RESEARCH ARTICLE

The Neuroprotective Effects of Germinated Black Glutinous Rice Diet on Aβ25-35 Peptide Induced Learning and Memory Deficits in Male Rats

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Abstract

Germinated black glutinous rice (GBGR) is a black glutinous rice (BGR) that has been soaked in water to initiate pre-germination. It is also known as pre-germinated brown rice (PGR). Important nutrients in GBGR are γ-aminobutyric acid (GABA), γ-oryzanol, and other bioactive lipids. GABA concentrations in GBGR are more than 15 times greater than in non-germinated rice. PGR has been reported to have neuroprotective effects in developing rats against the accumulation of lead and protects against neuronal cell loss and memory deficits, but there has been no such report regarding GBGR. We therefore investigated the effects and protective mechanisms of GBGR against Aβ25-35 peptide induced neurotoxicity in rats. In our in vivo studies, rats were fed with control, BGR, and GBGR diets throughout the experiment and were injected intraventricularly of 15 μL aggregated Aβ25-35 peptide on day 22. The effects on locomotor activities were evaluated by open field test, spatial navigation and recognition memory by Morris water maze and novel object recognition tests, respectively. Glutamate and GABA concentrations were analyzed by high performance liquid chromatography with electrochemical detection. Finally, the neuronal viability was counted by histological techniques. The results showed that rats given GBGR significantly increased spatial and recognition memory and increased GABA concentration in the hippocampus. Moreover, GBGR also improved neuronal viability. It can be concluded that GBGR has a potential role to protect against memory deficits in an Alzheimer’s rat model.

Keywords: Alzheimer’s disease, Aβ25-35 peptide, learning and memory, GABA, pre-germinated black glutinous rice
ผลของข้าวเหนียวด้าเพาะงอกต่อการป้องกันการเรียนรู้และความจำบกพร่องที่ถูกเหนี่ยวน้าด้วยสารอะไมลอยด์บีต้า 25-35 เปปไทด์ในหนูแรทเพศผู้

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บทคัดย่อ

ข้าวเหนียวด้าเพาะงอกคือ ข้าวเหนียวด้าที่ผ่านการแช่น้ำเพื่อกระตุ้นให้เกิดการงอก ซึ่งเป็นที่รู้จักโดยทั่วไปในชื่อข้าวกล้องงอก โดยข้าวกล้องงอกมีสารสำคัญ เช่น กรดอะมิโนบิวทิริกหรือกาบาร์นากรมาออริซานอล และไขมันที่มีฤทธิ์ทางชีวภาพสูง ๆ ข้าวกล้องงอกมีสารบาร์นากรมาออริซานอลมากกว่าข้าวที่ไม่ผ่านการเพาะงอก 15 เท่า มีรายงานการศึกษาว่าข้าวกล้องงอกสามารถป้องกันเซลล์ประสาทจากการเหนี่ยวน้าด้วยสารอะไมลอยด์บีต้า 25-35 เปปไทด์ได้ในหนูแรท มีรายงานว่าหนูแรทจะได้รับผลกระทบด้านความจำ การเคลื่อนที่และพฤติกรรมของข้าวเหนียวด้าเพาะงอก ดังนั้น ผู้วิจัยจึงได้ศึกษาสมมุติของข้าวเหนียวด้าเพาะงอกในการป้องกันภาวะความเสื่อมที่เกิดจากสารอะไมลอยด์บีต้า 25-35 เปปไทด์ในหนูแรท ในการศึกษา หนูแรทนี้จะได้รับการฉีดสารอะไมลอยด์บีต้า 25-35 เปปไทด์ 15 ไมโครลิตรเข้าไปในโพรงสมองในวันที่ 22 และทดสอบผลของข้าวเหนียวด้าเพาะงอกต่อการเคลื่อนที่ด้วยวิธี open filed ทดสอบความจำเกี่ยวกับสถานที่และวัตถุใหม่ด้วยวิธี Morris water maze และ novel object recognition ตามลำดับ ระดับความเข้มข้นของสารสื่อประสาทกลูตาเมตและกาบาร์นากรมาออริซานอลของเซลล์ประสาทที่มีชีวิตด้วยวิธีกลูตาเมตวิทยาศาสตร์เนื้อเยื่อ (histology) ภายใต้กล้องจุลทรรศน์ ผลการศึกษาพบว่าข้าวเหนียวด้าเพาะงอกสามารถเพิ่มการเรียนรู้และความจำเกี่ยวกับสถานที่และวัตถุใหม่ และเพิ่มระดับสารสื่อประสาทกลูตาเมตในสมองส่วนอับบิเคย์ปิส นอกจากนี้ข้าวเหนียวด้าเพาะงอกยังช่วยรักษาจำนวนเซลล์ประสาทที่มีชีวิตให้เทียบเท่ากับในหนูแรทปกติด้วย ดังนั้น จึงสามารถสรุปได้ว่าข้าวเหนียวด้าเพาะงอกมีประสิทธิภาพในการป้องกันภาวะความจำเสื่อมแบบอัลไซเมอร์ในหนูแรทได้

