EFFECT OF BLUEBERRY POLYPHENOLS ON 3T3-F442A PREADIPOCYTE DIFFERENTIATION

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To the Dean of the Graduate School:

I am submitting herewith a dissertation written by Shiwani Moghe "Effect of Blueberry Polyphenols on 3T3-F442A preadipocyte differentiation." I have examined this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science with a major in Nutrition.

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We have read this thesis and recommend its acceptance:

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Accepted: Jennifer Martin

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ABSTRACT

SHIWANI MOGHE

EFFECT OF BLUEBERRY POLYPHENOLS ON 3T3-F442A

PREADIPOCYTE DIFFERENTIATION

DECEMBER 2010

Today obesity is an epidemic and its prevalence has increased significantly over the last few decades. To avoid excessive accumulation of fat, optimum energy intake along with regular exercise is mandatory. Polyphenols present in green tea, grape seeds, orange and grapefruit combat adipogenesis at the molecular level and also induce lipolysis. However, very little is known regarding the role of blueberry polyphenols on adipocyte differentiation. Hence we tested the dose-dependent effects of blueberry polyphenols on mouse 3T3-F442A preadipocyte differentiation and lipolysis. 3T3-F442A preadipocytes were incubated with three doses of blueberry polyphenols (BB-150, BB-200 and BB-250 μg/mL) and intracellular lipid content, cell proliferation and lipolysis were assayed. Blueberry polyphenols showed a dose-dependent suppression of adipocyte differentiation determined by oil red-O staining and adipored assay. Intracellular lipid content in control (11,385.51 ± 1169.6, relative fluorescence units) was significantly higher ($P < 0.05$) than the three doses of blueberry polyphenols (8336.86 ± 503.57), (4235.67 ± 323.17), and (3027.97 ± 346.61). This corresponds to a reduction of 27%, 63% and 74%, respectively. Cell proliferation was observed to be significantly high in

control (0.744 ± 0.035, optical density units) than BB-150 (0.517 ± 0.031), BB-200 (0.491 ±0.023), and BB-250 (0.455 ± 0.012). However, when tested for lipolysis, there was no significant difference observed among the groups. We conclude that blueberry polyphenols may play an effective role in inhibiting adipogenesis and cell proliferation.

Keywords: Obesity; adipocyte differentiation; polyphenols; Blueberry; cell proliferation; lipolysis

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CHAPTER I

INTRODUCTION

All humans aspire for Good Health. Ideal body weight is essential for maintaining good health, which is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity (World Health Organization, 1948). Body weight is considered optimum when Body Mass Index (BMI) is between 18.5 - 24.9 kg/m^2. If it shifts away from normal, it predisposes a person to various disorders, thereby interfering with the normal metabolic processes. When energy consumption dominates energy expenditure, it contributes to an increase in body weight by increasing fat cells size (hypertrophy) and number (hyperplasia). Overweight (BMI: 25-29.9 Kg/m^2) and obesity (BMI $\geq$ 30 Kg/m^2) are conditions that fit the above criteria.

Obesity is becoming an epidemic in both children and adults (Poirier et al., 2006). Its prevalence has increased significantly over the last few decades (Ogden, Carroll, Curtin, McDowell, Tabak, and Flegal, 2006) and is the major risk factor for cardiovascular diseases and type 2 diabetes mellitus (Goran, Ball and Cruz, 2003). Today, heart disease ranks first and diabetes mellitus is the seventh leading cause of death in United States. The United States government spends approximately $147 billion every year to treat obesity (Finkelstein, Trogdon, Cohen and Dietz, 2009). Despite the

large annual spending on medical treatment, the rate of obesity related morbidity and mortality are on the rise and life expectancy is not improving. Sedentary life style and disordered eating habits are major factors contributing to obesity.

Surplus energy from a well fed state causes formation of triglycerides (TG) (Brito, Brito, and Bartness, 2008). TG is stored in fat cells called adipocytes and their number is directly proportional to percent of body fat. Adipogenesis is thus a consequence of both increased energy requirement and normal cell turnover (Prins and O'Rahilly, 1997).

Adipocyte differentiation is dependent on two critical events – mitotic clonal expansion and an irreversible commitment to differentiation (Ntambi and Young-Cheul, 2000; Tang, Otto and Lane, 2003). Adipocyte specific genes such as Peroxisome Proliferator-activated receptors gamma (PPAR-γ), CCAAT/enhancer binding protein alpha (C/EBPα) and adipocyte determination- and differentiation-dependent factor 1 / sterol regulatory element binding protein isoform (ADD1/SREBP1) regulate the sequence of adipocyte differentiation (Wu, Puigserver, and Spiegelman, 1999). Elevation in PPAR-γ and C/EBPα occurs during the early stages of differentiation (Spiegelman, Choy, Hotamisligil, Graves, and Tontonoz, 1993; Morrison and Farmer, 1999). Both, PPAR-γ and C/EBPα induce gene expression for adipocyte specific fatty acid binding proteins (aP2) and phosphoenol pyruvate carboxykinase (PEPCK). These genes in turn initiate gene expression for the enzymes associated with fatty acid synthesis, such as, fatty acid synthase, glycerophosphate dehydrogenase, acetyl COA carboxylase

(Spiegelman, Choy, Hotamisligil et al., 1993; Morrison and Farmer 1999). PPAR-γ has a considerable role to play, and hence is an indispensable marker in the process of adipogenesis.

Combating unnecessary adipogenesis at the molecular level will be beneficial to prevent diseases at a very early stage. Therefore, studies to identify agents that will interfere with the gene expressions and inhibit adipogenesis are warranted.

Fruits, vegetables, and legumes have LDL-cholesterol reducing effect. Not only are they significantly rich in fiber, but also abundant in antioxidants like polyphenols. Some polyphenols improve endothelial function (Stoclet et al., 2004), reduce total cholesterol and TG (Ruzaidi, Amin, Nawalyah, Hamid, and Faizul, 2005), and thereby are effective in the treatment of cardiovascular diseases (Vita, 2005). They also demonstrate anti-inflammatory effects (Yoon and Baek, 2005). Anthocyanins, compounds present in polyphenols, have antioxidant effect (Tsuda, Horio, and Osawa, 1998; Tsuda, Horio, and Osawa, 2002) and inhibit progression of obesity in mice fed a high fat diet (Tsuda, Horio, Uchida, Aoki, and Osawa, 2003). Polyphenols stimulate adipocytokine secretion (Tsuda et al., 2004) and attenuate gene expression of adipocyte specific genes like PPAR-γ and C/ EBPs alpha and beta in 3T3 L1 preadipocytes isolated from rat (Kubota, Morii, Kojima-Yuasa, Huang, Yano, and Matsui-Yuasa, 2009). Polyphenols present in green tea, grape seeds, orange, and grape-fruit inhibit the process of adipogenesis and initiate lipolysis (Lin, Della-Fera, and Baile, 2005; Pinent et al., 2005; Dallas, Gerbi, Tenca, Juchaux, and Bernard, 2008).

Blueberry polyphenol has shown promising results in the treatment of cognitive impairment (Shukitt-Hale, Lau, Carey, Galli, Spangler, Ingram, and Joseph, 2008), ischemic heart disease (Ahmet et al. 2009), oxidative stress and neurological degeneration (Joseph, Shukitt-Hale, and Casadesus, 2005). However, there are very few studies investigating the effect of blueberry polyphenols on adipogenesis. Therefore, in this research, we investigated the effect of blueberry polyphenols on adipocyte differentiation and lipolysis. We hypothesized that blueberry polyphenols will inhibit adipocyte differentiation and enhance lipolysis from mature adipocytes.

CHAPTER II

REVIEW OF THE LITERATURE

Obesity is one of the major causes of morbidity throughout the world. It is a disease associated with an excessive accumulation of body fat and is defined when body mass index (BMI) is greater or equal to 30 Kg/m^2. BMI determines nutritional status of an individual and is represented by the formula, weight in kilograms divided by square of height in meters.

$$BMI = \frac{\text{Weight (kgs)}}{\text{Height } (m^2)}$$

Classification

Depending upon the values of BMI, an adult is categorized as underweight (BMI < 18.5 Kg/m^2), normal (BMI: 18.5 – 24.9 Kg/m^2), overweight (BMI: 25 – 29.9 Kg/m^2), or obese. Obesity is further subdivided into grade I (BMI: 30 – 34.9 Kg/m^2), grade II (BMI: 35 – 39.9 Kg/m^2) and grade III – morbidly obese (BMI > 40 Kg/m^2) categories. Children and adolescents are said to be overweight and obese when their BMI is greater than 85^{th} percentile and 95^{th} percentile, respectively, based on the height and weight chart. (Centers for Disease Control and Prevention).

