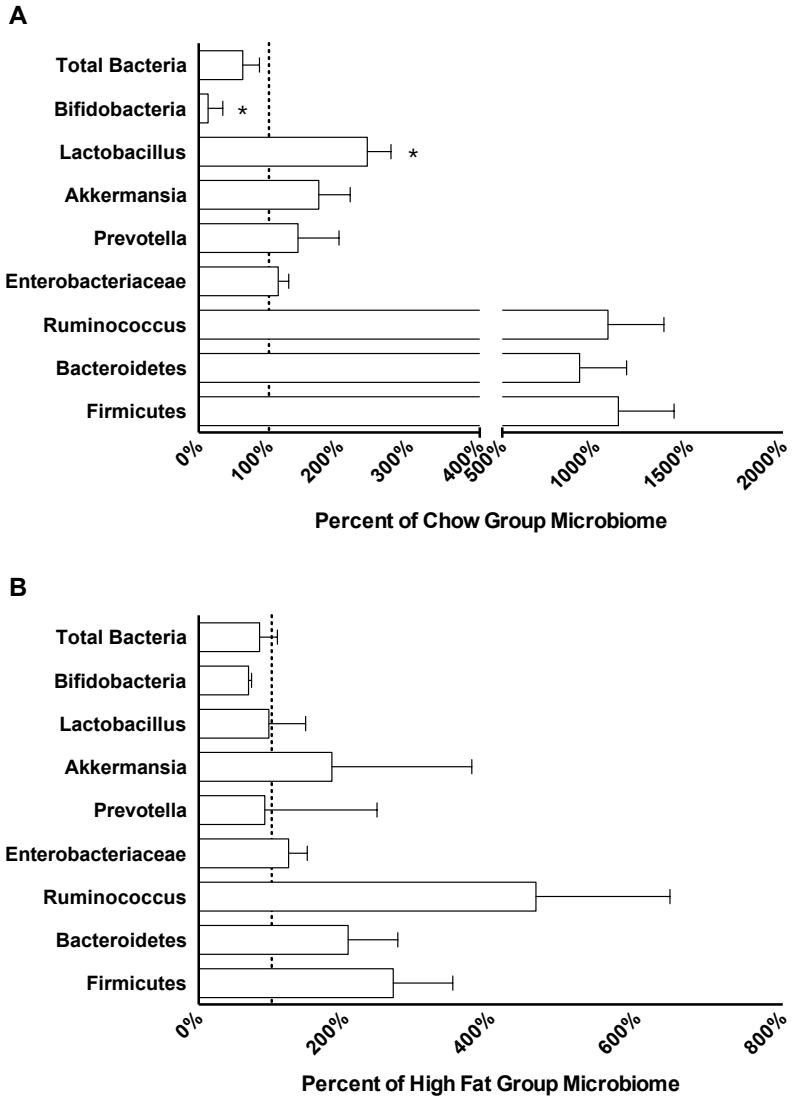

Fig. 2.9. Fecal microbiome in hamsters consuming high-fat, high-fat supplemented with glyceollins, or chow diet. Comparisons were made between chow and high-fat groups (A), and high-fat and high-fat supplemented with glyceollins (B), and presented as percent change. Statistical significance was defined at $p \leq 0.05$ and marked with asterisk.

Plasma Concentration of Glyceollins. In animals fed high-fat and chow diets, glyceollins were undetected in plasma by mass spectrometry. Average concentration of glyceollins was determined to be 0.14 ± 0.025 μM in supplemented animals, ranging from 0.05 to 0.32 μM. Daily consumption of glyceollins in the diet was calculated to be ~5.57 μmol, at the end of study, average animal body weight was 96.87 g, and hamster

has a blood volume of 78 mL/kg (Drexelmed.edu). Thus, about 19% of ingested glyceollins was detected in circulation in hamster model.

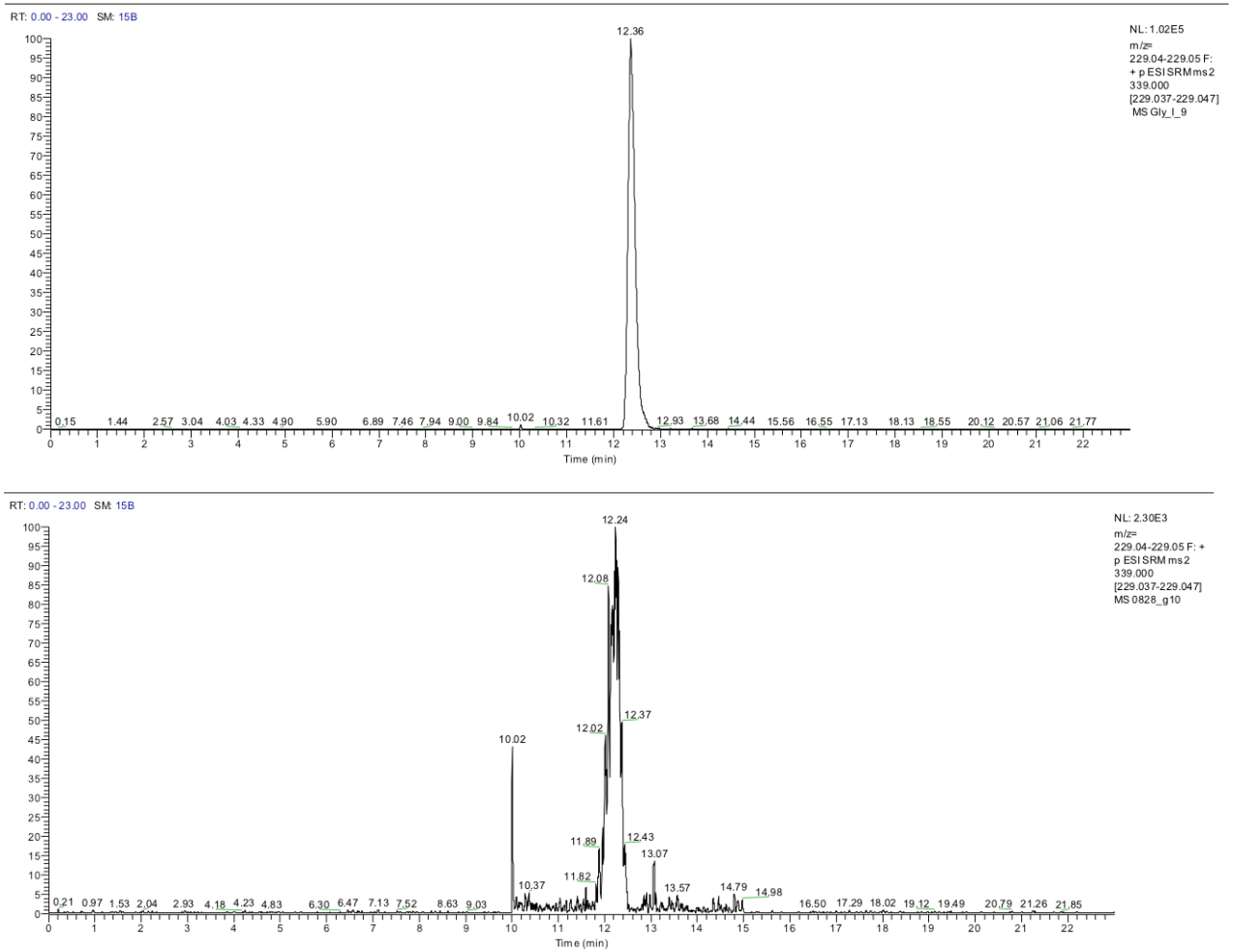

Fig. 2.10. Representative mass spectrometry chromatographs of glyceollin I standard (left) and plasma samples (right). Glyceollins peaks were detected at 12.3 min, and concentrations of plasma glyceollins were calculated based on peak area.

2.5. Discussion

Glyceollins are a family of major phytoalexins and phytoestrogens in stressed soy (Boue, Burow, Shih, Carter-Wientjes, & Cleveland, 2004; Kim, Lim, Kim, & Kim, 2012) (Fig. 2.1). We have previously shown that a soy protein diet enriched with isoflavones and glyceollins reduced cholesterol in postmenopausal female Cynomolgus monkeys (Wood, Boue, Collins-Burow, Rhodes, Register, Cline, et al., 2012), which suggested that glyceollin might be responsible for the effects in cholesterol reduction. This study assessed the short term (4 weeks) effect of glyceollins supplementation on lowering cholesterol level in animals fed with a high-fat western style diet. As shown in Fig. 2.2A, glyceollins significantly reduced the plasma concentration of VLDL by 30% in hamsters fed on high-fat diet. VLDL is responsible for transporting endogenous triglycerides, phospholipids, cholesterol, and cholesterol esters. Elevated VLDL is a critical factor in the onset and progression of cardiovascular conditions (Nielsen & Karpe, 2012; Shelness & Sellers, 2001). The higher circulating level of VLDL in animals consuming a high-fat diet is reflective of increased lipid and cholesterol intake. Our results suggested glyceollins supplementation, by reducing high-fat diet induced increase of VLDL, may be useful to prevent potential detrimental effects of high-fat intake. The plasma concentrations of LDL and total lipoprotein were also slightly lowered by 19% and 9% upon glyceollins supplementation (Figs. 2.2B, 2.2D), while HDL level was unaffected (Fig. 2.2C).

The consumption of the high-fat diet induced an increase of hepatic cholesterol esters and free cholesterol compared to the chow diet (Fig. 2.3). However, glyceollins supplementation significantly reduced hepatic cholesterol esters, free cholesterol, and

total lipid content. These effects of glyceollins suggest less cholesterol resided in liver, and less is available for packaging into VLDL, therefore leading to lower circulating VLDL level. Additionally, livers from glyceollins fed animals were dark red and smooth, were similar to chow fed animals. The high-fat diet animals' livers, on the other hand, were pale and mottled, which was suggestive of lipid accumulation. These results supported our previous conclusion suggesting that glyceollins supplementation may contribute to the reduction of cholesterol level in plasma (Wood, et al., 2012).

Typical dietary cholesterol reducing agents, such as cholestyramine, increase fecal bile acid excretion (Suckling, Benson, Bond, Gee, Glen, Haynes, et al., 1991). However, fecal excretion of bile acid and cholesterol appeared not to be responsible for glyceollins' effect on VLDL, and no change in these parameters was observed in this study (Fig. 2.4).

To further elucidate the mechanism of glyceollins action, we also examined the expression of genes involves in lipid metabolism. Our results indicated the changes in gene expression due to feeding glyceollins were limited but also appeared reflective of glyceollins effect on liver lipid and cholesterol contents. Since the LDL receptor functions to bind and internalize circulating LDL-cholesterol, and liver removes ~70% LDL from the circulation through LDL receptors, it can be a source of increased hepatic cholesterol. In this study, liver LDL receptor gene expression did not appear to change significantly regardless of the type of diet (Fig. 2.5A). ABCG5 and ABCG8, two transporters for cholesterol excretion, were significantly up-regulated by high-fat diet as compared to chow. However, expressions of both genes in animals fed with glyceollins were significantly lower than animals consuming high-fat only diet (Figs. 2.6A, 2.6B).

LPL, a dual function enzyme involved in cholesterol/lipid transport, was significantly up-regulated in high-fat diet fed animals compared to chow fed animals. The glyceollins supplemented diet significantly lowered hepatic LPL expression compared to the animals fed high-fat only diet. The changes in ABCG5, 8 and LPL appeared to be reflective of hepatic lipid/cholesterol status in glyceollins fed animals. We did observe a trend of bile synthetic enzyme CYP7A1 up regulation and increased bile acid excretion in glyceollins fed animals compared to high-fat only animals. However, there was a large difference between animal in these parameters and was not statistically significant. It is possible that a longer feeding period or increase in animal number might confirm this observation and warrant further study. One unique observation in our study was glyceollins fed animals appeared to have significantly lower adipose LPL mRNA level than high-fat or chow fed animals. This result suggested that glyceollins might have an effect on adipose tissues, such as prevent accumulation of lipid in adipose tissues. Since our study was designed to investigate short term effects and no body weight or adipose weight changes were observed, further long term studies would be necessary to elucidate the biological significance and mechanisms of glyceollins' effect on adipose LPL.

Consumption of a high-fat, high cholesterol diet can lead to gene changes that would compensate for changes in dietary intake. In our current hamster study, as expected, significant down regulation of cholesterol synthesis related gene HMG-CoA reductase and CYP51 (Fig. 2.5B, 2.5C) were observed in hamsters on the high-fat diets compared to the control group. This is consistent with the fact that abundant cholesterol is available to cells upon consumption of high-fat diet, de novo synthesis of cholesterol appeared to be unnecessary. As important as HMG-CoA reductase is in the process of

cholesterol synthesis, no effect was observed in this study, which suggested a lack of glyceollins influence on this cholesterol synthesis pathway both in a hamster model. Similar results were also observed for SREBP-1c, however in this case the high-fat diet lead to significant up-regulation of this gene as compared to the chow fed animals. Glyceollins did not affect SREBP-1c expression when compared to high-fat diet animals.

