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Research article

Caffeic acid protects against L-methionine induced reduction in neurogenesis and cognitive impairment in a rat model

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ABSTRACT

L-methionine (L-met) is a substantial non-polar amino acid for normal development. L-met is converted to homocysteine that leads to hyperhomocysteinemia and subsequent excessive homocysteine in serum resulting in stimulating oxidative stress and vascular dementia. Several studies have found that hyperhomocysteine causes neuronal cell damage, which leads to memory impairment. Caffeic acid is a substrate in phenolic compound discovered in plant biosynthesis. Caffeic acid contains biological antioxidant and neuroprotective properties. The neuroprotective reaction of caffeic acid can protect against the brain disruption from hydrogen peroxide produced by oxidative stress. It also enhances GSH and superoxide dismutase activities, which protect against neuron cell loss caused by oxidative stress in the hippocampus. Hence, we investigated the protective role of caffeic acid in hippocampal neurogenesis and cognitive impairment induced by L-met in rats. Six groups of Sprague Dawley rats were assigned including control, L-met (1.7 g/kg/day), caffeic acid (20, 40 mg/kg), and L-met + caffeic acid (20, 40 mg/kg) groups. Spatial and recognition memories were subsequently examined using novel object location (NOL) and novel object recognition (NOR) tests. Moreover, the immunofluorescence technique was performed to detect Ki-67/RECA-1, bromodeoxyuridine (BrdU)/NeuN and p21 markers to represent hippocampal neurogenesis changes. The results revealed decreases in vasculature related cell proliferation and neuronal cell survival. By contrast, cell cycle arrest was increased in the L-met group. These results showed the association of the spatial and recognition memory impairments. However, the deterioration can be restored by co-administration with caffeic acid.

1. Introduction

Chronic administration of L-methionine (L-met) leads to memory impairment. The impairment occurs via increased oxidative stress in the cell, which damages the vascular system. L-met, a non-polarity amino acid, can be found in general food such as fish, meat, poultry, cheese and milk [1]. L-met cannot be produced in the human body [2]. Notwithstanding, L-met can be changed to homocysteine leading to hyperhomocysteinemia when the homocysteine level increases in the bloodstream. Hyperhomocysteinemia can

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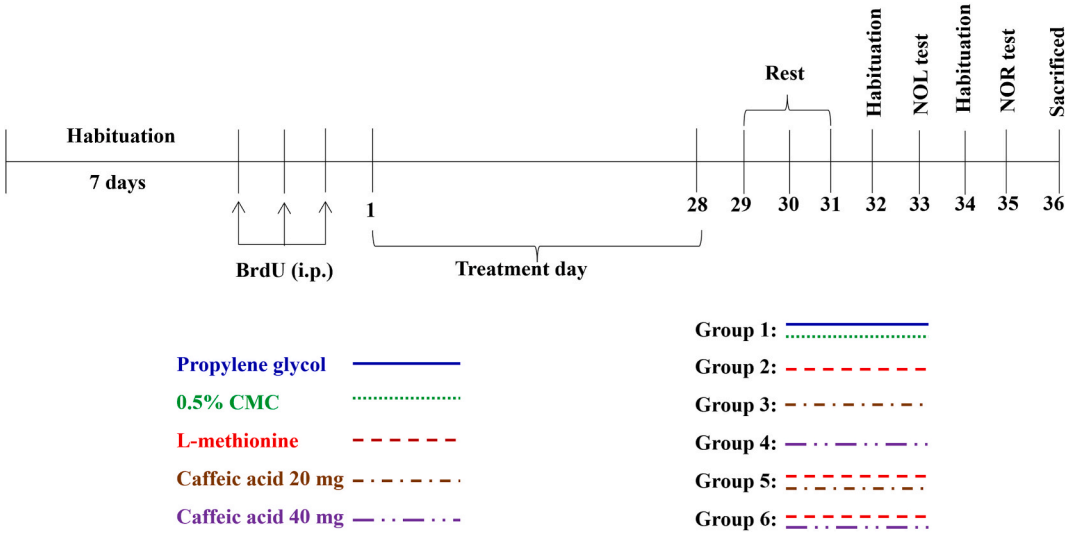

Fig. 1. Timeline of drug administration and behavioral testing.

trigger oxidative stress resulting in toxicity in the cell by decreasing nitric oxide in vascular endothelial cells. Besides, hyperhomocysteinemia increases lipid peroxidation associated with the inflammation process and causes memory deficits [3–5]. Toxicity of homocysteine in the cell is regulated by the redox potential of the cell. The clearance of homocysteine by methionine synthase is controlled by oxidative status [6]. There are several mechanisms of homocysteine neurotoxicity. An important mechanism to increase the level of homocysteine is to induce oxidative stress in the brain, which decreases nitric oxide leading to endothelial dysfunction. It is a cause of memory deficits, which are stimulated by decreasing of blood circulation [1,7] related to vascular dementia [8].

Neurogenesis is a circuit of synaptic plasticity that generates newborn neurons in the dentate gyrus. The dentate gyrus is an important region for cognition, learning and memory [9]. Neurogenesis occurs in adults that is regulated by numerous physiological and pathological stimuli including environment, learning, physical activity, stress, aging, irradiation and chemical substrates. Several studies have shown that enriched environment and extracted chemicals from plants such as hesperidin, asiatic acid, curcumin and S. amplexicaulis also increases hippocampal neurogenesis and neuroprotective properties, which take place in the hippocampus. This is a potential area associated with learning and memory [10–16].

Caffeic acid is a polyphenol that is classified in a group of hydroxycinnamic acid. Caffeic acid can be found in plants, such as tomato, mulberry, strawberry, tea, red wine and coffee [17]. Caffeic acid has an antioxidant property [18] and scavenges reactive oxygen species (ROS) [19]. In addition, caffeic acid has been described as a free radical scavenger as it has the antioxidant potential [20]. The pharmacological effect of caffeic acid is the ability to protect against cancer cells and cell inflammation [21]. Caffeic acid functions as an antioxidant in the circulatory system by decreasing vascular inflammation and atherosclerosis [22]. The neuroprotective features of caffeic acid can alleviate the disruption of the brain from oxidative stress [23]. Caffeic acid 30 mg/kg/day also pass through the blood-brain barrier, which results in attenuating neuroinflammation and cognitive impairment in Alzheimer's model [24]. Caffeic acid 10–50 mg/kg/day also improves the efficiency of acetylcholine neurotransmitter functioning, learning and memory [19,24–26]. However, caffeic acid triggers toxicity in animals receiving caffeic acid greater than 400 mg/kg/day [27]. Therefore, this research aims to investigate the neuroprotective effects of caffeic acid (20 and 40 mg/kg) on deteriorations of memory and neurogenesis caused by L-met in adult rats.

