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ORIGINAL ARTICLE: BIOLOGY

American ginseng improves neurocognitive function in senescence-accelerated mice: Possible role of the upregulated insulin and choline acetyltransferase gene expression

Shun Shi, Reika Shi and Kiyoshi Hashizume

Department of Aging Medicine and Geriatrics, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine, Matsumoto, Japan

Aim: To investigate the effects of American ginseng on neurocognitive function and glucose regulation in senescence-accelerated mice.

Methods: Male senescence-resistant inbred strains (SAMR1) and senescence-prone inbred strains (SAMP10) mice were divided into five groups and fed either a control diet or an American ginseng-supplemented diet (1% or 2% g/g) from 6 weeks to 10 months of age. Bodyweight, levels of fasting plasma glucose (FPG) and grading scores were monitored every month and neurocognitive functions were evaluated at 9 months of age with a KUROBOX apparatus using a stress-free positive cue task. Gene expressions of peroxisome proliferator-activated receptor delta (PPAR-δ), insulin, choline acetyltransferase (ChAT) and amyloid precursor protein (APP) in the brain were measured by real-time quantitative reverse transcription polymerase chain reaction assays.

Results: American ginseng decreased FPG in SAMR1 mice, but increased FPG in SAMP10 mice. Correct visit ratios were higher in both SAMR1 and SAMP10 strains consuming an American ginseng-supplemented diet. Gene upregulation of insulin and ChAT in the brain, but not of PPAR-δ or APP, was evident in American ginseng-fed groups.

Conclusion: Daily consumption of American ginseng induced an enhancement in neurocognitive function in senescence-accelerated mice, which could be related to the upregulation of insulin and ChAT gene expression in the brain. **Geriatr Gerontol Int 2012; 12: 123–130.**

Keywords: American ginseng, choline acetyltransferase, insulin, neurocognitive function, senescence-accelerated mouse.

Introduction

According to the statistics of the Japan Ministry of Internal Affairs and Communication, 23.1% of the population were 65 years or older in 2010 and this proportion is expected to increase to 39.6% by 2050. The morbidity risk of many diseases increases with aging, among which the consolidation of dementia and diabetes is drawing people's attention.[1,2] Two of the most common causes of dementia in elderly people are Alzheimer disease (AD) and vascular dementia (VD). Diabetes mellitus almost doubles the risk of dementia (relative risk 1.9),[1] while 81% of patients with AD have either type 2

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Correspondence: Dr Shun Shi MD, Ote 2-2-10-802, Matsumoto, Nagano 390-0874, Japan. Email: shijunworks@hotmail.com

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diabetes or impaired glucose regulation.[3] Although the correlation of diabetes with dementia has been reported in many papers,[1–3] the mechanism underlying these observations is still unclear. As a result, the progress of medical intervention has fallen far behind the rapidly increased cases of aging-related dementia.

The ginseng family of herbal medicines drew our attention as they were reported to have effects on both improving the function of the central nervous system (CNS) and regulating the levels of serum glucose.[4–9] Ginseng (Asia ginseng), the root of *Panax ginseng*, has been used for over 2000 years as a panacea and promoter of longevity. American ginseng, the root of *Panax quinquefolium*, was initially introduced to China as a succedaneum in 1694 when Asia ginseng production was insufficient to meet the public needs. Ginseng and American ginseng both have ginseng saponins as their important bioactive components, with ginsenoside Rg1, Rb2 and Rc being richer in ginseng, while Rb1, Re and Rd being more abundant in American ginseng. They have common pharmacological effects, such as enhancing learning and memory, anti-hyperglycemia and alleviating fatigue.[10,11] Because the stimulating property of ginseng may exacerbate hypertension, American ginseng is preferred by elderly people who are suffering from hypertension, hyperglycemia and impaired cognitive performance simultaneously.

