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Oxidative stress in the pathogenesis of Alzheimer's disease: Improvement of memory-deteriorating disease by administration of plant enzyme

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Abstract

Plant enzymes are natural substances with almost no side effects and reported effects on spatial learning and histopathological alterations of the hippocampus in senescence-accelerated mice. In senescence-accelerated mice (SAMP8), this in vivo study examined the effects of a plant enzyme on the brain hippocampus, memory and spatial cognition. In the Morris water maze, a comparison between the control group of SAMR1 mice and the sham control group of SAMP8 mice (SAMP8 without plant enzymes administration) showed a significant reduction in arrival time, and in the probe test, it was divided into 4 divisions. Moreover, there was a significant increase in dwell time and number of crossings of the virtual platform area. The plant enzyme-treated group tended to have a shorter arrival time than the sham group, but not significantly different. Serum peroxy radicals were significantly suppressed in the plant enzyme-treated group compared with the sham group. Plant enzyme inhibited the peroxy radical in comparison to a sham control. With plant enzymes treatment, the lipid peroxidation level was significantly lowered in the brain tissue compared with the sham control group (p < 0.05. Serotonin (5-HT) levels were significantly higher in the brain tissue of plant enzymes -treated mice than in that of sham control mice. There was no significant difference in the brain lipid peroxide concentration between the plant enzyme administration group and the sham group. In contrast, the serotonin concentration in the brain was significantly different between the plant enzymes administration group and the sham control group. The high neuron density (CA1 region) in the hippocampus of animals in the plant enzymes administration group indicates a possible improvement in memory.

Keywords: Alzheimer's disease; Plant enzymes; Antioxidant effects; Peroxidized fat level; Morris water maze test; Secretion

1. Introduction

Dementia is characterized by damage to the brain tissue resulting in cognitive impairment. The central symptom of dementia (core symptom) is intellectual disability, affecting not only memory but also cognitive functions such as

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language, calculation, understanding, cognition, and thinking. In particular, Alzheimer's disease (AD), affects recent memory formation and recall. In addition, patients suffer from executive dysfunction, apraxia, agnosia, and place and time disorientation. Furthermore, peripheral symptoms such as depression, hallucinations, delusions, anxiety, nighttime insomnia, rants, and haiku appear (Alzheimer, 1907, Alzheimer, 1902). Although not specific to dementia, these symptoms often increase the long-term care burden of affected patients.

Moreover, AD, often found in the elderly, is characterized by amyloid deposition and the formation of neurofibrillary tangles via tau protein hyperphosphorylation (Alzheimer, 1902, David *et al*., 2021, Selkoe, 1999). Moreover, low activity of the acetylcholine (ACh) synthase choline acetyltransferase was found in the autopsy of a brain with AD (Jessica *et al*., 2011, Allen *et al*., 1997, Perry *et al*., 1999).Some cognitive functions have been reported to correlate well with the Ach system and the degree of afferent fiber disorder in the cerebral cortex (Berger, 2003, Claus *et al*., 1997, Svensson *et al*., 1997). In addition, in lifestyle, overeating and stress cause chronic inflammation of cells due to oxidation from free radicals in the body. Therefore, it causes lipid peroxide and neurodegeneration in the brain due to the accumulation of activated microglia in brain cells. Amyloid plaque develops on proteins in the brain and has been reported to be associated with AD (Whitehouse *et al*., 1982, Buttini *et al*., 2002). ACh precursors are the major constituents of cell membranes of choline-containing phospholipids.

Oral administration of a combination of *bread enzyme* and cholinesterase inhibitor (AChE inhibitor) was reported to prevent aging by producing brain hormones and activating nerve cells, and improving memory in AD patients (Porter *et al*., 2002).

In addition, *plant enzymes* activate different types of phosphoroxidases. Unsaturated fatty acids, *plant enzyme* at a second place were removed by phospholipase A2 *in vitro*, which was reported to function as an intracellular messenger (Ferreira *et al*., 2016, Raber *et al*., 2004). ACh system activators are often used in AD, as it shows a decrease in ACh activity. Other options such as anti-inflammatory pharmaceuticals, antioxidants, steroids, etc. are also used (Probst *et al*., 1998, Lynch *et al*., 2019, Vuong *et al*., 2017, Zawia *et al*., 2009, Takasugi *et al*., 2011, Fathinajafabadi *et al*., 2014). Recently, AChE inhibitors are often used for AD treatment and prevention, but only donepezil hydrochloride is approved in Asia. In VD drug therapy, the focus of treatment is to control risk factors such as diabetes and hypertension to prevent their progression. For the elderly in particular, the interaction of drugs becomes a problem when multidrug combinations are used, which Moreover increases the effects. And treatment of VD and AD requires long-term measures, and it is important to select materials that do not have side effects so that patients can tolerate long-term treatment.

A study comparing global peroxidation activity in various brain regions in patients with AD and age-matched controls showed significantly decreased catalase activity in AD's temporal cortex, whereas superoxide dismutase (SOD) activity was significantly decreased in AD's frontal lobe and temporal cortex. This study supports the hypothesis that the AD brain is affected by increased oxidative stress.

Therefore, in this study, we focused on *plant enzymes*, natural materials with few side effects. We used senescence-accelerated mice (SAM) to investigate the effect of *plant enzymes* on the brain hippocampus and on memory *in vivo* (Fathinajafabadi *et al*., 2014, Gamache *et al*., 2020, Nilsson *et al*., 2014, Sakakibara *et al*., 2018, Dinan *et al*., 2017, Seo *et al*., 2019, Calvanese *et al*., 2009, Flax *et al*., 2011, Wang *et al*., 2008).

The development of treatment methods aiming at the fundamental treatment of AD is being actively promoted. It is said that Aβ gradually begins to accumulate in the brain, and it is known that Aβ is already accumulated even in the preliminary state of dementia called mild cognitive impairment. For this reason, the importance of starting treatment as early as possible is increasing. From the results of this mouse model experiment, it can be said that it may have a preventive effect even in a state where Aβ is accumulated. In this study, the results obtained were as follows: promotion of removal of Aβ by plant enzymes, prevention of Aβ production, Aβ antibody therapy, and inhibitor of Aβ-producing enzymes. In addition, radical scavengers reduce β-amyloid levels and lubricate internal hormones, which are thought to acutely modulate neuronal synaptic transmission involved in memory learning in the hippocampus (Seo et al., 2019, Calvanese et al., 2009, Flax et al., 2011, Wang et al., 2008).

