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# ผลของกรดโรสมารินิกต่อความบกพร่องของพฤติกรรมทางระบบประสาทที่ถูกเหนี่ยวนำด้วยแอล-เมไธโอนีนในหนูแรทโตเต็มวัย

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มหาวิทยาลัยขอนแก่น

# Beneficial Effect of Rosmarinic Acid on Neurobehavioral Deficits Induced by L-methionine in Adult Rats

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## บทคัดย่อ

**หลักการและวัตถุประสงค์:** การรับประทานอาหารที่มีแอล-เมไธโอนีนที่เป็นกรดอะมิโนจำเป็นในปริมาณมากส่งเสริมให้เกิดความเครียดออกซิเดชัน เนื่องจากการเพิ่มขึ้นของระดับโฮโมซิสเทอีน ซึ่งมีผลทำลายเซลล์ประสาทและนำไปสู่ภาวะความจำบกพร่อง กรดโรสมารินิกเป็นสารประกอบฟีโนลิก พบได้ในโรสแมรี่ ไธม์ และกะเพรา การศึกษาที่ผ่านมาพบว่ากรดโรสมารินิกมีฤทธิ์ในการกระตุ้นการเรียนรู้และความจำ ดังนั้นการศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลป้องกันระบบประสาทของกรดโรสมารินิกต่อภาวะความจำบกพร่องที่เกิดจากการเหนี่ยวนำด้วยแอล-เมไธโอนีน

**วิธีการศึกษา:** หนูแรทเพศผู้สายพันธุ์ Sprague Dawley ถูกแบ่งออกเป็น 6 กลุ่ม ได้แก่ กลุ่มควบคุม แอล-เมไธโอนีน (1.7 ก./กก.) กรดโรสมารินิก (10 และ 30 มก./กก.) และ แอล-เมไธโอนีน + กรดโรสมารินิก (10 และ 30 มก./กก.) หนูทดลองได้รับการป้อนสาร 1 ครั้ง/วัน เป็นเวลา 28 วัน ในระหว่างการให้สาร หนูจะถูกชั่งน้ำหนักและนำมาวิเคราะห์ ก่อนและหลังจากสิ้นสุดการให้สาร หนูทดลองได้ถูกทดสอบความจำเกี่ยวกับพื้นที่และความจำโดยรู้จำโดยวิธี novel object location (NOL) และ novel object recognition (NOR) ตามลำดับ

**ผลการศึกษา:** ผลการทดลองแสดงให้เห็นว่ากรดโรสมารินิกและแอล-เมไธโอนีนไม่มีผลด้านลบต่อน้ำหนักตัวและความสามารถในการเคลื่อนที่ ขณะที่การทดสอบ NOL และ NOR พบว่ากลุ่มควบคุม กรดโรสมารินิก (10 และ 30 มก./กก.) และ แอล-เมไธโอนีน + กรดโรสมารินิก (10 และ 30 มก./กก.) สามารถแยกความแตกต่างระหว่างวัตถุในตำแหน่งใหม่และตำแหน่งเก่าและสามารถแยกความแตกต่างระหว่างวัตถุใหม่และวัตถุเก่าได้อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มแอล-เมไธโอนีน

**สรุป:** การศึกษาในครั้งนี้แสดงว่ากรดโรสมารินิกมีฤทธิ์ป้องกันความจำเสื่อมที่เกิดจากเหนี่ยวนำด้วยแอล-เมไธโอนีน

**คำสำคัญ:** กรดโรสมารินิก, แอล-เมไธโอนีน, ความจำเกี่ยวกับพื้นที่, ความจำโดยรู้จำ

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## Abstract

**Background and Objective:** Food consumption that contains large quantities of L-methionine (L-met), an essential amino acid can cause oxidative stress by increasing levels of homocysteine. High levels of homocysteine contribute to neuronal cell damage, which can lead to memory impairment. Rosmarinic acid (RA) is a phenolic compound widely distributed in rosemary, thyme and basil. Several studies revealed that rosmarinic acid has the ability to enhance learning and memory function. Therefore, this study was designed to determine the neuroprotective effect of rosmarinic acid against L-methionine-induced memory impairments.

