Original Research

Anti-inflammatory and antiobesity effects of mulberry leaf and fruit extract on high fat diet-induced obesity

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Abstract

The purpose of this study was to investigate the anti-inflammatory and antiobesity effect of combinational mulberry leaf extract (MLE) and mulberry fruit extract (MFE) in a high-fat (HF) diet-induced obese mice. Mice were fed a control diet or a HF diet for nine weeks. After obesity was induced, the mice were administered with single MLE at low dose (133 mg/kg/day, LMLE) and high dose (333 mg/kg/day, HMLE) or combinational MLE and MFE (MLFE) at low dose (133 mg MLE and 67 mg MFE/kg/day, LMLFE) and high dose (333 mg MLE and 167 mg MFE/kg/day, HMLFE) by stomach gavage for 12 weeks. The mulberry leaf and fruit extract treatment for 12 weeks did not show liver toxicity. The single MLE and combinational MLFE treatments significantly decreased plasma triglyceride, liver lipid peroxidation levels and adipocyte size and improved hepatic steatosis as compared with the HF group. The combinational MLFE treatment significantly decreased body weight gain, fasting plasma glucose and insulin, and homeostasis model assessment of insulin resistance. HMLFE treatment significantly improved glucose control during intraperitoneal glucose tolerance test compared with the HF group. Moreover, HMLFE treatment reduced protein levels of oxidative stress markers (manganese superoxide dismutase) and inflammatory markers (monocyte chemoattractant protein-1, inducible nitric oxide synthase, C-reactive protein, tumour necrosis factor-α and interleukin-1) in liver and adipose tissue. Taken together, combinational MLFE treatment has potential antiobesity and antidiabetic effects through modulation of obesity-induced inflammation and oxidative stress in HF diet-induced obesity.

Keywords: inflammation, insulin resistance, mulberry fruit, mulberry leaf, obesity, oxidative stress

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Introduction

The obesity epidemic has increased due to lifestyle changes, including dietary patterns.[1] Obesity has been known as a risk factor for diverse diseases such as hypertension, type 2 diabetes, dyslipidemia, renal diseases, cardiovascular diseases and various cancers.[2] Recently, in addition to insulin resistance, obesity has been strongly regarded as a causative factor of type 2 diabetes,[3] suggesting that insulin resistance is caused by inflammation induced by macrophage infiltration in adipose tissue.[4]

Obesity is considered as a low-grade chronic inflammation.[5,6] Adipose tissue is not only a triglyceride (TG) storage tissue but is also a producer of adipokines,[6] which function as linkers between obesity and inflammation. Macrophage infiltration suppresses the production of anti-inflammatory cytokines such as adiponectin in adipose tissue.[7] The levels of pro-inflammatory adipokines and proteins such as tumour necrosis factor-α (TNF-α), interleukin (IL)-6 and inducible nitric oxide synthase (iNOS) in adipose tissues and C-reactive protein (CRP) in plasma are increased in obese people.[8] On the other hand, adiponectin, an anti-inflammatory adipokine, is reduced in the obese.[9] The imbalance between pro-inflammatory and anti-inflammatory adipokines generates local or systemic inflammation in obesity. Ultimately, an uncontrolled inflammatory response leads to a persistent pro-inflammatory state,[10] resulting in rising blood pressure, thrombosis, dyslipidemia and metabolic diseases in obesity.[11]

Obesity is associated with an increase in free fatty acids (FFAs) liberated from adipose tissue, leading to hyperglycemia accompanied by reduced insulin sensitivity in muscle.[12] Mainly, TNF-α, resistin and leptin released from adipose tissue lead to lipotoxicity, causing insulin resistance and dysfunction of endothelial cells

and pancreatic cells, which eventually results in metabolic syndrome.[13]

Moreover, the increase in fatty acids in obesity causes oxidative stress via NADPH oxidase (reduced form of nicotinamide adenine dinucleotide phosphate) activation, resulting in depletion of antioxidants and dysregulation of adiponectin and monocyte chemoattractant protein (MCP)-1.[14] Increased oxidative stress leads to biochemical changes including lipid peroxidation, DNA damage and altered enzyme activities in obesity.[15–17]

Mulberry leaves and fruits are especially drawing attention as materials of functional foods in Korea, China and Japan.[18] Mulberry leaves possess 50 species of inorganic components, 21 species of amino acids and flavonoid substances such as 1-deoxynojirimycin (DNJ), rutin, quercetin and isoquercitrin.[19,20] It has been reported that mulberry leaves have various benefits against obesity-related diseases such as dyslipidemia, diabetes, fatty liver and hypertension.[20,21] Mulberry fruits contain abundant amounts of anthocyanin pigments such as cyanidin-3-glucoside (Cy-3-glu) and cyanidin-3-rutinoside (Cy-3-rut),[22] which have both antioxidant and anti-inflammatory actions.[23,24] Moreover, another component of mulberry fruits, resveratrol, has been reported to induce apoptosis, inhibit adipogenic gene expression *in vitro*[25] and play an important role in improving pathophysiological conditions such as dyslipidemia, hyperinsulinemia and hypertension.[26] To date, antiobesity effect of mulberry leaf and fruits has not been fully examined yet and few studies have been conducted with respect to the beneficial effects of combined mulberry leaves and fruits in obesity.

Therefore, we investigated whether combined mulberry leaf and fruit extract may have beneficial effect on obesity through regulation of anti-inflammatory efficacy and antioxidant function in obesity.

Materials and methods

Plant extraction

Mulberry (*Morus alba*) leaves and fruits were collected in June 2009 in YangPyeong, Korea, and were generously provided by YangPyeong Agricultural Development & Technology center (YangPyeong, South Korea). The dried leaves and fruits were extracted with 70% ethanol. Each mixture was filtered, evaporated in a rotary evaporator and lyophilized. Using this procedure, the yield was 20% and 28% of the starting dry weight of mulberry leaves and fruits, respectively. The obtained ethanol extract of mulberry leaves and fruits were kept at −20°C until subsequent use.

