Protective Effect of Rice Bran Oil against β-Amyloid Protein-Induced Memory Impairment and Neuronal Death in Mice

Ji Yeon Jang,† Hong Kyu Lee,† Hwan-Su Yoo, and Yeon Hee Seong*

1College of Veterinary Medicine, Chungbuk National University, Cheongju 28644, Republic of Korea
2College of Pharmacy, Chungbuk National University, Cheongju 28160, Republic of Korea

Abstract – This study was undertaken to investigate the protective effect of rice bran oil (RBO) on amyloid β protein (Aβ) (25-35)-induced memory impairment and brain damage in an ICR mouse model. Memory impairment was produced by intracerebroventricular microinjection of 15 nmol Aβ (25-35) and assessed using the passive avoidance test. Treatment with RBO at 0.1, 0.5, or 1 mL/kg (p.o. daily for 8 days) protected against Aβ (25-35)-induced memory impairment. Furthermore, Aβ (25-35)-induced decreases in glutathione and increases in lipid peroxidation and cholinesterase activity in brain tissue were inhibited by RBO, and Aβ (25-35)-induced increases of phosphorylated mitogen-activated protein kinases (MAPKs) and inflammatory factors, and changes in the levels of apoptosis-related proteins were significantly inhibited by RBO. Furthermore, Aβ (25-35) suppressed the PI3K/Akt pathway and the phosphorylation of CREB, but increased phosphorylation of tau (p-tau) in mice brain; these effects were significantly inhibited by administration of RBO. These results suggest that RBO inhibits Aβ (25-35)-induced memory impairment by inducing anti-apoptotic and anti-inflammatory effects, promoting PI3K/Akt/CREB signaling, and thus, inhibiting p-tau formation.

Keywords – rice bran oil, Alzheimer's disease, amyloid β protein (25-35), memory impairment, neuroprotection

Introduction

Alzheimer's disease (AD) is the main cause of dementia in the elderly. The core features that lead to neuronal malfunction in AD are extracellular neuritic senile plaques composed of β-amyloid peptide (Aβ), intracellular neurofibrillary tangles containing hyperphosphorylated tau protein (p-tau), and loss of basal forebrain cholinergic neurons. Aβ, a causative factor of AD, is derived from amyloid precursor protein by β and γ secretases. Aβ inserted into the neuronal membrane bilayer increases the production of reactive oxygen species (ROS), decreases levels of glutathione (GSH) and superoxide dismutase (endogenous antioxidants), and thus, causes the peroxidation of neuron lipids and proteins. The oxidative stress generated leads to loss of Ca²⁺ homeostasis, disruption of signal pathways, and an inflammatory environment, which are the ultimate basis of neuron apoptosis. The phosphorylations of MAPKs (mitogen-activated protein kinases), such as ERK1/2 (extracellular signaling-regulating kinases) and P38, are induced during the development of AD and lead to the over-activations of pro-inflammatory molecules, such as cyclooxygenase-2 (COX-2) and iNOS (inducible nitric oxide synthase), which result in the formation of p-tau.

The PI3K (phosphatidylinositol-3 kinase)/Akt signaling pathway is one of the most important cell survival pathways in AD, and suppression of the PI3K/Akt signaling pathway by Aβ in the AD brain promotes neuron apoptosis and p-tau formation. CREB (cyclic AMP response element binding protein) is a crucial signaling factor of brain plasticity and learning, and the activation of CREB by its phosphorylation at Ser133 (p-CREB) is essential for memory consolidation and storage. Furthermore, it has been demonstrated that CREB signaling is dysregulated in mouse models of AD.

Rice bran oil (RBO) is considered highly nutritious because it is rich in fatty acids and biologically active anti-oxidants like γ-oryzanol, tocotrienols, tocopherols, and squalene. RBO has a balanced fatty acid profile and is composed of ~47% monounsaturated fat, ~33% polyunsaturated fatty acids (PUFAs), and ~20% saturated fatty acids. RBO has been reported to have hypocholeste-
terolemic effects in human and animal studies,\textsuperscript{17,18} and to lower blood pressure and improve lipid profile in hypertensive patients.\textsuperscript{19} RBO has also been shown to exhibit anti-inflammatory effects in rat models of arthritis and hypercholesterolemia.\textsuperscript{20,21} The long chain PUFAs are fundamental components of membrane lipids in the central nervous system (CNS) and are the precursors of various bioactive mediators. Interestingly, PUFAs in RBO have been reported to prevent lifestyle-related diseases such as cardiovascular disease and diabetes.\textsuperscript{22} In addition, low PUFA levels in rats have been suggested to be associated with poor learning behavior and reduced synaptic vesicle density in brain, particularly in hippocampus,\textsuperscript{23} and conversely, a diet rich in PUFA was found to benefit learning and memory functions in diabetic rats.\textsuperscript{24} In terms of brain function, it has been reported that RBO protects rats against stress-induced reductions in learning and memory and antipsychotic-induced movement disorders.\textsuperscript{25-27} The present study was conducted to confirm the neuroprotective effects of RBO on memory impairment and brain damage induced by a single intracerebroventricular (i.c.v.) injection of Aβ (25-35) in mouse model of AD-type amnesia.\textsuperscript{28,29}

