

Full Paper

Antidepressant-like Effect of Fuzi Total Alkaloid on Ovariectomized Mice

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Abstract. Recent studies in vivo and vitro have shown that Fuzi polysaccharide has an antidepressant-like effect. Polysaccharide and total alkaloid are the two most important components of Fuzi. However, little is known about the antidepressant-like effect of Fuzi total alkaloid. To investigate the antidepressant-like effect of Fuzi total alkaloid, behavioral studies were performed in the open field test and forced swimming test. Repeated intragastric administration of Fuzi total alkaloid for 7 days (10 mg/kg) to normal mice decreased immobility time compared to the vehicle group. Furthermore, repeated administration of Fuzi total alkaloid (10 or 30 mg/kg) to ovariectomized mice also decreased immobility time in a dose-dependent manner. However, these antidepressant-like behavioral effects were not simply due to locomotor hyperactivity. Further experiments showed that Fuzi total alkaloid enhanced the ratio of phospho-CREB/CREB (cAMP response element-binding) and BDNF (brain-derived neurotrophic factor) protein level in the frontal cortex and hippocampus in ovariectomized mice but not in normal mice. These results indicate that the CREB-BDNF pathway may be involved in the antidepressant-like effect of Fuzi total alkaloid in ovariectomized mice.

[Supplementary Figures: available only at <http://dx.doi.org/10.1254/jphs.12163FP>]

Keywords: alkaloid, ovariectomized mouse, forced swimming, BDNF (brain-derived neurotrophic factor)

Introduction

Depressive disorders are considered to be the most prevalent form of mental illness. It is estimated that one-tenth of the world population suffers from depression once in life (1). Fuzi (*Radix Aconiti Lateralis Preparata*) or its components is prescribed as treatment of depression frequently in Chinese medicine clinical practice (2, 3). It has been reported recently that Fuzi polysaccharide has an antidepressant-like effect (3). In addition to Fuzi polysaccharide, total alkaloid is a very abundant component of Fuzi (2). However, little is known about the anti-

depressant-like effect of Fuzi total alkaloid.

It is well known that antidepressant drugs up-regulate the CREB-BDNF (CREB, cAMP response element-binding; BDNF, brain-derived neurotrophic factor) pathway that contributes to the antidepressant activity, and CREB may act upstream of BDNF and is necessary for antidepressant-induced alterations in BDNF expression (4–6). Many studies demonstrate that CREB and phosphorylated CREB levels could be up regulated in rat and mouse brains during antidepressant administration (7–9). Therefore, these data indicate that phosphorylation of CREB protein is a common action of chronic antidepressant treatments that may lead to up-regulation of BDNF. On the other hand, in the past decades BDNF has been extensively studied and plays an important role in the maintenance and survival of neurons and in synaptic plasticity (4–6, 10, 11). The meta-analysis reveals that BDNF levels are lower in depressed subjects than in

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healthy controls (10), and BDNF mutant mice display a depressive phenotype when they are exposed to mild stress (11). Antidepressants restore both the level of BDNF and hippocampal size to the normal level (12). Furthermore, infusion of BDNF into the dentate gyrus produces antidepressant-like responses (4). These data indicate that BDNF play a critical role in depression. Recent studies have shown that the antidepressant-like effect of Fuji polysaccharide is more associated with BDNF (3). However, the role of the CREB-BDNF pathway in the antidepressant-like behavior effect of Fuji total alkaloid is unknown.

The incidence of depression in women is about twice than in men (13). Estrogen reduces depression-like behavior of aged female mice (14). Furthermore, CREB and BDNF levels seem to be higher with higher estrogen levels during the menstrual cycle and while using hormone therapy following menopause (15–17). These findings further suggest that menopausal depression may be related to down regulation of the CREB-BDNF pathway in the brain. Therefore, we hypothesize that BDNF could be responsible for the antidepressant-like behavior effect of Fuji total alkaloid. The immobility time was significantly increased in ovariectomized mice (18–21), and they displayed depressant-like behavior. Therefore, ovariectomy is a useful tool for investigating menopausal depression (20, 21). In the present study, we investigated whether Fuji total alkaloid has an antidepressant-like behavioral effect on immobility time in the ovariectomized mice. Additionally, we also determined the effect of Fuji total alkaloid on the CREB-BDNF pathway in the brain.

