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Neuroprotective Effect of *Yukmijihwang-tang(Liuweidihuangtang) Gamibang* on the Deficits of Learning and Memory in Trimethyltin-Intoxicated Rats

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트리메틸틴에 의해 유도된 흰쥐의 학습과 기억력 손상에 대한 육미지황탕가미방의 신경보호 효과

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목적 :
이 연구는 알쯔하이머 병에 대한 육미지황가미방의 효과를 조사하였다. 육미지황가미방은 산약, 산수유, 복령, 목단피, 구기자, 택사, 숙지황을 포함한 여러 가지 한약재의 전탕액으로 치매의 한약 치료제로 널리 사용되어 왔다.

방법 :
이 약물의 신경보호 작용을 조사하기 위해, 수중미로를 사용하여 학습과 기억에 대한 육미지황가미방의 효과를 평가했고, 트리메틸틴으로 신경과 인지 장애를 유발시킨 쥐의 해마의 중추성 콜린계에서의 작용을 연구했다. 트리메틸틴은 강력한 유독물질로 선택적으로 중추신경계와 면역계의 세포를 파괴시킨다. 트리메틸틴(6.0 mg/kg, i.p.) 주입 후, 쥐에게 육미지황가미방(400 mg/kg, p.o.)를 2주 동안 날마다 복용시켰으며, 수중미로를 수행시켰다.

결과 :
육미지황가미방을 트리메틸틴에 노출시킨 쥐에 투약했으며, 그들은 수중미로에서 학습과 기억의 향상을 보였고, 이는 육미지황가미방이 어떠한 환경에서는 트리메틸틴으로 유발된 신경퇴화 후 중추신경계의 결손을 감소시킬 수 있음을 보여준다.

결론 :
이러한 결론은 육미지황가미방이 인지능력을 증가시키고, 트리메틸틴으로 유발된 신경퇴화에서 콜린 아세틸전환효소의 정도를 변화시킬 수 있음을 보여준다.

주제어 :
트리메틸틴, 육미지황가미방, 학습과 기억, 신경퇴행, 콜린 아세틸전환효소

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I. Introduction

The consistent findings in Alzheimer's disease (AD) patients are impairment in cognitive performances such as attention, learning and memory, and change of cholinergic markers including levels of acetylcholine (ACh) and ChAT[1-3].

Since cholinergic deficits are found to be associated with cognitive decline or dementia, increase of the central cholinergic system by selective inhibition of cholinesterase is one of the more promising current therapeutic tools for treating AD[4].

TMT is an organotoxin compound with potent neurotoxicant effects. This substance is regarded as being particularly useful for studying the response to injury on account of the distinct pattern of degeneration it causes in rodent brain. In particular, the rat hippocampus constitutes the most suitable model for TMT-induced brain injury. The molecular basis of selective vulnerability of specific neuronal populations to neuronal insults has been a key focus in neurology and neuropathology. TMT-induced neurodegeneration is characterized bymassive neuronal death, mainly localized in the limbic system, especially in the hippocampus, accompanied by reactive gliosis, epilepsy and marked neurobehavioral alterations, and is considered a useful model of selective neuronal death[5-12].

Intoxication with TMT leads to profound neurodegeneration and behavioral and cognitive deficits in both humans and experimental animals[13]. In rats, TMT induces the degeneration of pyramidal neurons in the hippocampus and cortical areas (pyriform cortex, entorhinal cortex, subiculum) connected to the hippocampus, but there is also neuronal loss in the associatiated areas.[5,14-16] Furthermore, behavioral studies have shown increased locomotor activity, disruption in self-grooming and learning deficits in TMT-intoxicated rats[17-25]. TMT intoxication impairs the acquisition of water maze and Biel maze (water avoidance) task as well as Hebb-Williams maze and radial arm maze performance[11,26-29] .

