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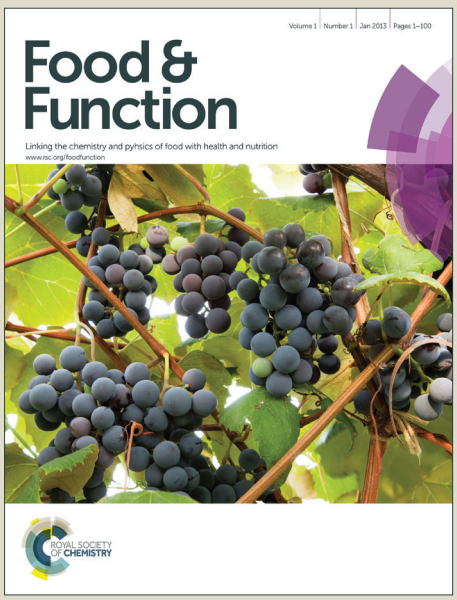

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Mulberry and mulberry wine extract increase the number of mitochondria during brown adipogenesis

Yilin You[1#], Xiaoxue Yuan[2, 3#], Hyuek Jong Lee[2], Weidong Huang[1], Wanzhu Jin[2], Jicheng Zhan[1*],

[1]College of Food Science and Nutritional Engineering, China Agricultural University, Tsinghua East Road 17, Haidian District, Beijing, 100083, China.

[2]Key Laboratory of Animal Ecology and Conservation Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China;

[3]The University of the Chinese Academy of Sciences, A13 Beijing 100049, China

* Corresponding author:

Jicheng Zhan

Tel: +86-10-62737553

Fax: +86-10-62737553

E-mail address: zhanjicheng@cau.edu.cn

Postal address: China Agricultural University, PO Box 301, No.17 Tsinghua East Road, Haidian District, Beijing 100083, P. R. China

# Yilin You and Xiaoxue Yuan have contributed equally to this work.

Abstract

Mulberry extract (ME) has been shown to possess beneficial effects towards obesity, but its mechanism is still unclear. In small mammals, mitochondria enriched brown adipose tissue (BAT) is known to convert proton electrochemical energy to heat and maintain a constant body temperature. Improving mitochondrial function or increasing the number of mitochondria could promote the metabolism of carbohydrate and fat. Thus, this study was designed to investigate the mitochondria function regulated by ME and mulberry wine extract (MWE) during the brown adipogenesis. The $C_3H_{10}T_{1/2}$ mesenchymal stem cell was treated with ME and MWE, which both significantly ($p < 0.05$) increased the expression levels of fatty acid oxidation related genes such as peroxlsome proliferator-activated receptor-γ coactlvator-1α, PR domain-containing 16 and carnitine palmitoyltransferase 1α during brown adipogenesis. These changes were accompanied with increases in mitochondrial oxidative complex proteins upon ME and/or MWE exposure. Notably, ME and/or MWE also significantly ($p < 0.05$) increased the expression of transcription factor A and nuclear respiratory factor-1, which are the key transcription factors of mitochondrial biogenesis. In parallel, mitochondrial copy number and brown adipose tissue specific gene—uncoupling protein-1 expression were dramatically ($p < 0.05$) elevated after ME or MWE treatment. Cyanidin-3-glucoside (Cy-3-glu), which was found to be one of the most abundant anthocyanins in ME and MWE. Therefore, the BAT regulatory activity of ME and MWE might be, at least in part, due to the effect of Cy-3-glu. These results suggested that ME and MWE could ameliorate metabolic disease through an improvement in mitochondrial functions.

Keywords: Mulberry extract, mulberry wine extract, cyanidin-3-glucoside, brown adipogenesis, mitochondria

Introduction

Mulberry is the fruit of *Morus alba* L. and is a traditional Chinese edible fruit that shows beneficial pharmacological effects including anti-inflammation[1-2], anti-oxidative stress[3], improvement of cardiac function[4], prevention of Alzeimer's disease[5], and anti-obesity[6]. Mulberry extracts contain high amounts of water-soluble anthocyanins, which are the most important antioxidative compounds in the plant kingdom[7]. Multiple lines of evidence suggest that anthocyanins have biological and pharmacological benefits including anti-bacterial, anti-inflammatory, anti-oxidative, and immune system-stimulating properties[8]. In addition, resveratrol—another component of mulberry fruits—inhibits adipogenesis *in vitro*[9] and improves pathophysiological conditions such as dyslipidemia, hyper-insulinemia and hypertension[10]. Similarly with grape wine, mulberry wine which we used is also made from 100% fresh mulberry fruit juice by yeast fermentation. In addition to containing many nutrients, such as amino acids, vitamins and minerals, mulberry wine also enriches a variety of bioactive substances, including organic acid, polyphenols, especially, anthocyanins and resveratrol[34]. However, to the best of our knowledge, the function of mulberry wine has been rarely studied.

