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Extract from Fructus cannabis activating calcineurin improved learning and memory in mice with chemical drug-induced dysmnesia[1]

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KEY WORDS Fructus cannabis; calcineurin; avoidance learning; maze learning; memory disorders

ABSTRACT

AIM: To investigate the effects of extract from Fructus cannabis (EFC) that can activate calcineurin on learning and memory impairment induced by chemical drugs in mice. **METHODS:** Bovine brain calcineurin and calmodulin were isolated from frozen tissues. The activity of calcineurin was assayed using *p*-nitrophenyl phosphate (PNPP) as the substrate. Step-down type passive avoidance test and water maze were used together to determine the effects of EFC on learning and memory dysfunction. **RESULTS:** EFC activated calcineurin activity at a concentration range of 0.01-100 g/L. The maximal value of EFC on calcineurin activity (35 %±5 %) appeared at a concentration of 10 g/L. The chemical drugs such as scopolamine, sodium nitrite, and 45 % ethanol, and sodium pentobarbital induced learning and memory dysfunction. EFC administration (0.2, 0.4, and 0.8 g/kg, ig×7 d) prolonged the latency and decreased the number of errors in the step-down test. EFC, given for 7 d, enhanced the spatial resolution of amnesic mice in water maze test. EFC overcome amnesia of three stages of memory process at the dose of 0.2 g/kg. **CONCLUSION:** EFC with an activation role of calcineurin can improve the impaired learning and memory induced by chemical drugs in mice.

INTRODUCTION

Protein phosphorylation and dephosphorylation are a major mechanism for controlling the activities of enzymes and other proteins[1], and it is involved in regulating many life processes, such as memory formation. Current neural network models of learning and memory formation support that bi-directional modifications of synaptic plasticity are particular essential to store information more effectively[2]. It is implicated that phosphatases are critical for many forms of synaptic plasticity and for learning and memory. Calcineurin regulates synaptic function through ion channels to transmitter release and gene transcription[3].

Calcineurin is a calcium/calmodulin binding protein that is very rich in the brain, and is also a key enzyme which stimulates T cells *in vivo*. It dephosphorylates some important neuronal substrates, including cytoskeletal proteins such as tau[4,5], *N*-methyl-*D*-aspartate (NMDA) channel constituents, and the protein kinase C substrates such as neurogranin and GAP-43 (neuromodulin). It also dephosphorylates inhibitor 1 and DARPP32 (dopamine and cAMP-regulated

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phosphoprotein). Dephosphorylated inhibitor 1 can activate PP-1. PP-1 has a broad range of substrates including important signal transduction proteins. So calcineurin can induce an endogenous phosphatase cascade via PP1.

The studies on roles of calcineurin in brain have progressed to the important discovery that it is the common target of the immunosuppressant drugs cyclosporin A (CsA) and FK506. CsA and FK506 inhibit calcineurin activity after forming complexes with cytoplasmic immunophilins, cyclophilins and FKBP12, respectively. These immunophilin-immunosuppressant complexes bind calcineurin and inhibit its function by sterically hindering the access of substrates to the catalytic site[6-8]. Many reported actions of CsA appear to be mediated by the inhibition of calcineurin phosphatase activity. Using CsA or FK506, most physiological studies including long-term potentiation (LTP), and long-term depression (LTD) are surveyed in the hippocampal slices[9-11]. Several studies reported that CsA prevented the expression of LTP in the hippocampus[12,13], suggesting the promotive role of calcineurin in the synaptic transmission. Up to now, there are few reports on single-trial, passive-avoidance task in day-old chicks that have applied both CsA and FK506 as antagonists of calcineurin, they have found that the intracranial administration of CsA disrupted memory formation. The effect was most prominent when administration took place between 10 min before and 40 min after learning. Importantly, the memory impairment induced by CsA persisted for at least 24 h post-training. It is speculated that protein phosphatases play the essential role in memory formation[14,15]. In recent years by using transgenic mice and knockout mice, it has been implicated that calcineurin seems to be critical for memory[16-18].

Due to the close connection between calcineurin and memory, an effective molecular screening model was established in our lab[19,20]. That is, using calcineurin as a target enzyme, its activity and its interaction with drugs were determined. Then, we got a few of traditional Chinese medicines (TCM) which can activate or inhibit the activity of calcineurin. During the past years, numerous articles on activators of calcineurin focused on metal ions, such as Mn^{2+}, Ni^{2+}. Our screening model can provide a new way to find new regulators of calcineurin, and new traditional Chinese medicines related to learning and memory. In this present study, the step-down passive avoidance task and water maze test were used to measure the effects of an active part extracted from Fructus cannabis (EFC), which can activate calcineurin, on learning and memory impairment induced by chemical drugs.

