생활과학박사학위논문

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유도한 비만 마우스와 당뇨병 모델 마우스의
염증반응과 지질대사에 미치는 영향

2016년 8월

서울대학교 대학원
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# Abstract

## Effect of Black Raspberry Seed Oil on Inflammation and Lipid Metabolism in High-Fat Diet-Induced Obese Mice and *db*/*db* Mice

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Increased interest in beneficial health effects of raspberries has raised the production of raspberries in Korea in recent years. The processing of black raspberry fruits for juice, wine, and puree typically produces the seeds as a byproduct. Black raspberry seed (BRS) oil, which can be obtained from black raspberry product pomace, contains about 30% α-linolenic acid (ALA), an n-3 fatty acid, and other beneficial phytochemicals such as tocopherols.

The ultimate objective of the study was to evaluate whether BRS oil has beneficial effects on inflammatory status and lipid metabolism in high-fat diet-induced obese mice and *db*/*db* (type 2 diabetes) mice.

The objective of the first part of the study was to determine the effect of BRS oil on inflammation and lipid metabolism in high-fat diet-induced obese mice. Five-

week old C57BL/6 mice were divided into two groups: 1) mice fed high-fat diet consisting of 50% calories from lard, 5% from soybean oil, and 5% from corn oil (control group), and 2) mice fed high-fat diet consisting of 50% calories from lard and 10% from BRS oil (BRS oil group). The BRS oil used in the study comprised 57.0% linoleic, 29.4% α-linolenic, and 9.80% oleic acids. Mice were fed the experimental diets for 12 weeks *ad libitum*. The content of ALA was significantly ($P<0.001$) higher in the BRS oil diet than the control diet. There were no significant ($P>0.05$) differences in initial body weight, final body weight, weight gain, food intake, and food efficiency between the two groups. The weights of liver, spleen, kidney, and epididymal adipose tissue of the BRS oil group were higher than those of the control group with no significant ($P>0.05$) differences. After 12 weeks, ALA in the liver of the BRS oil group accounted for 1.84% of the total fatty acids, which was significantly ($p<0.01$) higher than that of the control group (0.45%). Inflammation-involved proteins such as TLR4, NF-κB, phospho-NF-κB, COX2, I-κBα, and phospho-I-κBα were lower in the liver of the mice fed the BRS oil than those in the control. mRNA levels of pro-inflammatory markers including NF-κB, TNFα, IL-1β, IL-6, iNOS, COX2, and MCP1 in the liver and epipidymal adipose tissue of the BRS oil group were lower than those of the control. On the other hand, mRNA levels of anti-inflammatory markers including IL-10, arginase1, Chi3l3, and Mgl1 were higher in the liver and epipidymal adipose tissue of the BRS oil group than those of the control. Leptin level in serum was lower in the mice fed BRS oil diet than in the

control diet without significant (P>0.05) difference. mRNA level of leptin was lower in the epididymal adipose tissue of the mice fed BRS oil than in the control without significant (P>0.05) difference. mRNA level of adiponectin was significantly (P<0.01) higher in the epididymal adipose tissue of the BRS oil group than in the control. Whereas, adiponectin level in serum was higher in the mice fed BRS oil diet than in the control diet with no significant (P>0.05) difference.

Levels of TG in serum and liver of the mice fed BRS oil were 14.2% and 12.1%, respectively, lower than those of the control group. Serum non-esterified fatty acids (NEFA) and total cholesterol levels were 42.1% and 13.0%, respectively, lower (P<0.05) in the mice fed BRS oil diet than in the control. Serum HDL-C level was 4.10% higher in the BRS oil group than in the control group without significant (P>0.05) difference. Total lipid content in the liver of the BRS oil group was 13% lower than that of the control group without significant (P>0.05) difference. NEFA and total cholesterol levels in the liver of the BRS oil group were 25.7% (P<0.05) and 53.2% (P<0.001), respectively, lower than those of the control group. The mRNA levels of lipogenic markers such as CD36, FABP1, SREBP-1c, FAS, and SLC25A1 were lower in the liver of the BRS oil group than those of the control group. Whereas, mRNA levels of fatty acid oxidation markers including CPT1A, ACADL, HADHα, and ACOX were higher in the liver of the mice fed BRS oil than in the control mice. PPARα significantly increased both in mRNA (P<0.001) and protein (P<0.01) levels in the liver of the mice fed BRS oil diet compared with the control group. However,

PPARγ mRNA and protein levels showed no significant (P>0.05) differences between the two groups. PPARα mRNA level was significantly (P<0.05) higher in the epididymal adipose tissue of the mice fed BRS oil than in the control. However, there were no significant (P>0.05) differences in PPARα protein and PPARγ mRNA and protein expression levels between the two groups.

The objective of the second part of the study was to evaluate the status of the markers related to inflammation and lipid metabolism in *db*/*db* mice fed diets containing different concentrations of BRS oil. Mice were divided into four groups: 1) C57BL/6 mice fed 16% calories from soybean oil (normal CON); 2) C57BL/KsJ-*db*/*db* mice fed 16% calories from soybean oil (CON); 3) C57BL/KsJ-*db*/*db* mice fed 8% calories from soybean and 8% calories from BRS oil (BRS 50%); and 4) C57BL/KsJ-*db*/*db* mice fed 16% calories from BRS oil (BRS 100%). Mice were fed the experimental diets for 10 weeks *ad libitum*. There were no significant (P>0.05) differences in the food intake and initial body weight among the three *db*/*db* groups. The food intake and body weight of the normal CON were significantly (P<0.05) lower than those of the *db*/*db* groups. The final body weight of the BRS 50% was significantly (P<0.05) lower than that of the CON. Final body weight of the BRS 100% was lower than that of the CON without significant (P>0.05) difference. The weights of the liver, epididymal adipose tissue, spleen, and kidney were not significantly (P>0.05) different among the *db*/*db* mice. There was no significant (P>0.05) difference in blood glucose level among the *db*/*db* mice. Insulin level was

significantly ($P<0.05$) lower in the serum of the BRS 50% and BRS 100% than in the CON. After 10 weeks, n-6 to n-3 fatty acid ratios were significantly ($P<0.05$) lower in the livers and epididymal adipose tissues of the BRS 50% and BRS 100% mice than in the CON. Whereas, ALA and total n-3 fatty acids contents were significantly ($P<0.05$) higher in the livers and epididymal adipose tissues of the BRS oil-treated mice than in the normal CON and CON. Serum TNFα and IL-6 were significantly ($P<0.05$) lower in the BRS 50% and BRS 100% than in the CON. Serum IL-10 was significantly ($P < 0.05$) higher in the BRS 100% than in the CON. Protein expression levels involved in inflammation such as TLR4, NF-κB, and COX2 were lower in the epididymal adipose tissues of the BRS 50% and BRS 100% than those of the CON. In the liver and epididymal adipose tissue, mRNA levels of pro-inflammatory markers including TLR4, TNFα, IL-1β, IL-6, iNOS, COX2, MCP1, and CCR2 in the BRS 50% and BRS 100% were lower than in the CON. On the other hand, anti-inflammatory markers including IL-10, arginase1, Chi3l3, and Mgl1 were higher in the epididymal adipose tissues of the BRS 50% and BRS 100% than in the CON. In the epididymal adipose tissue, macrophage infiltration markers (F4/80 and CD68) and leptin mRNA were significantly ($P<0.05$) lower in the BRS 50% and BRS 100% than in the CON.

Levels of TG, NEFA, and total cholesterol in the serum were significantly ($P<0.05$) lower in the BRS oil-treated groups than in the CON. Serum HDL-cholesterol level was significantly ($P<0.05$) higher in the BRS 50% than in the CON. Levels of total

lipid, TG, NEFA, and total cholesterol in the liver were significantly ($P<0.05$) lower in the BRS oil-treated groups than in the CON. The mRNA levels of lipogenesis markers including CD36, FABP1, SREBP-1c, FAS, and SLC25A1 in the livers of the BRS oil groups were significantly ($p<0.05$) lower than in the CON. On the other hand, fatty acid oxidation markers were significantly (CPT1A and ACOX; $P<0.05$) or without significance (ACADL and HADHα; $P>0.05$) higher in the BRS oil groups than in the CON. PPARα mRNA level was significantly ($P<0.05$) higher in the liver of the mice fed BRS 50% diet than in the CON, whereas PPARα protein expression was significantly ($P<0.05$) higher in the liver of the mice fed BRS 100% diet than in the CON. PPARγ mRNA level was significantly ($P<0.05$) lower in the liver of the mice fed BRS 50% diet than in the CON, whereas PPARγ protein expression was significantly ($P<0.05$) lower in the liver of the mice fed BRS 100% diet than in the CON. These results are in agreement with the PPARα results. PPARα mRNA level was significantly ($P<0.05$) higher in the epididymal adipose tissue of the BRS 50% and BRS 100% than in the normal CON and CON. PPARα protein was significantly ($P<0.05$) higher in the epididymal adipose tissue of the BRS 100% than in the normal CON and CON. There were no significant ($P>005$) differences in PPARγ mRNA levels between the BRS oil groups and CON. To detect the effect of BRS oil on hepatic lipid accumulation, Oil Red O staining was performed. The Oil Red O staining showed that the lipid droplets in the livers of the BRS oil-treated mice were smaller and fewer than those of the CON, indicating reduced hepatic lipid

accumulation in the BRS oil groups.

This study demonstrated that dietary BRS oil treatment reduced pro-inflammatory markers and promoted anti-inflammatory markers in liver and epididymal adipose tissue of high-fat diet-induced obese mice and *db*/*db* mice. BRS oil also lowered lipogenesis markers and raised fatty acid oxidation markers in the livers of high-fat diet-induced obese mice and *db*/*db* mice.

Results of this study suggest that BRS oil may be a good source of ALA, an n-3 fatty acid, with anti-inflammatory effects on high-fat diet-induced obese mice and obese diabetic mice by ameliorating inflammatory responses. Also, BRS oil may improve lipid metabolism by inhibiting lipogenesis and promoting fatty acid oxidation in high-fat diet-induced obese and *db*/*db* mice. These results would promote utilization of BRS, a byproduct of beverage processing of black raspberries, and ultimately help black raspberry producers and manufacturers.

KEYWORDS: Black raspberry seed oil, α-Linolenic acid, Inflammation, Lipid metabolism, High-fat diet, *db*/*db* mouse

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## List of Abbreviations

AA: Arachidonic acid

ACADL: Acyl-CoA dehydrogenase

ACOX: Acyl-CoA oxidase

ALA: α-Linolenic acid

ATM: Adipose tissue macrophage

BRS: Black raspberry seed

CCR2: C-C motif chemokine receptor 2

CD: Cluster of differentiation

Chi3l3: Chitinase 3-like 3

COX2: Cyclooxygenase 2

CPT1A: Carnitine palmitoyltransferase 1A

DHA: Docosahexaenoic acid

EPA: Eicosapentaenoic acid

FABP: Fatty acid binding protein

FAS: Fatty acid synthase

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

HADHα: Hydroxylacyl-CoA dehydrogenase α

HDL-C: High-density lipoprotein cholesterol

IL: Interleukin

iNOS: Inducible nitric oxide synthase

LDL-C: Low-density lipoprotein cholesterol

LPS: Lipopolysaccharide

MCP1: Monocyte chemoattractant protein 1

Mgl1: Macrophage galactose N-acetyl-galactosamine-specific lectin 1

MUFA: Monounsaturated fatty acid

NAFLD: Non-alcoholic fatty liver disease

NEFA: Non-esterified fatty acids

NF-κB: Nuclear factor κ-B

PPAR: Peroxisome proliferator activated receptor

PUFA: Polyunsaturated fatty acids

SLC25A1: Solute carrier family 25 member 1

SREBP-1: Sterol regulatory element binding protein 1

TG: Triglyceride

TLR4: Toll-like receptor 4

TNFα: Tumor necrosis factor α

VLDL-C: Very-low-density lipoprotein cholesterol

# Chapter 1

# Introduction and Literature Review

## 1.1 Background

In the processing of berries for wine and juice, pomace and seeds are usually used as fertilizer or animal feed, or considered as a waste. Thus, their application in the food industry is limited (Radočaj et al. 2014). Since hard seeds are usually excreted undigested (Helbig et al. 2008), health promoting substances inside the seeds may not be used in the body.

Previous studies have detected significant levels of essential fatty acids in fruit seed oils. For instance, cold-pressed black raspberry seed (BRS) oil contains 56% linoleic acid (18:2n-6), followed by 35% α-linolenic acid (ALA, 18:3n-3) (Parry et al. 2004). Cranberry seed oil is also a rich source of these fatty acids, containing 44% linoleic acid and 22% ALA (Parker et al. 2003). Strawberry seed oil is composed of 40% linoleic acid and 38% ALA (Pieszka et al. 2013). It is well known that ALA has anti-inflammatory effects on human corneal epithelial cells (Erdinest et al. 2012), rodents (Jangale et al. 2013) and humans (Zhao et al. 2004; Mirfatahi et al. 2016).

Some fruit seed oils contain significant levels of other beneficial phytochemicals such as tocopherols. Adhikari et al. (2008) found that BRS oil had 175 mg tocols in 100 g oil, including α-, γ-, and δ-tocopherols at concentrations of 50.9, 113.7, and 11.1 mg in 100 g oil, respectively. Significant levels of tocopherols were also observed in blackberry (*Rubus fruticosus*) and raspberry (*Rubus ideaus*) seed oils (Radočaj et al. 2014). Previous studies reported that tocopherols may affect anti-

inflammation and lipid-lowering properties in rodents (Ash et al. 2011; Jiang et al. 2013). In addition, carotenoids, phytosterols, and phenolic compounds are present in the fruit seed oils (Parry et al. 2004; Ash et al. 2011; Pieszka et al. 2013). Tocopherols and polyphenols are natural anti-oxidants that may reduce radical-mediated cellular damage (Parry et al. 2005; Adhikari et al. 2008).

**1.2 Objectives**

Hypothesis of this study was that BRS oil removed as a by-product may contain health beneficial compounds. These substances might contribute beneficial effect on inflammatory status and lipid metabolism in high-fat diet-induced obese mice and *db*/*db* mice, two widely used animal models for obesity and type 2 diabetes.

The specific objectives were:

**Study 1**: To evaluate effect of BRS oil on inflammation and lipid metabolism in high-fat diet-induced obese mice; and

**Study 2**: To evaluate the status of the markers related to inflammation and lipid metabolism in *db*/*db* mice fed diets containing different concentrations of BRS oil.

## 1.3 Literature Review

### 1.3.1 Black raspberry seed oil

Black raspberry (*Rubus occidentalis*) fruits, one of the most widely cultivated *Rubus* fruits in Korea, have been extensively studied for their physiological properties due to a large amount of phenolic compounds in the fruits. Black raspberry fruits are mainly used to produce wine or juice. Pomace from black raspberry production, including pulp and seeds, has been used as fertilizer or animal feed. BRS constitute 7.3-9.6% (wet basis) of the fruit and contain 17.3-27.9% oil (dry basis), mainly consisting of linoleic (18:2n-6), followed by ALA, and oleic acid (Oh et al. 2007; Bushiman et al. 2004; Table 1.1). Over 30% ALA in BRS oil is a distinct characteristic compared to other vegetable oils (Oh et al. 2007).

Tocols (tocopherols and tocotrienols) are a group of lipid soluble compounds generally known as vitamin E (Adihikari et al. 2008). Tocols act as an antioxidant in vegetable oils. Oxidative stability of oil depends on its fatty acid composition and the content of other chemicals, such as antioxidants. BRS oil showed resistance to lipid oxidation despite its high content of polyunsaturated fatty acids (PUFA). This could be explained by the significant level of tocols present in the BRS oil (Parry et al. 2004). BRS oil is rich in tocopherols than commercial soybean, corn, olive, canola, perilla, and grape seed oils (Adhikari et al. 2008; Table 1.2). Adhikari et al. (2008) also suggested that the high content of tocopherols in BRS oil may promote high anti-inflammatory activity of BRS oil.

**Table 1.1 Fatty acid composition in seeds of *Rubus* fruits (unit: %, w/w)**

| Fatty acid | Red raspberry | Black raspberry | Boysenberry | Marion blackberry | Evergreen blackberry |
|---|---|---|---|---|---|
| 16:0 | 2.4 | 1.9 | 3.5 | 3.4 | 4.5 |
| 18:0 | 0.9 | 0.8 | 1.5 | 2.1 | 3.3 |
| 20:0 | 0.4 | 0.4 | 0.7 | 0.5 | 0.8 |
| 22:0 | 0.1 | 0.1 | 0.2 | - | - |
| 16:1 | 0.1 | - | - | - | - |
| 18:1n-7 | 0.7 | 0.6 | 0.7 | 0.7 | 0.7 |
| 18:1n-9 | 11 | 10.4 | 11.6 | 15.1 | 17.3 |
| 20:1 | 0.2 | 0.2 | 0.5 | 0.3 | 0.3 |
| 16:2 | 0.1 | - | - | - | - |
| 18:2 | 54.2 | 53.5 | 59.1 | 62.7 | 53.1 |
| 18:3n-6 | 0.2 | 0.2 | - | - | - |
| 18:3n-3 | 29.7 | 31.2 | 22.1 | 15.2 | 19.9 |
| 20:2 | - | 0.1 | 0.1 | - | - |

(Bushman et al. 2004)

**Table 1.2 Contents of tocols in black raspberry seed oils and commercial vegetable oils (mg/100 g oil)**

| Samples | α-Tocopherol | β-Tocopherol | γ-Tocopherol | δ-Tocopherol | α-Tocotrienol | γ-Tocotrienol | Total tocols |
|---|---|---|---|---|---|---|---|
| Black raspberry seed oil – extracted by Bligh and Dyer (1959) method | 48.6±9.3[ab] | ND[j] | 109±16.2[ab] | 8.2±9.4[ac] | ND | ND | 166±18.2[a] |
| Black raspberry seed oil - hot hexane extracted[f] | 50.9 ± 3.0[a] | ND | 113.7 ± 8.3[a] | 11.1 ± 3.5[a] | ND | ND | 175 ± 7.1[a] |
| Black raspberry seed oil - hot hexane extracted and refined[g] | 42.6 ± 3.2[b] | ND | 95.2 ± 5.3[b] | 3.9 ± 0.2[acd] | ND | ND | 142 ± 3.1[b] |
| Soybean oil[h] | 17.3 ± 3.4[c] | 6.9 ± 3.8[a] | 57.1 ± 8.7[c] | 18.6 ± 4.0[b] | ND | ND | 99.9 ± 16.5[c] |
| Corn oil[h] | 17.3 ± 1.5[c] | ND | 42.4 ± 7.6[d] | ND | 0.7 ± 1.2[a] | ND | 61.1 ± 8.2[d] |
| Olive oil[h] | 15.8 ± 1.2[c] | 1.5 ± 0.5b[c] | 2.1 ± 0.4[e] | 1.9 ± 0.6[cd] | 6.8 ± 1.3[b] | ND | 28.0 ± 3.2[e] |
| Canola oil[h] | 11.2 ± 6.0[c] | 2.5 ± 1.6b[c] | 13.2 ± 4.5[e] | ND | ND | ND | 27.0 ± 12.1[e] |
| Perilla oil[i] | 2.5 ± 0.8[d] | 3.9 ± 1.8[ac] | 37.4 ± 4.8[d] | 1.6 ± 2.0[cd] | ND | ND | 45.4 ± 7.0[de] |
| Grape seed oil[h] | 16.3 ± 2.2[c] | 5.0 ± 4.3[ac] | 8.1 ± 1.3[e] | ND | 11.2 ± 2.2[c] | 11.5±2.8 | 52.2 ± 7.7[d] |

a-e The same letter in a column is not significantly different (α=0.05).

f Mean value ± standard deviation of triplicates for black raspberry seed oils extracted using hot hexane.

g Mean value ± standard deviation of triplicates for black raspberry seed oils extracted using hot hexane and refined.

h Mean value ± standard deviation of three different commercial products.

i Mean value ± standard deviation of one commercial product and two extracted in the lab.

j ND: not detected.

(Adhikari et al. 2008)

### 1.3.2 ALA as an n-3 PUFA

n-3 PUFA are essential fatty acids. ALA, the simplest member of this family, can be converted to very long chain n-3 PUFA, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Calder 2012). They are converted to active metabolites, in particular, known as resolvins and protectins (Masterton et al. 2010). ALA is an essential fatty acid, along with linoleic acid because animals, including humans, cannot synthesize ALA and linoleic acid (Calder 2012). ALA and linoleic acid are precursors of n-3 and n-6 PUFA families, respectively. In mammals, the same enzymes (desaturates and elongases) participate in the synthesis of n-3 and n-6 long chain PUFA. Therefore, the conversion of ALA to EPA competes with the conversion of linoleic acid to arachidonic acid (AA, 20:4n-6) (Nguemeni et al. 2013).

ALA is a common constituent of green leaves. However, green leaves are not high in fat, so these are not usually a major dietary source of ALA. A number of different seed oils and some nuts contain substantial amounts of ALA. Flaxseed oil is the most well-known ALA source. Therefore, vegans use flaxseed oil as the major source of n-3 PUFA (Calder 2012). Flaxseed oil typically contains 45-55% of fatty acids as ALA, whereas soybean oil and rapeseed oil only typically contains about 10% of fatty acids as ALA. Perilla oil is also high in n-3 PUFA. Perilla oil consists of about 50-60% ALA in the form of triglyceride (TG) (Chang et al. 2008). Raspberry seed oil is composed of 29.0-33.0% ALA (Radočaj et al. 2014). Red raspberry seed oil contains about 32.4% fatty acids as ALA (Parry et al. 2005). Corn, sunflower, and safflower

oils are scarce in ALA, but rich in linoleic acid. Meanwhile, long chain n-3 PUFA, EPA and DHA, are found in fatty fish and fish oils (Calder 2012). EPA and DHA have been known to be associated with multiple positive health effects, proposing its use for the prevention of chronic diseases such as obesity, diabetes, inflammatory bowel disease, and cancer (Valenzuela et al. 2011).

Over the past 100 years, changes in the food supply in Western countries caused alterations in dietary fatty acid consumption, leading to a dramatic increase in the ratio of n-6 to n-3 PUFA in circulation system and in tissue. Increased n-6 to n-3 PUFA ratios are hypothesized to promote production of inflammatory mediators, bring about higher incidence of inflammatory diseases, and may influence inflammatory gene expressions (Weaver et al. 2009).

### 1.3.3 Fruit seed oils as a source of health beneficial compounds

The fruit seed oils may contain health beneficial compounds such as high level of unsaturation and high content of tocopherols, tocotrienols, carotenoids, and polyphenols (Parry et al. 2005; Adhikari et al. 2008; Pieszka et al. 2013; Radočaj et al. 2014; Fotschki et al. 2015). These substances have anti-oxidant and anti-inflammatory properties. Supplementation of the diet with raspberry seed oil and strawberry seed oil resulted in reduction of superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities compared with control diet in male Wistar rats (Pieszka et al. 2013). Pieszka et al. (2013) reported that these effect might be

positive and may suggest that the decreased activities of these anti-oxidant enzymes are enough to maintain oxidative homeostasis.

Tocols have anti-oxidant activity and nutritional value and exhibit protective effects in foods against lipid oxidation (Radočaj et al. 2014). Lotus germ oil showed distinct characteristics in composition in terms of abundance in phenolic compounds and tocopherols (Li et al. 2009). Mice fed lotus germ oil showed anti-oxidant activity in liver and kidney, suggesting that the anti-oxidant activity of lotus germ oil may be attributed to the high contents of phenolic compounds and tocopherols (Li et al. 2009). Jiang et al. (2013) demonstrated that γ-tocopherol significantly attenuated colon inflammation and suppressed inflammation-promoted colon tumorigenesis in male Balb/c mice. The mice supplemented with tocopherols had a high fecal excretion of tocopherols and vitamin E metabolites compared with mice fed control diet (Jiang et al. 2013).

Ash et al. (2011) reported that BRS oil showed lipid-lowering properties in male hamsters as a model of atherosclerosis. The presence of tocopherols in BRS oil may contribute additively or synergistically to their overall lipid-lowering efficacy (Ash et al. 2011). Phytosterols are plant sterols structurally similar to cholesterol, which decrease cholesterol absorption in the intestine (Pieszka et al. 2013). Ash et al. (2011) determined contents of phytosterols extracted from feces in hamsters after intake of BRS oil. Fecal excretion of phytosterols was significantly elevated in BRS oil group compared with the soybean and coconut oil groups. Phytosterols present in BRS oil

may contribute to lowering lipid in hamsters fed high levels of dietary TG and cholesterol, as phytosterols typically lower low-density lipoprotein cholesterol (LDL-C) in both hamsters and humans (Guderian et al. 2007; AbuMweis et al. 2008; Ash et al. 2011). Pieszka et al. (2013) also reported that phytosterols reduced blood cholesterol levels by inhibiting absorption of cholesterol in the intestine through competition with dietary and biliary cholesterol.

### 1.3.4 Obesity, inflammation, and diabetes

Obesity is a serious nutritional problem in industrialized countries and associated with metabolic diseases such as insulin resistance, cardiovascular disease, high blood pressure, and type 2 diabetes. Obesity is linked to a chronic low-grade inflammation in the adipose tissue (Calder 2005). Accumulation of excess fat is related to increased production of pro-inflammatory cytokines and decreased production of anti-inflammatory cytokines (Kalupahana et al. 2011). Overproduced pro-inflammatory cytokines and chemokines exacerbate diabetic deterioration (Chan et al. 2012). Previous studies demonstrated that expansion of adipose tissue during weight gain is related to inflammatory status by recruitment of inflammatory cells, mainly macrophages, in adipose tissue (Lumeng et al. 2007; González-Périz et al. 2009; Titos et al. 2011).

Previous studies showed that inflammation plays an essential role in the initiation and development of obesity and type 2 diabetes (Xu et al. 2003; Arkan et al. 2005).

Pro-inflammatory cytokines contributed to the development of insulin resistance by impairing insulin signaling via inhibitory phosphorylation of insulin receptor (Sowers 2008). Kalupahana et al. (2011) also reported that increased pro-inflammatory cytokines can induce insulin resistance by inhibition of insulin signaling.

### 1.3.5 Anti-inflammatory effect of n-3 PUFA

Long chain n-3 PUFA are known to have an anti-inflammatory effect. The other major group of PUFA, AA (n-6 PUFA), is known to be a pro-inflammatory and pro-thrombotic fatty acid. The intake of n-6 PUFA promotes the role of AA as a precursor of the 2 series prostanoids and the 4 series eicosanoids. They create a pro-inflammatory environment that may affect the progression of chronic diseases (Garófolo et al. 2006). On the other hand, intake of n-3 PUFA partially replaces AA in the eicosanoid metabolism, favoring the synthesis of less inflammatory 3 series prostanoids and 5 series leukotrienes (Surette 2008; Ellulu et al. 2015; Fig. 1.1).

It has been suggested that the ratio of n-6 to n-3 PUFA should be approximately from 4:1 to 1:1 (Simopoulos 2002). However, the ratio can be as high as 15:1 in the modern diet, which is rich in n-6 PUFA such as vegetable oils (corn, sunflower, and soybean oils) and meat (beef, chicken, and pork). The n-6 to n-3 PUFA ratio is significantly correlated with the quantity of hepatic TG (Masterton et al. 2010).

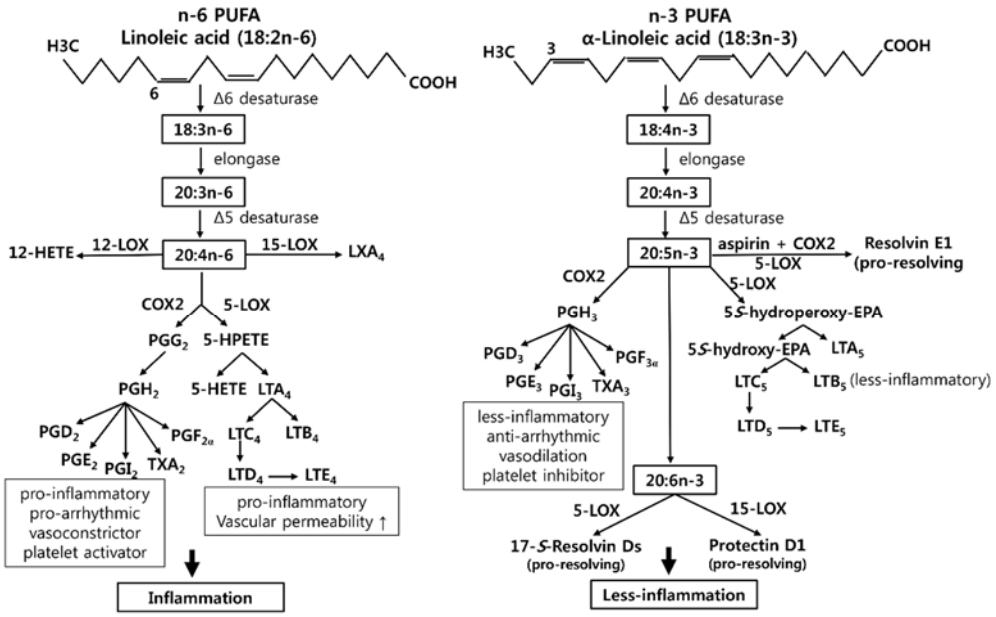


**Fig. 1.1 Metabolism of n-3 and n-6 PUFA and biosynthesis of their respective eicosanoids and proresolving mediators**

(Ellulu et al. 2015)

Altered fatty acid composition may influence several processes related to insulin resistance and obesity, such as hormone binding, signal transduction, and availability of precursor molecules for lipid synthetic and catabolic pathways (Tallman et al. 2003). Liver of mice fed ALA-rich linseed oil diet had higher n-3 PUFA and lower n-6 PUFA than that of the control diet (Perini et al. 2011). Rustichelli et al. (2012) also reported that diet with flaxseed oil significantly increased ALA and EPA contents in liver, brain, and adipose tissue of rats. Previous studies demonstrated that intake of ALA may ameliorate tissue lipid profile through increase in n-3 PUFA and decrease in n-6 PUFA (Perini et al. 2011; Rustichelli et al. 2012).

