Mitigation of Memory Impairment in Ovariectomized Rats Using Garlic Powder Treated with Subcritical Water

Akira Minami,*a Yasuyo Mikami,a Takahiro Kano,a Hiroshi Matsushita,b Yuka Fujita,a Marina Yoshimura,a Yuki Abe,a Hiromi Watanabe,a Masaomi Hara,a Yuki Kurebayashi,a Tadanobu Takahashi,a Hiroaki Kanazawa,d Akihiko Wakatsukib, and Takashi Suzuki*a

a Department of Biochemistry, School of Pharmaceutical Sciences; 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan; b Department of Obstetrics and Gynecology, Aichi Medical University, School of Medicine; 1–1 Yazakokarimata, Nagakute, Aichi 480–1195, Japan; c Nikken Foods Co., Ltd., 1–6–1 Nihonbashı Honcho, Chuo-ku, Tokyo 103–0023, Japan; and d Department of Functional Anatomy, School of Nursing, University of Shizuoka; 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan.

Women with estrogen deficiency are at the risk of suffering from neurological symptoms such as memory impairment. In the present study, we investigated the effect of garlic, Allium sativum L. (Asparagales: Amaryllidaceae), treated with subcritical water on memory impairment in ovariectomized (OVX) rats. OVX rats were administered garlic powder for 84 d. Hippocampus-dependent spatial memory was assessed using the Morris water maze test. Escape latency of the OVX rats increased compared with that of sham-operated rats. The prolonged escape latency of the OVX rats decreased to the level of that of sham-operated rats upon the administration of garlic powder (0.5% in feed). The weights of the body, uterus, and brain were not affected by the garlic powder administration. These results suggest that garlic powder treated with subcritical water mitigates memory impairment in OVX rats.

Key words garlic; subcritical water; memory impairment; depression; rat; postmenopausal syndrome

INTRODUCTION

Menopausal women with estrogen-deficiency experience hot flashes, osteoporosis, and metabolic syndrome. These symptoms can be reproduced in ovariectomized (OVX) rodents with estrogen deficiency. These symptoms subside with the administration of estradiol.1) Estrogen deficiency is also associated with the increased risk of neurological symptoms such as depression and memory deficit.2) The memory deficit exhibited in OVX-induced menopausal animal models is also improved by the administration of estrogen.3) Estrogen improves depression-like behavior in animals. Hormone replacement therapy (HRT) is effective in treating neurological symptoms associated with menopause. However, HRT has a slight risk of developing cancer. Long-term use of HRT with estrogen or estrogen/progesterin increases the risk of developing breast and uterine cancers. HRT exhibits a higher risk of breast cancer development in women who have previously had breast cancer.4) Based on these unfavorable effects of HRT, we investigated alternative therapeutics including alternate food materials, that are not associated with the risk of cancer and improve the neurological symptoms associated with menopause. In our previous studies, we found that ethanol extract from Pleurotus eryngii5) and royal jelly6) improve memory deficits in menopausal rat models.

Garlic, Allium sativum L. (Asparagales: Amaryllidaceae), contains lignans such as pinoresinol, lariciresinol, and secoisolariciresinol.7) These food-derived lignans are metabolized into phytoestrogens, enterodiol, and enterolactone by intestinal bacteria. Garlic also contains the phytoestrogen quercetin.8) Garlic also contains S-allyl-L-cysteine which has neuroprotective properties9) and an improvement effect in Alzheimer’s disease.10) In this study, we found that subcritical water treatment of raw garlic generates S-allyl-L-cysteine and cycloalliin. Thus, here, we investigated the effect of garlic powder processed using subcritical water treatment on the memory deficits in exhibited by OVX rats.

