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Antioxidant and cognitive promotion effects of anthocyanin-rich mulberry (*Morus atropurpurea* L.) on senescence-accelerated mice and prevention of Alzheimer's disease

Ping-Hsiao Shih[a], Yin-Ching Chan[b], Jiunn-Wang Liao[c], Ming-Fu Wang[b], Gow-Chin Yen[a,*]

[a]*Department of Food Science and Biotechnology, National Chung Hsing University, Taichung 40227, Taiwan*
[b]*Department of Food and Nutrition, Providence University, Taichung 43301, Taiwan*
[c]*Graduate Institute of Veterinary Pathology, National Chung Hsing University, Taichung 40227, Taiwan*

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Abstract

In the present study, we evaluated the beneficial effect of mulberry extracts (ME), which are rich in phenolics and anthocyanins, on the induction of antioxidant enzymes and on the promotion of cognition in senescence-accelerated mice (SAMP). Six-month old SAMP8 and SAMR1 mice were fed a basal diet supplemented with 0.18% and 0.9% ME for consecutive 12 weeks. The results showed that the mice fed the ME supplement demonstrated significantly less amyloid β protein and showed improved learning and memory ability in avoidance response tests. ME-treated mice showed a higher antioxidant enzyme activity and less lipid oxidation in both the brain and liver, as compared to the control mice. Furthermore, treatment with ME decreased the levels of serum aspartate aminotransferase, alanine aminotransferase, triglyceride and total cholesterol that increase with ageing. The hepatoprotective effect of ME appeared to occur through a mechanism related to regulation of the mitogen-activated protein kinases and activation of the nuclear factor-erythroid 2 related factor 2, where the latter regulates the induction of phase 2 antioxidant enzymes and reduction of oxidative damage. Overall, supplementation of ME might be advantageous to the induction of an antioxidant defense system and for the improvement of memory deterioration in ageing animals.

Keywords: SAMP8; Amyloid; Anthocyanin; Mulberry; Nrf2; MAPK

1. Introduction

Ageing has been recognized as an irreversible and inevitable process since ancient times. Ageing-associated disorders include immune dysfunction [1,2], cognition degeneration [3,4], carcinomatosis [5,6], cardiovascular disease [7,8] and metabolic syndrome [9,10]. Increasing evidence suggests that ageing increases the risk of degeneration of the nervous system, which mostly affects the moral and physiological life of the elderly. As a result of the development of medical science and health care, the average human life span is increasing; however, the future socioeconomic burden of the elderly must be a source of concern in developed countries.

The murine model of accelerated senescence was first developed in Japan [11] and has been widely discussed in their ageing progress. This strain of mouse was characterized by the senescence-accelerated prone mouse type (SAMP1, P2, P3, P6, P7, P8, P9 and P10) and normal-ageing mouse type (SAMR1, R4 and R5). With the exception of the SAMR strains, each SAMP strain has a specific age-dependent disorder, such as the impaired immune system observed in the P1 strain, senile osteoporosis seen in the P6 strain and the age-related cataracts found in the P9 strain [12]. The SAMP8 mouse, which is characterized by age-associated learning and memory deficits, anxiety, impaired immune system and the deposition of amyloid β-protein (Aβ), has been used as a model for investigating Alzheimer's disease (AD). However, the SAMR1 mouse shows normal ageing with nonthymic lymphoma, histiocytic sarcoma and ovarian cysts [12]. From the reports focusing on the relationship between natural phytochemicals and age-related cognitive deficiency [13,14], the behavior (i.e., locomotion ability, learning ability and memory) and appearance (such as hair, inflammatory index and ageing index) are absolutely different between SAMP8 and SAMR1 mice. Many important and naturally occurring flavonols and flavones have been shown to have a pharmacological benefit for AD because these compounds inhibit the formation of Aβ [15]. Therefore, natural phytochemicals might be potential treatments for patients with dementia.

Mulberry (*Morus atropurpurea* L.) has multiple biological and physiological effects, as well as hypoglycemic, hypotensive and diuretic effects because of the abundant content of the anthocyanins and phenolics. It is suggested that the supplementation of mulberry

* Corresponding author. Tel.: +886 4 2287 9755; fax: +886 4 2285 4378.
E-mail address: gcyen@nchu.edu.tw (G.-C. Yen).

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leaf extract diet has a preventive effect against an STZ-induced diabetic phenomenon in rats [16]. Recently, mulberry juice has been shown to have the ability to reduce elevated plasma levels of lipid peroxide in mice loaded with water immersion restraint stress [17]. Active compounds have been separated from the mulberries that have antidiabetic properties [18]. Furthermore, anthocyanin-rich fruits have been described that show memory improvement efficacy in animal models [19]. Importantly, mulberry leaf extracts prevents amyloid fibril formation and shows neuroprotective effects [20]. However, the suppressive or preventive effects of mulberry on age-associated deficits in memory and the antioxidant capacity of mulberries have not yet been discussed in SAMP8 mice.

Free radical theory strengthens the hypothesis that ageing occurs by the accumulation of oxidative damage, such as DNA mutation, and lipid and protein dysfunction. Epidemiological research reveals that the consumption of antioxidant phytochemicals shows benefits for oxidative stress-induced damage [21]. Herein, we investigated whether supplementation of mulberry extracts (ME), which is rich in nutraceuticals including phenolic compounds and flavonoids, could protect the liver from age-associated antioxidant decline and protect the brain from cognitive deficits in SAMP8 mice. The antioxidant capacity of the liver and brain was evaluated, and the serum biochemical values were examined. Moreover, the signal transduction molecules involved were also discussed.

2. Materials and methods

2.1. Mulberry extracts

Mature mulberry fruits were harvested from a local orchard in Tainan County, Taiwan. Freeze-dried mulberry fruits were blended and then extracted overnight with methanol (1/200, w/v, containing 0.1% HCl). Vacuum drying was then performed to remove any remaining solvent. The ME were dissolved in distilled water and then were mixed with the mouse feed.