Materials and Methods

Animals

ICR female mice (aged 6–10 weeks) were obtained from the Jilin University (Changchun, China). Mice were housed in plastic cages (25.5 × 15 × 14 cm), 23°C ± 1°C temperature, and kept under standard laboratory conditions of a 12-h light/dark cycle with free access to food and water. Behavioral studies were conducted during the light phase. All experiments were conducted in accordance with the Chinese Council on Animal Care Guidelines.

Drugs

Imipramine hydrochloride was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Imipramine (30 mg/kg, i.p.)-treated mice represent the positive control group as we previously reported (5, 6). Fuji was purchased from a traditional Chinese medicinal store and was identified as reported (22). Fuji total alkaloid was ex-

tracted according to a previous report (23, 24) and ingredient analysis was performed by Dr. Yue's group who reported the ingredient analysis of Fuji alkaloids before (24).

Purification and ingredient analysis

Purification of Fuji total alkaloids: Purification procedures reported by Wang et al. (23) were followed with some modifications. The dried fruits of Fuji (40 kg) were soaked in 95% ethanol and then sonicated for 45 min. The ethanol solution was separated from the solid residue by filtration and it was stored in a refrigerator (4°C) for further use. The ethanol extracts were evaporated at 60°C and then diluted 11-fold in 0.04% HCl. The resulting mixture was shaken vigorously for several minutes. Then the lower phase was separated and chloroform was added. The mixture was then washed twice with 0.04% HCl, and dried at 40°C by rotary vaporization under reduced pressure. The crude alkaloids were purified by silica gel column chromatography using the cyclohexane-ethanol (4:1) as the eluent to provide pure Fuji total alkaloid (70 g).

HPLC-ESI-MS analysis: The Thermo Accela HPLC system (Waters Corporation, Milford, MA, USA) was used to characterize samples. HPLC/ESIMS/MS experiments were performed using a LTQ ion trap mass spectrometer (Thermo, Waltham, MA, USA) equipped with an electrospray source in the positive ion mode. The electrospray voltage was set to 4.5 kV. The capillary temperature was 250°C. The HPLC was connected to the mass spectrometer via the UV cell outlet. Nitrogen gas was used as both a sheath gas and an auxiliary gas. A Waters BEH C18 (1.7 µm, 3 mm × 100 mm) column was used. The mobile phase used for HPLC separation consisted of various proportions of methyl cyanide (A), methanol (B), and ammonium bicarbonate plus ammonium hydroxide (C, pH 10.5). The elution gradient was as follows: 0–138 min (0% A, 35%–100% B, 65%–0% C); 139–149 min (100% A, 0% B, 0% C); 150–155 min (0% A, 100%–35% B, 0%–65% C). The flow rate was set at 0.3 ml/min and the column temperature was maintained at 23°C. Total alkaloid was dissolved in chloroform plus methanol (1:1) and loaded to the column as previously reported (24).

Open field test

The ambulatory behavior was assessed in an open field test. For the open-field test, mice were individually placed in an acrylic apparatus (48.8 cm in diameter × 16-cm-high wall) with the gray floor divided into 19 equal squares. Horizontal locomotor activity (grid lines crossed with the four paws) and vertical locomotor activity (rearing) were calculated during a period of 6

min. For more detailed information, refer to previous reports (5, 6).

Forced swim test

The forced swim test was performed 24 h after the last treatment of Fuzi total alkaloid for 7 days. It was carried out in a cylindrical container (11 cm in diameter, 25-cm-high) filled with water to the height of 20 cm. Water was maintained at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$. After swimming, animals were dried with a towel and kept warm before returning them to their home cage. Immobility times were recorded during the 6 min swim test using a digital video camera. The duration of immobility during the last 4 min of the trial (25–27) was measured by an observer who was kept unaware of the experimental conditions.

