CHAPTER IV

RESULTS AND DISCUSSION

Effect of VE on nicotine withdrawal symptoms in mice

1. Effect of VE on withdrawal symptoms

Nicotine withdrawal symptoms in mice were observed after nicotine discontinuation at day 15 and 21 of the experiment. Some of abstinence signs were shown in Figure 4. At day 15, all of concentration of VE significantly reduced total abstinence signs compare with NW and MEC groups. Total abstinence sign scores of mice received VE 250 and 500 mg/kg were significantly lower than control group (Figure 8). When separately considered each abstinence sign for every 5 minutes, VE250 and 500 mg/kg produced significant decrease in rearing compared with NW and MEC groups from the 15^{th} to 30^{th} minutes and control group exhibited more rearing than VE500 at the 10^{th} and 25^{th} minutes (Figure 9A).

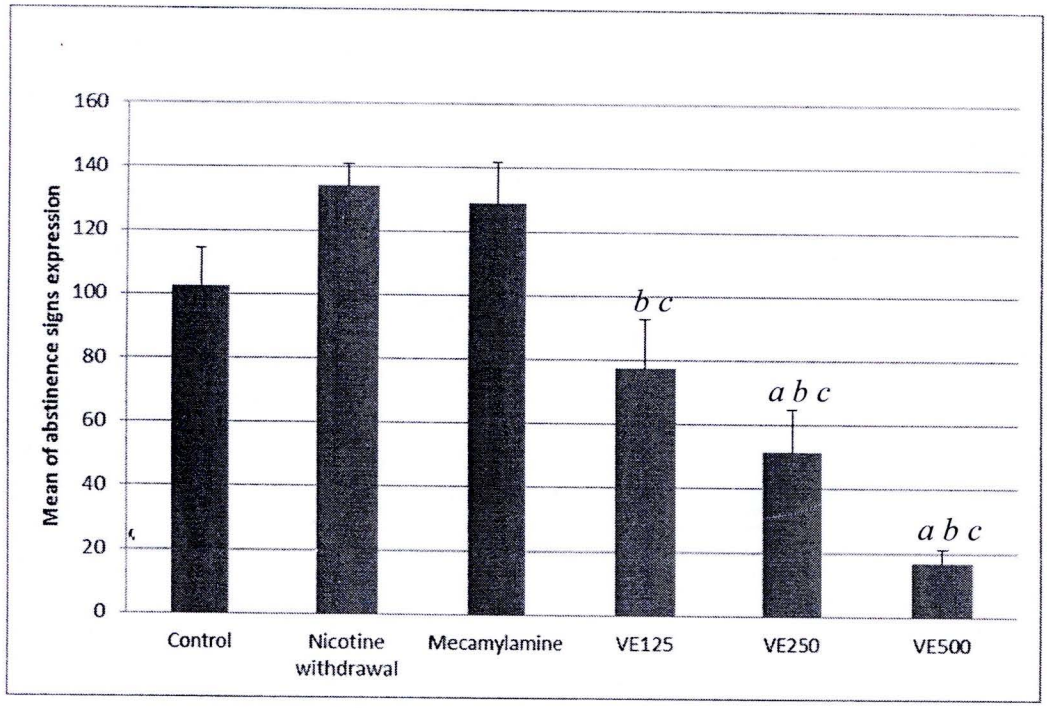


Figure 8 Total abstinence sign score of mice at the first day after nicotine withdrawal (day 15 of the experiment). (*a*, significant difference compared to control group; *b* significant difference compared to nicotine withdrawal group; *c* significant difference compared to mecamylamine received group, $p \leq 0.05$, n = 6)

