Beneficial effect of black rice (*Oryza sativa L.* var. *japonica*) extract on amyloid β-induced cognitive dysfunction in a mouse model

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Abstract. Alzheimer's disease (AD) is an age-dependent progressive neurodegenerative disease, resulting in memory loss and cognitive dysfunction. The accumulation of amyloid β (Aβ) has been identified as the most important risk factor for AD. Black rice (BR; *Oryza sativa L.* var. *japonica*), which is widely consumed in Asia, is a good source of bioactive compounds including anthocyanins. Therefore, the aim of the present study was to evaluate the protective effect of BR extracts against $A\beta_{25-35}$-induced memory impairment in an *in vivo* AD mouse model. After intracerebroventricular injection of $A\beta_{25-35}$, mice were treated with BR extract supplementation for 14 days. Memory and cognition function were evaluated over this period in both treated and untreated animals using T-maze, novel object recognition and Morris water maze tests. After behavioral tests, malondialdehyde (MDA) and nitric oxide (NO) concentrations in brain, liver and kidney tissues were analyzed. Mice treated with $A\beta_{25-35}$ had impaired memory and cognitive function; however, mice administered BR extract (100 mg/kg/day) demonstrated an improvement in cognition and memory function compared with the $A\beta_{25-35}$-injected control group. Furthermore, injection of $A\beta_{25-35}$ significantly increased MDA and NO generation in the brain, liver and kidney of mice. However, the group administered with BR extract had significantly inhibited lipid peroxidation and NO generation in the brain, liver and kidney. In addition, the protective effect of BR on lipid peroxidation and NO production by $A\beta_{25-35}$ was stronger in the brain compared with other tissues. Collectively, these findings suggested that BR supplementation may prevent memory and cognition deficits caused by $A\beta_{25-35}$-induced oxidative stress.

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Introduction

Alzheimer's disease (AD) is one of the most common neurodegenerative disorders in the elderly (1). AD is characterized by learning and memory dysfunction, cognitive impairment and personality changes (2). Moreover, this neurological disease affects >30 million individuals worldwide and this number is expected to double by the year 2030 (3). A main cause of neurodegeneration in AD is increased production and accumulation of amyloid β (Aβ) (4). Aβ changes its conformation to form aggregates, which are eventually deposited as senile plaques; the pathological hallmark of AD in the brain (5). The aggregated state of Aβ is of great importance in the induction of its toxic effects and Aβ is also implicated in increased free radical production, which in turn induces neuronal damage (6). Oxidative stress caused by overproduction of free radical transforms non-aggregated Aβ to aggregated Aβ and Aβ itself is also a source of free radicals (7). Consequently, oxidative stress induced by free radicals has been proposed to be a major contributing factor in neuronal dysfunction and AD (8). Thus, reducing Aβ neurotoxicity is one of the key strategies in improving AD outcomes and there are several studies that support this approach as a valid therapeutic strategy in treatment of AD pathogenesis (9-11).

Black rice (BR), *Oryza sativa L.* var. *japonica*, is predominantly grown and consumed in Korea, Japan and China (12). BR is known to serve several beneficial roles in mitigating the effects of pathological conditions such as cardiovascular disease, cancer and inflammation (13-15). Previous studies have also reported that the anthocyanins from BR, including cyanidin, malvidin and peonidin, have anti-oxidative, anti-bacterial and anti-cancer activities (16). Research on BR has mainly focused on its anthocyanin pigment, which has been shown to exert a positive effect in preventing arterial aging and lowering blood pressure (17). It has also been revealed that anthocyanin inhibits cholesterol absorption and reduces oxidative stress in cellular models (18,19). Previous studies on the beneficial effects of BR and its constituents have focused on the prevention of cancer and arteriosclerosis (13,14,20,21). However, to the best of our knowledge, the protective effect of BR in AD remain unknown and whether consumption of BR improves cognitive impairment is yet to be elucidated.

Therefore, the aim of the present study was to investigate the protective activity of BR extracts on cognition and memory impairment in an *in vivo* AD model induced by $A\beta_{25-35}$.

Materials and methods

Reagents. Malondialdehyde (MDA) was purchased from Sigma Aldrich (Merck KGaA). NaCl was purchased from Bio Basics, Inc. Thiobarbituric acid (TBA) was provided by Lancaster Synthesis Ltd. Phosphoric acid and 1-butanol were acquired from Samchun Pure Chemical Co., Ltd. Methanol (MeOH) was purchased from Duksan Pure Chemicals Co., Ltd.

Preparation of MeOH extracts of BR. BR was obtained from Jeon-ju National Agricultural Cooperative Federation Gongpanjang. Whole BR was washed with water and dried at 55˚C for 24 h and ground to powder. Then, 10 g BR powder was refluxed in 200 ml MeOH for 24 h at room temperature and vacuum-filtered through a Whatman no. 4 filter paper (pore size, 4 μm; Whatman; Cytiva). This was repeated three times and duration of each cycle was 24 h. The extract was concentrated using a rotary evaporator at 34˚C. The final yield of this extraction was 3.7% (w/w). The dried extract was stored in a deep freezer at -80˚C until further use. The extract was dissolved in PBS for the subsequent experiments.

