Original Article

Cyanidin-3-O-galactoside and Blueberry Extracts Supplementation Improves Spatial Memory and Regulates Hippocampal ERK Expression in Senescence-accelerated Mice*

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Abstract

Objective To investigate whether the antioxidation and the regulation on the Extracellular Regulated Protein Kinases (ERK) signaling pathway are involved in the protective effects of blueberry on central nervous system.

Methods 30 Senescence-accelerated mice prone 8 (SAMP8) mice were divided into three groups and treated with normal diet, blueberry extracts (200 mg/kg·bw/day) and cyaniding-3-O-galactoside (Cy-3-GAL) (50 mg/kg·bw/day) from blueberry for 8 weeks. 10 SAMR1 mice were set as control group. The capacity of spatial memory was assessed by Passive avoidance task and Morris water maze. Histological analyses on hippocampus were completed. Malondialdehyde (MDA) levels, Superoxide Dismutase (SOD) activity and the expression of ERK were detected.

Results Both Cy-3-GAL and blueberry extracts were shown effective functions to relieve cellular injury, improve hippocampal neurons survival and inhibit the pyramidal cell layer damage. Cy-3-GAL and blueberry extracts also increased SOD activity and reduced MDA content in brain tissues and plasma, and increased hippocampal phosphorylated ERK (p-ERK) expression in SAMP8 mice. Further more, the passive avoidance task test showed that both the latency time and the number of errors were improved by Cy-3-GAL treatment, and the Morris Water Maze test showed significant decreases of latency were detected by Cy-3-GAL and blueberry extracts treatment on day 4.

Conclusion Blueberry extracts may reverse the declines of cognitive and behavioral function in the ageing process through several pathways, including enhancing the capacity of antioxidation, altering stress signaling. Cy-3-GAL may be an important active ingredient for these biological effects.

Key words: Blueberry; Senescence-accelerated mice; Cognition; Cyanidin-3-O-galactoside; ERK; Oxidation

Biomed Environ Sci, 2014; 27(3): 186-196 *doi: 10.3967/bes2014.007* *ISSN: 0895-3988*

www.besjournal.com (full text) *CN: 11-2816/Q*

*This research was supported by the grant of National Natural Science Foundation of Tianjin (09JCYBJC12900).

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Received: January 5, 2013; Accepted: April 30, 2013

INTRODUCTION

China will face a dramatic transition from a young to an aged society in the coming decades. Currently more than 8.87% of the Chinese people are 65 years old, and the proportion will increase to 23% by 2050[1]. As the society becoming older, the prevalence of age-related diseases, such as Alzheimer's disease (AD) and Parkinson's disease increase rapidly[2-4]. Data showed that the prevalence of dementia was 4.4%-10% in elderly people, and 60%-80% cases of dementia aggravated as AD patients[5-6]. These diseases cause heavy social and economic burdens. Therefore, it is crucial to devise strategies to limited the harm of age-related diseases and improve the life quality of the elderly.

It is well known that oxidative stress (OS) plays an important role in mediating the deleterious effects of aging[7-8]. Plenty of evidence suggests that OS and inflammation may be a major factor in the generation and development of AD[9-10]. Mammals become less tolerant to stress with old age, both OS and inflammation[11-12] are contributing factors to the behavioral decrements seeing in aging. Evidences showed that plant extracts, such as mulberry, blueberry and grape, contain antioxidants which are able to induce the antioxidant defense system and improve memory deterioration in aging animals[13-14].

Mitogen-activated protein kinase ERK is required for memory formation, aging and neurodegeneration. Joseph showed that the increased susceptibility of aged neurons may be due to the activation of ERK and cyclic-AMP response element binding protein (CREB) pathway[15]. Other researches also showed that under OS or inflammatory conditions, some plants extracts such as polyphenols from red wine, tea or ginkgo biloba affected the elements activity of ERK pathway[13,16-18]. Similarly, the blueberry extract may be involved in the regulation of ERK pathway during protecting brain against aging.

Studies shown that dietary supplementation with blueberry extracts could reverse age-related behavioral deficits in aged rats[19-20]. Blueberry extracts supplementation was shown to improve the performance in motor function test. Some other studies replicated the finding and further showed the improvement of motor learning in the blueberry supplemented animals[21-22]. However, the active ingredients in the complex compounds playing dominant role in preventing motor and cognitive deficits in aging remains unknown. Since the blueberry has profound beneficial effects on several deficits in behavior for the senescent animals, it is very interest to assess the antioxidant activity of blueberry extracts *in vivo*, determining whether blueberry extracts have any effects on alteration in cellular signaling and identifying the active ingredients in the blueberry extracts.