คำสำคัญ: โรคความจำเสื่อม, อะไมลอยด์บีต้า 25-35 เปปไทด์, การเรียนรู้และความจำ, สารสื่อประสาท, ข้าวเหนียวด้าเพาะงอก
Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by a progressive decline in cognitive function particularly among the elderly. A primary risk factor for AD is increased age, with an incidence of 60-70% cases in the over 65 years old group. The prevalence of probable AD is 3% in people of age 65-74, 19% in those of age 75-84, and 47% in those over 85 years in age.1-3 Wimo et al.4 reported that the worldwide prevalence of AD is 35.6 million in 2010, predicted to increase up to 65.7 million in 2030 and up to 115.4 million in 2050. Total estimated worldwide AD patient care costs in 2010 were USD 604 billion.

Many studies have shown neuropathological changes such as neurofibrillary tangles, extracellular senile plaques, beta-amyloid (Aβ) protein deposits in the hippocampus and other brain areas.5 However, the mechanisms of neuronal damage leading to memory impairment have still been elucidated. One possible mechanism is excitotoxicity: glutamate toxicity due to an excess of glutamate in the central nervous system (CNS). Reducing or preventing excitotoxicity could be a target for improving the cognitive impairment. The γ-aminobutyric acid (GABA) is a major inhibitory neurotransmitter and co-function with glutamate. Therefore, the balance between the excitatory and inhibitory systems at the postsynaptic membrane ensures the nurturing of cognitive function.

Currently, symptomatic clinical treatments have been achieved by NMDA receptor blockers for the treatment of moderate to severe AD.6 However, patients treated this way experienced many side effects.7 Thus, to avoid the side effects from such medications, natural products have been searched as alternative treatment in the hope of creating modern therapeutic options and opportunities to focus on the disease modification aspects.

Rice is one of the most important grains in the world and is a staple in the diet of more than half of the world’s population. Rice has been identified as having medicinal properties which may be applied in alternative medicines. Glutinous rice is a type of Thai rice, known locally as sticky rice, contains a high level of dietary fiber and nutrients. The grains of red, brown, purple, and black rice have been reported to contain high levels of phenolic compounds, anthocyanin, and γ-oryzanol.8-10 Brown rice, when prepared by soaking in water to initiate slight germination, is called pre-germinated brown rice (PGR) or GABA rice has been reported to be low in calories and also low in fat. PGR is a well-known functional food due to its high content of vitamins, minerals and bioactive lipids such as γ-oryzanol and GABA. In addition, when PGR is compared to white rice, the γ-oryzanol content is higher by as much as 13 folds, the GABA content is 10-fold higher, the dietary fiber, vitamin E, niacin and lysine content are nearly 4-fold higher and the vitamin B1, vitamin B6, and magnesium content are about 3-fold higher.11 Germinated black glutinous rice (GBGR) is a black glutinous rice (BGR) that has been soaked in water to initiate pre-germination.

In animal studies, the benefits of PGR have been reported as decreasing accumulation of lead (Pb), reducing cognition deficits, and protecting against neuronal cell loss from Aβ.12 Previous studies demonstrated that PGR improves learning and memory in several rodent models.13-15 Furthermore, PGR has shown high anti-oxidative activities and suppression of cell death in SH-SY5Y cells by
blocking apoptotic mechanisms and reducing lipid peroxidation (in hypercholesterolemia rabbits)\textsuperscript{1,11,16,17}.

