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Study on the Ability of Extracts from C*ordyceps* Spp. Biomass to Prevent Long-Term Memory Impairment in Mice by Morris Water Maze

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The ability of three mycelial extracts of *Cordyceps* spp. (Poly DL0004, n-BuOH DL0015 and n-BuOH DL0006) to prevent long-time memory impairment of mice were tested by using Morris water maze model. Mice were induced memory impairment by Trymethyltin (TMT) as dose of 2.4 mg/kg (i.p. injection); then fed by solution of *Cordyceps's* mycelial extract as doses of 100 mg/kg (p.o) and 200 mg/kg (p.o); and test in Morris water maze model. The behavioral test of those mice was illustrated by concentration of neurotransmitter Acetylcholine (ACh) and its degraded enzyme Acetylcholinesterase (AChE) in their hippocampus. As results, Poly DL0004 and n-BuOH DL0015 had best effects on memory improvement at doses of 100 mg/kg in hidden platform test and probe test but no effect on working memory test. The concentrations of ACh and AChE in mice hippocampus were significant different between mice fed by *Cordyceps's* mycelial extract and negative control mice injected TMT dose of 2.4 mg/kg. No difference of concentrations of ACh and AChE were recorded between mice fed by extracts dose of 100 mg/kg and 200mg/kg. The memory improvement effects of *Cordyceps's* mycelial extract were the first step to potentially apply *Cordyceps* products for human healthcare in Vietnam

Keywords: Acetylcholine, Acetylcholinesterase, *Cordyceps*, memory impairment, Morris water maze.

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Introduction

Alzheimer's disease (AD) is one of the most common causes of mental deterioration in elder people (Paul *et al.*, 1999), AD's symptoms are memory or cognitive impairment, emotional and behavioral disorder. Currently, causes and progression of AD haven't been understood yet and there isn't really effective way to prevent the disease although having some hypothesis about AD causes. Currently, the basic of AD treatment method mainly relies on cholinergic hypothesis, which says that AD is caused by decrease in synthesis of the neurotransmitter (ACh), and increase in AChE activity in hippocampus. Many current drugs for AD only focus to reduce the progression of the disease rather than prevent degradation of nerve cells in hippocampus, one of the first injured areas of the brain in AD patients. When the nerve cells of the hippocampus died, secreted ACh was not enough to transmit information. In the new generation of drugd, anti-acetylcholinesterase group are used to prevent the decomposition of ACh for enhancing the nerved synaptic contacts.

Cordyceps is a rare medicinal herb in traditional Chinese, whose chemical composition was studied to contain all of the essential amino acids, vitamins, sugars, many complex polysaccharides, proteins, sterols, nucleosides, macro and microelements (Holliday and Cleaver, 2008). Some potentially bioactive constituents also were recognized: cordycepin, cordycepic acid, nucleosid, ergosterol, Δ-3 ergosterol, ergosterol peroxid, 3-sitosterol, daucosterol, campeasterol, etc. (Paterson, 2008). Nucleosid was recorded as a substance related to effects in regulating and adjusting various physiological processes in the nervous system. Nucleosides, especially adenosine used as markers of *Cordyceps*. Adenosine release of various neurotransmitters presynaptically and anticonvulsant activity (Li *et al*,. 2006). Inosine, a breakdown product of adenosine, recently has been shown to exert immunomodulatory and neuroprotective effects. Study in mice showed that the oral administration of inosine has antidepressant (Junko *et al.*, 2014). In addition, adenosine also stimulates axon growth *in vitro* and in the adult central nerve system and improves outcome in a rat stroke model (Irwin *et al.*, 2006; Lorber *et al.*, 2009). Additional benefits of inosine after brain injury include its anti-inflammatory effects (Hasko *et al.*, 2004), its ability to suppress glutamate-induced neural excitation (Shen *et al*., 2005); to enhance the ability of undamaged neurons to extend axon collaterals into areas that have lost their normal innervation (Peng *et al*., 2002; Zai *et al*., 2009).

In Vietnam, many researches about *Cordyceps* have focused on isolation and cultivation while bioactivity or pharmacology are limited. In our previous studies (Quyen *et al*., 2012), three extracts polyDL0004, n-BuOH DL0006 and n-BuOH DL0015 have the highest inhibited effect on acetylcholinesterase activity among 60 mycelial extract of *Cordyceps* spp. Poly DL0004 and n-BuOH DL0015 have also the positive effect on short term memory of mice in Y maze model and Novel object model (Quyen *et al*., 2014). Continuously in this research, we aimed to study the ability of Poly DL0004, n-BuOH DL0015 and n-BuOH DL0006 to prevent long-time memory impairment of mice.

