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Aronia melanocarpa reduced adiposity *via* enhanced lipolysis in high-fat diet-induced obese mice

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Abstract Obesity is a critical health issue in Korea, where half of all adults are overweight and a third obese. *Aronia melanocarpa* -rich in flavonoids and phenolics- with antioxidant and anti-inflammatory activities, could have anti-obesity activity and reduce body fat mass by upregulating lipolysis and β-oxidation in obese mice. Male C57BL/6J mice (n=12) were assigned into four groups: normal chow (18% kcal from fat); high-fat diet control (HFD, 45% kcal from fat); HFD +*A. melanocarpa* (200 mg/kg diet); HFD+Xenical (500 mg/kg diet, positive control). Antioxidant capacity of *A. melanocarpa* was established *in vitro* and *in vivo*. Weight loss was induced as decreased adiposity and lowered respiratory quotient at rest suggested oxidation of stored fat. Adiposity reduction, accompanied with elevated fat utilization, was owing to enhanced activity of hormone-sensitive lipase. Thus, *A. melanocarpa* lowered adiposity by enhancing lipolysis and utilization of fatty acids in visceral fat.

Keywords: anti-obesity therapeutic, *Aronia melanocarpa*, hormone-sensitive lipase, lipolysis, respiratory quotient

Introduction

Obesity is described as excessive or abnormal fat accumulation. The global prevalence of obesity in 2016 has nearly tripled since 1975; nearly 39% of adults are overweight, and 13% are obese (World Health Organization (WHO), 2018). The prevalence of obesity in Korea has increased; almost half of all adults are overweight and 32% obese, and more strikingly, 10% of children and adolescents, aged 6-18 years, are obese (Korean Society for the Study of Obesity (KSSO), 2016). The health consequences of overweight and obesity comprise cardiovascular diseases, type 2 diabetes, osteoarthritis, and some cancers (Centers for Disease Control and Prevention (CDC), 2017). Such diseases can be prevented by reducing undesirable body fat.

Natural functional foods have been found in the prevention and treatment of obesity. Reflecting on this regard, the market for health-functioning foods is growing throughout the world (National Institutes of Health (NIH), 2019). Especially materials and products that help reduce body fat are developing prosperous (Trigueros et al., 2013). The highest number of functional foods categories was in fact body fat reduction, with 91 cases as of 2018, according to the Ministry of Food and Drug Safety in Korea. In this respect, the use of bioactive supplements is considered to be beneficial.

Aronia melanocarpa (Michx.) Elliott, commonly known as black chokeberry, is a member of the rose family. It is native to eastern North America and has been planted widely across Europe and Asia (United States Department of Agriculture (USDA), 2005). The fruit of *A. melanocarpa* was used by Native Americans to recover from a cold (Kokotkiewicz et al., 2010). More recently, *A. melanocarpa* fruit (or berries) have been used for producing juice, which contains the beneficial constituents. *A. melanocarpa* contains multiple vitamins, minerals, organic acids, flavonoids (proanthocyanidins, anthocyanins, and flavanol), and phenolic acids (neochlorogenic and chlorogenic acids) (Szopa et al., 2017). These components have many beneficial properties, such as antioxidative, anti-inflammatory, and gastrointestinal protective activities (Jurikova et al., 2017).

More recently, *in vivo* studies have shown the potential of *A. melanocarpa* in treating metabolic syndromes. For instance, the juice of *A. melanocarpa* fruit improved plasma triglyceride and glucose levels in streptozotocin-induced diabetic rats (Valcheva-Kuzmanova et al., 2007). Similarly, dietary supplementation of *Aronia* juice reduced body weight, adiposity, and serum glucose and insulin levels in KKAy obese/diabetic mice (Yamane et al., 2016). Results obtained from *in vitro* studies strongly support these results by specifying target genes and proteins. An *A. melanocarpa* supplement inhibited *NPC1L1* and *ABCA1* in Caco-2 cells, potentiating the reduction of apical cholesterol absorption and basolateral cholesterol efflux (Kim et al., 2013a). Furthermore, the juice of *A. melanocarpa* induced the activation of nitric oxide synthase, a facilitator of endothelium-dependent relaxations, in porcine coronary artery endothelial cells, revealing its cardioprotective effect (Kim et al., 2013b).

However, the effect of *A. melanocarpa* supplements on white adipose tissue turnover and metabolic substrate utilization is not known. It has been hypothesized that *A. melanocarpa* supplements could reduce body fat by upregulating lipolysis and fatty acid β-oxidation in diet-induced obese mice. To test this hypothesis, we

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induced obesity in mice with a high-fat diet and fed them a diet containing *A. melanocarpa* extracts to evaluate growth performances, body composition, energy metabolism, blood profile, and molecular phenotypes.

