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Original Article

Effects of aged garlic extract on spatial memory and oxidative damage in the brain of amyloid-β induced rats

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Abstract

This study investigated the effect of aged garlic extract (AGE) on spatial learning and memory ability using Morris water maze (MWM) test in amyloid-β (Aβ) induced-neurotoxicity rats. Pretreatment of AGE at oral doses of 125, 250 and 500 mg/kg for 8 weeks significantly prevented the learning and short-term memory impairment in Aβ-induced neurotoxicity rats. Histological analysis has shown that pretreatment of AGE reversed the neuron loss in the CA1 and CA2 regions of hippocampus of Aβ-induced neurotoxicity in a comparable effect of ascorbic acid. By DPPH and FRAP determination, AGE had high antioxidative activity. Pretreatment of AGE caused significant increases of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, no significant change in catalase (CAT) activity, and a significant decrease of malondialdehyde (MDA) level of the Aβ-induced rat brain homogenate. The results suggest that AGE ameliorates the cognitive dysfunction in Aβ-induced neurotoxicity rats via its antioxidative effect.

Keywords: aged garlic extract, hippocampus, Morris water maze, oxidative stress, spatial memory

1. Introduction

The hippocampus, as a key structure in forming cognitive maps and the primary region that mediates the spatial abilities is affected in the initial stages of Alzheimer's disease (AD) (Moodley *et al*., 2015). Amyloid-β (Aβ), is a major component of neuritic plaques in the brain tissue of AD patients (Choi *et al*., 2014). The accumulation of Aβ induces an increase in intracellular reactive oxygen species (ROS), which are usually removed by the protective endogenous antioxidant systems. However, the overproduction of ROS can lead to a damaging cycle of lipid peroxidation, depletion of natural antioxidants, and disruption of normal cellular metabolism (Shishehbor and Hazen, 2004). Aβ-induced oxidative stress and neuronal death have been reported in AD brains (Harris, Hensley, Butterfield, Leedle, & Carney, 1995).With high oxygen demand and abundance of peroxidation-susceptible lipid cells, the brain is vulnerable to the effects of ROS(Kim *et al*., 2015) and the hippocampus is most sensitive to oxidative stress (Candelario-Jalil, Mhadu, Al-Dalain, Martínez, & León, 2001). Following oxidative stress and accumulation of ROS, there are hippocampal cell death and impairment of learning and memory, especially of

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the spatial type (Yang, Park, & Song, 2013). Morris water maze (MWM) test was commonly used for assessment of the impairment of hippocampus-dependent spatial memory in animal models (Frisch, Kudin, Elger, Kunz, & Helmstaedter, 2007). Dietary supplements containing antioxidant-rich plant extracts have been shown to improve cognitive function in both humans and animals (Casadesus *et al*., 2004; Cohen-Salmon *et al*., 1997). Furthermore, antioxidant enzymes and malondialdehyde are currently considered to be the most important markers of oxidative stress (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006). Antioxidant-enriched diets were shown to increase spatial memory scores in the MWM test and activity of antioxidant enzymes, including superoxide dismutase (SOD),catalase (CAT),and glutathione peroxidase (GPx), in aging animals (Rasoolijazi *et al*., 2015). Therefore, antioxidants may attenuate Aβ-induced neuro-toxicity and cell death, leading to the amelioration of AD-induced impairment of spatial memory.

