Potential Roles of Lutein in Mammary Gland Development and Breast Cancer Cell Growth

DISSERTATION

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By

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Abstract

Breast cancer is the most common form of malignancy and the second leading cause of cancer mortality in women. The most recent epidemiological studies have shown an inverse relationship between blood lutein concentration and breast cancer risk. However, it is currently unclear whether it is lutein itself, or a metabolite of lutein that plays a role in breast tissue protection. To better understand the impact of dietary lutein on breast development, we performed an in vivo feeding study by using a modified AIN-93G diet, in which lutein was added to reach the concentration 0.01%. C57/BL6 virgin female mice at 3-week of age were fed with diets with or without lutein supplementation. Plasma, mammary gland, and liver were collected after 5 weeks, 10 weeks, and 15 weeks of feeding. Plasma lutein levels were determined by high-performance liquid chromatography (HPLC). Whole mount staining, light microscopy, and immunohistochemistry (IHC) analyses were conducted to evaluate the development of mammary glands. Our results showed that plasma lutein concentration was close to human plasma lutein level, and lutein accumulated in mammary glands in the lutein supplemented group. In the control group no lutein was detected in plasma, nor mammary gland. Our data also showed that there was no difference with regards to branching of the glands, adipocyte area, or epithelial cell proliferation between the two dietary groups. In addition, to investigate the direct effects of lutein on breast cancer cellular growth, we performed an in vitro study by using multiple human breast cancer
cell lines (T47D, SKBR3, ZR-751, MCF7, MDA-MB-231). The cells were treated with lutein or β-carotene at the concentrations ranging from 0.1 µM to 10 µM, followed by assessing one measurement of cell proliferation and the uptake of carotenoid by the cells. Our in vitro studies showed that there was no significant effect of lutein on breast cancer cell growth with each concentrations we tested. β-Carotene, used as a positive control, led to around 30% decrease in cell growth at 1.0 µM and 10 µM. All five human breast cancer cell lines accumulated lutein to a greater extent than β-carotene. Taken together, our data indicate that there is no effect of lutein on normal mouse breast development or human cancer cell growth. The association between plasma lutein and breast cancer risk described in previous studies might result from lutein being a biomarker of vegetable intake, or a marker of other phytochemical intake, and not a direct effect of lutein itself.
Dedication

To my wonderful family for all of their love, support, encouragement, and every moment from their hearts.
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Chapter 1: Introduction
1.1 Introduction

Carotenoids, natural pigments ranging from yellow to red, are widely found throughout the vegetable kingdom and readily accumulated by vegetable-consuming animals and humans [1]. Currently more than 700 carotenoids have been identified, while the most well studied carotenoids include α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene. These compounds are very rich in human diets and reach comparatively high concentrations in plasma [2].

Lycopene, lutein, and zeaxanthin have been found to have important biological properties such as antioxidant and photoprotective activity. As a result, high intake of these compounds has been linked to reduced risk of numerous chronic diseases in observational studies [1]. Lutein is one of the major xanthophylls found in green leafy vegetables, and it is known as an antioxidant ingredient to protect the eyes from sunlight exposure. As a consequence, laboratory work have been done to explore the biological activities of dietary lutein.

Breast cancer is the most common form of malignancy and the second leading cause of cancer mortality in women [3]. It is a heterogeneous disease with numerous factors that influence the prognosis and the survival rate. The common symptoms of breast cancer include fatigue, depression, insomnia, and pain [4]. Over the past 30 years, special interest has been focused on the possibility that the high intake of fruits and vegetables may protect against the development of cancer, including breast cancer [5]. Those studies
have shown that antioxidant compounds can inhibit the carcinogenic process through a variety of mechanisms [6].

Carotenoids, one of the major antioxidant nutrients in fruits and vegetables, have attracted extensive attention from researchers because of their possible cancer preventive function. The epidemiological studies have shown an inverse relationship between the blood lutein concentration and breast cancer risk, however, it is currently unclear whether it is the lutein itself, or a metabolite of lutein that plays a role in breast tissue protection [7]. To better understand the impact of dietary lutein on breast development, we designed an in vivo feeding study by using a modified AIN-93G diet, in which lutein was added to reach the concentration 0.01%. Additionally, to investigate the direct effects of lutein on breast cancer cellular growth, we performed an in vitro study by using multiple human breast cancer cell lines (T47D, SKBR3, ZR-751, MCF7, MDA-MB-231). These cells were treated with lutein or β-carotene at the concentrations ranging from 0.1 µM to 10 µM. Based on our data generated from these studies, we did not observe an effect of lutein supplementation on the development of the mammary gland in vivo and on the growth of multiple breast cancer cell lines in vitro.
1.2 Aims

**Aim 1:** To determine whether dietary lutein, or a metabolite of lutein plays a role in mammary gland development in C57/B6 mice. The postnatal mammary gland develops extensively through cycles of proliferation, branching, involution, and remodeling. Mammary gland morphogenesis proceeds by expansion of the terminal end buds that result in the formation of tree-like structures made of interconnected hollow ducts and alveoli that are essential for the secretion and delivery of the milk [8]. Developing mammary gland displays properties associated with tumor progression, such as invasion and cell proliferation. A few previous studies demonstrated that mice fed with dietary lutein concentrations of 0.002% and 0.02% had lower development of breast tumors than the controls [9, 10]. To further assess the impact of lutein on postnatal mammary gland development, we treated virgin female mice from 3-weeks to 18-weeks of age with purified diets containing 0.01% lutein or the modified AIN-93G diet without lutein [11]. This 15-week window of mouse development is characterized by rapid body growth, sexual maturation, and mammary gland development, correlates with puberty in humans. By using this model, we evaluated whether this level of lutein supplementation could alter mammary gland development. Our working hypothesis for this aim is that dietary lutein supplementation has an effect on mammary gland branching and epithelial cell proliferation.

**Aim 2A:** To investigate whether lutein or β-carotene affects the proliferation of ER positive and ER negative breast cancer cells. Epidemiological studies have shown an
inverse relationship between plasma lutein concentration and breast cancer risk in both ER(+) and ER(-) subtypes. Studies showed estrogen-sensitive MCF7 and estrogen-resistant MDA-MB-231 breast cancer cells exhibit different sensitivities to treatment with retinoids [12] [13]. Studies also showed 10 µM β-carotene can reduce cell proliferation in both subtypes of breast cancer cell lines. However, no studies have been done to show how lutein treatment could affect the breast cancer cell growth. To further evaluate the effect of different concentrations of lutein on breast cancer cell proliferation, we treated both ER positive and ER negative human breast cancer cell lines with concentrations of lutein ranging from 0.1 µM to 10 µM. We compared the effects of lutein on cellular proliferation with the effects of β-carotene at the same concentrations. Our working hypothesis for this aim is that lutein treatment decreases breast cancer cell growth.

**Aim 2B: To evaluate the uptake and metabolism of lutein and β-carotene in ER positive and ER negative breast cancer cell lines.** A study was done in our lab showed lutein could by cleaved by β-carotene 9', 10'-dioxygenase (BCO2) and yield 3-hydroxy-β-apo-10'-carotenal and 3-hydroxy-α-ionone, or 3-hydroxy-α-apo-10'-carotenal and 3-hydroxy-β-ionone [14]. However, no studies have been found to show whether BCO2 is expressed in human breast cancer cell lines, nor whether lutein is metabolized by human breast cancer cells. The studies in this sub-aim addresses these issues.

Chapter 2 provides background and a review of literature related to physiological functions of carotenoids, carotenoids metabolism, lutein metabolism, mammary gland development, and breast cancer biology. This review provides a context for the research
aims and experiments. Chapter 3 includes the findings of mammary gland branching, proliferation, and lutein accumulation and BCO2 expression at tissue level from \textit{in vivo} study. This contains the bulk of results for research aim 1. Chapter 4 includes the findings on breast cancer cell proliferation, and the uptake and metabolism of lutein by these cells. This chapter contains the bulk of results for research under aim 2A and aim 2B.
Chapter 2: Literature Review
2.1 Carotenoids Introduction

2.1.1 Introduction

Carotenoids are widely distributed, lipophilic, naturally occurring yellow, orange, or red pigments. In total, over 700 of carotenoids, exclusive of \textit{trans} and \textit{cis} isomers, have been isolated and characterized from natural sources [15]. Interestingly, 70 of carotenoids are found in the human diet, while 20 of them are detectable in human blood and tissues [16, 17].

Carotenoids are a class of hydrocarbon compounds that consists of 40 carbon atoms (tetraterpenes), and have a unique structure characterized as an extensive conjugated double-bond system. Modifications of this basic structure, including hydrogenation, dehydrogenation, migration of double bonds, or rearrangement, result in an immense array of structures. Historically, carotenoids have been defined based on the biological source from which they are extracted. Currently, a semisystematic scheme has been developed to define carotenoids mainly on a basis of their structures. As a result, hydrocarbon carotenoids, such as \(\beta\)-carotene and lycopene, are collectively termed as carotenes; while derivatives containing oxygen, such as lutein and zeaxanthin, are referred to xanthophylls. [15].

2.1.2 Dietary sources

Carotenoids are synthesized in plants and certain microorganisms, e.g. some bacteria, algae, and fungi. They are widely present in the vegetable kingdom, and are readily accumulated by vegetable-consuming animals and humans [1]. In total, there are more
than 700 carotenoids present in nature, however, major carotenoids found in the plasma of subjects are limited to β-carotene, α-carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin, and related metabolites [2]. The chemical structures of these six most predominant carotenoids are shown in Figure 1. In addition, the percentage of each of these six main carotenoids in human serum is the following [18]: lutein (20%), lycopene (20%), β-carotene (10%), β-cryptoxanthin (8%), α-carotene (6%), and zeaxanthin (3%) [19, 20].

