

Protective effects of pre-germinated brown rice extract against amyloid β -peptide induced neurotoxicity in neuronal SK-N-SH cells

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Abstract: Amyloid β -peptide ($A\beta_{1-42}$) is the major component of amyloid plaques and is believed to induce oxidative stress and inflammation in brain, which are postulated to play important roles in the pathogenesis of Alzheimer's disease (AD). The present study investigated the effect of pre-germinated brown rice extract (*Oryza sativa* L., PGBR ex) which contains a much higher concentration of essential amino acids, such as lysine, isoleucine, methionine and several kinds of antioxidants than conventional brown rice, and over 8-13 times the amount of γ -aminobutyric acid (GABA) shown to protect against oxidative stress and cell death. Pre-treatment of SK-N-SH cells with 2000 μ g/ml PGBR ex significantly attenuated 10 μ M $A\beta_{1-42}$ -induced neuronal cells deaths. Apoptotic cells death was demonstrated morphologically as well as by detection of the presence of activated caspase-3, DNA fragmentation and the production of reactive oxygen species (ROS) which could be reduced by pre-treatment of PGBR ex and brown rice extract (BR ex). These results suggest that the protective effect of PGBR ex and BR ex against $A\beta_{1-42}$ -induced neuronal cell death might be due to its anti-oxidative and anti-apoptotic properties and could be useful as a value added functional food to prevent neurodegenerative diseases.

Keywords: Brown Rice Extract, SK-N-SH Cells, Antioxidant, GABA, ROS, $A\beta_{1-42}$, Apoptosis, DNA Fragmentation

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder. Amyloid β -peptide ($A\beta_{1-42}$) is the major component of amyloid plaques and is considered to have a causal role which initiator of a pathological cascade that leads to oxidative stress and neuronal dysfunction. $A\beta_{1-42}$ was shown to have the potential to induce oxidative stress and inflammation in the brain, which are postulated to play important roles in the pathogenesis of Alzheimer's disease [1, 2]. For example, $A\beta_{1-42}$ induces the production of hydrogen peroxide and lipid peroxide in neurons [3]. In addition, $A\beta_{1-42}$ has been reported to induce superoxide [4] and proinflammatory cytokines [5, 6] in astrocytes as well as in microglial cells. Antioxidants such as-tocopherol protect against

cytotoxicity in vitro [7] as well as learning and memory deficits induced by $A\beta$. Furthermore, -tocopherol and anti-inflammatory agents such as indomethacin reportedly slow the progression of Alzheimer's disease [9].

Rice is a major cereal food and a dietary staple worldwide, especially in Asian countries. Rice seeds and rice germ contain fiber and several kinds of antioxidants, such as ferulic acid, phytic acid, tocopherols, and oryzanols [10]. Brown rice (BR) is a rice seed form which only the hull is removed. Recently, we found that pre-germinated brown rice (PGBR) contains a much higher concentration of essential amino acids, such as lysine, isoleucine, methionine, than conventional brown rice, and over 13 times the amount of γ -aminobutyric acid (GABA) [11, 12]. PGBR is brown rice, which has been soaked in water for up to a day and had a germ of approximately 1 mm long.

During germination, nutrients in the brown rice change drastically. Nutrients that increase in content include GABA, dietary fiber, inositols, ferulic acid, phytic acid, tocotrienols, magnesium, potassium, zinc, γ -oryzanol, and prolyendopeptidase inhibitor. According to previous studies they reported that germinated brown rice contained more total ferulic acid (126%), total dietary fiber (145%), soluble dietary fiber (120%) and insoluble dietary fiber (150%) compared to the brown rice [13]. The neuroprotective activity of various natural extracts has been reported in the literature [14]. Ferulic acid (4-hydroxy-3-methoxycinnamic acid), a phenolic compound present in a variety of plants, has potent antioxidant and anti-inflammatory activities [15]. Among them rice bran is a by-product of the rice milling process and contains various antioxidant factors showing beneficial effects on human health. As well known antioxidants in rice bran, tocopherols, tocotrienols, oryzanols (ferulate esters of triterpene alcohols) are isolated from fat-soluble extracts of rice bran and they have potent hypocholesterolemic and antitumor properties. However, to date, no attempts have been made to investigate the effects of brown rice extracts containing high levels of GABA on proliferation and apoptosis of neuronal cells. The antioxidants of the water-soluble extract of rice bran have been poorly analyzed. Therefore, we hypothesized that the PGBR ex enhanced GABA level may have beneficial effect against $A\beta_{1-42}$ -induced neurotoxicity and apoptosis on SK-N-SH cells. To address this hypothesis, we investigated various methods to study by MTT assay, DNA fragmentation, Caspase-3 activity and ROS formation. The significance of this finding is discussed from the viewpoint of the preventive role of the rice bran against $A\beta_{1-42}$ related chronic diseases.