Obesity is a major risk factor for metabolic disorders including type 2 diabetes, hypertension, cardiovascular disease, and other related diseases[11]. In mammals, white adipose tissue (WAT) stores energy, whereas brown adipose tissue (BAT) burns energy. As a thermogenic organ, BAT maintains body temperature during cold acclimation. BAT has a large number of mitochondria and uniquely expresses the BAT-specific gene uncoupling protein-1 (UCP1) that uncouples chemical energy to produce heat and maintain body temperature[12-14]. BAT has received great attention in treating obesity and related diseases because of its important roles in energy metabolism and systemic triglyceride clearance.

The anti-obesity and anti-diabetic effects of mulberry extract are well known[4,15-16], but the molecular mechanism is not well studied[2,15]. A previous study suggested that mulberry fruit extract involves mitochondrial membrane potential and might work through a ROS-dependent mitochondrial pathway to regulate human glioma tumor cell growth[17]. Therefore, we hypothesized that mulberry fruit extract (ME) and mulberry fruit wine extract (MWE) might directly regulate the activity of BAT and mitochondrial function during brown adipogenesis. Finally, most of the research on BAT has been conducted on the $C_3H_{10}T_{1/2}$ mesenchymal stem cell, which is considered a suitable model for studying the function and pathophysiology of BAT. Thus, to explore this hypothesis, our study investigated the effects of ME and MWE on thermogenic genes and mitochondrial marker genes expressions using quantitative real time PCR in $C_3H_{10}T_{1/2}$ cells during brown adipogenesis. In addition, this article also measured the mitochondrial copy number by RT-PCR and quantified the mitochondrial specific oxphos protein using Western blotting after $C_3H_{10}T_{1/2}$ cells treated with ME and MWE. To the best of our knowledge, this is the first study of the effects of ME and MWE in brown adipogenesis.

Materials and Methods

Chemicals and reagents

An Amberlite XAD-7(Sigma, Sydney, Australia) and analytical reagent-grade solvents were used for the extraction of ME and MWE. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and N, N-bis (salicylidene)-o-phenylenediamine vanadium (IV) oxide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and streptomycine, penicillin were purchased from Gibco (BRL, Inchinnan, UK). Cyanidin-3-glucoside (Cy-3-glu, >99.0%), cyanidin-3-rutinoside

(Cy-3-rut, >99.0%), oligomycin, Carbonyl cyanide 4- (trifluoromethoxy) phenylhydrazone (FCCP), rotenone, anti-GAPDH and anti-beta actin were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Merck (Germany).

Plant materials and preparation of extracts

Mulberry (*M. alba* L.) fruit (100 g) was macerated and stirred with 95% ethanol (500 mL). The extract was then purified through an Amberlite XAD-7 column. The polyphenolics were eluted with ~600 mL absolute ethanol with 1% (v/v) formic acid. The eluent was concentrated under reduced pressure at 35 °C and freeze-dried. The MWE was obtained from mulberry wine, which was produced by Wine Technology Development Center, China Agricultural University. Mulberry wine was concentrated under reduced pressure at 35 °C and lyophilised. ME and MWE were stored at -20 °C before use.

Analysis of nutrient compositions of ME and MWE

The total polyphenol content in ME and MWE was determined with a modified Folin-Ciocalteu method[18]. The results are presented in mg gallic acid equivalent (GAE)/100 g of sample on a dry weight basis (DW). The total anthocyanin contents of ME and MWE were directly determined using a pH differential method described previously[19]. The results were expressed as weight percentages, which were calculated as follows: (Cy-3-glu equivalent/wet weight of extracts or fractions) × 100. The contents of Cy-3-glu and Cy-3-rut in ME and MWE were measured with reverse phase HPLC (Waters, Milford, MA, USA) equipped with a photo diode array detector (PDA 2996). Chromatographic separations were performed on a Merck LiChrospher 100RP-18e (Merck,

Germany) column (250 × 4.0 mm ID, 5 μm), protected by a Merck RP-18 (10 mm × 4.0 mm) guard column. The injection volume was 10 μL with a 0.4 mL/min flow. The mobile phases consisted of 2 phases (A) and (B). Mobile phase (A) was 0.1% (v/v) TFA in water and mobile phase (B) was 0.1% TFA in water/acetonitrile (1/3, v/v). ME and MWE was dissolved in mobile phase (A) and filtered through a 0.22 μm membrane filter. The elution conditions were as follows: isocratic elution 10% (B), 0 to 10 min; linear gradient from 10% (B) to 60% (B), 50 min; to 90% (B), 55 min; at 10% (B), 55 to 60 min. The column temperature was 40 °C and the detection wavelength was 520 nm.

