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ROLE OF BLUEBERRY IN PANCREATIC β-CELLS

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ROLE OF BLUEBERRY IN PANCREATIC β-CELLS

By

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List of abbreviations

AGE: Advanced glycation end-product

ANC: Anthocyanin

ATF3: Activating transcription factors

BSA: Bovine serum albumin

BrdU: Bromodeoxyuridine

GMD: Gestation diabetes mellitus

GTT: Glucose tolerance test

mTOR: Mammalian target of rapamycin

HFD: High-fat diets

HFD+B: High-fat diets with the freeze-dried whole blueberry powder

IL-6: Interleuk-6

ITT: Insulin resistance test

PDX-1: Pancreatic and duodenal homeobox 1

ROS: Reactive oxygen species

TNF-α: Tumor necrosis factor-alpha

T1D: Type 1 diabetes

T2D: Type 2 diabetes

Abstract

"We are what we eat." Imbalanced diet is a major reason for obesity and consequently type-2 diabetes. A healthy and attractive diet is key to the control and prevention of obesity and type-2 diabetes. Among many fruit products, blueberry is rich in bioactive substances and possesses powerful antioxidant potential, which can protect against oxidant-induced and inflammatory cell damage and cytotoxicity. Blueberry has been found to improve insulin sensitivity in muscle and adipose, and thus reduce the risk of developing type 2 diabetes. However, whether blueberry affects β-cell function and growth were not fully evaluated.

To study the effect of the whole blueberry on beta cell function, a modified high-fat diet supplemented with 4% (wt:wt) freeze-dried whole blueberry powder (HFD+B) was applied to the C57BL/6 male mice. Compared to the mice fed with high-fat diet (HFD), the addition of blueberry had no significant change in the body weight and glucose level. Interestingly, after 8 weeks feeding, the plasma insulin level was decreased significantly in mice fed with HFD+B compared to mice fed with HFD. In addition, mice fed with HFD+B had significantly increased glucose tolerance and insulin sensitivity. Moreover, the blueberry-supplemented diet prevents HFD-induced beta-cell expansion and preserves the islet structure. Taken together, our results indicated that blueberry-supplemented diet could significantly protect β-cell, restore HFD-induced impaired glucose homeostasis and attenuate the development of obesity, which will provide new insights into the effects of blueberry on beta-cell function and expand our understanding the importance of blueberry in treating and preventing obesity and diabete.

Chapter 1. Introduction

1.1 Glucose homeostasis

Glucose is an important energy source required for the normal functioning of the tissues and organs in the body. Abnormal blood glucose level can result in serious disease or even death. For example, low blood glucose level (hypoglycemia) can cause loss of consciousness and seizures. However, too much sugar in the blood (hyperglycemia) can result in diabetes, vascular disease and so forth. Several hormones are essential to maintain the blood glucose homeostasis at a steady state level. Insulin and glucagon, secreted form the pancreas, tightly regulate glucose homeostasis (Figure 1.1) (Freudenrich, C., 2008).

Insulin is synthesized and secreted from pancreatic beta cells found within the islets of Langerhans of the pancreas. Beta cells secret insulin in response to increasing levels of blood glucose after eating a meal. Secreted insulin enters the blood stream where it binds and activates the insulin receptor within target tissues such as liver, muscle and adipose (Figure 1.1). Insulin stimulates glucose uptake and storage as glycogen or lipids in these tissues, which lead to a reduction of blood glucose levels. When the glucose level reaches a set point, the stimulus of beta cell release diminishes and glucose level return to normal.

Glucagon promotes the opposite metabolic function that insulin stimulates. Glucagon is produced and secreted from pancreatic alpha cells within the islets of Langerhans of the pancreas. When blood glucose levels fall, glucagon is released from alpha cells. Like insulin, glucagon travels through the blood where it binds and activates the glucagon receptor within target tissues such as liver and muscle. In the liver, glucagon stimulates glycogen breakdown and converts stored glycogen into glucose, which is released into the blood. These events

allow blood glucose levels to increase, and thus, again maintain glucose homeostasis (Freudenrich, C., 2008)(Figure 1.1).

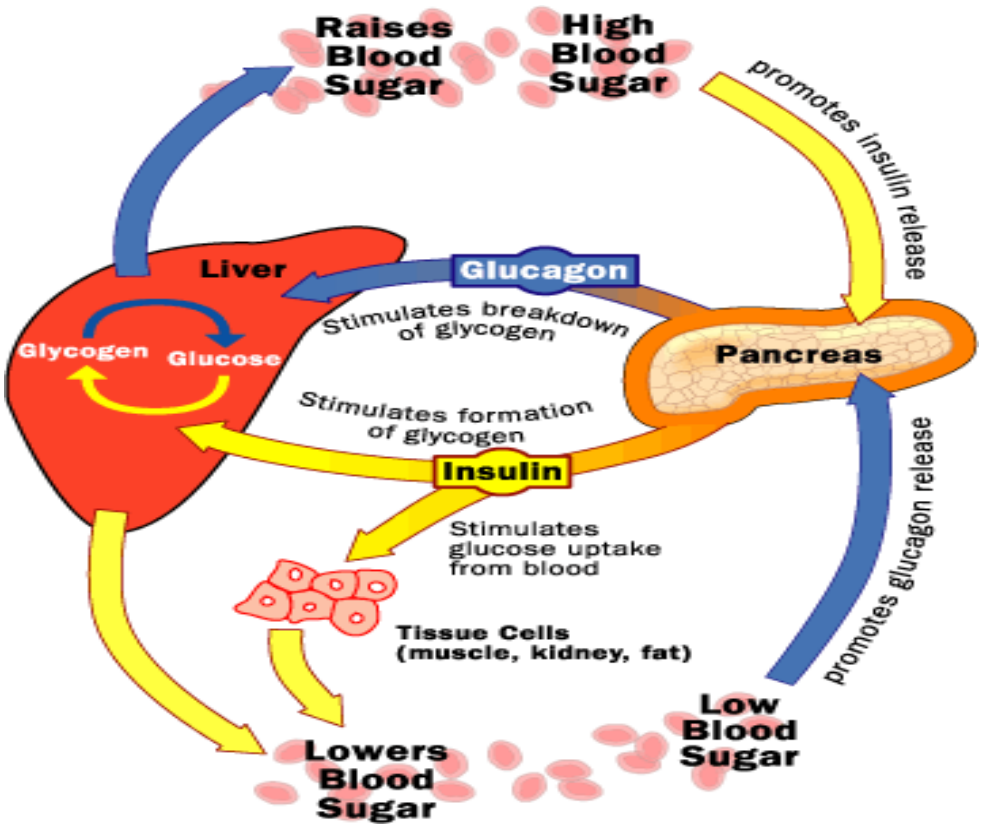


Figure 1.1 Insulin and glucagon have opposite actions in maintaining glucose homeostasis. Figure was adapted from Freudenrich, C., 2008

1.2 Pancreatic islets of Langerhans

The pancreatic islets of Langerhans (commonly referred to as islets) are very small and comprise only 2% of the entire pancreas. However, islets contain several types of cells, including Alpha, beta, delta, epsilon and PP cells that produce glucagon, insulin, somatostatin, ghrelin and islets amyloid polypeptide,

respectively. β-cell is the only source to release insulin and insulin is the only hormone to lower the blood glucose level. Insulin, a small protein composed of two polypeptides referring to A chain and B chain and containing 51 amino acids, is critical for glucose regulation (Mayer J. et al. 2007, White J. 2010).

