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Effect of *Monascus*-Fermented Products on Learning and Memory in the SAMP8 Mice

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Summary The purpose of this study was to evaluate the impact of *Monascus*-fermented products (MP) as regards certain changes in behavior for SAMP8 mice. Both male and female SAMP8 mice were fed a 0.03% MP diet from 3 mo of age to 11 mo of age. The results indicated that the grading score of passive avoidance behavior was significantly lower in the MP diet groups than in the control diet groups in both male and female SAMP8 mice ($p<0.05$). The MP diet-augmented test-animal body weight, feed intake and feed efficiency did not differ significantly from the corresponding values for control mice. The MP diet-fed mouse group revealed significantly improved learning and memory as revealed by average escape-response testing score when comparing with control mice ($p<0.05$). Further, the level of serum triglyceride and total cholesterol for the MP-fed group were shown to be significantly lower than for the control group of SAMP8 mice at 11 mo of age. The test mice fed an MP diet appeared to be significantly lower in aging score than the control group ($p<0.05$). The MP diet-fed mouse group revealed significantly improved total antioxidation of liver. Subsequent to supplementation of SAMP8 mice diets with MP for a period of 8 mo, these MP-fed mice revealed significantly lower lipofuscin-cell numbers within the hippocampus ($p<0.05$). The results suggest that dietary supplementation with MP might improve both learning and memory behaviour, and retard the aging process for SAMP8 mice.

Key Words *Monascus*-fermented products, SAMP8 mice, aging, learning and memory

Various members of the *Monascus* species of fungus have been traditionally used in food production and preservation in the Orient for centuries (*1*). *Monascus* has been demonstrated to produce many metabolites and is used as a food pigment or food additive (*2*). Certain members of this species demonstrate a variety of biological activities which are advantageous for human beings/human health (*3*), an example being the species' antihypertension effects (*4*, *5*). Monacolin K, a product extract from *Monascus*, has been demonstrated to act as a specific inhibitor of the activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-COA) reductase. Several reports also demonstrated the extract of *Monascus*, Monacolin K, was recognized as a cholesterol-lowering agent (*3*, *6*). The extracts of *Monascus anka* have also been associated with free radical-scavenging power and have been used to improve the hepatotoxicity induced by certain chemical compounds (*7*). It would appear that these authors' investigations were somewhat limited as regards the extracts' contribution to learning and memory ability, as well as its contribution to aging retardation.

In 1989, Sohal and Brunk (*8*) indicated that lipofuscin could be used as an intracellular marker for oxidative stress and aging. It has been suggested previously that lipofuscin deposits represent the accumulation of the peroxide-active action of free radicals (*9*, *10*). Reactive oxygen species (ROS) contribute to the pathogenesis of various diseases (*11*, *12*) and also to the aging process (*13*). Aging and age-related disease are associated with oxidative stress from the over-production of ROS. When a disturbance in the pro-oxidant-anti-oxidant balance of the cells (redox status) occurs in favor of the former, the potential for tissue damage due to oxidative stress ensues.

Various senescence-accelerated mouse (SAM) strains have previously been established by a number of investigators as an animal model of accelerated senescence (*14*, *15*). SAMP8 mice are characterized by signs of advanced senescence, reduced physical activity, hair loss, lack of hair glossiness, skin coarseness, increased lordokyphosis of the spine, and a shortened life span (*16*, *17*). SAMP8 mice used for this study constitute an animal model of the age-related premature deterioration of learning and memory (*18*). Some investigators conducting studies using SAMP8 mice have reported that the age-related morphological impairment of the brain commences, for such mice, at 6-to-8 mo of age (*19*, *20*). Aged SAMP8 mice typically exhibit learning

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and memory deficits associated with contextual fear conditioning due to hippocampal dysfunction (*21*). There would appear to be ever-increasing evidence presented in the literature that SAMP8 mice are an acceptable rodent model to demonstrate the cognitive deficits observed to be associated with aging such as those that appear for sufferers of Alzheimer's disease (*22*). The objective of this study was to evaluate the possibility that *Monascus*-fermented products (MP) diet supplied to SAMP8 mice could impact favourably upon the age-related impairment of learning and memory ability, and, in effect, retard the aging process.