2. Materials and Methods

2.1. Cells Culture

The human neuroblastoma SK-N-SH cells line was obtained from the ATCC (Rockville, MD, USA) and maintained in minimum essential medium (MEM) (Life Technology, Inc.) containing 2 mM L-glutamine in a humidified incubator at 37 °C and 5% CO₂. The medium was supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids (Life Technology, Inc.), 100 units/ml penicillin, and 100 mg/ml streptomycin (Biofluids, Rockville, MD). Cells were maintained for 24 h before treatments.

Cultures were plated at 1×10^5 cells/ml in 96 well plates for ROS assay, cells viability (MTT assay), and 1×10^6 cells/ml in 6 well plates for genomic DNA extraction using the Apoptotic DNA Kit, (Roche Applied Science, Mannheim, Germany) and caspase-3 activity assay using CaspACE™ assay kit (Promega; San Luis Obispo, CA).

2.2. Preparation of Rice Extracts

PGBR (*Oryza sativa* L.) was supplied by Innofood

(Thailand) Co., Ltd. PGBR was germinated by soaking in the following solutions at 25–26°C kept in the dark for 72 h: was air dried and frozen in liquid nitrogen. Rice extracts were prepared as previously described [16]. Briefly, PGBR, brown rice (BR) and white rice (WR) was ground with a grinder. The milling rice were weight 0.1 g in 1 mL of distilled water mix with Vortex for 10 min follow incubated in water bath at 70°C for 30 min. then centrifuged at 10,000 rpm at 4°C for 30 min. The supernatants were collected by passed through the filters (pore size 0.45 μ m) kept at -20°C and used as rice extracts (rice ex).

2.3. Determination of GABA Levels

GABA levels were quantified by high-performance liquid chromatography (2695 Separations Module, Waters Corporation, USA) with photodiode array detector (Waters 2996). Samples were separated on a Supelco LC-DABS HPLC column (4.6 mm \times 150 mm in length and 3 μ m beads). The milling rice was weight 0.5 g in plastic tube and added with deionized water 2 mL. The sample solution was fully mix, then centrifuged at 4500 rpm for 10 min. Supernatant was pipette 1 mL then added with 200 μ L of 0.4 M/L NaHCO₃ and 400 μ L of 6 mM/L DABSYL-Cl acetone solution. The reaction was performed at 70° C for 20 min. After derivatization, the sample was filtered to vial and 10 μ L of sample was injected into HPLC [17].

2.4. Effect of Rice Extracts on Viabilities of SK-N-SH Cells

To study the cytotoxicity of rice ex on cell viability which induced by $A\beta_{1-42}$. Cells were plated in 96 well plates. Rice ex were pre-treated with increasing concentrations (0 - 4000 μ g/ml) at 37°C for 24 h or treated with $A\beta_{1-42}$ alone to determine for time and concentrations dependent manner. In addition to study the protective effects of rice ex by pre-treatment 2000 μ g/ml for 3 h then added with $A\beta_{1-42}$ and incubated for 24 h. The cells viabilities were assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium-bromide] assay [18], MTT solution was added at a final concentration of 0.5 mg/ml and incubation for 3 h. The supernatant was removed and solubilize solution (DMSO) was added, the plates were shaken for 10 min and absorbance was read at 570 nm using a microplate reader (Synergy HT, BioTek Instrument).