Animals and experimental protocols. The animal protocol used in this study was reviewed and approved by the Pusan National University-Institutional Animal Care and Use Committee (approval no. PNU-2010-000142) on the Ethical Procedures and Scientific Care of Laboratory Animals. A total of 50 male ICR mice (age, 5 weeks; Orient Bio, Inc.; weight, 25-27 g) were housed in plastic cages at 20±2˚C, 50±10% humidity and 12 h light/dark cycle with *ad libitum* access to food and water. ICR mice were divided into four groups (n=8/group) as follows: Normal (0.9% NaCl injection + PBS), control ($A\beta_{25-35}$ injection + PBS), BR 50 ($A\beta_{25-35}$ injection + BR MeOH extract 50 mg/kg/day) and BR 100 ($A\beta_{25-35}$ injection + BR MeOH extract 100 mg/kg/day). There were no significant differences in body weight among the groups, which helped to eliminate physical differences due to body weight variation. The normal and control groups were orally administered 100 μl of PBS (n=8/group) and the BR 50 and BR 100 groups were orally administered BR extract at doses of 50 and 100 mg/kg/day for 14 days (n=8/group) via oral gavage. All the experimental and behavioral procedures are presented in Fig. 1.

Development of the $A\beta_{25-35}$-induced mouse model. To induce aggregation, $A\beta_{25-35}$ (Sigma Aldrich; Merck KGaA) was solubilized at a concentration of 5 nmol in 0.9% NaCl and incubated at 37˚C for 3 days. Non-aggregated $A\beta_{25-35}$ was dissolved in 0.9% NaCl at same concentration and incubated at 37˚C for 10 min. Mice were anesthetized with a mixture of Zoletil 50® (30 mg/kg) and Rompun (10 mg/kg) to reduce unnecessary pain. When Rompun (xylazine) is added to the Zoletil, this combination provides rapid induction, immobilization, good muscle relaxant and smooth recovery from anesthesia; thus, the Zoletil/Rompun mixture has been commonly used to anesthetize both wild animals and small laboratory animals (22-24). To ensure the animals were fully anesthetized, the pedal withdrawal reflex was assessed by pinching the skin between the toes and any toxic or side effect, such as muscle tremors, cardiac or respiratory arrest were not observed. Aggregated $A\beta_{25-35}$ was dissolved in saline solution (5 μl) and injected into the right ventricle using a 10 μl Hamilton microsyringe (Hamilton Company) fitted with a 26 gauge-needle, with the following stereotaxic coordinates from the Bregma (antero-posterior: -0.2 mm; mediolateral: +1.0 mm; dorsoventral: -0.22 mm; speed 1 μl/min). The volume of the injection was 5 μl (5 nmol/mouse) (25). In the preliminary study, to establish the AD model, mice underwent the same procedures and same volume (5 μl) of aggregated $A\beta_{25-35}$ or non-aggregated $A\beta_{25-35}$ (n=5, 5 nmol/mouse) were injected into the bregma. Mice in saline group was injected with 5 μl of 0.9% NaCl. After 3 days of injection, mice were scarified and measured the levels of MDA concentrations, as described in '*Measurement of lipid peroxidation*'. After establishment of AD model, mice in control and BR groups were given with aggregated $A\beta_{25-35}$ (5 μl). In the normal group, mice were injected with saline (5 μl) instead of $A\beta_{25-35}$. At day 6 post-$A\beta_{25-35}$ injection, BR 50 and 100 groups of mice were orally administered BR extract (50 and 100 mg/kg/day, respectively) via oral gavage once a day for 14 days. The normal and control groups were administered PBS for 14 days.

Novel object recognition test. Tasks were carried out in mice following 12 days of $A\beta_{25-35}$ injection and each mouse underwent one trial/day for 2 days. The object recognition test was performed in a black-painted square apparatus (40x30x20 cm), as described previously (26). A training session was performed using two identical objects (plastic bottles). The objects were placed at a fixed distance within a square field. The mice were placed at the center of the square field and the number of touch or sniffs each object was recorded for 10 min. After 24 h, the mice were placed back into the same field for the test session, in which one of the objects used in the training session was replaced with a new object (a differently shaped plastic bottle). The mice were allowed to explore freely for 10 min and the number of touch or sniffs of each object was manually recorded by two experienced independent observers who were blind to the groups (27). Object recognition ability (%) was calculated by comparing the number of touch or sniff for the old object and new object. All scores in behavioral tests were counted using the replay function in the digital camcorder mounted above the apparatus.