Cell culture and brown adipogenesis

$C_3H_{10}T_{1/2}$ cells, mouse mesenchymal stem cell line, were purchased from National Platform of Experimental Cell Resources (Sci-Tech, Shanghai, China). The cells were treated with brown adipogenic induction cocktails (DMEM containing 10% FBS, 1 μg/mL insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 0.12 mM indomethacin, and 1 nM 3,3′,5-Triiodo-L-thyronine (T_3))for the first two days. The medium was then replaced by medium supplemented with only insulin and T_3, which was changed every other day. The cells were treated with or without ME or MWE (10 μg/mL) for 6 days during brown adipogenesis. At day 6, the fully differentiated adipocytes were used for all experiments in this study.

Cell cytotoxicity assay

Cell viability was measured using the MTT assay according to a protocol described previously [20]. Briefly, the cells were treated with ME and MWE at various concentrations (0, 12.5, 25, 50, 100, 200, 400, 600, 800, and 1000 μg/mL) for 48 h; vehicle-treated cells served as a control. The 100 μL aliquots (0.5 mg/mL) of MTT were then added to each well. At the end of the experiment, the plates

were washed with phosphate buffered saline (PBS) buffer and lysed by adding 100 μL of 10% sodium dodecyl sulfate (SDS) in 0.1 M HCl. The absorbance was measured at 550 nm (650 nm as the reference wavelength) on a plate reader (Synergy Hybrid Reader H1, Gene Group Company). Cell survival rate (%) was calculated as follows: [(mean absorbance of the sample - reference absorbance)/mean absorbance of the control] × 100.

RNA Isolation and Real-Time PCR

Total RNA was extracted by using a Trizol reagent (Invitrogen). Reverse transcription of 2 μg total RNA was performed with the high-capacity cDNA reverse transcription kit (Promega Biotech Co., Ltd.). Real-time PCR in triplicate was performed with a SYBR Green Master Mix (Promega Biotech Co., Ltd.). The PCR reactions were run in triplicate for each sample and quantified using the ABI Prism VIIA7 real-time PCR (Applied Biosystems). See Supplementary Table 1 for the details of primer sequences.

Western Blotting

An equal amount of proteins from cell lysates were loaded in each well of a 12% SDS-PAGE gel after denaturation with SDS loading buffer. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes. The membranes were then incubated with blocking buffer (5% fat-free milk) for 1 h at room temperature, and then blotted with the following antibodies overnight: anti-UCP1, anti-OXPHOS (Abcam plc), anti-ERK1/2, anti-phospho ERK1/2, anti-AKT, anti-phospho AKT, anti-AMPK, anti-phospho AMPK, anti-p38 MAPK, anti-phospho p38 MAPK antibodies (Cell Signaling Technology, Inc.), anti-GAPDH and anti-beta actin. The membranes were incubated with HRP-conjugated secondary antibodies for 1 h at room

temperature. All signals were visualized and analyzed by densitometric scanning (Image Quant TL7.0, GE healthcare Bio-Sciences AB). Intensity values of the bands were quantified using Image J software (National Institutes of Health, USA).

Measurement of mitochondrial copy number

Total DNA Isolation-Total DNA (genomic and mtDNA) was isolated from $C_3H_{10}T_{1/2}$ cells using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. DNA concentration was assessed using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). The mtDNA copy number relative to the genomic DNA content was quantitatively analyzed with an ABI Prism VIIA7 real-time PCR (Applied Biosystems). The primers for COX-II and β-globin were as following:

COX-II: forward GCCGACTAAATCAAGCAACA, reverse CAATGGGCATAAAGCTATGG, β-globin: forward GAAGCGATTCTAGGGAGCAG, reverse GGAGCAGCGATTCTGAGTAG.

Measurements of oxygen consumption assay

Mouse Primary Immortalized Brown Adipocytes- BAT-cMyc cells were seeded in gelatin-coated XF24 culture microplates (Seahorse Bioscience), and cultured in DMEM with 20% FBS and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin) overnight at 37 °C with an atmosphere of 5% CO_2. The next day, the cells were treated with the Cy-3-glu (10 μM); DMSO served as the control. After 24 h, The O_2 consumption was measured with a Seahorse Bioscience XF24-3 extracellular flux analyzer. Basal respiration was also assessed in untreated cells. The cells were then treated with 10 μmol/L oligomycin to measure the ATP turnover. The maximum respiratory capacity was assessed by 1 μmol/L FCCP stimulation. Finally, mitochondrial respiration

was blocked with 1 μmol/L rotenone and the residual oxygen consumption rate (OCR) was considered non-mitochondrial respiration.