Our results indicated that several pathways appeared not to be affected by dietary perturbation at the transcriptional level. We did not observe significant differences in LXRα, PPARα, FAS or ACOX mRNA expression between the different diets. None of these pathways were affected by glyceollins supplementation. These data would support the notion that, under our experimental condition and bioavailability of glyceollins in hamster, cholesterol synthesis and excretion were the main pathways animals used to adapt to the high-fat diet.

In this study, high-fat diet and glyceollins' effect on microbiome was also investigated. High-fat inhibited Bifidobacteria and induced Lactobacillus in the fecal microbiome (Fig. 2.9A). Bifidobacteria and Lactobacillus are known to be involved in energy metabolism in gut microbiome (Cani & Delzenne, 2009; Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005), previous studies have also reported diet conversion high in fat content can alter population of Bifidobacteria and Lactobacillus (Brinkworth, Noakes, Clifton, & Bird, 2009; Zeng, Liu, Jackson, Yan, & Combs, 2013). However, there were other reports indicating opposite changes induced by high-fat diet in these microorganisms (Cani, Bibiloni, Knauf, Neyrinck, Delzenne, & Burcelin, 2008; Zhao, Liu, Xie, Wang, Cui, Yang, et al., 2011), which indicated that alterations in

microbiome may be highly dependent on animal models, diet compositions, and also fecal collection methods.

For the first time, our study gathered direct evidence that glyceollins supplementation in a high-fat diet significantly reduced VLDL, hepatic cholesterol esters, hepatic free cholesterol and hepatic total lipids, as well as hepatic inflammatory cytokines in animals. The effects of glyceollins on hepatic lipid and cholesterol levels appeared to contribute to molecular changes in hepatic ABCG5, 8 and LPLs mRNA levels. However, the actual molecular mechanism of cholesterol-lowering effect of glyceollins in-vivo remains unclear and needs further investigation. Given that glyceollins are naturally-derived phytochemicals in stressed soy, our results suggested that the inclusion of glyceollins in diet may benefits the population by lowering VLDL cholesterol and decreasing the risk for cardiovascular disease.

Chapter 3: Prostate cancer preventative effect of glyceollins in xenograft mouse model

3.1. Abstract

Glyceollins are soy-derived phytoalexins that have been proposed to be candidate cancer preventive compounds. Prostate cancer is identified as the most prevalent cancer in U.S. population. Previous research has shown that the glyceollins significantly inhibited the androgen-responsive LNCaP cell growth by regulating cell cycle and androgen-mediated pathway. The present study aimed to evaluate glyceollins' effect in reducing prostate cancer tumor growth in a xenograft model. Androgen responsive LNCaP cell and androgen independent PC-3 cell were used to establish cancer cell tumor xenograft in animals fed control or glyceollins supplemented diets. An initial delayed appearance of tumor was observed in PC-3 xenograft model, however, no difference in tumor sizes was observed in LNCaP xenograft. Extrapolation analysis of tumor measurements indicated that no difference in sizes was expected for both PC-3 and LNCaP tumors at 100 days after injection. Glyceollins showed no effect on androgen responsive, proliferation, cell cycle, and angiogenesis genes in tumor and xenobiotic metabolism, cholesterol transport, and inflammatory cytokine genes in liver. Glyceollins' low bioavailability (0.054 ± 0.013 μM) might have led to the ineffectiveness in reducing tumor growth in-vivo.

3.2. Introduction

Prostate cancer is identified as the most prevalent cancer and the third as a cause of cancer deaths in U.S. population in the past two decades, and accounts for 15% of all malignant cancer incidences (AACR, 2014; Fitzpatrick, Schulman, Zlotta, & Schroder, 2009; Palapattu, Sutcliffe, Bastian, Platz, De Marzo, Isaacs, et al., 2005). Many factors, including genetics, hormones, race, age, diet, and the environment have been identified to be responsible for the initiation and development of prostate cancer. However, the exact causes of prostate cancer remain largely unknown, and there is currently no effective cure for this disease (Hsing & Chokkalingam, 2006; Violette & Saad, 2012). Therefore, developing and validating preventive strategies becomes critical to the control of prostate cancer's occurrence and impact (Meadows, 2012; Umar, Dunn, & Greenwald, 2012). Both population and experimental studies have implicated the importance of diet in prevention and reduction of cancer, including prostate cancer (Llaverias, et al., 2010; Meadows, 2012; Wang, Khor, Shu, Su, Fuentes, Lee, et al., 2012). Vegetables, legumes, and fruits are known to be rich in phytochemicals and were found to contribute to a decreased risk of cancer (Androutsopoulos, Papakyriakou, Vourloumis, Tsatsakis, & Spandidos, 2010; R. Chan, Lok, & Woo, 2009; Willett, 1995). Therefore, food-derived products or compounds are of great interest and importance as chemopreventive agents.

Previous studies indicated a correlation between consumption of soy products in Asian population with a decreased incidence of chronic diseases, such as cardiovascular disease and cancer (Ahmad, et al., 2008; Azadbakht & Esmaillzadeh, 2008; Clair & Anthony, 2005; Hwang, Kim, Jee, Kim, & Nam, 2009). Soy isoflavones, such as genistein and daidzein, have been widely studied as candidate of diet-derived compounds

for disease prevention (Sarkar & Li, 2003; Taku, Melby, Nishi, Omori, & Kurzer, 2011; Verdrengh, Jonsson, Holmdahl, & Tarkowski, 2003). Glyceollins are a group of phytoalexins, derived from the precursor daidzein, and the biosynthesis of which is increased under stress, e.g. UV light, low temperature or fungal infection (Kim, Lim, Kim, & Kim, 2012; Zimmermann, et al., 2010). Recent studies have shown glyceollins' inhibitory effect on estradiol-induced proliferation and ERα signaling in breast cancer cells and xenograft animal model (Payton-Stewart, et al., 2010; Zimmermann, et al., 2010). Our previous research found that the glyceollins significantly inhibited the androgen-responsive LNCaP cell growth, while glyceollins exert little growth inhibitory effects on the androgen independent PC-3 cells. The growth inhibitory effects of the glyceollins on LNCaP cells appeared to be due to an inhibition of G1/S progression and correlated with an up-regulation of CDKN1A and 1B at mRNA and protein levels. In addition, glyceollins treatments led to down-regulated mRNA levels of several androgen-responsive genes including PSA, supporting a role of glyceollins in androgen-mediated pathway (Payton-Stewart, et al., 2009). These findings suggest that glyceollins may possess preventative properties towards prostate cancer. However, the exact effect and mechanisms remain unclear.

In this study, the hypothesis that glyceollins can prevent or reduce prostate cancer tumor growth in xenograft model was tested. Glyceollins' effect on androgen responsive genes was first determined in-vitro. Then androgen responsive LNCaP cell and androgen independent PC-3 cell were used in xenograft model fed formulated rodent diet supplemented with or without glyceollins. An initial delayed appearance of tumor was observed in PC-3 xenograft model, however, no difference in tumor sizes was observed

in LNCaP xenograft. Extrapolation analysis of tumor measurements indicated that no difference in sizes was expected for both PC-3 and LNCaP tumors at 100 days after injection. Mass spectrometry analysis revealed that glyceollins' low bioavailability might have led to the ineffectiveness in reducing tumor growth in-vivo.

3.3. Material and methods

Chemicals and diets. Soy (Asgrow Soybean Seed, Treatment code: 0, Germination: 85%, Origin: NC) was acquired from Monsanto Company (St. Louis, MO). The fermentation process of soy was modified and standardized in order to ensure the elicitation of glyceollins and maximize its concentration. Soy was cut to pieces with a food processor and fermented with baking yeast (1% w/w) for 96 h. Glyceollins were then purified following previously published methods (Payton-Stewart, et al., 2009). Powder AIN-93M diets with or without glyceollins (250 mg/kg diet) were prepared by Research Diets (New Brunswick, NJ) and stored at -20 ºC until weekly feedings.

Cell treatment and gene expression analysis in LNCaP and PC-3 cells. To determine the glyceollins' effect on the attachment of LNCaP and PC-3, cells were plated in 6 well plates (Costar, Corning Incorporated, Corning, NY) with glyceollins at the indicated concentrations (5, 10, and 25 μM). After 8 h, plates were washed twice with 1×PBS, attached LNCaP and PC-3 cells were harvested, and total protein was determined using BCA Protein Assay (Thermo Scientific Pierce, Rockford, IL).

To determine the mRNA expression levels of proliferation gene expressions, LNCaP cells were cultured in 6 well plates overnight. Glyceollins were added into the media at the indicated concentrations (1, 2, 5, 10, and 25 μM). LNCaP cells were

incubated in PRMI media with 10% fetal bovine serum and 1% antibiotic/antimycotic. After 24 h or 48 h, culture media were discarded, and cells were collected.

RNA isolation and real-time PCR were performed according to the previously published protocol (Huang, Fletcher, Niu, Wang, & Yu, 2012). Cells were washed with 1×PBS and TRIzol reagent was added for total RNA isolation. StrataScript First Strand complementary DNA Synthesis kit was used to reverse transcribe complementary DNA. Real-time PCR was performed on Applied Biosystems ViiA™ 7 Real-Time PCR System using TaqMan Universal PCR Master Mix. The TaqMan gene expression assays were acquired from Applied Biosystems (Carlsbad, CA) and used for gene detection. The mRNA amounts were normalized to an internal control, TATA-binding protein (Tbp) mRNA. The following amplification parameters were used for PCR: 50 °C for 2 min, 95 °C for 10 min, and 46 cycles of amplification at 95 °C for 15 sec and 60 °C for 1 min.

Tumor xenograft model. LNCaP and PC-3 human prostate cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI-1640 supplemented with 2 mM L-glutamine, 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37 °C in a 5% CO2 atmosphere. Male athymic nude mice (BALB/c nu/nu, 20-22 g, 5-6 weeks old; Charles River, Frederick, MD) were individually housed in filter-top cages at the USDA BHNRC animal facility and consumed food and fresh tap water ad libitum. Food consumption and body weights were recorded twice a week. After an acclimation period of 2 weeks, during which mice were fed control AIN-93M diet, the mice were randomized into 4 experimental groups, with 12 animals in each group. The animals (24 animals each) received control AIN-93M or glyceollins supplemented diet. Two weeks

later, LNCaP and PC-3 human prostate cancer cell xenografts were established in the mice by injection s.c. in the flank (one injection each flank) with LNCaP or PC-3 cells (2×10^6 cells) in 50 µL of phosphate-buffered saline (PBS) and 50 µL Matrigel (BD Biosciences, Mansfield, MA). Animals injected with LNCaP cells and fed control diet were designated as Group L, and animals fed glyceollins diet as Group LG. Animals injected with PC-3 cells and fed control diet were designated as Group P, and animals fed glyceollins diet as Group PG. Cancer preventive efficacy of the treatments was assessed twice a week by measuring tumor volume (cm^3) calculated as 0.523 x [length (cm) × width^2 (cm^2)] (Hudson, Perkins, Hursting, Young, Kim, Wang, et al., 2012). Mice were remained on their respective diets for 8 weeks after cell injection. All animal experimental protocols were performed in accordance with the National Institutes of Health guidelines and approved by the USDA, ARS, Beltsville Area Animal Care and Committee (BAACUC).

Plasma and tissue collection. Animals were sacrificed at the fourth (PC-3 xenograft) and eighth (LNCaP xenograft) weeks after tumor injection when tumors reached 2-3 cm^3 in volume. Animals were anesthetized with CO2 and blood were obtained by cardiac puncture with syringes previously rinsed with potassium EDTA solution (15% w/v), and plasma was separated after centrifugation at 1500 rpm for 30 min at 4 ºC. Liver and tumor were collected and quickly frozen in liquid nitrogen and stored at -80 ºC.