T-maze test. The T-maze test was conducted in mice following 14 days of $A\beta_{25-35}$ injections and each mouse was underwent one trial/day for 2 days (28). The apparatus was T-shaped and the walls and bottom of the maze were equipped with a black square board (length of start and goal stems, 50 cm; width, 13 cm; height, 20 cm). The T-maze used in the current study consisted of a start box, a left arm and a right arm with a block door that could be separated. On the first day, each animal was placed at the start box and the number of right arm entries was recorded during a 10 min period (training session, one trial per day). The mice were placed back into the same apparatus 24 h after the training session and allowed to explore freely for 10 min. The number of left or right arm entries was manually recorded by two experienced independent observers (test

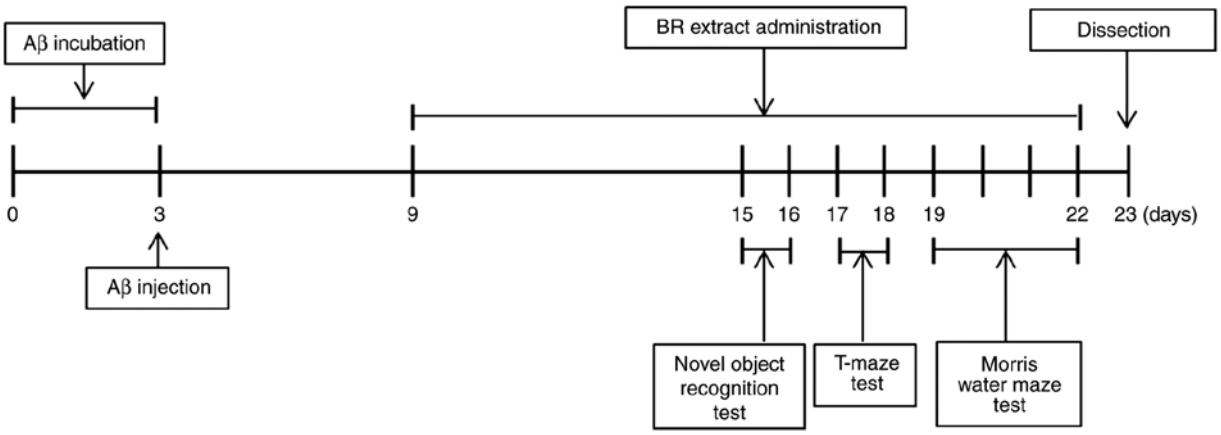

Figure 1. Experiment procedures for behavioral test. ICR mice (age, 5 weeks) were randomly allocated to the following groups (n=8/each groups): $A\beta_{25-35}$ (5 nmol/μl dissolved in 0.9% NaCl) was incubated for aggregation at 37˚C for 3 days before injection. Animals in the Aβ group were injected in the hippocampus with 5 μl of $A\beta_{25-35}$. Normal, 0.9% NaCl injection + oral administration of PBS; Control, $A\beta_{25-35}$ injection + oral administration of PBS; BR 50, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (50 mg/kg/day); BR 100, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (100 mg/kg/day). Aβ, amyloid β; BR, black rice; MeOH, methanol.

session, one trial per day). Space perceptive ability (%) was expressed as a ratio of the number of entries into either the right (old route) or left arms (new route) over the total number of arms entries (29). All scores in behavioral tests were counted using the replay function in the digital camcorder mounted above the apparatus.

Morris water maze test. The Morris water maze test was performed in mice after 16 days of $A\beta_{25-35}$ injection using a previous procedure established by Morris (30) with slight modification. The apparatus used in this study consisted of a dark plastic circular pool (diameter, 80 cm; surrounded by a 40 cm high wall), which was divided into quadrants with four visual cues on the walls to provide navigation. Milk powder was added into the pool to make the water opaque. The water temperature was maintained at 22±1˚C. A platform (diameter, 8 cm) was placed at 1 cm below surface of the water in one of the pool's quadrants. The position of the platform did not change during the training sessions. In total, three training trials per day were conducted for 3 days. In training trials, the mice were randomly placed in the water facing the pool wall and allowed to swim for a maximum of 60 sec. The latency time to find the platform was recorded. The mice that found the platform were allowed to rest on the platform for 15 sec. If a mouse did not reach the platform within 60 sec, it was guided to the platform and allowed to rest for 15 sec, before being returned to the cage. Then, 1 day after finishing the training trials (day 4), a probe trial was performed.

After completion of the probe trial of the Morris water maze task, a secondary test was conducted by removing the platform. The mice were placed in the pool and allowed to swim for 60 sec and the time spent in the target quadrant where the platform had been in the training trails was recorded. For the tertiary test, the time to reach the platform was recorded in transparent water. For the secondary and tertiary tests, only one trial was conducted for each mouse. At the end of each trial, all mice were dried and returned to the home cage. The time to reach the platform and the time spent exploring the target quadrant by the animals were recorded manually using a stopwatch and all scores in behavioral tests were counted using the replay function in the digital camcorder mounted above the apparatus.