2. Material and methods

2.1. Animal care

ICR male 5-week-old mice were purchased from CLEA Japan, Inc., Japan. Meanwhile, male SAMR1 mice and male SAMP8 were used in his SLC, Inc., Japan at 4 weeks of age (Raber *et al*., 2004, Nilsson *et al*., 2014, Sakakibara *et al*., 2018). Prior to the study, for acclimatization, mice were bred in a room with 14 h/day light (10 h/day dark) at a 22°C ± 3°C and a

humidity of 60%. Animals had free access to water (tap water) and food (provided by CLEA Japan, Inc.). Blood was collected from the fundus and placed in a Kubota hematocrit KH-120 centrifuge. For cardiac blood sampling, a Hitachi's Chibitan II Millipore was used. For enzyme immunoassay (EIA) measurements, LDN serotonin EIA RUO 10-0900 was used for serotonin (5-HT) measurements and LDN dopamine EIA RUO 10-0300 for dopamine measurements. When measuring noradrenaline, MP Biomedicals Noradrenalin ELISA 196594 was used for the enzyme-linked immunosorbent assay (ELISA). Other devices used were Taiyo's stirrer MONOSIN II, Star LC1011C for 450 nm absorbance measurements, and Toyo Soda's microplate reader MPRA 4 for 490 nm absorbance measurements.

SAM is an age-dependent aging score from a mouse colony in which an AKR/J strain mouse and an unknown strain have unexpectedly crossed by Takeda et al. in Kyoto University. It is a mouse strain established with a rapid increase as an index. SAM is roughly divided into P strain (prone), which indicates accelerated aging/short life, and R strain (resistant), which indicates normal aging. The P strain is further classified according to aging-related pathological conditions such as aging amyloidosis, learning / memory disorders, senile osteoporosis, and cataract.

Furthermore, animal experiments followed the guidelines of the Ministry of Education, Culture, Sports, Science and Technology's Notification No. 71 "Basic Guidelines on Implementation of Animal Experiments in Research Organizations" (June 1, 2006) and Ministry of the Environment's "Standards on breeding and storage of experimental animals and relief of pain."

All animal care and experimental protocols were approved by the Research Ethics Committee of Ref: 207/625/36.

Plant enzyme is commercially available and supplied as a heat-killed, dried powder by Validux Co. Ltd (Nagoya, Japan).

The *plant enzyme e*xtraction method is shown below. The *plant enzymes* used in this study were extracted under mild conditions. Plant enzymatic reactions generally progressed at room temperature (20°C–60°C), neutrality (around pH 7), and normal pressure, as their functions deteriorated under alkaline conditions.

Plant enzymes' catalyzed oxidation and reduction centered on the exchange of electrons between oxidoreductase substrates and coenzymes, depending on the type of reaction. Typical examples are reactions such as alcohol dehydrogenase (alcohol dehydrogenase) and aldehyde dehydrogenase, which catalyze the oxidation of alcohol shown in the reaction formula of Fig. 1, or the reduction reaction of acetaldehyde.

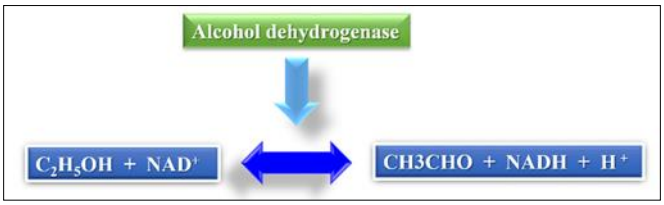


Figure 1 Plant enzyme produced by the alcohol dehydrogenase

The concentration of *plant enzymes* was 500 mg/kg/day in a dose of 0.2 mL, and the same amount of distilled water was administered to the control group. After one week of preliminary breeding, gavage by oral gastrectomy was performed every day until the end of the experiment (approx. 2 weeks). using the gastric zone for 4 weeks.

2.2. Hormone measurements in the brain of ICR mice

The control group and the *plant enzyme* treatment group each included 40 mice. Blood was drawn from the eye fundus and examined before administration and at 8 and 12 weeks thereafter.

SAM mice were weighed every three days. In addition, Morris water maze (MWM) experiments and brain hormone measurements were performed. Blood was collected for the last time 16 weeks after the last treatment. The collected blood was centrifuged at 10,000 rpm for 10 min and serum stored at −4°C. Brain hormones (5-HT, dopamine, and noradrenaline) were measured in serum samples using EIA and ELISA kits. In addition, the body weight of SAM mice was measured every 3 days.

2.2.1. MWM test

We used a typical MWM setup (Dinan *et al*., 2017, Seo *et al*., 2019, Calvanese *et al*., 2009, Flax *et al*., 2011, Wang *et al*., 2008, Vucetic *et al*., 2011).

The water labyrinth consists of a circular tank (diameter 150 cm, depth 50 cm) and a platform (diameter 12.5 cm, height 15 cm), to which the mouse needs to climb when the tank is filled with water.

The platform was made of crystalline acrylic and set 1 cm below the water surface; the water temperature was 23°C ± 2°C (water was mixed with 2% skim milk to conceal visual information on the platform location).

In addition, the wall surface of the circular tank was divided into four segments, north, south, east, and west; there were four off-center (northeast) platforms (Fig. 2A). In this experiment, multiple observations were made using a melanotic curtain called a circular tank. Contextualized data and motion tracking (Fig. 2B).

For the pretraining session in Step 1, the day before the test, the platform was set a few centimeters above the water surface in the center of the circular tank and the mouse could be held in a flat position.

After allowing the mouse to swim from the platform for ≥15 s, the experimenter returned the mouse to the platform. Thereafter, this operation was repeated 3 times at an interval of ≥10 s.

For the pretraining session in Step 2, the mouse was killed by drowning at three equidistant points with the mouse head being directed from the platform to the wall of the circular tank.

We measured the time it took to reach the platform and the time it took to drown when the mouse was left unattended for 10 s after delivery to memorize its trajectory. If the mouse did not reach the platform after 120 s, the experimenter forcibly moved to the platform and left it for 10 s to memorize the trajectory.

In this case, the delivery time for the mouse to reach the platform was 120 s (Vucetic *et al*., 2012). The study was conducted once and twice daily, first 30–60 min after oral administration and then after 60–60 min, respectively. It was done 90 min after the first trial was completed. This was repeated every day from 10:00 to 16:00 to measure the time to reach the platform. Then, the session was held for 9 days.

We calculated the average delivery time and screened the spatial cognitive ability twice over time. The probe test in Step 3 was performed once on the last day of the training session.

After recording the location, the platform was removed and the mice were placed in a tank on the water surface, similar to a training session. The swimming behavior was recorded for 120 s. The test targeted the frequency of dividing the dwell time into four periods, and the virtual platform area was established for data analysis and screening spatial awareness (Kaisho *et al*., 1994).

The test group included 10 mice in each of the SAMR1 mouse control group, the SAMP8 mouse control group, and the SAMP8 mice *plant enzyme* group. After preparation period, the plant enzyme group received 500 mg / kg of *plant enzyme* daily for 4 weeks, and the control group was administered the same amount of water. Eight weeks after the last dose, a MWM experiment was performed for one week.

The water maze device consists of a cylindrical tank (150 cm in diameter, 20 cm in depth) filled with water (water temperature 23°C) invisible acrylic platform (diameter 12 cm, height 25 cm) was installed 25 cm away. In addition, the area below the water surface was installed at 1 cm. The wall of the tank is divided into four different color zones (red, yellow, green, white) and the mice could learn its relative position in the tank.

The tests were conducted in a quiet and static environment. SAM mice are randomly selected to enter the tank at one of three fixed positions equidistant from the platform in the tank, with the head of each mouse facing the wall. The goal time from when the mouse first entered the water to when it reached the safety of the platform was measured. Once we reached the platform, we left the mouse on the platform for 10 s to give it time to remember its relative position.