Published data showed that senescence-accelerated mouse (SAM), as a model of aging, display many features that are occurred early in the pathogenesis of AD[23]. Thus, the hypothesis of this study was that the neural protective effects of blueberry may include antioxidative activity and the regulation of ERK signal pathway. To test this hypothesis, we investigated the protective effect of blueberry extracts on the cognitive and behavioral function in aging mice by investigating the actions of blueberry extracts and Cy-3-GAL on the abilities of learning and memory using Morris water maze and passive avoidance task; examining the histological change in hippocampus by morphological method; measuring the alternations in the oxidative/ antioxidative system by chemical colorimetry, and examining the expression of ERK by western blotting.

MATERIALS AND METHODS

Materials

Blueberry extracts (*Vaccinium uliginosum* L) were purchased from Daxinganling Lingongberry Organic Foodstuffs Co. Ltd., Harbin, Heilongjiang Province, China. Fresh blueberry were crushed and extracted four times with fresh ethanol (75%) at room temperature for 96 h. The four ethanolic phases were recovered, pooled, dried at 40 °C on a rotary evaporator and lyophilized. The content of anthocyanidins in the blueberry extracts was 25%. Blueberry monomer Cy-3-GAL (89.8%) was prepared and identified as previous described[24].

Animals and Experimental Protocols

Ten age-matched SAM resistance/1 (SAMR1) mice and thirty SAMP8 mice (3 months old) were provided by Animal Central Lab of the First Teaching Hospital of Tianjin University of Traditional Chinese Medicine. They were housed in 4 groups (5 per cage) under a 12:12 h light-dark cycle at a constant temperature (22-25 °C) and humidity (60%-70%), and were provided with *ad libitum* access to standard laboratory food (AIN-93 diet) and water. All mice were acclimated in this environment for 1 week prior to initiating the feeding protocols (Figure 1).

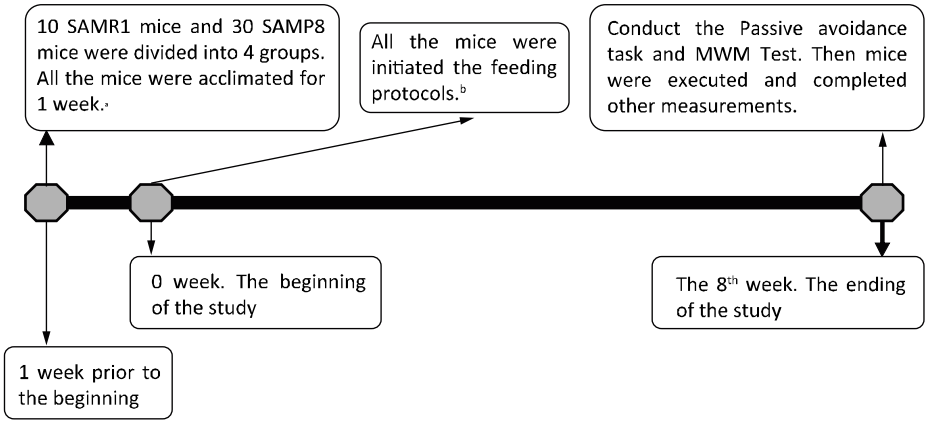

Figure 1. Experimental design. [a]: Ten SAMR1 mice were set as group control (R1). Thirty SAMP8 mice were randomly divided into group model (P8A), group model with blueberry extract treatment (P8B) and group model with Cy-3-GAL treatment (P8C). [b]: Group P8B was administered blueberry extracts at a dose of 200 mg/kg·bw/d. Group P8C was administered Cy-3-GAL at a dose of 50 mg/kg·bw/d. The control groups (R1, P8A) received equal volume of saline water (0.9%).

After the interval of acclimation, 10 SAMR1 mice were set as homologous control (R1), and 30 SAMP8 mice were assigned randomly to group of SAMP8 model (P8A), the blueberry extracts group (200 mg/kg·bw/d, P8B) and the Cy-3-GAL group (50 mg/kg·bw/d, P8C)[20,25]. The blueberry extracts (P8B) and Cy-3-GAL (P8C) were administered by daily gavage in a dose of 5 mL/kg body weight, and the control groups (R1, P8A) received the equal volume of saline water (0.9%). Mice in each group were treated once daily in the morning with Cy-3-GAL, blueberry extracts or sterilized water, respectively, for 8 weeks[26]. And this research was approved by the Animals Ethics Committee of Institute of Health and Environmental Medicine of Academy of Military Medical Sciences.

Passive Avoidance Task

At the end of treatment, the mice's learning and memory capacity was tested by passive avoidance task and Morris Water Maze (MWM). The passive avoidance box consists of two-grid floor compartments separated by a guillotine door (3 cm in diameter). The light compartment was illuminated with a lamp fixed to the lid of the box. The dark compartment was made of black Plexiglas. All the mice were habituated to the light and dark chamber for 3 min. For the acquisition trial, the mice were placed in the illuminated chamber opposite the guillotine door and allowed to move freely. When all four paws were on the grid floor of the dark compartment, a scrambled constant current foot shock (50 V, 50 Hz) was delivered to the grid. Every trial lasted for 5 min. 24 h after the acquisition trial, the mice were placed into the light compartment again for the retention trial, but without a foot shock. The latency time was recorded to a maximum of 300 s. The number of times the mice moved into the dark chamber, and latency to step into the dark chamber within 5 min were recorded automatically by computer. If a mouse did not enter the dark chamber within 300 s, the latency was recorded as the cut-off time of 300 s[27-28].

