

# Learning and memory enhancing effects of anthocyanin in black rice extract on cerebral ischaemia in mice

Napapan Kangwan, Komsak Pintha, Watcharaporn Preedapirom, Payungsak Tantipai boonwong, Orada Chumphukam, Maitree Suttajit\*

School of Medical Sciences, University of Phayao, Phayao 56000 Thailand

\*Corresponding author, e-mail: maitree.suttajit@gmail.com

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**ABSTRACT:** The protective effect of anthocyanins extracted from black rice against learning and memory impairment was investigated in mice. In a Morris water maze test, mice with transient cerebral ischaemia required a longer time to find the hidden platform than the sham-operated controls. Daily treatment of cerebral-ischaemia mice with black rice extracts (125, 250, and 500 mg/kg body weight) significantly decreased the escape latency and increased the percentage crossing the former platform quadrant in the probe trial. Furthermore, less lipid oxidation was found in the brains of cerebral-ischaemia mice fed with black rice extract. Overall, black rice extract supplementation could be advantageous in the protection against oxidative stress and oxidative damage in the brains of cerebral-ischaemia mice.

**KEYWORDS:** black rice, anthocyanins, lipid peroxidation

## INTRODUCTION

Oxidative stress is characterized by a high production of free radicals that the endogenous antioxidative defence system is unable to counterbalance by. This imbalance condition can cause severe damage to cellular macromolecules. It is widely accepted that increasing oxidative stress level is associated with several age-related degenerative diseases such as neurodegeneration and cognitive impairment<sup>1–4</sup>. In the absence of an effective therapy, brain ageing can increase the susceptibility to Alzheimer's disease, which is the most common cause of dementia<sup>5</sup>. From the report of the World Health Organization, there were nearly 36 million people who suffered from dementia in 2012 and this number will possibly be close to triple in 2050<sup>6</sup>.

Although there is not yet an effective therapy to cure dementia, the risk is however preventable. Intensive studies on preventive approaches have been performed to slow down or to delay the onset of diseases<sup>7–10</sup>. Dietary supplementation has been proposed as an effective and safe preventive approach. Daily consumption of coloured vegetables, fruits, and whole grains are not only energy sources but also promote good health due to their high content of biologically active phytochemicals such as polyphenol<sup>11–14</sup>. In plant themselves, phenolic

compounds are secondary metabolites mainly produced to protect plants from stresses such as photooxidation, reactive oxygen species, and UV light. Hence, to mimic the plant defensive mechanism, polyphenolic compounds have been used as potent antioxidants that can combat oxidative stress as well as maintain a balance between oxidants and antioxidants.

Rice grains (*Oryza sativa*) are the major dietary carbohydrate source in Thailand and are the main agricultural product exported to other countries. Pigmented rice, especially black rice, is known for its high antioxidant activity and high phenolic content. Anthocyanins are responsible for the black colour in pericarps. Black rice samples contain anthocyanins 18–26% of total antioxidant and the major anthocyanin is cyanidin-3-glucoside (58–95%)<sup>14</sup>. The positive correlation between a high level of antioxidant activity and a high content of anthocyanin in black rice triggered our interest. These phytochemicals are not only able to reduce oxidative stress but also to modulate cognitive function, especially enhancing memory as well as performing preventive roles in age-related declines of neural function. Thus the health-promoting effect of black rice crude extract was studied. We investigated whether an ethanolic extract from black rice could protect learning and memory impairment in cerebral ischaemia-