Statistical analysis

All experiments were performed at least three times, and data are expressed as means ±standard deviation (SD). Statistical significance was tested using a t-test. Statistical significance was set at $p < 0.05$.

Results

Analysis of nutrient contents of ME and MWE

The total phenols and total anthocyanin contents of ME and MWE are shown in **Table 1**. Polyphenols were the main component of the two extract samples. The total amounts of phenolic compounds in ME and MWE were 696.5 ± 33.9 GAE mg/g dry weight and 876.2 ± 36.1 GAE mg/g dry weight, respectively. The total anthocyanin contents in ME and MWE were 106.58 ± 3.34 Cy-3-glu mg/g and 65.69 ± 2.78 Cy-3-glu mg/g. Representative HPLC chromatographs of ME and MWE were shown in **Figure 1 A** and **B**. The two peaks at 520 nm were identified and quantified in relation to the authentic standard curves. ME and MWE contained Cy-3-glu (69.93 ± 1.31 mg/g of dry weight and 8.50 ± 0.12 mg/g of dry weight, respectively). The Cy-3-rut was 32.05 ± 0.69 mg/g of dry weight and 50.22 ± 0.63 mg/g of dry weight, respectively. The peaks were labeled (1) and (2) which correspond to Cy-3-glu and Cy-3-rut. The total anthocyanin and Cy-3-glu components in ME were higher than in MWE. There were more total phenolics in MWE than ME indicating that the high contents of the polyphenols resulted from yeast fermentation.

208

209 **Effects of ME and MWE on $C_3H_{10}T_{1/2}$ cell viability and brown adipogenesis**

210 The effect of ME and MWE on $C_3H_{10}T_{1/2}$ cell viability is shown in **Figure 2A**. The LC_{50} of ME
211 and MWE were calculated from a plot of dose dependent viability (%) (data not shown) with a
212 linear relationship of y = -89.618x + 298.31 (r^2 = 0.9718) and y = -76.741x + 253.36 (r^2 = 0.9946),
213 respectively. The LC_{50} values of $C_3H_{10}T_{1/2}$ cells were 589.9 ± 46.7 μg/mL for ME, and 446.6 ±
214 39.1 μg /mL for MWE. The growth of $C_3H_{10}T_{1/2}$ cells was inhibited in a dose-dependent manner
215 (**Figure 2A)**.

216 To investigate whether MWE or ME have an effect on brown adipogenesis, C_3H_{10}T1/2 cells were
217 treated with a brown adipogenic induction cocktail. The ME or MWE did not show significant
218 effect during the formation of mature adipocytes (**Figure 2B**). In parallel, the gene expression
219 levels of AP2, C/EBPα, C/EBPβ, and PPARγ2 (adipogenic markers) were not altered in the MWE
220 or ME treated groups. However, the level of C/EBPδ expression was significantly up-regulated (p
221 < 0.05) in the ME or MWE treated group (**Figure 2C**).

222

223 **ME and MWE enhanced the expression of thermogenic genes**

224 As adipogenesis was not affected by MW or MWE treatment, we interrogated whether there are
225 any changes in BAT-specific gene expression. Interestingly, ME or MWE (10 μg /mL)
226 significantly increased (p < 0.05) the expression levels of fatty acid oxidation related genes such
227 as PGC1α, PRDM16 and CPT1α during brown adipogenesis (**Figure 2D**). Furthermore, the
228 expression levels of UCP1 increased dramatically by 1.82- and 2.74-fold (p < 0.05) in the ME and
229 MWE treated groups, respectively (**Figure 2E**). These results indicated that ME and MWE
230 treatment specifically regulated the thermogenic gene expressions in $C_3H_{10}T_{1/2}$ cells during brown

adipogenesis.

ME and MWE increase mitochondrial copy number

BAT burns energy, whereas WAT stores energy. As a thermogenic organ, one of the characteristic of BAT is a higher number of mitochondria, which reminded us that ME and MWE maybe can regulate mitochondrial function. We analyzed the expression of mitochondrial transcription factor A (Tfam) and nuclear respiratory factor-1 (NRF-1) using a quantitative real time PCR in $C_3H_{10}T_{1/2}$ cells (**Figure 3A** and **3B**).