BMI focuses on degree of obesity, but does not emphasize on body fat distribution, which is an additional risk for developing diseases. Body fat greater than or equal to 25% for

men and greater than or equal to 35% for women symbolize obesity. There are several methods to measure body fat, including, dual energy X-ray absorptiometry, bioelectrical impedance analysis, underwater weighing, and anthropometric measurements like waist-hip ratio, waist circumference, and skinfold measurements. Waist-hip ratio and waist circumference are easy to measure and accurate in tracing fat deposition (Daniels, Daniels, Khoury, and Morrison, 2000).

An obese individual is classified as android (central or apple) shaped, or gynoid (pear) shaped. Apple shape or central obesity has fat accretion in the abdominal region of the body. When waist to hip ratio is greater than 0.8 for women and 0.9 for men, whereas abdominal circumference is greater than or equal to 35 inches (88 cm) for women and 40 inches (102 cm) for men (National Institutes of Health, National Heart, Lung, and Blood Institute,1998), they are classified as obese. Fat is stored on hips, buttocks and thighs in gynoid obesity. Men are more prone to have fat deposition in the abdomen, while in women it is mainly deposited on the buttocks and thighs. In the postmenopausal stage, women may also develop central obesity.

Prevalence of Obesity

Today, obesity is a World-wide epidemic (Poirier et al., 2006). and its prevalence has increased significantly since 1980 (Ogden, Carroll, Curtin, McDowell, Tabak, and Flegal, 2006). A study carried out in 2003 suggested a 74% increase in its incidence in the previous decade (Mokdad et al., 2003). According to the World Health Organization's (WHO) statistical report, about one billion people are overweight out of

which about 300 million are obese, globally (World Health Organization) and the figure can reach to 600 million by 2025, if it is not treated well (Formiguera and Cantón, 2004). The Centers for Disease Control and Prevention (CDC) denoted an elevated trend of obesity in United States and according to them, occurrence of obesity in most states in the United States in 1985 was below 10% and by 2008, it had reached 25-29% (Center of Disease Control and Prevention). Obesity is therefore an extremely important health issue.

In order to control or prevent the spread of any disease, it is important to know the underlying factors responsible for its development. Thus identifying the causative agents is the primary goal for treating obesity.

Causes

There are various factors responsible of developing obesity, namely, genetics and environmental factors, certain diseases or disorders that cause secondary problems, psycho-social aspects, peer pressure, and stress. However, one major cause of obesity is lack of physical activity and improper eating habits that cause excessive accumulation of body fat.

Development in technologies in the last several decades and further development in the field of science have improved our quality of life and standard of living. In this Technological Era, manual labor has almost vanished and is replaced by much efficient and more productive machines. Most of the work, today, is just a click away. Energy expenditure plummeted and sedentary lifestyle has become the norm.

Sedentary but hectic lifestyle has increased consumption of junk food (high in carbohydrates and fats), and unhealthy carbonated drinks that supply empty calories. Eating habits have thus changed considerably. Increased energy intake combined with lack of physical activity has led to storage of excess calories as fat.

Human body converts dietary calories into biological energy as adenosine triphosphate (ATP). Hydrolysis of the terminal high-energy phosphate bond of ATP into adenosine diphosphate (ADP) and inorganic phosphate releases energy to perform bodily functions.

$$ATP \longleftrightarrow ADP + Pi + ENERGY$$

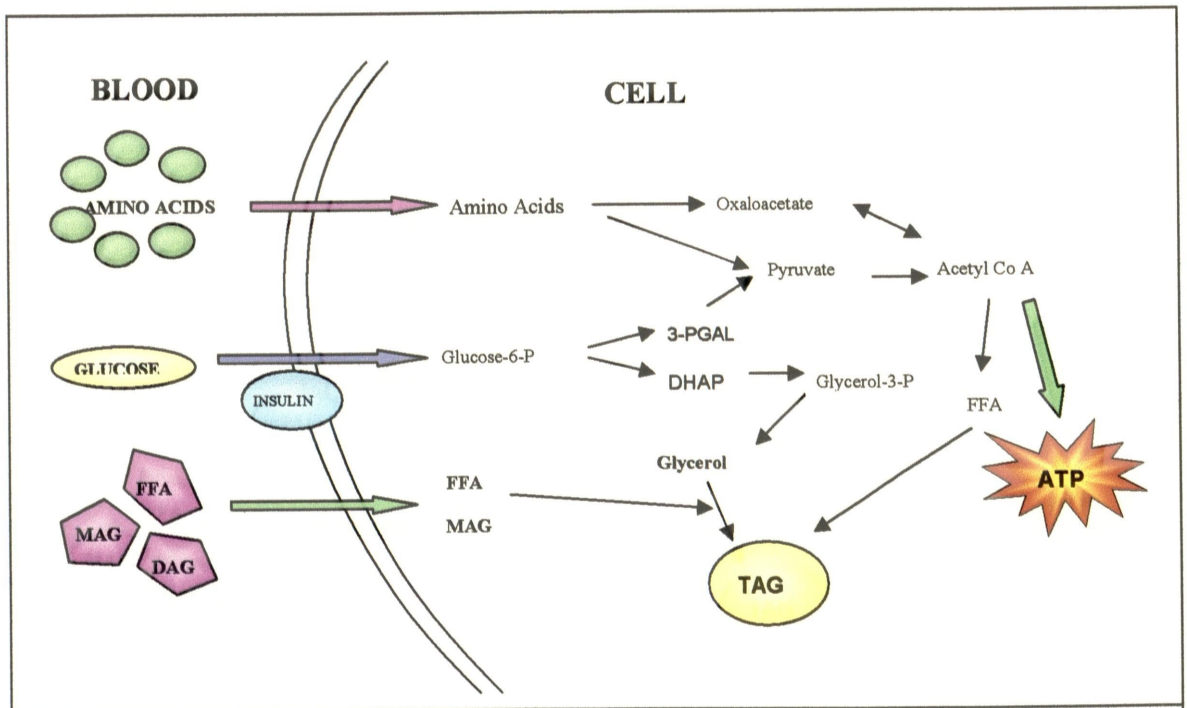

Figure 1: Energy requirement and normal cell turnover
3PGAL:3-phosphoglyceraldehyde, DHAP: dehydroxyacetonephosphate, FFA: free fatty acids, MAG: monoacyl glycerol, DAG: diacylglycerol

Surplus energy from a well-fed state is stored as fats (Brito, Brito and Bartness, 2008). Triglycerides (TGs) thus formed is stored in fat cells called adipocytes and their number is directly proportional to percent of body fat. Adipogenesis is thus a consequence of both increased energy requirement and normal cell turnover (Prins and O'Rahilly, 1997) as explained in figure 1.

Adipogenesis

The process of adipocyte differentiation produces adipocytes. In 1926, Wassermann observed that adipogenesis begins with a proliferating network of capillaries in loose connective tissues of subcutaneous region that later develop into adipose tissue; however the molecular marker that supported this process was unidentified (Wassermann, 1926). Research conducted over the past 20 years investigating cellular and molecular mechanisms of adipocyte differentiation suggests that fibroblasts are the precursors of preadipocytes, which then differentiate into adipocytes. Experiments in this field became more prominent after establishing immortal mouse preadipocyte cell lines such as 3T3-L1 and 3T3-F442A (Green and Kehinde, 1975).

Adipocyte differentiation depends on two critical events – mitotic clonal expansion (Ntambi and Young-Cheul, 2000) along with post mitotic growth arrest and an irreversible commitment to differentiation (Tang, Otto, and Lane, 2003). In vitro, growth arrest is due to cell-to-cell contact or addition of pro-differentiative agents like insulin; which is followed by another set of cell division called clonal expansion.

Adipocyte specific genes such as Peroxisome Proliferator-activated receptor gamma (PPAR-γ), CCAAT/enhancer binding protein alpha (C/EBPα) and adipocyte determination- and differentiation-dependent factor 1 / sterol regulatory element binding protein isoform (ADD1/SREBP1) regulate the process of differentiation (Wu, Puigserver, and Spiegelman, 1999).

Transcriptional Factors

Peroxisome Proliferator Activated Receptors (PPARs)

PPARs are members of nuclear hormone receptors superfamily. They are ligand activated transcription factors that control expression of various target genes.

Retinoic X receptors (RXR) bind to PPARs to form heterodimers. PPAR-RXR heterodimer binds to DR1, sequences present in the target genes. A ligand then binds itself to PPAR-RXR-DR1 complex that causes dissociation of the repressor molecule and activates PPAR transcription.

PPARs are of three types: PPAR-α, -β and -γ. They play important roles in regulating fat metabolism, adipogenesis, and energy balance (Staels, Schoonjans, Fruchart, and Auwerx, 1997; Lemberger, et al., 1996). PPAR-γ has two isoforms: PPAR-γ1 and PPAR-γ2. PPAR-γ2 has thirty extra amino acids as compared to PPAR-γ1 (Zhu, Qi, Korenberg, Chen, Noya, Rao, and Reddy, 1995; Fajas et al., 1997) and is highly expressed in the adipose tissues (Tontonoz, Hu, Graves, Budavari, and Spiegelman, 1994; Braissant, Foufelle, Scotto, Dauça, and Wahli, 1996).