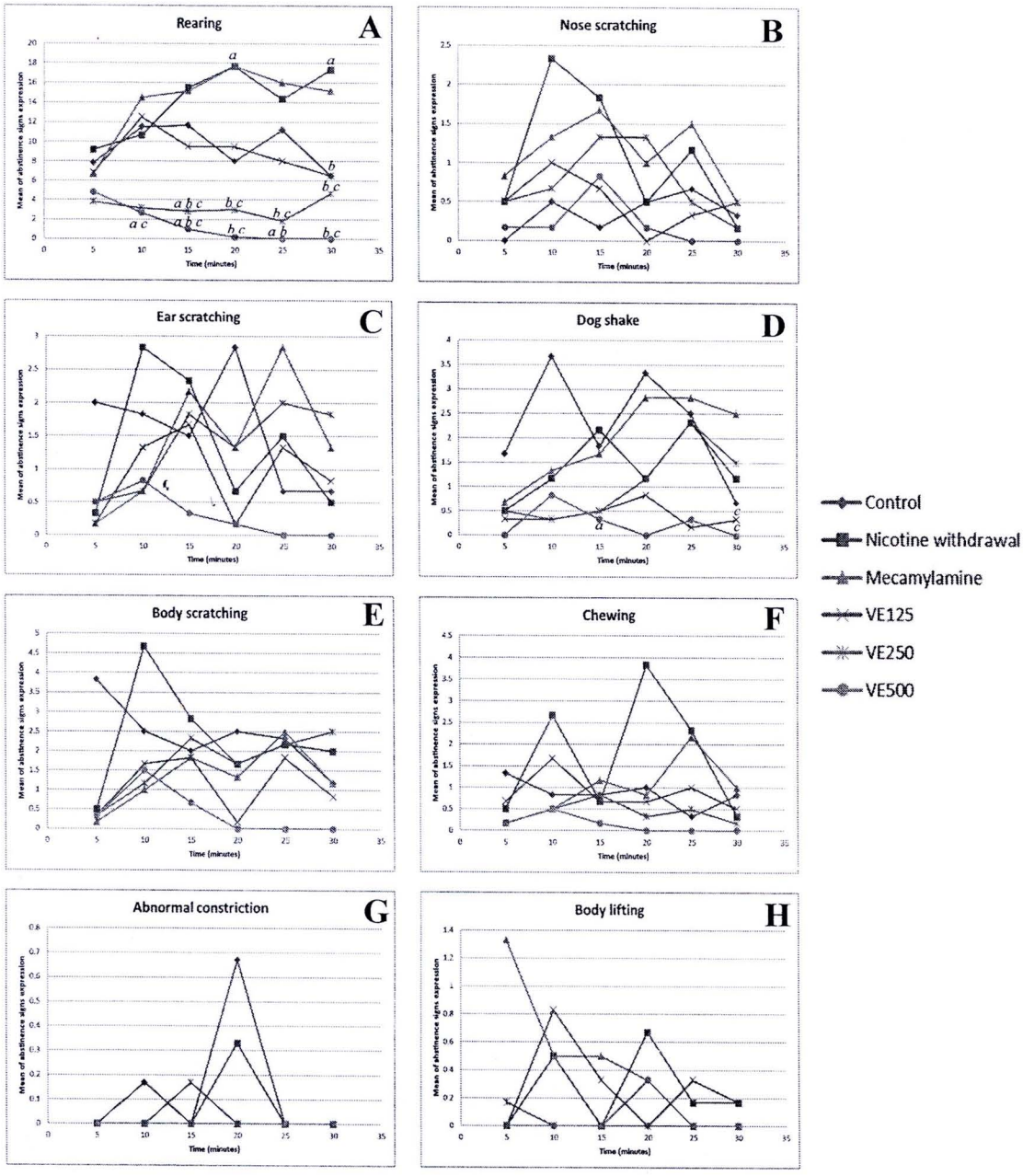


Figure 9 Mean of nicotine abstinence signs versus time, day 15 of the experiment. (A) Rearing (B) Nose scratching (C) Ear scratching (D) Dog shake (E) Body scratching (F) Chewing (G) Abnormal constriction (H) Body lifting. (*a* significant difference compared to control group; *b* significant difference compared to nicotine withdrawal group; *c* significant difference compared to mecamylamine received group, $p \leq 0.05$, n = 6)

At day 21, NW and MEC groups showed significant higher nicotine abstinence signs compared to control mice. VE 250 and 500 mg/kg significantly reduced the total abstinence signs compared to NW and MEC groups (Figure 10). When separately considered each abstinence signs, VE 250 and VE 500 mg/kg produced lower amount of rearing than MEC at the minute of 10 and significantly less rearing were also found in VE250 and VE500 groups compared with NW group at minute of 15, 20, and 30 (Figure 11).

Withdrawal from continuous nicotine injection produced many withdrawal symptoms including abstinence signs, anxiety, and hypolocomotion [61, 76, 77]. The observed abstinence signs at day 15^{th} is used for study in effect of VE on abstinence signs after the first day after nicotine withdrawal. *V. cinerea* is used for treatment of many diseases such as anti-inflammation, anti-malarial, cancer, and also used for reduction of smoking. Previous studies suggest that VE may be potential treatment alone or supplement for smoking cessation [17, 18]. The results showed the acute effect of VE in reducing of withdrawal symptoms with dose dependent manner and rearing was the most affected abstinence sign. At day 21 of experiment, total abstinence signs in mice received VE was still reduced compared with NW and MEC groups. These results suggest that effect of VE in reducing of nicotine withdrawal symptoms occur from the first day of administration and the effect still exhibit after the seventh day of administration.

