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Memory Enhancing and Antioxidant Properties of Fermented Chongmyung-tang

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Abstract – The Chongmyung-tang (CMT) has been used as an oriental herbal medicine for the purpose of enhanced learning and memory. Recently, since fermentation may give a positive effect on pharmacological actions of herbal medicine, many studies are focused to find fermented medicinal herbs with improved bioactivity. In the present study, memory enhancing, antioxidant and reducing power activity of CMT and fermented CMT with *Aspergillus oryzae* (FCMT-A) or *Saccharomyces cerevisiae* (FCMT-S) were determined. To evaluate the memory enhancing activities of CMT, FCMT-A and FCMT-S, we performed passive avoidance test using scopolamine induced amnesia model. Administration of CMT, FCMT-A and FCMT-S showed a significant memory enhancing effect about 72.5, 78.3, 71.8% of the normal group respectively. CMT, FCMT-A and FCMT-S also exhibited strong DPPH•, $\bullet O_2^-$, $NO^{\bullet}$, $ABTS^{\bullet+}$ scavenging activities and reducing power. It was also found that fermented CMT has slightly higher scavenging activities on DPPH•, $ABTS^{\bullet+}$ radicals compared to CMT. These results revealed that CMT, FCMT-A and FCMT-S had memory enhancing and radical scavenging activities. In addition, the fermentation of CMT was more or less important for elevated memory enhancing and antioxidant activities of CMT.
Keywords – Chongmyung-tang, Fermentation, Memory enhancing, Antioxidant

Introduction

Central cholinergic system is believed to be involved in learning and memory (Hasselmo, 2006) and dysfunction of cholinergic neurotransmitter system in the central nervous system (CNS) is one of the major factor of amnesia and age-related brain diseases (Fodale *et al*., 2006). Scopolamine is a non-selective muscarinic receptor antagonist, and therefore, many studies have been conducted scopolamine-induced amnesia model to investigate the effect of drugs on memory disorder induced by impaired function of cholinergic system.

Free radicals, a highly reactive molecules such as reactive oxygen species (ROS; $\bullet O_2^-$, •OH, H_2O_2) and reactive nitrogen species (RNS; $NO^{\bullet}$, HNO_2, $ONOO^-$) are generated as by-products during normal metabolism process under aerobic conditions. Since they can induce oxidative damage to biomolecules, the body has developed several antioxidant mechanism including SOD, GPx, catalase. However, an imbalance of prooxidants and antioxidants in the organism causes oxidative stress which raise the cell damages due to protein denaturation, lipid peroxidation and genetic variation (Halliwell, 1994). These damages could lead to pathological events such as aging, cancer, atherosclerosis, inflammation and diabetes mellitus (Dreher and Junod, 1996; Maxwell, 1997).

In particular, the brain and nervous system have a relatively weak free radical defense system. In addition, there is increasing evidences that ROS and RNS are implicated in many neurological pathogenesis such as Alzheimer's disease, Parkinson's disease, ischemia-reperfusion injury and stroke (Bland, 1995; Sen and Packer, 1996). Therefore, it seems quite reasonable to suggest that antioxidants, a free radical scavenger are a useful therapeutic agent for ROS-mediated neurodegenerative diseases with an ability to diminish oxidative stress in brain. Moreover, several studies have investigated the relation between fermentation and increased antioxidant activity (Georgetti *et al*., 2009; Chen *et al*., 2009; Lee *et al*., 2008).

Recently, many fermented foods have received the attention with functional properties on digestability, bioavailability and enhanced flavor. Chongmyung-tang (CMT) is an oriental herbal medicine, which has been frequently used with a beneficial effect in memory

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disorder and enhancing learning ability in Korea. CMT is consisted of three medicinal herbs of Polygalae Radix, Acori Graminei Rhizoma and Hoelen. Pharmacological studies of CMT are mainly focused on the neurodegenerative diseases and memory improvement. CMT have been found to possess neuroprotective activity (Kim *et al*., 2008; An *et al*., 2007; Yun *et al*., 2007), protective effect on Alzheimer's disease model (Gug *et al*., 2004), memory enhancing (Lee *et al*., 2006; Park *et al*., 2008) and inhibitory effect on acetylcholinesterase (Jung and Lee, 2006).