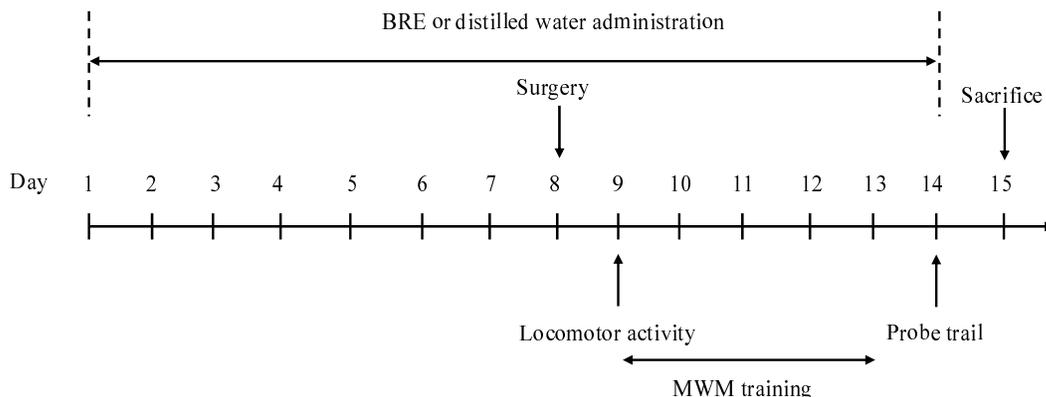


Fig. 1 Experimental design to study the effect of anthocyanin in BRE on cerebral ischaemia in mice model.

induced mice by improving antioxidative status. In addition, to prove that anthocyanins can decrease oxidative damage by lipid peroxidation in brain tissue, the whole brains of mice fed with black rice extract (BRE) were assayed for their malondialdehyde (MDA) levels.

## MATERIALS AND METHODS

### Preparation of black rice crude extracts

Thai black glutinous rice (*O. sativa*) was obtained from a local market in Phayao province. Dehusked rice grains were ground to a fine powder. Then 250 g of black rice sample powder was soaked in 1 l of 70% (v/v) ethanol in a shaker (150 rpm) for 12 h at room temperature. The ethanol extraction was performed twice under the same condition. Subsequently, the extracts were filtered through a Whatman No. 1 filter paper. The filtrates were combined and evaporated using a vacuum rotary evaporator at 70 °C (Buchi Rotavapor R-200, Flawil, Switzerland). The concentrated aqueous portion was lyophilized into a powder. The dark amorphous powder was then stored at -20 °C until use.

### Evaluation of phytochemical contents and antioxidant activity

Total phenolic content (TPC) of the BRE was determined using the Folin-Ciocalteu assay with some modifications as described in Saikia et al<sup>15</sup>. Quantification was expressed as mg of gallic acid equivalent (GAE) per gram BRE. Total flavonoid content (TFC) was also measured according to Saikia et al<sup>15</sup> using aluminium trichloride hexahydrate colorimetric method. The results are expressed as milligram catechin equivalent (CE) per gram sample. The estimation of total anthocyanin content (TAC) was

performed using the pH differential method as described by Sun et al<sup>16</sup>. The TAC was calculated and expressed as milligrams per litre extract (mg/l). Three replicates were performed for each sample. The composition of BRE was determined using reversed phase high performance liquid chromatography<sup>17</sup>. The free radical scavenging activity of BRE was measured by two methods, using the stable 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH<sup>•</sup>) assay<sup>18</sup> and reactive oxygen species (ROS) inhibition assay<sup>19</sup>. The results were shown as 50% of inhibition concentration (IC<sub>50</sub>) value.

### Animal grouping, diet, and experiment schedule

Male ICR mice (4 weeks old, 25–30 g body weight) were obtained from the National Laboratory Animal Centre, Mahidol University (Bangkok, Thailand). Mice were housed for a week under control conditions before initiating the experiments. They were given free access to food and water in control housing conditions including a constant temperature at 25 ± 2 °C, relative humidity at 60 ± 10% and a daily lighting for 12 h. After the acclimation period, the mice were randomly divided into five groups ( $n = 8$  per group) including (1) sham-operated control mice, (2) cerebral-ischaemic mice (CI-mice), (3) CI-mice plus BRE treatment at 125 mg/kg body weight (BW), (4) CI-mice plus BRE treatment at 250 mg/kg BW, and (5) CI-mice plus BRE treatment at 500 mg/kg BW. BRE doses were selected as referred to in a previous study<sup>20</sup>. The experiment schedule is shown in Fig. 1. Mice received either distilled water or BRE for 15 days administered orally. The cerebral-ischaemic process was performed on day 8. The Morris water maze (MWM) task was used to test the memory and learning ability of mice in each group from days 9–14 before

being sacrificed. All experiments were carried out under the approval of the Institutional Animal Ethics Committee, Naresuan University, Thailand.