2.2. Reagent

Blackcurrant extracts (BE) were purchased from Frutarom (Haifa, Israel). Anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK, anti-phospho-JNK, anti-p38 anti-phospho-p38 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-α-tubulin antibody was purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-nuclear factor erythroid 2-related factor 2 (Nrf2) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Aβ antibody was purchased from Abcam Inc. (Cambridge, MA, USA). Other reagents with the highest grade were purchased from commercial companies.

2.3. Evaluation of antioxidant capacity

Total phenolics and flavonoids of mulberry and BE were investigated spectrophotometrically as previously described [22]. In brief, different concentrations of mulberry or BE were mixed with 0.2 M Folin–Ciocalteau reagent, and the reaction was neutralized by adding 7.5% (w/v) sodium carbonate. The absorbance of the resulting blue color product was measured at 765 nm spectrophotometrically. Gallic acid was used as a standard, and total phenolic content was expressed as gallic acid equivalents per gram of extracts. To evaluate flavonoid content of mulberry and BE, different concentrations of extracts or standard (rutin) were mixed with 5% (v/v) $NaNO_2$, 10% $AlCl_3$ and 1 M NaOH. After mixing well, the absorbance was read at 510 nm and flavonoid contents were expressed as milligram of rutin equivalents per gram of extracts.

The total antioxidant capacity of mulberry and BE were measured using trolox equivalent antioxidant capacity (TEAC) assay previously described [23]. To measure antioxidant capacity, different concentrations of ME or BE were mixed well with green-blue 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$ radical), which was generated by the mix of peroxidase (4.4 U/ml), ABTS (100 μM) and H_2O_2 (50 μM). Absorbance was monitored at 734 nm after 10 min incubation in the dark. TEAC value is expressed as millimolar concentration of trolox equivalence.

2.4. Animal treatment

Six-month-old male SAMR1 and SAMP8 mice were used in this study. These strains of the senescence-resistant and senescence-accelerated prone mice were established previously [11] and bred in the Animal Center at Providence University, Taichung, Taiwan. SAMR1 and SAMP8 mice are considered young and old since their average life spans are about 24 and 12 months, respectively. The mice were randomly divided into five groups, where each group contained 10–12 mice. Group I was the SAMR1 control, while Group II was the SAMP8 control. These two groups were only fed the basal diet. Group III consisted of SAMP8 mice that were treated with a diet containing 0.09% (w/w) BE. Groups IV and V consisted of the SAMP8 mice that were treated with a diet containing 0.18% and 0.9% ME, respectively. All animals were housed under controlled experimental conditions (25±2°C, 65±5% relative humidity, and a 07:00–19:00 h lighting period) and provided with food and water ad libitum. The total duration of the experiment was 3 months. The study protocol was approved by the Animal Research Ethics Committee at Providence University, Taichung, Taiwan.

2.5. Single-trail passive avoidance test

To investigate the effects of supplementation with mulberry on learning and memory ability, the active and passive avoidance tests were performed dependently. Two weeks before scarification, animals were exposed to a single-trail passive avoidance test. Each animal was placed in a cage (35×17×20 cm, width×length×height, model E10-15, Coulbourn Instruments, Philadelphia, PA, USA) consisting of two equal compartments connected by a small opening (7.5×6.5 cm, Guillotine Door, model E10-15GD, Coulbourn Instruments). The platform was electrified. At the beginning, each animal was placed in the light compartment. After a brief orientation (10 s), the gate was opened to allow the mouse to enter the darkroom. Once the mouse was inside the darkroom, the gate was closed and the 0.5-mV, 0.5-s punishment shocks (for three cycles, with an interval of 5 s) were executed simultaneously. The time period that the mouse stayed in the light room was recorded. Each mouse was exposed to the test on the first, second, third and seventh day of the experiment. Upon the punishment, the time that the mouse hesitated before entering the darkroom again was observed. Each test was performed for no longer than 180 s.

2.6. Active (shuttle) avoidance test

The mouse was placed in the shuttle box (35×17×20 cm, width×length×height, model E10-15, Coulbourn Instruments), which was equally divided into two compartments that were connected by a gate (7.5×6.5 cm). Each mouse was allowed a 10-s brief orientation period and then the gate, which was controlled by a computer, was opened. At the same time, the conditional stimulus (CS), which consisted of a tone, and red, yellow and green lights, was presented. Following this stimulus, an unconditional stimulus consisting of a 0.3-mA, 0.5-s scrambled foot shock was carried out if the mouse had not escaped by entering the other compartment during the CS. Each mouse was exposed to 5 CS per session and two sessions per day, and the total experiment was performed for four consecutive days. Success on the first day of test meant acquisition of successful avoidance, and other avoidance responses signified retention. The avoidance responses of the mice were recorded automatically.

2.7. Serum analysis

After sacrifice, serum from each animal was analyzed for changes in aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), total cholesterol (TC), creatinine and blood urea nitrogen (BUN) levels. Specimens were examined by the Union Clinical Laboratory (Taichung, Taiwan).

2.8. Oxidant status assays

Glutathione peroxidase (GPx) activity was determined spectrophotometrically according to previously described [24]. The following solutions were pipetted into a cuvette: 0.1 ml of homogenate and 0.8 ml of 100 mM potassium phosphate buffer, pH 7.0. The potassium phosphate buffer consisted of 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U/ml GSH reductase and 1 mM GSH. This mixture was preincubated for 5 min at 37°C. Thereafter, the overall reaction was initiated by adding 0.1 ml of 2.5 mM H_2O_2. Enzyme activity was calculated by the change of the absorbance value at 340 nm for 5 min. The corresponding non-enzymatic reaction rate was assayed by replacing the homogenate sample with potassium phosphate buffer. GPx activity was expressed as nanomoles of NADPH per minute per milligram of protein.