In comparison to the control, the expressions of both Tfam and NRF1 increased more than 2-fold ($p < 0.05$) after treatment with ME (**Figure 3A**) and MWE (**Figure 3B**). Tfam and NRF1 are well-known key players in mitochondrial biogenesis [21]. Therefore, we measured the mitochondrial copy number in $C_3H_{10}T_{1/2}$ cells, and the results showed that both ME and MWE significantly increased ($p < 0.05$) the mitochondrial number in $C_3H_{10}T_{1/2}$ cells (**Figure 3C**).

To confirm this, the mitochondrial-specific oxphos protein was quantified with Western blotting. The levels of Ubiquinol-Cytochrome C Reductase Core Protein II (UQCRC2) and NADH Dehydrogenase (Ubiquinone) 1 Beta Subcomplex, 8 (NDUFB8) were significantly and consistently increased ($p < 0.05$) in both ME and MWE treated groups (**Figure 3D**). However, UCP1, ATP5A, and Succinate Dehydrogenase Complex, Subunit B (SDHB) protein expression increased ($p < 0.05$) only in the ME-treated group, but not in the MWE treated group (**Figure 3D**). These results indicated that ME and MWE significantly increased mitochondrial copy number during brown adipogenesis.

ME increased p38 MAPK phosphorylation

BAT is known to participate in blood glucose regulation and insulin sensitivity[22]. Our study has demonstrated that ME and MWE could dramatically increase the expression levels of UCP1, the key thermogenic gene, and other genes related to fatty acid oxidation. Therefore, we next tested whether ME or MWE influences insulin signaling pathways. Several proteins such as AMPK, AKT, ERK, and p-38, which were associated with the insulin signaling pathway, were analyzed by Western blotting (**Figure 3E**). Interestingly, the phosphorylation of p38 (P-p38) was significantly increased ($p < 0.05$) after the ME treatment in $C_3H_{10}T_{1/2}$ cells during brown adipogenesis. These results indicate that ME, not MWE, regulates mitochondria function at least in part through the p38 pathway.

Cy-3-glu increased mitochondrial copy number and enhanced cellular oxygen consumption

The ME and MWE contain large amounts of anthocyanin pigments such as Cy-3-glu and Cy-3-rut (**Table 1**, **Figure 1A** and **1B**). We examined the effects of Cy-3-glu on the expression of thermogenic genes and mitochondrial copy number. Cy-3-glu significantly up-regulated the UCP1 gene expression in a dose-dependent manner ($p < 0.05$) (**Figure 4A**). Meanwhile, Cy-3-glu markedly increased BAT-selective thermogenic genes including PRDM16, PGC1α and PGC1β mRNA expression during the brown adipogenesis ($p < 0.05$) (**Figure 4B**). Considered together, these data indicated that Cy-3-glu positively regulated BAT gene expression. Furthermore, the expression of NRF1 was significantly increased upon Cy-3-glu treatment ($p < 0.05$) (**Figure 4C**). In parallel, we observed that Cy-3-glu significantly increased mitochondrial number ($p < 0.05$) (**Figure 4D**). These results indicated that Cy-3-glu significantly increased mitochondrial copy number during brown adipogenesis. An important characteristic of brown fat cells is their extraordinarily high rates of oxygen (O_2) respiration, and uncoupled respiration in response to

277 FCCP in particular. As shown in **Figure 4E**, O_2 consumption was significantly increased by
278 Cy-3-glu treatment at the basal state ($p < 0.05$). It increased further in response to FCCP
279 stimulation ($p < 0.05$). These results suggest that Cy-3-glu could effectively improve the
280 respiratory function of brown fat cells.
281

282 ## Discussion

283 Our data demonstrate that the ME and MWE increase the number of mitochondria, the expression
284 levels of thermogeneic genes such as UCP1, PGC1α, and PRDM16, and the oxphos protein levels
285 on the mitochondria membrane of brown adipocytes during brown adipogenesis. These effects are,
286 at least in part, mediated by Cy-3-glu—an anthocyanin from ME and MWE.
287 ME has beneficial effects on metabolic syndrome through an unknown mechanism[6]. It may be
288 possible that these beneficial effects are associated with brown adipose tissue mitochondrial
289 function. ME reduces fasting blood sugar in type 2 diabetic paitients[23]. This has a positive effect
290 on various (patho) physiologic conditions including hypertension, hyper-glycemia, and
291 hyper-lipidemia.
292 In addition, overweight individuals that received ME for three months lost 10% of their initial
293 body weight[6]. ME has also been shown to inhibit lipogenesis and promote lipid clearance[24]. Our
294 data demonstrated that the ME and MWE increased the number of mitochondria, the expression
295 levels of thermogeneic genes such as UCP1, PGC1α, and PRDM16 expression and oxphos
296 protein levels on the mitochondria membrane in brown adipocytes during brown adipogenesis.
297 These effect at least in part, mediated by Cy-3-glu, the main anthocyanin from ME and MWE.

