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Inhibitory effects of berry anthocyanins on palmitic acid- or lipopolysaccharide- induced inflammation in human preadipocytes

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**INHIBITORY EFFECTS OF BERRY ANTHOCYANINS ON PALMITIC ACID- OR
LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION IN HUMAN
PREADIPOCYTES**

**A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment for the
Requirements for the degree of
Master of Science**

**In
The Department of Food Science**

**By
Chenfei Gao
B.S., Hebei University of Science and Technology, 2006
August 2011**

**Dedicated to my beloved parents
Mr. Yunshen Gao and Mrs. Zhenhua Chen**

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ABBREVIATIONS

| | |
|----------------|---|
| BAB | Anthocyanins-rich blackberry extract |
| BC | Anthocyanins-rich black currant extract |
| BMI | Body mass index |
| BUB | Anthocyanins-rich blueberry extract |
| BSA | Bovine serum albumin |
| C3G | Cyanidin-3-glucoside |
| COX-2 | Cyclooxygenase-2 |
| CRB | Anthocyanins-rich cranberry extract |
| CRP | C-reactive protein |
| CVD | Cardiovascular disease |
| FFA | Free fatty acid |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| IL-1 β | Interleukin-1 β |
| IR | Insulin resistance |
| LPS | Lipopolysaccharide |
| MCP-1 | Monocyte chemotactic protein-1 |
| NF- κ B | Nuclear factor kappa B |
| PA | Palmitic acid |
| PBS | Phosphate buffered saline |
| PVDF | Polyvinylidene fluoride |
| S.D | Standard deviation |
| T2DM | Type 2 diabetes mellitus |
| TBST | Tris buffered saline solution with Tween-20 |
| TNF- α | Tumor necrosis factor-alpha |

ABSTRACT

Obesity is an inflammatory disease associated with the development and progression of chronic degenerative diseases including insulin resistance, cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), and cancer. Circulating free fatty acid (FFA) are known risk factors promoting inflammation that can lead to CVD. Pre/adipocytes are known storage of low grade inflammatory biomarkers associated with obesity. However, the effects of dietary anthocyanins on inflammatory biomarkers in preadipocytes have not been reported. Berries are rich sources of naturally occurring antioxidant polyphenolics. There is increasing interest in the ability of berry anthocyanins to provide health benefits against obesity. Anthocyanins were isolated from blueberry, blackberry, cranberry and black currant and analyzed by spectrophotometry and liquid chromatography. The objective of this study was to test the effect of berry anthocyanins on palmitic acid (PA)-induced inflammatory biomarkers in human preadipocytes. Comparison was made with the effect of berry anthocyanins on lipopolysaccharide (LPS)-induced inflammation in similar human preadipocytes. Preadipocytes from obese women were incubated with different concentrations (10-40 μ M) of berry anthocyanins for 24 h followed by addition of 100 μ M PA and additional incubation for 24h or 100ng/ml of LPS for 8 h. In another treatment, preadipocytes were incubated with 100 μ M PA for 24h or 100ng/ml of LPS for 8 h and then incubated with 10-40 μ M anthocyanins for 24h. All incubations were performed at 37 °C in a 5% CO₂ humidified incubator. Pro-inflammatory cytokines IL-6, IL-8 and MCP-1 were analyzed in conditioned preadipocyte media by ELISA. NF- κ B and COX-2 from preadipocyte lysates were analyzed by Western Blot. PA or LPS induced up-regulation of the pro-inflammatory biomarkers. Berry anthocyanins significantly inhibited PA- or LPS-induced inflammation when anthocyanins were added to the preadipocytes prior to PA or LPS addition. However, the same anthocyanins were weak inhibitors of PA- or LPS-induced inflammation when preadipocytes were first

incubated with LPS or PA. The results of our investigation show that berry anthocyanins may be effective in preventing PA- or LPS-induced low-grade inflammation in preadipocytes.

Keywords: berry anthocyanins; human preadipocytes; inflammation; palmitic acid; lipopolysaccharide; cytokines

CHAPTER 1 INTRODUCTION

Obesity is a condition that contributes to human morbidity and mortality. It is associated with increased risk of chronic diseases including cardiovascular disease, type 2 diabetes mellitus (T2DM), high blood pressure, high cholesterol, and certain types of cancers (Ginsberg, 2000; James, 2008; Karlsen et al., 2007). Obesity-related diseases have significant economic impact on the United States health care system and the health systems in many other countries. A vast body of research exists demonstrating that obesity is a complex disorder with a strong genetic basis and a multifactorial etiology (Bray, 2008). The fundamental cause of obesity is an energy imbalance between calorie consumption and calorie expenditure. High-caloric intake and sedentary life style are two risk factors in the modern society that contribute to obesity.

Low grade or chronic inflammation is closely associated with the development and continuation of obesity. The development of inflammation is characterized by the excess production of cytokines and the activation of inflammatory signal pathways (Tsukumo et al., 2007). Obesity is frequently associated with chronic cytokines production in adipose tissue, including tumor necrosis factor- α (TNF- α), interleukin- β (IL-1 β), interleukin 6 (IL-6), interleukin 8 (IL-8), and monocyte chemotactic protein 1 (MCP-1) (Hotamisligil et al., 1993). Preadipocytes play important roles in the overall regulation of glycemia in T2DM. Preadipocytes cause persistently high levels of circulating free fatty acid, reduced peripheral glucose utilization, increased hepatic glucose production and decreased insulin sensitivity (Bays et al., 2004). Growing evidence has also demonstrated that pro-inflammatory cytokines such as TNF- α and MCP-1 impair adipocyte insulin sensitivity (Hotamisligil et al., 1994; Sartipy et al., 2003).

Edible berries such as blackberry, blueberry, cranberry and black currant are relatively low-calorie fruits. The bioactive compounds in these berries have many benefits to human

health. The bioactive phytochemicals from edible berries have been demonstrated to provide antioxidant, anti-inflammatory, cytoprotective and neurological benefits (Julie Beattie, 2005). Anthocyanins are critical phytochemicals in berries and possess anti-oxidant, anti-inflammation, anti-obesity, anti-diabetes, and anti-cancer properties (Lee et al., 2009; Wang et al., 2002a). However, few studies have investigated the anti-inflammatory effects of berry anthocyanins in human preadipocytes. Therefore, the main objective of the present study was to investigate the potential inhibitory effects of berry anthocyanins on PA- or LPS- induced (secretion) inflammatory biomarkers in human preadipocytes. Steps taken to achieve the objective were:

1. To isolate anthocyanins from blackberry, blueberry, cranberry or black currant
2. To determine the concentrations and profiles of anthocyanins in the berry extracts
3. To investigate the prevention effects of the berry anthocyanins on pro-inflammatory cytokines secretion in human preadipocytes
4. To study the intervention effects of berry anthocyanins on pro-inflammatory cytokines secretion by human preadipocytes
5. To establish possible links between anthocyanins and inflammation

CHAPTER 2 LITERATURE REVIEW

2.1 Definition of Obesity

Obesity is presently one of the greatest public health challenges around the world. Overweight and obese conditions generally are the result of abnormal or excessive fat accumulation in the body. Body mass index (BMI) is a simple index of weight-for-height that is commonly used to classify underweight, overweight and obesity in adults. It is defined as the weight in kilograms divided by the square of his or her height in meters (kg/m^2). A person with a BMI below 18.5 is underweight. The normal range of BMI is between 18.50 and 24.99. A person with a BMI equal to or more than 25 is considered overweight. A person with a BMI of 30 or more is regarded as obese.

2.2 Risk Factors of Obesity

A vast body of research exists demonstrating that obesity is a complex disorder with a strong genetic basis and a multifactorial etiology (Bray, 2008). Basically, the fundamental cause of overweight or obese condition is the energy imbalance between calorie consumption and calorie expenditure. Over the last 50 years, there has been a trend of increased intake of calorically dense foods that are high in fat, sugars and salt but low in vitamins, minerals and other micronutrients. There has been a simultaneous trend of decreasing physical activity due to the increasingly sedentary nature of many forms of work, changing modes of transportation, and increasing urbanization (French et al., 2001; Kumanyika, 2001). The combination of excessive food energy intake and lack of physical activity is the main cause of obesity.

Leptin is a protein hormone and plays an important role in the regulation of energy intake and expenditure. Leptin is almost exclusively expressed within adipocytes and serves as a signal of stored adipose to the brain. It has been reported that leptin influences food intake through a

direct effect on the hypothalamus (Halaas et al., 1995; Lee et al., 1996; Zhang et al., 1994). Leptin levels correlate with the amount of stored body fat in both humans and animals. The infusion of leptin decreases feeding and increases metabolic rate in rodents (Considine et al., 1996; Frederich et al., 1995; Halaas et al., 1995; Maffei et al., 1995; Pelleymounter et al., 1995).

In addition to the complex pathways, fat cell size also correlates with obesity (Avram et al., 2007) and with the degree of macrophage infiltration into the adipose tissue (Weisberg et al., 2003). The development of obesity is dependent on the coordinated interplay of adipocyte hypertrophy (increased fat cell size), adipocyte hyperplasia (increased fat cell number), and angiogenesis (Avram et al., 2007). The enlarged adipocytes of obese individuals recruit macrophages and promote inflammation and the release of a range of factors that predispose toward insulin resistance (Greenberg et al., 2006). Insulin is a hormone that regulates the metabolism of carbohydrate and fat in the body. Insulin resistance (IR) is a condition in which normal concentration of insulin fail to maintain normal blood glucose because of decreased responsiveness of peripheral tissues, including muscles (glucose uptake), liver (inhibition of gluconeogenesis) and fat cells (inhibition of lipolysis) (Zhuang et al., 2009).

Adiponectin, an adipocyte complement-related protein, enhances insulin sensitivity in muscle and liver and increases FFA oxidation in several tissues (Greenberg et al., 2006). Adiponectin also decreases serum FFA, glucose, and triacylglycerol concentrations (Fruebis et al., 2001). In terms of the risk factors associated with obesity, the methodologies controlling energy balance and adipocyte formation may achieve the purpose of prevention and intervention of obesity.

2.3 The Relation between Free Fatty Acid and Obesity

The average American diet is abundant in saturated fatty acids (SFA), such as palmitate and stearate. Foods high in SFA include fast foods, processed foods, high-fat dairy products, red meats, and pork (USDA, 2008). The mean daily per capita consumption of fat in the U.S. in 2003–2004 was 82.7 g, which exceeds the recommended amount by 16.0 g, based on a 8400 kJ diet (Kennedy et al., 2009). Overconsumption of fatty acid contributes to weight gain and inflammation (Lundman et al., 2007). The circulation of FFAs, especially saturated FFAs, increase obesity and diabetes (Lu et al., 2003). SFAs are also believed to contribute substantially to coronary artery disease (Jakobsen et al., 2009).

Palmitic acid (PA, C16:0) is one of the most common saturated fatty acid found in animals and plants. It also broadly exists in the daily diet including butter, cheese, milk and meat. The WHO claims that dietary intake of PA increases the risk of developing cardiovascular diseases (Geneva, 2003). In addition, PA is the major saturated fatty acid in serum FFA (23-32%) and adipose tissue TG (19.5-27%). Unbound PA at plasma physiological concentrations from 100 to 500 μ M induces the production of inflammatory biomarkers including IL-6, IL-8, MCP-1, and cyclooxygenase-2 (COX-2) (Ajuwon et al., 2005; Samokhvalov et al., 2009).

2.4 Obesity and Inflammation

2.4.1 Inflammation

Inflammation is a complex series of reactions executed by the host to prevent ongoing tissue damage, to activate repair processes and to provide defense mechanisms against infectious diseases (Karlsen et al., 2007). Prolonged inflammation can contribute to the pathogenesis of chronic diseases such as diabetes, neurodegenerative type 2 diabetes, cancers, hypertension, atherosclerosis, and cardiovascular disease. Dampening of inflammation may potentially retard

the development of such of diseases (Hoareau et al., 2009; Karlsen et al., 2007). The development and continuation of obesity is closely associated with a state of chronic, low-grade inflammation characterized by abnormal cytokine production and activation of inflammatory signaling pathways (Tsukumo et al., 2007). Obesity also exacerbates inflammation because visceral and subcutaneous fat store inflammatory cytokines including tumor necrosis factor- α (TNF- α), interleukin- β (IL-1 β), interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1), inducible nitric oxide synthase, transforming growth factor β 1, and procoagulant proteins (Fried et al., 1998; Grimble, 2002; Hotamisligil et al., 1993; Perreault et al., 2001; Samad et al., 1997; Sartipy et al., 2003; Weyer et al., 2002; Zhu et al., 2008).

2.4.2 Biomarkers of Inflammation

The primary risk factors for chronic inflammation include Interleukin-1 β (IL-1 β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), cyclooxygenase-2 (COX-2), transcription nuclear factor- κ B (NF- κ B), and tumor necrosis factor- α (TNF- α). In adipose tissue metabolism, cytokines are major regulators, particularly the IL-1 system. There are four main members of the IL-1 cytokine family: IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1ra) and IL-18. In general, the IL-1 family is considered to be pro-inflammatory and pro-atherogenic except IL-1ra which is regarded as an anti-inflammatory cytokine (Girn et al., 2007). IL-1 β is a major cytokine placed high in hierarchical (auto) inflammatory cascades. It is potentially involved in the pathogenesis of atherosclerosis and other cardiovascular diseases (Manica-Cattani et al., 2010). Elevated circulating levels of IL-1 β have been reported in obesity (Garanty-Bogacka et al., 2005). In vitro studies in human adipose tissue have suggested that endogenous release of IL-1 β and TNF- α up-regulate IL-6 production (Fain et al., 2005). Blocking IL-1 β results in a rapid and sustained reduction in the severity of most auto-

inflammatory diseases (Dinarello, 2011). IL-6 is a multifunctional inflammatory cytokine. It stimulates immune responses and is secreted from a wide variety of cell types (Ershler et al., 2000). Circulating levels of IL-6 are significantly higher in obese humans and animals. IL-6 increases FFA and fat oxidation in humans and plasma IL-6 concentrations are correlated with insulin resistance (Kern et al., 2001; van Hall et al., 2003). IL-6 acts synergistically with other regulatory factors to affect the development of several chronic diseases such as cardiovascular disease and Type 2 diabetes (Pradhan et al., 2001; Yudkin et al., 2000). Another pre-inflammatory marker TNF- α produced in adipose tissue is also associated with obesity and insulin resistance in patients with or without Type 2 diabetes (Hotamisligil et al., 1994; Kern et al., 2001). It was reported that TNF- α stimulates the IL-8 production in human adipocytes (Gerhardt et al., 2001). IL-8, a chemokine, is known to activate neutrophils, which is enhanced by inflammatory substances such as TNF- α , IL-1 β , and C-reactive protein (CRP). NF- κ B is a sequence specific transcription factor implicated in the transmission of different signals from cytoplasm to the nucleus, which plays an important role in regulating inflammatory and immune genes, apoptosis, and cell proliferation (de Winther et al., 2005). NF- κ B is also a pivotal factor in controlling the inflammatory and metabolic alterations associated with obesity (Sonnenberg et al., 2004; Wellen et al., 2005). NF- κ B mediates induction of pro-inflammatory cytokines, including IL-1, IL-6, and TNF- α .

2.5 Human Subcutaneous Adipocytes

Adipose tissue comprises 20% of the body weight of healthy individuals making it one of the largest organs in the body (Cinti, 2007). The main function of adipose tissue is an energy reservoir. Adipose tissue accumulates triacylglycerol when excess calories are intake (Tsuda, 2008). Adipose tissue also acts as an endocrine organ. It secretes a large number of proteins

known as adipokines. The adipokines impact a variety of biological functions, including energy balance, glucose homeostasis, lipid metabolism and inflammation (Greenberg et al., 2006; Meier et al., 2004; Siiteri, 1987; Trujillo et al., 2006; Zhang et al., 1994). Adipose tissue contains multiple cell types, including endothelial cells, macrophages, adipocytes and preadipocytes (Xu et al., 2003). Adipocytes, once considered solely as energy depot cells, are actually recognized as active endocrine cells that secrete cytokines, polypeptides and hormone-like molecules. In fact, their progenitors, preadipocytes, also secrete a large number of cytokines, polypeptides and hormone-like molecules (Kershaw et al., 2004; Trayhurn et al., 2001; Xu et al., 2003).