2. Materials and methods

2.1. Animals

Male Spraque-Dawley rats (4–5 weeks, 200–220 g) were purchased from the Nomura Siam International Co, Ltd., Bangkok, Thailand. The experiment methods were accepted by the Khon Kaen University Ethics Committee in animal Research (reference number AICUC-KKU-26/2562; Suppl Fig.1). Rats were divided and encased in a stainless cage (measuring 28 cm × 47 cm x 18 cm) under a 12:12 h light and dark cycle (28-30 °C) with food and water.

2.2. Drug treatment protocols

Rats were housed and freely habituated for 7 days prior drug treatment. Caffeic acid and L-met were purchased from Sigma Aldrich Chemical Co. (Saint Louis, MO, USA). Rats were randomly chosen into 6 groups (10 rats per group) and 5 rats per cage. Control rats (group 1) were fed with propylene glycol. Group 2: rats received L-met (1.7 g/kg/day) mixed in 0.5% w/v CMC by gastric gavage one

time a day on day 1–28. Group 3 and 4 rats received 20 and 40 mg/kg/day of caffeic acid dissolved in propylene glycol (1 mg/kg/day) by gastric gavage one time a day on day 1–28, respectively. In co-treatment groups (Groups 5 and 6), rats received L-met (1.7 g/kg/day) and caffeic acid (20 and 40 mg/kg/day) for 28 days. Before starting treatment, all animals were injected with 100 mg/kg BrdU (Sigma Aldrich, Inc., St. Louis, USA) freshly prepared in 0.9% saline solution by i. p. Injection (1 ml/kg/day) for 3 days (Fig. 1) [28].

2.3. Behavioral analysis

2.3.1. Novel object location (NOL) test

For spatial memory assessment, we used the novel object location (NOL) test, which is widely used to measure a behavioral index using rat spontaneous preferences for an object in a novel location. Prior the NOL test, each rat was habituated in a black square test box (50 cm^3 width x 50 cm^3 length x 50 cm^3 height) without objects for 30 min. One day later, each rat was individually allowed to accustom in the test box again for 3 min and then subsequently tested. In the familiarization phase, two objects were laid close to the edges (location A and B). Rats were permitted to explore those objects for 3 min and the exploratory behavior was recorded. In inter-trial interval, then, rats were brought to their home cages for 15 min. For the choice phase, two objects were set at the familiar location (location A) and a novel location (location C). The rats were permitted to survey the objects for 3 min [29–31].

2.3.2. Novel object recognition (NOR) test

One day after the NOL test, we used the novel object recognition (NOR) test to determine the ability to distinguish a novel object in an environment. This method was carried out using the same equipment as the NOL test and comprised of two trials. One day prior to the NOR testing, rats were habituated in the black square test box without objects for 30 min. One day later, the rats were accustomed in the test box again for 3 min and then two similar objects (object A and B) were placed at different corners in the test box. After 15 min of inter-trial interval, the rats were allowed to find the familiar object and a novel object (object C) for 3 min to test recognition memory [32–34].

The test box and objects were cleaned thoroughly using 20% alcohol solution to reduce scented hints between each NOL and NOR trial. The time spent to explore the objects in both tests were collected when a rat spend time exploring each object in the test box and actively directs its nose toward the object less than 2 cm, including sniffing, chewing, licking, or touching [29,31]. The rat behavior was monitored using an overhead video camera connected to EthoVision® XT tracking software (EthoVision®, XT Version 12, Noldus, Wageningen, Netherlands).

The preference index was determined by calculating the exploration time of novel locations or objects and revealed as percentage of the combined exploration time of the novel and familiar locations or objects. The choice trial was considered as a percentage when compared to 50% chance.

2.4. Immunohistochemical studies

2.4.1. Tissue preparation

One day after the behavioral assessment, animals were killed by rapid stunning and cervical dislocation. Then, the head was decapitated followed by removing the brains. Rat brains were divided into two halves in midsagittal plane. All brains were preserved in cryoprotective agent (30% sucrose solution) for 3 h at 4 °C followed by embedding in Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA). After that, the brains were immediately immersed in liquid nitrogen cooled isopentane and subsequently kept at −80 °C before further analysis.

2.4.2. Determination of cell proliferation and vascular association

Vascular association with cell proliferation was detected using endothelial antigen-1 (RECA-1) and Ki-67 markers to study the association of blood vessels and cell proliferation [35]. Right half of the brains were continuously cut (20 μm) between Bregma −2.3 to 6.3 mm to get total dentate gyrus using a cryostat (Cryostat Series HM550 Microm international, A.S. Science Co. Ltd., Walldrof, Germany). Every 15th section from the entire dentate gyrus was selected using a probability sampling method to get 9 sections from each brain [12,31]. The sections were first reacted with mouse Ki-67 monoclonal antibody (1:150, NOVOCASTRA, UK) for 60 min, then treated with rabbit anti-mouse Alexa Fluor 488 antibody (1:250, Invitrogen, USA) for 60 min. The sections were incubated with primary antibody monoclonal mouse RECA-1 (1:100), Invitrogen, USA) for 60 min and then reacted with goat anti-rat Alexa Fluor 568antibody (1:250, Invitrogen, USA). Each section was reacted with DAPI (1:6000, Molecular probes, Eugene, OR, USA) diluted with PBS about 30 s to enhance a contrasting background, thereafter each section was mounted with glycerol.