If the mouse could not find the platform within 120 s, it forced it to go to the platform and left it there for 10 s. The exam was conducted daily for a week. This test was conducted to evaluate spatial cognitive ability, a higher brain function in mice (Kaisho *et al*., 1994).

2.3. Preparation of frozen slide specimens

All mice in each group were euthanized by cervical dislocation 18 weeks after oral administration. Frozen specimens were embedded in white tissue coat (Ui Kasei Co., Ltd.) and then frozen at -80 °C for 1 minute with Histotech Pino (Sakura Finetech Japan Co., Ltd.) to prepare frozen blocks. Frozen sections were cut into 8 μm slices using a cryotome FE (Thermo Fisher Scientific) and attached to coated slides to prepare specimens. Fixation was performed under four conditions, and sufficient fixation and drying were performed.

This study was conducted by David et al., It refers to the hippocampal structure of the rat brain at the time of sectioning, as explained for graft manufacturing (David *et al*., 2021). It refers to the hippocampal structure of the rat brain at the time of sectioning, as explained by, and the tissue was sliced as shown in Fig. 3 (David *et al*., 2021, Allen *et al*., 1997).

2.4. Hematoxylin and eosin (HE) methods

Ethanol-based wet-fixing method (control method): Allow to stand in a wet-fixing solution (40 mL of 80% ethanol, 2 mL of acetic acid, and 4 mL of formalin stock solution) for at least 60 seconds. Dry fixation method: Leave to dry at room temperature for 10 minutes or longer. Dry fixation and rehydration method: After drying the sections at room temperature for 10 minutes or more, they were rehydrated by immersing them in physiological saline for 10 seconds.

Spray fixation method for fixing cytological specimens: After spraying Rapid Spray (Muto Kagaku Co., Ltd.) as a rapid coating agent, the samples were dried at room temperature for more than 60 seconds and then HE stained. The staining conditions were as follows: fixation, washing with warm water, hematoxylin for 30 seconds, warm water for 30 seconds, eosin for 30 seconds, dehydration, penetration, and encapsulation.

2.5. Klüver–Barrera (KB) staining

Kluver Barrera staining (KB staining) stains the myelin sheath and is an important special staining in neuropathology. In particular, in the separation operation of Luxol fast blue solution (LFB solution), it is easy for staining to vary between operators. It is thought that the separation operation differs depending on the length, which affects the staining results.

In this study, he examined the standing time for returning the LFB solution from heating to room temperature in KB staining.

The material used was an 8 μm section of cerebral autopsy tissue fixed in 10% buffered formalin. After the LFB solution containing the sections was heated at 60°C for 24 hours in a melting vessel, the sections were allowed to stand at room temperature after staining, and the time required for fractionation was investigated.

After leaving at room temperature for (1) 30 minutes, (2) 1 hour, (3) 2 hours, (4) 3 hours, and (5) 24 hours, the specimens were sorted appropriately and their stainability was examined.

2.6. Chemical structure of plant enzymes

The chemical structure of the *plant enzyme* is shown in Figure 3.

2.7. Statistical analysis

The significant differences were calculated at a $p < 0.05$ and $p < 0.01$ using Student's t-test, ANOVA and Tukey-Kramer test.

3. Results

3.1. Antioxidant action (SOD activity)

SOD activity was investigated in the mice serum 30 days after plant enzyme administration, as shown in Fig. 4A. Higher SOD-like activity was detected in the plant enzyme group (38.82 ± 6.2%) compared to the sham control group (25.8 ± 5.7%; $p < 0.05$).

3.2. Antioxidation effect (Luminol measurement)

The emission intensity measured with luminol is shown on the vertical axis in Fig. 4B. Thirty days after treatment, the plant enzyme had a positive effect on peroxyl radicals in mouse serum compared to the pseudo-control group. A

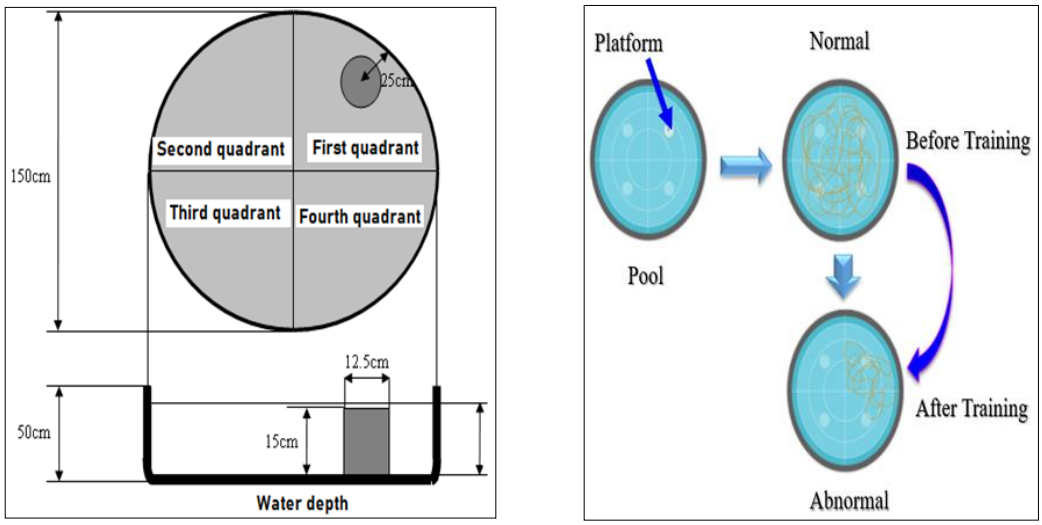
significant inhibitory effect was observed in the plant enzyme group (0.052 ± 0.003 Kcounts) compared to the bright intensity (0.074 ± 0.003 Kcounts) in the sham control group ($p < 0.05$). Therefore, a peroxy radical scavenging effect was confirmed in the plant enzyme group. In addition, a synergistic effect was suggested.

3.3. Fat peroxide levels in brain cells

The false control results of the water T-maze test and the density of fat peroxide in the mouse brain of the plant enzyme group were screened for tissue thiobarbituric acid-reactive substances (TBARS) (Fig. 5A).

A significant decrease in TBARS values was found in the false control group (3.1 ± 0.03 nmol / mL) and in the plant enzyme-treated group (1.6 ± 0.3 nmol / mL; $p < 0.05$). Therefore, the administration of plant enzyme suppresses lipids peroxidation in the brain.