High-performance liquid chromatography analysis to quantify functional ingredients

Samples were analysed on the Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, CA, USA) equipped with a quaternary solvent delivery system, an autosampler and a DAD detector. Separations were carried out on a J'sphere ODS-H80 column (250 × 4.6 mm, 4 μm, YMC Co., Japan). DNJ was analysed at 25°C and was detected at 265 nm in the gradient elution mode using acetonitrile (A) and 0.1% acetic acid in water (B). The elution profile was as follows: 0–15 min, 50% A in B; 15–20 min, 50–80% A in B; 20–25 min, 100% A in B. Anthocyanin (Cy-3-glu) was analysed at 35°C and was detected at 535 nm in the isocratic elution mode, using 0.4% trifluoroacetic acid (TFA) in acetonitrile (A) and 0.4% TFA in water (B). The elution profile was as follows: 0–30 min, 18% A in 82% B. Rutin was analysed at 30°C and was detected at 265 nm in the gradient elution mode, using methanol (A) and 0.5 M phosphoric acid in water (B). The elution profile was as follows: 0 min, 72% A in B; 0–12 min, 72–62% A in B; 12–17 min, 62–20% A in B; 17–20 min, 20–62% A in B; 20–30 min, 62–0% A in B. Resveratrol was analysed at 35°C and was detected at 310 nm in the gradient elution mode, using acetonitrile (A) and water (B). The elution profile was as follows: 0–13 min, 90% A in B; 13–14 min, 78–22% A in B; 14–17 min, 60–40% A in B; 17–17.5 min, 40–60%; 17.5–22 min, 10–90% B in A. Standards of DNJ, Cy-3-glu, rutin and resveratrol (Sigma, St. Louis, MO, USA) were prepared at 1 mg/mL, where 10 μL was injected as an external standard. For quantification of mulberry leaves and fruits extracts, the same solvent and conditions were applied to the analytical HPLC. For each sample, three replicate assays were performed.

Animals

Male C57BL/6 mice were obtained at four weeks of age (Orient Bio, Seongnam, South Korea) and were housed at constant temperature (22 ± 1°C), with a 12 h dark/light cycle and had access to tap water and food *ad libitum*. After one week of acclimation, the animals were randomly divided into two groups, a control diet (CON) group and a high-fat (HF) diet group. Each group was treated with either CON diet (D12450B, 10% kcal fat; Research Diets, New Brunswick, NJ, USA) or HF diet (D12451, 45% kcal fat; Research Diets) for nine weeks, respectively. All mice were used in accordance with animal protocols approved by Kyung Hee University Institutional Laboratory Animal Care and Use Committee.

Experimental design

After obesity was induced by HF diet for nine weeks, the animals were divided into six groups ($n = 6$ per group) and treated as follows: (a) lean control mice with distilled water (CON); (b) HF-fed obese mice with distilled water (HF); (c) HF-fed obese mice with low dose of mulberry leaf extract (MLE) only (133 mg/kg/day of MLE; LMLE); (d) HF-fed obese mice with high dose of MLE only (333 mg/kg/day of MLE; HMLE); (e) HF-fed obese mice with low dose of MLE and mulberry fruit extract (MFE) (133 mg/kg/day of MLE and 67 mg/kg/day MFE; LMLFE) and (f) HF-fed obese mice with high dose of MLE and MFE (333 mg/kg/day MLE and 167 mg/kg/day MFE; HMLFE). MFE-only treated groups were not included in this study because unpublished preliminary experiments in our lab showed increased food intake in MFE-treated group. MLE and MFE extracts were dissolved in distilled water and

treatments were administered daily via stomach gavage for 12 weeks. MLE and MFE for the combination treatment were mixed before daily treatment. During the experimental period, body weights and food intake were monitored. At the end of the experimental period, the animals were anesthetized by a mixture of Zoletil (Virbac, Carros, France) and Rompun (Bayer Korea, Seoul, South Korea) solution (3:1 ratio, 1 mL/kg, intraperitoneal). The blood was collected from a postcaval vein into heparin-coated tubes for analysis of biochemical parameters. Plasma was prepared by centrifugation at 1000 × g for 15 min and then frozen. Liver and adipose tissues were isolated, frozen in liquid nitrogen and stored at −80°C until subsequent analysis.

Intraperitoneal glucose tolerance test

After the treatment period, the animals were subjected to an overnight fast for 16 h before the intraperitoneal glucose tolerance test (IPGTT). The 50% glucose solution (2 g/10 mL/kg) was intraperitoneally injected and blood samples for the measurement of glucose levels were taken using a glucometer from the tail at 0, 15, 30, 60, 90 and 120 min after injection. The area under the curve (AUC) was calculated, which is the positive incremental area of blood glucose levels compared with basal fasting glucose levels.

Biochemical analysis

TG, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured enzymatically using a commercial kit (Bio Clinical System, Anyang, South Korea). Plasma glucose concentrations were measured by enzymatic assays (Sigma). Commercially available enzyme-linked immuosorbent assay (ELISA) kit was used to measure plasma insulin (Crystal Chem, Downers Grove, IL, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as follows: fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5.

Histological analysis

Histological sections (4 μm thickness) were prepared from liver and adipose tissue, fixed in 10% buffered formalin and embedded in paraffin. Histological sections were stained with haematoxylin and eosin. Stained tissues were observed using an optical microscope (HS-100, OPTICAL, China). The cell morphology, extents of fat accumulation and the presence of crown-like structure were observed. The average surface area for epididymal adipocytes was measured using Image J software (National Institute of Health, Bethesda, MD, USA).[27,28] Immature adipocytes were excluded for measurement of adipose size.