CCAAT/Enhancer Binding Proteins (C/EBP)

C/EBP is a member of the transcription factor family and consists of the basic leucine zipper (bZIP) domain on the C-terminal. Members of the C/EBP family (-α, -β, -γ and -δ) can form heterodimers with each other and are associated with adipocyte differentiation.

Adipocyte Determination-and Differentiation Dependent Factor (ADD)

ADD1 belongs to a helix-loop-helix-leucine zipper family (bHLH-zip) (Tontonoz, Kim, Graves, and Spiegelman, 1993). The bHLH protein binds to the DNA sites in the target genes (Benezra, Davis, Lockshon, Turner, and Weintraub, 1990) and the HLH part of the protein is associated with the formation of homodimers and heterodimers (Voronova and Baltimore, 1990). ADD1 has ability to bind to two DNA sequences at a time and a tyrosine residue in the main domain (Kim et al., 1995) is responsible for this property.

Tontonoz et al., observed an elevation in the mRNA activity of ADD1 in the preadipocytes that were determined to undergo differentiation and it was further increased when these cells differentiated. Increased activity of ADD1 also caused activation of certain promoters having ADD1 binding sequences that in turn cause an increased expression of certain enzymes like fatty acid synthase (Tontonoz, Kim, Graves, and Spiegelman, 1993), thereby enhancing fatty acid production and fat accumulation.

Expression of ADD1 is dominant in adipose tissue as well as liver, and is considered a homolog of sterol regulatory element binding protein (SREBP).

Sterol Regulatory Element Binding Protein (SREBP)

SREBP also belongs to a helix-loop-helix-leucine zipper family (bHLH-zip). It is synthesized in an inactive form and is attached to the endoplasmic reticulum (ER). Each SREBP molecule is made up of about 1150 amino acids (Horton, Goldstein, and Brown, 2002) and has the amino ($-NH_2$) terminal consisting of bHLH-zip (480 amino acids) for DNA binding, two hydrophobic trans-membrane domains (30 amino acids) connected by a loop, and the carboxyl (-COOH) terminal (590 amino acids) to perform regulatory functions (Horton, Goldstein, and Brown, 2002).

Newly synthesized SREBP is introduced in the ER membrane. Its carboxyl end binds to the carboxyl end of SREBP cleavage activating protein (SCAP) (Horton, Goldstein, and Brown, 2002) and water molecule is released. The SREBP-SCAP complex is incorporated into the ER transport vesicle and SCAP then escorts SREBP to the Golgi apparatus. Once inside the Golgi apparatus, site-1 protease (S1P) cleaves SREBP at Site-1 into two halves. The amino terminal (bHLH-zip), is released from the golgi apparatus by site-2 protease (S2P), which then enters the nucleus (Horton, Goldstein et al. 2002). Inside the nucleus, it binds to sterol response element (SRE) that enhances gene expression for fatty acid, TG and cholesterol formation.

SREBPs have 3 isoforms, namely, SREBP-1a, SREBP-1c and SREBP-2. Activity of SREBP-1c is restricted to fatty acid and TG formation, whereas SREBP-2 deals with cholesterol production alone (Horton, Goldstein, and Brown, 2002). However, SREBP-1a

has a key role in inducing gene expression of enzymes associated with both, cholesterol synthesis as well as TG and fatty acid synthesis (Horton, Goldstein, and Brown, 2002).

Inter-Relationship of the Transcriptional Factors

Certain changes occurring within the cell during the process of adipogenesis are highlighted in figure 2 (Ntambi and Young-Cheul, 2000; Takada, Kouzmenko et al. 2009).

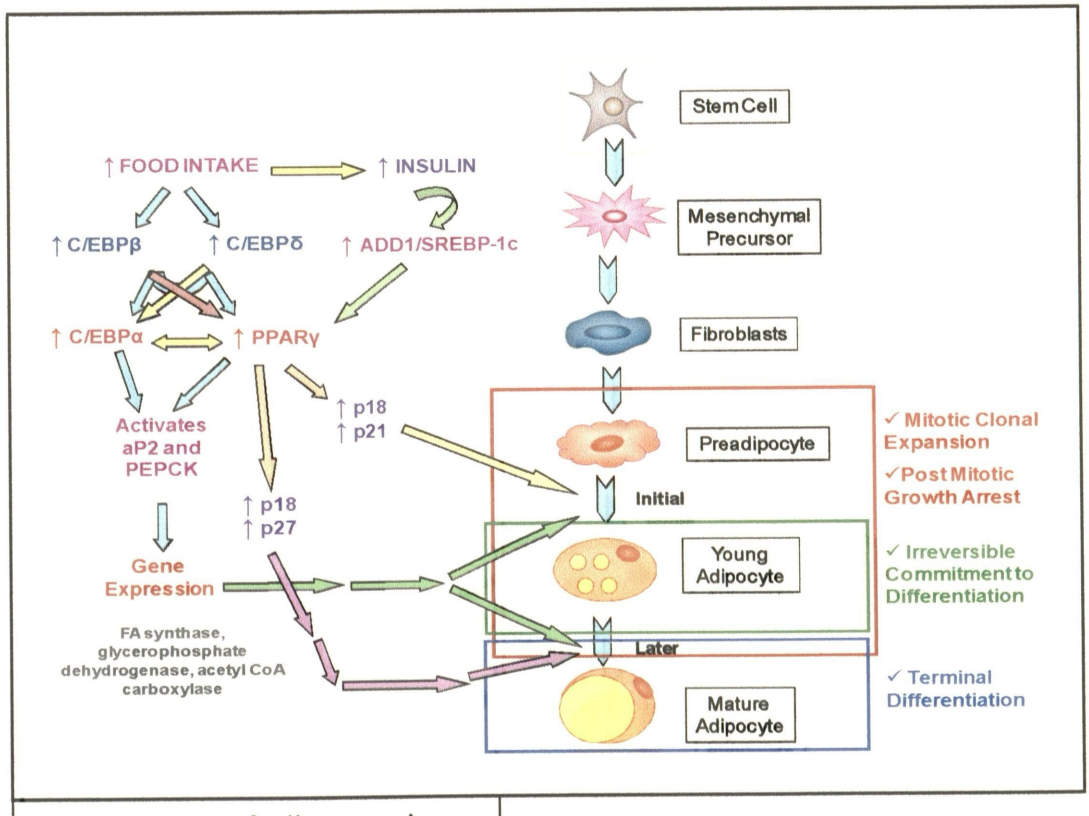

Fig 2: Process of adipogenesis

Increase in food intake elevates the activity of C/EBP-β and C/EBP-δ. PPAR-γ has C/EBP binding sites in its promoter region. As a result, activation of these two factors initiates expression of PPAR-γ, the latter then activates C/EBP-α that can in turn regulate

levels of PPAR-γ. C/EBP-β and C/EBP-δ also elevates C/EBPα levels. PPAR-γ and C/EBP-α act synergistically and their increased concentration is observed during the early stages of differentiation (Spiegelman, Choy, Hotamisligil, Graves, and Tontonoz, 1993). It denotes induction of mitotic clonal expansion and post mitotic growth arrest.
In 3T3-F442 cell line, elevated levels of PPAR-γ decreases phosphatase (PP2A) activity that results in an increase in phosphorylation of DP-1 (Spiegelman, Choy, Hotamisligil, 1993). This declines the performance of a transcriptional factor E2F/DP that is associated with DNA binding and cell growth (Altiok, Xu, and Spiegelman, 1997). This causes growth arrest.

Along with the initiation of PPAR-γ and C/EBPα activity, food consumption also instigates release of insulin that elevates activity of ADD1/SREBP-1c. Like C/EBPα, ADD1/SREBP-1c augments production of PPAR-γ.

Increased expression of PPAR-γ elevates levels of cyclin-dependent kinase inhibitors, p18 (INK4c) and p21 (Waf1/Cip1) during growth arrest. Although expression of p21 declines, high levels of p18 and p27 (Kip1) induced by PPAR-gamma mark the phase of terminal differentiation.

PPAR-γ also binds to the promoter regions present in the genes like adipocyte specific fatty acid binding protein (aP2) and phosphoenol pyruvate carboxykinase (PEPCK), and causes their activation. Induction of these proteins initiates gene expression for certain enzymes, namely, fatty acid synthase, glycerophosphate dehydrogenase, and acetyl COA carboxylase (Spiegelman et al. 1993; Morrison and

Farmer 1999). Increased activities of these enzymes stimulate fatty acid synthesis, thereby causing considerable accretion of TGs in the cells. Gene expression for PPAR-γ, aP2 and PEPCK is optimum throughout the young adipocytic phase that helps a young adipocyte to transform into a mature adipocyte and reach the stage of terminal differentiation.

As PPAR-γ plays a major role throughout the process of differentiation, it is an indispensable marker in the process of adipogenesis.