The glutathione reductase (GR) assay monitored the oxidation of NADPH consumed in the reduction of glutathione disulfide (GSSG) by measuring the change in absorbance at 340 nm, as previously described [25]. The following solutions were pipetted into a 1-cm spectrophotometric cuvette: 0.1 ml of homogenate and 0.9 ml of 0.10 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2 \bullet 6H_2O$, 50 mM GSSG and 0.1 mM NADPH. This mixture was preincubated for 5 min at 37°C. GR activity was calculated by the change of the absorbance value at 340 nm for 5 min; GR activity was expressed as nanomoles of NADPH per minute per milligram of protein.

Glutathione *S*-transferase (GST) activity was measured according to a previously described method [26], with a slight modification. One hundred microliters of homogenate was incubated with 880 μl of potassium phosphate buffer (100 mM, pH 6.5, containing 1 mM GSH) and mixed well with 20 μl of 1-chloro-2,4-dinitrobenzene (CDNB). GST activity was calculated by the change in the absorbance value at 340 nm for 3 min and was expressed as nanomoles of CDNB-GSH conjugate formatted per minute per milligram protein.

Catalase was measured according the method previously described [27]. The assay mixture consisted of 975 μl H_2O_2 (25 mM) and 25 μl of the tissue homogenates.

Changes in absorbance were recorded at 240 nm for 2 min. Catalase activity was expressed as nanomoles H_2O_2 per minute per microgram protein.

Isoprostane was examined with an ELISA commercial kit (Cayman Chemical Corporation, Ann Arbor, MI, USA) following the manufacturer's protocol.

2.9. Western blotting

The tissue homogenates were mixed with 5× sample buffer (8% SDS; 0.04% Coomassie blue R-250; 40% glycerol; 200 mM Tris, pH 6.8 and 10% 2-mercaptoethanol) and boiled for 10 min. Samples were electrophoresed in a 10% SDS-PAGE minigel and then transferred onto polyvinylidenedifluoride membranes (PVDF; Millipore Corp., Bedford, MA, USA) with transfer buffer (48 mM Tris; 39 mM glycine; 0.0037% SDS and 20% methanol) at 350 mA for 60 min. The membranes were blocked with 5% nonfat milk in PBS solution containing 0.1% Tween-20 (PBST) for 1 h. The membrane was immunoblotted, respectively, with primary antibodies of rabbit anti-rat Nrf2, anti-Aβ and anti-rat mitogen-activated protein kinase (MAPK) family in PBST solution containing 5% bovine serum albumin at 4°C overnight. After consecutive 30-min PBST washes, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 60 min at room temperature and then washed with PBST for 30 min. The final detection was performed using an enhanced chemiluminescence (ECL™ kit) Western blotting reagents (Amersham Pharmacia Biotech, NJ, USA).

2.10. Statistical analysis

All data are presented as means±S.D. The statistically significant differences were compared with the untreated groups and were calculated by one- or two-way analysis of variance (ANOVA). Statistically significant individual group means were then compared using Student's *t* test.

3. Results

3.1. Effect of mulberry on changes in body weight and relative organ weight

No significant ($P>.05$) difference was observed between SAMR1 (Group I) and SAMP8 (Group II) mice in terms of their initial body weights (Table 1). The body weights of mice in Group I increased by 3 g over the 3-month period of the experiment. In contrast, the body weights were almost constant in SAMP8 mice, and there was no significant difference ($P>.05$) between SAMP8 groups treated with or without ME. More importantly, the relative organ weight changes for the liver in Group II was significantly ($P<.05$) heavier than that of Group I, and treatment with the high ME dose (500 mg/kg body weight, Group V) decreased the liver tumidity that accompanies ageing. These data suggest that consumption of mulberry or BE did not influence the body and organ weights of mice; however, dietary supplementation with mulberry or BE did inhibit ageing-induced hepatomegaly.

3.2. Effect of mulberry on liver function

Table 2 shows serum biochemical parameters that are involved in liver and kidney function. The data revealed that the levels of ALT, TG and BUN in Group II were significantly ($P<.05$) higher than that of Group I. Consumption of mulberry or BE decreased the levels of AST, ALT, TG and TC. Furthermore, supplementation with ME did not influence the kidney function, as shown by the data for BUN and creatinine. The ageing-related liver injury was significantly suppressed by the treatment with ME.

Table 1
Body weight and relative organ weight of 6-month-old SAMP8 mice fed with or without ME for 12 weeks

Group	Body weight (g)			Relative organ weight (g/100 g bw)		
	0 month	2nd month	3rd month	Liver	Kidney	Spleen
I	29±1.2	31±2.2	32±2.2	5±0.2	1.6±0.19	0.36±0.05
II	29±0.9	30±0.4	30±1.9	6±0.3[a]	1.6±0.16	0.38±0.08
III	28±1.1	29±0.5	29±2.1	5±0.9	1.6±0.18	0.39±0.1
IV	28±0.7	29±0.7	29± 0.75	5±0.8	1.7±0.9	0.33±0.19
V	29±1.5	30±0.5	29±1.5	5±0.3[b]	1.6±0.2	0.32±0.09

Values are means±S.D and analyzed by one-way ANOVA. I, SAMR1 with normal diet; II, SAMP8 with normal diet; III, SAMP8 with BE (50 mg/kg bw); IV, SAMP8 with ME (100 mg/kg bw); V, SAMP8 with ME (500 mg/kg bw).
[a] Means with significant difference between Group I and II.
[b] Means with significant difference as compared to Group II at $P<.05$ ($n=6–8$).