MATERIALS AND METHODS

Animals Male Kunming mice (18-22 g, Grade II, Certificate No SCXK11-00-0004. Experimental Animal Center of Beijing University, China) were group housed in a regulated environment (20±1 ºC), with a 12-h light and 12-h dark cycle (08:00-20:00, light). Food and water were given *ad libitum,* except during behavioral experiments.

Extraction of Fructus cannabis Fructus cannabis (500 g) was purchased from Beijing Tongrentang Pharmaceutical Group. Materials were extracted two times with 90 % ethanol for 24 h and filtrated. The filtrate was concentrated to dry. The ethanol extract was subjected to column chromatography under reduced pressure on silica gel, eluted with a chloroform-methanol solvent system. The elution that can activate calcineurin was collected and dried (EFC), the total yield was 5 g (1 %) in terms of starting materials. EFC was dissolved in saline prior to administration at doses of 0.2, 0.4, and 0.8 g/kg.

Assays of phosphatase activity Bovine brain calcineurin and calmodulin were isolated from frozen tissues as previously described[21]. The activity of calcineurin was assayed using *p*-nitrophenyl phosphate (PNPP) as the substrate[22]. The assay contained Tris-HCl 50 mmol/L (pH 7.4), dithiothieitol (DTT) 0.5 mmol/L, $CaCl_2$ 0.2 mmol/L, $MnCl_2$ 0.5 mmol/L, bovine serum albumin 0.2 g/L, calmodulin 0.3 μmol/L, and PNPP 20 mmol/L. The enzyme was assayed at 30 ºC for 20 min in a final volume of 200 μL. Reactions were terminated by adding 1.8 mL of Na_2CO_3 0.5 mol/L, edetic acid 20 mmol/L and the absorbance at 410 nm was measured using a control lacking enzyme. One unit of activity was defined as that catalyzing the hydrolysis of 1 nmol of *p*-nitrophenyl phosphate/min. For determining the effects of EFC on calcineurin activity, EFC at various concentrations was incubated with the phosphatase in the reaction mixture at room temperature for 5 min.

Drugs Scopolamine (Shanghai Harvest Pharmaceutical Co, China), sodium nitrite (Beijing Chemical Co, China) and sodium pentobarbital (Beijing Chemical Co, China) were dissolved in sterile 0.9 % saline prior to injection. The injection volume was kept constant at

0.01 L/kg irrespective of dose. Ethanol was diluted in saline and orally administered in a volume of 0.02 L/kg.

Step-down type passive avoidance test The apparatus consisted of acrylic box with a stainless-steel grid floor. A platform was fixed in the end of the box. Electric shocks (36 V) were delivered to the grid floor for 3 s with an isolated pulse stimulator. At the beginning of training trial, mice were placed in the box to adapt for 3 min. When electric shocks were delivered, mice jumped to platform. The shocks maintained 5 min. After a 24-h training, mice were placed on the platform for testing trial. Step-down latency and the number of errors were recorded within 5 min.

Water maze EFC was orally administered every at the same hour of the day for 7 d. From d 8 after the first administration, water maze was used. The latencies to find the terminal platform, and number of errors entering non-exits in the maze were used to evaluate performances. The water maze apparatus was a double-layer opaque plastic box (80 cm×50 cm×20 cm) including a start point, a terminal platform, and four non-exits. Near the platform was the safe region and a ladder was located for rest. The maze was filled with water to depth of 12 cm and the temperature was kept 21±1 ºC. Sodium pentobarbital was injected ip 30 min before the start of the trial on d 8, d 9, d 10 to induce spatial memory impairment model[23]. On d 8, mice were placed at the first non-exit which is the nearest to the end. If mice did not climb the ladder and have a rest within 2 min, they were removed from the water and placed on the ladder for 15 s. On d 9 and d 10, mice were placed at the fourth non-exit which is the farthest to the end. Escape latency (time to find the ladder) and error numbers (numbers swimming to non-exits) were recorded.

Statistical analysis Statistical analysis of the data for multiple comparisons was performed by one-way ANOVA followed by Dunnett's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Effects of EFC on phosphatase activity of calcineurin The effect of EFC on activity of calcineurin was investigated. The activity of calcineurin was defined as 100 %. EFC activated calcineurin activity at a concentration range of 0.01-100 g/L. The maximal value of EFC on calcineurin activity (35 %±5 %) appeared at a concentration of 10 g/L (Fig 1).