However, there has been no research study of the effect of GBGR on learning and memory. Therefore, the purpose of this study was to evaluate the effects and mechanisms of GBGR diet on cognitive functions in Aβ\textsubscript{25-35} peptide-induced rat model of AD.

Materials and Methods

Plants material
Black glutinous rice (BGR) was collected from Phetchabun province, Thailand. The BGR was soaked in water at room temperature for 24 h to prepare GBGR. The water was then decanted to allow germination on cheesecloth for 40 h, and then dried by a hot air dryer. To maintain the quality of the specimens, BGR and GBGR were kept at 25°C.

Preparation of Aβ\textsubscript{25-35} peptide
Aβ\textsubscript{25-35} peptide (Sigma, St. Louis, MO, USA) at a concentration of 1 mg/mL was prepared by dissolving in phosphate buffer saline (PBS), and then incubating at 37°C for 6 days, resulting in the aggregation and formation of low molecular weight oligomers. PBS was also incubated under the same conditions to serve as the control. Aβ\textsubscript{25-35} peptide aggregation was observed under a light microscope to check the structures and globular aggregations.\textsuperscript{18} Fifteen microliter of Aβ\textsubscript{25-35} peptide solution was injected intracerebro-ventricularly (ICV) into the lateral ventricle of the rat.\textsuperscript{19}

Animals
Thirty-two male Sprague Dawley rats, aged 8 weeks, were purchased from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. The rats were acclimatized at the Naresuan University Center for Animal Research for a week before starting the experiment. Two rats were housed in each 8.5x16x8 inch polycarbonate case under standard fluorescent dark-light cycle (12:12 h) at temperature of 22±1°C and 55±10% humidity. The rats were allowed free access to a standard food pellet diet and reverse osmosis water. The experiment protocol was approved by the Ethics of Naresuan University Animal Care and Use Committee (NUACUC) no. 56 04 0044.

Surgery and treatments
The rats were randomly divided into four groups: control rats (Control), Aβ\textsubscript{25-35} peptide-treated rats (Aβ), Aβ\textsubscript{25-35} peptide-treated rats with BGR seed 6 g/day (Aβ+BGR) and Aβ\textsubscript{25-35} peptide-treated rats with GBGR seed 6 g/day (Aβ+GBGR).

All group of rats were administered their assigned diets by freely access once daily for 30 days. At day 22 of the experiment, aggregated Aβ\textsubscript{25-35} peptide was injected into the lateral ventricle under anesthesia by an intraperitoneal injection of sodium pentobarbital at 50 mg/kg body weight. All rats were allowed to recover in their home cage for 7 days before starting the next experiment (Figure 1).
Figure 1. The experimental design

Open field test
Open field test is a simply performed measurement of behaviors to detect the movement of the spontaneous locomotor and exploratory activities in rodents. Several measures of motor function in rats, including the distance covered, movement time and speed of progression can be recorded. This test is sensitive to detect the CNS, especially hippocampus dysfunction. In the present study, this test was conducted in the 50×50×40 cm open field box surrounded by walls to prevent escape. Total distance traveled and mean speeds were used to assess the activities of the rats. The rats were acclimatized in the apparatus for 5 min before testing. During the test, the rats were placed in the center of the arena and allowed to explore it for 5 min. This trial was video recorded (Watashi Engineering, Japan) and analyzed by using Smart Junior 3.0 video tracking software (Panlab, Spain).

Morris water maze test
The Morris water maze (MWM) test was first described by Richard G. Morris in 1981, in order to test the spatial memory that depended on the hippocampus. A circular pool with a diameter 100 cm and a height of 40 cm was filled with 22±2°C water, and a 20 cm diameter of platform was set inside the pool. The water level in the pool was 2 cm higher than the platform. The platform was located in a constant position in the middle of one quadrant equidistant from the center and the edge of the pool. For the test, the pool was divided into four quadrants marked with four different cues (circle, foursquare, triangle and star). Powder was added to the water to make the water semi-opaque hiding the platform from the rat view.