Aged garlic extract (AGE), prepared through the prolonged aging of garlic in an organic solvent, is a functional product of garlic (*Allium sativum* L.) and is rich in stable organosulfur compounds such as S-allyl cysteine (SAC), S-allylmercaptocysteine (SAM), and other substances with important biological activity(Amagase, Petesch, Matsuura, Kasuga & Itakura, 2001). Multiple components present in AGE are known to exert protective effects, as has been demonstrated in both *in vitro* and *in vivo* systems (Chauhan, 2006; Nishiyama, Moriguchi, & Saito, 1997). Aged garlic extract and SAC have been reported to be neuroprotective against Aβ-induced memory impairment in ICR mice, as evaluated by a Y-maze test and a passive avoidance task (Jeong *et al*., 2013). Although previous studies have demonstrated that SAC and AGE have anti-amnesic and neuroprotective effects (Hermawati, Sari, & Partadiredja, 2015; Jeong *etal*., 2013), little is known about the spatial learning and memory effects that result from the antioxidant activities of AGE in Aβ-induced animal models. The present study, thus, examines the effects of AGE in various doses on spatial learning and memory performance in rats. Moreover, we also investigated the effects of AGE on the alteration of malondialdehyde (MDA) and three antioxidant enzymes in the cerebral cortex, including SOD, CAT, and GPx.

2. Materials and Methods

2.1 Plant materials and chemicals

Aged garlic extract (AGE) was supplied by the Center for Research and Development of Herbal Health Products (CRD-HHP) at Khon Kaen University in Khon Kaen, Thailand. It was prepared by maceration fresh native garlic in 30% ethanol for 13 months in the dark at room temperature. After filtration and evaporation, a dried AGE powder was standardized to contain 30.96 mg/g of SAC and 32 μg/g of allicin (Petty patent No. 3506, Thailand). All chemicals in this study were analytical grade.

2.2 Antioxidant activity

The modified 2,2-Diphenyl-1-Picrylhydrazyl radical (DPPH) scavenging capacity assay was used (Sripanidkulchai & Fangkrathok, 2014)and the capability to scavenge DPPH radicals was expressed as a concentration to give 50% inhibition (IC_{50}) with vitamin C as a reference compound. The Ferric Reducing Antioxidant Power (FRAP) assay was used and the data were expressed as μmol Fe^{2+}/g of crude extract (Benzie & Strain, 1996).

2.3 Experimental animals

All experiments were conducted in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals, which were approved by the Ethics Committee of Khon Kaen University (Approval No. 0514.1.12.2/81). The animals obtained from the National Animal Center at Mahidol University (Bangkok, Thailand), were kept at 25±2°C with a relative humidity of 50-70% and maintained on a 12-h light /dark cycle (06:00–18:00 h) with *ad libitum* of food and water. A total of forty-eight male Wistar rats (180-220 g and 8-week age) were used in this study. After one week of familiarization with the sur-roundings, the animals were subjected to a five-day MWM training period. They were then housed at four rats per cage and classified into six groups (n=8) as follows:

Group 1 (V+ACSF) received distilled water and was injected with artificial cerebrospinal fluid (ACSF) into the lateral ventricle.

Group 2(V+Aβ) received distilled water and was injected with Aβ (1-42) into the lateral ventricle.

Group 3 (Vit C+Aβ) received Vitamin C (Black-mores, Australia) at 250 mg/kg BW.

Groups 4, 5 and 6 (A125+Aβ, A250+Aβ, A500+Aβ) received aged garlic extract at doses of 125, 250 and 500 mg/kg BW, respectively.

The animals were gastrically gavaged with bio-medical needles at 8.00 to 9.00 a.m. for 64 consecutive days. At day 56, the rats in groups 2-6 were injected with amyloid-β (Aβ1-42) into both sides of the lateral ventricle, whereas the rats in group 1received sham injections of ACSF. An MWM task was performed as a behavioral test for spatial learning and memory after seven days of Aβ injection. A probe test was conducted 24 h after each MWM test (Figure 1).

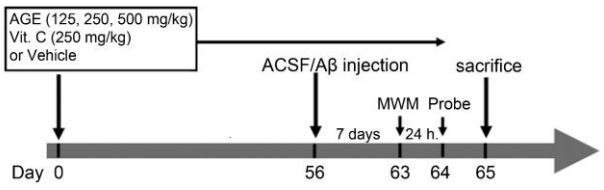

Figure 1. Schematic diagram of drug treatment and behavioral tests. (MWM: Morris water maze test, Aβ: amyloid-β (1-42), ACSF: artificial cerebrospinal fluid).