2.5. Antioxidant Activity Test

2.5.1. Effect of Rice Extracts on Apoptosis by DNA Fragmentation

Rice ex were pre-treated into the cultures at 2000 μ g/mL and incubated at 37°C for 3 h then added with $A\beta_{1-42}$ for 24 h. Cells were collected. Genomic DNA was extracted using the Apoptotic DNA Ladder Kit, with slightly modification. In brief, cells were washed twice with ice cold 1x PBS, then 200 μ L of lysis buffer was added and mixed immediately. After incubated at 22°C for 10 min, 100 μ L of 100% isopropanol was added and vortex for 10 sec. The

lysate was run through the column and washed twice with washing buffer. DNA was eluted with 200 μ L of pre-warmed (70°C) elution buffer and concentrated by a speedvac. Extracted DNA was subjected to gel electrophoresis, and the image was captured with GelDoc™ EQ (Bio-Rad Laboratories, Ltd. Hercules, CA).

2.5.2. Effect of Rice Extracts on Apoptosis by CASPASE-3 Activity Assay

Caspase-3 activity assay was determined using a colorimetric assay kit, CaspACE™ assay system, according to the manufacturer's instructions. Rice ex were pre-treated to the cultures 2000 μ g/mL and incubated at 37°C for 3 h then added with A β ₁₋₄₂ for 24 h. Culture was lysate and 100 μ g/well of total lysate protein were used. The specific cleavage of substrate DEVD-pNA by caspase-3 activity was measured at wavelength 405 nm. The results were expressed as the ratio comparing to the untreated control.

2.6. Antioxidant Activity Test

To detection of intracellular ROS, the production of ROS was estimated by using a fluorescent probe, 2', 7'-dichlorofluoresceindiacetate (DCFH-DA) [19]. DCFH-DA was transported across the cell membrane and hydrolyzed by intracellular esterase to form non-fluorescent 2', 7'-dichloro-fluorescein (DCFH), which is then rapidly converted to highly fluorescent 2', 7'-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is believed to be parallel to the amount of ROS formed intracellular. To determine the amounts of ROS induced by A β ₁₋₄₂, DCFH-DA (50 μ M final concentration) were added to the culture for 45 min and then rice ex (2000 μ g/ml) for 30 min before added with A β ₁₋₄₂ and incubation at 37°C for 3 h. The production of ROS was measured by microplate reader (Synergy HT, BioTek Instrument) with excitation and emission wavelength at 485 nm and 530 nm respectively.

2.7. Statistical Analysis

Results are expressed as the mean \pm SD (n=3). Data were analyzed by one-way ANOVA, followed by Dunnett's test for comparison between control and treated experimental groups. Differences were considered significant at a P-value of < 0.05.

3. Results and Discussion

3.1. Analysis of GABA from Rice Extracts by HPLC Method.

PGBR ex with enhanced GABA level were tested comparable with BR ex and WR ex. The content of GABA in the PGBR ex, BR ex and WR ex were compared with standard GABA. The concentration of GABA in PGBR ex (9.90 mg \pm 0.02/100g of flour) was 3 times and 8 times higher than the GABA content in BR ex (3.30 \pm 0.01 mg/100g of flour) and in WR ex (1.24 \pm 0.02 mg/100g of

flour) respectively, as shown in Table.1 (the data average are base on three measurement of each sample).

Table 1. The content of GABA from rice extracts

Type of rice	Content of GABA(mg/100g of flour)
PGBR ex	9.90 \pm 0.02 ^a
BR ex	3.30 \pm 0.01 ^a
WR ex	1.24 \pm 0.02 ^a

^aResults are given as Mean Values \pm SD (n=3)

3.2. The Cytotoxicity of Rice Extracts and A β ₁₋₄₂ on SK-N-SH Cells

First, we attempted to establish the cytotoxicity of rice ex by using the doses dependent on human neuronal SK-N-SH cells. PGBR ex, BR ex and WR ex was applied by increasing concentrations 0-4000 μ g/ml for 24 h. We found that the number of cells viability had no significantly different to decrease. The cells survival were up to 96.4 \pm 0.052% (p >0.48), PGBR ex 98.9 \pm 0.032% (p >0.30), BR ex 96.3 \pm 0.039% (p >0.39), and WR ex 95.3 \pm 0.037% (p >0.25) respectively. These data showed no toxicity of rice extracts affect on human neuronal SK-N-SH cells (Figure 1).