Adipocytes are major drug targets due to their central role in a vast array of pathophysiological processes that include obesity, diabetes, inflammation and cancer (Nawrocki et al., 2005). The expression levels of adipose-derived cytokines are postulated to be risk factors for cardiovascular disease, diabetes, hypertension, and other components of the metabolic syndrome (Trayhurn, 2005). Adipocytes are very strongly implicated in the inflammatory process associated with the development of obesity (Hoareau et al., 2009). Preadipocytes are present throughout adult life in adipose tissue and can proliferate and differentiate into mature adipocytes. Preadipocytes have the potential to “transdifferentiate” into macrophages (Charriere et al., 2003). Preadipocytes are adipogenic progenitor cells which possess a characteristic pathway of multipotent adult stem cells and are able to differentiate in vitro into adipogenic, osteogenic, chondrogenic, myogenic and neurogenic lineages under specific culture conditions (Scanarotti et al., 2010).

2.6 Dietary Approach to Reduce Obesity

2.6.1 Anthocyanins in Berries

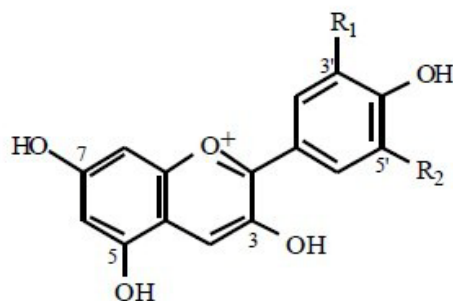
Bioactive compounds in foods have attracted much attention because of their potential

benefits to human health. Micronutrients in plants, animals or marine foods play an important role in maintaining human health. Edible berries have been a part of human's diet for centuries. As with other fruit and vegetables, berries are important dietary sources of fiber and essential vitamins and minerals. Berries also contain several phytochemicals, such as phenolic acids, proanthocyanidins, anthocyanins and other flavonoids. *In vitro* studies suggest that berry extracts can modulate cancer development processes by inhibiting the growth and proliferation of cancer cells, including cell death, and impairing angiogenesis through inhibition of expression of vascular endothelial growth factor VEGF (Roy et al., 2002). It has also been reported that blackberry, cherry, raspberry, blueberry, and bilberry extracts can inhibit LDL oxidation which is a key factor for the development of coronary arteries (Heinonen, 2007; Laplaud et al., 1997).

Anthocyanins are the largest group of water-soluble pigments responsible for the blue, purple and red color of many plant tissues in the plant kingdom (Prior et al., 2006). Anthocyanins are conjugated anthocyanidins, which provide the distinctive and vibrant palate of colors in berries. Anthocyanins are abundantly present in chokeberry, cherry, aubergine, blue grape, vaccinium, red cabbage, and the Usambara-violet (Tsoyi et al., 2008). They are widely distributed in the human diet through the consumption of crops, beans, fruits, vegetables, and red wine. Hollman and Katan (1999) and Scalbert and Williamson (2000) have found the average daily intake of anthocyanins by humans to range from 25 to 1000 mg.

Anthocyanins possess anti-oxidant, anti-inflammation, anti-obesity, anti-diabetes, and anti-cancer properties (Lee et al., 2009; Wang et al., 2002a). They also play a role in the reduction of coronary heart disease (Bridle and Timberlake, 1996). Anthocyanins can also increase visual acuity (Timberlake and Henry, 1988).

Much attention has focused on the ability of compounds such as anthocyanins and flavonols to act as antioxidants. The antioxidant effectiveness of anthocyanins and other polyphenols in vitro is essentially due to the ease with which a hydrogen atom from an aromatic hydroxyl group is donated to a free radical (Rice-Evans et al., 1997). In addition, anthocyanins can chelate transition metal ions involved in radical-forming processes such as Fenton reactions (Ferrali et al., 1997; Morel et al., 1993). They can also induce endogenous antioxidants (Fiander et al., 2000).



| Anthocyanins | R1 | R2 | Color |
|---------------------|------------------|------------------|---------------|
| Pelargonidin | H | H | orang-red |
| Cyanidin | OH | H | red |
| Delphinidin | OH | OH | pink |
| Peonidin | OCH ₃ | H | bluish purple |
| Petunidin | OCH ₃ | OH | purple |
| Malvidin | OCH ₃ | OCH ₃ | redish purple |

Fig.2.1 Structures of major anthocyanin

Blackberries (*Rubus* L. hybrids), blueberries (*Vaccinium* L. species), cranberry (*Vaccinium Macrocarpum*), and black currants (*Ribes nigrum* L.) are rich sources of dietary anthocyanins and antioxidants (Hong Wang, 1996; Kahkonen et al., 1999). The anthocyanins in the berries form conjugates with sugars including glucose, sophorose, rutinose, rhamnose, galactose, arabinose and xylose (Fig.2.1). Six anthocyanins most commonly found in plants are classified according to the number and position of hydroxyl groups on the flavan nucleus. They

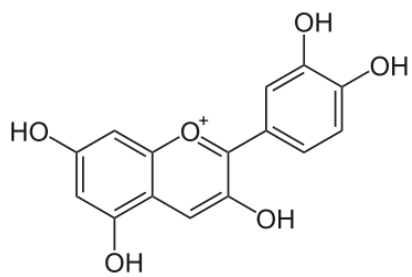
are named cyanidin (cy), delphinidin (dp), malvidin (mv), peonidin (pn), pelargonidin (pg), or petunidin (pt) (Valls et al., 2009) as shown in Fig.2.2.

Cyanidin-3-glucoside is the major anthocyanin found in blackberry (Garcia-Viguera et al., 1997; Goiffon et al., 1991). The major anthocyanins in blueberries are malvidin-3-galactose and malvidin-3-glucose, but other minor anthocyanins are also present (Seeram et al., 2006). Cranberries possess a distinctive flavor and a bright red color. The predominant anthocyanins in American cranberries are 3-O-galactosides and 3-O-arabinosides of cyanidin and peonidin (Mazza, 1993; Prior et al., 2001). Black currant skin is known to contain four major anthocyanins, namely cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside and delphinidin-3-O-rutinoside (Kapasakalidis et al., 2006).

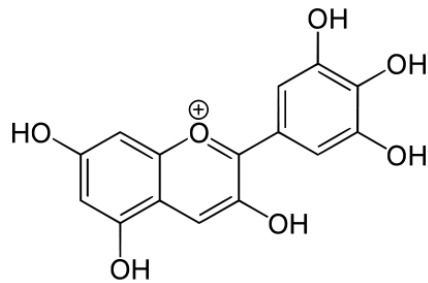
2.6.2 Anthocyanins and Inflammation

Numerous studies have proven the anti-inflammation effect of anthocyanins. Anthocyanin was reported to have inhibited the secretion of pro-inflammatory cytokines such as IL-8, MCP-1, IL-1 β , cytokine-induced neutrophil chemoattractant-1, IL-6, and TNF- α in cellular and animal models (Guo et al., 2008; Tsuda, 2008; Zhang et al., 2010). Similarly, Karlsen et al. (2007) observed that anthocyanins reduced the secretion of several molecules related to inflammatory modulation, specifically vascular endothelial growth factor and intracellular adhesion molecule-1 in cellular models (Karlsen et al., 2007).

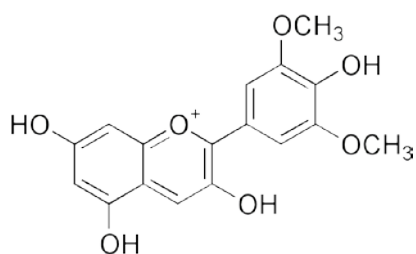
Anthocyanin-rich berry extracts showed considerable inhibitory effects on nitric oxide (NO) production in LPS/interferon- γ (IFN- γ) activated RAW264.7 macrophages (Wang et al., 2002b). Anthocyanin-rich blackberry extracts have potent antioxidant, anti-proliferative, and anti-inflammatory activities. Anthocyanin-rich berry extracts showed considerable inhibitory effects on nitric oxide (NO) production in LPS/interferon- γ (IFN- γ) activated RAW264.7



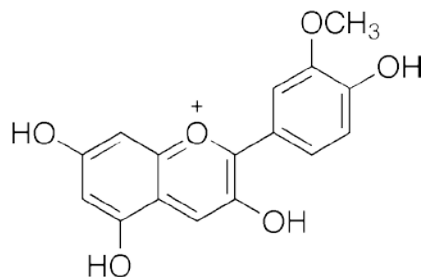
Cyanidin



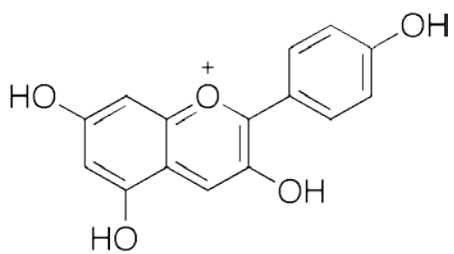
Delphinidin



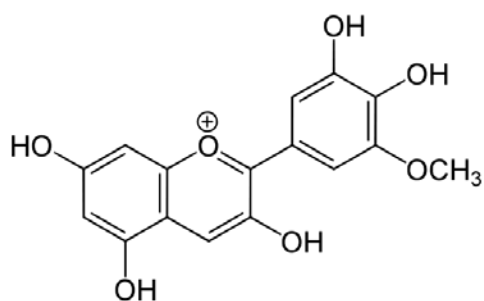
Malvidin



Peonidin



Pelargonidin



Petunidin

Fig.2.2 Chemical structures of anthocyanins

macrophages (Wang et al., 2002b). Anthocyanin-rich blackberry extracts have potent antioxidant, anti-proliferative, and anti-inflammatory activities. Blackberry extract-formulated products have the potential for the treatment and/or prevention of cancer and/or other inflammatory disease (Dai et al., 2007). Blueberries contain a wide range of bioactive compounds including anthocyanin, flavonols, flavanols, and stilbenes (de Pascual-Teresa et al., 2000). A study has shown that blueberry extract inhibited the production of the inflammatory mediator NO, the cytokines IL-1 β and TNF- α in BV2 cells activated to inflammation by LPS (Lau et al., 2007). Cranberries are an excellent source of anthocyanin, flavonol glycosides, proanthocyanidins and phenolic acids (Kandil et al., 2002; Prior et al., 2001). Polyphenolic extracts from cranberry inhibited the growth and proliferation of breast, colon, prostate, lung, and other tumors (Neto, 2007). Anthocyanin and hydroxycinnamic acids from blueberry and cranberry protected endothelial cells against TNF- α induced inflammatory responses (Youdim et al., 2002). Constituents present in black currant juice have been found to exert a number of health-promoting effects, including immune modulatory, antimicrobial and anti-inflammatory actions, inhibition of low-density lipoprotein, and reduction of cardiovascular diseases (Bishayee et al., 2010).

2.6.3 Anthocyanins and Preadipocytes

DeFuria et al. (2009) showed that the salutary effects of berries on adipocyte physiology may reflect the ability of anthocyanin to alter mitogen-activated protein kinase and nuclear factor-kappaB stress signaling pathways, which regulate cell fate and inflammatory genes (DeFuria et al., 2009). Cyanidin 3-glucoside has a significant potency of anti-obesity and ameliorates adipocyte function in the *in vitro* and *in vivo* systems. It has important implications for preventing metabolic syndrome (Tsuda, 2008). When preadipocytes were induced in the

presence of anthocyanins (1-40 $\mu\text{g/ml}$), the accumulation of cytoplasmic triglycerides was reduced in a dose- and time- dependent manner in 3T3-L1 preadipocytes (Bong-Gi Lee, 2007). Alaskan berries increased the expression levels of preadipocyte factor 1 and reduced lipid accumulation in 3T3-L1 adipocytes (Kellogg et al., 2010). However, little evidence has shown that anthocyanins-rich extracts from blackberry, blueberry, cranberry and black currant have anti-inflammatory effects induced by PA or LPS in human preadipocytes.

In summary, obesity is a health problem in the U.S and around the world. Currently pharmaceutical strategies are mainly used to lose weight; however, these are not suitable for every obese person. Therefore, other strategies must be developed to solve the obesity problem. From the cause of obesity, we know it is mainly because of the intake of too much high energy food. Hence, we need to utilize a more ideal diet in our daily life and also to increase physical activities to maintain an energy balance. In addition, obesity is closely associated with various chronic diseases especially inflammation which is a chronic risk factor for metabolic syndrome. This study focused on the interactions between inflammatory biomarkers in human preadipocytes and anthocyanins isolated from blackberry, blueberry, cranberry, and black currant.

CHAPTER 3 MATERIALS AND METHODS

3.1 Reagents

HPLC grade acetonitrile and methanol were purchased from Fisher Chemicals (Fair Lawn, N.J.). Trifluoroacetic acid (TFA) was purchased from Sigma-Aldrich (St. Louis, MO.). DMEM/F-12 (1:1) liquid media, Fetal bovine serum, trypsin, phosphate buffered saline were obtained from Fisher Scientific (Pittsburgh, PA). Lipopolysaccharide (*Escherichia coli* 0111:B4) and palmitic acid were purchased from Sigma (St. Louis, MO). Bio-Rad DC protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). PVDF membranes and 4-12% Bis-Tris gel were obtained from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA), primary antibodies, NF- κ B p65 and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). COX-2 primary antibody was purchased from Cayman Chemical (Ann Arbor, Michigan). Peroxidase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). X-ray film was purchased from Phoenix Research (Candler, NC).

3.2 Anthocyanin Extraction and Concentration Determination

Frozen blackberry (Marionberry, *Rubus L. subgenus*), blueberry (Duke, *Vaccinium corymbosum*), cranberry (Ben Lear, *Vaccinium macrocarpon*), and black currant (Jet, *Rubus nigrum*) fruits were supplied by Frito Lay, Inc (Dallas, TX). Frozen berry fruits were ground in a commercial blender. The liquefied samples were lyophilized in a Genesis XL-35 lyophilizer (VirTis Co., NY).

Twenty g of lyophilized berries were extracted with 100 ml acidified methanol containing 0.01% (v/v) HCl and gently shaken in laboratory shaker for 1 h at room temperature. The slurry was filtered through a Whatman No.1 filter paper by vacuum suction using a Buchner funnel. This extraction step was repeated for 5 times until all the pigment were extracted. The filtrates

were combined and evaporated in a rotary evaporator at 40 °C under vacuum. The resulting paste was stored at -20 °C until use.

The total anthocyanin concentration was determined by the pH-differential method (Giusti & Wrolstad, 2000). All measurements were performed in triplicate. Two buffer systems were used: 0.025 M potassium chloride buffer at pH 1.0 and 0.4 M sodium acetate buffer at pH 4.5. The berry extracts were diluted with pH 1.0 potassium chloride solution. The absorbance of the solution was read at 700 nm using the following formula: $A = (A_{520 \text{ nm}} - A_{700 \text{ nm}})_{\text{pH}1.0} - (A_{520 \text{ nm}} - A_{700 \text{ nm}})_{\text{pH}4.5}$

Monomeric anthocyanin pigment concentration in the original sample using the following formula: Monomeric anthocyanin pigment (mg/liter) = $\frac{A \times MW \times DF \times 1000}{\epsilon \times 1}$ where MW is the molecular weight and DF is the dilution factor. The ϵ value reported in the literature for anthocyanin pigment (cyanidin-3-glucoside) in acidic aqueous solvent was 26,900 and the MW is 449.2 (Giusti et al., 2001).

Anthocyanin pigments will combine with bisulfate to form a colorless sulfonic acid adduct. Polymerized colored anthocyanin-tannin complexes are resistant to bleaching by bisulfate, whereas the bleaching reaction of monomeric anthocyanins will rapidly go to completion. The ratio between polymerized color and color density is used to determine the percentage of the color that is contributed by polymerized material (Giusti et al., 2001). Color density of the sample treated with water was determined using the relation: $(A_{420 \text{ nm}} - A_{700 \text{ nm}}) + (A_{\text{vis-max}} - A_{700 \text{ nm}}) \times \text{DF}$. DF is the dilution factor. The polymeric color of sample treated with bisulfite was calculated as: $(A_{420 \text{ nm}} - A_{700 \text{ nm}}) + (A_{\text{vis-max}} - A_{700 \text{ nm}}) \times \text{DF}$: The percent polymeric color was determined as: $(\text{polymeric color}/\text{color density}) \times 100$

3.3 HPLC Analysis

3.3.1 Anthocyanins Profiles by HPLC Analysis

The HPLC system consisted of a Supelco (Bellefonte, Pa.) Discovery C18 column (internal diameter 3 mm × 25 cm), a Waters 2690 separation module, a 996 photodiode array detector, and a Millennium32 chromatography manager (Waters, Milford, Mass.). The mobile phase was a mixture of (A) 0.4% TFA in water and (B) acetonitrile, with the percentage of (A) 0.4% TFA in water ramped from 100% to 55% in 45 min with a constant flow rate of 0.8 ml/min. The peaks of anthocyanidins were confirmed by matching their retention times and UV–visible absorption spectrum with standards. The chromatograms obtained at a wavelength of 520 nm were used to quantify the anthocyanins and anthocyanidins. The concentrations were calculated using their standard or corresponding anthocyanidin standard calibration curves.