3.4. Fat peroxide levels in liver cells



A B

Figure 2 Cross section of experimental water tank (A), and contextualized data and motion tracking (B). The water maze was a cylindrical pool (diameter 50 cm) filled with water (depth 16 cm, 23 ±2°C) and skim milk for opacity. A hidden platform (12.5 × 12.5 cm2) of transparent acrylic was installed about 1 cm below the water surface. Four points around the aquarium were arbitrarily designated, dividing the pool area into four platforms: east, west, south, and north quadrants. The lab conditions included fluorescent lights on the ceiling and black curtains

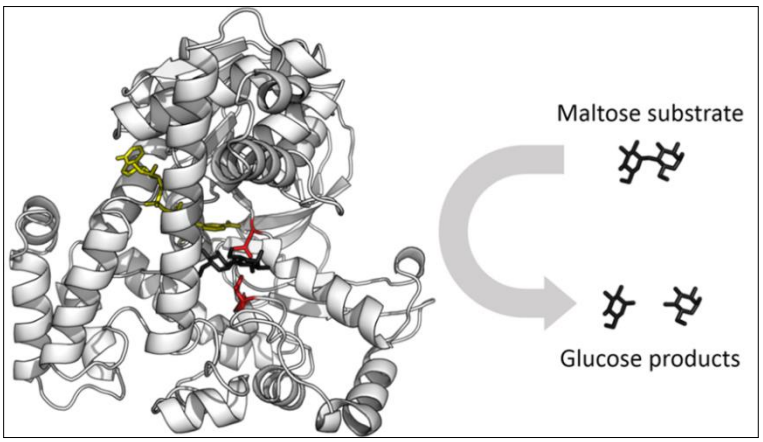
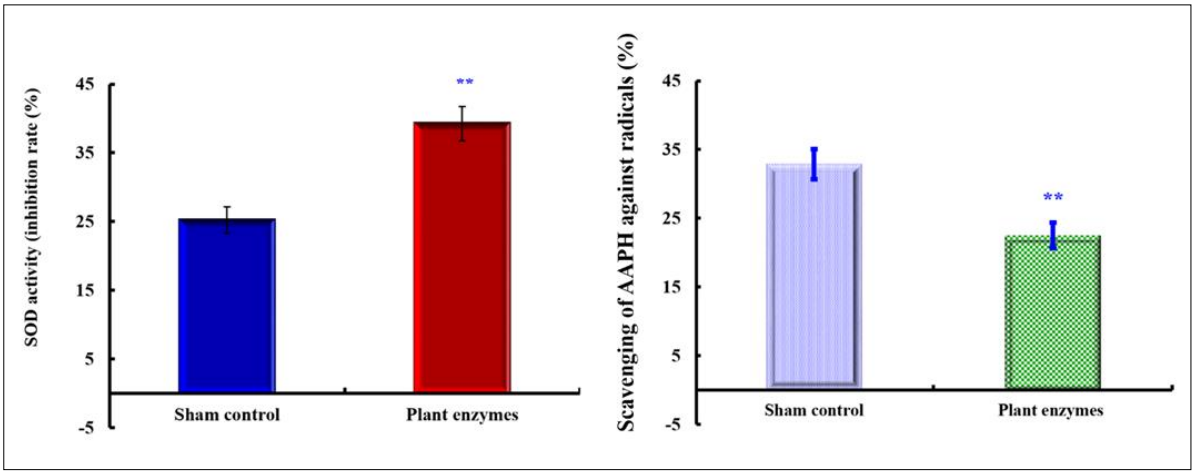


Figure 3 The *plant enzyme* glucosidase converts maltose into two glucose sugars. Active site residues are shown in red, maltose substrate in black, and the NAD cofactor in yellow. (PDB: 1OBB)

Pseudo-control group and mouse liver peroxidized fat density by T-maze test in aquarium the plant enzyme-treated group were screened by the tissue TBARS (Fig. 5B). The plant enzyme group (1.23 ± 0.4 nmol/mL) had a significantly decreased TBARS value compared with the sham control group (2.13 ± 0.4 nmol/mL; $p < 0.05$). Thus, lipids peroxidation inhibition in the liver appears to result from plant enzyme administration



A B

Figure 4 Effects of the *plant enzyme* group on SOD activity (inhibition rate,%) (A) and antioxidant activity (B) in the serum of the SAMP8 group. DW, the *plant enzyme* was administered for 30 days (p.o., 10 mL / kg dose per body weight). The resulting data is expressed as average ± S.E. (N = 10). The asterisks indicate significantly differences from sham control at $p < 0.05$ (**) compared with the t-test

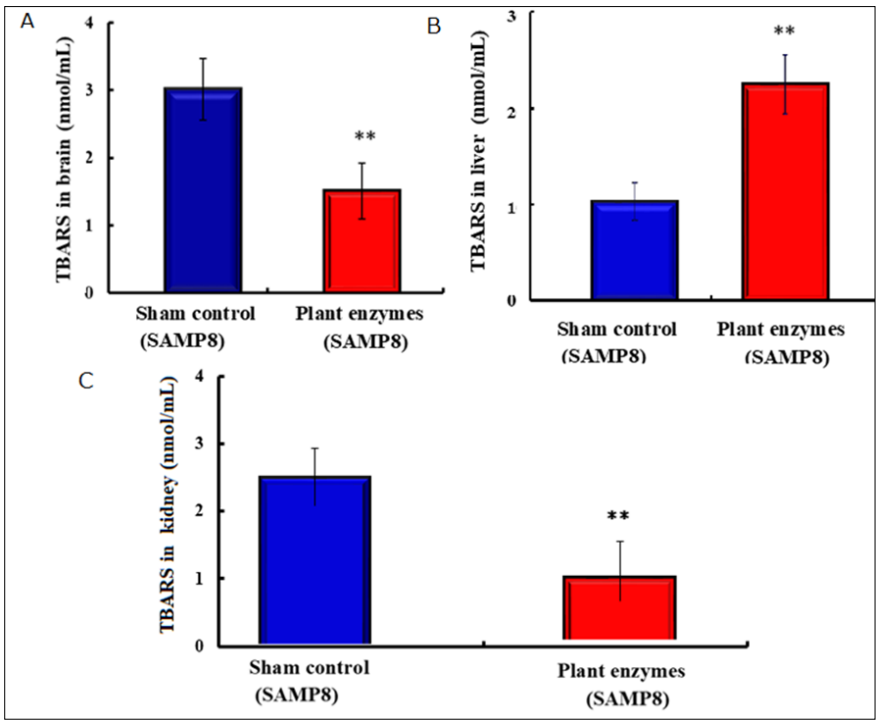


Figure 5 Effect of *plant enzymes* on TBARS levels in SAMP8 brain, liver, kidney, and homogenate. The results show the mean ± S.E. (n = 10). Asterisks indicate groups with significant differences from sham control at $p < 0.05$ (**) compared with the t-test