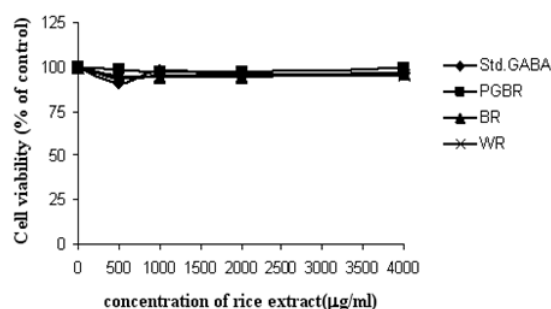


Figure 1. The Effects of rice ex on SK-N-SH cells viability using MTT assay. Pre-incubated cells with increasing concentrations of rice ex at 0-4000 μ g/ml. Results are expressed as percentages of the control value (CON) and data shown are means \pm S.D of three replicate experiments.

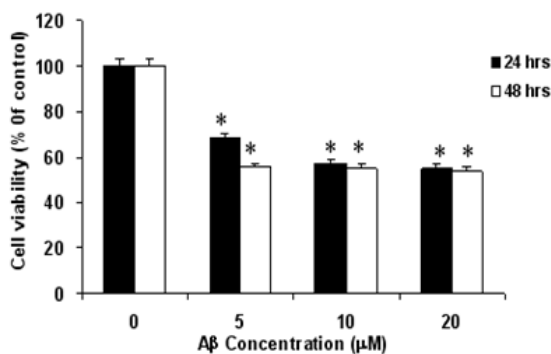


Figure 2. The Effects of A β ₁₋₄₂ on SK-N-SH cells viability using MTT assay. Cells viability was significantly decreased on time and concentration dependent manner. Results are expressed as percentages of the control value. * = P <0.05 compared to the untreated control.

Similar as Chan-Ho Oh and Suk-Heung Oh [16], they tested the effects of the BR ex on the viability of Hela cells,

by treated cells with the BR ex 2000 $\mu\text{g/ml}$ in cultured and the viability of the cells was assayed. They showed that BR ex had no effects on the retardation of HeLa cell survival and proliferation. To evaluated the cytotoxicity of $\text{A}\beta_{1-42}$ by using time and concentrations dependent manner. Cells were treated with 0-20 μM of $\text{A}\beta_{1-42}$ for 24 h and 48 h. The results showed that $\text{A}\beta_{1-42}$ caused cells death $57.3 \pm 0.03\%$ ($p < 0.05$) compared with control. These data indicated that $\text{A}\beta_{1-42}$ was toxicity to SK-N-SH cells depending on time and concentration as shown in Figure 2.

3.3. Protective Effect of Rice Extracts Inhibit Neuronal Cell Death Induced by $\text{A}\beta_{1-42}$

The protective effects of the rice ex on the viability of SK-N-SH cells were evaluated. The effects of PGBR ex with enhance levels of GABA against 10 μM $\text{A}\beta_{1-42}$ -induced cytotoxicity by compared with BR ex, and WR ex. Cells were pre-treated with the PGBR ex, BR ex and WR ex at 2000 $\mu\text{g/well}$ for 3 h and then added with $\text{A}\beta_{1-42}$ for 24 h. These results showed that PGBR and BR extracts can protect cells by significantly increase cell survival up to $30.74 \pm 0.02\%$ and $21.53 \pm 0.04\%$ ($p < 0.05$), but not WR ex comparable with $\text{A}\beta_{1-42}$ alone which caused cells death $57.3 \pm 0.03\%$ ($p < 0.05$), when compared with control as shown in Figure 3. Recently, it has been reported that the long-term administration of ferulic acid acts to protect against $\text{A}\beta_{1-42}$ -induced neurotoxicity and memory disturbance.