Measurement of lipid peroxidation. To evaluate MDA levels in the brain, liver and kidneys, mice were anesthetized using CO_2 gas and sacrificed under controlled chamber-replacement rate of 30% (chamber volume per minute) as previously reported (31,32). Death was confirmed by observation of the loss of the postural reflex and visible cessation of breathing. The brain, liver and kidneys were isolated immediately and placed on ice for 20 min. The dissected tissues were weighed and stored at -80˚C. The tissues were homogenized (12,000 x g; 15 min at 4˚C) in saline solution. The supernatant was collected and mixed with 1% phosphoric acid and 0.67% TBA, which was then heated at 100˚C for 45 min. After cooling on ice, 2 ml 1-butanol was added and the samples were centrifuged (1,150 x g; 10 min at 4˚C). The absorbance values for each supernatant were measured at 535 and 520 nm wavelength using a microplate reader. The yield of lipid peroxidase was calculated using an MDA standard curve (33).

Nitric oxide (NO) scavenging activity. The NO concentrations in the brain, liver and kidney tissues were determined according to a previously described method by Schmidt *et al* (34). Briefly, 150 μl tissue homogenate was mixed with 130 μl distilled water and then 20 μl mixed solution was added to the same amount of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylene-diamide dihydrochloride solution. The mixture was incubated at 37˚C for 30 min and the absorbance value was detected at 540 nm using microplate reader. The yield of NO production was calculated with a standard curve of $NaNO_2$ content.

Statistical analysis. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc analysis performed using SPSS version 23 software (IBM Corp.). In the T-maze and novel object recognition test, the perceptive ability between training and test sessions were compared using a

paired Student's t-test performed with SAS 9.4 software (SAS Institute, Inc.). Data are presented as the mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference. Each experiment was performed once.

Results

Establishment of injection of an AD mouse model. To establish the ideal conditions for the injection of $A\beta_{25-35}$ into the cerebral tissues of mice, a preliminary study was performed. The MDA levels of groups injected with $A\beta_{25-35}$ 3 days post-injection were significantly elevated compared with the control group and the group injected non-incubation $A\beta_{25-35}$ (Table I). These results were used to demonstrate that this method could reliably produce an $A\beta_{25-35}$-induced AD mouse model.

Effect of BR extract on the object recognition test. The same two objects were explored during training session and then a test session was conducted 24 h after the training session. In the testing session, one of the familiar objects was replaced with a novel object. The normal group demonstrated a higher number of touches for the novel object compared with the familiar object, showing 49.12 and 60.54% for familiar object and novel object, respectively (Fig. 2). However, the control group injected with $A\beta_{25-35}$ had no significant preference for either the familiar or novel object, while the 100 mg BR administered group had a significantly increased preference for the novel object compared with the familiar object, 48.57 (familiar object) and 56.56% (novel object), respectively. These results indicated that administration of BR (100 mg) extract protected against object recognition impairment induced by $A\beta_{25-35}$.

Effect of BR extract on the T-maze test. To investigate the protective effect of BR extract on cognitive dysfunction from $A\beta_{25-35}$ toxicity, a T-maze test was conducted (Fig. 3). The normal group approached the old and new routes at a rate of 43.94 and 56.06%, respectively, indicating a higher number of entries into the new route compared with the old route. However, the $A\beta_{25-35}$-injected control group exhibited no significant differences in the number of entries between old and new route, with rates of entries at 51.17 and 48.83%, respectively. However, mice in the BR 50 group did not exhibit significant differences between the old and new routes (47.66 and 52.34%, respectively), the administration of 100 mg BR significantly increased the rate of the new route entries compared with the old route, with 46.21 and 53.79% for the old and new routes, respectively. These results demonstrated that the oral administration of 100 mg BR protected spatial cognition impairments in mice induced by $A\beta_{25-35}$.

Effect of BR extract on the Morris water maze test. To assess the protective effect of BR extracts on spatial learning and memory impairment following $A\beta_{25-35}$ injection, a Morris water maze test was performed. The time taken to reach the platform was recorded consecutively during the test period. It was found that it took less time to reach the platform in all experimental groups as training time increased. However, the control group injected with $A\beta_{25-35}$ recorded a time of 20.25 sec at the final test, which indicated a relatively small decrease compared with the normal group record of 7.60 sec (Fig. 4). The groups administered with 50 and 100 mg BR extract recorded 11.67 and 10.00 sec at the final test, respectively, demonstrating a reduced latency time compared with the control group. There was no significant difference in the mean latency time when locating the exposed platform among the experimental groups (Fig. 5). However, when the platform was hidden, it took longer for the control group mice to find the platform compared with the normal and BR extract-administered groups. Thus, these results suggested that the differences in the time taken to locate platform in the experimental groups were related to memory ability rather than visual or physical abilities.

Inhibitory effect of BR extract on lipid peroxidation in the tissues. The results of inhibitory effect of BR extract from lipid peroxidation in tissues are presented in Table II. The MDA concentration of normal group in brain was 21.22 nmol/mg

Table I. Effect of injection of $A\beta_{25-35}$ peptide in mice brain on lipid peroxidation.

Group	MDA (nmol/mg protein)
Saline	2.57±0.1
Non-aggregated $A\beta_{25-35}$ peptide	2.80±0.4
Aggregated $A\beta_{25-35}$ peptide	3.75±0.7[a]

Data are presented as the mean ± SD. [a]$P<0.05$ vs. Saline, by Tukey's multiple range test. Saline, 5 μl 0.9% NaCl; Non-aggregated $A\beta_{25-35}$ peptide, 5 μl Saline solution containing $A\beta_{25-35}$ peptide at 37˚C for 15 min; Aggregated $A\beta_{25-35}$ peptide, 5 μl Saline solution containing aggregated $A\beta_{25-35}$ peptide at 37˚C for 3 days. Aβ, amyloid β; MDA, malondialdehyde.