Mecamylamine is nicotinic receptor antagonist that blockade binding of nicotine to nicotinic receptors [78]. Mecamylamine is used for decreasing blood pressure and has anti-rewarding effect of nicotine by reduced amount of nicotinic receptor [78]. Nowadays, mecamylamine is combined with NRT for improving smoking quitting rate [79]. Theoretically, 7 days exposing to mecamylamine should decrease nicotine abstinence sign [80]. However, mecamylamine received mice showed unchange in nicotine abstinence signs compared to nicotine withdrawal group in this study.

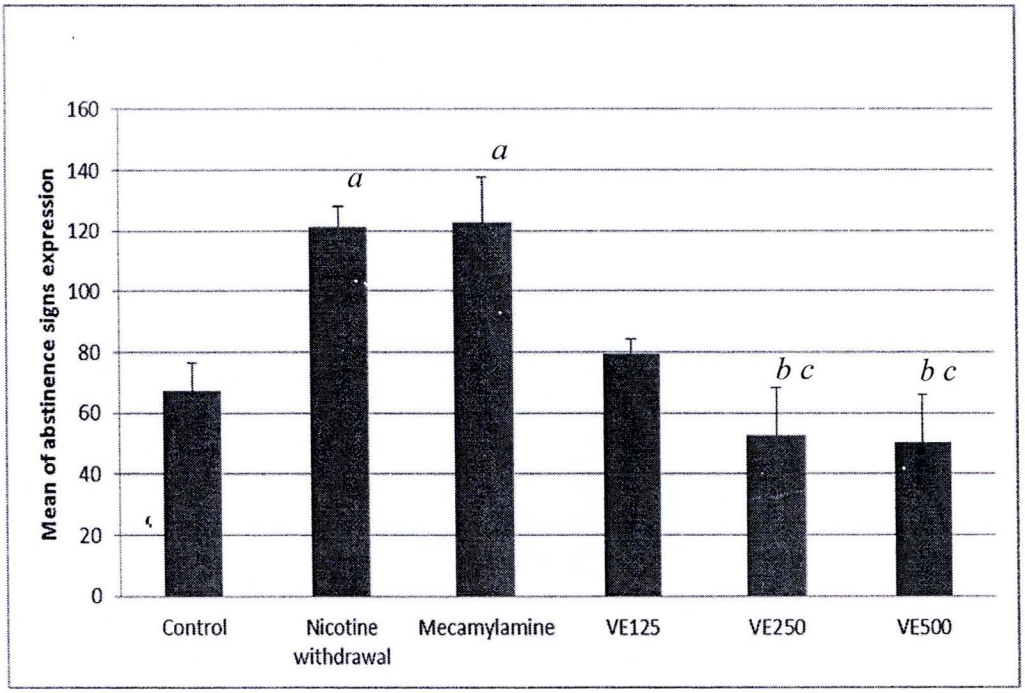


Figure 10 Total abstinence sign scoreof mice at the 7th day after nicotine withdrawal, day 21 of the experiment. (*a* significant difference compared to control group; *b* significant difference compared to nicotine withdrawal group; *c* significant difference compared to mecamylamine received group, $p \leq 0.05$, n = 6)