Materials and Methods

Preparation of *A. melanocarpa* extract

Frozen *A. melanocarpa* (10 g) was mixed with 100 mL 100% alcohol at 45°C with ultrasonication for 5 h. The yield value was 13.5% after being concentrated. The content of anthocyanin was estimated to be 1,007.3 mg/100 g by UV-visible spectroscopy. The *Aronia* extract was composed of 17.7 gm% (gram percent, w/w) carbohydrate, 0.3 gm% protein, and 0.4 gm% fat. The extract used in this study was provided by Sam Jung (Seoul, Korea).

Antioxidant capacity of *A. melanocarpa* extract

The *A. melanocarpa* extract was dissolved to prepare of 200, 400, 800, and 1,600 mg/L solutions, and DPPH (Sigma-Aldrich, St Louis, MO, USA) radical scavenging activity was assessed. The DPPH solution (in ethanol) was added to the samples and absorbance was measured at 515 nm after allowing the mixture to stand for 10 min in the dark. The percentage of DPPH radical scavenging activity was calculated as (1−absorbance of sample/absorbance of control) × 100. The data obtained from DPPH analysis were expressed as Trolox (Sigma-Aldrich) equivalents.

ABTS (Sigma-Aldrich) analysis was performed to measure the antioxidant capacity of the *A. melanocarpa* extract. ABTS and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) were mixed in phosphate-buffered saline. The sample was mixed with the ABTS solution. The results from ABTS analysis were expressed as Trolox (Sigma-Aldrich) equivalents. For estimating total phenolic content, the *A. melanocarpa* extract and Folin-Ciocalteu reagent (Sigma-Aldrich) were mixed with 5% Na_2CO_3. The absorbance of the solution was measured at 765 nm after incubating for 20 min at 40°C. Total phenolic content was reported as 1 mg/10 mL gallic acid (Sigma-Aldrich) equivalents (GAE).

Animals and diets

All animal experiments were approved by the Institutional Animal Care and Use Committee of Kookmin University (KMU-2016-04). Four-week old, male C57BL/6 mice were purchased from Dae-Han Biolink (Eumsung-si, Chungbuk, Korea). The mice were housed in individually ventilated cages with 50±10% humidity at 22±1°C with a 12 h light/dark cycle. After 1 week of adaptation, the mice were assigned into four groups (n=12): (1) normal chow (18 kcal% from fat), (2) high-fat diet control (HFD, 45 kcal% fat, negative control) (D12451, Research Diets, New Brunswick, NJ, USA) (3) HFD+*A. melanocarpa* (200 mg/kg diet), and (4) HFD+Xenical (Roche Korea, Seoul, Korea; 500 mg/kg diet). Xenical, a gastrointestinal lipase inhibitor, was used as the positive control. Detailed information about the feed ingredients is listed in Table 1. Feed and water were provided *ad libitum* for a 16-week period.

Table 1. Calorie composition of the experimental diets

Ingredients (kcal%)	High-fat diet (45 kcal% fat)			
	Chow	(−)	*Aronia melanocarpa*	Xenical
Carbohydrate	58	35	35	35
Protein	24	20	20	20
Fat	18	45	45	45
Total calories (kcal/100 g)	307	476	476	476

Growth performance and body composition

Body weight and feed intake were measured every week. Total energy intake (kcal) was estimated by calculating feed intake. Mice were anesthetized with ketamine (100 mg/kg body weight (bw)) and xylazine (10 mg/kg bw) after 4 h of fasting, to measure body composition. Body composition, including fat in tissue, lean mass, and bone mineral density, was evaluated using dual-energy X-ray absorptiometry (DEXA, Medikors, Seongnam-si, Gyeonggi-do, Korea) at 16 weeks of age.

Energy expenditure and respiratory quotient

Metabolic phenotypes, including energy expenditure and respiratory quotient, were analyzed by using the Oxylet indirect calorimetry system (Panlab, Barcelona, Spain). In brief, the Oxylet was calibrated using a mixture of gases, containing 50% O_2/1.5% CO_2/48.5% N_2 (high point) and 20% O_2/0% CO_2/80% N_2 (low point), as per the manufacturer's instructions. Individual animals were housed in cages, in which the amount of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) was monitored for the given time. Mice were provided with feed and water *ad libitum* during the experiment. The data were expressed as mL of O_2 or CO_2/h/animal, and the respiratory exchange ratio was determined. Energy expenditure and respiratory quotient were calculated from respiratory gas analysis using the METABOLISM® software (Panlab).

Lipid profiles and antioxidant biomarkers in serum

Whole blood was collected from the retro-orbital sinus after 4 h of fasting. The serum was separated by centrifuging (2,000×g at 4°C for 15 min). Total cholesterol, low-density lipoprotein cholesterol (LDL-c), and triglycerides present in the serum were measured using an enzymatic method by following the protocol of a commercial kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). High-density lipoprotein cholesterol (HDL-c) was calculated as follows: (total cholesterol−LDL-c−$\frac{TG}{5}$). Superoxide dismutase (SOD) and catalase levels, were measured in the serum collected from 8-week-old mice, using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA), as per the manufacturer's instructions.

