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ARTICLE

Red Mold Rice Promoted Antioxidase Activity against Oxidative Injury and Improved the Memory Ability of Zinc-Deficient Rats

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Zn deficiency is a common disease leading to memory impairment with increasing age. This study evaluated the protection effects of red mold rice (RMR) administration and Zn supplementation against memory and learning ability impairments from oxidative stress caused by Zn deficiency. Rats (4 weeks old) were induced to be Zn deficiency by a Zn-deficient diet for 12 weeks. After that, rats were administered Zn, 1× RMR, 5× RMR, and various dosages of RMR plus Zn, respectively. Decreases of antioxidant enzyme activities in the hippocampus and cortex were observed, and the levels of Ca, Fe, and Mg were increased in the hippocampus and cortex of Zn-deficient rats, leading to memory and learning ability injury. However, the administration of RMR (1- or 5-fold dosage) and with or without Zn significantly improved the antioxidase and neural activity to maintain cortex and hippocampus functions. This study demonstrates that RMR is a possible functional food for the prevention or cure of neural injury associated with Zn deficiency.

KEYWORDS: Zn deficiency; red mold rice (RMR); antioxidant enzymes; neural activity; functional food

INTRODUCTION

Zinc (Zn) deficiency is a prevalent disease around the world, including the United States (*1*). Approximately 50% of the world population does not get adequate Zn (*2*). Zn deficiency may result from several factors such as dietary restriction, gastrointestinal dysfunction, and elevated levels of dietary phytate or calcium (Ca). Clinical evidence suggests that Zn deficiency leads to growth retardation, osteopenia, and hypogonadism (*3*, *4*). In addition, Zn is the second most abundant trace metal in the brain and is required in the process of brain development (*5*). The level of Zn in the hippocampus is approximately 300 μM, which is relatively high in the brain. Consequently, the Zn concentration is decreased in rats fed a Zn-deficient diet (*6*, *7*). Localization of Zn in presynaptic areas is associated with the hippocampal function (*8*). On the other hand, the study indicates that the Zn level may be decreased in the brain with aging (*9*). Consequently, the function of the hippocampus is susceptible to Zn-deficient in the elderly. Zn deficiency has been demonstrated to lead to short-term memory impairment (*10*).

Zn is an essential microelement in the brain and plays several important roles, such as cofactors for antioxidant enzymes, including superoxide dismutase (SOD), and more than 300 metalloenzymes in a variety of animal species (*11*). Furthermore, Zn is assumed to be an antioxidant that inhibits lipid peroxidation (*12*). Chronic inflammation and the aging process are associated with the status of intracellularly available Zn, and continuous lack of Zn further increases reactive oxygen species (ROS) production (*13*). SOD activity is affected by Zn, indicating that Zn plays an important role in the antioxidant defense system. Thus, ROS are generated in abundance by a Zn-deficient diet. In addition, cognitive and behavioral impairments reflect brain dysfunction (e.g., deficits in learning and memory). Accurate spatial memory performance depends on the hippocampal formation (*14*); therefore, a simple object recognition task is used to investigate brain function in this study.

Recently, some studies have reported that several natural antioxidants such as flavanone and naringenin have neural protection activity (*15*), that anthocyanins ameliorate the memory and learning ability of ovariectomized rats (*16*), and that vitamin A promoted the level of nerve growth factor in the brain of Zn-deficient rats (*17*). These findings demonstrate that natural antioxidant inhibits ROS production and has neural protective effects to avoid memory impairment. In addition, Yokukansan (a herbal medicine) is used to prevent neural injury caused by Zn deficiency (*18*, *19*). Therefore, some substances in red mold rice (RMR) may prevent memory and learning injury caused by Zn deficiency. Zn supplementation effectively prevents recognition memory impairment, suggesting that Zn is beneficial to the function of the brain (*20*). Thus, the ability to improve memory by RMR administration in Zn-deficient rats (a neurological disease model) was examined in this study. Moreover, whether the memory ameliorative ability of RMR was higher than that of Zn supplementation in Zn-deficient rats was investigated.

The Zn-deficient diet significantly elicits serum glucocorticoid concentration, increasing glucocorticoids such as corticosterone through the activation of the hypothalamic−pituitary−adrenal (HPA) axis (*21*). Glucocorticoid increases

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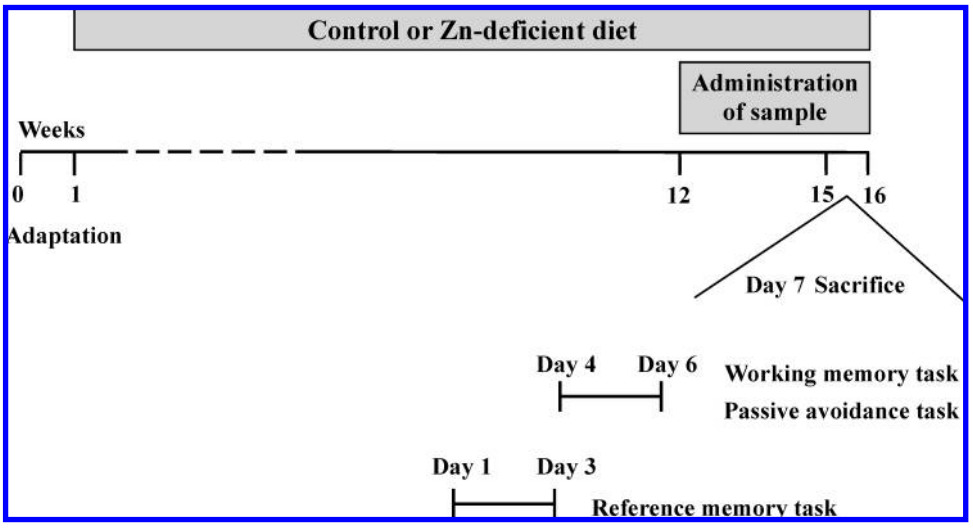

Figure 1. Experimental schedule.

in the levels of hippocampal cyosolic-free Ca and ROS in brain are conferred (*22*, *23*), which contribute to glucocorticoid-induced impairment of the synaptic function of the hippocampus (*24*). Increase in Ca level of the hippocampus injures neural function, which is observed after 2 weeks of Zn deficiency induction (*25*, *26*).