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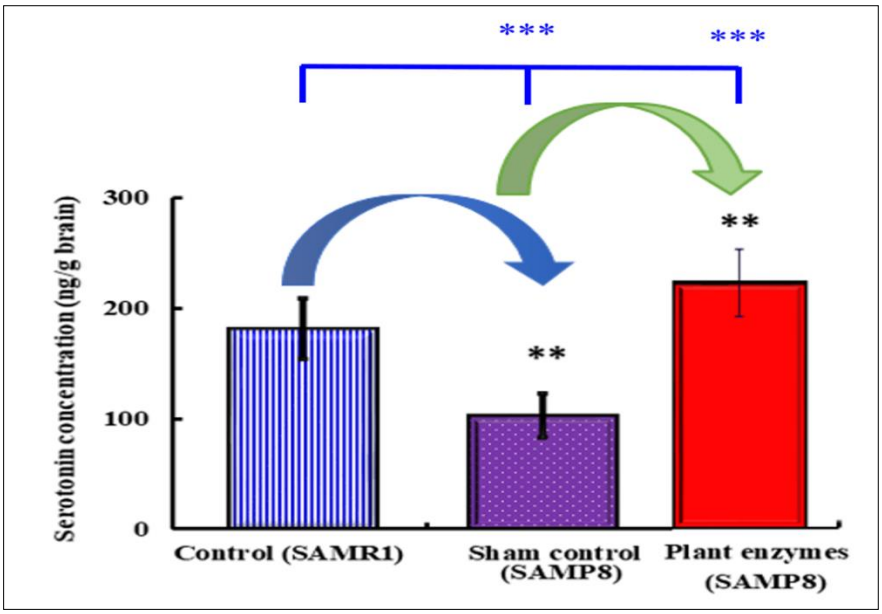


Figure 6 Serotonin concentration (ng/g brain) in SAM R1 and SAM P8 brains after the water maze test. Destilled water (Sham control) or *plant enzymes* were administered (p.o.) 30–60 min before the acquisition trial. The results are shown by the mean ± S.E. (n = 10). Asterisks indicate significant differences from sham control at $p < 0.05$ (**),control at $p < 0.05$ (***)compared with the t-test, ANOVA and Tukey-Kramer test

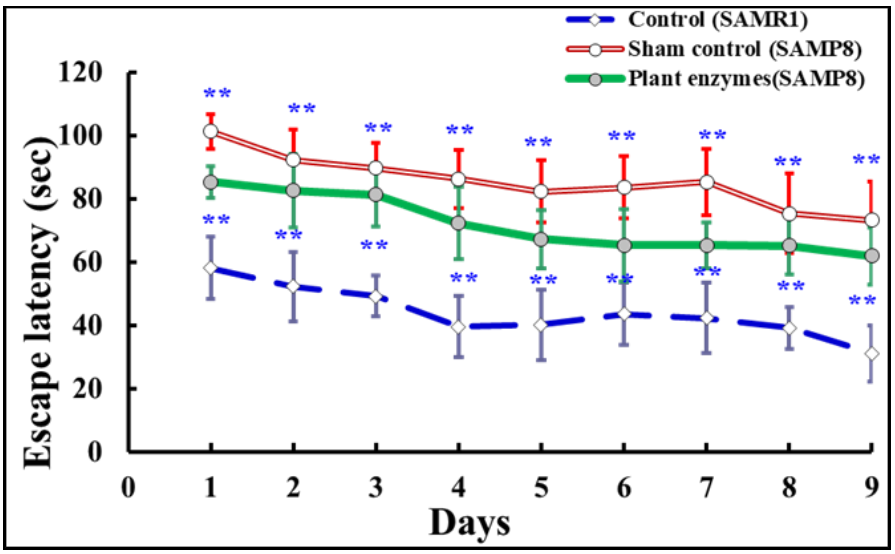
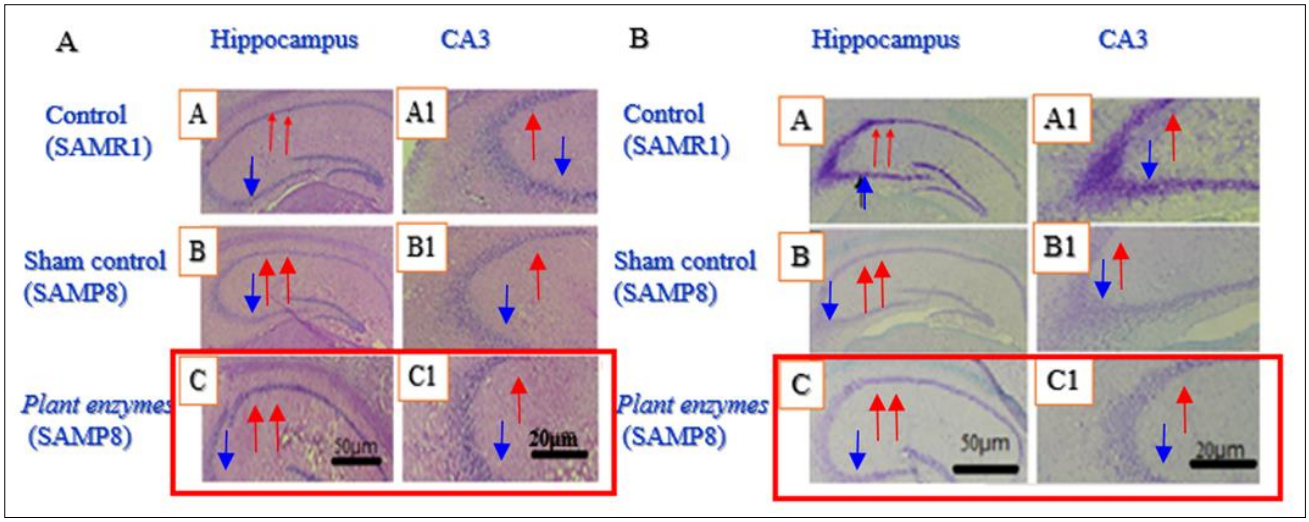


Figure 7 Waiting time to reach the platform from the water. Distilled water was administered 30–60 min before the experiment (p.o., 10 mL / kg each dose per body weight) and plant enzymes (p.o., 10 mL / kg each dose per body weight). The resulting data are expressed as mean ± S.E. (Control, sham control, *plant enzyme*). A 3 x 9 mixed (groups *v.s.* days) two-way ANOVA was analyzed for escaped latency time. Asterisk marks indicate significant differences from control at $p < 0.05$ (**) compared with the ANOVA and Tukey-Kramer test



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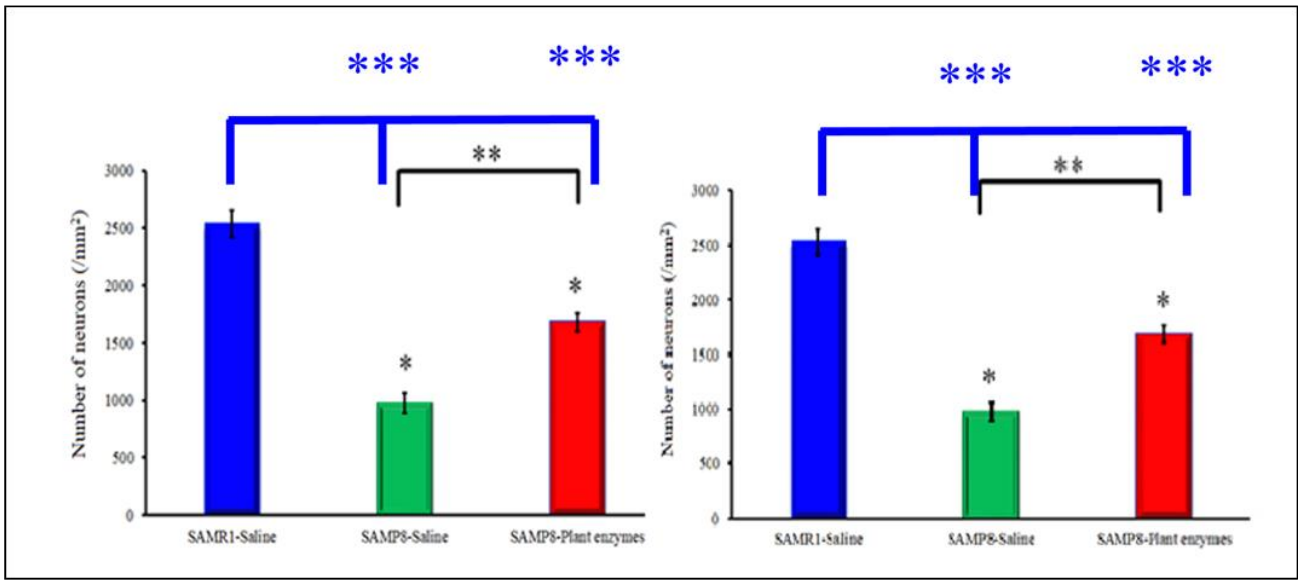