Types of Adipose Tissues

Adipocytes form the adipose tissue (AT) is the main site of energy storage in the body. There are two types of AT: brown adipose tissue (BAT) and white adipose tissue (WAT). Brown adipocytes are smaller in size with a diameter of approximately 30-40 μm (Fonseca-Alaniz, Takadia, Alonso-Vale, and Lima, 2007). They store lipid in small, but multiple droplets, have a large amount of cytoplasm, centrally located nuclei and a large number of mitochondria. BAT is associated with thermogenesis. (Lowell and Flier, 1997).

White adipocytes on other hand are larger compared to brown adipocytes, and have an average diameter of 60-100 μm (Fonseca-Alaniz, Takadia, Alonso-Vale, and Lima, 2007. The nucleus, cytoplasm and other organelles are at the periphery; and the major intracellular area (approximately 85-90%) is occupied by TGs (Fonseca-Alaniz, Takadia, Alonso-Vale, and Lima, 2007. It has one single lipid droplet. White adipocyte stores excessive amount of fat. When adipocyte accumulates fat, it increases in size and is

referred as hypertrophy. WAT has a capacity to hold 200,000-300,000 kilocalories of energy in a non-obese adult (Fonseca-Alaniz, Takadia, Alonso-Vale, and Lima, 2007). When the cell reaches its maximum size and can no longer expand to store lipid, it divides to form two new adipocytes. This multiplication in the cell number is referred as hyperplasia.

WAT functions as an energy reserve, shock absorber, friction reducer, and insulator of the body. However, along with these properties, WAT was recently found to secrete certain hormones, thus recognizing it as an important endocrine organ.

Endocrine Properties of WAT

AT secretes hormones associated with glucose homeostasis (leptin, resistin, and Adiponectin), the immune system (tumor necrotic factor alpha, and interleukins), and blood pressure (angiotensinogen) (Fruhbeck, Frühbeck, Gómez-Ambrosi, Muruzábal, and Burrell, 2001); and all these WAT secreted hormones are named adipokines. Of these, the most studied adipocyte hormones are leptin and Adiponectin.

Leptin

In humans, the ob(Lep) genes are located on chromosome 7. Leptin is a protein that consists of 167 amino acids.

When energy intake is optimum and circulating insulin level reaches maximum, leptin is secreted into the blood. It acts on certain receptors in the hypothalamus, generates a feeling of satiety, suppresses appetite, elevates basal metabolic rate (BMR),

and freezes the process of lipogenesis. Thus, it has an inverse relationship with BMI and weight gain.

Most obese people have an elevated trend of leptin as large numbers of adipocytes secrete leptin (Considine et al., 1996). Hyperleptinemia is associated with an increase in the secretion of suppressor of-cytokine signaling-3 (SOCS-3) that has adverse effects on the signals sent to the brain to stop leptin production (Münzberg, Flier, and Bjorbaek, 2004). This leads to leptin resistance.

Under normal conditions, elevated leptin levels counteract insulin resistance (IR), hyperinsulinemia, and hyperglycemia. Thus, it is a natural treatment for diabetes mellitus (DM).

Leptin, also increases the action of cyclic AMP, which inturn activates AMP activated protein kinase (AMPK) (Kahn, Alquier, Carling, and Hardie, 2005), enhances beta-oxidation in the skeletal muscles and increases glucose uptake (Kamohara, Burcelin, Halaas, Friedman, and Charron, 1997; Minokoshi and Kahn, 2003). It suppresses TG formation, supports lipolysis and helps to correct IR and thereby metabolic syndrome (MS) (Ruderman and Prentki, 2004). It also improves immune system functioning by enhancing T-cell proliferation (Fruhbeck, Frühbeck, Gómez-Ambrosi, Muruzábal, and Burrell, 2001).

Adiponectin

Adiponectin is also known as AdipoQ. It is secreted in large amounts by ATs. However, its effect depends on its concentration as well as the type of tissue.

It has an inverse relationship with IR and weight of an individual. High concentration is associated with improved insulin sensitivity in muscles and liver. It also helps to lower the plasma glucose levels and thus corrects hyperglycemia. Adiponectin improves glucose tolerance and insulin sensitivity in mice (Yamauchi et al., 2001). Drop in the Adiponectin level results in a steady weight gain (Arita et al., 1999) and IR (Hotta et al., 2001). Low Adiponectin level is closely associated with type-2 diabetes mellitus (Hara et al., 2002), and MS (Kissebah et al., 2000).

Elevated levels of Adiponectin reduce formation of adhesion molecules and conversion of macrophages to foam cells reducing the risk of atherosclerosis (Ouchi et al., 1999; Ouchi et al., 2001). It also increases beta-oxidation (Yamauchi et al., 2002) and thus helps to maintain free fatty acids and TG levels.

Increase in obesity increases the number of macrophages (Weisberg, McCann, Desai, Rosenbaum, Leibel, and Ferrante, 2003; Xu et al. 2003), which trigger production of certain proinflammatory cytokines (Vgontzas, Papanicolaou, Bixler, Kales, Tyson, and Chrousos, 1997), namely, tumor necrosiss factor-alpha (TNF-α) (Hotamisligil, Shargill, and Spiegelman, 1993), and interleukins (Fried, Bunkin and Greenberg, 1998). Production of these cytokines causes inflammation, which in turn causes hyperinsulinemia and IR.

Tumor Necrosis Factor-alpha (TNF-α)

TNF-α causes inflammation in the vascular region, increases production of adhesion molecules, and conversion of macrophages into foam cells. This initiates the

formation of fatty streaks in the arteries, which ultimately leads to the development of atherosclerosis. Adiponectin inhibits production of adhesion molecules and can help reduce fatty streaks formation; however, TNF-α suppress adiponectin levels, thereby reducing its protective function.

Elevated expression of TNF- α decreases insulin signaling, lessens formation and translocation of glucose transporter type 4 (GLUT-4) to the membrane, reduces insulin receptor subtrate-1 phosphorylation (Arner, 1995), and thus increases blood glucose and insulin concentration (Hotamisligil, Shargill, and Spiegelman, 1993). It also releases free fatty acids from the adipose tissue and increases TG concentration. All of these factors make TNF-α a potential risk factor for IR (Boden, Boden, Cheung, Stein, Kresge, and Mozzoli, 2002) and heart disease.

Interleukins-6 (IL-6)

Interleukin (IL-6) is a cytokine associated with inflammation. It induces lipolysis by initiating β-oxidation. Increase in β-oxidation causes an elevation in the glycerol and non esterified fatty acids (NEFA) concentrations in blood leading to IR (Boden, Boden, Cheung, Stein, Kresge, and Mozzoli, 2002).

Non-Esterified Fatty Acids (NEFA)

Apart from inducing development of IR (Boden, Boden, Cheung, Stein, Kresge, and Mozzoli, 2002) and diabetes mellitus, NEFA also stimulates TG formation that in turn enhances secretion of very low-density lipoprotein (VLDL) particles along with increased levels of apolipoprotein B and small low density lipoproteins (LDL) particles.

This is accompanied by a decrease in high density lipoprotein (HDL) that adds on to the risk of cardiovascular diseases (CVDs) (Matteo, Pascale et al. 2002).

Pathological Effects of Obesity

Obesity is associated with a number of pathological conditions including CVDs, renal failure, and DM.

Cardiovascular Diseases (CVDs)

Heart disease is the primary cause of mortality in the United States (Goran, Ball, and Cruz, 2003). An estimate by WHO in 2003 indicated that about 16.7 million people died of heart ailments, globally (World Health Organization, 2010). American Heart Association (AHA) estimated treatment cost of cardiovascular disease to be about $475.3 billion in 2009 (American Heart Association, 2010).

As discussed earlier, obesity elevates TNF-α levels in the blood and inflammation, thereby promoting formation of atheromatous lesions in the arterial walls. Progression of atherosclerotic plaque growth hampers blood flow to the heart and this causes an increase in blood pressure to maintain a normal blood supply. An impairment in blood supply to the heart and further atherosclerotic plaque burden lead to the development of angina and other cardiovascular complications.

Renal Failure

Elevated blood pressure causes an increase in blood circulation to the kidneys. Kidneys are overworked and this damages the functional unit of the kidney, the nephron; thereby impairing kidney function.

Diabetes Mellitus (DM)

In 2006, DM was the seventh leading cause of death in United States (Hernon 2008). In 2007, the American Diabetes Association reported that 23.6 million people in United States had diabetes (American Diabetes Association) and about 170 billion people suffered from the disease worldwide (World Health Organization, 2006). In the United States, treatment cost was approximately $174 billion in 2007 (American Diabetes Association).

Increased expression of TNF-α negatively affects insulin signaling. It also inhibits formation of GLUT-4 (Arner, 1995), thereby inducing hyperinsulinemia (Hotamisligil, Shargill, and Spiegelman, 1993) and IR. A decline in the glucose uptake by the cells initiates gluconeogenesis followed by hyperglycemia (Hotamisligil, Shargill, and Spiegelman, 1993). Excess glucose is removed from the body by frequent urination (polyuria), which is followed by an increase in thirst (polydipsia). As glucose cannot enter the cell, ketone bodies are generated due to incomplete oxidation of fatty acids. Increased concentration of ketone bodies cause ketoacidosis. High glucose level and ketoacidosis damage nephrons thereby causing diabetic nephropathy and ultimately death.