Monascus-fermented rice is known as red mold rice (RMR), which has been used for many centuries to enhance the color and flavor of food, as well as a traditional medicine for digestive and vascular functions (*27*, *28*). Several studies have been shown that RMR contains monacolins in abundance, which as an inhibitor of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, has hypolipidemic ability (*29*, *30*). Furthermore, RMR has various antioxidants such as dimerumic acid, tannin, and phenol (*31*). In our previous study, ethanol extracts of RMR were proved to show antioxidative ability, including reducing power and DPPH radical scavenging activity (*32*). Moreover, the monascin and ankaflavin in RMR are found to have some biological functions such as anti-inflammatory and antitumor activities (*31*−*35*).

There is a relationship between antioxidant activity and memory ability. Furthermore, Zn deficiency affects the antioxidase activities and glucocorticoid concentration of serum. Oxidative stress is generated by glucocorticoids of serum. We hypothesized that RMR may be a useful chemopreventive agent to promote antioxidase activity against oxidative stress in the brain of Zn-deficient rats, and the inhibition level of ROS production by RMR with or without Zn administration is explained in the present study.

MATERIALS AND METHODS

Materials and Chemicals. An SOD assay kit was purchased from Randox Laboratories Ltd. (Antrim, U.K.). Glutathione (GSH), glutathione reductase (GR), glutathione disulfide (GSSG), nicotinamide adenine dinucleotide phosphate (NADPH), and nitroblue tetrazolium (NBT) were purchased from Sigma (St. Louis, MO). Zinc dichloro, potassium dihydrogen phosphate (KH_2PO_4), and dipotassium hydrogen phosphate (K_2HPO_4) were purchased from Merck (Darmstadt, Germany). Hydrogen peroxide (H_2O_2) was purchased from Aldrich (Milwaukee, WI).

Sample Preparation. RMR is obtained from *Monascus purpureus* NTU 568 fermented rice. *M. purpureus* NTU 568 strain was maintained on potato dextrose agar (PDA) slants at 10 °C and transferred monthly. The preparation of RMR was carried out under the substrate of long-grain rice (*Oryza sativa*) purchased from a local supermarket in Taiwan and using the method of solid-state culture (*36*). Briefly, 500 g of rice was soaked in water for 8 h. After that, excess water was removed with a sieve. The rice was autoclaved (HL-341 model, Gemmy Corp., Taipei, Taiwan) for 20 min at 121 °C in a "koji-dish" (the koji-dish was made of wood with dimensions of 30 × 20 × 5 cm) that is a fermented-instrument tray of RMR during the fermentation process. After having been cooled, the rice was inoculated with a 5% (v/w) spore suspension. The inoculated rice was cultivated at 30 °C for 10 days. During the culturing stage, 100 mL of water was daily added to the rice from the second day to the fifth day. At the end of cultivation, the crushed and dried product with the mold was used for the experiments.

Animal and Diets. Male Wistar rats, 4 weeks old (90.7 ± 10.6 g), were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Animals were acclimatized for 1 week prior to use and then divided at random into seven treatment groups (nine rats/group) and provided with food and water ad libitum. Animals were subjected to a 12 h light/dark cycle with a maintained relative humidity of 60% and a temperature at 25 °C (protocol complied with guidelines described in the "Animal Protection Law", amended on January 17, 2001, Hua-Zong-(1)-Yi-Tzi-9000007530, Council of Agriculture, Executive Yuan, Taiwan, ROC). The Zn-deficient diet was designed in accordance with the dietary formula (*37*) and contained 200.0 g of egg white, 631.1 g of dextrose, 100.0 g of corn oil, 30.0 g of fiber, 9.9 g of calcium carbonate, 3.2 g of calcium phosphate, 0.002 g of cobalt chloride, 0.01 g of cupric sulfate, 0.9 g of ferric citrate, 3.4 g of magnesium sulfate, 0.009 g of manganese sulfate, 0.026 g of potassium iodide, 5.55 g of sodium chloride, 0.004 g of biotin, 0.02 g of vitamin B_{12}, 0.016 g of calcium pantothenate, 1.5 g of choline chloride, 0.25 g of chlortetracycline, 0.0005 g of folic acid, 0.0003 g of menadione, 0.25 g of niacin, 0.004 g of pyridoxine HCl, 0.006 g of riboflavin, 0.01 g of thiamin HCl, 10000 IU of retinyl palmitate, 1250 IU of ergocalciferol, and 110 IU of tocopheryl acetate per kilogram of diet. Rats were fed with the normal daily diet containing 60 mg/kg of Zn in the normal group, but approximately 0.3 mg/kg of Zn in the Zn-deficient feedstuff. The experimental schedule for the Zn-deficient model is shown in **Figure 1**. All animals were divided into two groups (normal and Zn-deficient) during the first 12 weeks, and the Zn-deficient rats were later divided into six groups (Zn-deficient, Zn compensation, 1× RMR, 5× RMR, 1× RMR + Zn, and 5× RMR + Zn groups, respectively). Animals were induced to be Zn deficient by the Zn-deficient diet for 12 weeks prior to 4 weeks of administering sample. At the 15th week, animals were evaluated for memory and learning capabilities, including reference memory task, working memory task, and passive avoidance task.