Carotenoids can also be divided into provitamin A carotenoid and nonprovitamin A carotenoid. Specifically, provitamin A carotenoids, such as α-carotene, β-carotene, and β-cryptoxanthin, can be converted to vitamin A by enzymatic cleavage. These carotenoids are mostly found in carrots, sweet potatoes, pumpkin, kale, spinach, collards and squash [21]. On the other hand, nonprovitamin A carotenoids, including lutein/zeaxanthin, capsanthin, astaxanthin, and fucoxanthin, cannot be converted to vitamin A [22]. Lutein/zeaxanthin is mostly found in parsley, spinach, kale, and egg yolk [23].
Figure 1. Chemical Structures of main carotenoids in human serum.
2.2 Absorption and Metabolism of Carotenoids

2.2.1 Digestion and Absorption of carotenoids

Carotenoids are fat soluble and follow the same intestinal absorption path as dietary fat. Carotenoids are released from food matrices and solubilized in the gut. This process is carried out in the presence of fat and conjugated bile acids. In terms of carotenoid absorption, as little as 3-5 grams of fat in a meal is sufficient. Chylomicrons are responsible for the transport of carotenoids from the intestinal mucosa to the bloodstream via the lymphatics. Carotenoids are transported in fasting plasma exclusively by lipoproteins, namely high-density lipoprotein (HDL) and low-density lipoprotein (LDL). The small intestinal phase is the most critical process that limits the bioaccessibility of carotenoids. Several crucial steps contribute to the intestinal absorption of carotenoids. After the release from the food matrix, solubilized carotenoids are incorporated into mixed lipid micelles in the lumen. Then the compounds can be taken up by intestinal mucosal cells and incorporated into chylomicrons. Finally the carotenoids and their metabolites that are associated with chylomicrons are secreted into the lymph. Recent studies have shown that SR-BI is involved in the uptake of β-carotene, lutein, and lycopene [24-27].

2.2.2 Enzyme Cleavage of Carotenoids

After enterocyte uptake, carotenoids can be metabolized by enzymatic cleavage. Two key enzymes have been identified to play roles in carotenoid metabolism in mammals. The β-carotene-15, 15′-oxygenase (BCO1), which is the key enzyme for retinoid production,
converts a limited number of provitamin A carotenoids such as β-carotene to retinaldehyde by central cleavage at C15/C15 [28]. The other enzyme, β-carotene 9′, 10′-dioxygenase (BCO2), catalyzes the cleavage of carotenoids at the C9/C10′, and displays broader substrate specificity. Lutein can be cleaved by BCO2 to yield 3-hydroxy-β-apo-10′-carotenal and 3-hydroxy-α-ionone, or 3-hydroxy-α-apo-10′-carotenal and 3-hydroxy-β-ionone. [8, 29]. Lutein is not cleaved by BCO1.

2.2.3 Lutein Metabolism

Previous studies have shown that lutein is present as esterified form in plant while as free from in plasma. This indicates that the esterified lutein is enzymatically cleaved during the process of digestion. Our previous work showed that lutein was cleaved at C9/C10′ carbon double bond by BCO2 and yielded 3-hydroxy-β-apo-10′-carotenal and 3-hydroxy-α-ionone, or 3-hydroxy-α-apo-10′-carotenal as well as 3-hydroxy-β-ionone [8]. As a result, the deficiency of BCO2 might impair lutein metabolism and cause problems in humans.

This is supported by an animal study with BCO2 knock-out mice. In this study, animals were supplemented with lutein at several concentrations for a period of 8 weeks. By the end of the study, the researchers observed abrogated lutein metabolism and lutein accumulation in several tissues in BCO2 knock-out mice, but not in control mice [8]. This indicates that these metabolite compounds were rapidly further metabolized and/or secreted in control mice. This study provided strong evidence that BCO2 is required for lutein metabolism in mice.
2.3 Mammary Gland Development

Murine mammary gland development is initiated at approximately embryonic day 10.5 (E10.5) with the formation of the mammary ridges, or milk lines. At E11.4, five pairs of placodes form along the milk lines in the position of the presumptive nipples. Placode formation requires signaling events within the epithelium, as well as paracrine signals originating from the underlying somites [30, 31]. By E16, the mammary bud elongates to a mammary sprout that invades the mammary fat pad precursor mesenchyme. Thereafter, a small amount of branching morphogenesis is initiated, which produces a rudimentary ductal tree that fills only a small portion of the mammary fat pad at birth. The rudimentary ductal tree present at birth is largely growth quiescent until the onset of puberty at 3-4 weeks of age in most strains of laboratory mice [30]. The terminal end buds (TEBs) are transient bulb-shaped structures which are positioned at the distal end of the ductal tree during puberty [32]. In addition, TEBs are the site of lumen formation, which is thought to be driven by apoptosis, and non-apoptotic cell death [33-35]. The mammary ductal tree reaches to the edge of the mammary fat pad and side branching is completed by 8-10 weeks of age [36, 37]. The mature virgin mammary duct consists of a single layer of luminal cells surrounded by a single layer of myoepithelial or basal cells, mammary stem cells, and luminal/basal progenitors [30, 36]. The mammary epithelium exists within the mammary fat pad stroma consisting of diverse cell types, including fibroblasts, mature adipocytes, neutrophils, macrophages, endothelial cells, and nervous tissue [30, 38].
In postnatal mammary tissue, most epithelial cells express receptors for estrogen and progesterone, which enables these hormones to stimulate ductal outgrowth and branching. These receptors have some overlapping effects, as a partial function of estrogen derives from the induction of progesterone receptors (PR) [36]. In the mammary gland, estrogen and progesterone control ductal outgrowth and alveolar expansion by regulating cell proliferation and cellular turnover respectively in the estrous cycle. Estrogen binds two distinct receptors, estrogen receptor (ER) $\alpha$ and ER $\beta$. ER $\alpha$ is required for normal ductal elongation and outgrowth during puberty, by contrast, the deletion of ER$\beta$ has no effects on ductal or alveolar development [39-41]. There are two isoforms of progesterone receptors (PR): PR-A, and PR-B. The PR-B form is responsible for the proliferative effects of progesterone on mammary epithelial cells [42-44].

During pregnancy, the hormones drive production and differentiation of the alveolar cells. This stage of development is characterized by dramatic stromal changes: extracellular matrix components remodeling, depleting of lipid from adipocyte, and augmenting vasculature [37]. After lactation, mammary gland involution is initiated, which is characterized by apoptosis and removal of a majority of the alveolar cells and epithelial remodeling [45, 46]. After involution, the mammary ductal tree resembles that of the adult virgin animal [47, 48]. This cycle of production of alveolar cells, lactation, and involution can occur through the lifespan of a mammal. In the laboratory studies, whole mount staining is widely used to differentiate each stages during this cycle (Figure 2) [36].
Figure 2. Schematic and whole mount presentation of the different stages of mammary gland development [36].
2.4 Benign Breast Diseases

Benign breast disease (BBD) represents a spectrum of breast disorders characterized as imaging abnormalities or as palpate lesion [49]. BBD can be classified histologically into three subtypes: nonproliferative, proliferative with atypia, or proliferative without atypia. Some BBD confers an increase in the risk for subsequent breast cancer [50, 51]. Surprisingly, proliferative BBD, with and without atypia, could be associated with a two- to four-fold increased risk of breast cancer [52].

To better understand the biological characteristics of BBD, the researchers have focused on ER, PR, and Ki67 as the biomarkers expressed in benign lesions that are not in proximity to concomitant breast cancer [53-55]. In a case-control study, the expressions of ER and PR in women with BBD were assessed. The results showed that increased risk of subsequent breast cancer was observed in the subjects with high expression of ER or PR [52]. In another study, Huh et al., investigated the association between BBD and the risk of breast cancer in women with proliferative disease. They observed a statistically significant increased risk of breast cancer in premenopausal women with the higher expression of Ki 67 [53]. Taken together, these studies suggest that the expressions of these molecules may be associated with the risk of breast cancer.

BBD can develop during adolescence and young adulthood, especially in the period between menarche and first birth. During this period, breast tissue is sensitive to environmental stress. In a prospective cohort study, higher intake of β-carotene was associated with lower risk of BBD in adolescent women. Additionally, the intake of α-
carotene and lutein/zeaxanthin were also inversely associated with BBD, but this trend was not statistically significant [56].

Carotenoids may reduce the risk of both BBD and breast cancer through several mechanisms. As antioxidants, carotenoids may reduce oxidative damage caused by environmental stress or abnormal metabolic processes in the body [57, 58]. In addition, carotenoids may play roles in multiple cellular processes, e.g. cell proliferation, cell growth, cell differentiation, and apoptosis [59]. However, the underlying mechanisms by which individual carotenoids mediate the protective effects for BBD and breast cancer remain largely unknown.
2.5 Breast Cancer

2.5.1 Introduction

Breast cancer is the most common invasive cancer found in women [60]. It is also the second leading cause of cancer death in the general population. [61]. A common pathway of breast cancer related to high mortality is the development of metastatic breast cancer. Approximately 40,000 women die of breast cancer each year in the United States [62].

The breast is made up of lobes, and ducts. Each breast has 15-20 lobes, which have many smaller sections called lobules. The lobes, lobules, and the terminal end buds are connected by thin tubes called ducts. Blood vessels and lymph vessels are also found in breast [63]. Breast cancer can spread to other parts of the body when the cancer cells get into the blood or lymph system. Axillary lymph nodes are the most common site where breast cancer metastasis initiates [64, 65]. Therefore, the pathologic status of the axilla provides important prognostic information for patients with breast cancer.

2.5.2 Neoplastic Development

Tumor development is a multistep process which involves mutation and selection for cells with progressively increasing ability for proliferation, survival, invasion, and metastasis [66]. Tumor initiation, the first step of tumor development, is generally thought to be the result of direct administration of a single dose of a chemical carcinogen or a genetic alteration that leads to abnormal cell proliferation [67, 68]. This step is followed by a stage of tumor promotion, where the application of a carcinogenic agent is not required [69]. Tumor promotion may be affected by a variety of environmental
factors, such as diet, age, hormonal balance, and sex. Tumor progression, the third step, continues as additional mutations occur within cancer cells. Some mutations confer a selective advantage to the cell, such as more rapid growth, and the descendants of a mutant cell consequently become dominant within the tumor population [70]. This process is called clonal selection, which occurs throughout tumor development [71].

Tumor initiation is triggered by a chemical, physical, or biologic agent that is capable of irreversibly altering genomic DNA. Such alterations may include a covalent reaction of DNA with the agent or with its metabolites [72]. In addition, the initiating agent may cause modification of DNA structure without covalent binding. Ultimately, the agent may cause one or more complete scissions of the DNA chain, a loss of part of DNA, abnormal DNA repair, or the insert mutation. However, DNA alterations are not absolutely required for the neoplastic transformation [73-75].