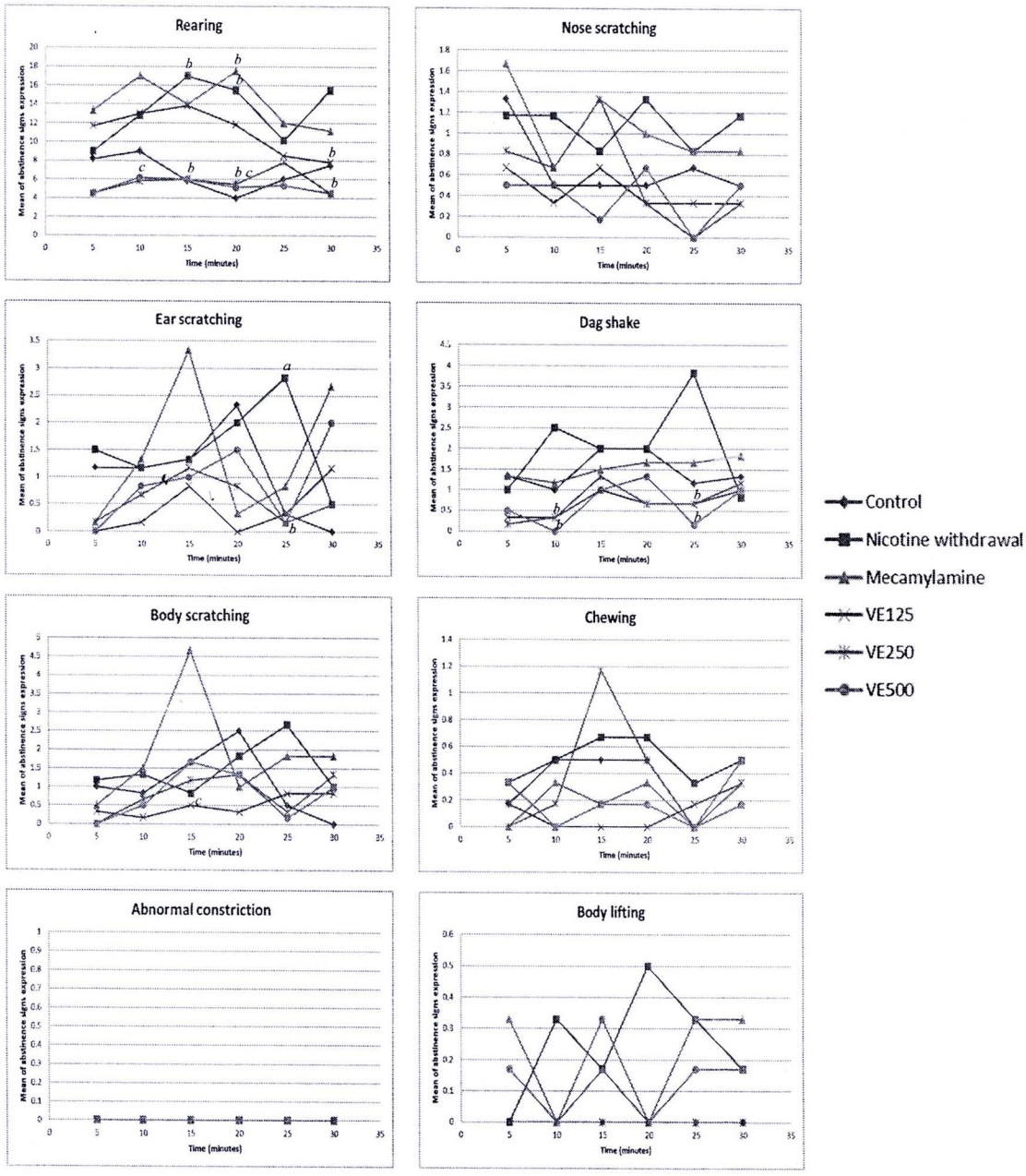


Figure 11 **Mean of nicotine abstinence signs versus time, day 21 of the experiment. (A) Rearing (B) Nose scratching (C) Ear scratching (D) Dog shake (E) Body scratching (F) Chewing (G) Abnormal constriction (H) Body lifting. (*a* significant difference compared to control group; *b* significant difference compared to nicotine withdrawal group; *c* significant difference compared to mecamylamine received group, $p \leq 0.05$, n = 6)**

2. Effect of VE on locomotors activity

At the first day of nicotine withdrawal, no significant differences of locomotor activity (LMA) were found between groups but nicotine withdrawal mice and mice received VE tended to have lower LMA than control group and mice received MEC. However, mice received MEC showed significant higher LMA than NW group at the first minute and VE 500 mg/kg produced the lowest LMA at the minute of 4 of the observation (Figure 12).