2.4.3. Determination of neuronal cell survival

Neuronal cell survivals were analyzed using BrdU-NeuN staining method [36]. Left half of the brains were continuously cut (40 μm) in the frontal plane throughout the dentate gyrus (between Bregma −2.3 to 6.3 mm) using a freezing microtome (freezing microtome Series HM430 Microm international, A.S. Science Co., Ltd., Walldorf, Germany). A probability sampling method was used to select every 8th section to obtain 9 sections from each brain [10,13]. The sections were first treated with 2 N HCL for 30 min at 37 °C followed by washing with 0.1 M borate buffer. Next, the sections were treated with BrdU monoclonal antibody (1:200, Abcam, UK) diluted with blocking solution at 4 °C overnight. On the second day, the sections were treated with Alexa Flour 568 goat anti-rabbit IgG antibody (1:200, Invitrogen, USA) for 2 h and performed incubation with primary *anti*-NeuN (1:500, Abcam, UK) at 4 °C overnight. Consequently, the sections were treated with Alexa Flour 488 rabbit anti-mouse IgG antibody (1: 500, Invitrogen, Carlsbad, CA, USA) for 2 h

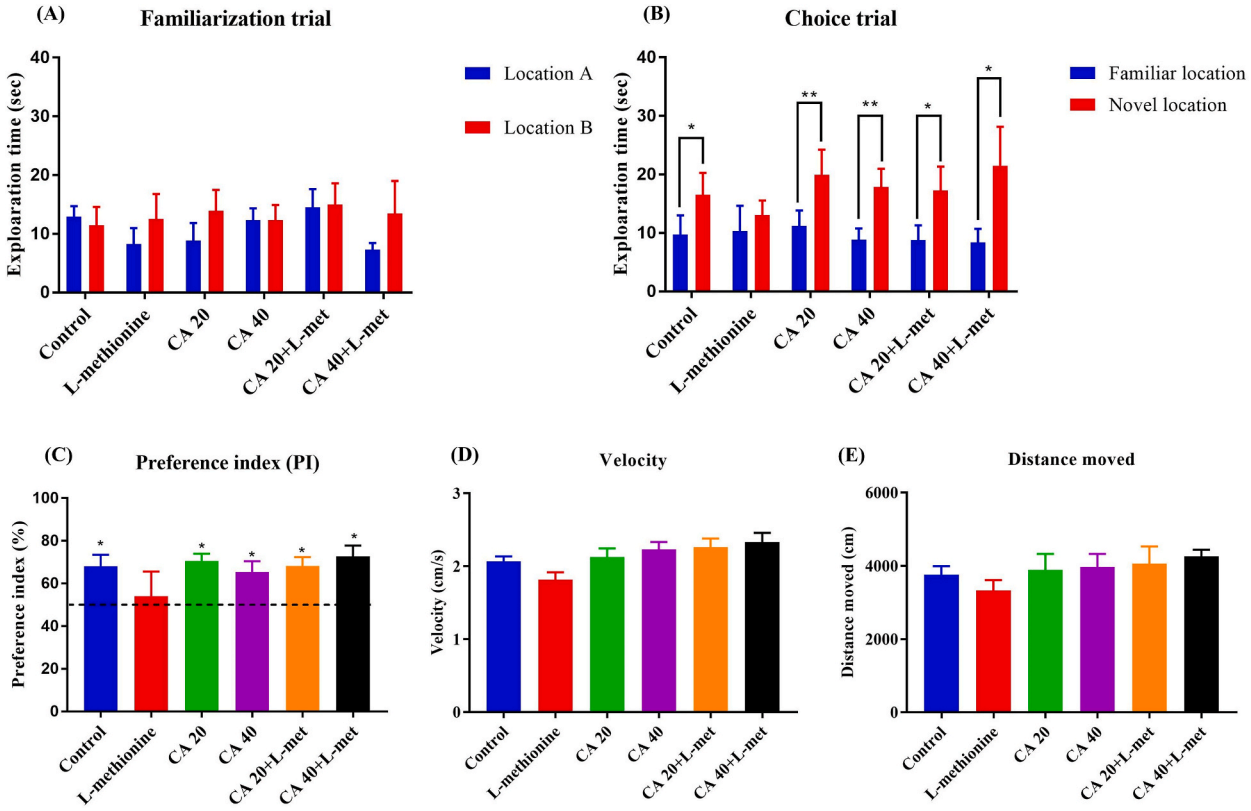

Fig. 2. Mean exploration time in the NOL test for the familiarization trial (A) and choice trial (B) after drug administration. The result showed that all rats explored the novel location significantly longer than the familiar location (*P < 0.05, **P < 0.01), except the L-met-treated rats (P > 0.05). The PIs in all groups were significantly higher than 50% chance, except L-met group (*P < 0.05, C). The results of mean velocity (D) and distance moved (E) were not significantly distinctive among the groups (p > 0.05).

and mounted with glycerol.

2.4.4. Determination of cell cycle arrest

Cell cycle arrest was investigated using the p21 staining method. The sections were cut using the same technique as the BrdU-NeuN staining. The sections were first treated with *anti*-p21 antibody (1:100 Santa Cruz Biotechnology, USA) at 4 °C overnight. The next day, the sections were treated with rabbit anti-mouse IgG Alexa Fluor 488 antibody (1:300, Invitrogen, USA) for 60 min and then performed counter staining with propidium iodide (1:6000, Sigma Aldrich, Inc., St. Louis, USA) for 30 s. Finally, glycerol was used to mount each section followed by cover-slipping for observation under fluorescence microscope.

Each section was observed under Nikon ECLIPSE 80i fluorescence microscope. Positive cells of Ki-67/RECA-1, BrdU-NeuN and p21 were examined within the inner edge of the dentate gyrus in the range of three cell diameter. Summation of Ki-67 positive cells was multiplied by 15 to determine proliferating cell number. Similarly, the total positive cell counts of BrdU/NeuN, and p21 of the whole dentate gyrus were multiplied by 8 to evaluate the number of neuronal cell survival and cell cycle arrest [37].

2.5. Statistical analysis

Data analysis was conducted using GraphPad Prism (Version 6.0; GraphPad Software Inc., San Diego, CA, USA). Statistical significances were considered by the probability level of p-value <0.05.

2.5.1 One-way ANOVA was used to analyze mean velocity, distance moved and positive cells of Ki-67/RECA-1, BrdU/NeuN and p21.

2.5.2 Paired Student's t-test was used to analyze mean exploration time for each object in familiar and choice trials of the NOL and NOR test.

2.5.3 One sample *t*-test was used to analyze the PI. Only the choice trial was considered as a percentage when compared to 50% chance.