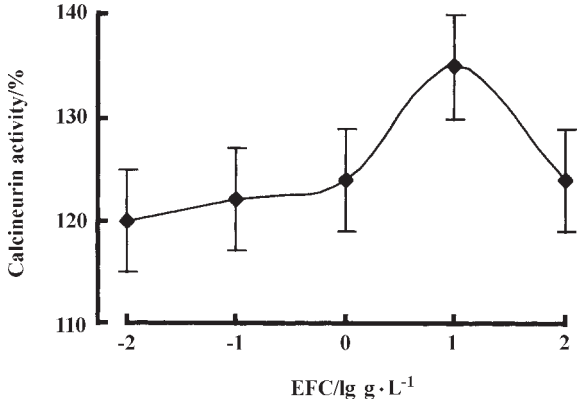

Fig 1. Effect of EFC on calcineurin activity. Calcineurin activity assayed in the absence of EFC was used as 100 %. *n*=3 independent experiments. Mean±SEM.

Effects of EFC on dysmnesic mice of aquired learning induced by scopolamine Scopolamine (1.5 mg/kg) injected ip 10 min before the training trial to induce acquired learning impairment model. In scopolamine-treated mice, the latencies shortened and the number of errors increased determined by the step-down test. EFC (0.2, 0.4, and 0.8 g/kg), given for 7 d, markedly improved the performance of mouse with dysmnesia (Fig 2).

Effects of EFC on dysmnesic mice of memory retention induced by sodium nitrite Sodium nitrite (120 mg/kg) was subcutaneously injected immediately after the training trial. After 24 h, mice were placed on the platform for testing. Sodium nitrite impaired the step-down type passive avoidance test performance of mice. EFC produced overall significant improvement in latency at doses of 0.2 and 0.4 g/kg, and markedly decreased the number of errors at the dose of 0.2 g/kg. The highest dose of EFC (0.8 g/kg) also produced improvement, but was not statistically significant (Fig 3).

Effects of EFC on dysmnesic mice of memory reappearance induced by 45 % ethanol Thirty mininutes before testing trial, 45 % ethanol was orally administered. The 45 % ethanol impaired the step-down type passive avoidance test performance of mice. EFC significantly prolonged the latency at the dose of 0.2 g/kg and decreased the number of errors at the dose of 0.2 and 0.8 g/kg. But the dose of 0.4 g/kg had no effect (Fig 4).

Effects of EFC on dysmnesia mice of spatial memory deficits induced by sodium pentobarbital Sodium pentobarbital (20 mg/kg) was injected ip 30 min before the trial on d 8, d 9, d 10 to induce spatial

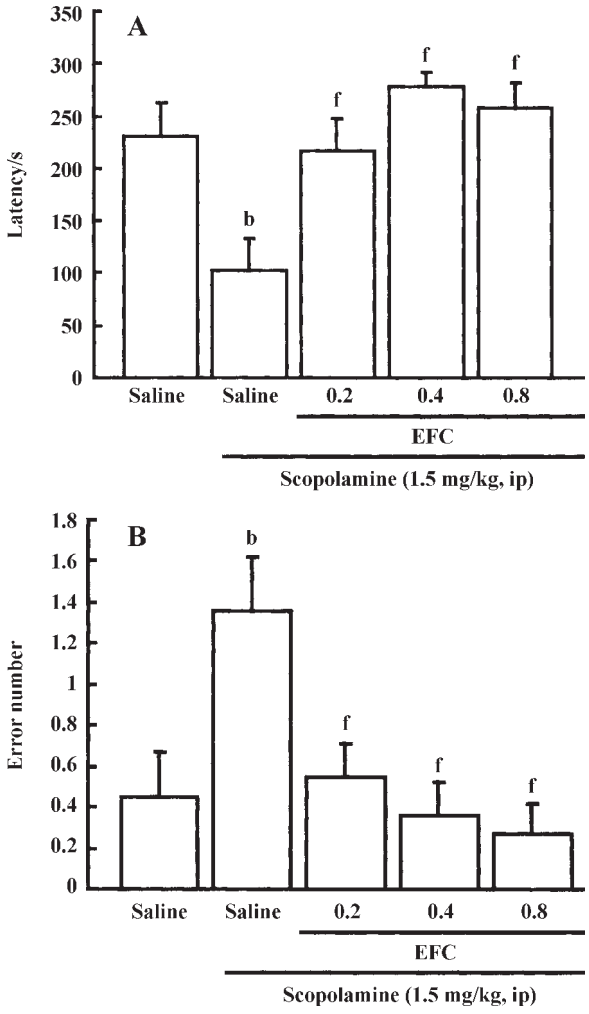

Fig 2. Effects of EFC on dysmnesic mice of acquired learning induced by scopolamine as evaluated by step-down type passive avoidance test in mice. (A) Latency; (B) Error number. *n*=11 mice. Mean±SEM. [b]*P*<0.05 *vs* saline-treated group. [f]*P*<0.01 *vs* (scopolamine+saline)-treated group.