How elevated glucose stimulates insulin releasing form beta cells? Glucose is uptake by cells through the glucose transporters (GLUT-1 1 or GLUT-2) and is phosphorylated by glucokinase (GCK). The phosphorylated glucose is converted into ATP by the subsequent metabolic reaction. Increased ATP levels lead to triggering the closure of potassium channels, membrane depolarization and the opening of calcium channels. The rising of intracellular calcium level result in the exocytosis of insulin –containing granules and eventually elevated insulin in adjacent blood vessels (Pagliuca, F.W. et al. 2013).

1.3 Diabetes and beta cell dysfunction

1.3.1 Prevalence of diabetes

Diabetes mellitus is a group of metabolic disorder diseases and is characterized by high blood glucose level (hyperglycemia). The situation of diabetes is getting worse all the time, and it is recognized as one of the most prevalent diseases. The number of diabetics has been increased rapidly since 1980. Report from World Health Organization stated the number of people lived with diabetes in 2014 is quadrupled compare to 1980 (422 million v.s 108 million) (WHO 2016).

There is three main type of diabetes mellitus include type 1 diabetes (T1D), type 2 diabetes (T2D) and gestation diabetes mellitus (GDM). The difference and characters are listed below (Table 1). T2D is one of most common chronic disease worldwide, and the T2D patients are increasing rapidly. 382 million people suffered diabetes in 2013 and were raised to 592 million in 2015

(Guariguata et al. 2014).

Table 1. The definition, characters, population and treatment of Type 1 diabetes, Type 2 diabetes and gestational diabetes mellitus.

Type	Definition	Characters	Population	Treatments	References
Type 1 diabetes (T1D)	An autoimmune disease result from dysfunction of β-cells in the pancreatic islets	• No insulin secretion by β-cells.	5%-10%	Insulin injection	• Rewers M. (2004) • American Diabetes Association(2004)
Type 2 diabetes (T2D)	The combination of β-cells defection and insulin resistance.	• Insulin resistance. • Insulin section deficiency.	90-95%	• Lifestyle • Antihyperglycemic treatment.	• DeFronzo RA. (1988) • National Collaborating Centre for Chronic Conditions (UK).(2008) • Klein S. et al (2004)
gestational diabetes	Glucose tolerance occurs during pregnancy.	• Only detected during pregnancy. • Temporary condition	2-9% of all pregnancy woman.	• Regular exercise • Specify diets • Glucose monitor.	• Kjos S. L. et al(1999) • Crowther C.A.(2005) • American Diabetes Association (2015)

1.3.2 Type 2 diabetes and β-cells dysfunction

The defect of insulin biosynthesis and action cause hyperglycemia and finally lead to diabetes. In the fasting state of nondiabetic, low glucose level stimulates glycogenolysis under the direction of glucagon and diminishes the suppression of gluconeogenesis and glycogenolysis by insulin action (Aronoff et. Al., 2004). Under the fed state of nondiabetic individuals, insulin decreases blood glucose level through Suppressing gluconeogenesis and glycogenolysis in the liver and inhibiting glucagon secretion and promoting properly. In the fed state of diabetes, insulin is ineffective in inhibiting glucagon secretion, which causes the

elevation of hepatic glucose production. The imbalance of the appearance of glucose and the disappearance of glucose in the circulation lead to hyperglycemia. Adapted from (Aronoff et. Al.2004).

Type 2 diabetes is associated with pancreatic β-cell dysfunction and insulin resistance. Insulin resistance is a complex pathological disease, and it is described as the resistance of insulin to uptake the glucose in insulin target tissues such as live muscle and fat. If insulin resistance exists, normal β-cells release a greater amount of insulin and/or increase β-cells mass to compensate for insulin resistance. An inadequate insulin compensation might lead to hyperglycemia that results in the gradually deteriorates of β-cells function and the aggravation of insulin resistance (Poitout & Robertson 2002;.Weir et al. 2001). The processes of deleterious effect induced by high-level glucose and free fatty acid call glucotoxicity and lipotoxicity respectively. Excess of reactive oxygen species (ROS) included superoxide, hydrogen peroxide, hydroxyl radical, nitric oxide, hypochlorite and peroxynitrite (Vincent et al. 2006) is contributed to the deleterious effect of hyperglycemia. The excess of ROS is harmful to the body that leads to DNA damage, protein, cell function (Yu 1994). The imbalance of ROS and antioxidants induces oxidative stress (Figure 1.6) (Kaneto et al. 2006). Oxidative stress has been reported the association with progression of T2D. It agrees with the existence of increased oxidative stress in patients with diabetes and its complications (Baynes et al. 1999, Baynes 1991). In addition of T2D, oxidative stress is also involved in carcinogenesis, inflammation, atherosclerosis.

In diabetic conditions, oxidative stress provoked in β-cells (Gorogawa et al. 2002). β-cells is valuable to oxidative stress because of the extremely low expression levels of antioxidant enzymes such as catalase, and glutathione (Tiedge et al. 1997). ROS accumulate by four major metabolic pathways during

the period of hyperglycemia: elevated polyol pathway flux, elevated advanced glycation end-product (AGE) pathway, elevated hexosamine pathway flux and activation of the isoform of protein kinase C (PKC) (Brownlee M. 2001).

The reduction of transcription factor activation in β-cells has been observed under the oxidative stress. PDX-1, involving in the homeodomain-containing transcription factor family, plays a critical role in pancreas development and differentiation (Cao et al. 2004; Dutta et al. 1998; Ferber et al. 2000; Hollan, et al. 2002; Horb et al., 2003;Jonsson, et al., 1994; Kaneto et al. 2005; Miyatsuka et al. 2003; Stoffers et al. 1997; Taniguchi et al. 2003). An in vitro study showed the oxidative stress inhibited insulin gene expression, and this suppression may cause by the PDX-1 activate reduction (Kajimoto Y. et al. 2004). Taken together, oxidative stress suppresses insulin biosynthesis through the reduction of PDX-1 activity (Kaneto et al. 2006).

c-Jun N-terminal Kinase (JNK), known as stress-activated protein kinase (SAPK), involve in several signal transduction pathways. Oxidative stress activates JNK pathway in β-cells, and the activation of JNK pathway reduces the insulin gene expression (Kaneto et al. 2002). The inhibition of JNK pathway protects oxidative stress induced β-cells dysfunction (Kaneto et al. 2002). The isolated rat islets were exposed to oxidative stress, the overexpression of wild-type JNK1 inhibited the insulin gene expression and secretion. Conversely, the Adenovirus-media overexpression of dominant-negative type JNK1 protects the oxidative induced decreased insulin gene expression and secretion (Kaneto et al., 2002). The resultant reduced in insulin gene expression induced by the activation of JNK pathway is associated with the decreased activation of PDX-1 DNA-binding (Kaneto et al. 2002). A potential mechanism for JNK mediated PDX-1 inactivation described that oxidative stress trigger the translocation of PDX-1 from the nuclei

to cytoplasm (Kaneto et al. 2006). Cyclin-dependent kinase inhibitor p21, involved in cell cycle regulation, can be induced by oxidative stress and associated to diabetes development by increased the expression level in islets (Kaneto H. et al. 1999). During the period of oxidative stress, activating transcription factors (ATF3), known as an oxidative stress-inducible gene, is induced in β-cell associated with β-cell apoptosis (Hartman M. et al. 2004).