Tissue preparation

At the end of the treatment (24 h after the last treatment), the animals were sacrificed by decapitation. The brains were quickly removed and dissected on ice into the frontal cortex and hippocampus. Samples were frozen at -80°C before homogenization. Sections were homogenized in a lysis buffer [137 mM NaCl, 20 mM TRIS, 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 mM sodium vanadate]. The homogenates were centrifuged at $10,000 \times g$ for 20 min, and then the supernatants were collected and processed for quantification of BDNF with a BDNF Emax ImmunoAssay System kit (Promega, Madison, WI, USA).

Measurement of BDNF protein by enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed using the BDNF Emax Immuno Assay System Kit (Promega) according to the manufacturer's instructions (26). Nunc Maxisorp 96 well immunoplates were coated with 100 μl /well of anti-BDNF monoclonal antibody (mAb) and incubated overnight at 4°C . The plates were incubated in a block and sample buffer at room temperature for 1 h. Then the samples were added to the coated wells (100 μl) and shaken for 2 h at room temperature. The antigen was incubated with an anti-Human BDNF polyclonal antibody (pAb) for 2 h at room temperature with shaking and then incubated with an anti-IgY antibody conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. The plates were then incubated with tetramethylbenzidine solution for 15 min and 1 M hydrochloric acid was added to the wells. The colorimetric reaction product was measured at 450 nm. Standard curves were plotted for each plate. BDNF concentrations were determined from the regression line for the BDNF standard ranging from 7.8 to 500 pg/ml

using the BDNF standard provided by Promega. Values of sections were above 16 pg/ml for each plate. More detailed information about the procedures are given in the previous reports (5, 6, 28).

Western blot of CREB and phospho-CREB (pCREB)

Tissue lysates containing CREB/pCREB were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting. CREB and pCREB were immunostained with phospho-CREB-ser133 (1:1000, rabbit polyclonal; Cell Signaling, Danvers, MA, USA) and CREB (1:1000, rabbit monoclonal; Cell Signaling). After several washes with TBST buffer, the membranes were incubated with the respective peroxidase labeled secondary antibodies (1:400; Solarbio, Beijing, China). Specific bands were quantified densitometrically and the ratio between the intensity of CREB and phospho-CREB from the same homogenate was calculated (29).

Statistical analyses

All values are presented as the means \pm S.E.M. The significance of the data was analyzed using one-way analysis of variance (ANOVA). When significant differences were obtained, post hoc comparisons within logical sets of means were performed using Dunnett's test. *P*-values less than 0.05 were considered significant.

Results

HPLC–ESI–MS analysis

In Table 1, HPLC–ESI–MS analysis shows the identified alkaloids in Fuzi total alkaloid (see ion chromatograms in Supplementary Figures, available in the online version only), and these data are consistent with those in the previous report (24).

Effect of repeated administration of Fuzi total alkaloid on body weight

Figure 1, A and B shows that 7-day repeated administration of Fuzi total alkaloid has no effect on body weight in the normal and ovariectomized mice. Furthermore, there is no body weight change in imipramine-treated mice that represent the positive control group.

Effect of repeated administration of Fuzi total alkaloid on immobility time in the forced swim test

In Fig. 2A, the immobility time is significantly shortened by repeated administration of total alkaloid at 10 mg/kg but not 3 and 30 mg/kg in the forced swim test (One-way ANOVA: $F = 4.5$, $P < 0.01$; post hoc Dunnett's test: $P < 0.05$). Figure 2B shows that the immobility time is significantly enhanced in the ovariectomized

Table 1. Alkaloids identified in Fuzi total alkaloid by HPLC–ESI–MS

No.	tR	[M+H] ⁺	Alkaloids
1	52.56	858	8-esp-Benzoylhypaconine, 8-pal-10-OH-Benzoylaconine
2	68.67	854	8-ndc-Benzoylhypaconine, 8-ole-Benzoylmesaconine
3	67.36	852	8-ndn-Benzoylhypaconine, 8-lino-Benzoylmesaconine, 8-ole-Benzoyldeoxyaconine
4	111.19	822	8-ole-Benzoyl-15-dexoyhypaconine
5	64.06	798	8-pdc-Benzoylhypaconine
6	42.92	770	unknown
7	26.01	700	unknown
8	72.50	668	unknown
9	54.82	662	10-OH-aconitine
10	74.39	648	10-OH-mesaconitine
11	86.82	632	Mesaconitine
12	44.41	614	3,13-Deoxyaconitine, Chasmaconitine
13	63.15	558	14-Benzoyl-3,13-deoxyaconine
14	71.95	464	14-Acetylaltatizamine
15	43.42	454	Fuziline
16	40.02	452	Chasmanine