Table 2
Serum biochemical values of 6-month-old SAMP8 mice fed with or without ME for 12 weeks

Group	I	II	III	IV	V
AST (U/l)	130±8.8	130±9.3	70±8.7[a]	80±13.3[a]	60±18.6[a]
ALT (U/l)	40±9.7	60±5.9[b]	40±10.1[a]	40±3.8[a]	30±9.3[a]
TG (mg/dl)	50±3.6	140±38.9[b]	70±22.9[a]	120±18.1	50±7.8[a]
TC (mg/dl)	85±0.1	100±14.4	80±9.5[a]	80±7.8[a]	90±11.9
BUN (mg/dl)	18±0.99	15±1.4[b]	15±1.4	20±5.7	18±2.2
Creatinine (mg/dl)	0.3±0.04	0.3±0.06	0.2±0.05[a]	0.3±0.05	0.2±0.06[a]

Values are means±S.D. and analyzed by one-way ANOVA. I, SAMR1 with normal diet; II, SAMP8 with normal diet; III, SAMP8 with BE (50 mg/kg bw); IV, SAMP8 with ME (100 mg/kg bw); V, SAMP8 with ME (500 mg/kg bw).
[a] Means with significant difference as compared to Group II at $P<.05$ ($n=6–8$).
[b] Means with significant difference between Group I and II.

3.3. Effect of mulberry on antioxidant capacity in liver and brain

As shown in Table 3, the antioxidant enzymes expressed in the liver and brain were examined. Activities of antioxidant enzymes, including GPx and catalase, in Group II were significantly less than that of Group I. Liver GPx and catalase were significantly ($P<.05$) promoted by treatment with mulberry or BE, and cerebral GST and catalase were also significantly elevated following these treatments. Moreover, significantly lower levels of isoprostane were observed in the liver and brain of mice that had been treated with high-dose ME. The data suggest that the antioxidant status of the SAMR1 group (Group I) was better than that of the SAMP8 group (Group II) and that the supplementation with ME reduced the lipid oxidation and promoted antioxidant capacity, thus improving antioxidant defense.

3.4. Effects of mulberry on cognitive ability

As shown in Table 4, the ME contained about 10% phenolic compounds, and about one third of these phenolic compounds were anthocyanins. Trolox equivalent antioxidant capacity demonstrated that the majority of the contents found in phenolic compounds were

Table 3
Oxidant status of 6-month-old SAMP8 mice fed with or without ME for 12 weeks

Group	I	II	III	IV	V
GPx (U/mg protein)					
Liver	130±23.8	40±15.4[a]	90±23.9[b]	50±21.2	90±14.5[b]
Brain	33±3.8	20±5.3[a]	30±3.9[b]	20±4.2	30±3.5[b]
GST (U/mg protein)					
Liver	50±12.5	30±9.4	40±5.2	40±6.7	50±8.9[b]
Brain	30±2.5	13±1.4[a]	24±2.2[b]	20±4.7[b]	22±3.9[b]
GRd (U/mg protein)					
Liver	2±0.3	2±0.2	2±0.2	2±0.2	2±0.41
Brain	0.08±0.033	0.04±0.019	0.098±0.029[b]	0.05±0.017	0.05±0.024
Catalase (U/mg protein)					
Liver	0.6±0.14	0.3±0.13[a]	0.2±0.02	0.3±0.07	0.5±0.02[b]
Brain	0.05 ±0.003	0.02 ±0.003[a]	0.04±0.002[b]	0.04±0.006[b]	0.05±0.009[b]
Isoprostane (mg/mg protein)					
Liver	6±2.8	4±1.6	3±0.4	3±1.3	2±0.5[b]
Brain	2±0.1	1.5±0.5	0.9±0.08[b]	0.7±0.06[b]	0.7±0.04[b]

Values are means±S.D. and analyzed by one-way ANOVA. I, SAMR1 with normal diet; II, SAMP8 with normal diet; III, SAMP8 with BE (50 mg/kg bw); IV, SAMP8 with ME (100 mg/kg bw); V, SAMP8 with ME (500 mg/kg bw).
[a] Means with significant difference between Group I and II.
[b] Means with significant difference compared to Group II at $P<.05$ ($n=6–8$).

Table 4
Antioxidant capacity and components of ME and BE

Antioxidant capacity	Mulberry	Blackcurrant
Total phenolics[a]	90±2.8	900±78
Total flavonoids[b]	50±3.4	376±28
Total anthocyanins[c]	30 ±2.5	260±11.8
TEAC[d]	0.46±0.017	4±0.19

Data are presented from three independent experiments.
[a] Total phenolic contents are presented as milligram of gallic acid per gram of extracts.
[b] Total flavonoid contents are presented as milligram of rutin per gram of extracts.
[c] Total anthocyanin contents are presented as milligram of kuromanin per gram of extracts. Kuromanin means cyaniding-3-*O*-glucoside.
[d] Trolox equivalent antioxidant capacity value is presented as millimole of trolox per gram of extracts.

attributable to the antioxidant capacity of ME. No statistically significant difference ($P>.05$) was observed between food intake profiles from the different animal groups (data not shown). Furthermore, a locomotion activity test was executed prior to memory evaluation, and these data suggested that ambulatory activity was similar between the groups (data not shown). The learning and recognition examinations were performed at the 11th week of the study. The results from avoidance trials showed that SAMR1 (Group I) had significantly superior cognition over SAMP8 (Group II) (Fig. 1). The passive avoidance time in Group I was significantly ($P<.05$) longer than the time in Group II. Although consumption of blackcurrant or ME improved the successful avoidance time of SAMP8 mice by Day 3, only Group V (500 mg/kg body weight, high dose) demonstrated superior memory compared to the other groups by Day 7. Furthermore, the successful avoidance times of SAMP8 mice supplemented with mulberry or BE were greater than those of SAMP8 mice with a normal diet (Fig. 2). These data revealed that SAMR1 mice had better memories than SAMP8 mice, suggesting that supplementation with ME helped to counteract the deficits of learning and recognition that accompany aging.