MATERIALS AND METHODS

Subcritical Water Treatment of Garlic Powder Garlic (Allium sativum cv. Fukuchi-howaito produced in Aomori prefecture in Japan) was harvested between mid-June 2016 and July and was turned into paste after enzyme inactivation in boiling water for 15 min, followed by continuous circulation processing in a subcritical water treatment device (Tohzai Chemical Industry, Osaka, Japan). Garlic was then powdered using a spray dryer. The subcritical water treatment conditions were 145°C and, 3 MPa, with 5 circulations. Dextrin was used as an excipient with a solid ratio of 70% during spray drying.

Quantitative Analysis of Garlic Powder Components The garlic powders (500 mg) before and after treatment with subcritical water, were extracted in 25.0 mL of methanol/water/formic acid (50:50:1) for 5 min using an ultrasonic cell disrupter. After centrifugation at 4500 × g for 10 min (radius of gyration: 128.4 mm), the supernatant was filtered using a 0.45 µm membrane filter. Then, quantitative analysis of S-allyl-L-cysteine and cycloalliin was performed by HPLC (absorbance: 210 nm, standard: 0.02–5.0 g, 8 points, r² > 0.999) using a Capcell Pak SCX UG80 column (4.6 ID × 250 mm, particle size: 5 µm. Osaka Soda, Osaka, Japan) and a mobile phase (1.0 mL/min at 45°C) of 10mM potassium dihydrogen phosphate solution (pH 2.5).

Animals Female Wistar rats (Japan SLC, Hamamatsu,
Japan) had *ad libitum* access to standard feed (MF; Oriental Yeast, Tokyo, Japan) and tap water. They were maintained in 12 h light/dark cycles (temperature 23 ± 1°C, humidity 55 ± 5%). The rats were used for the experiment after 2 weeks of acclimatization. All animal experiments were conducted in compliance with the guidelines established by the University of Shizuoka. All animal experiments were pre-approved by the animal ethics committee of the University of Shizuoka (authorization number: 176283).

**Ovariectomy and Administration** Both ovaries were excised from the rats (10 week old) under anesthesia, using a mixture of butorphanol tartrate (Meiji Seika Pharma Co., Ltd., Tokyo, Japan; 2.5 mg/kg body weight), medetomidine hydrochloride (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; 0.375 mg/kg body weight) and midazolam (FUJIFILM Wako Pure Chemical Corporation, 2 mg/kg body weight) that was administered by intraperitoneal injection. The sham group underwent the same procedure without the ovariectomy. In the 17β-estradiol (E2) group, rats were implanted with a small pellet (0.25 mg/90 d, Innovative Research of America, Sarasota, FL, U.S.A.) under their skin. OVX rats were administered a MF containing 0.5% (w/w) garlic powder from 4 d after the ovariectomy (garlic powder treatment group, n = 11). The sham group (n = 11) and OVX group (n = 11) were given standard MF without garlic powder. Food intake was measured for 7 d from the day 78 after the ovariectomy.

**Morris Water Maze Test** The Morris water maze test was started on day 88 after the ovariectomy. A transparent platform was installed under the water surface in a pool (130 cm diameter) filled with water (22 ± 2°C). The rat was placed in the pool and the time taken to reach the platform was measured (up to a maximum of 40 s). Four trials were performed on one day at one minute intervals, and this was repeated for 4 d. The location of releasing the rat in the pool was randomly changed after each trial. Memory for the platform location was assessed by quantifying the mean latency to reach the platform on day 4.

In the initial trial on day 1, the pool was virtually divided into 4 divisions of North, South, East, and West, and the number of times that the rat crossed each division was measured to evaluate motility. If rats got to the platform by chance, the number of crossing times was corrected to the number of crossing times per 40 s.

The behavior of the rats during the experiment was recorded using a video camera (Sony, Tokyo, Japan). The latency time to reach the platform and motility in the Morris water maze test was measured by analyzing the video data using a video tracking system (ANY-maze Ver. 5.3; Muromachi Kikai, Tokyo, Japan).

**Measurement of Body, Uterine and Brain Weights** The weights of body, uterus and brain were measured on day 99 after the ovariectomy.