Measurement of lipid peroxidation

Levels of malondialdehyde (MDA) were measured as a marker of lipid peroxidation by the thiobarbituric acid (TBA) method.[29] Briefly, liver tissues (0.1 g) were homogenized with 0.15 M KCl buffer; 0.2 mL of homogenated liver tissues, 0.2 mL of 8.1% sodium dodecyl sulphate (SDS), 3 mL of 20% acetic acid–0.8% TBA mixture and 600 μL of distilled water were added and then heated at 95°C for 60 min. After cooling on ice, 1 mL of distilled water and 5 mL mixture of n-butanol and pyridine (15:1, v/v) were added and centrifuged at 4000 rpm for 10 min. The absorbance of the upper aqueous phase was measured at 532 nm and compared with 1,1,3,3-tetramethoxypropane using an ELISA reader.

Western blot analysis of liver and epididymal adipose tissue

Tissues were homogenized in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, pH 7.5, 1% NP40, 0.5% Na-deoxycholate stock, 1 mM ethylenediaminetetraacetic acid and 0.1% SDS) and then centrifuged at 18,000 × g at 4°C for 30 min. For Western blot analysis, 60 μg of protein was separated by SDS-polyacrylamide gel electrophoresis, followed by electroblotting of the proteins onto polyvinylidene fluoride membranes (Millipore, Marlborough, MA, USA). The membranes were then blocked in 5% defatted dry milk in PBS-Tween 20, incubated with polyclonal antibodies against TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500), CRP (Abcam, Cambridge, UK; 1:200), IL-1β (Santa Cruz; 1:100), iNOS (Stressgen, Victoria, BC, Canada; 1:1000), MCP-1 (Cell signaling, Beverly, MA, USA; 1:1000), adiponectin (Cell signaling; 1:1000), manganese superoxide dismutase (MnSOD; Stressgen; 1:5000), heme oxygenase-1 (HO-1; Stressgen; 1:1000) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz; 1:200), washed and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz). Detection was performed using enhanced chemiluminescence (ECL Western Blotting Substrate, Santa Cruz), according to the manufacturer's instructions. Western blot images were obtained with a luminescent image analyzer (Gbox, Syngene, Cambridge, UK) and band intensities were quantified. GAPDH was used as a loading control in the quantification of band intensity.

Statistical analysis

The results were expressed as mean ± SEM. Statistical significance was determined by a one-way ANOVA followed by a Duncan's test for multiple comparisons using SPSS 20. $P < 0.05$ was deemed statistically significant.

Results

Composition analysis of MLE and MFE

The functional ingredients of MLE and MFE were analysed by HPLC. HPLC analysis of the standard substances showed that the retention times of DNJ, Cy-3-glu, rutin and resveratrol were 1.855, 3.716, 17.025 and 17.512 min, respectively. The bioactive ingredients in each extract were analysed and their contents are shown in Table 1.

Effects of single MLE and combinational MLFE treatments on body weight and food intake

The body weight gain of HF group was significantly greater than that of CON group. The body weight gain was significantly reduced in combinational MLFE treatment groups compared with HF group regardless of doses, whereas there was no significant difference in single treatment groups compared with HF group. The food intake did not significantly differ among groups (Table 2). Regarding liver functions, HF diet treatment significantly increased plasma ALT concentrations, which was restored to those of controls with combinational MLFE treatments (Table 3). Also, plasma AST was significantly reduced with the combinational MLFE treatments compared with HF groups (Table 3).

Table 1 Contents of functional ingredients in each extract

	Contents (mg/mL)			
	DNJ	Cy-3-glu	Rutin	Resveratrol
LMLE	4.99	–	–	0.02
HMLE	12.49	–	–	0.05
LMLFE	5.93	0.88	0.29	0.03
HMLFE	14.83	2.20	0.72	0.07

Cy-3-glu: cyanidin-3-glucoside; DNJ: 1-deoxynojirimycin; MLE: mulberry leaf extract; MFE: mulberry fruit extract; MLFE: combinational MLE and MFE; LMLE: single MLE at low dose; HMLE: single MLE at high dose; LMLFE: MLFE at low dose; HMLFE: MLFE at high dose.

Effects of single MLE and combinational MLFE treatments on glucose tolerance

Glucose tolerance shown by IPGTT was impaired in HF group (Figure 1(a)). Furthermore, the AUC of IPGTT was significantly greater in HF group compared with that of CON group. However, the AUC of IPGTT was significantly reduced by 30% in HMLFE group compared with HF group (Figure 1(b)). Fasting plasma glucose and insulin levels were reduced in LMLFE and HMLFE groups compared with HF group, which is accompanied by the improved HOMA-IR with combinational LMLFE and HMLFE treatments (Table 3).

Effects of single MLE and combinational MLFE treatments on plasma lipid profiles

The plasma level of TC was higher in HF group than that of CON group. However, there were no significant differences in TC in all MLE and MLFE treatment groups compared with HF group. In addition, the plasma HDL-C concentration did not differ among groups. The plasma TG concentration in all MLE and MLFE treatment groups was significantly reduced compared with that of HF group (Table 4).