In each trial, the rat was placed in the water facing the wall of the pool, in one of the three starting locations to find the platform. The position was the same in all repetitions of the activity conducted daily. Each rat participated in 3 trials per day and repeated daily for 7 days. In each daily session the rat was allowed 90 sec to find the platform. The time taken to find the platform is called the escape latency (sec). On day 22, each rat was put in the pool without the platform and their action was recorded by a video camera (Watashi Engineering, Japan). The time spent in the quadrant that used to house the platform is called the retention time (sec).
**Novel object recognition test**

Novel object recognition (NOR) is a task designed for detection of disruption or improvement of non-spatial memory, such as recognition memory. The test is based on the premise that a rat has a tendency to interact more with a novel object than with a familiar object.\(^{23,24}\)

In this process, a 100×100×40 cm box was used in two separate sessions. For the training session, two identical objects (identified as A and B) were placed in the box. Each rat was allowed to explore in the center of the apparatus for 10 min. After a 10 min training session, the testing session was started. In this testing session, object B was replaced by a novel object (C) and the rat was placed to explore the objects again for 10 min. In addition to the arena, all objects were cleaned with 70% ethanol between each session. Their action was recorded by a video camera (Watashi Engineering, Japan). The amount of time spent exploring the object A and C were calculated as the percentage of the recognition index.

\[
\text{Recognition index} = \left[ \frac{TC}{(TA+TC)} \right] \times 100
\]

Prescriptive:
- TA - total duration of exploration with object A in testing session
- TC - total duration of exploration with object C in testing session

**Glutamate and GABA concentrations measurement**

After completion of the behavioral tests, the rat was sacrificed and its cranium was rapidly removed and dissected out. The hippocampal tissue was homogenized in 0.1 M of perchloric acid and then centrifuged at 14,000 rpm at 4°C for 15 min. The sample (50 µL) was mixed with 100 µL of derivative agents for 1 min at room temperature before being placed in auto-sample injector system. The derivative agents comprised of 6.75 µL of O-phthalaldehyde in 0.25 mL of methanol and 1.25 µL of 2-mercaptoethanol in 9.75 mL of 0.1 M sodium tetraborate (pH 9.4). The mobile phase consisted of 0.1 M disodium hydrogen phosphate and methanol (60:40, v/v) at pH 6.7 and filtered through 0.45 µm durapore membrane filter and degassed by vacuum prior to using. Glutamate and GABA concentrations were quantified by HPLC, LC-20AT (Shimadzu Corporation, Japan) with Coulochem III electro-chemical detector (Thermo). All samples were separated on a C18 column (4.6×250 mm in length and 10 µm beads) and analyzed at 25±2°C. The compounds were eluted by the isocratic mode over 45 min of runtime at a flow rate 0.6 mL/min. Glutamate and GABA were eluted at the peak retention time of 8.45 min and 38.30 min, respectively. The levels of glutamate and GABA were expressed as µg/mL.

**Histology**

The brains were kept in 10% NBF for 48 h. Coronal sections at the level of hippocampus were cut with the brain matrix. Sections were processed and embedded in paraplast. Sections of 5 µm thickness were stained with hematoxylin and eosin and pre-mounted before being evaluated under a light microscope (Olympus BX51, Olympus) for the histological changes. The pyramidal layer of the CA1 sub-regions of the hippocampus (approximately -3.24 mm from bregma) were collected for counting under 10x magnification in an area 600×200 µm. The number of completed pyramidal neurons of each slide were counted using image analysis software (ImageJ, US National
Institutes of Health) under the criteria of cell viability, as defined, pyramid-shaped, clear cytoplasmic membrane-intact cells, without any nuclear condensation or distorted aspect.²⁵

**Statistical analysis**

Data were analyzed using the SPSS statistical software version 23 and expressed as mean±SEM. Groups were compared using one-way ANOVA followed by LSD test. Differences were considered significant when $P$-value $< 0.05$.

**Results**

**Effects of GBGR on locomotor activities**

The locomotor activities were evaluated by using the open field test. Figure 2 shows the total distance traveled per 5 min and mean speeds. Both total distance traveled and mean speeds were not significantly different among treatment groups.

