Review

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Anti-aging effects of oligomeric proanthocyanidins isolated from persimmon fruits

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ABSTRACT: Senescence-accelerated mouse prone/8 (SAMP8), a murine model of accelerated senescence, shows age-related deficits in learning and memory. The oral administration of oligomers improved spatial and object recognition impairment in SAMP8. The expression of phosphorylated neurofilament-H was significantly elevated in the hippocampal CA1. This indicates that oligomers induce an increase in the density of axons. To investigate the protective mechanisms of oligomers against brain dysfunction with aging, we carried out a receptor tyrosine kinase phosphorylation antibody array, and clarified that the administration of oligomers led to an increase in the phosphorylation of vascular endothelial growth factor receptor (VEGFR)-2, suggesting the neuroprotective role of oligomers. The phosphorylation of VEGFR-2 was more markedly increased in the hypothalamus and choroid plexus than in other brain regions of SAMP8. Memory in oligomer-treated mice was impaired by SU1498, a VEGFR-2-specific antagonist. Elucidating the relationship between memory impairment with aging and VEGFR-2 signaling may provide new suggestions for protection against memory deficit in the aging brain. In addition, we revealed that the administration of oligomers extended the life span of SAMP8. Oligomers elevated SIRT1 expression, which is recognized as an essential factor for life span extension in the brain. However, the administration of oligomers did not induce stereotypical behaviors such as rearing, jumping, or hanging from the lid of a cage, while food restriction increased these frequencies without a significant change in motor function. The present study suggests the promising role of oligomers as an anti-aging agent to extend life span.

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1. Introduction

The senescence-accelerated mouse (SAM), a murine model of accelerated senescence, was developed by Takeda *et al*. (*1*). The phenotype shows a shortened life span with an acceleration of several distinctive disease processes such as osteoporosis (P6), degenerative joint disease (P3), cataract (P9), hyperinflammation of the lungs (P1), and hearing impairment (P1) (*2*). One of these strains, SAMP8, was found to have age-related deficits in learning and memory. Numerous age-dependent alterations have been found in the brain of SAMP8, such as cortical atrophy in the pyriform cortex, increased axonal dystrophy in the gracile nucleus, spongiform degeneration in the brainstem reticular formation, periodic acid Schiff-positive granular structures in the hippocampus, βA4 protein-like immunoreactive granular structures in various regions, and blood-brain barrier dysfunction (*3*). There is increasing evidence showing that SAMP8 has a similar pathology to the human brain in regard to Alzheimer's disease as well as normal aging (*2,4*). Acetyl-L-carnitine (*5*), α-lipoic acid (*6*), and caloric restriction (*7*) ameliorated memory impairment in SAMP8. Anti-oxidative activity limited to the cerebral cortex was suggested as the underlying mechanism of those treatments (*8*). Considering neuronal degeneration in various brain regions of SAMP8, amelioration of injury in the cerebral cortex may not be sufficient. Therefore, identifying compounds which improve memory deficits in SAMP8 and analyzing the underlying mechanism other than the anti-oxidative effect are important.

Proanthocyanidins are known as condensed tannins, members of a specific group of polyphenolic compounds, and they have been reported to exhibit powerful antioxidant activity (*9,10*). Although proanthocyanidin is the most abundant dietary polyphenol, its high-level

polymerization results in limited absorption *in vivo* (*11*). We previously isolated oligomeric proanthocyanidins from persimmon peel, which is usually discarded even though it is rich in phenolic compounds (*12*). The amount of proanthocyanidin in the peel is higher than in the rest of the fruit. It was reported that oligomeric proanthocyanidins (oligomers) isolated from persimmon peel increased the expression of silent information regulator two ortholog 1 (SIRT1), which is recognized as an essential factor in life span extension, in an H_2O_2-induced cellular senescence model. Oligomer treatment also decreased the expression level of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidation in the model (*13*). In the present study, we hypothesized that the oligomeric form of proanthocyanidins exerts a beneficial effect on memory dysfunction and neuroprotection in the aged brain. Using the SAMP8 model, we investigated the effect of oligomers on spatial and object recognition memory, and the densities of axons, dendrites, and synapses were observed. Furthermore, to evaluate the neuroprotective effect, vascular endothelial growth factor receptor (VEGFR)-2 and its phosphorylation were also investigated. Moreover, we investigated the possibility of oligomers extending the life span of SAMP8 mice. Since dietary restriction extends the life span of rodents, we compared food-restricted with oligomer-treated mice regarding longevity and behavioral characters.

2. Oligomeric proanthocyanidins improve memory and enhance phosphorylation of VEGFR-2 in SAMP8

SAMP8 developed age-related cognitive deficits as early as 4 months and had a short life span relative to SAMR1. SAMP8 show decreased release of acetylcholine and noradrenaline in comparison with age-matched SAMR1 (*14,15*). Many age-dependent alterations in various brain regions such as the cerebral cortex and hippocampus at an early stage in SAMP8 were suggested as causes of memory deficits (*16,17*). In the hippocampus, an increase of glial fibrillary acidic protein as an astrocyte marker was observed in the CA1-CA3 regions of SAMP8 compared to age-matched SAMR1, indicating enhanced reactive gliosis in aged SAMP8 (*18*). Tanaka *et al.* (*19*) reported severe loss of oligodendrocytes in the hippocampal CA1 of SAMP8 mice. Moreover, neuronal loss and lower expression of glial cell line-derived neurotrophic factor in the hippocampal CA1 associated with memory impairment of SAMP8 were reported (*20*). Therefore, hippocampal dysfunction of SAMP8 has been considered as a major cause of age-dependent memory impairment in the brain. Various candidate therapeutic agents for memory dysfunction in SAMP8 were reported, such as acetyl-L-carnitine, α-lipoic acid, and Choto-san (a herbal formula), along with caloric restriction (*5-7,21*). In those studies, oxidative stress was focused on as a cause of memory impairment of SAMP8, although change of oxidative stress was limited to the cerebral cortex. Additionally, neuronal morphological evaluations were insufficient in those studies.