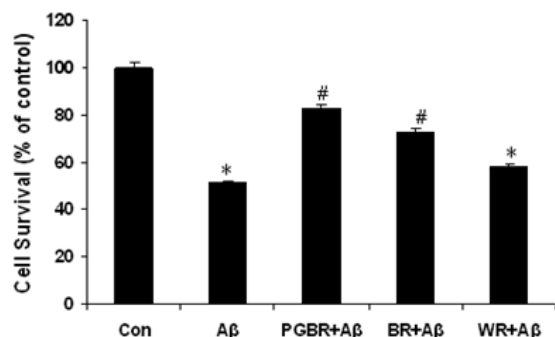


Figure 3. The Protective effects of rice ex on SK-N-SH cells viability induced by $\text{A}\beta_{1-42}$ by MTT assay. Pre-treated with rice ex significantly increase cells viability. Results are expressed as percentages of the control value. The data shown are means \pm SD ($n=3$). * = $P < 0.05$ compared to the untreated control; # = $P < 0.05$ compared to the $\text{A}\beta_{1-42}$ -treated group, respectively.

3.4. Effect of Rice Extracts on Apoptosis and DNA Fragmentation of SK-N-SH Cells

To verify the possible involvement of apoptosis in the $\text{A}\beta_{1-42}$ induced death of neuronal SK-N-SH cells, DNA fragmentation was tested on these cells. We investigated its inhibitory effect on the apoptosis induced by $\text{A}\beta_{1-42}$ by observing DNA fragmentation levels in SK-N-SH cells. As a control, we used DNA isolated from untreated cells. Only the degraded DNA was extracted, not the intact chromosomal DNA, from apoptotic and non-apoptotic cells.

Treatment with $\text{A}\beta_{1-42}$ to cells resulted in the characteristic apoptotic DNA ladder (Figure 4). These DNA fragmentation patterns are entirely consistent with the molecular weight patterns expected to result from internucleosomal DNA cleavage (20). DNA fragments smeared the whole lane in the condition of $\text{A}\beta_{1-42}$ treated alone and smear band was decrease in the condition of pre-treatment with PGBR ex and followed added by $\text{A}\beta_{1-42}$. According to administration of PGBR ex alone and non-treated cells (control) did not cause cell death. Similar as the previous studies have shown that intracellular ROS have been implicated with DNA fragmentation and apoptosis [21].

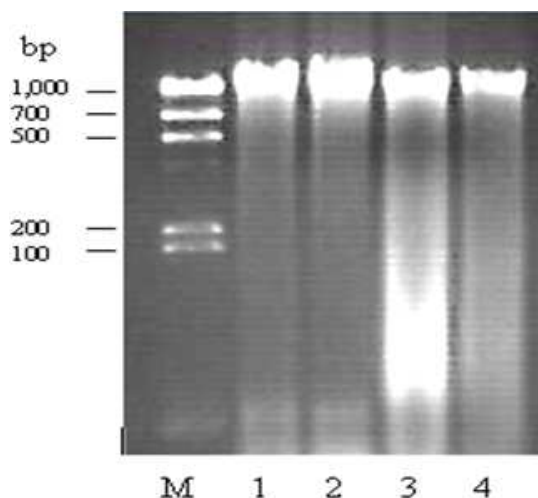


Figure 4. PGBR ex protect SK-N-SH cells against $\text{A}\beta_{1-42}$ -induced apoptosis examined by DNA fragmentation. Pre-treatment with PGBR ex were reduced DNA fragmentation. Genomic DNA was then extracted and subjected to gel electrophoresis. The image was captured with GelDoc™ EQ (Bio-Rad Laboratories, Ltd. Hercules, CA). Marker; M., control (nontreated-cells); lane 1, PGBR ex; lane 2, 10 μM $\text{A}\beta_{1-42}$; lane 3, PGBR ex and 10 μM $\text{A}\beta_{1-42}$; lane 4.