The 1999 estimate of total obesity related mortality in United States was 300,000 in one year (Allison, Fontaine, Manson, Stevens, and VanItallie, 1999). To treat obesity, the United States government spends approximately $147 billion every year (Finkelstein,

Trogdon, Cohen and Dietz, 2009). To avoid these complications and health related expenses, it is necessary to treat and prevent obesity.

Treatment

To decrease excessive accrual of body fat, quality and quantity of food consumed along with optimum exercise should be the goal. Modification of the diet includes consumption of complex carbohydrates, high fiber, and low fat food along with other dietary alterations depending upon the types of health issues, secondary to obesity. Proteins obtained from plants are deficient in one or more amino acids. Therefore, they should be complimented with another source of proteins to ensure consumption of all the essential amino acids in adequate amounts. A healthy body weight is maintained when energy intake is equal to energy expended. Combating unnecessary adipogenesis at the molecular level will be beneficial to prevent diseases at a very early stage. Adipogenesis can be hindered by inhibiting preadipocyte differentiation. Therefore, studies to identify agents that can inhibit adipogenesis are warranted.

Certain drugs can inhibit the process of differentiation. However, they have adverse side effects. Naturally occurring substances that will not only reduce lipogenesis but also avoid side effects are always welcome.

Food groups like fruits, vegetables and legumes reduce weight (Crujeiras, Goyenechea and Martínez, 2010) and LDL-cholesterol (Estruch et al. 2009). They are not only rich in fiber, but also abundant in antioxidant – polyphenol.

Polyphenols

Polyphenols are a group of chemical substances having one or more phenol rings in a single molecule and are classified as tannins, lignins and flavonoids. Some polyphenols improve endothelial function (Stoclet et al., 2004), reduce cholesterol and triglycerides (Ruzaidi, Ruzaidi, Amin, Nawalyah, Hamid, and Faizul, 2005), and thereby are effective in the treatment of cardiovascular diseases (Vita 2005). They also demonstrate anti-inflammatory effects (Yoon and Baek 2005) and thus can combat inflammation-induced diabetes mellitus, metabolic syndrome and cardiovascular diseases.

Flavonoids are further classified into flavonols, flavones, catechins, flavanones, anthocyanins and isoflavonoids. Fruits like berries, apples, grapes, pears and plum are rich in flavonoids as they contain the coloring compound anthocyanins; and are considered good sources of polyphenols.

Anthocyanins, the water-soluble pigments, are stable at acidic pH, but highly unstable under neutral conditions (Brouillard, 1988). They have antioxidant effect (Tsuda, Horio, and Osawa, 1998; Tsuda, Horio, and Osawa, 2002) and inhibit progression of obesity in mice fed a high fat diet (Tsuda, Horio, Uchida, Aoki, and Osawa, 2003). Polyphenols stimulate adipocytokines secretion (Tsuda et al., 2004) and attenuate gene expression of adipocyte specific genes like PPAR-γ and C/ EBPs alpha and beta in preadipocytes isolated from rat (Kubota, Morii, Kojima-Yuasa, Huang, Yano,

and Matsui-Yuasa, 2009). This suggests that anthocyanins may cause alterations in adipocyte gene expression and thereby have a regulatory effect on adipogenesis. Green tea polyphenol (epigallocatechin gallate) inhibits the process of adipogenesis and induce apoptosis in mature adipocytes (Lin, Della-Fera, and Baile, 2005). Grape seed procyanidins reduce fat accumulation in adipocytes (Pinent et al., 2005). Red orange, orange, and grape-fruit polyphenols stimulate lipolysis in adipocytes (Dallas, Gerbi, Tenca, Juchaux, and Bernard, 2008).

Blueberries have shown promising results in the treatment of cognitive impairment (Shukitt-Hale, Lau, Carey, Galli, Spangler, Ingram, and Joseph, 2008), ischemic heart disease (Ahmet et al., 2009), oxidative stress, and neurological degeneration (Joseph, Shukitt-Hale, and Casadesus, 2005). Blueberries have also been reported to attenuate diet-induced atherosclerosis in mice (Wu et al., 2010). Moreover, purified blueberry anthocyanins and blueberry juice are effective in preventing development of obesity in C57BL mice (Prior, Wilkes, Rogers, Khanal, Wu, and Howard, 2010). However, to our knowledge, there have been very few studies investigating the effect of blueberry polyphenols on adipogenesis or lipolysis, and therefore in this research we examined the effect of blueberry polyphenols on adipocyte differentiation and lipolysis.

We hypothesized that blueberry polyphenols inhibited adipocyte differentiation and enhanced lipolysis from mature adipocytes.

CHAPTER III

METHODOLOGY

Blueberry Polyphenol Extraction

Polyphenol extraction was performed by modification of a previously described procedure by Kim et al. (Kim, Chun et al. 2003). Blueberry powder was obtained from US Highbush Blueberry Council, California; and was stored at -20° C, in the dark. Ten gm of blueberry powder was mixed with 100 mL (80%) aqueous ethanol in a 500 mL Erlenmeyer flask in subdued light. The mixture was then sonicated for 20 minutes at room temperature under nitrogen gas with continuous stirring. The sonicated mixture was then filtered through a Buchner funnel using Whatman no 2 filter paper. The residue was rinsed three times using 100% ethanol to ensure complete extraction of polyphenols. The filtrate was concentrated by rotary evaporation at 62 ° C and lyophilized. The lyophilized polyphenol extract was stored in an amber colored bottle at -20° C.

Polyphenol Assay

Polyphenol content of blueberry extract was determined using gallic acid as a standard and expressed as Gallic acid equivalent (GAE) using a previously described procedure (Kim, Chun et al. 2003). Briefly, lyophilized blueberry extract (0.10 gm) was dissolved in 5 mL of deionized water and aliquots were used for the assay. Varying

concentrations of gallic acid standard and blueberry extract were incubated with Folin-Ciocalton reagent for 5 minutes at room temperature. Five milliliter of sodium bicarbonate (7%) was then added to each tube followed by 2 mL of deionized water. Solutions were mixed well and aliquots of 200 μL were transferred into a 96-wells plate and allowed to incubate for 90 minutes at room temperature. Absorbance at 750 nm was measured after 90 minutes in a spectrophotometer. The polyphenol concentration of blueberry extract (mg/L) was determined from the standard curve.

Cell Culture

3T3-F442A cells were obtained from Dr Howard Green (Harvard Medical School) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. After 24-hours, that is on day zero (d 0), differentiation was initiated with DMEM containing 10% fetal bovine serum (FBS) and 167 nM insulin in the presence and absence of different concentrations (150 μg, 200 μg and 250 μg per mL of media) of blueberry polyphenol (BB-150, BB-200, BB-250, respectively). After 48 hours (day 2), the cells were switched to DMEM and 10% FBS with or without blueberry polyphenol. Thereafter, the culture medium was replaced every 48 hours with fresh media of identical constitution. Analyses were performed on day 8.

Oil Red-O Staining

Cells were plated in a 6-wells plate at a density of 4×10^4 cells per well. They were allowed to undergo differentiation in the presence and absence of blueberry

polyphenols. On day 8, cells were fixed with 10% formalin and then stained with Oil Red-O by modification of a previously described procedure (Kinkel et al. 2004).

AdipoRed Assay

Cells were plated in 6-wells plates at a density of 4×10^4 cells per well and allowed to differentiate in the presence and absence of blueberry polyphenols. On day 8 of differentiation, cells were treated with AdipoRed, (Lonza, Houston, TX) and total lipid content was determined as per the manufacturer's instructions.

Cell Titer

The effect of Blueberry extract on cell proliferation was determined by the MTS assay (Promega, Madison, WI) as per the manufacturer's instructions. Briefly, 4×10^4 cells were plated per well of a 6-wells plate. Cells were allowed to undergo differentiation in the presence and absence of blueberry extract as mentioned in the above procedure. Cell viability was determined on day 8. The cells were washed with Hank's Buffered Salt Solution (HBSS) and incubated with MTS reagent for 1-4 hours at 37° C in a CO_2 (5%) incubator. Absorbance was then measured at 490 nm in a plate reader.

Fatty Acid Assay

Cells were plated in 6-wells plates at a density of 4×10^4 cells per well and allowed to undergo differentiation without blueberry. On day 8, varying doses of blueberry polyphenols were added to the wells and the cells were placed at 37°C in a 10% humidified CO_2 incubator for three days. On day 11, free fatty acid content of the media

was determined using the Free fatty acid Assay Kit from BioVision (San Francisco, CA). Values were corrected for the absorbance obtained for cell-free incubations.