Nuclear factor κ-B (NF-κB) is a key transcription factor involved in inflammation. It regulates a range of genes and proteins including several pro-inflammatory cytokines (tumor necrosis factor α (TNFα), IL-6, and monocyte chemoattractant protein 1 (MCP1)), adhesion molecules, inducible nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX2) (Onai et al. 2004). NF-κB present in the cytosol is activated via phosphorylation of I-κB, an inhibitor protein which leads to its degradation. Subsequently, the degraded NF-κB is translocated to the nucleus and binds to the promoter or enhancer of specific genes, including inflammation-involved ones (Onai et al. 2004). n-3 PUFA inhibit the production of inflammatory mediators via decreased I-κB phosphorylation and inactivation of NF-κB (Novak et al. 2003).

Siriwardhana et al. (2012) demonstrated that EPA has anti-inflammatory effect in 3T3-L1 adipocytes through mediation of NF-κB pathway. They reported that

increasing EPA to AA ratio decreased MCP1 and IL-6 secretions and dose-dependently decreased NF-κB activation in 3T3-L1 adipocytes. Inflammatory response in white adipose tissue induced by a high-fat diet in obese diabetic mice is prevented by inclusion of n-3 PUFA. Plasma IL-6 and mRNA levels of MCP1 and TNFα in adipose tissue were lower in *db*/*db* mice fed high-fat diets rich in EPA and DHA than mice fed high-fat diets rich in n-6 PUFA (Todoric et al. 2006). Treatment with DHA alleviated adipose tissue inflammation by decreasing mRNA levels of TNFα, IL-6, and MCP1 and increasing IL-10 (Titos et al. 2011). Treatment of ALA significantly decreased protein content of TNFα, IL-1β, and IL-6 on human corneal epithelial cells. Similar results were demonstrated at the mRNA level (Erdinest et al. 2012). Administration of ALA-rich perilla oil significantly reduced pro-inflammatory cytokines including TNFα, IL-1β, and IL-6 compared with corn oil in BALB/c mice with ovalbumin-challenged allergic inflammation (Chang et al. 2008). Dietary flaxseed or fish oil decreased NF-κB, TNFα, IL-6, and MCP1 mRNA levels in liver of diabetic rats (Jangale et al. 2013). Zhao et al. (2004) reported that a diet high in ALA confers cardiovascular benefits, in part by eliciting an anti-inflammatory effect. Increased intake of dietary ALA resulted in anti-inflammatory effects by inhibiting TNFα, IL-1β, and IL-6 production in cultured peripheral blood mononuclear cells (Zhao et al. 2004). Dietary ALA also reduced cardiovascular disease risk by decreasing inflammation. ALA diet decreased C-reactive protein (CRP), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin more than linoleic acid diet in

hypercholesterolemic subjects (Zhao et al. 2004). Changes in CRP and VCAM-1 were inversely associated with changes in serum EPA concentration (Zhao et al. 2004).

It is well known that adipose tissue macrophages (ATM) infiltrate into adipose tissue during inflammatory processes (Khazen et al. 2005; Lumeng et al. 2007). ATM infiltration into adipose tissue is a prominent source of pro-inflammatory cytokines such as TNFα and IL-6 (Lumeng et al. 2007). Therefore, pro-inflammatory mediators increased in adipose tissue as the macrophage infiltration into the adipose tissue progresses (Kanda et al. 2006; Clària et al. 2011). Rodent studies suggest that inflammatory factors produced by ATM contribute to insulin resistance and type 2 diabetes (Koppaka et al. 2013). MCP1 plays a role in the recruitment of macrophages into adipose tissue. Kanda et al. (2006) reported that the plasma concentration of MCP1 was higher in diabetic mice than in normal mice. C-C motif chemokine receptor 2 (CCR2) is a receptor of MCP1. Increased production of MCP1 appears to recruit circulating macrophages through interaction with CCR2, and MCP1 expression increases in human adipose tissue from obese subjects (Oh et al. 2012). Lumeng et al. (2007) observed that high-fat diet-fed CCR2 knock out mice had lower inflammatory cytokine expression in adipose tissue than high-fat diet-fed control mice. n-3 PUFA are known to prevent macrophage infiltration. Cluster of differentiation 68 (CD68) is known for discriminative marker in macrophages and CD11b is a macrophage antigen. The CD68 and CD11b (macrophage antigen-1)

mRNA levels were lower in *db*/*db* mice with high-fat diets rich in n-3 PUFA than *db*/*db* mice with high-fat diets rich in saturated (lard) or n-6 PUFA (Todoric et al. 2006). Treatment with DHA inhibited the F4/80 expression which is also known as discriminative markers in macrophages and induced mRNA level of anti-inflammatory mediators including IL-10, arginase1, and chitinase 3-like 3 (Chi3l3) in adipose tissue of high-fat diet-induced obese mice (Titos et al. 2011).

Increased adiposity leads to a chronic low-grade inflammation in adipose tissue, and inflammation of obesity-induced adipose tissue is a unique process characterized by an inflammatory response (Clària et al. 2011). The chronic low-grade inflammation occurring in adipose tissue is regarded as a major factor in pathogenesis of obesity-induced insulin resistance (Kalupahana et al. 2011). Inflammation of adipose tissue leading to insulin resistance also has negative results on the liver. Increased lipid deposition in skeletal muscle and liver is also considered to be a factor linked to pathogenesis of insulin resistance (Schenk et al. 2008). On the other hand, obese, insulin-sensitive individuals have lower skeletal muscle and liver lipids than obese, insulin-resistance individuals (Stefan et al. 2008). There are some mechanisms associated with dysfunction of adipose tissue and insulin resistance with metabolic liver disease. First, increased lipolysis from visceral fat causes increased free fatty acid efflux to the liver. Second, secretion of pro-inflammatory and insulin-resistant adipokines (TNFα and IL-6) increases. Finally, a combined hepatic dysregulation in free fatty acid oxidation and lipogenesis alters hepatic insulin sensitivity (Sanyal

2005; Tilg et al. 2008). n-3 and n-6 PUFA have opposite effects on insulin resistance. n-3 PUFA attenuate the inflammation and ameliorate the insulin resistance induced by saturated fatty acids, while n-6 PUFA promote the development of insulin resistance (Liu et al. 2013).

Adipose tissue, once considered a mere storage depot of energy in the form of fat, is regarded as an important endocrine organ that secretes adipokines involved in homeostasis of the body and inflammatory response (Clària et al. 2011). Adipokines including leptin and adiponectin are produced and secreted by adipose tissue. Leptin increases production of TNFα and IL-6. Leptin levels in the serum and adipose tissue increase in response to pro-inflammatory stimuli, including TNFα and lipopolysaccharide (LPS) (Ouchi et al. 2003). Leptin deficiency protects liver damage and reduces serum TNFα in mice with T-cell-mediated hepatitis (Faggioni et al. 2000). Thus, leptin is generally regarded as a pro-inflammatory adipokine (Ouchi et al. 2003). On the other hand, adiponectin is expressed at the highest levels by functional adipocytes that are found in lean organisms, but its expression is down-regulated in the obesity-associated dysfunctional adipocytes (Ouchi et al. 2003). Adiponectin production in adipocyte is inhibited by pro-inflammatory factors, such as TNFα and IL-6 (Berg et al. 2005). Plasma adiponectin level negatively correlates with visceral fat accumulation (Ryo et al. 2004). Plasma adiponectin levels decrease in type 2 diabetes patients and adiponectin inhibits expression of TNFα in adipose tissue (Ouchi et al. 2003). Adiponectin stimulates production of anti-inflammatory cytokine,

IL-10, in human monocyte-derived macrophages (Kumada et al. 2004). Wu et al. (2013) reported that, in randomized placebo-controlled clinical trials, fish oil supplementation moderately increased circulating adiponectin.

### 1.3.6 Effect of n-3 PUFA on lipid metabolism

Hepatic steatosis is a state of fat accumulation in the liver. Obesity generates low-grade inflammation and this condition is often associated with the development of insulin resistance. Insulin resistance is related with a defect in insulin signaling and results in defective lipid metabolism (Bargut et al. 2014). Inflammation mediators such as TNFα and IL-6 have been found to be associated with development of obesity. These inflammatory mediators are involved in regulating lipid metabolism and consequently affect lipid accumulation (Tai et al. 2010)

Major effects of n-3 PUFA on lipid metabolism are to promote lipolysis and to inhibit lipogenesis. n-3 PUFA are important regulators of hepatic gene expressions, thereby changing metabolism from lipogenesis and storage to fatty acid oxidation and catabolism (Fig. 1.2). Therefore, they reduce hepatic steatosis and markers of inflammation and improve insulin sensitivity in animal study and human clinical trial (Masterton et al. 2010). n-3 PUFA are well documented for reducing plasma TG (Batetta et al. 2009; Yang et al. 2011; Fink et al. 2014) and showed anti-obesity effects on humans (Pettinelli et al. 2011) and rodents (Berlanga et al. 2014; Devarshi et al. 2013).

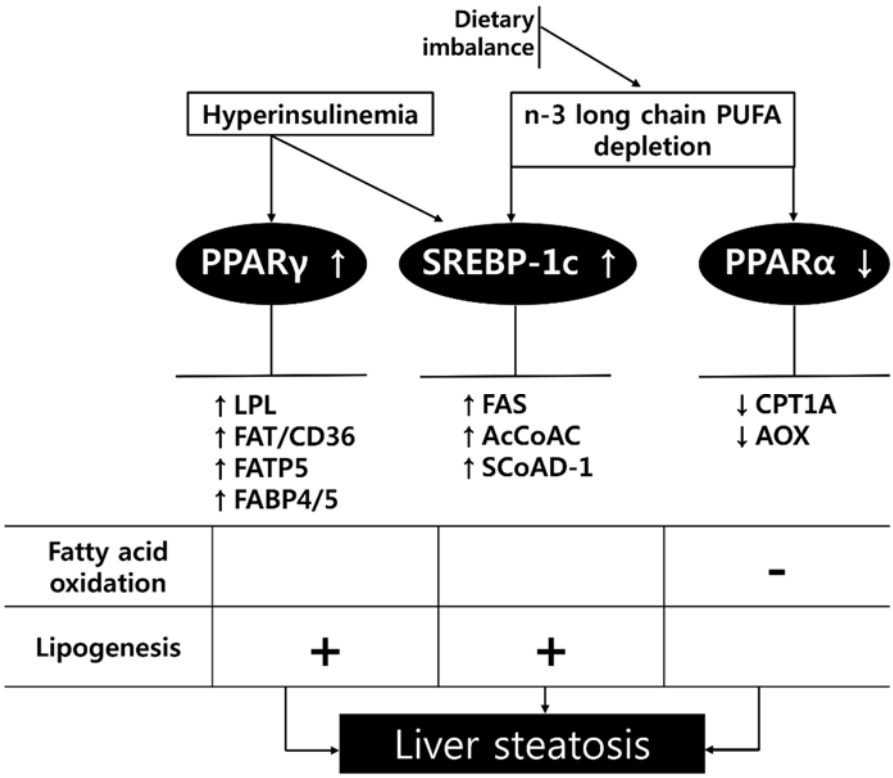


**Fig. 1.2 Depletion of n-3 PUFA leading to enhancement in hepatic SREBP-1c/PPARα ratio that favors de novo lipogenesis over fatty acid oxidation**

(Valenzuela et al. 2011)

Increased level of lipids in serum, caused by high-fat diet, could lead to fat accumulation in the liver, as well as oxidative stress and inflammation, which result in hepatic steatosis (Han et al. 2015). Dietary supplementation of flaxseed oil containing flaxseed oil esters of plant sterols reduced expressions of genes related to inflammation and improved lipid metabolism, and thus ameliorated hepatic steatosis (Han et al. 2015). Riediger et al. (2008) reported that consumption of ALA was associated with improvements in the levels of plasma TG and high-density lipoprotein cholesterol (HDL-C). In a randomized, double-blind, placebo-controlled trial study, EPA and DHA supplementation significantly decreased serum TG in comparison with control (Capanni et al. 2006). Circulating AA and n-6 to n-3 PUFA ratio was also reduced in EPA and DHA-treated NAFLD patients (Capanni et al. 2006). Zhu et al. (2008) reported that n-3 PUFA from seal oil is efficacious for patients with NAFLD associated with hyperlipidemia and can improve their total symptom scores and serum lipid levels.

It is well-known that free fatty acids are taken up into cells by passive diffusion and by protein-mediated mechanisms involving a number of fatty acid transporters, of which cluster of differentiation (CD36) is the best characterized (Berlanga et al. 2014). Uptake of free fatty acids is an important contributing factor to hepatic steatosis because free fatty acids can be converted to TG (Zhou et al. 2008). Therefore, fatty acid uptake in liver, for example, mediated by CD36, plays an important role in steatosis. Fatty acid binding protein (FABP) are a family of lipid-

binding proteins in the regulation of lipid metabolism and inflammation. Among these, FABP1 is abundant in liver cytoplasm (Shi et al. 2012). FABP1 has been hypothesized to be involved in liver lipid transport and lipoprotein metabolism. FABP1 null mice showed decreased fatty acid-binding capacity in the cytosol, which resulted in significantly ($P<0.05$) reduced hepatic TG deposition (Newberry et al. 2009). Newberry et al. (2009) also demonstrated that FABP1-deficient mice were protected against obesity and hepatic steatosis, and accumulation of TG was reduced in liver when fed a high saturated fat diet and a high saturated fat diet containing cholesterol. Fatty acid synthase (FAS) is highly regulated by a transcriptional factor, sterol regulatory element binding protein-1c (SREBP-1c) which is involved in fatty acid synthesis, and plays an important roles in metabolism of fatty acids (Berlanga et al. 2014). On the other hand, carnitine palmitoyltransferase 1A (CPT1A) is considered as the master regulator of hepatic mitochondrial β-oxidation because it is the gateway for fatty acid entry into the mitochondrial matrix. (Bargut et al. 2014). In the liver of obese NAFLD patients, SREBP-1c and FAS mRNA expressed more and CPT1A mRNA expressed less compared with the control (Pettinelli et al. 2009).

Long chain n-3 PUFA are potent activators of peroxisome proliferator-activated receptor α (PPARα), which up-regulates several genes associated with fatty acids and lipid metabolism (Jump 2008). In addition to improvements in steatosis, n-3 PUFA may have anti-inflammatory effect via PPARα-mediated suppression of TNFα and IL-6 (Stienstra et al. 2007). ALA also has been well known as a natural ligand of

PPARα, a key transcriptional regulator of fatty acid β-oxidation, thereby reducing hepatic fat accumulation (Han et al. 2015). Devarshi et al. (2013) demonstrated that dietary flaxseed oil improved lipid metabolism through up-regulation of PPARα and down-regulation of SREBP-1c in streptozotocin-nicotinamide-induced diabetic rats. EPA ameliorated hepatic steatosis in mice which have steatohepatitis based on decreased expression of SREBP-1c and increased PPARα expression (Ishii et al. 2009). EPA-treated group exhibited less severe chronic hepatic inflammation than the control group, resulting in dramatically low ratio of AA to EPA (Ishii et al. 2009). EPA significantly suppressed hepatic TG content in mice fed high-fat and high-sucrose diet compared to control diet through decreased mRNA and protein of hepatic SREBP-1c (Kajikawa et al. 2009). EPA also altered the composition of fatty acids in liver by lowering monounsaturated fatty acids (MUFA) and increasing n-3 PUFA in the same model (Kajikawa et al. 2009). Oliveira et al. (2006) reported that rats fed a high-fat diet with fish oil protected against the severe hepatic steatosis and increased lipid oxidation compared with rats fed the same diet without fish oil. Deficiency of n-3 PUFA results in hepatic steatosis, whereas supplementation of n-3 PUFA appears to safely reduce hepatic steatosis in adults (Shapiro et al. 2011). A number of studies have demonstrated an ameliorative effect of supplemental fish oil and seal oil (rich in EPA, DPA and DHA), reducing hepatic lipid content in non-alcoholic fatty liver disease (NAFLD) (Yamazaki et al. 2007; Martin et al. 2007; Zhu et al. 2008). Long chain n-3 PUFA depletion in liver of morbid obese patients with steatosis or

nonalcoholic steatohepatitis is evidenced by 50% diminution in EPA and DHA level (Valenzuela et al. 2011). Therefore, depletion of long chain n-3 PUFA in the liver of obese patients may favor fatty acid and TG synthesis with derangement in the capacity for fatty acid oxidation and TG export from the liver, leading to SREBP-1c up-regulation, PPARα down-regulation, and hepatic steatosis promotion (Valenzuela et al. 2011). Pettinelli et al. (2011) reported that level of hepatic PPARγ mRNA was higher in steatotic people than that of the control and hepatic PPARγ mRNA level was positively correlated with hepatic SREBP-1c mRNA level. Morán-Salvador et al. (2011) also reported that PPARγ expression is up-regulated in steatotic livers compared with healthy livers. PPARγ increases in liver of obese NAFLD patients in association with insulin resistance, a transcription factor favoring the expression proteins involved in fatty acid uptake, binding, and intracellular transport (Pettinelli et al. 2011).

# Chapter 2

# Effect of Black Raspberry Seed Oil on Inflammation and Lipid Metabolism in High-Fat Diet-Induced Obese Mice (Study 1)

## 2.1 Introduction

BRS constitute 47.8-53.5% (dry basis) of fruits and contain 12.8-17.1% moisture. Oil content of BRS is 17.3-27.9% (dry basis) (Oh et al. 2007). The oil extracted from BRS is composed of 53.2-53.5% linoleic, 31.2-33.6% α-linolenic, and 8.3-10.4% oleic acids (Oh et al. 2007). More than 90% of the total fatty acids in BRS oil are unsaturated. BRS oil has an oxidation stability because it has considerably large amount of tocopherols (Adhikari et al. 2008). Over 30% ALA in BRS oil is a noticeable characteristic compared to other common vegetable oils such as corn, sunflower, soybean, and olive oil.

Previous studies have demonstrated that significant levels of PUFA and natural antioxidants are present in fruit seed oils (Parker et al. 2003; Parry et al. 2005; Pieszka et al. 2013). Cranberry seed oil is a rich source of essential fatty acids, containing 35-44% linoleic acid and 23-35% ALA, along with a significant level of tocopherols (Parker et al. 2003). Cold-pressed blueberry, red raspberry, marionberry, and boysenberry seed oils contained significant amounts of ALA, carotenoids, and tocopherols (Parry et al. 2005). Oils obtained from strawberry, blackcurrant, raspberry, and apple seeds are rich sources of PUFA, tocopherols, tocotrienols, and phytosterols (Pieszka et al. 2013). These substances generally have positive effects on human health. Therefore, seed oils are considered value-added products that can be used for functional and nutraceutical food products (Radočaj et al. 2014).

n-3 Fatty acids are able to disrupt n-6 fatty acid-induced inflammatory status,

including pro-inflammatory mediators produced from AA (Calder 2005). On the contrary, EPA produces eicosanoids that have less inflammatory response compared with those produced from AA (Calder 2012). ALA, mainly found in nuts, seeds, and vegetable oils, is a possible precursor of EPA and DHA (Chang et al. 2008). Erdinest et al. (2012) reported that ALA has anti-inflammatory effects on human corneal epithelial cells. The protein and mRNA of TNFα, interleukin-1β (IL-1β), IL-6, and IL-8 levels significantly ($P<0.05$) decreased following treatment with ALA in the cells. Treatment with EPA and DHA favorably modulated adipose tissue and systemic inflammation in severely obese nondiabetic patients and improved lipid metabolism by significantly ($P<0.05$) reduced serum TG concentration compared with control treatment (Itariu et al. 2012). In hypercholesterolemic patients, administration of n-3 fatty acid inhibits platelet aggregation and alters inflammatory status by significantly ($P<0.05$) decreased IL-6 concentration in serum (Doenyas-Barak et al. 2012). Expressions of several pro-inflammatory cytokines were reduced in mononuclear cells of healthy humans after supplementation with fish oil for 4 weeks (Weaver et al. 2009). Intake of 1.8 g EPA and DHA per day for 26 weeks changed the expressions of 1040 genes involved in inflammatory- and atherogenic-related pathways, such as NF-κB signaling, eicosanoid synthesis, and adipogenesis in human mononuclear cells (Bouwens et al. 2009).

ALA-rich flaxseed oil effectively protected against ethanol-induced liver injury in mice. This protective effect might be associated with suppressing inflammation via

toll-like receptor 4 (TLR4) mediated NF-κB signaling pathway (Wang M et al. 2016). Daily consumption of 6 g flaxseed oil reduced inflammation markers including serum C-reactive protein (CRP) and vascular cell adhesion protein 1(VCAM-1) in hemodialysis patients (Mirfatahi et al. 2016). Flaxseed oil supplementation for 21 days down-regulated the expression of IL-1β in white blood cells of dogs (Purushothaman et al. 2014). Plasma ALA and EPA levels significantly ($P<0.05$) increased after treatment with flaxseed oil. Plasma ALA and EPA levels showed negative correlation with IL-1β mRNA expression of white blood cells (Purushothaman et al. 2014).

Tocopherols are common lipophilic antioxidants abundant in some oils and nuts (Bushman et al. 2004). BRS oil contains higher amounts of total tocols than soybean, corn, olive, canola, perilla, and grape seed oils (Adhikari et al. 2008). Pieszka et al. (2013) reported that raspberry seed oil, which is substantially similar with BRS oil in the fatty acid composition, is a rich source of tocols and displays anti-oxidant properties. Pieszka et al. (2013) also reported that raspberry seed oil contains some anti-oxidant and anti-inflammatory substances such as carotenoids, flavonoids, phytosterols, phenolic acids, and ALA. Male rats receiving raspberry seed oil showed improved antioxidant status via reducing activity of glutathione peroxidase and superoxide dismutase.

Type and quantity of fats consumed regulate hepatic lipid composition and gene expression and different sources of fats drive different amounts of fat accumulation in

liver (Bargut et al. 2014). Catta-Preta et al. (2012) reported that the lipid source is as important as the quantity of food consumed. Ratio of n-6 to n-3 PUFA significantly ($P<0.05$) correlated with the quantity of hepatic TG in NAFLD patients (Vuppalanchi et al. 2007). Patients with NAFLD not only showed a higher intake of saturated fatty acids, but also increased n-6 PUFA consumption while n-3 PUFA uptake is reduced, leading to a shift toward increased dietary n-6 to n-3 PUFA ratio (Fink et al. 2014). Araya et al. (2004) also reported that excess of n-6 PUFA in liver of the patients with NAFLD is associated with hepatic fat accumulation.

Long chain n-3 PUFA are known to improve several metabolic problems associated with obesity, including insulin resistance, liver and heart steatosis, and hypertension (Batetta et al. 2009). Liver TG concentration was significantly ($P<0.05$) lower in the rats fed fish and krill oil, which are abundant in n-3 PUFA, than in the rats fed control diet (Batetta et al. 2009). Balogum et al. (2016) reported that n-3 PUFA prevented obesity by down-regulating key genes involved in adipocyte hypertrophy. n-3 PUFA regulate hepatic gene transcriptions, with PPARα and SREBP-1 being best known. These have diverse effects on lipid metabolism and alter the function of specific response elements in target genes (Masterton et al. 2010). n-3 PUFA are potent activators of PPARα, which up-regulate several genes associated with fatty acids and lipid metabolism that stimulate fatty acid oxidation (Jump 2008). On the other hand, SREBP-1 is a key regulator of fatty acid synthesis.

Poudyal et al. (2013) compared the hepatic and metabolic response to individual dietary n-3 PUFA (ALA, EPA, and DHA) in a high-carbohydrate, high-fat diet-induced model of metabolic syndrome in rats. ALA-rich chia seed oil did not reduce total body fat but induced lipid redistribution away from the abdominal area and favorably improved insulin sensitivity and dyslipidemia. EPA and DHA reduced abdominal adiposity and total body fat and attenuated insulin sensitivity and dyslipidemia. Moreover, ALA, EPA, and DHA all reduced inflammation in both heart and liver and hepatic steatosis (Poudyal et al. 2013). Fink et al. (2014) provided effect of walnut oil, which has 4:1 ratio of n-6 to n-3 PUFA, on obese rats. Intake of walnut oil decreased hepatic TG compared with control diet. Consumption of walnut oil also inhibited hepatic lipid accumulation along with modulated hepatic gene expression implicated in fatty acid oxidation and lipogenic pathway. Hepatic TG and total cholesterol concentrations were lower in male mice fed high-fat diet containing pollock oil than mice fed control diet, although body and liver mass did not differ between the two groups (Yang et al. 2011). Pollock oil contains considerable amounts of n-3 PUFA. Total cholesterol, LDL-C, and TG concentrations in plasma decreased in pollock oil group compared with control group. Expressions of hepatic genes involved in cholesterol metabolism and lipogenesis were suppressed in the pollock oil group, and might favorably affect hyperlipidemia and hepatic steatosis induced by the high-fat diet (Yang et al. 2011).

Ash et al. (2011) reported that BRS oil is a promising potential lipid-lowering dietary therapeutic because of their high levels of PUFA, polyphenolic compounds, tocopherols, and phytosterols. Unrefined BRS oil diet significantly ($P<0.05$) lowered TG in plasma than coconut oil diet. TG concentration in liver was lower in the unrefined and refined BRS oil-fed male hamsters than in coconut oil-fed group (Ash et al. 2011).

In this study, the effects of BRS oil on inflammatory response and lipid metabolism in high-fat diet-induced obese mice were determined. High-fat diets induce obesity and metabolic disorders in rodents that resemble human metabolic syndrome (Buettner et al. 2007). Liver plays a central role in maintaining energy balance and contributing to energy storage (Zhang et al. 2014). Thus, fatty acid composition of liver of the mice fed BRS oil for 12 weeks as well as protein and mRNA levels related with inflammation in liver and adipose tissue were determined in this study. Lipid profile in serum and liver and protein and mRNA levels related to lipogenesis and fatty acid oxidation in the liver were also measured.

## 2.2 Materials and methods

### 2.2.1 Sample preparation

Black raspberry (*Rubus occidentalis*) fruits harvested in 2012 in Gochang, Korea were purchased from a farm. Seeds were separated from the fruits by hand and pigments on the seeds were washed off with tap water until the color disappeared. BRS was powdered with a blender (Hanil Co., Bucheon, Korea) until enough sample

was collected. Hexane (1,000 mL, extra pure grade; Samchun Chemical Co., Ltd., Seoul, Korea) was added to 200 g powdered BRS followed by stirring for 2 h for extraction of oil. After extraction, the seed powder-hexane mixture was filtered with Whatman No. 2 filter paper (Whatman International Ltd., Maidstone, England). Filtrate was concentrated using a vacuum rotary evaporator (A-10005; Eyela Co., Tokyo, Japan). The concentrated extract (BRS oil) was stored at -20ºC after flushing with nitrogen gas.

2.2.2 Animals

Male C57BL/6 mice (5 weeks old) were purchased from Orientbio (Seongnam, Korea) and individually housed in cages in an animal facility with conventional (CV) rooms (College of Veterinary Medicine, Seoul National University, Seoul, Korea). After 1 week of acclimation with the control diet, the mice were randomly assigned into 2 groups (BRS oil group (n=9) and control group (n=9)) (Fig. 2.1). The CV room was controlled with constant temperature (23±2ºC), humidity (55±10%), and 12 h dark/light cycle. Food intake and body weight were measured twice a week. After 12 weeks on the experimental diets, the mice were fasted for 12 h and euthanized with $CO_2$ asphyxiation. All the animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (approval no: SNU-120630-4).