Liquid chromatography-mass spectrometry analysis of plasma concentration of glyceollins. LC-ESI-MS and LC-ESI-MS/MS analyses were conducted on an Agilent 1100 series LC system (Agilent, Santa Clara, CA) coupled to a TSQ Vantage™ Triple

Quadrupole Mass Spectrometer (Thermo Scientific, Middletown, VA). Separation was performed on an Agilent Eclipse XDB C18 column (4.6 × 150 mm ID, 5 μm). Ten microliters was injected onto the column held at 25 °C. The binary mobile phase consisted of mobile phase A (water with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid). The gradient was 0-4 min 30% A to 2% A and hold at 2% A for 10 min; 14-16 min 2% A to 30% A and hold at 30% A for 7 min; the flow rate was 0.200 mL/min. The UV absorbance detector was set at 285 nm. For positive ion LC-ESI-MS and LC-ESI-MS/MS analyses, electrospray parameters were set at the following: sheath gas pressure of 60 psi, ionspray voltage of 3500 V, auxiliary gas pressure of 15 psi, collision gas pressure of 1.5 mTorr, capillary temperature of 268 °C. Declustering potential and collision energy were maintained at -12 V and 16 eV, respectively. Parent and product mass scan were performed at 339.000 and 229.042.

Total RNA isolation, cDNA synthesis and gene expression analysis from liver and adipose tissue. To determine the gene expression changes, liver and adipose tissues preserved in RNALater were cut into 0.1 to 0.2 g pieces and homogenized using a Precellys 24 (Bertin Technologies, Villeurbanne, France). RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) were used for total RNA isolation for liver and adipose, respectively. StrataScript First Strand complementary DNA Synthesis kit from Stratagene (Santa Clara, CA, USA) was used to reverse transcribe complementary DNA. Real-time PCR was performed on an Applied Biosystems ViiA™ 7 Real-Time PCR System using TaqMan Universal PCR Master Mix and TaqMan® Probe-Based Gene Expression assays by Applied Biosystems (Carlsbad, CA, USA). Relative mRNA expression levels were calculated using the delta Ct method (J. S. Yuan,

Reed, Chen, & Stewart, 2006). TATA-binding protein (Tbp) expression was used as the house-keeping gene for calculations.

Statistical analysis. All end point assays for each sample were conducted in triplicate and the average was used for group analysis, data for each treatment group were presented as mean ± standard error. Significance level of differences in means was detected using one-way ANOVA and Tukey's test. Statistics analysis was performed using IBM SPSS Statistics 19.0 (2010, IBM Corporation, Armonk, NY, USA) or Graphpad Prism 6 (2012, Graphpad Software, La Jolla, CA, USA). Statistical significance was defined at $p \leq 0.05$.

3.4. Results

Glyceollins' Effect on Androgen Dependent Pathway in LNCaP Cells. Glyceollins, as low as 1 μM, significantly reduced androgen responsive genes PSA and NKX3.1 expression in LNCaP cells, 70% and 75% respectively (Fig. 3.1).

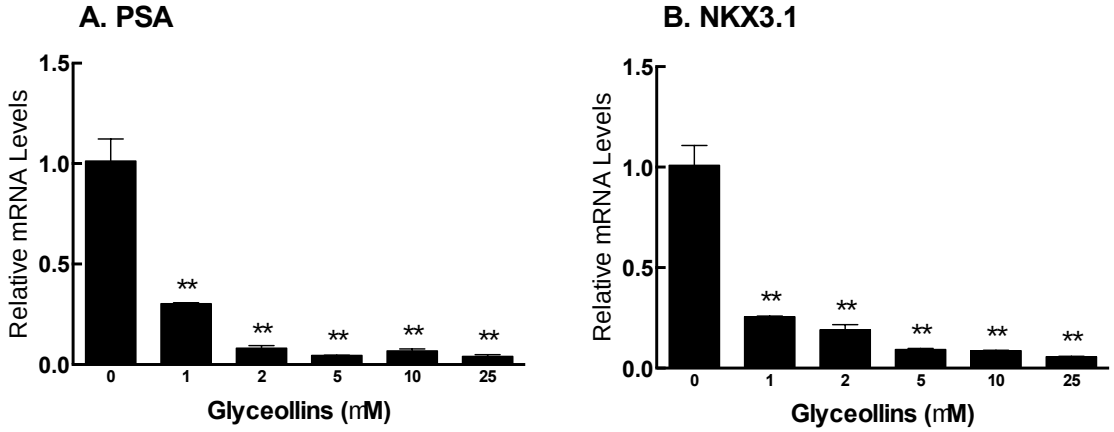

Fig. 3.1. Glyceollins' effect on PSA and NKX3.1 gene expression in LNCaP cells. Significant difference in expression level was marked with asterisk (*, $p \leq 0.05$; **, $p \leq 0.001$).

Body Weight and Food Intake. There were no difference in body weight or body weight gain between any of the diet treatments during the experimental period except for the last measurement, in which group LG animals showed a significant higher food intake and body weight (Fig. 3.2). Food intake of group L and LG animals significantly decreased at the eighth week after injection, as the tumors on the flanks started to hinder the movement of the animals.

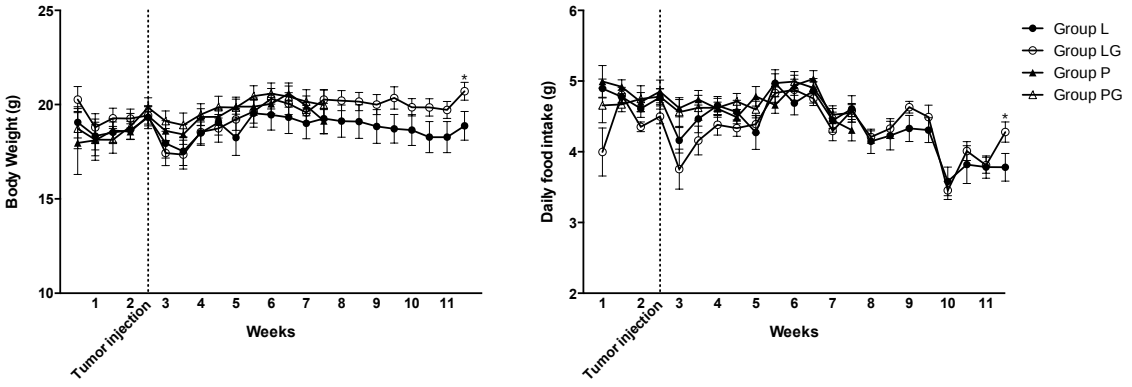

Fig. 3.2. Body weight and food intake. Animals injected with LNCaP cells and fed control diet was designated as Group L, and animals fed glyceollins diet as Group LG. Animals injected with PC-3 cells and fed control diet was designated as Group P, and animals fed glyceollins diet as Group PG.

LNCaP and PC-3 Tumor Growth. Palpable and measureable LNCaP tumors were recorded on the fourth week after the injection, and PC-3 tumors were recorded on the sixth day after the injection (Fig. 3.3). PC-3 tumor was solid, pale, and no blood vessel were observed in the tumor; whereas LNCaP tumor was dark red with dense blood vessel. Although control group (L) showed higher tumor volume than the glyceollins treatment group (LG), no significant difference in LNCaP tumors size were detected throughout the experiment period. On the other hand, PC-3 tumors of glyceollins treatment group (PG) were significantly smaller than that of the control group (P). PC-3

tumors grew at a much fast rate and reached 930 and 688 mm3 5 weeks after the injection, and LNCaP tumors reached 419 and 266 mm^3 9 weeks after the injection.

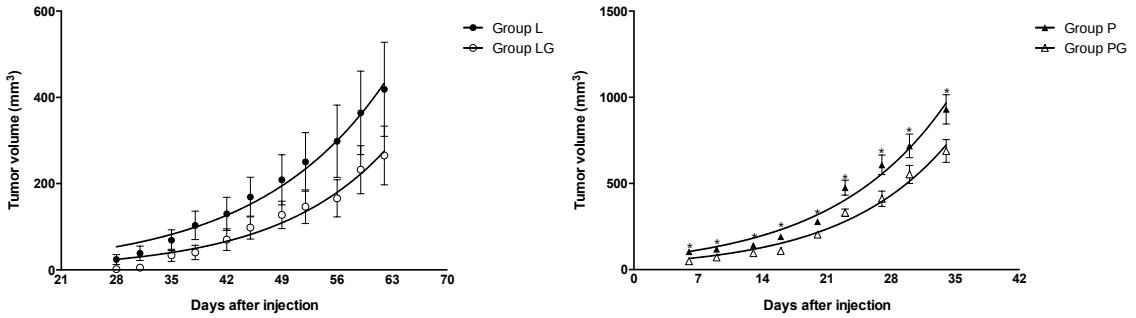

Fig. 3.3. Tumor growth in LNCaP and PC-3 xenograft. Tumor size was measured twice a week. Significant difference in tumor volume between control and glyceollins treatment was marked with asterisk ($p \leq 0.05$).

Extrapolation of tumor volume data to 100 days after injection indicated that no difference in of tumor size between the control and treatment groups (Fig. 3.4). LNCaP tumors reach similar sizes in control and glyceollins treated animals at around 110 days, and PC-3 tumors reach similar at around 75 days.

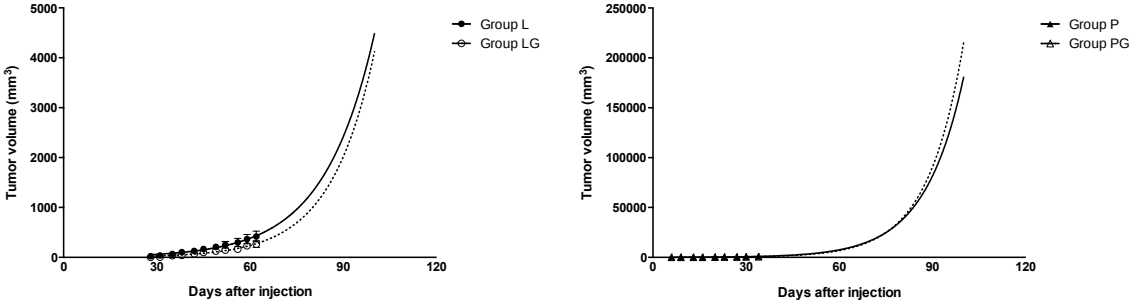

Fig. 3.4. Extrapolation of LNCaP and PC-3 tumor growth. Extrapolation analysis was performed using the measurement data in this study and performed using Prism Graphpad.

Attachment Assay of LNCaP and PC-3 Cells. LNCaP and PC-3 cells at the same passage (passage 12) as the cells used in the injection were plated with glyceollins

in the media. After 8 h of incubation, attached cells were harvested and it was determined that 25 μM and 10 μM glyceollins reduced attachments of LNCaP and PC-3 in-vitro, respectively (Fig. 3.5), lower concentrations did not affect their attachments.

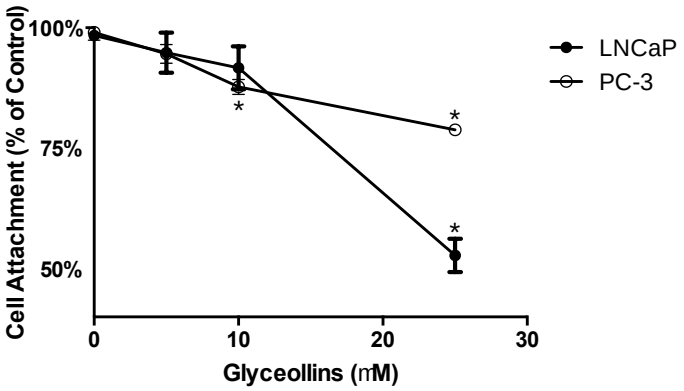

Fig. 3.5. In-vitro LNCaP and PC-3 attachment assay. LNCaP and PC-3 cells were plated in 6-well plates with indicated concentration of glyceollins. Attached cells were measured by total protein and significant difference between control and glyceollins treatment was marked with asterisk ($p \leq 0.05$).

Glyceollins' Effect on Gene Transcription in LNCaP Tumors. LNCaP tumors were analyzed for the androgen responsive (PSA), proliferation (Ki-67, PCNA), apoptosis (Bcl-2, Bax), cell cycle (CDKN1A, CDKN1B) and angiogenesis (VEGF, PECAM) genes to determine glyceollins' effect on these pathways (Fig. 3.6). A significant but minor (9%) reduction in PCNA gene expression was observed in tumors of glyceollins supplemented animals. No change was observed in the expression of PSA, Ki-67, Bcl-2, Bax, CDKN1A/1B, VEGF and PECAM.