C D

Figure 8 Histopathological alterations of the hippocampal CA1 (double arrow↑↑) and CA3 (arrow↑) regions in 26–27 week-old SAM. Microphotographs of coronal sections of the hippocampus in control (A), sham control (B), and *plant enzyme* (C) stained with Klüver-Barrera. Scale bar, 50 μm × 1 (A-C), 20 μm × 5 (A1-C1). (A) representative photomicrographs of Klüver–Barrera's stained brain sections of SAMRI mice (a, b and c), SAMP8 mice treated with *plant enzyme*. Panels (b, e, and h) are representative. The number of Klüver- Barrera positive cells in CA-1 (B) and CA-3 (C) for each treatment group are shown in the representative images, and typical CA-3 images, respectively. Number of Klüver–Barrera's positive cells in CA-3 (C1) for each treatment group (n = 5 for each group, *$p<0.05$, **$p<0.05$, ***$p<0.05$, in comparison with age- matched SAMR1-saline, "$p<0.05$, SAMP8-saline *v.s.* SAMP8- *plant enzyme*), in compared with the t-test, ANOVA and Tukey-Kramer test

3.5. Fat peroxide levels in kidney cells.

Fat density peroxide concentration of mice kidney cells in sham control group from aquarium T-maze test of the plant enzyme-treated group were screened in terms of the tissue TBARS value (Fig. 5C). The plant enzyme treatment group (1.2 ± 0.4 nmol/mL) had a significantly lower TBARS value than the sham control group (2.6 ± 0.4 nmol/mL; $p < 0.05$). This indicates that lipid peroxidation in the kidney was inhibited by plant enzyme administration.

3.6. 5-HT concentration

Immediately after the T-maze test, the 5-HT concentration of cells in the mouse brain was measured (Fig. 6B).

Memory training tests were compared between the aging-promoting model control group (SAMR1 mice) and the aging-promoting model pseudo-control group (SAMP8 mice). As shown in Fig. 6A, the 5-HT concentration in the SAMP8 group was significantly lower (105 ± 18 ng / g brain) than SAMR1 (183 ± 27 ng / g brain; $p < 0.05$).

A significant increase in 5-HT concentration was found in the plant enzyme treatment group (225 ± 33 ng/g brain) compared with the sham control group (106 ± 59 ng/g brain; $p < 0.05$). Moreover, the range of brain 5-HT concentrations between the control group (SAMR1) and the plant enzyme treatment group were similar, compared with Fig. 6A. This suggested that the decrease in 5-HT concentration induced by accelerating senescence was inhibited by plant enzyme administration.

3.7. MWM test

In the MWM test, the spatial cognitive ability of SAMP8 mice was evaluated compared with SAMR1 mice (Fig. 7).

A significant delay in the platform delivery time was found for the sham control group and the plant enzyme treatment group compared with the control group ($p < 0.05$). In contrast, there was no significant difference in the platform delivery time between the sham control group and the plant enzyme treatment group.

3.8. Brain tissue staining

3.8.1. HE method

The histological changes in the hippocampus after chronic administration of plant enzymes are shown in Fig. 8A; histological changes were screened in the CA3 domain. The density of pyramidal cells in the hippocampal CA3 region of the sham control group (SAMP8 mice) decreased compared to the control group (SAMR1 mice) (Fig. 8A1, B1).

However, the cell density in the CA3 region was similar to that of the hippocampal domain in the control group and very similar to the plant enzyme group (Fig. 8C1). It was more prominent in the entire hippocampal region, including the CA1 domain of the plant enzyme-treated group and the CA3 region of the pseudo-control group; the density of pyramidal cells was high in the CA1 domain. In the CA1 domain, the density of pyramidal cells in the CA1 domain of the plant enzyme treatment group was significantly higher than that of the sham control group ($P < 0.05$).

3.8.2. KB method

KB staining in the hippocampus after chronic administration of the plant enzyme is shown in Fig. 8B, C, and D. Histological changes were observed in the CA3 domain. Tissue slices from the control, pseudo-control, and plant enzyme groups are shown in A, B, and C, and the hippocampal domain is shown in Fig. 8B, C, and D. The density of pyramidal cells throughout the hippocampus, including the CA1 and CA3 regions, was significant. This was reduced in the sham control group (SAMP8 mice) compared to the control group (SAMR1 mice) (Fig. 8A and B). In contrast, in the CA1 and CA3 regions of the plant enzyme-treated group, the cell density (C) was very similar to that of the hippocampal domain of the control group.

4. Discussion

The SAM model mouse is an inbred mouse strain developed by repeated mating and dividing by aging score selected based on pathological findings (Saito et al., 2016). SAM model mice compare four SAMR (accelerated aging-resistant mice) systems with 14 SAMP (accelerated aging-prone mice) systems in terms of accelerated aging and dementia aging. In addition, the SAMP Quadok system is a relatively aging-specific system in terms of promoting dementia-type aging. Therefore, it is possible to spontaneously develop various aging conditions (Saito et al., 2016, Merkenschlager et al., 2013). The production of mice with such a condition is often used in a model system of physiological aging in the elderly.

The SAMP8 mice used in this study show abnormalities in areas such as learning, mneme disorders, emotional disorders, immune dysfunction, and daily rhythm disorders, and show more aging-promoting symptoms than SAMR1 mice (Zuo et al., 2017, Siegel et al., 2012, Krueger et al., 2011, Wang et al., 2011). In addition, SAMR1 mice show natural aging, and a relatively low degree of aging compared to SAMP mice. Therefore, age-related increases in aging scores screened by the senile scoring system often use conventional-fed SAMR1 mice because they have a 40% longer survival of 50% compared to SAMP mice (Merkenschlager et al., 2013).

Dementia tends to increase with aging; one of the causes is OS, closely related to degenerative changes with aging, and characteristic of Alzheimer's disease (AD) (Akalin et al., 2014, Quinlan et al., 2010). Since reactive oxygen species (ROS) are generated when mitochondria are acidified with energy, the causes are said to be overwork, overeating, and stress.