**Experimental**

**Reagents** – Rice bran was produced by milling Cheongwon Saengmyeong rice (Chungbuk, Korea) in Ochang Rice Processing Center (Chungbuk, Korea). The rice bran (18 kg) was extracted under pressure (60 Mpa) at 75 °C using an oil press (National Hanaro Oil Press, National ENG, Seoul) to yield 1.35 L of oil. The oil was re-extracted with n-hexane (3.6 L) at 37 °C for 12 h in agitation shaker, after which it was subjected to rotary vacuum evaporator to remove hexane and to yield 1.26 L of rice bran oil (RBO). RBO was stored in the dark at 4 °C. Aβ (25-35) was purchased from Bachem (Bubendorf, Switzerland). The bicinechonic acid protein assay kit and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Prepar buffer was from iNiRONBio. Inc. (Gyeonggi-Do, Korea), and thiobarbituric acid (TBA) was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Rabbit polyclonal antibodies against Bel-2, Bax, active caspase-3, procaspase-3, iNOS, phosphorylated ERK1/2 (p-ERK1/2), phosphorylated P38 (p-P38), PI3K, and β-actin and goat polyclonal antibody against COX-2 and horseradish peroxidase-conjugated anti-rabbit secondary antibody were purchased from Millipore, Inc. (Bedford, MA, USA). Rabbit polyclonal antibodies against Akt, p-Akt, CREB and P-CREB were acquired from Cell Signaling, Inc. (Danvers, MA, USA). Horseradish peroxidase-conjugated anti-goat secondary antibody was obtained from Assay Designs (Ann Arbor, MI, USA). All other chemicals were of the highest grade available.

**Experimental animals** – Male ICR mice (5-week-old) were supplied by Daehan BioLink Co., Ltd. (Chungbuk, Korea) and housed in an environmentally controlled room at 22 ± 2 °C and 55 ± 5% RH under a 12-h light/dark cycle with ad libitum access to food and water. Mice were allowed to adapt to the experimental environment for a week. All procedures involving experimental animals complied with the animal care guidelines issued by the U.S. National Institute of Health and those issued by the Animal Ethics Committee of Chungbuk National University (CBNUA-928-16-02).

**Induction of memory impairment in mice and administration of RBO** – Memory impairment was induced by i.c.v. injection of 15 nmol of the aggregated form of Aβ (25-35), as previously described.\textsuperscript{30} RBO was administered orally 30 min before Aβ (25-35) injection (on experimental day 0; ED0) and then further administered daily for 7 days prior to passive avoidance test. RBO was dissolved in corn oil (Sigma Chemical Co.) by brief ultra-sonication (W-380, Heat systems, Inc., Farmingdale, NY, USA). Sham-controls were injected ICV with physiological saline instead of 15 nmol of Aβ (25-35). Mice were randomly divided into five groups; sham-operated (the sham control group; n = 12), the 15 nmol Aβ (25-35) group (n = 13), the 15 nmol Aβ (25-35) + 0.1 mL/kg RBO group (n = 10), the 15 nmol Aβ (25-35) + 0.5 mL/kg RBO group (n = 13), and the 15 nmol Aβ (25-35) + 1 mL/kg RBO group (n = 13) groups. Corn oil as vehicle was administered orally to the sham control and Aβ (25-35) control groups using the same schedule as RBO administration.

**Memory assessment** – Passive avoidance test was performed using a two-compartment shuttle chamber device (Avoidance System version 1.1, B. S Technolab INC, Seoul, KOREA), one chamber was illuminated and the other was unlit and equipped with a grid floor and shock generator.\textsuperscript{30} Thirty minutes after administering RBO on experimental day 7 (ED7), the mice were trained to perform the step-through passive avoidance task with the electric shock system activated (0.5 mA, 3 s; the acquisition trial). Retention trials were performed 24 h after the acquisition trial and the step-through latencies were recorded (maximum test time was set at 300 s).