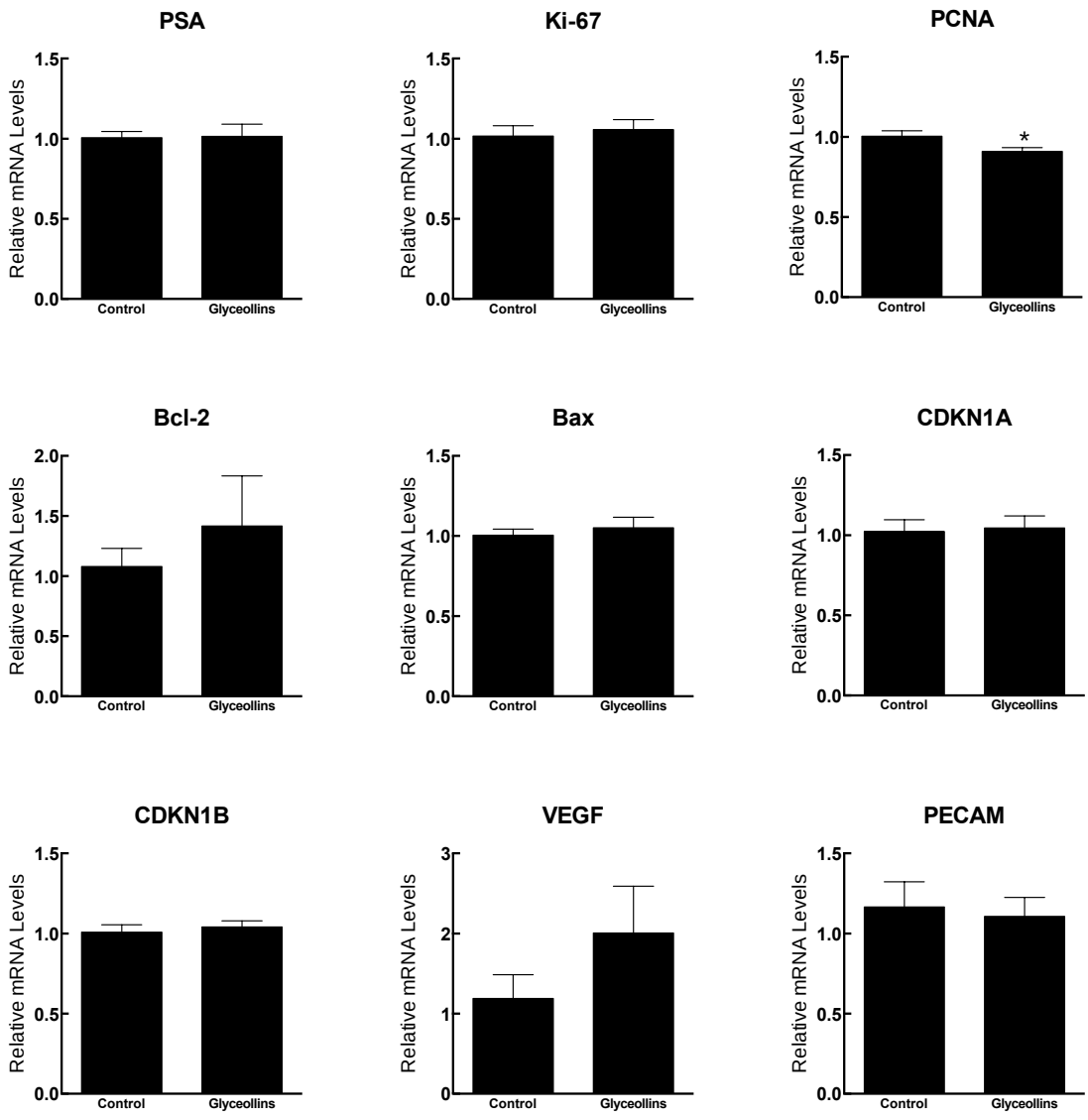

Fig. 3.6. Expression of Androgen Responsive, Proliferation, Apoptosis, Cell Cycle, and Angiogenesis Markers in LNCaP Tumors. Significant difference between control and glyceollins treatment was marked with asterisk ($p \leq 0.05$).

LNCaP tumors were analyzed for inflammatory gene markers (TNFα, IL-1β, IL-6, COX-2, and EMR1) expressions. No change was observed in inflammatory cytokine expressions of TNFα, IL-1β, IL-6, and COX-2, and there was no change in macrophage marker EMR1 between control and glyceollins treatment groups.

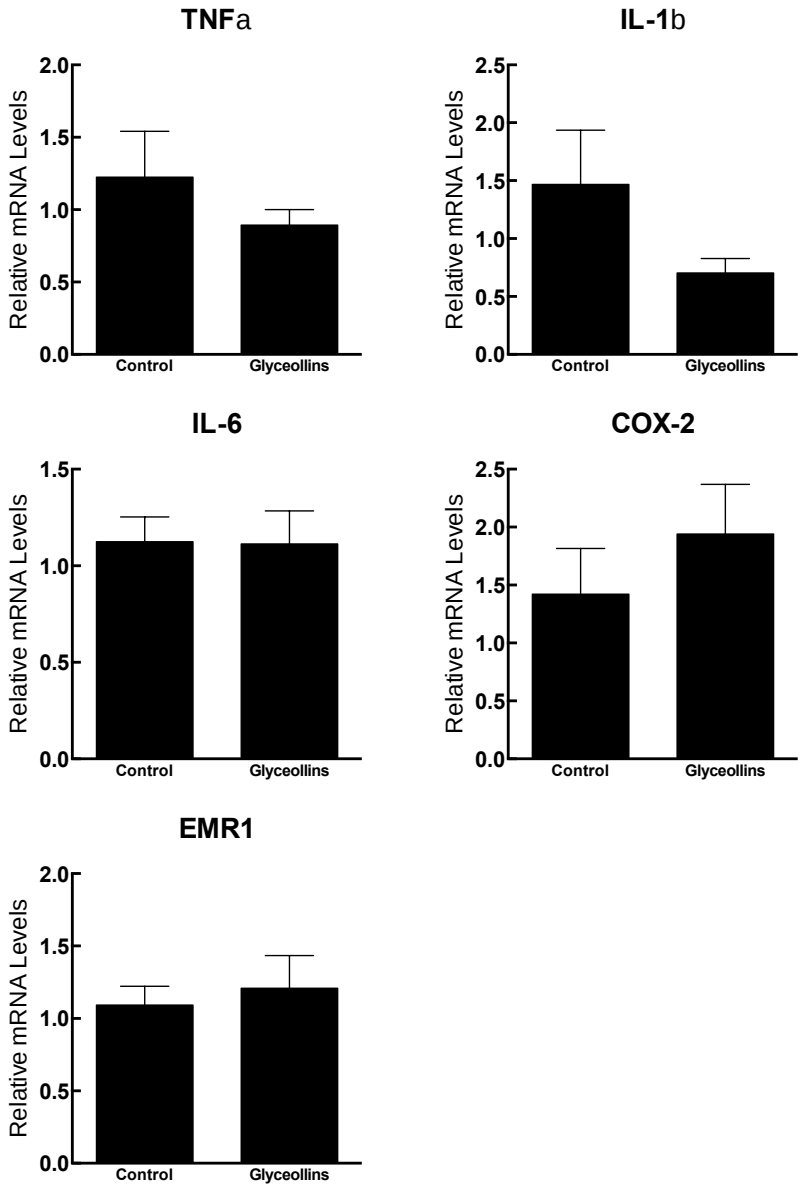

Fig. 3.7. Expression of Inflammatory Markers in LNCaP Tumors. Significant difference between control and glyceollins treatment was marked with asterisk ($p \leq 0.05$).

Proliferation Markers in PC-3 Tumors. PC-3 tumors were analyzed for the proliferation marker, Ki-67, to determine glyceollins' effect on cell proliferation (Fig. 3.8). In this study, no change was observed in the expression of Ki-67 in PC-3 tumors.

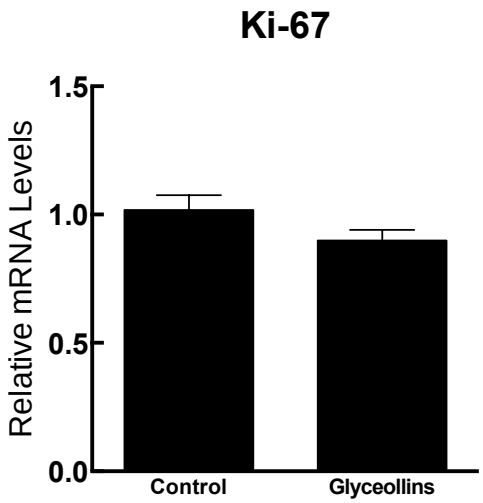

Fig. 3.8. Expression of Proliferation Markers in PC-3 Tumors. Significant difference between control and glyceollins treatment was marked with asterisk ($p \leq 0.05$).

Glyceollins' Effect on Gene Transcription in LNCaP Tumor Animal Livers.

Gene expressions of phase I and phase II enzymes in livers of LNCaP tumor animals were analyzed in this study. No difference in expression levels of CYP1A1, CYP2D10, CYP2C55, CYP3A11 (Fig. 3.9) and NQO1, UGT1A1 (Fig. 3.10) was detected.

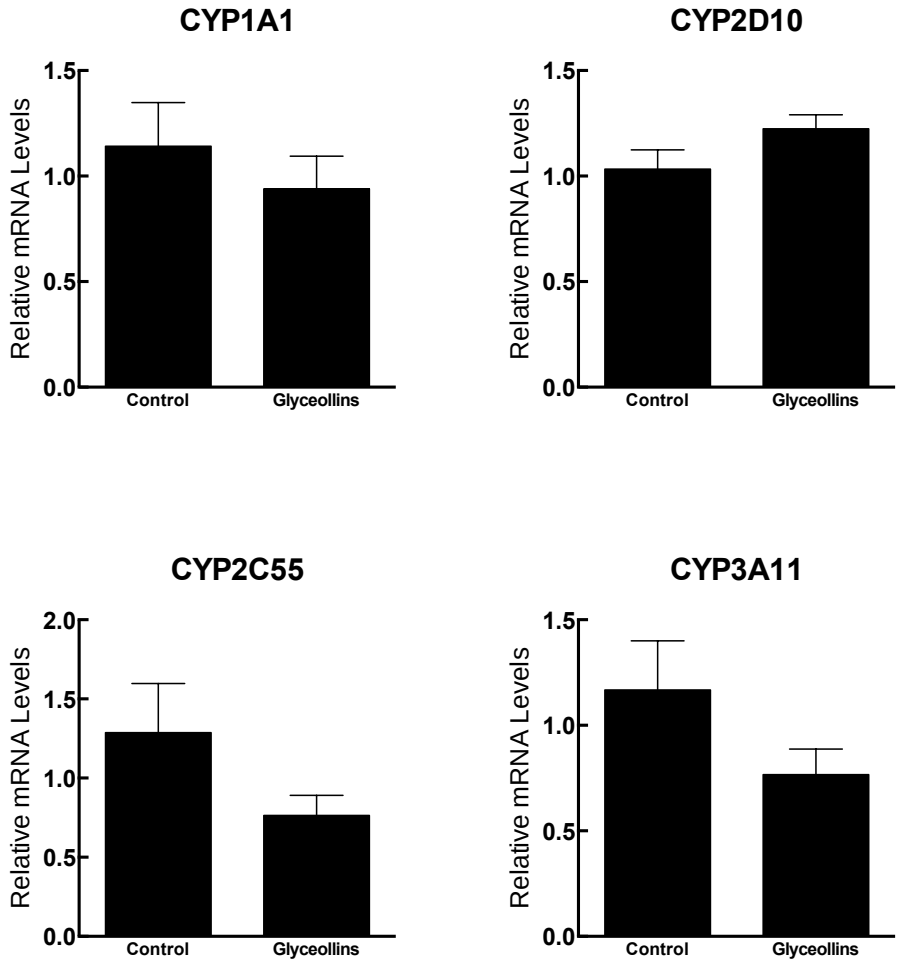

Fig. 3.9. Expression of CYP450 genes in Livers of LNCaP Tumor Animals. Significant difference between control and glyceollins treatment was marked with asterisk ($p \leq 0.05$).

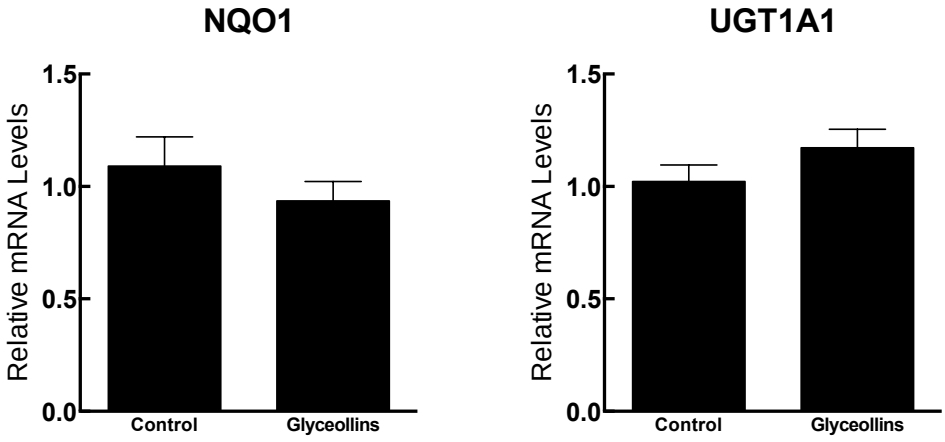

Fig. 3.10. Expression of Phase II Enzyme Genes in Livers of LNCaP Tumor Animals. Significant difference between control and glyceollins treatment was marked with asterisk ($p \leq 0.05$).