Materials and methods

Animals

All the experiments were carried out using male of *Swiss albino* mice provided by Pasteur Institute of Ho Chi Minh City. All in same age of 5-6 weeks, weigh of 25-30 g and strong healthy. Mice was kept by group of 6 individual in plastic box (white, 28 cm× 30 cm× 15 cm). After moving to laboratory, mice were kept in 3 days before doing experiments.

Mice were fed by animal food (content of rice bran, rice flour, corn starch and vitamins) which provided by Institute of Vaccine and Biomedical Products in Nha Trang City.

Extracts

All extracts were conducted from *Cordyceps* spp.'s dried mycelial biomass at Nguyen Long Joint Stock Company, Lam Dong Province following previous studies (Quyen *et al.*, 2012, 2014). All *Cordyceps* strains used in this research were collected in southern Vietnam.

Abbreviation

DL0015, DL0006 and DL0004 mean name of *Cordyceps* strains which mycelial biomass used to extract. n-BuOH and poly mean the extraction's fragment, n-BuOH is the final extract conducted by n-Butanol in liquid phase, poly is polysaccharide extract from solid phase. p.o. means per os/per oral. i.p. means intraperitoneal injection.

Model

The Morris water maze was design as a circular pool (1.5 m diameter, 0.8 m height). The pool was made from stainless steel with a black inner surface; conceptually divided into quadrants such as northeast, southeast, northwest, and southwest; and filled to a depth of 25.1 cm with water. A small platform (12 cm in diameter and 25 cm in height) was placed in one of the pool quadrants; submerged 2 cm below the water surface; and invisible at water level. Four images were placed at four different positions around the pool as the oriented objects for mice.

Preparing for Moriss Water Maze test

Mice were divided into 9 groups, each group included 10 mice. At 10 days before test, 7 groups of mice were drunk Galantamine (Gal.) or *Cordyceps*'s extracts following: Gal. (10 mg/kg, p.o) as a positive control, DL0004.1 (poly DL0004, 100 mg/kg, p.o); DL0004.2(poly DL0004, 200 mg/kg, p.o); DL0006.1 (n-BuOH DL0006, 100 mg/kg, p.o), DL0006.2 (n-BuOH DL0006, 200 mg/kg, p.o), DL0015.1(n-BuOH DL0015, 100 mg/kg, p.o), DL0015.2 (n-BuOH DL0015, 200 mg/kg, p.o). The Saline solution (Sal.) group and negative control group were kept normally. At 7 days before test, Sal. group was injected saline solution (i.p injection) while all other groups were induced memory impairment by Trimethyltin (TMT) injection (dose of 2.4 mg/kg, i.p. injection).

The Moriss Water Maze test

The test was conducted in total 8 days. During the test, the pool was strictly monitored temperature in the range 24 - 28^{o}C, in darkness as 30 - 40 lux, and quiet condition. Investigators were hidden from the view of the mice in all test.

Hidden platform test (4 days, 1st day to 4th day): 5 trials were carried out every day, maximum time for each trial is 60 s with the same release points for all mice. In each trial, mice were let to swim as maximum time as 60 s. If couldn't find platform after 60 s, mice were guided to the platform and located in it for 15 s. If be able to reach the platform in 60 s, mice was let to remain on it for 15 s. Next trials were carried similarly with different released point. All test were recorded by video camera system

Probe test (1 day, 5th day): Platform was taken out of the pool, 2 trials are carried out in that day. Trials were conducted similarly the Hidden platform test. Recording the times that mice swim across the previous location where the platform was placed.

Working memory test (3 days, 6th day to 8th day): 5 trials are carried out each day. Trials were conducted similarly the Hidden platform test with the platform position changed every day. The time mice taken to find the platform was recorded.

Quantify ACh, AChE concentration and total protein of mice brain hippocampus

Mice were divided into 9 groups, each group included 8 mice. Before testing 5 days, all groups were treated similarly as the preparation at 10 days before testing in Morris water maze. Then, at 2 days before test, all groups except Sal. group were induced memory impairment by Trimethyltin (TMT) injection (dose of 2.4 mg/kg, i.p. injection). Sal. group was injected saline solution (i.p injection). Finally, hippocampus were collected from mice, grinded with PBS pH 7.4 (1:20), collected suspension, centrifuged 12000 rpm in 15 minutes at 4 ^{o}C, and got homogeneous solution contented ACh and AChE. ACh and AChE activity was detected by Amplex Red kit. Simultaneously, protein concentration of hippocampus was also determined by Bradford method.

Statistics

Values are expressed as mean ± SE. Data was analyzed by One-way analysis of variance (One way ANOVA) and Fisher protected least significant difference (PLSD).

Results and Discussions

Morris water maze

Hidden platform test

The change of time to find the platform in hidden platform test was shown in Fig. 1. The time to find the hidden platform of Sal. (Positive control group) is lowest and declines through testing days. By statistical analysis, there is significant difference between TMT and Sal. ($p < 0.001$), this demonstrated TMT group exhibited memory impairment.