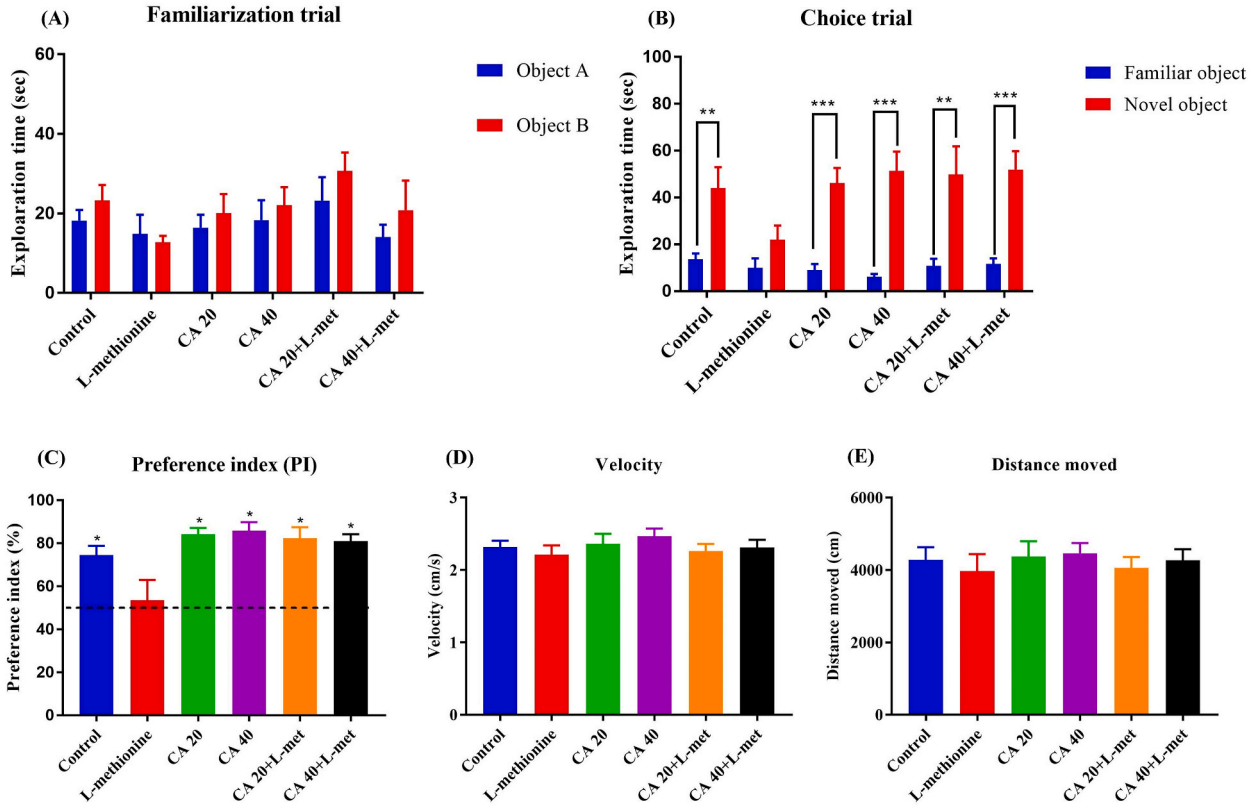

Fig. 3. Mean exploration time of the familiarization trial (A) and choice trial (B) in the NOR test after drug administration. The results showed that all rats explored the familiar object significantly longer than the novel object (**P < 0.01, ***P < 0.001), except the L-met group (P > 0.05). The PIs of all groups showed significantly higher than 50% chance, except the L-met group (*P < 0.05, C). The results of mean velocity (D) and distance moved (E) showed no significant differences among the groups (P > 0.05).

3. Results

3.1. Effects of caffeic acid and L-met on spatial memory

After drug administration, the distance moved and velocity were not significantly different among groups (velocity; F 9, 45 = 0.8288, P = 0.5933, distanced moved; F 9, 45 = 1.762, P = 0.1027, one-way ANOVA, Fig. 2D and E). It indicates that the drug did not interfere with locomotor movement. Then, each rat performed the NOL. The rats in all groups did not display a significant difference in exploring the objects in both locations in the familiarization trial (P > 0.05, paired Student t-test, Fig. 2A). In the choice trial, the results demonstrated a significant difference in the time spend exploring between the old and novel locations in all groups (P > 0.05, Fig. 2B), except the L-met group. The rats received only L-met failed to distinguish between the old and novel locations (P > 0.05). These results indicate that the L-met affected spatial memory impairment while co-treatment with caffeic acid could prevent and improve this impairment.

The preference index (PIs) was used to differentiate the degree of preference of animals in the novel location. This index was considered as the percentage of the time used to explore the novel location in the choice trial correlated to 50%. The results demonstrated that the PIs of the rats in all groups were significantly higher than 50% (P < 0.05, one sample *t*-test Fig. 2C), but it was not found in the L-met group (P > 0.05, one sample *t*-test). The results demonstrated that the rats, which received L-met induced spatial memory impairment, however the caffeic acid could prevent and improve this impairment.

3.2. Effects of caffeic acid and L-met on recognition memory

In the NOR test, the distance moved and velocity were not significantly different among the groups (velocity; F 9, 45 = 1.017, P = 0.4412, distance moved; F9, 45 = 0.4857, P = 0.8765, one-way ANOVA, Fig. 3D and E). The results demonstrated no impairment of locomotor activity after drug administration. After the locomotor examination, the rats in all groups showed no dissimilarities in the exploration time spent exploring two objects in the familiarization trial (P > 0.05, paired Student t-test, Fig. 3A). In the choice trial, the exploration time spend on the novel object was significantly different from the familiar object in all groups (P < 0.05, Fig. 3B), except the L-met group (P > 0.05). The results indicate that L-met induced recognition memory impairment, however caffeic acid could

