



Restoration of Declined Immune Responses and Hyperlipidemia by *Rubus occidentalis* in Diet-Induced Obese Mice

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Abstract

Hyperlipidemia, which is closely associated with a fatty diet and aging, is commonly observed in the western and aged society. Therefore, a novel therapeutic approach for this disease is critical, and an immunological view has been suggested as a novel strategy, because hyperlipidemia is closely associated with inflammation and immune dysfunction. In this study, the effects of an aqueous extract of *Rubus occidentalis* (RO) in obese mice were investigated using immunological indexes. The mice were fed a high-fat diet (HFD) to induce hyperlipidemia, which was confirmed by biochemical analysis and examination of the mouse physiology. Two different doses of RO and rosuvastatin, a cholesterol synthesis inhibitor used as a control, were orally administered. Disturbances in immune cellularity as well as lymphocyte proliferation and cytokine production were significantly normalized by oral administration of RO, which also decreased the elevated serum tumor necrosis factor (TNF)- α level and total cholesterol. The specific immune-related actions of RO comprised considerable improvement in cytotoxic T cell killing functions and regulation of antibody production to within the normal range. The immunological evidence confirms the significant cholesterol-lowering effect of RO, suggesting its potential as a novel therapeutic agent for hyperlipidemia and associated immune decline.

Key Words: *Rubus occidentalis*, Hyperlipidemia, Immune restoration, *In vivo* CTL assay

INTRODUCTION

Our society has currently adopted a westernized diet and lifestyle and, therefore, the prevalence of metabolic diseases has increased. Metabolic diseases are regarded as the leading cause of mortality in the US. In particular, hyperlipidemia is a symptom of metabolic disease and is significantly associated with inflammation. Excessive lipoproteins initiate partial inflammation by modulating leukocyte activity and disturbing cytokine regulation, which worsens over time (Emanuela *et al.*, 2012; van Diepen *et al.*, 2013). Furthermore, immune cells and their signaling processes are likely to be impaired by lipids, which is deleterious to the normal immune functions. Therefore, long-term hyperlipidemia-induced inflammation possibly contributes to immune disturbances or immune function decline. Diet-induced obese (DIO) mice, develop various metabolic dysfunctions such as hyperlipidemia and systemic inflammation, and their immune functions are considerably im-

paired (Sato Mito *et al.*, 2009; Im *et al.*, 2010). Although it is difficult to determine the full extent of the effect of hyperlipidemia on global immune function, its relevance to the host defense system has been partly reported (Ludewig *et al.*, 2001).

Rubus occidentalis (RO), commonly called the "black raspberry," is one of the most popular fruits used as a flavorant and additive. It has been used for nourishment and as traditional remedies, especially in the immature form in Asian countries (Cha *et al.*, 2001; Lee *et al.*, 2011). Numerous studies have reported that the active compounds present in this fruit include various antioxidants such as flavonoids and organic acids, and have investigated its significant antitumor, anti-inflammatory, and lipid metabolism effects (Yoon *et al.*, 2003; Bhandary *et al.*, 2012; Lee *et al.*, 2014a). However, for commercial use, the quality of RO needs to be enhanced and this process has been achieved by using the black raspberry crop harvested from Gochang, Korea, the largest producer of black raspberry in Korea. Through a series of studies, various factors such as

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the sampling period or extraction conditions were optimized to produce fruits with a high content of bioactive principles (Choi *et al.*, 2013; Lee *et al.*, 2014b). The aqueous extract of immature RO investigated in the present study was chemically analyzed in a previous study (Choi *et al.*, 2013). The major active compound of the extract was confirmed to be ellagic acid, which is well known for its antioxidative and anticancer effects (Saleem *et al.*, 2002; Seeram *et al.*, 2005). Recent studies have also demonstrated the lipid lowering effects of ellagic acid both *in vitro* and *In vivo* (Yoshimura *et al.*, 2013; Okla *et al.*, 2015). Ellagic acid as a marker compound of RO, was independently analyzed using a standardized method (Kim *et al.*, 2012). The aqueous extract of RO used in this study contained 3.2% ellagic acid.

The lipid-lowering effect of RO was demonstrated *in vitro* by Choi *et al.* (2013), who reported that RO not only regulated the mRNA of low-density lipoprotein (LDL) receptor-related and apolipoprotein (APO) genes, but also reduced 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity. However, the immune enhancing effect of RO has not been clearly demonstrated. Therefore, we designed this study to determine the restorative effect of RO on hyperlipidemia-associated immune decline in DIO mice.

MATERIALS AND METHODS

Preparation of aqueous extract of immature RO

The RO used in this study was provided by the Berry and Biofood Research Institute (Gochang, Korea). The aqueous extract of the immature RO was prepared in accordance with a previous report (Choi *et al.*, 2013). In brief, the immature fruits of RO were collected 28 days before the flower bloomed (Gochang, Korea) and subsequently extracted twice with a 10-fold volume of distilled water using a heating mantle at 100°C for 2 h. The extract was then filtered, freeze-dried (PVTFD 500R, Il-sinbiobase, Dongduchen, Korea), and the lyophilized RO was dissolved in 0.5% carboxymethylcellulose (CMC) solution to the appropriate concentrations (125 and 250 mg·kg⁻¹·day⁻¹) before use.