In this study, we investigated the influence of American ginseng on neurocognitive function and glucose regulation using senescence-accelerated mice (SAM). The SAM strains, established by Takeda *et al.*, are a group of related strains consisting of senescence-prone (SAMP) and senescence-resistant (SAMR) inbred strains.[12,13] The SAMP series exhibit accelerated aging with a shortened lifespan compared to the SAMR series.[11] SAMP10, a substrain of SAMP developed by Shimada *et al.*, showed accelerated brain atrophy and cognitive dysfunction.[14–16] The brain atrophy of SAMP10 mice was characteristic of synaptic loss in the frontal cortex.[17,18] Because the loss of neocortical synapses is believed to be one of the causes of intellectual decline in aged people, we used this strain as a model of aging-related cognitive disorder. SAMR1, one of the substrains of normal aging, was used as a control.

Methods

Animals and experimental design

Four-week-old male SAMR1/TaSlc and SAMP10 /TaSlc mice were purchased from Japan SLC (Shizuoka Japan) and bred three per cage under conventional conditions in a temperature and humidity controlled room with a 12 h dark/12 h light cycle (7:00 to 19:00) and free access to food and drinking water.

Woods grown American ginseng (*P. quinquefolium* L.) was purchased from Hsu's Ginseng Enterprises (Wausau, WI, USA) and made into powder with a grinder. The custom preparations of normal CE-2 diet and American ginseng-supplemented CE-2 diet containing 1% or 2% (g/g) of American ginseng powder were ordered from Clea (Tokyo, Japan).

Twenty-four SAMR1 and 36 SAMP10 mice were divided into two groups of R1 and three groups of P10, respectively. They were fed either a normal CE-2 diet (R1C, P10C) or a CE-2 diet containing 1% or 2% (g/g) American ginseng (R1G1, P10G1, P10G2) from 6 weeks of age. Diet was weighted and added twice a week. Bodyweight and levels of fasting plasma glucose were measured every month from 1 month of age. A grading score system was used to evaluate the degree of senescence once a month from 3 months of age.[12,13] Neurocognitive function was tested at 9 months of age. Mice were killed by withdrawing blood from the abdominal aorta after being anaesthetized with diethyl ether, and the whole brains were rapidly excised and cut into two parts along the longitudinal cerebral fissure and submerged immediately in RNAlater solution (Ambion; Applied Biosystems, Foster City, CA, USA). After being kept at 4°C overnight, brain samples were stored at −20°C until the isolation of total RNA. In compliance with the national regulations and guidelines, all experimental procedures were reviewed by the Committee for Animal Experiments and finally approved by the president of Shinshu University.

Monitoring the degree of senescence

The senescence degree was evaluated according to a grading score system which had been established by Takeda *et al.*[12,13] Mice were examined by inspection and palpation every month. Each mouse was scored according to the criteria set for 11 individual categories: reactivity, passivity, glossiness, coarseness, loss of hair, skin ulcers, periophthalmic lesions, corneal opacity, ulcer of the cornea, cataract and lordokyphosis of the spine. The scores in each category were summed to give a total grading score for each mouse from 3 months of age.

Monitoring the levels of fasting plasma glucose (FPG)

From 1 month of age, blood samples of fasted mice were acquired monthly from the tail vein and analyzed with the FreeStyle FREEDOM blood glucose monitoring system (NIPRO, Osaka, Japan).

Neurocognitive functional test

The neurocognitive function was tested at 9 months of age in the KUROBOX Memory Version (Phenotype

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Analyzing, Nagasaki, Japan).[19,20] The apparatus used in this experiment consists of five individual boxes, a controller and a computer. Each box consists of two parts; the back part is used as a nest and the front part for observation, where the locomotion of a mouse is monitored by infrared photosensors. The mouse can move freely from the nest to the observation field to drink water from a nozzle or eat food from the food station. Four food stations are set at the four corners of the observation field, but the mouse can get food from only one of the stations and this correct food station changes in a counterclockwise direction every 4 h. These four corners are called regions of interest (ROI) and attempts to visit ROI were counted when the mouse stayed in an ROI for more than 6 s. The mouse was put into the nest of the box at 17:00 for a 2-h adaption and the experiment was finished at 19:00 of the following day. A correct visit ratio (the ratio of visits to the correct ROI where food is accessible to the number of visits to all ROI) was calculated according to the locomotion record monitored from the first 19:00 to the second, namely, just one dark and light cycle. The box was washed and dried before the next mouse was tested.