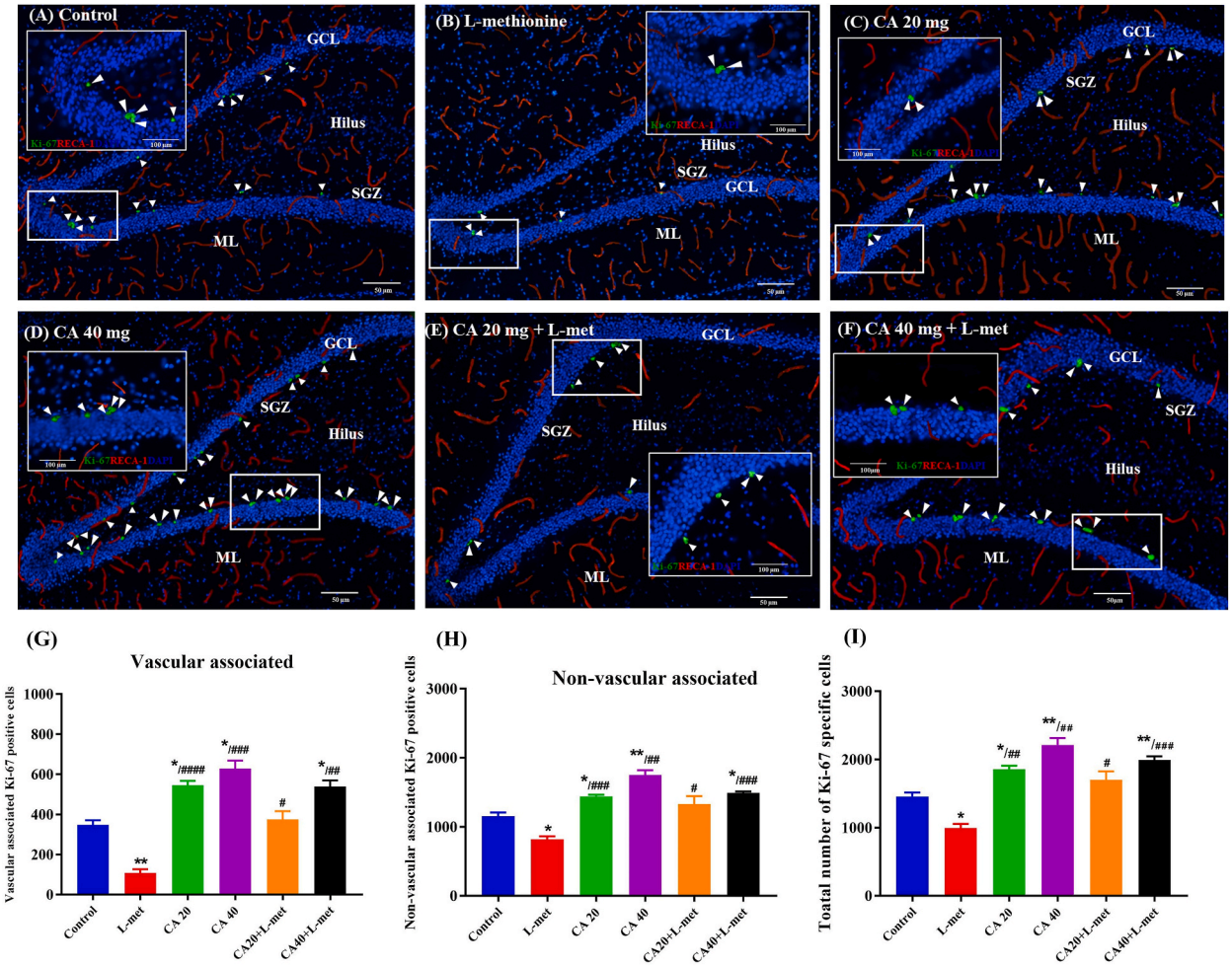

Fig. 4. Images of Ki-67 (green) and RECA-1 (red) specific in all groups (A–F). Each section was reacted with DAPI (blue) that is a counterstain. The white arrowheads demonstrate Ki-67 specific cells in the SGZ of the hippocampal dentate gyrus. Vascular associated Ki-67 (G), non-vascular associated Ki-67 (H) and total Ki-67 specific cells (I) were evaluated by cell counting compared to the control group (*P < 0.05, **p < 0.01) and the L-met group (#p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.001, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

prevent and improve this impairment.

The PIs of the control, caffeic 20, caffeic 40, caffeic 20+L-met, and caffeic 40+L-met groups exhibited significantly higher than 50% chance ($P < 0.05$, one sample *t*-test, Fig. 3C), except the L-met group ($P > 0.05$). These results indicate that the L-met-treated rats had memory deficits, however caffeic acid could prevent and improve memory impairment.

3.3. *Effects of caffeic acid and L-met on the association of vascular and cell proliferation*

The association of vascular and proliferating cells was analyzed using Ki-67/RECA-1 immunohistochemistry staining technique (Fig. 4A–F). The positive cells of Ki-67 in rats receiving L-met revealed a significant decline when related to the control group ($F5, 25 = 0.9667$, $P < 0.05$, one-way ANOVA, Bonferrani's post-hoc test, Fig. 4I). While the other groups significantly exceeded the L-met group ($P < 0.05$). Interestingly, the data of Ki-67 specific cells in the caffeic acid 20, caffeic acid 40 and caffeic acid 40+L-met exceeded in the control group (Fig. 4I). This shows that caffeic acid itself could increase proliferating cells and receiving L-met and caffeic acid could simultaneously ameliorate a reduction of Ki-67 positive cells induced by L-met.

The vascular associated Ki-67 specific cells in rats given only L-met demonstrated a significant reduction when examined according to the control group ($F5, 25 = 0.7512$, $P < 0.01$ one-way ANOVA, Bonferrani's post-hoc test, Fig. 4G), whereas the other groups significantly exceeded the L-met group ($p < 0.05$). Furthermore, vascular associated Ki-67 specific cell numbers in the caffeic acid and caffeic acid 40+L-met groups significantly exceeded the control group (Fig. 4G). These postulates that caffeic acid could increase vascular associated Ki-67 specific cells and co-treatment with caffeic acid could ameliorate a decline of vascular associated Ki-67 specific cells induced by L-met.