Statistics

One-way analysis of variance (ANOVA) along with Tukey's Post Hoc test was performed to assess significant difference between the groups. Data was presented as Means ± SE. Values were considered significant when $P < 0.05$.

CHAPTER IV

EFFECT OF BLUEBERRY POLYPHENOLS ON 3T3-F442A PREADIPOCYTE DIFFERENTIATION

A PAPER TO BE SUBMITTED TO BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS (BBRC)

SHIWANI S. MOGHE, SHANIL JUMA, PARAKAT VIJAYAGOPAL

ABSTRACT

Today obesity is an epidemic and its prevalence has increased significantly over the last few decades. To avoid excessive accumulation of fat, optimum energy intake along with regular exercise is mandatory. Polyphenols present in green tea, grape seeds, orange and grapefruit combat adipogenesis at the molecular level and also induce lipolysis. However, very little is known regarding the role of blueberry polyphenols on adipocyte differentiation. Hence we tested the dose-dependent effects of blueberry polyphenols on mouse 3T3-F442A preadipocyte differentiation and lipolysis. 3T3-F442A preadipocytes were incubated with three doses of blueberry polyphenols (BB-150, BB-200 and BB-250 μg/mL) and intracellular lipid content, cell proliferation and lipolysis were assayed. Blueberry polyphenols showed a dose-dependent suppression of adipocyte differentiation determined by oil red-O staining and adipored assay. Intracellular lipid content in control (11,385.51 ± 1169.6, relative fluorescence units) was significantly

higher ($P < 0.05$) than the three doses of blueberry polyphenols (8336.86 ± 503.57), (4235.67 ± 323.17), and (3027.97 ± 346.61). This corresponds to a reduction of 27%, 63% and 74%, respectively. Cell proliferation was observed to be significantly high in control (0.744 ± 0.035, optical density units) than BB-150 (0.517 ± 0.031), BB-200 (0.491 ±0.023), and BB-250 (0.455 ± 0.012). However, when tested for lipolysis, there was no significant difference observed among the groups. We conclude that blueberry polyphenols may play an effective role in inhibiting adipogenesis and cell proliferation.

Keywords: Obesity; adipocyte differentiation; polyphenols; Blueberry; cell proliferation; lipolysis

Introduction

Obesity is becoming an epidemic in both children and adults [1]. Its prevalence has increased significantly over the last few decades [2] and is the major risk factor for cardiovascular diseases (CVD) and type 2 diabetes mellitus [3]. The rate of obesity related morbidity and mortality is on the rise in the United States, and is contributing to an exponential increase in health care costs. Sedentary life style and disordered eating habits are major factors contributing to obesity. Surplus energy from a well fed state is stored as triglycerides (TG) in the adipocytes [4]. Combating unnecessary adipogenesis at the molecular level can be beneficial to prevent diseases at a very early stage. Therefore, in vitro studies to identify compounds that will interfere and inhibit adipogenesis are warranted.

Fruits, vegetables, and legumes have LDL-cholesterol reducing effect. Not only are they significantly rich in fiber, but also abundant in antioxidants like polyphenols. Some polyphenols improve endothelial function [5], reduce total cholesterol and triglycerides [6], and thereby are effective in the treatment of cardiovascular diseases [7]. They also demonstrate anti-inflammatory effects [8]. Anthocyanins, compounds present in polyphenols, have antioxidant effect [9,10] and inhibit progression of obesity in mice fed a high fat diet [11]. Polyphenols stimulate adipocytokines secretion [12] and attenuate gene expression of adipocyte specific genes like PPAR-γ and C/ EBPs alpha and beta in 3T3 L1 preadipocytes isolated from rat [13]. Polyphenols present in green tea, grape seeds, orange, and grape-fruit have been shown to inhibit adipogenesis and initiate lipolysis [14,15,16]

Blueberry polyphenol has shown promising results in the treatment of cognitive impairment [17], ischemic heart disease [18], oxidative stress and neurological degeneration [19]. Blueberries have been reported to attenuate diet-induced atherosclerosis in mice[20]. Moreover, purified blueberry anthocyanins and blueberry juice are effective in preventing development of obesity in C57BL mice [21]. However, to our knowledge, very few studies have investigated the effect of blueberry polyphenols on adipogenesis. Therefore, we examined the effect of blueberry polyphenol extract on adipocyte differentiation and lipolysis.

Materials and methods

Blueberry Polyphenol Extraction. Polyphenol extraction was performed by modification of a previously described procedure by Kim et al. [22]. Blueberry powder

was obtained from US Highbush Blueberry Council, California; and was stored at -20° C, in the dark. Ten gm of blueberry powder was mixed with 100 mL (80%) aqueous ethanol in a 500 mL Erlenmeyer flask in subdued light. The mixture was then sonicated for 20 minutes at room temperature under nitrogen gas with continuous stirring. The sonicated mixture was then filtered through a Buchner funnel using Whatman no 2 filter paper. The residue was rinsed three times using 100% ethanol to ensure complete extraction of polyphenols. The filtrate was concentrated by rotary evaporation at 62° C and lyophilized. The lyophilized polyphenol extract was stored in an amber colored bottle at -20° C.

Polyphenol Assay. Polyphenol content of blueberry extract was determined using gallic acid as a standard and expressed as Gallic acid equivalent (GAE) using a previously described procedure [22]. Briefly, lyophilized blueberry extract (0.10 gm) was dissolved in 5 mL of deionized water and aliquots were used for the assay. Varying concentrations of gallic acid standard and blueberry extract were incubated with Folin-Ciocalton reagent for 5 minutes at room temperature. Five milliliter of sodium bicarbonate (7%) was then added to each tube followed by 2 mL of deionized water. Solutions were mixed well and aliquots of 200 µL were transferred into a 96-wells plate and allowed to incubate for 90 minutes at room temperature. Absorbance at 750 nm was measured after 90 minutes in a spectrophotometer. The polyphenol concentration of blueberry extract (mg/L) was determined from the standard curve.

Cell Culture. 3T3-F442A cells were obtained from Dr Howard Green (Harvard Medical School) and cultured in Dulbecco's modified Eagle's medium (DMEM)

containing 10% calf serum. After 24-hours, that is on day zero (d 0), differentiation was initiated with DMEM containing 10% fetal bovine serum (FBS) and 167 nM insulin in the presence and absence of different concentrations (150 μg, 200 μg and 250 μg per mL of media) of blueberry polyphenol (BB-150, BB-200, BB-250, respectively). After 48 hours (day 2), the cells were switched to DMEM and 10% FBS with or without blueberry polyphenol. Thereafter, the culture medium was replaced every 48 hours with fresh media of identical constitution. Analyses were performed on day 8.

Oil Red-O Staining. Cells were plated in a 6-wells plate at a density of 4×10^4 cells per well. They were allowed to undergo differentiation in the presence and absence of blueberry polyphenols. On day 8, cells were fixed with 10% formalin and then stained with Oil Red-O by modification of a previously described procedure [23].

AdipoRed Assay. Cells were plated in 6-wells plates at a density of 4×10^4 cells per well and allowed to differentiate in the presence and absence of blueberry polyphenols. On day 8 of differentiation, cells were treated with AdipoRed, (Lonza, Houston, TX) and total lipid content was determined as per the manufacturer's instructions.

Cell Titer. The effect of Blueberry extract on cell proliferation was determined by the MTS assay (Promega, Madison, WI) as per the manufacturer's instructions. Briefly, 4×10^4 cells were plated per well of a 6-wells plate. Cells were allowed to undergo differentiation in the presence and absence of blueberry extract as mentioned in the above procedure. Cell viability was determined on day 8. The cells were washed with Hank's

Buffered Salt Solution (HBSS) and incubated with MTS reagent for 1-4 hours at 37° C in a CO_2 (5%) incubator. Absorbance was then measured at 490 nm in a plate reader.

Fatty Acid Assay. Cells were plated in 6-wells plates at a density of 4×10^4 cells per well and allowed to undergo differentiation without blueberry. On day 8, varying doses of blueberry polyphenols were added to the wells and the cells were placed at 37^0C in a 10% humidified CO_2 incubator for three days. On day 11, free fatty acid content of the media was determined using the Free fatty acid Assay Kit from BioVision (San Francisco, CA). Values were corrected for the absorbance obtained for cell-free incubations.

Statistics. One-way analysis of variance (ANOVA) along with Tukey's Post Hoc test was performed to assess significant difference between the groups. Data was presented as Means ± SE. Values were considered significant when $P < 0.05$.

Results

When the cells were examined on day 8 of differentiation, blueberry polyphenols caused a dose-dependent suppression of intracellular lipid accumulation within the 3T3-F442A cells (Figure 1: I-IV).

In order to highlight actual lipid accumulation, cells were fixed with 10% formalin, and then stained with Oil Red-O. The result showed the dose-dependent decrease in triglyceride content with increasing concentrations of blueberry polyphenols (Figure 1: V-VIII). When BB-250 group was observed under a high-resolution microscope, cells had fine red colored lipid droplets surrounding the inner lining of the cell membrane.

