Yokukansan Ameliorates Hippocampus-Dependent Learning Impairment in Senescence-Accelerated Mouse

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Yokukansan (YKS) is a traditional Japanese herbal medicine. It has been currently applied for treating neurosis, insomnia, and children’s night crying which has been approved by the Japanese government for the treatment of neurosis, insomnia, and children’s night crying. It could also improve the behavioral and psychological symptoms of dementia. It has been indicated that the hippocampus is a key target for preventing and treating this disease. The hippocampus plays a pivotal role in processes of spatial learning and memory, which is one of the current issues for elucidating mechanisms of dementia.

The clinical effect of traditional medicine has been reevaluated with applied for treating diseases, including dementia. Yokukansan (YKS) is a traditional Japanese herbal medicine, which has been approved by the Japanese government for the treatment of neurosis, insomnia, and children’s night crying and irritability. It could also improve the behavioral and psychological symptoms of dementia.

It was reported that senescence-accelerated mouse prone-8 (SAMP8) mice revealed age-related behavioral alterations from 4 months of age. Hippocampal-dependent learning ability began to decline in these mice as early as 2 to 4 months, as shown by impaired performance in the water maze test. SAMP8 mouse has been used as a model of Alzheimer’s disease, the most common type of dementia, which are registered in the Pharmacopeia of Japan ver. 17.

Table 1 shows the herbal constituents and contents of YKS. The dried extract powder of YKS was supplied by Tsumura & Co. (Tokyo, Japan). Table 1 shows the herbal constituents and contents of YKS. The dried extract powder of YKS was supplied by Tsumura & Co. (Tokyo, Japan).

| Botanical plant name | Contents (g) |
|----------------------|-------------|
| Atractylodes lancea rhizome | Atractylodes lancea De Candolle 4.0 |
| Poria sclerotium | Poria cocos WOLF 4.0 |
| Cnidium rhizome | Cnidium officinale MAKINO 3.0 |
| Angelica radix | Angelica acutiloba KITAGAWA 3.0 |
| Uncaria uncis cum ramulus | Uncaria rhynchophylla MIQUEL 3.0 |
| Bupleurum radix | Bupleurum falcatum LINNÉ 2.0 |
| Glycyrrhizae radix | Glycyrrhiza uralensis FISHER 1.5 |

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produced in accordance with the formulation reported previously.\textsuperscript{7,10} The components of YKS has been identified by three-dimensional HPLC (see Supplementary Fig. 1). The representative compounds include ferulic acid, glycyrrhizin and saikosaponin b2.\textsuperscript{10} The extract quality is standardized on the basis of Good Manufacturing Practices defined by the Japanese Ministry of Health, Labor and Welfare.\textsuperscript{10}

**Animals and Experimental Design** Male SAMP8 mice at 5 months of age were obtained from Japan SLC, Inc. (Shizuoka, Japan). Five mice per cage were housed under controlled temperature (23±1°C), humidity (55±2%) and light (12h light/dark cycle, lights on at 06:00 and off at 18:00). We used 36 mice in the present study. The mice were maintained on a standard rodent chow (CE-2, CLEA Japan, Inc., Tokyo, Japan) \textit{ad libitum}. This study was approved by the ethics committee of Asahi University School of Dentistry. All experiments were in compliance with the guidelines for laboratory animal care and use of Asahi University. The mice were randomly assigned to control and experimental groups. In the experimental group, YKS of 0.15\% aqueous solution was treated orally for eight weeks. The concentration of YKS was determined by body weight and the average daily water intake of each mice. The dosage of YKS was decided based on the previous studies.\textsuperscript{7,10} The control group had drug-free water \textit{ad libitum}.

**Morris Water Maze Test** The water maze was carried out for both control and experimental mice \((n=6/group)\) as reported previously.\textsuperscript{22,23} A circular stainless pool (90 cm in diameter and 30 cm high) was filled with water (\textit{ca.} 23°C) to a height of 23 cm. A platform (12x12 cm) was submerged 1 cm under the water surface in the center of the tank. Mice were placed into the water from one of four randomly selected positions around the pool, and given 4 acquisition trials per day for 5 days continuously. Escape latency and swimming path were recorded and analyzed with the aid of a software (Move\textsuperscript{er}/2D, Library Co., Ltd., Tokyo, Japan). All animals underwent a visible probe test 2h after the last training trial on the last day of training.