We previously reported that oligomers attenuated the expression level of 8-OHdG as a DNA damage maker and increased the expression level of SIRT1, which is recognized as an essential factor in lifespan extension in an H_2O_2-induced cellular senescence model (*13*). These bioactivities were stronger in the oligomer-treated group than the group treated with non-oligomerized proanthocyanidins showing a high-level of polymerization. Aging is a progressive physiological change in an organism that leads to senescence, or a decline in biological functions and the organism's ability to adapt to metabolic stress. The process decreases the prevalence of learning and increases memory deficits. For example, in aged rats, a loss of synapses in the dentate gyrus and an alteration of Ca^{2+} regulation in the CA1 area lead to a decline of synaptic plasticity, resulting in a change in interactions among hippocampal networks and deficits in the storage and retrieval of information regarding spatial organization of the environment (*22*). Therefore, we expected the anti-aging effect of oligomers to improve age-associated memory impairment. In the present study, oligomers improved spatial memory and object recognition memory in SAMP8. The memory improvements seen in 18-week-old and 38-week-old SAMP8 led to memory levels almost the same as those of SAMR1 (Figures 1 and 2). To investigate neurological changes brought about by the oral administration of oligomers, we carried out an immunohistological analysis in the brain of 59-week-old SAMP8. The oral administration of oligomers increased expression levels of phosphorylated neurofilament-H (p-NF-H), microtubule-associated protein 2 (MAP2), and synaptophysin in the hippocampus, but this was not observed in regions of the cerebral cortex and striatum of SAMP8. In particular, expression of p-NF-H significantly increased in the hippocampal CA1 with oligomer administration (Figure 3). p-NF-H is used as a marker of axons, since the phosphorylated form of NF-H is translocated into axons (*23*). In the hippocampus of aged mice, fragments of degenerated axons were also increased, although reductions of neuronal numbers were small in this region (*24*). Axonal termination to the spine is a necessary step for synaptogenesis. Considering synaptic losses in the hippocampal CA1 and CA3 and the parietal cortex in SAMP8 (*25*), as well as in the hippocampal CA1, CA3, and dentate gyrus in aged rats (*26*), axonal regeneration is important for improving hippocampal function. Therefore, the increased density of axons in the hippocampal CA1 was suggested to perform a protective role against memory deficit with aging.

Other previous studies suggested that oxidative stress is a major cause of memory impairment in SAMP8. Hippocampus-specific modulation by oligomers is

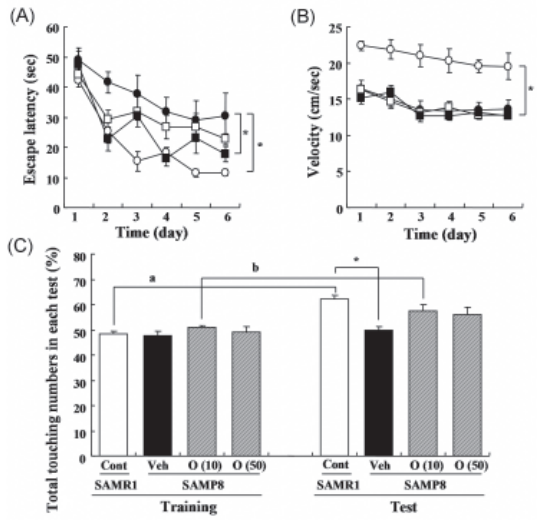


Figure 1. Effects of oligomers on spatial memory deficit in SAMP8. Eighteen-week-old SAMP8 were administered vehicle (Veh, water *p.o.*, $n = 6$; closed circles) or oligomers (10 mg/kg body weight/day, *p.o.*, $n = 6$; open squares or 100 mg/kg body weight/day, *p.o.*, $n = 5$; closed squares) for 5 weeks. SAMR1 were used as a control (Cont, $n = 5$; open circles). Fifteen days after administration started, memory acquisition tests were continued for 6 days in a Morris water maze. Administration was continued during the tests. Escape latencies to a hidden platform were measured **(A)**. Swimming velocities of mice in the memory acquisition test are shown **(B)**. Thirty-eight-week-old SAMP8 were administered vehicle (Veh, water *p.o.*, $n = 7$) or oligomers (O (10), 10 mg/kg body weight/day, *p.o.*, $n = 7$ or O (50), 50 mg/kg body weight/day, *p.o.*, $n = 7$). Age-matched SAMR1 were used as a control (Cont, $n = 7$). Twenty eight days after administration started, an object location test was performed. The preference index was defined as the number of times a mouse made contact with any one of the objects (training session) or the moved object (test session) out of the total number of times the mouse made contact with both objects (%) **(C)**. * $p < 0.05$ *vs.* Veh. (**A** and **B**: Repeated measures two-way ANOVA followed by Dunnett's or Bonferroni's *post-hoc* test); * $p < 0.05$ (**C**: One-way ANOVA followed by Bonferroni's *post-hoc* test); [a] $p = 0.0005$; [b] $p = 0.0213$ (**C**: paired *t*-test).