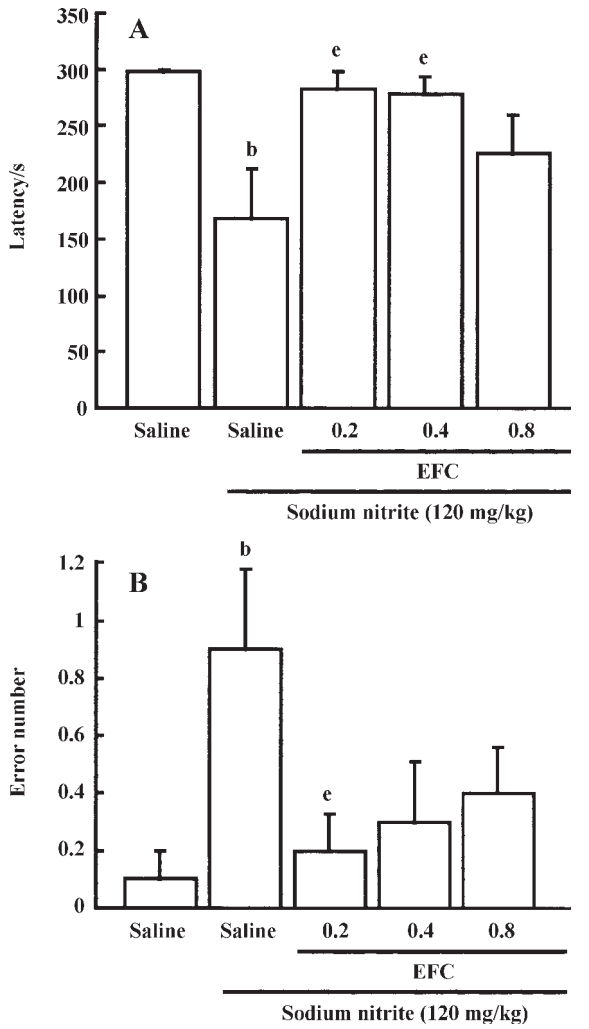

Fig 3. Effects of EFC on dysmnesia mice of memory retention induced by sodium nitrite as evaluated by step-down type passive avoidance test in mice. (A) Latency; (B) Error number. *n*=10 mice. Mean±SEM. [b]*P*<0.05 *vs* saline-treated group. [e]*P*<0.05 *vs* (sodium nitrite+saline)-treated group.

memory impairment model. On d 9 and d 10 in sodium pentobarbital-treated mice, escape latencies and error numbers increased. This means that sodium pentobarbital-treated mice took longer time to find the ladder than the saline-treated mice. EFC, given for 7 d, significantly improved dysmnesic mouse performance. In EFC-treated mice, escape latencies significantly decreased at the dose of 0.2 g/kg and the number of errors decreased at the doses of 0.2, 0.4, and 0.8 g/kg on d 10 (Tab 1).

DISCUSSION

In general, memory processes are divided into three stages: learning acquisition, memory retention, and reappearance. Chemical agents such as scopolamine, sodium nitrite, and 45 % ethanol impaired memory in mice trained on a step-down type passive avoidance[24], which is used to measure the three stages of memory process depending on drug-treated period. Sodium pentobarbital could impair spatial memory determined by water maze[25,26], which is often used to test the capabilities of spatial memory. Scopolamine injected 10 min before training, sodium nitrite subcutaneously injected immediately after training trial, 45 % ethanol orally administered 30 min before testing trial induced learning and memory impairment. The administration of EFC can improve the performance to some degree. EFC (0.2, 0.4, and 0.8 g/kg) produced a U-shaped dose effect on acquired learning of mice with scopolamine-induced dysmnesia. But the observed results of EFC at