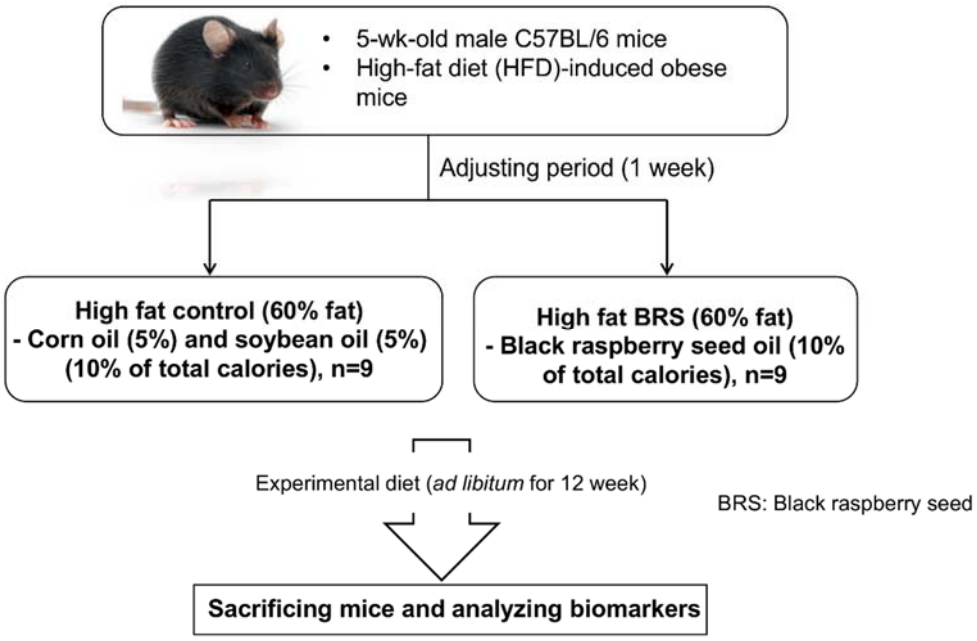


**Fig. 2.1 Overview of the study design**

2.2.3 Experimental diets

High-fat-purified diet was customized (Feed Lab, Guri, Korea). We provided the BRS oil to Feed Lab. The control diet consisted of 50% calories from lard, 5% from soybean oil, and 5% from corn oil. The BRS oil diet consisted of 50% calories from lard and 10% from BRS oil. Experimental diets were stored at -20ºC until provided to animals. Animals were fed the diets *ad libitum* for 12 weeks. Table 2.1 shows the compositions of the experimental diets.

**Table 2.1 Compositions of the diets**

| | Control diet | | Black raspberry seed oil diet | |
|---|---|---|---|---|
| | Weight (%) | Energy (%) | Weight (%) | Energy (%) |
| Protein | 27% | 20% | 27% | 20% |
| Carbohydrate | 25% | 20% | 25% | 20% |
| Fat | 36% | 60% | 36% | 60% |
| Ingredient | Weight (g) | Energy (kcal) | Weight (g) | Energy (kcal) |
| Caseins | 200 | 800 | 200 | 800 |
| Corn starch | 47.536 | 190 | 47.536 | 190 |
| Dextrose | 132 | 528 | 132 | 528 |
| Cellulose | 50 | 0 | 50 | 0 |
| Black raspberry seed oil | - | - | 45 | 405 |
| Corn oil | 22.5 | 202.5 | - | - |
| Soybean oil | 22.5 | 202.5 | - | - |
| Lard | 225 | 2025 | 225 | 2025 |
| Mineral mixture | 35 | 0 | 35 | 0 |
| Vitamin mixture | 10 | 40 | 10 | 40 |
| TBHQ | 0.014 | 0 | 0.014 | 0 |
| L-Cystine | 3 | 12 | 3 | 12 |
| Choline bitartrate | 2.5 | 0 | 2.5 | 0 |
| Total | 750.1 | 4,000 | 750.1 | 4,000 |

2.2.4 Tissue collection

After mice were euthanized by asphyxiation with $CO_2$, about 1 mL blood was collected by cardiac puncture. Serum was separated by centrifugation (Micro 17R+, Hanil Science Industrial Co., Incheon, Korea) at 387×g at 4ºC for 20 min after coagulation at room temperature for 2 h. Liver, spleen, kidney, and epididymal adipose tissue were collected and washed with phosphate-buffered saline (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All tissues were stored at -80ºC until analysis.

2.2.5 Lipid extraction from diets

Lipid was extracted from experimental diets by modified Bligh and Dyer (1959) method. Thirty g diet was mixed with 50 mL chloroform (Samchun Chemical Co., Ltd.), 50 mL methanol (Samchun Chemical Co., Ltd.), and 40 mL water. The mixture was agitated for 1.5 h and centrifuged at 3,500 rpm for 20 min by a centrifuge (Hanil Science Industrial Co.). Lower layer (chloroform layer) was filtered with Whatman No. 2 filter paper (Whatman International Ltd.). Filtrate was concentrated using a vacuum rotary evaporator (A-10005; Eyela Co.) and used for analyzing fatty acid composition.

2.2.6 Lipid extraction from livers

Lipid was extracted from livers of the experimental mice by modified Bligh and Dyer (1959) method. Five hundred mg liver tissue was mixed with 10 mL chloroform

(Samchun Chemical Co., Ltd.) and 5 mL methanol (Samchun Chemical Co., Ltd.), followed by homogenizing with a homogenizer (Daihan Scientific Co., Ltd., Wonju, Korea), stirring for 20 min and filtering with Whatman No. 2 filter paper (Whatman International Ltd.). The filter paper were washed with 1 mL 0.9 % NaCl (Samchun Chemical Co., Ltd.), collecting lower layer (chloroform layer) with NaCl solution, followed by concentration using nitrogen gas. The concentrated lipid was used for analyzing fatty acid composition.

2.2.7 Fatty acid composition analysis

The BRS oil and extracted lipid were methylated using $BF_3$-methanol (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) according to the AOCS method (1989). Fatty acid composition was analyzed by GC (6890; Agilent Technologies, Palo Alto, CA, USA) equipped with flame ionization detector and a DB-23 column (30 m × 0.25 mm × 0.25 μm; J&W Scientific, Folsom, CA, USA). The oven was programmed at 120ºC for 1 min, to 160 ºC at 25/ºC min, to 240ºC at 4ºC/min, held for 2 min, and to 250ºC at 25ºC/min, held for 5 min. Injector and detector temperatures were set at 250ºC and 270ºC, respectively. Helium was used as the carrier gas with an injection split ratio of 50:1 (v/v). Peak identification and quantification were performed with reference retention times and peak area as weight percent of standard FAME mixture (Nu-Chek-Prep, Inc., Elysian, NM, USA).

2.2.8 Protein extraction from liver and western blotting

One mL of the solution made from radioimmune precipitation assay (RIPA) buffer (Biosesang Inc., Seongnam, Korea) and protease inhibitor cocktail #6 (Biosesang Inc.) at the ratio of 100:1 was added to 100 mg liver tissue, followed by homogenizing for 30 sec. After agitation for 30 min, the mixture was centrifuged at 4ºC at 12,000×g using a centrifuge (Micro 17R+, Hanil Science Industrial Co). The supernatant was transferred to a new tube and protein concentration was determined by modified Lowry protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA). Protein solution was adjusted to contain equal concentrations of the proteins using RIPA buffer. The samples were then separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Electrophoresis was done at 75 V for 30 min and 125 V for 90 min. After electrophoresis, the proteins on the gel were transferred to nitrocellulose (NC) transfer membrane (Whatman International Ltd.) at 100 V for 90 min. The transferred membrane was blocked with 5% skim milk solution (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Primary and secondary antibodies were added to the NC membrane, followed by stirring for 2 h and washing three times at 10 min intervals using tris-buffered saline (TBS; Bio-Rad Laboratories) containing 0.1% tween 20. Results were visualized using a chemiluminescent substrate kit (West-Q chemiluminescent substrate kit; GenDEPOT Inc., Barker, TX, USA) in an X-ray film. Relative protein levels were

determined via scanning densitometry analysis using Quantity One software (Bio-Rad, Richmond, CA, USA).

2.2.9 Total RNA extraction, cDNA synthesis, and real-time polymerase chain reaction (PCR)

RNA from liver and epididymal adipose tissue were extracted with TRIzol reagent (Sigma Chemical Co.). Fifty mg of liver tissue or 100 mg of epididymal adipose tissue was homogenized in 1 mL TRIzol reagent using a Tissuelyser (QIAGEN, Hilden, Germany) at room temperature. The homogenized sample was incubated for 5 min at room temperature. After 0.2 mL chloroform (Sigma Chemical Co.) was added, the sample was shaken vigorously for 15 sec, followed by incubated for 3 min at room temperature, and centrifuged at 12,000×g for 15 min at 4ºC. The colorless aqueous phase was transferred to a fresh tube, followed by adding 0.5 mL isopropanol (Sigma Chemical Co.). After incubation for 10 min, the sample was centrifuged at 12,000×g for 10 min at 4ºC. Supernatant was removed, and 1 mL 75% ethanol (Sigma Chemical Co.) was added to wash the RNA pellet. The ethanol solution was discarded, followed by drying the RNA pellet. The RNA pellet was dissolved in 50 μL 0.1 % (w/w) diethyl pyrocarbonate (DEPC; Sigma Chemical Co.) aqueous solution. Absorbance of the diluted RNA solution was measured at 260 nm using a spectrophotometer (Beckman DU 530, Bechman Coulter Inc., Fullerton, CA, USA) to determine the concentration of RNA. cDNA was synthesized using a GoScriptTM Revers Transcription kit (Promega, Madison, WI, USA). mRNA levels

were quantified by Applied Biosystems StepOne Real-Time PCR system (Life Technologies Co., Carlsbad, CA, USA). PCR reactions were carried out at 95ºC for 2 min for initiation, at 95ºC for 15 sec for denaturation, and at 60ºC for 60 sec for annealing up to 40 cycles. To normalize the results, Δthreshold cycle (Ct) was calculated by subtracting the Ct value of house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression of the genes was calculated from $2^{-\Delta\Delta Ct}$. Specific primer sequences used in this study are shown in Table 2.2.

**Table 2.2 Primer sequences used in real-time quantitative PCR**

| | | |
|---|---|---|
| GAPDH | Forward (5'-3') | GGAGAAACCTGCCAAGTA |
| | Reverse (5'-3') | AAGAGTGGGAGTTGCTGTTG |
| NF-κB | Forward (5'-3') | ATTTGCTTTGTGTTGTTA |
| | Reverse (5'-3') | TTACAGTAGATGGCTAGA |
| TNFα | Forward (5'-3') | AAGCCTGTAGCCCACGTCGTA |
| | Reverse (5'-3') | GGCACCACTAGTTGGTTGTCTTTG |
| IL-1ß | Forward (5'-3') | CAACCAACAAGTGATATTCTCCATG |
| | Reverse (5'-3') | GATCCACACTCTCCAGCTGCA |
| IL-6 | Forward (5'-3') | ATGGATGCTACCAAACTGGAT |
| | Reverse (5'-3') | TGAAGGACTCTGGCTTTGTCT |
| iNOS | Forward (5'-3') | CCCTTCCGAAGTTTCTGGCAGCAGC |
| | Reverse (5'-3') | GGCTGTCAGAGAGCCTCGTGGCTTTGG |
| COX2 | Forward (5'-3') | GGAGAGACTATCAAGATAGTGATC |
| | Reverse (5'-3') | ATGGTCAGTAGACTTTTACAGCTA |
| MCP1 | Forward (5'-3') | CCACTCACCTGCTGCTACTCAT |
| | Reverse (5'-3') | TGGTGATCCTCTTGTAGCTCT CC |
| IL-10 | Forward (5'-3') | GGTTGCCAAGCCTTATCGGA |
| | Reverse (5'-3') | ACCTGCTCCACTGCCTTGCT |
| Arginase-1 | Forward (5'-3') | TGGCTTGCGAGACGTAGAC |
| | Reverse (5'-3') | GCTCAGGTGAATCGGCCTTTT |
| Chi3l3 | Forward (5'-3') | AGAAGGGAGTTTCAAACCTGGT |
| | Reverse (5'-3') | GTCTTGCTCATGTGTGTAAGTGA |
| Mgl1 | Forward (5'-3') | TGAGAAAGGCTTTAAGAACTGGG |
| | Reverse (5'-3') | GACCACCTGTAGTGATGTGGG |
| Leptin | Forward (5'-3') | CCGCCAAGCAGAGGGTCAC |
| | Reverse (5'-3') | GCATTCAGGGCTAACATCCAACT |
| Adiponectin | Forward (5'-3') | GGCTCTGTGCTGCTCCATCT |
| | Reverse (5'-3') | AGAGTCGTTGATGTTATCTGCATAG |

Continued

| | | |
|---|---|---|
| CD36 | Forward (5'-3') | CCAAGCTATTGCGACATGATT |
| | Reverse (5'-3') | TCTCAATGTCCGAGACTTTTCA |
| FABP1 | Forward (5'-3') | GAACTCATTGCGGACCACTT |
| | Reverse (5'-3') | CATCCAGAAAGGGAAGGACAT |
| SREBP-1c | Forward (5'-3') | GCCCACAATGCCATTGAGA |
| | Reverse (5'-3') | GCAAGACAGCAGATTTATTCAGCTT |
| FAS | Forward (5'-3') | CCTGGATAGCATTCCGAACCT |
| | Reverse (5'-3') | AGCACATCTCGAAGGCTACACA |
| SLC24A1 | Forward (5'-3') | TTCCCTTTAGCCCTTGTTCC |
| | Reverse (5'-3') | TGACCAGACTTCCTCCAACC |
| CPT1A | Forward (5'-3') | GATGTTCTTCGTCTGGCTTGA |
| | Reverse (5'-3') | CTTATCGTGGTGGTGGGTGT |
| ACADL | Forward (5'-3') | TCGCAATATAGGGCATGACA |
| | Reverse (5'-3') | ACTTGGGAAGAGCAAGCGTA |
| HADHα | Forward (5'-3') | CCCTTTGAACACTTGCTGCT |
| | Reverse (5'-3') | GCCCAGGTCTCTGTGGATAA |
| ACOX | Forward (5'-3') | CTTGTTCGCGCAAGTGAGG |
| | Reverse (5'-3') | CAGGATCCGACTGTTTACC |
| PPARα | Forward (5'-3') | GCAGTGGAAGAATCGGACCT |
| | Reverse (5'-3') | CAACCCGCCTTTTGTCATAC |
| PPARγ | Forward (5'-3') | CAGCAGGTTGTCTTGGATGTC |
| | Reverse (5'-3') | AGCCCTTTGGTGACTTTATGG |

2.2.10 Serum analysis

Serum leptin and adiponectin (BioVendor Laboratory Medicine, Inc., Modrice, Czech Republic) concentrations were determined using commercial kits based on enzyme-linked immunosorbent assay. In detail, 100 μL standard, 100 μL blank, and 100 μL diluted serum were added to 96-well plate coated with mouse-specific antibody. After 1 h incubation at room temperature, each well of 96-well plate was washed 3 times with 350 μL washing buffer. 100 μL biotin labelled antibody solution was added to each well and incubated for 1 h at room temperature. After incubation, each well of 96-well plate was washed 3 times with 350 μL washing buffer and 100 μL streptavidin-HRP (horseradish peroxidase) conjugate was added to each well. After 30 min incubation at room temperature, each well of 96-well plate was washed 3 times with 350 μL washing buffer. One hundred μL substrate solution was added, followed by incubating for 10 min at room temperature. One hundred μL acidic stop solution was added to stop the reaction. Absorbances were measured at 450 nm and 630 nm using a microplate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA). Leptin and adiponectin concentrations were calculated using corresponding standard curves.

Serum TG concentration was determined using a commercial kit (Asan Pharmaceutical Co., Ltd., Seoul, Korea) based on enzymatic assay. Two μL serum or standard was added to each well of 96-well plate and 300 μL enzyme solution was added to each well, followed by incubating for 10 min at 37 ºC. Absorbance was

measured at 550 nm using a spectrophotometer (Spectramax 190, Molecular Devices). Concentration of TG was calculated using corresponding standard curve.

Serum total cholesterol concentration was determined using a commercial kit (Asan Pharmaceutical Co., Ltd.) based on enzymatic assay. Two μL serum or standard was added to each well of 96-well plate and 300 μL enzyme solution was added to each well, followed by incubating for 5 min at 37 ºC. Absorbance was measured at 500 nm (Spectramax 190, Molecular Devices). Concentration of cholesterol was calculated using corresponding standard curves.

Serum HDL-C concentration was determined using a commercial kit (Asan Pharmaceutical Co., Ltd.) based on enzymatic assay. Twenty μL serum was incubated with 20 μL dextran sulfate to precipitate apo B-containing lipoprotein. Ten μL supernatant was added to each well of 96-well plate and 300 μL enzyme solution was added to each well, followed by incubating for 5 min at 37 ºC. Absorbance was measured at 500 nm (Spectramax 190, Molecular Devices). Concentration of cholesterol was calculated using corresponding standard curves.

Serum non-esterified fatty acids (NEFA) concentration was determined using a commercial kit (Wako Pure Chemical Industries, Osaka, Japan). Four μL serum or standard was added to each well of 96-well plate, followed by incubating for 10 min at 37 ºC with 80 μL enzyme solution A. One hundred sixty μL enzyme solution B was added, followed by incubating for 10 min at 37 ºC. Absorbance was measured at 550

nm (Spectramax 190, Molecular Devices). Concentration of NEFA was calculated using corresponding standard curves.

2.2.11 Lipid extraction from liver and analysis of liver lipid content

Lipid was extracted from livers of the experimental mice by modified Bligh and Dyer (1959) method. Three hundred mg of the liver tissue was mixed with 6 mL chloroform (Samchun Chemical Co., Ltd.) and 3 mL methanol (Samchun Chemical Co., Ltd.), followed by homogenizing with a homogenizer (Daihan Scientific Co., Ltd.), stirring for 16 h and filtering with Whatman No. 2 filter paper (Whatman International Ltd.). The filter paper were washed with 1 mL 0.9 % NaCl (Samchun Chemical Co., Ltd.), collecting lower layer (chloroform layer) with NaCl solution, followed by concentration using nitrogen gas. The concentrated lipid was used for determining lipid content of liver.

2.2.12 Hepatic lipid analysis

Total lipids for lipid profile of liver were extracted according to the Bligh and Dyer (1959) method with modification. Fifty mg of liver tissue was homogenized in 400 μL methanol. Eight hundreds μL chloroform was added to homogenized sample, followed by incubating for 17 h at room temperature. After centrifugation at 2,000×g for 10 min at 4°C, bottom layer (lower transparent part) was transferred into a fresh tube and evaporated with nitrogen gas. One hundred μL isopropanol was added to the

lipid pellet and amounts of TG, NEFA, and total cholesterol from lipids were determined using methods mentioned above in 2.2.10.

2.2.13 Statistical analysis

The experiments were repeated 3 times and the values were expressed as mean ± standard deviation. Statistical analyses were processed with SPSS program (version 21.0, SPSS Chicago, IL, USA) using unpaired t-tests ($p<0.05$, $p<0.01$, or $p<0.001$).

**2.3 Results**

2.3.1 Fatty acid compositions of BRS oil and experimental diets

Major fatty acids composing the BRS oil used in the study were linoleic (57.0%), α-linolenic (29.4%), and oleic (9.80%) acids (Table 2.3). Soybean and corn oils used in the control diet were also analyzed for comparison. Linoleic (56.0%) and oleic (20.0%) acids were the major fatty acids in soybean oil. Linoleic acid (56.1%) was the most abundant in corn oil, followed by oleic acid (27.8%). Fatty acid compositions of the experimental diets are shown in Table 2.4. ALA content was significantly ($P<0.001$) higher in BRS oil diet than in the control diet.

**Table 2.3 Fatty acid compositions of oils (unit: %, w/w)**

| Fatty acid | Black raspberry seed oil | Soybean oil | Corn oil |
|---|---|---|---|
| 16:0 | 2.01 ± 0.01 | 10.6 ± 0.05 | 11.6 ± 0.00 |
| 18:0 | 0.83 ± 0.01 | 4.00 ± 0.2 | 2.16 ± 0.00 |
| 20:0 | 0.40 ± 0.01 | 0.34 ± 0.00 | 0.48 ± 0.00 |
| Saturated | 3.78 | 14.9 | 14.2 |
| 18:1 | 9.80 ± 0.01 | 20.0 ± 0.07 | 27.8 ± 0.05 |
| 20:1 | 0.37 ± 0.09 | 0.10 ± 0.15 | 0.28 ± 0.00 |
| MUFA | 10.2 | 20.1 | 28.1 |
| 18:2n-6 | 57.0 ± 0.01 | 56.0 ± 0.06 | 56.1 ± 0.03 |
| 18:3n-3 | 29.4 ± 0.08 | 8.23 ± 0.02 | 1.23 ± 0.00 |
| 20:4n-6 | 0.20 ± 0.00 | 0.39 ± 0.01 | 0.20 ± 0.01 |
| PUFA | 86.6 | 64.6 | 57.5 |

Values are means and standard deviations (n=3).

MUFA: mono unsaturated fatty acid; and PUFA: poly unsaturated fatty acid

**Table 2.4 Fatty acid compositions of the diets (unit: %, w/w)**

| Fatty acid | Control | Black raspberry seed oil |
|---|---|---|
| 8:0 | 0.09 ± 0.01 | 0.08 ± 0.01 |
| 10:0 | 0.11 ± 0.00 | 0.10 ± 0.01 |
| 12:0 | 0.82 ± 0.01 | 0.82 ± 0.02 |
| 14:0 | 1.32 ± 0.01 | 1.34 ± 0.01 * |
| 16:0 | 28.5 ± 0.15 | 28.1 ± 0.05 ** |
| 18:0 | 12.3 ± 1.75 | 13.4 ± 1.13 |
| 20:0 | 0.53 ± 0.03 | 0.54 ± 0.05 |
| 22:0 | 0.13 ± 0.02 | 0.12 ± 0.01 |
| 24:0 | 0.29 ± 0.10 | 0.28 ± 0.03 |
| Saturated | 44.1 | 44.7 |
| 14:1 | 0.04 ± 0.00 | 0.04 ± 0.00 |
| 16:1 | 1.19 ± 0.01 | 1.22 ± 0.02 * |
| 18:1 | 35.9 ± 1.76 | 33.2 ± 1.11 |
| 20:1 | 0.1 ± 0.03 | 0.07 ± 0.01 |
| 22:1 | 0.07 ± 0.01 | 0.06 ± 0.01 |
| 24:1 | 0.31 ± 0.06 | 0.22 ± 0.05 |
| MUFA | 37.6 | 34.9 |
| 18:2n-6 | 17.0 ± 0.25 | 15.6 ± 0.08 ** |
| 18:3n-3 | 1.09 ± 0.01 | 4.53 ± 0.10 *** |
| 20:2n-6 | 0.06 ± 0.02 | 0.08 ± 0.00 |
| 20:3n-6 | 0.13 ± 0.01 | 0.13 ± 0.01 |
| 20:4n-6 | 0.11 ± 0.01 | 0.1 ± 0.01 |
| PUFA | 18.3 | 20.4 |
| n-6/n-3 | 15.9 ± 0.09 | 3.51 ± 0.06*** |

Values are means and standard deviations (n=3). *, **, *** Statistical significance by unpaired t-test ($P<0.05$, $P<0.01$, or $P<0.001$).

MUFA: mono unsaturated fatty acid; and PUFA: poly unsaturated fatty acid

### 2.3.2 Body weight, food intake, and organ weight of experimental animals

There were no significant ($P>0.05$) differences in initial body weight, final body weight, weight gain, food intake, and food efficiency between the two groups (Table 2.5). The weights of liver, epididymal adipose tissue, and kidney were higher in the BRS oil group than those of the control group with no significant ($P>0.05$) difference.

### 2.3.3 Fatty acid compositions of livers of experimental animals

ALA in the liver of the BRS oil group accounted for 1.84% of the total fatty acids, whereas ALA content of the control group was 0.45% (Table 2.6). These two values were significantly ($P<0.01$) different. Sum of n-3 fatty acids was also significantly ($P<0.01$) higher in the BRS oil group than in the control group. Content of AA, the substrate of 2 and 4 series of eicosanoids known to cause inflammatory responses, was lower in the liver of the BRS oil group (2.42%) than in the control group (3.40%) with no significant ($P>0.05$) difference. The mice fed the BRS oil diet had significantly ($P<0.001$) lower n-6 to n-3 fatty acid ratio than the mice fed the control diet. These differences in the fatty acid composition of the livers between the two groups might be due to the source of the oils as BRS oil contains higher amount of ALA than soybean and corn oils used in the control diet.

**Table 2.5 Body weight, food intake, and organ weights of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

| | Control | Black raspberry seed oil |
|---|---|---|
| Initial weight (g) | 19.8 ± 1.17 | 19.9 ± 1.07 |
| Final weight (g) | 44.9 ± 3.34 | 45.4 ± 1.83 |
| Weight gain (g) | 24.7 ± 3.11 | 25.3 ± 1.52 |
| Food intake (g/day) | 2.69 ± 0.16 | 2.74 ± 0.15 |
| FER (%) | 11.3 ± 0.94 | 11.6 ± 0.53 |
| Liver (g) | 1.94 ± 0.54 | 2.05 ± 0.34 |
| Epididymal adipose tissue (g) | 2.20 ± 0.92 | 2.58 ± 0.30 |
| Spleen (g) | 0.10 ± 0.03 | 0.10 ± 0.01 |
| Kidney (g) | 0.49 ± 0.08 | 0.52 ± 0.07 |

Values are means and standard deviations. No statistical significance by unpaired t-test ($P>0.05$).

FER (food efficiency ratio, %) = (weight gain (g)/food intake (g)) × 100

**Table 2.6 Fatty acid compositions of livers of high-fat diet-induced obese mice fed control and black raspberry seed oil diets (unit: %, w/w)**

| Fatty acid | Control | Black raspberry seed oil |
|---|---|---|
| 14:0 | 0.54 ± 0.05 | 0.46 ± 0.04 * |
| 16:0 | 27.2 ± 0.87 | 28.2 ± 0.41 |
| 18:0 | 4.06 ± 0.76 | 3.53 ± 0.58 |
| 20:0 | 0.35 ± 0.05 | 0.34 ± 0.18 |
| 24:0 | 0.54 ± 0.23 | 1.17 ± 0.12 ** |
| Saturated | 32.7 | 33.7 |
| 16:1 | 2.09 ± 0.53 | 2.23 ± 0.35 |
| 18:1 | 41.3 ± 4.31 | 38.8 ± 3.79 |
| 20:1 | 0.88 ± 0.16 | 0.84 ± 0.14 |
| MUFA | 46.3 | 45.3 |
| 18:2n-6 | 15.7 ± 2.39 | 15.9 ± 2.35 |
| 18:3n-3 | 0.45 ± 0.21 | 1.84 ± 0.53 ** |
| 20:2n-6 | 0.52 ± 0.09 | 0.36 ± 0.02 * |
| 20:3n-6 | 0.94 ± 0.05 | 0.83 ± 0.04 * |
| 20:4n-6 | 3.4 ± 0.73 | 2.42 ± 0.55 |
| 22:6n-3 | 2.05 ± 0.71 | 3.38 ± 0.48 ** |
| PUFA | 21.0 | 21.4 |
| ∑6 | 20.6 ± 3.13 | 19.4 ± 2.51 |
| ∑3 | 2.50 ± 0.91 | 5.22 ± 0.86** |
| n-6/n-3 | 8.67 ± 1.69 | 3.73 ± 0.17*** |

Values are means and standard deviations (n=6/group). *, **, *** Statistical significance by unpaired t-test ($P<0.05$, $P<0.01$, or $P<0.001$).

MUFA: mono unsaturated fatty acid; and PUFA: poly unsaturated fatty acid

2.3.4 Protein expressions involved in inflammation in liver

Protein expressions of TLR4, NF-κB, phospho-NF-κB, COX2, I-κBα, and phospho-I-κBα in the livers of the mice are shown in Fig. 2.2. TLR4, NF-κB, phospho-NF-κB, COX2, and phospho-I-κBα protein expressions were lower in the BRS oil group than those of the control group with no significant ($P>0.05$) difference. Whereas, I-κBα, which inhibits the activation of NF-κB expression, was higher in the BRS oil group than in the control group without significant ($P>0.05$) difference.