Doses and Groups. Rats were divided at random into seven treatment groups, including normal, Zn-deficient (ZD), Zn-compensative (ZC), 1× RMR, 5× RMR, 1× RMR + Zn (RZ), and 5× RMR + Zn (5RZ) groups, and administered sample per oral (po). The dose of RMR powder was calculated in accordance with Body's formula of body surface area as recommended by the U.S. Food and Drug Administration (FDA). The

daily dietary dosage of RMR is usually recommended at 1.0−2.0 g for adults (*38*). Therefore, 2 g of RMR was used as the 1-fold dosage for an adult with a weight of 65 kg and a height of 170 cm; 151 mg/kg of bw (1-fold dosage) of RMR was used as a frame of reference for conversion of the dosage into a rat model, and 755 mg/kg bw of RMR was used as 5-fold dosage, relatively. On the other hand, *M. purpureus* NTU 568 strain had shown neural protection activity in our previous study, and the employment of the 5× RMR dosage significantly improved memory ability in an Alzheimer's disease rat model (*39*). In addition, the level of Zn in RMR was 25.9 mg/kg. Thus, the administration level of 1× RMR contained 0.004 mg of Zn/kg of bw, and 0.02 mg of Zn/kg of bw was administered in the 5× RMR group. However, the zinc gluconate was employed to compensate in the ZC group (the administration level of Zn was 1.1 mg/kg of bw), which was in accord with the recommended dietary allowances (RDA). Accordingly, a level of 0.004 + 1.1 mg/kg of bw of Zn was administered in the 1× RMR + Zn (RZ) group, and a level of 0.02 + 1.1 mg/kg of bw of Zn was administered in the 5× RMR + Zn (5RZ) group.

Apparatus for Water Maze. The Morris water maze task with some modifications was used to evaluate memory and learning ability (*40*). A black circular tank (diameter = 140 cm, height = 45 cm) was used as the apparatus of the water maze in which a movable escape platform (diameter = 10 cm, height = 25 cm) was located inside the tank. The tank was filled to a height of 27.5 cm with water of approximately 23 °C, and the surface of the platform was below the surface of the water by about 2.5 cm. The circular tank was divided into four quadrants (I, II, III, and IV), and a position with equal distance from the center and edges in the middle of each quadrant was marked for the location of the platform.

Reference Memory Task. According to the procedure of Yamaguchi et al. (*41*) with some modifications, the reference memory task was carried out from day 1 to day 3 of the 15th week and included four continuous trials per day. In each training trial, the rat was put into one of four different starting positions in the water tank. The platform was located at the set position in the middle of quadrant I during the period of the reference memory task. The latency time from starting position to escape platform was recorded in each training session. The maximal latency time was assigned as 90 s. If a rat was unable to find the platform within 90 s, the rat was guided to the platform. After each trial, the rat was allowed to remain on the platform for 30 s and then was returned to its home cage, and the next trial was started after a rest of 30 s.

Working Memory (Repeated Acquisition) Task. The working memory task modified from the procedure of Yamaguchi et al. (*41*) was performed from day 4 to day 6 of the 15th week and consisted of five trials per day. The rules and apparatus for the working memory task were the same as the standard training of the reference memory task, except that the platform was located in a new quadrant each day. The rat was put into the water tank at one of the five different starting positions in each trail. The first trial of each session per day was recorded as an informative practice trial. The rat was allowed to remain on the platform for 15 s and then was returned to its home cage. The next trial was started after a rest of 60 s. The platform was located in the same position, and the practice was repeated until the finish of five trials of a day. The working memory task was designated the mean escape latency (sec) in the second to fifth trials. The escape latency (sec) in the working memory for each rat was assessed by the mean performance on three consecutive days.

Passive Avoidance Task. The passive avoidance task was carried out from day 4 to day 6 of the 15th week and with some modifications according to Yamaguchi et al. (*41*). The apparatus consisted of light and dark chambers. A door was set for separation of the two chambers. Electric wires arranged with a parallel interval of 1 cm were set through the floor of the dark chamber and delivered an electric shock (100 V, 0.3 mA, 2 s) when the door was closed. In each trial, a rat was placed into the light chamber first, and the shuttle door was opened. After the rat entered the dark chamber, the door was immediately closed and the retention time was recorded, and an inescapable electric shock (100 V, 0.3 mA, 2 s) was delivered through the electric wires. If the rat did not enter the dark chamber after 300 s, the rat was compelled to go into the dark chamber and receive electric shock. After receiving the foot shock, the rat was removed from the dark chamber. After 24 and 48 h, the passive avoidance task was repeated.

Assay for Antioxidant Enzymes and ROS. The animals were sacrificed with CO_2 asphyxia. The cortex, hippocampus, and blood were then removed. Blood were placed at room temperature for 1 h and then centrifuged at 1000*g* for 10 min to obtain serum. The cortex and hippocampus tissues were respectively homogenized in ice-cold 20 mM Tris-HCl (pH 7.4) (1:10, w/v). The cortex and hippocampus homogenates were centrifuged at 2500*g* for 30 min at 4 °C. The homogenates were collected and stored at −80 °C for the following experiments. Glutathione peroxidase (GPx) activity was determined as previously described (*42*). Briefly, 0.1 mL of homogenate was mixed with 0.8 mL of 100 mM potassium phosphate buffer (1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GR, and 1 mM GSH, pH 7.0) and incubated for 5 min at room temperature. Thereafter, the reaction was initiated after the addition of 0.1 mL of 2.5 mM H_2O_2. GPx activity was calculated by the change of the absorbance at 340 nm for 5 min. In another reaction containing 0.1 M phosphate buffer (1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG, and 0.1 mM NADPH, pH 7.0) was added 0.1 mL of homogenate for GR activity determination (*43*). The decreased absorbance at 340 nm was measured for 3 min. The catalase (CAT) activity was determined according to the method of Aebi (*44*). Fifty microliters of homogenate was mixed with 950 μL of 0.02 M H_2O_2 and incubated at room temperature for 2 min. CAT activity was calculated by the change of the absorbance at 240 nm for 3 min. The assay of SOD activity was accomplished with a commercial kit (Randox Laboratories Ltd.). The level of ROS was assayed with NBT. NBT is reduced to form blue-black formazan by ROS and dissolved with dimethyl sulfoxide (DMSO). Therefore, it demonstrates the higher absorbance level of NBT−formazan when ROS is produced in abundance. In the measurement of ROS, 100 μL of homogenates was added to 96-well plates, and 10 mg/mL of NBT was added and measured by the absorbance at 570 nm.