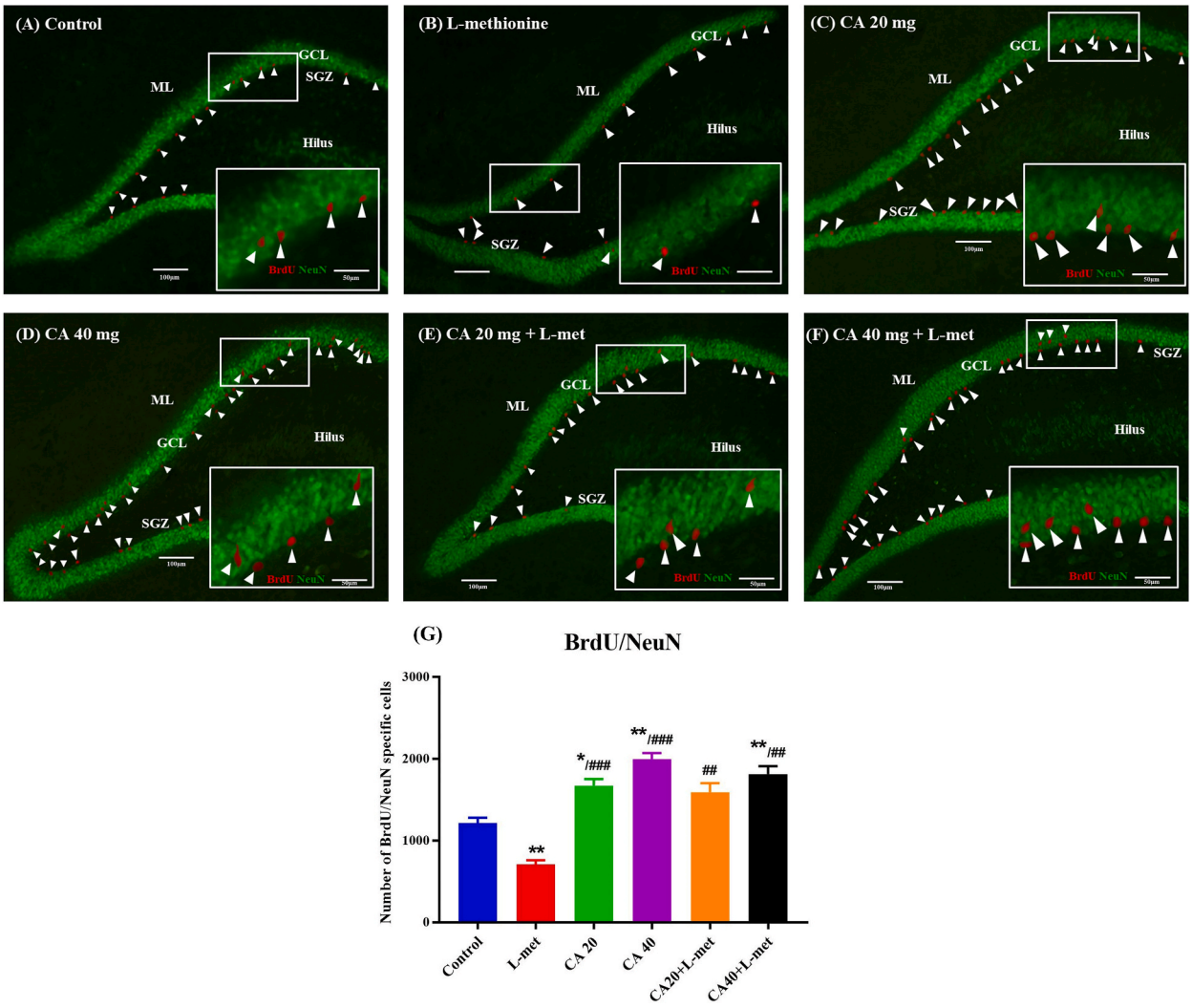

Fig. 5. Images of BrdU/NeuN specific cells (red) in each group (A–F). Sections were counterstained with NeuN (green). The white arrowheads indicate BrdU/NeuN specific cells in the SGZ zone of the hippocampal dentate gyrus. Total number of BrdU/NeuN specific cells of the caffeic acid 20, caffeic acid 40, and caffeic acid 40+L-met groups was exceeded the control group (*P < 0.05, **P < 0.01) and the L-met group (##P < 0.01, ###P < 0.001, respectively, G). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Likewise, rats in the L-met group had significantly lesser vascular associated Ki-67 specific cells, non-vascular associated Ki-67 and total Ki-67 specific cells, but it was not displayed in the controls ($F_{5, 25} = 0.8038$, $P < 0.0001$ one-way ANOVA, Bonferrani's post-hoc test, Fig. 4H). Conversely, non-vascular associated Ki-67 specific cells in the other groups significantly exceeded the L-met group ($P < 0.05$). Moreover, the number of non-vascular associated Ki-67 specific cells in the caffeic acid 20, caffeic acid 40 and caffeic acid 40+L-met groups was higher than the control group (Fig. 4H). These consequences demonstrate that caffeic acid itself (20 and 40 mg/kg) could increase vascular associated Ki-67 specific cells and co-treatment with caffeic acid could prevent the reduction of vascular associated Ki-67 specific cells induced by L-met in adult rats.

3.4. *Effects of caffeic acid and L-met on neuronal cell survival*

Brdu/NeuN immunostaining technique was done to evaluate neuronal cell survival in the SGZ zone of the dentate gyrus using immunohistochemistry staining technique (Fig. 5A–F). The results showed a significant decrease in BrdU/NeuN specific cells in rats treated with L-met compared to the control rats ($F_{5, 25} = 0.0063$, $P < 0.01$ one-way ANOVA, Bonferrani's post-hoc test, Fig. 5G). By contrast, the other groups were significantly increased in Brdu/NeuN specific cells when compared the L-met group ($P < 0.05$). Interestingly, a significant increase in BrdU/NeuN specific cells in the caffeic acid 20, caffeic acid 40, and caffeic acid 40+L-met groups was found, but not in the control group (Fig. 5G). This demonstrates that caffeic acid could improve and prevent the reduction of