3.5. Effect of mulberry on MAPK expression

Figs. 3 and 4 show the effects of ME on the expression of the MAPK family in liver and brain samples, respectively, from SAM mice. In comparison to Group I, Group II showed significantly ($P<.05$) greater

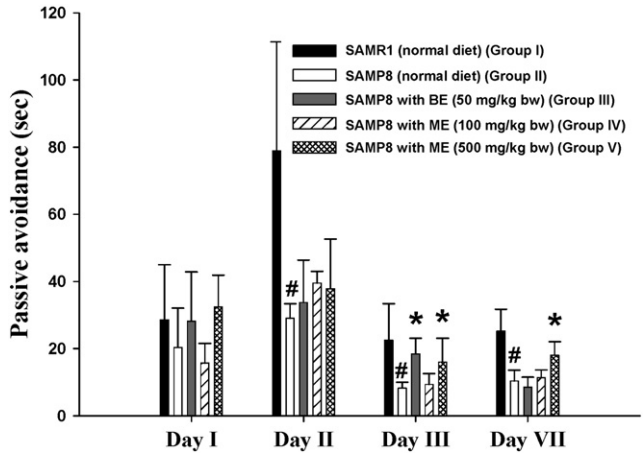

Fig. 1. Passive avoidance ability of 6-month-old SAMP8 mice fed with or without ME for 12 weeks. Avoidance tests were performed for three consecutive days as well as on the seventh day of the study. #Significant difference compared to group SAMR1 control. *Significant difference compared to group SAMP8 with normal diet at $P<.05$ ($n=6–8$).

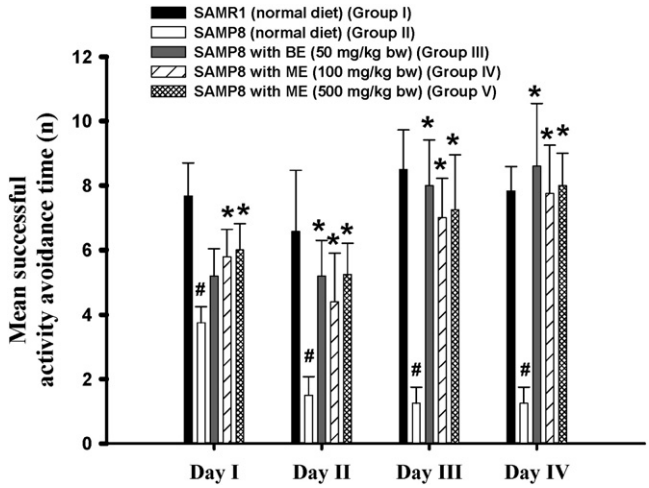

Fig. 2. Active avoidance ability of 6-month-old SAMP8 mice fed with or without ME for 12 weeks. Avoidance tests were performed 10 times per day for four consecutive days. #Significant difference compared to group SAMR1 control at $P<.05$. *Significant difference compared to group SAMP8 with normal diet at $P<.05$ ($n=6–8$).

expression of phosphorylated p38 and JNK in the liver, and decreased phosphorylated p38 and JNK expression was noted in mice that were fed with ME. Furthermore, significantly ($P<.05$) higher expression levels of phosphorylated ERK1/2 were also noted in the liver and brain in SAMP8 mice treated with the high-dose ME. These results suggest that supplementation with ME might influence signal transduction pathways by down-regulation of p38 and JNK in the liver and by up-regulation of ERK1/2 both in the brain and liver.

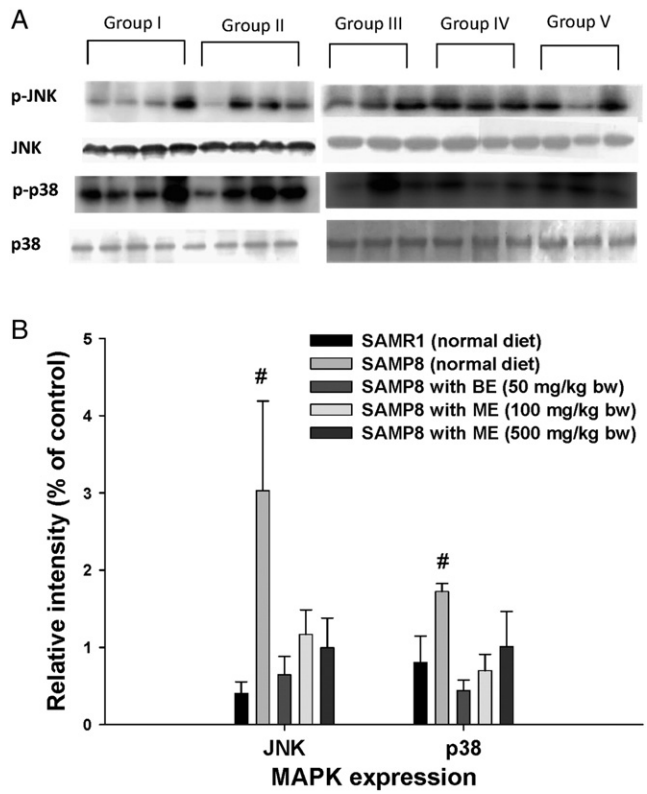

Fig. 3. MAPK expression in the liver of 6-month-old SAMP8 mice fed with or without ME for 12 weeks. (A) Western blotting profile and (B) Western blot results were quantified using a densitometer for five to six mice per group. #Significant difference compared to other groups at $P<.05$.