298 Several interesting compounds including resveratrol were identified during ME analysis[25]. An

299 animal study showed that long-term treatment with resveratrol dramatically improves
300 dyslipidemia and hyperinsulinemia[10]. Mulberry fruits contain large amounts of anthocyanin
301 pigments such as Cy-3-glu and Cy-3-rut[26], which have both antioxidant and anti-inflammatory
302 actions[3]. Anthocyanin has been shown to block lipid accumulation, and thereby decrease body
303 weight gain in a high fat diet[27]. Our results consistently indicate that as an active constituent,
304 Cy-3-glu could significantly (p <0.05) increase mitochondria copy number and O_2 consumption
305 during brown adipogenesis. The HPLC data (**Table 1**) suggested that the concentration of
306 Cy-3-glu (10 μM) was higher than the concentration in ME/MWE at 10 μg/mL. However, our
307 results demonstrated that both ME/MWE (10 μg/mL) and Cy-3-glu (10 μM) could significantly (p
308 <0.05) up-regulate UCP1, PGC1α, PRDM16, Tfam and NRF1 gene expression levels and
309 increase the number of mitochondria (**Figure 2, Figure 3, and Figure 4**). In addition to Cy-3-glu,
310 there were a number of other bioactive substances (such as resveratrol and Cy-3-rut) in
311 ME/MWE[25-26]. Therefore, other bioactive compounds in ME/MWE may work in concert with
312 single purified Cy-3-glu to improve BAT activity.

313 It has been suggested that ME could be involved in mitochondrial function[17]. BAT is
314 characterized by multi-locular lipid droplets and is enriched with mitochondria; the total weight of
315 BAT in man is around 50 g[29]. As a thermogenic organ, BAT contributes 20% of the total resting
316 energy expenditure when maximally stimulated[30]. Cold exposure dramatically activates BAT and
317 clears excessive triglycerides in the plasma by increasing lipid uptake into BAT improving energy
318 metabolism and weight loss[31-32].

319 These results suggest that the increase in BAT thermogenic function could contribute to the
320 clearance of excessive lipid contents. Our results indicated that both ME and MWE increased

mitochondria copy number and mitochondrial membrane protein expression during brown adipogenesis (**Figure 3**). However, ME and MWE did not accelerate brown adipogenesis *in vitro* without significant changes in the expression of adipogenic genes (**Figure 2**). Our results highlight that ME and/or MWE might improve BAT function by increasing mitochondria number. Future studies should specify which compositions from ME and MWE would be beneficial for improving BAT activity.

In this study, we demonstrated that ME regulated mitochondria function at least in part through the p38 pathway (**Figure 3E**). P38 has previously been identified as an inducer of UCP1[33], and this report provides evidence that the p38-AMPK-PGC1a pathway could regulate mitochondrial function[33]. Our results also confirmed that ME could increase p38 phosphorylation and mitochondrial function.

In conclusion, we show that ME and MWE could increase mitochondria number during brown adipogenesis. These results may open new avenues to treat obesity and attendant disease.

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Figure captions

Figure 1. A representative HPLC chromatogram of mulberry extract (A) and mulberry wine extract (B) with peaks corresponding to cyanidin-3-glucoside (1) and cyanidin-3-rutinoside (2).

Figure 2. Effects of ME and MWE on $C_3H_{10}T_{1/2}$ cell viability and brown adipogenesis. The MTT assay shows that ME and/or MWE inhibits the growth of $C_3H_{10}T_{1/2}$ cells in a dose-dependent manner (**A**). ME or MWE treatment did not show significant change in the formation of mature adipocytes (**B**). The expression levels of adipogenic marker genes are shown in (**C**). ME or MWE significantly increased (p <0.05) the expression levels of fatty acid oxidation related genes such as PGC1α, PRDM16, CPT1α (**D**), and brown adipose tissue specific gene UCP1 (**E**) during brown adipogenesis. ME, mulberry extract; MWE, mulberry wine extract.

Figure 3. ME or MWE increase mitochondrial copy number and increase p38 MAPK phosphorylation. Tfam and NRF1, mitochondrial biogenic genes, were significantly increased (p <0.05) after treatment with ME (**A**) and MWE (**B**). Mitochondrial copy number was significantly increased (p <0.05) by both ME and MWE (**C**). The mitochondrial oxphos complex proteins were also significantly increased (p <0.05) in both ME and MWE treated groups (**D**). The expression levels of proteins involved in insulin signaling were analyzed after ME or MWE treatment. The phosphorylation of p38 (P-p38) was significantly increased (p <0.05) after the ME treatment (E). * The number shown in Figure 3D and 3E is the intensity value of the band. The asterisk shown in Fig. 3E indicates the top bands for the p-AMPK. ME, mulberry extract; MWE, mulberry wine extract.