Gene expressions of ABC family transport were analyzed in this study. A significant but minor (22%) increase in ABCG5 gene expression was observed in livers of glyceollins supplemented animals (Fig. 3.11). No change was observed in the expression of ABCA1, ABCG1, and ABCG8 (Fig. 3.11).

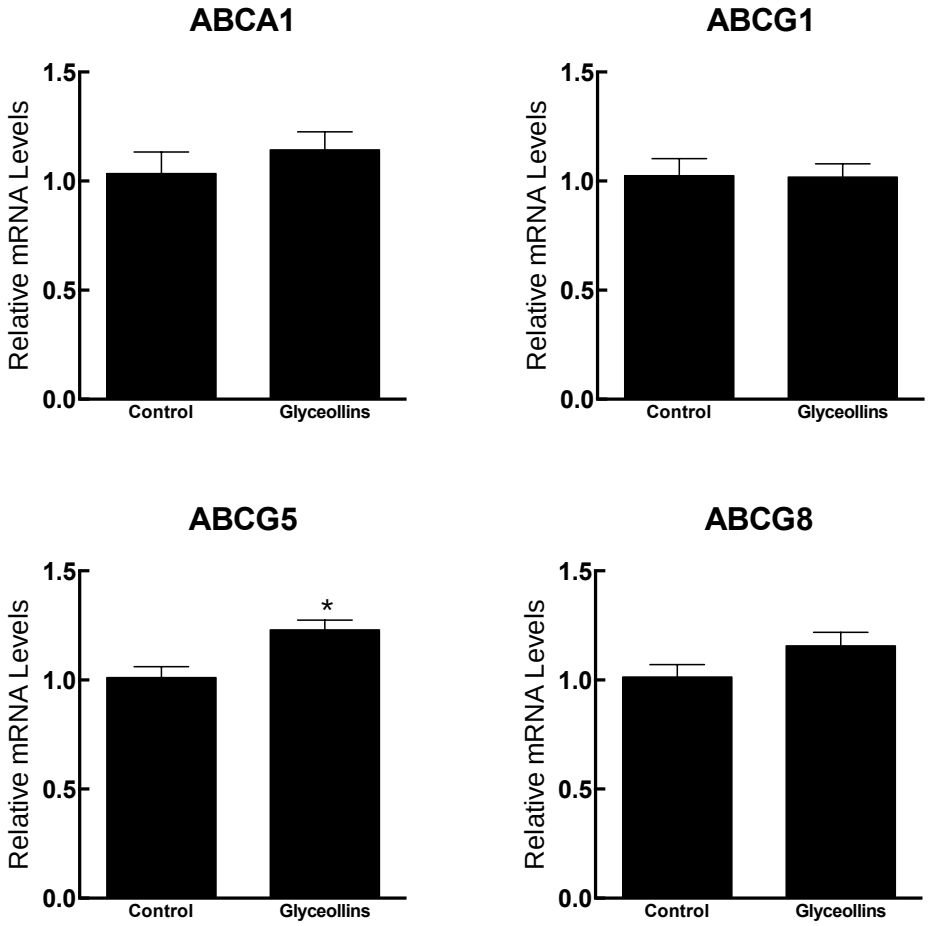

Fig. 3.11. Expression of Cholesterol Transport Genes in Livers of LNCaP Tumor Animals. Significant difference between control and glyceollins treatment was marked with asterisk ($p \leq 0.05$).

Gene expressions of IL-1β, IL-6, COX-2, and TGF-β were analyzed in livers of LNCaP tumor animals. No difference in expression levels of these inflammatory markers was detected (Fig. 3.12).

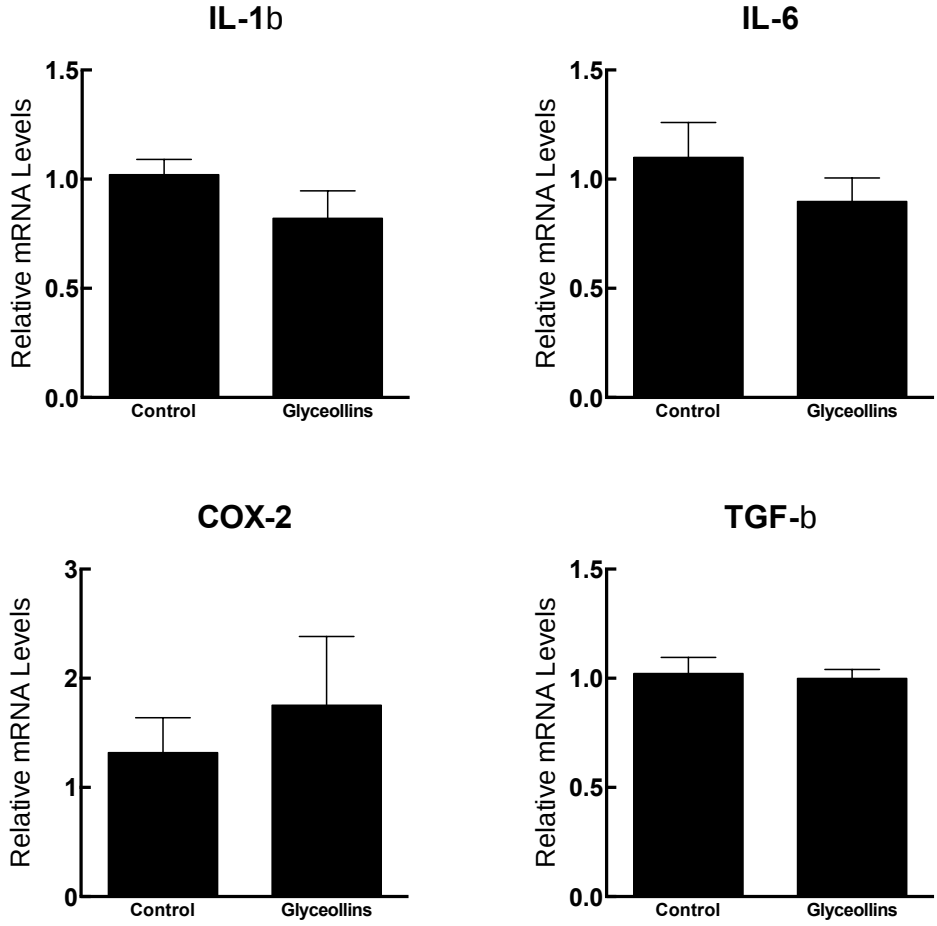

Fig. 3.12. Genes Expressions of Inflammation Markers in LNCaP Tumor Animal Livers. Significant difference between control and glyceollins treatment was marked with asterisk ($p \leq 0.05$).

Plasma Concentration of Glyceollins. In animals fed with the control diet, glyceollins were undetected in plasma by mass spectrometry. Average concentration of glyceollins was determined to be 0.054 ± 0.013 μM in supplemented animals, ranging from 0.004 to 0.12 μM. Daily consumption of glyceollins in the diet was calculated to be ~3.61 μmol, at the end of study, average animal body weight was 20.04 g, and mouse has a blood volume of 79 mL/kg (Drexelmed.edu). Thus, about 2.3% of ingested glyceollins was detected in circulation in this athymic nude mouse model.

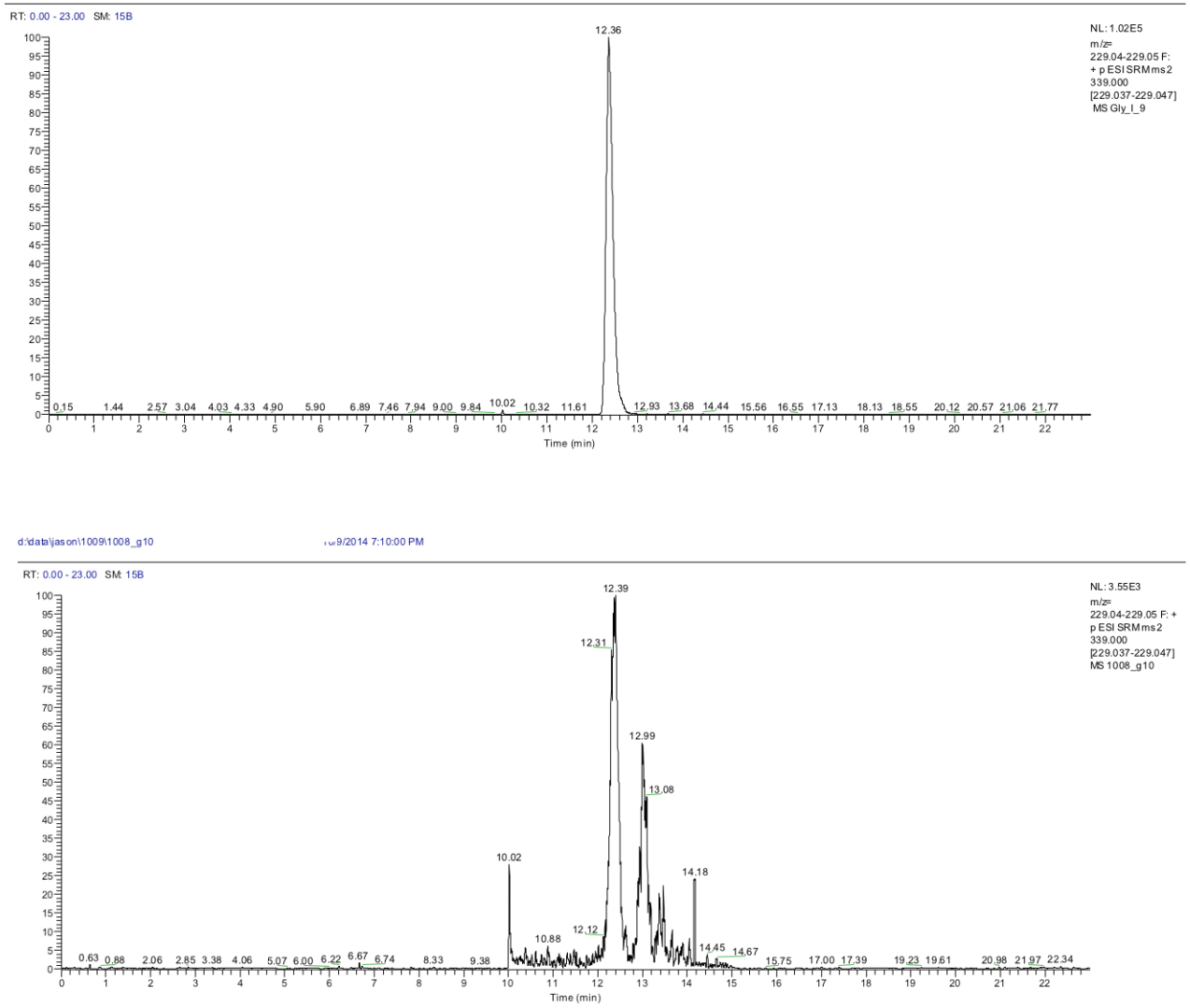

Fig. 3.13. Representative mass spectrometry chromatographs of glyceollin I standard (left) and plasma samples (right). Glyceollins peaks were detected at 12.3 min, and concentrations of plasma glyceollins were calculated based on peak area.

3.5. Discussion

This study examined the anti-tumor effects of glyceollins on androgen responsive LNCaP and androgen independent PC-3 human prostate cancer cells in-vivo. An initial delayed appearance of tumor was observed in PC-3 xenograft model, however, no difference in tumor sizes was observed in LNCaP xenograft. Extrapolation analysis of tumor measurements further support that no difference in tumor sizes between the diet group for both PC-3 and LNCaP tumors even at 100 days after injection. In-vitro analysis showed that, glyceollins was able to significantly reduce the mRNA expression of androgen responsive genes PSA and NKX3.1 in LNCaP prostate cancer cells at as low as 1 μM (Fig. 3.1). This observation was consistent with our previous report, in which glyceollins was able to inhibit LNCaP cell growth through inhibition of G1/S progression and up-regulation of CDKN1A/1B (Payton-Stewart, et al., 2009). However, no effect was observed in LNCaP tumor growth in xenograft model (Fig. 3.3) and androgen responsive genes (Fig. 3.6). The disconnection between in-vitro and in-vivo data may be explained by the low bioavailability of glyceollins in the athymic nude mouse model. Analysis of plasma revealed that only 2.3% of glyceollins contained in the diet was absorbed into the circulation, achieving 0.054 ± 0.013 μM in the blood stream. In this study, glyceollins were able to affect the androgen responsive genes in-vitro at as low as 1 μM, while, in our animal model, oral intake of 250 mg/kg glyceollins merely achieved 0.054 ± 0.013 μM in circulation, which is about 5% of the concentration used in in-vitro assays.