High-performance liquid chromatography (HPLC) analysis of marker compound in RO

The ellagic acid content of the lyophilized RO was analyzed as a marker compound by using a standardized high-performance liquid chromatography (HPLC) method (Kim *et al.*, 2012). Briefly, the RO was assayed on an HPLC system (ACQUITY H-class, Waters, Co, Milford, MA, USA) using an ultraviolet (UV) detector (370 nm) and the separation was performed on a Shiseido capcellpak C18 UG column (5- μ m, 4.6×250 mm, Shiseido, Tokyo, Japan) with a mobile phase of 0.1% phosphoric acid in water and methanol.

Animals and experimental treatments

The male C57BL/6 mice (4-week-old) were purchased from Samtako Inc. (Osan, Korea), and allowed to acclimatize for 1 week before the experiments were conducted. The mice were housed in a laboratory animal facility at a temperature and humidity of approximately 20–24°C and 30–70%, respectively, and a 12-h light-dark cycle, with free access to commercial rodent feed and sterile water. All the experimental procedures were performed in strict compliance with the Guidelines for the

Care and Use of Laboratory Animals issued by the Sahmyook University (Seoul, Korea).

To induce hyperlipidemia, the mice were fed a 45-kcal high-fat diet (HFD) for 23 weeks, and the disease progression was confirmed by monitoring the body weight and blood cholesterol levels. Then, RO and rosuvastatin were orally administered for 16 weeks, after which the mice were euthanized under ether anesthesia.

Measurement of cholesterol indexes in peripheral blood

The relevant biomarkers such as body weight and blood cholesterol levels were used as indicators of hyperlipidemia. During the HFD feeding, blood samples from each mouse were collected by retro-orbital bleeding, and a specific amount of serum was isolated for cholesterol analysis by using a biochemical analyzer (AU480, Beckman Coulter, CA, USA). The mice were selected and grouped for further experiments based on their individual cholesterol parameters including total cholesterol, high-density lipoprotein (HDL), LDL, and triglyceride levels. The mice were subsequently euthanized, and their whole blood was collected for analysis.

Lymphocyte cellularity analysis

The mouse splenocytes were prepared in phosphate-buffered saline (PBS). Prior to staining the lymphocytes, non-specific binding was prevented by Fc receptor blocking, and then they were incubated with monoclonal antibodies. The immunostained cells were fixed with 1% paraformaldehyde in PBS, and each sample was analyzed using a fluorescence-activated cell sorting (FACS) system (Beckman Coulter).

Splenocyte proliferation assay

The splenocytes were incubated in the presence of lipopolysaccharide (LPS, 100 ng/mL) or concanavalin A (ConA, 1 μ g/mL), and the cell proliferation was measured by using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) cell viability assay. The absorbance values were read at 560 nm using an automated VERSAmix microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cytokine assay

The levels of pro-inflammatory cytokines in the lymphocyte culture medium and mouse serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience Inc., San Diego, CA, USA). The experimental process was performed according to the manufacturer's instructions, and the absorbance values were measured at 450 and 570 nm using a microplate reader (Molecular Devices, Sunnyvale). The values obtained at 570 nm were subtracted from those at 450 nm, and the concentration of each cytokine was calculated according to a standard curve constructed by using each of the recombinant cytokines in the ELISA kit.

Immunoglobulin G (IgG) assay

The mice in each group were injected subcutaneously with ovalbumin (OVA) peptide suspended in complete Freund's adjuvant (CFA, Sigma-Aldrich Corp., St. Louis MO, USA), followed by a secondary injection of OVA peptide in incomplete Freund's adjuvant (IFA, Sigma-Aldrich Corp.). After the secondary immunization, the mice were euthanized, whole blood samples were collected, the serum was separated, and then immunoglobulin G (IgG) levels were measured by using an