1.4 Obesity and insulin resistance

Obesity, a high-risk factor for diabetes, is a prevalent health problem, resulting from the increased ratio of energy intake and energy output. Obesity is classified a BMI (body mass index) of 30 or greater (WHO 2011). The contribution of besity is related to gender, age, ethnicity, diet and physical inactivity (WHO 2011; Wang et al. 2007). Most T2D patients are obese, and some studies showed obesity itself could cause some degree of insulin resistance. Insulin resistance is referred to the resistance of target tissues to responses to insulin action. Adipose tissue is one of these insulin target tissue, and adipose tissue has been proposed to be a side of insulin resistance (Kahn and Flier 2000). Insulin promoted storage of triglycerides in adipose tissue through multiple pathways, involving in stimulating the glucose transportation, increase the uptake of fatty acid derived from circulating lipoproteins and lipogenesis in mature adipocytes, promotes the differentiation of preadipocytes to adipocytes, and inhibiting lipolysis (Kahn & Flier 2000). Insulin binding to the insulin receptor on the membrane of adipose cells which lead to the activation of insulin receptor substrate (IRS). The activation of insulin receptor substrate protein is involved in the activation of the Ras-mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K)-AKT/protein kinase B (PKB) pathway (Jung

& Choi 2014).The PI3K-AKT/PKB pathway is associated with most insulin metabolic action. IRS1 is the phosphorylated insulin receptor substrate and activate PI3K by binding to its SH2 domain (Jung, 2014). The activated PI3K generates a lipid second messenger, phosphatidylinositol-(3,4,5)-triphosphate, leading to the activation of several phosphatidylinositol-(3,4,5)-triphosphate-dependent serine/threonine kinases, including AKT/PKB (Jung 2014). These signaling events trigger the translation of glucose transporter 4 (Glut 4) to plasma membrane and ultimately increase the glucose uptake by adipocytes resistance. In contrast to HFD fed wild-type mice, JNK-KO mice (JNK1 gene knockout) attenuated HFD-induced obesity and decreased the glucose levels. Moreover, the intraperitoneal insulin tolerance test and intraperitoneal glucose tolerance test indicated the improvement of insulin sensitive and glucose tolerance in JNK-KO mice.

Studies showed that some factors involve tumor necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6) play an importance role in obesity-induced IR. TNF-α, a potent pro-inflammatory cytokine, is primarily produced by monocytes and macrophage. In obese state, the adipocyte involves the development of obesity-induced inflammation by releasing the pro-inflammatory cytokine and chemokines include TNF-α and IL-6 (Hotamisligil et al.1993; Rotter et al. 2003). The production of TNF-α through the activation of MAPK and NF-κB signaling pathways stimulate the release of other inflammation such as IL-1β and IL-6 (Chen et al. 2002; De Luca. 2006). TNF-α has reported as the first obesity-induced IR related inflammatory mediator (Hotamisligil et al. 1993). TNF-α has been reported the inhibition of activating insulin receptor and IRS1. Knockout TNF-α in diet-induced obese mice significantly improves the insulin sensitivity

(Uysal K. 1997). Moreover, IL-6, known as one of the important inflammatory mediators, is released by white adipose tissue (WAT), skeletal muscle, and liver and activated by the JAK/STAT and MAPK pathways (Heinrich et al., 2003). IL-6 have been found the expression level of IL-6 is related to obesity-induced insulin resistance (Kern P. 2001). IL-6 also have been reported the association of suppressor of cytokine signalling-3 (SOCS-3) through the inhibition of insulin signal transduction in hepatocytes (Senn. J.J. et al. 2002).

An elevated level of FFA is common in the obese and T2D individual (Gordon G. et al. 1960, Reaven G 1988). Plasma FFA, the major circulating lipid fuel, usually originates from adipose tissue lipolysis (Jensen 2002).The increased plasma FFA is likely associated with an expansion in adipose tissue mass (Bjorntorp et al. 1969). Studies showed the increased FFA contributed to the development of insulin resistance in skeletal muscle and liver (Boden 1997; Shulman 2000).

In skeletal muscle, insulin stimulates increased glucose transportation and glycogen. Elevation of glucose intake, insulin stimulate the increased glucose uptake by the translocating of GLUT-4 from intracellular vesicle to the plasma membrane and increased glycogen synthesis by activating glycogen synthesis in the healthy insulin-sensitive skeletal muscle (Saltiel & Kahn 2001). One possible explanation of FFA-induced IR in human is that the increased plasma FFA-induced IR by inhibiting the glucose transporter activity (Kovacs & Stumvoll 2005). As we mention above, PI3K is required for the insulin signaling and the decreased glucose transporter activity lead to the decreased IRS-1 related PI3K (Kovacs & Stumvoll 2005).

A study found that increased oxidative stress caused by the elevated ROS production from fat accumulation in obesity (Shigetada 2004). This study also

found the mRNA expression level of NADPH oxidase increased while the mRNA expression level and activity of the antioxidant enzyme is declined (Shigetada F. 2004). Ros accumulation in obesity is achieved by polyol pathway, increased advanced glycation end-products and glucose autooxidation (Figure 1.2) and the increased ROS result in oxidative stress (Vincent et al. 2006). An in vitro study illustrated that Oxidative stress caused by ROS also play a major role in insulin resistance (Houstis 2006).

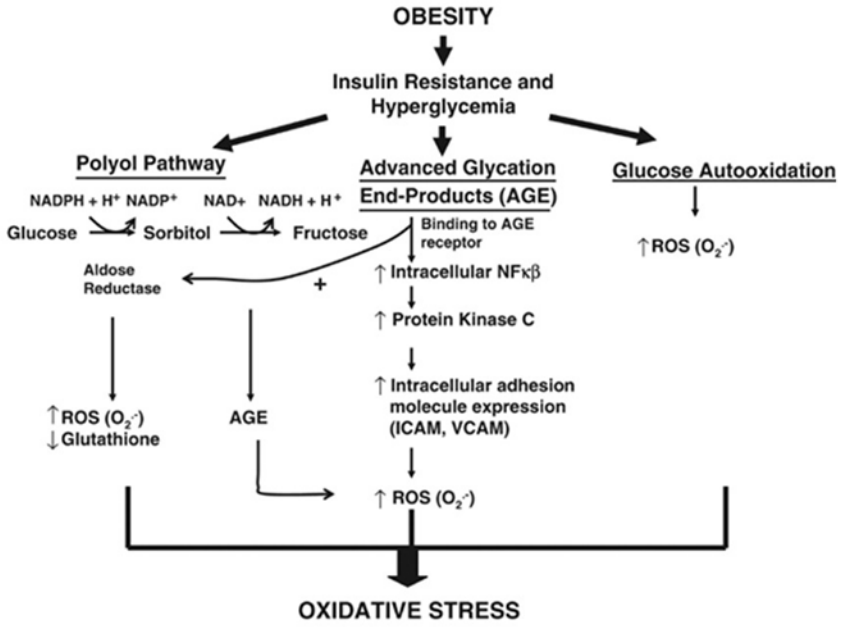


Figure 1.2 Reactive oxygen species (ROS) accumulation pathways in Obesity-induced hyperglycemia and IR. Accumulation of ROS by the polyol pathway, the advanced glycation end-products pathway and glucose autooxidation pathway lead to oxidative stress in obese people. Figure was adpated by Vincent et al. 2006.

1.5 Prevention and Treatment of Type 2 diabetes and obesity

Excessive caloric intake is an important reason to develop obesity and consequently T2D. An adequate and balanced diet could prevent obesity and T2D. Obesity associated with inflammation and oxidative stress, the dietary restriction, and weight loss might lead to the decreased of inflammation and oxidative stress. In both Esposito K, et al. (2002) and Mohanty, P. et al. (2001) studies showed 3 hours of excess glucose intake cause acute oxidative stress and inflammation at cellular and molecular level. Contrarily, four weeks of dietary restriction lead to a significant reduction of reactive oxygen species in obesity (Dandona et al. 2001).