Figure 4. Cyanidin-3-glucoside increased the mitochondrial copy number and enhanced cellular oxygen consumption. Cyanidin-3-glucoside significantly increased (p <0.05) the expression levels of brown adipose tissue specific gene UCP1 (**A**) and fatty acid oxidation related genes such as PRDM16, PGC1α, and PGC1β (**B**)

456 during brown adipogenesis. NRF1 was significantly increased (p <0.05) after treatment with
457 cyanidin-3-glucoside (**C**). Mitochondrial copy number was significantly increased (p <0.05) by
458 cyanidin-3-glucoside (**D**). Cyanidin-3-glucoside significantly increased (p <0.05) the total and uncoupled
459 respiration of BAT-cMyc cells (**E**).

Table 1. Compositional analysis of ME and MWE.

Ingredients	Mulberry Extract (mg/g)	Mulberry Wine Extract (mg/g)
Total phenolics	696.5 ± 33.9	876.2 ± 36.1
Total anthocyanin	106.58 ± 3.34	65.69 ± 2.78
Cyanidin-3-glucoside	69.93 ± 1.31	8.50 ± 0.12
Cyanidin-3-rutinoside	32.05 ± 0.69	50.22 ± 0.63

ME, mulberry extract; MWE, mulberry wine extract.

Figure 1

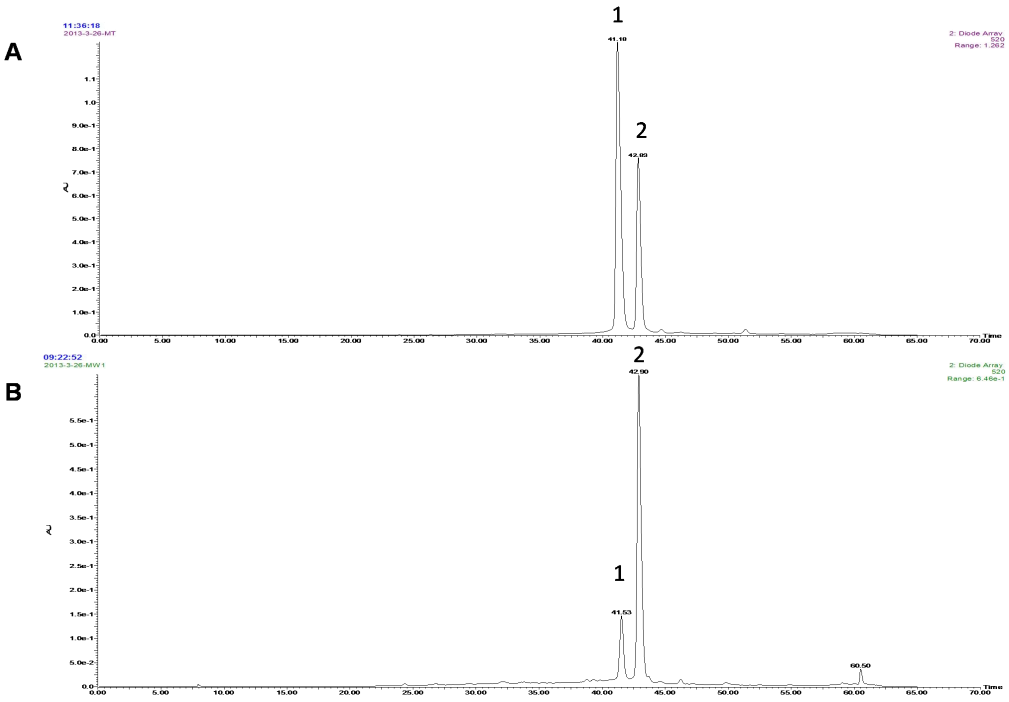

Figure 2

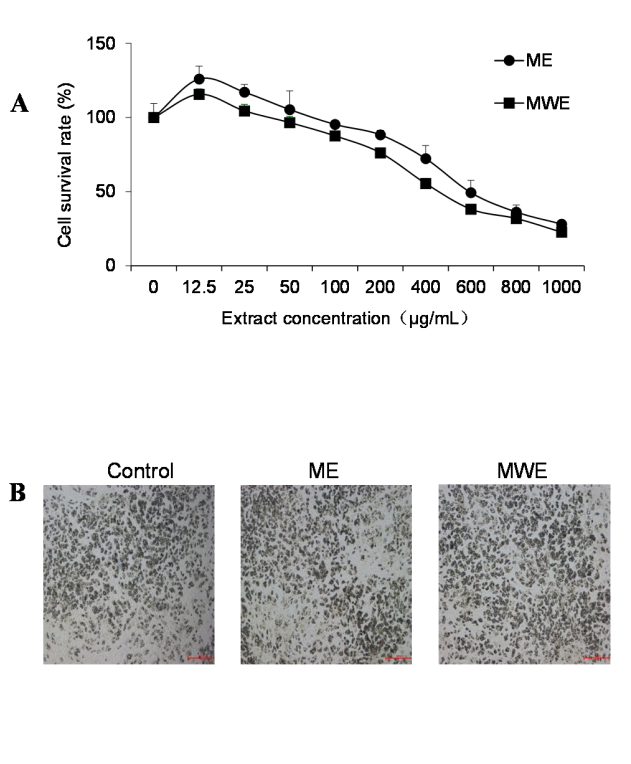

Figure 3

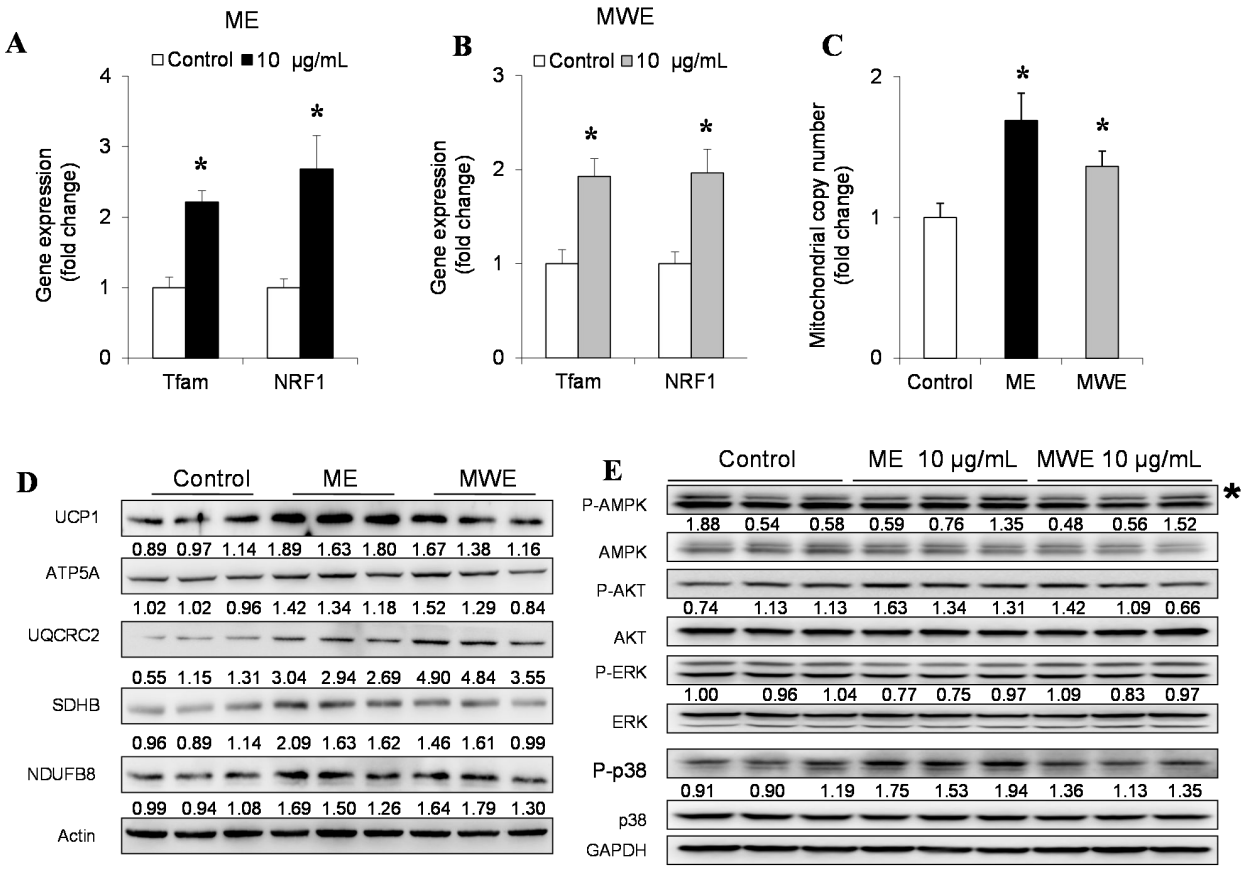

Figure 4