3.3.2 Phenolics Profiles by HPLC Analysis

The phenolics profiles analysis used the same HPLC system and Discovery C18 column. The chromatograms monitored at all wavelengths from 200 to 600 nm. Gradient elution was performed with solution A, composed of 50 nM sodium phosphate (pH3.3) and 10% methanol, and solution B, comprising 70% methanol, delivered at a flow rate of 1.0 mL/min as follows: initially 100% of solution A; for the next 15 min, 70% A; for another 30 min, 65% A; for another 20 min, 60% A; for another 5 min, 50% A; and finally 0% A for 25 min. The injection volume for the extract was 25 µl.

3.4 Cell Culture

Human subcutaneous preadipocytes from four donors (Table 3.1) were obtained from the Stem Cell Biology Laboratory, Pennington Biomedical Research Center. Liposuction aspirates were obtained from female subcutaneous adipose tissue sites (abdomen, flank, thighs)

undergoing elective plastic surgical procedures. Tissues were washed with phosphate buffered saline (PBS) three to four times and suspended in an equal volume of PBS with 1% bovine serum albumin and 0.1% collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ) prewarmed to 37 °C. The tissues were continuously agitated for 60 min at 37°C and centrifuged for 5 min at 300 × g at room temperature (Aust et al., 2004; Dubois SGHY-D, 2005; Halvorsen et al., 2001; Sen et al., 2001). The supernatant containing mature adipocytes were removed. The precipitated pellet was identified as the stromal vascular fraction (SVF). The SVF was suspended and plated in T225 flasks in Stromal Media at a density of 0.156 ml of tissue digest/cm² of surface area for expansion and culture (DMEM/F-12 Ham's. 10% fetal bovine serum (Hyclone, Logan, UT), 100 U penicillin/100 µg streptomycin/0.25 µg Fungizone).

The initial passage of the primary cell culture was referred to as “passage 0” (P0) for the adherent adipose-derived stem cell (ASC) population. Following the first 48 h of incubation at 37 °C and 5% CO₂, the cultures were washed with PBS and maintained in Stromal Media until 75-90% confluence was achieved (approximately 6 days). The cells were passaged by trypsin (0.05%) digestion and plated at a density of 5,000 cells/cm² (“passage 1”). The cells were maintained in DMEM/F-12 medium containing 10% BFS and 100 U penicillin/100 µg at 37°C in humidified atmosphere containing 5% CO₂. The cells were seeded at a density of 5×10⁴/cm². After 48 h, the cells were treated.

Table 3.1 Human preadipocytes donor demographics: age, gender and Body Mass Index

| Donor | Gender | Age (Year) | Body Mass Index |
|-------------|--------|------------|-----------------|
| L070711 | Female | 41 | 28.22 |
| L080909 | Female | 47 | 31.61 |
| L090724 | Female | 47 | 26.65 |
| L090330 | Female | 45 | 26.46 |
| Mean ± S.D. | | 45±2.8 | 28.2 ±2.4 |

3.5 Crystal Violet Cell Viability Assay

Human preadipocytes were treated with 100 μ M or 250 μ M palmitic acid (PA) or 100 ng/ml lipopolysaccharide (LPS), and incubated for 2, 4, 8, 24, or 48 h in a humidified incubator containing 5% CO₂ at 37°C. Each treatment was carried out in triplicate. Cell viability was determined by the crystal violet assay (Wosikowski et al., 1993). Cell viability was expressed as the percentage of control cells.

3.6 Interaction between PA or LPS and Human Preadipocytes

Human preadipocytes were seeded in 12 well plates and incubated over night at 37°C and 5% CO₂ in a humidified atmosphere. Blackberry (BAB), blueberry (BUB), cranberry (CRB) or black currant (BC) extracts (0, 10, 20, and 40 μ M) were added and incubated for 24 h; Palmitic acid (PA) was then added to a final concentration of 100 μ M. The cells were incubated for an additional 24 h. The concentration of anthocyanin was determined in this procedure.

Two approaches were tested to emulate the intervention and prevention of inflammation. In the prevention approach, berry anthocyanins were incubated with preadipocytes for 24 h followed by 100 μ M PA treatment for 24 h. Similarly, berry anthocyanins were incubated with preadipocytes for 24 h followed by 100 ng/ml LPS for 8 h. In the intervention approach, human preadipocytes were treated with 100 μ M PA for 24h, followed by addition of berry anthocyanins for 24 h. In the comparison group, LPS was used to induce inflammation. Human preadipocytes were treated with 100 ng/ml LPS for 8 h, followed by addition of berry anthocyanins and 24 h incubation.

3.7 Enzyme Linked Immunosorbent Assay for Pro-inflammatory Cytokines

ELISA for human IL-6, IL-8 and MCP-1 levels in the conditioned media for control and treated cells were performed using commercially available kits from Peprotech (Rock Hill, NJ) according to the manufacturer's instructions. Human IL-6, IL-8 and MCP-1 ELISA development

kit contains the key components required for the quantitative measurement in a sandwich ELISA format. Recombinant standard human IL-6, IL-8 and MCP-1 served as the standard for quantitative evaluation. All standards and samples were tested in triplicate.

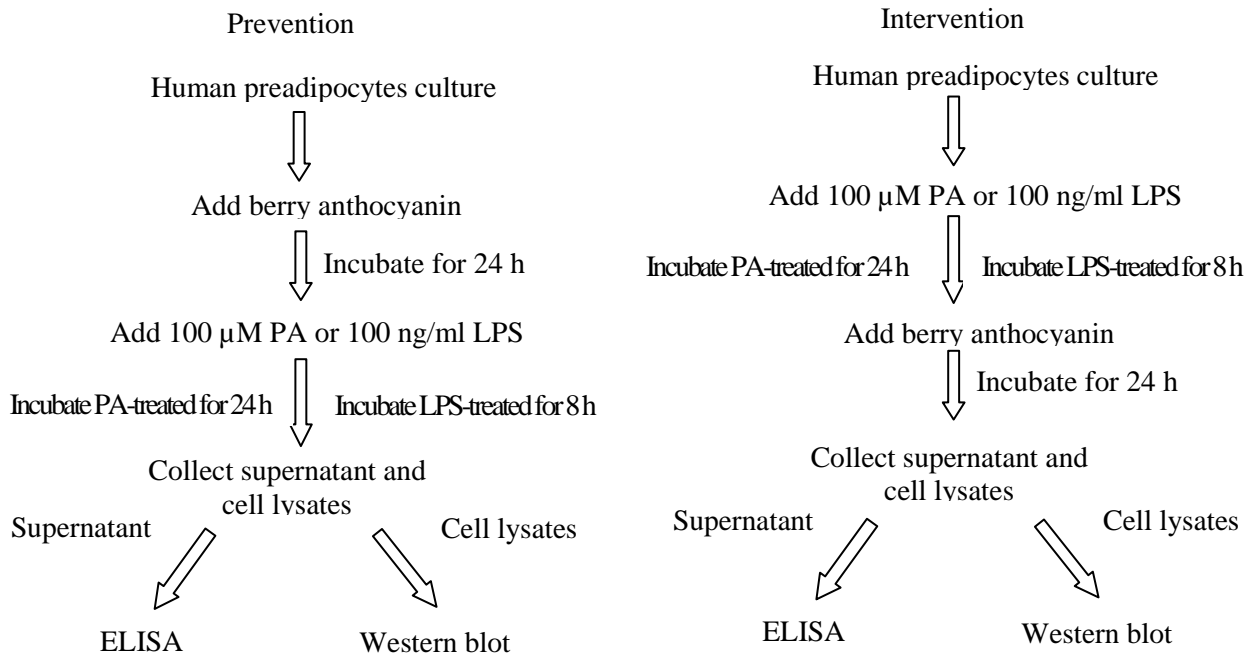


Figure 3.1 Interactions of anthocyanins with PA or LPS

3.8 Western Blot

Control and treated cells were washed with ice cold PBS, lysed with ice cold RIPA (Radio-Immunoprecipitation Assay) buffer (50 mM Tris-HCL, pH7.5, 1% Nonidet P-40, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% SDS, 0.1% deoxycholic acid), and centrifuged at 14,000 ×g for 10 min at 4 °C. The supernatants were collected. Protein concentrations of the supernatants were determined by Bio-Rad DC protein assay following manufacturer's instructions (Bio-Rad Laboratories, CA). Briefly, pipet 5 μl of standards and samples into 96 wells plates; add 25 μl of reagent A into each well; add 200 μl reagent B into each well. After 15 minutes, absorbance can be read at 655 nm. For Western blot analyses, 30 μg protein lysates per sample were denatured in

9 μ l 4 \times NuPAGE LDS sample buffer. The volume of the sample was adjusted to 36 μ l with water, and then boiled at 100°C for 5 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run with 4-12% Bis-Tris gel. The proteins separated were then transferred to polyvinylidene fluoride (PVDF) membrane. Residual binding sites on the membrane were blocked by incubation in TBST (10 mM Tris, 100 mM NaCl, 0.1% Tween20) with 3% (w/v) bovine serum albumin (BSA) for 1 hour at room temperature. The membranes were probed with specific primary antibodies at 4°C overnight, followed by peroxidase-conjugated secondary antibodies. After incubation with 1st and 2nd antibodies, the blots were washed five times with 10 ml tris buffered saline solution (TBST) for 5 min each time. The blots were visualized with SuperSignal West Pico Chemiluminescent substrate (Fisher Scientific) and exposed to X-ray film (Kodak X-omat 1000A processor).

3.9 Statistical Analyses

The results are expressed as means \pm standard deviation. Each experiment was conducted using (3-4) donors and each treatment was carried out independently in triplicates. For comparison of the differences among the groups, ANOVA was used, followed by Tukey's post hoc test. Statistical significance was set at P-value < 0.05.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Anthocyanins Concentration Determination

To determine the anthocyanins concentration in blackberry, blueberry, cranberry or black currant, we performed the test by using the spectrophotometric method reported by Giusti & Wrolastad (2001). The results are presented in Table 4.1. The results show that black currant contained the highest level of anthocyanins among the four fruits, whereas the cranberry used contained the lowest levels. Marcin Horbowicz (2008) reported that the anthocyanins content in blackberry were 83-326 mg/100g and blueberry had 25-495 mg anthocyanins per 100 g fruit (Marcin Horbowicz, 2008). Our results show that anthocyanins concentration in blackberry was 98.1 ± 4.1 mg in 100 g fresh fruits and blueberry had 56.0 ± 5.9 mg anthocyanins per 100 g fresh fruits (Table 4.1). Timberlake et al. (1988) reported that the total anthocyanin of black currant ranges from 130 to 400 mg/100g fresh fruits (Timberlake, 1988). Our results show that the anthocyanins in black currant are 136.5 ± 2.0 mg/100g. The total content of anthocyanins in cranberry fruits range from 180 to 656 mg/Kg of fresh weight (Wang et al., 2001). Cranberry had 39.2 ± 5.7 mg/100g in fresh fruits. The results of anthocyanin concentration in this study are consistent with previous reports. The concentrations of anthocyanins in berries vary according genotype, growth environment, storage time, storage conditions, extraction methods and the detection methods (Dai et al., 2009; Yue et al., 2008).

Polymeric anthocyanins were determined by spectrophotometer and the results are presented in Table 4.1. Blueberry and black currant have 22.8% and 29.7% polymeric anthocyanins, respectively. The values are relatively higher than blackberry or cranberry which had a concentration of 5.98% and 5.15% polymeric anthocyanins, respectively. Processing may result in increase in polymeric color values and losses of monomeric anthocyanins (Hager et al.,

2008). Polymers have a higher affinity for cellular material than lower molecular weight compounds due to the presence of multiple binding sites on large molecules (Le Bourvellec et al., 2004; Renard et al., 2001).

Table 4.1 Monomeric anthocyanins and percentage of polymeric anthocyanins in berries

| | Monomeric Anthocyanin in frozen fruits (mg/100g) | Polymeric Anthocyanin in frozen fruits (%) |
|---------------------------|--|--|
| Black berry (Marionberry) | 98.1 ± 4.1 | 6.0 ± 2.0 |
| Blueberry (Duke) | 56.0 ± 5.9 | 22.8 ± 3.7 |
| Cranberry (Ben Lear) | 39.2 ± 5.7 | 5.2 ± 3.2 |
| Black currant(Ben Tirran) | 136.5 ± 2.0 | 29.7 ± 0.5 |

4.2 Polyphenols Content by HPLC Analysis

4.2.1 Anthocyanins Content in Berry Extracts

To determine the profile of anthocyanins in the four berries, we performed HPLC analysis of the extracts. The results of blackberry are presented in Fig.4.1A. Three anthocyanins were found in blackberry extracts (Table 4.2). The total anthocyanin content was 267.0 mg/100g of the extract. The main anthocyanin in blackberry extract was cyanidin glucoside which represented 92.2% of the total anthocyanin content.

The anthocyanins profile in the blueberry extract by HPLC method is shown in Fig. 4.1B. Ten different anthocyanins were found in blueberry extracts (Table 4.2). The main anthocyanins in blueberry extract included cyanidin 3-arabinoside, petunidin 3-glucoside, peonidin 3-galactoside, and malvidin 3-galactoside which is cyanidin glucoside. For the blueberry extract, malvidin 3-galactoside represented 11.5% of the total anthocyanins (107.4 mg/100g).

The anthocyanins profile in the cranberry extract by HPLC method is shown in Fig. 4.1C. Six anthocyanins were found in the cranberry extract (Table 4.2). The main anthocyanins in cranberry extract included cyanidin 3-galactoside, cyanidin 3-arabinoside, peonidin 3-galactoside, and peonidin 3-arabinoside. The content of each of the anthocyanins was more than

10% of the total compounds extracted. The total anthocyanin content was 57.5 mg/100g of the extract.

The HPLC analysis of anthocyanins in the black currant extract is shown in Fig. 4.1D. Seven anthocyanins were found in black currant extract (Table 4.2). The main anthocyanins in black currant extract were delphinidin 3-glucoside, delphinidin 3-rutinoside, and cyanidin 3-rutinoside. The content of each of the anthocyanins mentioned above was more than 10% in the total anthocyanins. The total anthocyanins content was 137.9 mg/100g.

Briefly, both the pH differential and HPLC methods are commonly used methods to quantify anthocyanins in berries. Comparison of the results from the two analytical methods shows that overall values by HPLC method were higher than the pH differential method. This trend is in agreement with a reported study (Lee et al., 2008).

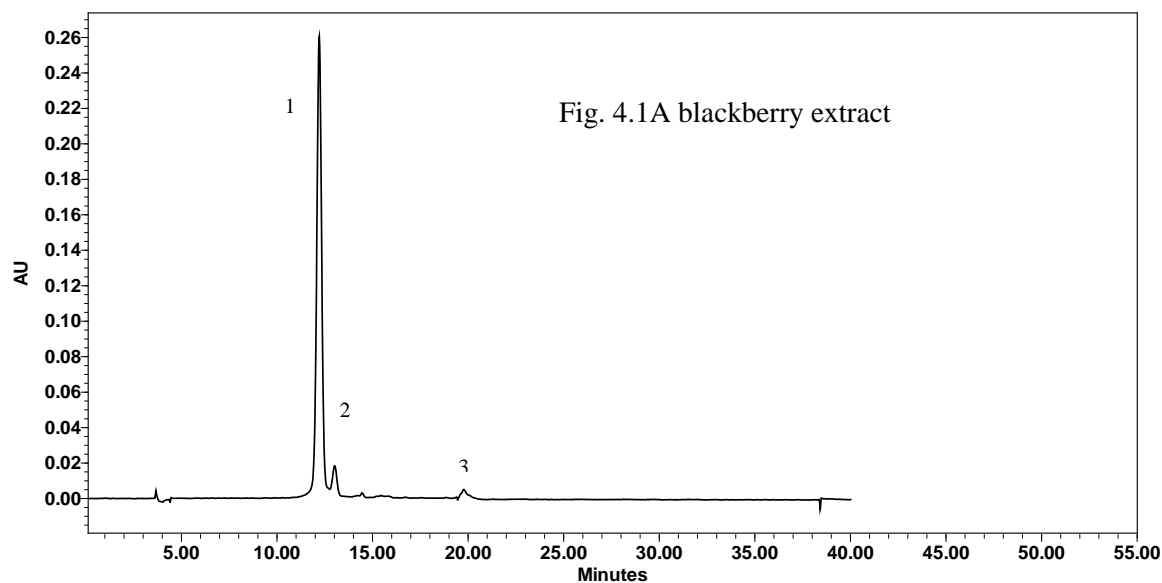


Fig.4.1. Anthocyanins profiles in the berry extracts by HPLC analysis. A: blackberry extract; B: blueberry extracts; C: cranberry extract; D: black currant extract.