MATERIALS AND METHODS

Animals and diets. The SAMP8 mice used for this study featured the characteristics of aging and memory-retardation common to such mice and were fully developed in our animal laboratory in this study. Fifty-two SAMP8 mice of both genders were weaned at 4 wk of age, and were then housed under controlled environmental conditions (25±2°C 70% RH, 08:00–20:00 lighting period). The mice were allowed free access to water and diet. They were weighed weekly and, at 3 mo of age, were separated into four individual groups (two gender groups × two different diets) and maintained according to the conditions for the accorded group over the period from 3 to 11 mo of age. At the commencement of the experimental procedures, each animal was weighed and examined according to a variety of behavioral paradigms. The control diet contained protein, corn starch, sucrose, soy bean oil, cellulose, mineral mixture, and vitamin mixture in this study (Table 1). The experimental diet contained 0.03% MP, a mixture of solid and liquid-fermented *Monascus* powder (Table 1), was formulated by Standard Chemical and Pharmaceutical Co., Ltd. (Tainan, Taiwan) for the purposes of this study, and was combined with the relevant animals' diet. The chemical compositions of MP, analysed in triplicate for moisture, protein, and lipid contents by using Association of Official Analytical Chemists' approved methods, were 925.10, 963.09 and 923.03, respectively (*23*). The Monacolin K (lovastatin equivalent) content was analysed according to the method of Wang et al. (*24*). The SAMP8 mice were fed the control and MP diet starting at 3 mo of age for 8 mo diet treatment. The locomotion activity test of SAMP8 mice was conducted at the age of 4 mo and 10 mo, when mice were classified as young and old mice, respectively, according to the average life span of 12 mo in SAMP8 mice. At the age of 10 mo, the grading score and active shuttle-avoidance test of SAMP8 mice were evaluated. After these learning and memory examinations, the SAMP8 mice were sacrificed at 11 mo old for serum biochemical assay, antioxidant activity and morphological analyses. The study protocol was approved by the Animal Research Ethics Committee at Providence University, Taiwan.

Table 1. The composition of experimental diets.

	Diet group (g%)	
	Control	MP
Casein[1]	20	20
α-Corn starch[2]	43.5	43.2
Sucrose	21.5	21.5
Soy bean oil	7	7
Cellulose[3]	1	1
Mineral mixture[4]	5	5
Vitamin mixture[5]	2	2
Monascus[6]	none	0.03

[1] The protein content of casein powder is 90.64%.
[2] α-Corn starch : sucrose=2 : 1 ratio.
[3] Obtained from Sigma Inc. (USA).
[4] Obtained from ICN Pharmaceuticals Inc. AIN-93G (USA).
[5] Obtained from ICN Pharmaceuticals Inc. AIN-93VX (USA).
[6] A mixture of solid-fermented *Monascus* powder and liquid-fermented *Monascus* powder (2 : 8 ratio).

Locomotion activity test. Before the grading score and active shuttle-avoidance test, the locomotion activity of all the mice was measured by using an activity monitor video path analyzer (Model E61-21, Coulbourn Instruments, USA). The mice were placed individually in the box of the open field apparatus (25×25×25 cm^3) and introduced into the center of the apparatus. The open field activity was counted and recorded by the secants of locomotion for the initial 5 min and the remaining 5 min (6–10 min) (*25*).

Evaluation of grading score. The criteria used for determining the grading score relating to accelerated senescence were applied according to the method of Takeda et al. (*14*) and Hosokawa et al. (*16*). The various parameters assessed to provide the final grading score included behavior (individual reactivity, passivity), skin and hair (glossiness, coarseness, loss of hair) and spinal characteristics (lordokyphosis). Each parameter was graded according to a scale incorporating five levels (grades 0–4; where grade 4 is the most severe) and criteria that had been developed and evaluated by a specific laboratory researcher, who had been extensively trained and who had undertaken such assessment activity for a period of 2 y prior to the conduct of this study.