Morris Water Maze (MWM) Test

The MWM was used to monitor spatial learning and memory in rodents[29]. The MWM consisted of a circular pool filled with mixture of water and ink kept at 23-25 °C. A video camera, mounted in the center above the water maze, provided a picture of the pool on a TV monitor (Because the white mice could not readily be seen on the monitor against the background of the water tank, a black background was placed on the floor of the pool, and the escape platform was covered by black cloth to increase the animal-background contrast). A small round Perspex escape platform was placed at a fixed position in the centre of one quadrant and was hidden 1 cm beneath the water surface[30]. The MWM test consisted of acquisition trial and probe trial. Briefly, each mouse was tested 2 daily sessions on 4 successive days during the acquisition phase[31]. A session consisted of 4 trials, in which a mouse was started once from each of 4 start position. The order

of start positions was randomized[32]. At each session's beginning, the mouse was placed on the platform for 10 s. A trial began with removing the mouse and placing it in the water facing the maze wall. It was terminated when the mouse had escaped onto the platform or when 60 s had elapsed, whichever event occurred first[33-34]. The latency before reaching the platform was recorded (Ethovision 3.1). If the mouse failed to climb onto the platform within 60 s, the trial was stopped and the mouse was removed from the water and placed upon the platform for 10 s. The escape latency (the time taken to escape onto the submerged platform) was measured in seconds[34].

After the fourth trial of the eighth daily session, an additional trial was given as a probe trial. In the probe trial, the platform was removed from the pool. All mice started from the same start position, opposite to the quadrant where the escape platform had been positioned during acquisition. All of the mice swam 60 s in the absence of the training platform. The probe test trial was analyzed by measuring the time spent in the quadrant in which the platform placed and the times crossing the platform area.

Histological Analyses

The mice were executed after behavioral test, the brains were removed, bisected sagittally. One half of the brain was rapidly frozen, and the other half was immersed in 4% buffer paraformmaldehyde for 48 h, cut in 2 mm thick coronal sections, processed, and embedded in paraffine. For each blocks, 6 serial sections of 5 μm thick were made. The serial sections were stained with hematoxylin and eosin for the observation of the cellular morphology of hippocampus[35].

The average cell number in each hippocampal CA1 and CA3 sector was derived from ten view fields under a 400× microscope magnification lens (Olympus BX40). Hippocampal CA1 and CA3 pyramidal cell counts were derived using systematic random sampling techniques. Briefly, the involved selecting sections at a regular interval along the entire hippocampus after the first section had been selected at random from the first interval[36-37]. Neurons were counted using high-definition color medical image analysis software (HMIAS2000).

Measurements of MDA Levels and SOD Activity

The MDA level in plasma (quantified as nmol/mL) and brain tissues (nmol/mg·protien) were analyzed by the thiobarbituric acid-reactive substances (TBARS) assay kits, and the SOD activity in plasma (quantified as U/mL) and brain (U/mg protein) were determined by xanthine oxidase kits, both of which were purchased from Nanjing Jiancheng Bioengineering Institute, China. The assays were performed according to the commercial kit's procedures.

Western Immunoblotting

Total protein was extracted from hippocampal tissue using protein lysis kit (Beyotime, China). After protein quantitation, each sample was diluted with sample buffer (5×) and then boiled for 5 min. Protein samples were separated by SDS-PAGE on a 12% polyacrylamide resolving gel and transferred to a PVDF membrane (Millipore, USA). The membrane was blocked with 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST) overnight at 4 °C, followed by incubation with rabbit anti-p-ERK1/2 and total ERK antibodies, (1:1000 dilution, Cell Signaling, USA) for overnight at 4 °C and then an additional incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000 dilution, Santa Cruz, USA) for 1 h at room temperature. Protein bands were visualized on a Kodak film using the ECL kit (Santa Cruz, USA) and the film was subjected to Image Master^{R} VDS (Pharmacia, Sweden). The density of protein p-ERK1/2 bands were quantified by Tatallab software/Quantity One software (Bio-Rad, USA) and normalized against the density of total ERK[38].

Statistical Analyses

Data of continuous variables are presented as means±SD. Student *t*-test was used to detect the group effects between group R1 and P8A. One-way analysis of variance (One-way *ANOVA*) followed by *LSD* and *S-N-K* post hoc test were used to test for differences among the groups of SAMP8 mice. The data on the count of apoptotic neurons is shown as medians and quartiles and analyzed by rank sum test. Statistics were performed using SPSS 15.0. A value of $P<0.05$ was considered to be significant.