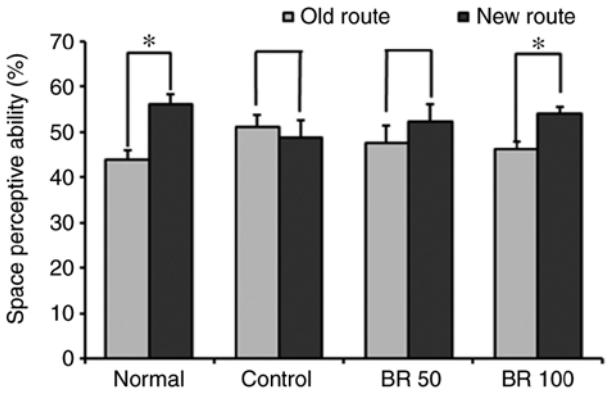

Figure 2. Effect of BR on novel object recognition test. After training with two identical objects to touch, the mice were allowed to explore one familiar object from training and one novel object. The time that the mice spend with the novel object was recorded. Data are presented as the mean ± SD. The object perceptive abilities for familiar and novel objects were significantly different as determined by Student's t-test (*$P<0.05$). Normal, 0.9% NaCl injection + oral administration of PBS; Control, $A\beta_{25-35}$ injection + oral administration of PBS; BR 50, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (50 mg/kg/day); BR 100, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (100 mg/kg/day). Aβ, amyloid β; BR, black rice; MeOH, methanol.

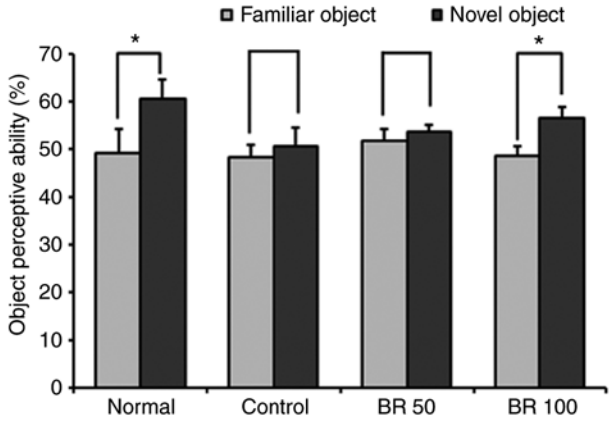

Figure 3. Effect of BR on spatial alternation test in the T-maze. After training to explore the right arm in T-maze for 10 min, the number of touch and exploration times to right and left maze sides were calculated. Data are presented as the mean ± SD. The space perceptive abilities for the old and new routes were significantly different as determined by Student's t-test ($^{*}P<0.05$). Normal, 0.9% NaCl injection + oral administration of PBS; Control, $A\beta_{25-35}$ injection + oral administration of PBS; BR 50, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (50 mg/kg/day); BR 100, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (100 mg/kg/day). Aβ, amyloid β; BR, black rice; MeOH, methanol.

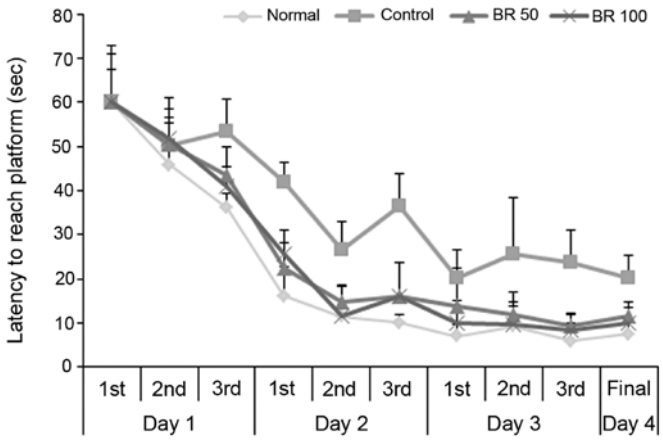

Figure 4. Effect of black rice on spatial learning in Morris water maze test. Mice were trained to swim and locate the platform for 3 days. The latency time to reach platform during training and final test day was calculated. Normal, 0.9% NaCl injection + oral administration of PBS; Control, $A\beta_{25-35}$ injection + oral administration of PBS; BR 50, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (50 mg/kg/day); BR 100, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (100 mg/kg/day). Data are presented as the mean ± SD. Aβ, amyloid β; BR, black rice; MeOH, methanol.

protein, while the control group injected with $A\beta_{25-35}$ had a notably higher MDA level at 69.10 nmol/mg protein. However, the MDA values in the BR extract 50 and 100 mg groups were significantly reduced (51.52 and 46.88 nmol/mg protein, respectively), suggesting that BR extract was exerting a protective effect against lipid peroxidation in the brain.