Active shuttle-avoidance test. The active shuttle-avoidance test was conducted at the age of 3 mo, the initial stage of diet treatment, and 10 mo, according to the method of Chen et al. (*25*). The shuttle cage (35×17×20 cm, width×length×height, respectively) (Model E10–15, Coulbourn Instruments) was divided into two equal compartments connected by a small opening (7.5×6.5 cm). Each mouse was exposed to the shuttle box for 10 s prior to administering the conditioned stimulus (CS), which consisted of a tone and green, yellow and red light. Each mouse was subjected to the test of conditioned stimulus for 10 s. After the CS presentation, an unconditioned stimulus (UCS), which consisted of a 0.3 mA, 5 s scrambled foot shock, was given if the tested mouse had not made an avoidance response by moving to the other compartment. The

escape response was scored if the mouse moved from one compartment to the other after 5 s scrambled shock. Each animal received four daily sessions of a combination of five CS/UCS trials, for a total of 20 trials each day. The CS/UCS trials were run continuously for 4 d. Mice were allowed to rest for 20–25 min between the test sessions. The escape responses displayed represent a mouse's display of unsuccessful avoidance for the active shuttle-avoidance test, an outcome that resulted in a scramble shock for the test mouse after presentation of CS. Therefore, it could be concluded that a lower frequency of active escape responses reflected the better learning ability for test SAMP8 mice.

Serum biochemical analyses. The quantitative assay for serum biochemical analyses including triglyceride, total cholesterol, LDL-cholesterol, and HDL-cholesterol involved analysis of samples by means of the use of a commercial assay kit (COBAS INTEGRA 700, COBAS® INTEGRA, Roche, USA).

DPPH free radical-scavenging capacity. The DPPH free radical-scavenging capacity of *Monascus*-fermented products was estimated according to the method of Yamaguchi et al. (*26*). The solution consisted of α,α-diphenyl-β-picrylhydrodrate (DPPH; 0.5 mL, 0.25 mM), Tris-HCl buffer (0.4 mL, pH 7.4; 100 mM), MP-extract and/or ethanol (0.1 mL). The mixture was shaken vigorously and incubated at room temperature in dark room for 20 min. The radical-scavenging activity of MP-extract was measured spectrophotometrically at 517 nm (Hitachi U2001, Tokyo, Japan). The DPPH free radical scavenging capacity was calculated by the following equation: Scavenging capacity (%)=[1−(absorbance of sample at 517 nm/absorbance of control at 517 nm)]×100%. The half-inhibition concentration (IC_{50}) was calculated by the first-order kinetics equation as the antioxidant concentration required for providing 50% of the antioxidative activity.

Total antioxidant activity. Total antioxidant activity was measured by using a commercial kit (*27*) (Randox Laboratories Ltd., Antrim, UK) in which 2,2′-azino-di-(3-ethylbenzthiazolin sulphonate) (ABTS) was incubated with a peroxidase and H_2O_2 to produce the radical cation ($ABT^{\cdot+}$). The total antioxidant activity was detected spectrophotometrically at 600 nm (Hitachi U2001).

Morphological analyses. The mice were sacrificed by cervical dislocation at 11 mo of age, subsequent to completion of the memory test period. Their brains and selected tissues of SAMP8 mice were removed rapidly, weighed and then fixed in 10% buffered formalin. The brain specimens containing the hippocampal formation were embedded in paraffin and sectioned and stained with periodic acid-Schiff's regent (PAS) for lipofuscin measurements. Based on the landmarks matching those of a mouse brain atlas, six consecutive sections of the CA3 region of the hippocampus, 1.6 mm behind Bregma, were used for lipofuscin measurement (*28*). The level of lipofuscin accumulation was determined using light microscopy, the microscope (Model BX40F, Olympus Optical Co., Japan) being fitted with a video camera. The cell number of lipofuscin in the hippocampus was studied.

Statistical analysis. All data are expressed as means of triplicate measurements. One-way and two-way ANOVA were used to analyze the parameter mean for each treatment. A statistical level of less than 0.05 for p value was accepted as representing a statistically significant difference between compared data sets.

RESULTS

The protein contents of MP powder were high, reaching 13.57% in this study. The MP powder also contained 7.06% lipid and 8.21% moisture as well as 1.36% Monacolin K (lovastatin equivalent). In this study, the MP-supplemental dietary group consumed 1.5 mg MP/d for each mouse, which is approximately 0.023 mg/d Monacolin K per mouse. The results of our investigation revealed that no significant difference was apparent between the different treatment groups (gender and diet) as regards mouse body weight, quantity of food intake or feeding efficiency (data not shown). The results of locomotion activity testing are presented in Table 2. All young mice exhibited a more substantial level of locomotion activity than was the case for old mice in both male and female mice. In the comparison of diet effect on locomotion activity, the young female

Table 2. Effect of *Monascus*-fermented products diet on locomotion activity at different physiological ages of SAMP8 mice.[1]