In view of the several reports of CMT described above, CMT was proposed to have protective effect on neurodegenerative diseases and it might be due to its antioxidant activity, at least in part. However limited information is available concerning the fermented CMT and its memory enhancing and antioxidant capacity. Hence, here in our study we evaluated the memory enhancing and radical scavenging activities of CMT and determined whether fermentation of CMT with *A. oryzae* or *S. cerevisiae* affect memory improvement and antioxidant activity.

Experimental

Microorganism – *A. oryzae* KCCM 11371 and *S. cerevisiae* KCCM 11693, obtained from the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea), was used in the experiments. These fungi and yeast were maintained on steamed-rice and YM broth (Yeast extract 13.0 g, Malt extract 3.0 g, peptone 5.0 g, dextrose 10.0 g/L) at 30 ºC for 3days.

Preparation of CMT and fermented CMT – The plant samples (Polygalae Radix 12 g, Acori Graminei Rhizoma 12 g, Hoelen 12 g) were obtained from the Hanul herb company, and a voucher specimen was deposited at the College of Pharmacy, Woosuk University. The plant samples were mixed with D.W and pre-cultures of *A. oryzae*. or *S. cerevisiae* were used to inoculate (1%, v/v). The fermentation was carried out during 72 h at 30 ºC and the mixtures were then heated at 105 ºC for 1 hour. After centrifugation, the supernatant were collected and freeze-dried.

Animals and drug treatment – Male albino mice (ICR), weighing 20 - 25 g, were used in this study. The animals were housed 5 per cage and allowed access to water and food *ad libitum* under controlled temperature (22 ± 2 ºC) and lighting conditions. CMT, FCMT-A and FCMT-S were dissolved in saline and administered orally in the dose of 300 mg/kg with a volume of 2.5 mL/kg and control group were treated equal volume of saline. Drug treatment was conducted 30 min before the acquisition trial.

Passive avoidance test – The passive avoidance test was performed by a step-through method. The shuttle box, consists of light and dark compartment, separated by automatic guillotine door. Briefly, In a training trial, a mouse was placed in the light room. After 10 sec, the door was opened and then shut again when the mouse had entered the dark room. The transfer latency time was recorded and the mice within 60 sec were used for the experiment. After training trial, we injected scopolamine (1 mg/kg). The acquisition trial was conducted 30 min after this injection. All the procedures were similar to the training trial, except for the punishment of 1.0 mA electrical shock when the subject transferred to the dark room. Twenty-four hours later, latency times to reenter the dark room were determined and the maximum observation time was 300 sec.

1,1-diphenyl-2-picrylhydrasyl (DPPH) radical scavenging assay – The scavenging effect of CMT and fermented CMT on DPPH radical was measured according to the method of Gyamfi *et al*. (Gyamfi *et al*., 1999) with some modification. A 5 μL aliquot of the different concentrations of samples were added to 495 μL of DPPH in absolute ethanol solution (0.25 mM). After incubation for 20 min, the absorbance of each solution was determined at 520 nm using microplate reader.

Superoxide scavenging by NBT method – The superoxide scavenging ability of CMT and fermented CMT was studied by xanthine/xanthine oxidase/NBT method according to Ibrahim *et al*. (Ibrahim *et al*., 2007) with some modification. The reaction mixture contained 0.5 mL of 1.6 mM xanthine, 0.48 mM NBT in 10 mM phosphate buffer (pH 8.0). After pre-incubation at 37 ºC for 5 minutes, the reaction was initiated by adding 1 mL of xanthine oxidase (0.05 U/mL) and incubation at 37 ºC for 20 min. The reaction was stopped by adding 1 mL of 69 mM SDS, and the absorbance at 570 nm was measured.