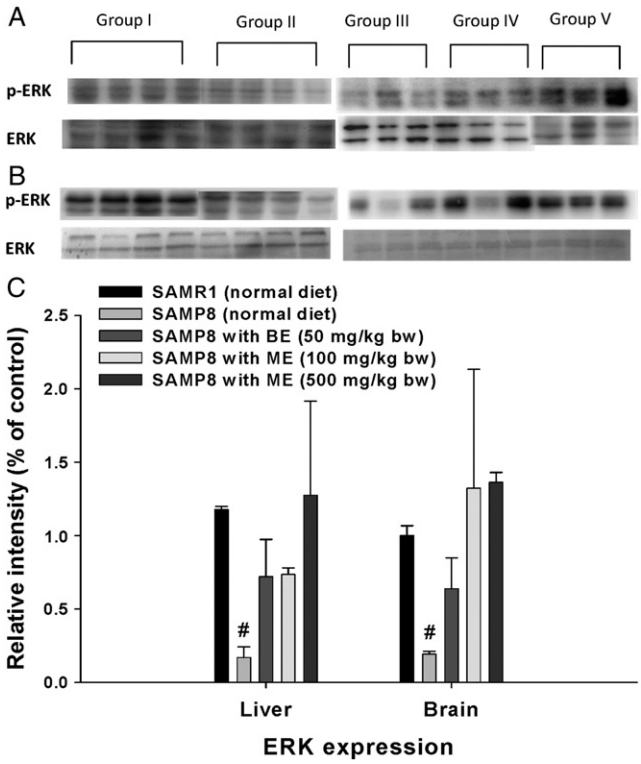

Fig. 4. MAPK expression in the brain of 6-month-old mice treated with or without ME for 12 weeks. Western blotting profile of liver (A) and brain (B) were presented. (C) The Western blot results were quantified using a densitometer. #Significant difference compared to other groups at $P<.05$.

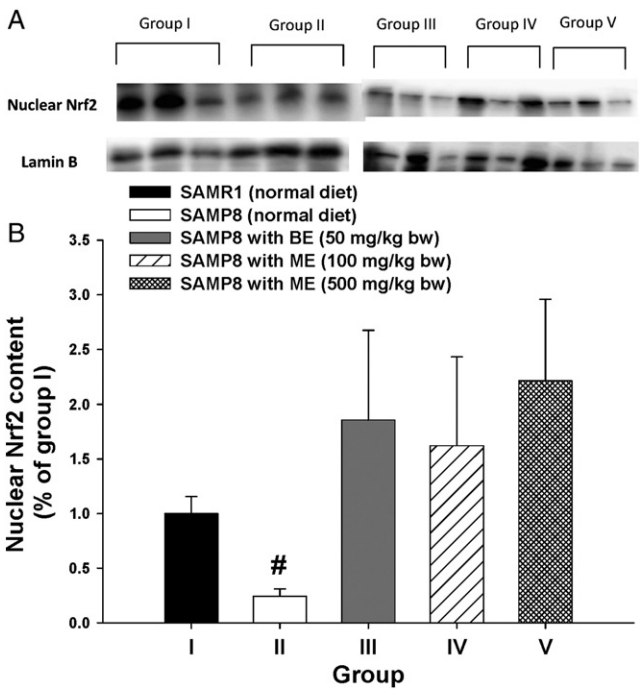

Fig. 5. Effects of ME supplementation on Nrf2 expression in the liver. SAMP8 mice were treated continuously administered blackcurrant or ME for 12 weeks. The nuclear fractions of the livers were separated and Western blot analysis was performed. (A) Western blotting profile and (B) the Western blot results were quantified using a densitometer. #Significant difference compared to other groups at $P<.05$.

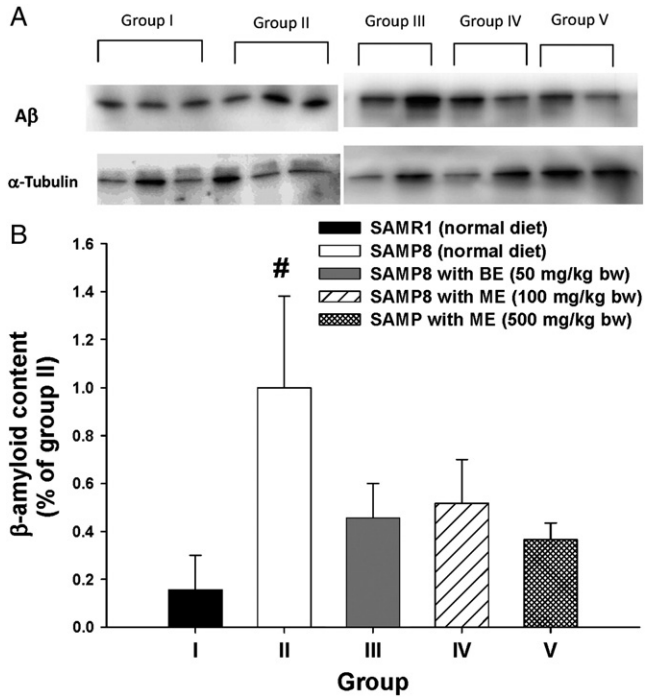

Fig. 6. Effects of ME supplementation on Aβ expression in the brain. SAMP8 mice were treated continuously administered BE or ME for 12 weeks. The hippocampal fraction of the brain was separated and Western blot analysis was performed. (A) Western blotting profile and (B) the Western blot results were quantified using a densitometer. #Significant difference compared to other groups at $P<.05$.

3.6. Effect of mulberry on Nrf2 translocation

The transcription factor Nrf2 plays an important role in modulating responses to oxidative stress. Fig. 5 shows the effects of ME on Nrf2 levels in the liver of SAM mice. In comparison to Group I, significantly ($P<.05$) lower nuclear Nrf2 was observed in Group II; however, the nuclear Nrf2 level was increased upon treatment with mulberry or BE. These data suggested that the consumption of mulberry or BE promoted Nrf2 expression and transcription factor translocation to the nucleus, thus increasing the expression of antioxidant enzymes.

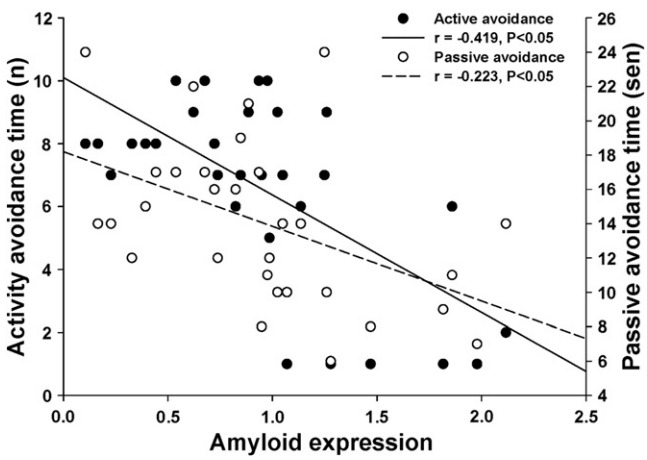

Fig. 7. Scatter plots of the relationship between cognitive performance and Aβ expression. Active and passive avoidance ability of 6-month-old SAMP8 mice fed with or without ME for 12 weeks.