All surgical procedures were conducted under aseptic conditions and sodium pentobarbital (35 mg/kg, i.p., Sigma-Aldrich, Germany) anesthesia. Aβ1-42 or Aβ (Enzo Life Science, Switzerland) was dissolved in glacial acetic acid at a concentration of 1 mg/ml and stored at −18°C in aliquot tubes. Before injection, Aβ-aggregation was performed by incubation at 37 °C for 24 hrs (Nillert *et al*., 2017). Rats were restrained using a stereotaxic holder. A midline sagittal incision was made in the scalp and a hole was drilled in the

skull over the lateral ventricle using the following coordinates: 0.8 mm posterior to Bregma and 1.5 mm lateral to the midline(Lin *et al*., 2009). All injections were made using a 10-µl Hamilton syringe equipped with a 26-gauge needle. The dura was perforated with the needle of the micro syringe, which was inserted 3.8 mm beneath the dura mater. Animals were injected with 1 µl of aggregated Aβ into each side of the lateral ventricles at a rate of 1µl/min. The sham-operated rats were injected with ACSF. The syringe was left in place for 5 min after injection before being slowly removed.

2.4 Morris water maze test

Spatial learning and memory were measured using the MWM task that tests the animal's ability to learn the spatial location of a platform submerged below the surface of a pool of water. A large blue plastic circular pool, 180 cm in diameter and 60 cm in height, was placed in the center of the testing square room. The pool was divided into four equal quadrants and filled with water (25±2 ℃) to a depth of 45 cm. A plastic circular platform (10 cm in diameter) was always positioned 30 cm from the wall of a quadrant and hidden 2 cm below the water surface. White talcum powder was then scattered on the water's surface to make it opaque. Objects of various shapes (such as circular, triangular, square and hexagonal) and colors were hung on the wall in the testing room as visual spatial cues. The testing consisted of an acquisition (learning) phase and a probe (retention) phase. The acquisition phase was used to test the rats' learning and short-term memory abilities; whereas the probe phase was used to test the rats' long-term memory. Each phase contained four trials with a 10-min interval between each trial. For the learning phase, the rats were given 60 s to find the hidden platform. When successful, the rat was allowed 15 s on the platform. If unsuccessful within 60 s, the rat was placed on the platform by the experimenter for 15 s and given a score of 60 s. For the probe phase, four trials (60 s each) were conducted with no platform present 24 h after the last learning trial. The swimming activity of each rat was tracked via a camera linked to a computer monitoring system. A video camera was positioned directly above the center of the pool, where it could monitor the entire surface area of the pool. Latency to find the platform and time spent in the target quadrant were recorded and analyzed using the free track analyzer program (Wolfer & Lipp, 1992) to compare spatial memory and learning ability between groups.

2.5 Tissue preparation

At the end of the experiment, all rats were anesthetized by injection with sodium pentobarbital (60 mg/kg, i.p.) and transcardially perfused with 0.9 % normal saline solution. The whole brain of each rat was then immediately cut into two hemispheres. The left hemisphere was cryopreserved in sucrose solution (30%) and fixed in ice-cold 4% paraformaldehyde solution for histological investigation. The cortical tissue from the right hemisphere of each brain was separated from the white matter on an ice-cold surface. The brain homogenate in an ice-cold 0.04 M sodium phosphate buffer (pH 7.4) was immediately prepared (Carrillo, Kanai, Nokubo, & Kitani,1991). The homogenate was centrifuged at 15,000 g at 4°C for 10 min, then the protein content of the supernatant was determined (Lowry, Rosebrough, Farr,& Randall, 1951) and stored at –80 °C until further biochemical analysis.

2.6 Histological procedure

Serial coronal sections were cut on a freezing microtome at 35µM, and every fifth section was stained with cresyl violet (Deitch & Moses, 1957). The brain sections were then rinsed in 0.01 M phosphate buffer and mounted on slides coated with 0.01% aqueous solution of high molecular weight poly-L-lysine. The slides of brain sections were stained with 0.75% cresyl violet, dehydrated through graded alcohols, xylene and cover-slipped using DPX mountant (Sigma, St. Louis, MO). Slides were examined under a light microscope at 40X objective.