Tumor promotion involves an agent capable of altering the expression of genetic information of the cell. Such agents include hormones, drugs, plant products, and so on. Of note, these agents do not directly alter the genomic DNA but rather affect its expression [76, 77]. For example, some agents interact with cell surface receptors or the cytoplasmic and nuclear protein receptors. Upon binding to these receptors, the cells transduce the external signals into intracellular and activate/repress the expression of a certain gene [78].

Tumor progression is characterized as major changes with regards to uncontrolled cell growth, increased invasiveness, metastases, as well as biochemical and morphologic characteristics of the neoplasm [79, 80]. It is now known that in numerous neoplasms
with high degree of malignancy or anaplasia, abnormal or missing regulation of cellular replication is the principal abnormality of the neoplastic population that can be lethal. There are many pathways responsible for transferring metastatic cells, and the major one is the circulation through blood or lymphatics [79, 81]. Previous studies have shown that both endogenous and exogenous factors could alter metastatic growth. Burger et al. have reported that certain cell lines with an increased activity of the enzyme fucosyltransferase were resistant to metastasize [82]. Moreover, in another article, Giraldi and Sava summarized the drugs that inhibit metastases mostly in experimental situation [83]. However, the precise mechanisms of metastasis remain elusive.

2.5.3 Breast cancer prevalence, incidence, and mortality

Breast cancer is the most frequently diagnosed noncutaneous malignancy among women worldwide. It accounts for 1% of all cancer cases and 25% of all female cancer cases. The incidence rates vary among countries: the lowest incidence is found in the developing countries, while highest found is in the developed countries [84]. In 2016, a total of 1,685,210 new cases of breast cancer were reported, and 14% of these patients died of breast cancer in the United States [85, 86]. The majority of the deaths from breast cancer are due to the drug resistance and potential of metastasis to distant organs, such as the lymph nodes, bone, lung, and liver [62, 87]. Overall, the mortality rate of breast cancer dropped from 1989 to 2007, because of the timely screening, early diagnoses, and better treatments for breast cancer. Currently, there are more than 3.1 million breast
cancer survivors in the United States, which include women still being treated and those who have completed the treatment.

2.5.4 Breast cancer risk factors and screening

Breast cancer is a heterogeneous disease, and its etiology is multifactorial, including both environmental stress and genetic factors [88]. The risk factors for breast cancer include early menopause, late menarche, late childbirth, obesity, nulliparity, a high-fat diet, oral contraceptives, age, environmental toxins, and alcohol and cigarette use [89].

Mammography is the most common screening test for breast cancer. The mammogram is an X-ray of the breast, which may recognize small tumors. This technique may also identify ductal carcinoma in situ (DCIS), which indicates the growing of abnormal cells in the lining of a breast duct [63, 90]. Clinical breast exam is an exam of the breast by a doctor or other health professional to check the breast for lumps or other changes [91, 92]. MRI is widely used as a screening test for women who have certain genetic defects, family history, or other health problems [93].

Women who suffer from benign breast disease have an increased risk of breast cancer [94, 95]. The biopsies from these patients reflect a spectrum of histologic conditions, from normal breast tissue of varying physiologic states at one extreme, to changes approximating in situ carcinoma at the other. The researchers are trying to subdivide this spectrum into more homogeneous and prognostically relevant categories [96-99]. These work suggest that the risk of breast cancer is not identical in women with benign breast disease but is concentrated in those with proliferative lesions. However, the number of
patients with proliferative disease in these studies is not sufficient to perform a detailed subgroup analysis [100].

A retrospective cohort study, with sufficient sample size, was conducted to determine the breast-cancer risk associated with histologically reproducible subcategories of proliferative disease, and to estimate the interaction between these diagnoses and nonanatomical risk factors for breast cancer [100]. This study suggests that the high degree of histologic heterogeneity in benign breast-biopsy specimens is associated with large variations in the subsequent risk of breast cancer. Additionally, most of these histologic patterns are not related to the increased risk of cancer. Of the 10,366 benign lesions that they evaluated, 69.7% were nonproliferative and 26.7% were proliferative without atypia. The relative risk of breast cancer for patients in these two categories was 0.89 and 1.6, respectively [96, 100, 101]. More importantly, this study indicates that biopsy specimens lacking proliferative disease do not confer women an increased risk of cancer, and that a family history of breast cancer does not substantially increase the risk for these patients if their specimens lack cysts [100].

2.5.5 Dietary factors
Emerging evidence shows that nutrition and lifestyle factors account for about 25% of all breast cancer cases [102, 103]. Soy is one of the dietary components that is well studied in childhood and adolescence. A meta-analysis of 7 studies showed that high intake of soy (20 mg per day of isoflavone) in Asian women was associated with a reduced risk for breast cancer, compared to those consuming lower amounts (5 mg daily) [104]. A
subsequent prospective study confirmed this protective effect of soy intake on breast cancer in Asian populations [105].

Moreover, several other aspects of adolescent and early adult diet have been studied to evaluate their effects on the risk for both proliferative BBD and invasive breast cancer. A prospective study showed that high fiber intake in adolescence was inversely related to the risk of proliferative BBD [106]. A population-based case control study from Ontario suggested a strong inverse relation between adolescent dietary fiber, vegetable protein, or nut intake and invasive breast cancer risk [107, 108]. In addition, intake of fruit and vegetables has also been hypothesized to reduce the risk of breast cancer [109].

Carotenoids can act as antioxidants, pro-oxidants, and may reduce tumor formation via IGF-receptor inhibition [110]. High intake of carotenoid-rich fruit and vegetables has been associated with a significant reduction in oxidative stress biomarkers and lipid peroxidation [111]. β-carotene is one of carotenoids that have been most extensively studied in terms of cancer prevention [112]. Preclinical studies have shown that β-carotene inhibits tumorigenesis in head/neck, mammary, colon, bladder, and skin cancer models in vivo [113-115].

The researchers found that adolescent intake of a β-carotene-rich fruits and vegetables reduced mammary tumor multiplicity in the adult rat. They also observed that dietary intervention, limited to the time interval for sexual maturation, significantly altered pubertal gland development. This demonstrated that the development of mammary gland was affected by the nutrient microenvironment [116]. In addition, in a prospective cohort study, the researchers showed that higher adolescent β-carotene intake was associated
with lower risk of BBD in young women. This may related to its antiproliferative properties, inhibiting cell growth and angiogenesis and increasing cell differentiation and apoptosis [117].

2.5.6 Breast cancer subtypes

Human breast tumors are histologically complex tissues, containing a variety of cell types as well as the carcinoma cells [118]. There are two distinct types of epithelial cells found in the human mammary gland: basal (or myoepithelial) cells and luminal epithelial cells. These two cell types are conveniently distinguished by immunohistochemistry [119]. Genomic studies have established four major breast cancer intrinsic subtypes: luminal A, luminal B, HER2-enriched, basal-like [119-121]. Understanding the epidemiology of breast cancer by subtypes is critical for guiding treatment, predicting survival, and developing preventive strategies [122, 123].

Luminal A breast cancer is the most common subtype, which accounts for 70% of breast cancers [124]. It is hormone-receptor (ER and/or PR) positive, HER2 negative, and has low Ki 67 protein expression. Luminal A cancers are low-grade, slowly growing, and have the best prognosis [125].

Luminal B breast cancer is hormone-receptor (ER and/or PR) positive, HER2 positive and/or negative with high ki67 protein expression. Luminal B breast cancers grow faster than luminal A subtype [125]. However, it is difficult to distinguish luminal A and B subtypes since the expression of genes defining these two groups is a continuum.
HER2-enriched breast cancer is hormone-receptor (ER and PR) negative, and HER2 positive with faster growing than luminal cancers. This subtype of breast cancer has a worse prognosis, but often successfully treated with targeted therapies aimed at the HER2 protein \([121, 126]\). Basal subtype represents around 15% of invasive ductal breast cancers. It display specific epidemiological, phenotypic, and molecular features \([127, 128]\). The basal-like breast tumor is characterized by low expression of luminal genes, HER2 gene cluster, and high expression of basal epithelial genes. The basal-like subtype is more common among younger and African-American women.
2.6 Human Breast Cancer Cell Lines

Breast cancer is a complex and heterogeneous disease. BT-20 was the first human breast cancer cell line that was established in 1958 [129]. MCF7 is the most commonly used breast cancer cell line that was established in 1973 at Michigan Cancer Foundation [130]. Because of its exquisite hormone sensitivity through the expression of estrogen receptor (ER), MCF7 is a popular model to study hormone-responsive breast cancer [131].

The development of molecular profiling using DNA microarrays have advanced the classification of breast cancer subtypes on the basis of the expression of ER, PR, and HER2 [132]. The characteristics of the four breast cancer subtypes were previously described. The key cell lines for each subtypes are listed in Table 2.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Molecular Expression</th>
<th>Other Feature</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER(+), PR(+/-),</td>
<td>Low ki67 expression, endocrine responsive</td>
<td>MCF7, T-47D</td>
</tr>
<tr>
<td></td>
<td>HER2(-)</td>
<td>chemotherapy responsive</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER(+), PR(+/-),</td>
<td>High ki67 expression, endocrine responsive</td>
<td>ZR-75-1</td>
</tr>
<tr>
<td></td>
<td>HER2(+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal-like</td>
<td>ER(-), PR(-),</td>
<td>High ki67 expression, endocrine nonresponsive</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td></td>
<td>HER2(-)</td>
<td>chemotherapy responsive</td>
<td></td>
</tr>
<tr>
<td>HER2</td>
<td>ER(-), PR(-),</td>
<td>High ki67 expression, chemotherapy responsive</td>
<td>SK-BR-3</td>
</tr>
<tr>
<td></td>
<td>HER2(+)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ER: estrogen receptor; PR: progesterone receptor.
2.7 Lutein and Breast Cancer

In the past 30 years, special interest has been focused on the possibility that a high intake of fruits and vegetables may protect against the development of cancer [5]. Studies have shown that antioxidant compounds can inhibit the carcinogenic process through a variety of mechanisms [6]. Carotenoid, one of the major antioxidant nutrients in fruits and vegetables, therefore, has been extensively studied to elucidate its cancer preventive function. However, those studies mainly focus on the relationship between carotenoids and the risk of lung cancer [133].