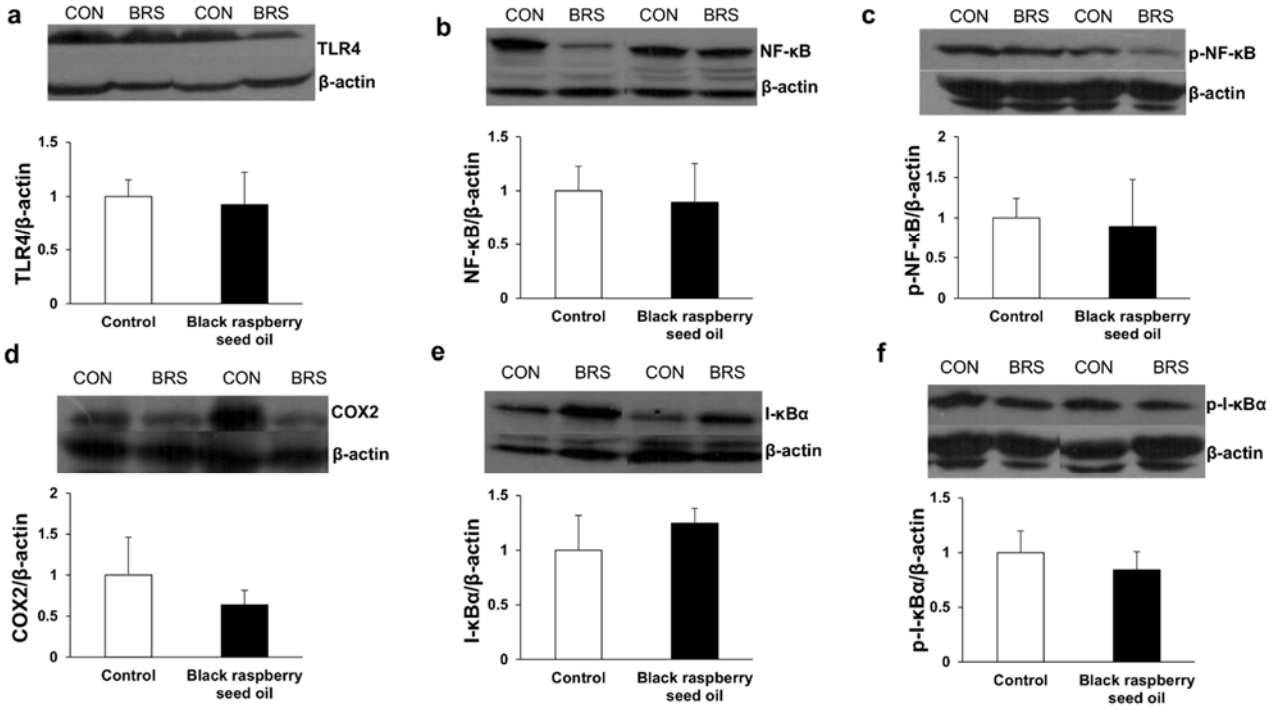


**Fig. 2.2 Protein expressions involved in inflammation in the livers of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=4/group).

CON: control; and BRS: black raspberry seed oil

### 2.3.5 mRNA levels involved in pro-inflammation in liver and epididymal adipose tissue

Relative mRNA levels involved in pro-inflammation including NF-κB, TNFα, IL-1β, IL-6, iNOS, COX2, and MCP1 in the livers of the mice are shown in Fig. 2.3. mRNA levels of NF-κB, TNFα, and COX2 were lower in the liver of the mice fed the BRS oil diet than those of the control diet without significant ($P>0.05$) difference. mRNA levels of IL-1β, iNOS, MCP-1 ($P<0.05$), and IL-6 ($P<0.01$) were significantly lower in the liver of the BRS oil group than those of the control group.

Relative mRNA levels associated with pro-inflammation in the epididymal adipose tissue of the mice are shown in Fig. 2.4. mRNA levels of NF-κB, IL-6, iNOS, COX2, and MCP1 were lower in the BRS oil diet group than those of the control diet with no significant ($P>0.05$) difference. TNFα and IL-1β mRNA levels in the BRS oil group were significantly ($P<0.01$) lower than those of the control group.

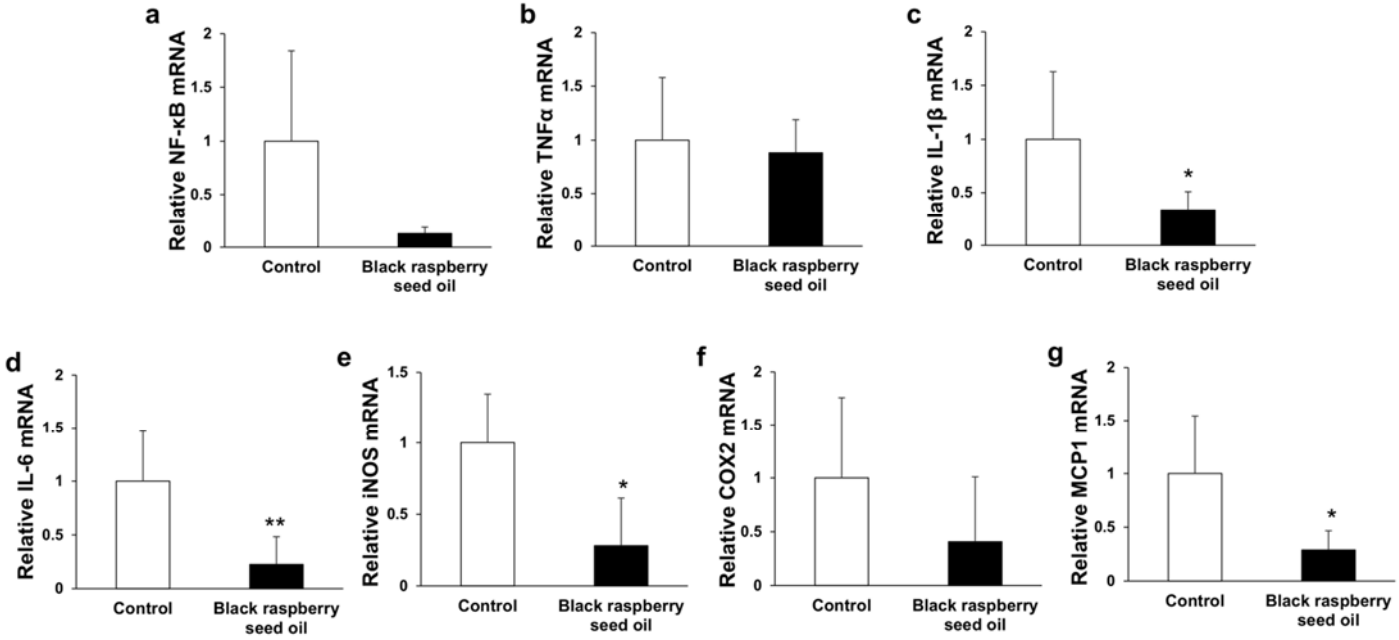


**Fig. 2.3 Relative mRNA levels involved in pro-inflammation in the livers of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=8/group). *, ** Statistical significance by unpaired t-test ($P<0.05$, or $P<0.01$).

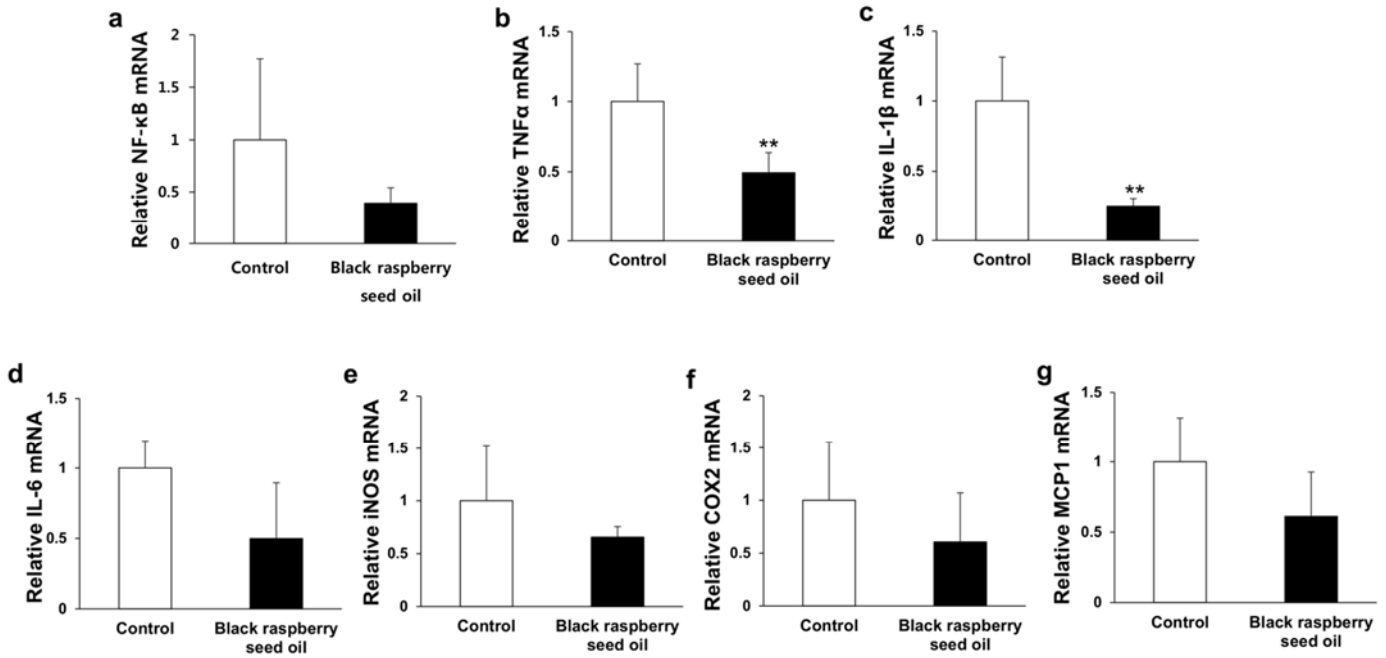


**Fig. 2.4 Relative mRNA levels involved in pro-inflammation in the epididymal adipose tissues of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=8/group). ** Statistical significance by unpaired t-test ($P<0.01$).

### 2.3.6 mRNA levels involved in anti-inflammation in liver and epididymal adipose tissue

Relative mRNA levels involved in anti-inflammation such as IL-10, arginase1, Chi3l3, and macrophage galactose N-acetyl-galactosamine-specific lectin 1 (Mgl1) in the liver of the mice are shown in Fig. 2.5. mRNA levels of arginase 1 and Chi3l3 were significantly ($P<0.05$) higher in the liver of the BRS oil group than those of the control. IL-10 and Mgl1 mRNA levels were higher in the liver of the BRS oil group than those of the control without significant ($p>0.05$) difference.

All of the four anti-inflammatory response markers (IL-10, arginase 1, Chi3l3, and Mgl1) were significantly higher in the epieieymal adipose tissue of the BRS oil group than those of the control (IL-10, Chi3l3, and Mgl1: $P<0.05$; and arginase1: $P<0.01$) (Fig. 2.6).

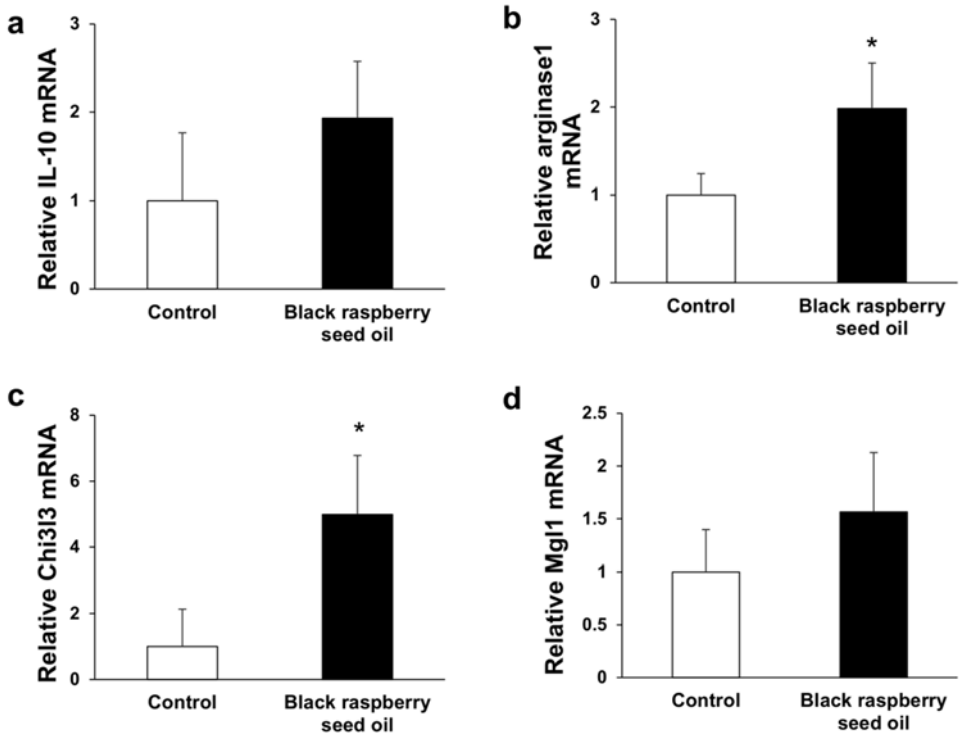


**Fig. 2.5 Relative mRNA levels involved in anti-inflammation in the livers of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=8/group). * Statistical significance by unpaired t-test ($P<0.05$).

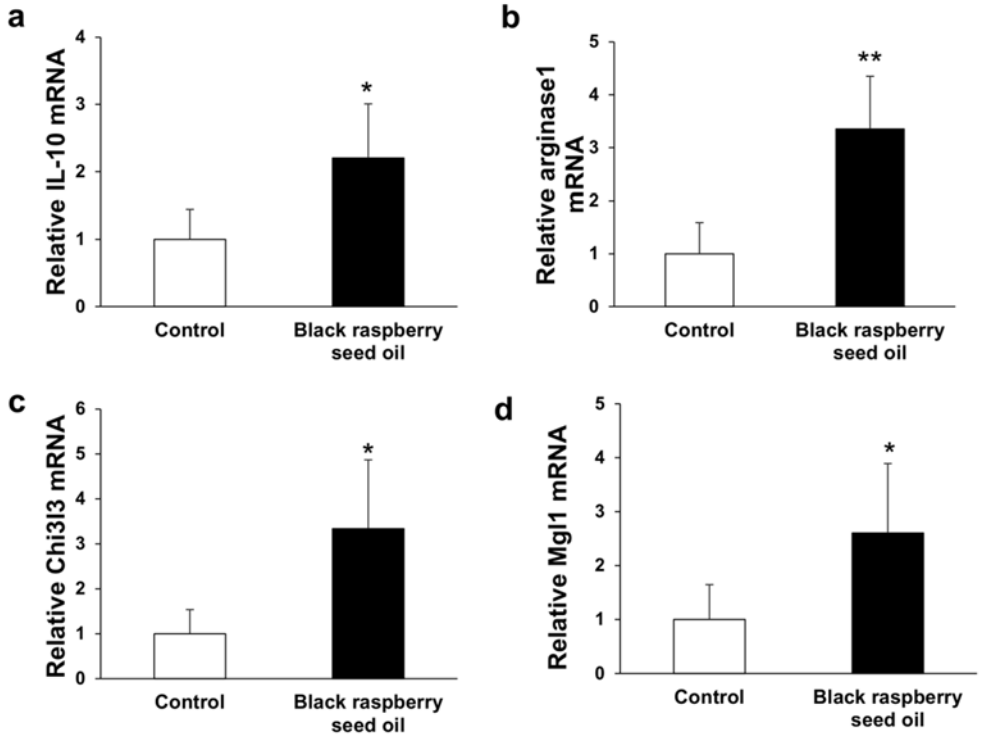


**Fig. 2.6 Relative mRNA levels involved in anti-inflammation in the epididymal adipose tissues of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=8/group). *, ** Statistical significance by unpaired t-test ($P<0.05$, or $P<0.01$).

### 2.3.7 Leptin and adiponectin levels in serum and epididymal adipose tissue

Leptin level in serum was lower in the mice fed BRS oil diet than in the control diet without significant ($P>0.05$) difference. mRNA level of leptin was lower in the epididymal adipose tissue of the mice fed BRS oil than in the control without significant ($P>0.05$) difference. mRNA level of adiponectin, which stimulates anti-inflammatory cytokine production, was significantly ($P<0.01$) higher in the epididymal adipose tissue of the BRS oil group than in the control. Whereas, adiponectin level in serum was higher in the mice fed BRS oil diet than in the control diet with no significant ($P>0.05$) difference (Fig. 2.7).

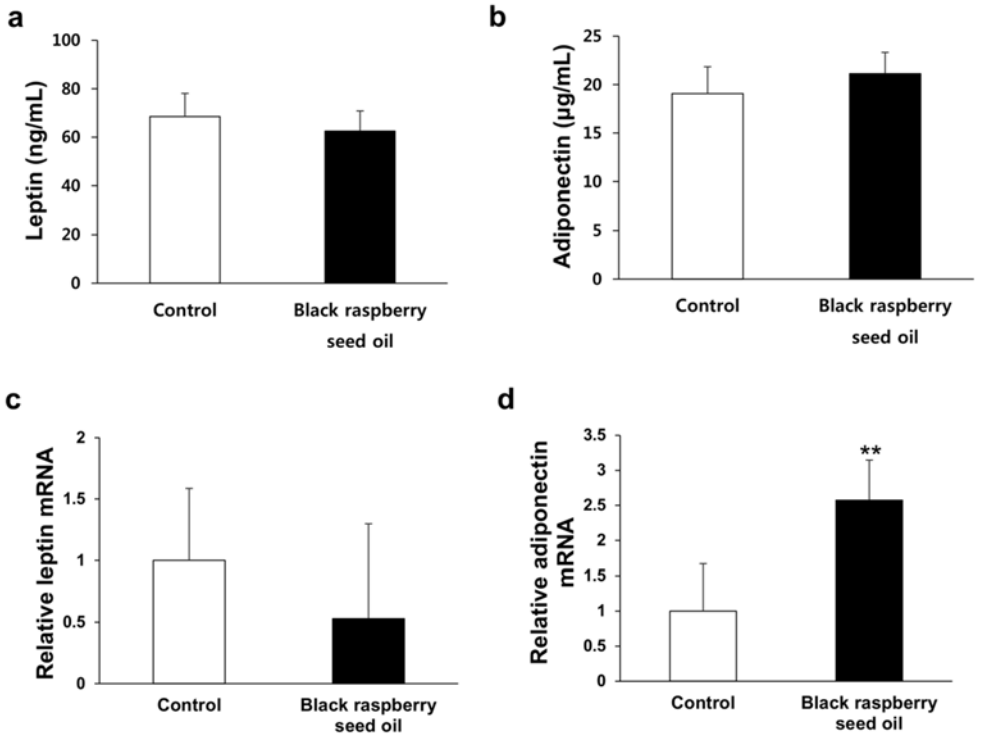


**Fig. 2.7 Leptin and adiponectin levels in serums (a and b) and epididymal adipose tissues (c and d) of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=8/group). ** Statistical significance by unpaired t-test ($P<0.01$).

2.3.8 Lipid profiles of serum and liver

As shown in Fig. 2.8, levels of TG in the serum and liver of the mice fed BRS oil were 14.2% and 12.1%, respectively, lower than those of the control group. NEFA, a primary factor that causes hepatic lipid accumulation, in the serum and liver of the mice fed BRS oil were 42.1% and 25.7%, respectively, lower ($P<0.05$) than in the control diet-fed mice. Total cholesterol in the serum and liver of the BRS oil group was also 13.0% ($P<0.05$) and 53.2% ($P<0.001$), respectively, lower than that of the control group. Liver total lipid was 13.0% lower in the BRS oil group than in the control group without significant ($P > 0.05$) difference. The serum of the BRS oil-treated group showed slightly higher HDL-C level than that of the control group, although the difference is not significant ($P>0.05$).

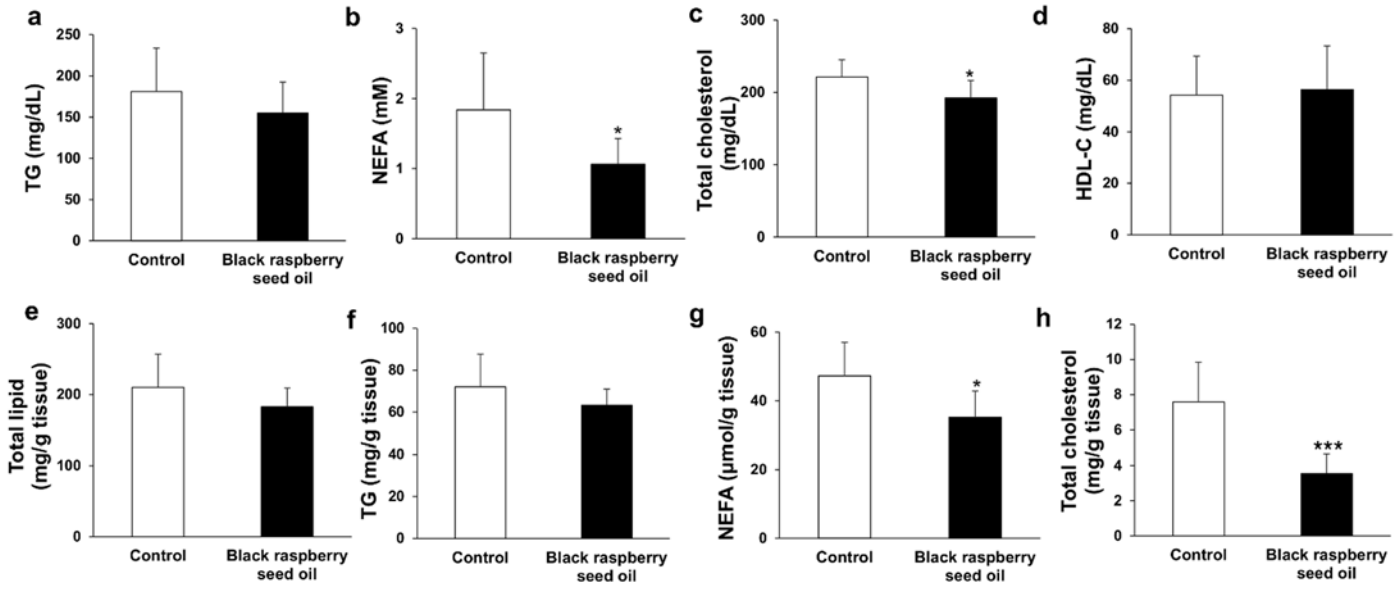


**Fig. 2.8 Lipid profiles of serums (a-d) and livers (e-h) of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=9/group). *, *** Statistical significance by unpaired t-test ($P<0.05$, or $P<0.001$).

2.3.9 mRNA levels involved in lipogenesis in liver

To determine the effect of BRS oil on lipogenic pathways in liver of the high-fat diet-induced obese mice, mRNA levels of lipogenic markers were analyzed (Fig. 2.9). It was observed that administration of BRS oil significantly ($P<0.05$) lowered mRNA levels of CD36 and SREBP-1c compared with the control. mRNA levels of FABP1, FAS, and solute carrier family 25 member 1 (SLC25A1) were also lower in the BRS oil-treated group than those of the control group, although the difference is not significant ($P>0.05$).

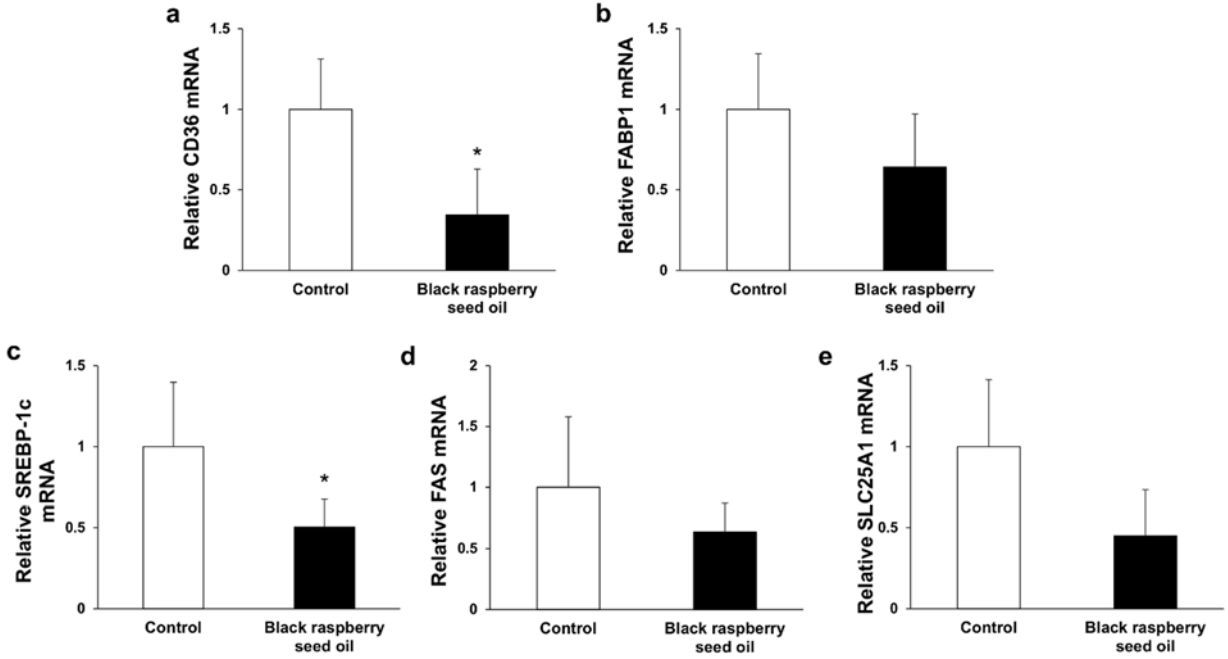


**Fig. 2.9 Relative mRNA levels involved in lipogenesis in the livers of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=8/group). * Statistical significance by unpaired t-test ($P<0.05$).

2.3.10 mRNA levels involved in fatty acid oxidation in liver

Fatty acid oxidation markers including CPT1A, acyl-CoA dehydrogenase (ACADL), hydroxylacyl-CoA dehydrogenase α (HADHα), and acyl-CoA oxidase (ACOX) were examined to determine the effect of BRS oil on fatty acid oxidation in liver of the high-fat diet-induced obese mice (Fig. 2.10). mRNA levels of CPT1A and ACADL were significantly ($P<0.01$) higher in the liver of the mice fed BRS oil than those of the control mice. mRNA level of ACOX, which is an enzyme of peroxisomal β-oxidation of fatty acids, was also significantly ($P<0.001$) higher in the BRS oil-treated group than that of the control group. HADHα mRNA level was higher in the liver of the mice fed the BRS oil diet than the control diet without significant ($P>0.05$) difference.

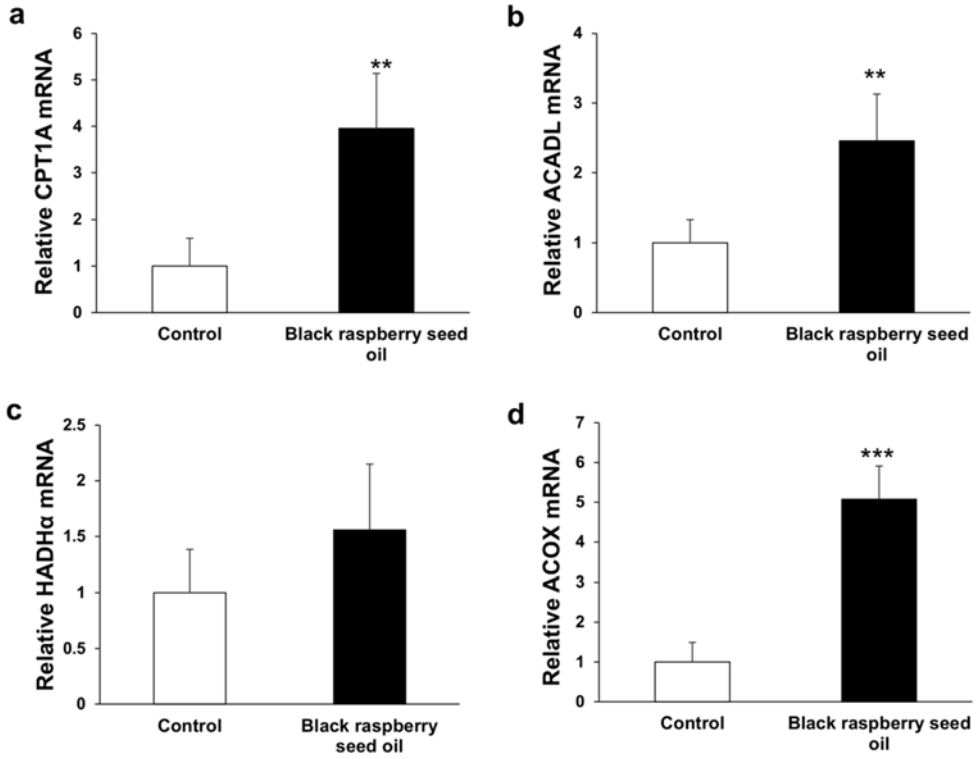


**Fig. 2.10 Relative mRNA levels involved in fatty acid oxidation in livers of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=8/group). **, *** Statistical significance by unpaired t-test ($P<0.01$ or $P<0.001$).

2.3.11 mRNA and protein levels of PPAR in liver and epididymal adipose tissue

PPARα and PPARγ have contrasting functions in regulation of lipid metabolism. PPARα promotes fatty acid oxidation, while activation of PPARγ promotes lipogenesis (Berlanga A et al. 2014). PPARα significantly increased both in mRNA ($P<0.001$) and protein ($P<0.01$) levels in the liver of the mice fed BRS oil diet compared with the control diet. However, PPARγ mRNA and protein levels in the liver showed no significant ($P>0.05$) differences between the two groups (Fig. 2.11).

PPARα mRNA level was significantly ($P<0.05$) higher in the epididymal adipose tissue of the mice fed BRS oil than that of the control. However, there were no significant ($P>0.05$) differences in PPARα protein and PPARγ mRNA and protein expression levels in the epididymal adipose tissue between the two groups (Fig. 2.12).