The consumption of cereal fiber and polyunsaturated fat is highly recommended because of the beneficial for decrease the risk of T2D, whereas the higher dietary trans fat and glycemic load is associated with the increased risk of T2D (Salmetron et al. 2006, Salmetron et al. 1997, Salmetron et al. 2001; Meyer et al. 2000,). The higher of sugar-sweetened beverages is also a risk factor for obesity and T2D. Evidence indicated the individuals with highest sugar-sweetened beverages intake had 26% greater risk to develop T2D compared to those with lower sugar-sweetened beverages intake (Malik et al. 2010). In addition to weight gain, sugar-sweetened beverages contain larger quantities of rapidly absorbable carbohydrates could cause the increased risk of obesity due to the quickly elevated blood glucose(Malik et al. 2010). Reduced absorption of nutrient is one of obesity and T2D strategies.

Health diets can prevent obesity and type 2 diabetes, popular dietary approaches have generated to treat obesity, such as low-fat, high protein diets, or antioxidant supplements. A study with HFD induced-obese rat showed that the administration of a dietary with oral gingerol once a day for 30 days significantly

reduced the glucose level, body weight, and insulin compared to rat only fed with HFD(Saravanan et al. 2014). Either obesity or obesity-induced type 2 diabetes has been described as a state of oxidative stress. Dietary antioxidants supplementation including vitamin C, vitamin E, and carotenoids is widely believed as a potential therapeutic strategy. Studies have been indicated diets with antioxidants supplementation have a positive effect on anti-obesity and anti-diabetes include reducing the oxidative stress, improving beta-cell function(Jayaprakasam 2006), increasing insulin secretion and decreasing insulin resistance(Guo et al. 2007, Lai 2008, Prior et al. 2010)

1.6 Health Benefits of Blueberries

Berries are excellent sources of antioxidants. Blueberries are receiving growing interest due to the beneficial effect on health with delicious taste and flavor. Blueberries are as species from the family "Ericaceae" (Gough 1994). The remarkably high antioxidant properties of blueberries are related to the richness bioactive substance especially natural phenolic compounds (You Q., et al. 2011). There are two major groups of phenolic compound in blueberry: non-flavonoids and flavonoid (Nicoletti et al. 2015). Anthocyanins is a world research fruit because of the high antioxidant capacity.

Anthocyanin

Anthocyanin, belong to flavored, is a world-researched bioactive compound, due to its powerful antioxidant action. It exists in blueberry as glycosidic and aminoglycosidic forms of anthocyanidins (Routray & Orsat 2011). The basic structure of anthocyanins is showed in Figure 1.3 left and the 6 most common anthocyanins are showed on right of figure 1.3. The most function of

anthocyanins is to give color to fruits, vegetables, and plants. Anthocyanins also can act as antioxidant because of its excellent antioxidant capacity. (Kong et al. 2003).

Bioavailability refers to "the proportion of the nutrient that is digested, absorbed and metabolized" (McGhie and Walton 2007). Some studies have demonstrated the low bioavailability of anthocyanins (Bitsch et al. 2004; Felgines 2003, Felgines et al. 2005). This low bioavailability is associated with the instability chemical structure of anthocyanins (Giovanelli et al. 2009). The oral anthocyanins can be quickly absorbed as glycosides by the stomach and small intestine. The systemic bioavailability of oral anthocyanins administration is around 0.26-1.8% in animal studies (Felgines et al. 2002; Felgines et al. 2003; Ichiyanagi T et al. 2006; Marcyzlo T. et al. 2009; Matsumoto et al. 2006). Surprisedly, a study found the high bioavailability of long-term anthocyanin extraction or anthocyanins supplementation feeding (Kalt et al. 2008). In (Kalt et al. 2008), Kalt et al. fed the pig with four weeks' diets supplemented with blueberry and found anthocyanins accumulation in tissues but none in plasma and urine (Speakman et al. 2007).

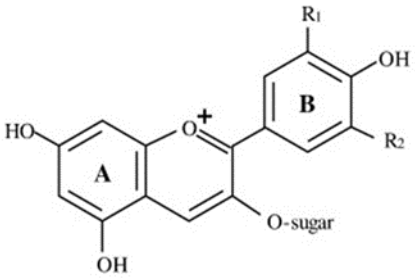
As we are mentation before, Obesity can induce oxidative stress, and β-cell is very sensitive to oxidative stress because of the low expression of antioxidant enzymes. Bioactive substances such as anthocyanins in Blueberry may act as an exogenous antioxidant to prevent or slow obesity-induced oxidative stress through eliminating free radical (Veberic et al. 2015). Studies showed both anthocyanins purification and blueberry supplements reduce oxidative stress and increased antioxidant capacity (Guo et al. 2007; Kay et al. 2002; Nizamutdinova et al. 2009,).

In addition to the reduction of oxidative stress, Low dose of Anthocyanin

extraction prevented obesity. HFD supplemented with purified blueberry anthocyanins in the drinking water inhibited diet-induced obesity. Moreover, purified anthocyanins restored β-cell function(Prior 2010).

The whole blueberry also has reported the protection of β-cell function. An oral administration of freeze-dried wild blueberry power protected β-cell function by reducing the oxidative stress (Kay et al. 2002). The C57BL/6 mice fed an HFD diet supplemented whole Freeze-dried blueberry power reduced insulin resistance (Defuria et al. 2009).

The beneficial effect of anthocyanins may associate with the anthocyanins metabolites. For example, protocatechuic acid is the main metabolite of anthocyanins in humans, and it serves as an antioxidation and anti-inflammatory. In addition to anthocyanin, other bioactive substance in blueberry such as flavonoids, vitamin C also has antioxidative properties and are contribute to the antioxidant action of blueberry (Giovanelli et al. 2009).



Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvinidin	OCH_3	OCH_3

Figure 1.3 Basic structure and common types of anthocyanins.The differentiation of anthocyanin depend on the number of hydroxyl

groups, the number and position of sugar attach to molecular and the present aliphatic and aromatic acid link to the sugar molecular .

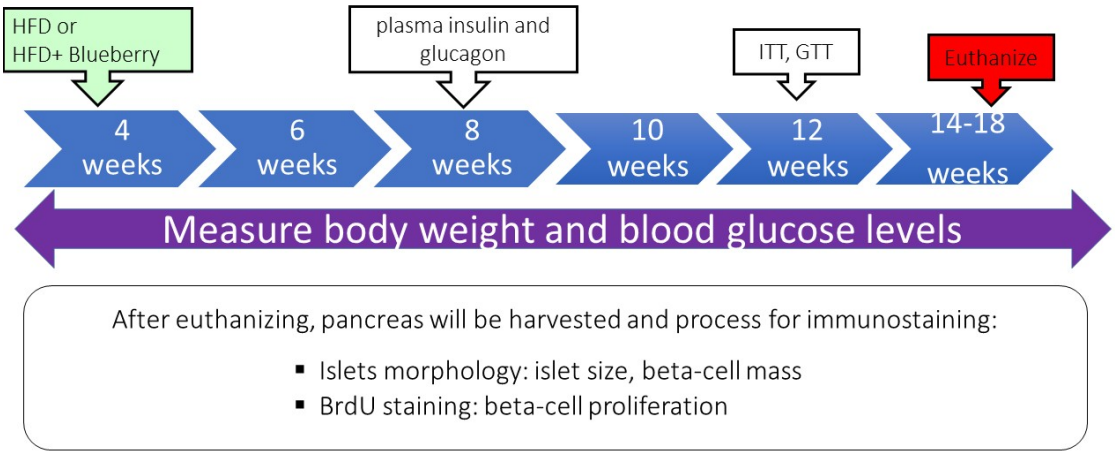
Delphinidin, The table on the right showed the six most common anthocyanin. Cyanidin, malvidin, and petunidin are most common anthocyanin found in blueberries (Sancho & Pastore 2012; Wang et al. 2012). Figure was Adapted from Sancho & Pastore 2012.