Immunoblotting

The total protein present in white adipose tissue was evaluated by lysing the tissue in radioimmunoprecipitation assay buffer

(Biomax, Seoul, Korea), containing a phosphatase and protease inhibitor cocktail (Cell signaling, Danvers, MA, USA). The concentration of protein in the lysates was determined by the Bradford assay. Thereafter, the protein samples were mixed with 2× Laemmli buffer (Bio-Rad, Hercules, CA, USA), and proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the separated proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% bovine serum albumin (GenDEPOT, Barker, TX, USA) in Tris-buffered saline, containing 0.1% Tween 20. Primary antibodies (1:1,000), including hormone-sensitive lipase (HSL), HSL-pS563, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling), were used for incubation overnight at 4°C. Thereafter, the membrane was incubated with the HRP-conjugated secondary antibody (1:3,000) for 1 h at 20°C. The proteins were quantified using Image Lab software (Bio-Rad).

Statistical analysis

All data obtained from the animal experiments were expressed as the mean±standard error of the mean. The statistical difference between groups and descriptive statistics were analyzed using a Prism 6.0 (GraphPad Software, San Diego, CA, USA). An unpaired *t*-test was employed to examine the difference between the chow group and the negative control. One-way analysis of variance was applied to test the difference between the negative control vs. treatment groups. If a difference was verified between groups ($p<0.05$), Tukey's multiple comparison tests were performed.

Results and Discussion

Antioxidant capacity of *A. melanocarpa* extract

The antioxidant capacity of *A. melanocarpa* extract is shown in Table 2. DPPH and ABTS have been extensively used to assess the antioxidant capacity of foods and biological samples (Floegel et al., 2011). Both tests are based on the reduction of colored oxidants *via* electron transfer, which correlates with the total phenolic content in samples (Ainsworth and Gillespie, 2007). DPPH radical scavenging activity was presented as percentage inhibition relative to Trolox, which increased in a dose-dependent manner in *A. melanocarpa* extracts (the square of Pearson's correlation coefficient, $r^2=0.83$). The results of the ABTS assay have been presented as Trolox equivalent antioxidant capacity, which showed a correlation with the content of the extract ($r^2=1.00$). The total phenolic content of the extract was determined by GAE, which increased upon addition of the extract ($r^2=1.00$). Similar studies have been conducted using different extraction methods in *A. melanocarpa*. The solvent character affected the contributing composites and antioxidant capacity of *A. melanocarpa*. Alcohol (ethanol and methanol) extracts contained an approximately two-fold greater amount of anthocyanin, total phenolics, and flavonoid than that in the water extract (Park and Hong, 2014). The *A. melanocarpa* ethanol extract has greater DPPH activity, up to 200 μg/mL, in a dose-dependent manner, and ABTS activity of the alcohol extract was found to be greater, up to 100 μg/mL, than that of the water extract (Ghosh et al., 2018). Moreover, processing of *A. melanocarpa* considerably increased the range of total phenolic content, for example, juice, 3,002-6,639 mg GAE/L; powder 1,620-1,906 mg GAE/100 g dry matter (dm); capsule, 4,511-5,292 mg GAE/100 g dm; dried berries 1,954-2,466 mg GAE/100 g dm (Toliæ et al., 2015). Together, these findings suggest that the *A. melanocarpa* extract used in this study had considerable antioxidant capacity.

Growth performance and body composition

Having found that *A. melanocarpa* extract exhibited antioxidant capacity in a dose-dependent manner, we explored its physiological benefits. For *in vivo* studies on obesity and its intervention, male C57BL/6J mice were fed a high-fat diet (45 kcal% fat) with or without *A. melanocarpa* extract (200 mg/kg bw) for a 16-week period. A standard purified rodent diet (chow, 18 kcal% fat) was fed to maintain healthy normal mice. Xenical, a pancreatic lipase inhibitor, was used as the positive control drug for weight loss. Body weight was measured weekly to monitor weight loss (Fig. 1A). The body weight of animals fed a high-fat diet (35.48±0.80 g) was significantly higher ($p<0.05$) than that of the normal group (32.45±0.28 g), from 8 to 16-week period, indicating that high-fat diet-induced obesity was successfully established. The Xenical treated (+) control showed a modest body weight increase, similar to the control group ($p<0.05$). Dietary provision of *A. melanocarpa* resulted in a significant decrease in body weight, relative to the negative control, after a 4-week period ($p<0.05$). The potency of *A. melanocarpa* on weight loss was as compelling as that of Xenical in this study. The details of weight loss by *A. melanocarpa* were assessed and are shown in the following section.