Pal, pdc, lino, ole, ndn, ndc, and esp represent the residues of palmitic acid, pentadecanoic acid, linoleic acid, oleic acid, nonadecenoic acid, nonadecanoic acid, and eicosapentaenoic acid, respectively.

mice ($P < 0.01$), and this enhancement was also inhibited by Fuzi total alkaloid in a dose-dependent manner (10 mg/kg, $P < 0.05$; 30 mg/kg, $P < 0.01$). However, we did not observe the effect of imipramine in immobility time after 7 days treatment.

Effect of repeated administration of Fuzi total alkaloid on locomotor activity and rearing in the open field test

Figure 3, A and B show that repeated administration of Fuzi total alkaloid or imipramine has no effect on the locomotor activity and rearing in the open field test. It indicates that locomotor activity is not responsible for the decreasing effect of immobility time induced by Fuzi total alkaloid.

Effect of repeated administration of Fuzi total alkaloid on BDNF protein level in the brain

Figure 4A shows the effect of repeated administration of Fuzi total alkaloid on the BDNF protein level in the normal mouse brain. Total alkaloid did not alter the BDNF protein level in the hippocampus and frontal cortex of normal mice as shown in Fig. 4A. However, BDNF significantly decreased in the ovariectomized mice (frontal cortex, $P < 0.05$; hippocampus, $P < 0.01$), and repeated administration of Fuzi total alkaloid at 10 and 30 mg/kg significantly elevated BDNF protein level in the frontal cortex and hippocampus of ovariectomized

mice compared to the control group as shown in Fig. 4B (frontal cortex One-way ANOVA: $F = 4.55$, $P < 0.01$; Dunnett's test, 10 mg/kg: $t = 3.20$, $P < 0.05$; 30 mg/kg: $t = 3.22$, $P < 0.05$ and hippocampus One-way ANOVA: $F = 5.729$, $P < 0.001$; Dunnett's test, 10 mg/kg: $t = 3.675$, $P < 0.05$; 30 mg/kg: $t = 3.163$, $P < 0.05$).

Effect of repeated administration of Fuzi total alkaloid on the ratio of pCREB/CREB in the brain

Figure 5A shows that Fuzi total alkaloid or imipramine did not change the ratio of the pCREB/CREB in the normal mice. In Fig. 5B, the ratio of pCREB/CREB was significantly reduced in the ovariectomized mice ($P < 0.05$), and Fuzi total alkaloid (10 or 30 mg/kg) and imipramine (30 mg/kg) tended to increase the ratio of pCREB/CREB in the frontal cortex and significantly increased it in the hippocampus in the ovariectomized mice (One-way ANOVA: $P < 0.05$). In the frontal cortex, Fuzi (3, 10, and 30 mg/kg) dose dependently increased the ratio of pCREB/CREB (Dunnett's test: 3 mg/kg, $P < 0.05$; 10 mg/kg, $P < 0.05$; 30 mg/kg, $P < 0.05$) compared to the ovariectomized mice with vehicle treatment. In the hippocampus, Fuzi at 10 or 30 mg/kg and imipramine at 30 mg/kg significantly increased BDNF compared to the ovariectomized mice as the control group (Dunnett's test: 10 mg/kg, $P < 0.05$; 30 mg/kg, $P < 0.05$; imipramine, $P < 0.01$).