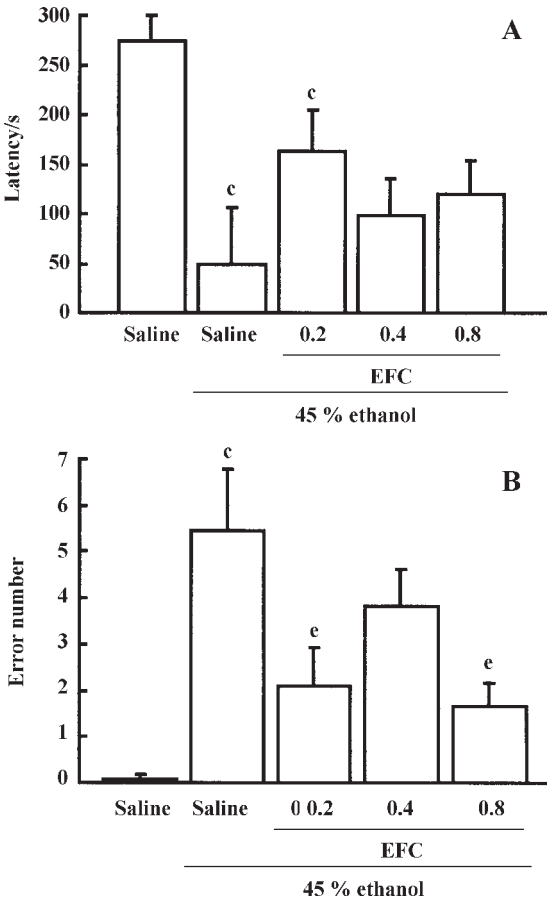

Fig 4. Effects of EFC on dysmnesic mice of memory reappearance induced by 45 % ethanol as evaluated by step-down type passive avoidance test in mice. *n*=11 mice. (A) Latency; (B) Error number. Mean±SEM. $^{c}P<0.01$ *vs* saline-treated group. $^{e}P<0.05$ *vs* 45 % (ethanol+saline)-treated group.

Tab 1. Effects of EFC on dysmnesia mice of spatial memory deficits induced by sodium pentobarbital (Pento) as evaluated by water maze. (1) Latency; (2) Error number. *n*=13 mice. Mean±SEM. $^{b}P<0.05$, $^{c}P<0.01$ *vs* saline-treated group. $^{e}P<0.05$, $^{f}P<0.01$ *vs* (sodium pentobarbital+saline)-treated group.

Drugs	d 9		d 10	
	Latency/s	Error numbers	Latency/s	Error numbers
Saline	89±10	3.6±0.5	69±13	3.5±0.6
Pento+saline	95±7	6.0±0.9^{b}	104±7^{b}	7.2±1.0^{c}
Pento+EFC 0.2 g/kg	79±11	3.3±0.6^{e}	74±10^{e}	2.9±0.5^{f}
Pento+EFC 0.4 g/kg	97±10	4.4±0.4	82±9	3.5±0.6^{f}
Pento+EFC 0.8 g/kg	93±9	3.9±0.5	84±8	3.5±0.5^{f}

the same doses on other memory stages did not appear in whole bell shape, such as retention and reappearance. Because EFC is an effective part of Fructus cannabis and contains a mixture of active components, EFC may therefore be able to exert its improving effects through more than one component. It suggested that EFC might exert a high effective action with an appropriate amount. Taking all these observations into account including latencies and error numbers, we proposed that EFC overcome amnesia of three stages of memory process at the dose of 0.2 g/kg. Because calcineurin potentially plays an essential role in activity-dependent modulation of synaptic efficacy, it is speculated that the improvement of memory by EFC is related to activation of calcineurin.

In the molecular screening model as described by Yan *et al*, the assay of phosphatase activity needs RII peptide as a substrate[19,20]. In our present study, it has been found that during the TCM screening, the phosphatase activity using PNPP as a substrate was coincident with the result using RII peptide as a substrate. Out of consideration for EFC as a mixture of active components, PNPP as a substrate was suitable for assay of phosphatase activity. Our results clearly demonstrate that EFC activates calcineurin activity. It may be absorbed and activate calcineurin. The activation of calcineurin exerts subtle roles in brain via influencing LTP and LTD which have been widely accepted as the synaptic models of learning and memory[9-11]. Further more the activated calcineurin may store memory more effectively. The calcineurin-mediated effects of EFC on memory are just one possible explanation for the observed results. Other mechanism of action still can not be excluded. The potential mechanism of EFC improvement of memory may be a synergistic effect of active ingredients.

In sum, the administration of EFC significantly improves memory deficits in amnesic mice induced by chemical substance. At this stage, any conclusion that the improving effects of EFC are due to activation of calcineurin must remain somewhat speculative. It is thus imperative to investigate the changes of activity and content of calcineurin in brain. The precise mechanisms of action by which EFC improves memory also need further investigation. But the present study provided direct pharmaco-behavioural evidence for regulator screening from Chinese herbal medicine. More importance is that calcineurin is an efficient enzyme for screening. The natural products we obtained from our

screening offer the clues for further development of lead compounds.

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