Total energy intake over the experiment period was calculated to examine whether the *A. melanocarpa* supplement modulated appetite or satiety (Fig. 1B). A high-fat diet significantly increased

Table 2. The antioxidant capacity of the *Aronia melanocarpa* extract

Item	*Aronia melanocarpa* extract (mg/L)				
	0	200	400	800	1,600
DPPH radical scavenging activity (%)	0.0±0.0	23.5±7.9	29.8±14.0	41.9±21.1	44.8±37.3
ABTS (μM TE[1])/g)	25.4±13.9	108.0±39.7	184.7±91.5	333.7±143.6	605.1±233.5
Total phenolic content (g GAE[2])/L dw)	0.0±0.0	4.0±1.1	9.2±2.6	18.1±4.1	34.2±5.6

[1])TE, Trolox equivalent
[2])GAE, gallic acid equivalent
The data are presented as the mean±standard deviation

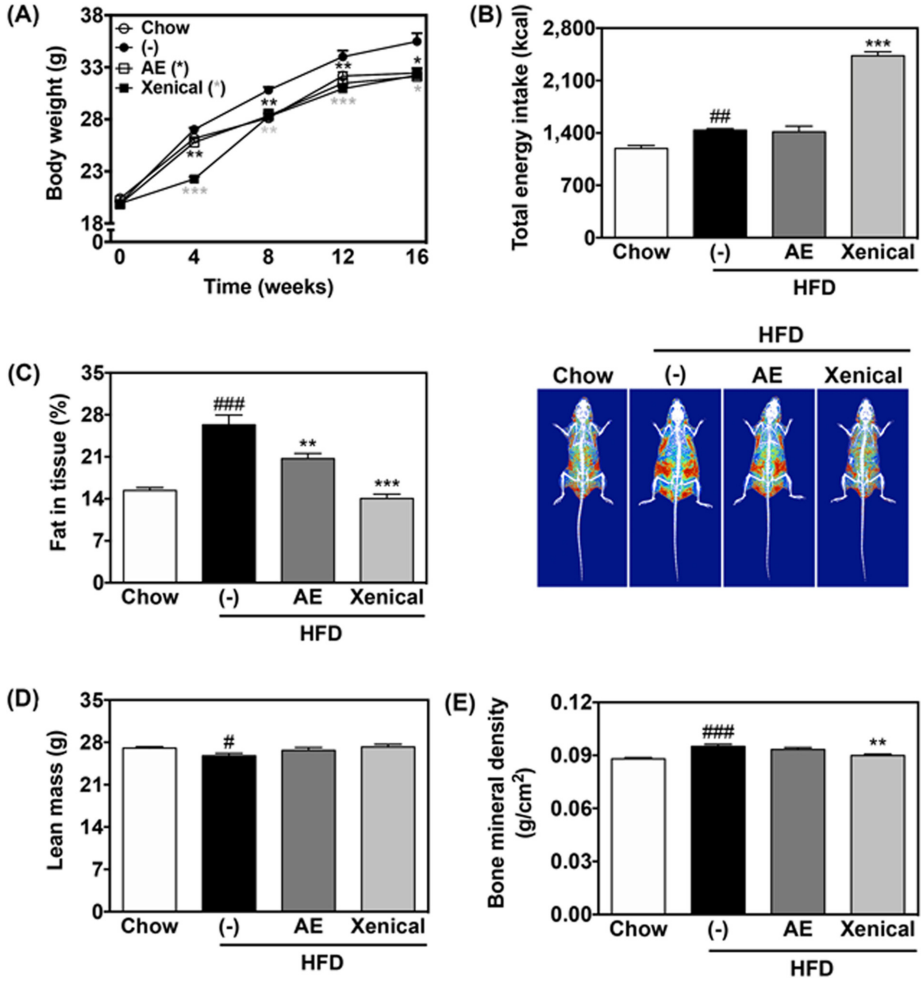

Fig. 1. Growth performance and whole-body composition of mice fed experimental diets for a 16-week period. (A) Body weight, (B) total energy intake, (C) tissue fat, (D) lean mass, and (E) bone mineral density. A high-fat diet (HFD, 45 kcal% fat)-induced obesity model was established in male C57BL/6J mice. In contrast, a standard purified rodent diet (chow, 18 kcal% fat) was fed to maintain healthy normal mice. Obesity model mice were fed either null (–), *Aronia melanocarpa* (AE, 200 mg/kg diet), or Xenical (500 mg/kg diet). Xenical was used as a positive control for weight loss. Dual X-ray energy absorptiometry was used to quantify whole-body composition. The data are presented as mean±standard error of the mean (n=12). Statistical significance between chow and negative control was analyzed by the unpaired t-test ($^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$). The difference, between negative control and treatments, was analyzed by one-way analysis of variance with Tukey's multiple comparisons ($^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$).

(p<0.01) total energy intake (1,437±20.80 kcal) relative to the normal chow diet (1,193±40.85 kcal). Xenical (2,431±52.71 kcal) further boosted (p<0.001) total energy intake to compensate for the caloric loss of fat-soluble nutrients (Lucas et al., 2020). Isocaloric feed uptake was observed between the negative control and the *Aronia* group, suggesting that the *A. melanocarpa* supplement did not alter energy uptake in obese mice. Previously, another study using high-fat diet-induced mice showed that body weight decreased by *A. melanocarpa* juice supplement without changing the feed intake (kcal/day) (Baum et al., 2016).