As a result, ROS damages cell function by modifying functional molecules such as proteins, lipids, and DNA. Moreover, OS can cause damage inside and outside the cell if it cannot adequately handle the overproduced ROS. The contribution of ROS causes neurodegenerative diseases, including AD (Gloor et al., 2017). In addition, organic OS protection includes oxidative damage repair enzymes such as SOD, free radical scavengers, glutathione peroxidases, SH groups, antioxidants such as vitamin C and vitamin E, and free radical supplement antioxidants such as flavonoids and lipases (Gu et al., 2021). By measuring the ability to remove peroxy radicals from AAPH as an indicator of antioxidant activity in this study, the SAMP8 group had more luminescence than that of the control group (SAMR1). Furthermore, in this study, it is derived from SOD-like activity involving O2・-degrading enzyme and AAPH, which has an index of antioxidant activity, from the viewpoint of the effect of plant enzyme on antioxidant activity compared to the fake control group (SAMP8). The ability to remove peroxy radicals was investigated.

SOD-like activity was not significantly different in the plant enzyme treatment group but slightly higher and may be involved in the removal of peroxy radicals. Given the significant suppression in the release of light intensity luminol derived from AAPH, OS was attenuated in vivo by antioxidants derived from mouse serum such as AAPH. Therefore, plant enzymes are thought to prevent the progression of dementia and related diseases.

Potential enzymes for the prevention and treatment of Alzheimer's disease are polyphenols (GPPs). The effect of GPP on neuronal injury was investigated on cell viability, lactate dehydrogenase (LDH), lipid peroxidation (MDA), production of intracellular antioxidant enzymes, reactive oxygen species (ROS), mitochondrial membrane potential (MMP), Decrease the level of cellular inflammation(Tan et al., 2022).

The accumulation of TBARS in each measuring organ decreased with plant enzyme administration. In a longitudinal study using SAM mice, increased fat peroxide preceded symptoms (Chen et al., 1971). In addition, the serum TBARS of SAMP mice shows an increasing tendency compared with SAMR mice at 2 to 8 months after birth, showing similar changes in liver and skin tissues (Glavind et al., 1967). Moreover, Glavind et al. first measured TBARS in the brain of Wistar-derived rats reporting an increase in fat peroxide with aging (Glavind et al., 1967, Weglicki et al., 1968).

Dayan et al. reported an increase in TBARS with aging in rat serum, liver microsomes, and rat brain, liver, and kidney (David et al., 2021).

We think that the increase in TBARS in the liver is due to the promotion of liver metabolism due to recovery, but stabilized at medium–long-term.

In this study, we measured the brain, liver, and kidney TBARS of SAMP8 mice and investigated the effects of plant enzymes on lipid peroxidation in each organ(Tan et al., 2022, Chen et al., 1971, Glavind et al., 1967, Weglicki et al., 1968). Since the amount of peroxy radicals caused by AAPH is expected to correlate with that of fat peroxide, the decrease in peroxy radicals caused by plant enzyme led to a decrease in the amount of fat peroxide in the brain (Dayan et al., 1993). There was a significant difference between the sham control group and the radical enzyme treatment group in the liver and kidneys (Tappel et al., 1978).

Brain memory is made up of very complex phases between many nervous system structures and neurotransmitter systems. The cholinergic and glutamine-based systems are medically associated with cognitive processes such as attention, learning, and memory function, and 5-HT is reported to play a more important role in advanced cognitive processes (Benjamini et al., 2001, Kowalski et al., 2019). 5-HT of 1A receptors are predominantly highly distributed in the 5-HT receptor subtypes, especially in the hippocampal region. It is clear that the limbic system plays a particularly important role in learning and memory (Mazmanian et al., 2005, Mazmanian et al., 2008).

In addition, 5-HT is thought to be associated with emotional disorders with dementia as a peripheral symptom. This is because 5-HT is associated with such effects and has been shown to reduce anger and aggression with 5-HT agonists as measured by psychological tests (Deacon et al., 2006). However, it is known that there are many abnormalities in neurotransmitters in the AD brain, and it has been reported that 5-HT is related to the deep raphe nucleus as well as the midline of the raphe nucleus. The posterior pedunculopontine nucleus is reduced (Winkler et al., 1995). In addition, Gu et al reported to increase serum 5-HT levels in SAMP8 mice (Winkler et al., 1995). Therefore, in this study, we investigated the effect of plant enzymes on 5-HT concentrations in the brain of SAM mice.

A significant effect for the intracerebral 5-HT concentration was found in the two groups. As plant enzyme's antioxidant action resulted in an increase in the serum 5-HT level, we considered its effect on neurotransmitters such as acetylcholine. In this study, considering the regulatory mechanism of extrapyramidal disorders by the nervous system, it was shown that increasing 5-HT can alleviate exacerbation of extrapyramidal disorders caused by stimulation of 5-HT receptors (Anandatheerthavarada et al., 2007).

Recent literature has observed the accumulation of the Alzheimer's disease amyloid precursor protein (APP) and its C-terminal cleavage product β-amyloid (Abeta) in the mitochondrial compartment. Oxidative stress has been suggested to play an important role in the pathogenesis of neurodegenerative diseases. Alzheimer's disease is an age-related neurodegenerative disease recognized as the most common form of dementia. AD is characterized histopathologically by the presence of extracellular amyloid plaques, intracellular neurofibrillary tangles, oligomers of amyloid beta peptide, and synaptic loss.

Plant enzyme have shown that the low serum peroxyl radicals in the plant enzyme group are due to the enhancement of the dilution and degradation of β-amyloid by enzymatic radical scavengers (Bhat et al., 2015).

ROS and RNS are typically produced by tightly regulated enzymes. Excessive stimulation of NAD(P)H and electron transport chains leads to overproduction of ROS and oxidative stress, which are excellent mediators of damage to cellular structures, lipids, proteins, and DNA. Moderate concentrations of ROS/RNS also play a role in the normal physiology of many processes, including signaling pathways, induction of mitotic responses, and defense against infectious agents. Oxidative stress has been thought to be a major contributor to the pathogenesis of many diseases, including Parkinson's disease and Alzheimer's disease.

Therefore, it is suggested that it had a promising neuroprotective effect that would allow prevention or treatment of AD (Miller et al., 1980).

This study demonstrated progress with a specific landmark, indicating that allied learning is necessary for motion in the MWM (Miller et al., 1980). In this study, we used the MWM to examine cognitive function. Changes in spatial memory can be screened by testing the ability to acquire spatial recognition information. In this study, SAM mice were used to screen for spatial cognitive ability after plant enzyme treatment in a tank T-maze test. From the training sessions on 1 day to the last day, there was a significant increase in delivery time in the sham control group of SAMP8 mice compared to the control group of SAMR1 mice. In addition, in the probe test, the number of intersections increased significantly during the dwell time and was divided into four areas and a virtual platform area. In addition, the sham control group showed no reduction in delivery time until day 7, with no other changes over time. These results confirmed impaired learning of spatial perception and memory in SAMP8 mice.