In our previous study, hamster fed with 250 mg/kg glyceollins diet achieved 0.14 ± 0.025 μM glyceollins in circulation, and cholesterol-lowering effect was observed. At the same supplementation level, hamster absorbed 19% glyceollins from diet, whereas mouse only absorbed 2.3%. Two factors may have significantly affected the bioavailability of glyceollins in the animal models: 1) species differences between hamster and athymic nude mouse; and 2) composition of a diet, such as fat content. Species differences exist in animal models, which can significantly affect the bioavailability of exogenous compounds, such as phytochemicals and drugs (Gad, 2007). Thickness and length of the small intestine, gut transit time, and differences in facilitated or active transport all play a role in gastrointestinal absorption of exogenous compounds. Blood flow to organs, extent and avidity of compounds binding to plasma proteins, and the extent of clearance of the compounds can affect the distribution process (Gad, 2007). Distinct xenobiotic metabolism systems (Phase I and Phase II enzymes) in different animal models can also impact the metabolism and retention of specific compounds (Liska, 1998). Thus, physiological differences in athymic nude mouse and hamster may significantly affect the bioavailability of glyceollins. On the other hand, the composition of a diet may also affect the uptake of glyceollins in the animals. Hamsters were fed high diet (36% calorie from fat) supplemented with 250 mg/kg glyceollins, while low fat diet (10% calorie from fat) was used in the mouse study (Huang, Xie, Boue, Bhatnagar, Yokoyama, Yu, et al., 2013). The differences in fat contents may affect the absorption of glyceollins in the diet. Further study is needed to understand the extent of the impact of animal models and diet composition on the bioavailability of glyceollins.

This study indicated that glyceollins supplementation could delay the initial appearance of PC-3 tumor (Fig. 3.3). PC-3 is an androgen independent cell line, and analysis of proliferation factor Ki-67 in the PC-3 tumor showed no change of expression, thus, the delayed appearance of tumor might be the result of reduced attachment of PC-3 cells. In-vitro analysis did show reduced cell attachment, however, a significant reduction was only observed at concentration higher than 10 μM. Considering the limited bioavailability and plasma concentration of glyceollins, 10 μM was a concentration unlikely to achieve in-vivo.

Inhibition of PCNA, a cell proliferation factor, was observed in glyceollins supplemented LNCaP tumor. However, the minor change may be of little biological significance, and no difference was detected in LNCaP tumor growth and other transcriptional factors. An increase of ABCG5 in LNCaP tumor animal livers was observed, which indicated glyceollins may facilitate the efflux of cholesterol from the liver through ABCG transport. In our previous study in hamster, ABCG5 was reduced in glyceollins treated animals compared to high-fat diet animals (Huang, et al., 2013). The discrepancy may result from the difference in diet compositions and physiological conditions. Hamster was fed high fat diet while the mouse was fed low-fat diet. The increased consumption of dietary fat and cholesterol led to an increase in circulating and hepatic lipid and cholesterol in hamster. The decreased of ABCG5 in the hamster model was concluded to be the consequence of reduced circulating and hepatic cholesterol (Huang, et al., 2013).

Based on the previous observation in cell model, the present study aimed to analyze glyceollins' anti-tumor effect in LNCaP and PC-3 prostate cancer xenograft

models. The growth inhibition effect in-vitro was not observed in the xenograft models, regardless of their androgen responsiveness. The ineffectiveness of glyceollins in-vivo might result from the limited bioavailability of glyceollins in athymic nude mouse model through ingestion with low-fat diet. Further study in the attempt to increase the bioavailability and elucidate high-fat diet's effect on absorption of glyceollins is warranted to evaluate glyceollins' potential in prevention and relief of prostate cancer.

Chapter 4. Effect of Glyceollins on gut Microbiome

4.1. Abstract

Microbiome has emerged as an important and integral part of the human physiology with a significant role in human health and disease. Diet and dietary components have been shown to shape and alter the population and composition of human gut microbiome. A relation between soy consumption and change in microbiome has been reported previously. However, the precise component(s) contributed to soy's effect on microbiome remain unclear. The current study aimed to evaluate the effect of glyceollins, a group of phytoalexin isolated from soy, on the gut microbiome in animal models. Fecal and cecal samples collected from mouse feeding studies were analyzed for microbial population and composition. Glyceollins supplementation did not alter gut bacteria groups in cecal sample examined in this study. Glyceollins significantly affected total Enterobacteriaceae and Ruminococcus population in fecal samples collected at 24 h, indicating the impact and importance of time of collection in interpreting gut microbiome data in fecal analysis.

4.2. Introduction

Research in human microbiome has demonstrated that microbial cells (~10^{14} cells) outnumber human cells by about 10 times, which makes it a very large and complex ecosystem between the residing microorganisms and human body (Hattori & Taylor, 2009; Savage, 1977). Among the bacteria in the gastrointestinal tract,

Bacteroidetes and Firmicutes are the dominant phyla and account for over 90% of gut microbiome in mouse and human, with Ruminococcus, Lactobacillus and Prevotella being the major families or genera in these phyla (Eckburg, Bik, Bernstein, Purdom, Dethlefsen, Sargent, et al., 2005; Frank, Amand, Feldman, Boedeker, Harpaz, & Pace, 2007; Ley, Backhed, Turnbaugh, Lozupone, Knight, & Gordon, 2005; Ley, Peterson, & Gordon, 2006). Researches have shown a correlation of increase in Firmicutes to Bacteroidetes ratio to an increase in energy harvesting from diet and the pathology of obesity (Turnbaugh, Hamady, Yatsunenko, Cantarel, Duncan, Ley, et al., 2009; Turnbaugh, Ley, Mahowald, Magrini, Mardis, & Gordon, 2006; Turnbaugh, Ridaura, Faith, Rey, Knight, & Gordon, 2009). High fat diet was shown to induce an increase in Lactobacillus, and it was also involved in simple sugar degradation (Guarner & Malagelada, 2003; Zeng, Liu, Jackson, Yan, & Combs, 2013; Zhao, et al., 2011). Ruminococcus and Prevotella were shown to be involved in polysaccharides metabolism, especially fiber, in the gastrointestinal tract (Walker, et al., 2011; Wu, et al., 2011). More importantly, Enterobacteriaceae, Akkermansia and Bifidobacteria have been shown to be involved in energy metabolism and balance, and an increase in carbohydrate intake was shown to elevate Enterobacteriaceae and Bifidobacteria population, while Akkermansia population was negatively associated with consumption of polysaccharides (Amar, Burcelin, Ruidavets, Cani, Fauvel, Alessi, et al., 2008; Backhed, et al., 2004; Sonoyama, Fujiwara, Takemura, Ogasawara, Watanabe, Ito, et al., 2009; Turnbaugh, Baeckhed, Fulton, & Gordon, 2008; Turnbaugh & Gordon, 2009). Bifidobacteria population was also shown to

correlate positively with cholesterol intake and metabolism (Martinez, Wallace, Zhang, Legge, Benson, Carr, et al., 2009).

The link between long-term dietary pattern and chronic diseases has been well documented (Galland, 2010; Tyrovolas & Panagiotakos, 2010; WHO/FAO, 2002), and the gut microbiome forms the interface between diet and human body (Cho & Blaser, 2012). The interaction between bacteria and human cells is central to the protective role of intestinal commensal bacteria for maintenance of health (Brestoff & Artis, 2013). Putative effects of microbiome on pathology or prevention of metabolic diseases, such as obesity, diabetes, and cardiovascular disease are mounting attention in recent years (Cani, 2013). Although the exact influence and mechanism remains unknown, previous studies have gathered evidence that human microbiome may affect human development, physiology, immunity, and nutrition (Dethlefsen, McFall-Ngai, & Relman, 2007). Clinical trials and comparisons of intestinal microbiomes between diseased and healthy subjects have identified biomarkers associated with inflammation and functional metagenomic changes (Karlsson, et al., 2013). Recent studies demonstrated that the composition of the microbiome, along with its gene expression and functional metabolic pathways, could change rapidly when animals were switched from low-fat to high-fat diets (Turnbaugh & Gordon, 2009; Turnbaugh, Ridaura, Faith, Rey, Knight, & Gordon, 2009). Hence, understanding the interaction between diet components and microbiome may be a critical part in studying their influence on human health.

A relation between soy consumption and change in microbiome has been reported. 3-month consumption of soymilk has been shown to alter the microbiome in

overweight and obese men including a potentially beneficial alteration of the Firmicutes to Bacteroidetes ratio (Fernandez-Raudales, Hoeflinger, Bringe, Cox, Dowd, Miller, et al., 2012). However, the precise component(s) contributed to soy's effect on microbiome remain unclear. Phytoalexins are a group of phytochemicals induced by stress factors, such as fungal and bacterial infection, wounding, freezing and UV light exposure (Darvill & Albersheim, 1984). Glyceollins are phytoalexins accumulated in stressed soy to defend the plant from bacteria and fungi infection (Schmidt, Parniske, & Werner, 1992), and previous researches have reported glyceollins' in-vitro anti-bacterial activity (Fett & Osman, 1981; Tzi, Ye, Wong, Fang, Chan, Pan, et al., 2011). Therefore, we hypothesize that phytoalexin, such as glyceollins, may contribute to soy's effect on the microbiome.

In our literature review, we found one critical and unresolved problem in microbiome research appeared to be variable collection time of the fecal sample between studies. There appeared to be a wide discrepancy in control of fecal sample collection procedure and time among human and animal model studies. Some studies used instructions or laboratory protocol to guide the participants to collect fecal samples (David, et al., 2014; Karlsson, et al., 2013; Matsuki, Watanabe, Fujimoto, Takada, & Tanaka, 2004), while others did not specify the collection procedure (Eckburg, et al., 2005; Walker, et al., 2011; Wu, et al., 2011; Zimmer, et al., 2012). Most reports did not mention the collection time point in terms of length of time elapsed after excretion. A previous study indicated that the fecal microbiome was stable over a 72 h period (Roesch, Casella, Simell, Krischer, Wasserfall, Schatz, et al., 2009). However, other study showed that a change of gastrointestinal physiology

could rapidly alter microbiome within 24 h (David, et al., 2014; Li, Wu, Baldwin, Li, & Li, 2012). Hence, further study warranted to further elucidate the stability of the fecal microbiome and optimal collection time.

The primary aim of this study is to evaluate glyceollins' effect on the gut microbiome in a mouse model. The secondary goal is to compare fecal and cecal samples microbial population and composition. Lastly, we seek to evaluate the impact of collection time on the fecal microbiome.

4.3. Materials and methods

Mouse diet and feeding. Male athymic nude mice (BALB/c nu/nu, 20-22 g, 5-6 weeks old; Charles River, Frederick, MD) were fed control AIN-93M diet and AIN-93M supplemented with 250 mg/kg glyceollins (12 animals each group). Mice were remained on their respective diets for 10 weeks. At the end of the tenth week, 2 h and 12 h fecal samples were collected from the bottom of the cage. At the time of animals sacrifice, 24 h fecal samples were collected from the bottom of the cage, and cecal samples were collected from the ceca, flash frozen in liquid nitrogen and kept at -80 °C.

Gut microbiome analysis. Microbial DNA will be extracted using QIAamp DNA Stool Mini Kit from Qiagen following manufacturer's protocol. Briefly, 100 mg of fecal sample will be weighed and placed in a 2 mL tube, homogenized with Precellys (Bertin Technologies, France). DNA will be eluted from the column with 200 µL AE buffer. The concentration of DNA elution will be determined by its absorbance at 260 nm, followed by serial dilutions to the final concentration of 0.4

ng/mL. Real-time PCR was performed on an Applied Biosystems ViiA™ 7 Real-Time PCR System using Fast SYBR Green Master Mix by Applied Biosystems (Carlsbad, CA). A reaction system of 10 μL SYBR® Green Real-Time PCR Master Mix, 0.25 μL 500 nM custom-made oligo primers, 4.5 μL water and 5 μL DNA was used. Total bacteria were measured using primers detecting sequence universal in 91 bacteria species (Parnell & Reimer, 2012). Primers specific for Bifidobacteria (Parnell & Reimer, 2012), Lactobacillus (Parnell & Reimer, 2012), Akkermansia (Parnell & Reimer, 2012), Prevotella (Parnell & Reimer, 2012), Enterobacteriaceae (Parnell & Reimer, 2012), Ruminococcus (Wang, Bose, Kim, Hong, Kim, Kim, et al., 2014), Bacteroidetes (Wang, et al., 2014), Firmicutes (Wang, et al., 2014) were used to determine the population of respective microorganisms. Primer sequences are listed in Table 4.1. The following amplification parameters were used for PCR: 50 °C for 2 min, 95 °C for 10 min, and 46 cycles of amplification at 95 °C for 15 sec and 60 °C for 1 min, followed by a dissociation stage.