Table 2 Effect of single MLE and combinational MLFE treatment on body weight and food intake in C57BL/6 mice fed a HF diet*

	Body weight (g)			
	Before treatment (g)	After treatment (g)	Weight gain during treatment period (g for 12 weeks)	Food intake (g/day)
CON	26.08 ± 0.56^{a}	30.58 ± 0.46^{a}	4.50 ± 0.29^{a}	2.45 ± 0.09
HF	34.20 ± 1.40^{b}	48.27 ± 1.51^{c}	14.07 ± 0.81^{b}	2.36 ± 0.07
LMLE	31.91 ± 1.88^{b}	46.36 ± 3.04^{bc}	14.45 ± 1.41^{b}	2.53 ± 0.11
HMLE	32.47 ± 1.35^{b}	45.54 ± 1.82^{bc}	13.07 ± 0.73^{b}	2.57 ± 0.01
LMLFE	35.88 ± 1.15^{b}	42.42 ± 1.53^{b}	6.54 ± 1.38^{a}	2.32 ± 0.09
HMLFE	36.28 ± 1.14^{b}	42.40 ± 1.67^{b}	6.12 ± 0.92^{a}	2.55 ± 0.30

MLE: mulberry leaf extract; MFE: mulberry fruit extract; MLFE: combinational MLE and MFE; CON: control diet; HF: high-fat diet; LMLE: single MLE at low dose; HMLE: single MLE at high dose; LMLFE: MLFE at low dose; HMLFE: MLFE at high dose.
*Values are mean ± SEM. Mean values with unlike letters were significantly different ($P < 0.05$).

Table 3 Effect of single MLE and combinational MLFE treatments on plasma metabolic parameter in C57BL/6 mice fed a HF diet*

	Fasting glucose (mmol/L)	Fasting insulin (μU/mL)	HOMA-IR	AST (U/L)	ALT (U/L)
CON	6.13 ± 0.76^{ab}	1.99 ± 0.61^{ab}	0.61 ± 0.25^{ab}	144 ± 19^{ab}	22 ± 2^{a}
HF	7.44 ± 0.29^{c}	4.63 ± 1.45^{ac}	1.58 ± 0.53^{ac}	187 ± 22^{b}	97 ± 20^{b}
LMLE	7.19 ± 0.28^{ac}	5.92 ± 1.25^{c}	1.86 ± 0.36^{c}	117 ± 16^{ab}	53 ± 12^{ab}
HMLE	7.75 ± 0.16^{c}	5.98 ± 1.32^{c}	2.05 ± 0.44^{c}	125 ± 8^{ab}	46 ± 22^{ab}
LMLFE	5.74 ± 0.45^{bd}	0.42 ± 0.08^{b}	0.11 ± 0.02^{b}	70 ± 8^{a}	21 ± 5^{a}
HMLFE	6.06 ± 0.48^{ad}	1.65 ± 0.72^{b}	0.39 ± 0.15^{b}	90 ± 22^{a}	31 ± 12^{a}

MLE: mulberry leaf extract; MFE: mulberry fruit extract; MLFE: combinational MLE and MFE; CON: control diet; HF: high-fat diet; LMLE: single MLE at low dose; HMLE: single MLE at high dose; LMLFE: MLFE at low dose; HMLFE: MLFE at high dose; HOMA-IR: homeostasis model assessment of insulin resistance; AST: aspartate aminotransferase; ALT: alanine aminotransferase.
*Values are mean ± SEM. Mean values with unlike letters were significantly different ($P < 0.05$).

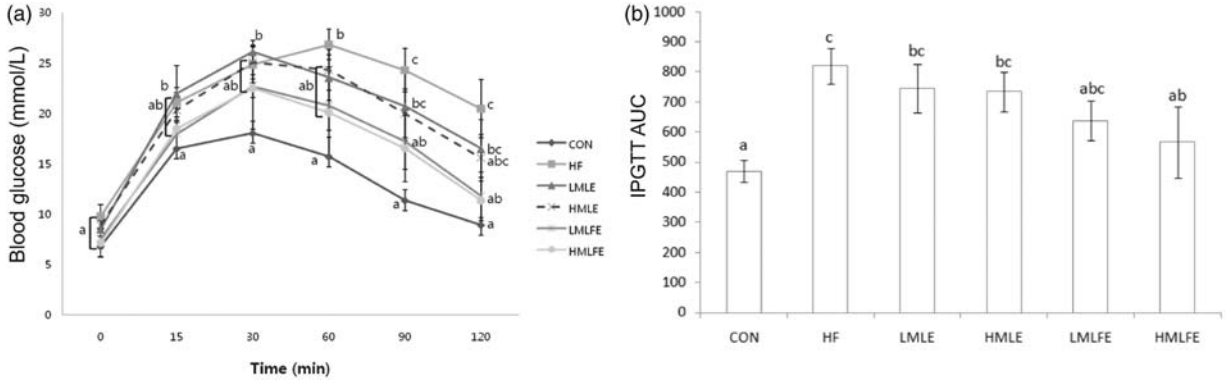

Figure 1 Effect of single MLE and combinational MLFE on: (a) glucose tolerance and (b) intraperitoneal glucose tolerance test (IPGTT) area under the curve (AUC) in C57BL/6 mice fed a high-fat diet. Values are mean ± SEM. Mean values with unlike letters were significantly different ($P < 0.05$). MLE: mulberry leaf extract; MFE: mulberry fruit extract; MLFE: combinational MLE and MFE; CON: control diet; HF: high-fat diet; LMLE: single MLE at low dose; HMLE: single MLE at high dose; LMLFE: MLFE at low dose; HMLFE: MLFE at high dose

Table 4 Effect of single MLE and combinational MLFE treatments on plasma lipids in C57BL/6 mice fed a HF diet.*

	TC (mmol/L)	HDL-C (mmol/L)	TG (mmol/L)
CON	2.83 ± 0.28^{a}	1.80 ± 0.16	0.84 ± 0.05^{a}
HF	4.35 ± 0.24^{b}	1.57 ± 0.12	1.74 ± 0.23^{b}
LMLE	3.50 ± 0.34^{ab}	2.13 ± 0.21	1.25 ± 0.12^{a}
HMLE	3.25 ± 0.31^{ab}	1.93 ± 0.24	1.02 ± 0.26^{a}
LMLFE	3.69 ± 0.44^{ab}	1.99 ± 0.15	1.06 ± 0.12^{a}
HMLFE	3.53 ± 0.42^{ab}	2.14 ± 0.26	1.05 ± 0.13^{a}

MLE: mulberry leaf extract; MFE: mulberry fruit extract; MLFE: combinational MLE and MFE; CON: control diet; HF: high-fat diet; LMLE: single MLE at low dose; HMLE: single MLE at high dose; LMLFE: MLFE at low dose; HMLFE: MLFE at high dose; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol.