RESULTS

Passive Avoidance Tasks

The retention trial of the passive avoidance task for the latency into the dark chamber differed between group R1 and P8A ($t=2.50$, $P<0.05$). The

number of errors was increased although not significantly. The difference suggested the retention deficits in P8A mice. One-way ANOVAs showed that both Cy-3-GAL (P8C) and blueberry extracts (P8B) prolonged the latency time (F=51.61, P=0.000) and decreased the number of errors (F=13.91, P=0.000). The pairwise comparisons, however, showed that only the Cy-3-GAL affected the retention significantly (q=14.14, P<0.05) and decreased the number of errors (q=7.41, P<0.05) (Figures 2 and 3).

Morris Water Maze Test

The result for the acquisition sessions are depicted in Figure 4. All mice improved their performances during the four learning sessions. Compared to animals in group R1, the escape latency of P8A was significantly longer on day 3 and 4 (P<0.05). Mice from group P8B and P8C, which were fed Cy-3-GAL and blueberry extract, respectively showed a trend toward shorter latency. A significant decrease in latency was detected on day 4 in group P8B and P8C (F=3.61, P<0.05), although the differences in previous days were not significant.

In the probe test, the numbers of platform crossing and time spent in quadrants (platform quadrant) were analyzed. An apparent quadrant effect between group R1 and P8A was found, although, both the decreases in numbers of platform

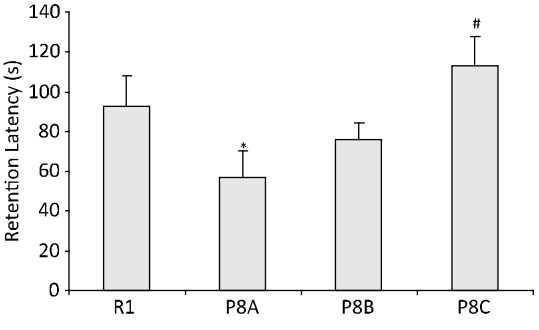

Figure 2. The retention latency changes of mice were assessed in passive avoidance test. Group R1 was normal control. Group P8A was model which treated with saline water, same as control. Group P8B and P8C were administered with blueberry extracts and Cy-3-GAL for 8 weeks, respectively. The differences between group R1 and P8A were analyzed by t test. *: P8A *vs* R1, P<0.05. The differences among groups of SAMP8 mice were analyzed by one way *ANOVA* and multiple range test was by *LSD* and *S-N-K* post hoc test. $^{\#}$: *vs* P8A, P<0.05. Values are means±SD, n=10.

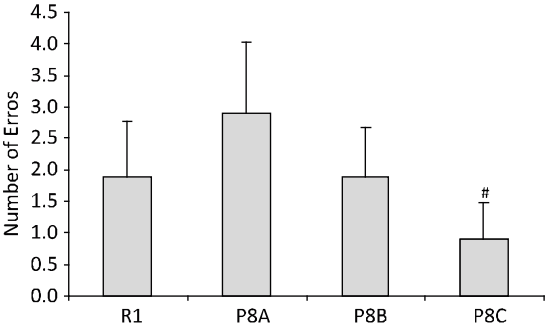

Figure 3. The improvements of the number of errors were measured in passive avoidance test. The differences between group R1 and P8A were analyzed by t test. The differences among groups of SAMP8 mice were analyzed by one way *ANOVA* and multiple range test was by *LSD* and *S-N-K* post hoc test. $^{\#}$: vs P8A, P<0.05. Values are means±SD, n=10. Data showed P8A emerged more errors than R1, however, t test did not detect the difference because of the big variance. The number of errors in SAM mice decreased with the blueberry extracts supplementation (P8B) and occurred a further decline with the Cy-3-GAL supplementation.

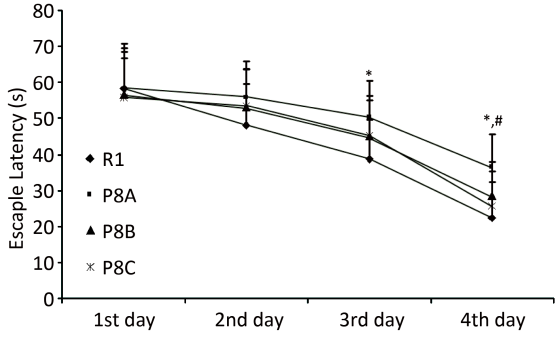

Figure 4. Effects of blueberry extracts or Cy-3-GAL supplementation on the escape latency among four groups in 4-day acquisiton sessions of MWM test. The learning capacity and memory were assessed according the escape latency. Generally, the normal animals will spend less and less time to reach the platform as the training last. The animals with defective learning capacity will spend more time in the water. *: P8A *vs* R1 by t test, P<0.05. $^{\#}$: *vs* P8A, P<0.05, the differences among groups of SAMP8 mice were analyzed by one way *ANOVA* and multiple range test was by *LSD* and *S-N-K* post hoc test. Values are means±SD, n=10. The data of escape latency every single day was analyzed. There was no statistical difference between R1 and P8A and among the three groups SAMP8 mice in the 1-2 d training. Compared with R1, the latency of P8A prolonged in 3-4 d (P<0.05). The latency of P8C shortened sharply in 4 d.