**Methods:** Male Sprague Dawley rats were divided into 6 groups: control, L-met (1.7 g/kg), RA (10 and 30 mg/kg) and L-met + RA (10 and 30 mg/kg) groups. Subjects were treated once a time per day for 28 days. Body weight was recorded every day. Before and after drug administration, spatial and recognition memory were investigated using the novel object location (NOL) and the novel object recognition (NOR) tests, respectively.

**Results:** The results revealed that rosmarinic acid and L-met did not have a negative effect on the body weight and locomotor activity. Whereas NOL and NOR tests revealed that control, RA (10 and 30 mg/kg), L-met + RA (10 and 30 mg/kg) groups were significantly different in the time spent exploring between the familiar and novel location or object compared with the L-met group.

**Conclusion:** This study demonstrates that rosmarinic acid had a protective effect against memory impairment induced by L-met.

**Keywords:** Rosmarinic acid, L-methionine, spatial memory, recognition memory

## Introduction

L-methionine (L-met) is one of the nine essential amino acids[1]. It can be found in natural products like fish, eggs and grains. However, high levels of L-met have been associated with deleterious effects such as vascular and neurological dysfunction[2-3]. High doses of methionine consumption can promote oxidative stress by increasing homocysteine levels. Methionine is converted into homocysteine after serving as a methyl group donor in methylation. Increased homocysteine levels in the blood stream lead to hyperhomocysteinemia[4]. Hyperhomocysteinemia causes endothelial injury by interfering with endothelial function and causing oxidative damage, which leads to cardiovascular, cerebrovascular and kidney diseases[5-7]. In addition, hyperhomocysteinemia could increase lipid peroxidation and decrease antioxidant enzyme activity that are associated with the memory deficit. It has a deleterious impact on the neurons of the brain, which can lead to impaired learning and memory[8-10].

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid that occurs in nature as phenolic compounds. It is commonly found in the *Lamiaceae* family, which contains rosemary, thyme, thai basil and perilla[11]. Rosmarinic acid has numerous biological activities, including anti-inflammatory and neuroprotective properties. It also has an antioxidant activity to scavenge free radicals[12]. Previous report revealed that rosmarinic acid alleviates the impairment of memory performance by decreasing oxidative stress levels[13-15]. However, no evidence of neuroprotective effects of Rosmarinic acid against memory impairments caused by L-methionine. Therefore, this study was designed to determine the neuroprotective effect of Rosmarinic acid against L-met-induced memory impairments in adult rats. The spatial and recognition memory were evaluated using the novel object location (NOL) and novel object recognition (NOR) tests, respectively.

## Methods

### Animals

Fifty-four male Sprague Dawley rats (age 4-6 weeks, body weight 180-200 grams) were obtained from Nomura Siam International Co., Ltd. Pathumwan, Bangkok. The experimental protocol was approved by the Ethics Committee in Animal Research of Khon Kaen University (project number AICUC-KKU-70/2565). The environment where the test subjects were kept was controlled to achieve light/dark cycle time of 12 hr each and the ambient temperature between 23-35 ℃. Food and water were provided to all subjects throughout the study period.

### Drug administration

The animals were divided into 6 groups (9 rats per group) and 4-5 rats per cage. Animals were allowed to acclimatize in a standard animal room at Khon Kaen University's Northeast Laboratory Animal Center for one week before drug administration. The control rats received 1 ml/kg propylene glycol (Ajax Finechem Pty Ltd., Australia) and 0.1% w/v carboxymethyl cellulose (CMC) 5 ml/kg (Sigma Aldrich Chemical Co., Saint Louis, MO, USA). The L-met group received L-methionine 1.7 g/kg suspended in 0.1% w/v CMC (Sigma Aldrich Chemical Co., Saint Louis, MO, USA). The rosmarinic acid 10 and 30 groups received rosmarinic acid (Sigma Aldrich Chemical Co., Saint Louis, MO, USA) 10 and 30 mg/kg dissolved in propylene glycol. The L-met + rosmarinic acid 10 and 30 groups received L-methionine 1.7 g/kg and rosmarinic acid 10 and 30 mg/kg for 28 days. Three days after drug administration, novel object location (NOL) and novel object recognition (NOR) tests were performed to assess spatial and recognition memory (Figure 1).