Fig.4.1 Continued

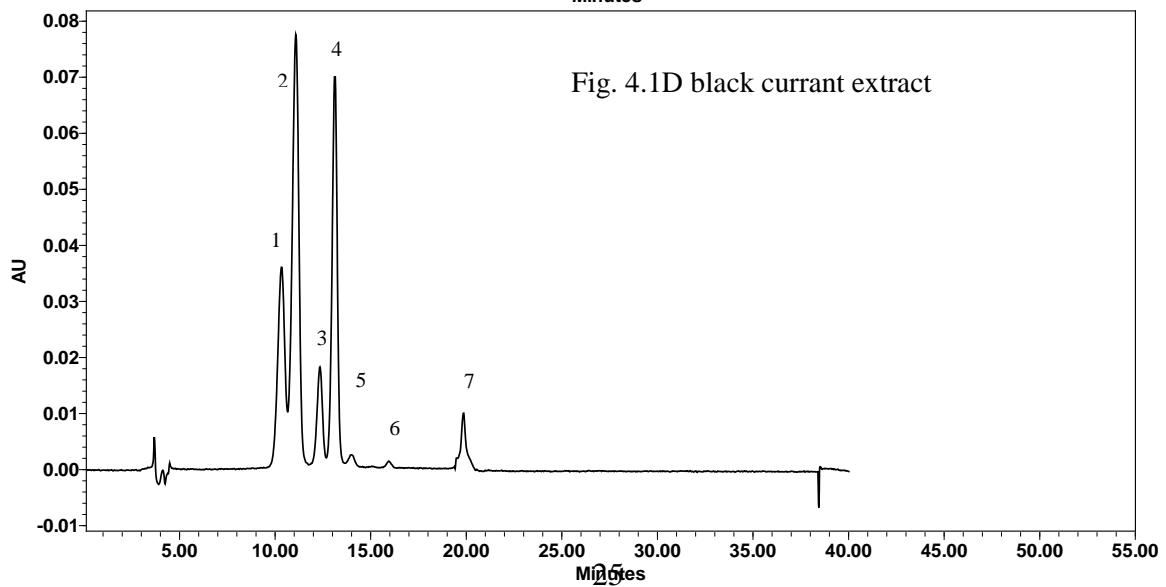
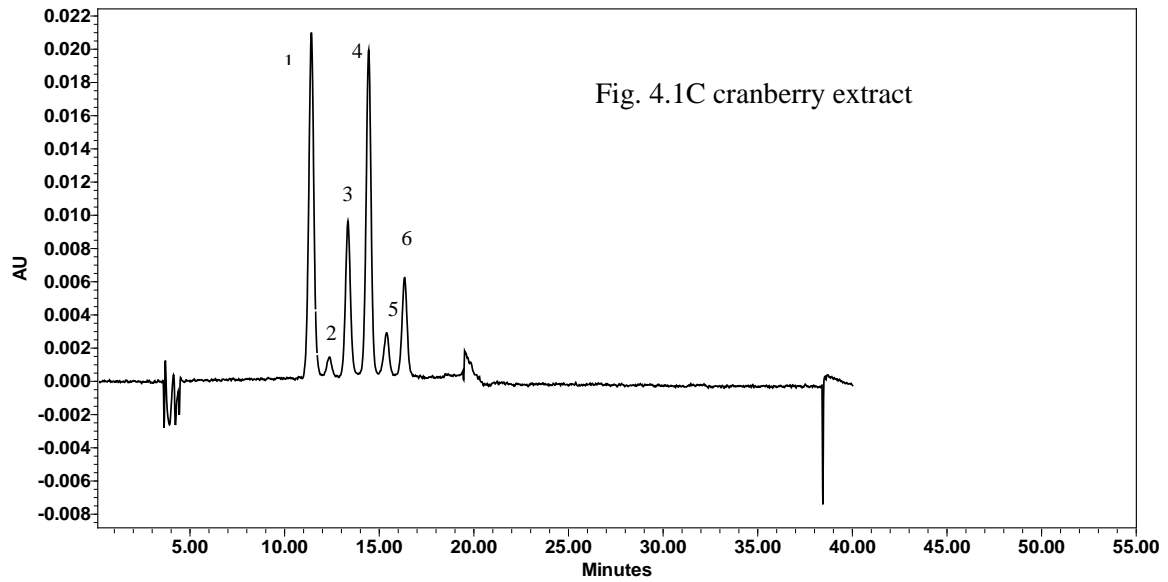
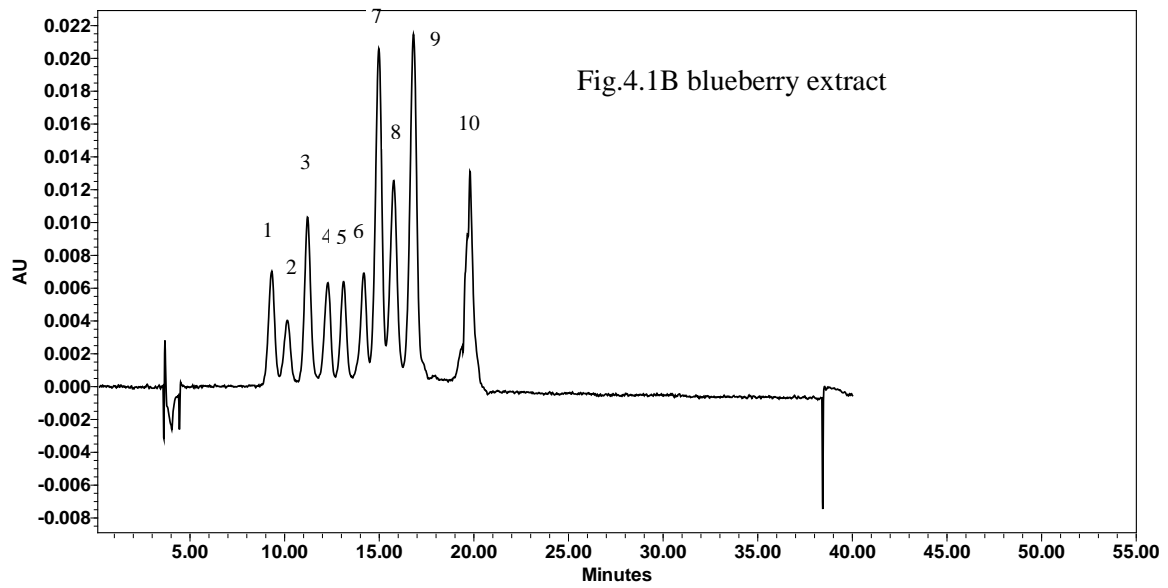


Table 4.2 Anthocyanin profile and content in the berry extracts

| | Compounds | Retention time (min) | Content mg/100g | % of total anthocyanin |
|------------------------------------|-------------------------------|-------------------------|--------------------|---------------------------|
| Black berry (Marionberry) extract | | | | |
| 1 | cyanidin 3-glucoside | 12.2 | 246.2 | 92.2 |
| 2 | cyanidin 3-rutinoside | 13.1 | 16.1 | 6.0 |
| 3 | cyanidin 3-dioxalyglucoside | 19.7 | 4.7 | 1.8 |
| Total | | | 267.0 mg/100g | |
| Blueberry (Duke) extract | | | | |
| 1 | delphinidin 3-galactoside | 9.4 | 6.6 | 6.2 |
| 2 | delphinidin 3-glucoside | 10.2 | 3.8 | 3.5 |
| 3 | cyanidin 3-galactoside | 11.3 | 9.5 | 8.9 |
| 4 | delphinidin 3-arabinoside | 12.1 | 5.7 | 5.3 |
| 5 | cyanidin 3-glucoside | 13.2 | 6.2 | 5.8 |
| 6 | petunidin 3-galactoside | 14.3 | 6.6 | 6.2 |
| 7 | cyanidin 3-arabinoside | 14.9 | 24.3 | 22.7 |
| 8 | petunidin 3-glucoside | 15.9 | 11.8 | 11.0 |
| 9 | peonidin 3-galactoside | 16.9 | 20.4 | 19.0 |
| 10 | malvidin 3-galactoside | 19.7 | 12.3 | 11.5 |
| Total | | | 107.4 mg/100g | |
| Cranberry (Ben Lear) extract | | | | |
| 1 | cyanidin 3-galactoside | 11.3 | 19.9 | 34.6 |
| 2 | cyanidin 3-glucoside | 12.2 | 1.4 | 2.4 |
| 3 | cyanidin 3-arabinoside | 13.3 | 9 | 15.7 |
| 4 | peonidin 3-galactoside | 14.5 | 18.6 | 32.3 |
| 5 | peonidin 3-glucoside | 15.4 | 2.4 | 4.2 |
| 6 | peonidin 3-arabinoside | 16.2 | 6.2 | 10.8 |
| Total | | | 57.5 mg/100g | |
| Black currant (Ben Tirran) extract | | | | |
| 1 | delphinidin 3-glucoside | 10.6 | 34.1 | 16.4 |
| 2 | delphinidin 3-rutinoside | 11.1 | 73.9 | 35.6 |
| 3 | cyanidin 3-glucoside | 12.4 | 17 | 8.2 |
| 4 | cyanidin 3-rutinoside | 13.3 | 68.2 | 32.9 |
| 5 | petunidin 3-rutinoside | 14.2 | 2.8 | 1.4 |
| 6 | peonidin 3-rutinoside | 16.1 | 1.9 | 0.9 |
| 7 | cyanidin 3-coumaroylglucoside | 19.8 | 9.5 | 4.6 |
| Total | | | 137.9 mg/100g | |

4.2.2 Other Polyphenols Content in the Berry Extracts

Polyphenolics in vegetables, fruits, and tea are able to prevent degenerative diseases through antioxidative action (Sakakibara et al., 2002). Other polyphenols content in the same berry extracts from blackberry, blueberry, cranberry or black currant were also determined by HPLC methodology described in Chapter 3.3.2. The results of other polyphenols are showed in the Table 4.3. The mainly polyphenols in the berry extracts using acidified methanol are anthocyanins. Other polyphenols are trace amount compared with anthocyanins. This observation is consistent with previous studies (Fan-Chiang et al., 2005; Wrolstad, 2001). Therefore, the anthocyanins in the berry extracts are the major functional polyphenols for the potential benefits of human health.

Table 4.3 Other polyphenols content in the berry extracts

| Berry extract | Polyphenols | Wavelength for determination (nm) | Content (mg/100g) |
|----------------|--------------|-----------------------------------|-------------------|
| 0.325 0.007 | Cafechin | 280 | 32.5 |
| | Chlorofeuc | 320 | 0.7 |
| 0.023 | Chlorofeuc | 320 | 2.3 |
| | Caffeic acid | 320 | 0.2 |
| Cranberry | Cafechin | 280 | 4.3 |
| | Chlorofeuc | 320 | 3.6 |
| Black currant | Gallic acid | 250 | 3.9 |
| | Protocateuc | 280 | 0.1 |
| | Cafechin | 280 | 13.4 |
| | Chlorofeuc | 320 | 0.6 |
| | Caffeic acid | 320 | 1.0 |

4.3 Effects of PA or LPS on Cell Viability Assay

To determine the effects of PA or LPS on cell viability in preadipocytes, the crystal violet assay was performed on human preadipocytes treated with 100 or 250 μM PA or 100 ng/ml LPS. The cell viability results are shown in Fig 4.2. There was no significant difference ($P>0.05$) between 100 μM PA and control treatments. There was also no significant difference ($P>0.05$) between 250 μM PA and control treatments. Similarly, the viability of 100 ng/ml LPS treated cells shows no significant difference ($P>0.05$) compared to control cells. The results suggest that PA or LPS at the concentrations used had no cytotoxic effects on human preadipocytes at the dosages uses.

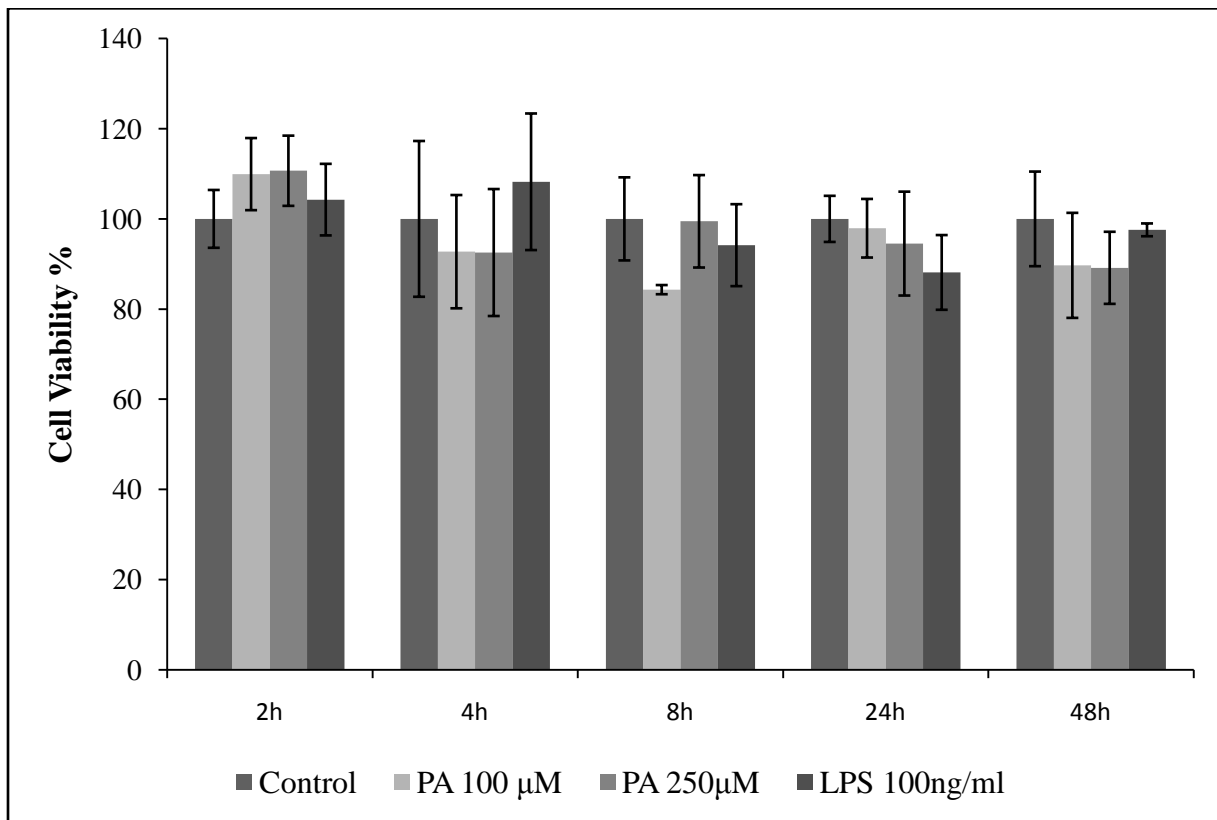
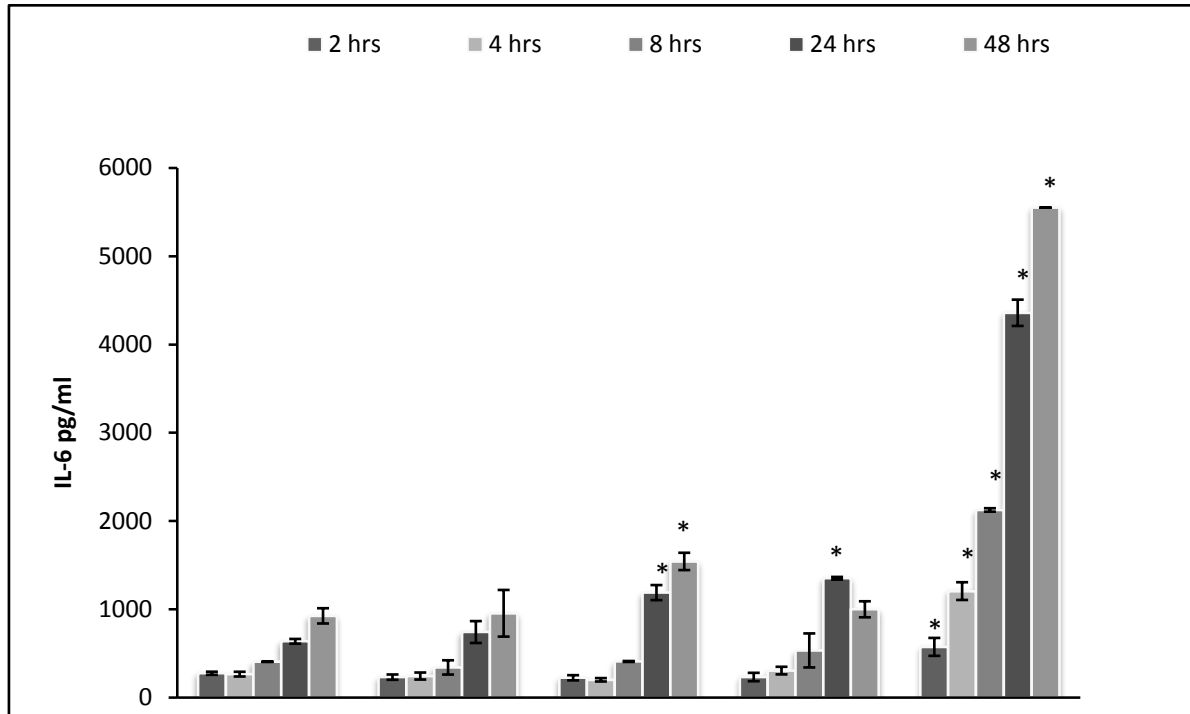


Fig.4.2 Effects of palmitic acid (PA) or lipopolysaccharide (LPS) on cell viability. PA (100 μM or 250 μM), and LPS (100 ng/ml) treated human preadipocytes for 0-48 hours. The values were expressed as percentage of untreated control cells.