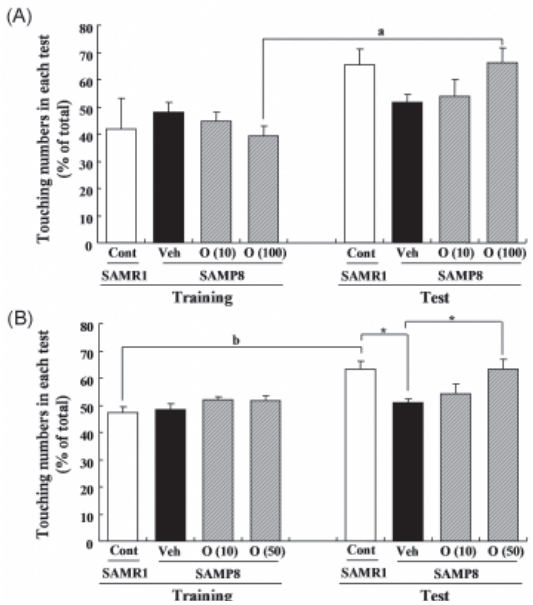


Figure 2. Effects of oligomers on object recognition memory deficit in SAMP8. Eighteen-week-old SAMP8 were administered vehicle (Veh, water *p.o.*, $n = 6$) or oligomers (O (10), 10 mg/kg body weight/day, *p.o.*, $n = 6$ or O (100), 100 mg/kg body weight/day, *p.o.*, $n = 5$). Age-matched SAMR1 were used as a control (Cont, $n = 5$). Twenty four days after administration started, a novel object recognition test was performed **(A)**. Thirty-eight-week-old SAMP8 were administered vehicle (Veh, water *p.o.*, $n = 7$) or oligomers (O (10), 10 mg/kg body weight/day, *p.o.*, $n = 7$ or O (50), 50 mg/kg body weight/day, *p.o.*, $n = 7$). Age-matched SAMR1 were used as a control (Cont, $n = 7$). Twenty three days after administration started, a novel object recognition test was performed **(B)**. The preference index was defined as the number of times a mouse made contact with any one of the objects (training session) or the novel object (test session) out of the total number of times the mouse made contact with both objects (%). * $p < 0.05$ (One-way ANOVA followed by Bonferroni's *post-hoc* test); [a] $p = 0.0174$; [b] $p = 0.0014$ (paired *t*-test).

not explained by an antioxidative effect, since only the cerebral cortex is susceptible to oxidative stress in SAMP8 and not the hippocampus (*8*). Therefore, to investigate target molecules following the oral administration of oligomers in the brain of SAMP8, we performed a receptor tyrosine kinase phosphorylation antibody array, and clarified that oligomer treatment increased phosphorylation of VEGFR-2 (Figure 4). Expression of VEGFR-2 was identified in the cerebral cortex, hippocampus, and choroid plexus of adults as well as neonatal rodents (*27*,*28*). However, the localization of VEGFR-2 in the brain of SAMP8 has yet to be clarified. In the present study, VEGFR-2 expression was also detected in the striatum and hypothalamus of SAMP8, as well as the cerebral cortex, hippocampus, and choroid plexus. In neurons, stimulation by VEGFR-2 among protein tyrosine kinase receptors of VEGF is linked to Akt/protein kinase B activation and neuronal protection in hypoxic preconditioning (*29*). Moreover, VEGFR-2 mediated a protective effect through phosphatidylinositol-3-kinase/Akt- and mitogen-activated protein/extracellular signal-regulated kinase-signalling pathways in glutamate-induced toxicity (*30*). In particular, the memory enhancement shown by mice injected with recombinant adeno-associated viral vectors expressing human VEGF was inhibited by injection of dominant-negative mutant VEGFR-2 (*31*). This indicates that VEGF/VEGFR-2 is directly associated with neuronal signaling. VEGF also exerts indirect effects on neurons. Moreover, the topical administration of VEGF to the surface of the brain reduces the infarct size, and intraventricular VEGF enhanced the survival of newly generated neurons in the dentate gyrus and subventicular zones after focal cerebral ischemia (*32*). In this study, we first showed that memory enhancement through oligomer treatment was eliminated by SU1498 (Figure 5). Considering that VEGF-E-induced memory was also inhibited by SU1498, oligomers or their metabolites may regulate memory by activation of VEGFR-2.

We elucidated that administration of oligomers increased phosphorylation of VEGFR-2 in the hippocampal CA3 regions (Figure 6), suggesting that oligomeric metabolites directly affect the hippocampus, like the VEGFR-2 ligand. It has been reported that