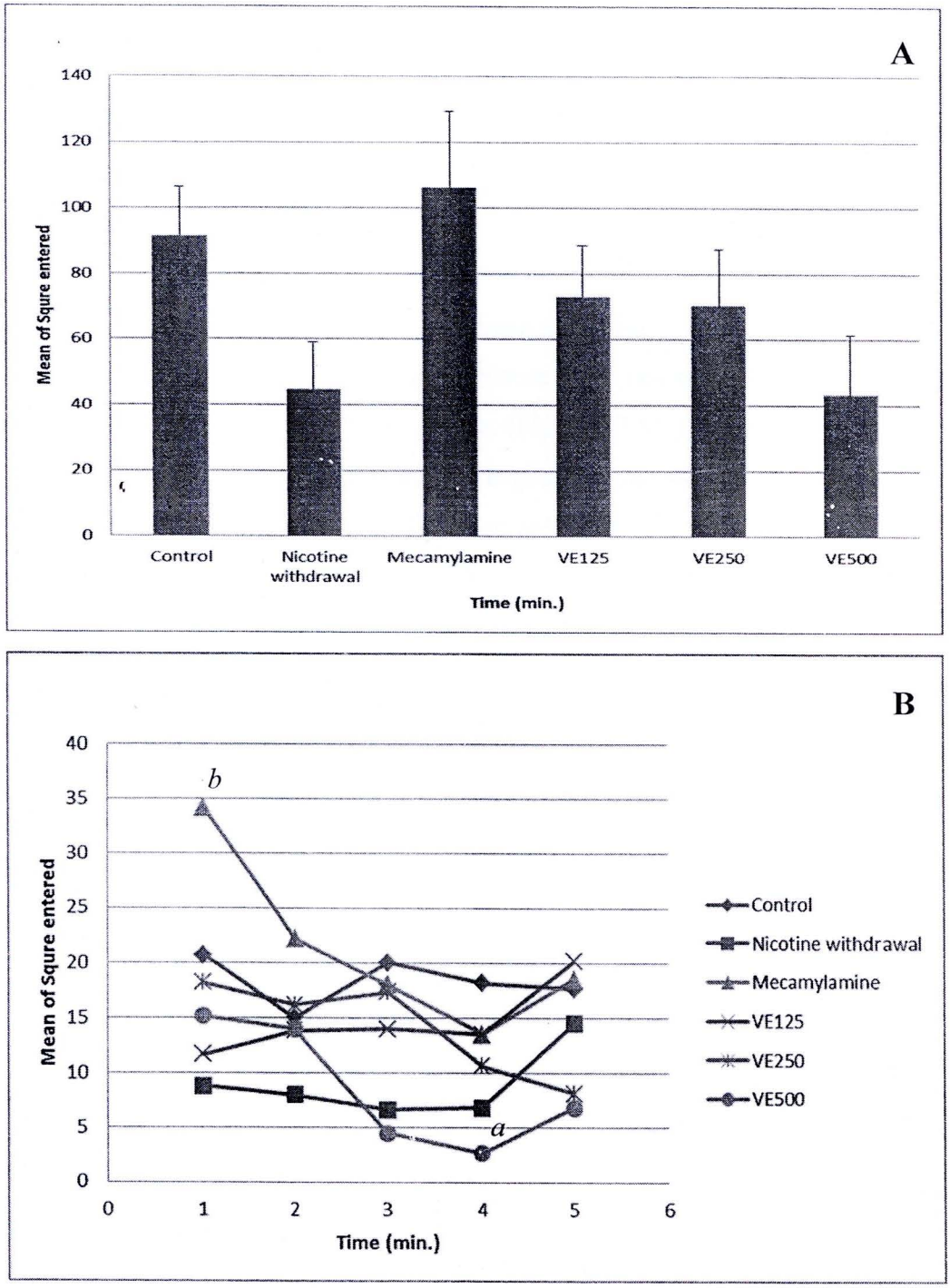


Figure 12 Locomotor activity (LMA) of mice at 1 day after nicotine withdrawal (day 15). (A) Mean of total squre enter. (B) Mean of squre enter versus time. (*a* significant difference compared to control group; *b* significant difference compared to nicotine withdrawal group, $p \leq 0.05$, n = 6)

At the 7th day of nicotine withdrawal, mice received VE 125, 250, and 500 mg/kg showed significant LMA reduction compared with control group. Mice received VE 125 mg/kg also showed significant decrease in LMA compared with NW mice. Although reduction in LMA in mecamylamine-treated mice was not be seen in the first day of nicotine withdrawal, less number of square enter was observed after mecamylamine administration at the 7th day of nicotine withdrawal. The result may be due to the onset of mecamylamine action is longer than pre-habituation period [81], hence any significances were not to be found after the first dose compared with control and NW groups (Figure 13A). Figure 13B showed LMA of mice at day 21 of experiment from the beginning (the minute of 0) to the end of experiment (the minute of 5). Significant reduction of LMA was observed in mice received VE compared to control and MEC groups at the first minute, whereas LMA of MEC, VE125, and VE500 groups were significantly lowered than NW group at 4th minute.