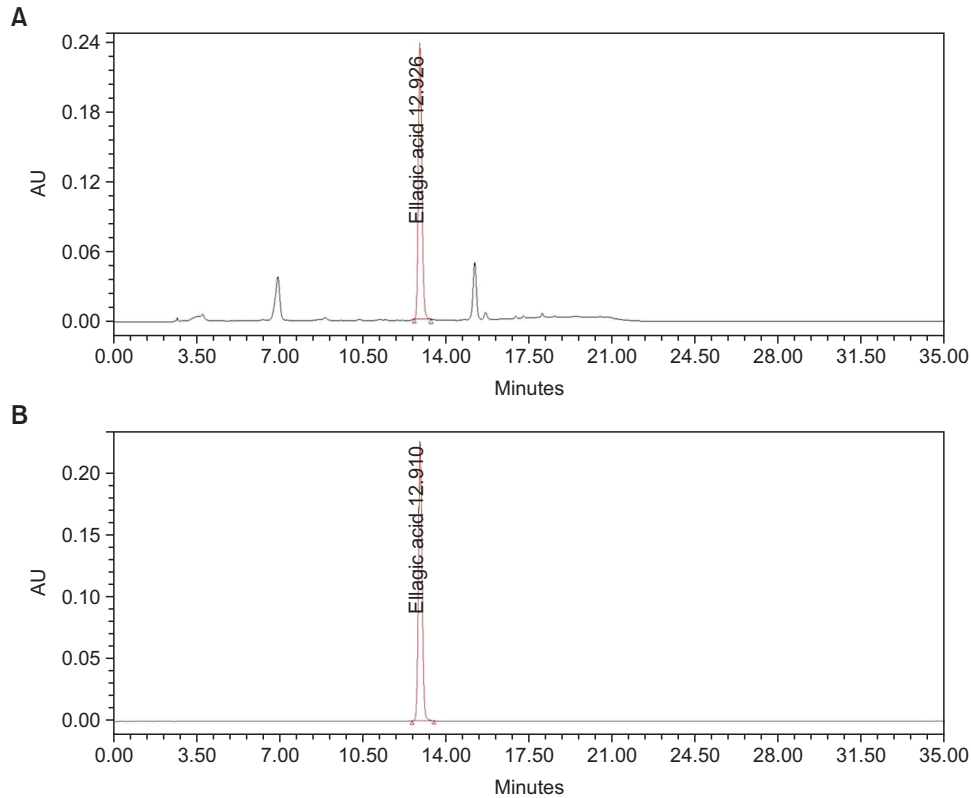


Fig. 1. Chromatogram of the standard and aqueous extract of immature *Rubus occidentalis* (RO). Ellagic acid standard solution (A) and RO sample solution (B) were analyzed using high-performance liquid chromatography (HPLC), and the total amount of ellagic acid in the aqueous extract of RO was calculated based on comparison with the authentic standard.

ELISA. In brief, an immuno-plate (NUNC, Roskilde, Denmark) was coated overnight with 300 mg/mL of OVA solution (Sigma-Aldrich Corp.) followed by blocking with 10% fetal bovine serum (FBS) in PBS. The serum samples and standard were dropped onto the plate and incubated, followed by the addition of the horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (Sigma-Aldrich, Corp.) for specific binding, and then incubation with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution to determine the IgG concentration.

In vivo cytotoxic T lymphocyte (CTL) assay

According to the method of Im *et al.* (2010), four mice per group were immunized with soluble OVA (100 µg/mouse), intravenously (*i.v.*). After 7 days, the splenocytes from naïve syngeneic mice were prepared as target cells, and were pulsed with OVA (257-264) peptide for 1 h at 37°C, followed by labeling with a high concentration of carboxyfluorescein succinimidyl ester (CFSE, 5 µM, CFSE_{high}) for 10 min at room temperature. The same number of unpulsed syngeneic cells was prepared and labeled with a low concentration of CFSE (1 µM, CFSE_{low}). A 1:1 mixture of each target cell was injected via the tail vein of the OVA-immunized recipient mice, which were then sacrificed, their splenocytes were harvested, and then subsequently analyzed by using FACS.

Statistical analysis

All the results are presented as mean ± standard error of mean (SEM), and the significant differences between the con-

trol and treated groups were statistically analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test for each parameter of interest. This study used a *p*<0.05 significance level.

RESULTS

HPLC analysis of RO

The major components of the RO were previously elucidated (Choi *et al.*, 2013) with ellagic acid as a marker compound, which was analyzed by HPLC. The total ellagic acid was identified based on its retention times and quantified using an authentic standard. The results revealed that the aqueous extract of RO prepared in this study contained 3.2% ellagic acid (32.2 mg/g, Fig. 1).

Effect of RO on body weight and hyperlipidemia indexes

To monitor the changes in body weight and lipid parameters of the mice, we measured their body weight every two weeks and blood lipids at the middle and end of experimental period. As shown in Table 1, HFD feeding for 23 weeks successfully induced obesity and hyperlipidemia in mice, but no significant change was observed in the triglyceride level. As shown in Table 2, the oral administration of RO significantly decreased the total cholesterol in the hyperlipidemic mice and improved the other indexes.

Effect of RO on lymphoid organ weights

We also determined the weights of the spleens and thymuses of the mice as crucial indexes of the host immune state. As shown in Fig. 2A, the weight of the mouse thymuses decreased with age and HFD feeding, but there were no significant differences between the HFD-fed groups. In contrast, the HFD-induced hypertrophy of the spleen was normalized by oral administration of RO and rosuvastatin (Fig. 2B).