		Locomotion activity (times/5 min)			
		First period (0–5 min)		Second period (6–10 min)	
		Young mice	Old mice	Young mice	Old mice
Male	Control diet	121.8 ± 2.4^{Ba}	103.2 ± 7.0^{Ab}	96.0 ± 3.2^{Bbc}	82.0 ± 2.9^{ABc}
	MP diet	115.1 ± 7.0^{Ba}	95.1 ± 6.2^{Ab}	95.6 ± 6.1^{Bb}	71.7 ± 0.2^{Bc}
Female	Control diet	123.5 ± 5.1^{Ba}	108.7 ± 5.8^{Ab}	104.2 ± 3.3^{Ab}	91.8 ± 0.7^{Ac}
	MP diet	136.2 ± 7.9^{Aa}	99.0 ± 7.5^{Ab}	106.0 ± 4.8^{Ab}	82.2 ± 7.0^{ABc}

[1] Young and old mice are at the ages of 4 mo and 10 mo, respectively.
[A-C] Means with different letters within the same column differed significantly (mean±SE, $n=13$) ($p<0.05$).
[a-c] Means with different letters within the same row differed significantly ($p<0.05$).

Table 3. Effect of *Monascus*-fermented products diet on the grading score of aging of SAMP8 mice.

Grading items		Male		Female	
		Control	MP	Control	MP
Behavior	Reactivity	0.25±0.12[a]	0.18±0.12[a]	0.09±0.09[b]	0.00±0.00[b]
	Passivity	0.58±0.15[a]	0.36±0.20[b]	0.64±0.20[a]	0.08±0.08[c]
Appearance of skin and hair	Glossiness	1.00±0.00[a]	0.91±0.09[a]	0.91±0.09[a]	0.46±0.14[b]
	Coarseness	1.25±0.19[a]	0.73±0.14[b]	0.64±0.15[b]	0.23±0.12[c]
	Hair loss	1.33±0.13[a]	1.60±0.20[a]	1.36±0.15[a]	1.31±0.13[a]
Spine		0.92±0.20[a]	0.36±0.15[b]	0.82±0.18[a]	0.85±0.25[a]
	Total	5.58±0.30	3.91±0.49	4.64±0.43	2.92±0.47

[a-c] Means with different letters within the same row differed significantly (mean±SE, $n=13$) ($p<0.05$).