Locomotion in rodent probably increase after activation of DA receptor on NAcc [82]. Activation of DA receptor in NAcc caused DA release and project to other site espacially VTA which is the brain region contributing about cognition, motivation, and reward [83, 84]. Acute nicotine administration can depress locomotor activity and chronic nicotine administration can increase locomotor activity [54, 85]. Short term of nicotine withdrawal after chronic nicotine can also depress locomotor activity [85, 86]. In this experiment, raising of square enter means stimulation of locomotor activity and decreasing of square enter means reduction of locomotor activity [61]. VE and MEC received mice only showed decrease LMA both the first and the 7th day after nicotine withdrawal. Therefore, VE has effect on locomotor activity in nicotine withdrawal mice by reduced activity of DA.

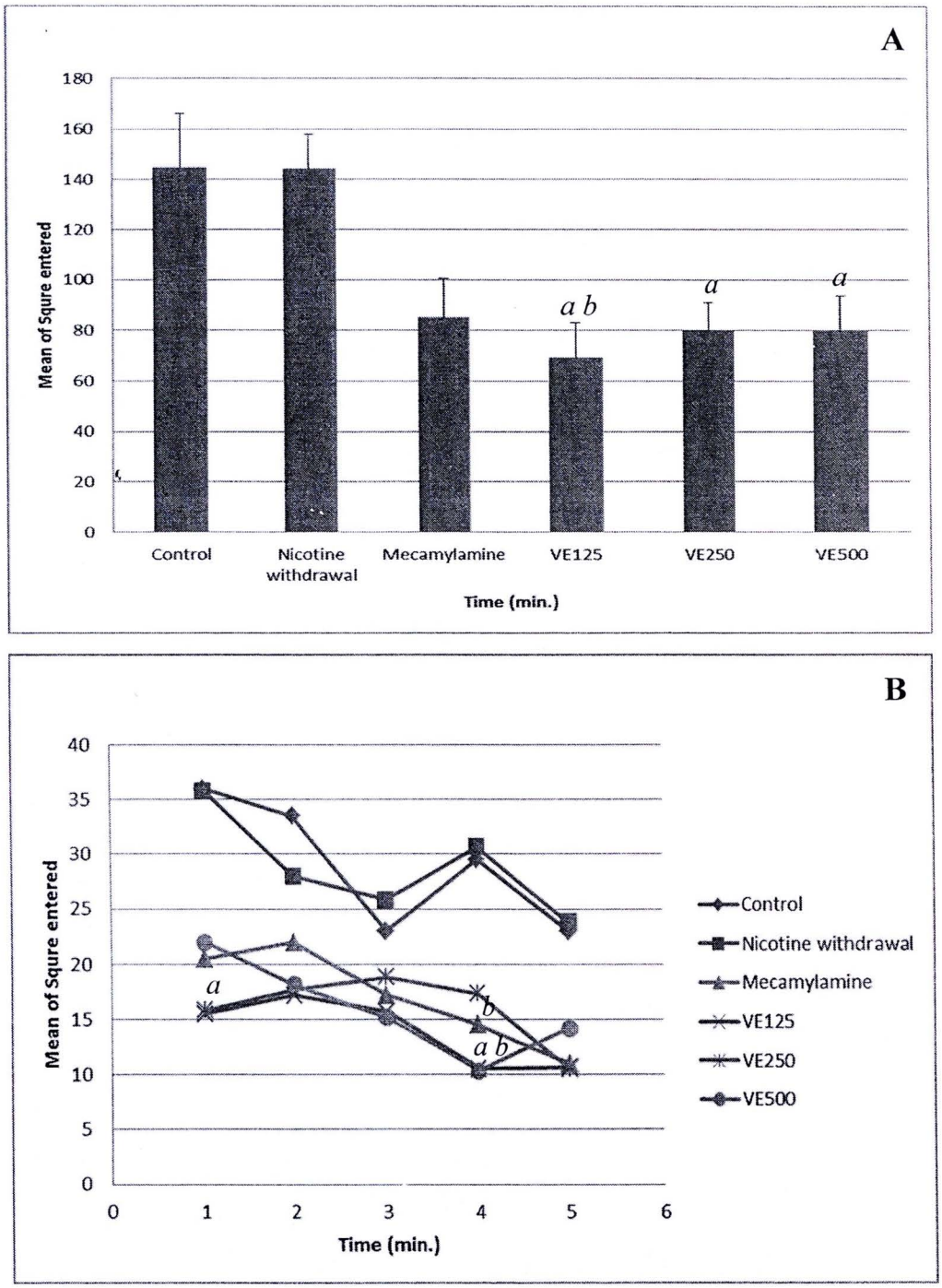


Figure 13 Locomotor activity (LMA) of mice at 7 days after nicotine withdrawal (day 15). (A) Mean of total squre enter. (B) Mean of squre enter versus time. (*a* significant difference compared to control group; *b* significant difference compared to nicotine withdrawal group, $p \leq 0.05$, n = 6)