Herbal medicines, such as Ginko biloba, Ginseng, or Melisa officinalis, have been commonly used as memory or cognition enhancers. The effects of these enhancers have been demonstrated scientifically.[34,35] *Yukmijihwang-tang* (YMJ or *Liuweidihuangtang*) is another memory or cognition enhancer. *Yukmijihwang-tang* (YMJ or *Liuweidihuangtang*) is composed of 6 herbal medicines, including steamed *Rehmannia radix*, *Discoreae radix*, *Corni fructus*, *Hoelen*, *Mountain cortex radicis*, and *Alismatis radixand* has long been applied in the treatment of diabetes mellitus and neurosis. *Yukmijihwang-tang* (YMJ or *Liuweidihuangtang*) is composed of 7 herbal medicines which are 6 herbal medicines of YMJ and *Lycii fructose*. YJG has a significant effect on memory enhancement and the expression of genes associated not only with the prevention of neuronal degeneration but also with neuronal growth events.[36] Recent double-blind placebo-controlled trials have

also demonstrated that YJG significantly enhances cognitive abilities in normal human subjects.[37] However, there have been few reported studies on accessing learning and memory enhancement triggered by treatment with YJG in dementia animal models. Thus, we examined the effect of YJG on learning and memory ability in TMT-induced amnesia rats using the Morris water maze, and the relationship between the cholinergic marker in the Hippocampus and the neural mechanism underlying its improving effect on memory is discussed.

II. Experiment

A. Materials

1. Animals

Male Sprague-Dawley rats weighting 250-280 g each were purchased from Samtaco Animal Corp. (Kyungki-do, Korea). The animals were allowed to acclimatize themselves for at least 7 days prior to the experimentation. The animals were housed in individual cages under light-controlled conditions (12/12-hr light/dark cycle) and at 23℃ room temperature. Food and water were available ad libitum.

2. Preparation of Yukmijihwang-tang(Liuweidihuangtang) gamibang

The prescription and the ratio of each component in YJG are shown in Table 1.

3. Reagents

Trimethyltin chloride (TMT; Sigma-Aldrich Inc., St. Louis, MO)

Cholinacetyl transferase (ChAT; Cambridge Research Biochemicals, Wilmington, DE)

ABC complex Kit (Vectastain Elite Kit; Vector Lab., Burlingame, CA, USA)

4. Apparatus

Brain matrix (ASI Instruments, Warren, MI, USA)

pH meter (Corning Inc., USA)

Peristaltic Pump (Gilson Inc., France)

Heating pad (Biomed S.L., Spain)

Hamilton syringe (Reno, NV, USA)

Microinjection pump (Pump 22; Harvard Apparatus, South Natick, MA, USA)

Cryostat (Leica, Germany)

Scion image program (Scion Corp., Frederick, MD, USA)

S-MART program (S-MART; Pan-Lab, Barcelona, Spain)

Axio Vision 3.0 imaging system (Zeiss, Oberkochen, Germany)

B. Methods

1. Experimental design

The rats were divided into three groups (n=10 per group): the normal group (untreated animals), the TMT-lesioned +saline group (1 ml/kg per day, the control group) and TMT-lesioned + YJGgroup (400 mg/kg treated animals, the YJG group). The rats were injected intraperitoneally (i.p.) with TMT (6.0 mg/kg, body weight) dissolved in 0.9% saline and then returned to their home

cages. YJG was orally administered for two weeks after TMT-induced neurodegeneration. The water maze test was performed for one week from the 15th day after the injection of TMT.

2. Water maze test

The swimming pool of the Morris water maze was a circular water tank 200 cm in diameter and 35 cm deep. It was filled to a depth of 21cm with water at 23 ± 2℃ A platform 15 cm in diameter and 20cm in height was placed inside the tank with its top surface being 1.5 cm below the surface of the water. The pool was surrounded by many cues that were external to the maze. A CCD camera was equipped with a personal computer for the behavioral analysis. Each rat was received four daily trials. For 6 consecutive days, the rats were tested with three acquisition tests. They also received retention tests on the 7th day. For the acquisition test, the rat was allowed to search for the hidden platform for 180 seconds and the latency to escape onto the platform was recorded. The animals were trained to find the platform that was in a fixed position during 6 days for the acquisition test, and then for the retention test, they received a 1 min probe trial in which the platform was removed from the pool. The interval time was 1 min. Performance of the test animals in each water maze trial was assessed by a personal computer for the behavioral analysis (S-mart program, Spain).