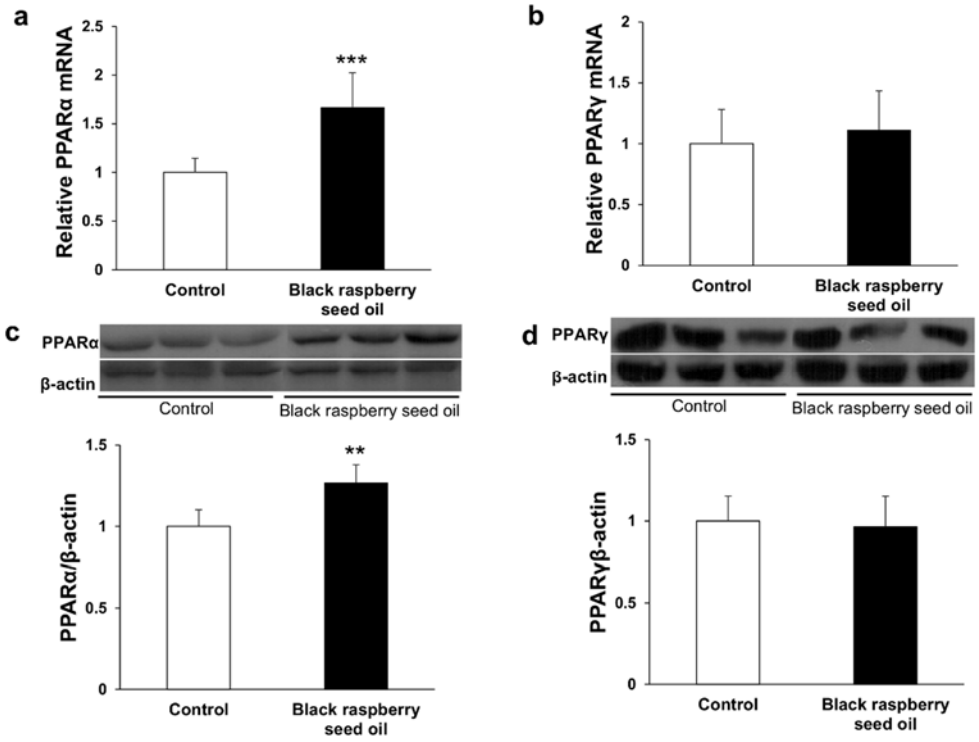


**Fig. 2.11 Relative mRNA and protein levels of PPAR in the livers of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=8/group (a, b), n=4/group (c, d)). **, *** Statistical significance by unpaired t-test (P<0.01 or P<0.001).

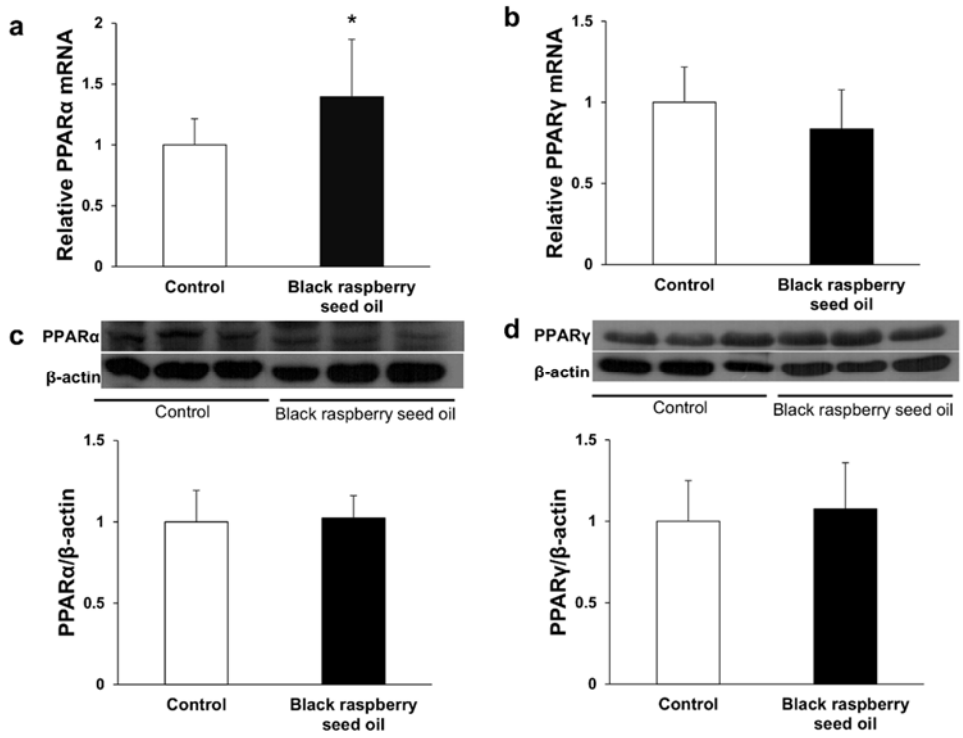


**Fig. 2.12 Relative mRNA and protein levels of PPAR in the epididymal adipose tissues of high-fat diet-induced obese mice fed control and black raspberry seed oil diets**

Each bar represents means and standard deviations (n=8/group (a, b), n=4/group (c, d)). * Statistical significance by unpaired t-test ($P<0.01$).

## 2.4 Discussion

In this study, ALA content in the liver of the mice fed BRS oil diet was higher than that of the control diet. On the other hand, AA content was lower in the BRS oil group than in the control group. It has been reported that higher ratio of n-6 to n-3 PUFA causes increased rate of heart disease and arteriosclerosis and mortality of these diseases (Simopoulos 2002; Margioris 2009; Russo 2009). Inflammatory cells typically contain a high proportion of AA. AA is the major substrate for inflammatory eicosanoids (Calder 2005). Calder (2005) reported that contents of AA and n-3 PUFA can be altered through oral administration of n-3 PUFA.

González-Périz et al. (2009) reported that pro-inflammatory mediators decreased in obese mice after intake of DHA-fortified diet. TNFα, IL-1β, and IL-6 production significantly ($P<0.05$) decreased in mice with ovalbumin-induced allergy when fed 5% ALA-rich perilla oil diet compared with 5% linoleic acid rich corn oil diet (Chang et al. 2008). In this study, NF-κB mRNA level decreased when BRS oil was fed high-fat diet-induced obese mice. The mRNA levels of TNFα, IL-1β, IL-6, iNOS, COX2, and MCP-1, which are regulated by NF-κB, were also lower in the BRS oil group than those of the control group. These results suggest that intake of BRS oil might reduce inflammation induced by obesity through inhibition of NF-κB signaling.

BRS oil also had an anti-inflammatory effect through promoted expressions of anti-inflammatory mediators such as IL-10, arginase1, Chi3l3, Mgl1, and adiponectin. Lumeng et al. (2007) reported that mRNA expressions of IL-10, arginase 1, Mgl1,

and Chi3l3 in adipose tissue macrophages isolated from lean mice were higher than those from mice fed high-fat diet. These results are consistent with those of a previous study that the expressions of Mgl1, arginase 1, and Chi3l3 tend to show similar trends (Ohashi et al. 2010).

BRS oil contains a lot of tocopherols besides ALA. These substances have anti-oxidant and anti-inflammatory properties (Pieszka et al. 2013; Radočaj et al. 2014; Fotschki et al. 2015). Anti-inflammatory effect of BRS oil in this study might be relevant to large amounts of ALA and tocopherols present in the BRS oil although tocopherol content was not measured in this study.

Adipose tissue, once considered as a mere storage depot of energy in the form of fat, has been recently recognized as an important endocrine organ that secretes adipokines associated with homeostasis of the body and inflammatory response (Clària et al. 2011). Adipokines such as leptin and adiponectin are produced and secreted from adipose tissue. Leptin and adiponectin show opposite functions each other. Leptin in blood and adipose tissue is increased by pro-inflammatory stimulus. Whereas, adiponectin is recognized to have anti-inflammatory property (Ouchi et al. 2003). Leptin showed significant ($P<0.05$) correlations with body weight, body fat, LDL-C, and TG in female students (Choi et al. 2009). Blum (1997) also reported that blood leptin concentration increased in obese people. On the contrary, adiponectin concentration was lower in blood and adipose tissue of obese people than in the normal people (Ryo et al. 2004). Ohashi et al. (2010) reported that mRNA levels of

arginase 1, Mgl1, and IL-10 significantly ($P<0.05$) decreased and TNFα, MCP1, and IL-6 mRNA levels significantly ($P<0.05$) increased in adiponectin knock-out mice compared with wild type. DHA-rich oil increased adiponectin concentration in adult with abdominal obesity compared with n-6 PUFA-rich oil (Baril-Gravel et al. 2015). In this study, intake of BRS oil reduced leptin mRNA level, and increased adiponectin mRNA level in epididymal adipose tissue.

Dietary n-3 PUFA have been shown to have positive effects on lipid metabolism. Hashimoto et al. (2013) reported that total cholesterol, TG, and NEFA levels were lower in plasma and liver of mice fed high-fat diet containing fish oil than those of high-fat diet containing soybean oil and lard. NEFA, TG, total cholesterol, and HDL-C levels of plasma were significantly ($P<0.05$) lower in mice fed high n-3 PUFA diet than those of medium and low n-3 PUFA diet groups (Balogum et al. 2016). Major source of hepatic lipids is circulating NEFA. Therefore, an increase in the release of NEFA from adipocytes results in accelerated uptake of fatty acids by liver and increased production of TG, cholesterol, and LDL-C in serum (Balogum et al. 2016). In this study, NEFA, TG, and total cholesterol levels in both of the serum and liver were lower in the mice fed BRS oil than those of the control.

High-fat diet rich in fish oil reduced serum TG, total cholesterol, and LDL-C compared with high-fat control diet (Bargut et al. 2014). Also, PPARα and CPT1A mRNA levels in liver of mice fed high-fat fish oil were significantly ($P<0.05$) higher, and SREBP-1c and FAS mRNA levels were significantly ($P<0.05$) lower than in

high-fat control diet group (Bargut et al. 2014). Fukumitsu et al. (2013) reported that ALA suppressed the levels of mRNA related to lipogenic pathway including SREBP-1c and FAS in 3T3-L1 adipocytes. On the other hand, ALA enhanced the level of CPT1A mRNA which is related to the fatty acid oxidation in differentiated 3T3-L1 adipocytes (Fukumitsu et al. 2013). In this study, lopogenic pathway-related genes such as CD36, FABP1, SREBP-1c, FAS, and SLC25A1 in the liver of the mice fed BRS oil diet were lower and fatty acid oxidation-related genes including CPT1A, ACADL, HADHα, and ACOX were higher than those of the mice fed control diet.

PPARα is a member of the ligand-activated nuclear hormone receptor super family, and is regarded as a principal regulator of fatty acid oxidation (Giby et al. 2014). On the other hand, PPARγ activates genes involved in lipid storage. Fatty acid oxidation and *de novo* lipogenesis are considered as two key metabolic pathways that control hepatic lipid metabolism (Han et al. 2015). PPARα mRNA and protein expressions elevated in epididymal adipose tissue of mice fed high-fat diet containing fish oil compared with high-fat diet containing lard (Bargut et al. 2015). DHA and EPA have beneficial effects on fatty acid oxidation in the liver through increased fatty acid oxidation-related genes and proteins such as PPARα, CPT1A, and ACOX (Xu et al. 2015). Han et al. (2015) observed that administration of flaxseed oil with flaxseed oil esters of plant sterols significantly ($P<0.05$) up-regulated mRNA and protein expressions of hepatic PPARα and down-regulated SREBP-1c in mice with hepatic steatosis induced by high-fat diet. Hepatic PPARγ overexpression has been linked to exacerbated steatosis by mechanisms involving activation of lipogenic genes and *de*

*novo* lipogenesis and increased hepatic TG concentrations. Deletion of PPARγ in hepatocytes and macrophages reduced high-fat diet-induced hepatic steatosis in mice. SREBP-1c, CD36, and FABP1 were significantly ($P<0.05$) reduced in PPARγ null mice (Morán-Salvador et al. 2011). Berlanga et al. (2014) also reported that activation of PPARγ in adipose tissue has been proposed to increase storage of fat in adipose tissue. Expression levels of PPARγ mRNA and protein in muscle and adipose tissue of pigs fed diets with n-6 to n-3 PUFA ratios of 1:1 and 2.5:1 were significantly ($P<0.05$) reduced compared to pigs fed diets with n-6 to n-3 PUFA ratios of 5:1 and 10:1 (Duan et al. 2014). In this study, protein and mRNA levels of PPARα in liver and epididymal adipose tissue of the mice fed BRS oil were higher than those of the control. However, there were no differences in PPARγ levels between the two groups.

Results of this study suggest that BRS oil might reduce obesity induced chronic inflammatory responses and improve lipid metabolism by inhibiting lipogenesis and promoting fatty acid oxidation in high-fat diet-induced obese mice.

# Chapter 3

# Effect of Black Raspberry Seed Oil on Inflammation and Lipid Metabolism in *db*/*db* Mice (Study 2)

### 3.1 Introduction

One of the most important consequences of inflammation is the development of insulin resistance (González-Périz et al. 2010). Previous studies have shown that inflammation plays an essential role in initiation and development of insulin resistance, which is responsible for the development of obesity and type 2 diabetes (Liu et al. 2013). Pro-inflammatory cytokines including TNFα and IL-6 contribute to the progress of insulin resistance by impairing insulin signaling which consequently can lead to type 2 diabetes (Sowers 2008). Devarshi et al. (2013) also reported that TNFα and IL-6 are instrumental in contributing toward pathogenesis of insulin resistance. Type 2 diabetes patients have higher plasma levels of TNFα and IL-6 than in the normal subjects (Pickup et al. 2000). Popa et al. (2007) reported that TNFα contributed to development of insulin resistance and hypertriglyceridemia and increased free fatty acid level. TNFα has been suggested to have effects on lipid metabolism, such as increased lipogenesis, thus contributing to hyperlipidemia (Chen et al. 2009). Inflammatory cytokine levels including TNFα, IL-6, and MCP1 were higher in type 2 diabetes patients than normal subjects and inflammatory markers were inversely correlated with insulin sensitivity (Daniele et al. 2014).

Obesity is a major health problem worldwide and associated with metabolic syndrome, which is characterized by hyperglycemia, elevated plasma TG, and reduced plasma HDL-C levels. It is well established that obesity is associated with a state of chronic low-grade inflammation, characterized by alterations in circulating immune-modulatory factors which may increase adiposity and insulin resistance

(Hotamisligil 2006). In addition, subclinical inflammation plays an important role in pathogenesis of insulin resistance and type 2 diabetes (Daniele et al. 2014).

Previous studies reported that n-3 and n-6 PUFA have opposite effects on insulin resistance each other. n-3 PUFA attenuated the inflammation and ameliorated saturated fatty acid-induced insulin resistance, while n-6 PUFA promoted development of insulin resistance (Siriwardhana et al. 2012). Hussein et al. (2012) reported that flaxseed oil administration had a beneficial effect on decreasing fasting blood glucose and insulin resistance in diabetic rats.

Anti-inflammatory effect of EPA and DHA, as well as their precursor, ALA, has been widely investigated. The roles of n-3 PUFA are inflammation antagonists, while n-6 PUFA are precursors for inflammation (Ellulu et al. 2015). It was reported that dietary ratio of n-6 to n-3 PUFA influenced controlling markers of obesity, insulin resistance, inflammation, and lipid profiles (Chang et al. 2008; Liu et al. 2013; Yang et al. 2016). Liu et al. (2013) reported that diet with n-6 to n-3 PUFA ratio of 1:1 reduced TLR4 protein and mRNA levels in gastrocnemius muscle of rats compared to diet with n-6 to n-3 PUFA ratio of 4:1. Diets with low n-6 to n-3 PUFA ratios of 1:1 and 5:1 showed more anti-inflammatory effect than in diet with a higher n-6 to n-3 PUFA ratio of 20:1 (Yang et al. 2016). EPA and DHA supplementation decreased serum IL-6 and TNFα compared to typical American diet in healthy middle-aged and older adults (Kiecolt-Glaser et al. 2012). Plasma EPA and DHA levels were significantly ($P<0.05$) higher and n-6 to n-3 PUFA ratio was significantly ($P<0.05$)

lower after EPA and DHA supplementation (Kiecolt-Glaser et al. 2012). ALA-rich perilla oil administration significantly ($P<0.05$) reduced pro-inflammatory mediators in allergic asthma mice compared with corn oil (Chang et al. 2008).

The quantity and quality of dietary fat also play an important role in the development of obesity (Balogum et al. 2016). Diet with a higher ratio of n-6 to n-3 PUFA may increase the pathology of the metabolic syndrome such as type 2 diabetes. Therefore, lowering the n-6 to n-3 PUFA ratio in diets is beneficial for health of animals and humans (Duan et al. 2014). In various species, including humans, rodents, and pigs, it has been shown that n-3 PUFA decreased hepatic lipogenesis and stimulated fatty acid oxidation (Pettinelli et al. 2011; Devarshi et al. 2013; Berlanga et al. 2014; Duan et al. 2014). Flaxseed oil and fish oil diets increased n-3 PUFA levels in plasma and erythrocytes of streptozotocin-nicotinamide-induced diabetic rats. Flaxseed oil diet also significantly ($P<0.05$) up-regulated the key transcription factor PPARα and down-regulated SREBP-1c in diabetic rats (Devarshi et al. 2013). Flaxseed oil would increase fatty acid oxidation and concomitantly reduce lipogenesis, thereby reducing serum TG and very-low-density lipoprotein cholesterol (VLDL-C) levels (Devarshi et al. 2013). ALA-rich linseed oil treatment significantly ($P<0.05$) increased level of serum HDL-C and decreased levels of total cholesterol, TG, and LDL-C in diabetic rats in comparison to normal control (Kaithwas et al. 2012).

The liver, a key metabolic organ, plays an important role in lipid metabolism (Park

et al. 2013). Type 2 diabetes is associated with a varied array of biochemical and physiological anomalies, especially in the liver, including abnormal lipid metabolism. The abnormalities in lipid metabolism result in higher level of circulating free fatty acids and hypertriglyceridemia and lower HDL-C level in type 2 diabetes patients (Devarshi et al. 2013).

In this study, diets containing different concentrations of BRS oil were fed *db*/*db* mice, which genetically have diabetes. The markers involved in inflammation were analyzed in serum, liver, and epididymal adipose tissue of the mice to determine the effect of BRS oil on inflammatory response in insulin resistant status. Lipid profiles in serum and liver as well as mRNA levels involved in lipid metabolism in liver were determined.

## 3.2 Materials and methods

### 3.2.1 Sample preparation and BRS oil extraction

Black raspberry (*Rubus occidentalis*) fruits harvested in 2013 in Gochang, Korea were provided from a farm. Seeds were separated from the fruits by hand and pigments on the seeds were washed off with tap water. BRS was powdered with a blender (Hanil Co.) and oil was extracted by hexane (extra pure grade; Samchun Chemical Co.). The BRS oil was stored at -20ºC after flushing with nitrogen gas.

### 3.2.2 Experimental diets

There were three types of experimental diets (Feed Lab). Control (AIN93G) diet

consisting of 16% calories from soybean oil. BRS oil substituted for soybean oil in BRS oil 50% and BRS oil 100% diets. That is, BRS oil 50% diet consisted of 8% calories from soybean and 8% calories from BRS oil, and BRS oil 100% diet consisted of 16% calories from BRS oil. Diet compositions were not different among the diets except the fat source. All diets were made as solid pellets and stored at -20°C until provided to animals. Animals were fed the diets and water *ad libitum* for 10 weeks. The compositions of experimental diets are shown in Table 3.1.

### 3.2.3 Animals

Male C57BL/6 mice and C57BL/KsJ-*db*/*db* mice (6 weeks old) were purchased from Orientbio and individually housed in cages in an animal facility with conventional (CV) rooms (College of Veterinary Medicine, Seoul National University). After 1 week of acclimatization, the mice were randomly divided into the following four groups (Fig. 3.1): normal mice fed control diet (normal CON, n=15); C57BL/KsJ-*db*/*db* mice fed control diet (CON, n=12); C57BL/KsJ- *db*/*db* mice fed BRS oil 50% diet (BRS 50%, n=12); and C57BL/KsJ-*db*/*db* mice fed BRS oil 100% diet (BRS 100%, n=12). The CV rooms were controlled with constant temperature (23±2ºC), humidity (55±10%), and 12 h dark/light cycle. Food intake and body weight were measured twice a week. After 10 weeks on the experimental diets, the mice were fasted for 12 h and euthanized with $CO_2$ asphyxiation. All the animal procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (approval no: SNU-130617-2).

**Table 3.1 Compositions of the diets**

| | Control (AIN 93G) | | BRS oil 50% | | BRS oil 100% | |
|---|---|---|---|---|---|---|
| | Weight (%) | Energy (%) | Weight (%) | Energy (%) | Weight (%) | Energy (%) |
| Protein | 20 | 20 | 20 | 20 | 20 | 20 |
| Carbohydrate | 64 | 64 | 64 | 64 | 64 | 64 |
| Fat | 7 | 16 | 7 | 16 | 7 | 16 |
| kcal/kg | 4,000 | | 4,000 | | 4,000 | |
| Ingredient | g | kcal | g | kcal | g | kcal |
| Casein | 200 | 800 | 200 | 800 | 200 | 800 |
| Corn starch | 397.486 | 1590 | 397.486 | 1590 | 397.486 | 1590 |
| Sucrose | 100 | 400 | 100 | 400 | 100 | 400 |
| Dextrose | 132 | 528 | 132 | 528 | 132 | 528 |
| Cellulose | 50 | 0 | 50 | 0 | 50 | 0 |
| Black raspberry seed oil | - | - | 35 | 315 | 70 | 630 |
| Soybean oil | 70 | 630 | 35 | 315 | | - |
| Mineral mixture | 35 | 0 | 35 | 0 | 35 | 0 |
| Vitamin mixture | 10 | 40 | 10 | 40 | 10 | 40 |
| TBHQ | 0.014 | 0 | 0.014 | 0 | 0.014 | 0 |
| L-Cystine | 3 | 12 | 3 | 12 | 3 | 12 |
| Choline bitartrate | 2.5 | 0 | 2.5 | 0 | 2.5 | 0 |
| Total | 1000 | 4,000 | 1000 | 4,000 | 1000 | 4,000 |

Control (AIN93G): fed normal CON and CON groups; BRS oil 50%: fed BRS 50% group; and BRS oil 100%: fed BRS 100% group

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil)

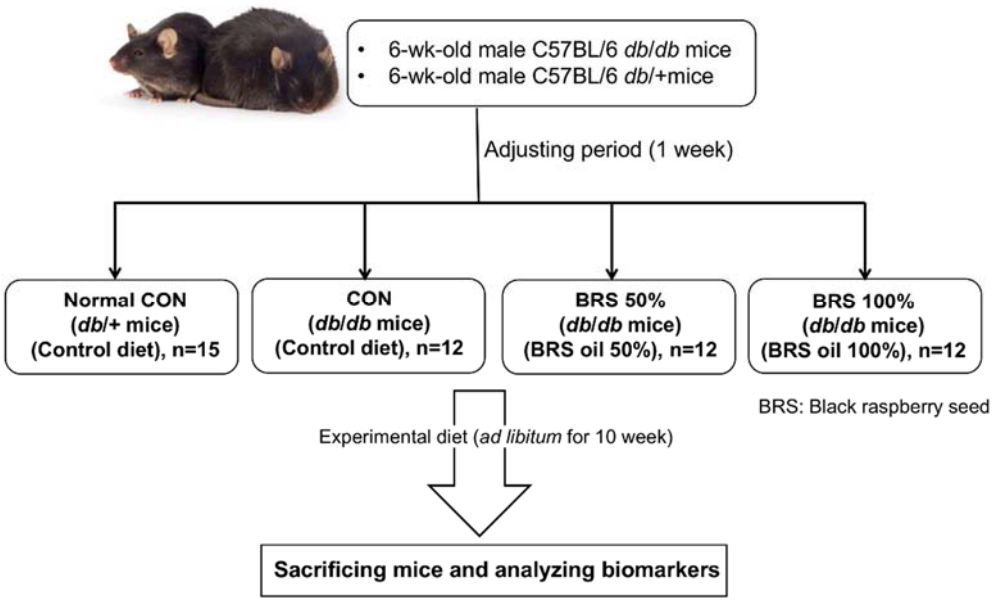


**Fig. 3.1 Overview of the study design**

3.2.4 Tissue collection and blood glucose analysis

After the mice were euthanized by asphyxiation with $CO_2$, about 1 mL blood was collected by cardiac puncture. Serum was separated by centrifugation (Micro 17R+, Hanil Science Industrial Co.) at 387×g at 4ºC for 20 min after coagulation at room temperature for 2 h. Liver, epididymal adipose tissue, kidney, and spleen were collected and washed with phosphate-buffered saline (Bio-Rad Laboratories). All tissues were stored at -80ºC until analysis. Fasting glucose levels were measured using an Accu-Chek® Active (Roche Diagnostics GmbH, Mannheim, Germany).

3.2.5 Fatty acid composition analysis

Lipid was extracted from livers and epididymal adipose tissues of the experimental mice according to the Blight and Dyer (1959) as described in 2.2.6. The extracted lipid were methylated using BF3-methanol (Sigma-Aldrich Chemical Co.) and fatty acid composition was analyzed by GC (6890; Agilent Technologies) equipped with flame ionization detector and a DB-23 column (J&W Scientific) as described in 2.2.7.

3.2.6 Serum analysis

Serum TNFα, IL-6, IL-10 (Invitrogen, Camarillo, CA, USA), leptin, adiponectin (BioVendor Laboratory Medicine, Inc.), and insulin (Mercodia, Uppsala, Sweden) concentrations were determined using commercial kits based on enzyme-linked immunosorbent assay as described in 2.2.10.

### 3.2.7 Protein extraction and western blotting

Protein extraction and western blotting were done as in the method section of 2.2.8.

### 3.2.8 Total RNA extraction, cDNA synthesis, and PCR

Total RNA extraction, cDNA synthesis, and PCR were done as in the method section of 2.2.9. Specific primer sequences used in this study are shown in Table 2.2 and Table 3.2.

### 3.2.9 Liver lipid content and hepatic lipid analysis

Lipids in the liver were analyzed as in the method sections of 2.2.11 and 2.2.12.

### 3.2.10 Statistical analysis

Values were expressed as means ± standard deviations. Statistical analyses were processed with one-way analysis of variance (ANOVA) of SPSS program (version 22.0, SPSS). Significant differences identified using ANOVA were subjected to Duncan's multiple range test for analysis of differences between mean values at $P<0.05$.

**Table 3.2 Primer sequences used in real-time quantitative PCR**

| | | |
|---|---|---|
| GAPDH | Forward (5'-3') | GGAGAAACCTGCCAAGTA |
| | Reverse (5'-3') | AAGAGTGGGAGTTGCTGTTG |
| TLR4 | Forward (5'-3') | GGGCCTAAACCCAGTCTGTTTG |
| | Reverse (5'-3') | GCCCGGTAAGGTCCATGCTA |
| CCR2 | Forward (5'-3') | CAATATGTTACCTCAGTTCATCCACGG |
| | Reverse (5'-3') | AAGGCTCACCATCATCGTAGTCA |
| F4/80 | Forward (5'-3') | TTTCCTCGCCTGCTTCTTC |
| | Reverse (5'-3') | CCCCGTCTCTGTATTCAACC |
| CD68 | Forward (5'-3') | AGGGTGGAAGAAAGGTAAAGC |
| | Reverse (5'-3') | AGAGCAGGTCAAGGTGAACAG |
| CD14 | Forward (5'-3') | GGCTTGTTGCTGTTGCTTC |
| | Reverse (5'-3') | CAGGGCTCCGAATAGAATCC |

### 3.3 Results

#### 3.3.1 Fatty acid composition of the diets, and body weight, food intake, and organ weight of the animals

Linoleic acid was the most abundant in all the diets (Table 3.3). Oleic (15.7%) and stearic (13.8%) acids were the major fatty acids in the control diet. The contents of ALA in the BRS oil diets were significantly ($P<0.05$) higher than in the control diet. The ratio of n-6 to n-3 PUFA was the highest in the control diet (7.92), followed by the BRS 50% (2.80) and BRS 100% (1.65). There were no significant ($P>0.05$) differences in the food intake and initial body weight among the three *db*/*db* groups. The food intake and body weight of the normal CON were significantly ($P<0.05$) lower than those of the *db*/*db* groups. The final body weight of the BRS 50% was significantly ($P<0.05$) lower than that of the normal CON. Final body weight of the BRS 100% was lower than that of the CON without significant ($P>0.05$) difference. The weights of the liver, epididymal adipose tissue, spleen, and kidney were not significantly ($P>0.05$) different among the *db*/*db* mice (Table 3.4).

#### 3.3.2 Blood glucose level and insulin level in serum

There was no significant ($P>0.05$) difference in blood glucose level among the *db*/*db* mice. Blood glucose level of the normal CON was significantly ($P<0.05$) lower than that of the *db*/*db* mice. Insulin level was significantly ($P<0.05$) lower in serum of the BRS 50% and BRS 100% than that of the CON (Table 3.5).