Effect of aqueous extract of immature RO on lymphocyte subset population

We monitored the changes in the ratio of immune cell subtypes to investigate the effects on the balance between them. As shown in Fig. 3A, the numbers of CD4⁺ and CD8⁺ T cells in the thymus were dramatically decreased by the fatty diet while the population of CD4⁺CD8⁺ double-positive cells was not affected. The oral administration of the RO extract significantly increased the population of these lymphocytes. The same subtypes in the splenocytes were examined, and the calculation of the ratio of CD4⁺ to CD8⁺ T cells showed that it was significantly altered by HFD feeding and restored following oral RO treatment (Fig. 3B). However, the absolute number of CD4⁺ and CD8⁺ T cells was not significantly modified by RO treatment. The CD11b⁺ cells in the spleen significantly increased in the HFD-fed mice while this effect was considerably diminished by treatment with both concentrations of RO

Table 1. Body weight and serum lipid level in high-fat diet (HFD) and regular diet (RD) fed mice

	RD	HFD
Body weight (g)	31.7 ± 1.8	45.7 ± 3.2***
Total cholesterol (mg/dL)	120.9 ± 21.5	312.3 ± 38.1***
HDL (mg/dL)	87.1 ± 14.8	207.7 ± 18.1***
LDL (mg/dL)	18.1 ± 7.2	55.3 ± 9.4***
Triglyceride (mg/dL)	137.0 ± 49.5	116.8 ± 20.5

Mice were fed a 45-kcal HFD or RD for 23 weeks and their average body weight and serum lipid levels were determined. Peripheral blood samples for biochemistry analysis were collected from live mice by using retro-orbital bleeding. RD: regular diet-fed mice, HFD: 45 kcal high fat diet-fed mice. Values are means ± standard error of the mean (SEM; RD, n=7; HFD, n=28), ****p*<0.001 compared with RD.

Table 2. Effect of aqueous extract of *Rubus occidentalis* (RO) on the body weight and hyperlipidemia indexes

	RD	HFD	HFD+RO125	HFD+RO250	HFD+Rosuva
Body weight (g)	31.5 ± 1.6	48.9 ± 2.3***	47.8 ± 5.0	48.5 ± 2.5	50.8 ± 4.5
Total cholesterol (mg/dL)	86.3 ± 12.0	252.6 ± 21.8***	205.7 ± 27.4 [†]	207.1 ± 38.8 [†]	197.1 ± 27.8 ^{††}
HDL (mg/dL)	68.6 ± 8.6	187.4 ± 26.6***	153.7 ± 15.3	158.3 ± 25.0	154.3 ± 21.5
LDL (mg/dL)	13.1 ± 2.0	25.7 ± 3.1***	26.3 ± 6.0	23.7 ± 3.7	21.7 ± 3.9
Triglyceride(mg/dL)	61.1 ± 17.4	73.1 ± 7.2*	76.9 ± 10.1	74.3 ± 13.5	70.3 ± 8.9
Perirenal fat (g)	0.752 ± 0.543	1.238 ± 0.462***	1.475 ± 0.229	1.440 ± 0.226	1.041 ± 0.579
Epididymal fat (g)	1.037 ± 0.481	1.396 ± 0.272**	1.528 ± 0.382	2.036 ± 0.746 [†]	1.362 ± 0.520

Mice were orally administrated test substances for 16 weeks, followed by 23 weeks of experimental diet feeding. RD: regular diet-fed mice, HFD: high-fat diet-fed mice, HFD+RO 125: HFD-fed mice treated with low dose (125 mg·kg⁻¹·day⁻¹) of aqueous extract of *Rubus occidentalis* (RO), HFD+RO 250: HFD-fed mice treated with high dose (250 mg/kg/day) of RO, HFD+Rosuva: HFD-fed mice treated with rosuvastatin (15 mg·kg⁻¹·day⁻¹). Values are means ± standard error of the mean (SEM, n=7/group), **p*<0.05, ***p*<0.01, ****p*<0.001 compared with RD mice; [†]*p*<0.05, and ^{††}*p*<0.01 compared with HFD mice.

extract (125 and 250 mg¹·kg⁻¹·day⁻¹). No significant difference was observed between the groups in the splenic CD11c⁺ subset analysis.