Chapter 2. Research Objective

Recent findings indicated blueberrie supplementation reduces the risk of obesity and obesity-induced disease. However, the effect of the whole blueberries on β-cell function needs to be determined. The objective of this study focuses on the effects of whole blueberry on pancreatic β-cell function in mice fed with high-fat diet.

Chapter 3: Materials and Methods

3.1 Experimental design



3.2 Animals and diet

All animal protocols were approved by the Animal Care Committee at the Michigan Technological University. The C57BL/6 mice housed in an animal facility of Michigan Technological University with 12-h light / dark cycle and unlimited ad libitum water. The male wild-type mice, which are more susceptible to obesity and diabetes than female mice, at age of 4 weeks will be assigned to cohorts (n = 5 mice/cohort) that received two different kinds of diets including: 1) a high-fat diet (HFD) containing 60% of energy from fat (Research Diets, no. D12492); 2) a modified HFD supplemented with 4% (wt:wt) freeze-dried whole blueberry powder (HFD+B). The blueberry powder will be purchased from the U.S. Highbush Blueberry Council.

The following metabolic parameters were analyzed biweekly in two groups of mice: Body weight, blood glucose, plasma insulin and glucagon.

3.3 Methods

Plasma insulin and glucagon

Fasting blood samples were collected from orbital venous sinus while mice were anesthetized with isoflurane. 3 ul 2% Ethylenediaminetetraacetic acid (EDTA) were applied to blood samples to avoid clot. Samples were centrifuged at 4000rmp for 4 min twice at 4C. After centrifugation, plasma were transferred to a new microcentrifuge tube and stored in -80C refrigerator. Plasma insulin and glucagon levels were measured by Ultrasensitive plasma insulin ELISA kit and glucagon ELISA kit (Mercodia).

Glucose Tolerance Test (GTT)

The GTT were performed following 8 weeks of diet feeding and mice were anesthetized with isoflurane after 16 hours starving. Glucose solution (1.5 g/ kg of body weight) was injected into the intraperitoneal cavity. Blood glucose level was measured by the tail tip and recorded at the following time point：0,15,30, 45, 60, 90 min and blood samples were collected from the orbital vein at 0,15,30 min. Blood samples were centrifuged at 4000rmp for 4 min twice at 4°C. Plasma (supernatant) was transferred to a new microcentrifuge tube and stored in -80°C until for analyzing insulin level. The area under the curve of GTT was calculated to analyze the glucose tolerance. Plasma insulin and glucagon levels during GTT period were also measured as described.

Insulin Tolerance Test (ITT)

Insulin tolerance test (ITT) was performed on mice that had been fasted for 6 hours. Mice were injected with insulin (0.5 units/ kg body weight) by

intraperitoneal (I.P) injection. Blood glucose was measured before (time=0) and 15, 30, 45, 90 and 120 mins after injection.

Evaluation of β-cell mass and islet size

Mice at 18-week-old were sacrificed through CO2 asphyxiation and performed whole body perfusion with PBS and 4% paraformaldehyde respectively. The isolated pancreas was fixed in 4% Paraformaldehyde for overnight. Entire paraffin blocks containing pancreas were processed and serially sectioned at 5 μm.

Insulin and glucagon staining: Section slides were dewaxed and rehydrated in Ethanol series (xylene, 100% Ethanol, 95% Ethanol, 90% Ethanol 79% Ethanol 50% Ethanol and ddH2O). Treated the sections with 4% citrate buffer with Tween 20 (antigen retrieval) for 14 min at a high level of microwaves. After rinsing the section by dH2O and washing with PBS, incubated sections with 5% Bovine serum albumin (BSA) in PBS for 1 hours in a humid chamber at room temperature and treated with primary antibody (anti-insulin and anti-glucagon) overnight at 4°C in a humid chamber. Treated the sections with fluorescence secondary antibody (Alexa 488 and 594) after washing with PBS for 2 hours in a cover humid chamber at room temperature on the following day. Staining DAPI for 2 min after Washing slides by PBS and rinsing in ddH2O. Cover the section with a coverslip after rinsing in ddH2O. Let the slices dry overnight in dark.

The entire pancreatic sections were scanned and the fraction of the insulin- or glucagon positive areas were determined using Image-Pro Premier software. The mass was calculated by multiplying this fraction by the initial pancreatic wet weight.

β-cell proliferation

Bromodeoxyuridine staining: 18-week old mice were sacrificed after intraperitoneal injection of Bromodeoxyuridine (BrdU, 100mg/kg) for seven consecutive days. Pancreas was dissected and fixed. The section slide was immunostained with BrdU antibody as described. Blocks needed to treat with hydrochloric acid for 20 min before antigen retrieval. Immunofluorescent microscopy was used to determine fluorescence signal. All images were taken under the 20X magnification. BrdU-positive cells were counted to determine cell proliferation.

3.4 Statistical analysis

Two-tailed Student t-test was used to analyze statistical significance, and a p-value of lower than 0.05 was considered as statistically significant.

Chapter 4: Results

4.1 Effect of blueberry diet on body weight

To study the effect of the blueberry diet on mouse body weight and fed glucose levels, we measured the body weight and glucose level biweekly. There were no significant differences in body weight between these two groups (Figure 4.1 A). We analyzed the accumulative body weight gains between these two groups. We measured the body weight of 4 weeks old mice prior to diets changed. The mice fed with HFD+B gain slightly less weight (>3.5 g) than mice fed with HFD at 12 weeks old (Figure 4.1 B). Results indicated that the addition of blueberry power to HFD did not increase body weight and may protect against HFD induced weight gain because of the less cumulative body weight gain. We also examined the effect of blueberry on fed glucose level. The addition of blueberry did not attenuate the HFD-induced high fed glucose level (Figure 4.1C).

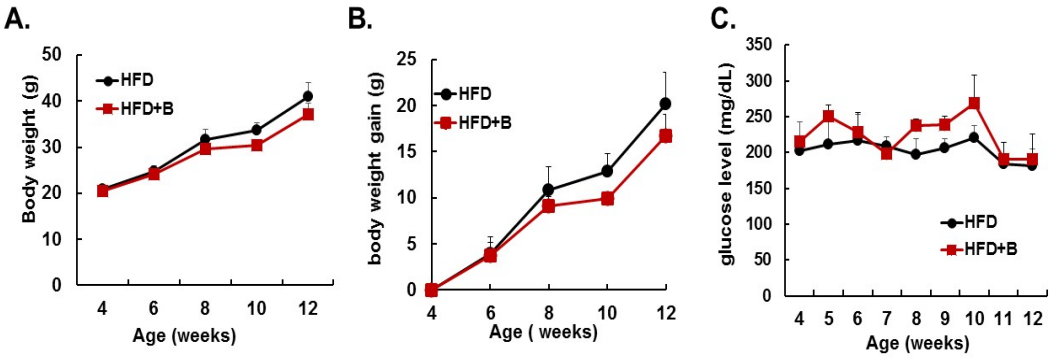


Figure 4.1 Effect of blueberry diet on body weight. Biweekly and accumulative Body weight (g) of mice fed with HFD or HFDB for 8 wk. The Body

weight gains were normalized by the body weight of 4-week old mice. Values are presented by mean + STDEV. A: Biweekly body weight. B: Cumulative body weight. C. Weekly glucose level (mg/dL)