**Bromodeoxyuridine (BrdU) Treatment** We examined the hippocampal newborn cell proliferation in the dentate gyrus (DG) region after intraperitoneal injection of BrdU (50mg/kg; 10mg/mL dissolved in 0.9\% sodium chloride, Sigma-Aldrich, St. Louis, MO, U.S.A.) into the mice \((n=7/group)\) 5 times at 3-h intervals.\textsuperscript{22} The next day after the last injection of BrdU, mice were perfused \textit{via} the ascending aorta with 0.9\% physiological saline followed by Karnovsky's fixative (2\% paraformaldehyde and 2.5\% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4), as reported previously.\textsuperscript{21} The brain was carefully dissected and further fixed in the same fixative solution for 24 h at 4°C. The specimens were postfixed in 1\% OsO\textsubscript{4} for 1 h. After dehydrating through an ascending graded acetone series, specimens were embedded in epoxy resin. The ultrathin sections (80nm thick) were obtained with glass knives on a Porter Blum MT-1 ultramicrotome (Ivan Sorvall, Inc., Norwalk, CT, U.S.A.) and collected onto copper mesh grids. The sections were observed using a transmission electron microscope (JEM-1400Plus, JEOL Ltd., Tokyo, Japan), after staining with 0.1\% uranyl acetate and lead salts.

Myelin sheathes in the hippocampal Cornu Ammonis (CA) 1 region were observed at 10000X magnification. Twenty images containing 200 axons per mouse were obtained for quantitative evaluation. The G-ratio (the ratio of the inner to the outer diameter of the myelin sheath) was determined, as previously described.\textsuperscript{23,26} Synaptic structural analyses were performed at 30000X magnification. Synapses were confirmed by clearly visible synaptic vesicles and postsynaptic density (PSD). Fifty synapses per mouse were selected for measuring PSD length, as previously described.\textsuperscript{23,27}

**Statistical Analysis** All values are expressed as means±standard deviation (S.D). The statistical analysis was performed by using SPSS version 22. Statistical significance was determined using a Wilcoxon signed-rank test or Mann–Whitney test. \(p\) value of less than 0.05 was considered to be statistically significant.

**RESULTS**

**Morris Water Maze Test** All mice demonstrated improved performance during acquisition based on the decrease in the escape latency and swimming path length over the five training days (Fig. 1). On day 1, there was no significant difference between the control and experimental groups regarding the latency and path length, indicating both groups have similar motor and visual capabilities. Compared with the control group, the escape latency and the swimming path length were significantly shorter in the experimental group for days 3 to 5. We did not find any significant differences between
the control and experimental groups with regard to the performance in the visible probe test, indicating both groups had similar motor and visual capabilities (Fig. 1).

**Hippocampal Cell Proliferation in the DG Region**  Representative photomicrographs of BrdU immunohistochemistry in the hippocampus DG region are shown in Fig. 2A. BrdU-positive cells were observed within the subgranular zone of the DG region in both control and experimental groups. The number of BrdU-positive cells of the experimental group was around 40% higher than that of the control group ($p<0.01$, Fig. 2B). This finding suggests that treatment with YKS improved the hippocampal newborn cell proliferation in SAMP8 mice.

**Ultrastructural Feature of the Hippocampal CA1 Neurons**  The ultrastructural features of the hippocampal CA1 neurons were examined using transmission electron microscope. The neuron of both control and experimental groups exhibited conspicuous nucleus with prominent euchromatin (Fig. 3). The cytoplasm contained cisternae of the rough endoplasmic reticulum, Golgi apparatus, and mitochondria with intact cristae. There were a lots of lipofuscin inclusions in the control group (Fig. 3). Lipofuscin inclusions were found in a great many varieties, small or large, round, oval or irregular. However, little were observed in the experimental group (Fig. 3). We did not find any obvious ultrastructural alterations of other organelles for both groups.

**Myelin Sheath**  The ultrastructural features of the myelin sheaths were examined under the electron microscope. The morphology of axons in the control and experimental groups appeared grossly normal (Fig. 4A). The control group showed various irregular profiles of the myelin sheaths. Some were loosely organized with a disordered texture, and others exhibited stratification, collapse, or disruption, as well as disordered arrangements (Fig. 4A). Myelin sheaths in the experimental group appeared normal, exhibiting concentric compact multilamellar structure around the axon (Fig. 4A). The thickness of the myelin sheaths correlated with the axon diameter. Morphometric analysis showed that the G-ratio differed significantly between groups. Compared with the control group, the G-ratio was significantly lower in the experimental group, indicating thicker myelin sheaths in the experimental group (Fig. 4B).

**PSD Length**  Figure 5 shows the representative electron micrographs of the hippocampal synapses in CA1 region. Compared with the control group, the estimated PSD length was significantly longer in the experimental group. There were no significant differences between the groups regarding the synaptic vesicles and synaptic cleft between the control and experimental groups.