Several epidemiological studies have shown that plasma lutein or lutein intake is inversely associated with the risk of breast cancer (Table 2). In addition, a previous study explored the association between circulating carotenoids and mammographic density, which is one of the strongest predictors of breast cancer risk [134]. The researchers observed a 30-40% reduction in breast cancer risk for women who consumed the highest levels of lutein/zeaxanthin, α-carotene, and β-carotene compared with those who consumed the lowest [135]. Inverse associations with breast cancer for dietary and circulating carotenoids have also been observed in two pooled analyses of prospective studies [7, 136]. However, there is equal evidence showing that lutein intake has no effects on the development of breast cancer (Table 2). Therefore, direct empirical evidence from animal and human studies might help to address these discrepant observations.
Table 2. Summary of epidemiological studies on lutein and breast cancer.

<table>
<thead>
<tr>
<th>Major Author</th>
<th>Subject</th>
<th>Study Design</th>
<th>Major Finding</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohen K</td>
<td>3537 women; \leq 50 years old; no history of cancer or PBD</td>
<td>Nested case control study</td>
<td>No significant association between plasma lutein and the risk of PBD</td>
<td>[137]</td>
</tr>
<tr>
<td>Sisti JS</td>
<td>1,179 breast cancer subjects; 1,179 control subject</td>
<td>Case control study</td>
<td>No significant association between plasma premenopausal lutein and the risk of breast cancer</td>
<td>[138]</td>
</tr>
<tr>
<td>Hu F</td>
<td>33 research studies</td>
<td>Meta analysis</td>
<td>No significant association between lutein intake and the risk of breast cancer</td>
<td>[139]</td>
</tr>
<tr>
<td>Bae JM</td>
<td>1,028,438 women; followed for up to 26 years</td>
<td>Pooled analysis of 18 prospective cohort studies</td>
<td>Inverse association between plasma lutein and the risk of ER-breast cancer</td>
<td>[140]</td>
</tr>
<tr>
<td>Yan B</td>
<td>521 breast cancer subjects; 521 control subjects; 25-70 years old</td>
<td>Case control study</td>
<td>Inverse association between serum lutein and the risk of ER+ and ER-breast cancer</td>
<td>[141]</td>
</tr>
<tr>
<td>Wang L</td>
<td>561 breast cancer subjects; 561 control subjects; 25-70 years old</td>
<td>Case control study</td>
<td>Inverse association between lutein intake and the risk of ER+ and ER-breast cancer</td>
<td>[142]</td>
</tr>
<tr>
<td>Eliassen AH</td>
<td>3055 case subjects; 3956 matched control</td>
<td>Pooled analysis of 8 cohort studies</td>
<td>Inverse association between serum lutein and the risk of ER+ and ER-breast cancer</td>
<td>[7]</td>
</tr>
<tr>
<td>Tamimi RM</td>
<td>604 breast cancer subjects; 636 control subjects</td>
<td>Nested case control study</td>
<td>Inverse association between plasma lutein and the risk of breast cancer among women with high mammographic density</td>
<td>[135]</td>
</tr>
<tr>
<td>Mignone LI</td>
<td>5,707 breast cancer subjects; 6389 control subjects</td>
<td>Case control study</td>
<td>Inverse association between lutein intake and the risk of breast cancer among premenopausal women</td>
<td>[143]</td>
</tr>
<tr>
<td>Sato R</td>
<td>295 breast</td>
<td>Case control</td>
<td>Inverse association</td>
<td>[144]</td>
</tr>
</tbody>
</table>
Hulten K 201 breast cancer subjects; 290 control subjects Case control study Inverse association between plasma lutein and the risk of breast cancer among premenopausal women [145]

PBD: premalignant breast disease ER: estrogen receptor.
Chapter 3: Effect of Dietary Lutein on Mouse Mammary Gland Development
3.1 Abstract

Recent epidemiological studies have shown an inverse relationship between the blood lutein concentration and breast cancer risk. However, it is currently unclear whether it is the lutein itself, or a metabolite of lutein that plays a role in breast tissue protection. To better understand the impact of dietary lutein on breast development, we performed an \textit{in vivo} feeding study by using a modified AIN-93G diet, in which lutein was added at a concentration 0.01%. C57/BL6 virgin female mice were fed diets with or without lutein supplementation for 15 weeks. Plasma, mammary gland, and liver were collected at 5 weeks, 10 weeks, and 15 weeks after feeding. Whole mount staining and immunohistochemistry showed no significant difference in proliferation and branching of the glands between the two dietary groups. H&E staining showed no significant difference in adipocyte area in the mammary fat pad between two dietary groups. HPLC analysis showed mouse plasma lutein concentration was close to human plasma lutein level, and lutein accumulated in mammary gland in the lutein supplemented group. No lutein was detected in control group. β-carotene 9′, 10′-dioxygenase (BCO2) is the only enzyme known that catalyzes lutein cleavage. BCO2 protein expression in liver and mammary gland was also unaffected by lutein feeding.
3.2 Introduction

Breast cancer is defined as abnormal growth of cells in breast, which can be initiated from different parts of the breast, and can be felt as a lump or tested by x-ray. Although most breast lumps are benign, they can dramatically increase the patient’s risk of getting breast cancer in the future. Breast cancer can spread to other parts of the body when the cancer cells enter into the blood or lymph system, thereby developing metastases. Breast cancer remains a major problem with regards to its prevalence, incidence, and mortality. Therefore, the effective strategies for its prevention and/or treatment are urgently needed.

Carotenoids, a group of fat-soluble pigments that are present in red, orange, and yellow fruits and vegetables, are hypothesized to reduce risk of breast cancer due to their antioxidative or antiproliferative properties [56, 146]. The data from previous studies are inconsistent regarding the relationship between plasma carotenoids and breast cancer risk. In addition, these studies have reported the different levels of protective effects for specific carotenoids on tumor development [7, 147-152]. A recent pooled analysis on 3055 subjects with breast cancer showed significant inverse association of breast cancer risk and circulating levels of \( \alpha \)-carotene, \( \beta \)-carotene, lutein, zeaxanthin, lycopene, and total carotenoids [153].

Carotenoids may also reduce risk of development benign breast disease (BBD), a group of breast lesions that can develop during adolescence and young adulthood [154]. BBD are associated with higher risk of breast cancer. During adolescence, especially in the period between menarche and first birth, breast tissue is particularly sensitive to environmental exposures because cells are not fully differentiated [155, 156]. In this
period, carotenoids may play a role in preventing hyperplasia, cyst development, and other proliferative conditions [155, 156]. Carotenoids protect cells against oxidative stress via absorbing free radicals, and also inhibit cell growth and angiogenesis by increasing apoptosis [157]. β-Carotene has been shown to reduce mammary tumor growth in the adult rat, and higher adolescent β-carotene intake is also related with lower risk of BBD in young women [117].

No previous studies have been conducted to investigate the effects of dietary lutein on mammary gland development. We examined this potential role of lutein in mammary gland development by conducting a mouse feeding study with adding 0.01% lutein. We hypothesized that lutein supplementation can decrease epithelial proliferation, mammary gland branching, and adipogenesis in mammary fat pad.

This chapter provides the detailed methods and results for Specific Aim 1.
3.3 Materials and Methods

3.3.1 Animals and diet

Three-week old C57/BL6 female mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and housed 5 per cage at 22±0.5°C on a 12-hour light, 12-hour dark cycle with free access to water and food. Thirty female mice were randomly divided into two groups upon arrival, and body weights were weekly recorded. One group of mice were fed with AIN 93-G diet (D100102G, Research Diets Inc. New Brunswick, NJ, USA), and the other group of mice were fed with 0.01% lutein supplemented diet prepared with 5% lutein beadlets a gift from DSM Nutritional Products Ltd (Basel, Switzerland) (D15013001, Research Diets Inc. New Brunswick, NJ, USA). The composition of these purified diets is shown in Table 3. This concentration of lutein supplementation is based on a previously published study in mice showing that mice fed with lutein concentrations at 0.002% and 0.02% were more effective on lowering mammary tumor incidence, tumor growth than the concentrations at 0.2% and 0.4% [10]. Body weight and food intake were recorded weekly. After 5 weeks, 10 week, and 15 weeks feeding, five mice from each dietary group were anesthetized with isoflurane for blood collection, and then euthanized by CO₂ and cervical dislocation. Blood was collected into EDTA-treated blood collection tubes, and centrifuged for 10 minutes at 2000 rpm using a refrigerated centrifuge. Following centrifugation, plasma was aliquoted and transferred to a clean polypropylene tube. Tissues, including liver, thoracic mammary gland, and perigonadal adipose tissue, were excised and snap frozen in liquid nitrogen, and stored at -80°C until further analysis. The left side abdominal mammary gland was
dissected, fixed in 10% formalin, and embedded in paraffin for histology and immunohistochemistry (IHC) analysis. The right side abdominal mammary gland was dissected for whole mount staining. All procedures followed institution guidelines and were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Table 3 Composition of Experimental Diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>gm%</th>
<th>kcal%</th>
<th>gm%</th>
<th>kcal%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nutrient</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Fat</td>
<td>7</td>
<td>16</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>kcal/gm</td>
<td>4.0</td>
<td></td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td><strong>Ingredient</strong></td>
<td></td>
<td>kcal</td>
<td></td>
<td>kcal</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>800</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>L-cystine</td>
<td>3</td>
<td>12</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>397.486</td>
<td>1590</td>
<td>397.486</td>
<td>1590</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>132</td>
<td>528</td>
<td>132</td>
<td>528</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>400</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>50</td>
<td>0</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>70</td>
<td>630</td>
<td>70</td>
<td>630</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.014</td>
<td>0</td>
<td>0.014</td>
<td>0</td>
</tr>
<tr>
<td>Mineral Mix S10022G</td>
<td>35</td>
<td>0</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin Mix V10037</td>
<td>10</td>
<td>40</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>Lutein Beadlets (5% Lutein)</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>4000</td>
<td>1002</td>
<td>4000</td>
</tr>
</tbody>
</table>
3.3.2 Whole mount staining

The right abdominal mammary gland was removed from the area close to the nipple towards the distal end of the gland. The gland was spread immediately onto a labeled glass slide, fixed with Carnoy's fixative (25% glacial acetic acid and 75% absolute ethanol) at room temperature for 48 hours. Slide was washed with 70% ethanol for 1 h and distilled water for 30 min. Then, slides were stained with carmine alum (1 g carmine and 2.5 g aluminum potassium sulfate in 1000 mL distilled water) for two days. Excess dye was washed using increasing concentrations of ethanol (70, 95, 100%) (slides were kept in each concentration of alcohol for 1 h) and cleared with xylene and kept room temperature at least for 48-72 hours. Then slides was washed using decreasing concentrations of ethanol (100, 95, 70%) for 30 min each, and rinsed in distilled water for 10 min. The whole gland was covered by mount media, and completely dried at room temperature. Mammary gland whole mounts was examined under a light microscope.