3. ChAT and cAMP immunohistochemistry

At the end of the behavioral observation, the rats were deeply anesthetized with a sodium pentobarbital (100 mg/kg, i.p.) and then perfused through the ascending aorta with normal saline (0.9%), followed by 900 ml 0f 4% paraformaldehyde in 0.1 M phosphate-buffered saline(PBS). The brains were removed, post-fixed overnight and cryostat as 30-um coronal sections, which were processes immunohistochemically as free-floating sections.

The brain sections were washed in PBS containing 0.3% Triton X-100. The primary rabbit polyclonal antibodies against the following specific antigen were used: ChAT or cAMP (concentration 1:2000; Cambridge Research Biochemicals, Wilmington, DE). The primary antibodies were diluted with blocking solution (10% fetal bovine serum in PBS, pH=7.4) and the tissues were incubated for 72 h at 4℃ with constant agitation. Following rinsing in PBS, the sections were incubated for 2 h at room temperature in biotinylatedrabbit anti-serum (Vector Laboratories, Burlingame, CA) that was diluted 200:1 in PBST containing 2% normal rabbit serum. The sections were placed in Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA) for 2 h at room temperature. Followinga further rinsing in PBS, the tissue was developed using diaminobenzidine chromogen with nickel intensification. The sections were mounted on gelatine-coated slides, air-dried and coverslipped for microscopic observation.

C. Statistical analysis

The data were presented as means ±S.E. Behavioral data (acquisition and retention data) were statistically analyzed by ANOVA testing with repeated measures on the time factor. Immunohistochemical data were analyzed by one-way ANOVA testing. The origin of the significant effects was further examined by post-hoc comparisons using the Tukey technique. The criterion for statistical significance was considered to be P values <0.05.

Table Ⅰ. The Contents of YJG and the Amounts of Standard Materials

Herbal Medicines	Dosage(g)
Rehmanniae radix Preparant (熟地黃)	16
Cornii fructus (山茱萸)	8
Discoreae radix (山藥)	8
Hoelen (茯苓)	6
Mountain Cortex radix (牧丹皮)	6
Alismatis radix (澤瀉)	6
Lycii Fructus (枸杞子)	6
Total amount	56

III. Results

A. Effect of YJG on performance in water maze task

The control group showed a worse performance than did the normal group ($p<0.05$ at the Day 1, 2, 3 respectively). The latency to find the hidden platform of the normal and YJG were significantly decreased compared with the control group. TMT severely impaired the rats'spatial cognition in the water maze test, and the YJG were ameliorated TMT induced learning and memory deficits in the water maze ($p<0.05$ at the Day 1, 2, 3, $p<0.01$ at the Day 4, respectively) (Fig. 1.)

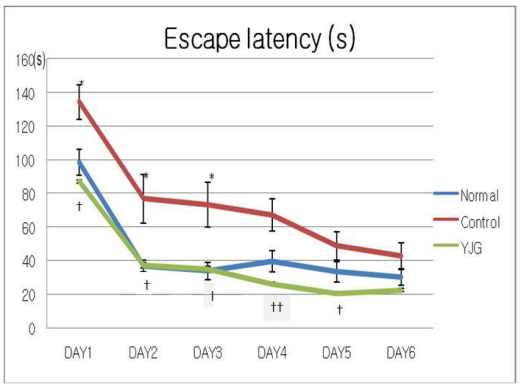


Fig. 1. The latency to escape onto the hidden platform during the Morris water maze. The task was performed with 3 trials per day during 6 days for the acquisition test. The values are presented as means ± S.E.M. * $p<0.05$, compared with the normal group, † $p<0.05$, †† $p<0.01$, compared with the control group.