**Table 3.3 Fatty acid compositions of the diets (unit: %, w/w)**

| Fatty acid | Control (AIN93G) | BRS oil 50% | BRS oil 100% |
|---|---|---|---|
| 14:0 | 0.14 ± 0.03 | 0.10 ± 0.02 | 0.10 ± 0.02 |
| 16:0 | 11.2 ± 0.23 | 6.95 ± 0.14 | 2.44 ± 0.13 |
| 18:0 | 13.8 ± 2.88 | 8.47 ± 1.42 | 4.49 ± 0.93 |
| 20:0 | 0.44 ± 0.01 | 0.40 ± 0.02 | 0.42 ± 0.04 |
| 22:0 | 0.43 ± 0.01 | 0.31 ± 0.01 | 0.24 ± 0.05 |
| 24:0 | 0.20 ± 0.06 | 0.12 ± 0.01 | 0.09 ± 0.03 |
| Saturated | 26.2 | 16.4 | 7.8 |
| 16:1 | 0.09 ± 0.03 | 0.11 ± 0.01 | 0.12 ± 0.01 |
| 18:1 | 15.7 ± 2.38 | 12.0 ± 1.68 | 6.41 ± 1.32 |
| 20:1 | 0.26 ± 0.01 | 0.28 ± 0.01 | 0.34 ± 0.01 |
| 22:1 | 0.14 ± 0.08 | 0.13 ± 0.01 | 0.17 ± 0.00 |
| MUFA | 16.2 | 12.5 | 7.0 |
| 18:2n-6 | 51.2 ± 0.66 | 52.4 ± 0.14 | 53.0 ± 0.16 |
| 18:3n-3 | 6.46 ± $0.16^{c}$ | 18.7 ± $0.35^{b}$ | 32.2 ± $0.53^{a}$ |
| 20:2n-6 | 0.06 ± 0.02 | 0.07 ± 0.00 | 0.09 ± 0.00 |
| PUFA | 57.7 | 71.2 | 85.2 |
| n-6/n-3 | 7.92 ± $0.11^{a}$ | 2.80 ± $0.04^{b}$ | 1.65 ± $0.02^{c}$ |

Values are means and standard deviations (n=3). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Control (AIN93G): fed normal CON and CON group; BRS oil 50%: fed BRS 50% group; and BRS oil 100%: fed BRS 100% group.

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

**Table 3.4 Body weight, food intake, and organ weights of the mice fed control and BRS oil diets**

| | Normal CON | CON | BRS 50% | BRS 100% |
|---|---|---|---|---|
| Initial weight (g) | 18.7 ± 0.40$^{b}$ | 38.2 ± 1.73$^{a}$ | 38.0 ± 1.49$^{a}$ | 38.1 ± 1.31$^{a}$ |
| Final weight (g) | 26.5 ± 2.32$^{c}$ | 44.0 ± 3.81$^{a}$ | 40.3 ± 4.95$^{b}$ | 43.1 ± 3.56$^{ab}$ |
| Food intake (g/day) | 2.94 ± 0.19$^{b}$ | 7.65 ± 0.60$^{a}$ | 7.62 ± 0.47$^{a}$ | 7.38 ± 0.82$^{a}$ |
| Liver (g) | 0.96 ± 0.11$^{b}$ | 2.20 ± 0.43$^{a}$ | 2.25 ± 0.43$^{a}$ | 2.41 ± 0.34$^{a}$ |
| Epididymal adipose tissue (g) | 1.46 ± 0.39$^{b}$ | 3.07 ± 0.43$^{a}$ | 2.68 ± 0.53$^{a}$ | 3.03 ± 0.37$^{a}$ |
| Spleen (g) | 0.06 ± 0.01 | 0.07 ± 0.02 | 0.06 ± 0.01 | 0.06 ± 0.01 |
| Kidney (g) | 0.39 ± 0.13$^{b}$ | 0.60 ± 0.05$^{a}$ | 0.60 ± 0.13$^{a}$ | 0.56 ± 0.07$^{a}$ |

Values are means and standard deviations (n=15/group (Normal CON), n=12/group (CON, BRS 50%, and BRS 100%)). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

**Table 3.5 Blood glucose and insulin levels in serum of the mice fed control and BRS oil diets**

| | Normal CON | CON | BRS 50% | BRS 100% |
|---|---|---|---|---|
| Blood glucose (mg/dL) | 166 ± 33.7[b] | 717 ± 62.3[a] | 687 ± 118[a] | 660 ± 121[a] |
| Insulin (μg/L) | 0.50 ± 0.14[c] | 1.81 ± 1.08[a] | 1.15 ± 0.34[b] | 1.16 ± 0.36[b] |

Values are means and standard deviations (n=10/group). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

### 3.3.3 Fatty acid compositions of the livers and epididymal adipose tissues of experimental animals

ALA contents in the livers of the BRS oil-treated groups were significantly ($P<0.05$) higher than that of the control group (Table 3.6 (a)). The liver of the BRS100% group had the highest ALA, followed by that of the BRS 50%. The contents of EPA and DHA in the livers of the BRS oil-treated groups were higher than those of the CON without significant ($P>0.05$) differences. Contents of AA were higher in the livers of the normal CON and CON than that of the BRS 100%. There was no significant ($P<0.05$) difference in the total amounts of n-6 PUFA between the four groups. However, the total amounts of the n-3 PUFA were significantly ($P<0.05$) higher in the BRS oil-treated groups than in the CON. ALA and total n-3 PUFA contents were significantly ($P<0.05$) higher in epididymal adipose tissues of the BRS oil-treated groups than those of the normal CON and CON (Table 3.6 (b)). The mice fed the BRS oil diet had significantly ($P<0.05$) lower n-6 to n-3 PUFA ratio in the liver and epididymal adipose tissue than the mice fed the control diet.

**Table 3.6 Fatty acid compositions of livers (a) and epididymal adipose tissues (b) of the mice fed control and BRS oil diets (unit: %, w/w)**

(a)

| Fatty acid | Normal CON | CON | BRS 50% | BRS 100% |
|---|---|---|---|---|
| 14:0 | 0.42 ± 0.14 | 0.52 ± 0.10 | 0.45 ± 0.09 | - |
| 16:0 | 22.2 ± 1.12 | 20.9 ± 1.71 | 16.7 ± 0.84 | 16.8 ± 2.21 |
| 18:0 | 6.41 ± 1.75 | 7.32 ± 1.19 | 7.75 ± 1.99 | 5.74 ± 1.11 |
| 20:0 | 0.16 ± 0.11 | 0.06 ± 0.07 | 0.08 ± 0.02 | 0.12 ± 0.01 |
| 22:0 | 0.29 ± 0.29 | 0.28 ± 0.38 | 0.02 ± 0.02 | 0.04 ± 0.05 |
| 24:0 | 0.48 ± 0.15 | 0.44 ± 0.38 | 0.83 ± 0.38 | 0.94 ± 0.46 |
| Saturated | 30.0 | 29.5 | 25.9 | 23.7 |
| 16:1 | 3.86 ± 1.60 | 3.10 ± 1.06 | 2.61 ± 0.63 | 3.47 ± 1.56 |
| 18:1 | 27.2 ± 4.80 | 27.1 ± 5.78 | 24.7 ± 5.26 | 28.7 ± 8.18 |
| 20:1 | 0.68 ± 0.42 | 0.28 ± 0.30 | 0.60 ± 0.38 | 0.79 ± 0.32 |
| 22:1 | 0.51 ± 0.49 | 0.46 ± 0.61 | 0.14 ± 0.06 | 0.10 ± 0.06 |
| MUFA | 32.2 | 31.0 | 28.1 | 33.1 |
| 18:2n-6 | 28.8 ± 2.92 | 30.2 ± 3.13 | 33.5 ± 3.02 | 30.0 ± 6.76 |
| 18:3n-3 | 1.90 ± 0.47$^{c}$ | 1.87 ± 0.49$^{c}$ | 4.23 ± 0.39$^{b}$ | 8.37 ± 2.06$^{a}$ |
| 20:2n-6 | 0.33 ± 0.06 | 0.68 ± 0.49 | 0.86 ± 0.82 | 0.59 ± 0.35 |
| 20:3n-6 | 0.88 ± 0.34 | 0.90 ± 0.34 | 0.77 ± 0.29 | 0.65 ± 0.19 |
| 20:4n-6 | 5.39 ± 2.91$^{a}$ | 4.76 ± 2.25$^{ab}$ | 4.63 ± 1.28$^{ab}$ | 2.69 ± 0.70$^{b}$ |
| 20:5n-3 | 0.31 ± 0.33 | 0.53 ± 0.53 | 0.81 ± 0.70 | 0.94 ± 0.51 |
| 22:6n-3 | 2.26 ± 1.44 | 2.05 ± 1.04 | 3.16 ± 1.50 | 2.85 ± 0.37 |
| PUFA | 39.8 | 40.9 | 48.0 | 46.1 |
| ∑n-6 | 34.8 ± 3.90 | 36.4 ± 5.65 | 39.5 ± 4.14 | 35.5 ± 6.48 |
| ∑n-3 | 3.24 ± 1.79$^{c}$ | 3.50 ± 2.07$^{c}$ | 7.53 ± 1.82$^{b}$ | 10.8 ± 3.15$^{a}$ |
| n-6/n-3 | 13.7 ± 7.38$^{a}$ | 13.3 ± 7.36$^{a}$ | 5.49 ± 1.41$^{b}$ | 3.42 ± 0.60$^{b}$ |

(b)

| Fatty acid | Normal CON | CON | BRS 50% | BRS 100% |
|---|---|---|---|---|
| 12:0 | 0.05 ± 0.00 | 0.10 ± 0.00 | 0.11 ± 0.00 | 0.12 ± 0.03 |
| 14:0 | 1.08 ± 0.06 | 0.98 ± 0.08 | 0.93 ± 0.07 | 1.13 ± 0.11 |
| 16:0 | 18.3 ± 1.24 | 18.7 ± 0.79 | 17.8 ± 1.05 | 18.6 ± 0.91 |
| 18:0 | 1.42 ± 0.62 | 5.22 ± 2.69 | 3.42 ± 2.66 | 1.63 ± 0.65 |
| 20:0 | 0.26 ± 0.32 | 0.10 ± 0.01 | 0.10 ± 0.01 | 0.11 ± 0.01 |
| 22:0 | 0.04 ± 0.02 | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.08 ± 0.04 |
| 24:0 | 0.10 ± 0.00 | 0.06 ± 0.06 | 0.05 ± 0.00 | 0.06 ± 0.01 |
| Saturated | 21.3 | 25.1 | 22.4 | 21.8 |
| 14:1 | 0.16 ± 0.04 | 0.13 ± 0.01 | 0.09 ± 0.02 | 0.11 ± 0.03 |
| 16:1 | 8.84 ± 0.59 | 6.68 ± 1.12 | 6.87 ± 1.10 | 7.45 ± 0.68 |
| 18:1 | 35.6 ± 1.16 | 33.6 ± 3.90 | 29.2 ± 2.32 | 29.5 ± 1.18 |
| 20:1 | 0.62 ± 0.28 | 0.46 ± 0.03 | 0.49 ± 0.03 | 0.51 ± 0.02 |
| 22:1 | 0.07 ± 0.02 | 0.04 ± 0.01 | 0.04 ± 0.01 | 0.09 ± 0.01 |
| 24:1 | 0.03 ± 0.00 | 0.01 ± 0.01 | 0.01 ± 0.00 | 0.03 ± 0.03 |
| MUFA | 45.3 | 41.0 | 36.7 | 37.7 |
| 18:2n-6 | 29.9 ± 1.11 | 32.7 ± 1.12 | 35.2 ± 2.87 | 31.9 ± 2.70 |
| 18:3n-3 | 2.32 ± 0.09$^{c}$ | 2.16 ± 0.27$^{c}$ | 5.26 ± 0.51$^{b}$ | 8.22 ± 0.35$^{a}$ |
| 20:2n-6 | 0.23 ± 0.02 | 0.17 ± 0.03 | 0.17 ± 0.00 | 0.18 ± 0.03 |
| 20:3n-6 | 0.27 ± 0.02 | 0.11 ± 0.03 | 0.11 ± 0.02 | 0.11 ± 0.02 |
| 20:4n-6 | 0.51 ± 0.17$^{a}$ | 0.14 ± 0.05$^{b}$ | 0.13 ± 0.03$^{b}$ | 0.09 ± 0.03$^{b}$ |
| 20:5n-3 | 0.04 ± 0.00$^{b}$ | 0.01 ± 0.00$^{c}$ | 0.03 ± 0.01$^{bc}$ | 0.07 ± 0.02$^{a}$ |
| 22:6n-3 | 0.05 ± 0.04 | 0.06 ± 0.03 | 0.06 ± 0.02 | 0.07 ± 0.05 |
| PUFA | 33.3 | 35.3 | 40.9 | 40.6 |
| ∑n-6 | 30.7 ± 1.29$^{b}$ | 33.1 ± 1.28$^{ab}$ | 35.6 ± 2.84$^{a}$ | 32.3 ± 3.07$^{ab}$ |
| ∑n-3 | 2.63 ± 0.16$^{c}$ | 2.22 ± 0.35$^{c}$ | 5.35 ± 0.52$^{b}$ | 8.36 ± 0.33$^{a}$ |
| n-6/n-3 | 11.7 ± 0.50$^{b}$ | 15.3 ± 2.91$^{a}$ | 6.73 ± 1.25$^{c}$ | 3.86 ± 0.35$^{d}$ |

Values are means and standard deviations (n=6/group). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

### 3.3.4 Inflammatory markers in serums of the mice

Serum TNFα levels in the BRS 50% and BRS 100% were 31% and 19%, respectively, lower than in the CON. Noticeable reduction in serum IL-6 levels was observed in the BRS oil groups. IL-6 levels in the serums of the BRS 50% and BRS 100% were 69% and 58%, respectively, lower than that of the CON. Serum IL-6 levels of the BRS oil-treated groups were statistically ($P>0.05$) the same as that of the normal CON. Serum IL-10 was the highest in the BRS 100%. Its level was 17% and 76% higher than those of the normal CON and CON groups, respectively. IL-10 level was 38% higher in the BRS 50% than in the CON without significance ($P>0.05$) (Table 3.7).

### 3.3.5 Leptin and adiponectin levels in serum

Serum leptin was the highest in the CON among the four groups. Leptin in the BRS 50% was significantly ($P<0.05$) lower than in the CON. Leptin in the BRS 100% was lower than in the CON without significance ($P>0.05$). Serum adiponectin levels in the BRS 50% and BRS 100% were higher than in the CON without significance ($P>0.05$). Adiponectin in the normal CON was significantly ($P<0.05$) higher than in the three *db*/*db* groups (Table 3.8).

**Table 3.7 Inflammatory markers in the serums of the mice fed control and BRS oil diets**

| | Normal CON | CON | BRS 50% | BRS 100% |
|---|---|---|---|---|
| TNFα (pg/mL) | 14.5 ± 1.72$^{ab}$ | 15.0 ± 3.51$^{a}$ | 10.3 ± 2.40$^{c}$ | 12.1 ± 2.62$^{bc}$ |
| IL-6 (pg/mL) | 10.5 ± 6.50$^{b}$ | 22.4 ± 10.9$^{a}$ | 6.9 ± 5.16$^{b}$ | 9.37 ± 6.25$^{b}$ |
| IL-10 (pg/mL) | 47.0 ± 15.0$^{ab}$ | 31.2 ± 7.34$^{b}$ | 43.0 ± 9.98$^{ab}$ | 54.9 ± 29.8$^{a}$ |

Values are means and standard deviations (n=10/group). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

**Table 3.8 Leptin and adiponectin levels in serums of the mice fed control and BRS oil diets**

| | Normal CON | CON | BRS 50% | BRS 100% |
|---|---|---|---|---|
| Leptin (ng/mL) | 3.35 ± 2.85$^{c}$ | 65.2 ± 17.6$^{a}$ | 47.1 ± 12.1$^{b}$ | 56.5 ± 14.3$^{ab}$ |
| Adiponectin (μg/mL) | 30.0 ± 3.65$^{a}$ | 16.9 ± 2.63$^{b}$ | 17.8 ± 3.30$^{b}$ | 18.8 ± 3.20$^{b}$ |

Values are means and standard deviations (n=10/group). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

### 3.3.6 Protein and mRNA levels involved in pro-inflammation in liver and epididymal adipose tissue

Protein expressions of TLR4 and NF-κB in the livers and epididymal adipose tissues of the mice are shown in Fig. 3.2. There were no significant ($P>0.05$) differences in TLR4 and NF-κB protein expressions in the livers among the *db*/*db* groups. However, I-κBα, an inhibitor protein which leads to prevent NF-κB activation, was significantly ($P<0.05$) higher in the liver of the BRS 100% than that of the CON. This level was not statistically ($P>0.05$) different from the normal CON. TLR4 and NF-κB protein expressions in the epididymal adipose tissues of the BRS 50% and BRS 100% were significantly ($P<0.05$) lower than those of the CON. COX2 protein was expressed significantly ($P<0.05$) lower in the BRS 100% than in the CON; however, that of the BRS 50% was not significantly ($P>0.05$) different from that of the CON.

Relative mRNA levels involved in pro-inflammation such as TLR4, TNFα, IL-β, IL-6, iNOS, COX2, MCP1, and C-C motif chemokine receptor2 (CCR2) were significantly ($P<0.05$) lower in the liver of the BRS oil-treated groups than in the CON (Fig.3.3). IL-6 mRNA level was slightly lower in the BRS 50% than in the BRS 100%, which was in agreement with those of the serum result. mRNA levels of TLR4, IL-6, iNOS, MCP1, and its receptor CCR2 were not significantly ($P>0.05$) different between the normal CON and CON groups.

TLR4 mRNA levels in the epididymal adipose tissues of the BRS 50% and BRS

100% were significantly ($P<0.05$) lower than that of the CON (Fig. 3.4a). TLR4 mRNA level was the lowest in the normal CON, which was significantly ($P<0.05$) different from those in the other three groups. TNFα and IL-6 mRNA levels were significantly ($P<0.05$) lower in both of the BRS oil groups than in the CON (Fig. 3.4b and d). The trend of the IL-6 mRNA level was in a close agreement with the serum IL-6 level. IL-1β mRNA level was lower in the BRS 50% and BRS 100% than in the CON without significant ($P>0.05$) difference (Fig. 3.4c). mRNA level of iNOS was significantly ($P<0.05$) lower in the BRS 50% and BRS 100% than in the CON and was not significantly ($P>0.05$) different from that of the normal CON (Fig. 3.4e). COX2 mRNA level reduction was observed in the BRS 100% group only, which was in agreement with the western blot result (Fig. 3.4f). Level of CCR2 mRNA was significantly ($P<0.05$) lower in the normal CON than in the other three *db*/*db* groups, which was not in agreement with the liver results. BRS oil showed significantly ($P<0.05$) lowering effect of the CCR2 mRNA levels in the BRS 50% and BRS 100% (Fig. 3.4h). However, MCP1 mRNA level was significantly ($P<0.05$) lower only in the BRS 50% than in the CON (Fig. 3.4g).

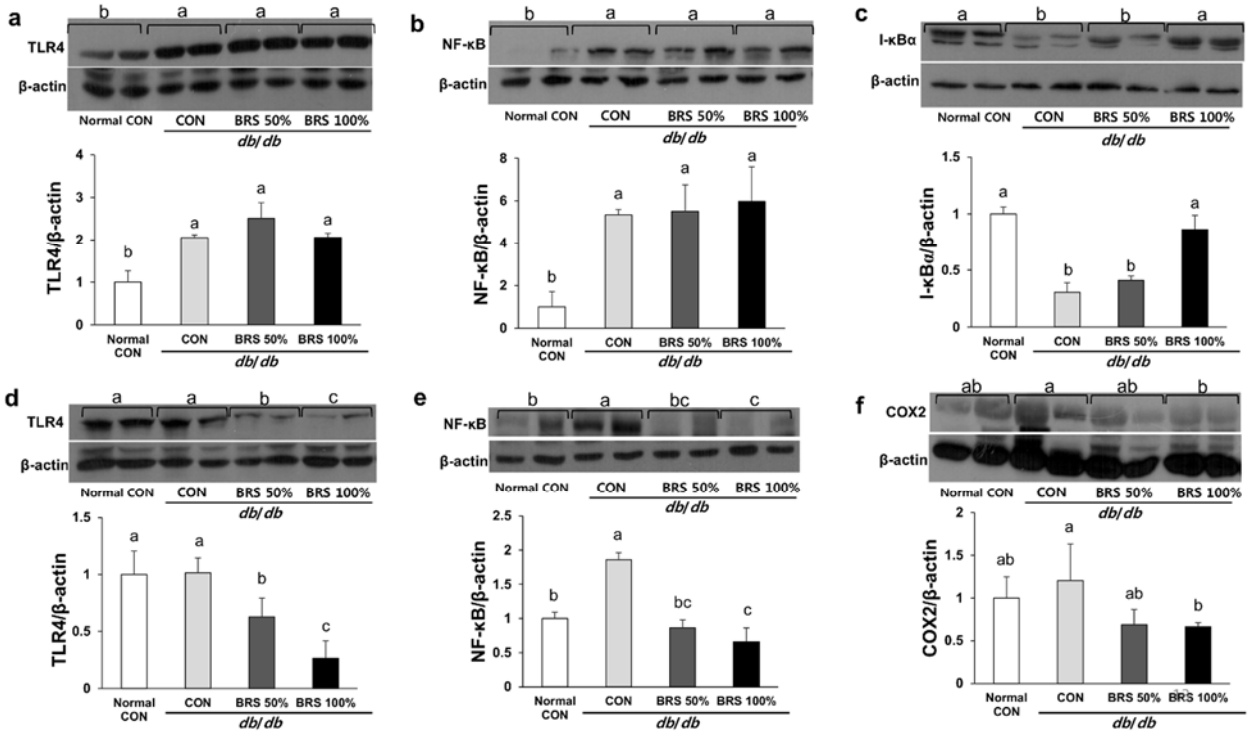


**Fig. 3.2 Protein expressions involved in inflammation of the livers (a-c) and epididymal adipose tissues (d-f) of the mice fed control and BRS oil diets**

Each bar represents means and standard deviations (n=4/group). Values with different letters in the same markers are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

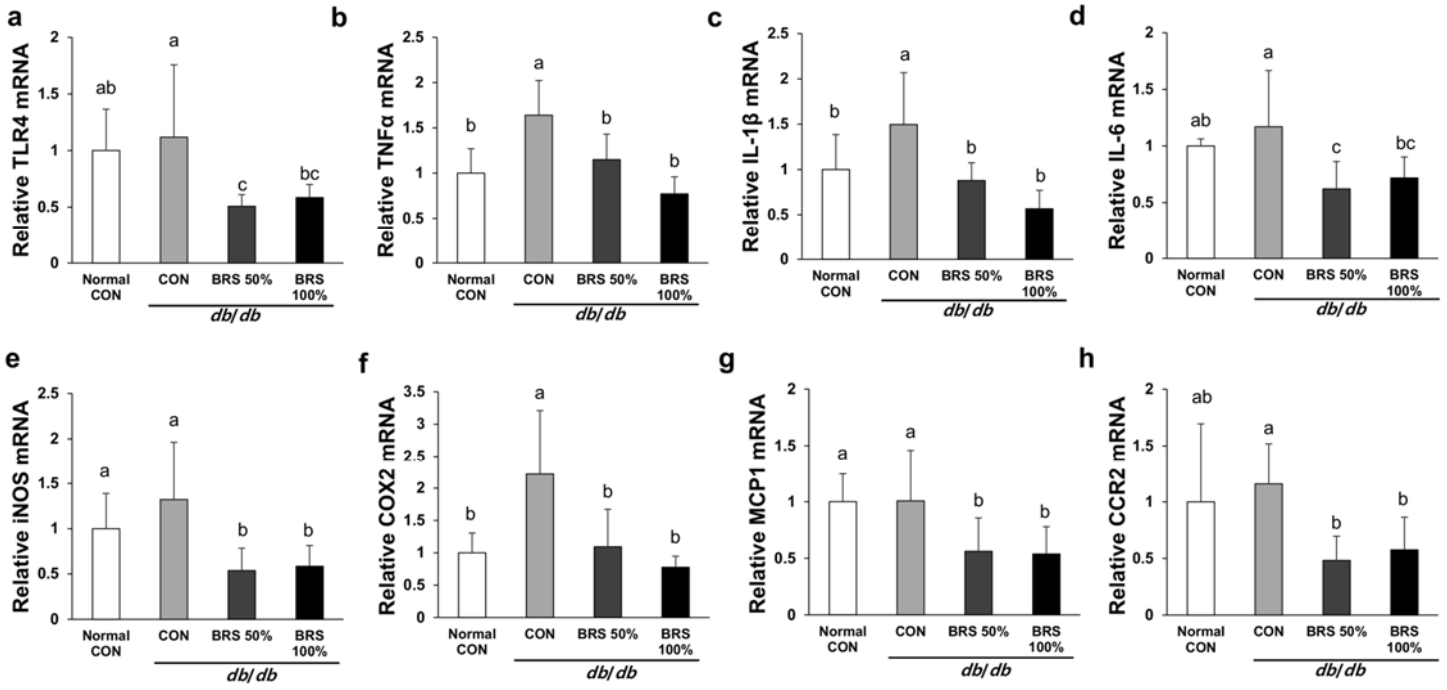


**Fig. 3.3 Relative mRNA levels involved in pro-inflammation in the livers of the mice fed control and BRS oil diets**

Each bar represents means and standard deviations (n=8/group). Values with different letters in the same markers are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

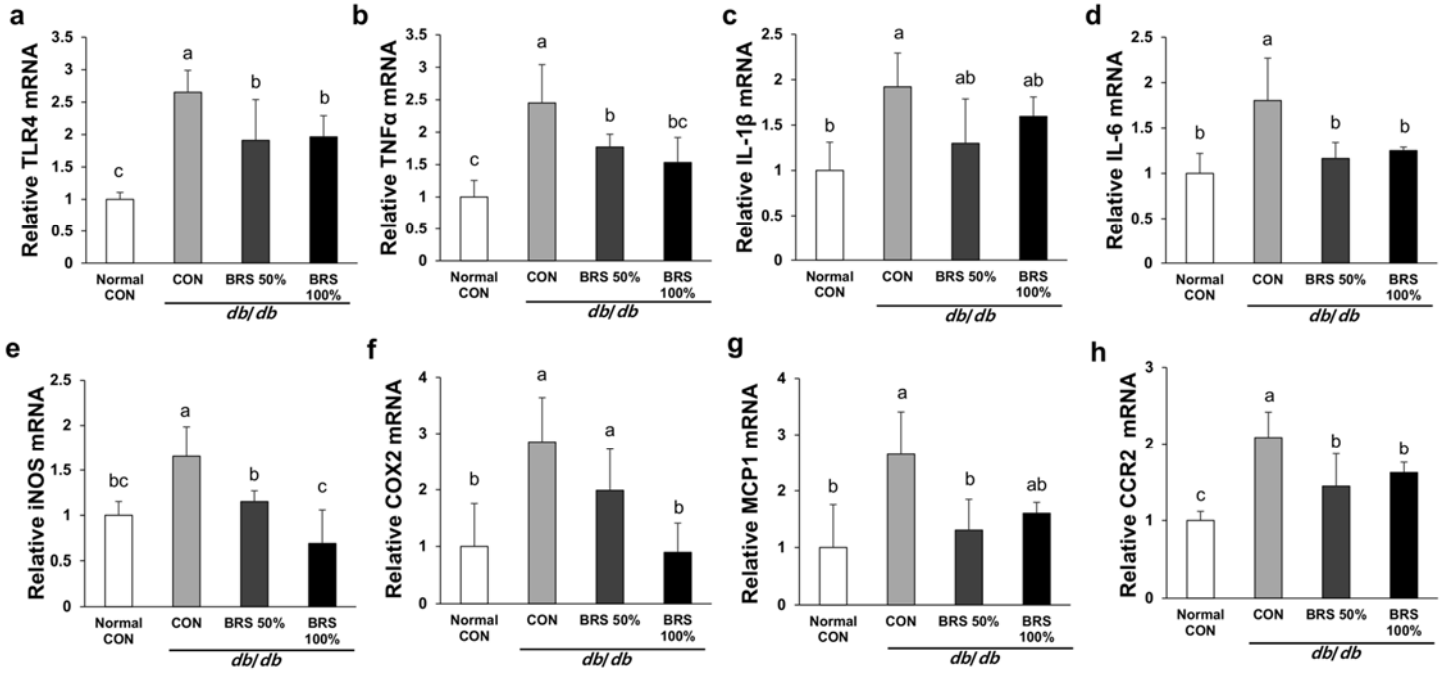


**Fig. 3.4 Relative mRNA levels involved in pro-inflammation in the epididymal adipose tissues of the mice fed control and BRS oil diets**

Each bar represents means and standard deviations (n=8/group). Values with different letters in the same markers are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

### 3.3.7 mRNA levels involved in anti-inflammation in liver and epididymal adipose tissue

mRNA levels of anti-inflammatory markers including IL-10, arginase1, Chi3l3, and Mgl1 in the liver and epididymal adipose tissue were measured (Fig. 3.5). Only IL-10 was significantly ($P<0.05$) higher in the liver of the BRS 100% than that of the CON and even significantly ($P<0.05$) higher than that of the normal CON. IL-10 mRNA level in liver was similar to that of the serum IL-10 level. Arginase1 and Chi3l3 mRNA levels were not significantly ($P>0.05$) different among the four groups. Mgl1 mRNA levels were not significantly ($P>0.05$) different among the *db*/*db* mice, where these levels were significantly ($P<0.05$) lower than in the normal CON. The mRNA levels of arginase1 and Mgl1 in the epididymal adipose tissue of the BRS 50% and BRS 100% were significantly ($P<0.05$) higher than those of the CON and were similar to the normal CON. The IL-10 mRNA level was significantly ($P<0.05$) higher in the BRS 50% than in the CON, and Chi3l3 mRNA level was higher in the BRS oil groups than in the CON without significance ($P>0.05$).