3.3.3 Immunohistochemistry

Formalin-fixed, paraffin-embedded mouse mammary gland sections of 3–5 µm were prepared by Veterinary Histology Laboratory Services, The Ohio State University (Columbus, OH, USA). Before proceeding with staining, paraffin sections were first deparaffinized in xylene and rehydrated in graded ethanol. Sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity and blocked with 3% BSA
and 1.28% NDS in PBS (pH 7.4). After blocking, sections were incubated with primary antibody for Ki67 (ab 16667, Abcam, Cambridge, MA, USA). Sections were then be incubated with biotin-SP conjugated secondary antibody. An anti-biotin conjugated horseradish peroxidase (HRP) tertiary antibody was used followed by diaminobenzidine (DAB, Dako, Carpinteria, CA) and counterstained with Gill’s Hematoxylin (Fisher Scientific, Fair Lawn, NJ). Sections were examined using Olympus BX51 light microscope, and DP Manager Software was used to digitally record images.

3.3.4 Histology

Formalin-fixed, paraffin-embedded mouse abdominal mammary gland sections of 3-5 µm were prepared, and stained with Hematoxylin and eosin (H&E) by the Veterinary Histology Laboratory Services, The Ohio State University (Columbus, OH, USA). Images were captured by using a light microscope (Olympus BX51 6 headed microscope), and digital camera (Olympus SC30 Digital Camera), with a CellSens Standard imaging system (Olympus Corporation, Tokyo, Japan). Three random fields were captured from each animal, and evaluated by using ImageJ software (NIH) to quantify the cross-sectioned area of 150 adipocytes for each animal (n=3/group).

3.3.5 Protein isolation and immunoblotting

Tissues (liver and mammary glands) were homogenized with Radio-Immunoprecipitation Assay buffer (RIPA, Thermo Scientific, Rockford, IL) with protease inhibitor cocktail (PI, Roche Diagnostics, Indianapolis, IN) in a ratio of 1 g:5 ml with an Omni Tissue
Homogenizers (Kennesaw, GA). The homogenates were incubated at 4°C for 1 hour, and then the total protein concentration was determined using the BCA protein assay (Thermo Scientific, Rockford, IL). Proteins (50-100 µg/sample) were resolved by SDS–PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After transfer, membranes were blocked with 5% non-fat dry milk in PBS with 0.1% Tween-20 (PBST) for 1 hour at room temperature. The membranes were incubated with primary antibodies BCO2 (14324-1-AP, Proteintech Group, Rosemont, IL, USA) and β-actin (60008-1-lg, Proteintech Group, Rosemont, IL, USA) overnight at 4 °C on a rocker. After washing, membranes were incubated with infrared dye conjugated secondary antibody for 30 min at room temperature. Membranes were protected from direct light during secondary antibody (LI-COR, Lincoln, NE, USA) incubation and observed with the Odyssey LI-COR Infrared Imaging System (LI-COR biosciences, Lincoln, NE). β-actin was used as a loading control.

3.3.6 Lutein extraction

Lutein was extracted from food pellets, plasma and animal tissues as previously described with slight modifications [158, 159]. For lutein extraction from plasma, 200 µL of plasma was treated with 600 µL 2-propanol-dichloromethane (2:1, v/v) and vortexed. All samples were kept on ice under yellow light to prevent oxidation during extraction. The mixture was centrifuged for 5 minutes at 2,000 RPM. The top layer was removed and dried under nitrogen. The resulting residue was re-suspended in 200 µL of mobile phase, filtered through a 0.22 µm pore sized filter and run on HPLC. For lutein extraction from food pellets and animal tissues, 5 µl of ice-cold PBS was added to every 1 mg of
food pellet or tissue, and homogenized with an Omni Tissue Homogenizers (Kennesaw, GA). 200 µL of the homogenized solution was treated with 600 µL 2-propanol-dichloromethane (2:1, v/v) and vortexed, and followed with the method described above. Extracts were then collected, dried under nitrogen, re-suspended in mobile phase, filtered through a 0.22 μM pore sized filter and run on HPLC.

3.3.7 HPLC analysis

Plasma and tissue lutein levels were analyzed using an Agilent Technologies 1200 Series Diode Array and Multiple Wavelength Detector HPLC system using a method previously described [160]. A column C30 Type Carotenoid, 4.6 X 250 mm, 3 μm (YMC, Inc., Milford, MA) was used with methanol-methyl-tert-butyl-ether (90:10, v/v) at a flow rate of 0.9 ml/min as mobile phase. Lutein was monitored at 453 nm and quantified using external standard curves established for each test.

3.3.8 Statistical analysis

All data are presented as mean ± SEM. Student’s t-test is used to determine the statistical significance in the differences between 2 groups. One-way ANOVA followed by Tukey’s post-hoc test is used to compare multiple treatment groups. Two-way ANOVA is used to assess the statistical differences between multiple treatment groups at different time-points. Data is analyzed using a Graph-pad Prism; p < 0.05 is considered statistically significant.
3.4 Results

3.4.1 Effects of dietary lutein on body weight and food intake

The animal body weight per cage (5 mice per cage) was recorded, and feeding started upon arrival. The weekly body weight (Figure 3A) was not significantly different between AIN-93G (control) diet group and 0.01% lutein supplement (lutein) group during the whole 15 weeks feeding period. There was no significant difference in body weight gain (Figure 3B) between control group and lutein treatment group after 15 weeks feeding. In addition, there was no significant difference between two dietary groups in weekly food intake (Figure 3C).
Figure 3. Body weight and food intake in response to AIN-93G diet (control diet) and lutein supplemented diet (lutein diet). To be continued.
C57/B6 female mice were received at 3-weeks of age, and randomly divided into two groups (n=5/group). Animal body weight was recorded upon arrival, and feeding with either Ain-93G diet (control), or 0.01% lutein supplement diet (lutein) started upon arrival. The feeding period lasted for 15 weeks. The weekly body weight of the two groups was recorded (A), and body weight gain after 15-week feed was determined (B). The average weekly food intake of the two dietary groups was recorded (C). Values are presented as mean±SEM. No significant difference were observed.
3.4.2 Effects of dietary lutein on mammary gland development

Mice from each dietary group (n=5/group) were sacrificed after every 5-week feeding, and the right abdominal mammary gland from each animal was collected for whole mount staining. Over the feeding period, the tree-like structure of the mammary glands developed normally in both the control group (Figure 4A and 4C) and lutein group (Figure 4B and 4D). The epithelial elongation is the distance from the nipple to the end of the epithelial tree structure. The length of the nipple to the end of the epithelial structured was manually measured from each animal under microscope. The length in each group increased over the feeding period (Figure 4E). However, there was no significant difference between two dietary groups. Another marker to evaluate the mammary gland development from whole mount staining is the number of terminal end buds (TEB), which is the largest bulb located only at the distal end of the mammary epithelial tree. Whole mount staining photographs were taken, and the numbers of terminal end buds were manually counted (Figure 4F). Over the feeding period, the numbers of TEB increased in both dietary groups which were consistent with the development of the epithelial tree structure. There was no significant difference between to control group and lutein supplemented group.
Figure 4. Effects of dietary lutein on mammary gland development. To be continued.
After 5, 10, 15-week feeding, the right abdominal mammary gland from each dietary group (n=5/group) were collected for whole mount staining. Representative photographs from control group (A) and lutein supplement group (B) were taken under 2X magnification and 10 X magnification (C and D). Epithelial elongation (E) was measured from the whole mount staining slides, and number of terminal end bud (F) was counted from the whole mount staining pictures. Values are presented as mean±SEM. *Indicates the significant difference v.s. control diet at 5 week time-point. # Indicates the significant
difference v.s. lutein diet at 5 week time-point. No significant difference were observed between two dietary groups at any time point.
3.4.3 Effect of dietary lutein on mammary gland histology

Mice from each dietary group (n=5/group) were sacrificed after every 5-week feeding, and the left abdominal mammary gland from each animal was collected for histology. Hematoxylin and eosin (H&E) staining showed normal mammary fat pad composition in both control group (Figure 5A) and lutein treatment group (Figure 5B). The cross-sectioned area of 150 adipocytes from each animal (n=3/group) was quantified by using ImageJ software (Figure 5E). It showed there was no significant difference in the adipocyte area between two dietary groups. The ki67 protein is a cellular marker of proliferation. The mammary gland ki67 immunohistochemistry (IHC) showed normal epithelial cell proliferation in both control group (Figure 5C) and lutein supplement group (Figure 5D). Adipose tissue, lactiferous duct, and connective tissue were shown in both groups. The percentage of ki67 staining was calculated by the number of ki67 positive cells divided by the total number of epithelial cells around the lactiferous duct (Figure 5F). There was an increase of the percentage of ki67 staining in both dietary groups as the animal grew, however, there was no significant difference between two dietary groups.
Figure 5. Effects of dietary lutein on mammary gland histology. To be continued.
After 5, 10, 15-week feeding, the left abdominal mammary gland from each dietary group (n=5/group) were collected for histology. H&E staining of the mammary fat pad for the control group (A) and lutein supplement group (B) were shown over the feeding period respectively. Ki67 IHC staining of the mammary gland for both groups (C and D) showed epithelial cell proliferation. Adipocyte area (E) of was quantified for both dietary groups (n=3/group) by Image J software. The percentage of ki67 staining was quantified for four image fields of each animal for both dietary groups (n=5/group). Values are presented as mean±SEM. *Indicates the significant difference v.s. control diet at 5 week.
time-point. # Indicates the significant difference v.s. lutein diet at 5 week time-point. No significant difference were observed between two dietary groups at any time point.
3.4.4 Lutein levels in diet, plasma, and mammary gland

Lutein was monitored at 453 nm, and external standard curve was established separately (Figure 6A) for quantification. Lutein concentration in food pellet (Figure 6B) was confirmed after exposed to the same environment as in mouse feeding cage. After 5, 10, and 15 weeks feeding, plasma and mammary gland lutein levels from both dietary groups were measured by HPLC. Mouse plasma chromatogram showed multiple peaks, indicating the metabolism of lutein to more polar compounds that were not identified (Figure 7A). Peak c showed the same UV-visible spectra and retention time as a lutein. No lutein was detected in the plasma from the control diet group, while the plasma lutein concentration was between 0.1 µM and 0.2 µM from the lutein supplement group (Figure 7B). The mouse plasma lutein level from the treatment group was very close to the human plasma lutein concentration, which was measured by our lab previously [161]. Similarly, mouse mammary gland chromatogram showed three peaks (Figure 8A), and peak c displayed the same UV-visible spectra and retention time as lutein. As expected, no lutein was detected in the mammary gland from the control diet group (Figure 8B). The mammary gland lutein concentration increased over 15 weeks of feeding period (Figure 8B).
Figure 6. Lutein standard and lutein in food pellet.