Assay for Fe, Ca, Mg, and Zn Concentrations. Prior to analysis, the whole hippocampus and cortex were minced finely with a razor blade, and the minced tissue was solubilized in 25% tetramethylammonium hydroxide. The cortex and hippocampus homogenates were mixed with 1 mL of HCl solution, diluted to 10 mL with distilled water, and directly nebulized in an air/acetylene flame under optimal instrumental parameters. The analyte addition technique was used for the determination. Analytical calibration solutions for Zn, Fe, Ca, and Mg were prepared by suitable dilution of stock standard solutions by $ZnCl_2$, $Fe(NO_3)_3$, $Ca(NO_3)_2$, and $Mg(NO_3)_2$ (Merck). The levels of Zn, Fe, Ca, and Mg were determined by atomic absorption spectrophotometry (AAS) in an air/acetylene flame (Z-8200 model, Hitachi Corp., Tokyo, Japan) using an aqueous standard calibration curve.

Corticosterone Assay. Blood was centrifuged (37 °C, 1000*g*) for 10 min, and the obtained serum was frozen at −20 °C until analysis. Serum corticosterone was accomplished with commercial kits (Cayman Chemical Co., Ann Arbor, MI).

High-Performance Liquid Chromatography (HPLC) Assay. Red mold rice (0.5 g) was extracted with 50 mL of ethanol at 37 °C for 24 h, and then the extracts were centrifuged (4 °C, 1000*g*) for 10 min. The filtrate was then filtered with a 0.45 μm pore size filter, and the filtered liquid was analyzed by HPLC (PU2089 plus, Jasco Co., Tokyo, Japan). Chromatographic separation was conducted on a C_{18} column (25 cm × 4.6 mm i.d., 5 μm, Discovery, Bellefonte, PA). An acetonitrile/water (80:20, v:v) solution was used as the mobile phase. The eluent was pumped at a flow rate of 0.5 mL/min. Monascin and ankaflavin were detected by ultraviolet detector (UV2075 plus, Jasco Co.) set at 231 nm (*45*).

Statistical Analysis. The above data are expressed as means ± SD. ANOVA software was used to evaluate the difference between multiple groups. If significance was observed between groups, Duncan's multiple range was used to compare the means of two specific groups. $p < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Trace Metals (Ca, Zn, Mg, and Fe) Assay. Numerous investigations have reported that Zn is an adjuvant factor during the development of the central nervous system and brain (*46*, *47*) and involved in learning, behavioral performance, and cognitive functions (*10*, *48*). A study indicates that the level of Zn is decreased in brain with aging (*9*), which appeared to affect the brain and resulted in brain dysfunctions by the Zn-deficient

Table 1. Variations of Trace Element Levels in the Hippocampus and Cortex of Zn-Deficient Rats by RMR Administration

	concentration[a] (ppm)							
	cortex				hippocampus			
group[b]	Fe	Zn	Ca	Mg	Fe	Zn	Ca	Mg
normal	46.5 ± 6.7	14.8 ± 6.4	83.1 ± 43.0	144.2 ± 30.8	55.9 ± 22.5	20.4 ± 9.1	48.4 ± 30.1	165.2 ± 24.0
ZD	56.5 ± 34.5∗	13.6 ± 4.8	141.2 ± 43.1∗	166.2 ± 31.1∗	98.0 ± 36.7∗	16.7 ± 4.4∗	141.5 ± 62.9∗	184.5 ± 18.6∗
ZC	46.2 ± 10.0#	14.8 ± 3.1	127.7 ± 32.8	151.9 ± 22.2	87.6 ± 28.5	21.4 ± 6.5#	101.6 ± 75.8#	164.8 ± 13.7#
1× RMR	38.9 ± 5.5#	14.2 ± 4.8	117.1 ± 54.1#	131.3 ± 15.6#	95.6 ± 54.5	15.3 ± 4.9	88.5 ± 58.5#	162.2 ± 13.8#
5× RMR	41.9 ± 12.6#	16.3 ± 3.7	65.4 ± 32.1#	135.9 ± 19.0#	74.5 ± 48.7#	18.1 ± 9.1	79.6 ± 23.6#	139.8 ± 37.7#
RZ	47.2 ± 17.8#	15.9 ± 6.3	90.6 ± 42.7#	149.7 ± 54.8	96.8 ± 32.3	15.5 ± 3.1	97.8 ± 57.8#	165.5 ± 11.7#
5RZ	33.0 ± 14.2#	15.2 ± 8.4	58.9 ± 29.3#	120.0 ± 55.6#	65.5 ± 11.6#	20.1 ± 8.9#	81.1 ± 27.9#	126.0 ± 25.0#

[a] Each value is expressed as mean ± SD ($n = 9$). ∗, significantly different from the normal group, $p < 0.05$. #, significantly different from the ZD group, $p < 0.05$. [b] Rats were divided into normal, Zn-deficient (ZD), Zn-compensative (ZC), 1× RMR, 5× RMR, RMR ± Zn (RZ), and 5× RMR ± Zn (5RZ) groups.