3.5. Effect of Rice Extracts Inhibits Caspase-3 Activity

Caspase-3 belongs to a family of evolutionally conserved cysteine proteases that play a key role in regulating programmed cell death, or apoptosis, a normal process required for maintenance of tissue homeostasis and the regulation of physiological functions [22]. Once caspase-3 is activated, a series of irreversible events are set in motion that lead to the death of the cell, including activation of the CAD endonuclease that degrades DNA within the nucleus and initiates chromatin condensation. Increasing evidence suggests involvement of caspase-3 in Alzheimer's Disease (AD) via cleavage of amyloid precursor protein (APP) and tau and the formation of beta-amyloid ($\text{A}\beta$) and neurofibrillary tangles (NFTs), making caspase-3 an interesting therapeutic target [23]. The evidence of apoptotic cell death by $\text{A}\beta_{1-42}$ in this study was showed by increased the level of caspase-3 activity compared to untreated controls. According to pre-treatment with 2000 $\mu\text{g/ml}$ of PGBR ex and BR ex significantly inhibited the content of activated caspase-3 (Figure 5).

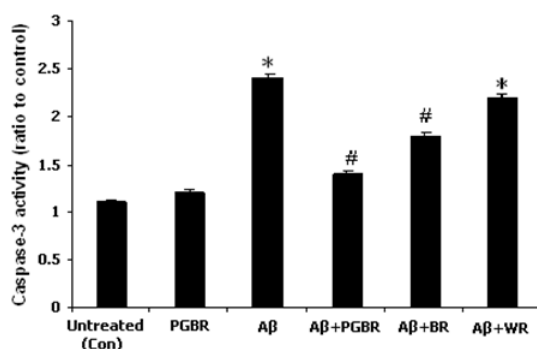


Figure 5. Effects of rice ex induced by A β_{1-42} on caspase-3 activity in SK-N-SH cells. The caspase-3 activity was measured at 3 h after treatment with 10 μ M A β_{1-42} in the presence or absence of the 2000 μ g/ml of rice ex and represents as the ratio to the caspase activity of the untreated control. The data shown are means \pm SD (n=3). * = P<0.05 indicated statistically significant differences compared with the untreated control and # = P<0.05 indicated statistically significant differences compared with the A β_{1-42} treated group, respectively.

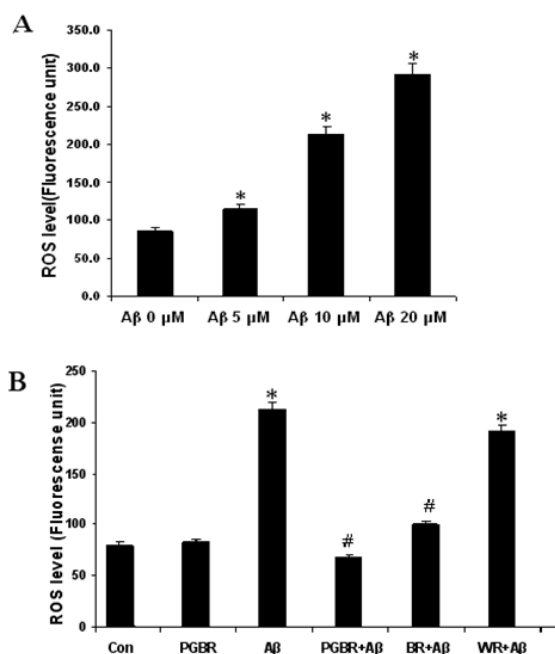


Figure 6. Intracellular ROS generation using the DCFHDA assay, to measure DCF in neuronal SK-N-SH cells. ROS levels were increased by induction with A β_{1-42} in a dose dependent manner (A). Intracellular ROS levels are significantly reduced by pre-treatment of PGBR ex and BR ex (B). The data shown are means \pm SD (n=3). * = P<0.05 compared to the untreated control; # = P<0.05 compared to the A β_{1-42} -treated group, respectively.