Nitric oxide radical scavenging assay – A 5 μL aliquot of the CMT and fermented CMT were added to 495 μL of sodium nitroprusside solution (5 mM). After incubation at room temperature for 150 min, 100 μL aliquots were removed from reaction mixture and incubated with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride, 2.5% H_3PO_4). The absorbance at 540 nm was determined and the standard was determined by using sodium nitrite.

Trolox equivalent antioxidant capacity (TEAC) – The experiments were carried out using a modified ABTS

decolorisation assay (Obo'n, *et al*., 2005) and it involves the generation of ABTS radical chromophore by the oxidation of ABTS with potassium persulfate. The ABTS radical cation was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stable for at least 16 h in the dark at room temperature before use. The ABTS radical solution was diluted to an absorbance of 0.70 ± 0.1 at 734 nm with distilled water. Absorbance was measured 3 min after the initial mixing of different samples with ABTS radical solution. Trolox, was used as a reference standard.

Reducing Power – The reducing power of CMT and fermented CMT were determined according to the method of Athukorala *et al*. (2006) with some modifications. CMT and fermented CMT (500 μg/mL) were was mixed with phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 mL, 30 mmol/L). The mixture was incubated at 50 °C for 20 min. A 2.5 ml TCA (0.6 mol/L) was added to the mixture, which was then centrifuged for 10 min at 12,000 rpm. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 6 mmol/L), and the absorbance was measured at 700 nm in a spectrophotometer.

Statistical analysis – All measurement are expressed as the mean ± S.D. of independent experiments. Data between groups were analyzed by a paired Student's *t*-test and *P*-values less than 0.05 were considered significant.

Results and Discussion

The present study was designed to evaluate the influence of CMT and fermented CMT with *A. oryzae* (FCMT-A) or *S. cerevisiae* (FCMT-S) on scopolamine-induced memory impairment and its radical scavenging activities. Cholinergic neurons in the CNS possess an important role in learning and memory (Bartus *et al*., 1982). Moreover, a major factor in age-related brain disease may be a disorder in the cholinergic neurotransmitter system. It is well known that scopolamine induces dysfunction of cholinergic activity and may causes impairment of memory and cognitive function. Weingartner, (1985) also reported that the young scopolamine-treated humans suffering from memory and cognitive deficits being similar to those exhibited by the elderly. Thus, scopolamine-induced amnesia model has been extensively used as an experimental tool for screening effective drugs on age-related brain diseases such as Alzheimer's diseases (AD) (Whitehouse *et al*., 1981).

Table 1. Effect of CMT and fermented CMT on scopolamine-induced amnesia model in mice.

Group	Scopolamine (1 mg/kg)	Latency time (sec)	
		Acquisition trial	Retention trial
Normal	–	20.57 ± 4.05	268.69 ± 19.91*
Control	+	21.02 ± 7.68	20.90 ± 8.56
CMT	+	25.04 ± 7.29	194.69 ± 23.45#
FCMT-A	+	28.74 ± 8.65	210.46 ± 24.84#
FCMT-S	+	20.32 ± 2.68	192.95 ± 27.44#

Each value in the table represents the mean ± S.E.M. *$p < 0.01$, #$p < 0.05$ (student's *t*-test) significantly different from control group.

Moreover, the passive avoidance test using scopolamine-induced murine amnesia model is a useful experimental method for the measurement of learning and memory performance.

Here in our study, the latency time of the normal group in retention trial was significantly increased about 13-fold compare to that of acquisition trial while the scopolamine-treated group did not showed any change of latency time between retention trial and acquisition trial. These results demonstrate that scopolamine induced a memory impairment by suppression of cholinergic neurotransmitter system. The group treated with CMT (300 mg/kg), FCMT-A (300 mg/kg) and FCMT-S (300 mg/kg) improved the reductions of latency time induced by scopolamine to 72.5%, 78.3% and 71.8% of the vehicle treated control group respectively. In comparison to CMT-treated group, administration of FCMT-A produced a slight increase of latency time in retention trial while, fermentation with *S. cerevisiae* did not show any increased latency time. However, this result was not statistically significant. Since increased latency time may reflect the improvement memory function, these results suggest that CMT, FCMT-A and FCMT-S have potent antiamnesic activity.