*Values are mean ± SEM. Mean values with different letters were significantly different ($P < 0.05$).

Effects of single MLE and combinational MLFE treatments on liver and adipose tissue morphology

Histology analysis showed that livers of HF group had a lot of fat droplets compared with CON group (Figure 2(a)). The MLE and MLFE treatments decreased fat droplet accumulation. The combinational MLFE treatment effectively inhibited hepatic fat deposition compared with the single MLE treatments, regardless of doses (Figure 2(a)). Moreover, histological examination of epididymal adipose tissue showed that the adipocytes in the HF group were irregular in shape and was larger in size compared with lean controls (Figure 2(b) and (c)). Moreover, a number of crown-like structures were observed in HF adipose tissues, which were reduced by the combinational MLFE treatments (Figure 2(b)). Also, it markedly suppressed mean adipocyte size (Figure 2(c)).

Effects of single MLE and combinational MLFE treatments on lipid peroxidation in liver

MDA level as an indicator of lipid peroxidation was significantly increased in HF group compared with CON group, while levels significantly decreased by 33%, 34% and 35% in LMLE and all MLFE treatment groups, respectively, compared with HF group (Figure 3).

Effects of single MLE and combinational MLFE treatments on protein levels of inflammatory and oxidative stress markers in adipose tissue

The protein levels of MCP-1, adiponectin, iNOS, MnSOD and HO-1 were explored in adipose tissue. The protein level of MCP-1 increased in HF group compared with CON group and the level decreased in the combinational treatment groups (Figure 4(a)). In contrast, the protein level of adiponectin in HF group was significantly decreased compared with CON group. Adiponectin levels did not significantly differ in all MLE and MLFE treatment groups compared with HF group (Figure 4(b)). Furthermore, iNOS protein levels were reduced in HMLFE group compared with HF group (Figure 4(c)). The protein level of MnSOD in HF group was increased compared with that of CON group, while the levels significantly decreased in LMLE and HMLFE groups compared with HF group (Figure 4(d)). The protein level of HO-1 was significantly higher in HF group than that of CON group, whereas the level was significantly lower in LMLFE group (Figure 4(e)).

Effects of single MLE and combinational MLFE treatments on protein levels of inflammatory markers in liver

The protein expression levels of TNF-α, pro IL-1β and CRP were measured in the liver. Although the protein levels of TNF-α and CRP were not significantly different between CON and HF group, they were significantly reduced in HMLFE group (Figure 5(a) and (c)). The protein level of pro IL-1β was significantly increased in HF group compared with CON group. In contrast, pro IL-1β levels of HMLFE were significantly reduced to control levels (Figure 5(b)).