3.6. Effect of Rice Extracts Inhibits A β_{1-42} Induced ROS Generation

The intracellular radical scavenging activity of a given substance can be evaluated by DCFHDA assay. To determine whether rice ex inhibits the ROS generation induced by A β_{1-42} in neuronal SK-N-SH cells, and to determine whether the protection of SK-N-SH neuronal cells from apoptosis is accompanied by free radicals scavenging by the rice ex, changes in the concentrations of

ROS in whole cell suspensions were analyzed over a 2 h period after treatment with PGBR ex and BR ex and/or A β_{1-42} . Non-treated SK-N-SH cells showed relatively low levels of basal fluorescence, while treated with A β_{1-42} alone for 3 h, a marked increase in fluorescence was observed. However, this increased fluorescence by A β_{1-42} rapidly returned to the control level after pre-treatment with in PGBR ex and BR ex (2000 μ g/ml) to the cells as shown in Figure 6. Similar study had reported that the addition of A β_{1-42} to neural cells leads to the rapid production of H $_2$ O $_2$ and lipid peroxidation, suggesting that A β_{1-42} toxicity is mediated by free radical damage [24].

4. Conclusion

The main objective of present study was to evaluate the protective effects of rice ex to protect neuronal SK-N-SH cells which induced by A β_{1-42} . By taking into account, these results demonstrated that PGBR ex and BR ex are potent scavengers of free radical. The results showed protective effects by increased cells survival and inhibit the induction of apoptosis by decrease level of DNA fragmentation ladder and caspase-3 activity. Recently, it has been reported that the long-term administration ferulic acid acts to protect against A β_{1-42} -induced neurotoxicity and memory disturbance [25]. Previously reports had hypothesized that oxidative stress is one of the mechanisms of A β_{1-42} -induced neurotoxicity. *In vitro* studies have shown that α -tocopherol, a representative antioxidant, inhibits A β_{1-42} induced neuronal cell death and lipid peroxidation [26]. Ferulic acid also possesses free radical scavenging activity and reduces peroxidative damage [27]. Thus, PGBR may ameliorate A β_{25-35} -induced learning and memory deficits by scavenging oxidative stress.

Very recently, it has been reported that GABA and its receptor agonist improve cognitive decline that accompanies aging in monkeys [28]. Taken together, the large amount of GABA in PGBR may regulate the glutamatergic system by enhancing glutamate release and/or the sensitivity of NMDA receptors, resulting in memory enhancement. PGBR ex contains approximately 13 times the amount of GABA than in WR ex. Ferulic acid ester is one of main components of oryzanol, a phytosterol derived from rice bran. Natural antioxidants that can neutralize ROS include cysteine, reduced glutathione, polyphenolic compounds carotenoids, ascorbic acid (vitamin C), α -tocopherol (vitamin E) and indolecarbinols. Some researcher have suggested that germination may bring about changes in nutrients and physiologically active substances. During the germination of wheat [29] and Pangium edule Reinw [30], vitamin C, vitamin E, ferulic acid, and total phenolic acid contents increased. Upon malting of finger millet, changes in both free and bound phenolic acid contents were observed and these reflected their antioxidant properties. During the germination of rice seeds, alanine and γ -aminobutyrate have been reported to increase significantly [31]. We speculate that, during

germination, as seed moisture increases, the seed coat has the potential for injury by oxidation and/or microorganism infiltration. Induced saccharolytic enzymes to hydrolyze starch would produce free phenolic compounds having more effective antioxidant activity from hydroxycinnamate sucrose esters. As a result, the content of hydroxycinnamate sucrose esters decreases, whereas that of free phenolic compounds increases. This hypothesis requires verification through further experiments; however, the changes in content and form of phenolic compounds in germinated brown rice suggest that appropriate germination of brown rice may be a method to improve health-related benefits. From previous studies have shown that A β is associated with ROS generation, which leads to mitochondrial dysfunction, lipid peroxidation and apoptosis [5, 17]. Therefore, suppression of ROS generated by antioxidants may be an effective means in preventing oxidative cells death. The present study demonstrated that A β_{1-42} -induced ROS production and apoptosis of neuronal cultured is attenuated by PGBR ex and BR ex. Thus, PGBR and BR have a potential role in the future in alleviating neuronal apoptotic death associated with neurodegenerative disease.

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Conflict of interest: The authors declare no conflict of interest.

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