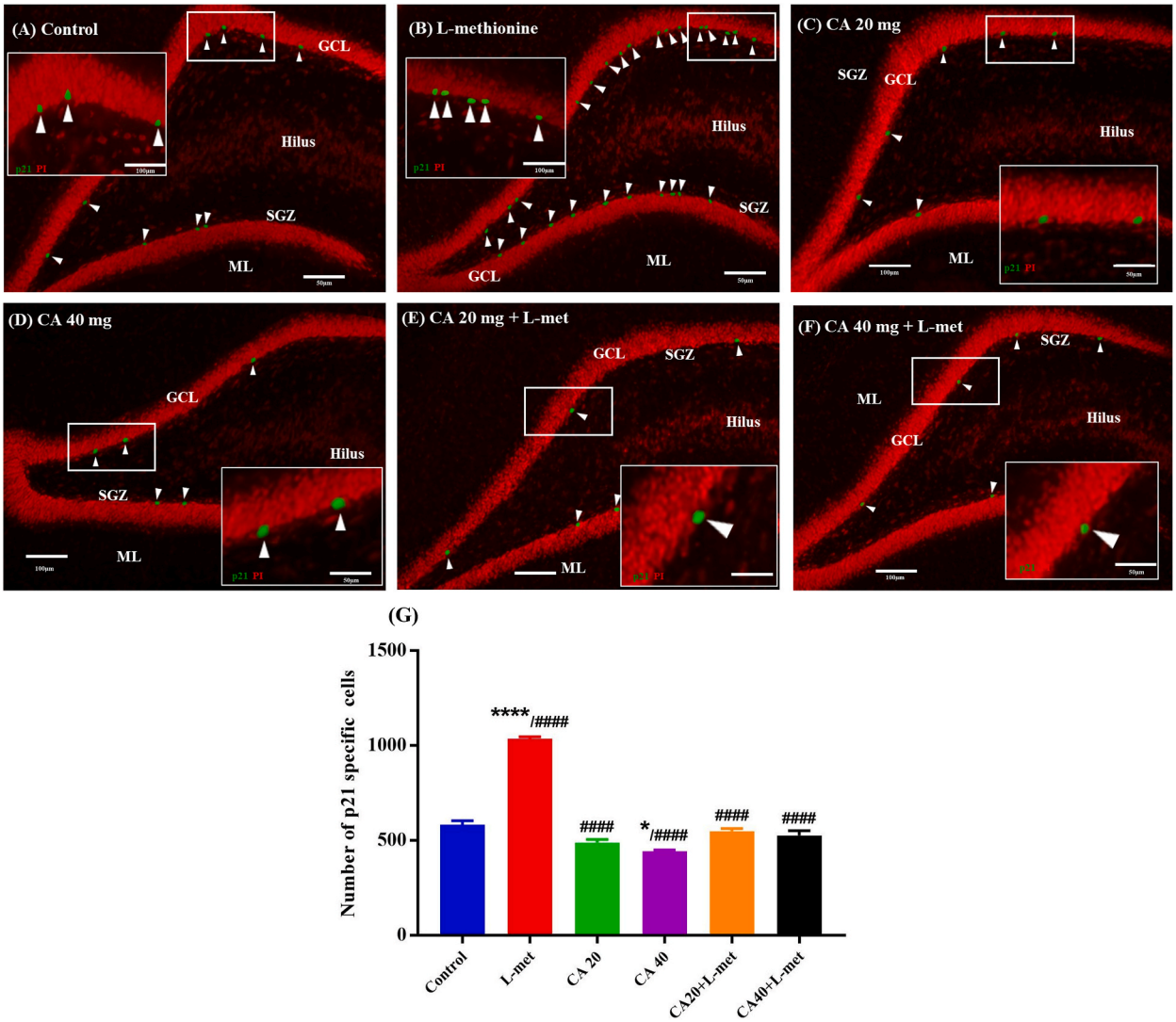

Fig. 6. The p21 specific cells exceeded in the L-met group when compared to control group (*P < 0.05, ****P < 0.0001) and the co-treated group (####P < 0.001). Images of p21 specific cells (green) in each group (A–F). Each section was stained with propidium iodide (red), which is a nuclear dye. The white arrowheads indicate p21 specific cells in the SGZ zone of the hippocampal dentate gyrus. The p21 specific cells in the caffeic acid and caffeic acid + L-met groups were lower than the L-met group (G). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

neuronal cell survival caused by L-met.

3.5. *Effects of caffeic acid and L-met on cell cycle arrest*

The p21 expression was implemented to represent DNA impairment and cell cycle arrest in the dentate gyrus using immunohistochemistry staining technique (Fig. 6A–F). The result of the L-met group revealed a significant difference in p21 specific cells in comparison with the control group (L-met: F5, 25 = 2.288, P < 0.0001, one-way ANOVA, Bonferrani's post-hoc test, Fig. 6G). Nevertheless, p21 specific cells in the caffeic acid 20, caffeic acid 40, caffeic acid 20+L-met and caffeic acid 40+L-met groups were significantly diminished in comparison with the L-met group (Fig. 6G). These consequences suggest that caffeic acid could inhibit cell cycle arrest caused by L-met.

4. Discussion

This study purposefully evaluated the reaction of caffeic acid on memory deficits activated by L-met in a rat model. The behavioral analysis presented that L-met (1.7 g/kg) administration led to initiate spatial and recognition memory deficits in adult rats [38]. In this

study the novel object location (NOL) and novel object recognition (NOR) tests, were conducted to assess the changes in animal behavior, that had been widely used to investigate the neurological mechanisms underlying memory formation [13,31,34]. The results of this study reveal that rats treated with L-met showed an impairment to discriminate objects located in the familiar and novel locations in the NOL test. Similar to the NOR test, rats receiving L-met could not distinguish between the familiar and novel objects. The present study showed the animals receiving co-treated with caffeic acid were able to discriminate between objects and locations in the NOL and NOR tests. This confirms an earlier report that caffeic acid treatment could alleviate L-met induced memory deficit. Furthermore, the result of the PIs did not significantly exceed 50% chance in the NOL and NOR tests in the L-met group. The data indicates that rats had spatial and recognition memory impairments after receiving L-met, which are in line with previous studies [1,3,39]. A study of chronic L-met administration in adult rats has shown that receiving L-met (1.7 g/kg) by oral gavage has a negative effect on cognitive activity tested by radial arm water maze (RAWM) test [7]. A previous study has demonstrated that oral feeding of L-met (1.7 g/kg) stimulates spatial memory loss using the RAWM test in adult rats [38]. Moreover, L-met (1 g/kg) melted in drinking water could promote learning and memory performance deficits caused by hyperhomocysteinemia [40]. Similarly, increasing blood homocysteine levels induces cognitive function impairment in rats receiving oral feeding of L-met (1 g/kg) for 30 days using Morris water maze test [41]. Locomotor activity is used to assess the voluntary movement in animal models [37]. The present study exhibited that caffeic acid and L-met had no negative actions on locomotor activity as shown by the velocity and distance moved. These findings reveal that the animals had the ability to explore the object in both the NOL and NOR tests without negative reinforcement [30,33,42].