**Cholinesterase activity and oxidative stress in mouse brain** – After the passive avoidance test retention trial on
ED8, mice were deeply anesthetized with diethyl ether, brains were quickly removed, and brain homogenates were prepared using five volumes of 0.1 M phosphate buffer (pH 7.4) in ice bath. Cholinesterase activity and levels of reduced GSH in mouse brain were determined spectrophotometrically using Ellman reagent. Acetylthiocholine iodide was used as a substrate to measure cholinesterase activity. Extent of lipid peroxidation was assayed by measuring thiobarbituric acid reactive substance (TBARS) levels at 532 nm as described by Yoshioka et al. using 1,1,3,3-tetramethoxypropane as a standard. Protein concentrations were determined using Lowry’s method.

Western blotting – Mice were anesthetized with diethyl ether on ED8 and brains were removed. Hippocampi were homogenized in Pro-Prep buffer, and total proteins were extracted according to the manufacturer's instructions and quantified using bicinchoninic acid assays. Western blot analyses of Bcl-2, Bax, active caspase-3, procaspase-3, iNOS, COX-2, p-ERK 1/2, p-P38, Akt, P-Akt, PI3K, CREB, P-CREB, and p-tau expressions were conducted as previously described. Briefly, approximately 50 μg of total protein was loaded on 12.5% SDS-PAGE and then transferred to a PVDF membrane (Perkin Elmer Co., MA, USA). The membranes were incubated with the primary antibodies, followed by horseradish-peroxidase conjugated secondary antibodies. Protein expression was detected with an enhanced chemiluminescence detection reagent (Santa Cruz Biotechnology INC., CA, USA) and band images were quantified using Image J software (Image J 1.45S, a freely available application in the public domain for image analysis and process, developed and maintained by Wayne Rasband at the Research Services Branch, National Institutes of Health).

Statistical analysis – Results are presented as means ± standard errors (SEMs). The significances of differences were assessed by one-way analysis of variance (ANOVA) followed by Tukey’s tests. P values of < 0.05 were considered significant.

Results and Discussion

Since AD patients display learning and memory deficits, we investigated the inhibitory effect of RBO on memory impairment in Aβ (25-35)-treated mice using passive avoidance test. In the acquisition trial, group step-through latencies were similar in the five groups. However, in the retention trial, step-through latency in the Aβ (25-35) control group was significantly shorter than in the sham control group (44.2 ± 10.2 s versus 203.0 ± 27.1 s) indicating that Aβ (25-35) had induced memory impairment. Mice administered Aβ (25-35) and RBO at 0.1, 0.5 or 1 mL/kg daily 8 days showed significant memory improvements (141.1 ± 29.5, 165.7 ± 23.6, and 203.6 ± 23.7 s for 0.1, 0.5 and 1 mL/kg, respectively) as compared with mice in the Aβ (25-35) control group (Fig. 1). Cholinergic neuron damage is the fundamental pathological change caused by Aβ accumulation in the AD brain and is known to be related to cognitive impairment. Thus, some cholinesterase inhibitors to increase acetylcholine concentration, such as tacrine (Cognex®) and donepezil (Aricept®), have been developed for the treatment of AD. In the present study, cholinesterase activities in the brains of mice in the Aβ (25-35) group were significantly higher than in the sham control group, and these increases were inhibited by RBO (at 0.5 and 1 mL/kg) (Table 1), which suggested that RBO might ameliorate
memory impairment associated with the inhibition of cholinesterase activity.

Oxidative stress is a potential contributor to the development of AD and occurs when free radical production exceeds antioxidant capacity. Extracellular Aβ accumulation may directly or indirectly alter N-methyl-D-aspartic acid (NMDA) type glutamate receptor-mediated cytosolic increases in Ca^{2+}, which is rapidly taken up by mitochondria and endoplasmic reticulum. Ca^{2+} overload of mitochondria causes ROS generation, and excessive production of ROS and impairment of the ROS removal system in the AD brain result in protein, nucleic acid, and neuronal membrane lipid damage, which in turn, cause neuronal cell death. GSH is a central antioxidant and the most important free radical scavenger in the cellular armamentarium against oxidative stress. Lipid peroxidation is a major outcome of ROS-mediated injury, and results in the generation of stable end products.