To quantify the qualitative results obtained with Oil Red-O staining, we performed the AdipoRed assay. AdipoRed aids in quantifying the intracellular lipid droplets formed during adipogenesis. The values obtained confirmed that control had highest amount of lipid concentration measured in relative fluorescence units (Figure 2). All blueberry polyphenols concentrations had significantly reduced cellular lipid content compared to control ($P < 0.05$). The reduction was 26.8%, 62.8%, and 73.4% for blueberry doses 150, 200 and 250, respectively. When three blueberry doses were compared among themselves, BB-150 had significantly higher intracellular lipid content than BB-200 and BB-250 ($P < 0.05$); however, there was no significant difference between BB-200 and BB-250 ($P > 0.05$).

Cell titer was analyzed by MTS assay to determine dose dependent effect of blueberry polyphenols on cell proliferation (Figure 3). When cell titer was performed on the differentiated cells, compared to all the blueberry groups, cell proliferation was observed to be significantly high in control ($P < 0.05$) However; there was no significant difference within three doses of blueberry polyphenol. Thus, blueberry polyphenol seems to inhibit cell proliferation.

Free fatty acid assay was performed to determine the effect of blueberry polyphenol on adipocyte lipolysis. The results indicated that there was no significant difference between the groups (Figure 4). Hence, blueberry polyphenols at each of the doses had no effect on free fatty acid concentration.

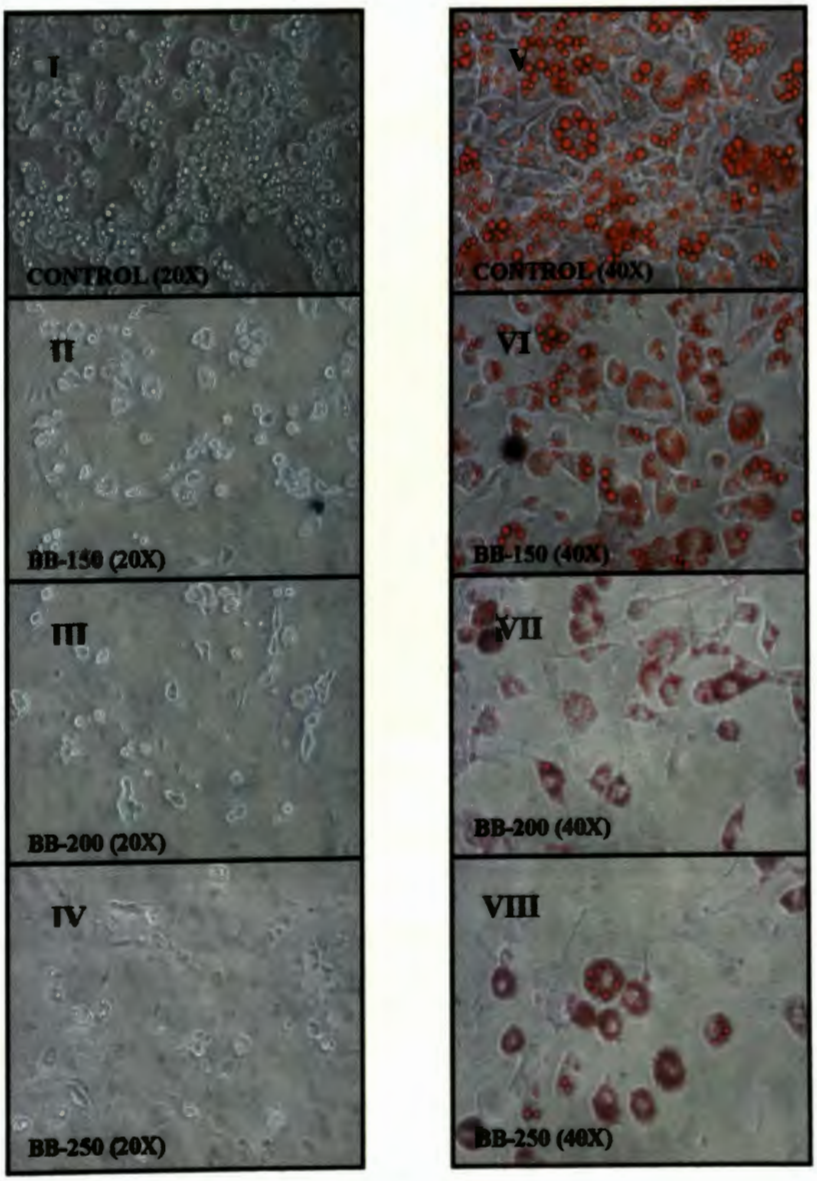

Figure 1: Dose-dependent inhibition of intracellular lipid accumulation. Pictures (I-IV) are the differentiated cells on Day 8 at the magnification of 20X; whereas, pictures (V-VIII) are the Oil Red-O stained cells at 40X. BB-150, BB-200 and BB-250 denotes blueberry polyphenol concentrations of 150 μg, 200 μg and 250 μg per mL of media. n = 18. These are representative photographs from 3 independent experiments, each performed in duplicate.

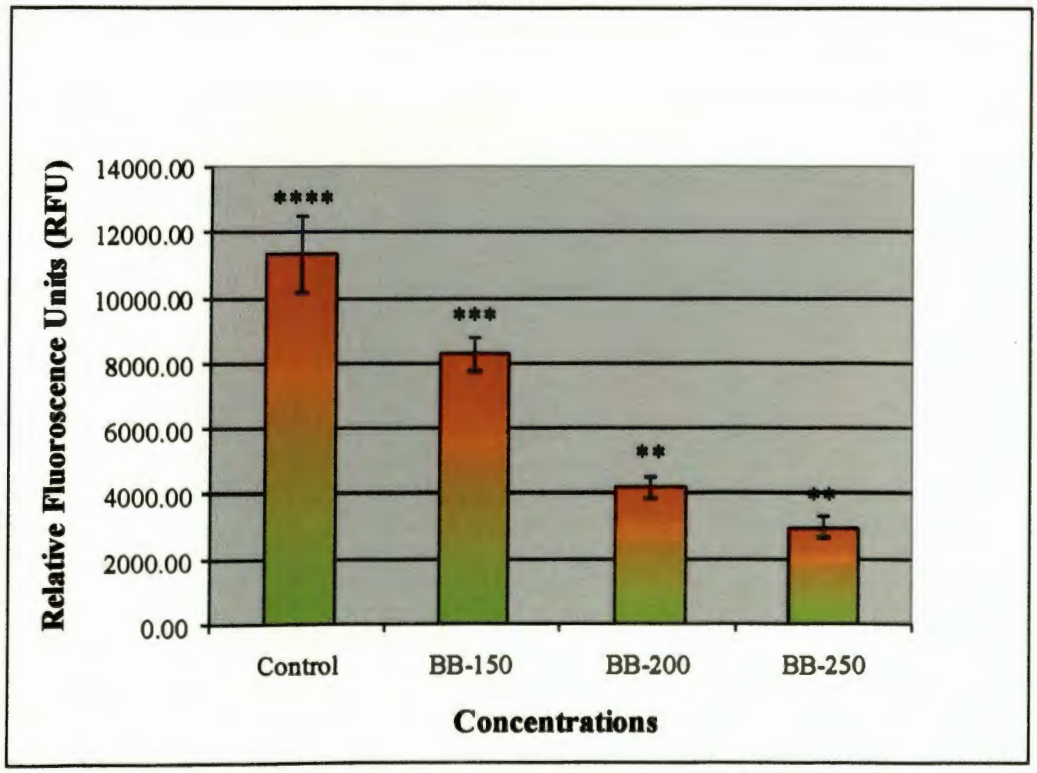

Figure 2: Quantification of dose-dependent inhibition of intracellular lipid accumulation. Cells were differentiated with or without blueberry polyphenol. Adipored assay was performed on day 8. Histograms with different superscripts are significantly different from each other ($P < 0.05$). n = 12