Given that the *A. melanocarpa* supplement caused weight loss in obese mice without altering caloric intake, we examined whether weight loss was owing to fat loss. DEXA was employed to assess tissue fat (Fig. 1C). A high-fat diet resulted in substantially higher fat accumulation by 71% in the whole-body than that by the standard diet (p<0.001). Xenical successfully prevented fat accumulation, as low as that in the normal diet group (p<0.001). Notably, dietary supplementation of *A. melanocarpa* caused a significant reduction in adiposity in 21% obese mice (p<0.01). DEXA scan image displayed a dramatic reduction of fat (shown in red in the images) in the *Aronia* supplemented group, as shown in the bar graphs. Some studies have shown that *A. melanocarpa* is capable of reducing fat accumulation. For instance, fat accumulation in obese mice was reduced in the *A. melanocarpa* supplemented group (Kim et al., 2018). Supplementation of *A. melanocarpa* juice significantly reduced epididymal, mesenteric, retroperitoneal, and subcutaneous white adipose tissue in KKAy mice (Yamane et al., 2016). The mechanisms of *Aronia*-related fat loss *in vivo*, however, remains unknown. However, *in vitro* data have shown that *A. melanocarpa* suppressed the differentiation of 3T3-L1 adipocytes (Kim et al., 2018). Thus, the degree of inhibition of adipogenesis attributed to weight loss is not known.

Lean mass is associated with the basal metabolic rate at rest. A higher lean mass can prevent obesity by increasing energy

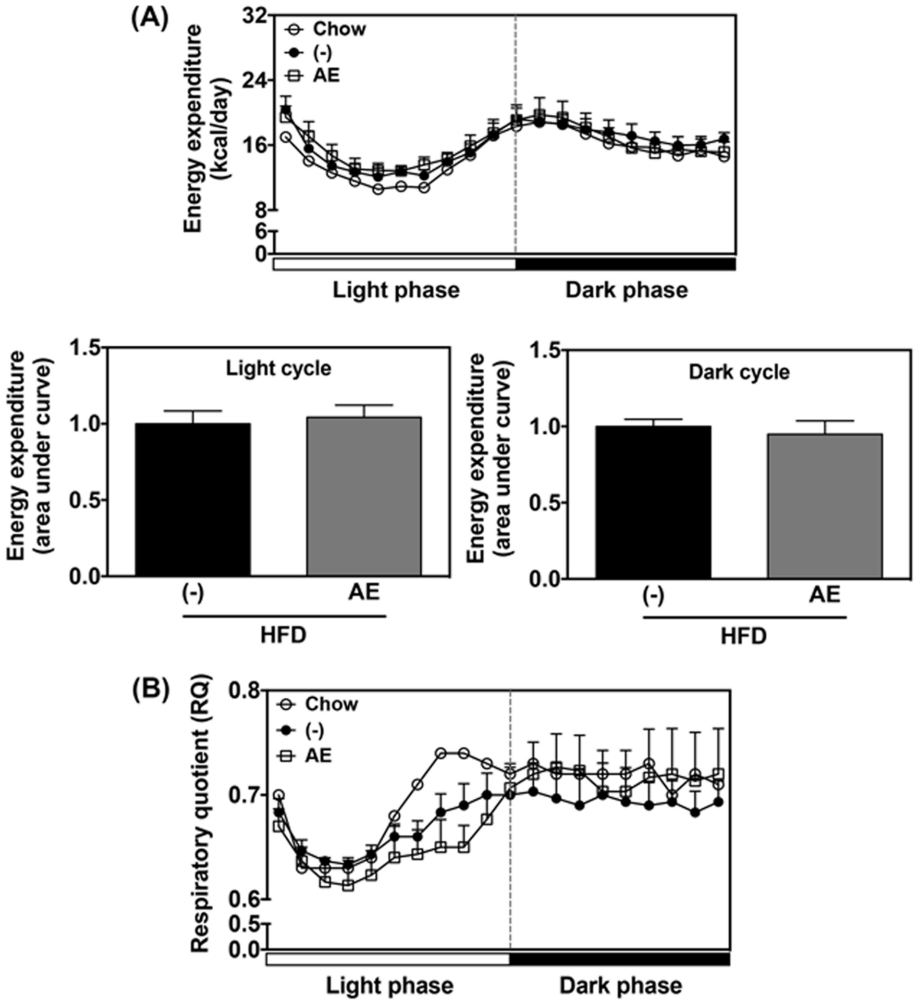

Fig. 2. Study of the effect of the *Aronia melanocarpa* supplement on energy metabolism and substrate utilization, using a gas analyzer. (A) Energy expenditure and (B) respiratory quotient. A high-fat diet (45 kcal% fat)-induced obesity model was established in male C57BL/6J mice. In contrast, a standard purified rodent diet (chow, 18 kcal% fat) was fed to maintain healthy normal mice. Obesity model mice were fed either null (–) or *Aronia melanocarpa* (AE, 200 mg/kg diet). Individual mice were placed in the metabolic chamber of the gas analyzer. After 24 h of acclimatization, O_2 consumption (VO_2) and CO_2 production (VCO_2) were monitored for 24 h. The data are presented as mean±standard error of the mean (chow, n=4; control and AE, n=12). The difference between samples was analyzed by the unpaired t-test (p<0.05).