There is a significant difference in time to find hidden platform ($p < 0.001$) between Gal. and TMT groups. It means Galantamine has effect to attenuate memory impairment, and acted as positive control in this test.

Following a significant difference in how long for mice to find the platform between first day and second day ($p < 0.001$), second day and third day ($p < 0.01$), third day and fourth day ($p < 0.01$), the time factor (testing day) could effect to the change (declining) of the time to find the platform of mice. Through training process with unchanging platform position during all trials, mice progress in shortening the time to find the platform.

There is significant difference between DL0004.1 and TMT ($p < 0.05$), DL0015.1 and TMT ($p < 0.01$). Thus, mice of two groups DL0004.1 and DL0015.1 have a memory improvement in this test. Therefore, extracts of Poly DL0004 and n-BuOH DL0015 proved protective effects on preventing memory impairment of mice at dose of 100 mg/kg.

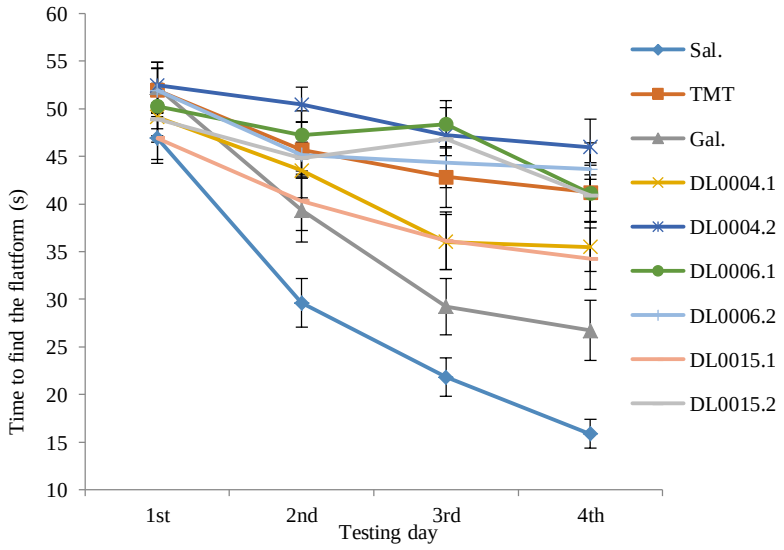

Fig. 1. The change of time when mice used to find the hidden platform among experiment groups. Gal,, TMT, Sal,, DL0004.1, DL0004.2, DL0006.1, DL0006.2, DL0015.1, DL0015.2 is the groups of mice which prepared following *2.2.1*

Probe test (Figure 2A)

The number of times which mice swim across the platform position of Sal. group is highest. Comparing to TMT group, Gal., DL0004.1 and DL0015.1 groups have significant difference in a number of times which mice swim across the platform position with $p < 0.001$, < 0.05 and < 0.05 in sequentially. Combining with hidden platform test, polyDL0004 and n-BuOH DL0015 extract should effect to memory improvement of mice in dose of 100mg/kg, p.o.

Working memory test (Figure 2B)

In this test, there is a change of platform position among quadrants of the pool. The results showed that there is no significant difference when comparing other groups with TMT (unless Sal. and Gal. group). This means dosage of extracts and model of preventing disease are not enough to maintain effect to protect brain.

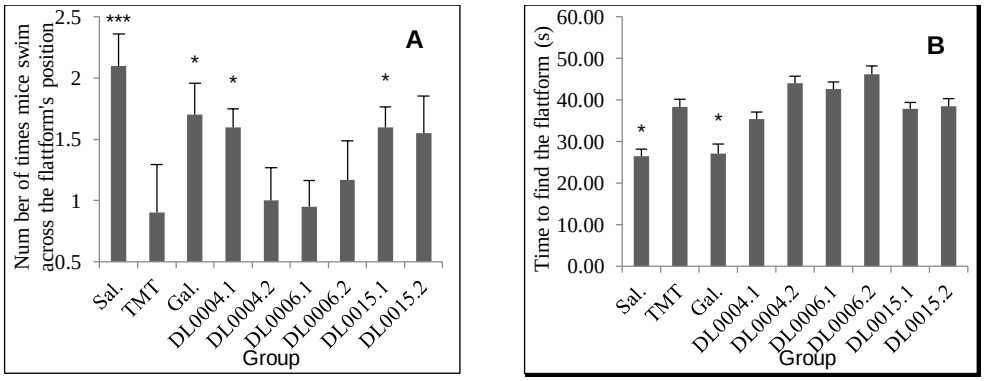

Fig. 2. The change in the number of times mice swim across the platform position on fifth day of probe test (A), and the difference in time to find the platform on sixth, seventh and eighth day in working memory test (B). Statistical significant difference compared with TMT group: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

In previous experiments, evaluating by Y-maze and Novel object recognition models, the results showed that dose of 100 mg/kg of Poly DL0004 and n-BuOH DL0015 extract has also an effect on preventing from memory impairment caused by TMT (Quyen *et al.*, 2014). However, in the working memory test, this effect was not detected. We suggest that dose of extracts should be changed (50 mg/kg or 150 mg/kg, p.o) to find better dosage.Otherwise increasing the number of days for treatment more than 3 days, that can be 7 or 14 days; or time point for behavioral test were started, extract oral therapy will be still continuous.