3. Effect of VE on anxiety-like behaviors

Behavioral dimension that related to anxiety consist of avoidance of unprotected area, risk assessment, exploration, food intake inhibition, and cognition [87]. This experiment used avoidance of unprotected area to test for anxiety-like behavior and measured on EPM by scoring the number of both open and close-arm entry of mice and total time spent in open-arm. Increase of open-arm entry means decrease of anxiety-like behavior whereas increase of close-arm entry means increase of anxiety-like behaviors [86].

3.1 Open-arm entry

After the first and the final day of nicotine withdrawal, no significant differences were found in both open-arm entry and time spent in open-arm in total 5 minutes of experiment. Moreover, all groups of mice showed no significant differences in percent time spent in open-arm (Figure 14 and 15).

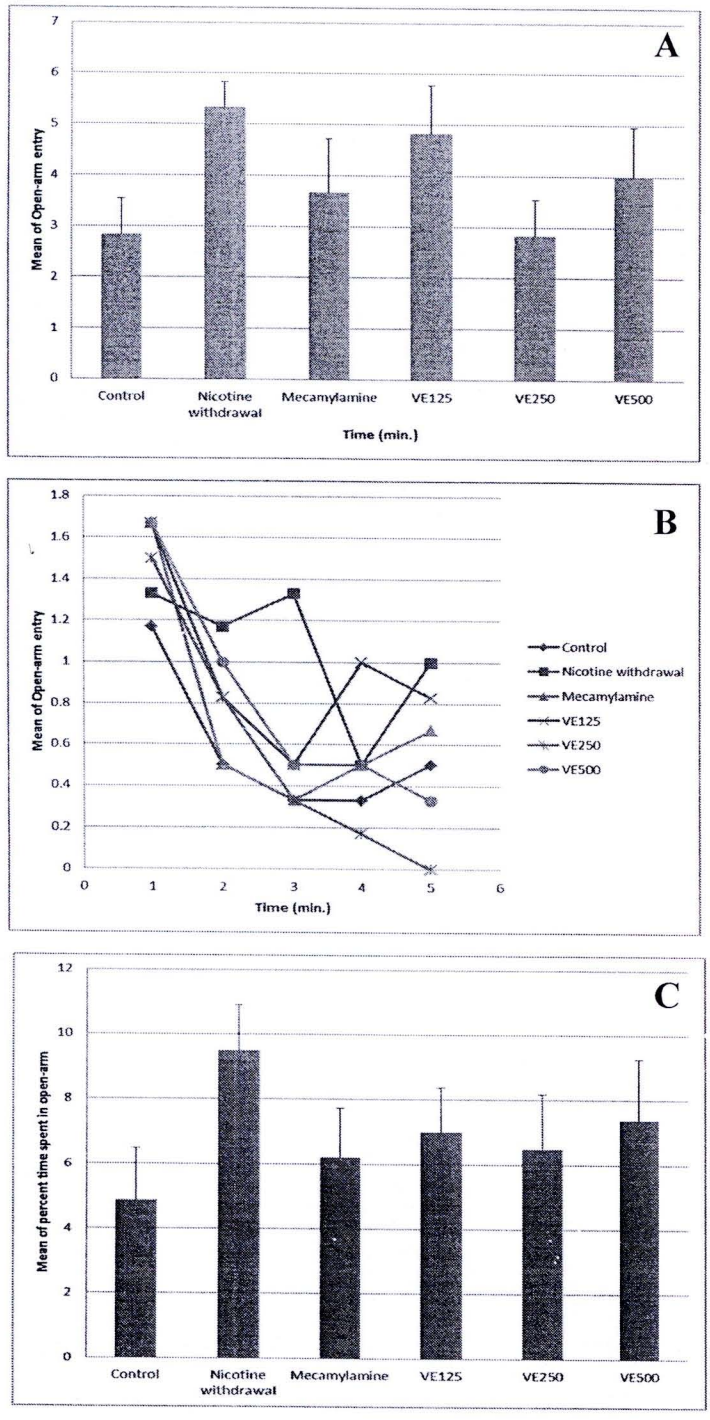


Figure 14 Anxiety-like behaviors of mice at 1 day after nicotine withdrawal. (A) Total open-arm entry. (B) Open-arm entry versus time. (C) Percent time spent in open-arm.