diet (*6*). The Zn concentration was markedly decreased in the hippocampus of rats fed a Zn-deficient diet (**Table 1**). However, the cortical and hippocampal levels of Zn in the 5× RMR and 5RZ groups (cortical levels = 16.3 and 15.2 ppm, respectively, and hippocampal levels = 18.1 and 20.1 ppm, respectively) returned to normal. Ca and Fe ions in high concentrations exert toxic effects and cause neuronal damage (*7*). The hippocampal levels of Fe, Ca, and Mg showed significant increase after the rats were fed a Zn-deficient diet, and these toxic concentrations induced neuronal death. The Zn dosage of the 5× RMR administered group (0.02 mg/kg of bw) was markedly lower than that of the ZC group (1.1 mg/kg of bw). However, the inhibitory effects of 5× RMR on the hippocampal concentrations of Fe, Ca, and Mg were superior to those of the ZC group. A similar decrease was seen in the 1× RMR administered group with respect to the Ca and Mg concentrations in the hippocampus. These observations indicated that certain components of RMR could mitigate the Zn deficiency-induced increase in the hippocampal concentrations of Fe, Ca, and Mg. Among these components, the major ones may be monascin and ankaflavin. In addition, the cortical levels of Fe, Ca, and Mg in the ZC, 1× RMR, and 5× RMR groups were less than those in the ZD group. A similar pattern was observed in the hippocampal levels of Fe, Ca, and Mg. However, the decreases in the cortical and hippocampal levels of Fe, Ca, and Mg in the RZ and 5RZ groups were more significant than those in the ZC, 1× RMR, and 5× RMR groups. Experimental results support the hypothesis that RMR administration inhibited the increase in the Fe, Mg, and Ca levels, thus preserving the cortical and hippocampal functions. A previous study supports the present findings of increased intracellular Ca levels in the hippocampus and cortex with the Zn-deficient diet (*7*). Administration of RMR with or without Zn inhibited the Zn deficiency-induced increase in the levels of the above-mentioned elements. This inhibition can be attributed to the Zn-induced decrease in the level of serum corticosterone (**Table 2**).

Corticosterone Assay. Inflammation always associates with oxidative stress, and Zn deficiency leads to ROS overproduction. Furthermore, corticosterone is a glucocorticoid, which is related to inflammation. Therefore, anti-inflammatory reduction in oxidative damage was demonstrated in this study. Levels of the anti-inflammatory agents monascin and ankaflavin, respectively, are 2177.3 and 3444.2 ppm in RMR, indicating that RMR may have potential in preventing inflammation of the brain due to oxidative injury caused by Zn deficiency. In addition, the corticosterone level in serum was significantly increased in the Zn-deficient (ZD) group (**Table 2**). These results are consistent with a reporter (*25*). However, no significant differences were observed in corticosterone level in the Zn compensation (ZC), 1× RMR, and 5× RMR groups compared with the ZD group, but the coadministration of RMR (1- and 5-fold dosages) plus Zn inhibited the increase in the level of serum corticosterone. Furthermore, Zn also lowers the level of serum corticosterone in Zn-deficient rats (*49*). Therefore, the inhibition effects of RMR with Zn (RZ and 5RZ administrations) on corticosterone are superior to those of the administration of ZC or RMR alone, indicating that several substances of RMR such as monascin and ankaflavin inhibited corticosterone as well as Zn. Notably, ROS and corticosterone are associated with inflammatory reaction, indicating that monascin and ankaflavin may reduce oxidative stress. Moreover, corticosterone increased the cytosolic-free Ca concentration in cultured hippocampal neurons (*22*, *23*), and an increased Ca level was observed in hippocampal slices from mice and rats deprived of Zn for 2 weeks (*25*), indicating that Zn deficiency resulted in Ca rise in the brain through the regulation of corticosterone.

Table 2. Regulative Effects of RMR on the Serum Corticosterone Level of Zn-Deficient Rats

group[a]	concentration[b] (ng/dL)
normal	1220 ± 510
ZD	2750 ± 960∗
ZC	2160 ± 790
1× RMR	2340 ± 780
5× RMR	2100 ± 840
RZ	1990 ± 880#
5RZ	1900 ± 340#

[a] Rats were divided into normal, Zn-deficient (ZD), Zn-compensative (ZC), 1× RMR, 5× RMR, RMR ± Zn (RZ), and 5× RMR ± Zn (5RZ) groups. [b] Each value is expressed as mean ± SD ($n = 9$). ∗, significantly different from the normal group, $p < 0.05$. #, significantly different from the ZD group, $p < 0.05$.

Learning-Related Behavior Evaluation. Glucocorticoids result in a reduction of hippocampal long-term potentiation (LTP) expression (*50*, *51*). A study demonstrates that there is a negative effect of glucocorticoids on spatial learning (*52*). Thus, the increase in glucocorticoid secretion from the adrenal gland might affect LTP induction by altering Ca homeostasis in the hippocampus. The increase in corticosterone secreted from the adrenal gland may influence hippocampal function via the increase in basal Ca level in Zn-deficient rats and be linked to memory impairment. The effects of RMR administration to Zn-deficient rats on the reference memory-related learning ability is shown in **Figure 2**. The ZD group always had longer escape latency from 12 trials compared with the normal group. However, the administration of RMR (1× RMR, 5× RMR, RZ, and 5RZ groups) significantly decreased the escape latency time compared with the ZD group from the results of the 5th to 12th trials. Furthermore, the escape latency of RMR administration (1× RMR, 5× RMR, RZ, and 5RZ groups) from the 9th to the 12th trials was similar to that of the normal group. These findings suggested that RMR