The time spent around the platform was 3.25 ± 0.84% in the normal group, 1.93 ±0.53% in the control group and 5.77 ±0.95% in the YJG. The normal and YJG spent more time around the platform than did the control group. The YJG showed a significantly increase ($P<0.001$) in retention time compared to the control group (Fig. 2).

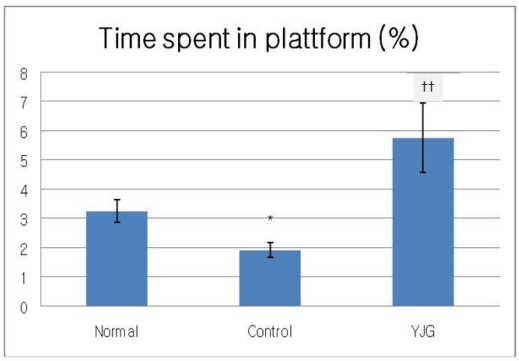


Fig. 2. Retention performance was tested on 7th day. The rats received a 1 min probe trial in which the platform was removed from the pool for retention testing. The values are presented as means ± S.E.M. * p<0.05, compared with the normal group, † † p<0.01, compared with the control group.

B. ChAT immunoreactive neurons of the hippocampus

The mean numbers of ChAT-positive neurons (number/4 cm^2) in the examined regions of the CA1 and CA3 were 20.33 ± 6.1 and 17 ± 3.2 for the normal group, respectively; and 16.5 ± 0.3 and 16.5 ± 2.1 for the control group, respectively. ChAT-positive neurons in the controls significantly reduced compared normal in CA1 (P<0.05). However, for the YJG group, the mean numbers of ChAT-positive neurons were 20 ± 3.8 and 16 ± 3.6 in each region, respectively. The density of ChAT-positive neurons of the YJG group in the hippocampus was increased compared to the control group in CA1 (P<0.05) as seen in Fig. 3, 4.

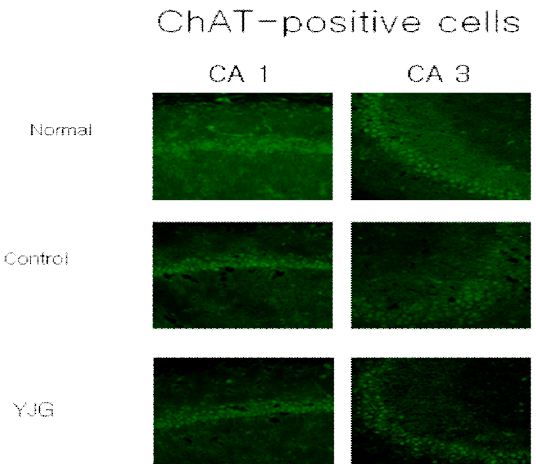


Fig. 3. Photographs showing the distribution of ChAT immunoreactive cells in the hippocampal CA1 and CA3 region of normal, control and YJGgroup after TMT-induced neurodegeneration. Sections were cut coronally 30 μm and the original magnification 200X.

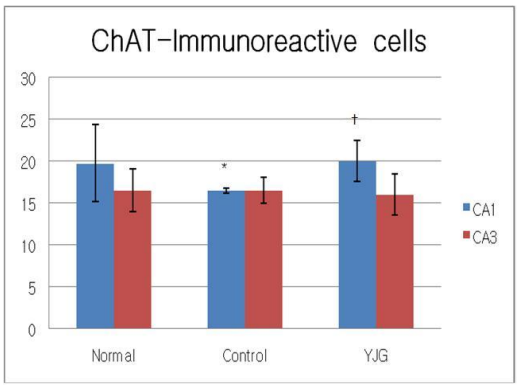


Fig. 4. The number of ChAT (ChAT) immunostained nuclei in different hippocampal CA1 and CA3 of the experimental groups. Each values represents the ±S.E.M. * p<0.05, compared with the normal group, † p<0.05, compared with the control group.