In the studies on the constituent herbs of CMT, polygalasaponin (Xue *et al*., 2009) and acylated oligosaccharides (Ikeya *et al*., 2004) from Polygalae Radix improve cognitive function. However, Hsieh *et al*. (2000) determined no action of Acori Graminei Rhizoma on scopolamine-induced amnesia model. Oh *et al*. (2004) also revealed that Acori Graminei Rhizoma and Hoelen did not affect the cholinesterase activity. Thus, it is strongly suggested that the components of Polygalae Radix such as polygalasaponin and acylated oligosaccharides are responsible for cognitive enhancing action of CMT. In addition, there are several reports on the beneficial effect of *A. oryzae* fermentation in soybean and ginseng which have lots of saponin and glycoside

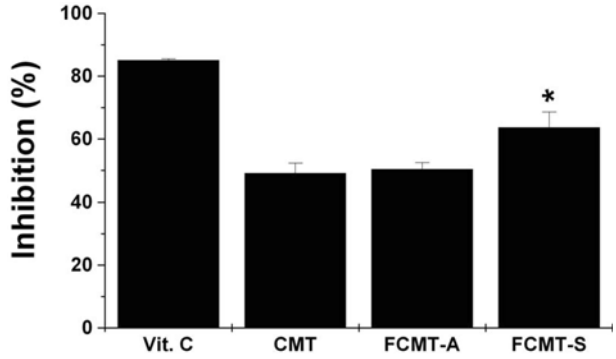

Fig. 1. DPPH radical scavenging effect of CMT and fermented CMT. The values are % inhibition compared with vehicle treated group. Ascorbic acid (100 μg/mL) used as positive control. CMT : Chongmyungtang (500 μg/mL); FCMT-A : fermented CMT with *A. oryzae* (500 μg/mL); FCMT-S : fermented CMT with *S. cerevisiae* (500 μg/mL). Results are means ± S.D of three repeated experiments and the symbol * represents statistically difference from CMT treated group (*$p < 0.01$).

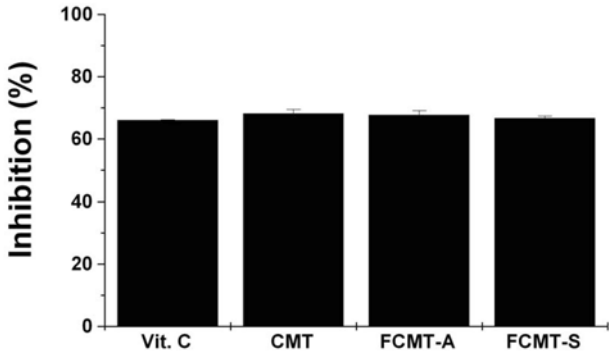

Fig. 2. Inhibition effect of CMT and fermented CMT on NBT reduction in the xanthine oxidase/xanthine system. The values are % inhibition compared with vehicle treated group. Ascorbic acid (100 μg/mL) used as positive control. CMT : Chongmyungtang (500 μg/mL); FCMT-A : fermented CMT with *A. oryzae* (500 μg/mL); FCMT-S : fermented CMT with *S. cerevisiae* (500 μg/mL). Results are means ± S.D of three repeated experiments.