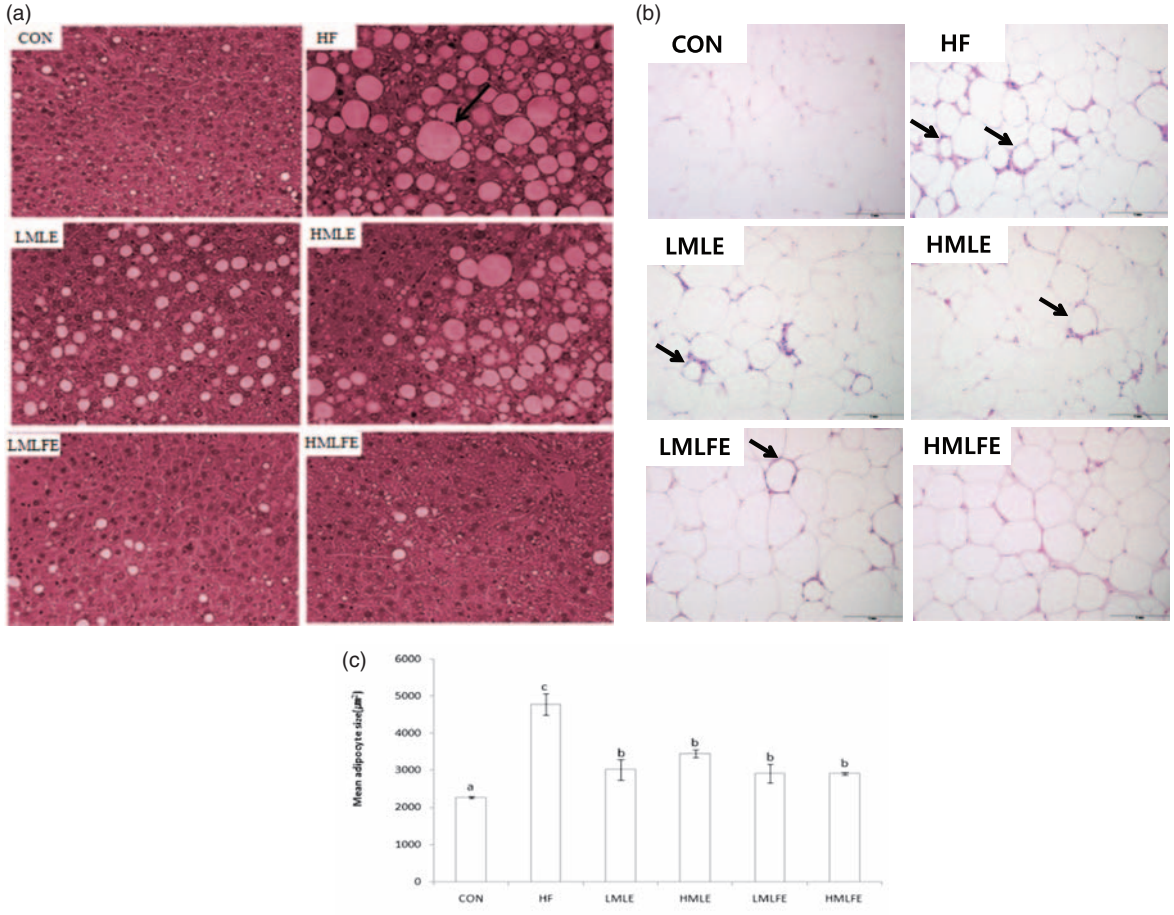

Figure 2 Effect of single MLE and combinational MLFE on: (a) liver morphology (magnification × 400); arrow indicates big lipid vacuole in HF group, (b) epididymal adipose tissue morphology (magnification × 200); arrow indicates crown-like structure, and (c) mean adipocyte size in C57BL/6 mice fed a HF diet. Histological sections in liver and adipose tissue were stained with haematoxylin and eosin. MLE: mulberry leaf extract; MFE: mulberry fruit extract; MLFE: combinational MLE and MFE; CON: control diet; HF: high-fat diet; LMLE: single MLE at low dose; HMLE: single MLE at high dose; LMLFE: MLFE at low dose; HMLFE: MLFE at high dose. (A color version of this figure is available in the online journal)

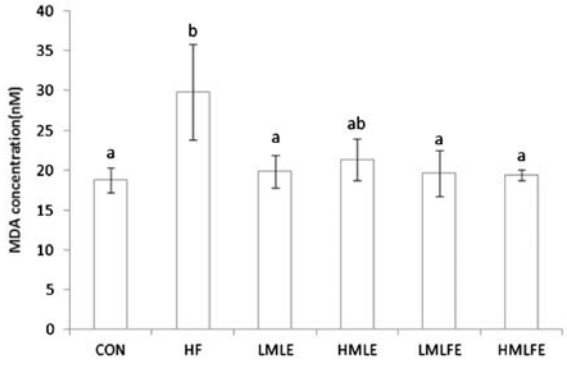

Figure 3 Effect of single MLE and combinational MLFE on lipid peroxidation in liver in C57BL/6 mice fed a HF diet. Values are mean ± SEM. Mean values with unlike letters were significantly different ($P < 0.05$). MLE: mulberry leaf extract; MFE: mulberry fruit extract; MLFE: combinational MLE and MFE; CON: control diet; HF: high-fat diet; LMLE: single MLE at low dose; HMLE: single MLE at high dose; LMLFE: MLFE at low dose; HMLFE: MLFE at high dose

Discussion

In this study, we investigated the anti-inflammatory and antiobesity effects of combined MLFE treatment in a HF diet-induced obese mouse model. The study clearly demonstrated that combinational MLFE treatment with a HF diet led to significant decreases in body weight gain, the extents of crown-like structure in adipose tissue and adipocyte size, and improved glucose control, plasma insulin, HOMA-IR, plasma TG, fatty liver and hepatic lipid peroxidation. In particular, HMLFE treatment significantly reduced obesity-induced pro-inflammatory proteins including MCP-1 and iNOS in adipose tissues and TNF-α, IL-1β and CRP in liver. However, the single treatment of MLE did not reduce body weight gain and obesity-related inflammatory proteins levels, although single MLE treatment selectively improved plasma TG at a high dose and decreased levels of hepatic lipid peroxidation and MnSOD in adipose tissues at a low dose.

Our data demonstrated that combinational MLFE treatment decreased body weight gain without changing food intake, regardless of treatment doses. A previous study that showed that MLE treatment did not modify body weight gain and the food efficiency ratio in obese fa/fa male Zucker rats[21] is in accordance with our results, which suggest that MFE treatment combined with MLE may have beneficial effects on reducing body weight gain.