2.7 Neurons quantification

A Nikon Eclipse *Ci* Upright Microscope (Nikon Corp, Japan) equipped with Image Frame Work (Tarosoft®, Inc, USA) was used with a Prosilica GT digital camera (Dynatech Inst, Thailand) connected to a computer. Neuron counts were derived from 15 histological sections spaced at 245 µm intervals, through the entire rostrocaudal extent of one hippocampus from each brain. A systematic random sampling scheme was used for determination of the counting frame (Korbo *et al*., 1990; Royet, 1991). Cell counting was confined to small areas in each region of the hippocampus and began in the left part of the section. The neurons visualized inside the frame were quantified and those cells that touched the boundary line were eliminated (West, Slomianka, & Gunder sen, 1991).

2.8 Biochemical analysis

The activity of SOD was determined based on inhibition of superoxide-dependent reactions (McCord & Fridovich, 1969).The data were expressed as units/mg protein. CAT activity was determined and the data were expressed as units/mg protein. (Goldblith & Proctor, 1950). The activity of GPx was determined by an indirect method and expressed as unit/mg protein (Gunzler & Flohe, 1985). MDA levels as an oxidative damage indicator (Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003) were determined and the data were expressed as nmol/mg protein**.**

2.9 Statistical analysis

All data were expressed as standard error of the mean. Statistical analysis of the experimental data was carried out using SPSS (version 11.0). The significance of differences among groups was analyzed using a one-way ANOVA and a Newman-Keuls post hoc test. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1 Antioxidant activity of AGE

According to DPPH assay, AGE exhibited high activity compared to standard vitamin C (IC_{50} = 3.23 ± 0.57versus 3.11 ± 0.02 µg/ml). With the calibration curve of

standard ferrous sulfate (y = 0.2307x + 0.0872, R^2 = 0.9995), the reducing ability of the AGE was 119.44 ± 18.79 µmol Fe^{2+}/g crude extract (Table 1).

Table 1. Antioxidant activities of aged garlic extract.

	FRAP µmol/g extract (in Fe^{2+})	DPPH IC_{50}(µg/ml)
Vit. C	-	3.11 ± 0.01
Aged Garlic Extract	119.44 ± 10.84	3.23 ± 0.33

3.2 Effects of AGE on spatial learning and memory

When compared to the V+ACSF group, Aβ induction caused significant deficits in both the spatial learning and probe phases ($p < 0.05$). Pretreatment with AGE at doses of 250 and 500 mg/kg BW for 56 days significantly improved learning and prevented short-term memory loss. The rats received vitamin C or AGE at a dose of125 mg/kg BW had shorter times in reaching the platform than those in the V+Aβ group without statistical significance. In contrast, in the probe phase, there was no significant difference in the time to spent among all treated groups of rats. However, all AGE-treated rats displayed higher retention time than those in the V+Aβ group, similar to those in the positive control (Vit C-treated) group (Figure 2). In addition, there was no significant difference in the average swimming speed among groups (data not shown).

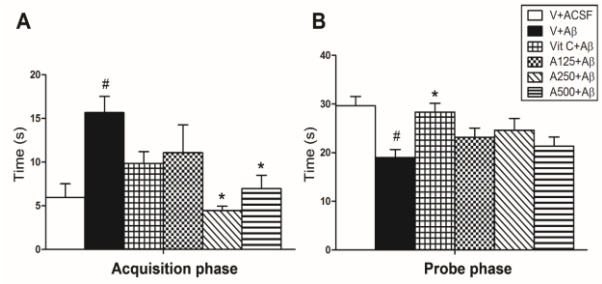

Figure 2. Effects of AGE on spatial learning and memory performance in Aβ-induced rats as measured by latency time to reach the platform (A) and time to spent in the target quadrant (B) during the MWM task. Data are presented as mean ± S.E.M. (n = 8/group), # = significant differences from V+ACSF group at p < 0.05; * = significant differences from V+Aβ group at p < 0.05.