4.2 Effect of blueberry diet on fasted blood glucose level and plasma insulin level.

To understand the effect of blueberry diet on fasted blood glucose level and fasting plasma insulin level. We examined the body weight, glucose level, and plasma insulin before and after 16 hours fasting. Either fed or 16 hours fasted, there were no significant differences in body weight and glucose level between these two groups at 8 wk old and 12 wk old (Figure 4.2 A-D). However, the fasted glucose levels of mice fed with HFD with freeze-dried blueberry powers were slightly lower than mice fed HFD diet (141.75 mg/dL vs. 174.75 mg/dL). Fasted plasma insulin level was similar between these two groups at 8 wk old. Compare to the mice fed with HFD, mice fed with HFDB had significant lower fasted plasma insulin level at 12 wk old (Figure 4.2 F). Results demonstrated the improvement of whole Blueberry supplement on insulin efficiency.

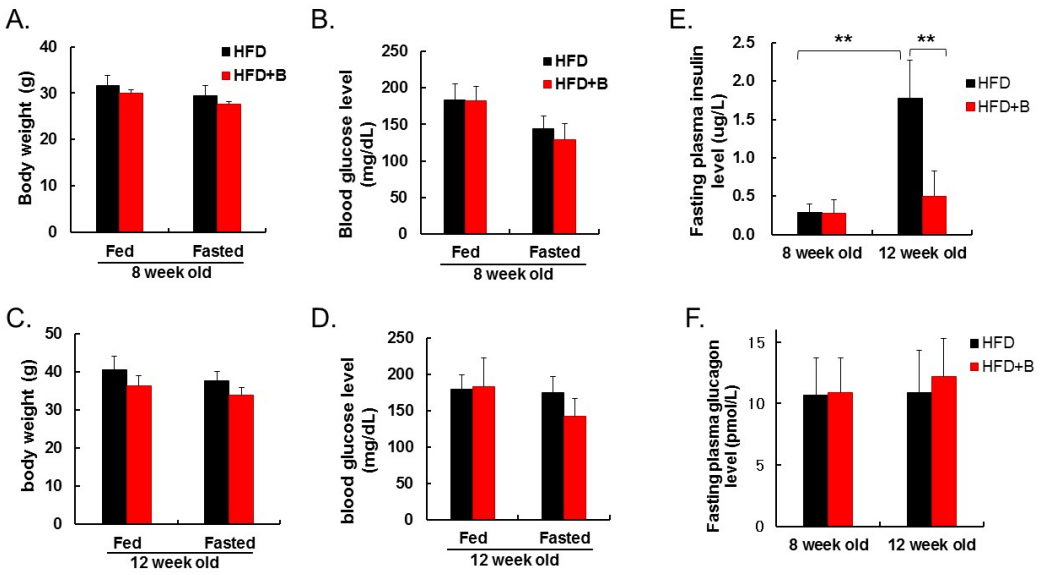


Figure. 4.2 Effect of blueberry diet on blood glucose level and plasma insulin level. Body weight and blood glucose level were measured before and fasted at 8 wk old and 12 wk old. Plasma samples were collected from orbital vein blood at 8 wk old and 12 wk old after 16 hours fasted. Value are mean + STDEV. ** $p<0.01$. A, B: body weight and glucose level before and after fasted at 8 wk old. C, D: body weight and glucose level before and after fasted at 12 wk old. E. Fasted plasma insulin level at 8 wk old and 12 wk old.

4.3 Blueberry improves insulin sensitivity and glucose tolerance in high-fat diet treat mice.

HFD+B fed male had a significant low plasma insulin at 12-week old compared to HFD fed mice. We suggested the addition of blubbery in HFD might increase insulin efficacy. To further study the effect of the whole blueberry on insulin efficacy, we performed ITT after 8 wk of HFD or HFDB treatment. As expected, mice fed with HFD+B had less insulin resistance (IR) compared with those fed the HFD (Figure 4.3 C). HFD+B fed mice had less IR than HFD fed mice at 45, 60

and 90 min. The area under the curve (AUC) during ITT demonstrated the HFD+B fed mice had significant less IR than HFD fed mice (figure 4.3 E). The addition of blueberry in HFD could decrease the HFD induce IR. Compare to mice fed with HFD, mice fed with HFD+B had a high glucose tolerance (Figure 4.3 A) and lower insulin level (Figure 4.3 C). At 30 min and 45 min after glucose injection, blood glucose levels in HFD+B fed mice were significantly lower than HFD fed mice. In contrast, no significant changes of plasma insulin level in HFD fed mice, and HFD+B fed mice at 30 min after glucose chandelle as well as 15 min. The plasma insulin level of HFD+B fed mice were significantly higher than HFD fed mice at 16 hours fasting. We calculated the AUC under the GTT, the AUC of HFD+B fed mice were significantly lower than HFD fed mice (Figure B). Taken together, these results suggested Blueberry might decrease HFD-induced IR and increase glucose tolerance.

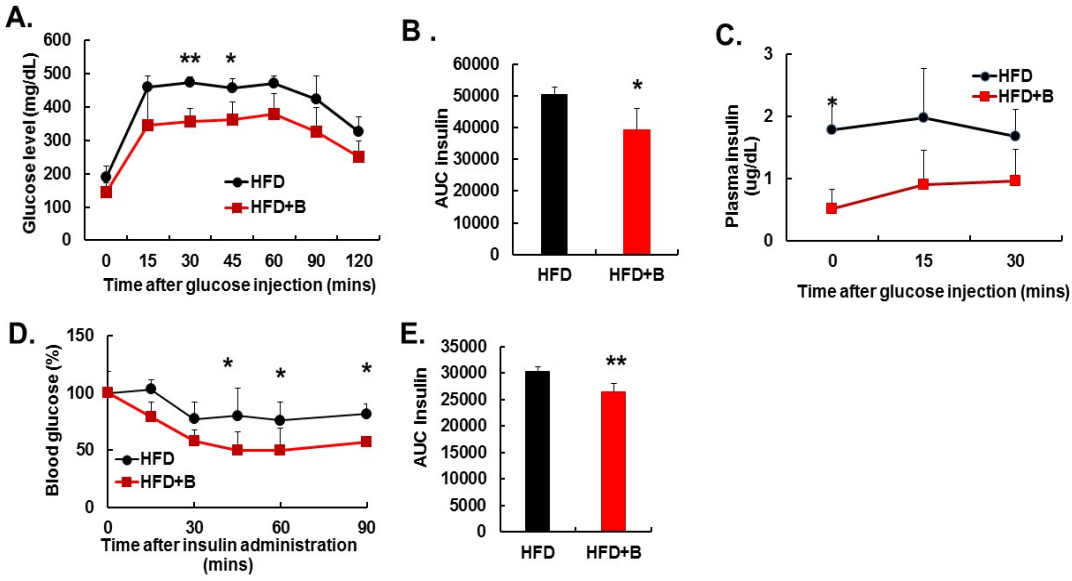


Figure 4.3 Diet supplemented blueberry reduced insulin resistance and glucose tolerance. GTT an ITT were performed on mice with 8 wk HFD or HFD+B treatment through I.P injection (n=5). HFD and HFD+B fed mice fasted