RNA extraction

Total RNA from brain samples was extracted with a combination of TRIZOL® Reagent (Invitrogen, San Diego, CA, USA) and RNeasy® Mini Kit (QIAGEN, Hilden, Germany). An on-column DNase digestion was performed with the RNase-Free DNase Set (QIAGEN). RNA integrity was checked by electrophoresis on 1% agarose gel. The concentration of RNA was determined by measuring the absorbance at 260 nm (A_{260}) in an Ultrospec 3000 UV/visible spectrophotometer (Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and purity was confirmed by the ratio of A_{260}/A_{280}. Total RNA was stored at –20°C until the synthesis of cDNA.

Real-time quantitative reverse transcription polymerase chain reaction (qRT–PCR) assays of gene expression

The mRNA levels of peroxisome proliferator-activated receptor (PPAR)-δ, insulin, choline acetyltransferase (ChAT) and amyloid precursor protein (APP) were measured by real-time qRT–PCR using a 7300 Real Time PCR System (Applied Biosystems). The rationales for selecting these four genes were:

1. PPAR-δ, a nuclear receptor of the PPAR family playing important roles in adipocyte growth and insulin responsiveness, is found to be significantly lower in the brain of patients with AD.[21,22]
2. Gene expression and function of insulin deteriorates with progression of AD.[23,24]
3. Aging-related cognitive deficits are probably due to cholinergic hypofunction appearing with advancing age.[25] Gene expression of ChAT, the enzyme responsible for acetylcholine synthesis, is reduced in AD.[23]
4. APP is the precursor of amyloid β-peptide (Aβ), which is believed to be a main contributor to the dysfunction and degeneration of neurons that occur in AD.[26] Increased expression of APP is linked to learning and memory loss in SAMP8 mice (another substrain of SAMP). However, there are as yet no reports concerning the APP or Aβ in SAMP10 mice.[18,27]

Total RNA (0.5 μg) was reversely transcribed using the QuantiTect Reverse Transcription Kit (QIAGEN). PCR amplifications were performed in 50 μL reactions containing 2 μL aliquots of cDNA generated from 20 ng of original RNA template, 300 nM each of gene-specific forward and reverse primers (Table 1),[23,28]

Table 1 Primer pairs used for real time quantitative reverse transcription polymerase chain reaction

Primer	Direction	Sequence (5′→3′)	Position (mRNA)	Amplicon size (bp)
PPAR-δ	For	TGT CAA CAA AGA CGG ACT GCT G	1136	107
PPAR-δ	Rev	CGA ACT TGG GCT CAA TGA TGT C	1242	
Insulin	For	TTC TAC ACA CCC AAG TCC CGT C	145	135
Insulin	Rev	ATC CAC AAT GCC ACG CTT CTG C	279	
ChAT	For	TCA CAG ATG CGT TTC ACA ACT ACC	478	106
ChAT	Rev	TGG GAC ACA ACA GCA ACC TTG	583	
APP	For	GCA GAA TGG AAA ATG GGA GTC AG	278	199
APP	Rev	AAT CAC GAT GTG GGT GTG CGT C	476	
18S	For	GGA CAC GGA CAG GAT TGA CA	1278	50
18S	Rev	ACC CAC GGA ATC GAG AAA GA	1327	

Table of forward (For) and reverse (Rev) primer pairs used to measure mRNA levels of specific genes expressed in mice brains using real-time quantitative reverse transcription polymerase chain reaction. APP, amyloid precursor protein; ChAT, choline acetyltransferase; PPAR-δ, peroxisome-proliferator activated receptor-δ.

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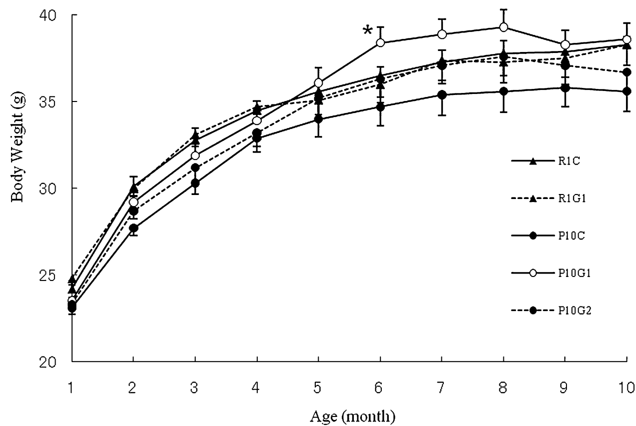

Figure 1 Growth pattern of R1C, R1G1, P10C, P10G1 and P10G2. Data are shown as the mean ± standard error of bodyweight ($n = 9–12$). *$P < 0.05$ (vs P10C).