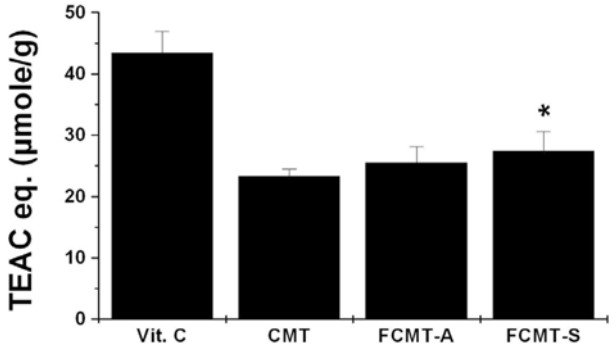

Fig. 3. Scavenging effect of CMT and fermented CMT on ABTS radical. The values are expressed as Trolox equivalent (μmole/g). Ascorbic acid (100 μg/mL) used as positive control. CMT : Chongmyungtang (500 μg/mL); FCMT-A : fermented CMT with *A. oryzae* (500 μg/mL); FCMT-S : fermented CMT with *S. cerevisiae* (500 μg/mL). Results are means ± S.D of three repeated experiments and the symbol * represents statistically difference from CMT treated group (*$p < 0.01$).

(Fernandez-Orozco *et al*., 2007; Hung *et al*., 2007; Hong *et al*., 2004; Kim *et al*., 2006). Therefore, the improved latency time of FCMT-A could be explained by increased biological activity through fermentation-induced structural modification.

Brain and nervous system could easily get damaged by free radicals due to several reasons. Not only has brain high concentration of polyunsaturated fatty acid, the most peroxidizable fatty acid, but it also has a high content of iron and copper ions which are used as cofactor in radical reactions. In addition, it is well known that brain utilizes extra high amounts of oxygen per weight (Floyd and Hensley, 2002). During this aerobic metabolic processes, just as other parts of the body, free radicals are generated in brain as well. High free radical formation and low radical protective defenses increase the oxidative stress of the brain (Harman, 1993) and leading to neurodegenerative diseases including ischemic stroke, Alzheimer's disease, Parkinson's disease, Lou Gehrig's disease, Huntington's disease and Cognitive dysfunction in the elderly (Gilgun-Sherki *et al*., 2001). Thus, the application of antioxidants with an ability to scavenge free radicals might be useful in oxidative stress-mediated neurodegenerative diseases.

The radical scavenging activity of CMT and fermented CMT was determined from the reduction of absorbance at 520 nm due to scavenging of stable DPPH free radical. Scavenging activity in fermented CMT with *S. cerevisiae* (FCMT-S) was higher ($p < 0.01$) than that in CMT and fermented CMT with *A. oryzae* (FCMT-A) (Fig. 1) and these results suggest that fermentation of CMT with *S. cerevisiae* increases the radical scavenging capacity. In the xanthine/xanthine oxidase-NBT system, the generated superoxide anion react with NBT and form a formazan. The reduction of absorbance at 570 nm represents the scavenging of superoxide anion by antioxidants. Fig. 2 shows strong superoxide radical scavenging ability of CMT and fermented CMT compared with ascorbic acid, a positive control. However, no significant differences found between CMT and fermented CMT treated group. The ABTS radical scavenging activities of CMT and fermented CMT are shown in Fig. 3. Like the DPPH radical scavenging abilities described above, fermentation with *S. cerevisiae* had a positive influence on ABTS radical scavenging. The unfermented CMT exhibited

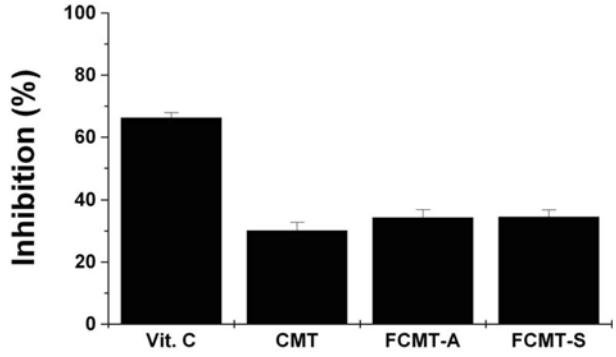

Fig. 4. Scavenging effect of CMT and fermented CMT on nitric oxide, generated with 5 mM SNP. The values are % inhibition compared with vehicle treated group. Ascorbic acid (100 μg/mL) used as positive control. CMT : Chongmyungtang (500 μg/mL); FCMT-A : fermented CMT with *A. oryzae* (500 μg/mL); FCMT-S : fermented CMT with *S. cerevisiae* (500 μg/mL). Results are means ± S.D of three repeated experiments.

