Beneficial Effects of Different Polygonum multiflorum Thunb.
Extracts on Memory and Hippocampus Morphology

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(Received May 17, 2002)

Summary

Four groups of 1-mo-old male senescence-accelerated mice (SAMP8) were fed Polygonum multiflorum Thunb. (PM) extract for 18 wk to determine the effect of PM on memory ability and histopathological changes in mice. The baseline diet consisted of a casein diet group, and the three test diets were supplemented with 50% ethanol, 95% ethanol, or water extracts of PM. It was found that the mice fed with PM extracts had better active shuttle avoidance response, fewer vacuole numbers, less lipofuscin in the hippocampus, and lower MDA concentrations in the brain. Our data showed that the ethanol PM extract groups (both 50% and 95% groups) had lower lipofuscin percentages and MDA concentrations, and higher total thiol concentrations than the water PM extract group. The 50% ethanol PM extract group showed significantly lower total cholesterol and triglyceride values than the other groups, but the HDL cholesterol level was the same. These results suggest that dietary supplementation with either ethanol or water extracts can reduce brain pathological changes and promote learning and memory ability. The performance of PM extracts depended on the extraction method, with ethanol extraction tending to obtain better results.

Key Words lipofuscin, malondialdehyde, memory, Polygonum multiflorum Thunb., vacuoles

Free-radical-involved oxidative stress has been implicated in the aging process, such as the development of age-related cardiovascular disorders including atherosclerosis and hypertension (1). Many chemical compounds in Chinese herbs have antioxidant activity. For example, yukmi extracts have been found to increase the activities of hepatic superoxide dismutase (SOD) and catalase, and effectively block the paraquat-induced effects on malondialdehyde (MDA) levels in mouse liver (2). Polygonum multiflorum Thunb. (PM), the root of a Chinese herb, has been used for centuries in the Orient as a tonic and anti-aging agent. In clinical studies, PM has been shown to improve hypercholesterolemia, coronary heart disease, neurosis, and other diseases commonly associated with aging (1, 3, 4). It has also been shown to promote vasorelaxation (5), act as an antibacterial agent (6), as an antimutagen (7), and to suppress lipid peroxidation in rat heart mitochondria (8).

The medicinal effects of PM in treating disorders, especially age-related diseases, are possibly mediated by the antioxidative ability of PM (9). Researchers have demonstrated that the antioxidant activity of PM extracts can promote cellular antioxidant activity, increase the function of SOD, inhibit the formation of oxidized lipids, and repress lipid peroxidation in the mitochondria of rat heart (4, 8).

Aging is associated with impairments in a variety of biological functions. One of the most striking is the impairment of cognitive functions, such as deficits in motor learning and memory. The impairment probably relates to the vulnerability of the hippocampus during the aging process and can be reversed with antioxidants, as several cognitive functions rely on the integrity of this brain area (10). In our previous study we demonstrated that 50% ethanol PM extracts can improve the learning and memory functions of mice, and concluded that the antioxidative ability of PM might be the most significant factor because decreases in MDA concentration and the percentage of lipofuscin in the brain were observed after administering PM extracts to mice (11). Diamond et al. (12) also reported that ginkgo can reverse the age-related deficits in motor learning and memory, and one of the mechanisms may include its antioxidative ability.

The biological effects of PM extracted with different solvents may vary because the active compounds in the extracts may not be the same. Chen et al. (9) identified the antioxidant compounds in 95% ethanol PM extracts, and found that the gallic acid, catechin, and 2,3,5,4'-tetrahydroxystilbene-2-O-β-D-glucoside contained in the ethyl acetate fraction showed strong antioxidant activity. Yim et al. (6) reported that the ethyl acetate extract of PM and its anthraquinone-containing fraction could enhance the myocardial glutathione antioxidant under ischemia-reperfusion induced oxidative stress, as indicated by reducing glutathione (GSH) depletion and inhibition of Se-glutathione peroxidase (GPx) and glutathione reductase (GRD) activities. Ip et
MATERIALS AND METHODS