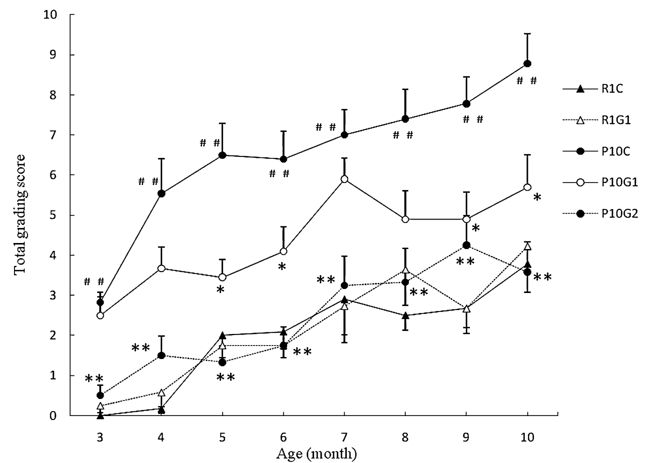

Figure 2 Changes of total grading scores of R1C, R1G1, P10C, P10G1 and P10G2 with advancing age. Data are shown as the mean ± standard error ($n = 9–12$). *$P < 0.05$ (vs P10C), **$P < 0.01$ (vs P10C), $^{##}P < 0.01$ (vs R1C).

25 μL 1 × FastStart Universal SYBR Green Master (ROX; Roche Diagnostics, Mannheim, Germany) and 13 μL Nuclease-Free Water (QIAGEN). The amplification protocol was as follows: initial activation at 95°C × 15 min, 45 cycles of 94°C × 15 s, 60°C × 30 s and 72°C × 45 s. Serial twofold dilutions of cDNA from 80 ng of RNA template were used for building the regression curves for each mRNA. Ribosomal 18S RNA levels measured in parallel reactions were used to calculate relative abundance of each mRNA (mRNA/18S RNA ratio). Control studies included analysis of template-free reactions and RNA that had not been reversely transcribed. All assays were repeated in triplicate and the intrarater reliability was checked by using the intraclass correlation coefficients (ICC) test.

Statistical analysis

Statistical analysis was performed using SPSS ver. 18.0J. Data depicted in the graphs represent the means ± standard error for each group, with 9–12 animals being studied per group. Difference between R1C and R1G1 was analyzed using an independent sample Student's *t*-test. One-way ANOVA and Tukey's honest significant difference multiple comparisons were used to identify the difference between P10C, P10G1 and P10G2 groups. Significance tests were two-sided, with an α-level of 0.05.

Results

Changes in bodyweight

Bodyweight rapidly increased without significant differences among all groups up to 4 months of age (Fig. 1). Although statistical difference was not observed, bodyweight of P10C was constantly lower than that of R1C during the whole breeding period. Furthermore, it was noticed that American ginseng treatment increased the bodyweight of SAMP10 mice; however, no obvious influence was observed in SAMR1 mice.

Changes in senescence grading score

The total grading score of P10C was significantly higher than that of R1C from 3 months of age, suggesting that the aging process is accelerated in P10C mice (Fig. 2). P10G1 and P10G2 showed lower grading scores compared to that of P10C, with P10G2 being significantly lower than P10C at any age from 3 months of age. However, R1G1 and R1C showed similar grading scores throughout the breeding period.

Changes in FPG

American ginseng showed different effects on FPG levels between SAMR1 and SAMP10 mice. As shown in Figures 3 and 4, American ginseng decreased FPG in SAMR1 mice, but increased FPG in SAMP10 mice.

Changes in neurocognitive function

Neurocognitive function determined by correct visit ratio was significantly lower in P10C compared to that of R1C. Because the correct visit ratio is an indicator for detecting the ability of learning and memory, it is possible to speculate that these activities were markedly diminished in P10C. Correct visit ratios were increased in both SAMR1 and SAMP10 mice fed with an American ginseng-supplemented diet (Fig. 5).