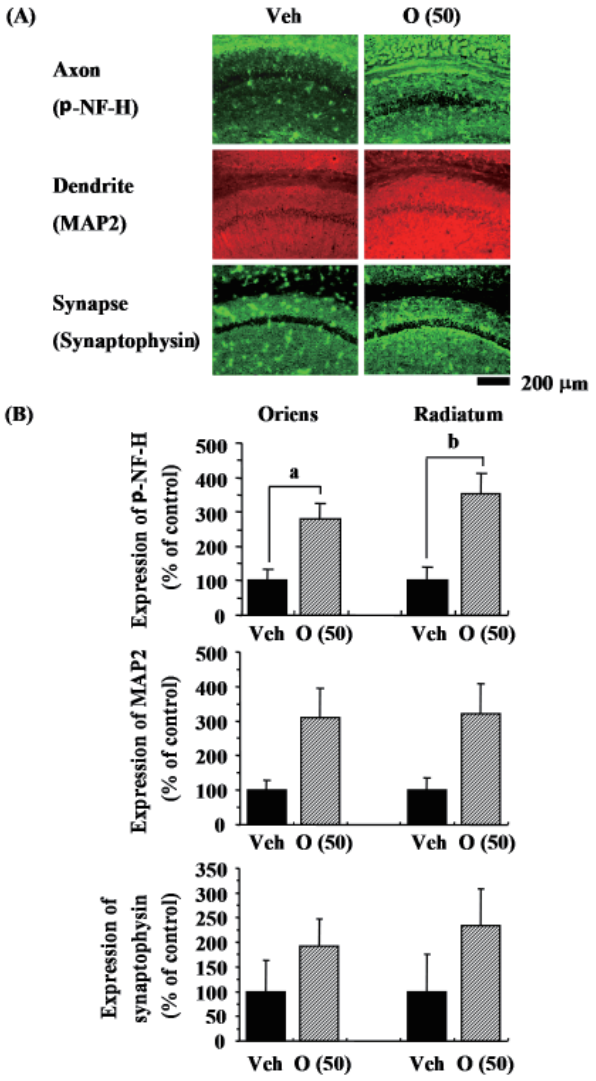


Figure 3. Effects of oligomers on the decrease of axons, dendrites, and synapses in the hippocampal CA1. Fifty-nine-week-old SAMP8 were administered vehicle (Veh, water *p.o.*, $n = 3$) or oligomers (O (50), 50 mg/kg body weight/day, *p.o.*, $n = 3$). After seven days of administration, brain slices were immunostained with p-NF-H, MAP2, and synaptophysin antibodies **(A)**. Intensities of immuno-positive areas in the stratum oriens and stratum radiatum in CA1 were quantified (**B**). [a] $p = 0.0243$; [b] $p = 0.0344$ (Student's *t*-test).

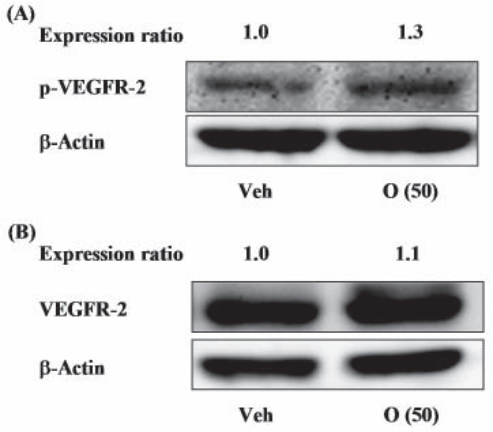


Figure 4. Effects of oligomers on p-VEGFR-2 and VEGFR-2 expressions. Fifty-nine-week-old SAMP8 were administered vehicle (Veh, water *p.o.*) or oligomers (O (50), 50 mg/kg body weight/day, *p.o.*). After seven days of administration, brain lysates were immunoblotted with antibodies for p-VEGFR-2 **(A)** or VEGFR-2 **(B)**. Expression intensities were divided by β-actin expression to generate ratios.

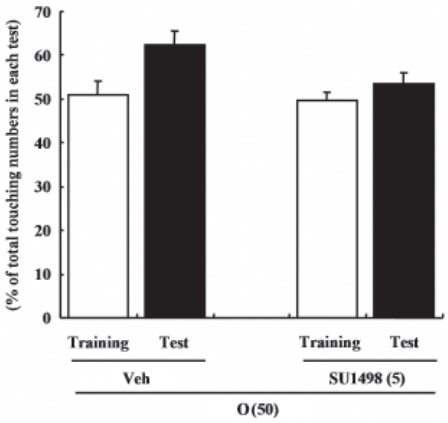


Figure 5. Effects of oligomers and VEGFR-2 on memory. Male ddY mice (6 weeks old) were administered oligomers (O (50), 50 mg/kg body weight/day, *p.o.*, $n = 4$) for 7 days. Then, the vehicle (Veh, 5% DMSO in 0.9% NaCl) was injected intracerebroventricularly at 60 min after the final administration of oligomers. Five days after vehicle injection, SU1498 (5 nmol/μL, solution is 5% DMSO in 0.9% NaCl) injected intracerebroventricularly at 60 min after the final administration of oligomers.

Ca^{2+} influx and synaptic transmission by VEGF in the hippocampus influences generation of long-term changes in synaptic efficacy (*33*). VEGF also stimulates neurite outgrowth *via* Rho/Rho kinase signaling in cerebral cortical neurons (*34*). Interestingly, changes in the synapes and neurites induced by VEGF are caused by activation of VEGFR-2 rather than VEGFR-1. Therefore, we speculated that phosphorylation of VEGFR-2 induced by administration of oligomers within the hippocampus may be related to an increase in densities of neurites and synapses in the hippocampus.

Administration of oligomers increased phosphorylation of VEGFR-2 in the hypothalamus and choroid plexus as well as hippocampus. The hypothalamus is contained in the Papez circuit. The Papez circuit is a sensory circuit involving the thalamus, sensory cortex (especially the cingulate region), hippocampus, and mammillary body of the hypothalamus (*35*). It has been reported that lesions in the Papez circuit are associated with amnesia and impairment of recognition memory (*36*). Therefore, we speculate that the hypothalamus is activated by phosphorylation of VEGFR-2, which may affect the hippocampus through the Papez circuit.