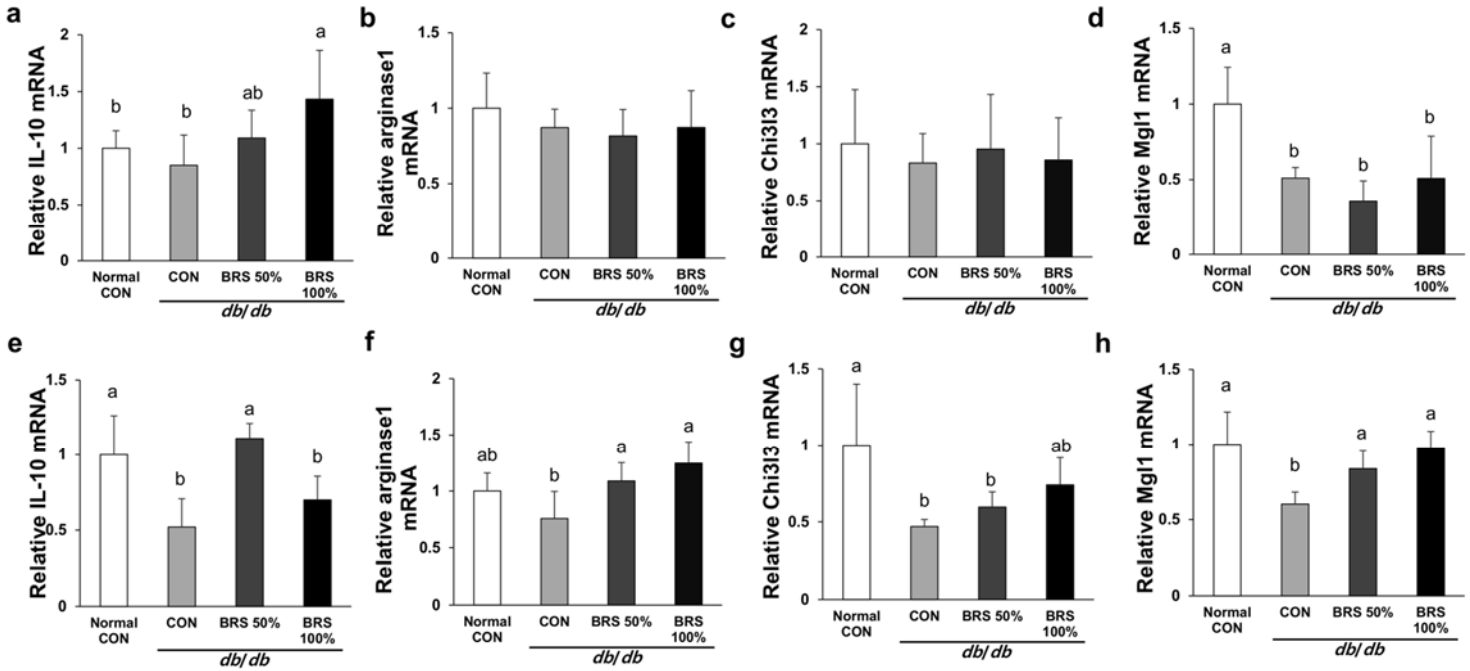


**Fig. 3.5 Relative mRNA levels involved in anti-inflammation in the livers (a-d) and epididymal adipose tissues (e-h) of the mice fed control and BRS oil diets**

Each bar represents means and standard deviations (n=8/group). Values with different letters in the same markers are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

### 3.3.8 Macrophage markers, leptin, and adiponectin mRNA levels in epididymal adipose tissue

The mRNA levels of F4/80 and CD68 (macrophage marker) in the epididymal adipose tissue of the BRS 50% and BRS 100% were significantly ($P<0.05$) lower than those of the CON (Fig. 3.6). However, CD14 mRNA level was not significantly ($P>0.05$) different among the *db*/*db* mice. BRS oil significantly ($P<0.05$) reduced the leptin mRNA level compared with the CON. mRNA level of adiponectin showed significant ($P<0.05$) elevation only in the BRS 50%. Adiponectin mRNA was higher in the BRS 100% than in the CON without significance ($P>0.05$) and statistically ($P>0.05$) the same as in the normal CON.

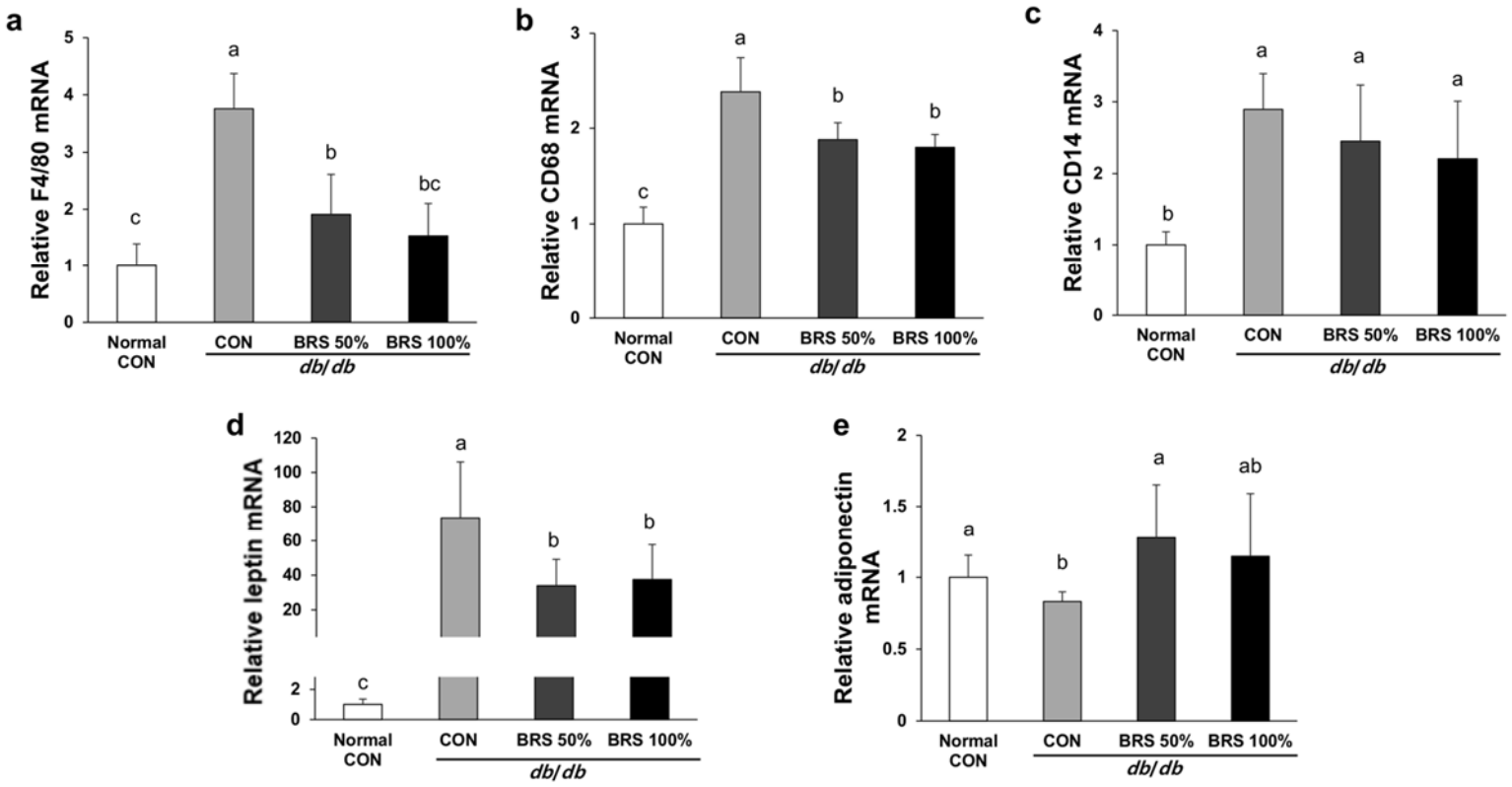


**Fig. 3.6 Relative mRNA levels in the epididymal adipose tissues of mice fed control and BRS oil diets**

Each bar represents means and standard deviations (n=8/group). Values with different letters in the same markers are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

3.3.9 Lipid profiles of serum and liver

Levels of TG in the serums of the BRS 50% and BRS 100% were 24.1% and 21.4%, respectively, lower ($P<0.05$) than that of the CON. Serum NEFA levels of the BRS 50% and BRS 100% were the same. These value was 23.5% lower ($P<0.05$) than that of the CON. The BRS 50% and BRS 100% groups showed 18.0% and 17.6%, respectively, lower ($P<0.05$) serum total cholesterol levels thanthe CON. Serum HDL-C level was 46.0% higher ($P<0.05$) in the BRS 50% than in the CON. Total lipid levels in the livers of the BRS 50% and BRS 100% were 27.1% and 26.0%, respectively, lower ($P<0.05$) than that of the CON and these values reached closer to that observed in the normal CON. Levels of TG in the liver were 27.5% and 53.4%, respectively, lower ($P<0.05$) in the BRS 50% and BRS 100% than in the CON and these levels were statistically the same as in the normal CON. NEFA levels in liver were 48.7% and 42.0%, respectively, lower ($P<0.05$) in the BRS 50% and BRS 100% than in the CON. Total cholesterol level in liver of the BRS 50% was 35.6% lower ($P<0.05$) than that of the CON (Fig. 3.7).

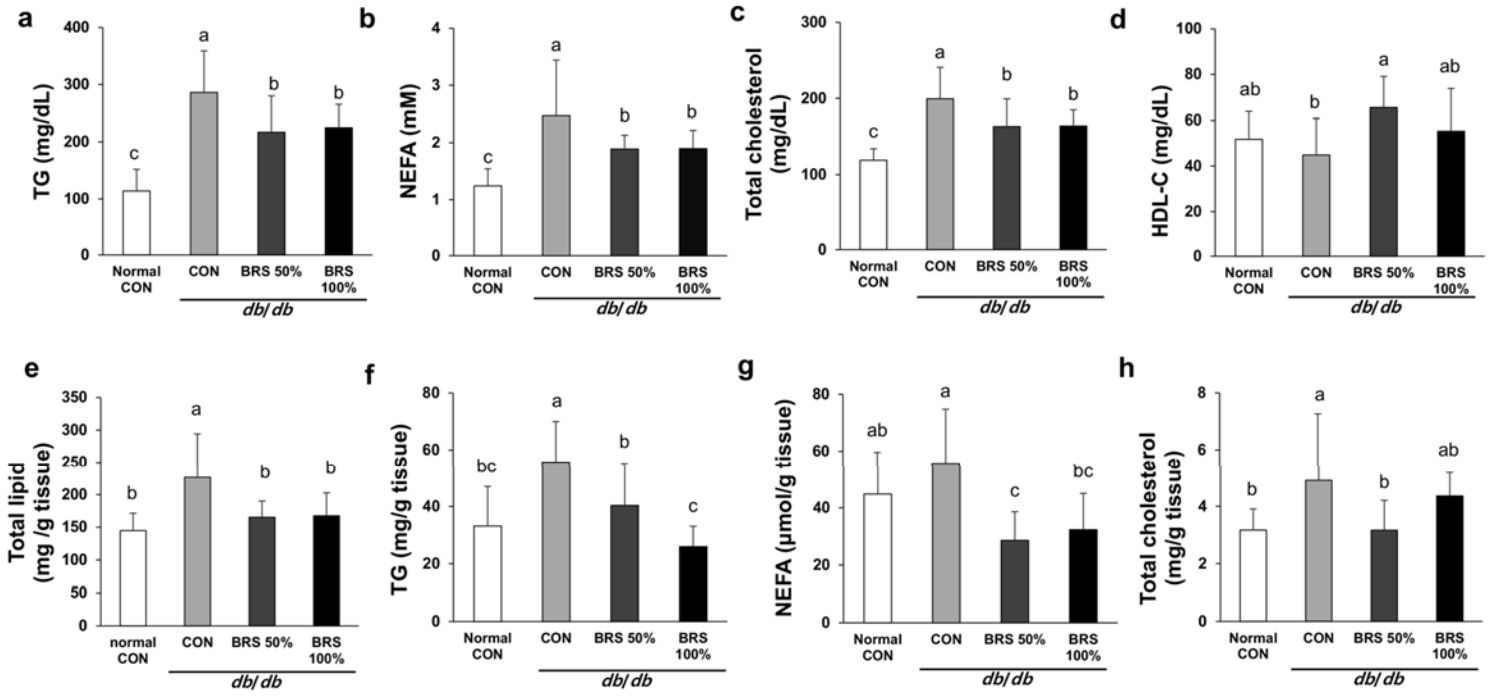


**Fig. 3.7 Lipid profiles of serums (a-d) and livers (e-h) of the mice fed control and BRS oil diets**

Values are means and standard deviations (n=10/group). Values with different superscripts in the same rows are significantly

different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

### 3.3.10 mRNA levels involved in lipogenesis in liver

To determine the effect of BRS oil on lipogenic pathways in liver, mRNA levels of lipogenic markers were examined (Fig. 3.8). mRNA levels of lipogenic markers including CD36, FABP1, SREBP-1c, FAS, and SLC25A1 in the livers of the BRS 50% and BRS 100% were significantly ($P<0.05$) lower than those of the CON. FABP1 and SLC25A1 mRNA levels in the BRS oil-treated groups were even significantly ($P<0.05$) lower than those of the normal CON. CD36 and FAS mRNA levels in the BRS oil-treated groups were statistically the same as in the normal CON.

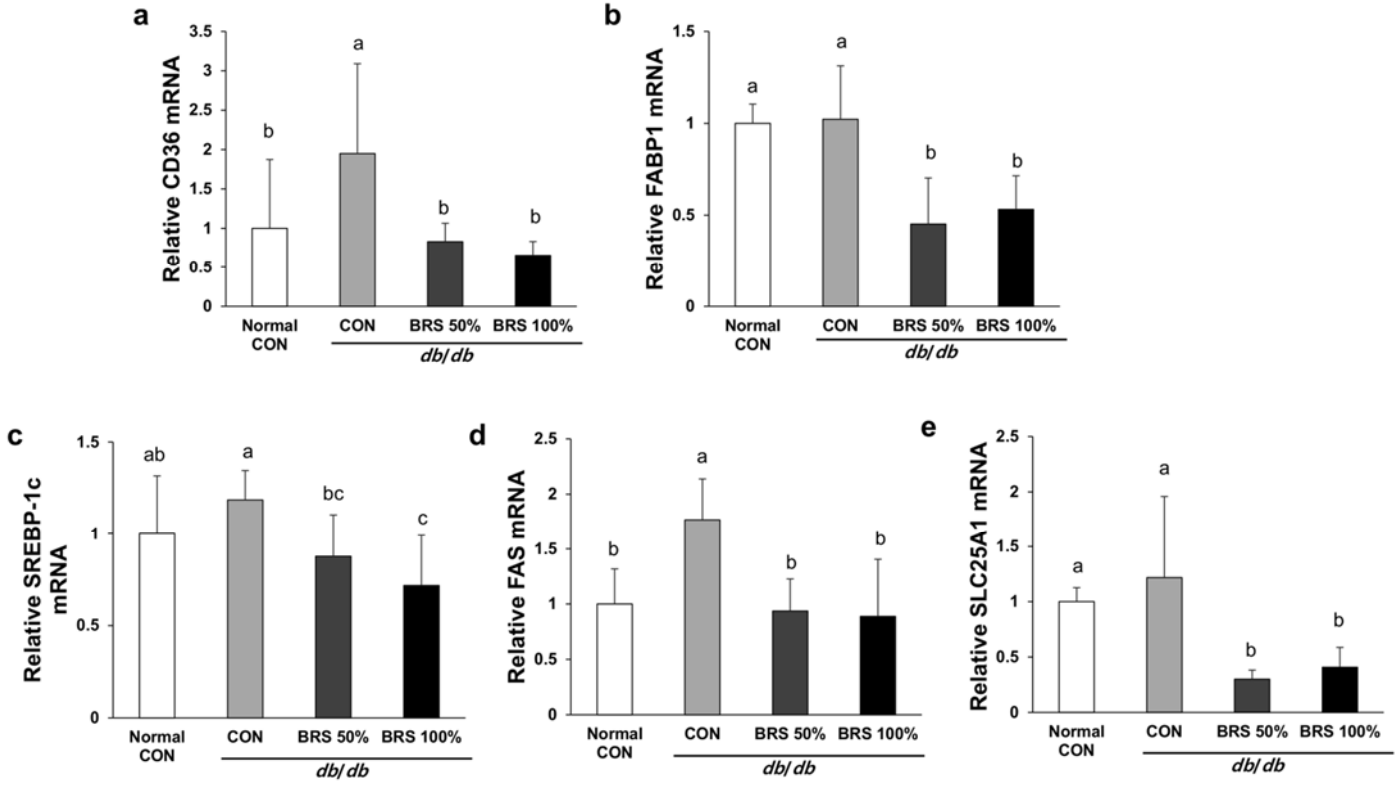


**Fig. 3.8 Relative mRNA levels involved in lipogenesis in the livers of the mice fed control and BRS oil diets**

Values are means and standard deviations (n=8/group). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

### 3.3.11 mRNA levels involved in fatty acid oxidation in liver

CPT1A, ACADL, HADHα, and ACOX known as fatty acid oxidation markers were used to determine the effect of BRS oil on fatty acid oxidation in liver (Fig. 3.9). mRNA levels of CPT1A and ACOX in the livers of the BRS 50% and BRS 100% were significantly ($P<0.05$) higher than those of the CON and these levels were statistically ($P>0.05$) the same as in the normal CON. However, ACADL and HADHα mRNA levels were significantly ($P<0.05$) higher only in the BRS 50% than in the CON.

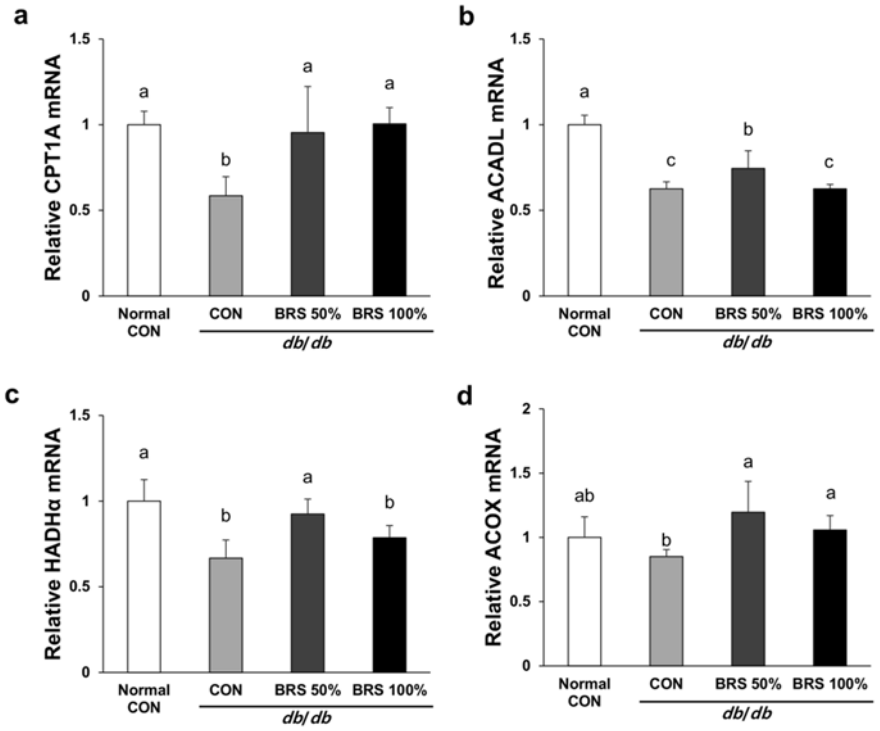


**Fig. 3.9 Relative mRNA levels involved in fatty acid oxidation in the livers of the mice fed control and BRS oil diets**

Values are means and standard deviations (n=8/group). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

3.3.12 mRNA and protein levels of PPAR in liver and epididymal adipose tissue

PPARα mRNA level was significantly ($P<0.05$) higher in the liver of the BRS 50% than that of the CON. However, PPARα mRNA level in the BRS 100% group was higher than in the CON without significance ($P>0.05$). Trend of expressions of PPARα proteins in the livers were different from mRNA result. PPARα protein was significantly ($P<0.05$) higher in the BRS 100% than in the CON, while PPARα protein in the BRS 50% group was higher than in the CON without significance ($P>0.05$). Expressions of PPARα proteins in the BRS oil-treated groups were higher than in the normal CON, although the difference is not significant ($P>0.05$). PPARγ mRNA level was significantly ($P<0.05$) lower in the liver of the BRS 50% than that of the CON. PPARγ protein was significantly ($P<0.05$) lower in the liver of the BRS 100% than in the CON (Fig. 3.10). PPARα mRNA levels were significantly ($P<0.05$) higher in the epididymal adipose tissues of the BRS 50% and BRS 100% than those of the normal CON and CON. PPARα protein was significantly ($P<0.05$) higher in the epididymal adipose tissue of the BRS 100% than those of the normal CON and CON. PPARγ mRNA level was significantly ($P<0.05$) lower in the epididymal adipose tissue of the normal CON than those of the *db*/*db* groups. PPARγ mRNA level was higher in the epididymal adipose tissue of the BRS 50% than that of the CON, although the difference is not significant ($P>0.05$). Protein expression level of PPARγ in the epididymal adipose tissue of the BRS 50% was significantly ($P<0.05$) lower than that of the CON (Fig. 3.11).

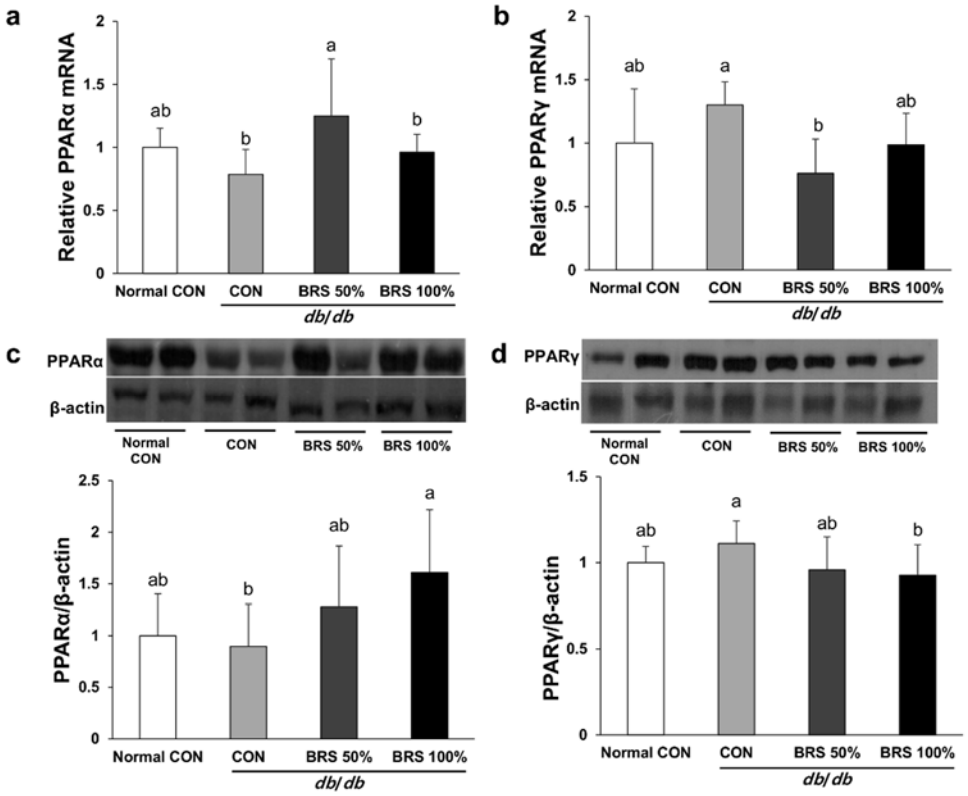


**Fig. 3.10 Relative mRNA and protein levels of PPAR in the livers of the mice fed control and BRS oil diets**

Values are means and standard deviations (n=8/group (a, b), n=4/group (c, d)). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

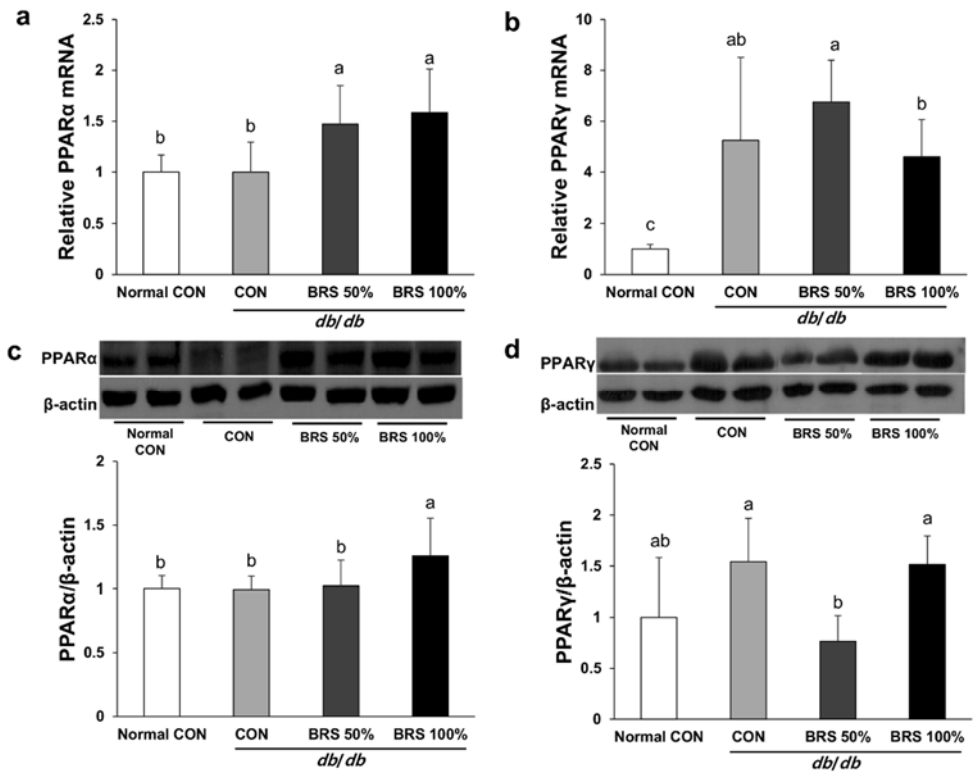


**Fig. 3.11 Relative mRNA and protein levels of PPAR in the epididymal adipose tissues of the mice fed control and BRS oil diets**

Values are means and standard deviations (n=8/group (a, b), n=4/group (c, d)). Values with different superscripts in the same rows are significantly different (ANOVA and Duncan's multiple range test, $P<0.05$).

Normal CON: normal mice fed control diet (16% calories from soybean oil); CON: *db*/*db* mice fed control diet; BRS 50%: *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and BRS 100%: *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

### 3.3.13 Improvement of hepatic lipid accumulation

Effect of BRS oil on hepatic steatosis was observed by histological analysis of liver sections staining with Oil Red O (Fig. 3.12). Oil Red O staining showed that the liver of the CON mice had severe hepatic steatosis. However, the lipid droplets in the livers of the BRS oil-treated mice were smaller and fewer than in the CON, indicating that hepatic lipid accumulation was reduced in the mice fed BRS oil.