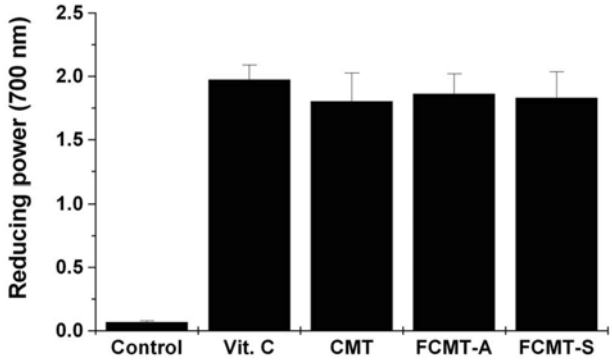

Fig. 5. Reducing power of CMT and fermented CMT. The values are absorbances at 700 nm. Ascorbic acid (100 μg/mL) used as positive control. CMT : Chongmyungtang (500 μg/mL); FCMT-A : fermented CMT with *A. oryzae* (500 μg/mL); FCMT-S : fermented CMT with *S. cerevisiae* (500 μg/mL). Results are means ± S.D of three repeated experiments.

ABTS radical scavenging activity of 23.3 μM/L trolox eq. while FCMT-A and FCMT-S showed that of 25.5 and 27.3 μM/L trolox eq. respectively. CMT and fermented CMT also quenched NO released by sodium nitroprusside (SNP), a NO donor (Fig. 4). Incubation of 5 mM SNP for 120 min produced about 40 μM of NO and it was decreased by CMT and fermented CMT. The inhibitory ratio (%) of CMT was 30.13 and that of FCMT-A and FCMT-S were 34.27 and 34.57 respectively. The fermented group showed slightly increased NO radical scavenging activity compared to unfermented group without significant differences. Superoxide anion may react with NO radical resulting in the formation of peroxynitrite ($ONOO^-$), a powerful oxidant. In the present study, CMT and fermented CMT showed potent radical scavenging activities on superoxide anion and NO radical, and therefore, they may suppress peroxynitrite generation. Fig. 5 shows that all of the groups have significant reducing capacity and it was similar to ascorbic acid, a positive control. However, the influence of fermentation on reducing capacity was not detected.

The values obtained with several radical scavenging assay showed that CMT and fermented CMT have strong antioxidant properties as natural ROS scavenger and this result is supported by several reports about the antioxidant activities of the consists of CMT including Polygalae Radix, Acori Graminei Rhizoma and Hoelen (Park *et al*., 2000; Goo and Lee, 2001; Kim *et al*., 2002). In addition, FCMT-S has slightly higher scavenging activities on DPPH•, $ABTS^{\bullet+}$ radicals compare to CMT. These results revealed that the fermentation of CMT with *S. cerevisiae* was more or less important for increased antioxidant properties of CMT.

As described above, oxidative stress in brain is associated with age-related neurodegenerative diseases. Moreover, El-Sherbiny *et al*. (2003) noted that dysfunction of memory induced by scopolamine administration in rats is involved in altered levels of GSH and with the activities of antioxidant enzymes in the brain. It is also demonstrated that scopolamine administration causes an increase in TBARS, an important marker for lipid peroxidation, and decrease in both glutathione reductase and SOD activities in the cortex and hippocampus of amnesic mice (Jeong *et al*., 2008). Therefore, we suggest that protective activities of CMT, FCMT-A and FCMT-S against oxidative damage in brain induced by scopolamine may be involved in its antiamnesic activity in scopolamine-induced amnesia model.

Hence in the present study, we demonstrated that CMT, FCMT-A and FCMT-S had potent memory enhancing in scopolamine-induced amnesia model and antioxidant properties by scavenging DPPH•, $\bullet O_2^-$, $NO^{\bullet}$, $ABTS^{\bullet+}$ radicals with strong reducing power. In addition, our results revealed that fermentation of CMT has some positive effect on the memory enhancing and antioxidant activities.

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