The choroid plexus is made up of numerous villi that project into the ventricles of the brain. Each villus is composed of a single layer of epithelial cells overlying a core of connective tissue and blood capillaries (*37*). The choroid plexus is involved in the most basic aspects of neural function, including maintaining the extracellular milieu of the brain by actively modulating chemical exchange between the cerebrospinal fluid and brain parenchyma, surveying the chemical and immunological

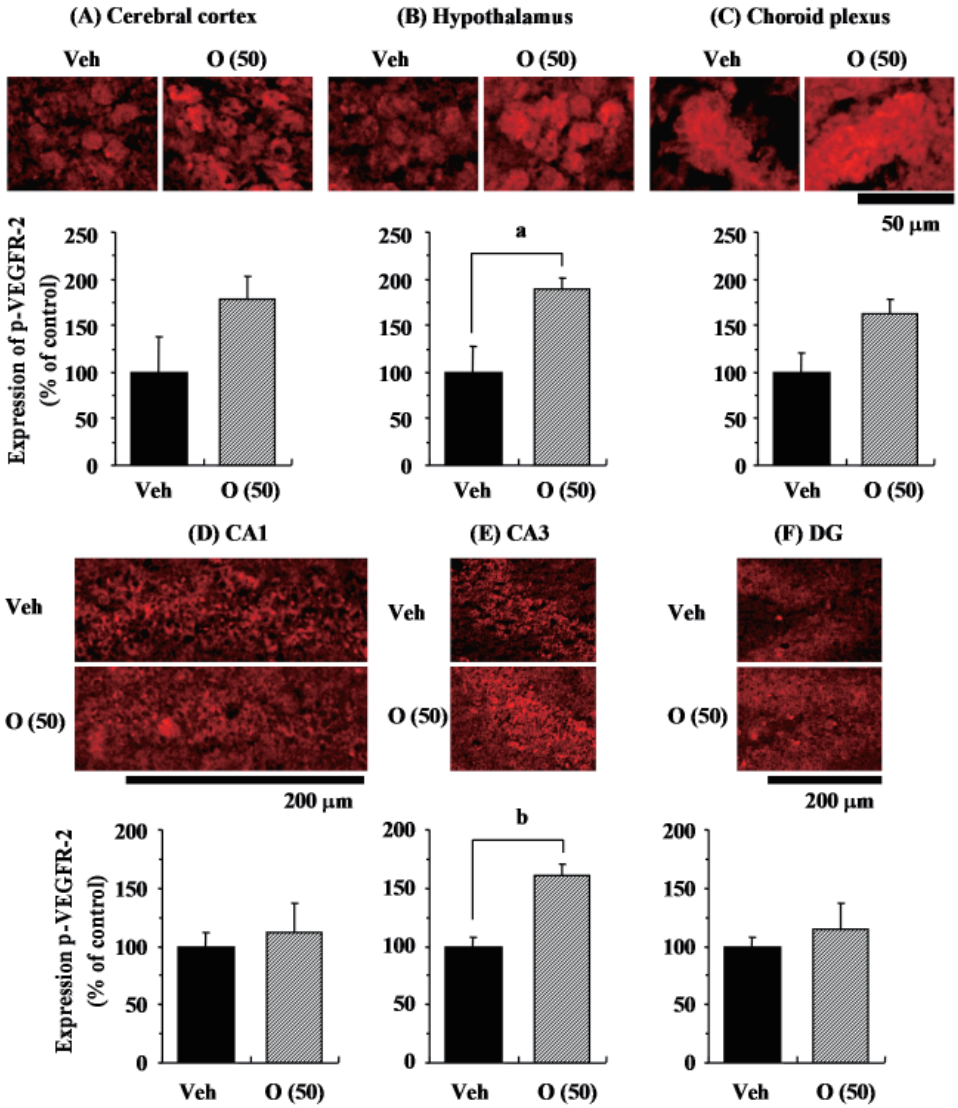


Figure 6. Effects of oligomers on p-VEGFR-2 expression in various brain regions. Fifty-nine-week-old SAMP8 were administered vehicle (Veh, water *p.o.*, $n = 3$) or oligomers (O (50), 50 mg/kg body weight/day, *p.o.*, $n = 3$). After seven days of administration, brain slices were immunostained with p-VEGFR-2 antibody. Intensities of p-VEGFR-2-positive areas were quantified in the cerebral cortex **(A)**, hypothalamus **(B)**, and choroid plexus **(C)**, and CA1 **(D)**, CA3 **(E)**, and dentate gyrus **(F)** of the hippocampus. [a] $p = 0.0429$; [b] $p = 0.0076$ (Student's *t*-test).

status of the brain, detoxifying the brain, secreting a nutritive cocktail of polypeptides, and participating in repair processes following trauma. This diversity of functions may mean that even modest changes in the choroid plexus can have far-reaching effects (*38*). Actually, a host of growth factors and other neuroprotective agents supplied by the cerebrospinal fluid can minimize the adverse effects of stroke on the rat hippocampus. Multiple functional failures including a decrease of cerebrospinal fluid as well as atrophy of choroidal epithelial cells shown in normal aging as well as advanced Alzheimer's disease indicate that the maintenance of cerebrospinal fluid through the choroid plexus may have beneficial effects against neurodegenerative diseases (*39*). Moreover, it was reported that the intracerebroventicular injection of nerve growth factor or insulin-like growth factor-1 improved memory deficit and hippocampal deterioration (*40,41*). Therefore, we speculate that oligomers induce the secretion of some peptides after the phosphorylation of VEGFR-2 in the choroid plexus, and then these peptides induce changes in the hippocampus.