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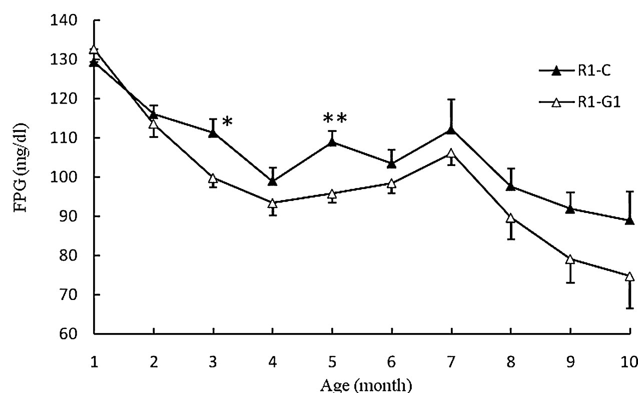

Figure 3 Changes of fasting plasma glucose (FPG) of R1C and R1G1 with advancing age. Data are shown as the mean ± standard error ($n = 9$–12). *$P < 0.05$ (vs R1C), **$P < 0.01$ (vs R1C).

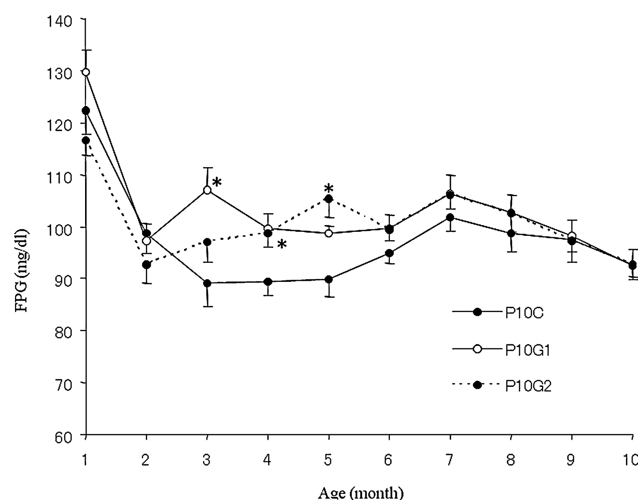

Figure 4 Changes of fasting plasma glucose (FPG) of P10C, P10G1 and P10G2 with advancing age. Data are shown as the mean ± standard error ($n = 9$–12). *$P < 0.05$ (vs P10C).

Changes in gene expression of PPAR-δ, insulin, ChAT and APP

Significant changes were observed in insulin and ChAT gene expression, but not in PPAR-δ and APP (Fig. 6). American ginseng treatment increased the expression of insulin in both SAMR1 and SAMP10 mice, with R1G1 and P10G1 being statistically different from the relative control groups. The expression of ChAT was also increased by American ginseng treatment, significant differences were observed between treatment groups (R1G1 and P10G2) and the respective control groups (R1C and P10C). Differences of the expression of insulin and ChAT between R1C and P10C were not significant.

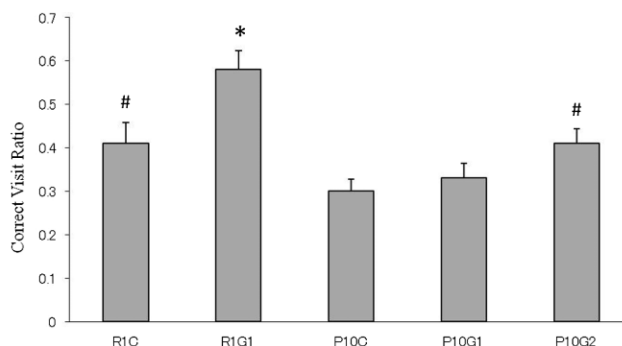

Figure 5 Correct visit ratios of R1C, R1G1, P10C, P10G1 and P10G2. Graph depicts the mean ± standard error ($n = 9$–12). *$P < 0.05$ (vs R1C), $^{\#}P < 0.05$ (vs P10C).