Lutein standard (A), and extract of food pellet (B).
Figure 7. Plasma lutein. To be continued.
HPLC chromatograms were monitored at 453 nm. Mouse plasma chromatogram showed four peaks (A), and peak c had similar UV spectra and retention time as lutein. Mouse plasma lutein was measured after 5, 10, 15-week feeding (B) from both dietary groups (n=5/group). Values are presented as mean±SEM.
Figure 8. Mammary gland lutein concentration. To be continued.
HPLC chromatograms were monitored at 453 nm. Mouse mammary gland chromatogram showed several peaks (A), and peak c had similar UV spectra and retention time as lutein. Mouse mammary gland lutein was measured after 5, 10, 15-week feeding (B) from both dietary groups (n=5/group). # Indicates the significant difference v.s. lutein diet at 5 week time-point. Values are presented as mean±SEM.
3.4.5 BCO2 protein expression in mouse liver and mammary gland

BCO2 protein expression was detected in mouse liver in both control group and lutein supplement group (Figure 9A). There was no significant difference of BCO2 expression at each time point between two groups. And there was no significant different among three time point within each group. Similarly, BCO2 expression was observed in mouse mammary gland (Figure 9B). There was no significant difference between two dietary groups at any time point, nor within each dietary group.
Figure 9. BCO2 expression in mouse liver and mammary gland.

BCO2 expression (green signal) by western blot in mouse liver (A) and mammary gland (B) from both dietary groups over 15-week feeding. β-actin (red signal) was used as loading control.
3.5 Discussion

In summary, we want to highlight several findings in the present studies. 1) lutein supplement has no effects on body weight and food intake. 2) we are able to reach the similar lutein concentrations in plasma and mammary gland as that in humans 3) lutein supplement does not influence mammary gland development with regards to the tree-like structure, the epithelial elongation, the length of the nipple, the number of TEB, adipocyte area, and adipocyte proliferation. 4) lutein supplement has no influence on BCO2 expression in liver and mammary gland. 5) BCO2 expression in liver and mammary gland is the same in the presence or absence of lutein supplement.

Numerous epidemiological studies have come to the conclusion regarding the relationship between plasma lutein concentration and breast cancer risk [7, 151]. A pooled analysis of 8 prospective studies observed significant inverse association of circulating levels of β-carotene and lutein with the risk of breast cancer [7]. Benign breast disease (BBD) is a group of breast lesions that can develop during adolescence and young adulthood, and are associated with higher risk of breast cancer. An analysis of carotenoid intake among adolescent girls demonstrated significant inverse association between β-carotene consumption and risk of benign breast disease (BBD) [56]. Lutein/zeaxanthin was also inversely associated with BBD, but the association was not statistically significant [56]. Therefore, it is not clear whether it is dietary lutein, or a metabolite of lutein, that may lower the breast cancer risk. As a result, our current studies were designed to address this important question.
A previous study in mice showed that low concentrations of lutein supplementation (0.002 and 0.02%) lowered mammary tumor incidence, tumor growth, and increased tumor latency, whereas high concentrations of lutein supplementation (0.2 or 0.4%) were less effective [10]. Thus, in our present study, we chose 0.01% lutein supplementation for the feeding study. Our data showed that the plasma lutein levels had reached similar concentrations as observed in humans. In the meantime, we observed a similar body growth and food intake between two groups of animals.

In order to elucidate the direct effects of lutein supplementation on mammary gland development, we carried out analyses on our mouse mammary glands. First, we analyzed the tree-like structure and the epithelial elongation. The breast ductal network follows a tree-shaped morphology. To compare the tissue samples from the two diet groups, we analyzed these tree-like structures in terms of the branching frequency, the tortuosity, and the spatial distribution of branching. Interestingly, we did not see any significant difference. Then we quantitated the epithelial elongation by measuring the length of the nipple to the end of the epithelial structure. The epithelium of mammary gland exists in a highly dynamic state and undergoes dramatic morphogenetic changes throughout life. We observed the increased epithelial elongation over time in both diet groups. This indicates a normal breast development in these animals. However, we did not see a significant difference between the two diet groups.

In humans, the mammary gland starts to grow as a blank fat pad with a primary duct at the nipple. This primary duct branches posteriorly throughout the fat pad and progressively infiltrate the mammary gland over time. These ducts are led by areas of
high cell proliferation, termed terminal ductal lobular unites (TDLU) and eventually differentiate into lobular-alveolar unites (LAU) when the mammary gland becomes fully matured [54, 162]. In rodents, TEB and alveolar buds have equivalencies to TDLU and LAU, respectively. Interestingly, TEB has been identified as a primary site of tumor initiation and therefore a point of interest related to the development of breast cancer [163]. To further evaluate the mammary gland development, we calculated the number of TEB in the mice fed with or without lutein supplement. We also observed an increased number of TEB in each group over time, which indicates the maturation of mammary gland in our mice. However, there was no difference between the diet groups.

Adipocytes constitute a major component of breast tissue, and are proposed to be obligate partners in breast cancer progression [164, 165]. Adipocytes can release several cytokines, e.g. TNF-α, adiponectin, and IL-6, that regulate cell proliferation, migration, and invasion [166]. In our studies, we quantitated the area of adipocytes and evaluated the cell proliferation by ki67 IHC methods. Again, there was no difference in animals as the control diet and lutein supplement. Taken together, these data strongly suggest that lutein supplement does not influence the development of mammary gland and the risk of breast cancer in this mouse model.

We detected the expressions of BCO2, the only enzyme that is known to cleave lutein [14]. Our data showed the expression of BCO2 in liver and mammary gland tissues, and the lack of influence of lutein supplement on expression.

In summary, our current studies provide evidence that lutein supplement does not influence the development of mammary gland in this mouse model. However, our studies
have limitations. We used healthy young female mice in the current studies. Thus, it is
difficult to extend our findings directly to cancer prevention. In breast cancer subjects,
the microenvironment within the breast is very different from that of our animals. In
addition, women who are diagnosed with breast cancer are often above 40-year-old, and
combined with many other risk factors, e.g. obesity, alcohol abuse, genetic defects, and
reproductive history. Our animal model can’t mimic those conditions. Future studies
using more humanized animal models will provide insight into the physiological roles of
this compound.
3.6 Acknowledgement

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Chapter 4: Effects of Lutein and β-Carotene on ER(+) and ER (-) Breast Cancer Cell Growth
Breast cancer is the most common form of malignancy and the second leading cause of cancer mortality in women. There are four subtypes of breast cancer with different characteristics of molecular expression. The inverse relationship between plasma lutein concentration and breast cancer risk was observed in both estrogen receptor positive and estrogen receptor negative subtypes. To further investigate the function of lutein on breast cancer cell growth, we performed *in vitro* studies by using human breast cancer cell lines (T47D, SKBR3, ZR-751, MCF7, MDA-MB-231). These cells were treated with lutein or β-carotene, then one measurement of cell proliferation over 72 hours was determined by MTT assay. The cellular uptake of carotenoids was analyzed by HPLC. No significant difference in cell proliferation was observed with concentrations of 0.1 µM to 10 µM of lutein from MTT assay. In contrast, β-carotene treatment, as a positive control, led to a 30% decrease in cell growth at concentrations of 1.0 µM and 10 µM incubation. The cellular uptake of lutein was greater than that of β-carotene, in spite of the growth inhibitory effects of the latter. BCO2 protein expression was observed in all five cell lines, however, no cleavage products of lutein were detected. Taken together, our data showed that lutein has no effects on the growth of multiple breast cancer cell lines, although the compound accumulates in the cells.
4.2 Introduction

Breast cancer has claimed the highest incidence rate and the second highest mortality rate of all cancers in American women. Great efforts to lower these rates have been made and mainly focused on the development of chemoprevention, which is a pharmacological approach to arrest or reverse the process of multi-step carcinogenesis [146, 157]. A number of compounds naturally present in foods, particularly antioxidative compounds in plants, have shown the potentials as chemopreventive agents [167, 168]. According to the epidemiological studies, vegetable and fruit consumption has constantly been associated with a reduced incidence of a variety of cancer, and dietary carotenoid intake from these sources has similarly been correlated with a reduced cancer risk [168-171]. Carotenoids could lower cancer risk probably due to their antimutagenic properties, the ability to scavenge free, as well as improving immune response [172, 173].

β-Carotene is the major source of vitamin A as a provitamin A carotenoid. Two metabolic pathways exist for its conversion to vitamin A: central cleavage by BCO1 and excentric cleavage by BCO2. Previous research has shown modest relationships between the consumption of nutrients found in carotenoid rich foods, such as α-carotene and β-carotene, and a reduced risk of breast cancer [174, 175]. Several in vitro studies also showed β-carotene and its excentric cleavage products could inhibit cellular proliferation in human breast cancer cell [176].