We previously elucidated that the oligomers consisted of various combinations of 4 types of monomer: epigallocatechin (EGC), epicatechin (EC), epigallocatechin 3-*O*-gallate (EGCg), and epicatechin 3-*O*-gallate (ECg). Oligomers containing dimers, trimers, and tetramers of EGC, EC, EGCg, and ECg are considered to exert a stronger activity than polymers. de Boer *et al*. (*42*) demonstrated that ECg and EGCg stimulated SIRT1 more effectively than EC and EGC, since galloyl and catechol groups are essential for the repair of DNA damage. Therefore, the structural difference between oligomers and polymers is considered to be an important factor regarding proanthocyanidin's action, and it also affects utilization in biological systems. It is absorbed through the gut barrier, and its absorption

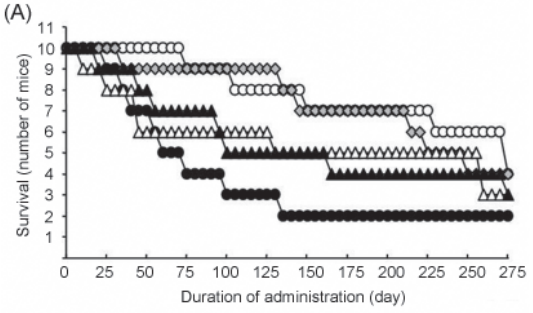
depends on the degree of polymerization. Low-molecular-weight proanthocyanidins are known as sustained-release antioxidants; on the contrary, high-molecular-weight proanthocyanidins can exert their anti-oxidant activity in the digestive tract and protect lipids, proteins, and carbohydrates from oxidative damage during digestion and spare soluble antioxidants (*43*). Furthermore, EC and EGCg are distributed in the brain by their absorption into plasma after oral administration, and these monomeric forms may act as potential therapeutic agents in neurodegenerative diseases (*44*). This suggests that those monomeric forms can act as potential therapeutic agents against neurodegenerative diseases in the brain. In addition, it has been reported that dimers and trimers of proanthocyanidins can be absorbed into epithelial cells such as Caco-2 cells (*45*), suggesting that oligomers act both oligomerically and monomerically. We must elucidate the similarities and differences in activities and functional mechanisms between oligomers and metabolites including monomers *in vivo*.

3. Oligomeric proanthocyanidins extend life span of SAMP8

Increased longevity is one of the most common desires of human beings. Therefore, research on anti-aging is ultimately focused on life span extension. However, no convincing strategy based on scientific evidence has been suggested, except for dietary restriction (*46*). Life span extension by dietary restriction has been observed over the years in many species, including rats, mice, hamsters, dogs, fish, invertebrates, and yeast. The inhibition of reactive oxygen molecule formation in isolated mitochondria and microsomes was considered an important mechanism for life span extension in food-restricted rodents (*47*). In particular, it has been reported that the attenuation of oxidative stress by dietary restriction is involved in the protective role against degenerative diseases related to the aging process such as diabetes, cardiovascular diseases, and neurodegenerative diseases (*48*). Despite these very encouraging results, clinical application is complex and limited. Regarding this point, although various dietary restriction mimetics, such as glycolytic inhibitors and antioxidants, have been suggested, scientific evidence must be accumulated to support their application (*46*). For this reason, the search for novel anti-aging agents to elicit the same beneficial effects as caloric restriction without side effects and toxicity has attracted much attention. Recently, we showed that oligomers have more effective anti-aging activities than polymers in a cellular senescence model (*13*). Although polymers structurally contain many more phenolic groups compared to free radical scavengers than oligomers, oligomers are more easily absorbed than polymers, which is associated with their bioactivity (*43*).

The administration of oligomers extended life span, as shown in Figure 7. On the other hand, life span does not extend in response to an increase in oral dose of oligomers. Probably, absorption of proanthocyanidins is related to the effect on life span. Bioactivity of catechin derivatives is related to their structural phenolic groups. Increase in the level of polymerization means a rise in phenolic group content. Previously, we demonstrated that proanthocyanidins showed strong antioxidative activities compared with monomeric catechin derivatives *in vitro* (*49*). Many researchers have suggested that antioxidative activities are associated with a delay in the aging process and extension of life span in various organisms (*50*). Actually, we demonstrated that oligomers increased SIRT1 expression, associated with extended longevity, in a cellular senescence model (*13*). Therefore, we expected oligomeric proanthocyanidins to exert stronger activity to extend life span, compared with monomeric forms such as catechin derivatives. Further study has to be carried out to elucidate differences between monomeric and oligomeric forms.

To elucidate related mechanisms, expression of SIRT1 was observed. Sir2 is an NAD^+-dependent deacetylase implicated in regulation of lifespan in species as diverse as yeast, worms, and flies (*51*). Yeast Sir2 is a heterochromatin component that silences transcription at the silent mating loci, telomeres, and ribosomal DNA (*52*). In addition, it suppresses recombination in rDNA and modulates longevity of most organisms including mammals (*53*,*54*). Therefore, the enzymatic activity of Sir2 may indicate its usefulness as an effective



(B)

Group		Life span index
SAMR1		2,207
SAMP8	Veh (water)	1,065
	60% Food restriction (water)	2,105
	Oligomers (1 mg/kg B.W./day)	1,590
	Oligomers (10 mg/kg B.W./day)	1,566