3.3 Neuroprotective effects of AGE

In the V+ACSF group, most of the neurons were in fact having round or oval nuclei located in the center of the perikaryon surrounded by pale cytoplasm (Figures 3A, 3a and 3g.). In contrast, the rats injected with Aβ showed morphological alterations in most of their neurons, including irregular shape, some dark staining due to condensation of cytoplasm and nucleoplasm, and changes to their nuclear cell boundaries (Figures 3B, 3b. and 3h.). Moreover, neuronal cell loss was visualized as the absence of the Nissl staining, which showed that the Aβ induced the loss of neurons in all regions of the hippocampus. Pretreatment with vitamin C and all doses of AGE showed significant restoration of neuronal density and reversal of morphological changes to neurons in the hippocampus, to the point where these features were similar to those observed in the ACSF control rats. This indicates that AGE had a neuroprotective effect against Aβ-induced neurotoxicity (Figures 3c.-f., 3i.-l., 3C., and 3D.). Counting of neurons revealed that the neuron densities in the CA1 and CA3 regions of Aβ-induced rats were at 42.58% and 44.64%, respectively, of those of the control rats. Vitamin C and AGE at all doses are able to reverse the loss of neurons that had occurred due to Aβ toxicity in the hippocampus (Figure 3C. and 3D.).

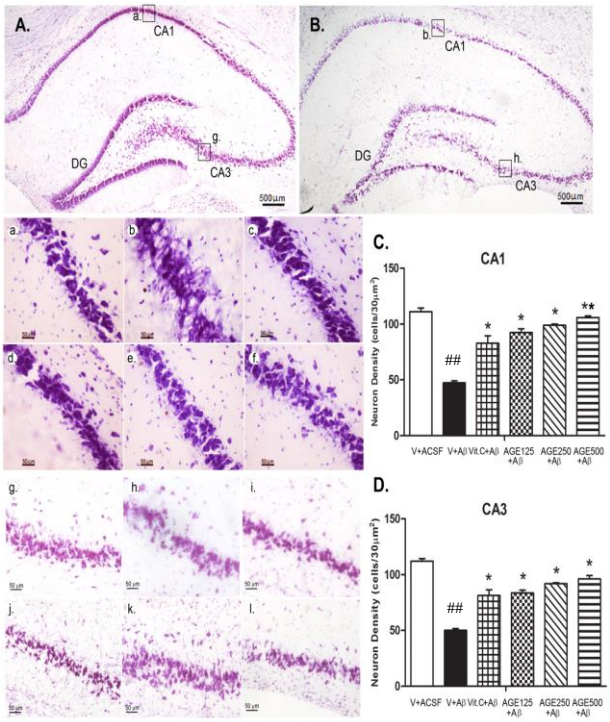

Figure 3. A low-power micrograph of the hippocampal sections of an ACSF-induced rat (A) and an Aβ-induced rat (B) showing the distribution of neurons in the regions of the hippocampus using Nissl staining with cresyl violet. The small square boxes in Figures 3A and 3B represent the CA1 and CA3 regions of the hippocampus. Figures 3a.- 3f. and Figure 3g. - 3l. represent the neurons in the region of CA1 and CA3 of the hippocampus of V+ACSF (3a, 3g), V+Aβ (3b, 3h), Vit C+ Aβ (3c, 3i), AGE125+Aβ (3d, 3j), AGE250+Aβ (3e, 3k), AGE500+Aβ (3f, 3l), respectively, under high magnification. Figures 3C and 3D show the neuron density in the CA1 and CA3 regions of the hippocampus. Data are presented as means ±S.E.M. (n = 8/group), ## = significant differences from V+ACSF group at p<0.01; *, ** = significant differences from V+Aβ group at p < 0.05 and 0.01, respectively.