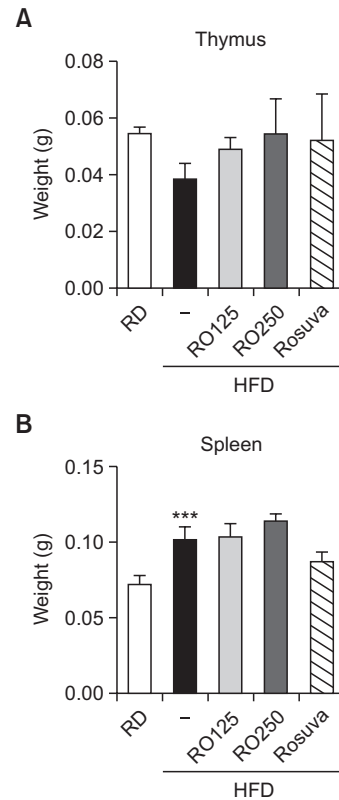


Fig. 2. Effect of aqueous extract of immature *Rubus occidentalis* (RO) on lymphoid organ weights. Thymus (A) and spleen (B) were excised and weighed after euthanasia. RD: regular diet-fed mice, HFD: high-fat diet-fed mice, HFD+RO 125: HFD-fed mice treated with low dose (125 mg·kg⁻¹·day⁻¹) of aqueous extract of immature RO, HFD+RO 250: HFD-fed mice treated with high dose (250 mg·kg⁻¹·day⁻¹) of RO. Rosuva: HFD-fed mice treated with rosuvastatin (15 mg·kg⁻¹·day⁻¹), n=7/group. Values are mean ± standard error of the mean (SEM) of three experiments, ****p*<0.001 compared with RD mice.

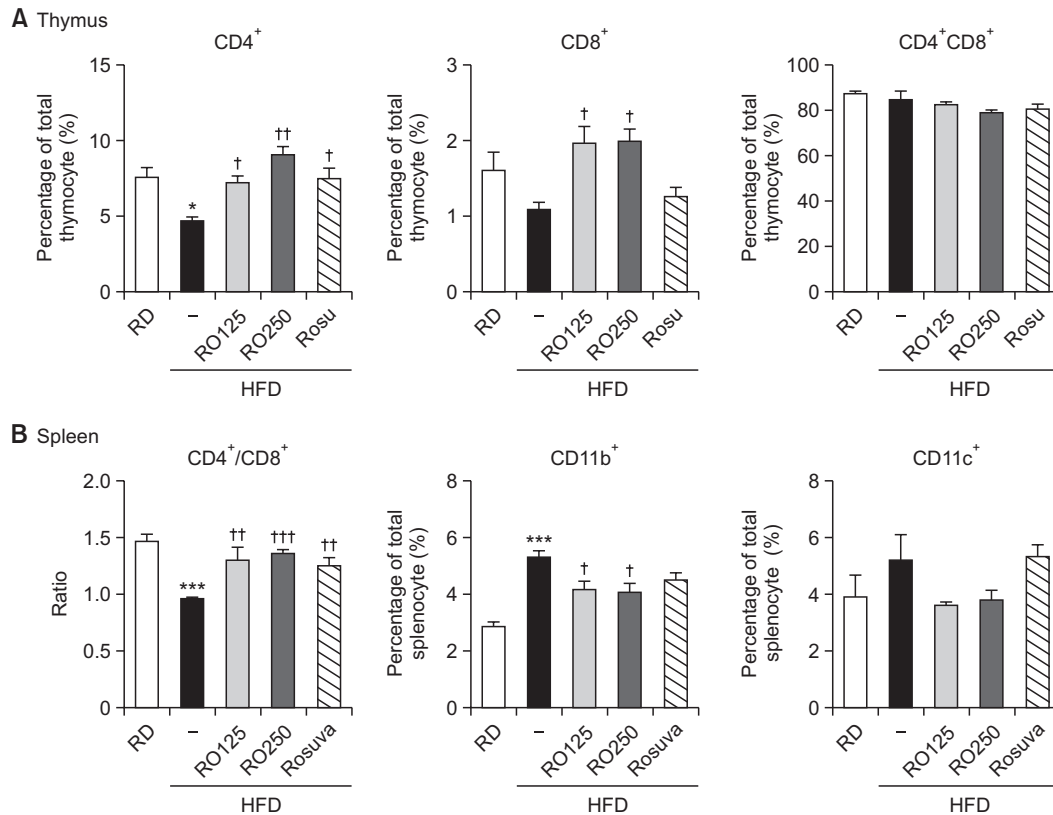


Fig. 3. Effect of aqueous extract of immature *Rubus occidentalis* (RO) on lymphocyte subset population. Different subsets of lymphocytes in the thymus (A) and spleen (B) were investigated using flow cytometry. Mice were grouped as shown in Fig. 2. Values are mean \pm standard error of the mean (SEM) of three experiments, * $p < 0.05$, *** $p < 0.001$ compared with RD mice; [†] $p < 0.05$, ^{††} $p < 0.01$, and ^{†††} $p < 0.001$ compared with HFD-fed mice.

Effect of aqueous extract of immature RO on lymphocyte proliferation

To confirm the viability and proliferative activity of the lymphocytes, we performed an *ex vivo* proliferation assay using mice splenocytes from each group. As shown in Fig. 4, the lymphocyte proliferation significantly decreased in the HFD-fed mice compared to that in the regular diet (RD)-fed mice following exposure to the mitogens. Furthermore, this effect was significantly inhibited in the RO-treated mice, regardless of the mitogen treatments. To present the *ex vivo* activity of the lymphocytes as the proliferation index, we expressed the value as the ratio of stimulated to non-stimulated lymphocyte viability.