4.4 Effects of PA or LPS on Pro-inflammatory Cytokine IL-6

To determine the effects of PA or LPS on IL-6, ELISA was performed to detect the IL-6 levels in the supernatants from the control and PA- or LPS- treated preadipocytes. The results for the IL-6 analysis are presented in Fig.4.3. PA at 100 μ M induced IL-6 secretion in a time-dependent manner. The maximal mean levels of IL-6 reached 1542 pg/ml in conditioned media after 24 h of PA exposure and it is significantly higher ($P < 0.05$) than the IL-6 level in the control. The levels of secreted IL-6 did not increase in a strict time dependent manner following exposure to PA at 250 μ M. IL-6 level exposed to 250 μ M PA at 48 h is even lower than 24 h, and the statistical analysis showed that the IL-6 level was not significantly different ($P > 0.05$) with the IL-6 level in the control. PA or LPS are potent agonists for activated inflammation and express the multiple cytokines in adipocytes (Ajuwon et al., 2005; Corre et al., 2006; Kilroy et al., 2007; Samokhvalov et al., 2009). Based on the preliminary results from this study and the results reported in literature (Samokhvalov et al., 2009), I decided to expose human preadipocytes for 24 h.

LPS at 100 ng/ml increased IL-6 secretion in conditioned human preadipocytes media in a time-dependent manner (Fig.4.3). The IL-6 level secreted by Human preadipocytes was significantly increased ($P < 0.05$) after two hours incubation with 100 ng/ml LPS. Following the exposure to LPS, the IL-6 accumulation reached to a maximal mean level of 5549 pg/ml in conditioned media after 48 h (Fig. 4.3). The IL-6 level in the conditioned media incubated with 100 ng/ml LPS for 8 hours was five times of the control. LPS at the concentration of 100 ng/ml was able to induce acute inflammation in human preadipocytes in 8 hours. Based on the result obtained from this study and the result reported in the literature (Kilroy et al., 2007), I chose 8 h exposure time in 100 ng/ml LPS.



| | | | | | |
|-------------|---|---|-----|-----|-----|
| 0.1% DMSO | - | + | + | + | - |
| PA (μM) | - | - | 100 | 250 | - |
| LPS (ng/ml) | - | - | - | - | 100 |

Fig.4.3 Effect of PA or LPS on IL-6 secretion. Human preadipocytes were treated by PA 100 μM or 250 μM or LPS 100 ng/ml for 0-48 hours. The columns represent the mean ± S.D. of triplicate determinations, *: P < 0.05.

4.5 Effects of Anthocyanins on the Cell Viability of the Cells Treated with PA or LPS

To determine the prevention effects of anthocyanins on preadipocytes viability treated with PA, crystal violet analysis was performed as the described in chapter 3.5. For prevention method, the results in Fig.4.4 demonstrate that the anthocyanins did not significantly affect ($P>0.05$) preadipocytes viability when cells were pretreated with anthocyanins extracts from BAB, BUB, CRB, and BC (0- 40 μM) for 24 h, followed by the treatment of 100 μM PA for 24 h. The intervention effects of anthocyanins on preadipocytes viability are shown in Fig. 4.5. There was also no significant difference ($P>0.05$) in viability of preadipocytes when they were stimulated with 100 μM PA for 24 h, followed with anthocyanins from BAB, BUB, CRB, and BC (0-40 μM) for 24 h. The results demonstrated that anthocyanins (0-40 μM) were not cytotoxic to human preadipocytes. Previous studies have also shown that an anthocyanin, cyanidin-3-glucoside, at 40 μM was not cytotoxic to adipocytes (Guo et al., 2008).

To determine the effects of anthocyanins on preadipocytes viability treated with LPS, the crystal violet analysis was performed as the method in chapter 3.5. The viability results of the prevention method are shown in Fig.4.6. The results of crystal violet assay demonstrates that there was no significant difference ($P>0.05$) in preadipocytes viability when they were pretreated with anthocyanins from BAB, BUB, CRB, and BC (0-40 μM) for 24 h, followed by 100 ng/ml LPS for 8 h. The viability results of intervention are shown in Fig. 4.7. There was also no significant difference ($P>0.05$) in viability of preadipocytes when they were stimulated with 100 ng/ml LPS for 8 h, followed with anthocyanins from BAB, BUB, CRB, and BC (0-40 μM) for 24 h. The results confirmed that at anthocyanins at concentration between 0 and 40 μM were not cytotoxic to human preadipocytes.

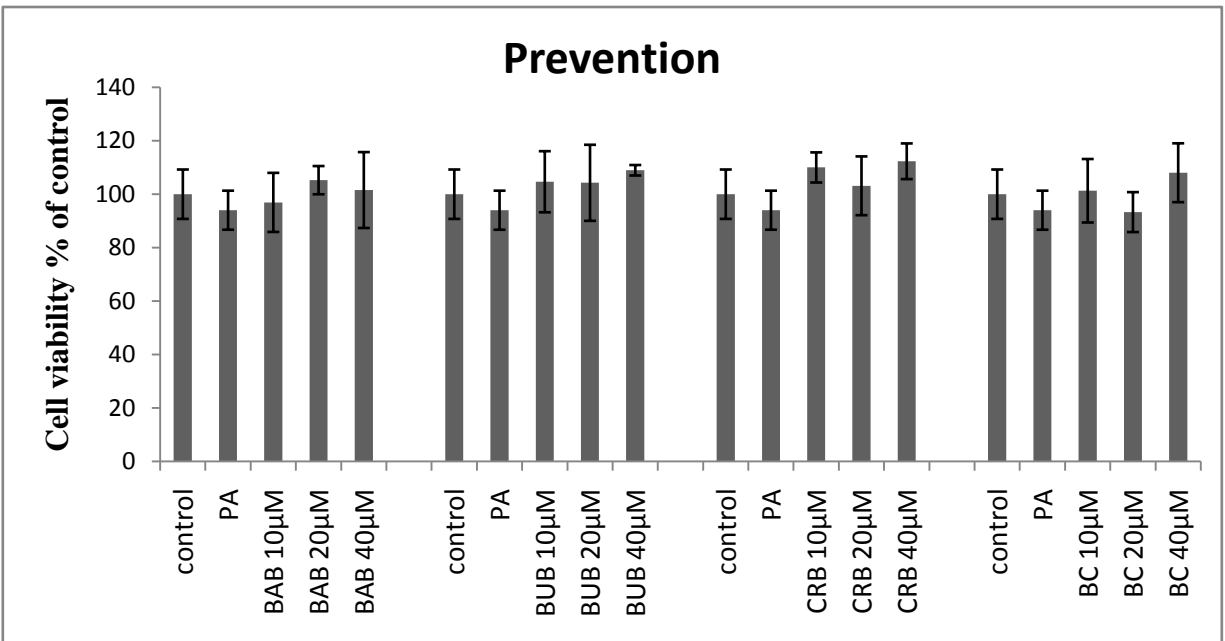


Fig. 4.4 Prevention effects of anthocyanins on viability of human preadipocytes stimulated by PA. Human preadipocytes were pretreated with different concentrations of anthocyanins for 24 h, followed by the treatment of 100 μ M PA for 24 h. The human preadipocytes were from one donor. Results are representatives of three independent experiments. The columns represent the mean \pm S.D. of triplicate determinations.

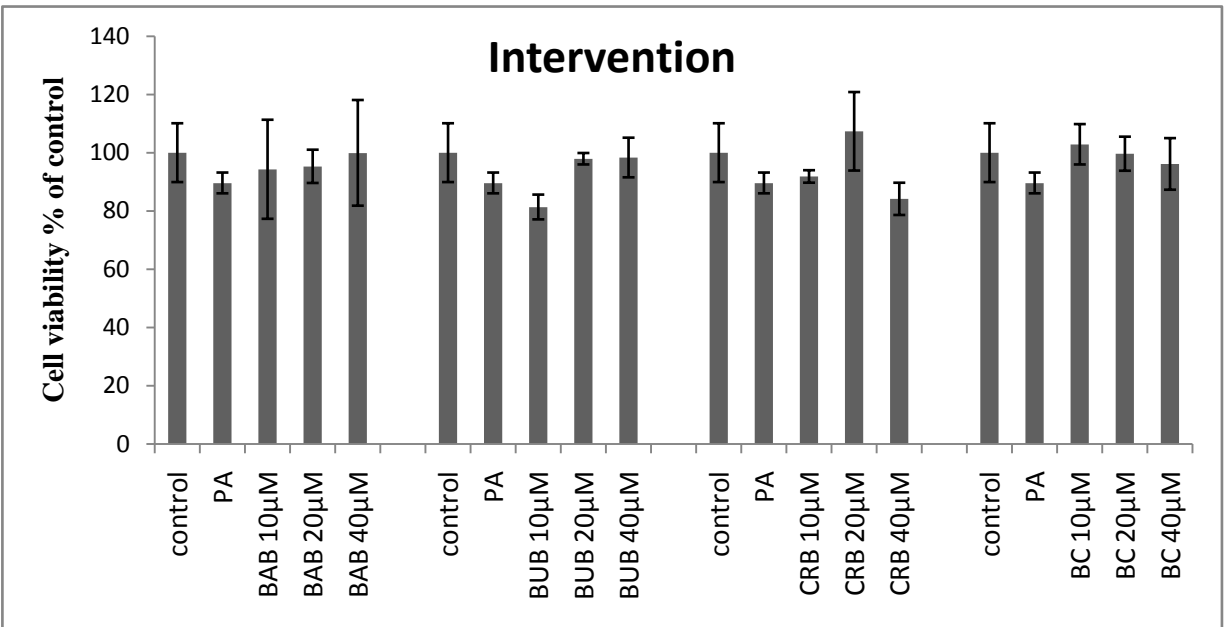


Fig. 4.5 Intervention effects of anthocyanins on viability of human preadipocytes stimulated by PA. Human preadipocytes were stimulated with 100 μ M PA for 24 h, followed with the addition of different concentrations of anthocyanins extracted from BAB, BUB, CRB, and BC. The viability of the cells was determined for 24 h after the addition of the anthocyanins. The human preadipocytes were from one donor. Results are representatives of three independent experiments. The columns represent the mean \pm S.D. of triplicate determinations.

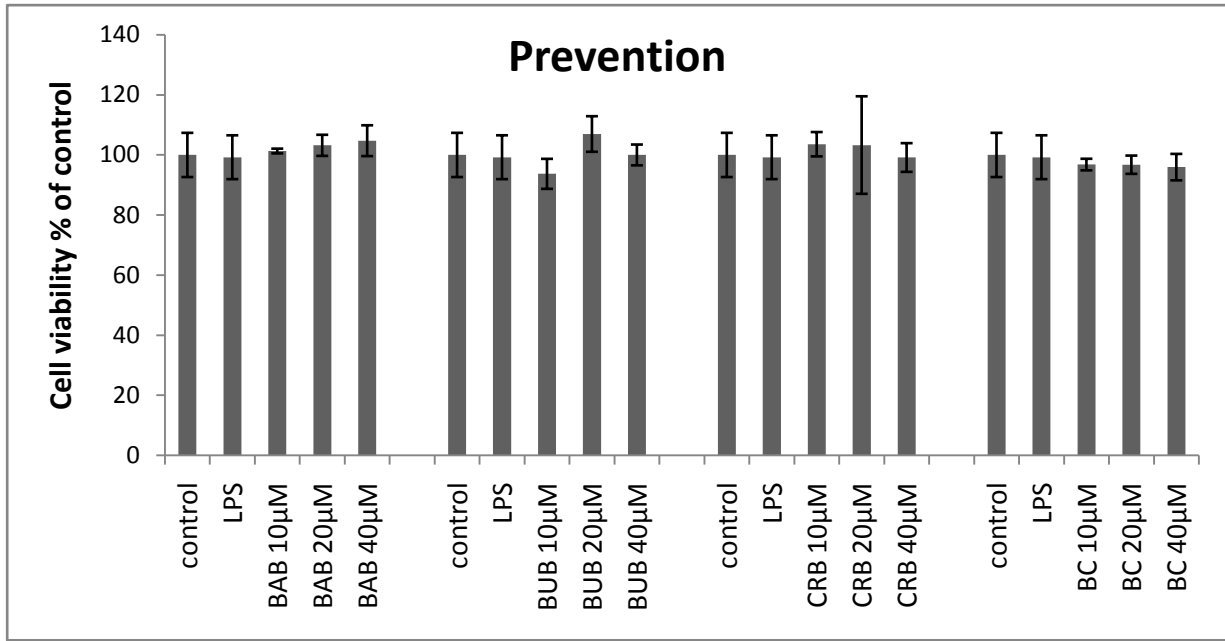


Fig. 4.6 Prevention effects of anthocyanins on the viability of human preadipocytes stimulated by LPS. Human preadipocytes were pretreated with different concentrations of anthocyanins extracted from BAB, BUB, CRB, and BC for 24 h, followed by the treatment of 100 ng/ml LPS for 8 h. The human preadipocytes were from one donor. Results are representatives of three independent experiments. The columns represent the mean \pm S.D. of triplicate determinations.

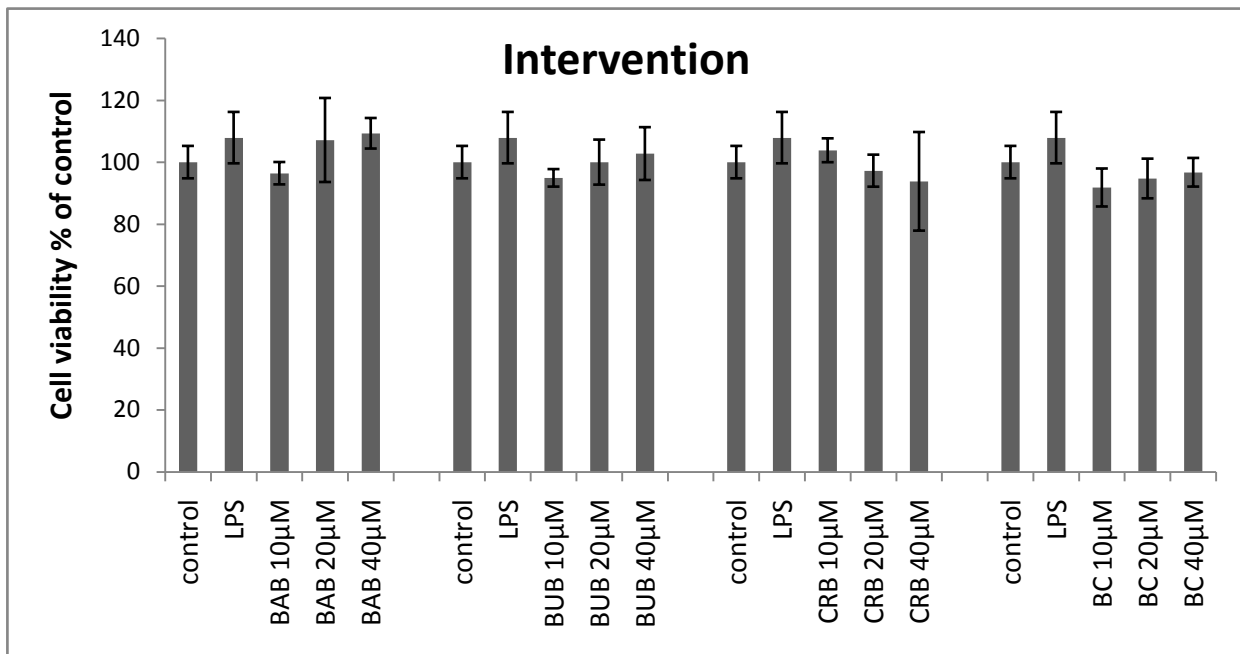


Fig. 4.7 Intervention effects of anthocyanins on the viability of human preadipocytes stimulated by LPS. Human preadipocytes were stimulated with 100 ng/ml LPS for 8 h, followed with the treatment of different concentrations of anthocyanins extracted from BAB, BUB, CRB, and BC for 24 h. The human preadipocytes were from one donor. Results are representatives of three independent experiments. The columns represent the mean \pm S.D. of triplicate determinations.