crossing and time spent in quadrants were not significant because of the scattering data (Figure 5). There were an improvement of time spent in quadrants in the group P8B (F=13.71, P<0.05), however, there was no significant difference in the platform crossing number among the SAMP8 mice (P>0.05) (Figure 6).

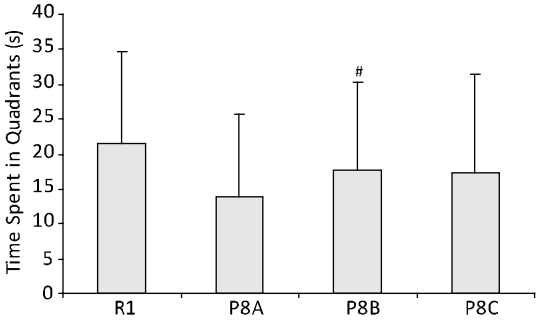

Figure 5. The effect of blueberry extracts or Cy-3-GAL supplementation on the time spent in platform quadrant was examined in probe test of MWM. #: *vs* P8A, P<0.05. The differences between group R1 and P8A were analyzed by *t* test. The differences among groups of SAMP8 mice were analyzed by one way *ANOVA* and multiple range test was by *LSD* and *S-N-K* post hoc test. Values are means±SD, n=10. Compared with group R1, mice in group P8A spent less time in the target quadrant. However, the difference was not significant because of the big variance. Both of the two treated groups (P8B and P8C) spent more time in the quadrant although only group P8B prolonged significantly.

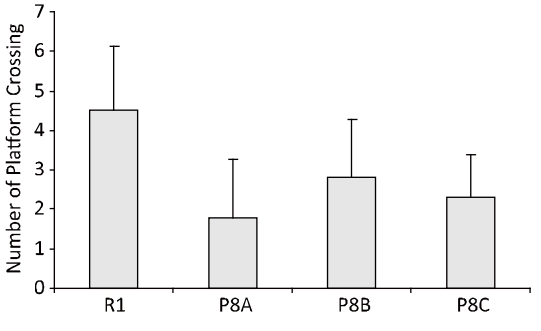

Figure 6. Effect of blueberry extracts or Cy-3-GAL feeding on the numbers of platform crossing were analyzed in probe test of MWM. There was no significant difference, though it had an obvious reduction in group P8A compared with R1. A relatively big variation in the data may cause the differences insignificant. Values are means±SD of measurements from 10 mice each group.

Histological Analyses and Hippocampal Neuron Number Counting

HE staining showed robust cellular injuries in pyramidal cell layer regions CA1 and CA3 in group P8A mice. Cellular loss in the dorsal regions of CA1 and CA3 appeared to be focal, primarily affecting the region of pyramidal cell layer. The pyramidal cells underwent pyknosis, apoptosis, and the pyramidal cell layer is loosely packed. Interestingly, much more karyopyknosis fragments were detected throughout the hippocampus CA1 region and these pyknosis fragments decreased in size to a greater extent than the nuclei of CA3. As shown in Figure 7 in group P8B and P8C, the features of neurons are alleviated apparently and approximate to group R1 in pyramidal cell layer regions CA1 (Figure 7A) and CA3 (Figure 7B), with less cellular pyknosis and apoptotic bodies. The result indicated an 8-week Cy-3-GAL or blueberry extracts supplementation significantly affected the morphological feature and survival of hippocampal neurons. The effects on improving hippocampal morphological features were more intensive with blueberry extracts supplementation.

As indicated in Table 1, significant CA1 and CA3 cell loss occurred in group P8A when compared to the control mice (P<0.01). The CA1 and CA3 apoptotic neurons were significantly decreased in both group p8b and P8C (P<0.01).

Table 1. The Counts of Apoptotic and Alive Pyramidal Neurons in CA1 and CA3 Area of Hippocampus [Median (interquartile range), n=10]

Groups	CA1[a]	CA3
R1	1/43.5 (1/45.5-1/38.5)	2.5/59.5 (2/58-3/57)
P8A	13.5/41 (14/39-19.5/34)*	23.5/59 (18.5/52.5-24.5/57.5)*
P8B	2.5/36.5 (2/44-3.5/36)#	3/43.5 (3/57.5-4/42.5)#
P8C	5/44 (2.5/42.5-6/35)#	3/41 (3/50-4.5/42)#

Note. [a]: Data are presented as fraction. The numerators in the fractions mean the numbers of the apoptotic neurons. The denominators in the fractions are the numbers of the alive hippocampal CA1 and CA3 pyramidal neuron counts. All the numbers were counted based on the HE staining images (400×). *: The significantly differences of the hippocampal neuron counts between group R1 and P8A were detected by Mann-Whitney test (P<0.01). The differences among group P8A, P8B, and P8C on the apoptotic and alive neuron number were analyzed by Kruskal-Wallis test. #: The hippocampal neuron counts in this group was significantly different from group P8A (P<0.01).