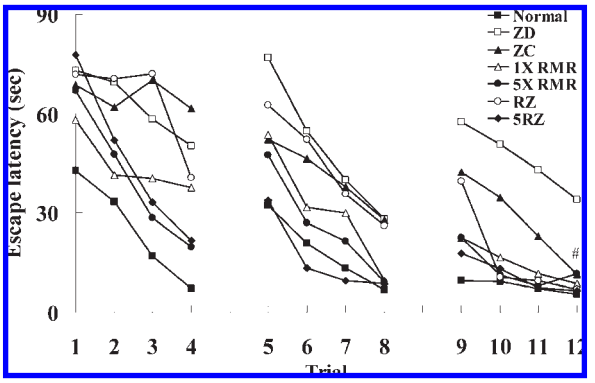

Figure 2. Protective effects of red mold rice (RMR) administration on reference memory task in Zn-deficient rats. Animals were induced Zn deficiency for 12 weeks and then were administered sample (Zn and/or RMR) for 4 consecutive weeks. Rats were divided into normal, Zn-deficient (ZD), Zn-compensative (ZC), 1× RMR, 5× RMR, RMR + Zn (RZ), and 5× RMR + Zn (5RZ) groups. Data are expressed as means ± SD ($n = 9$). #, significantly different ($p < 0.05$) versus the Zn-deficient group.

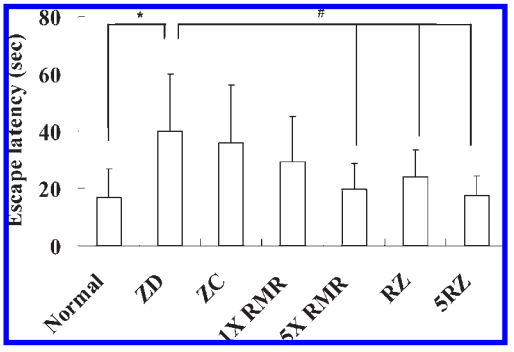

Figure 3. Protective effects of red mold rice (RMR) administration on working memory task in Zn-deficient rats. Animals were induced Zn deficiency for 12 weeks and then were administered sample (Zn and/or RMR) for 4 consecutive weeks. Rats were divided into normal, Zn-deficient (ZD), Zn-compensative (ZC), 1× RMR, 5× RMR, RMR + Zn (RZ), and 5× RMR + Zn (5RZ) groups. Data are expressed as means ± SD ($n = 9$). ∗, significantly different ($p < 0.05$) versus the normal group. #, significantly different ($p < 0.05$) versus the Zn-deficient group.

had neural protection for the Zn-deficient rats. In addition, the escape latency in the working memory task of short-term learning ability was shown in **Figure 3**. Most escape latency time was spent in Zn-deficient rat compared with the normal group ($p < 0.05$). Both 5× RMR and 5RZ groups were able to perform learning as well as the normal group, in which escape latency time decreased by 50.5 and 56.5% compared with the ZD group ($p < 0.05$). The RZ group significantly decreased escape latency by 40% compared with the ZD group, but the effect was weaker than for the 5× RMR and 5RZ groups. However, no significant amelioration effect was observed on working memory task in the ZC group compared to the ZD group. These findings demonstrated that the administration of RMR improved the ZD rats' learning the task quickly, indicating adequate learning capabilities and confirming that their short-term memory functions were promoted after RMR administration in the Zn-deficient rat model.

Passive Avoidance Task. We further evaluated the neuronal protection of RMR by the passive avoidance task in Zn-deficient rats. Rats were subjected to electric shock when they went into the dark chamber from the light chamber, and their memory ability was evaluated at 24 and 48 h after the electric shock. The time of

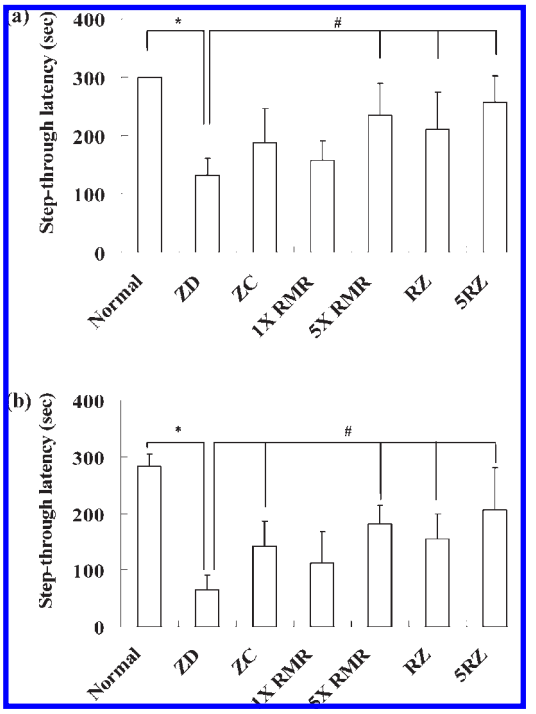

Figure 4. Protective effects of red mold rice (RMR) administration on step-through latency of multiple-trial passive-avoidance task in Zn-deficient rats. The passive avoidance task was carried out for 24 (**a**) and 48 (**b**) h after an electric shock treatment. Animals were induced Zn deficiency for 12 weeks and then were administered sample (Zn and/or RMR) for 4 consecutive weeks. Rats were divided into normal, Zn-deficient (ZD), Zn-compensative (ZC), 1× RMR, 5× RMR, RMR + Zn (RZ), and 5× RMR + Zn (5RZ) groups. Data are expressed as means ± SD ($n = 9$). ∗, significantly different ($p < 0.05$) versus the normal group. #, significantly different ($p < 0.05$) versus the Zn-deficient group.

step-through latency from light chamber to dark chamber was used as a marker to the memory and learning ability in the passive avoidance task. The result is shown in **Figures 4**. The functions of the hippocampus and cortex were damaged by Zn deficiency; thereby, the step-through latency (sec) of the ZD group was shown to be the most significant different compared with the normal group after 24 and 48 h. However, 5× RMR and 5RZ administration, respectively, increased the time of step-through latency by 44 and 53.8% compared with the ZD group after 24 h. After 48 h, the results clearly indicated that Zn-deficient rats still spent a shorter time in the light chamber than normal rats. However, the administration of RMR (1× RMR, 5× RMR, RZ, and 5RZ groups) was able to ensure staying in the light chamber for a longer time compared with the ZD group. These results demonstrated that RMR improved memory ability for Zn-deficient rats.