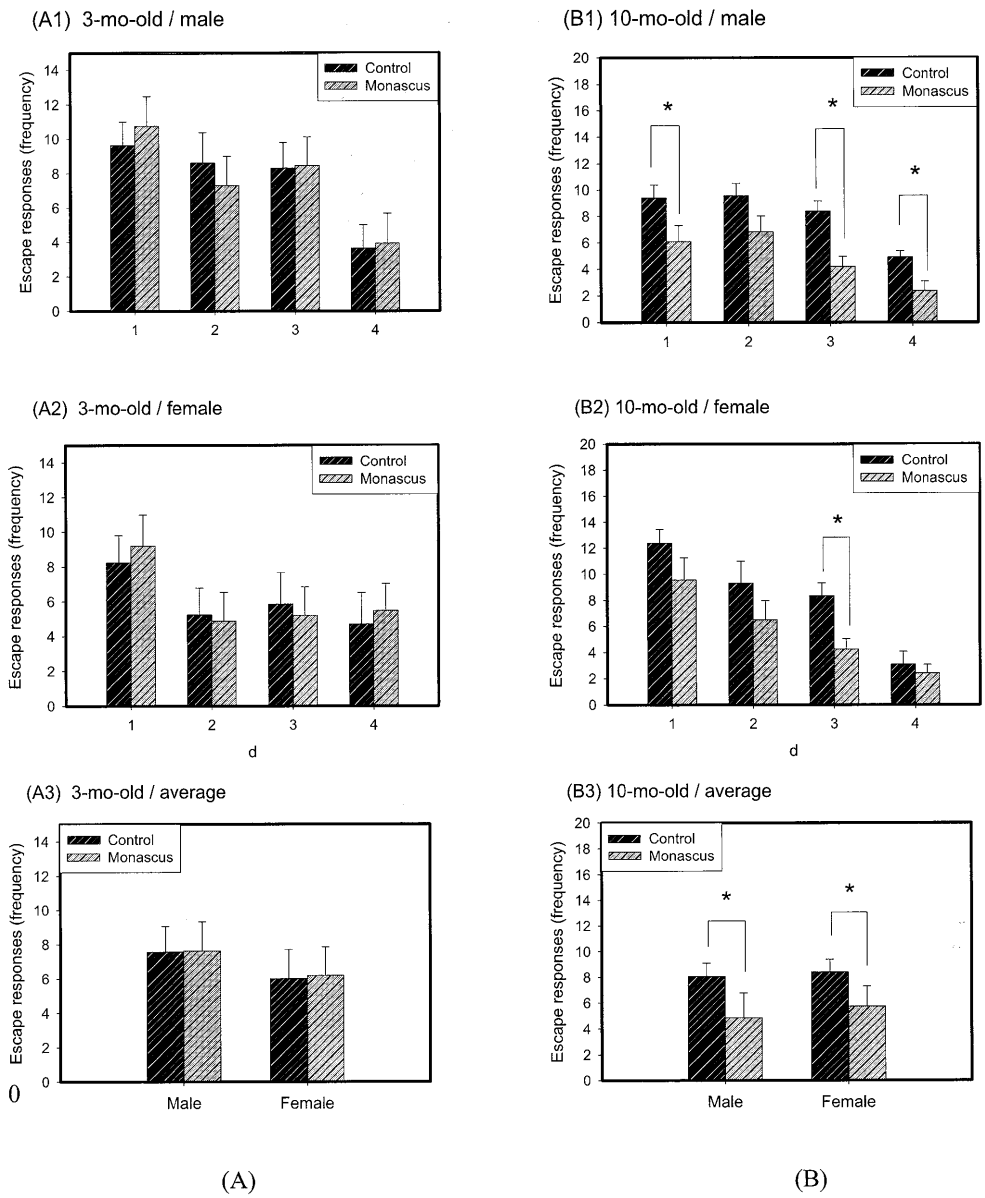

Fig. 1. Effect of *Monascus*-fermented products (MP) diet on the escape response of active shuttle avoidance test among 20 trials in 3-mo-old (A) and 10-mo-old (B) male (1) and female (2) of SAMP8 mice. The bar graph represents the mean±SE ($n=13$, $^*p<0.05$).

Table 4. Effect of *Monascus*-fermented products diet on the serum biochemical analysis of 11-mo-old SAMP8 mice.

Biochemical analysis	Male		Female	
	Control	MP	Control	MP
TG[1] (mg/dL)	177.3±13.2[a]	132.0±12.5[b]	116.4±9.6[c]	95.3±11.5[c]
Total Chol.[1] (mg/dL)	113.5±7.1[a]	90.9±4.8[b]	79.9±5.6[c]	67.1±9.6[d]
LDL-C[2] (%)	25.3±3.8[a]	26.7±3.0[a]	29.8±2.3[a]	20.6±2.9[b]
HDL-C[3] (%)	50.3±3.6[c]	48.5±5.9[c]	62.0±1.4[a]	56.3±1.6[b]

[1] TG, triglyceride; Total Chol., total cholesterol.
[2] LDL-C=(LDL-C)/T-Chol.×100%.
[3] HDL-C=(HDL-C)/T-Chol.×100%.
[a-c] Means with different letters within the same row differed significantly (mean±SE, n=13) (p<0.05).

SAMP8 mice fed the MP diet exhibited a greater level of locomotion activity (for the first period of observation, i.e., 0–5 min) than was the case for young male and female mice fed the control diet, but no significant difference as regards locomotion activity was apparent in old mice for either the first or second period.

The results of the grading scores for behavior, appearance of skin and hair, and spine condition are presented in Table 3. Supplementation of the dietary complement with MP for the preceding 7 mo (individuals aged 10 mo old) was shown to have resulted in a significant decrease in passive behaviour, and a reduced level of hair glossiness and coarseness for the female SAMP8 mice as compared to the controls (p<0.05). For the male SAMP8 mice, a diet supplemented with MP was shown to reduce the grading score of appearance of hair coarseness and lordokyphosis of the spine as compared to control mice (p<0.05), suggesting that MP-fed male SAMP8 mice were more youthful in appearance than their control analogues. In summary, a diet supplemented with MP for SAMP8 mice resulted in a significantly reduced grading score that meant less aged for a variety of test aspects for both male and female mice when compared to control mice (p<0.05).