Table 2. Inhibitory effects of RBO on Aβ (25-35)-induced reductions in GSH levels and increases in TBARS levels in mouse brains

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mL/kg)</th>
<th>GSH (nmol/mg protein)</th>
<th>TBARS (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>-</td>
<td>9.8 ± 0.4</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Aβ (25-35) 15 nmol</td>
<td>7.6 ± 0.1^{2*}</td>
<td>2.3 ± 0.2^{2*}</td>
<td></td>
</tr>
<tr>
<td>+ RBO 0.1</td>
<td>9.3 ± 0.7^{*}</td>
<td>1.8 ± 0.1^{*}</td>
<td></td>
</tr>
<tr>
<td>+ RBO 0.5</td>
<td>8.9 ± 0.4^{*}</td>
<td>1.5 ± 0.1^{**}</td>
<td></td>
</tr>
<tr>
<td>+ RBO 1.0</td>
<td>10.2 ± 0.6^{**}</td>
<td>1.6 ± 0.1^{*}</td>
<td></td>
</tr>
</tbody>
</table>

GSH and TBARS levels were measured after the passive avoidance test retention trial. Values are expressed as means ± SEMs. (n = 6-8 mice/group). ^{*} P < 0.05 and ^{**} P < 0.01 vs. sham control; ^{##} P < 0.05 and ^{##*} P < 0.01 vs. 15 nmol Aβ (25-35) control.

Fig. 2. Inhibitory effect of RBO on the Aβ (25-35)-induced expressions of apoptosis-associated proteins. (A) Representative western blot results for protein levels in hippocampi after the passive avoidance test retention trial. Bar graphs show Bcl-2/Bax (B) and active caspase-3/procaspase-3 (C) expression ratios versus sham controls. Values are expressed as means ± SEM (n = 4). ^{##} P < 0.01 vs. sham control; ^{*} P < 0.05 and ^{**} P < 0.01 vs. 15 nmol Aβ (25-35) control.
products like malondialdehyde, which reacts with TBA and is quantified by measuring TBARS levels. In the current study, brain GSH contents in the Aβ (25-35) group were significantly lower than in the sham control group, and RBO administration suppressed these decreases. On the other hand, TBARS levels in the Aβ (25-35) group were significantly higher than in the sham group, and RBO also suppressed these increases (Table 2). This result suggests that the antioxidant effect of RBO inhibits Aβ (25-35)-induced neuronal cell damage. Active antioxidants like γ-oryzanol, tocotrienols, tocopherols, and squalene are probably involved in the mechanism of

Fig. 3. Inhibitory effects of RBO on the Aβ (25-35)-induced expression of iNOS, COX-2, p-ERK 1/2, and p-P38. (A) Representative western blot analysis of proteins in hippocampi after the passive avoidance test retention trial. Bar graphs show i-NOS/β-actin (B), COX-2/β-actin (C), p-ERK 1/2/β-actin (D), and p-P38/β-actin (E) expression ratios versus sham controls. Values are expressed as means ± SEMs (n = 4). # P < 0.05 and ## P < 0.01 vs. sham control; * P < 0.05 and ** P < 0.01 vs. 15 nmol Aβ (25-35) control.
neuroprotective effects of RBO against Aβ (25-35)-induced neurotoxicity.

Furthermore, the association between Aβ-induced oxidative stress and mitochondrial dysfunction is critical in terms of causing neuronal apoptosis in AD patients.6 Bcl-2 family proteins are involved in the regulation of neuronal apoptosis by modulating mitochondrial permeability.39 Bax is a member of the Bcl-2 protein family and activates apoptosis, whereas Bcl-2 has anti-apoptotic effects.39,40 Bcl-2 prevents apoptotic cell death by preserving mitochondrial membrane permeability for cytochrome c, whereas pro-apoptotic proteins such as Bax and Bad trigger release of cytochrome c into cytosol. Cytochrome c released from the mitochondrial intermembrane space into cytosol mediates the activation of caspase-3, the executor of apoptosis.5,41 The expression levels of Bcl-2 (anti-apoptotic) were significantly lower in the Aβ (25-35) group than that in the sham-operated group. However, the expression level of Bcl-2 was significantly higher in animals administered RBO (0.1, 0.5, or 1 mL/kg) than in the Aβ (25-35) (Fig. 2A). Furthermore, Aβ (25-35) caused marked increases in the levels of pro-apoptotic proteins, Bax, and activated caspase-3, and RBO (0.1, 0.5, or 1 mL/kg) significantly inhibited these increases (Fig. 2B). Our results show that Aβ (25-35) reduced Bcl-2 expression and enhanced the expressions of Bax and active caspase-3 in hippocampus, and that RBO significantly suppressed these pro-apoptotic changes.