Discussion

The results of this study showed a protective effect of American ginseng on SAMP10 mice, reflected in better neurocognitive function, suppressed senescence and increased bodyweight. The bodyweight of P10C was constantly lower than that of R1C during breeding. American ginseng treatment increased the bodyweight of SAMP10 mice, showing a similar growth pattern to that of SAMR1 mice. However, the upregulation of growth was not due to more food consumption, as no obvious difference in food consumption was observed (data not shown). A similar result was reported in the study of the anti-aging effect of garlic by Moriguchi *et al*.[29] Lower total grading scores were found in SAMP10 mice consuming an American ginseng diet, which suggested that accelerated senescence was suppressed by the administration of American ginseng. American ginseng and its components (ginsenoside) were found to have antioxidative effects on cells and animals.[30–33] Because oxidative stress within mitochondria has been recognized as one of the most important causes of aging,[34] it was thus assumed that the antioxidative properties of American ginseng may be related to this anti-aging effect on SAMP10 mice.

Mice are usually nocturnal, even though the SAMP10 mice showed a somewhat altered circadian rhythm. Traditional learning and memory tests of mice including the fear conditioning task, water maze test and radial arm maze test use stressful or negative cues and cannot be performed during the dark period. Here, we evaluated the neurocognitive performance of mice with a KUROBOX apparatus using stress-free positive cue tasks.[19,20] Human interventions were reduced to the greatest extent to get more reliable evaluation on the performance of mice. In this experiment, American ginseng treatment improved the neurocognitive performance of both SAMR1 and SAMP10, showing higher correct visit ratios compared to their control. For more in-depth understanding of the mechanism of this

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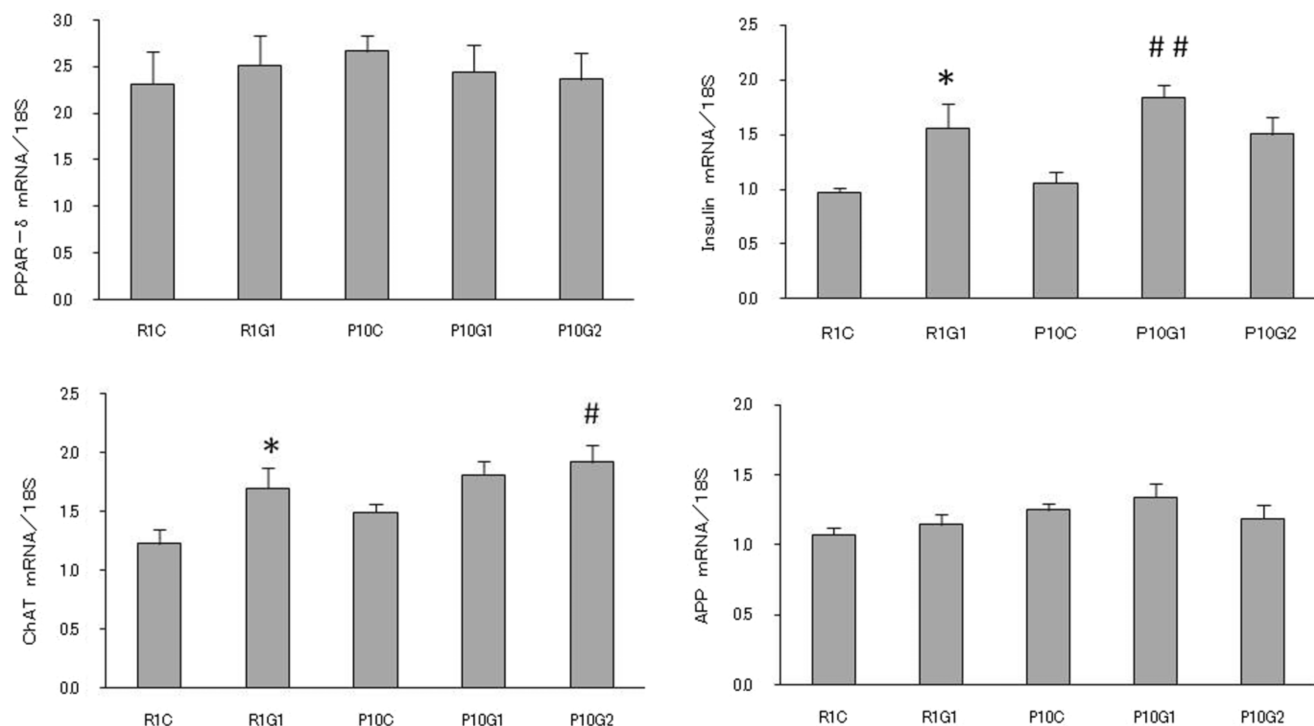