**Statistical Analysis** Multiple group comparisons were conducted using one-way ANOVA followed by Newman–Keuls multiple comparison test.

**RESULTS**

**Garlic Powder Component Analysis** The content of S-allyl-L-cysteine in garlic powder before and after treatment with subcritical water were 15.9 mg/100 g powder and 64.9 mg/100 g powder, respectively. The content of cycloalliin in the garlic powder before and after treatment were 47.2 mg/100 g powder and 136.5 mg/100 g powder, respectively.

The concentrations of S-allyl-L-cysteine and cycloalliin in garlic powder after treatment with subcritical were similar to those observed in black garlic.11)

**Effect of Garlic Powder on Body, Uterine and Brain Weights** The body weight of OVX rats measured on the day 99 after ovariectomy was higher than that of sham-operated rats. The intake of feed containing garlic powder for 95 d did not affect the body weight of OVX rats. However, the body weight of OVX rats was decreased after E2 administration (Fig. 1A).

The uterine weight of OVX group, measured on day 99 after ovariectomy, was lower than the uterine weight of the Sham group. The decrease in uterine weight of OVX rats was not affected by garlic powder, but was recovered to the levels of sham-operated rats after administration of E2 (Fig. 1B). Brain weight was not affected by ovariectomy nor by the administration of garlic powder and E2 (Fig. 1C).

**Daily Intake of Garlic Powder** On days 78–84 after the

![Fig. 1. Effect of Garlic Powder Treated with Subcritical Water on Weights of Body (A), Uterus (B) and Brain (C)](image-url)

Each line represents the mean ± standard error of the mean (S.E.M.) (n = 11). ***p < 0.001.
ovariectomy, daily average amount of garlic powder ingested by each rat was 66.5 ± 1.2 mg. Food intake during this term was not changed between the sham and OVX group, but it was higher in the garlic powder treatment group compared to other two groups (Fig. 2).

Effect of Garlic Powder on Memory Deficit in OVX Rats

We investigated the effect of garlic powder on the memory deficit exhibited by OVX rats using the Morris water maze test. Escape latency i.e., time taken to reach the escape platform, on day 4 was significantly higher in the OVX group compared to that of the sham group. Increased escape latency exhibited by the OVX group was significantly improved by the intake of either garlic powder or E2 (Figs. 3A, B).

We investigated the effect of garlic powder on motility to confirm that the shortened escape latency observed in the garlic powder treatment group in the Morris water maze test was not caused by changes in motility and distance covered. During the first trial Morris water maze test on the first day, the pool was virtually divided into 4 divisions of North, South, East and West, and the number of times the rats crossed each division was measured (equivalent to moving distance). There was no significant change in the distance covered by the rats in all the groups, which confirmed that garlic powder does not affect motility in OVX rats (Fig. 3C).

DISCUSSION

It is widely known that cooking or processing foods increases the absorption and enhances the function of the active ingredients in the food material. Black garlic is produced by placing raw garlic in a high-temperature, high-humidity environment for 3–4 weeks, and the black color appears as a result of the sugar and amino acid compounds in the garlic undergoing a Maillard reaction. In comparison with raw garlic, black garlic contains an abundance of Maillard compounds, and S-allyl-L-cysteine and cycloalliin.12) S-Allyl-L-cysteine has neuroprotective properties 9) and has exhibited an improvement effect in Alzheimer’s disease.10) Thus, black garlic is also reported to function as antioxidant, anti-diabetic, anticancer, anti-allergy, anti-inflammatory agent and beneficial effects on the heart, liver and lipid metabolism. In addition to black garlic, the amount of S-allyl-L-cysteine in garlic can be also increased by immersion in ethanol. Garlic treated with ethanol (aged garlic) is reported to have a neuroprotective effect. Administration of aged garlic to rats improves short-term memory, working memory, and cognitive ability.13)

Subcritical water treatment may help in enhancing the food quality through hydrolysis of proteins and polysaccharides in food and through the amino-carbonyl reaction.14) In this study, we found that subcritical water treatment of raw garlic generates S-allyl-L-cysteine and cycloalliin. The garlic powder used in this study was subjected to subcritical water treatment. Concentrations of S-allyl-L-cysteine and cycloalliin in the powder were similar to those found in black garlic and aged garlic. Thus, we investigated the beneficial effect of garlic powder processed using subcritical water treatment on memory deficits in menopausal rats.