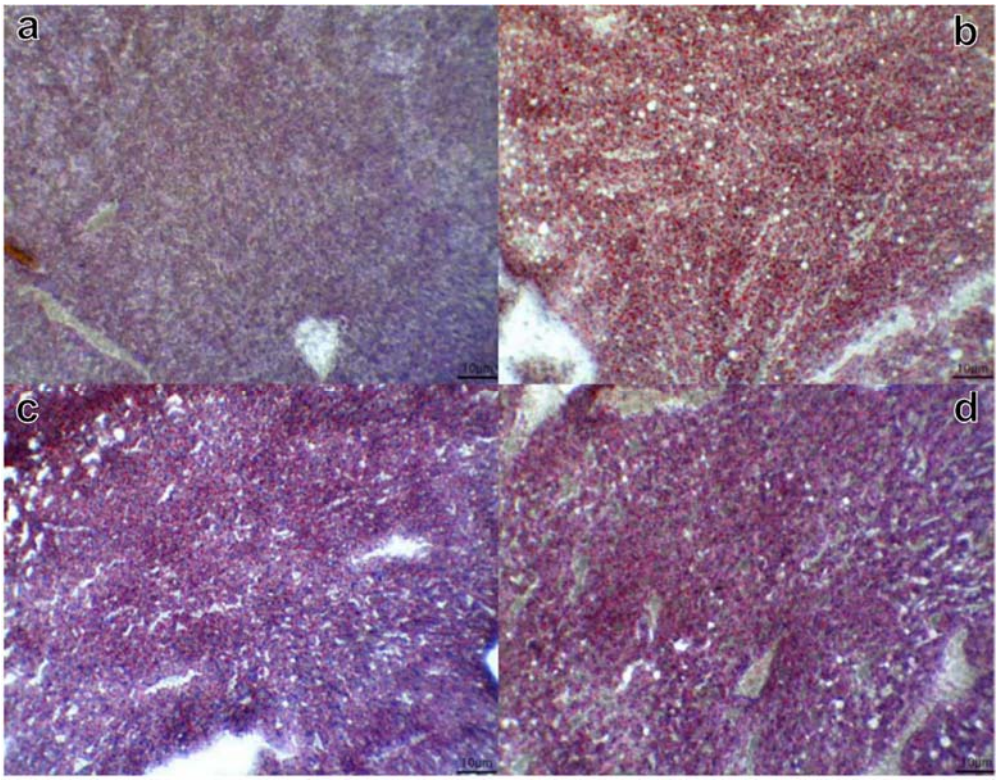


**Fig. 3.**12 Oil Red O-stained frozen sections of livers

Scale bar indicates 10 μm (×100). (a) Normal mice fed control diet (16% calories from soybean oil); (b) *db*/*db* mice fed control diet; (c) *db*/*db* mice fed BRS oil 50% diet (8% calories from soybean and 8% calories from BRS oil); and (d) *db*/*db* mice fed BRS oil 100% diet (16% calories from BRS oil).

### 3.4 Discussion

This study demonstrated that consumption of BRS oil improved inflammatory response in obese diabetic mouse model. The BRS oil diets had more ALA than in the control diet. Concomitantly, the ratio of n-6 to n-3 PUFA decreased with the addition of BRS oil in the experimental diets. An increased ratio of dietary n-6 to n-3 PUFA has been linked to the risk of chronic inflammatory diseases (Grimble 1998). The amount of AA in inflammatory cells may also be influenced by dietary intake of its precursor, linoleic acid (Calder 2005). Replacement of AA with n-3 PUFA in the eicosanoid metabolism leads to less inflammation (González-Périz et al. 2009). The contents of n-3 PUFA increased and n-6 PUFA decreased in the liver of the mice after treatment with ALA-rich linseed oil (Perini et al. 2011). Dietary n-3 PUFA treatment significantly ($P<0.05$) increased long chain n-3 PUFA (20:5, 22:5, and 22:6) in severely obese nondiabetic patients compared with control group. Consequently, n-6 to n-3 PUFA ratio decreased after treatment with n-3 PUFA compared with control group (Itariu et al. 2012). In this study, the BRS oil diets significantly ($P<0.05$) increased ALA and total n-3 PUFA in the liver and epididymal adipose tissue of the diabetic mice compared with control diet. Intake of high amount of n-3 PUFA may be favorable in tissue lipid profile through increase in n-3 PUFA and reduction in AA.

In the present study, TNFα, IL-6, and leptin levels of the serum were lower and IL-10 and adiponectin levels were higher in the BRS oil-treated groups than in the control group. TNFα is able to activate other cytokine networks, including the release of IL-1 and IL-6, thereby amplifying inflammatory response and tissue injury (Tacke

et al. 2009). On the other hand, adiponectin stimulates secretion of anti-inflammatory cytokines (i.e., IL-10), blocks NF-κB activation, and inhibits release of TNFα, IL-6, and inflammatory chemokines (Tilg et al. 2006). Type 2 diabetes and metabolic syndrome have been associated with decreased IL-10 production and circulating IL-10 level is positively correlated with insulin sensitivity (Straczkowski et al. 2005).

This study demonstrated that the dietary BRS oil reduced pro-inflammatory markers (TLR4, TNFα, IL-1β, IL-6, iNOS, COX2, MCP1, and CCR2) and promoted anti-inflammatory markers (IL-10, arginase1, Chi3l3, and Mgl1) in the liver and epididymal adipose tissue of *db*/*db* mice. Stimulation of TLR4 induced expressions of TNFα and IL-6 and TLR4-deficient female mice had less TNFα, IL-6, and MCP1 mRNA levels in liver and adipose tissue than in the wild type mice (Shi et al. 2006). Treatment with DHA alleviated adipose tissue inflammation via decreasing mRNA levels of TNFα, IL-6, and MCP1 and increasing IL-10 (Titos et al. 2011). Yoshihira et al. (2015) also reported that TNFα, MCP1, and IL-1β mRNA levels decreased in aorta of abdominal aortic aneurysms mouse model supplemented with EPA and DHA.

n-3 PUFA are known to prevent ATM infiltration. Titos et al. (2011) observed that the expression of F4/80 was inhibited and IL-10, arginase1, and Chi3l3 expressions were induced in obese mice with the treatment of DHA. To determine the effect of BRS oil on macrophage infiltration in adipose tissue, expressions of F4/80, CD68, and CD14, which are known as discriminative markers in macrophages, were analyzed in this study. F4/80 and CD68 mRNA levels were significantly ($P<0.05$)

lower in the BRS oil-treated mice than in the control mice. This effect is related to decreased expression of inflammatory markers in adipose tissue. These results are in agreement with Clària et al. (2011) and Kanda et al. (2006)'s reports. They reported that pro-inflammatory mediators increases in adipose tissue as the macrophage infiltration into the adipose tissue progresses.

Leptin has been shown to modulate immune responses by stimulating pro-inflammatory immune response and secreting of pro-inflammatory markers such as TNFα and IL-1 (Cava et al. 2004). Ouchi et al. (2003) reported that leptin levels in serum and adipose tissue increase in compliance with pro-inflammatory stimuli and progression of obesity. Feeding EPA and DHA to obese male rats reduced plasma leptin concentration compared to control group (Hassanali et al. 2009). On the other hand, adiponectin plays an important role in regulating insulin sensitivity, and glucose and lipid metabolism besides its anti-inflammatory property (von Frankenberg et al. 2014). Frankenberg et al. (2014) reported that low level of adiponectin was found in patients with obesity, type 2 diabetes, and coronary artery disease. Hassanali et al. (2009) found that concentration of plasma adiponectin increased in obese male rats fed n-3 PUFA diet compared with control. These results are in consistent with the present study, in which leptin decreased and adiponectin increased by intake of BRS oil diets.

In this study, the mice fed BRS oil diet not only lowered serum TG and total cholesterol but also elevated HDL-C compared with the CON. Serum TG and VLDL-

C levels in diabetic rats receiving flaxseed oil or fish oil were significantly ($P<0.05$) lower than those of the control (Devarshi et al. 2013). On the other hand, flaxseed oil or fish oil diet caused significant ($P<0.05$) increase in serum HDL-C level in diabetic mice (Devarshi et al. 2013). DHA-rich fish oil supplementation for 8 weeks reduced serum TG level in normotriglyceridemic and hypertriglyceridemic subjects compared with placebo group (Mansoori et al. 2015). Dietary feeding of ALA-rich garden cress oil to rats significantly ($P<0.05$) decreased total cholesterol, TG, and LDL-C in their liver and serum compared to control (Umesha et al. 2012). Yang et al. (2016) demonstrated that a low n-6 to n-3 PUFA ratio had a beneficial effect on serum lipid metabolism. Low n-6 to n-3 PUFA ratios of 1:1 and 5:1 decreased serum TG, total cholesterol, and LDL-C levels compared with those on the diets with high n-6 to n-3 PUFA ratios of 10:1 and 20:1 (Yang et al. 2016).

Dietary n-3 PUFA have been shown to have positive effects on lipid metabolism. Flaxseed oil diet decreased serum TG level, along with down-regulation of hepatic SREBP-1c mRNA in diabetic rats (Devarshi et al. 2013). Down-regulation of SRBEP-1c reduces FAS, leading to decreased *de novo* lipogenesis (Devarshi et al. 2013). Pachikian et al. (2011) reported that n-3 PUFA depletion diet leads to activation of hepatic SREBP-1c mRNA and lipogenesis, which contribute to hepatic steatosis, whereas flaxseed oil diet decreases hepatic SREBP-1c mRNA in diabetic mice. Dietary EPA supplementation did not significantly ($P<0.05$) influence body weight compared to control diet-fed animal; however, feeding EPA significantly ($P<0.05$) decreased plasma and hepatic TG level (Atek-Mebarki et al. 2015). Also

feeding EPA significantly ($P<0.05$) lowered hepatic SREBP-1c mRNA (Atek-Mebarki et al. 2015). In this study, the mRNA levels of lipogenesis markers including CD36, FABP1, SREBP-1c, FAS, and SLC25A1 in the livers of the BRS oil groups were significantly ($p<0.05$) lower than in the CON. On the other hand, consumption of the diet containing BRS oil increased fatty acid oxidation markers such as CPT1A, ACADL, HADHα, and ACOX.

PPARα induces several markers involved in fatty acid oxidation. n-3 PUFA are key regulators of gene transcription in liver with PPARα and SREBP-1 being best known (Masterton et al. 2010). Flaxseed oil diet up-regulated hepatic PPARα, which leads to decrease in the levels of TG and NEFA and also increases HLD-C level in diabetic rats (Devarshi et al. 2013). n-3 Long chain PUFA depletion leads to substantial enhancement in hepatic SREBP-1c to PPARα ratio that favors *de novo* lipogenesis over fatty acid oxidation (Valenzuela et al. 2011). On the other hand, n-3 long chain PUFA supplementation may be involved in PPARα up-regulation and SREBP-1c down-regulation (Valenzuela et al. 2011). Although hepatic PPARγ is not abundantly expressed under normal conditions, high expression of PPARγ in liver is related to increase of FAS (Pettinelli et al. 2011). As an increased PPARγ expression has been found in steatotic livers, it has been suggested that PPARγ may contribute to the development of steatosis (Berlanga et al. 2014). In this study, expressions of PPARα mRNA and protein were higher and expressions of PPARγ mRNA and protein were lower in the liver and epididymal adipose tissue of the mice fed BRS oil than those of the CON.

Possibility that the BRS oil treatment would ameliorate liver steatosis was investigated by Oil Red O staining in the present study. Lipid droplets were reduced in the livers of the mice fed BRS 50% and BRS 100% compared with the CON. The result of the histological data is consistent with the lipid profiles of serum and liver as well as genes and proteins. Oral administration of EPA ameliorated hepatic fat accumulation in mice fed a Western style diet through down-regulating the expressions of several lipogenic genes such as SREBP-1 and FAS (Kajikawa et al. 2009). Alwayn et al. (2005) also observed that n-3 PUFA supplementation prevented hepatic steatosis in mice with NAFLD.

Raspberry seed oil contains health-beneficial compounds such as high levels of ALA, tocopherols, and phenolic acids (Radočaj et al. 2014). Dietary raspberry seed oil improved plasma lipid profile and liver functions and reduced low-grade inflammation in rats (Fotschki et al. 2015). In this study, consumption of BRS oil, which is abundant in ALA, tocopherols, and phenolic compounds, might affect the results of this study similar to those of the study by Fotschki et al. (2015).

In conclusion, BRS oil might improve inflammatory status in *db*/*db* mice. Potent anti-inflammatory effect of BRS oil might be related with disrupting production of inflammatory mediators and promoting production of anti-inflammatory markers. BRS oil might elevate ALA and total n-3 PUFA levels and lower AA and n-6 to n-3 PUFA ratio in the liver and epididymal adipose tissue of *db*/*db* mice. In addition, BRS oil might inhibit lipogenesis and promote fatty acid oxidation in *db*/*db* mice.

# Chapter 4

# Summary and Conclusion

Black raspberry (*Rubus occidentalis*) fruits, most widely cultivated among *Rubus* fruits in Korea, are mainly used to produce wine or juice. Pomace from processing of the fruits, which contains pulp and seeds, has been used as fertilizer or animal feed. However, the seeds in the by-product may be an attractive source of functional compounds. BRS contains considerable amounts of oil. BRS oil contains significant levels of essential fatty acids, especially ALA, and other health beneficial compounds.

The aim of this study was to evaluate whether dietary BRS oil has beneficial effect on inflammatory status and lipid metabolism in high-fat diet-induced obese and *db*/*db* mice, two widely used animal models for obesity and type 2 diabetes.

Study 1: Fatty acid compositions of the liver, protein and mRNA levels related to inflammation, and lipid metabolism were measured in the liver and epididymal adipose tissue of the mice fed high-fat control and high-fat BRS oil diets.

1) BRS oil increased ALA ($P<0.01$) level and lowered AA level in the liver of the high-fat diet-induced obese mice.

2) mRNA levels of pro-inflammatory markers including NF-κB, TNFα, IL-1β, IL-6, iNOS, COX2, and MCP1 in the liver and epididymal adipose tissue of the BRS oil group were lower than those of the control group.

3) Correspondingly, mRNA levels of anti-inflammatory markers including IL-10, arginase1, Chi3l3, and Mgl1 were higher in the liver and epididymal adipose tissue of the BRS oil group than those of the control group.

4) mRNA levels involved in lipogenesis such as CD36, FABP1, SREBP-1c, FAS, and SLC25A1 in the liver of the BRS oil group were lower than in the control group.

5) mRNA levels involved in fatty acid oxidation such as CPT1A, ACADL, HADHα, and ACOX in the liver of the BRS oil group were higher than those of the control group.

6) PPARα, which stimulates fatty acid oxidation, significantly increased both in mRNA ($P<0.001$) and protein ($P<0.01$) levels in the liver of the mice fed BRS oil diet compared with control diet.

Study 2: Fatty acid composition in the liver and epididymal adipose tissue and protein and mRNA levels related to inflammation in the liver and epididymal adipose tissue of the *db/db* mice were determined. Lipid profiles in serum and liver as well as mRNA levels related to lipid metabolism in liver also analyzed to evaluate the effect of BRS oil on lipid metabolism in *db/db* mice.

1) ALA contents in the liver and epididymal adipose tissue of the BRS oil-treated groups were significantly ($P<0.05$) higher than those of the control group. The mice fed the BRS oil diet also had significantly ($P<0.05$) lower n-6 to n-3 PUFA ratio in the liver and epididymal adipose tissue than the mice fed the control diet.

2) Protein and mRNA levels of pro-inflammatory markers in the liver and epididymal adipose tissue of the BRS oil group were lower than those of the control group.

3) Consequently, mRNA levels of anti-inflammatory markers in the liver and epididymal adipose tissue of the BRS oil group were higher than those of the control group.

4) BRS oil ameliorated macrophage infiltration through decreased mRNA levels of F4/80 and CD68 in the epididymal adipose tissue.

5) The BRS oil diet significantly ($P<0.05$) lowered serum TG, NEFA, and total cholesterol compared with the control diet. The BRS oil diet also significantly ($P<0.05$) lowered hepatic total lipid, TG, and NEFA compared with the control diet.

6) mRNA levels related to lipogenesis were lower in the liver of the mice fed BRS oil than those of the control, while mRNA levels related to fatty acid oxidation were higher in the liver of the mice fed BRS oil than those of the control.

7) Protection effect of BRS oil on hepatic steatosis in *db*/*db* mice was observed by histological analysis of liver sections staining with Oil Red O.

Results of this study suggest that BRS oil may increase n-3 PUFA and lower n-6 PUFA in the liver and ameliorate inflammatory consequences in high-fat diet-induced obese mice and *db/db* mice. Inflammatory response attenuation effect of BRS oil might be related to disrupting the production of pro-inflammatory mediators and elevating the production of anti-inflammatory markers. The anti-inflammatory properties of BRS oil might be related to inhibition of TLR4 and NF-κB pathway, which then inactivates the transcription of many pro-inflammatory genes that encode

pro-inflammatory molecules including cytokines, chemokines, and enzymes. BRS oil might also improve lipid metabolism and hepatic steatosis by inhibiting lipogenesis and promoting fatty acid oxidation in high-fat diet-induced obese mice and *db/db* mice. Effect of BRS oil on lipid metabolism might be related to PPARα up-regulation and SREBP-1c down-regulation favoring fatty acid oxidation over lipogenesis. Further research involving glucose metabolism and insulin resistance might be needed to understand how BRS oil affects inflammation-induced insulin resistance.

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# 국문 초록

## 블랙라즈베리 씨앗 기름이 고지방식이로 유도한 비만 마우스와 당뇨병 모델 마우스의 염증반응과 지질대사에 미치는 영향

이희재

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국민소득이 증가됨에 따라 건강에 대한 관심이 높아지고 있으며 만성질환의 치료 및 예방을 위하여 천연물, 특히 식품성분을 이용하는 연구가 활발히 이루어지고 있다. 블랙라즈베리(*Rubus occidentalis*)는 현재 우리나라에서 널리 재배하는 나무딸기류의 품종으로 과일 수확량이 많고 다른 품종에 비해 폴리페놀 함량이 높은 것으로 알려져 있다. 블랙라즈베리는 생과일을 그대로 섭취하기도 하지만 대부분 발표주 등의 술로 제조하고 있으며, 일부 음료나 퓨레 등으로 제조하기도 한다. 이러한 식품 제조 후에 남는 씨앗과 과육을 폐기하거나 거름 혹은 가축 사료로 사용하고 있다. 블랙라즈베리 씨앗(BRS)에는 n-3 지방산인 알파-리놀렌산

(ALA)이 약 30% 함유되어 있는데, 이는 콩기름, 카놀라유, 옥수수기름 등의 다른 식물성 기름과의 차이점이라 할 수 있다. 또한 BRS 기름에는 콩기름, 옥수수기름, 올리브유, 카놀라유, 들기름, 포도씨유보다 총 토콜 함량이 높으며, 폴리페놀과 파이토스테롤과 같은 기능성 성분이 풍부한 특징이 있다.

본 연구의 최종 목적은 BRS 기름을 고지방 식이로 비만을 유도한 마우스와 제 2형 당뇨병 모델 마우스인 *db*/*db* 마우스에 섭취시켰을 때 이 동물의 염증상태와 지질대사에 미치는 영향을 확인하는 것이다.

본 연구의 첫 번째 부분에서는 블랙라즈베리 씨앗 기름 섭취가 고지방 식이로 비만을 유도한 마우스의 염증반응과 지질대사에 미치는 영향을 확인하였다. 5주령 C57BL/6 마우스를 1) 총 식이의 50% 열량을 라드에서, 5% 열량을 콩기름과 5% 열량을 옥수수기름에서 제공받는 대조군과 2) 총 식이의 50% 열량을 라드와 10% 열량을 BRS 기름에서 제공받는 BRS 기름 군 등 두 군으로 나누었다. 총 12주 동안 식이를 공급하였다. 본 연구에서 사용한 BRS 기름의 지방산은 리놀레산이 57.0%, ALA가 29.4%, 올레산이 9.80%였다. BRS 기름 식이가 대조군 식이에 비해 ALA 함량이 유의적으로 높았다($P<0.001$). 실험 시작 전의 동물의 몸무게, 실험 종료 후의 몸무게, 식이 섭취량, 식이 효율은 두 군 간의 유의적인 차이가 없었다($P>0.05$). 실험 종료 후 간, 비장, 신장, 부고환

지방조직의 무게는 BRS 기름 군이 대조군보다 높았지만 유의적인 차이가 없었다(P>0.05). 12주 후 간조직의 ALA 함량은 BRS 기름 군이 1.84%로 0.45%인 대조군보다 유의적으로 높았다(P<0.01). BRS 기름 군의 간조직의 TLR4, NF-κB, phospho-NF-κB, COX2, I-κBα, phospho-I-κBα와 같은 염증반응과 관련된 단백질 발현은 대조군의 경우보다 낮았다. BRS 기름 군의 간조직과 부고환지방조직의 NF-κB, TNFα, IL-1β, IL-6, iNOS, COX2, MCP1과 같은 염증반응과 관련된 mRNA는 대조군의 경우보다 낮았다. 한편, BRS 기름 군의 간조직과 부고환지방조직의 항염증반응과 관련된 IL-10, arginase1, Chi3l3, Mgl1의 mRNA는 대조군의 경우보다 높았다. BRS 기름 군의 부고환지방조직의 leptin의 mRNA는 대조군보다 낮았지만 유의적인 차이가 없었고(P>0.05), 부고환지방조직의 adiponectin mRNA는 BRS 기름 군이 대조군에 비하여 유의적으로 높았다(P<0.01).

BRS 기름 군의 혈청과 간조직의 TG는 대조군의 경우보다 각각 14.2%와 12.1% 낮았다. BRS 기름 군의 혈청의 NEFA와 총 콜레스테롤은 대조군의 경우보다 각각 42.1%와 13.0% 낮았다(P<0.05). BRS 기름 군의 혈청의 HDL-C은 대조군의 경우보다 약간(4.10%) 높았지만 유의적인 차이는 없었다(P>0.05). BRS 기름 군의 간조직의 총 지방함량은 대조군의 경우보다 13.0% 낮았지만 유의적인 차이는 없었다(P>0.05). BRS 기

름 군의 간조직의 NEFA(25.7%: P<0.05)와 총 콜레스테롤은 대조군의 경우보다 53.2% 낮았다(P<0.001). BRS 기름 군의 간조직의 CD36, FABP1, SREBP-1c, FAS, SLC25A1과 같은 지방 합성에 관련된 mRNA는 대조군의 경우보다 낮았다. 반면 BRS 기름 군의 간조직의 CPT1A, ACADL, HADH$\alpha$, ACOX와 같은 지방 산화와 관련된 mRNA는 대조군의 경우에 비해 높았다. BRS 기름 군의 간조직의 PPAR$\alpha$의 mRNA(P<0.001)와 단백질(P<0.01) 모두 대조군보다 높았다. 그러나 두 군간에 간조직의 PPAR$\gamma$의 mRNA와 단백질은 유의적인 차이가 없었다. BRS 기름 군의 부고환지방조직의 PPAR$\alpha$의 mRNA는 대조군에 비해 유의적으로 높았다(P<0.05). 그러나 부고환지방조직의 PPAR$\alpha$의 단백질과 PPAR$\gamma$의 mRNA와 단백질은 두 군간에 유의적인 차이가 없었다(P>0.05).

본 연구의 두 번째 부분에서는 블랙라즈베리 씨앗 기름을 당뇨병 모델 마우스에 섭취시켰을 때의 염증반응과 지질대사에 미치는 영향을 확인하였다. 6주령 마우스를 네 군으로 나누어 총 10주간 식이를 공급했다: 1) 총 식이의 16%의 열량을 콩기름에서 제공받는 C57BL/6 마우스(normal CON); 2) 총 식이의 16%의 열량을 콩기름에서 제공받는 C57BL/KsJ-*db*/*db* 마우스(CON); 3) 총 식이의 8% 열량을 콩기름과 8%의 열량을 BRS 기름에서 제공받는 C57BL/KsJ-*db*/*db* 마우스(BRS

50%); 4) 총 식이의 16% 열량을 BRS 기름에서 제공받는 C57BL/KsJ-*db*/*db* 마우스(BRS 100%). 동물의 식이 섭취량과 실험 시작 전의 몸무게는 *db*/*db* 마우스 세 군간에 유의적인 차이가 없었다 (P>0.05). Normal CON 군의 식이섭취량과 몸무게는 *db*/*db* 마우스 군보다 유의적으로 낮았다(P<0.05). 실험 종료 후의 몸무게는 BRS 50% 군이 CON 군보다 유의적으로 낮았다(P<0.05). BRS 100% 군의 최종 몸무게는 CON 군보다 낮았지만 유의적인 차이는 없었다(P>0.05). 간, 부고환지방조직, 신장, 비장의 무게는 *db*/*db* 마우스 세 군간에 유의적인 차이가 없었다(P>0.05). BRS 50%와 BRS 100% 군의 혈청의 인슐린 농도는 CON 군의 경우보다 유의적으로 낮았다(P<0.05). 10주 후, BRS 50%와 BRS 100% 군의 간조직과 부고환지방조직의 n-6 지방산과 n-3 지방산의 비율이 CON 군에 비하여 유의적으로 낮았다(P<0.05). 반면 BRS 50%와 BRS 100% 군의 간조직과 부고환지방조직의 ALA와 총 n-3 지방산의 함량은 normal CON과 CON 군의 경우보다 유의적으로 높았다(P<0.05). BRS 50%와 BRS 100% 군의 혈청의 TNF$\alpha$와 IL-6는 CON 군의 경우보다 유의적으로 낮았으며(P<0.05), BRS 100% 군의 혈청 IL-10은 CON 군보다 유의적으로 높았다(P<0.05). BRS 50%와 BRS 100% 군의 부고환지방조직에 있어서의 염증반응과 관련된 TLR4, NF-$\kappa$B, COX2의 단백질 발현은 CON 군보다 유의적으로 낮았다

(P<0.05). BRS 50%와 BRS 100% 군의 간조직과 부고환지방조직에 있어서의 염증반응과 관련된 TLR4, TNF$\alpha$, IL-1$\beta$, IL-6, iNOS, COX2, MCP1, CCR2의 mRNA가 CON 군의 경우보다 낮았다. 반면 BRS 50%와 BRS 100% 군의 부고환지방조직에 있어서의 항 염증반응과 관련된 IL-10, arginase1, Chi3l3, Mgl1의 mRNA는 CON 군보다 높았다. 또한 BRS 50%와 BRS 100% 군의 부고환지방조직의 F4/80, CD68, leptin의 mRNA가 CON 군에 비해 유의적으로 낮았다(P<0.05).

BRS 기름 군의 혈청 TG, NEFA, 총 콜레스테롤은 CON 군에 비해 유의적으로 낮았으며(P<0.05), BRS 50% 군의 혈청 HDL-C은 CON 군에 비해 유의적으로 높았다(P<0.05). BRS 기름 군의 간조직의 총 지질 함량, TG, NEFA, 총 콜레스테롤은 CON 군에 비해 유의적으로 낮았다(P<0.05). BRS 기름 군의 간조직에 있어서의 CD36, FABP1, SREBP-1c, FAS, SLC25A1와 같은 지방 합성 관련 지표의 mRNA는 CON 군에 비해 유의적으로 낮았다(P<0.05). 한편, BRS 기름 군의 간조직에 있어서의 지방 산화에 관련된 지표인 CPT1A, ACADL, HADH, ACOX의 mRNA는 CON 군에 비해 높았다. BRS 50% 군의 간조직의 PPAR$\alpha$의 mRNA는 CON 군의 경우보다 유의적으로 높았으며(P<0.05), BRS 100% 군의 간조직의 PPAR$\alpha$의 단백질 발현은 CON 군의 경우보다 유의적으로 높았다(P<0.05). BRS 50% 군의 간조직의 PPAR$\gamma$의 mRNA는

CON 군의 경우보다 유의적으로 낮았으며(P<0.05), BRS 100% 군의 간조직의 PPAR$\gamma$의 단백질 발현은 CON 군의 경우보다 유의적으로 낮았다(P<0.05). BRS 50%와 BRS 100% 군 모두의 부고환지방조직에 있어서는 PPAR$\alpha$의 mRNA가 normal CON과 CON 군의 경우에 비해 유의적으로 높았으며(P<0.05), BRS 100% 군의 PPAR$\alpha$의 단백질 발현은 CON 군의 경우보다 유의적으로 높았다(P<0.05). 부고환지방조직에서의 PPAR$\gamma$의 mRNA는 CON 군과 BRS 기름 군 간에 유의적인 차이가 없었으며(P>0.05), PPAR$\gamma$의 단백질 발현은 BRS 50% 군의 경우가 CON 군의 경우보다 유의적으로 낮았다(P<0.05). 간조직을 Oil Red O 시약으로 염색하여 조직학적으로 관찰한 결과, BRS 기름을 처리한 군이 CON 군에 비해 지방입자의 크기와 수가 감소한 것을 확인하였다.

본 연구에서는 고지방 식이로 비만을 유도한 마우스와 당뇨병 모델 마우스에게 BRS 기름을 제공하였을 때 간조직과 지방 조직에서 염증성 물질의 생성이 감소하고, 항염증성 물질의 생성이 증가하는 것을 확인하였다. 또한, 고지방 식이로 비만을 유도한 마우스와 당뇨병 모델 마우스에게 BRS 기름을 섭취하도록 했을 때 간조직에서 지방 합성 물질이 감소하고 지방 산화 물질이 증가하는 것을 확인하였다. 본 연구 결과, BRS 기름은 n-3 지방산인 ALA의 좋은 급원이 되며, 고지방 식이로 비만을 유도한 마우스와 당뇨병 모델 마우스에서 염증반응을 개선한다는 것을

확인하였다. 또한 BRS 기름은 지방 합성을 억제하며 지방 산화를 촉진하여 비만과 당뇨 상태에서 지질 대사를 개선하는 것으로 보인다. 따라서 블랙라즈베리 가공품의 부산물인 씨앗의 활용도를 높일 수 있을 것이라고 생각한다.

주요어: 블랙라즈베리 씨앗 기름, 알파-리놀렌산, 염증, 지질대사, 고지방 식이, *db*/*db* 마우스

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