Table 4.1. Sequence of Real-time PCR Primers

Bacteria	Direction	Sequence (5'-3')
Total bacteria	Forward	ACTCCTACGGGAGGCAG
	Reverse	GTATTACCGCGGCTGCTG
Bifidobacteria	Forward	TCGCGTCYGGTGTGAAAG
	Reverse	CCACATCCAGCRTCCAC
Lactobacillus	Forward	GAGGCAGCAGTAGGGAATCTTC
	Reverse	GGCCAGTTACTACCTCTATCCTTCTTC
Akkermansia	Forward	CAGCACGTGAAGGTGGGGAC
	Reverse	CCTTGCGGTTGGCTTCAGAT
Prevotella	Forward	TCCTACGGGAGGCAGCAGT
	Reverse	CAATCGGAGTTCTTCGTG
Enterobacteriaceae	Forward	CATTGACGTTACCCGCAGAAGAAGC
	Reverse	CTCTACGAGACTCAAGCTTGC
Ruminococcus	Forward	GGCGGCCTACTGGGCTTT
	Reverse	CCAGGTGGATAACTTATTGTGTTAA
Bacteroidetes	Forward	GGARCATGTGGTTTAATTCGATGAT
	Reverse	AGCTGACGACAACCATGCAG
Firmicutes	Forward	GGAGYATGTGGTTTAATTCGAAGCA
	Reverse	AGCTGACGACAACCATGCAC

Statistical analysis. All end point assays for each sample were conducted in triplicate, and the average was used for group analysis, data for each treatment group were presented as mean ± standard error. Significance level of differences in means was detected using one-way ANOVA and Tukey's test. Statistics analysis was performed using IBM SPSS Statistics 19.0 (2010, IBM Corporation, Armonk, NY, USA) or Graphpad Prism 6 (2012, Graphpad Software, La Jolla, CA, USA). Statistical significance was defined at $p \leq 0.05$.

4.4. Results

Comparison of Cecal Microbiome in Animals Fed Control or Glyceollins Diet. We determined the relative bacterial abundance in cecal samples. No change was observed in selected bacteria species examined after 10 weeks of glyceollins supplementation as compared to the control diet (Fig. 4.1). Although an average of 45% decrease and 62% increase in Bifidobacteria and Akkermansia population, respectively, were observed in cecal sample (Fig. 4.1), due to the large individual difference in bacteria population, no statistical significance was detected.

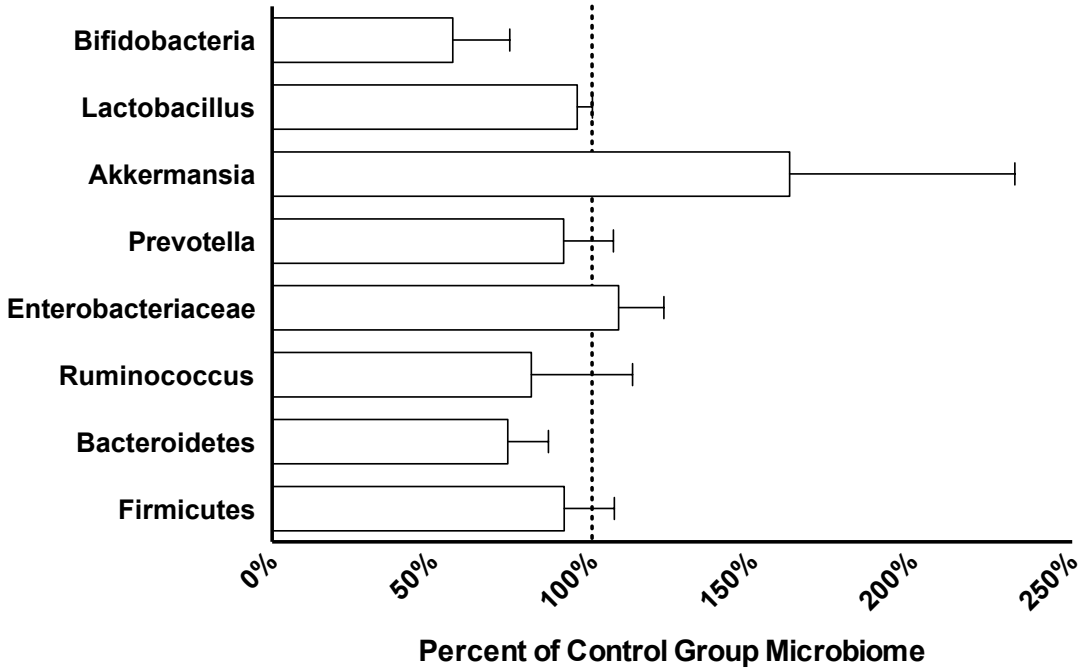

Fig. 4.1. Cecal microbiome in mouse consuming control or glyceollins supplemented diet. Comparisons were made between control and glyceollins supplemented diet, and presented as percent change ± SE. Statistical significance was defined at $p \leq 0.05$ and marked with asterisk.

Temporal Change of Fecal Microbiome Population in Animals Fed Control or Glyceollins Diet. To determined optimal collection time for the fecal

microbiome analysis, a temporal comparison of fecal samples collected at 2, 12 and 24 hours after changes of bedding were analyzed for their microbiome content. No significant change in the microorganism population was observed between the 2 h and 12 h fecal samples of the two diet groups (Fig. 4.2). Although an average of 506% and 264% increases in Akkermansia were observed at 2 h and 12 h, respectively, no statistical significance was detected due to large inter animal differences. Significant increases of Enterobacteriaceae (96%), and Ruminococcus (708%) were observed in fecal samples collected over a 24 h period (Fig. 4.2). Akkermansia showed the most variance between individual animals in cecal or fecal samples. Average Akkermansia in the 24 hours fecal samples from glyceollins treated group was 1000-fold higher comparing to that of the control group. However, the individual difference resulted in no statistical significance (Fig. 4.2).

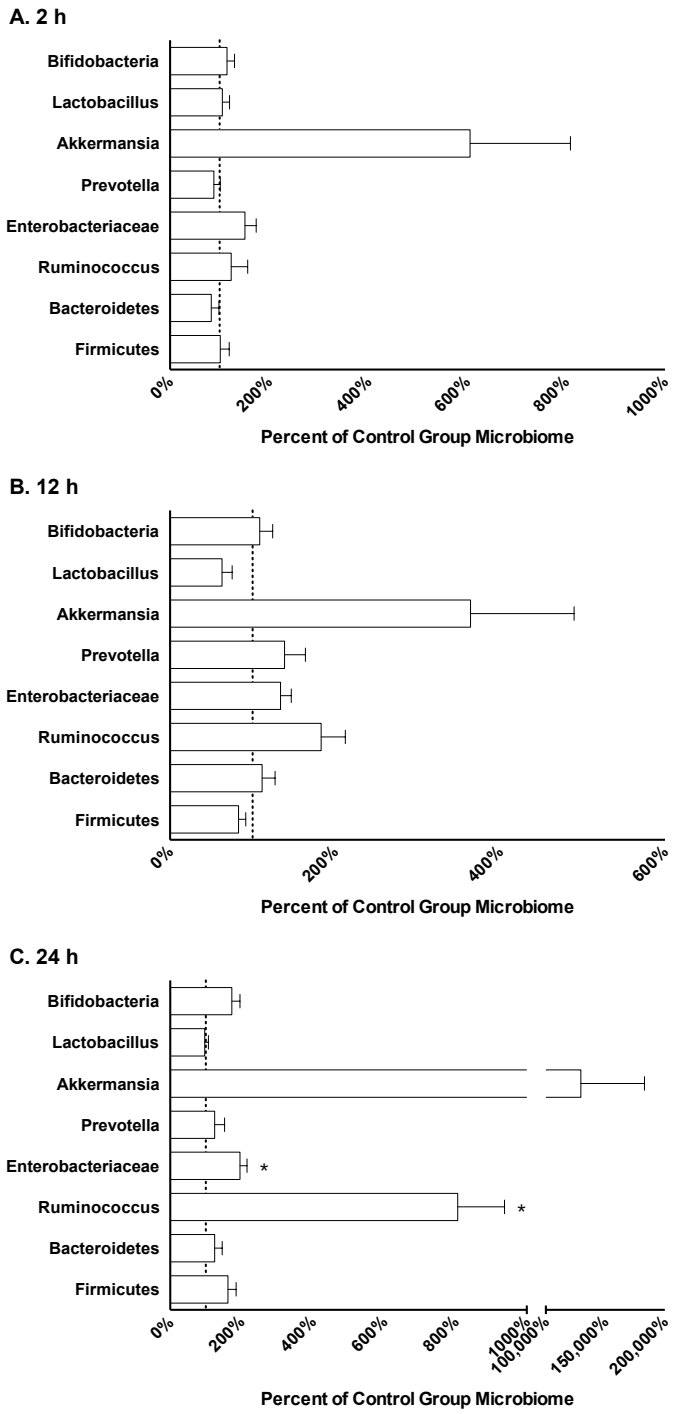

Fig. 4.2. Fecal microbiome in mouse consuming control or glyceollins supplemented diet accumulated in 2 h, 12 h, and 24 h. Comparisons were made between control and glyceollins supplemented diet, and presented as percent change. Statistical significance was defined at $p \leq 0.05$ and marked with asterisk.

Comparison of Cecal and Fecal Microbiome. Total bacteria in cecal and fecal samples were further compared (Fig. 4.3). Cecal samples were designated as 0 hour and compared with the 2, 12, and 24 hours fecal samples. Total bacteria population from animals consumed control diet showed no significant change over 24 h period. On the other hand, there was a significant decrease in total bacteria in fecal samples from animals fed with glyceollins supplemented diet at 24 h (Fig. 4.3). The decreased total bacteria in glyceollins supplemented animal feces were also significantly lower than that in control animal feces.

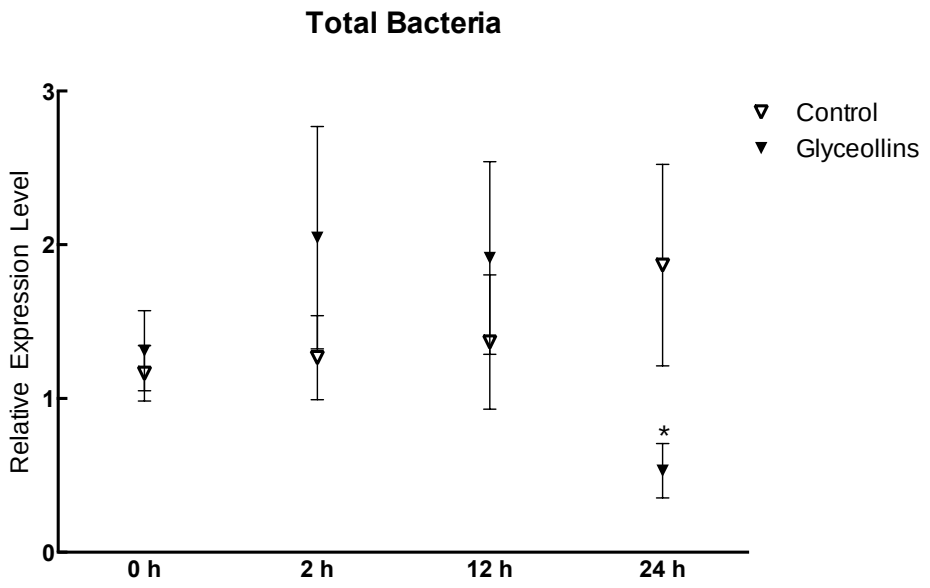

Fig. 4.3. Changes of total bacteria in mouse consuming control or glyceollins supplemented diet over a 24 h period time course. Relative bacteria levels were normalized to cecal bacteria. Comparisons were made between control and glyceollins supplemented diet. Statistical significance was defined at $p \leq 0.05$ and marked with asterisk.