expenditure. In our study, high-fat diet-fed mice (25.78±0.40 g) showed notably lower lean mass than that in the normal group (27.04±0.24 g) (p<0.05) (Fig. 1D). In contrast, dietary supplementation of *A. melanocarpa* tended to increase the lean mass by 4% (p=0.07). These findings suggest there was weak evidence that *A. melanocarpa* improved the lean mass, which has the potential to increase energy utilization. Related observations are shown in the following sections.

A recent study showed a positive association between obesity and bone mineral density, which is called the "obesity paradox", that is, obese or overweight subjects exhibited greater bone mineral density than the normal weight group (Salamat et al., 2016). Therefore, we investigated whether dietary supplementation of *A. melanocarpa* modulated bone mineral density in obese mice (Fig. 1E). High-fat diet-induced obese mice showed higher bone mineral density (p<0.001) than the normal and Xenical groups, consistent with the obesity paradox observation. The *A. melanocarpa* supplement did not reduce bone mineral content despite the relatively low-fat accumulation. These findings suggest that *A. melanocarpa* supplement is a fat reducing compound without side effects on bone health.

Energy expenditure and respiratory quotient

Indirect calorimetry is one of the most advanced techniques to date to examine metabolic assimilation, such as energy expenditure and respiratory quotient. Respiratory quotient, in particular, provides qualified information about the type of fuel that is being metabolized (e.g., carbohydrate vs. fatty acid). In this study, we employed state-of-the-art techniques to understand energy catabolism in obesity-induced mice fed the *A. melanocarpa* supplement. Integrated energy expenditure and respiratory quotient over 24 h are shown in Fig. 2. Mice fed a high-fat diet showed slightly higher total energy expenditure at the light phase than that in normal mice (Fig. 2A). Total energy expenditure, both at the light and dark phase, did not differ between mice fed *A. melanocarpa* vs. the negative control. Strikingly, dietary supplementation of *A.*