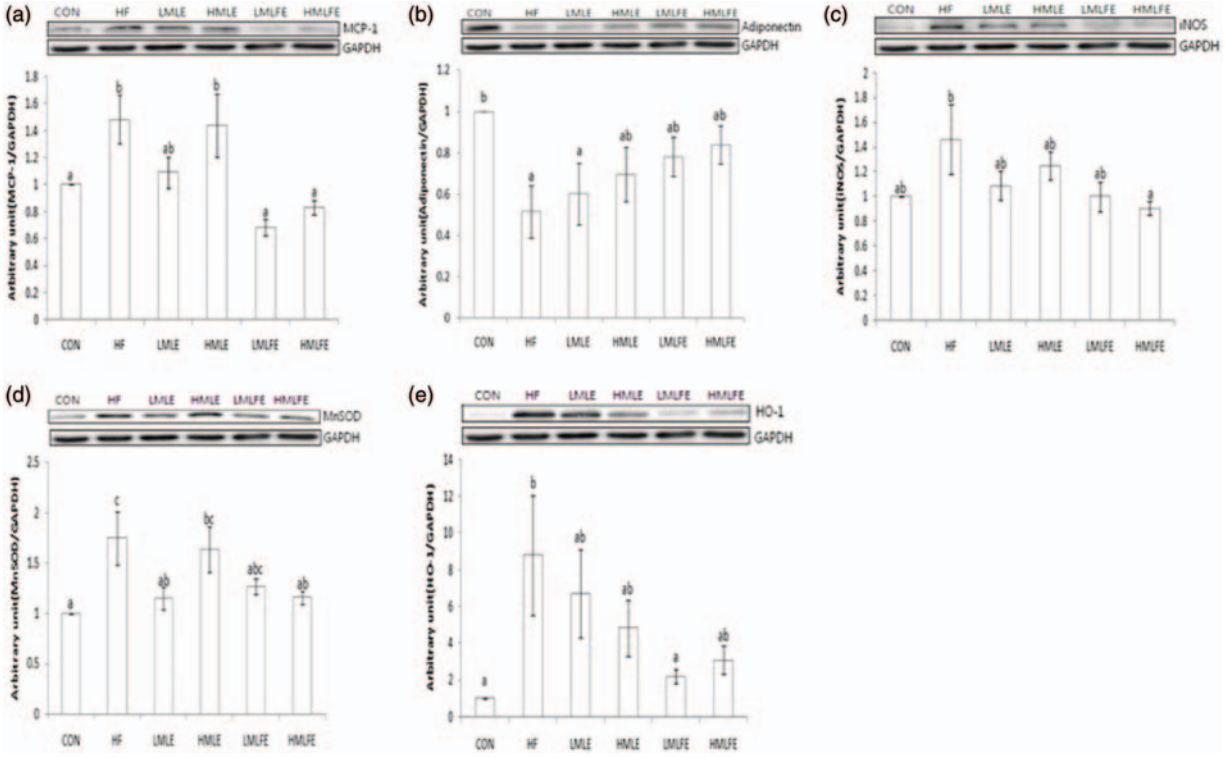

Figure 4 Effect of single MLE and combinational MLFE on: (a) monocyte chemoattractant protein-1 (MCP-1), (b) adiponectin, (c) inducible nitric oxide synthase (iNOS), (d) manganese superoxide dismutase (MnSOD) and (e) heme oxygenase-1 (HO-1) protein levels in adipose tissue in C57BL/6 mice fed a high fat diet. Values are mean ± SEM. Mean values with unlike letters were significantly different ($P < 0.05$). MLE: mulberry leaf extract; MFE: mulberry fruit extract; MLFE: combinational MLE and MFE; CON: control diet; HF: high-fat diet; LMLE: single MLE at low dose; HMLE: single MLE at high dose; LMLFE: MLFE at low dose; HMLFE: MLFE at high dose; GAPDH: glyceraldehyde 3-phosphate dehydrogenase

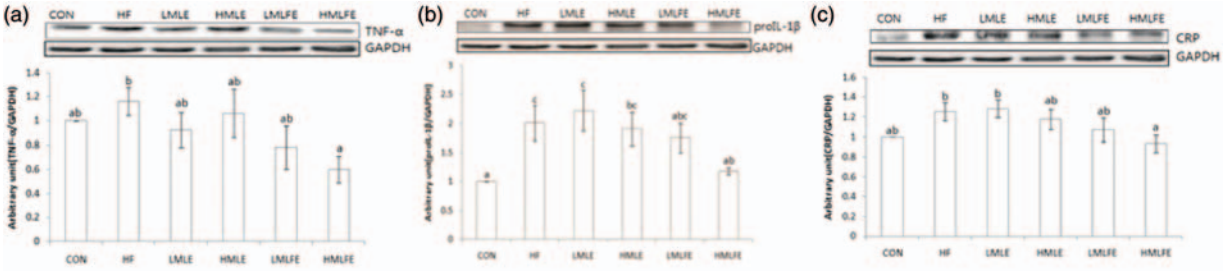

Figure 5 Effect of single MLE and combinational MLFE on: (a) tumour necrosis factor-α (TNF-α), (b) pro interleukin-1β (IL-1β) and (c) C-reactive protein (CRP) protein levels in liver in C57BL/6 mice fed a HF diet. Values are mean ± SEM. Mean values with unlike letters were significantly different ($P < 0.05$). MLE: mulberry leaf extract; MFE: mulberry fruit extract; MLFE: combinational MLE and MFE; CON: control diet; HF: high-fat diet; LMLE: single MLE at low dose; HMLE: single MLE at high dose; LMLFE: MLFE at low dose; HMLFE: MLFE at high dose; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Obesity is associated with an increase in FFAs liberated from adipose tissue, leading to hyperglycemia by reduction of insulin sensitivity in muscle.[12] Mainly, TNF-α, resistin and leptin released from adipose tissue lead to lipotoxicity, causing insulin resistance and dysfunction of endothelial and pancreatic cells, which eventually results in metabolic syndrome.[13] Glucose control was impaired by HF diet feeding but was significantly improved by HMLFE treatment. A previous study demonstrated that DNJ, a rich flavonoid in mulberry leaves, plays a role as a glycosidase inhibitor that decreases blood glucose levels.[30,31] A combination of mulberry leaves, fruits and silkworm powder has preventive effects against diabetes by regulating the antioxidant system and lipid metabolism in streptozotocin-induced diabetic rats.[32] Another study showed that MLE treatment decreased the plasma levels of TC, low-density lipoprotein cholesterol and TG in HF diet-induced obese rats.[33] Resveratrol reduced visceral fat-pad weights and the levels of plasma FFAs, TG and TC by suppressing visceral adipogenesis in HF diet-induced obese animals.[34]

Morphological changes in hepatic and adipose tissues are critical in the metabolic complications of obesity.[35] The increased mass of liver fat in obese individuals results in the development of non-alcoholic fatty liver disease.[5] Single MLE and combinational MLFE treatments reduced large fat particles and a widespread deposition of fat globules

in liver shown in HF group. In particular, the combinational treatment of MLFE markedly suppressed hepatic fat accumulation. As pro-inflammatory condition persists chronically, inflamed adipocytes are surrounded by M1 macrophages forming the crown-like structure.[36] In this study, combinational MLFE treatment reduced the extents of crown-like structure and adipocyte size compared with HF groups. These findings indicate that combinational MLFE treatment exhibits protective effects against inflammation and steatosis induced by abnormal lipid metabolism and adipocyte enlargement in obesity.