Over a 24 h period, fluctuations in bacteria population were observed (Fig 4.4) and samples from control or glyceollins diet groups showed a similar trend of change (Fig. 4.4). In animals fed a control diet at the first 2 h after excretion, an increase in

population was detected in Lactobacillus. By contrast, population of Bifidobacteria, Akkermansia, Prevotella, Ruminococcus, Bacteroidetes, and Firmicutes remained at the same level. A decrease in Enterobacteriaceae was observed (Fig. 4.4). At the 12 h time point, increased bacteria population were observed for Lactobacillus, with Prevotella, Ruminococcus, Akkermansia, Bacteroidetes. Firmicutes remained at the same level, and decreases in population were observed in Bifidobacteria and Enterobacteriaceae (Fig. 4.4). At 24 h, population of Bifidobacteria, Prevotella, Enterobacteriaceae, Bacteroidetes, and Firmicutes were lower than that in 0 hour (cecal) samples, while Lactobacillus, Akkermansia, and Ruminococcus remained at the same level as that in 0 hour (cecal) samples (Fig. 4.4). In samples from animals fed the glyceollins diet, similar trend of changes as that of the control diet animals were observed except that no significant change was observed in Bifidobacteria at 12 and 24 h, and no change was observed in Bacteroidetes at 24 h.

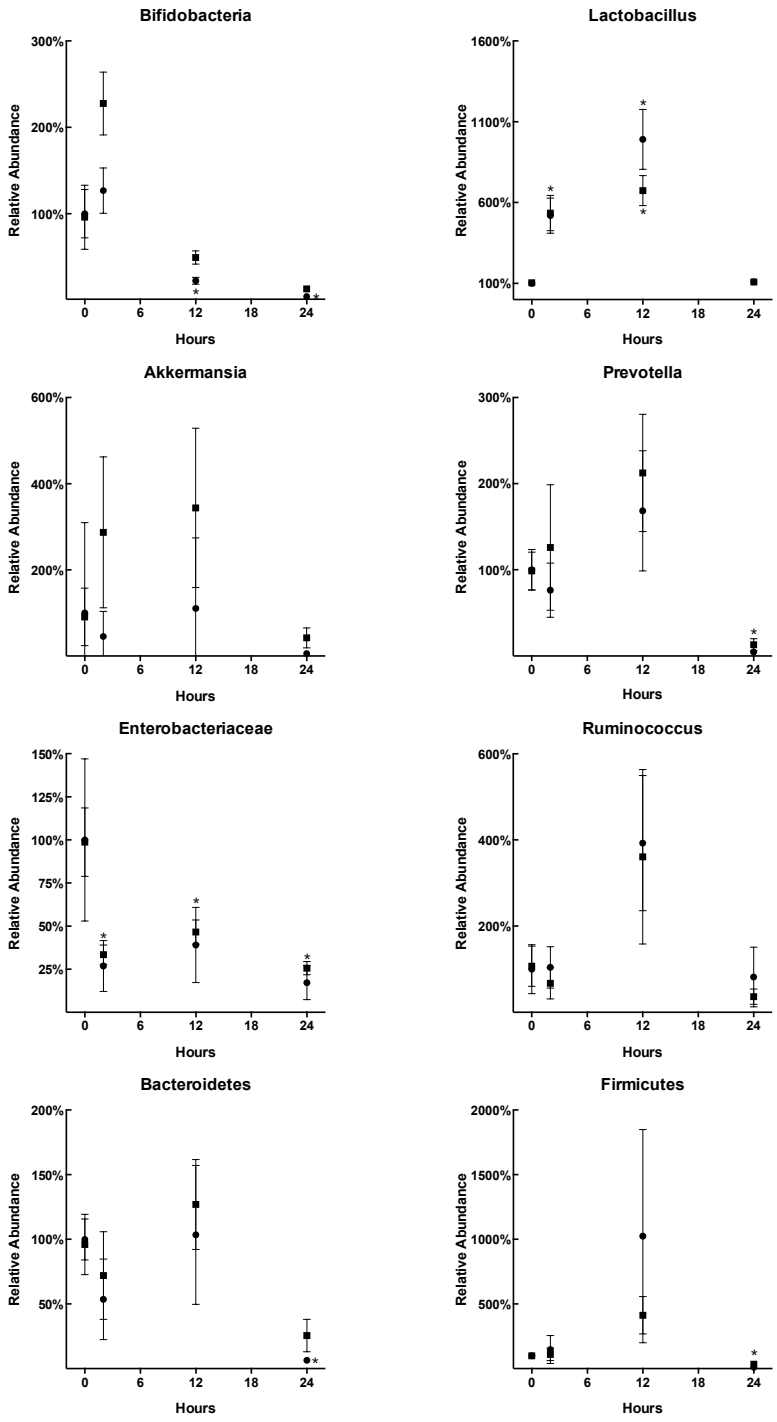

Fig. 4.4. Changes of microbiome in mouse consuming control or glyceollins supplemented diet over a 24 h period time course. Relative bacteria levels were normalized to cecal bacteria. Comparisons were made between 0 h cecal samples and fecal samples at different time points, and statistical significance was defined at $p \leq 0.05$ and marked with asterisk. ● stands for control group and ■stands for glyceollins supplemented group.

4.5. Discussion

There are several interesting observations in our study on the gut microbiome. In this study, we first evaluated glyceollins' effect on the gut microbiome in cecal samples. Soy has been reported to alter microbiome, however, the precise active component has been not identified (Fernandez-Raudales, et al., 2012). This is the first report on the effect of a specific soy component on the gut microbiome. Despite the phytoalexin properties of glyceollins, we did not observe any significant change in microbiome as compared to the cecal samples from animals fed a control diet. Glyceollins have been reported to possess anti-bacterial activities and growth inhibitory effect on some bacteria. Previous study has shown that glyceollins inhibited growth of certain strains of Bacillus, Staphylococcus, and Xanthomonas (Fett & Osman, 1982). However, the bacteria strains determined in this study did not match the ones tested in the previous study, and the exact species or stains affected by glyceollins require further elucidation.

Another interesting observation was fecal samples collected from animals consuming a glyceollins supplemented diet showed significant changes over a 24 h period (Fig. 4.3). A sharp decrease in total bacteria after 24 h was observed in the glyceollins supplemented group, and was significantly less than that of the control diet group. Total bacteria in fecal samples from animals fed control diet did not change significantly over a 24 h period. Therefore glyceollins appeared to affect total bacteria, albeit after 24 h outside of gut. Hence, the sample collecting process (time of collection) couple with a treatment might in part contribute to an observed change in the fecal microbiome.

Previous study has shown that the fecal microbiome was relatively stable after excretion to 72 h, and the change in bacterial communities was about 10% within 24 h (Roesch, et al., 2009). Interestingly, our study showed that specific groups of fecal bacteria changed significantly as early as 2 h. Species specific changes in bacteria were observed in this study (Fig. 4.4), which might result from 1) compositions of aerobic and anaerobic bacteria in each phyla, families or genera, or 2) the roles of specific bacteria in energy metabolism and balance of diet-derived component. Lactobacillus and Enterobacteriaceae showed significant increase and decrease, respectively, comparing to the cecal microbiome (Fig. 4.4). Cecal samples were directly collected from ceca dissected from the animals and immediately frozen in liquid nitrogen. Thus, cecal microbiome is likely to be more reflective of the gut microbiome in-vivo, while the fecal microbiome was subject to environmental factors, such as oxygen exposure. Gut microbiome are composed of aerobic and anaerobic bacteria, with anaerobic bacteria being the predominant population. Firmicutes are facultative anaerobic and Lactobacillus is microaerophilic (Ichimura, 1962), which can survive and grow with or without oxygen. Majority of Enterobacteriaceae are anaerobic, and Bifidobacteria, Ruminococcus, Bacteroidetes, and Prevotella are strictly anaerobic (Evaldson, Heimdahl, Kager, & Nord, 1982; Janssen, 1991; Mahowald, Rey, Seedorf, Turnbaugh, Fulton, Wollam, et al., 2009; Walker, et al., 2011). Thus, exposure to oxygen may reduce the growth or affect the survival of anaerobic bacteria (Evaldson, Heimdahl, Kager, & Nord, 1982; Peterson, 1997). However, no precise pattern of change in specific bacteria population could be concluded based on their aerobic/anaerobic properties and exposure time. Hence,

according to our finding, the influence of collection time on the fecal microbiome cannot be neglected, and if possible, cecal samples should be used to assess the gut microbiome in animal study.

The oxidation and fermentation of diet-derived fecal components might shift the energy metabolism of microbiome and affect their population, leading to an increase or decrease of specific bacteria (Albenberg & Wu, 2014; David, et al., 2014). AIN-93M diet used in this study was a casein-based diet, which could preferentially support the growth of Lactobacillus (Zhang, Ren, Zhao, Zhao, Xu, & Zhao, 2011). Lactobacillus was also known to be involved in lipid and simple sugar metabolism (Guarner & Malagelada, 2003; Zhao, et al., 2011), and the increase of Lactobacillus would lead to faster degradation of lipid and carbohydrate in the feces. Bifidobacteria and Enterobacteriaceae were also active in metabolizing polysaccharides (Backhed, et al., 2004; Turnbaugh, Baeckhed, Fulton, & Gordon, 2008; Turnbaugh & Gordon, 2009). The increased degradation of polysaccharides by Lactobacillus might have led to the decrease in population of Bifidobacteria and Enterobacteriaceae.

Trend of changes were observed in several species at all time points, for example, 2, 6, 4, and over 1200 folds increase were observed in Akkermansia at 0, 2, 12, and 24 h, respectively (Fig. 4.1), however, no statistical significance was detected. This appeared to result from the huge individual differences between animals in this study. Akkermansia was relatively abundant and readily detected in fecal microbiome. The exact reason of such discrepancy is not clear. Therefore, the consistency of gut microbiome between individual animals is critical to controlling the quality and reliability of a study. To this end, a gnotobiotic mouse model was

developed by transplanting gut microbiome from a single human donor into germ-free mice (from same litter), appeared establish consistent microbiome in experimental animals (Gootenberg & Turnbaugh, 2011; Turnbaugh, Baeckhed, Fulton, & Gordon, 2008). The animals used in our study were from an identical genetic background (BALB/c nude mouse, strain: CAnN.Cg-Foxn1nu/Crl inbreed), raised in the same laboratory environment, and fed rodent chow diet. We believe that diversity in the gut microbiome would mimic more of a real life situation and suggest that, in human population, the microbiome may be more challenging to dissect.

In summary, this study examined the effect of dietary intake of glyceollins on major phyla, families and genera of microbiome. However, no effect was observed for glyceollins on the microbiome under our experimental conditions. Change in microbiome can occur to specific species (Turnbaugh, Baeckhed, Fulton, & Gordon, 2008), and a more global investigation using metagenomic techniques may be necessary to pinpoint the specific change in gut microbiome induced by glyceollins treatment. Time of fecal collection could play an important role in the outcome of microbiome population and composition.

Summary

It is promising and important to investigate soy's health promoting potential as an important agriculture commodity. The present study focused on characterizing the soy-derived phytoalexins glyceollins' bioactivities in alleviating cholesterol dysregulation, prevention of prostate cancer, and regulating gut microbiome. Glyceollins supplementation in high-fat diet was shown to significantly reduce circulating and hepatic cholesterol, as well as hepatic inflammatory cytokine expressions. Glyceollins' effects in preventing prostate cancer and regulating gut microbiome were not observed in our experimental condition, however, further study may be performed to conclude glyceollins biological efficacies.

This project provided scientific evidence for the health beneficial effects of the readily accessible soy phytochemicals, and may promote the use of soy as healthy dietary component and enhance food and agriculture economy.

Future perspective

Molecular mechanism of glyceollins' cholesterol-lowering and anti-inflammatory effect and glyceollins' long-term effect on metabolic disorder induced by western style diet may be investigated in future studies. The in-vivo absorption, distribution and metabolism of glyceollins upon ingestion need to be characterized to pinpoint glyceollins' molecular target(s). Deep sequencing and metabolomic tools may be used to elucidate glyceollins' mechanism of actions at transcriptional and translational levels.

Improvement of glyceollins' bioavailability may enhance glyceollins' biological efficacy, including anti-tumor effect. Modification of diet formulation and delivery mechanisms may help to increase the absorption of glyceollins and prevent the degradation or metabolism of glyceollins in the gastrointestinal tract or in the liver.

Glyceollins may affect specific group(s) or species of gut microorganisms in the gut and may induce temporal change(s) in gut microbiome. Metagenomic tools can be used to reveal the specific change(s) in gut microbiome and multiple time points may be studied and correlated to the health outcomes in the animals to understand the potential health implication of alteration of gut microbiome.

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