### Transient cerebral ischaemia

Transient cerebral ischaemia (CI) was induced in mice by bilateral common carotid occlusion as described in Xu et al<sup>11</sup>, with slight modifications. In brief, mice were anaesthetized by intraperitoneal injection (40 mg/kg BW). The bilateral common carotid arteries were exposed and occluded by artery clips for 20 min. Blood was then withdrawn from the cutting off tip of the tail while the arteries were clamped. The cerebral blood flow was then restored by removing the clips and the skin incision was closed. The mice were then kept in a room with the temperature maintained at 25 °C with a lamp on. Mice in the sham-operated group were subjected to the same process, except that there were no carotid clamping or bleeding.

### Locomotor activity and Morris water maze

To establish whether mice were affected by the cerebral ischaemia, their locomotor activity was tested 24 h after the operation. A spontaneous movement of each mouse by ambulation and rearing was monitored in an open-field and was recorded for 5 min. After that, spatial learning and memory test was carried out using a Morris water maze (MWM) as described in Morris<sup>21</sup> and Xu et al<sup>11</sup>, with modifications. The test was performed in a soundproof room surrounded by various visual cues. The apparatus in the room consisted of a black circular pool (70 cm in diameter and 30 cm in height) divided into four quadrants. The pool was filled with cloudy water (starch solution) to a depth of 23 cm and the water temperature was maintained at approximately at 25 °C. A 10 cm diameter platform was fixed at the centre of one quadrant and was hidden 1 cm below the water surface. The mice were subjected to a three-trial daily training for five consecutive days (days 9–13). The experiment was started by gently placing each mouse in the water facing against the wall of the pool from one of the three starting points in a random manner. The mouse was allowed to swim until it found and climbed onto the platform and the escape latency or time to reach the platform was recorded. If it was unable to reach the platform within 60 s, the trial was stopped and the mouse was placed on the platform. Each mouse was allowed to remain on the platform for 10 s before being removed from the pool. On day 14 for probe trial, the platform was removed. The mouse was again

**Table 1** Phytochemical components in a BRE.

Phytochemical components <sup>†</sup>	mg/g extract
TPC (total phenolic content)	104.3 ± 3.4*
TFC (total flavonoid content)	21.1 ± 2.9*
TAC (total anthocyanin content)	11.83 ± 0.61*
Cyanidin-3-O-glucoside	4.59
Peonidin-3-O-glucoside	1.94

The % yield of BRE was 18.7.

<sup>†</sup> TPC as gallic acid equivalent; TFC, as catechin equivalent; TAC, as cyanidin-3-O-glucoside equivalent.

\* Mean ± SD.

allowed to swim while the crossing time from the former platform quadrant and the crossing time of all quadrants were recorded for 1 min. The ratio of these two crossing times is shown as the percentage of crossing the quadrant of the former platform, which is a representative of spatial memory.

### Lipid peroxidation assay in brain homogenates

On day 15, the mice were sacrificed by neck dislocation and the brain tissues were collected for lipid peroxidation assay. A lipid peroxidation is related to the formation of a reactive carbonyl product, MDA, which derives from the oxidation of polyunsaturated lipids. To monitor the lipid peroxidation, the level of MDA was measured using thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical Company, USA). The whole mice brain was dissected and homogenized in ice-cold RIPA buffer supplemented with protease inhibitors. The homogenate was centrifuged at 1600g for 10 min at 4 °C. The supernatant was collected to determine the content of MDA.