The results from the measurement of the kidney MDA levels identified that the control group treated with $A\beta_{25-35}$ had 37.29 nmol/mg protein, which was higher compared with the normal group (21.85 nmol/mg protein). However, the administration of BR 50 and 100 mg inhibited lipid peroxidation in the kidney, with levels of 23.99 and 17.64 nmol/mg protein, respectively.

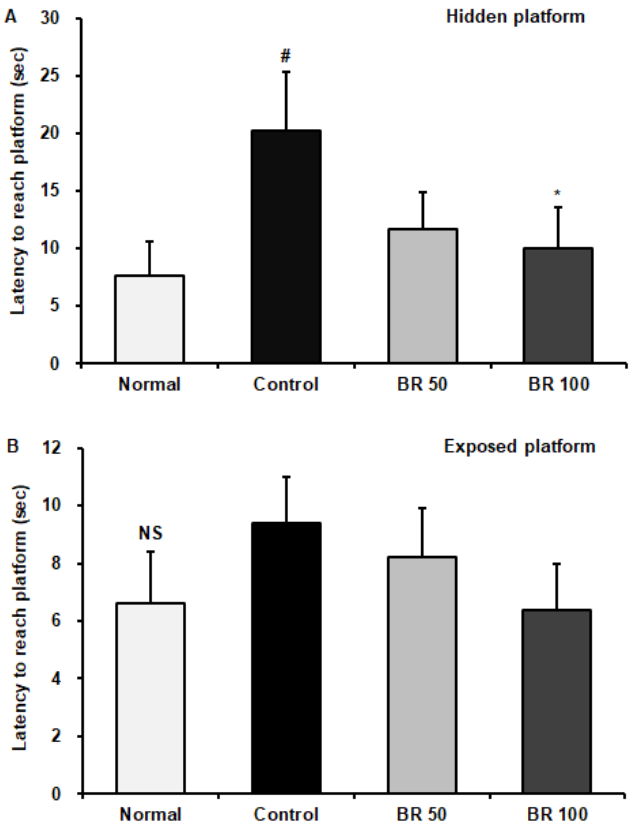

Figure 5. Latency to reach the hidden and exposed platform in Morris water maze test. Time to find (A) hidden and (B) exposed platform was recorded on final test day in water maze test. Data are presented as the mean ± SD. $^{\#}P<0.05$ vs. Normal, $^{*}P<0.05$ vs. Control, by Tukey's multiple range test. NS, no significance. Normal, 0.9% NaCl injection + oral administration of PBS; Control, $A\beta_{25-35}$ injection + oral administration of PBS; BR 50, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (50 mg/kg/day); BR 100, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (100 mg/kg/day). Aβ, amyloid β; BR, black rice; MeOH, methanol.

The MDA concentration in the liver of the control group was 6.18 nmol/mg protein, which was 2.4 times higher compared with the normal group (2.59 nmol/mg protein). However, 50 and 100 mg of BR extract-administered group had significantly lower MDA values compared with the control group, with 3.71 and 3.34 nmol/mg protein, respectively. These results indicated that administration of BR extract protected lipid peroxidation induced by $A\beta_{25-35}$ in brain, kidney and liver.

Effect of BR extract on NO production in the tissues. Table III presents the scavenging effect of BR extract from NO generation induced by $A\beta_{25-35}$ in tissues. The NO level of the normal group was 27.49 nmol/mg protein, while that of the control group was significantly increased to 63.68 nmol/mg protein. However, the groups administered with 50 and 100 mg BR extract demonstrated lower NO levels compared with the control group, 37.02 and 27.79 nmol/mg protein, respectively.

The NO levels in the kidney, (normal group, 22.99 nmol/mg protein; control group, 40.35 nmol/mg protein; BR 50 mg group, 33.28 nmol/mg protein; BR 100 mg group, 31.71 nmol/mg protein) and the liver (normal group, 9.53 nmol/mg protein; control group, 58.03 nmol/mg protein; BR 50 mg group, 39.77 nmol/mg protein; BR 100 mg group, 34.23 nmol/mg

Table II. Protective activity of BR from lipid peroxidation in mice brain induced by $A\beta_{25-35}$.

Sample	MDA (nmol/mg protein)		
	Brain	Kidney	Liver
Normal	21.22±4.22	21.85±5.19	2.59±0.77
Control	69.1±5.49[a]	37.29±5.6[a]	6.18±1.41[a]
BR 50	51.52±7.22[b]	23.99±3.66[b]	3.71±0.63[b]
BR 100	46.88±8.48[b]	17.64±4.44[b]	3.34±0.73[b]

Data are presented as the mean ± SD. [a]P<0.05 vs. Normal, [b]P<0.05 vs. Control, by Tukey's multiple range test. Normal, 0.9% NaCl injection + oral administration of PBS; Control, $A\beta_{25-35}$ injection + oral administration of PBS; BR 50, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (50 mg/kg/day); BR 100, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (100 mg/kg/day). Aβ, amyloid β; BR, black rice; MeOH, methanol; MDA, malondialdehyde.