Figure 6 Relative gene expression levels of peroxisome proliferator-activated receptor (PPAR)-δ, insulin, choline acetyltransferase (ChAT) and amyloid precursor protein (APP). Graphs depict the mean ± standard error of relative ratios of specific mRNA to 18S RNA (n = 9–12). $^{*}P < 0.05$ (vs R1C), $^{\#}P < 0.05$ (vs P10C), $^{\#\#}P < 0.01$ (vs P10C).

cognitive improving activity, we analyzed the gene expression of PPAR-δ, insulin, ChAT and APP, which are considered to play important roles in neurocognitive function or neurodegenerative pathologies. The importance of insulin action in the CNS has been highlighted in recent years. Insulin is neurotrophic and can promote neuronal survival by inhibiting apoptosis.[24,35] Its improving activity on cognitive performance has been proved in humans and animal models.[24,36] AD is associated with brain insulin/insulin-like growth factor (IGF) deficiency and/or resistance, and even recognized as a "type 3 diabetes".[37–39] ChAT (the enzyme responsible for acetylcholine synthesis), which is expressed in insulin receptor-positive cortical neurons, is found to be reduced in AD.[23] It has been reported that ginsenoside Rb1, one of the main constituents of American ginseng, increased the expression of ChAT in the basal forebrain.[40] A similar result was found from our experiments that gene expressions of insulin and ChAT were upregulated in mice having an American ginseng-supplemented diet. We think that the neurocognitive improving activity of American ginseng may be closely related to the gene upregulation of insulin and ChAT in the brain. More in-depth experiments are under planning to determine which cells of which parts of the brain were involved in the upregulated expression of insulin and ChAT.

The anti-hyperglycemic effect of ginseng and American ginseng has long been recognized and used in traditional medicine for diabetes-like conditions.[41,42] American ginseng reduced postprandial glycemia in non-diabetic subjects and subjects with type 2 diabetes mellitus.[8] Mechanisms of the hypoglycemic effect of American ginseng were suggested to be its activity of increasing insulin production and reducing apoptosis of pancreatic β cells.[43] An interesting effect of American ginseng on FPG was observed in this study. FPG was decreased in SAMR1 but increased in SAMP10 mice by American ginseng treatment. Whether it is a decreasing or increasing effect seems to depend on the pathophysiological status of the subject. American ginseng has multiple constituents and a living body's different adaption abilities may allow it to exhibit different effects. Such phenomenon is termed "dual modulation" in traditional Chinese medicine (TCM) and has been observed in other herbal medicines as well.[44,45] This is the first report of an increasing effect of American ginseng on FPG in SAMP10 mice, a model of accelerated aging. There is evidence that impaired cognition was associated with impaired glucose regulation in aged animals. Following the glucose injection, improved cognitive performance in aged animals was found to correlate with the elevated blood glucose levels.[46] That FPG of P10C was constantly lowest among all groups before

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8 months of age (Figs 3,4) might suggest an impaired glucose regulation in SAMP10 mice. American ginseng may have different effects on FPG or pancreatic insulin secretion in different models. Mechanisms underlying this dual modulation of American ginseng on FPG and the consequent influence on cognitive performance are of great interest for further exploration.

Daily consumption of American ginseng showed anti-aging effect and improved the neurocognitive function in SAMP10 mice. The gene upregulation of insulin and ChAT in the brain may be associated with this cognitive improvement. Enhancement of neurocognitive function by American ginseng on healthy adults was confirmed in an acute, randomized, placebo-controlled, cross-over study recently.[5] More in-depth study of American ginseng on aging-induced neurodegeneration models or populations, which focus on the insulin/IGF signaling, mitochondrial function and glucose utilization, will contribute to the better understanding of senile dementia and shed light on medical interventions.

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