L-met is changed to homocysteine by metabolic methylation pathway and then causes an increase in blood homocysteine level to induce hyperhomocysteinemia [43]. Homocysteine increases oxidative stress in the brain, which is the most likely pathway leading to neuronal damage and memory deficits [44]. High levels of homocysteine induce cytotoxicity and inhibit cell proliferation in hippocampal neural stem cells [45]. Elevated homocysteine can inhibit new neuron formation in the brain via neural stem and precursor cells [46]. For further observation, levels of homocysteine in blood should be evaluated to confirm the effect of L-met in this model. In present study, the immunofluorescence technique was performed to assess a reduction of cell proliferation and neuronal cell survival generated in the SGZ of the hippocampal dentate gyrus after drug administration. The result demonstrates that L-met is a cause of decreased hippocampal neurogenesis. A significant decrease in cell proliferation and neuronal cell survival was found in only the L-met administration group compared to the control group using Ki-67 and BrdU-NeuN labelling, respectively. Moreover, NeuN was used to counterstain with BrdU to detect surviving of mature neurons developed from immature neurons [44,47,48]. The present work is agreeable to several studies, which reveals an induction of hyperhomocysteinemia caused by neuroinflammation after receiving L-met with low level of folate, vitamins B6 and B12 for 3 months leading to vascular dementia tested by RAWM test in aged mice [49]. Along with other studies, homocysteine can potentially stimulate oxidative stress, cytotoxicity and cellular apoptosis and endothelial dysfunction [2,50]. Furthermore, rat endothelial cell antigen or RECA-1 represents specialized simple squamous epithelium lining in the interior surface of blood vessels. RECA-1 is important to determine vascular associated proliferating cells on clusters associated with the neurovascular stem cell niche of adult neurogenesis [51]. In this study, vascular associated and non-vascular associated proliferating cells were measured via the expression of Ki-67 and RECA-1 positive cells. The results of vascular associated and nonvascular associated proliferating cells demonstrated the significant declines in the L-met group when compared to the control group, however, these declines were ameliorated by receiving co-treatment with caffeic acid. A previous report has shown that chronic administration of L-met diminishes cell proliferation by several mechanisms including neuronal damage [44], oxidative stress and increasing p53 signaling protein in apoptosis and cell cycle arrest pathway [52]. Likewise, decreasing vascular associated proliferating cells is revealed in this study. Moreover, neurogenic niches in the mammalian hippocampus are an important contributor in hippocampal neurogenesis. Proliferating cells reside closely to blood vessels in the dense cluster of neurogenic niches and vasculature in the SGZ of hippocampal dentate gyrus to serve as conduits for oxygen, nutrients and fulfill perfusion in neurogenesis [53]. On the other hand, p21 (cell cycle arrest) is significantly increased in the L-met group compared to the other groups. The p21 is also known as cyclin/CDK complex inhibitors, which can bind with cyclin and stop cell cycle progression [54]. Therefore, p21 expression represents DNA damage caused by cell cycle arrest in the SGZ of the hippocampal dentate gyrus. Similarly, neuronal damage and apoptosis after L-met administration promotes memory impairment [55].

Caffeic acid is a polyphenol produced through the secondary metabolism of coffee beans, olives, fruits, vegetables and propolis [22]. Polyphenols can reduce the generation of free radicals, reactive oxygen species, oxidative stress, DNA damage and age-related diseases [56]. Caffeic acid has the most potential antioxidant activity to trap free radicals such as hydroxyl group [17,18]. Caffeic acid has been reported to inhibit lipid peroxidation and reduce DNA damage by the pathway of free radical scavenging and electron donors [23]. Caffeic acid can pass through the blood-brain barrier by supporting the monocarboxylic acid transporter on endothelial cell surface and methylation of phenolic compound and then it is contained in the neurons [57]. Caffeic acid could decrease levels of hydrogen peroxide (H_2O_2) and free radical scavenging abilities, inhibit lipid peroxidation and protect polyunsaturated fat in neurons [58,59]. Interestingly, in this study, the rats that received combination of caffeic acid (20, 40 mg/kg) and L-met can protect the reduction of cell proliferation and neuronal cell survival compared to the L-met group. The rats that received only caffeic acid either 20 or 40 mg/kg also showed significant increases in cell proliferation and neuronal cell survival in the SGZ of the hippocampal dentate gyrus compared to the control group. On the other hand, p21 positive cells representing cell cycle arrest were significantly decreased in the rats receiving only caffeic acid and L-met + caffeic acid. These results showed that the ability of caffeic acid increased hippocampal neurogenesis and prevented reduction of hippocampal neurogenesis induced by L-met associated with the behavioral test results. However, further studies are necessary to determine the mechanism of caffeic acid in the brain using animal models to reveal neurodegenerative or antioxidant activities.

In conclusion, the results of this study demonstrated that treatment with L-met can induce cognitive dysfunction. It also decreases vascular and non-vascular associated proliferating cells and neuronal cell survival in the hippocampal dentate gyrus, but increases cell

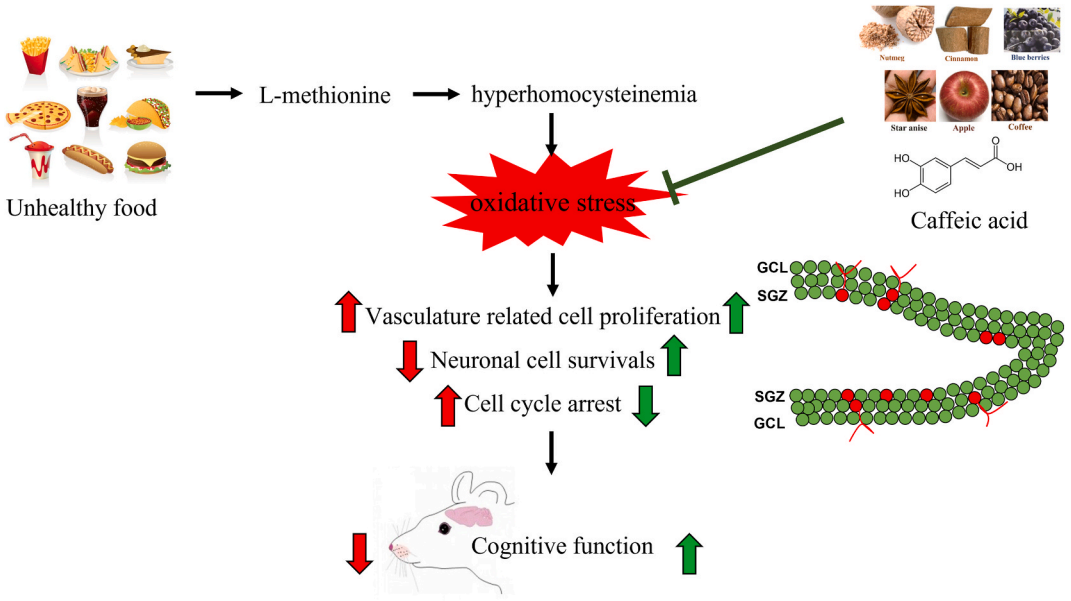

Fig. 7. The conclusion of this study.

cycle arrest. However, caffeic acid can reverse negative effects of L-met (Fig. 7). The results from this study may be beneficial to develop an alternative drug to treat memory deficits in the future.

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CRediT authorship contribution statement

Apiwat Sirichoat: Writing – original draft, Software, Formal analysis, Data curation. **Oabnithi Dornlakorn:** Investigation, Formal analysis, Data curation. **Rasa Saenno:** Data curation. **Anusara Aranarochana:** Formal analysis. **Nataya Sritawan:** Data curation. **Wanassanun Pannangrong:** Resources. **Peter Wigmore:** Writing – review & editing. **Jariya Umka Welbat:** Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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