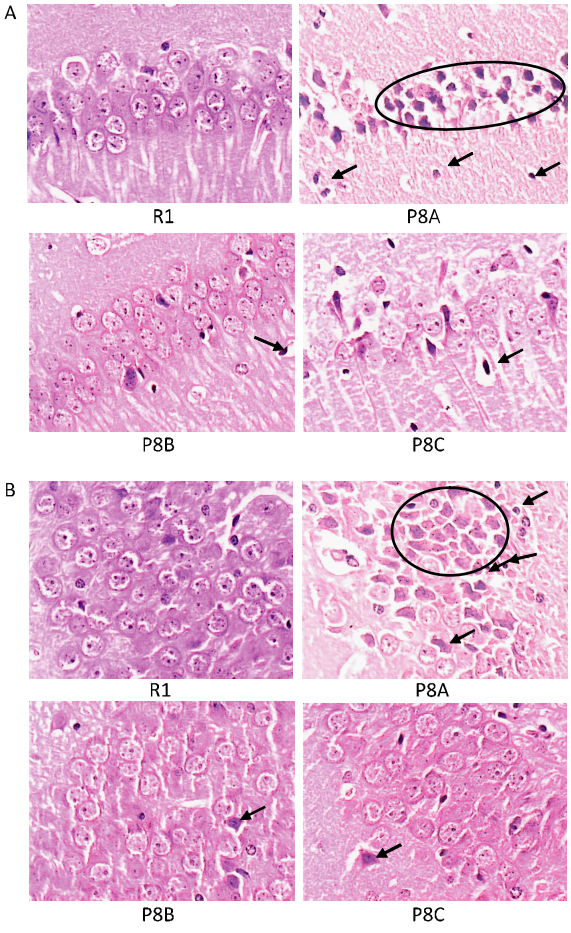

Figure 7. (A&B) A: Histological section of CA1 hippocampus (HE, 400×). The representative CA1 pyramidal neuron from a fixed tissue section of the mouse hippocampus was stained with H&E. (R1) a normal photo micrograph of CA1 pyramidal neuron. (P8A) the lesion in the model hippocampus was more extensive and serious compared with the treated groups. Many nuclei have become pyknotic (the black circle noted) and even occurred karorrhexis and apoptosis body (the black arrow noted). (P8B, P8C) lesions in the two groups treated with blueberry extracts or monomer were relieved significantly and approached to the morphological character of normal control. B: Histological section of CA3 hippocampus from SAM mice treated with Blueberry (HE, 400×). (P8A) it appeared remarkable pyknoticnucleus and apoptosis bodies scattered over the CA3 pyramidal cell layer (arrow). (P8B, P8C) most of the pyramidal neuron presented normal profile as control (R1).

MDA Levels and SOD Activity

Results of SOD and MDA measurements were shown in Figure 8. The MDA contents in brain increased significantly when compared with R1 (t=3.92, P<0.05), while there was no significant difference in plasma MDA between group R1 and P8A. MDA contents in both plasma (F=47.69, P<0.01) and brain (F=26.77, P<0.01) were significantly reduced by blueberry extracts (plasma: q=13.20, P<0.05; brain: q=8.54, P<0.01) or Cy-3-GAL treatments (plasma: q=10.12, P<0.01; brain: q=9.33, P<0.01) (Figure 8A). Compared with R1, SOD activity in plasma in P8A group were significantly decreased (t=2.16, P<0.05), however, the decrease in SOD activity in brain was not significant in group P8A. An opposite variation of the plasma and brain SOD activities to MDA was detected among the treated groups. SOD activities in plasma (F=14.96, P<0.01) and brain (F=26.77, P<0.01) were significantly increased by blueberry extracts (plasma: t=5.45, P<0.05; brain: t=8.54, P<0.01) or Cy-3-GAL treatments (plasma: t=7.48, P<0.01; brain: t=9.33, P<0.01) (Figure 8B). There were no differences in MDA content and SOD activity in plasma and brain between blueberry extracts and Cy-3-GAL treatments.