C. cAMP immunoreactive neurons of the hippocampus

The mean numbers of cAMP-positive neurons (number/4cm^2) in the examined regions of the CA1 and CA3 were 19.5 ± 1.2 and 25.3 ± 1.65 for the normal group, respectively; and 15.6 ± 1.3 and 18.9 ± 1.2 for the control group, respectively. cAMP -positive neurons in the controls significantly reduced compared normal in CA1and CA3 ($P<0.05$). The mean numbers of cAMP -positive neurons of the YJG group were 18.5 ± 1.5 and 19.25 ± 1.7 in each region, respectively. The density of cAMP -positive neurons of the YJG group in the hippocampus was not significantly different from the control group in CA1 and CA3 as seen in Fig. 5, 6.

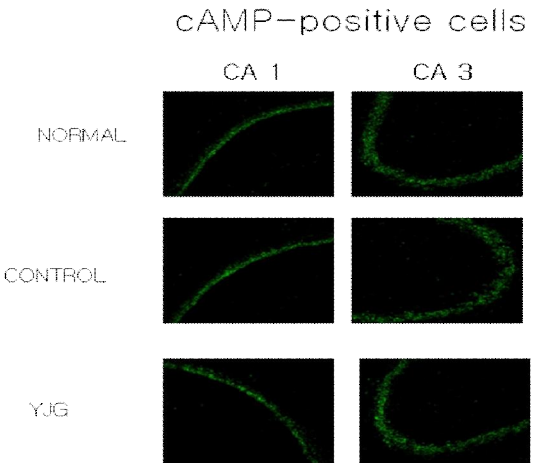


Fig. 5. Photographs showing the distribution of cAMP immunoreactive cells in the hippocampal CA1 and CA3 region of normal, control and YJGgroup after TMT-induced neurodegeneration. Sections were cut coronally 30 μm and the original magnification 200X.

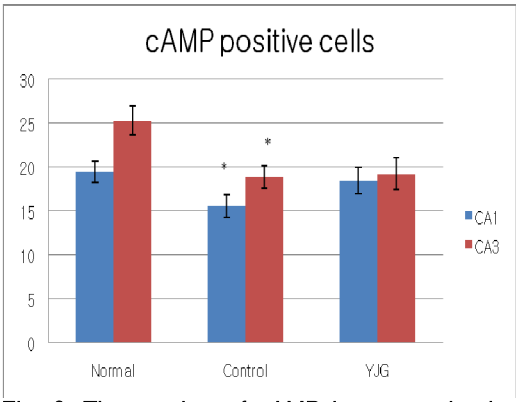


Fig. 6. The number of cAMP immunostained nuclei in different hippocampal CA1 and CA3 of the experimental groups. Each values represents the ±S.E.M. * $p<0.05$, compared with the normal group.

IV. Discussion

The present results demonstrated that TMT produced severe deficits in the performance on the Morris water maze and hippocampal cell losses. Pretreatment with YJG ameliorate learning and memory deficits in Morris water maze and have a protective effect against TMT-induced cell damage in the brain.

TMT intoxication produces deficits in passive avoidance retention, but not in the acquisition of the passive avoidance response[27,29,20,31]. Furthermore, deficits in acquisition of active avoidance at the beginning of training have been reported[20]. Moreover, TMT has been shown to produce effects on operant behavior, since TMT-intoxicated rats had higher lever pressing rats under a fixed-ratio schedule of

food presentation[32] and TMT impaired the performance of differential reinforcement at low response rates in an operant schedule[25]. These anatomical and behavioral findings have made TMT-intoxicated rats an attractive model for degenerative diseases such as AD, the most common cause of dementia[33].

As expected, the findings from Morris water maze test showed that memory impairment was demonstrable in the hippocampus injury after TMT. This study thus strongly propose TMT-treated rat to be a good model for studying neuronal degeneration. This model may be also useful for elucidating mechanisms underlying resistance to dementia and excitotoxic injury in the hippocampus.