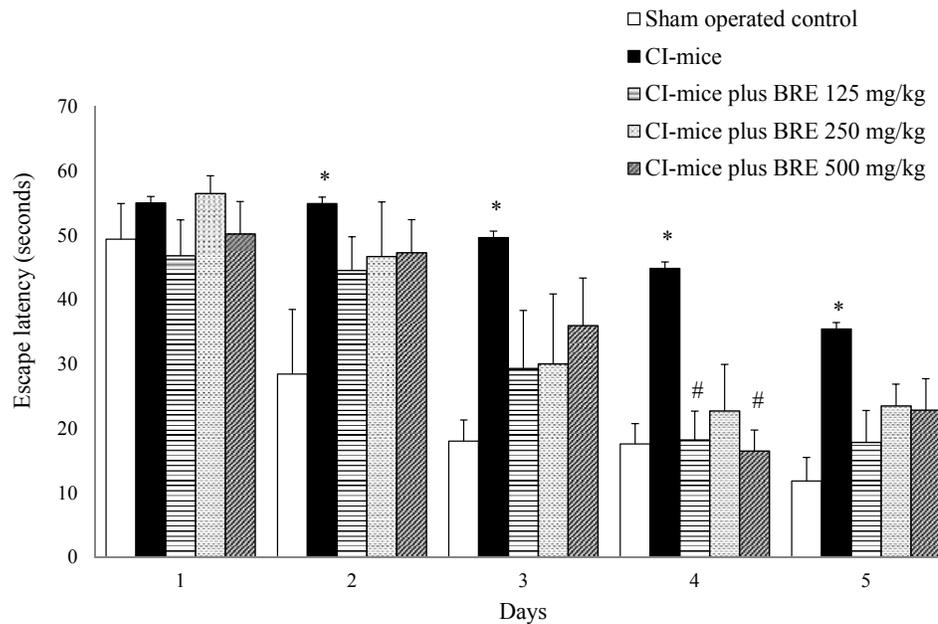
### Statistical analysis

All data are presented as mean ± SEM. The statistically significant differences compared between groups were calculated by one-way ANOVA followed by an LSD post-hoc test. The differences were considered significant at the level of  $p < 0.05$ .

## RESULTS

### Phytochemical contents and antioxidant activity of ethanolic extract of black rice

The TPC and TFC were 104.3 ± 3.4 mg GAE/g extract and 21.1 ± 2.9 mg CE/g extract, respectively (Table 1). The TAC, estimated using the pH differential method, was 11.83 ± 0.61 mg/g extract. The two major anthocyanins were found to be cyanidin-3-O-glucoside (predominant) and



**Fig. 2** Effect of BRE on the escape latency in MWM after cerebral ischaemia. Mice were pretreated with distilled water in sham-operated control and in cerebral ischaemia group (CI-mice), and with BRE at doses of 125, 250, and 500 mg/kg BW once daily for 15 days. The escape latency was evaluated on 5 consecutive days. Data represent the mean of three trials ( $n = 8$ ). \*:  $p < 0.05$  versus sham-operated control; #:  $p < 0.05$  versus cerebral ischaemia group (one-way ANOVA-LSD post hoc).

peonidin-3-O-glucoside as shown in an HPLC chromatogram (data not shown). The antioxidant capacity of BRE was evaluated by measuring its DPPH and ROS scavenging activity. The antioxidant effect is proportional to the decolourization of the stable organic radicals. The  $IC_{50}$  value of BRE, measured by the former method, was  $45.7 \pm 2.5 \mu\text{g/ml}$  and for the latter method was  $80.2 \pm 3.1 \mu\text{g/ml}$ .

#### Locomotor activity of mice

Locomotor activity of mice, 24 h after the cerebral ischaemia operation, was monitored prior to memory evaluation. The control group was the sham-operated mice. The experimental groups were divided into the CI-mice and CI-mice with BRE daily treatment at different doses. It was found that the cerebral ischaemia did not affect the ambulatory or rearing activity.

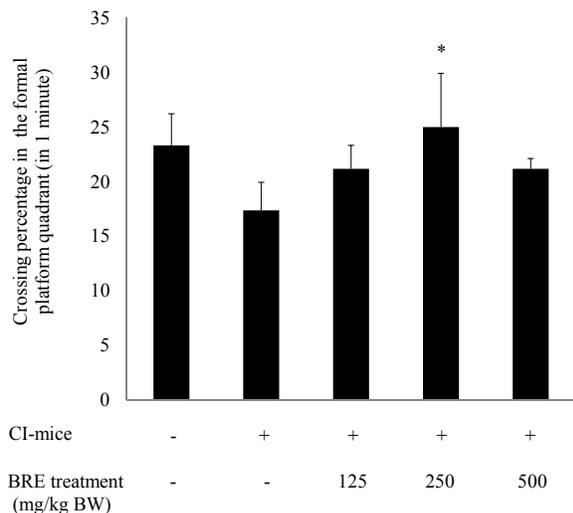
#### Learning and memory assessment

The learning and memory assessment was performed using MWM test where the mice from each group were given training sessions before a probe trial. The escape latency time was recorded and the average time within the group computed. The data revealed that, during the training trials, the