Animals and diets. The P8 strain of the senescence-accelerated mouse (SAMP8) was established by Takeda in 1994 as a murine model for testing accelerated aging (14). Four groups of 1-mo-old male SAMP8 were prepared, each living on a different diet for 18 wk. Casein was used for the baseline diet (casein diet group, control group) and the other three groups were fed test diets supplemented with water, 50% ethanol, or 95% ethanol extracts of PM. Precise components of the experimental diets are shown in Table 1. The mice were housed about five per cage and allowed free access to the diets and tap water under controlled environmental conditions (25±1°C, 55±10% relative humidity, 0700–1900 h lighting period). The study protocol was approved by the Animal Research Ethics Committee at Providence University, Taichung, Taiwan.

Procedures of PM extraction. Dry PM root imported from China was purchased from a local traditional Chinese medicine store (Tong-Youn-Fan) and authenticated by a specialist in Chinese Medical College, Taichung, Taiwan. The dried PM root was cut into small pieces, weighed, and extracted three times with water, 50% ethanol, or 95% ethanol extracts of PM. Precise components of the experimental diets are shown in Table 1. The mice were housed about five per cage and allowed free access to the diets and tap water under controlled environmental conditions (25±1°C, 55±10% relative humidity, 0700–1900 h lighting period). The study protocol was approved by the Animal Research Ethics Committee at Providence University, Taichung, Taiwan.

Locomotion activity. The ambulatory activities of all the mice were recorded using a video activity monitor (E61-21, Coulbourn Instruments, Philadelphia, PA, USA) 1 wk before the memory examination. The mice were individually placed in a box (25×25×25 cm; L×W×H) and the time(s) of ambulatory activity was measured for 10 min.

Memory examination. An active (shuttle) avoidance test was performed to evaluate the learning and memory ability after feeding the experimental diets for 18 wk. The shuttle box used in the test (E10-15, Coulbourn Instruments) was 35×17×20 cm (W×L×H) and divided into two equal compartments connected by a small opening (7.5×6.5 cm). Each mouse in this test was placed in the shuttle box for 10 s to adapt to the new environment before receiving subsequent stimulus. A successful avoidance response was scored if the mouse moved from one compartment to the other in response to a conditioned stimulus (CS), a tone and red, yellow, and green light. If the mouse did not display a successful avoidance response, an unconditioned stimulus (UCS), a 0.3-mA, 5 s scrambled foot shock was given during the CS. Each mouse participated in four daily sessions consisting of five CS/UCS trials, for a total of 20 trials/d for 4 d. Between the sessions, the mice were allowed to rest for 15–20 min. The avoidance response (running to the other compartment of the test apparatus after presentation of CS) before the shock was delivered was recorded automatically.

Examination of serum lipids and hippocampus morpholgy. After the learning and memory examination, the mice were anesthetized, and then sacrificed. Blood was withdrawn and centrifuged at 1,200×g for 10 min to obtain serum for analysis of triglycerides and total and HDL cholesterol levels at a commercial analytical service center (Lian-Ming Co., Taichung, Taiwan). Entire brain tissues of mice were fixed in 10% phosphate-buffered formalin at room temperature overnight. The brain tissues were sliced into 4–6 mm thick pieces, dehydrated in ethanol, embedded in paraffin wax, and finally sectioned. Then the morphological changes in the hippocampus areas were observed. The samples were stained by hematoxyline and eosin (H&E) for determination.
ing the number of vacuoles, and reacted with periodic acid Schiff (PAS) for measuring the lipofuscin deposits (15). The vacuole numbers and lipofuscin deposits in the hippocampus were measured, and the percentage of cells with lipofuscin were calculated using the following equation (16):

\[
\text{Lipofuscin} \, (\%) = \frac{\text{number of positive lipofuscin}}{\text{number of total nerve cells}} \times 100.
\]