Figure 7. Effects of oligomers on the life span of SAMP8. Forty-five- or forty-six-week-old SAMP8 mice were administered vehicle (Veh, water *p.o.*, $n = 10$), while another two groups were administered oligomers orally at doses of 1 or 10 mg/kg body weight/day ($n = 10$) using a stomach tube until death. For the remaining group of mice, the mean food intake was restricted to 60% until death ($n = 10$). SAMR1 mice (45-46 weeks old, $n = 10$) were used as a control group. **(A)** Effect of oligomers on survival of SAMP8. **(B)** Life span index based on survival data. Open circle: SAMR1; Closed circle: SAMP8 (Veh); Open triangle: SAMP8 (oligomers at 1 mg/kg body weight/day); Closed triangle: SAMP8 (oligomers at 10 mg/kg body weight/day); Gray square: SAMP8 (60% food restriction).

caloric restriction mimetic (*55*). Among the seven mammalian homologues of Sir2, SIRT1 is the human orthologue of yeast Sir2, and the best-characterized member of mammalian sirtuins. Recently, we showed that pretreatment with oligomers significantly increased SIRT1 expression in a cellular senescence model (*13*). Therefore, in the present study, we investigated the effect of oligomers on the expression of SIRT1 in the SAM model.

Resveratrol, as an activator of SIRT1, has been reported to extend the fitness and survival of simple organisms such as *Saccharomyces cerevisiae* (*56,57*) as well as mice fed high-calorie diets (*58,59*). Moreover, we previously clarified that oligomers increased SIRT1 expression in a cellular senescence model (*13*). Therefore, the effect of oligomers on SIRT1 was compared with resveratrol *in vivo*. We expected administration of oligomers to also increase expression and activation of SIRT1 in the brain to slow aging-related deteriorations of SAMP8. In this study, the administration of oligomers slightly elevated SIRT1 expression in the brain of SAMP8, whereas resveratrol did not show a significant effect (Figure 8). On the other hand, another report demonstrated that acetylated p53, a well-characterized target of SIRT1 deacetylase activity, is decreased by resveratrol treatment (*60*).

We previously elucidated that oligomers reversed up-regulation of oxidative stress-related gene expression such as inducible nitric oxide synthase and cyclooxygenase-2 in type 1 and 2 diabetic rodent models (*61,62*). In addition to activation of SIRT1, attenuation of oxidative stress would play a crucial role in the anti-aging effect of oligomers. Since oxidative stress plays an important role in modulation of life span in SAMP8, the antioxidative effects of oligomers may also be involved with life span extension in SAMP8.

Dietary restriction as an effective method for the extension of longevity has also been reported to induce stereotypical behaviors such as rearing and jumping independent of life span (*63*). In behavioral analyses, we showed that rearing, jumping, and hanging from the lid of the cage in 60% food-restricted SAMP8 markedly increased compared with vehicle-treated SAMP8. Surprisingly, oligomer-treated SAMP8 did not show an increase in these stereotypical behaviors (Figure 9). Moreover, in the inclined plane and voluntary running tests performed to observe differences in motor function, we found no significant difference in motor function among all SAMP8 groups (Figure 10). These results indicate that stereotypical behaviors shown in the 60% food-restricted group have no relation with motor function. It has been reported that dietary restriction may induce anxiety-like behavior by down-regulation of corticotrophin-releasing factor (*64*). Diet-restricted rats showed stereotypy by an increase of dopamine receptor signaling (*65*). Chen *et al.* (*63*) demonstrated that stereotypical behaviors brought about by caloric restriction were eliminated in SIRT1-knockout mice, indicating that SIRT1 activation may cause stereotypical behaviors with dietary restriction. In our study, although life span was extended by oligomers as well as 60% food restriction, mice administered oligomers did not

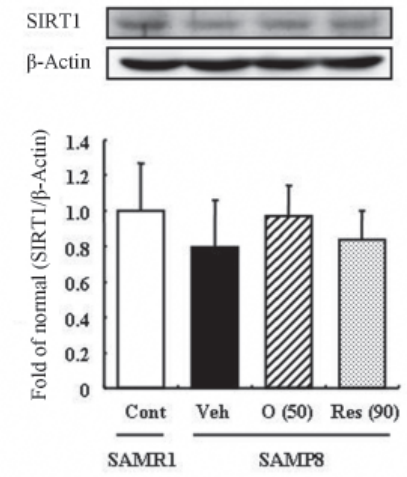


Figure 8. Effects of oligomers on SIRT1 expression in the brain of SAMP8. Forty-five-week-old SAMP8 were administered vehicle (Veh, water *p.o.*, $n = 5$), oligomers (O (50), 50 mg/kg body weight/day, *p.o.*, $n = 5$), or resveratrol (Res (90), 90 μmol/kg body weight/day, *p.o.*, $n = 5$). Five weeks after administration, brain lysates were immunoblotted with antibodies for SIRT1. SIRT1 expression intensities were divided by β-actin expression. SAMR1 were used as a control (Cont, $n = 4$).