![Figure 2. Effects of GBGR on locomotor activities: the total distance traveled (A) and mean speed (B). Data were expressed as mean±SEM (n = 8).](image)

**Effects of GBGR on spatial memory ability**

Spatial memory was evaluated by MWM test. Figure 3 shows the performance in the MWM test. In training session, the escape latency time of rats in all groups were slightly shortened by 7 days repeated trials. However, there were not significantly different among groups. In testing session, there was no significant different between control and Aβ groups whereas the Aβ+GBGR group was significantly increased escape latency time in a comparison with the control, Aβ and Aβ+BGR groups (31.89±1.53 sec, 22.87±1.07 sec, 20.96±0.66 sec and 24.32±0.99 sec, respectively).
Figure 3. Effects of GBGR on spatial memory: the escape latencies in training session (A) and the retention time (B). Data were expressed as mean±SEM (n = 8). **P < 0.001 compared with control, ††P < 0.001 compared with Aβ and ##P < 0.001 compared with Aβ+BGR.

Effects of GBGR on recognition memory ability

Recognition performance was evaluated by NOR test and was showed in Figure 4. The Aβ treated group did not discriminate the novel and familiar objects by showing a significantly decreased exploration to the new object in a comparison with the control group (50.15±1.63% vs. 61.90±3.27%), whereas BGR and GBGR significantly reversed the recognition index (61.65±2.32% and 71.45±1.48%) impaired by Aβ treatment, and only GBGR could improve the recognition index significantly better than control group (71.45±1.48% vs. 61.65±2.32%).

Effects of GBGR on glutamate and GABA concentrations in the hippocampus

The concentrations of glutamate and GABA in the hippocampus were measured by using HPLC with electrochemical detection analysis. The glutamate concentration in the Aβ and the Aβ+BGR groups were significantly decreased in a comparison with the control group (136.4789±2.7902 μg/mL and 125.7815±2.2915 μg/mL, respectively vs. 145.3326±1.8997 μg/mL). Only GBGR treatment could normalize glutamate concentration depleted by Aβ to control level (Figure 5A). The GABA concentration in the Aβ group significantly decreased in a comparison with the control group (17.3911±0.7921 μg/mL vs. 29.3606±2.3103 μg/mL). GBGR
treatment significantly and fully prevented GABA depletion caused by Aβ toxicity while BGR could only exert partial prevention (Figure 5B).

**Figure 4.** Effects of GBGR on recognition memory. Data were expressed as mean±SEM (n = 8). *P < 0.05 compared with control, †P < 0.05 compared with Aβ, ‡P < 0.001 compared with Aβ, and #P < 0.05 compared with Aβ+BGR.

![Figure 4](image)

**Figure 5.** Effects of GBGR on concentrations of glutamate (A) and GABA (B). Data were expressed as mean±SEM (n = 5). *P < 0.05 compared with control, †P < 0.001 compared with control, ‡P < 0.05 compared with Aβ, §P < 0.001 compared with Aβ+BGR.

![Figure 5](image)

**Effects of GBGR on neuronal viability in hippocampus CA1 subfield**

Figure 6 shows neuronal viability in the CA1 subfield of the hippocampus. The number of viable pyramidal cells/120,000 µm² in the Aβ group were significantly decreased in a comparison with the control group (37.10±1.28 vs. 42.40±0.87). BGR significantly and fully protected pyramidal cells from Aβ toxicity (42.05±0.64 vs. 37.10±1.28 but GBGR could only partially and not significantly exert protective effects.
Figure 6. Effects of GBGR on neuronal viability of pyramidal neurons in CA1 subfield of hippocampus. Data were expressed as mean±SEM (n = 4). *P < 0.05 compared with control, †P < 0.05 compared with Aβ.