Memory deficit in OVX rats was ameliorated upon the administration of estradiol.15) In this study, spatial memory, eval-

---

**Fig. 2.** Effect of Garlic Powder Treated with Subcritical Water on Food Intake in OVX Rats

Each line represents the mean ± S.E.M. (n = 11). *p < 0.05.

**Fig. 3.** Mitigation of Memory Impairment in OVX Rats Using Garlic Powder Treated with Subcritical Water

(A) Latency time until arriving at the platform in the Morris water maze test is shown. (B, C) Statistical analysis at day 4 and the motility in the initial trial on day 1 are shown in panels B and C, respectively. Each line represents the mean ± S.E.M. (n = 11). *p < 0.05.
uated using the Morris water maze, had significantly declined in rats 88 d after ovariectomy. We confirmed that the OVX rats used in this study could be used as a model for menopausal memory deficit. This reduction in memory function was improved by the intake of subcritical water treated garlic powder and E2 for 84 d. Although more detailed investigation is required, S-allyl-l-cysteine may be involved in the improved memory function of OVX rats with the garlic powder.

The main estrogen receptors are type α (ERα) and type β (ER/β). When ERα is activated, body weight decreases and uterine weight increases. This accounts for the increased body weight seen in postmenopausal women. Overactivation of ERα is associated with the development of breast cancer, uterine cancer, and cardiovascular disease. Activation of ERβ improves depression-like behavior and memory function and is involved in the protection of neurons. The estradiol used in HRT activates both ERα and ERβ, and is therefore associated with a risk of developing cancer. The selective activation of ERβ can improve neurological symptoms associated with menopause.

The OVX rats in this study had increased body weight and decreased uterine weight, which confirmed the estrogen deficiency in OVX rats. These phenotypes were not affected by the intake of garlic powder. This suggested that garlic powder does not activate ERα. The diallyl trisulfide present in garlic has been reported to inhibit ERα expressed in human breast cancer cells. Although the daily food intake after ovariectomy was higher in the garlic powder treatment group compared to sham and OVX groups, body weight remained unchanged after administration of garlic. This is considered to be due to the improvement effect of garlic on lipid metabolism and the anti-obesity effect of garlic. Overall, garlic processed using subcritical water treatment may be used as a functional food for improving neurological symptoms associated with menopause, without the risk of developing cancer due to ERα activation.

Acknowledgments We are grateful to Oriental Medicine Research Club and Herbal Medicine Research Club for the daily support they provided to our experiments. This research was supported by the Japan Society for Menopause and Women’s Health under the JMWH Bayer Grant to A.M. and by the Japan Menopause Society under the JMS Bayer Schering Pharma Grant to H.M.

Conflict of Interest Hiromi Watanabe and Masaharu Hara are employees of Nikken Foods Co., Ltd. They performed the subcritical water treatment of garlic powder. The other authors declare no conflict of interest.