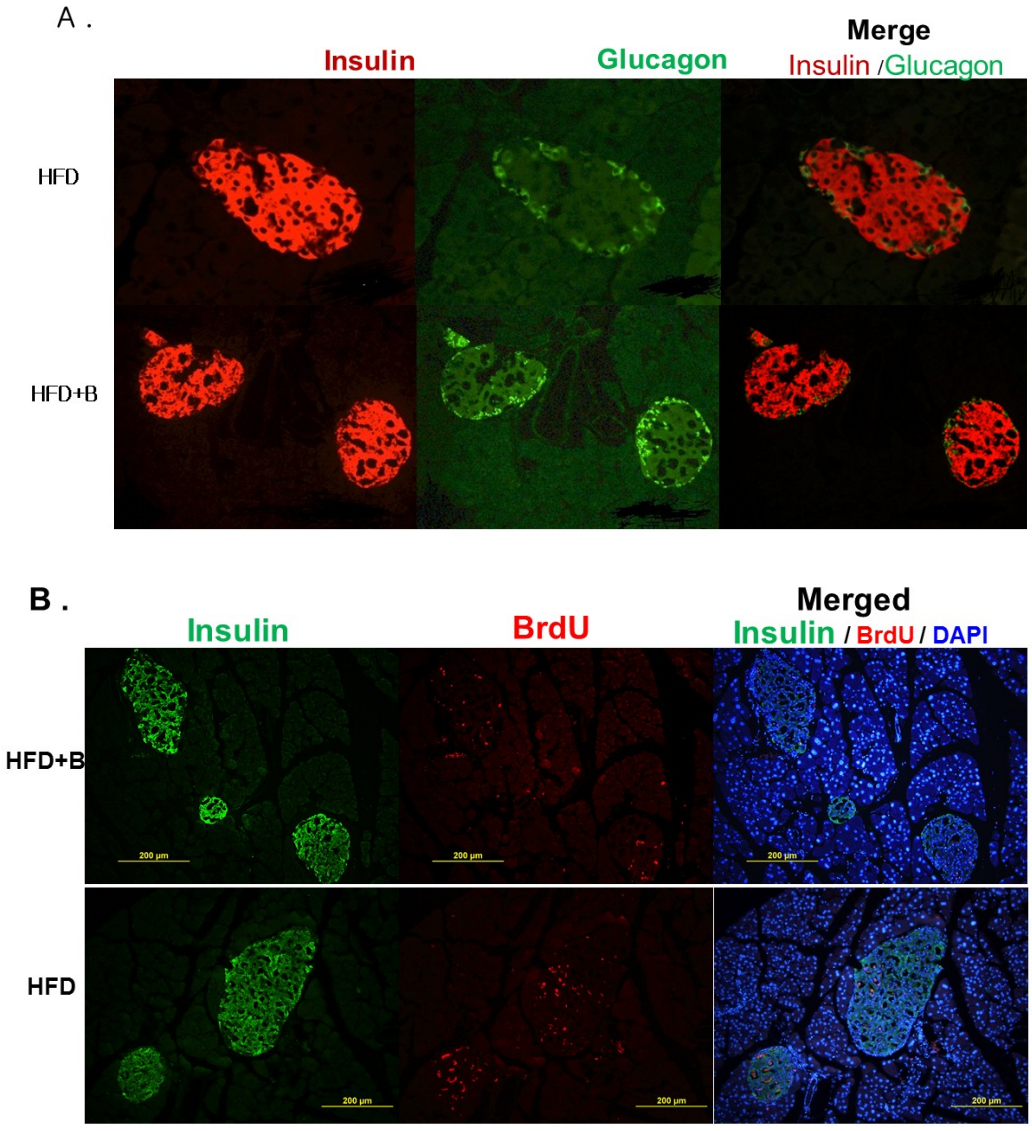
16 hours for GTT and 6 hours for ITT. The injection amount of glucose was 1.5g/kg body weight in GTT, and the injection amount of insulin was 0.5IU in ITT. The Values are mean +- STDEV. * p<0, 05 and **p<0.01. A: GTT. B: The insulin area under the curve (AUC) during GTT, C: ITT. D: The glucose area under the curve (AUC) during ITT. E: 16 hours fasted plasma insulin (ug/L) during GTT at 0, 15, 30 min.

4.4 Blueberry protects pancreatic β-cell function

Long-term HFD feeding might lead to β-cell expansion, in contrast, anthocyanins extracts have been reported for associated with preserved islet architecture (45). To determine whether whole blueberries protect islet architecture, we examined the islets morphology by immunolabeling and quantify the islet sizes, beta-cell mass. Islets in mice fed HFD+B showed preserved the islet morphology (Figure 4.4 A). Islet size and beta cell mass were no significant different between HFD-fed or HFD+B fed mice (Figure C). However, the average islets size in HFD+B fed mice was slightly smaller than HFD-fed mice (18874.10 um2 vs. 21815.87 um2). We analyze the range of islet size distribution. The number of Islets belong to 5000-10000um2 had a significant increased and the number of islets size 40000-5000um2 had a significant decrease in HFD+B (Figure 4.4 D). The blueberry supplement significant increased the smaller islets and decreased the number of larger islet size (figure 4.4 D).

We suggested the blueberry supplement might increase the β-cell proliferation based on The increasing number of smaller islets in the blueberry supplement group. We did 7 consecutive days BrdU (100mg/kg) I.P injection to determine the cell proliferative in the pancreas. The β-cell proliferation rate was slightly higher in mice fed HFD with freeze-dried blueberries but no significant changes

between these two group (figure B).



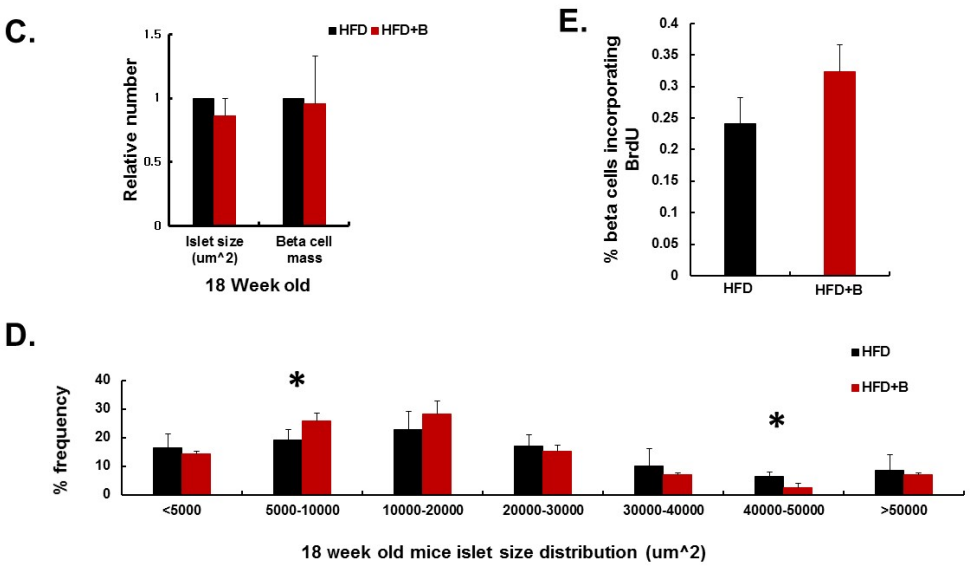


Figure 4.4 The addition of blueberry preserved islet architecture.