Effect of RO on the level of pro-inflammatory cytokines in the serum

We conducted a pro-inflammatory cytokine assay using the mouse host serum samples since inflammation is considered as a key factor in the development of obesity-associated immune dysfunction. The serum cytokines, tumor necrosis factor (TNF)- α and interleukin (IL)-6, were measured to determine the degree of systemic inflammation. The level of these cytokines remained high in the serum of the mice that were subjected to prolonged HFD feeding while the TNF- α but not IL-6 level was significantly restored following treatment with the RO extract (Fig. 5).

Effect of RO extract on IgG production

To determine whether RO extract treatment enhanced the humoral immune function *in vivo*, we measured the antigen-specific IgG production level. The mice were previously immunized with the OVA peptide, which induced the production of a large amount of IgG in all groups after secondary immunization (Fig. 6). Excessive antibody production was observed in the HFD-fed mice compared to that in the RD-fed mice. The dysregulation of antibody production was significantly alleviated by oral administration of both RO (250 mg·kg⁻¹·day⁻¹) and rosuvastatin.

Effect of RO extract on CTL activity

To determine whether RO extract treatment enhanced the cellular immune function *in vivo*, we assessed the CTL activity. As shown by the two peaks in the histogram in Fig. 7, specific cell killing was achieved by CTL only in the OVA- but not the PBS-immunized mice. The generation of the potent CTLs was considerably suppressed by prolonged HFD feeding, whereas the administration of the RO extract considerably enhanced the CTL function. These results are clearly shown as percentages in Fig. 7.

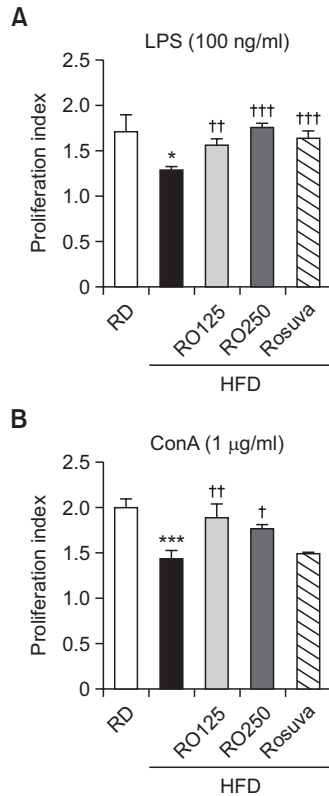


Fig. 4. Effect of aqueous extract of immature *Rubus occidentalis* (RO) on lymphocyte proliferation. Isolated cells from mice spleen were co-cultured with LPS (100 ng/mL) (A) or ConA (1 µg/mL) (B). Viability of splenocytes was measured by using the XTT assay and indicated as a ratio of nonstimulated lymphocytes. Mice are grouped as described in Fig. 2. Values are mean \pm standard error of the mean (SEM) of three experiments, * p <0.05, *** p <0.001 compared with RD mice. † p <0.05, †† p <0.01, ††† p <0.001 compared with HFD-fed mice. LPS: lipopolysaccharide, ConA: concanavalin A, HFD: high-fat diet, RD: regular diet, XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide.

DISCUSSION

Metabolic diseases such as obesity, diabetes, and hyperlipidemia are mutually regulated and accelerated by systemic inflammation (Lumeng *et al.*, 2011; Emanuela *et al.*, 2012). Furthermore, the progression of these metabolic-related diseases is significantly associated with immune dysfunctions (Gottschlich *et al.*, 1993; Geerlings and Hoepelman, 1999; Lei *et al.*, 2013) in particular, hyperlipidemia was shown to exacerbate the host's ability to generate an adequate immune response to external pathogens (Lei *et al.*, 2013). Additionally, statins, the major frontline drug treatment for hyperlipidemia, were reported to show positive effects on immune regulation in a recent study (Yoshimura *et al.*, 2015). Immunological assay has been suggested as a novel method for evaluating the efficacy of therapeutic agents in the treatment of these metabolic disorders. Therefore, we designed this study to focus on immunological analyses, with rosuvastatin as a positive control.

Black raspberry (RO), which is well known for its high content of polyphenols and other antioxidants, is thought to be a prominent therapeutic candidate for metabolic diseases. A