3.7. Effect of mulberry on Aβ deposition is correlated with cognitive performance

The hippocampus, which is an area of the brain that is crucial for learning and memory, has also been established as a common location of pathologic Aβ accumulation. In this study, we used Western blot assays to examine whether treatment with ME decreased the accumulation of Aβ typically occurring with age. As shown in Fig. 6, a significantly ($P<.05$) higher amount of Aβ was observed in the brains of Group II mice than in Group I, and supplementation with ME was able to significantly reduce Aβ accumulation. Furthermore, regression analysis reported significant negative correlation between the cognitive performance and the expression of Aβ (Fig. 7). Aβ negatively significantly correlated with active avoidance response ($r=-0.419$, $P<.05$) and passive avoidance response ($r=-0.223$, $P<.05$) of tested mice. These data suggested that the observed improvement in age-related cognitive decline secondary to consumption of ME might be through a reduction in Aβ levels.

4. Discussion

Reactive oxygen species (ROS) are highly reactive molecules or intermediates that are continuously produced by all aerobic organisms, primarily as a consequence of aerobic respiration. Accumulation of ROS results in oxidative damage to important molecules such as proteins, lipids, and DNA, which may result in cellular dysfunction and mutations, thus promoting the development of disease.

Ageing is a physiologic process associated with many structural and functional changes in different organs and tissues, including the liver [28]. The liver plays an important role in metabolism and detoxification. Furthermore, it has been revealed in the literature that the majority of Phase II antioxidant and detoxification enzymes are regulated by the antioxidant response element (ARE)-Nrf2 pathway [29]. Suh et al. [30] have also demonstrated age-related declines in GSH levels and GSH biosynthetic capacity, thus further promoting oxidative damage as a consequence of ageing. Recently, we have discovered that varying levels of antioxidants in ageing rats might be related to alterations in the Nrf2 and MAPK signaling pathway [31]. On the other hand, age-related disorders in lipid metabolism are also likely to contribute to these effects [32]. An increase in oxidative damage and a decrease in the antioxidant capacity of the liver suggest that oxidative stress may have an impact on age-related liver disorders. In this study, we investigated whether anthocyanin-rich mulberry might have an effect on the antioxidant capacity and age-associated disorders observed in senescence-accelerated-prone mice (SAMP8), which is a strain of mice that has gained attention in gerontological dementia research due to its characteristic learning and memory deficits when compared to the SAMR strain, which shows normal aging [12].

Dietary supplementation of antioxidant-rich berries (e.g., blueberry, strawberry) can positively affect cognition and memory in the aged animal [33]. Cyanidin-3-*O*-glucopyranoside (Cy-3G), an anthocyanin that abundant in colorful vegetables and fruits, has recently been identified as neuroprotective phytochemicals. It decreases Aβ peptide-mediated cytotoxicity in SH-SY5Y neurocytes [34]. Cy-3G also could reduce cerebral ischemia and ageing-related neuronal deficits in animal model [35]. Dietary supplementation with anthocyanin-rich *Vaccinium myrtillis* L. suppresses stress-induced protein carbonyl formation and lipid oxidation in the brain [36]. Dietary supplementation with *Vaccinium* spp. (blueberry) for consecutive 12 weeks also shows enhancing effects on spatial working memory test in aged rats (21-month-old) through cAMP-response element-binding protein and brain-derived neurotrophic factor pathways [37]. Furthermore, blackcurrant, which is known to be rich in anthocyanins, is already used as food additive and has been shown to have health benefits that could enhance the antioxidant status in vivo [38]. Therefore, the blackcurrant-treated group is used as a positive control to verify the biological and physiological efficacy of anthocyanin-rich fruit in this study.

Six-month-old adult mice from SAMR1 and SAMP8 strains were randomly divided into five experimental groups. Table 1 demonstrates that there were no significant ($P>.05$) differences in body weight between the mice in the experimental groups at the beginning of the study. Three months later, the body weights continued to increase in the SAMR1 group but remained constant in the SAMP8 group. Given these data, it appears that ageing might influence activity levels and appetite. Furthermore, data from learning and memory tests showed that SAMP8 mice (Group II) had inferior cognitive ability compared to SAMR1 mice (Group I) (Figs. 1 and 2). Additionally, the passive avoidance times for Group II were significantly less than those for Group I, and the successful avoidance times for Group II were also lower than those for Group I. However, when consistently fed with mulberry or BE, the memory deterioration observed in the SAMP8 mice was greatly improved. These data suggested that consumption of mulberry could improve the learning and cognition capacities of mice in the setting of age-related dementia.

After sacrificing the mice, blood was collected and biochemical parameters in the serum were further examined. SAMP8 mice were found to have significantly ($P<.05$) higher ALT and TG levels than the SAMR1 control group. Following continuous treatment with blackcurrant or ME for 12 weeks, the AST, ALT, TG and TC levels in the SAMP8 mice were significantly reduced. It has been previously demonstrated that there is a significant correlation between age and serum ALT and AST activity [39,40]. Recently, it has been found that SAMP8 mice have hypertriglyceridemia and hepatic steatosis [41]. In our study, we observed similar findings with regards to liver dysfunction and went on to demonstrate that dietary supplementation with ME may suppress age-related hepatic disorders.