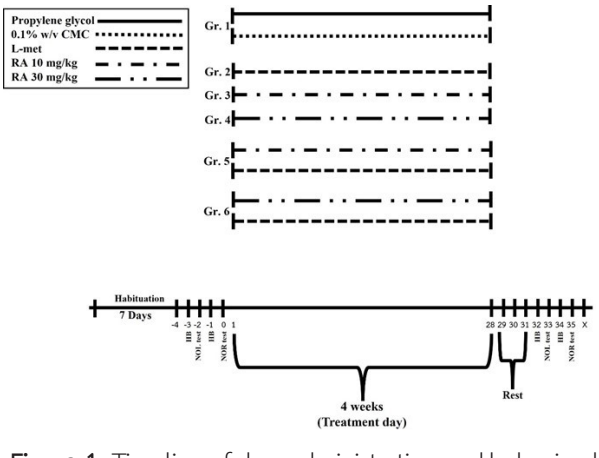


**Figure 1** Timeline of drug administration and behavioral testing

### Behavioral tests

The NOL and NOR tests were performed before and after drug administration. The equipment consists of an open field arena (50 cm x 50 cm x 50 cm) and water-filled plastic bottles. Movement patterns were detected by an overhead video camera connected to EthoVision® XT software (EthoVision®, XT Version 12, Noldus, Wageningen, Netherlands).

### Novel object location (NOL) test

The NOL test consists of habituation, familiarization and choice trials. In the habituation, the animals were placed in the open field arena without any objects for 30 minutes. The next day, the animals were habituated again for 3 minutes. In the familiarization trial, two identical objects were deposited in different corners. The animals were allowed to explore two objects for 3 minutes and then returned to their cage for 15 minutes. In the choice trial, one of the objects was placed in the same corner (familiar location: FL) and the other one was moved to a novel corner (novel location: NL). The animals were allowed to freely explore the objects in the arena for 3 minutes.

### Novel object recognition (NOR) test

In the NOR test, the habituation and familiarization trials are the same as the procedure of the NOL test. In the choice trial, the animals were placed in an arena with one of the familiar objects (FO) and a novel object (NO). The animals were allowed to freely explore the objects in the arena for 3 minutes. The exploration time in both tests was recorded when animals explored each object and actively directed its nose toward the object less than 2 cm using EthoVision® XT software.

### Statistical analysis

All statistical analyses were analyzed using GraphPad Prism (version 9.0; GraphPad Software Inc., San Diego, CA, USA). The data were expressed as mean ± standard error of mean (SEM). A probability level of p-value < 0.05 was considered statistically significant. Two-way repeated measure analysis of variance (ANOVA) was used to analyze body weight. One-way ANOVA was used to determine total exploration time. The discrimination index (DIs) were compared using one sample t-test.

## Results

### Effects of rosmarinic acid and L-methionine on body weight

The body weight of animals was continuously measured daily during the experiment. The body weight data showed no significant differences among groups ($p > 0.05$). This finding suggests that rosmarinic acid and L-met did not have a negative effect on body weight (Figure 2).

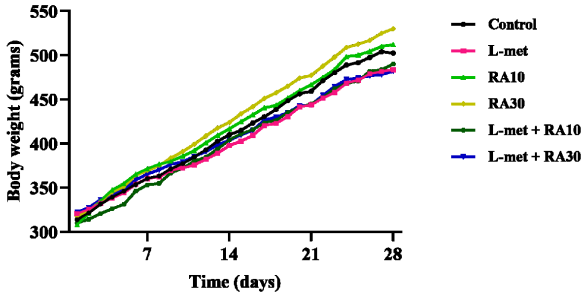


**Figure 2** The body weight of animals in each group during the experiment (day 1 to day 28).

### Effects of rosmarinic acid and L-methionine on spatial memory

The locomotor activity of animals was evaluated using the total exploration time. Total exploration time is defined as the exploration time of the familiarization and choice trials. The data showed no significant differences among groups before and after drug administration ($p > 0.05$, one-way ANOVA, Figure 3A and 3B). This result indicates that animals had no locomotor activity impairment in the NOL tests.

In addition, the DIs of all groups before drug administration were significantly higher than zero ($p<0.05$, one sample t-test, Figure 4A). The results indicate that all animals could discriminate between two identical objects in different locations. After drug administration, the DIs of the animals in all groups were significantly higher than zero except the L-met group ($p < 0.05$, one sample t-test, Figure 4B). These indicated that co-treated with rosmarinic acid could counteract L-met-induced spatial memory deficits.