**Oxidative status analyses.** Entire brain tissues were homogenized in a 100 mM sodium phosphate buffer, and then centrifuged at 3,000 × g for 10 min in a high-speed centrifuge. The supernatant was collected to determine the extent of lipid peroxidation and total thiol concentration. The lipid peroxides were determined by malondialdehyde–thiobarbituric acid (MDA-TBA) adduct according to the method described by Ohkawa et al. (17). The mixture was prepared by mixing the supernatant, 2′-thiobarbituric acid (0.4% in 0.2 M HCl), and butylated hydroxytoluene (0.2% in 95% ethanol) at a ratio of 1 : 2 : 0.3, and then heating at 90°C for 45 min. After cooling, 5 mL of n-butanol was added to the mixture, thoroughly mixed, and then the n-butanol layer was separated by centrifugation at 1,000 × g for 10 min. The n-butanol layer containing MDA production was measured spectrophotometrically at 532nm (Hitachi U2001) using tetramethoxypropane as the external standard. The total thiol concentrations in the tissues were measured at 412nm after their reaction with 5,5′-dithio-nitrobenzoic acid (18). All chemicals were analytical grade, and all solutions were prepared using triple-distilled water.

**Statistics.** All data were expressed as mean ± standard error of the mean and analyzed using SPSS 8.0 software (Spss Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for the comparison of means between the different groups. Differences were considered to be significant at p<0.05.

**RESULTS**

The body weight change and food intake of different diet groups are shown in Table 2. There were no differences among the control and PM extract groups, and there was no significant difference among the results of the three different PM extract groups (p>0.05). These results indicate that the intake amounts of the four groups were similar.

Table 3 shows the results of locomotion tests in SAMP8 mice fed with different diets. No differences in locomotion activity were observed among the PM and control groups (p>0.05), and there were also no significant differences found between the three PM extract groups. Figure 1 shows the mean successful avoidance scores of the experimental groups. The scores of all three PM extract groups were significantly higher than those of the control group (p<0.05), but there were no significant differences among the three different PM extract groups in the second, third and fourth days. These results indicate that PM can improve the learning and memory abilities of the mice.

Table 2. Body weight change and food intake in SAMP8 mice fed different diets for 18 wk.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body weight</th>
<th>Food intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial (g)</td>
<td>Final (g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>20</td>
<td>23.4±0.7</td>
<td>27.6±0.5</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>22.1±0.6</td>
<td>27.4±0.4</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>21.8±0.8</td>
<td>28.4±0.6</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>22.7±0.6</td>
<td>27.6±0.4</td>
</tr>
</tbody>
</table>

Values are mean±SE and analyzed by one-way ANOVA. A: casein, B: Polygonum multiflorum extracted by water, C: Polygonum multiflorum extracted by 50% ethanol, D: Polygonum multiflorum extracted by 95% ethanol.

Table 3. The locomotion in SAMP8 mice fed different diets.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Time interval (min)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–5</td>
</tr>
<tr>
<td>A</td>
<td>20</td>
<td>90.13±5.70</td>
</tr>
<tr>
<td>B</td>
<td>20</td>
<td>98.88±3.58</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>95.63±4.50</td>
</tr>
<tr>
<td>D</td>
<td>20</td>
<td>98.45±3.63</td>
</tr>
</tbody>
</table>

Values are mean±SE and analyzed by one-way ANOVA. * Recorded time indicates recording every 5 min by E61-21 monitor. A: casein, B: Polygonum multiflorum extracted by water, C: Polygonum multiflorum extracted by 50% ethanol, D: Polygonum multiflorum extracted by 95% ethanol.

The vacuole numbers and lipofuscin percentage in the hippocampus of SAMP8 mice fed different diets for 18 wk are shown in Table 4. All the PM extract groups had lower vacuole numbers than the control diet group (p<0.05), while no significant difference among the PM extract groups was observed. The lipofuscin percentages in the hippocampus were also lower in all the PM extract groups (p<0.05), while the 50% and 95% ethanol PM extract groups showed lower percentages than the water PM extract group (p<0.05).