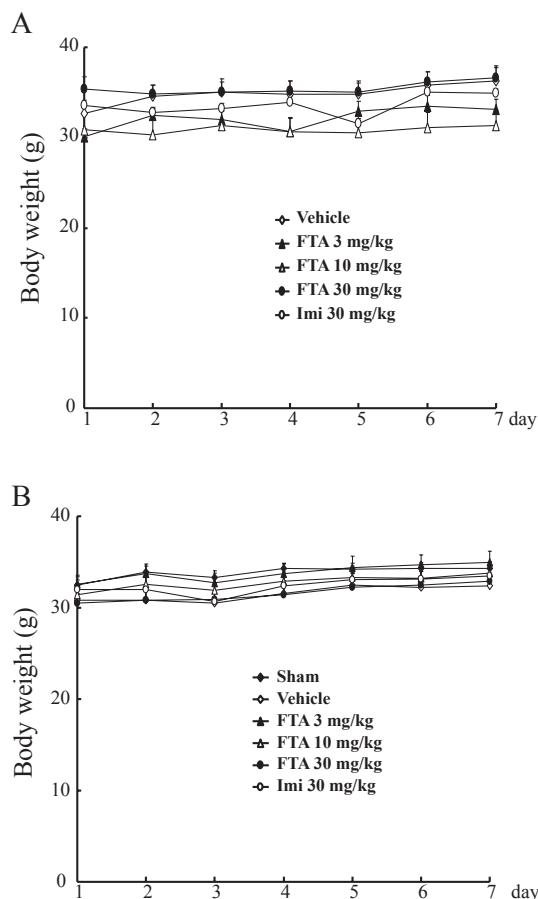


Fig. 1. Effect of repeated administration of Fuzi total alkaloid (FTA) on body weight. A: Effect of repeated administration of FTA on body weight in the normal mice. Columns represent the mean \pm S.E.M.; Imi, imipramine; $n = 8$ per group. B: Effect of repeated administration of FTA on body weight in the ovariectomized (OV) mice. Columns represent the mean \pm S.E.M.; Imi, imipramine; $n = 7 - 10$ per group.

Discussion

Recent studies in vivo and vitro have shown that Fuzi polysaccharide has antidepressant-like effect (3). Polysaccharide and total alkaloid are the two most important components of Fuzi (2, 3). However, little is known about the antidepressant-like effect of Fuzi total alkaloid. In the present study, we have demonstrated that repeated administration (7 days) of Fuzi total alkaloid significantly reduces the immobility time in the forced swim test in the normal mice. Decrease of immobility time was not directly due to locomotor activity. These data indicate that Fuzi total alkaloid has an antidepressant-like effect. To further examine the antidepressant-like behavioral effects of Fuzi total alkaloid, it was administered to ovariectomized mice, which is a useful tool for investigating menopausal depression (20, 21). It has been extensively

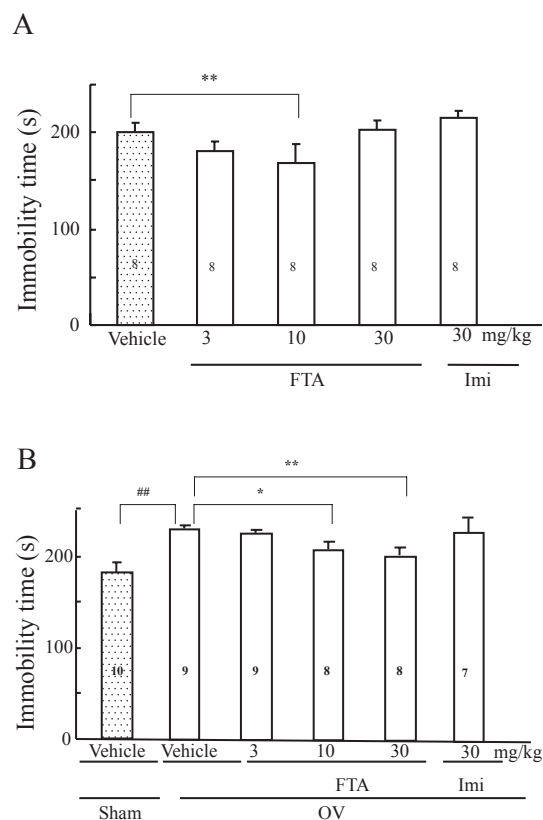


Fig. 2. Effect of repeated administration of FTA on immobility time. A: Effect of repeated administration of FTA on immobility time in the normal mice. Columns represent the mean \pm S.E.M.; $n = 8$ per group; Imi, imipramine; $**P < 0.01$. B: Effect of repeated administration of FTA on immobility time in the OV mice. Columns represent the mean \pm S.E.M.; Imi, imipramine; $n = 7 - 10$ per group; $*P < 0.05$, $**P < 0.01$, $##P < 0.01$.