YJG has been widely used as herbal treatment for disorder of chronic cognition. From the result of time spent in platform, YJG may be more effective in the treatment of long term memory disease(Fig. 2).

The animal model used in this study clearly exhibits the functional significance of the hippocampal neurodegeneration by TMT.

The cholinergic system is known to be involved in information processing related to hippocampal learning and memory[38]. The hippocampus possesses information derived from the associated brain regions that are involved in learning and memory, emotion and motivation[39], and any damage to the hippocampal cholinergic system may result in altered behavioral responses[40]. In particular, the loss of cholinergic function has been associated with a decline in cognition during aging and also in AD[41]. Several studies have demonstrated learning and memory deficits after TMT-induced neurodegeneration[11,13,26-9], which is consistent with results of this study.

The present results demonstrated that YJG improve spatial recognition after administering with TMT-induced neurodegeneration. The latency of the YJG group to find the hidden platform was significantly decreased on the acquisition testing, suggesting that YJG could improve the acquisition and retention deficits on the Morris water maze testing.

The present results suggest that the ChAT immunoreactivity in the hippocampus of the YJG group may explain the better performances for the learning and memory testing. This suggestion is strengthen by the fact that local implementations of acetylcholine-releasing cells into the cortical and hippocampal target areas of the basal forebrain improved age-induced learning and memory deficits[42]. Taken together, the results of the present study demonstrated that YJG ameliorated learning and memory deficits through their effects on the central nervous system, and this herbal medicine therapy may have beneficial effects on TMT-induced cognitive impairment.

Although the precise mechanisms for recovery of such behavioral deficits following the administering of YJG are not clear, one possibility is that anti-oxidant and anti-inflammatory effects from components in YJG.

Mountain cortex radicis, a component of YJG decreased ROS generation and

cytotoxicity in hydrogen peroxide stimulated neuronal cells through gene expressions of heme oxygenase (HO) and COMT, which play a major role in regulating ROS production[43]. It was shown that *Rehmanniae Radix*, a component of YJG improved the function of learning and memory of monosodium glutamate (MSG) treated rats through anti-oxidation and the increase of the expression of hippocampal cfos and NGF and intelligence in human[44,45]. Also, it was reported that *Lycii Fructus* and *Corni fructus*, components of YJG presented a strong anti-oxidative effects. In addition to such anti-oxidant effects from some components of YJG, it was found that *Lycii Fructus*,[46] *Rehmannia radix*[47], *Discoreaeradix*[48], *Corni fructus*[49] and *Mountain cortex radicis*[50], components of YJG showed anti-inflammatory effects. Thus, such anti-oxidative and anti-inflammatory effects from those components in YJG may have been responsible for the protection against TMT-induced neurodegeneration and cognitive deficits shown in the present study.

The cAMP-dependent pathway is a ubiquitous communication device within neurons that is extremely efficient, versatile, and multimodal, and is able to integrate two major cellular messengers, cAMP and Ca2+. Because cAMP-dependent signaling is so prominent and essential to cells, multiple means of regulation have evolved to insure its proper functioning[51]. The present study shows that the density of cAMP -positive neurons of the YJG group in the hippocampus was not significantly different from the control group in CA1 and CA3 as seen in Fig.5, 6. From this result, cAMP dependent signaling pathway may not relate with the protection against TMT-induced neurodegeneration and cognitive deficits.

In conclusion, herbal medicine therapy using YJG in the TMT-induced neurodegeneration brain may have a solid therapeutic potential as a treatment for dementia and amnesia.

V. Conclusion

1. In TMT exposed rats YJG improved spatial learning and memory in water maze, suggesting that herbal medicine can in some circumstances reduce spatial deficits on the central nervous system after TMT-induced neurodegeneration.

2. Anti-oxidative and anti-inflammatory effects from those components in YJG may have been responsible for the protection against TMT-induced neurodegeneration and cognitive deficits shown in the present study.

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