4.6 Effects of Anthocyanins on Pro-inflammatory Cytokine IL-6

To determine the preventative effects of anthocyanins on the levels of IL-6 in PA- treated human preadipocytes, the cells were pretreated with 0, 10, 20, and 40 μM of the anthocyanins extracted from the four different types of berries. The cells were then treated with 100 μM PA for 24 h. IL-6 ELISA was performed on the supernatants of the control and treated preadipocytes. The results of the IL-6 levels in the conditioned media are shown in Fig.4.8. The anthocyanins extracted from four different berries reduced the IL-6 secretion induced by 100 μM PA in a dose-dependent manner and the effectiveness also varied with the types of berries. Blackberry anthocyanins significantly ($P < 0.05$) attenuated the IL-6 levels induced by PA at a dosage of 40 μM . However, 10 and 20 μM blackberry anthocyanins did not significantly decrease ($P > 0.05$) the IL-6 level. For the anthocyanins extracted from blueberry, significant IL-6 reduction was observed at a dosage of 20 and 40 μM . For the anthocyanins extracted from cranberry and black currant, all dosages, 10, 20 and 40 μM , significantly reduced the production of IL-6. Previous studies by other researchers demonstrated that anthocyanins at a concentration range from 0 to 40 μM can inhibited the production of inflammatory cytokines induced by H_2O_2 in 3T3 adipocytes (Guo et al., 2008).

To investigate the effects of anthocyanins on IL-6 secretion induced by LPS, a similar experiment was carried out. In the experiment, human preadipocytes were pretreated with 10, 20 and 40 μM of anthocyanins extracted from the different berries for 24 h. A control (no anthocyanin addition) was also included. The cells were then treated with 100 ng/ml LPS for 8 h. The IL-6 in the supernatant of the samples was tested using the IL-6 ELISA mentioned before. The results from the experiment are presented in Fig. 4.9. For blackberry, blueberry and black currant, all the dosages used, 10, 20 and 40 μM , significantly reduced the production of IL-6

induced by 100 ng/ml LPS ($P < 0.05$). For the anthocyanins extracted from cranberry, the lowest effective concentration required was 20 μM .

Lipopolysaccharide has been demonstrated in previous studies by other researchers to have the ability to induce acute inflammation in various cell models and animal studies (Kilroy et al., 2007; Lu et al., 2008; Samokhvalov et al., 2009). Based on the results obtained from this study, it was decided that 20 μM of berry anthocyanins was to be used for the treatment of human preadipocytes in the following experiments described in Chapters 4.7, 4.8 and 4.9.

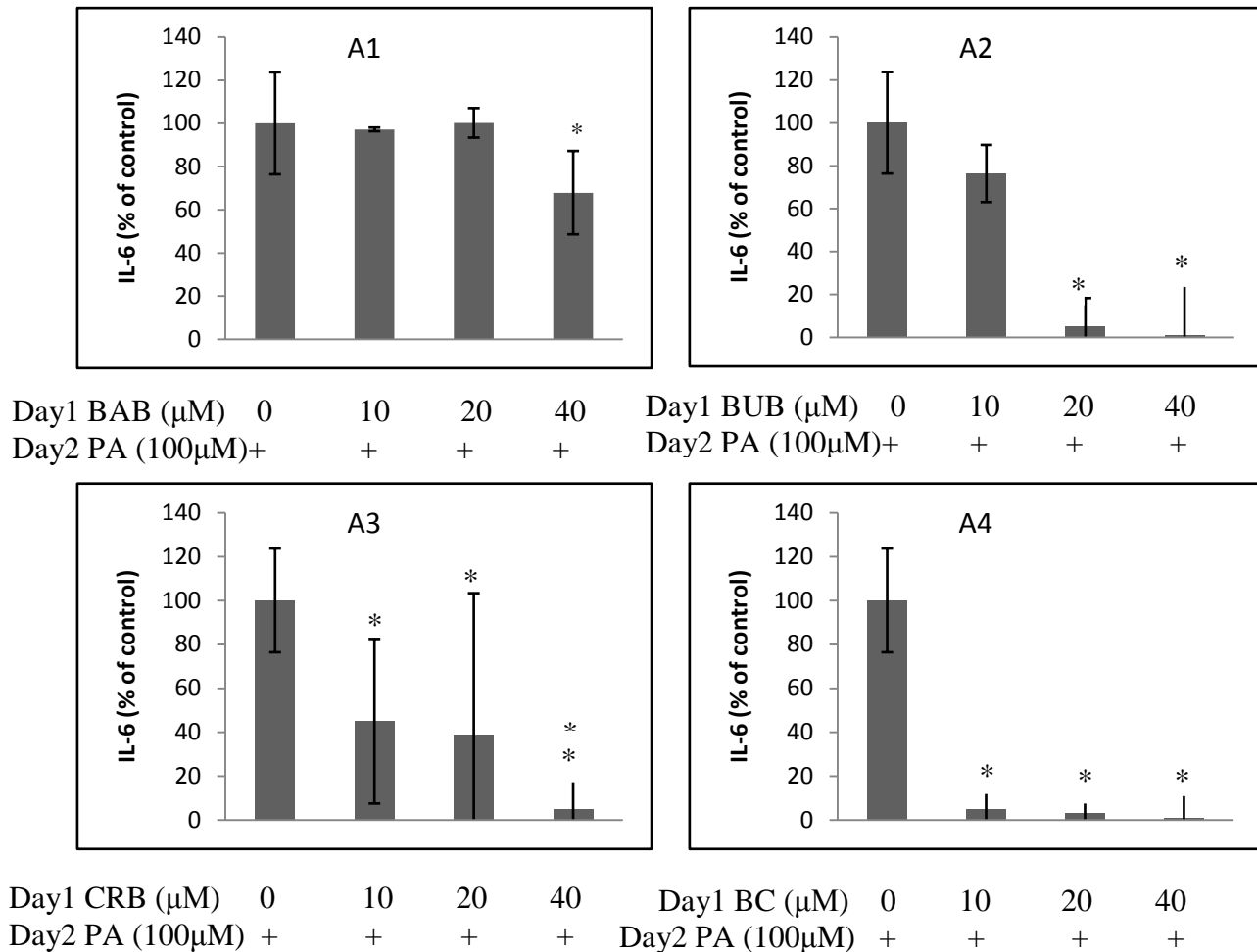
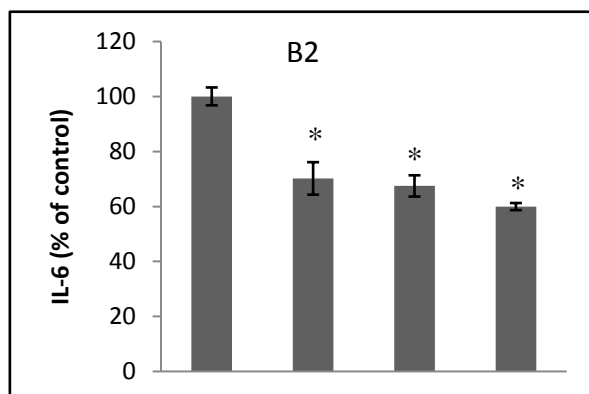
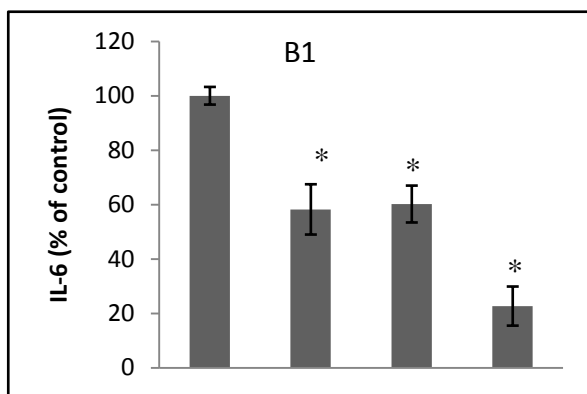
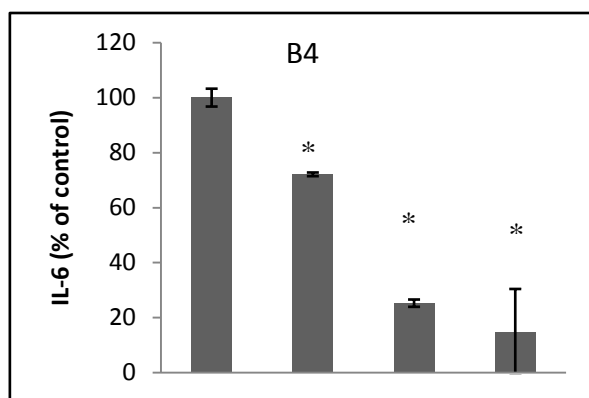
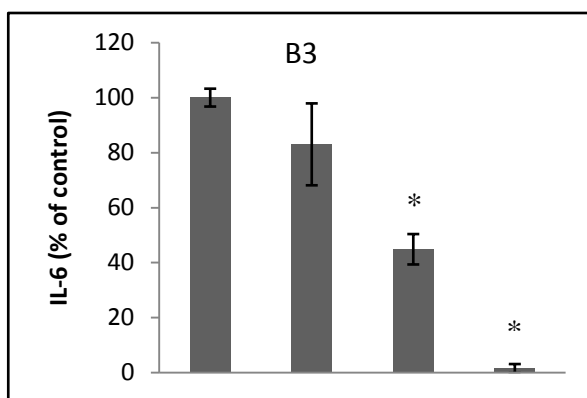


Fig. 4.8 Effects of anthocyanins on IL-6 secretion in human preadipocytes media. Cells were pretreated with BAB (A1), BUB (A2), CRB (A3), and BC (A4) anthocyanins (0, 10, 20, and 40 μM) for 24 h, and followed by incubation with 100 μM PA for 24 h. BAB: blackberry; BUB: blueberry; CRB: cranberry; BC: black currant. The IL-6 levels of the anthocyanin treated samples are expressed as percentage of preadipocytes treated with 100 μM PA alone. Values represent mean \pm S.D of three independent experiments. *: $P < 0.05$.



Day1 BAB (μM) 0 10 20 40
Day2 LPS (100ng/ml)+ + + +

Day1 BUB (μM) 0 10 20 40
Day2 LPS (100ng/ml)+ + + +



Day1 CRB (μM) 0 10 20 40
Day2 LPS(100ng/ml)+ + + +

Day1 BC (μM) 0 10 20 40
Day2 LPS (100ng/ml)+ + + +

Fig. 4.9 Effects of anthocyanins on IL-6 secretion in human preadipocytes media. Cells were pretreated with BAB (B1), BUB (B2), CRB (B3), and BC (B4) anthocyanins (0, 10, 20, and 40 μM) for 24h, and followed by incubation with 100 ng/ml LPS for 8 h. The IL-6 levels of the anthocyanin treated samples are expressed as percentage of preadipocytes treated with 100 ng/ml LPS alone. Values represent mean ± S.D of three independent experiments. *: P < 0.05.

4.7 Effects of Berry Anthocyanins on NF- κ B

To determine the effects of anthocyanins on NF- κ B induced by PA- or LPS- in human preadipocytes, Western blot assay was performed on the cell lysates of the control and treated samples as described in Chapter 3.6. The results presented in Fig.4.10 A and B show the levels of NF- κ B in the cell lysates of the control and treated cells. In the prevention treatment, the levels of NF- κ B in the preadipocytes were pre-incubated with BAB, BUB, CRB, or BC extracts, followed by 100 μ M PA treatment were $66.1 \pm 24.2\%$, $72.1 \pm 19.3\%$, $74.3 \pm 14.5\%$, and $83.1 \pm 3.0\%$ of the control, respectively. The levels of NF- κ B in preadipocytes pretreated with BAB, BUB, CRB or BC extracts, followed by 100ng/ml LPS treatment were $83.4 \pm 7.6\%$, $69.4 \pm 28.3\%$, $69.6 \pm 16.6\%$, and $64.3 \pm 11.2\%$ of the control, respectively. These results show that the NF- κ B levels in all the samples were significantly ($P < 0.05$) decreased when human preadipocytes were pretreated with anthocyanins. There was no significant difference ($P > 0.05$) between the PA- and LPS- induced inflammation.

In the intervention strategy, human preadipocytes were exposed to PA or LPS followed by the addition of anthocyanins. The NF- κ B levels in human preadipocytes induced by PA- and followed by the incubation with BAB, BUB, CRB, or BC extracts were $85.3 \pm 18.4\%$, $104.6 \pm 19.7\%$, $102.1 \pm 20.1\%$, and $76.2 \pm 31.0\%$ of the control, respectively. The levels of NF- κ B in human preadipocytes treated by LPS and followed by BAB, BUB, CRB or BC treatments were $88.9 \pm 13.9\%$, $90.0 \pm 6.3\%$, $82.4 \pm 23.5\%$, and $87.0 \pm 6.1\%$ of the control, respectively. In this strategy, BAB, BUB and CRB did not show the similar inhibitory effect as observed in the prevention treatment. Only the treatment with BC anthocyanins significantly ($P < 0.05$) decreased the NF- κ B level in the human preadipocytes.

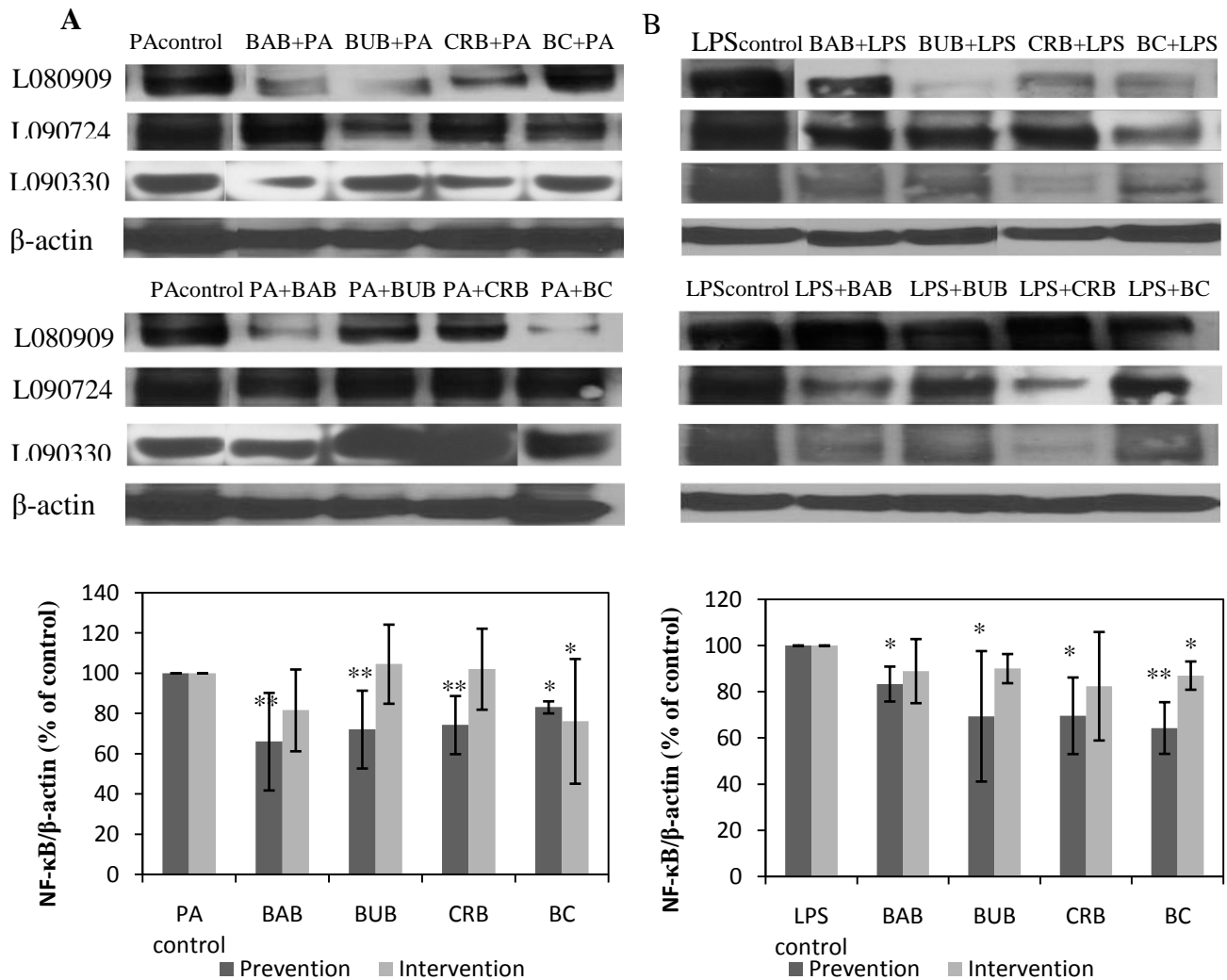


Fig. 4.10 Effects of anthocyanins on the production of NF- κ B induced by PA (A) or LPS (B) in human preadipocytes as preventative and intervention measures. In the prevention treatment, human preadipocytes were pretreated with anthocyanins at 20 μ M for 24h, followed by incubation with 100 μ M PA for 24h or incubation with 100 ng/ml LPS. In the intervention treatment, human preadipocytes were treated with PA 100 μ M or LPS 100 ng/ml, followed by incubation with 20 μ M anthocyanins for 24 h. NF- κ B levels were determined by Western blot and quantified by “Quantity one”. Results are representatives of three donors’ experiments. The values of NF- κ B are expressed as percentage of preadipocytes treated with 100 μ M PA alone or 100 ng/ml LPS alone. The value represents the mean \pm S.D. of triplicate determinations, * P < 0.05, ** P < 0.001.