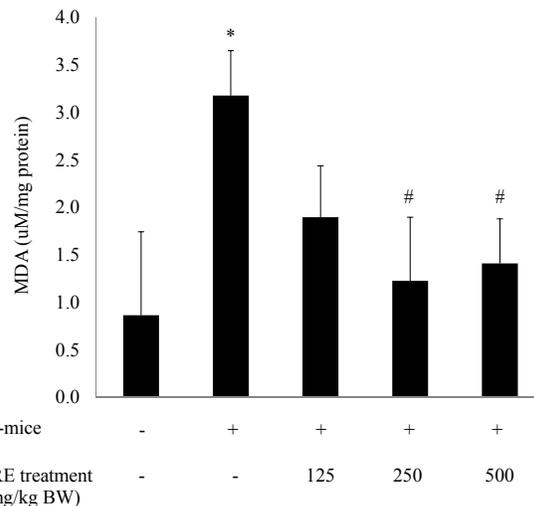
CI-mice required longer time to find the hidden platform than the sham-operated control group. Interestingly, the group of CI-mice that were supplemented with different doses of BRE (125, 250, and 500 mg/kg BW) were significantly faster in finding the platform on day 4 of the training trial (Fig. 2). During five days training, the CI-mice plus BRE treatment showed no significantly different performance when compared to the sham-operated control group. To measure the spatial memory, the probe trial was performed on day 14. Two factors were recorded: the time that mice used to cross the quadrant of the former platform and the total time of crossing all quadrants in the water maze. The ratio between these two represents the time that the mice spent searching for the previous platform location and was calculated as the percentage of crossing the quadrant of the former platform (Fig. 3). The percentage of crossing the former platform location increased significantly in the CI-mice group treated with BRE, especially with BRE at 250 mg/kg BW.

#### Lipid peroxidation in brain tissue

To investigate the level of lipid peroxidation in the brain, the MDA content was determined. It was



**Fig. 3** Effect of BRE on the crossing percentage in the former platform quadrant in probe trial of MWM. Mice were pretreated with distilled water in sham-operated control (CI-mice with negative sign) and in cerebral ischaemia group (CI-mice with positive sign), and with BRE at doses of 125, 250, and 500 mg/kg BW once daily for 15 days. The crossing percentage in the former platform quadrant was measured after 24 h training trail. \*:  $p < 0.05$  versus cerebral ischaemia group (one-way ANOVA-LSD post hoc).



**Fig. 4** Effect of BRE on the level of MDA product in brain. Mice were pretreated with distilled water in sham-operated control (CI-mice with negative sign) and in cerebral ischaemia group (CI-mice with positive sign), and with BRE treatment at doses of 125, 250, and 500 mg/kg BW. Data represent the mean  $\pm$  SEM, ( $n = 8$ ). \*:  $p < 0.05$  versus sham-operated control; #:  $p < 0.05$  versus cerebral ischaemia group (one-way ANOVA-LSD post hoc).

used as a marker of oxidative stress. As shown in Fig. 4, the level of MDA increased in the brain of CI-mice, which was higher than that of the sham-operated control group and the BRE treated group. A significant decrease in the level of MDA was found in the brain of mice treated with high doses of BRE (250 and 500 mg/kg BW). It implies that the BRE administration was able to alter the concentration of MDA in the brain of mice after cerebral ischaemia.

**DISCUSSION**

Numerous studies have shown that various fruits and vegetables contain high amounts of polyphenolic compounds which display high total antioxidant activity. Anthocyanins are responsible for a dark-purple colour and have been reported as potent sources of phytonutrients that exhibit excellent antioxidant capacity<sup>20,22-24</sup>. Pigmented rice or black rice has been reported to have a very high content of total anthocyanins mainly cyanidin-3-glucoside and peonidin-3-glucoside<sup>14,22-24</sup>, which corresponds to our investigation. Recent studies show that anthocyanins play an important role in neuroprotection by reducing oxidative stress, pre-

serving cognitive performance as well as limiting or reversing the deleterious effects of brain ageing. As a daily carbohydrate source for Southeast Asia, we had focused on the investigation of the protective effect of anthocyanin-rich extract from black rice on spatial memory as well as its potential impact against lipid peroxidation in memory deficit mice that have been induced by transient middle cerebral artery occlusion.