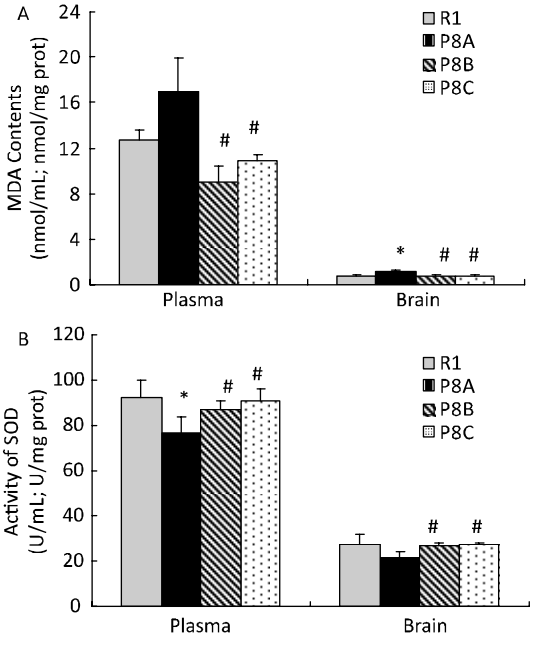

Figure 8. MDA contents (A) and activity of SOD (B) in plasma and brain tissue. Two independent samples were analyzed by t test. *: P8A *vs* R1, P<0.05. $^{\#}$: *vs* P8A, P<0.05, the differences among groups of SAMP8 mice were analyzed by one way *ANOVA* and multiple range test was by *LSD* and *S-N-K* post hoc test. Data points represent means±SD, n=10.

The MDA contents of brain in P8A increased significantly, compared with R1. Both MDA contents of plasma and brain were significantly reduced by blueberry extracts or Cy-3-GAL treatments (P<0.05). Compared with R1, SOD activity in plasma were decreased significantly (P<0.05), however, the decrease of SOD activity in brain was not significant in group P8A. Activities of SOD in plasma and brain were significantly increased by blueberry extracts or Cy-3-GAL treatments (P<0.05). There were no differences in MDA content and SOD activity of plasma and brain between blueberry extracts and Cy-3-GAL treatments.

Expression of P-ERK 1/2

Normalized densities of p-ERK 1/2 were quantified, and the protein expression in group R1 was standardized as 1. As shown in Figure 9 (A&B), the activity of p-ERK 1/2 decreased significantly in the SAMP8 mice (t=5.03, P<0.05). Both Cy-3-GAL and blueberry extracts supplementation enhanced phosphorylation of hippocampal ERK in SAMP8 mice (F=34.15, P<0.01). No difference in pERK levels was seen between group P8B and P8C.

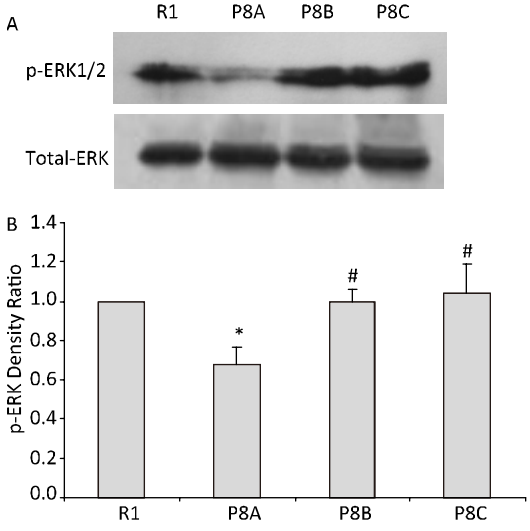

Figure 9. (A&B) (A) ERK phosphorylation in hippocampus was probed for levels of p-ERK1/2 and total ERK by Western immunoblotting. Representative immunoblots showing ERK levels in R1 mice, P8A, P8B supplemented with blueberry extracts diet and P8C mice with Cy-3-GAL are presented. (B) Levels of p-ERK1/2 and total ERK were measured in the same brain lysates. Band intensities for p-ERK1/2 and total ERK were determined by densitometric analysis using Bio-Rad Quantity One 1-D Analysis. It indicates a significant decrease in p-ERK (Ser133) in SAMP8A mice relative to SAMPR1 mice. While the expressions of p-ERK1/2 were upregulated significantly by the blueberry extracts and Cy-3-GAL supplementation. *: P8A *vs* R1 by *t* test, P<0.05; $^{\#}$: *vs* P8A, P<0.05, the differences among groups of SAMP8 mice were analyzed by one way *ANOVA* and multiple range test was by *SNK-q*. Data points represent means±SD, n=10.