Effects of RMR-Promoted Antioxidase Activity on the Cortex and Hippocampus of Zn-Deficient Rats. Notably, Zn deficiency leads to ROS overproduction and results in brain oxidative damage, adversely affecting the functions of the cortex and hippocampus, including memory and learning ability. Some specific antioxidative enzymes are inhibited when free radical over-expression can break bioactive molecules such as lipids, protein, and DNA are overproduced (*53*). The report indicates indicate that antioxidants reduced the reactive oxygen species (ROS) level (*54*). An imbalance between the antioxidant system and ROS level results in oxidative stress. This study supports previous findings that Zn deficiency induces oxidative stress and increases malondialdehyde synthesis in the cortex and hippocampus, which further impairs memory and learning activity (*55*, *56*).

Table 3. Antioxidase Activities of Cortex in Zn-Deficient Rats by RMR Administration[a]

group[b]	CAT (nmol of H_2O_2/min/mg of protein)	GR (nmol of NADPH/min/mg of protein)	GPx (nmol of NADPH/min/mg of protein)	SOD (U/mg of protein)	ROS (%)
normal	14.5 ± 9.3	3722.3 ± 307.8	39.2 ± 2.3	5.8 ± 0.5	100.0 ± 0.0
ZD	9.7 ± 2.6*	1249.5 ± 603.3*	19.6 ± 5.1*	5.3 ± 0.4	166.5 ± 40.9*
ZC	11.8 ± 2.0	1815.8 ± 806.1	29.2 ± 4.8#	5.2 ± 0.2	149.3 ± 22.3
1× RMR	12.7 ± 4.8	2269.8 ± 203.6#	40.0 ± 4.9#	5.5 ± 0.2	136.5 ± 45.7
5× RMR	15.4 ± 3.3	3694.8 ± 213.8#	58.8 ± 11.7#	5.7 ± 0.8	131.7 ± 16.1
RZ	16.6 ± 3.8	3221.8 ± 120.7#	39.1 ± 14.5#	5.6 ± 0.4	126.2 ± 32.7#
5RZ	15.7 ± 2.2	3842.4 ± 451.6#	97.4 ± 38.4#	6.0 ± 0.7	97.6 ± 11.7#

[a] Each value is expressed as mean ± SD ($n = 9$). *, significantly different from the normal group, $p < 0.05$. #, significantly different from the ZD group, $p < 0.05$. CAT, catalase; GR, glutathione reductase; GPx, glutathione peroxidase; SOD, superoxide dismutase; ROS, reactive oxygen species. [b] Rats were divided into normal, Zn-deficient (ZD), Zn-compensative (ZC), 1× RMR, 5× RMR, RMR ± Zn (RZ), and 5× RMR ± Zn (5RZ) groups.

Table 4. Antioxidase Activities of Hippocampus in Zn-Deficient Rats by RMR Administration[a]

group[b]	CAT (nmol of H_2O_2/min/mg of protein)	GR (nmol of NADPH/min/mg of protein)	GPx (nmol of NADPH/min/mg of protein)	SOD (U/mg of protein)	ROS (%)
normal	14.9 ± 5.0	3555.1 ± 300.0	71.4 ± 26.1	5.5 ± 0.8	100.0 ± 0.0
ZD	12.3 ± 3.7	1940.0 ± 158.1*	0.6 ± 0.0*	4.9 ± 0.4	175.0 ± 10.5*
ZC	16.4 ± 1.1	2463.5 ± 563.8#	19.9 ± 2.5#	5.5 ± 0.6	156.5 ± 43.8
1× RMR	15.2 ± 5.3	2821.7 ± 783.3#	18.7 ± 2.3#	5.9 ± 0.5	89.1 ± 31.7#
5× RMR	23.1 ± 1.9#	4687.7 ± 779.1#	46.2 ± 18.7#	6.0 ± 1.5	75.6 ± 13.4#
RZ	16.4 ± 3.3	3744.8 ± 398.2#	49.5 ± 10.1#	5.8 ± 0.3	86.8 ± 28.5#
5RZ	37.3 ± 1.6#	4990.1 ± 313.2#	78.4 ± 21.3#	7.0 ± 2.6#	64.7 ± 29.4#

[a] Each value is expressed as mean ± SD ($n = 9$). *, significantly different from the normal group, $p < 0.05$. #, significantly different from the ZD group, $p < 0.05$. CAT, catalase; GR, glutathione reductase; GPx, glutathione peroxidase; SOD, superoxide dismutase; ROS, reactive oxygen species. [b] Rats were divided into normal, Zn-deficient (ZD), Zn-compensative (ZC), 1× RMR, 5× RMR, RMR ± Zn (RZ), and 5× RMR ± Zn (5RZ) groups.

Table 5. Correlation between Cortex Antioxidant Properties and Memory Tasks by Administration of Various Samples

correlation[a]	CAT	GR	GPx	SOD	ROS	reference memory task	working memory task	passive avoidance task	corticosterone
CAT		0.903	0.645	0.773	−0.899	−0.522	−0.875	0.681	−0.538
GR			0.727	0.916	−0.900	−0.778	−0.991	0.864	−0.654
GPx				0.834	−0.719	−0.562	−0.709	0.502	−0.260
SOD					−0.934	−0.858	−0.934	0.762	−0.582
ROS						−0.792	−0.931	−0.896	0.842
reference memory task							0.846	0.727	0.626
working memory task								0.882	0.713
passive avoidance task									−0.865
corticosterone									

[a] CAT, catalase; GR, glutathione reductase; GPx, glutathione peroxidase; SOD, superoxide dismutase; ROS, reactive oxygen species.