The transcription nuclear factor- κ B (NF- κ B) plays an important role in controlling the inflammation and metabolic alterations associated with obesity (Sonnenberg et al., 2004; Wellen et al., 2005). It has been reported that anthocyanins or berry extracts attenuated the inflammation by NF- κ B cell signaling in different cell models including murine macrophage RAW 264 cells, macrophages, adipocytes, preadipocytes, endothelioma cell line and also in animal models (Afaq et al., 2005; Atalay et al., 2003; Karlsen et al., 2007; Wang et al., 2008; Zafra-Stone et al., 2007).

It is noteworthy that other phytochemicals such as procyanidins, lycopene, resveratrol and curcumin have also been reported to display similar anti-inflammatory properties in various models including macrophages, monocytes or adipocytes, via a modulation of the NF- κ B pathway (Chacón et al., 2009; Gonzales et al., 2008; Karlsen et al., 2007). In our study, NF- κ B signaling pathway was activated by PA or LPS. The western blot results confirmed that anthocyanins cause down-regulation of NF- κ B when human preadipocytes were pretreated with anthocyanins. Black currant anthocyanins may be speculated to have potent pharmacological ability because of its inhibitory effects on NF- κ B in human preadipocytes.

4.8 Effects of Berry Anthocyanins on COX-2

To determine the effects of anthocyanins on COX-2 induced by PA or LPS in human preadipocytes, Western blot assay was performed on the cell lysates of control and treated samples described in Chapter 3.6. The results are presented in Fig.4.11 A and B. In the prevention treatment, the levels of COX-2 in the preadipocytes pretreated with BAB, BUB, CRB, or BC extracts, followed by PA-stimulation were $79.2 \pm 20.8\%$, $81.2 \pm 9.6\%$, $77.1 \pm 17.6\%$, and $67.9 \pm 13.1\%$ of the control, respectively. The levels of COX-2 in the preadipocytes pretreated with BAB, BUB, CRB, or BC extracts, followed by LPS-incubation were $71.7 \pm 17.1\%$, $80.4 \pm 17.2\%$, $81.1 \pm 24.4\%$, and $86.7 \pm 9.8\%$ of the control, respectively. Statistical

analysis of these results indicates that anthocyanins extracted from all four berry types significantly ($P < 0.05$) suppressed the expression of COX-2. There was no significant ($P > 0.05$) difference in the COX-2 levels in the samples stimulated by PA and LPS.

In the intervention experiment, the COX-2 levels in human preadipocytes treated with PA, followed by BAB, BUB, CRB, or BC extracts were $89.3 \pm 10.9\%$, $110.8 \pm 27.3\%$, $100 \pm 34.4\%$, and $88.1 \pm 10.3\%$ of the control, respectively. The COX-2 levels in human preadipocytes treated with LPS and followed by the incubation with BAB, BUB, CRB, or BC extracts were $91.6 \pm 12.1\%$, $92.6 \pm 4.9\%$, $96.2 \pm 9.0\%$, and $92.2 \pm 5.7\%$ of control, respectively. In this strategy, the levels of COX-2 induced by PA or LPS did not significantly decrease ($P > 0.05$) when the human preadipocytes were treated with BAB, BUB, and CRB anthocyanins. However, BC anthocyanins significantly ($P < 0.05$) decreased COX-2 expression in human preadipocytes. No significant difference ($P > 0.05$) was found between PA- and LPS- treatments. Again, there was no significant difference ($P > 0.05$) between the PA and LPS treatments.

COX-2 is another highly induced inflammatory mediators (Sellers et al., 2010). COX-2 enzyme activation was modulated by NF- κ B cell signaling pathway (Chuang et al., 2011; Gonzales et al., 2008; Hou et al., 2005). Anthocyanins or anthocyanin-rich berry extracts from cranberry, grape, or blueberry were reported to be able to inhibit COX-2 expression in several cell models including human adipocytes, murine macrophages, mouse epidermal cells, RAW 264.7 cell line, THP-1 macrophages, and BV2 cells (Chuang et al., 2011; Hou et al., 2005; Kwon et al., 2007; Wang et al., 2008). Resveratrol and other phytochemicals also cited to have inhibited COX-2 gene expression in adipocytes (Gonzales et al., 2008). In the present study, the Western blot results confirmed the inhibitory effects of berry anthocyanins on COX-2 in human preadipocytes.

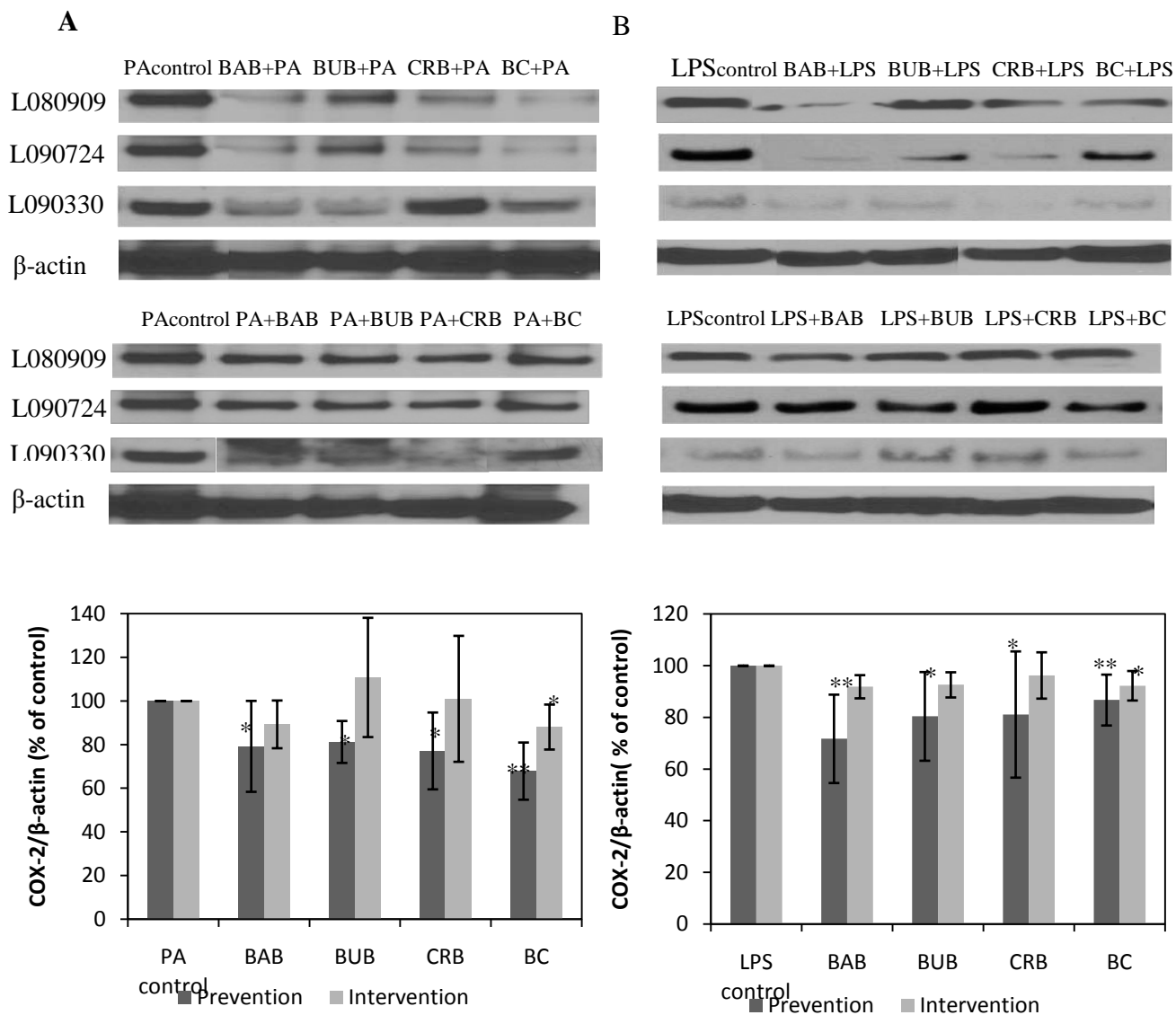


Fig. 4.11 Effects of anthocyanins on the production of COX-2 induced by PA (A) or LPS (B) in human preadipocytes. In the prevention treatment, human preadipocytes were pretreated with anthocyanins at 20 μ M for 24h, followed by incubation with 100 μ M PA for 24h or incubation with 100 ng/ml LPS. In the intervention treatment, human preadipocytes were treated with PA 100 μ M or LPS 100 ng/ml, followed by incubation with 20 μ M anthocyanins for 24 h. Results are representatives of three donors' experiments. The values of COX-2 are expressed as percentage of preadipocytes treated with 100 μ M PA alone or 100ng/ml LPS alone. The value represents the mean \pm S.D. of triplicate determinations, *: P < 0.05, **: P<0.001.

Thereby, the results of this study showed that in the prevention treatment berry anthocyanins from BAB, BUB, CRB and BC at a concentration of 20 μ M significantly ($P < 0.05$) inhibited the expression of NF- κ B and COX-2 either induced by PA or LPS in human preadipocytes. The mechanisms of the inhibitory effects of anthocyanins on NF- κ B activation are not fully understood. One possible mechanism is that anthocyanins or other metabolites serve as redox buffers capable of suppressing oxidative stress and thereby dampening the inflammatory response by direct ROS scavenging (Karlsen et al., 2007).

4.9 Effects of Berry Anthocyanins on PA- or LPS- Activated Inflammatory Cytokines

4.9.1 Effects of Berry Anthocyanins on PA- or LPS- Induced IL-6

To further investigate the effects of anthocyanins on inflammatory cytokines, the IL-6 ELISA assay was performed in the supernatant of human preadipocytes media. The ELISA results of IL-6 are shown in Fig.4.12 A and B. In the prevention treatment, the IL-6 levels in the supernatant of human preadipocytes pretreated with BAB, BUB, CRB, or BC anthocyanins, followed by incubation with PA were $64.4 \pm 24.5\%$, $41.6 \pm 40.0\%$, $62.6 \pm 33.8\%$, and $55.1 \pm 17.7\%$ of the control, respectively. The IL-6 levels in human preadipocytes pretreated by BAB, BUB, CRB, or BC anthocyanins, followed by LPS- treatment were attenuated to $65.2 \pm 31.2\%$, $57.9 \pm 26.7\%$, $81.43 \pm 18.6\%$, and $70.7 \pm 16.1\%$ of the control, respectively. Statistical analysis of the data indicate that BAB, BUB, CRB or BC anthocyanins all significantly ($P < 0.05$) suppressed the IL-6 expression in human preadipocytes media. There was no significant ($P > 0.05$) difference between PA and LPS treatments.

In the intervention treatment, the IL-6 levels for the samples treated with BAB, BUB, CRB, or BC anthocyanins were $116.1 \pm 22.4\%$, $96.8 \pm 23.5\%$, $80.3 \pm 21.2\%$, and $67.8 \pm 22.9\%$ of the control, respectively. In the parallel treatment with LPS, the IL-6 levels for the samples treated

with BAB, BUB, CRB, or BC anthocyanins were $104.3 \pm 7.7\%$, $86.1 \pm 22.7\%$, $87.4 \pm 21.5\%$, and $84.7 \pm 16.2\%$ of the control, respectively. Statistically analysis of the results show that BAB, BUB and CRB anthocyanins did not significantly ($P > 0.05$) inhibit the IL-6 expression in human preadipocytes media and there was no significant ($P > 0.05$) difference between the PA and LPS treatments. However, BC anthocyanins significantly ($P < 0.05$) suppressed IL-6 expression either induced by PA or LPS in this intervention strategy.

IL-6 is one of the pro-inflammatory cytokines and an important regulator of the acute phase response. IL-6 is shown to be positively correlated with the degree of obesity as assessed by body mass index (BMI) and type 2 diabetes patients (Tsuda, 2008; Vgontzas et al., 1997). Anthocyanins or anthocyanins-rich berry extracts including grape, cranberry, or blueberry down-regulate of IL-6 at protein or gene level in various cell models including adipocytes, macrophages, and *in vivo* in mice plasma or adipose tissue (Bodet et al., 2006; Chuang et al., 2011; DeFuria et al., 2009; Tsuda, 2008; Tsuda et al., 2006; Zhang et al., 2010). Other phytochemicals such as lycopene, curcumin, resveratrol, or flavonols have also showed the inhibitory effect on IL-6 in mice adipose tissue, adipocytes, or macrophages (Chacón et al., 2009; Gonzales et al., 2008; Gouranton et al., 2010). In current study, anthocyanins extracted from the four berries can efficiently suppress the IL-6 expression in human preadipocytes when anthocyanins were used in the prevention treatment. The anthocyanins extracted from BC also suppressed the IL-6 expression when used in the intervention treatment.

4.9.2 Effects of Berry Anthocyanins on PA- or LPS- Induced IL-8

The ELISA results of IL-8 are shown in Fig. 4.13 A and B. In the prevention trial with PA, IL-8 levels were significantly ($P < 0.05$) decreased to $43.6 \pm 35.0\%$, $12.5 \pm 12.8\%$, $15.9 \pm 16.7\%$ and $21.9 \pm 18.4\%$ of the control for the treatments with BAB, BUB, CRB, and BC

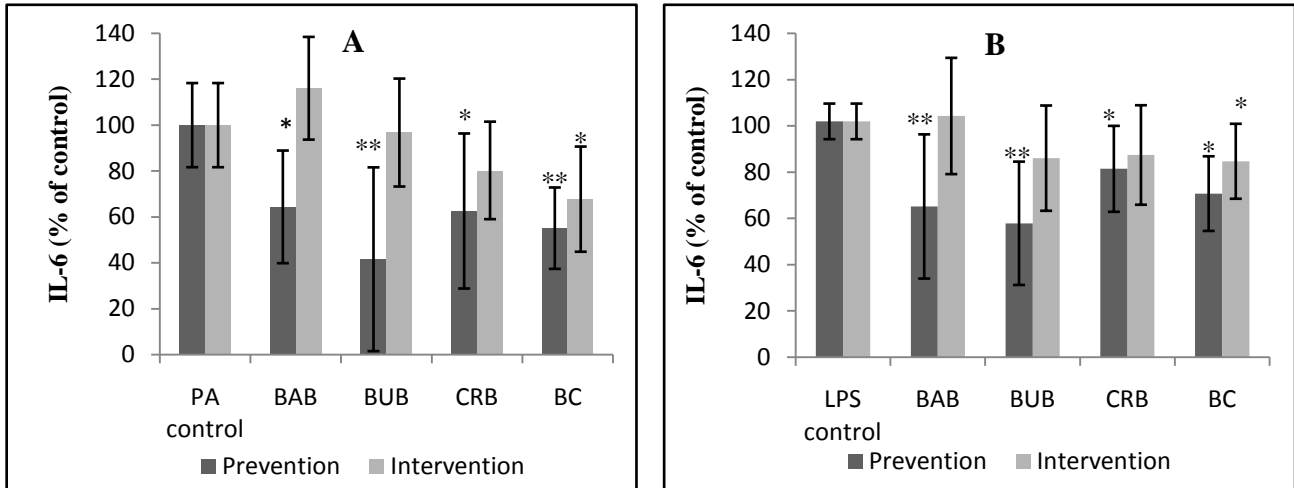


Fig. 4.12 Effects of anthocyanins on IL-6 induced by PA (A) or LPS (B) in human preadipocytes. In the prevention treatment, human preadipocytes were pretreated with anthocyanins at 20 μ M for 24 h, followed by incubation with 100 μ M PA for 24 h or incubation with 100 ng/ml LPS. In the intervention treatment, human preadipocytes were treated with PA 100 μ M for 24 h or LPS 100 ng/ml for 8h, followed by incubation with 20 μ M anthocyanins for 24 h. The levels of IL-6 in the supernatant of human preadipocytes were determined by ELISA. Results are representatives of four donors' experiments. The values of IL-6 are expressed as percentage of preadipocytes treated with 100 μ M PA alone or 100 ng/ml LPS alone. The value represents the mean \pm S.D. of triplicate determinations, *: P < 0.05, **: P < 0.001.