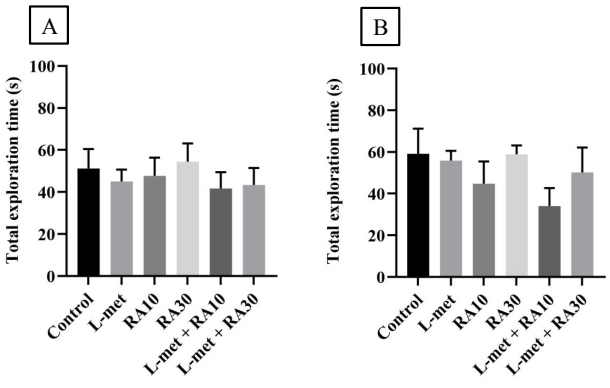


**Figure 3** Total exploration time of the NOL test before (A) and after (B) drug administration.

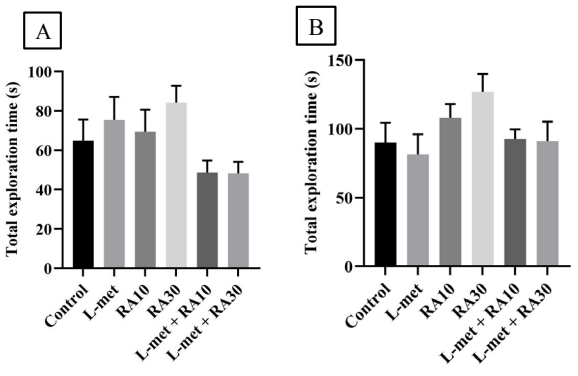


**Figure 5** Total exploration time of the NOR test before (A) and after (B) drug administration.

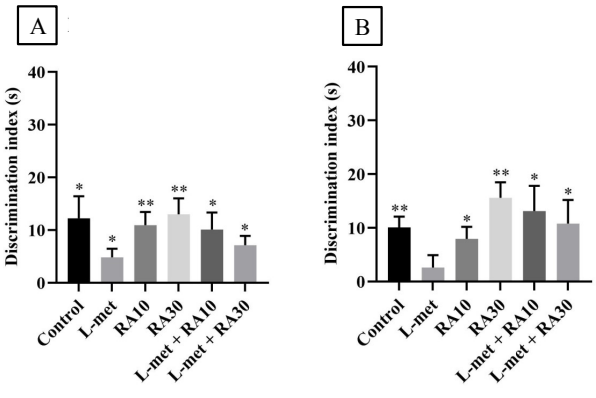


**Figure 4** The discrimination index of the NOL test before (A) and after (B) drug administration (*p < 0.05, **p < 0.01 significant difference compared to zero).

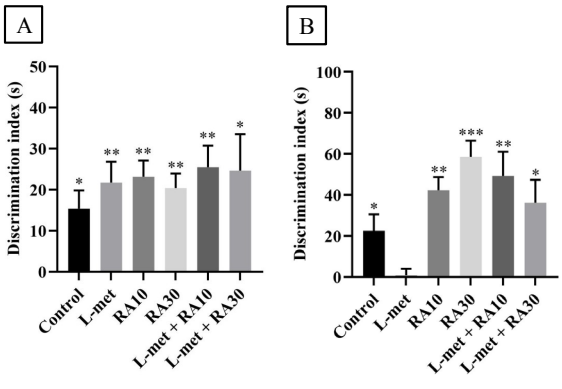


**Figure 6** The discrimination index of the NOR test before (A) and after (B) drug administration (*p < 0.05, **p<0.01, ***p<0.001 significant difference compared to zero).

**Effects of rosmarinic acid and L-methionine on recognition memory**

In NOR test, the total exploration time was not significantly difference among groups before and after drug administration (p > 0.05, one-way ANOVA, Figure 5A and 5B). This result suggests that the drug administration did not affect on locomotor activity.