To examine the effects of MLE and MLFE on oxidative damage, we also measured liver MDA levels. Increased MDA levels with HF diet were significantly reduced by single or combinational treatments, except in HMLE group. This result is in partial accordance with the previous study showing that the oral administration of MLE alleviated the plasma levels of lipid peroxidation in rats fed a HF diet.[37] In addition, protein levels of oxidative stress-related markers, including MnSOD and HO-1, in adipose tissue were decreased by the combinational MLFE treatment. Recent studies have reported that anthocyanins, including Cy-3-glu and Cy-3-rut, in mulberry fruits possess the ability to scavenge free radicals.[38] In addition, rutin, which is the main functional component of mulberry fruits, reduced lipogenesis and oxidative stress in hepatocytes.[39] These results suggest that HMLFE has strong antioxidant activity, which suppresses reactive oxygen species production mediated by oxidative stress in obesity due to bioactive ingredients, including anthocyanin and rutin in MFE.

Obesity is described as a low-grade chronic inflammation accompanied by increased oxidative stress. The increase in obesity-associated oxidative stress is probably due to macrophage infiltration and excessive adipose tissue. MCP-1 is secreted from macrophage and adipose tissue and causes the recruitment of macrophages in obesity.[40] These macrophages release pro-inflammatory cytokines such as TNF-α, expanding insulin resistance to other susceptible organs.[41] Our data showed that the higher MCP-1 levels in HF group were lowered by the combinational treatment of MLFE regardless of doses. TNF-α is one of the main cytokines in systemic inflammatory status, which is also linked to the development of insulin resistance in obesity.[42] We showed that the protein level of TNF-α was significantly reduced in the HMLFE group. On the other hand, adiponectin is produced from adipose tissue and plays an important role in suppressing the inflammatory response.[43] Moreover, the overproduction of anti-inflammatory adipokines, such as adiponectin, protects from HF diet-induced insulin resistance *in vivo*.[44] Yamauchi *et al*. also reported that the level of adiponectin is positively correlated with insulin sensitivity.[45] In this study, we confirmed that HF group had a lower adiponectin level compared with CON group but single MLE and combinational MLFE treatments did not show significant changes in adiponectin levels, although HMLFE treatment improved glucose control and reduced MCP-1 levels in adipose tissues. The results suggest that the combinational treatment of MLFE reduces the recruitment of inflammatory molecules and inhibits chronic inflammatory processes but is not directly correlated with adiponectin.

Nuclear factor kappa B (NF-κB) is activated by oxidative stress and pro-inflammatory stimuli,[46,47] which regulate inflammatory cytokines including TNF-α and IL-1β.[48] IL-1β, one of various pro-inflammatory cytokines, plays a role as a crucial mediator of inflammation in obesity. IL-1β production is regulated by two distinct signals for activation and release. A pro-inflammatory signal stimulates NF-κB activation and pro-IL-1β expression, a precursor of mature IL-1β. The potent induction of biologically active IL-1β can lead to excessive inflammation in the innate immune response.[49] Our result supports the conclusion that HMLFE treatment leads to a decrease in mature IL-1β levels. The iNOS, a downstream gene of NF-κB, is an enzyme that produces NO, which leads to several obesity-associated abnormalities.[50] HMLFE decreased the protein level of iNOS, which was increased in HF group. A previous study reported that aqueous fractions of mulberry leaves suppressed TNF-α-induced lectin-like oxidized LDL receptor-1 expression, a cell-surface receptor for oxidized LDL, by inhibiting NF-κB activation in vascular endothelial cells.[51] Our results support the fact that the combinational HMLFE treatment may attenuate the obesity-induced inflammatory response through the modulation of several inflammatory markers. Furthermore, CRP, an acute-phase protein, is a sensitive marker of acute inflammation and tissue damage.[52] The increased plasma CRP level is associated with insulin resistance, obesity and metabolic syndrome.[53] Our data showed that CRP level was decreased, combined with improved glucose control and dyslipidemia in HMLFE group. Previous studies have reported that anthocyanins modulate inflammatory responses *in vitro* and *in vivo* by inhibiting the secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, CRP and iNOS.[54–56] Moreover, another study showed that resveratrol significantly attenuated pro-inflammatory cytokines such as TNF-α and IL-6 and their upstream signalling molecules in obese animals.[34] Our study demonstrated that the anti-inflammatory effect of the combinational HMLFE treatment might be due to beneficial effects of phytochemicals, including anthocyanin, rutin and resveratrol, which are rich in MFE. Taken together, we clearly demonstrated that HMLFE treatment containing DNJ, anthocyanin, rutin, flavonoids and resveratrol has potential benefits in reduction of the protein levels of oxidative stress (MnSOD and HO-1) and inflammatory markers (MCP-1, iNOS, TNF-α, IL-1β and CRP) in liver and adipose tissues.

In conclusion, combinational MLFE treatment has potential antiobesity and antidiabetic effects by reducing body weight gain and improving glucose control through modulation of obesity-induced inflammation and oxidative stress in HF-induced obesity. This research may provide critical insights with respect to overweight or obese people in nutritional intervention strategies to prevent further weight gain and potential metabolic complications.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data

and review of the manuscript; HHL performed the experiments, analysed the data and wrote the manuscript, SOL and SYK performed the sample extraction and the HPLC analysis, SJY analysed the data and revised the manuscript and YSL designed the study, analysed the data and wrote the manuscript.

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