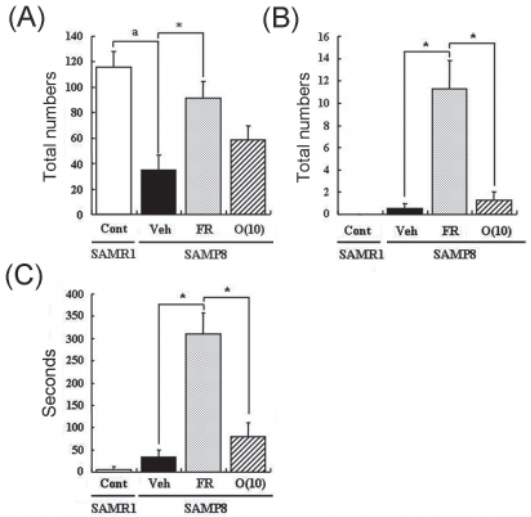


Figure 9. Effects of oligomers on stereotypical behaviors. Forty-five- or forty-six-week-old SAMP8 were administered vehicle (Veh, water *p.o.*, $n = 4$), while another two groups were administered oligomers orally at doses of 10 mg/kg body weight/day (O (10), $n = 4$) using a stomach tube until death. For the remaining group of mice, the mean food intake was restricted to 60% until death (FR, $n = 4$). SAMR1 mice (Cont, 45-46 weeks old, $n = 4$) were used as a control group. One hundred and thirty nine days after administration, actions of rearing up on the hindlimbs and jumping from the bottom of the cage were counted for 15 min **(A, B)**. The time spent hanging from the lid was measured for 10 min **(C)**. Administration was continued during the tests. * $p < 0.05$ (One-way ANOVA, *post-hoc* Bonferroni's test); [a] $p = 0.0034$ (Student's *t*-test).

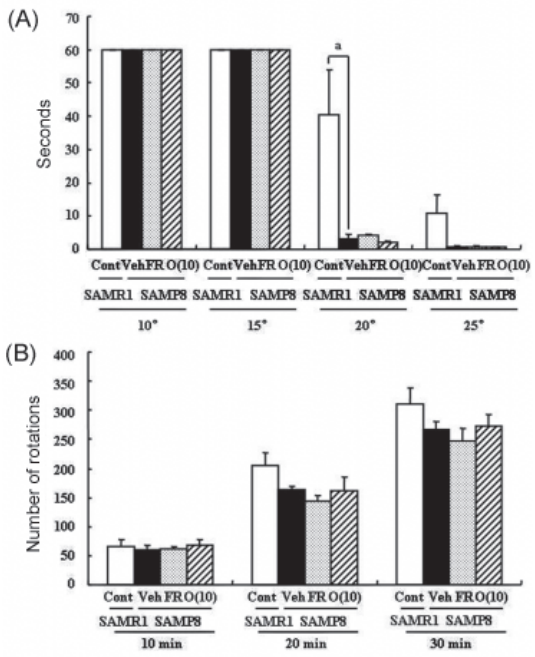


Figure 10. Effects of oligomers on motor function. Forty-five- or forty-six-week-old SAMP8 were administered vehicle (Veh, water *p.o.*, $n = 4$), while another two groups were administered oligomers orally at doses of 10 mg/kg body weight/day (O (10), $n = 4$) using a stomach tube until death. For the remaining group of mice, the mean food intake was restricted to 60% until death (FR, $n = 4$). SAMR1 (Cont, 45-46 weeks old, $n = 4$) were used as a control group. One hundred and thirty nine days after administration, the time spent on the inclined surface without dropping was measured **(A)**. The number of rotations was measured for 30 min **(B)**. Administration was continued during the tests. [a] $p = 0.0159$ (**A**: Student's *t*-test).

show stereotypical behaviors, like SIRT1-knockout mice undergoing food restriction. We revealed that oligomers consist of various combinations of 4 types of monomer: EGC, EC, EGCg, and ECg. Their absorption rates and bioactivities were distinguished by their types (*44*). Therefore, composition of oligomers may play an important role in neuronal signaling.

We also carried out an investigation of side effects or toxicity of oligomers. The results showed normal ranges of hematological data such as alanine aminotransaminase, aspartate aminotransaminase, and blood urea nitrogen as well as changes in body and tissue weights, although the maximum concentration for oral administration was at a higher level (500 mg/kg body weight/day) than the average dietary intake of proanthocyanidins of 58 mg/day of humans in the United States (*66*). Therefore, we suggest that oligomeric proanthocyanidins are safe and novel anti-aging agents associated with life span extension. Further studies are needed to elucidate molecular mechanisms associated with extension of life span by oligomers, as well as to clarify the contribution of SIRT1 to the aging process of SAM.

4. Conclusion and perspectives

The present study indicated that oral administration of oligomers improved memory impairment in SAMP8. In particular, the density of axons in the hippocampal CA1 was significantly increased by oligomer administration. Moreover, the administration of oligomers increased phosphorylation of VEGFR-2 in the hippocampal CA3, hypothalamus, and choroid plexus. We speculate that memory improvement accompanied with histological changes may be induced directly in the hippocampus and indirectly in the hypothalamus and choroid plexus through VEGFR-2 signaling. In the present study, we elucidated the protective effect of oligomers against memory impairment with aging. VEGFR-2 signaling may provide new insight into ways to protect against memory deficit in the aging brain. The present study also indicated that the oral administration of oligomers extended the life span of SAMP8 with a slight up-regulation of SIRT1 in the brain. On the other hand, oligomer-treated SAMP8 did not show stereotypical behavior.

Proanthocyanidin is known as a condensed tannin, a member of a specific group of polyphenolic compounds, and it has been reported to exhibit powerful antioxidant activity (*67*,*68*). Although this is the most abundant dietary polyphenol, its marked polymerization leads to limited absorption *in vivo* (*69*). Many researchers have focused on the oligomeric form with its lower level of polymerization in foodstuffs such as grape seeds and blackberry (*70*). In this study, oligomers are suggested to be novel anti-aging agents.

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