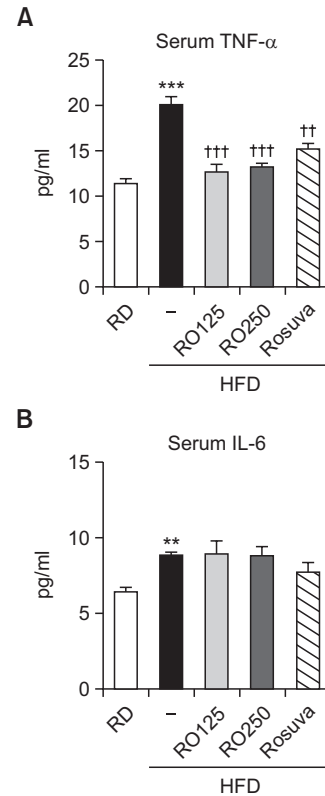


Fig. 5. Effect of aqueous extract of *Rubus occidentalis* (RO) on serum cytokines. Blood samples were collected by cardiac puncture and centrifuged at 4°C. Pro-inflammatory cytokines in serum were monitored; TNF- α (A) and IL-6 contents (B) were determined by (ELISA). Mice are grouped as shown in Fig. 2. Values are means \pm standard error of the mean (SEM) of three experiments, ** p <0.01, *** p <0.001 compared with RD mice; †† p <0.01, ††† p <0.001 compared with HFD mice. TNF- α : tumor necrosis factor- α , IL-6, interleukin-6, ELISA: enzyme-linked immunosorbent assay.

series of studies recently elucidated the effect of the *Rubus* species on metabolic diseases; however, the immune functional assay has rarely been performed for investigating the metabolic effects of potential drug candidates (Nam *et al.*, 2014; Jeong *et al.*, 2015). This prompted us to investigate the beneficial effect of RO on hyperlipidemia using an immunological approach.

Most importantly, the increased total cholesterol level, which is the metabolic phenotype of hyperlipidemia, was significantly ameliorated by the oral administration of RO extract in our study (Table 2). In addition, the immune-related effect of the RO extract was investigated to demonstrate the improvement in hyperlipidemia in a mouse model of DIO.

The host defense system can be quantitatively and qualitatively evaluated by determining the number of immune cells based on the prevalent subtypes and by assessing the immune cell functions associated with proliferation or cell killing. This can also be evaluated at the protein level by measuring the levels of immune-related proteins such as antibodies or cytokines. Based on these premises, the immunological analysis was designed and performed.

The lymphoid organs, which are the fundamental source

of the entire array of immune cells, undergo changes in size based on the host immune status (Pope, 1991), and their weights can be used as a quantitative immunological index. The thymus, which is the site of immature T cell growth and maturation, tends to diminish in size as the host ages, and this effect is reported to be further accelerated by an HFD (Yang *et al.*, 2009). As shown in Fig. 2A, 3A, a decrease in the size as well as in the number of CD4⁺ and CD8⁺ T cells was observed in the HFD-fed mice, which was restored by oral administration of the RO extract. However, the CD4⁺CD8⁺ immature T cell population in the thymus was not affected by the diet or oral treatment, whereas its maturation process was disturbed by the HFD (Fig. 3A). In addition, the spleen plays a role in the peripheral defense systems of the body and is the assembly site of immune cells for the exchange of antigen

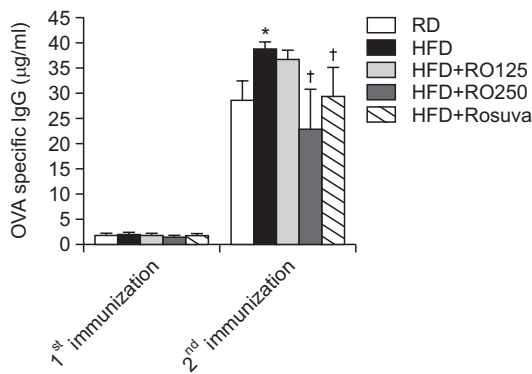


Fig. 6. Effect of aqueous extract of immature *Rubus occidentalis* (RO) on immunoglobulin G production. Immunization of ovalbumin (OVA) peptide was achieved in mice (subcutaneous, s.c.). OVA-specific immunoglobulin G (IgG) level in serum was monitored using ELISA analysis followed by primary and secondary immunization. Mice are grouped as shown in Fig. 2. The values are mean \pm standard error of the mean (SEM) of three experiments, * p <0.05 compared with RD-fed mice. † p <0.05 compared with HFD-fed mice. HFD: high-fat diet, RD: regular diet, ELISA: enzyme-linked immunosorbent assay.