Cerebral ischaemia condition results from the blockage of blood vessels and thus decreases the blood supply (as well as oxygen and glucose) to the brain or parts of the brain. It is accompanied by an imbalance of energy metabolism which is promptly followed by the dysfunction of energy dependent ion pumps, depolarization of the neurons, and the generation of reactive oxygen species (ROS). ROS are highly reactive molecules and their accumulation can cause a severe damage such as DNA mutation and lipid and protein dysfunction<sup>2-5</sup>. This condition can cause severe damage to the brain and contributes to numerous neurological illnesses such as Alzheimer’s disease and stroke.

A mouse-brain injury model with transient cerebral ischaemia-induced memory deficit is important to the evaluation of the functional consequences of

cerebral ischaemia. This is also vital to the analysis of the potential therapeutic effect of drugs and food supplements on behavioural testing. Several studies also support the notion that transient cerebral ischaemia could induce learning and memory deficit and neurodegeneration<sup>2-4</sup>. The reason behind this induction is that the damaged brain region was mainly the hippocampus. The hippocampus is a brain area highly associated with spatial learning and memory. This area is also shown to display the most characteristic neuropathological damage in Alzheimer's disease. That is to say, the neuronal injury in the hippocampus may have a positive correlation to the impairment of spatial learning<sup>3</sup>.

In the present study, the normal behaviour of all mice were checked by locomotor activity after surgery. We found that the cerebral ischaemia affected neither general behaviour nor locomotor activity. In addition, the cerebral ischaemia did not affect the swimming ability. Mice were then started training in the MWM after 24 h surgery (1 day) because we need baseline of MWM on the first day to compare with other days of training. If mice were trained with MWM before surgery, that would not be the baseline study. Furthermore, the results in this study demonstrate that black rice consumption may improve spatial memory in the CI-induced mice using MWM test. The occlusion of bilateral common carotid arteries for 20 min plus hypertension affected neither general behaviour nor locomotor activity<sup>11</sup>. In this study, mice were performed a transient cerebral ischaemia, produced by bilateral common carotid occlusion with removal of 0.3 ml of blood from the tip of the tail. This method produced transient cerebral ischaemia, which affected neither general behaviour nor locomotor activity. However, it produced a deficit in spatial cognition (MWM task). These observations concur with previous reports that transient cerebral ischaemia impairs behavioural performance in a learning and memory tasks in gerbil and mice. It induced however memory deficit as the CI-mice required longer escape latency than the sham-operated control group during the training trials of MWM. The CI-mice that were treated with BRE at 125, 250, and 500 mg/kg BW significantly shortened the escape latency on day 4 of training trial of MWM (Fig. 2). In addition, the probe trial was tested according to the fact that a mouse with good spatial memory and learning ability should spend much time in the target quadrant where the platform was previously located. The crossing percentage in the previous platform quadrant in the CI-mice was insignificantly

lower than the sham-operated control group but significantly higher in the BRE-treatment, especially at 250 mg/kg BW group during the probe trial of MWM (Fig. 3). This indicates that the supplementation with BRE-anthocyanins resulted in memory-enhancing effects.

Further study on in vivo function of BRE on neuroprotection was demonstrated. As described above, the ROS was induced in brain injury through cerebral ischaemia. The process then induced the generation of lipid peroxides that were degraded to reactive aldehyde products such as MDA. The result of this present study demonstrated that oral intake of BRE slightly inhibited the elevation of MDA levels in CI-mice. Anthocyanin-rich extract has been described for its protective effect against oxidative stress by reducing ROS and increasing antioxidant enzyme activities both in vitro and in vivo. This assumption correlates with the finding that polyphenolic compounds can cross the blood brain barrier. Anthocyanins are delivered and distributed in various brain regions, particularly in the cortex and hippocampus to perform a protection as it is well known that the hippocampus is vulnerable to cerebral ischaemia and related with memory deficit<sup>7</sup>.

To summarize, the anthocyanins in black rice could improve learning and memory as well as reduce the level of lipid peroxidation damage to the brain of CI-mice. Hence black rice may be beneficial for slowing down the memory loss induced or caused by oxidative stress. The protective mechanism of anthocyanins from black rice needs to be studied more intensively. Moreover, further studies on human subjects who are at risk of or at early stage of cognitive problem are required using long-term dietary approach.

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