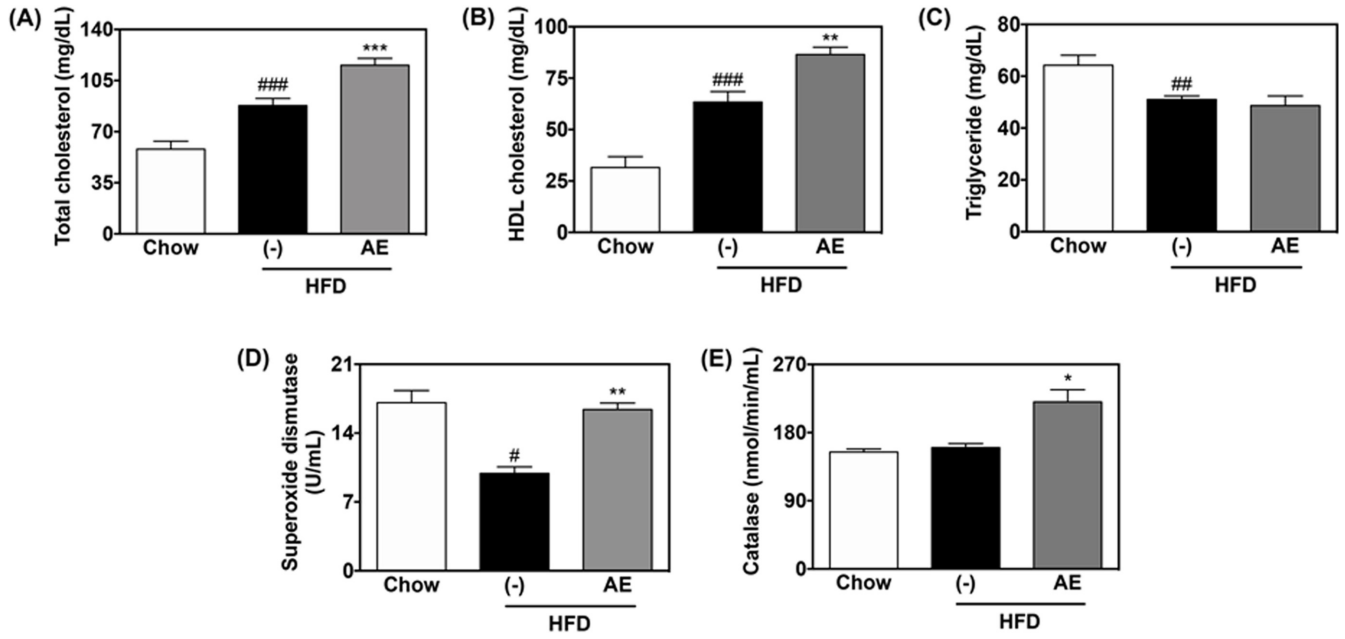

Fig. 3. The effects of *Aronia melanocarpa* on the lipid profile and antioxidant activity in mice serum. (A) Total cholesterol, (B) HDL cholesterol (calculated), (C) triglyceride, (D) superoxide dismutase, and (E) catalase. A high-fat diet (45 kcal% fat)-induced obesity model was established in male C57BL/6J mice. In contrast, a standard purified rodent diet (chow, 18 kcal% fat) was fed to maintain healthy normal mice. Obesity model mice were fed either null (−) or *Aronia melanocarpa* (AE, 200 mg/kg diet). The data are presented as mean±standard error of the mean (A-C, n=12; D-E, n=4). The difference between samples was analyzed by the unpaired t-test ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ chow vs. control; $^{*}p<0.05$, $^{**}p<0.01$, $^{**}p<0.001$ control vs. AE).

melanocarpa decreased the respiratory quotient at the light phase, indicating utilization of more fat at rest (Fig. 2B). In contrast, in the dark phase, mice fed *A. melanocarpa* had an increased respiratory quotient than the control, showing that carbohydrates are readily utilized. The previous study showed that *A. melanocarpa* improved insulin sensitivity, including oral glucose tolerance test and intraperitoneal insulin tolerance test, in high-fat induced glucose intolerance mice (Kim et al., 2018). Similarly, *A. melanocarpa* supplementation improved the homeostatic model assessment-insulin resistance (HOMA-IR) score, a homeostasis model assessment of insulin resistance, in dyslipidemia-induced mice (Jeong and Kim, 2019). Therefore, our finding findings support to identify *A. melanocarpa* as a nutraceutical for obesity-induced insulin resistance.

Lipid profiles and antioxidant biomarkers in serum

Obese individuals readily develop dyslipidemia such as hypercholesterolemia and hypertriglyceridemia (Poirier et al., 2005). Cardiovascular diseases driven by dyslipidemia predominantly contribute to increasing the mortality of obese people (Global Burden of Disease Study 2015 (GBD, 2015) Obesity Collaborators, 2017). Therefore, we assessed whether *A. melanocarpa* improved these aspects, in addition to its antioxidant capacity. Dietary supplementation of *A. melanocarpa* resulted in a significantly higher total cholesterol by 1.3-fold than that in the negative control (p<0.001), which was mostly associated with the increase of HDL-c (Fig. 3A and 3B). In fact, HDL-c was significantly higher by 1.4-fold (p<0.01) in *A. melanocarpa* supplemented mice (86.46±3.61 mg/dL) than in the control (63.39±5.05 mg/dL). The increase of HDL-c by *A. melanocarpa* could play a cardioprotective role. However, no conclusive evidence of the effect of *A. melanocarpa* on the increase HDL-c level has been shown in previous studies.

LDL-c, a prevalent risk factor of cardiovascular disease, has not been evaluated in this study. The obesity model employed in this study presents a potential limitation in developing LDL-c induced hypercholesterolemia. This is because LDL-c is not the primary lipoprotein in rodents; instead, approximately 80% of all cholesterol exists as HDL-c (Go et al., 2014). However, previous clinical studies have shown a significant reduction of LDL-c by *A. melanocarpa*. For instance, consumption of *A. melanocarpa* for a 12-week period lowered LDL-c by 11% in a randomized controlled trial (Xie et al., 2017). Similarly, a meta-analysis of five randomized controlled trials presented that *A. melanocarpa* consumption decreased LDL-c level by 5.84% (Rahmani et al., 2019). Further studies are necessary to examine the role of *A. melanocarpa* in cholesterol trafficking using *Ldlr*$^{-/-}$ or *Apoe*$^{-/-}$ mice on a high-cholesterol/cholic acid diet.

Hypertriglyceridemia is not induced in high-fat-fed control mice (51.05±1.32 mg/dL) vs. chow mice (62.24±3.88 mg/dL) (Fig. 3C). This was the most significant observation in this study. There was no significant difference in triglycerides between the negative control and *A. melanocarpa*-fed mice. Collectively, unexplored LDL-c outcomes and abnormal triglyceride observation remain the limitation of this study.