studied that ovariectomy decreases estrogen level in the rodent (15, 16). The prolongation of immobility shown in the forced swim test in mice after ovariectomy is also consistent with the previous study (20, 21). Furthermore, the prolongation of immobility was also inhibited by repeated administration of Fuzi total alkaloid. These results support the antidepressant-like behavior effects of Fuzi total alkaloid in the ovariectomized mice. In addition, we did not observe the antidepressant effect of imipramine as a positive control drug in this study, but previous reports of another laboratory and our group indicated that imipramine shows an antidepressant effect after at least 14 days administration (5, 30). However, in the present study imipramine was administered for 7 days. Lalrem-ruta et al. (31) also shows that immobility time was also not affected by 7 days of imipramine administration as a positive control drug, but 14 and 21 days of imipramine treatment reduced immobility time significantly. These findings are consistent to our present data and previous

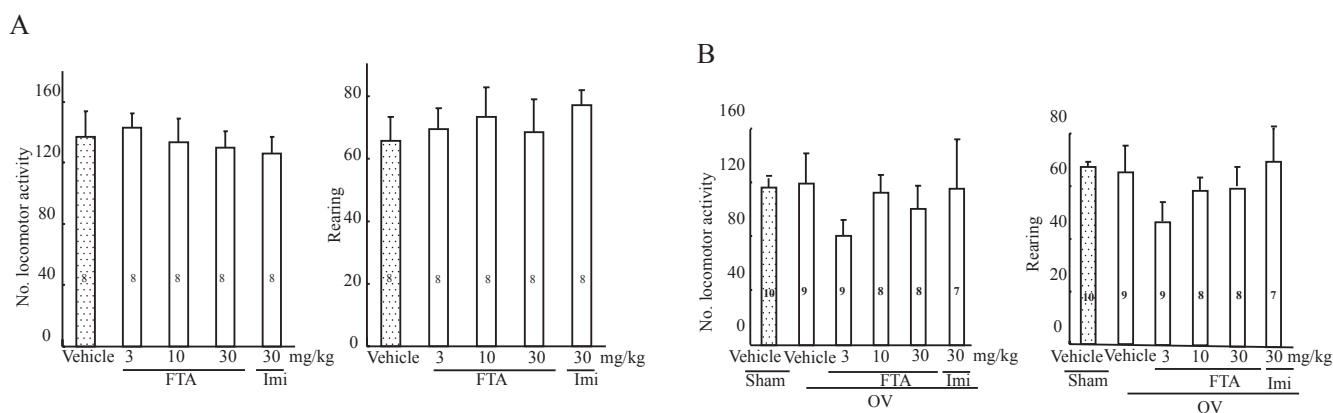


Fig. 3. Effect of repeated administration of FTA on locomotor activity and rearing. A: Effect of repeated administration of FTA on locomotor activity and rearing in the normal mice. Columns represent the mean \pm S.E.M.; Imi, imipramine; $n = 8$ per group. B: Effect of repeated administration of FTA on locomotor activity and rearing in OV mice. Columns represent the mean \pm S.E.M.; Imi, imipramine; $n = 7 - 10$ per group.