Lutein, highly presents in dark green leafy vegetables (spinach, kale, broccoli), quenches peroxy radicals and demonstrates antioxidant properties against oxidative damage in vitro [177, 178]. Lutein can be cleaved by BCO2 but not by BCO1. The potential protective
effect of lutein on the development of breast cancer has been a great of interest recently. A recent study found inverse correlation between plasma lutein and oxidative stress by analyzing 37 women [179]. Lutein has also been shown to induce apoptosis in transformed human mammary cells, but protect normal mammary cells from apoptosis in *in vitro* cell culture studies [180].

To the best of our knowledge, no previous studies have been conducted to explore the effects of lutein on both ER positive and ER negative human breast cancer cell lines. We examined this potential anti-tumor function of lutein in multiple cell lines, and compared the cellular proliferation as well as cellular uptake of lutein with that of β-carotene. We hypothesized that lutein treatment can inhibit cellular proliferation in a concentration dependent manner.

This chapter provides the detailed methods and results for Specific Aims 2A and 2B.
4.3 Materials and Methods

4.3.1 Materials

All-trans-lutein was purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ), and all-trans-β-carotene was purchased from Sigma Aldrich (Saint Louis, MO). Fetal bovine serum was purchased from Gibco Thermo Scientific. Dimethyl sulfoxide (DMSO) and solvents used for HPLC were purchased from Sigma Aldrich. BCO2 primary antibody and β-actin were purchased from Proteintech Group (Rosemont, IL, USA), and infrared dye conjugated secondary antibodies were purchase from LI-COR (Lincoln, NE, USA).

4.3.2 Cell culture

MCF7 cells (ATCC® HTB-22™) were purchased from the American Type Culture Collection (Rockville, M). T-47D cells, ZR-75-1 cells, SK-BR-3 cells, and MDA-MB-231 cells were generous gifts from Dr. Lisa Yee (The Ohio State University, OH). All cells were maintained in Ham’s F12 Media: DMEM (1:1) (Dulbecco’s modified Eagle’s medium) with 10% fetal bovine serum (FBS) (Gibco, Life Technologies, Inc.). Cells were plated as monolayers in T-75 flasks, and maintained at 37°C with 5% CO₂. The cell medium was changed 2-3 times per week.

4.3.3 Carotenoid enrichment of fetal bovine serum

Fetal bovine serum was enriched with carotenoids by using a method described previously with slightly modification [181]. Lutein was first dissolved in ethanol, and β-carotene was dissolved in tetrahydrofuran, and then added to fetal bovine serum. The
volume of ethanol and tetrahydrofuran was at less than 2% of the final volume. The solution was mixed and incubated under nitrogen gas overnight at 4°C in the dark. Aliquots of the lutein-enriched fetal bovine serum, and β-carotene-enriched fetal bovine serum were collected and analyzed to confirm the initial concentrations of carotenoids added to the cells prior to the experiments.

4.3.4 Addition of carotenoids to human breast cancer cell lines

Human breast cancer cells were plated at 2 x 10⁴ cells/well of 6-well plate with serum-free medium. For both cell viability assay and cell uptake assay, carotenoid-enriched fetal bovine serum was added to serum-free medium (10% of final volume) to reach the desired carotenoid concentrations.

4.3.5 Cell viability assay

Human breast cancer cells were plated at 2x10⁴ cells/well of 6-well plate, and immediately treated with lutein or β-carotene at different concentrations. Cells were incubated for 0, 24, 48, or 72 hours. MTT solution (5 mg/ml) was added to each well to achieve the final concentration of 0.45 mg/ml at each time point. Cells were incubated with MTT solution for 1 hour at 37°C, followed by removal of the supernatant. Around 300 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and the absorbance was recorded at 570 nm.

4.3.6 Carotenoid extraction
Extractions of lutein and β-carotene from cells and medium were performed as previously described with slight modifications [158, 159]. For extractions from medium, 200 µL of medium was treated with 600 µL of 2-propanol-dichloromethane (2:1, v/v) and vortexed. All samples were kept on ice under yellow light to prevent oxidation during extraction. The mixture was centrifuged for 5 minutes at 2,000 RPM. The top layer was removed and dried under nitrogen gas. The resulting residue was re-suspended in 200 ul of mobile phase, filtered through a 0.22 μm pore sized filter and run on HPLC. For extractions from cells, cell medium was removed and cells were rinsed twice with ice-cold PBS. Addition of 1 ml of 2-propanol-dichloromethane (2:1, v/v) for 30 minutes to the cell, and this procedure was repeated twice at room temperature. The extracts were collected and dried under nitrogen gas, and followed with the method described above.

4.3.7 HPLC analysis of Carotenoid

Carotenoids were analyzed using an Agilent Technologies 1200 Series Diode Array and Multiple Wavelength Detector HPLC system using a method previously described [160]. For lutein measurement, a column C30 Type Carotenoid, 4.6 X 250 mm, 3 μm (YMC, Inc., Milford, MA) was used with methanol: methyl-tert-butyl-ether (90:10, v/v) at a flow rate of 0.9 ml/min as mobile phase. When β-carotene was measured, the same column was used, with methanol: methyl-tert-butyl-ether (75:25, v/v) at a flow rate of 1.4 ml/min as mobile phase. Both lutein and β-carotene were monitored at 453 nm and quantified using external standard curves established for each test.
4.3.8 Protein isolation and immunoblotting

Human breast cancer cells were collected, washed with PBS, and centrifuged at 1000 rpm for 5 minutes. Cell pellets were resuspended in Radio-Immunoprecipitation Assay buffer (RIPA, Thermo Scientific, Rockford, IL) with protease inhibitor cocktail (PI, Roche Diagnostics, Indianapolis, IN), and kept on ice for 1 hour. Cell lysates were centrifuged, and supernatant was collected. The total protein concentration was determined using the BCA protein assay (Thermo Scientific, Rockford, IL). Proteins (100 µg/sample) were resolved by SDS–PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After transfer, membranes were blocked with 5% non-fat dry milk in PBS with 0.1% Tween-20 (PBST) for 1 hour at room temperature. The membranes were incubated with primary antibodies BCO2 (14324-1-AP, Proteintech Group, Rosemont, IL, USA) and β-actin (60008-1-lg, Proteintech Group, Rosemont, IL, USA) overnight at 4 °C on a rocker. After washing, membranes were incubated with infrared dye conjugated secondary antibody for 30 min at room temperature. Membranes were protected from direct light during secondary antibody (LI-COR, Lincoln, NE, USA) incubation and observed with the Odyssey LI-COR Infrared Imaging System (LI-COR biosciences, Lincoln, NE). β-actin was used as a loading control.

4.3.9 Statistical analysis

All data are presented as mean ± SEM. Student’s t-test is used to determine the statistical significance in the differences between 2 groups. One-way ANOVA followed by Tukey’s post-hoc test is used to compare multiple treatment groups. Two-way ANOVA is used to
assess the statistical differences between multiple treatment groups at different time-points. Data is analyzed using a Graph-pad Prism; p < 0.05 is considered statistically significant.
4.4 Results for Aim 2A

4.4.1 Effect of lutein on ER(+) and ER (-) breast cancer cell proliferation

ER positive human breast cancer cells MCF7 (Figure 10A), T-47D (Figure 10B), and ZR-75-1 (Figure 10C), and ER negative human breast cancer cells SK-BR-3 (Figure 10D) and MDA-MB-231 (Figure 10E), were treated with 10 µM lutein and incubated for 24, 48, and 72 hours at 37°C. Cell proliferation was monitored by MTT assay. At this concentration, lutein had no effect on the proliferation of these cell lines. In order to see if lower concentrations of lutein might affect cell growth, one ER positive (MCF7) cell line and one ER negative (MDA-MB-231) cell line were selected and treated with different concentrations of lutein ranging from 0.1 µM to 10 µM over 72 hours. Cell proliferation was assessed at 0, 24, 48, and 72 hours by MTT assay. Both MCF7 cells (Figure 11A-E) and MDA-MB-231 (Figure 12A-E) cells proliferated after 24 hours, and growth plateaued between 24 hours and 72 hours. MDA-MB-231 cells continued to proliferate up to 72 hours. However, there was no significant difference observed between lutein treatment group and control group.
Figure 10. Effect of 10 µM lutein on ER(+) and ER(-) breast cancer cellular proliferation. To be continued.
MCF7 (Figure 9A), T-47D (Figure 9B), ZR-75-1 (Figure 9C), SK-BR-3 (Figure 9D) and MDA-MB-231 (Figure 9E) cellular proliferation after incubation with 10 µM lutein over 72 hours. Values are presented as mean±SEM. No significant difference were observed between control and 10 µM lutein treatment group in any cell lines.
MCF7 (Figure 10A) cell proliferation after incubation with lutein ranging from 0.1 µM to 10 µM over 72 hours was detected (Figure 10B-E). Values are presented as mean±SEM. No significant difference were observed between control group and lutein treatment group at any concentration of lutein.
Figure 12. Effect of different concentrations of lutein on MDA-MB-231 cell proliferation.

MDA-MB-231 (Figure 11A) cell proliferation after incubation with lutein ranging from 0.1 µM to 10 µM over 72 hours was detected (Figure 11B-E). Values are presented as mean±SEM. No significant difference were observed between control group and lutein treatment group at any concentration of lutein.
4.4.2 Effect of β-carotene on ER(+) and ER(-) breast cancer cell proliferation

A previous study had shown that β-carotene had an effect in reducing breast cancer cell growth, so we repeated these experiment and tested the effect of different concentrations of β-carotene on ER(+) and ER (-) cell growth. The same ER positive (MCF7) cell line and ER negative (MDA-MB-231) cell line from lutein experiment were selected and treated with different concentrations of β-carotene ranging from 0.1 µM to 10 µM over 72 hours. Cell proliferation was detected by MTT assay (Figure 13A and Figure 14A).

For MCF7 cells, 0.1 µM β-carotene treated group (Figure 13B) and 0.5 µM β-carotene treated group (Figure 13C) proliferated up to 24 hours, but there was no significant difference was observed between control group and treatment group on cell growth. In contrast, 1µ M β-carotene (Figure 13D) and10 µM β-carotene (Figure 13E) treatment led to a 20-30% decrease in cell proliferation at 24, 48, and 72 hours.