**DISCUSSION**

In this study, we provide the first evidence that treatment with YKS for 8 weeks ameliorates hippocampal morpho-
logical changes and learning impairment in SAMP8 mice. SAMP8 mice exhibit age-related learning impairment and rapid advancement of senescence.\(^{18,21,28}\) We found that treatment with YKS ameliorated learning impairment in SAMP8 mice. YKS was reported to prevent cognitive disturbances in Tg2576 mouse, the transgenic model of Alzheimer’s disease,\(^{13,14}\) intracerebroventricular amyloid β protein (Aβ)-injection mice,\(^{16,29}\) animal models of schizophrenia,\(^{30,31}\) and aged rats.\(^{32}\)

Previous animal studies suggested that YKS could ameliorate hippocampal neurogenesis associated with cognition.\(^{33}\) Our study showed that the hippocampal neurogenesis in SAMP8 mice was maintained by administration with YKS. Several studies confirmed the correlation of the newborn neurons in the hippocampal DG region with the spatial learning ability.\(^{34,35}\) Hippocampal neurogenesis is related to the formation of associative memories. Animals with impaired hippocampal neurogenesis perform worse than the control in hippocampal-dependent forms of spatial learning test.\(^{36,37}\) Therefore, the relationship between hippocampal neurogenesis
and spatial learning ability indicates that preserved hippocampal neuronal proliferation after treatment with YKS is involved in the maintenance of learning ability in SAMP8 mice. It was reported that the number of BrdU-positive cells reduced in the hippocampal DG region of the aged rats, and this decrease was improved by YKS treatment, which may influence the proliferation of neural stem cells in the hippocampal DG region. It is conceivable that YKS has pharmacological potency for preserving the hippocampal neurogenesis in aged animals.

Lipofuscin accumulations of the hippocampal neurons are commonly found in aged animals. Lipofuscin accumulations were observed in the hippocampal neurons of SAMP8 mice, especially after treatment with kainic acid. It was considered that SAMP8 mice were susceptible to kainic acid-induced oxidative damage, which might be one of the causal factors in lipofuscin accumulation of the hippocampal neurons. The present study confirmed that numerous lipofuscin inclusions were presented in the hippocampal neurons of the control SAMP8 mice. However, there were little lipofuscin inclusions after administration of YKS. We consider that YKS might play a role in delaying neuronal aging process, involved in lipofuscin accumulations. However, direct evidences for the contribution of lipofuscin accumulation, lipid peroxidation and protein oxidation to aging process are still unidentified. The precise mechanism of this phenomenon needs further investigation.

The myelin sheaths facilitate the rapid conduction of nerve impulses in myelinated fibers. Thinning or deformation of the myelin sheaths leads to a decrease in nerve conduction velocity. The hippocampal abnormal myelin sheaths are involved in behavioral impairments, inducing delayed cognitive development. Accordingly, we consider that prevention of learning impairment in SAMP8 mice by treatment with YKS may be associated with the maintenance of the hippocampal myelin sheaths. Previous study showed that oligodendrocytes in the hippocampal CA1 region decreased progressively with age. Myelin sheaths of the central nervous system are formed by oligodendrocytes. We recently found that both myelin sheaths and oligodendrocytes in the hippocampus were influenced by prenatal stress and chewing stimulation. We presume that the alteration of myelin sheath is associated with age-dependent degeneration of oligodendrocytes in SAMP8 mice, though the oligodendrocytes were not assessed in the present study.

Synapse is a highly specialized structure designed to guar-
antec precise and efficient communication among neurons.\textsuperscript{52)} The synaptic structural plasticity reflects the physiological function and plays a critical role in spatial learning ability.\textsuperscript{43)} Synaptic morphological changes, including the PSD length is closely related to synaptic functional plasticity. Furthermore, synaptic functional alterations are always accompanied by the structural changes.\textsuperscript{44)} PSD contains a lot of receptors, scaffolding proteins, and signaling complexes involved in synaptic plasticity and transmission. It was reported that induction of long-term potentiation is associated with an increase in PSD length.\textsuperscript{45)} Synaptic size can be determined by the measurement of PSD length, which is an important factor related to the synaptic plasticity and neurobehavioral activities.\textsuperscript{46)} Our study showed that administration with YKS increased the PSD length in the hippocampal CA1 region of SAMP8 mice. We speculate that the amelioration of learning and memory impairment by YKS involved in the alteration of the hippocampal PSD length in SAMP8 mice.

In conclusion, our results indicate that treatment with YKS for 8 weeks prevents age-related learning impairment in SAMP8 mice, mediated at least partially via delaying neuronal aging process, neurogenesis, myelin sheath, and synaptic plasticity in the hippocampus. Further studies are needed to clarify the detailed mechanisms underlying the neuroprotective effects of YKS.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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