REFERENCES
1) Geary N, Asarian L, Korach KS, Pfaff DW, Ogawa S. Deficits in E2-dependent control of feeding, weight gain, and cholecystokinin satiation in ER-alpha null mice. Endocrinology, 142, 4751–4757 (2001).
2) Hyde JS, Mezulis AH, Abramson LY. The ABCs of depression; integrating affective, biological, and cognitive models to explain the emergence of the gender difference in depression. Psychol. Rev., 115, 291–313 (2008).
3) Henderson VW. Cognitive changes after menopause: influence of estrogen. Clin. Obstet. Gynecol., 51, 618–626 (2008).
4) Holmberg L, Anderson H. HABITS (hormonal replacement therapy after breast cancer—is it safe?), a randomised comparison: trial stopped. Lancet, 363, 455–455 (2004).
5) Minami A, Matsushita H, Horii Y, Ieno D, Matsuda Y, Saito M, Kanazawa H, Ohyama Y, Wakatsuki A, Takeda A, Hidari KI, Sabaratnam V, Suzuki T. Improvement of depression-like behavior and memory impairment with the ethanol extract of Pleurotus eryngii in ovariectomized rats. Biol. Pharm. Bull., 36, 1990–1995 (2013).
6) Minami A, Matsushita H, Ieno D, Matsuda Y, Horii Y, Ishii A, Takahashi T, Kanazawa H, Wakatsuki A, Suzuki T. Improvement of neurological disorders in postmenopausal model rats by administration of royal jelly. Cimicifuga, 19, 568–573 (2016).
7) Milder IE, Fesken S, Arts IC, Bueno de Mesquita HB, Hollman PC, Kronhout D. Intake of the plant lignans sesaminolresin, matairesinol, lariciresinol, and pinostrobin in Dutch men and women. J. Nutr. 135, 1202–1207 (2005).
8) Sengupta A, Ghosh S, Das S, Tomato and garlic can modulate azoxymethane-induced colon carcinogenesis in rats. Eur. J. Cancer Prev., 12, 195–200 (2003).
9) Imai T, Kosuge Y, Saito H, Uchiyama T, Wada T, Shimba S, Ishige K, Miyairi S, Makishima M, Ito Y. Neuroprotective effect of S-allyl-l-cysteine derivatives against endoplasmic reticulum stress-induced cytotoxicity is independent of calpain inhibition. J. Pharmacol. Sci., 130, 185–188 (2016).
10) Ishige K, Takagi N, Imai T, Rausch WD, Kosuge Y, Kihara T, Kusama-Eguchi K, Ikeda H, Cools AR, Waddington JL, Koshikawa N, Ito Y. Role of caspase-12 in amyloid beta-peptide-induced toxicity in organotypic hippocampal slices cultured for long periods. J. Pharmacol. Sci., 104, 46–55 (2007).
11) Research report. Bull. Agr. Produc. Process. Res. Inst., Vol. 13, Aomori Pref. Industr. Tech. Res. Centr., Aomori, p. 42 (2019).
12) Liang T, Wei F, Lu Y, Kodani Y, Nakada M, Miyakawa T, Tanokura M. Comprehensive NMR analysis of compositional changes of black garlic during thermal processing. J. Agric. Food Chem., 63, 683–691 (2015).
13) Nifert N, Pannangrong W, Welbat JU, Chajaroonkanharak W, Sripanidkulchai K, Sripanidkulchai B. Neuroprotective effects of aged garlic extract on cognitive dysfunction and neuroinflammation induced by beta-amyloid in rats. Nutrients, 9, 24 (2017).
14) Kruse A, Dinjus E. Hot compressed water as reaction medium and reactant: properties and synthesis reactions. J. Supererit. Fluids, 39, 362–380 (2007).
15) Jacome LF, Gaultreux A, Inagaki T, Mohan G, Alves S, Lubbers LS, Luine V. Estradiol and ERbeta agonists enhance recognition memory, and DPN, an ERbeta agonist, alters brain monoamines. Neurobiol. Learn. Mem., 94, 488–498 (2010).
16) Hahn ER, Singh SV. Diallyl trisulfide inhibits estrogen receptor-alpha activity in human breast cancer cells. Breast Cancer Res. Treat., 144, 47–57 (2014).
17) Schwingshackl L, Missbach B, Hoffmann G. An umbrella review of garlic intake and risk of cardiovascular disease. Phytomedicine, 23, 1127–1133 (2016).