Figure 2 shows the MDA and total thiol concentrations among the different diet groups. The MDA concentrations in the brain of both 50% and 95% ethanol PM extract groups were significantly lower than that in the control group (p<0.05), but no difference was found between the water PM extract group and the control group. The total thiol concentrations were higher in both the 50% and 95% PM extract groups, but not significantly different in the water PM extract group and control group (p>0.05).

Figure 3 shows the serum lipids of the different diet groups. The total cholesterol and triglyceride levels in the 50% ethanol PM extract group were significantly lower as compared to the other groups (p<0.05), but there was no difference in the HDL cholesterol levels.
DICUSSION

SAMP8 is a murine model system that exhibits remarkable age-related deterioration in learning and memory when challenged with various learning tasks such as passive avoidance, two-way active avoidance, and water maze tests (19, 20). Thus, it is thought to be an excellent animal model for studying deficits in learning and memory. The active avoidance (shuttle avoidance) test was used to evaluate the learning and memory functions in this study. In our experiments, the successful avoidance scores of the control group were significantly lower than those of all three PM extract groups (p<0.05) (Fig. 1), suggesting that chronic oral administration of PM might decrease age-related learning and memory deterioration. However, there was no significant difference among the three different PM extract groups.

Humans with dementia attributed to Alzheimer’s disease (AD) have a degree of disturbance in antioxidant balance that may predispose increases in oxidative stress (21). AD involves neuronal degeneration in the...
an age-dependent increase in TBA reactivity and a decrease in the GSH level in the brain, suggesting that the mechanism of senescence acceleration was, to some extent, related to free radical damage. Our findings suggest that the mechanism of PM activity on reducing morphological changes in the brains of SAMP8 mice might be due to decreasing the free radical damage.

Free radicals are known to oxidize the polyunsaturated free fatty acids of the cellular membrane and thiol groups of free amino acids that subsequently result in cell damage (27, 28). Rodriguez-Martinez et al. (29) reported that the MDA levels increased two- and threefold in 24- and 30-mo-old rats when compared to 6-mo-old rats, and therefore concluded that lipid peroxidation increases with age. From our data, both the 50% and 95% ethanol PM extract groups had significantly lower MDA concentrations in the brain after 18 wk of feeding, indicating that the ethanol PM extracts were more effective for inhibiting lipid peroxidation. The processes of neurodegeneration with aging have been proposed to be associated with the generation of free radicals (30, 31). For protection from damage due to oxidative stress, a well-coordinated network of antioxidant defense systems is essential.

Kim et al. (30) found that the total thiol level in old rats is significantly lower than that observed in young rats, and the reduction of thiols was exacerbated by the lowering of GSH levels. Rao et al. (32) reported that the SOD and catalase (CAT) activities of the brain in Fisher F344 rats significantly decreased with age, while glutathione peroxidase (GPx) was not changed. The fact that antioxidant systems are not equal among different brain regions has been reported (31, 33). Ansari et al. (33) found that the GPx and CAT activities were different among 5 CNS regions in Fisher F344 rats, while illustrating that the activities of SOD, CAT, and GPx did not change with age. Hussain et al. (31) demonstrated that the antioxidant enzyme level varies in different brain regions of C57BL/6N mice, but the overall antioxidant enzyme activities tend to increase with age. However, the relationships between antioxidant defense with aging and the different regions of the brain are complex. Our data shows that the ethanol PM extract groups (both 50% and 95% groups) had a higher total thiol concentration in the entire brain than the water PM extract group. Wang (34) found that both the 50% and 95% ethanol PM extract diet groups had significantly higher GSH-Px and SOD activities in the brain of SAMP8 mice than those in the water PM extract group. Further studies are necessary to fully understand the effects of PM on the antioxidant status in different brain regions.

Our results show that the ethanol PM extract groups (both 50% and 95% groups) had lower lipofuscin percentage and MDA concentration, and higher total thiol concentration than the water PM extract group. When comparing the food intake between different PM diet groups, no significant difference was found, suggesting that the differences between the ethanol and water PM extract groups were not cause by the intake amount.