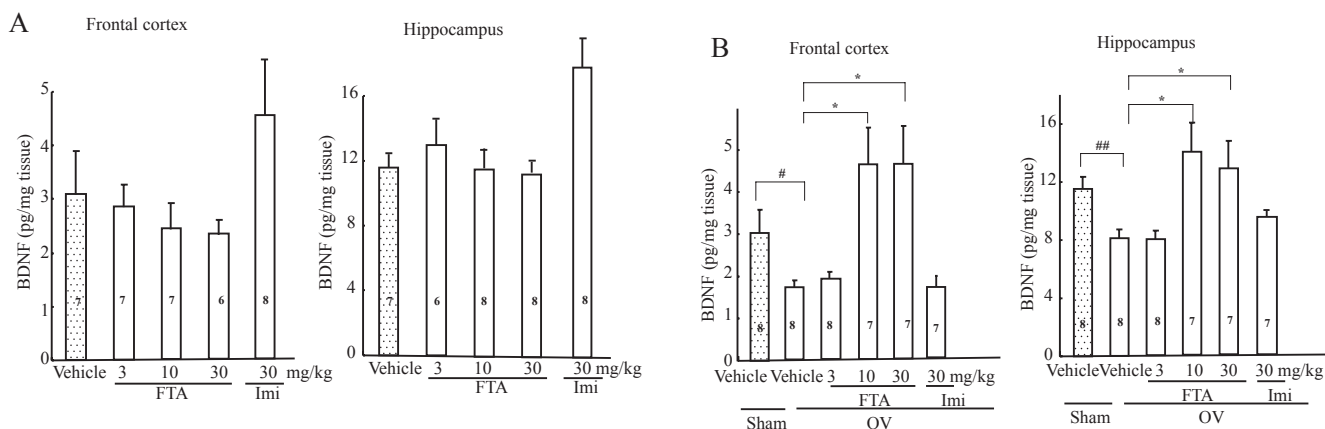


Fig. 4. Effect of repeated administration of FTA on BDNF level in the frontal cortex and hippocampus. A: Effect of repeated administration of FTA on BDNF level in the frontal cortex and hippocampus of normal mice. Columns represent the mean \pm S.E.M.; Imi, imipramine; $n = 6 - 8$ per group. B: Effect of repeated administration of FTA on BDNF level in the frontal cortex and hippocampus of OV mice. Columns represent the mean \pm S.E.M.; Imi, imipramine; $n = 7 - 8$ per group; $\#P < 0.05$ or $*P < 0.05$, $##P < 0.01$.

report (5). However, the effect of imipramine in 7 and 14 days of treatment requires further exploration. This data also means that Fuji total alkaloid has a faster antidepressant-like effect compared to imipramine. Another interesting finding is that middle doses (30 mg/kg) of Fuji total alkaloid show an antidepressant-like effect but not low doses (10 mg/kg) or high doses (30 mg/kg). Fuji total alkaloid maybe has dual action like some drugs, such as nicotine. Therefore, this may explain why middle dose of Fuji total alkaloid could cause antidepressant-like but not high doses, and low doses of Fuji total alkaloid is too low to induce this kind of effect.

Many studies have shown that the CREB-BDNF

pathway was involved in the depression (4, 5, 17). We found that the CREB-BDNF pathway was down-regulated significantly in ovariectomized mice, and the CREB and BDNF level of the hippocampus and frontal cortex was raised by Fuji total alkaloid in the ovariectomized mice but not normal mice. These data indicate that the mechanism of the antidepressant-like effect of Fuji total alkaloid may be different in normal mice from that in ovariectomized mice. It is also consistent with previous reports that estrogen modulates the CREB and BDNF level in the adult hippocampus and cortex (17, 32 - 35). Therefore, the CREB-BDNF pathway is responsible for the antidepressant-like behavioral effects of the total al-