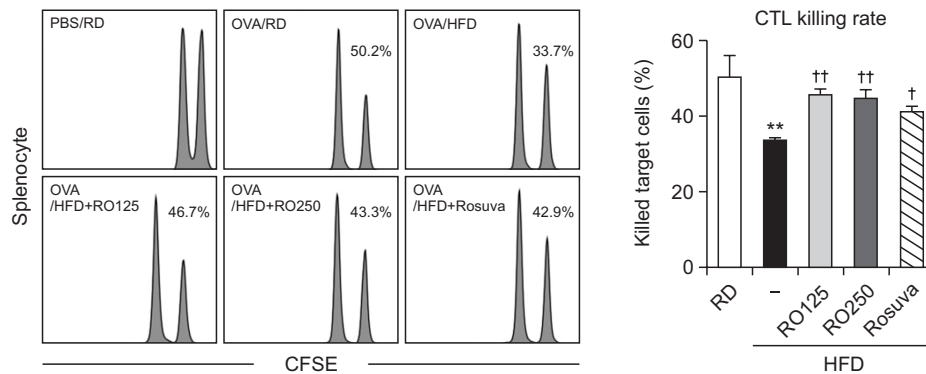


Fig. 7. Effect of aqueous extract of immature *Rubus occidentalis* (RO) on cytotoxic T lymphocyte (CTL) activity. Immunization of ovalbumin (OVA) peptide was achieved in mice (intravenous, i.v.). Specific killing of CTL is shown by histogram peaks and killing rates (%) of OVA (257-264) peptide-pulsed target cells in the spleen. Ratio of CFSE_{high} and CFSE_{low} was calculated as a numerical value to present specific killing. Mice are grouped as shown in Fig. 2. Values are mean \pm standard error of the mean (SEM) of three experiments, ** p <0.01 compared with RD-fed mice; † p <0.05 and †† p <0.01 compared with HFD-fed mice. HFD: high-fat diet, RD: regular diet. CFSE_{high}: carboxyfluorescein succinimidyl ester 5 μ m, CFSE_{low}: carboxyfluorescein succinimidyl ester 1 μ m.

information. Changes in the spleen are considerably different from those that occur in the thymus because peripheral immune cells are more influenced by altered cytokine patterns, which are involved in hyperlipidemia or adipocyte hypertrophy (Feingold and Grunfeld, 1992; Wisse, 2004). The hypertrophic spleen and splenocyte phenotypic changes were assumed to be associated with the immune disturbances caused by the HFD-related disease progression (Fig. 2B, 3B), which are especially associated with obesity-related inflammation. The ratio of CD4⁺ to CD8⁺ T cells and the number of macrophages and dendritic cells (DCs) were abnormally altered in the HFD-fed mice and restored in the RO extract-treated group (Fig. 3B). These alterations were considered to be attributable to the restorative effects of the RO extract on the imbalanced lymphocyte population and peripheral inflammation.

The decline in the lymphocyte functions was determined by their proliferation level during the process of counteracting antigens, LPS- and Con A-targeted T cells. The HFD-fed mice exhibited an impairment in the lymphocyte proliferative activity, which was significantly restored by the oral administration of the RO extract (Fig. 4). Furthermore, the analysis of TNF- α , which was accompanied by a proliferation assay, showed high TNF- α levels in the HFD-fed mice compared to that in the RD-fed mice. This observation suggests that the lymphocytes of the HFD-fed mice were more likely to have initiated the inflammatory process, and the elevated TNF- α production was decreased by the oral administration of the RO extract (Supplementary Fig. 1). The increased levels of serum TNF- α and IL-6 in the HFD-fed mice, which were indicative of systemic inflammation, were decreased by RO extract treatment (Fig. 5), and this was assumed to be a novel finding.

TNF- α is a key regulator of the inflammatory response, and its blockade is used as a potent therapeutic strategy for the treatment of inflammatory diseases such as rheumatoid arthritis (Popa *et al.*, 2007). Moreover, this cytokine contributes to the progression of atherosclerosis in hyperlipidemia, initiated by the inflammatory cascade (Bradley, 2008). A small number circulating cytokines in the bloodstream can lead to undesirable immune reactions with detrimental effects including those on the serum TNF- α level. The regulation of the TNF- α level

by RO treatment was considered to have mediated the synergic restoration of the declined immune functions and hyperlipidemia by ameliorating the systemic inflammation.

To evaluate the specific immune function, the mice in each group were immunized with OVA, a model antigen. Since the antigen-specific defense system is associated with cellular and humoral immunity, we designed appropriate experiments, which involved the determination of IgG production level and target cell killing activity, respectively (Fig. 6, 7). The IgG production in B cells, which is generally regarded as the efficient defense strategy, has been found to be fundamentally pathogenic in DIO mice (Winer *et al.*, 2011). In addition, the functional decline in cell-mediated target cell killing was previously reported in DIO mice (Im *et al.*, 2010). This pattern of immune dysfunction was observed and subsequently restored following treatment with RO in our studies. The deterioration of the cell killing functions in the HFD-fed mice was noticeably improved by the oral administration of the RO extract (Fig. 7), and their upregulated IgG levels were also attenuated (Fig. 6). It is thought that the fatty diet induced a chronic disturbance in cytokines, leading to inflammation with a consequent dysfunction in both the cellular and humoral immunity. Furthermore, we hypothesized that the oral administration of the RO extract significantly restored this disease pattern.

These results consistently suggest that the oral administration of the RO extract attenuated the hyperlipidemia associated-immune decline in the HFD-fed mice. Furthermore, the RO extract was not only shown to improve hyperlipidemia, but its immune-enhancing effect was also newly elucidated based on the quantitative and qualitative evidence. In addition, the beneficial effects of the RO extract appeared to be mediated by its potential regulation of the TNF- α level.

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