3.4 Biochemical analysis

As shown in Figure 4A, Aβ injection produced a significant increase in MDA levels (147.81% of the ACSF control group at $p<0.05$). The Aβ-treated rats that were pretreated with Vit C and AGE at all doses showed significant

reductions in MDA levels ($p < 0.05$). The rats treated with Aβ showed significant decreases in brain SOD and GPx activity($p < 0.05$) as compared with ACSF animals (Figures 4B and 4D). All doses of AGE pretreatment were able to protect against the neurotoxicity of Aβ as observed in that there were significant increases in SOD and GPx activity in the brain supernatant ($p<0.05$). Although all AGE doses tended to increase CAT activity in the Aβ-induced rat brain, these increases were not statistically significant (Figure4C). In contrast, pretreatment of the standard drug, Vit C, significantly increased the activities of all antioxidant enzymes ($p<0.05$).

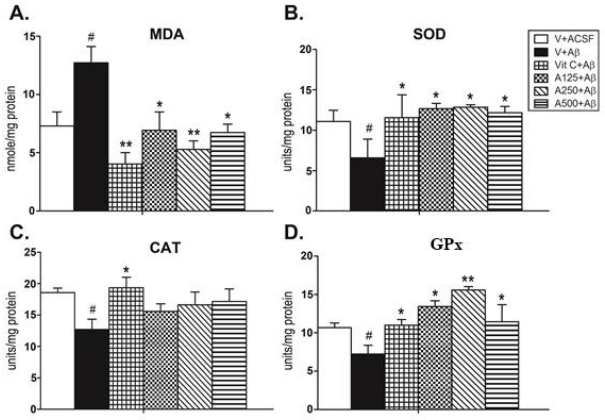

Figure 4. The effect of AGE on MDA levels (A) and activation of antioxidant enzymes [SOD (B), CAT (C), and GPx (D] in the rat brain homogenates. Data are presented as mean ± S.E.M. (n = 8). # = significant differences from V+ACSF group at $p < 0.05$; *, ** = significant differences from V+Aβ group at $p < 0.05$ and 0.01, respectively.

4. Discussion

In this study, high antioxidant activities of AGE were confirmed in comparison with the activity of standard vitamin C as measured by the DPPH assay. The results on FRAP assay also showed high antioxidative of AGE activity as previous report (Wong *et al*., 2006).

MWM task was chosen to evaluate the spatial memory performance of the AGE-treated rats in this study because it has proven to be a powerful and reliable test that is strongly correlated with hippocampal spatial function in rodents (Vorhees & Williams, 2006) and deficits in MWM performance may relate to the intellectual decline in human Alzheimer patients (D'Hooge & De Deyn, 2001). In animal models, several studies have focused on the effects of Aβ peptides on the spatial memory and hippocampal neurons. The injection of Aβ impaired memory performance in MWM tests in both mice (Yan *et al*., 2001) and rats (Yamada *et al*., 1999)and caused neuronal degeneration in the hippocampus (Nitta, Fukuta, Hasegawa & Nabeshima, 1997). In this study, the impaired learning and memory, as well as loss of neurons in various hippocampal regions of the rats' brains were detected after bilateral Aβ injections. Pretreatment of AGE at doses of 250 and 500 mg/kg was able to protect against neurotoxicity caused by Aβ induction, as demonstrated by improvements in short-term spatial memory. Although AGE did not enhance long-term spatial memory, it tended to restore it. We also evaluated swimming speed and locomotor behavior in the locomotor test, but there were no significant differences among groups (data not shown), indicating that AGE, especially at medium and high doses, may facilitate spatial learning and memory without any motor effects. The neuron density in the CA1 and CA3 regions of all AGE-treated groups was significantly higher than that of the Aβ-treated group and did not differ from the V+ACSF group. Furthermore, the cell morphology of the Aβ-induced rats was difficult to identify because most of the cells had dark staining and cell boundaries were not clear. This indicates that Aβ both damaged and caused a reduction in the numbers of neurons in the CA1 and CA3 regions and that pretreatment with AGE can reverse this deficit.