For MDA-MB-231 cells, we have similar observations on cell growth. 0.1 µM β-carotene treated group (Figure 14B) proliferated up to 72 hours, but there was no significant difference was observed between control group and treatment group on cell growth. In contrast, 0.5 µM β-carotene (Figure 14C), 1µ M β-carotene (Figure 14D) and10 µM β-carotene (Figure 14E) treatment led to a 20-30% decrease in cell proliferation at 24, 48, and 72 hours.
Figure 13. Effect of different concentrations of β-carotene on MCF7 cell proliferation.

MCF7 (Figure 12A) cell proliferation after incubation with β-carotene ranging from 0.1 µM to 10 µM over 72 hours was detected (Figure 12B-E). Values are presented as mean±SEM. P<0.05 was considered as statistically significant. *P<0.05, **P<0.01.
Figure 14. Effect of different concentrations of β-carotene on MDA-MB-231 cell proliferation.

MDA-MB-231 (Figure 13A) cell proliferation after incubation with β-carotene ranging from 0.1 µM to 10 µM over 72 hours was detected (Figure 13B-E). Values are presented as mean±SEM. P<0.05 was considered as statistically significant. *P<0.05, **P<0.01.
4.5 Results for Aim 2B

4.5.1 Human breast cancer cellular uptake of lutein

To determine whether lutein or its metabolites enter the cells, we looked carefully at the uptake and recovery of lutein.

We first studied the uptake of lutein in the same ER positive (MCF7) cell line and one ER negative (MDA-MB-231) cell line. Cells were treated with different concentrations of lutein ranging from 0.1 µM to 10 µM over 72 hours. Lutein uptake was analyzed at 24, 48, and 72 hours. Chromatograms of initial lutein added (1 µM), cellular lutein extracted over 72 hours are shown in Figure 15 A-G. Lutein uptake is expressed as a percentage of the initial amount added to the cells.

For MCF7 cells incubated with 0.1 µM lutein (Figure 16A) and 0.5 µM lutein (Figure 16B), most cellular uptake happened between 48 and 72 hours, and were highest at 72 hour. For MCF7 cells incubated with 1 µM lutein (Figure 16C) and 10 µM lutein (Figure 16D), most cellular uptake happened within 24 hours, and were highest at 72 hour. After 72 hours incubation, there was 50% recovery of lutein in the cell, and 50% conserved in the medium.

For MDA-MB-231 cells incubated with 0.1 µM lutein (Figure 17A) and 0.5 µM lutein (Figure 17B), most cellular uptake happened between 0 and 48 hours, and were highest at 72 hour. For MDA-MB-231 cells incubated with 1 µM lutein (Figure 17C) and 10 µM lutein (Figure 17D), most cellular uptake happened within 24 hours, and were highest at 72 hour. After 72 hours incubation, there was over 50% recovery of lutein in the cell.
Figure 15. Chromatograms: cell extract after 72 hours incubation with 1 µM lutein. To be continued.
72-hour MDA-MB-231 cell extract-1 μM Lutein treated
Figure 16. MCF7 cellular uptake of lutein: delivery in fetal bovine serum.

MCF7 cells were incubated with 0.1µM (A), 0.5 µM (B), 1.0 µM (C), and 10 µM (D) of lutein. Cellular lutein uptake was analyzed and expressed as a percentage of the initial amount added to the cell. Values are presented as mean±SEM.
MDA-MB-231 cells were incubated with 0.1 µM (A), 0.5 µM (B), 1.0 µM (C), and 10 µM (D) of lutein. Cellular lutein uptake was analyzed and expressed as a percentage of the initial amount added to the cell. Values are presented as mean±SEM.
4.5.2 Human breast cancer cellular uptake of \(\beta\)-carotene

To compare the cellular uptake of different carotenoids, we next studied the uptake of \(\beta\)-carotene in the same ER positive (MCF7) cell line and same ER negative (MDA-MB-231) cell line. Cells were treated with different concentrations of \(\beta\)-carotene ranging from 0.1 \(\mu\)M to 10 \(\mu\)M over 72 hours. \(\beta\)-carotene uptake was analyzed at 24, 48, and 72 hours. Chromatograms of initial \(\beta\)-carotene added (10 \(\mu\)M), cellular \(\beta\)-carotene extractions over 72 hours are shown in Figure 18 A-G. \(\beta\)-carotene uptake is expressed as a percentage of the initial amount added to the cells.

For MCF7 cells incubated with 0.1 \(\mu\)M \(\beta\)-carotene (Figure 19A), 0.5 \(\mu\)M \(\beta\)-carotene (Figure 19B), and 1.0 \(\mu\)M \(\beta\)-carotene (Figure 19 C), only a very small amount of \(\beta\)-carotene was taken by the cells over 72 hours incubation. For MCF7 cells incubated with 10 \(\mu\)M \(\beta\)-carotene (Figure 19D), cell uptake of \(\beta\)-carotene was highest (20% of initial) after 24 hours incubation.

For MDA-MB-231 cells incubated with 0.1 \(\mu\)M \(\beta\)-carotene (Figure 20A), 0.5 \(\mu\)M \(\beta\)-carotene (Figure 20B), and 1.0 \(\mu\)M \(\beta\)-carotene (Figure 20C), only a very small amount of \(\beta\)-carotene was taken by the cells over 72 hours incubation. For MDA-MB-231 cells incubated with 10 \(\mu\)M \(\beta\)-carotene (Figure 20D), cell uptake of \(\beta\)-carotene was highest (20% of initial) after 72 hours incubation.
Figure 18. Chromatograms: cell extract after 72 hours incubation with 10 µM β-carotene. To be continued.
G 72-hour MDA-MB-231 cell extract-10 μM β-Carotene treated
Figure 19. MCF7 cellular uptake of β-carotene: delivery in fetal bovine serum.

MCF7 cells were incubated with 0.1 µM (A), 0.5 µM (B), 1.0 µM (C), and 10 µM (D) of β-carotene. Cellular β-carotene uptake was analyzed and expressed as a percentage of the initial amount added to the cell. Values are presented as mean±SEM.
Figure 20. MDA-MB-231 cellular uptake of β-carotene: delivery in fetal bovine serum.

MDA-MB-231 cells were incubated with 0.1 µM (A), 0.5 µM (B), 1.0 µM (C), and 10 µM (D) of β-carotene. Cellular β-carotene uptake was analyzed and expressed as a percentage of the initial amount added to the cell. Values are presented as mean±SEM.
4.5.3 BCO2 protein expression in ER(+) and ER(-) breast cancer cells

BCO2 is the only enzyme that cleaves the asymmetric lutein molecular and yield two groups of product. Our chromatograms did not show the appearance of lutein metabolites at any concentration. Thus, we tested BCO2 protein expression in all five human breast cancer cell lines (Figure 21). The western blot showed BCO2 protein at molecular weight of 60-65 kDa, and the expression (green signal) was observed in all five cell lines. β-actin was used as loading control (red signal). Thus, although BCO2 was expressed in the cells, we did not detect any of the primary metabolites of lutein in the cell or medium.
Figure 21. BCO2 expression in ER(+) and ER(-) cell lines.

BCO2 protein expression (green signal) was detected in all five breast cancer cell lines. β-actin (red signal) was used as loading control.
4.6 Discussion

In summary, in the current studies we found the followings 1) lutein (0.1-10 µM) had no effect on one measurement of cell proliferation in multiple human breast cancer cell lines; 2) β-carotene (0.1-10 µM) inhibited cell growth of MCF-7 and MDA-MB231 cells; 3) above 50% of initially added lutein was recovered by the cells after 72 hours, while around 20% of β-carotene was recovered; 4) BCO2 was detected in these cell lines.

As previously discussed, we did not observe the significant effects of lutein supplement on the development of the mammary gland and the risk of breast cancer in our animal studies. We then designed in vitro cell culture studies using multiple human breast cancer cell lines. Based on the previous epidemiological studies, plasma lutein levels are negatively associated with the risk of human breast cancer in both ER positive and negative cell lines [7, 148]. Therefore, both ER positive and negative cell lines were used in the current studies.

Our cell proliferation MTT assays showed that lutein had no effects on the cell growth in multiple human breast cancer cell lines. The initial concentration we used was 10 µM, which is much higher than the levels found in human studies (0.1-0.2 µM). To explore the effects of lower concentrations, we treated MCF7 and MDA-MB-231 cells with lutein at the concentrations ranging from 0.1-10 µM. Similar to the highest concentration, we did not observe any major difference of cell growth at all the doses we tested between control and lutein groups. In order to confirm our methods, we treated the two cell lines with β-carotene independently. As shown in the previous study [182, 183], β-carotene
can inhibit cell proliferation in both MCF7 and MDA-MB-231 cells. Our study confirms these previous results on these cell lines.

Intracellular accumulation of lutein is important for its biological function [184-186]. To determine whether the lack of inhibitory effects of lutein on cell viability was due to the low level of intracellular accumulation, we conducted the uptake assay by using HPLC. Our data showed that about 50% of the initially added lutein was recovered in the cells after 72 hours, while about 20% of β-carotene was recovered only with the highest concentration. Thus the cellular uptake of lutein was higher than that of β-carotene. Thus, the lack of inhibitory effects of lutein on cell proliferation was not due to the lack of cellular uptake.

Our previous work showed that BCO2 is the only enzyme that is known responsible for lutein cleavage. In the current studies, we detected the expression of BCO2 in the human breast cancer cell lines using western blot analysis. This enzyme may rapidly cleave lutein into several metabolites that influence the potential effects of lutein on cell viability. However, we did not observe any significant metabolites of lutein over 72 hours of incubation. It is possible that after initial cleavage by BCO2, the products are rapidly degraded to compounds undetectable by our method.

Interestingly, a recent publication from an independent group reported the synergistic effects of astaxanthin, lutein and β-carotene on cytotoxicity and oxidative stress in MCF-7 cells [183]. In this study, the researchers treated the cells with either individual compounds or the combination, at the concentrations ranging from 10-50 µM. They found that lutein treatment, higher than 20 µM concentration, can reduce the cell viability
over 48-hours incubation. However, the concentrations in this study were much higher than the physiological levels in human, and could be toxic to the cells.
4.7 Acknowledgement

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