Microglia activation is considered central to the risk and pathogenesis of AD. Reactive microglia and astrocytes surround Aβ plaques and secrete numerous pro-inflammatory cytokines.7,42 Furthermore, the over-activation of glial cells in association with Aβ-induced neuroinflammation can reduce glutamate uptake and further increase the productions of pro-inflammatory molecules like COX-2 and iNOS, and thus, contribute to synaptic dysfunction and neuronal death.43 COX-2 expression is notably enhanced in the AD brain, and seems to be associated with Aβ plaque formation and advanced cognitive impairment.44 The synthesis of NO by iNOS and its subsequent release from astrocytes and microglia also induces neuroinflammation and can directly influence neuronal apoptosis.45 Moreover, Aβ-induced neuroinflammation might be involved in the excessive activation of MAPks, including ERK1/2 and P38, which can cause apoptosis, neurodegeneration, and memory impairment in AD.46,47 Therefore, inhibitions of the phosphorylations of ERK1/2 and P38 might reduce Aβ-induced neurotoxicity.48 In the present study, the expressions of iNOS and COX-2 in the hippocampi were increased by Aβ (25-35), and these increases were significantly inhibited by RBO. The expressions of p-ERK1/2 and p-P38 were higher in the Aβ (25-35) group than in sham controls, and RBO administration (at 0.1, 0.5, or 1 mL/kg) suppressed these Aβ (25-35)-induced increases (Fig. 3). These results suggest that the anti-oxidant and anti-inflammatory effect of RBO might be responsible for its neuroprotective effect on Aβ (25-35)-induced apoptotic neuronal damage in the AD brain.

PI3K enhances neuroprotection through the phosphorylation and activation of Akt,49 which is a well-known prosurvival kinase that is activated by its phosphorylation at Ser 473 via the PI3K pathway, and PI3K has also been demonstrated to play a critical role in neuronal survival.50 p-Akt can also promote neuronal survival by enhancing the functions of anti-apoptotic molecules like Bcl-2.51 PI3K/Akt signaling is downregulated in the AD brain, and activation of this pathway has been shown to prevent Aβ-induced neurotoxicity.10,52 Moreover, suppression of the PI3K/Akt signaling pathway can cause p-tau production and the formation of neurofibrillary tangles, and cause neuronal death.9,53 CREB mediates transcription regulation related to synaptic plasticity in memory and has a neuroprotective effect against ROS-induced cell toxicity,54,55 and CREB signaling is diminished in the hippocampus of the AD brain.56 CREB can be activated by various kinases such as protein kinases A and C, MAPks, and PI3K/Akt signaling pathways in neurons.57 CREB is a downstream target of PI3K/Akt signaling, and is therefore important for neuronal survival and memory.56,58 Various neuroprotectants activate the PI3K/Akt/CREB axis, and thus, improve cognitive function and promote neuronal survival.59,60 In the present study, the expression levels of p-Akt, PI3K, and p-CREB in the hippocampi were significantly lower in the Aβ (25-35) group than in the sham control group, but higher in animals administered RBO at 0.1, 0.5, or 1 mL/kg than in the Aβ (25-35) group. Furthermore, RBO administration significantly suppressed Aβ (25-35)-induced p-tau increases (Fig. 4). These observations suggest RBO might protect against cognitive impairment by upregulating p-CREB, increasing PI3K/Akt signaling, and consequently inhibiting neuronal apoptosis and p-tau formation in Aβ (25-35)-treated mice.

RBO contains a variety of anti-oxidants such as γ-oryzanol, tocotrienols, tocopherols, and squalene.13-16 Dietary γ-oryzanol plays a significant role in the anti-inflammatory activity of RBO by decreasing pro-inflammatory mediators secretion by macrophages.51 γ-Oryzanol has been reported to improve cognitive function
and prevent LPS-induced brain inflammation and cognitive impairment in mice. In addition, the vitamin E in RBO has anti-oxidative and anti-inflammatory activities, for example, RBO dietary supplementation improved neurological symptoms in vitamin E-deficient mice. Furthermore, RBO is rich in PUFAs, which can benefit learning and memory functions, and in a previous study, RBO-treated rats showed spatial memory
enhancements in a Morris water maze test. Therefore, we surmise that the favorable effects of RBO on Aβ (25-35)-induced cognitive deficits observed in the current study, are attributable to the anti-oxidative and anti-inflammatory activities of these constituents of RBO.

In summary, the current study demonstrates that Aβ (25-35)-induced cognitive decline in our mouse model was accompanied by p-tau protein up-regulation in the brain. Daily administration of RBO inhibited Aβ (25-35)-induced cognitive deficits observed in the current study, are attributable to the anti-oxidative and anti-inflammatory activities of these constituents of RBO.

Accordingly, we suggest RBO be considered a promising agent for preventing the progression of AD.

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Received June 04, 2020
Revised August 07, 2020
Accepted August 10, 2020