Elevation of β-cell proliferation, islet size, and β-cell mass by immunohistochemistry. HFD or HFD+B fed mice were killed by CO2 asphyxiation at 18-week old (n=3-5). A: anti-Insulin (red) and anti-glucagon (green) immunostaining on HFD or HFD+B treated mouse pancreas at 18wk old. B. anti-insulin(green) and anti-BrdU (red) and anti-glucagon (green) immunostaining on HFD or HFD+B treated mouse pancreas at 18wk old (images).. C. beta cell mass and islet size of 18-week old HFD or HFD+B fed mice. Beta cell mass was normalized by HFD treated beta cell mass. D. Islet size distribution. Islets graded by size, The Value is mean +- STDEV. E. Positive β- cell incorporation BrdU (left)

Chapter 5: Discussion

Our findings suggested that blueberry supplementations reduce insulin resistance and glucose tolerance through increased insulin sensitivity. Moreover, the blueberry supplements attenuate the HFD-induced obesity and protect beta cell function by preserving islet morphology and stimulating β-cell proliferation. Taken together, the blueberry supplement may have a therapeutic potential in preventing and treating obesity and T2D.

In this study, we chose to study blueberries because of it is most available and more attractive. Blueberries contain 5 of 6 most common anthocyanins and other bioactive compounds. Bioactive substances such as flavonoids, vitamin C contributing to antioxidant action as well as anthocyanins.

Our finding that blueberry supplement decreased insulin resistance is in agreement with the previous study suggesting the effect of the whole blueberry on decreasing insulin resistance (DeFuria et al., 2009). Mice was subjected to insulin tolerance test, mice treated with HFD+B show a significant low insulin resistance, especially at 45, 60 90 mins after insulin injection. In DeFuria et al. study, the decreased insulin resistance also have been observed in mice treated with blueberry freeze powder supplement. In addition, other anthocyanins rich fruit or anthocyanins extract also have been found the effect of decreased HFD-induced insulin resistance (Guo et al. 2007; Nizamutdinoza et al., 2009; Seymour et al. 2009). Taken together, the anthocyanins supplement improve the HFD-induced IR. However, what is not know is whether a particular single anthocyanin or several anthocyanins is a response to this effect. Whether other bioactive substances in anthocyanin-rich fruit have the similar effect on the IR is required to determined.

β-cell expansion can result from long-term HFD feeding. In the previous study, C57BL/6 mice were subjected to HFD with anthocyanins supplement, islets morphology were preserved as well as glucose tolerance improvement (Jayaprakasam et al. 2009). Our findings related to glucose tolerance and islets morphology is consistency with this. The purified anthocyanins used in this study (Jayaprakasam et al. 2009) is the mixture of Cyanidin 3-O-galactoside, pelargonidin 3-O-galactoside, and delphinidin 3-O-galactoside. Blueberry contain two of them: Cyanidin 3-O-galactoside and delphinidin 3-O-galactoside. Either Cyanidin 3-O-galactoside or delphinidin 3-O-galactoside or both of them contribute to the ability of islet structure preservation. Furthermore, the whole blueberry also increases glucose tolerance. We subjected mice to GTT on 12-week old and measured plasma insulin during GTT. The fasted plasma insulin levels were significantly lower in HFD+B group. The increased glucose tolerance with low insulin level indicated that the blueberry supplements increase glucose tolerance through the elevation of insulin sensitivity.

Diet-induced obesity in an animal model has been used to mimic obesity like condition in humans. Long-term HFD feeding contributes to obesity present increased body weight and fat accumulation. C57BL/6J mouse is impressionable to HFD-induced obesity (Peakman J. et al. 2007). In our study, the results showed the cumulative weight gain of mice fed HFD supplemented freeze-dried blueberry powder was slightly less than mice fed HFD. Base on these results, blueberry supplementation may attenuate the HFD-induced obesity. In contrast to our results, a study showed C57BL/6J fed HFD with freeze-dried blueberry powder supplementation increased body weight (Prior R. L. 2008). However, another study claimed 12-week blueberry juice intake inhibited obesity (Wu T., et al. 2013). Moreover, purified anthocyanins from Cornus mas also demonstrated

amelioration of obesity (Jayaprakasam et al. 2006). This conflict results may cause by the differential purified preparation methods and content of anthocyanins or the differential of total consumed anthocyanins in different individuals.

Compared to HFD feeding, feeding with HFD+B for 12 weeks dramatically decreased the fasted plasma insulin level (Figure 4.2 E). A previous study showed blueberry juice did not alter the plasma insulin level (Wu et al. 2013). The differences may result from the difference of blueberry supplements. We purchased the HFD and HFD+B from the research lab. The fructose levels were normalized in HFD and HFD+B. Compare to HFD+B we used in our experiment, blueberry juice may have a higher amount of fructose. The higher blueberry juice may have an effect on insulin secretion.

β -cell expansion and proliferation might result from long-term HFD feeding. To examined the role of blueberry in islet structure and β-cell proliferation. We observed the islet morphology by immunostaining technique. Diet supplement blueberry slightly increase β-cell proliferation (Figure 4.4 E). An in vitro study showed the fruit extract of different parts of blueberry plants increased β-cell proliferation and replication in β TC-test-cells (Martineau et al. 2006). The bioactive substances have not been defined, but the anthocyanins present in this fruit was supposed involved in this process (Prior et al. 1998, Martineau et al. 2006). In the previous study, anthocyanin extracts prevent β-cell apoptosis through down-regulation of pro-apoptotic Proteins (Caspase 3 and Bax) (Nizanutdinova et al. 2009). If the blueberry supplement has a similar effect as anthocyanins, blueberry may indicate a potential protection of β-cell through an increase in β-cells proliferation and a decrease in β-cell apoptosis.

Chapter 6: Future work

The beneficial effects of anthocyanins on obesity, diabetes, vascular disease and cancer have been observed. However, the effect of the whole blueberry has not been completely evaluated on these diseases. Since blueberry is most available and consumption compares to the anthocyanin extracts, it is necessary to determine whether consumption of the whole blueberry would have the similar effect to those observed for anthocyanins extract.

The previous study indicated anthocyanin extracts inhibited β-cell apoptosis. Whether the whole blueberry has the similar effect on β-cell apoptosis needs to be determined. If the whole blueberry has a similar effect on decreasing β-cell apoptosis, The whole blueberry supplement can be considered as the potential treatment of diabetes.

Studies showed either anthocyanin extraction or anthocyanins rich food have anti-inflammatory property and reduce the expression level of TNF-a. MCP-1, IL-6 in adipose tissue (Defuria et al . 2009; Wu et al. 2013). The effects of whole freeze-dried blueberry supplements on anti-inflammatory factors in pancreas islet have not been determined.

ROS induces β-cell apoptosis because of the low antioxidant enzymes. Anthocyanin extraction reduces oxidative stress maker and increases antioxidant enzymes activities in the pancreas (Nizamutdinova et al. 2009). It is possible that blueberry supplements may have the same effect as anthocyanin extraction on antioxidant defense. It is important to observe the effect of blueberry on the expression of anthocyanin enzymes. Increased antioxidant defense can protect β-cell function and reduces apoptosis.

Chapter 7: Conclusion

Dietary supplementation with whole blueberries can not only improves insulin sensitivity in muscle and adipose tissues, but also protects β-cell function and β-cell proliferation. Long time of HFD consumption leads to impaired glucose homeostasis. Blueberry supplements can attenuate the HFD-induced impaired glucose homeostasis. Our findings strongly suggest that the whole blueberry has a therapeutic potential for improving diet-induced β-cell dysfunction.

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