ACh and AChE concentration in hyppocampus (Fig. 3A)

The significant difference in ACh concentration between TMT and Sal. ($p < 0.01$) showed that ACh concentration of TMT group declined (Fig. 3A). This demonstrated TMT is an agent causing memory impairment. Between Gal. and TMT group ($p < 0,001$), Gal. group has a better ACh concentration, so galantamine has positive effect on mice.

In addition, comparing TMT with other groups, results are also different from DL0004.1 ($p < 0.001$), DL0004.2 ($p < 0.01$), DL0006.2 ($p < 0.01$). Specially, ACh concentration of DL0015.1 group has no significant difference comparing with TMT group. Although dose of extracts of DL0004.2 and DL0006.2 group doesn't affect on behavior tasks, ACh concentration still be remained. This result showed cholinergic hypothesis also has a limited role on enhancing memory in mice.

AChE concentration in hippocampus (Fig. 3B)

AChE concentration of TMT group is highest, and significantly differentiate to other groups (unless DL0006.1). According to this results, AChE activity could be increased when induced with TMT.

ACh and AChE concentration showed no correlatation with behavioral tasks. The mechanism of preventing memory impairment in mice is very complex. It might be beyond the influence of the neurotransmitter (ACh) with several other mechanisms.

Concentration of ACh and AChE in hippocampus of mice in different dosages of same extract had no significant significance (between DL0004.1 and DL0004.2; DL0006.1 and DL0006.2, DL0015.1 and DL0015.2).

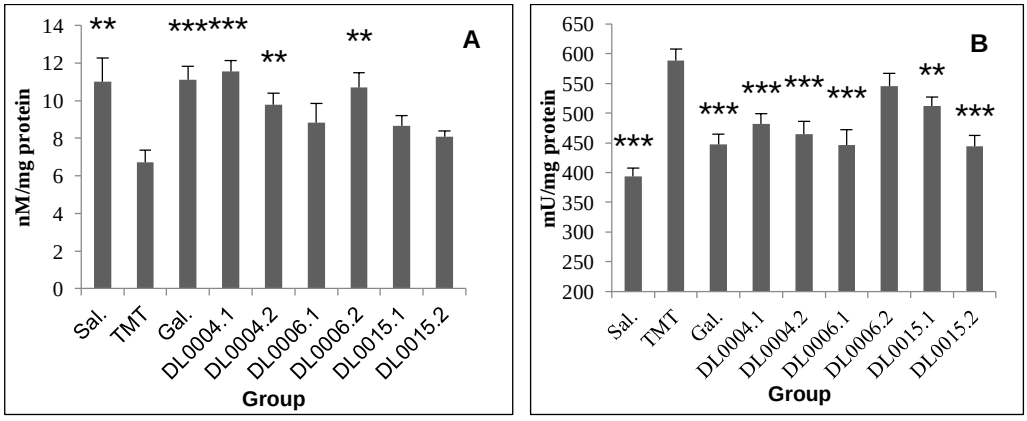

Fig. 3. The difference in acetylcholine concentration (nM/mg protein) (A) and acetylcholinesterase concentration (mU/mg protein) (B) of hyppocampus among testing groups. Statistical significant difference compare with TMT group: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Some studies showed that the cause of AD is related to oxidative stress, the injured brain area has many free radicals (Orhan *et al.*, 2011; Windelborn *et al.*, 2008). Therefore, the drugs used to treat AD must have high antioxidant activity. In 2007, Yu *et al.* (2007) compared ability against oxidative damage between

two strains of cultured *C. militaris* and natural *C. sinensis* on lipids, proteins and lipoproteins, the results show that both of them contain high antioxidant activity. In another research, Gu *et al.* (2007) concluded both natural and cultured *Cordyceps* have the potential to product antioxidant compounds. Along with the research about antioxidant activity of extracts from *Cordyceps* isolated and cultured in Vietnam, extracts by n-buthanol and the polysaccharide extract also have high antioxidant activities (Zai *et al*., 2009). Parallel to results above, it showed positive effects of extracts on the brain in mice can through not only anti-acetylcholinesterase but also antioxidant activity.

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