**Fig. 3.** The comparison of triglyceride (TG), total cholesterol (TC) and HDL concentrations among different diet groups. Data are expressed as mean±SE and analyzed by one-way ANOVA. Data with different superscripts are significantly different at p<0.05. A: casein, B: Polygonum multiflorum extracted by water, C: Polygonum multiflorum extracted by 50% ethanol, D: Polygonum multiflorum extracted by 95% ethanol.

In SAMP8 mice, the spongy degenerations (vacuolization) in the brain stem and spinal cord, especially in the reticular formation, have been shown to be closely related to learning and memory deficits (23). Old mice usually have higher vacuole numbers than young mice, indicating rapid development of the sponge caused by aging. In this study, the number of vacuoles in the hippocampus was lower in all the PM extract groups than in the control group, suggesting that PM extract could inhibit the spongy production.

The lipofuscin percentages in the hippocampus were also lower in all PM extract groups, while the 50% and 95% ethanol PM extract groups showed lower percentages than the water PM extract group (Table 4). It has been suggested that lipofuscin deposits represent the accumulation of the peroxide active action of free radicals, and can be used as a marker for oxidative stress and aging (24, 25). Since PM extract can decrease the lipofuscin percentage, it was suggested that PM might protect the hippocampus from the attack of free radicals. Our data shows that all PM extracts could significantly decrease the vacuole number, lipofuscin percentage, and MDA concentration in the brain of SAMP8 mice.

Liu and Mori (26) stated that SAMP8 mice showed
Wang (34) evaluated the antioxidative activities of water, 50% ethanol, and 95% ethanol PM extracts by determining their DPPH radical scavenging abilities. In an in vitro study, they found the DPPH radical scavenging abilities of the PM extracts followed the order of 50% ethanol PM extract > 95% ethanol PM extract > water PM extract. In the in vivo study, Wang (34) found that both the 50% and 95% ethanol PM extract diet groups had significantly higher antioxidant enzyme activity, including GSH-Px and SOD, in the brain of SAMP8 mice than did the water PM extract group. These findings all demonstrate that ethanol PM extracts (both 50% and 95% ethanol PM extracts) tend to have higher antioxidant ability than water PM extract.

It is well known that nonpolar substances have a higher possibility to pass the blood-brain barrier (BBB) directly, whereas most of the polar soluble substances have to depend on the assistance of carriers. The authors suggest that the 50% and 95% ethanol PM extracts may contain more nonpolar compounds, and these compounds can pass the BBB more effectively and protect the nerve cells from oxidative stress. Another possible explanation is that the antioxidant components in the PM extracts were different due to different extraction methods. Wang (34) reported that the gallic acid level in the 50% ethanol PM extract was 136.3 mg/g, which was significantly higher than that in the water PM extract (51.4 mg/g) and 95% ethanol PM extract (39.53 mg/g).

The total cholesterol and triglyceride levels in the 50% ethanol PM extract group were significantly lower as compared to the other groups, but there was no difference in HDL cholesterol level (Fig. 3). Our results are consistent with those of Zhang et al. (3), whose data showed that PM extract could reduce the plasma cholesterol levels in rats. It was reported that PM could affect the activity of HMG-CoA reductase in the liver, and can therefore reduce cholesterol synthesis in the liver (35). However, the mechanisms by which 50% ethanol PM extract lowers the serum lipids need further study.

In summary, our results indicate that supplementation with water, 50% ethanol, and 95% ethanol PM extracts can reduce pathological changes in the brain of SAMP8 mice, prevent oxidative damage, and decrease the deterioration of cognitive performance. The ethanol PM extracts tended to show better performance than the water PM extract. Further research is necessary to determine the specific compounds in the PM extracts responsible for slowing the aging process.

Acknowledgments
We are grateful to Dr. Cheng-Kuang Hsu, Dr. Su-Tu Chou, Dr. Ken-Feng Huang, and Ms. Hsu-Ching Chang for their help in this research. This study was supported by the National Science Council, Taiwan (NSC-89-2316-B-126-002), the Republic of China.

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