The DIs before drug administration of animal in all groups were significantly higher than zero (p < 0.05, one sample t-test, Figure 6A). These indicated that all animals were able to discriminate between the novel and familiar objects. After drug administration, the DIs were significantly higher than zero in the control, rosmarinic acid 10 and 30 mg, L-met + rosmarinic acid 10 and 30 mg groups but it was not found in the L-met group (p < 0.05, one sample t-test, Figure 6B). The results demonstrate that L-methionine-treated rats had memory deficits. While the rosmarinic acid could ameliorate L-met-impaired recognition memory.

## Discussion

The present study demonstrates the effect of rosmarinic acid on memory impairment induced by L-met. We found that L-met could induce memory impairment in rats. Nevertheless, this impairment was improved by rosmarinic acid co-administration. The result after drug administration demonstrates that the body weight of animals was not significantly different among groups. The body weight of animals that received L-met were tended to decrease but not significantly difference when compared to the other groups, indicating that rosmarinic acid and L-met did not affect on body weight which is consistent with the previous study[16]. In some similar studies reported that the body weight of animal that received rosmarinic acid did not significantly different when compared to the control group[13]. In contrast, animals receiving L-met for 56 days showed a decrease in body weight when compared to controls. The

decrease in body weight may be associated with the duration of induction L-met that linked to alterations in methionine metabolism[17].

Locomotor activity is a spontaneous activity and an important movement in rodents. The term locomotor refers to movement from one place to another, which is a key component of spatial exploration in rodents. It is often measured to assess the physical and mental status of animal[18-19]. In the present study, the animals that received both L-met and rosmarinic acid did not effect on movement ability before and after drug administration. According to previous study, the exploration times were used to evaluate the locomotor activity in memory study in cerebral hypoperfusion paradigm[20].

Adult neurogenesis is the process of generating new neurons. The new neurons are generated in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. It plays a crucial role in learning and memory process[21]. The NOL and NOR tests were used to assess memory that is dependent on the hippocampus. The NOL test is a spatial memory assessment that focuses on hippocampus-dependent memory, such as spatial locations, configurations or routes[22]. The NOR test is a recognition memory test related to the hippocampus and perirhinal cortex. The hippocampus is involved in recollection, whereas the perirhinal cortex is involved in familiarity[23]. Spatial and recognition memory performance is related to adult neurogenesis in the subgranular zone[24-26]. The discrimination index is an ability to discriminate between novel and familiar locations or objects. If the DI value is not different from zero, this indicates that animals are unable to distinguish between novel and familiar locations or objects[27-28]. Results from the present study show that the DI in the L-met group was not significantly different from zero in both the NOL and NOR tests. This data indicates that animals receiving L-met showed memory impairments. Similarly, several studies have shown that the number of Ki-67, BrdU and NeuN positive cells in the hippocampus was significantly decreased after L-met administration. L-met induces learning and memory impairment by elevating oxidative stress. Oxidative stress can impact cell proliferation and survival during adult neurogenesis and associated with memory formation[29-32]. However, the animals in the co-administrated groups could discriminate between the novel and familiar locations or objects. Therefore, rosmarinic acid can attenuate the negative effects on spatial and recognition memory.

A high dose of dietary methionine can produce homocysteine. Hyperhomocysteinemia plays a role in neurotoxicity, which impairs cell division function and stimulates apoptosis within the SGZ of the DG in the hippocampus and leads to memory impairment[33]. Furthermore, hyperhomocysteinemia is capable of triggering neuronal damage via oxidative stress by up-regulating reactive oxygen species formation. These subsequently activate p53, resulting in apoptosis[34]. Rosmarinic acid can mitigate the effects of L-met-induced memory deficits. It exhibits strong antioxidant capacity by scavenging free radicals and reactive oxygen species[35]. Previous studies have reported that rosmarinic acid could improve memory and cognitive functions by neutralizing free radicals and inhibiting lipid peroxidation[14,36]. As oxidative stress is known to play an essential role in the development of memory impairment caused by L-met, the antioxidant properties of rosmarinic acid may be responsible for the observed memory improving effects[13,14,37-39]. These findings suggest that rosmarinic acid could be employed as a natural nutritional intervention for memory impairment.

## Conclusion

This study reveals that L-met can negatively affect spatial and recognition memory in adult rats. However, administration of rosmarinic acid can protect the deterioration of memory. Therefore, the result of this study postulates the beneficial effect of rosmarinic acid to protect against spatial and recognition memory deficits.

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