Table III. Effect of oral administration of BR on $A\beta_{25-35}$ induced nitric oxide formation in organ.

Sample	$NaNO_2$ (μmol/mg protein)		
	Brain	Kidney	Liver
Normal	27.49±2.76	22.99±3.65	29.53±3.89
Control	63.68±4.93[a]	40.35±5.48[a]	58.03±5.65[a]
BR 50	37.02±4.89[b]	33.28±5.20	39.77±4.38[b]
BR 100	27.79±3.01[b]	31.71±4.05	34.23±4.43[b]

Data are presented as the mean ± SD. [a]P<0.05 vs. Normal, [b]P<0.05 vs. Control, by Tukey's multiple range test. Normal, 0.9% NaCl injection + oral administration of PBS; Control, $A\beta_{25-35}$ injection + oral administration of PBS; BR 50, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (50 mg/kg/day); BR 100, $A\beta_{25-35}$ injection + oral administration of BR MeOH extract (100 mg/kg/day). Aβ, amyloid β; BR, black rice. MeOH, methanol.

protein) were found to follow a similar pattern. Collectively, these findings suggested that supplementation of BR extract can inhibit $A\beta_{25-35}$-induced NO formation in the brain, kidney and liver.

Discussion

AD is one of the most common age-dependent neurological disorders, affecting mental function, memory and other cognitive dysfunction, resulting in changes in personality and behavior (35). Deposition of Aβ plaques in the brain is the most important risk factor for the development of AD (36). Previous studies have reported that acute or continuous injections of Aβ into the brain of mice can cause neurodegeneration and impair learning and memory abilities (37,38). Therefore, the AD mouse model induced by $A\beta_{25-35}$ is widely used to study the pathology and screen therapeutics against AD. $A\beta_{25-35}$ is the core fragment of full-length Aβ and exerts several of the characteristics of full-length Aβ peptides, including the neurotoxic properties described in patients with AD (39). According to previous study, $A\beta_{25-35}$ is more toxic compared with the full-length peptide and often causes oxidative damage more rapidly than full-length Aβ (40). Moreover, the injection of $A\beta_{25-35}$ into the brain leads to learning and memory dysfunction via the deposition and dissemination of Aβ in the cortex and hippocampus of mice (41). The aggregation of Aβ also induces oxidative stress via the overproduction of free radicals and Aβ transforms itself from its non-aggregated to its aggregated form (7). Thus, the $A\beta_{25-35}$-injected mice model is an effective method of examining functional improvements and pathological effects. Furthermore, the inhibition of Aβ accumulation and attenuation of oxidative stress are important strategies in the treatment of AD. Therefore, efforts to identify dietary supplements with antioxidant activities to help prevent AD have attracted increased attention in recent years.

A previous study revealed that BR extract (125 and 250 mg/kg body weight) did not significantly influence liver function as demonstrated by the non-significant changes in the serum levels of alanine aminotransferase and aspartate transaminase, which are enzymatic bio-marker for liver toxicity (42). BR extract is also rich in polyphenols with anthocyanins, which have no toxic effects at doses ≤20 mg/kg/day in rat and 25 mg/kg/day in mice (43). Previous studies have reported that bread containing BR contributes to the reduction of Aβ peptide concentrations in the plasma of aged mice (44). In addition, BR and its constituent, cyanidin, have been shown to attenuate Aβ-induced neuronal cell death via modulation of the mitochondrial death pathway in SK-N-SH cells (45). Anthocyanin, a major component of BR, prevents Aβ-induced neurotoxicity by inhibiting reactive oxygen species production and regulating Ca^{2+} homeostasis (46). Moreover, anthocyanin has been revealed to block β-secretase activation, which is a key enzyme in Aβ production (46). However, to the best of our knowledge, there is limited evidence of BR efficacy against Aβ-induced cognitive impairment and oxidative damage *in vivo*. Therefore, the present study investigated the neuroprotective effects of BR extract on cognitive dysfunction in an $A\beta_{25-35}$-induced AD mice model.

It has been previously demonstrated that neither the reversed nor scrambled peptide of $A\beta_{25-35}$ can induce neurodegenerative changes in animal brain (47,48). Therefore, to establish the incubation method for Aβ, a preliminary study was performed in mice. It was found that injection of $A\beta_{25-35}$ into brain after aggregation at 37˚C for 3 days led to lipid peroxidation. The MDA level of groups injected with aggregated $A\beta_{25-35}$ was significantly elevated compared with the control and non-incubated $A\beta_{25-35}$ injected groups. Thus, the present study injected $A\beta_{25-35}$ after 3 days of incubation at 37˚C to investigate the protective effect of BR against AD-associated memory impairment.