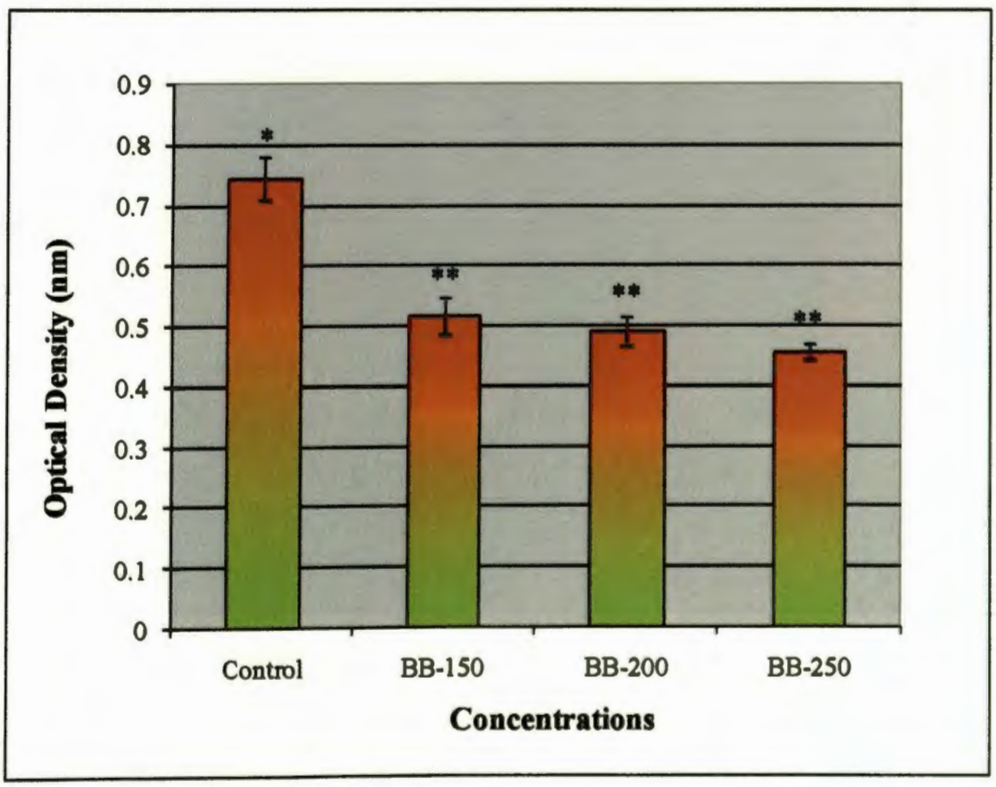

Figure 3: Dose-dependent response of blueberry polyphenols on cell proliferation. Cells were differentiated in the presence or absence of blueberry polyphenol until day 8 when cell titer assay was performed. Histograms with different superscripts are significantly different from each other ($P < 0.05$)

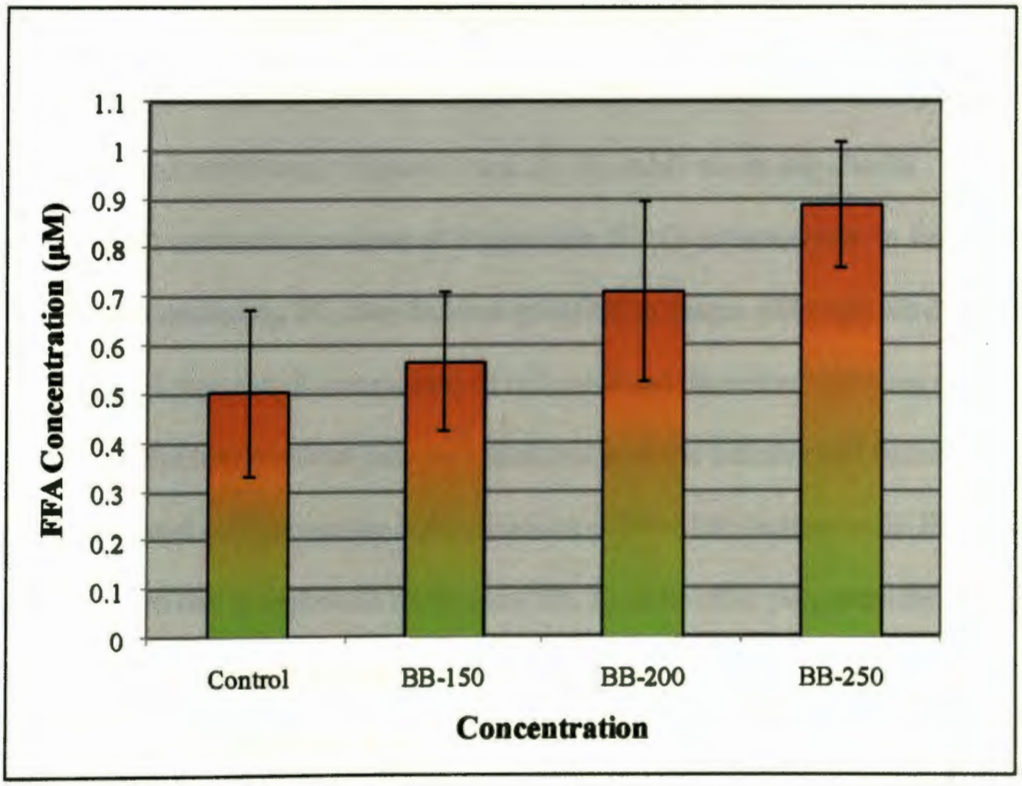

Figure 4: Effect of blueberry polyphenols on lipolysis. Cells were differentiated with differentiating media until day 8. Blueberry polyphenol was added on day 8. Lipolysis assay was performed on day 11. Values were corrected for free fatty acid content of media from cell-free incubations with and without blueberry polyphenols. There was no significance difference ($P > 0.05$) observed when comparisons were made between the groups $n = 12$

Discussion

Our study demonstrated that polyphenols extracted from blueberry inhibit 3T3-F442A preadipocyte differentiation. Cells incubated with blueberry polyphenols showed a dose-dependent reduction in intracellular lipid accumulation as demonstrated by Oil red-O staining and adipored assay (Figures 1 and 2). Oil red-O stains only the fat droplets, and gives a qualitative estimate of triglyceride (TAG) accumulation in the cell. When cells start accumulating TG, they become spherical in shape. Although we did not directly measure cell size, visual comparison of cell sizes and fat content between the control cells and polyphenol treated cells indicated that both the cell size and cellular lipid content decreased with increasing concentrations of blueberry polyphenols. Previous studies have reported that polyphenols from green tea, as wells other polyphenolic compounds, such as resveratrol, genistein, and quercetin, also inhibited adipogenesis in 3T3-L1 cells [14,24]. While green tea polyphenols mainly belong to the catechin family, blueberry polyphenols are a mixture of different phenolic compounds, including flavonoids, anthocyanins, proanthocyanidins, and hydrocinnamic acids, [25,26,27,28]. Thus, polyphenols in general appear to inhibit adipogenesis regardless of the nature of the individual compound.

In this study we did not investigate the mechanism of blueberry polyphenol-induced adipocyte differentiation. 3T3 preadipocyte differentiation involves the activation of several adipocyte genes, including peroxisome proliferator activated receptor gamma (PPAR-γ), CCAAT/enhancer binding protein alpha (C/EBPα), and differentiation-dependent factor 1/sterol regulatory element binding protein isoform

(ADD1/SREBP1). Collectively, they regulate the sequence of adipocyte differentiation. Polyphenolic compounds such as epigallocatechin gallate from green tea [14], resveratrol from red grapes [24], and curcumin from turmeric [29] have been shown to suppress one or more of these genes and thus inhibit differentiation of 3T3-L1 cells. It is likely that blueberry polyphenols also inhibit adipocyte differentiation by one of the above mechanisms.

Blueberry polyphenols decreased cell viability determined by the MTS assay (Figure 3). In this procedure, 3T3-F442 preadipocytes were differentiated in the presence and absence of varying doses of blueberry polyphenols and cell proliferation was determined on the eighth day of differentiation. Although cell proliferation was reduced significantly in the blueberry-treated cells compared to control cells, cell proliferation did not differ across the three doses of blueberry polyphenols. We also observed inhibition of cell proliferation in cultures incubated with polyphenol concentration as low as 75 μg/ml (data not shown). Recent studies have shown that epigallocatechin gallate from green tea [14] and a combination of genistein, quercetin, and resveratrol also inhibited proliferation of 3T3-L1 preadipocytes in culture [24]. It appears that blueberry polyphenols reduce adipocyte lipid accumulation not only by inhibiting intracellular lipid content but also by also reducing adipocyte number by preventing cell proliferation.

The net intracellular lipid contents in differentiated adipocytes represent a balance between total triacylglycerol synthesis and lipolysis. Increased triglyceride synthesis accompanied by reduced beta-oxidation will result in substantial intracellular lipid accumulation. Therefore, in order to determine whether blueberry polyphenols affect

adipocyte lipolysis in our study, we performed lipolysis assay in mature adipocytes following exposure to different polyphenol doses. The result indicates that blueberry polyphenols have no effect on adipocyte lipolysis. While this may be true, there is also the possibility that the assay was not sensitive enough to detect very small changes in lipolysis. The sensitivity limit of the current assay was 2 μM free fatty acid. Use of a more sensitive method might have detected even minute changes in free fatty acid and given different results.

Blueberry polyphenols have been shown to improve insulin sensitivity in 3T3-L1 adipocytes [28] and obese, insulin-resistant men and women [30] and decrease cardiovascular risk factors in people with metabolic syndrome [31]. Our study shows for the first time that blueberry polyphenols also inhibit adipocyte differentiation in 3T3-F442 preadipocytes. Thus, blueberry polyphenols have the potential to become an effective supplemental therapy in the treatment of obesity.

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APPENDIX A

Publication in Biochemical and Biophysical Research Communications