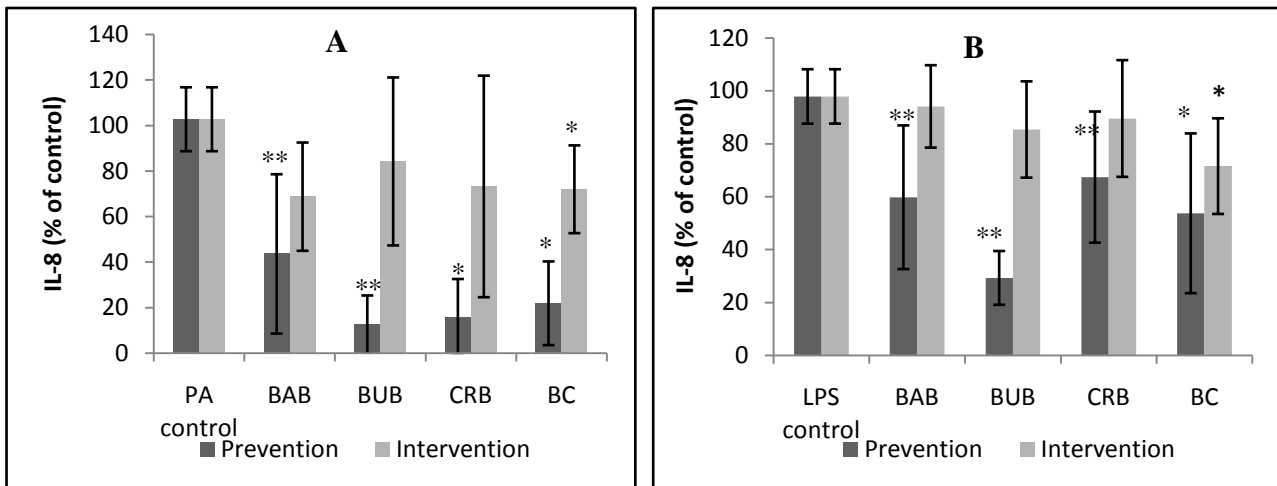


Fig.4.13 Effects of anthocyanins on IL-8 induced by PA (A) or LPS (B) in human preadipocytes. In the prevention treatment, human preadipocytes were pretreated with anthocyanins at 20 μ M for 24 h, followed by incubation with 100 μ M PA for 24 h or incubation with 100 ng/ml LPS. In the intervention treatment, human preadipocytes were treated with PA 100 μ M for 24 h or LPS 100 ng/ml for 8h, followed by incubation with 20 μ M anthocyanins for 24 h. The levels of IL-8 in the supernatant of human preadipocytes were determined by ELISA. Results are representatives of four donors' experiments. The values of IL-8 are expressed as percentage of preadipocytes treated with 100 μ M PA alone or 100 ng/ml LPS alone. The insert represents the mean \pm S.D. of triplicate determinations, *: P < 0.05, **: P < 0.001.

anthocyanins, respectively. IL-8 levels were reduced to $59.8 \pm 27.1\%$, $29.3 \pm 10.2\%$, $67.4 \pm 24.8\%$ and $53.7 \pm 30.2\%$ of the controls with BAB, BUB, CRB, and BC anthocyanins, respectively when LPS- was added to treat human preadipocytes. Statistical analysis of the results demonstrates that BAB, BUB, CRB and BC anthocyanins all significantly ($P < 0.05$) attenuated IL-8 expression in human preadipocytes media when they were used at $20 \mu\text{M}$ pre-treated the human preadipocytes. There was no significant difference ($P > 0.05$) between the PA and LPS treatments.

In the intervention trial with PA, the IL-8 results for the treatments with BAB, BUB, CRB, or BC were $68.8 \pm 23.8\%$, $84.3 \pm 36.9\%$, $73.3 \pm 48.7\%$, and $72.1 \pm 19.3\%$ of the control, respectively. In the parallel intervention trial with LPS, the IL-8 results of BAB, BUB, CRB, or BC was decreased to $94.1 \pm 15.6\%$, $85.4 \pm 18.2\%$, $89.6 \pm 22.1\%$, and $71.6 \pm 18.1\%$ of the control, respectively. BAB, BUB and CRB anthocyanins did not significantly ($P > 0.05$) inhibit the IL-8 expression in human preadipocytes media and this is true for both trials with PA and LPS. However, BC anthocyanins significantly ($P < 0.05$) suppressed IL-8 expression either induced by PA or LPS.

Previous studies have shown that anthocyanins or anthocyanins-rich berry extracts from various berries such as blueberry, cranberry, or grape, attenuated IL-8 expression in adipocytes, macrophages, or endothelial cells. Anthocyanins also showed inhibitory effects on IL-8 levels in human or experimental mice (Beltran-Debon et al., 2010; Bodet et al., 2006; Chuang et al., 2011; Youdim et al., 2002; Zafra-Stone et al., 2007). Fig. 4.13 A and B show that anthocyanins extracted from the four berries significantly ($P < 0.05$) attenuated pro-inflammatory cytokine, IL-8, expression in supernatant of human preadipocytes media in the prevention treatment, but not

in the intervention treatment. The anthocyanins extracted from BC also suppressed the expression in the intervention treatment.

4.9.3 Effects of Berry Anthocyanins on PA- or LPS- Induced MCP-1

The ELISA results of MCP-1 levels are shown in Fig. 4.14 A and B. In the prevention treatments, MCP-1 levels of BAB, BUB, CRB or BC anthocyanins treated samples were $63.2 \pm 20.3\%$, $49.3 \pm 36.6\%$, $43.1 \pm 35.5\%$, and $48.9 \pm 41.7\%$ of the PA- control, respectively. The MCP-1 levels for the BAB, BUB, CRB, and BC anthocyanins treated samples were $90.7 \pm 16.8\%$, $69.2 \pm 35.7\%$, $62.2 \pm 32.9\%$, and $74.5 \pm 23.1\%$ of the LPS control, respectively. Further statistical analysis of the results show that BAB, BUB, CRB and BC anthocyanins significantly ($P < 0.05$) decreased MCP-1 expression in the human preadipocytes treated by PA. For the samples induced with LPS, BUB, CRB, and BC significantly ($P < 0.05$) decreased MCP-1 levels in the samples but BAB did not significantly ($P > 0.05$) suppress the expression of MCP-1 in the cells.

In the intervention treatment, the MCP-1 levels for the samples treated with BAB, BUB, CRB, or BC were $76.6 \pm 24.0\%$, $99.3 \pm 50.9\%$, $59.9 \pm 40.0\%$, and $45.1 \pm 35.2\%$ of the PA control, respectively. The MCP-1 levels in LPS-induced samples were $85.9 \pm 18.9\%$, $79.6 \pm 10.6\%$, $74.3 \pm 14.0\%$, and $72.6 \pm 22.9\%$ of the control, respectively. Statistical analysis of the data show that the MCP-1 expression in BAB, BUB, or CRB anthocyanins treated human preadipocytes conditioned media was not significantly different ($P > 0.05$) from the control in both cases with PA- and LPS- induced samples. However, BC anthocyanins significantly ($P < 0.05$) decreased the MCP-1 expression in both PA- and LPS- induced samples.

Obese individuals have higher circulating concentrations of MCP-1 compared to their lean counterparts (Van Gaal et al., 2006). Pro-inflammatory adipokine, MCP-1, is expressed in

the adipose tissue and induces macrophage infiltration which largely participates in the low-grade inflammation of adipose tissue (Fasshauer et al., 2004; Gerhardt et al., 2001; Malavazos et al., 2005). *In vitro* data showed that anthocyanins or anthocyanins-rich products such as blueberry, cranberry, grape, or red wine inhibited MCP-1 expression in different cell models including endothelium cells, human adipocytes and macrophage (Atalay et al., 2003; Chuang et al., 2011; Garcia-Alonso et al., 2009; Youdim et al., 2002; Zafra-Stone et al., 2007). *In vivo* study also showed that MCP-1 circulating levels in human plasma were decreased by red wine anthocyanins or anthocyanins-rich Hibiscus sabdariffa (Beltran-Debon et al., 2010; Garcia-Alonso et al., 2009). The results from the present study confirmed that anthocyanins down-regulated MCP-1 secretion induced either by PA or LPS in primary culture of human preadipocytes. The attenuation of MCP-1 expression in human preadipocytes may decrease macrophage infiltration.

The antioxidant effects of anthocyanins have been observed *in vivo* experimental models of healthy animals exposed to severe oxidative stress. *In vitro* experiments also illustrated the anti-inflammatory capacity of anthocyanins may be due to their antioxidant property for protecting cells from oxidative damage (Jensen et al., 2008; Tarozzi et al., 2005; Tsuda et al., 1999). Elevated levels of pro-inflammatory cytokines in adipose tissue are causative factors for the cardiovascular disease, diabetes, hypertension, obesity, insulin resistance, and other chronic diseases (Atalay et al., 2003; Cimino et al., 2006; Herath et al., 2003; Hou et al., 2005; Karlsen et al., 2007; Trayhurn, 2005). Dietary strategies for alleviating the metabolic complications of obesity are being pursued as alternatives to pharmaceutical interventions (Cefalu et al., 2008). NF- κ B is a transcription factor that is important for the activation of many inflammatory mediators, cytokines (e.g., IL-6, IL-8, and IL-1 β), and the COX-2 enzyme (Dendorfer et al.,

1994; Galien et al., 1996; Slogoff et al.). Anthocyanins can attenuate NF- κ B actions and limit the inflammatory response (e.g. IL-6, IL-8, MCP-1 and COX-2). Thereby, their application can be a potential strategy to prevent the prevalence of obesity-associated pathologies.

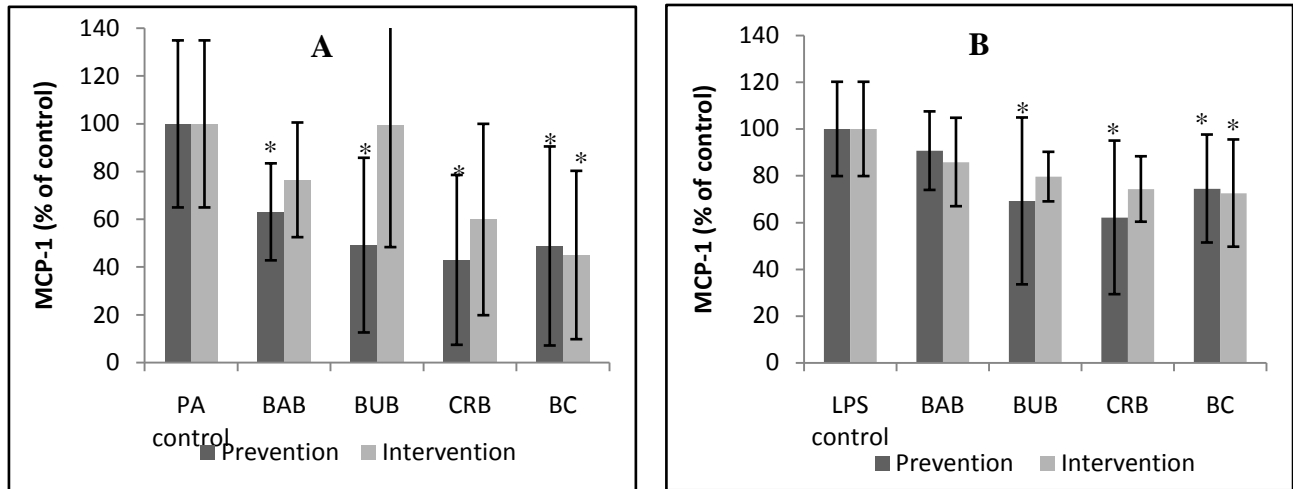


Fig.4.14 Effects of anthocyanins on MCP-1 induced by PA (A) or LPS (B) in human preadipocytes. In the prevention treatment, human preadipocytes were pretreated with anthocyanins at 20 μ M for 24 h, followed by incubation with 100 μ M PA for 24 h or incubation with 100 ng/ml LPS for 8 h. In the intervention treatment, human preadipocytes were treated with PA 100 μ M for 24 h or LPS 100ng/ml for 8h, followed by incubation with 20 μ M anthocyanins for 24 h. The levels of MCP-1 in the supernatant of human preadipocytes were determined by ELISA. Results are representatives of four donors' experiments. The values of MCP-1 are expressed as percentage of preadipocytes treated with 100 μ M PA alone or 100 ng/ml LPS alone. The insert represents the mean \pm S.D. of triplicate determinations, *: $P < 0.05$, **: $P < 0.001$.

The results from the present study indicate the inhibitory effects of anthocyanins on the production of pro-inflammatory cytokines including IL-6, IL-8, MCP-1, NF- κ B and COX-2. In addition, the prevention treatment with berry anthocyanins inhibited PA- or LPS- induced pro-inflammatory cytokines expression in human preadipocytes. The intervention treatment with the berry anthocyanins did not have significant reduction ($P > 0.05$) on the expression of the cytokines.

The berry anthocyanins attenuated the inflammatory effects of saturated fatty acids or LPS stimulation. The results of the present study may suggest that consumption of berries may

prevent obesity-associated inflammation and associated chronic diseases. The data from the prevention treatment indicate better attenuation effects on pro-inflammatory cytokines expression than from the intervention treatment. This suggests that berries may possess disease preventing properties rather than therapeutic and pharmacologic properties. The attenuation of inflammation in human preadipocytes especially MCP-1 may decrease the infiltration of macrophages into adipose tissue. Previous studies by other researchers have illustrated that attenuated inflammation in human preadipocytes may diminish the accumulation of triglyceride in the process of differentiation of human preadipocytes into adipocytes (van Tienen et al., 2011; Yi et al., 2011). Therefore, an increase in consumption of berries may be a good method for weight loss.

Because of the association between inflammatory cytokines and insulin resistance in adipose tissue, reduction of the pro-inflammatory cytokines secretion in human preadipocytes may prevent insulin resistance in adipose tissue and attenuate the insulin resistance related diseases.

4.10 Future Work

The present study tested the expression of some pro-inflammatory cytokine proteins in human preadipocytes treated with berry anthocyanins in absence or presence of PA or LPS. Future work should address mRNA expression. The results demonstrated that berry anthocyanins modulated the pro-inflammatory cytokines expression by NF- κ B signaling pathway; however, the molecular mechanism of this process is not completely understood. In future studies human preadipocytes will be differentiated into adipocytes. The effects of anthocyanins on pro-inflammatory cytokines in human adipocytes will also be studied. In addition, an in vivo test is necessary to understand the mechanism by which anthocyanins modulate inflammation in

preadipocytes. Mice will be fed with berries or berry products and the changes of several pro-inflammatory cytokines will be evaluated in blood and adipose tissue.

CHAPTER 5

SUMMARY AND CONCLUSION

Inflammation plays an important role in the development of obesity. Free fatty acids produced by adipocytes or ingested from diets can induce low-grade, chronic inflammation. Anthocyanins, the natural pigments of berries, are novel, safe, and proven antioxidants and chemopreventive agents. The results from the present study have illustrated the anti-inflammatory ability of anthocyanins-enriched berry extracts including blackberry, blueberry, cranberry, and black currant. In general, the prevention strategy by the application of berry extracts significantly ($P < 0.05$) decreased the PA- or LPS-induced pre-inflammatory cytokines. However, most pre-inflammatory cytokines expressions in the intervention studies were not significantly decreased ($P > 0.05$) by berry extracts. From this study we might conclude that dietary treated with anthocyanins alleviates the inflammation state induced by PA or LPS in human preadipocytes. Thereby, berry rich diets might be a good strategy for preventing obesity and alleviating chronic diseases. The reduction in pre-inflammatory cytokines expression in human preadipocytes pre-treated with BAB, BUB, CRB and BC suggests that berry extracts may inhibit one of the primary steps in this inflammatory pathway.

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