Significant difference was found between the sham control group and the plant enzyme treatment group in comparison with the control group. Moreover, there was a significant difference between the sham control group and the plant enzyme treatment group. The inhibition of hippocampal cone cell death with plant enzyme administration suggests an improvement of spatial perception learning and memory impairment.

Most dementias apparently develop due to a remarkable neuronal decrease, and this is checked in the animal experiment case where the hippocampus is strongly associated with memory.

The hippocampal region of the brain consists of CA1, CA3, and the dentate gyrus (the lowest ranked region). The most important input site in the hippocampus is the entorhinal cortex, where neurons with axons are located in the dentate gyrus, CA1, and CA3 (McNaughton et al., 1989). In addition, the hippocampus gains important spatial information and acts as input to the hippocampus via a direct combination of the entorhinal cortex and CA1 (Miller et al., 1980). In the MWM, NMDA receptor antagonists were administered to the cone cell domain of CA1 in hippocampal slice specimens, as shown from the results of the T-maze test in the aquarium and from anatomical experiments (Wang et al., 2008). In addition, Riedel et al. also found hippocampal damage suppression, showing that the hippocampus is necessary not only for fixation but also for spatial memory recall (McNaughton et al., 1989). Furthermore, a decrease in nerve cells in the hippocampal CA1 region of SAMP8 mice compared to SAMR1 at 10 months of age has been reported (Riedel et al., 1999, Tanaka et al., 2005).

Observations of histological changes in this study were performed by the CA1 and CA3 domains in the SAM mice hippocampus. It was performed on the hippocampal structure of the rat brain described by David et al(David et al., 2021). The rodent hippocampal CA3 pyramidal cells are arched, and the pyramidal cells are thickly packed, but the pigment is very dark. In contrast, the ganglion cell layer of the optic nerve in the CA1 domain is thinly blocked. It was

determined to be even higher in the CA3 domain in Fig. 7. This is because CA1 cells are clearly reduced compared to CA3 pyramidal cells (Mitrovic et al., 1994). This significant effect throughout the hippocampus includes the CA1 domain of the sham control group (SAMP8 mice) and the CA3 domain of the control group (SAMR1 mice), with lower density of pyramidal cells as shown by HE and KB staining. Therefore, it was speculated that accelerated aging reduced spatial cognitive abilities, and as a result, hippocampal neurons decreased and as well as the input of spatial information. On the other hand, the cell density of the CA3 domain in the plant enzyme-treated group was higher than in the pseudo-control group. Furthermore, this was more pronounced in the entire hippocampus containing the CA1 domain of the CA3 region in the plant enzyme-treated group than in the sham control group. The plant enzyme-treated group also had significantly higher cone cell densities in the CA1 domain than the sham control group (Li et al., 2010).

Therefore, the nerve cells (CA1 domain) in the hippocampus become dense, and memory improvement is expected by a direct combination of CA1 effects. The reason why plant enzymes increased CA1 neuron density in the hippocampus is that radical scavengers reduce β-amyloid levels and lubricate with hormones to acutely modulate neural synaptic trans-mission involved in memory learning in the hippocampus (Gu et al., 2010).

Therefore, we speculate that plant enzyme improved learning and memory ability in this Aβ1-42 animal model by anti-neuroinflammation through its antioxidative effects (Matsubara et al., 2006).

Amyloid β (a is a 36–43 amino acid peptide that plays a significant role in Alzheimer's disease as the main component of amyloid plaques found in the brains of Alzheimer's disease patients. In this study, the peroxy radical was eliminated by of plant enzyme administration. Lipid hydroperoxides in the SAM mouse brain decreased, inhibiting lipid peroxidation in the brain (Rice-Evans et al., 1996).α-Glucosidase, of which glucosidase inhibitors are of particular interest, is essential for the breakdown of glycogen to glucose. It acts on complex carbohydrate molecules to produce monosaccharide units that are readily absorbed into the bloodstream. Inhibition of α-glucosidase appears to result in reduced glucose release into the bloodstream, thereby ameliorating AD.

Furthermore, the nerve cells of the hippocampus were activated, and it was suggested that the recognition function was improved. In addition, the intracerebral 5-HT level was normalized, which enabled an effect on the affective disorders that were related to the recognition symptoms, by changing the stability (Ballasch et al., 1996).

Furthermore, an effect on lipid peroxidation inhibition and learning and improvement effect in terms of memory impairment were found with plant enzyme administration. Therefore, plant enzyme was clearly effective in memory improvement in terms of the recognition symptoms.

List of abbreviations

- SAM: Senescence-Accelerated Mouse,
- HE: Hematoxylin and eosin,
- TBARS: Thiobarbituric acid reactive substances,
- SOD:Superoxide dismutase,
- KB: Klüver–Barrera

5. Conclusion

Plant enzyme was administered in a senescence-accelerated model mouse (SAMP8), and the effects on spatial cognition and the cerebral hippocampus were investigated. In the Morris water labyrinth experiment, a comparison between the control group of SAMR1 mice and the sham control group of SAMP8 mice (SAMP8 without *plant enzyme* administration) showed a significant reduction in arrival time, and in the probe test, it was divided into 4 divisions. Moreover, a significant increase in dwell time and number of virtual platform area crossings was observed. The *plant enzyme* group tended to have a shorter, but not significantly so, arrival time than the sham control group. Serum peroxy radicals were significantly suppressed in the *plant enzyme* group as compared with the sham control group. However, there was no significant difference in brain lipid peroxide concentration between the *plant enzyme* administration group. In addition, the serotonin concentration in the brain significantly differed between the *plant enzyme* group and the sham control group. Given the density of nerve cells (CA1 region) in the hippocampus in the *plant enzyme* group, we expect an improvement in memory.

Compliance with ethical standards

Acknowledgments

In this research, the Ministry of Education, Culture, Sports, Science and Technology Notification No. 71 "Basic Guidelines on Implementation of Animal Experiments in Research Organizations" (June 1, 2006), Ministry of the Environment "Standards on breeding and storage of experimental animals and relief of pain" with reference to the reference.

Disclosure of conflict of interest

The authors declare no conflict of interest, financial or otherwise.

Statement of ethical approval

This study was approved by the Suzuka University of Medical Science Animal Research Ethics Committee (Ref: 18/721/32).

Human and animal rights

In this study, *plant enzyme* were administered using the Society for Senescence Acceleration Model (SAMP8), and the effects on spatial cognition and the cerebral hippocampus were investigated. We Moreover examined the evaluation of memory capacity in the Morris water maze experiment. Twenty animals were used to evaluate hippocampal cell density, antioxidative measurements, and brain serotonin levels in *plant enzyme* in SAM mice with Alzheimer's disease. *Plant enzyme* were used to measure lipid peroxide levels in the brain, liver and kidneys of 20 animals. This animal experiment must comply with the Basel Declaration of Ethical Guidelines, and the International Council for Laboratory Animal Science (ICLAS) has Moreover published the ethical guidelines. In addition, based on the above animal rights, we obtained the approval of Suzuka University of Medical Sciences and the approval of the Animal Ethics Science Committee.

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