DISCUSSION

The SAMP8 mouse, which shows accelerated aging, is a non-transgenic model with great use. The model provides early evidence and insights of the deleterious impact of amyloid-beta on the neurons[39]. MWM is a classical and wide used facility for study of hippocampal-dependent spatial learning and memory. Performance in the MWM depends on several elements, ranging from attention, learning and memory to vision and motor coordination[30]. Blueberry supplementation was demonstrated to reverse the deleterious effects of aging on motor behavior and neuronal signaling[20,40]. Results of present study showed that the SAMP8 animals displayed significant memorial and cognitive defect by MWM test and step-down passive avoidance test in 5 month. Both Cy-3-GAL and blueberry extracts supplementation significantly enhanced behavioral performance on the passive avoidance test. Joseph JA et al.[22] treated aged rats with blueberry diet and tested their object recognition memory on the visual paired comparison task. The results showed that young rats and aged blueberry-supplemented rats performed similarly and significant better than the aged non-supplemented group. However, the slightly increased memory on MWM, by blueberry supplementation was not potent[41]. Data from our study showed that the latency on day 4 was shortened and the time spent in quadrants was decreased in the probe test suggesting that blueberry is beneficial for improving the learning and memory ability in AD mice. It also suggested that the different animal strains may have different sensitivity and adaptability to the MWM test.

The morphologic results showed that marked cellular loss happened in the hippocampus of SAMP8 mice. The damage was associated with a decrease in the cognitive and behavioral capacity. Damage within the hippocampus was confined primarily to the pyramidal cell regions, especially in the CA1 region. The hippocampi are unique and vital regions that primarily mediate learning and memory[42]. AD is neurodegenerative disease and is characterized by the loss of neurons in the brain, especially in the CA1 region of hippocampus[43]. The pathologic results

showed that both blueberry extracts and Cy-3-GAL can ease the neuronal loss effectively. It is concordant with the conclusion from Joseph[19, 44-46]. Additionally, our research also suggested that Cy-3-GAL might play a dominant role in the extracts to the cellular protection.

Researches[20,24,47-49] suggested that the increased OS during aging may be one of the most important mechanisms involved in the cognitive decline. Antioxidant-rich blueberry is beneficial to reduce the impact of OS in CNS. Cy-3-GAL, an anthocyanin that abundant in blueberry, may be the main components as neuroprotective phytochemicals. Antioxidant enzyme activity and oxidative in the brain and blood were examined to evaluate the antioxidant capacity of mice with blueberry extract or Cy-3-GAL supplementation[13]. In our study, both higher MDA level and lower SOD activity were found in brain and plasma. We also showed that the activity of SOD was effectively increased and MDA concentration was effectively decreased by blueberry supply. Numerous studies revealed that blueberry contains a large amount of polyphenolic compounds and anthocyanin which has powerful antioxidant activity[50-54]. Experiment *in vivo*[55] showed that blueberry extract-treated cells were more resistant to H_2O_2 exposure. Blueberry also inhibited kainic acid-impaired learning performance in rats. Our study demonstrated that the OS increased significantly in AD mice. OS play a crucial role in neuronal loss and neurodegenerative diseases. The increased SOD activity and decreased MDA level by Cy-3-GAL and blueberry extracts provided a distinct evidence to illustrate the protective effect of blueberry to CNS in aging. Cy-3-GAL may be a dominant component of blueberry extracts against OS.

Evidences showed that the MAPK/ERK pathway is involved in OS, aging and neurodegeneration, including AD. In AD, Aβ stimulates transit MAPK/ERK2 activation while oxidative stress constantly stimulates ERK2 for a long term[56]. High level of activated p-ERK and p-CREB pathways can increase neurons' susceptibility to Aβ toxicity[13]. Previously published study showed that the relationship between ageing and MAPK family expression was subtle and inconsistent[13,15,57]. Experiment in SAM mice showed that down-regulation of p-ERK might be involved in the ageing process[15]. Expression of p-ERK was determined to investigate whether blueberry extracts modulate the kinases pathway. We showed that, compared with group SAMR1, the expression of p-ERK1/2 decreased significantly in SAMP8 mice suggesting that mitosis in hippocampus was restrained in AD brain. However, the expressions of p-ERK1/2 were both reversed by Cy-3-GAL and blueberry extracts treatments. The results suggested that blueberry extract can promote the function of CNS by activate cell mitotic signaling pathway, including ERK pathway. Cy-3-GAL may be the dominating ingredient in the blueberry extract that induced the activation of the pathway.

In conclusion, the present results suggest a beneficial effect of blueberry extracts or Cy-3-GAL supplementation on the antioxidative system and ERK signal pathway. However, it should be noted that we just detected the tendency of improvement to probe test in these mice. It weakened the result power to our hypothesis in the study. Further studies are, therefore, needed to assess the effects of blueberry extracts on the behavioral and cognitive capacity. The primary mechanisms of protection may be the enhancement of antioxidative capacity, which facilitates the clearance of free radicals, alters stress signals, and even partially affects the metabolism of neurotransmitters. Cy-3-GAL, the main components in anthocyanin 'family', may be the dominant active constituent for the beneficial effects of the extracts. As the intensifying OS with age, oxidation may be a primary and direct factor in the decline of cognition and memory with AD.

ACKNOWLEDGMENTS

We thank all participants who took part in the work described in this report. The authors declare that there are no conflicts of interest.

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