Increased attention has been given to induction of phase II enzymes as an important mechanism for chemoprevention against carcinogenesis, but there appear to be fewer studies on the correlation between antioxidant enzymes and ageing. In the present study, we examined antioxidant enzyme activity and oxidative status in the liver and brain to evaluate the antioxidant capacity of spontaneously ageing mice. As shown in Table 3, not all parameters followed the same trend; however, antioxidant enzyme expression in Group I (SAMR1 control) was greater than that in Group II (SAMP8 control). Additionally, administration of ME could be observed to reverse antioxidant enzymes expression. Furthermore, the measured levels of isoprotane, a derivative of lipid oxidation, suggested that consumption of ME also reduced oxidative stress-induced lipid oxidation. These phenomena may help to explain the relationship between the antioxidant content in ME and its observed physiological effects. As shown in Table 4, mulberry and BE were both rich in phenolics, and anthocyanin contents were approximately 30 and 260 mg/g in the respective extracts, with TEAC values showing that mulberry and blackcurrant have potential antioxidant capacities. We further examined the phytochemicals in ME using HPLC-DAD (diode array detector) and found that kuromanin (cyaniding-3-*O*-glucoside) and oenin (malvidin-3-*O*-glucoside) were two of the major anthocyanin component in ME, and that blackcurrant was rich in delphinidin-3-*O*-rutinoside (data not shown). The abundant antioxidants found in these fruits might explain their antioxidant capacities against free radical-induced cellular damage and may also promote an increase in the expression of antioxidant enzymes. It has been previously shown that dietary supplementation with antioxidants may augment the decreased antioxidant capacity commonly seen in SAMP8 mice [14], and it is important that the signaling mechanism responsible for this observation be examined in greater detail.

The MAPK family is a well-known group of enzymes that is involved in the regulation of cellular survival, proliferation and death [42,43]. The MAPK cascade includes proteins such as ERK, JNK and p38, which have been described to be involved in the activation of ARE [44]. Previously, we also suggested that the expression of MAPK and Nrf2 were significantly distinct from rats with different age [31]. The interactions of flavonoids within neuronal signaling pathways are suggested through modulating phosphatidylinositol-3 kinase (PI3K)/Akt, protein kinase C and other signaling cascades [45,46]. Herein we focused on the expression of downstream signaling cascades that modulate both antioxidant capacity and neurotransduction. In our study we found that the hepatic levels of phospho-p38 and phospho-JNK in SAMP8 control mice were significantly ($P<.05$) higher than those in SAMR1 mice and that the administration of ME reduced the activation of p38 and JNK (Fig. 3). Furthermore, as compared with SAMR1 mice, lower expression of cerebral and hepatic phospho-ERK were observed in SAMP8 control mice, and the activated ERK levels were also increased upon dietary supplementation with ME (Fig. 4). A previously published study showed that the relationship between ageing and MAPK family expression was subtle and inconsistent [47]. However, in the present study, we demonstrated that up-regulation of JNK and p38 and down-regulation of ERK might be involved in the ageing process, and that dietary supplementation with antioxidants such as mulberry may help modulate the expression of these signal transduction molecules.

MAPKs are key regulators in regulating the translocation and activation of Nrf2 [48]. Since the expression of MAPKs in SAMR1 and SAMP8 mice suggest that ageing might play a role in the regulation of Nrf2 activation, we decided to further examine nuclear Nrf2 levels to confirm this causal relationship. We observed that the concentration of nuclear Nrf2 in SAMP8 controls was significantly less than that seen in SAMR1 controls (Fig. 5). Furthermore, consumption of ME was demonstrated to significantly promote the activation and translocation of Nrf2 into the nucleus. These data might help to explain how chemopreventive compounds such as anthocyanins modulate the expression of MAPKs and influence the activation of Nrf2. Similarly, it has been reported that down-regulated Nrf2 expression is accompanied by ageing [49]. These data suggested that ageing and oxidative stress might be the major determinants that regulate Nrf2 representation.

Finally, we examined the accumulation of Aβ in the hippocampus of the mice to demonstrate that the positive effects of mulberry on learning and cognition are potentially mediated by the inhibition of Aβ formation. The data obtained showed that the SAMP8 controls contained significantly higher Aβ accumulation when compared to SAMR1 controls and that supplementation with ME was able to reduce the Aβ deposition (Fig. 6). Oxidative stress is strongly implicated in the progressive decline of cognition associated with aging and neurodegenerative disorders [50]. It is suggested that antioxidants such as α-tocopherol and phenolics found in *Toona sinensis* Roemor could lessen the memory deterioration and cognition deficient, possibly much closely involved in their antioxidant capacity [14,51]. Much importantly, naturally occurring flavonoids, particularly for anthocyanins, are the new inhibitors against β-amyloid aggregation, suggesting that phytochemicals with the particular C_6-linkers-C_6 structure could be potent inhibitors [52]. Furthermore, Aβ-stimulated decrease in antioxidant capacity is positively correlated with spatial cognitive learning impairment of rats; however, administration of green tea catechins significantly increases antioxidant activity of plasma and consequently prevents from Aβ-induced cognitive deficits [53]. In the present study, we observed that isoprostane, which is a hallmark of lipid oxidation, showed a high tendency of positive correlation with Aβ content in hippocampus of SAM mice ($r=.277$, $P=.057$, data not shown), and the cognitive performance was significantly correlated with Aβ expression negatively (Fig. 7). Thus, the antioxidant effects and Aβ-inhibiting potential of anthocyanin-rich ME might be the key causations that improve cognitive performance in SAMP8.

In conclusion, the current study illustrated that administration of ME that are rich in anthocyanins promoted age-dependent antioxidant protection and reduced oxidative stress-induced damage, as shown by the hepatoprotective effect of mulberry in a spontaneously aging mouse model. Antioxidant enzyme expression was shown to be modulated through the up-regulation of ERK and down-regulation of JNK and p38, thus promoting the activation of Nrf2. Furthermore, supplementation with ME was observed to inhibit the accumulation of Aβ and improve learning and cognitive ability. Overall, mulberry is able to protect against ageing-induced oxidative damage and cognitive deficits; however, the physiological and molecular mechanisms underlying this conclusion warrant further investigation.

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