A summary of the results for the escape responses exhibited by SAMP8 mice participating in the active shuttle-avoidance test is presented in Fig. 1. The escape responses displayed represent a mouse's display of unsuccessful avoidance for the active shuttle-avoidance test, an outcome that resulted in a scramble shock for the test mouse. Therefore, it was concluded that a lower number of active escape responses demonstrated by a mouse reflected an enhance memory ability for test SAMP8 mice. Our results indicated that no significant difference was shown in the escape response of the active shuttle-avoidance test at the initial stage of day 1 to day 4 (3-mo-old) between control and MP diet in SAMP8 mice (p<0.05) (Fig. 1, A1–A3). After the 7-mo long-term diet treatment, the results revealed that male SAMP8 mice, the diet of which had been supplemented with MP, exhibited a significantly lower escape-response value than was the case for the control diet group of mice (p<0.05) on days 1, 2 and 4 of the testing period. The same results were noted for female SAMP8 mice fed an MP-supplemented diet as compared to control mice,

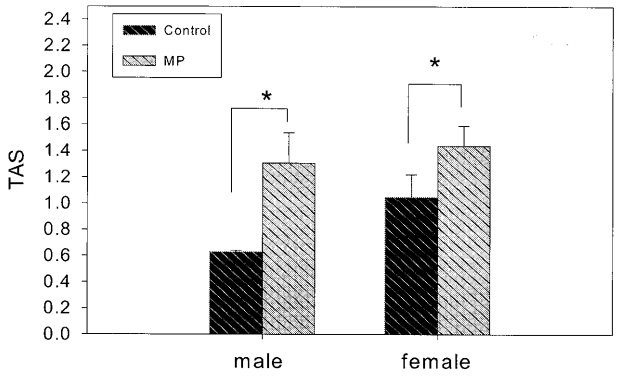

Fig. 2. Effect of *Monascus*-fermented products (MP) diet on the total antioxidation status (TAS) of liver of SAMP8 mice. The bar graph represents the mean±SE (n=13, *p<0.05).

but only for day 3 of testing. Regardless of gender, the MP-supplemented diet group demonstrated a significantly lower level of escape responses than did the control diet group (p<0.05). These results would appear to suggest that an MP-supplemented diet may improve the memory ability of SAMP8 mice as tested by application of a shuttle-avoidance test.

The results of serum biochemical analyses for the 11-mo-old study-participating SAMP8 mice are presented in Table 4. The male and female mice fed a diet supplemented with MP exhibited significantly lower triglyceride and total cholesterol levels than was the case for the control-diet mice (p<0.05). The female SAMP8 mice fed an MP-supplemented diet also presented a lower serum LDL-C and HDL-C level than was the case for their male analogues (p<0.05).

For the male and female mice, a diet supplemented with MP was shown to increase the total antioxidant status for the liver as compared to control mice (p<0.05) (Fig. 2), suggesting that MP-fed SAMP8 mice were more stable to oxidation than their control analogues. The numbers of lipofuscin cells present in the hippocampus region of the brain, as assessed at 11 mo of age, proved to be significantly lower for male SAMP8 mice that had been fed a diet supplemented with MP than was the case for control-diet mice (p<0.05), We did note, however, that no such corresponding significant difference was apparent for female SAMP8 mice

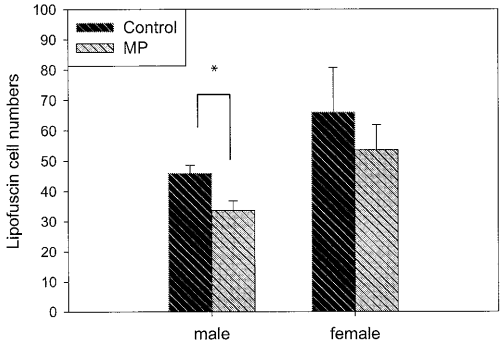

Fig. 3. Effect of *Monascus*-fermented products (MP) diet on cell numbers of lipofuscin in the hippocampus region of SAMP8 mice. The bar graph represents the mean±SE (n=13, *p<0.05).

fed an MP-supplemented diet as compared to control female mice (Fig. 3).