Recent studies have reported important differences in the function of the CA1 and CA3 regions of the hippocampus in spatial memory (Lee, Yoganarasimha, Rao & Knierim, 2004). The CA1 region plays a critical role in memory regarding sequences of events, in addition to its well-described role in spatial navigation memory (Hunsaker, Lee & Kesner, 2008; MacDonald, Lepage, Eden & Eichenbaum, 2011). In contrast, the CA3 region has been found to principally contribute to memory regarding the association of items with spatial information (Kesner & Warthen, 2010). Therefore, any damage to the CA1 or CA3 regions impair spatial learning and memory, which can be measured by the MWM task (Bartsch *et al*., 2010; Tsien *et a*l., 1996). Although it has been argued that the CA3 region contributes to the retrieval of memory using pattern completion rather than spatial memory acquisition (Nakazawa, McHugh, Wilson,& Tonegawa, 2004),many theoretical models of hippocampal function have proposed different roles for the hippocampal subfields in memory encoding and retrieval (Treves & Rolls, 1994; Wiebe, Stäubli,& Ambros-Ingerson, 1997). In our study, all AGE-treated groups performed better than the V+Aβ groups in the acquisition phase of the MWM test, but not in the retention phase, suggesting that this better performance may have been caused by alterations in the CA1 and CA3 regions of the rats' hippocampi.

Prevention of memory and neuronal loss in the Aβ-induced rats may relate to antioxidant activity of AGE. Aβ has the potential to induce oxidative stress, including increased production of hydrogen peroxide and lipid peroxides in neurons (Behl, 1997; Varadarajan, Yatin, Aksenova, & Butterfield, 2000). SOD, GPx and CAT were reported to involve in cellular protection against damage caused by oxygen-derived free radicals (Crack, Cimdins, Ali, Hertzog, & Iannello, 2006) and malondialdehyde, an end product of lipid peroxidation, has been considered as a late biomarker of oxidative stress and cellular damage (Vaca, Wilhelm,& Harms-Ringdahl, 1988).To assess the effect of AGE on oxidative stress, these four parameters were monitored. In our study, the brain homogenates from Aβ-induced rats elevated MDA levels and decreased SOD, CAT and GPx activity. The increase in MDA levels suggests that this event is necessary to scavenge the free radicals induced by Aβ. A significant reduction of SOD activity in Aβ-treated rats might be responsible for increased concentrations of superoxide radicals. A significant reduction in both CAT and GPx

activities can increase the production of highly deleterious H_2O_2. Therefore, the elevation of oxidative stress and reduction of antioxidant enzyme (SOD, GPx, and CAT) activities may cause neuronal loss and play a role in memory impairment. Furthermore, AGE at all doses was able to restore MDA levels and increase the SOD and GPx activity in the brain homogenates of Aβ-treated rats. However, there was no increase in CAT activity. In term of phytochemicals, the process of macerating fresh garlic in 30% ethanol for 13 months to obtain AGE caused a considerable increase in SAC and decrease in allicin (Seanthaweesak, 2006).However, other thiosulfinates, such as S-allylmercaptocysteine, have also been reported to exhibit significant antioxidant activity (Banerjee, Mukherjee,& Maulik, 2003). Therefore, antioxidant activity of AGE in the present study maybe resulted from not only SAC, but also other compounds or combination among various organ sulfides.

5. Conclusions

AGE provides beneficial effects in terms of both cognitive enhancement and neuroprotection, and these benefits can occur in Aβ-induced cognitive deficit conditions. The mechanism may partially relate to the antioxidant properties of AGE, which can scavenge free radicals through SOD and GPx activity, allowing for the enhancement of spatial learning and memory performance and protection of neurons against damage caused by oxygen-derived free radicals. However, further studies are needed to investigate the molecular mechanisms underlying this phenomenon.

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