Table 6. Correlation between Hippocampal Antioxidant Properties and Memory Tasks by Administration of Various Samples

correlation[a]	CAT	GR	GPx	SOD	ROS	reference memory task	working memory task	passive avoidance task	corticosterone
CAT		0.812	0.654	0.921	−0.618	−0.398	−0.575	0.890	−0.134
GR			0.847	0.839	−0.880	−0.646	−0.891	0.676	−0.372
GPx				0.694	−0.758	−0.722	−0.943	0.917	−0.762
SOD					−0.818	−0.479	−0.676	0.437	−0.296
ROS						0.726	0.881	−0.581	0.480
reference memory task							0.846	0.727	0.626
working memory task								0.882	0.713
passive avoidance task									−0.865
corticosterone									

[a] CAT, catalase; GR, glutathione reductase; GPx, glutathione peroxidase; SOD, superoxide dismutase; ROS, reactive oxygen species.

As seen in **Table 3**, the CAT, GR, and GPx activities in the cortex were decreased in the ZD group, thereby increasing the ROS level in the cortex. However, an increase of only cortical GPx activity was seen in the ZC group, indicating that Zn compensation partially restored the activity of antioxidant enzymes. Although the levels of Zn in the 1× RMR (dietary Zn dosage was 0.004 mg/kg of bw) and 5× RMR (dietary Zn dosage was 0.02 mg/kg of bw) groups were lower than those in the ZC group (1.1 mg/kg of bw), the increase in the activity of antioxidant was higher in the former than in the latter. The presence of a small amount of Zn in RMR (1× and 5× RMR), which led to a higher activity of the antioxidant enzymes in the RMR-administered groups than in ZC group, indicating that the extent of inhibition of oxidative damage by RMR is similar to that by Zn. Moreover, the activity levels of antioxidases in the RZ and 5RZ groups were the same as in the control group. Activity levels of hippocampal antioxidases were similar to those in the cortical antioxidases (**Table 4**). CAT, GR, and GPx activities were greater in the groups administered 5× RMR and 5RZ than in the control group. Thus, these administrations brought about a reduction in the levels of ROS

production in the cortex and hippocampus against oxidative damage caused by Zn deficiency (**Tables 3** and **4**). However, SOD activity was not significantly increased by administration of RMR with or without Zn. This could be because Zn was needed for transcription of SOD; therefore, long-term Zn deficiency led to a decrease of SOD activity. Hence, administration of RMR with or without Zn did not significantly increase SOD activity. These results suggested that the increase in the antioxidase activity in the cortex and hippocampus brought about by RMR compensates for Zn deficiency, and this is because of monascin, a major component of RMR that has antioxidative and anti-inflammatory effects (*31*−*33*).

The brain functions are affected by feeding the Zn-deficient diet, and learning behavior during normal development and aging was also studied in association with Zn of the brain. Zn is necessary for the formation and is stored in synaptic vesicles in the hippocampal formation and cerebral cortex. Zn in the cerebral cortex and hippocampal formation may function for learning. These results demonstrated that a proper Zn supply to the mature brain is necessary for maintenance of learning ability. On the other hand, there is a negative correlation between the antioxidase activity (CAT, GR, GPx, and SOD) and ROS (**Tables 5** and **6**). The activation of antioxidase always showed a negative correlation with corticosterone level, indicating that antioxidase could affect corticosterone level and indirectly affected Ca, Mg, and Fe concentrations in the cortex and hippocampus. In addition, the activation of antioxidase had negative and positive correlations on reference memory task, working memory task, and passive avoidance task, respectively (**Tables 5** and **6**). These findings demonstrated that the Ca and corticosterone both are related to memory and learning ability, as well as the antioxidase activities of the cortex and hippocampus.

In conclusion, we observed increased activity of the antioxidases such as CAT, GR, GPx, and SOD by RMR administered to rats deficient in Zn for 12 weeks. These antioxidants markedly decreased the ROS level and helped protect neuronal function (**Tables 3** and **4**). Glucocorticoids increase the ROS levels, which induces oxidative stress (*56*, *57*), thus resulting in hippocampal neuronal death. The results of this study indicate that administration of RMR led to a decrease in oxidative stress by reducing the level of glucocorticoids (corticosterone) (**Table 2**). Recently, some studies have reported that several natural antioxidants have neural protection (*15*−*19*). On the other hand, the concentration of Zn in the brain is not significantly decreased by 12 weeks of Zn deficiency, whereas the level of Zn in the hippocampus is clearly lowered (*6*). These findings demonstrate that the hippocampus is responsive to Zn deficiency and that it is associated with memory and learning ability. The cognitive memory impairment was reversed, including reference memory task, working memory task, and passive avoidance task by RMR administration, and various dosages of RMR plus Zn significantly improved the cognitive performance of previously impaired rats more efficiently than the Zn-compensative group. Furthermore, the administration of lovastatin (monacolin K) was not beneficial in the memory and learning ability of Zn-deficient rats (data not shown).

Zn deficiency results in an increase in the serum corticosterone levels, leading to inflammation and oxidative stress, which causes brain dysfunction (*21*, *31*−*34*). Because both monascin and ankaflavin exhibit anti-inflammatory activity, they can effectively reverse cognitive impairment in Zn-deficient rats. On the basis of our findings that RMR containing monascin and ankaflavin can prevent hippocampal and cortical damage caused by Zn deficiency, RMR can be considered as a possible functional food for the prevention or cure of neural diseases associated with Zn deficiency.

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