DISCUSSION

Some further investigation of the biological effects of the secondary metabolites of *Monascus* species is clearly warranted. For our study, the total aging score of the MP-supplemented dietary group was significantly lower than was the case for the control groups for, separately, both male and female mice (p<0.05). Several reports indicated that the SAMP8 strain exhibited an age-related deterioration in leaning and memory when challenged with various learning tests such as passive avoidance (*29*, *30*). Our results demonstrated that the MP supplement is associated with a reduction in the age-related deterioration of skin coarseness, hair loss, and lordokyphosis of spine as well as learning and memory ability in both male and female SAMP8 mice. The results of locomotion activity testing indicated that dietary supplementation of MP over the period 3 mo was noted to be able to contribute to an improvement in the locomotion activity of such female mice.

Herein, the active shuttle-avoidance test was utilized for the purposes of assessing the learning and memory abilities of 11-mo-old SAMP8 test mice. Our results would appear to strongly suggest that dietary MP supplementation is able to improve a test animal's escape responses and significantly decrease lipofuscin-cell numbers present in the hippocampus, at 11 mo of age, for both male and female SAMP8 mice fed such a diet over the preceding 8-mo period.

The results for serum lipid level indicated that dietary supplementation with MP resulted in a significantly lower triglyceride and total cholesterol level amongst 11 mo-old male and female mice fed such a diet, as compared to control-diet mice (p<0.05). Cholesterol plays an important role in the pathophysiology of Alzheimer's disease (AD). For instance, an elevated serum cholesterol level has been shown to be a risk factor for AD. Epidemiological studies show that patients with elevated cholesterol levels have an increased risk of AD (*31*, *32*). Aging and age-related changes are known to be associated with increased oxidation stress. Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion, hydroxyl radical, and nitric oxide, are formed in the body as a consequence of aerobic metabolism, damaging all intracellular components, including nucleic acids, proteins and lipids (*33*, *34*). Disturbances in the serum redox status by weakened defense systems can lead to age-related, vascular diseases such as atherosclerosis (*35*), coronary heart disease (*36*), and cancer (*37*).

Lipofuscin accumulation may be a good histochemical marker for central nervous system cell degeneration because, as has been reported previously, the number of lipofuscin cells present in the brain correlates well with the level of memory impairment for aged rats (*38*). Further, an increase in the level of lipofuscin depositing in the hippocampus of SAMP8 mice, as compared to control mice, has been previously reported by Flood et al. (*39*), and SAMP8 mice are reported to be more susceptible to oxidative stress and seizure activities than is the case for normal mice (*22*). Oxidative damage and neuronal degeneration mediated by seizures partially contribute to the formation of lipofuscin in the hippocampus (*40*). Associated with this observation comes the hypothesis that oxidative stress could be one of the primary factors in the accumulation of lipofuscin (*41–47*). Several reports have demonstrated that lipofuscin deposits represent the accumulation of the peroxidative action of free radical on membrane lipid, resulting at a considerably earlier age than for normal mice (*9*, *10*, *28*, *48*). Our present study has also provided evidence suggesting that the MP diet delivered to test SAMP8 mice resulted in individuals that exhibited enhanced levels of metabolites that elicited in vitro antioxidant properties (data not shown). The scavenging power of MP metabolites upon the DPPH free radical has been reported to be strongly correlated with the concentration of MP in a test animal's diet. In 2000, Bickford et al. (*40*) reported that antioxidant-rich diets improved cerebellar physiology and learning ability amongst aged rats. Moreover, the results of this study appear to suggest that certain nutritional interventions were able to retard the decline in the cerebellar physiology of recipient test mice, and also enhanced learning ability. We find that an MP-supplemented diet is able to significantly reduce lipofuscin accumulation within brain cells of test mice receiving such a diet. These results would appear to indicate that the normal rate of age-related deterioration of learning and memory for SAMP8 mice could be retarded somewhat by long-term dietary supplementation with MP.

In conclusion, dietary supplementation with MP can improve the locomotion activity of male SAMP8 mice. The total aging-grading score for SAMP8 mice, as assessed at 11 mo of age, was significantly lower for mice having received a diet supplemented with MP for both male and female SAMP8 mice. It would appear that certain age-related changes as regards learning and memory can be improved by application of an MP-supplemented diet for SAMP8 mice. In the present study, these results have suggested that dietary supplementation with *Monascus*-fermented products (MP) for

test mice could be considered to effectively contribute to the retardation of the aging process for such individuals, as well as contributing to an improvement in their learning ability, and encouraging a reduction in memory deterioration with age.

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