Previous studies have reported that BR has anti-oxidant, anti-inflammatory and anti-hyperlipidemic activities (49,50) Furthermore, anthocyanins, such as cyanidin and malvidin, from BR have been shown to serve a protective role in numerous pathological conditions via their induction of superoxide dismutase and catalase (51). However, to the best of our knowledge, studies on the protective effect of BR against aging and aging-related degenerative diseases including AD have not

been performed. In the current study, BR extracts significantly improved the cognitive impairments induced by $A\beta_{25-35}$ in the T-maze test, object recognition test and Morris water maze test. A T-maze test is used to evaluate the short-term memory of mice (52), while a novel object recognition test is used to obtain information on the amnesiant potential of functional substances (29). Moreover, since patients with AD exhibit deficits in object recognition, this task is considered as a useful tool to investigate learning ability and memory function in animal models (53). In the novel object recognition test, the exploration of a previously seen object and a novel object is measured and used as an index of memory performance (54). The present results indicated that cognitive dysfunction was observed in the $A\beta_{25-35}$-induced mice, as demonstrated by the lack of preference for the new routes and objects compared with the familiar route and object. However, groups administered BR extracts had significantly increased preference for new routes and objects compared with the familiar route and object, suggesting BR can protect the impairment of learning and memory function against $A\beta_{25-35}$.

The Morris water maze test is well-known for the assessment of spatial cognition ability and long-term memory (55). In training trials, the latency of mice administered BR was significantly shortened by training repeatedly for 3 days compared with control group mice. Furthermore, the groups that were BR exhibited a considerable decrease in the time to reach the platform compared with the control group in the final test (day 4), indicating that the mice administered with BR extract could recognize the location of the platform, even when it was removed. In addition, the time to reach the exposed platform was not significantly different among the groups, while the time was shorter in BR treated group compared with the control group when the platform was hidden. These experimental results indicated that BR exerts a protective effect against cognitive impairment induced by $A\beta_{25-35}$ and this effect is not related to the visual or exercise abilities.

Lipid peroxidation is widespread in the AD brain and is a marker for oxidative stress (56). Previous studies have revealed that lipid peroxidation is an important mechanism for neurodegeneration in AD and Aβ causes lipid peroxidation in the brain (57-59). Moreover, the injection of $A\beta_{25-35}$ into the brain of mice leads to notable increases in MDA levels in the hippocampus, indicating that $A\beta_{25-35}$ results in lipid peroxidation (57-59). NO is involved in neuronal death in AD and other neurodegenerative disorders (60). Furthermore, NO can generate peroxynitrite via its reaction with O_2^-, which induces various chemical reactions producing compounds such as nitrotyrosine (61). It has also been reported that the overproduction of nitrotyrosine is correlated with increased levels of cerebral Aβ and NO-mediated oxidative damage in the brain contributes to the neurotoxicity and cognitive impairments (62). Cleavage of the Aβ precursor protein (APP), one of the most abundant proteins present in central nervous system, can produce Aβ (63). APP is ubiquitously expressed in muscle, epithelial and several circulating cells (64,65). Furthermore, deposition of Aβ is detectable in the brain and several other tissues, including the skin, intestine and other organs, and Aβ is circulated in the blood and cerebrospinal fluid (66,67). Accumulation of Aβ is also strongly associated with oxidative stress, leading to pathological conditions in the peripheral tissues. For instance, our previous studies revealed that the injection of $A\beta_{25-35}$ significantly elevated the levels of MDA and NO in the brain and liver of mice (68-72). In the present study, groups administered with BR extract had significantly reduced MDA and NO contents in the brain, liver and kidney. Moreover, the protective effect of BR extract against lipid peroxidation and NO production was greatest in the brain. Collectively, these findings suggested that BR supplementation may exert a positive effect on cognitive improvement by attenuating oxidative stress induced by $A\beta_{25-35}$.

A limitation with the present study was that only cognitive improvement by oral administration of BR extract was observed. This may be associated with attenuation of oxidative stress *in vivo* model. However, the molecular mechanisms by which BR extract ameliorates Aβ-induced cognitive deficit through anti-oxidative pathway remains unclear. In addition, the present study emphasized the effect of BR MeOH extract; however, active compounds, including anthocyanins, were not examined. Therefore, characterizing the specific active compound and elucidating the mechanism of action, which responsible for learning and memory improvement property of BR, should be investigated further.

In conclusion, it was demonstrated that supplementation with BR extracts resulted in improved cognitive function, as indicated by behavioral tests in the $A\beta_{25-35}$-induced AD mouse model. BR administration also significantly inhibited the generation of MDA and NO in the brain, kidney and liver following $A\beta_{25-35}$ injection. Although further studies are required to evaluate the underlying mechanism involved in the neuroprotective effects of BR on Aβ-induced cognitive impairment and oxidative damage, BR may have a role as a protective agent against Aβ-induced learning and memory impairment, which may be mediated by attenuating oxidative stress.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AYL, JMC and SHS were responsible for data acquisition, analysis and interpretation. AYL wrote the manuscript and prepared the figures and tables. YAL participated in the design of the study and assisted in certain experiments. AYL and YAL were responsible for the critical revision of the manuscript. EJC was responsible for the research creation and design, interpretation of data and critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental procedures were approved and permitted using the guidelines established by the Pusan National University Institutional Animal Care and Use Committee (approval no. PNU-2010-000142).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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