Having found that *A. melanocarpa* extract presented a powerful antioxidant potential *in vitro*, we investigated its antioxidant capacity in the serum. Superoxide dismutase and catalase play a

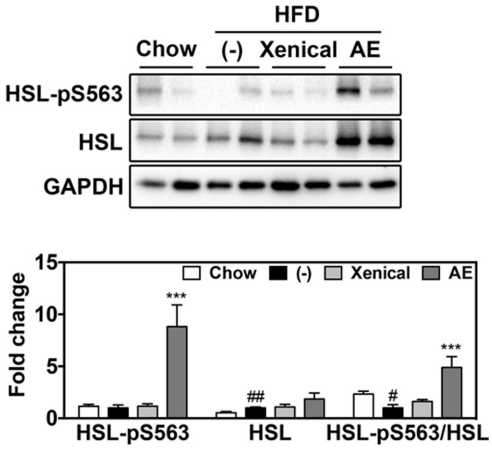

Fig. 4. Level of lipolytic enzymes in visceral white adipose tissue of mice fed *Aronia melanocarpa*. A high-fat diet (45 kcal% fat)-induced obesity model was established in male C57BL/6J mice. In contrast, a standard purified rodent diet (chow, 18 kcal% fat) was fed to maintain healthy normal mice. Obesity model mice were fed either null (−), *Aronia* melanocarpa (AE, 200 mg/kg diet), or Xenical (500 mg/kg diet). Visceral fat, dissected from the abdomen, was used for immunoblotting. The ratio of phosphorylated protein to total protein has estimated by densitometry. The quantified data are presented as mean ± standard error of the mean (n=6). The difference between chow and negative control was analyzed by the unpaired t-test ($^{\#}p<0.05$ and $^{\#\#}p<0.01$). The difference between sample vs. negative control was analyzed by one-way ANOVA, followed by Tukey's multiple comparisons ($^{***}p<0.001$). HSL, hormone-sensitive lipase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

pivotal role in defense against reactive oxygen species. These enzymes neutralize superoxide and hydrogen peroxide, thereby protecting the body against the toxic effects of oxidative stress (Peng et al., 2014). In the present study, the *A. melanocarpa* supplement recovered the level of superoxide dismutase ($p<0.01$), which had decreased owing to the high-fat diet (Fig. 3D). Catalase activity was significantly increased in *A. melanocarpa* compared to the (-) control ($p<0.05$) (Fig. 3E). These results are consistent with those of a previous study where the activity of superoxide dis'mutase and catalase in the blood was shown to recover in the presence of *A. melanocarpa* (Kim et al., 2013c). Thus, *A. melanocarpa* exhibited a robust antioxidant activity *in vivo*.

Activity of lipolytic enzymes in white adipocytes of mice

Adiposity is regulated by the interplay between hepatic lipogenesis and peripheral adipogenesis in the fed state and lipolysis in the fasting state or during exercise (Yu and Ginsberg, 2005). In the current study, lowered of adiposity by *A. melanocarpa* was marked by enhanced fatty acid oxidation; therefore, lipolysis in white adipose tissue was hypothesized to be the key mechanism. Triglycerides stored in white adipose tissue are hydrolyzed to release free fatty acids by hormone-sensitive lipase (HSL) (Ahmadian et al., 2007). The action of HSL is mediated by its protein kinase A (PKA)-dependent phosphorylation, which is the rate-limiting step (Melzer, 2011). Therefore, we evaluated the rate-limiting mechanism in visceral white adipose tissue using western blot (Fig. 4).

Phosphorylation of HSL (at serine 563) was 8-fold higher in *A. melanocarpa*-fed mice than in the negative control ($p<0.001$). Total HSL also seemed to increase in *A. melanocarpa*-fed mice, as shown in the blots; however, a significant difference was not seen upon quantification ($p<0.21$) The ratio of phosphorylated HSL to total HSL was 5-fold higher in mice that were fed *A. melanocarpa* than in the negative control ($p<0.001$). A previous study showed that the *A. melanocarpa* supplement could inhibit transcriptional modulation of CCAAT-enhancer-binding proteins alpha (C/EBPα), peroxisome proliferator-activated receptor gamma (PPARγ), and sterol regulatory element-binding protein 1c (SREBP-1c, followed by the lowering of adipogenesis (Lim et al., 2019). Another study showed that the phenolic-rich extract from blueberry promotes the hydrolysis of stored triglycerides in white adipose tissue (Li et al., 2020). However, the role of *A. melanocarpa* in stimulating lipolysis in white adipose tissue has not yet been investigated. Thus, our results give a new insight into the anti-obesity aspect of *A. melanocarpa*.

Conclusion

In conclusion, *A. melanocarpa* supplements reduced body weight and adipocyte mass in mice with high-fat-induced obesity, *via* the up-regulation of lipolysis in white adipose tissue. Our findings are consistent with results of other studies implicating *A. melanocarpa* in reducing adiposity. Further, the antioxidant capacity of the *A. melanocarpa* supplement was established *in vitro* and *in vivo*. *A. melanocarpa* supplement induced weight loss as adiposity decreased. The low respiratory quotient at rest suggested that stored fat was being oxidized in the presence of the *A. melanocarpa* supplement. Adiposity reduction, accompanied with elevated fat utilization, was shown to be owing to the enhanced activity of HSL in visceral fat. In conclusion, the dietary *A. melanocarpa* supplement reduced adiposity by enhancing lipolysis, which was followed by fatty acid utilization, in visceral fat.

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