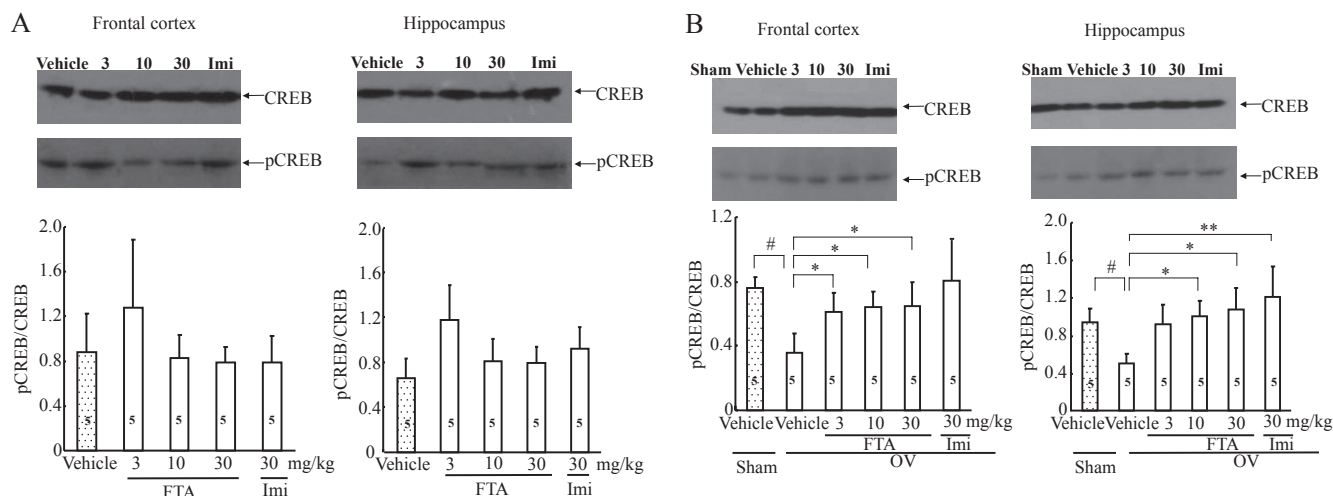


Fig. 5. Effect of repeated administration of FTA on ratio of pCREB/CREB level in the frontal cortex and hippocampus. A: Effect of repeated administration of FTA on ratio of pCREB/CREB level in the frontal cortex and hippocampus of normal mice. Columns represent the mean \pm S.E.M.; Imi, imipramine; $n = 5$ per group. B: Effect of repeated administration of FTA on ratio of pCREB/CREB level in the frontal cortex and hippocampus of OV mice. Columns represent the mean \pm S.E.M.; Imi, imipramine; $n = 5$ per group, $^{\#}P < 0.05$ or $^{*}P < 0.05$, $^{**}P < 0.01$.

kaloid of Fuzi in ovariectomized mice. However, in normal mice we observed that Fuzi total alkaloid caused a significant antidepressant-like effect, but it did not affect CREB-BDNF pathways. It is considered that the mechanisms of depression are quite complex, and, in the past decades, several major hypothesis of depression have been raised including the monoamine hypothesis, Hypothalamic–Pituitary–Adrenal (HPA) axis hypothesis, or CREB-BDNF hypothesis (5, 20, 32). Therefore, other mechanisms should be further explored in normal mice. On the other hand, we have also found that in imipramine-treated mice the ratio of pCREB/CREB is increased but not BDNF in the hippocampus of ovariectomized mice. It is possible that CREB is more sensitive to imipramine in ovariectomized mice. However further studies are required to assess the exact mechanism. In the present study, Fuzi (10 mg/kg) produced a decreasing effect on immobility time, but there was no correlation between BDNF or pCREB/CREB, so there may be a different mechanism for it. Further study is needed to confirm its exact mechanism.

Total alkaloid and polysaccharide are the two most important components of Fuzi. We have found that acute administration of Fuzi total alkaloid and polysaccharide has no antidepressant-like effect in our study (data not shown here) and other ones (3). However, in the chronic study, Fuzi total alkaloid showed the antidepressant-like effect more rapidly (7 days) in our studies compared to the data of polysaccharide (14–28 days) (3), which suggests that Fuzi total alkaloid has a more rapid action,

while polysaccharide has a long-lasting effect. Furthermore, we also observed that the effect of the hippocampus and frontal cortex was involved in the antidepressant-like effect of Fuzi total alkaloid, and hippocampus is more responsible for the effect of polysaccharide (3). This may be the reason why Fuzi total alkaloid has more rapid action via influence of more brain regions.

In summary, Fuzi total alkaloid has an antidepressant-like effect, and particularly the CREB-BDNF pathway is likely responsible for antidepressant-like effect in the ovariectomized mice.

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