Discussion

In this study, we found the neuroprotective effects of the GBGR diet. It could restore learning and memory impairment induced by Aβ25-35 peptide in male rats. The MWM and NOR tests are well-known and frequently used to determine spatial navigation and recognition memories in rodents, and are widely used to investigate the pharmacological actions of herbal medications on laboratory behaviors.22,23 In the MWM test, the escape latency time of rats in all groups were slightly shortened by 7 days repeated trials. Aβ25-35 did not affect spatial memory in MWM test. These result similar to previous report that Aβ25-35 did not induce impairment in testing session in MWM test.13 Although, Aβ25-35 did not effect on target quadrant occupancy in MWM test but Aβ25-35 effect on the impairment of spatial memory by increasing of the opposite target quadrant (data not showed). This study related with previous report26 that Aβ25-35-treated rats had a larger number of crossings to the opposite quadrants during the memory test. In the NOR test, our study demonstrated that Aβ25-35-treated rats had toxic effect on recognition memory by decreasing the percentage of recognition index. Our study is agree with previously reported27, which indicated Aβ25-35-treated impaired novel object recognition in mice. However, GBGR could improve the spatial working memory and enhance exploration time of novel object. These results are consistent with previous report that PGR attenuates the decrease in exploratory preference induced by Aβ25-35 peptide.15 The recognition memory has been associated with regions that include the entorhinal and temporal association cortex, the prefrontal cortex, and the hippocampus. Since the spatial memory is dependent on the integrity of the hippocampus, it might be suggested that the mechanism of the GBGR diet to improve cognitive function could have been related to the protection of hippocampus regions associated with novelty and spatial memory responsible for new cognitive processing.28,29
We explored the protective role of GBGR further by determining neuronal viability in hippocampus, which is an important brain area for spatial cognition. The mechanism underlying the protective effect of GBGR might be related to the neuronal viability located in the hippocampus. Previous studies suggest the loss of the novelty relates with neuronal impairment in the hippocampus, especially in CA1 sub-field of hippocampus, which plays a role in synaptic function. The decrease in the number of undamaged neurons in the CA1 could indicate that Aβ25-35 peptide caused a cognitive deficit. This result is in agreement with previous reports that confirm the neurotoxicity of Aβ25-35 peptide. In contrast, BGR and GBGR diets restored the number of pyramidal neurons equal to control group. Suggesting that both BGR and GBGR diets protect against cognitive decline thereby preventing neuronal cell death in the CA1. Our results are consistent with the BR and PGR extracts that protect SK-N-SH cells against Aβ1-42 induced oxidative stress and neurotoxicity reported. However, the possible underlying mechanism of neuroprotective effects on enhancing performance of BGR and GBGR might also be correlated with the effects of antioxidants and GABA.

To clarify the possible mechanism in enhance memory further, the glutamate and GABA concentrations in the hippocampal were determined. Previous studies indicate that Aβ peptides enhance the release of glutamate from glutamatergic neurons resulting in the neuronal degeneration. Furthermore, the smaller Aβ oligomers cause neuronal loss in the hippocampus and disrupt glutamatergic/GABAergic balance thereby impairing synaptic plasticity that leads to memory disturbance. Our data showed the decreasing of glutamate and GABA in Aβ25-35 peptide treatment, similar to previous study that showed the lower levels of glutamate and GABA in the temporal cortex of AD patients. This suggests the toxic effect of Aβ related to the neuronal cell loss in the hippocampal CA1 region. In contrast, GBGR normalized glutamate and GABA concentrations to control level thereby restoring the glutamatergic/GABAergic balance. This finding added to the possible mechanism of neuroprotection of GBGR in hippocampus by balancing the excitatory and inhibitory neurotransmitter levels in Aβ25-35 peptide induced neuronal toxicity. The abundant of GABA in GBGR improved spatial navigation and recognition memory as seen in MWM and NOR tasks. These results are in line with the report of GABA levels in the hippocampus of rats fed with BR and PGR were increased in comparison with lead-induced memory deficit.

A previous study indicated that high doses of GABA affect spontaneous locomotor activity because of GABA inhibition on dopaminergic neurons involved in motility regulation. However, our results showed that GBGR improved cognitive performance without impairment on the motor movement in rodents, may be the GABA concentrations in GBGR are not high enough to cause such impairment.

Conclusions

Our research results demonstrate that the 6 g/day dose of GBGR diet protects learning and memory deficits against Aβ25-35 peptide-induced by increasing of GABA levels and maintaining neuronal viability in the hippocampus. GBGR diet should be promoted as a supplementary food for protection against memory deficits in AD.
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