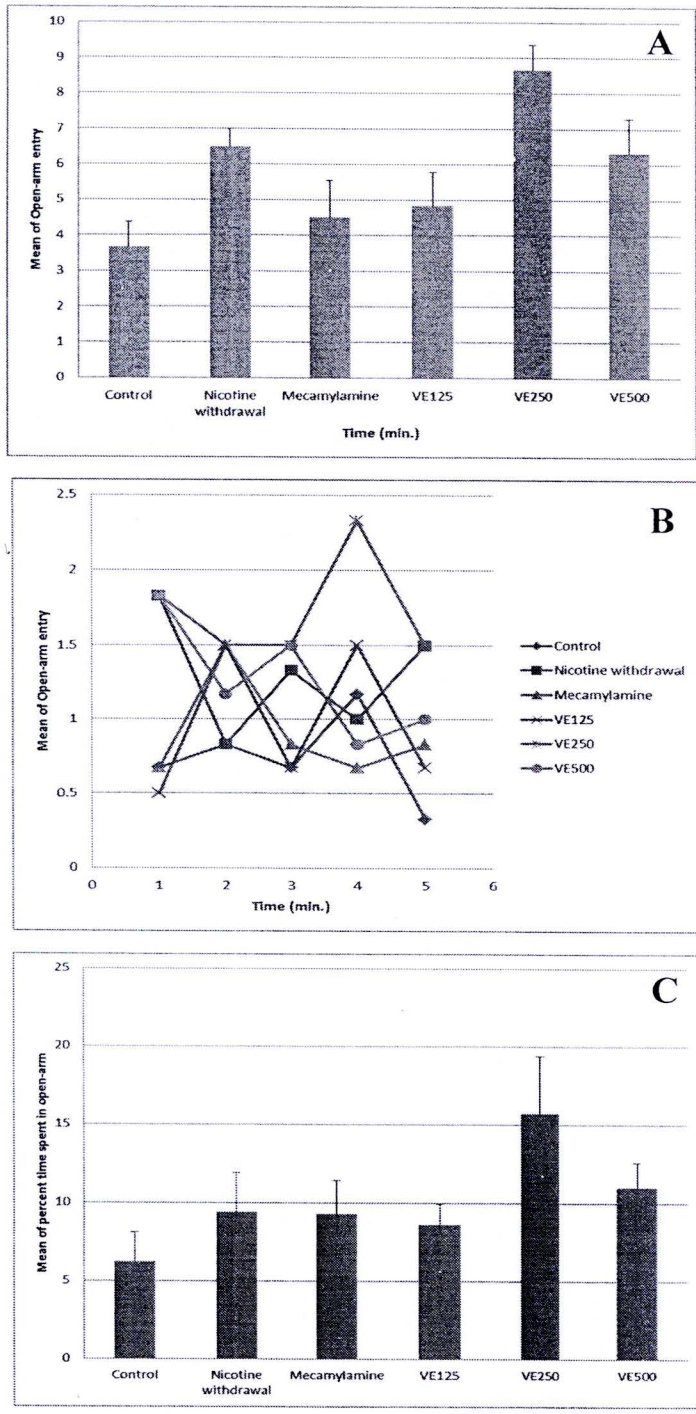


Figure 15 Anxiety-like behaviors of mice at 7 days after nicotine withdrawal. (A) Total open-arm entry. (B) Open-arm entry versus time. (C) Percent time spent in open-arm.

3.2 Close-arm entry

VE 500 mg/kg significantly reduced total close-arm entry compared to MEC after the first day of nicotine withdrawal (Figure 16). However, no significant differences in total close-arm entry on the last day of nicotine withdrawal, although VE250 group exhibited significant increase in close-arm entry compared to C group at the minute of 2 (Figure 17).

The results from EPM tested indicate that VE has no effect on anxiety-like behavior. This may be due to nicotine withdrawal produced mild anxiogenic effects [79] and VE may have no participate to mouse brain region that modulation of anxiety [88].

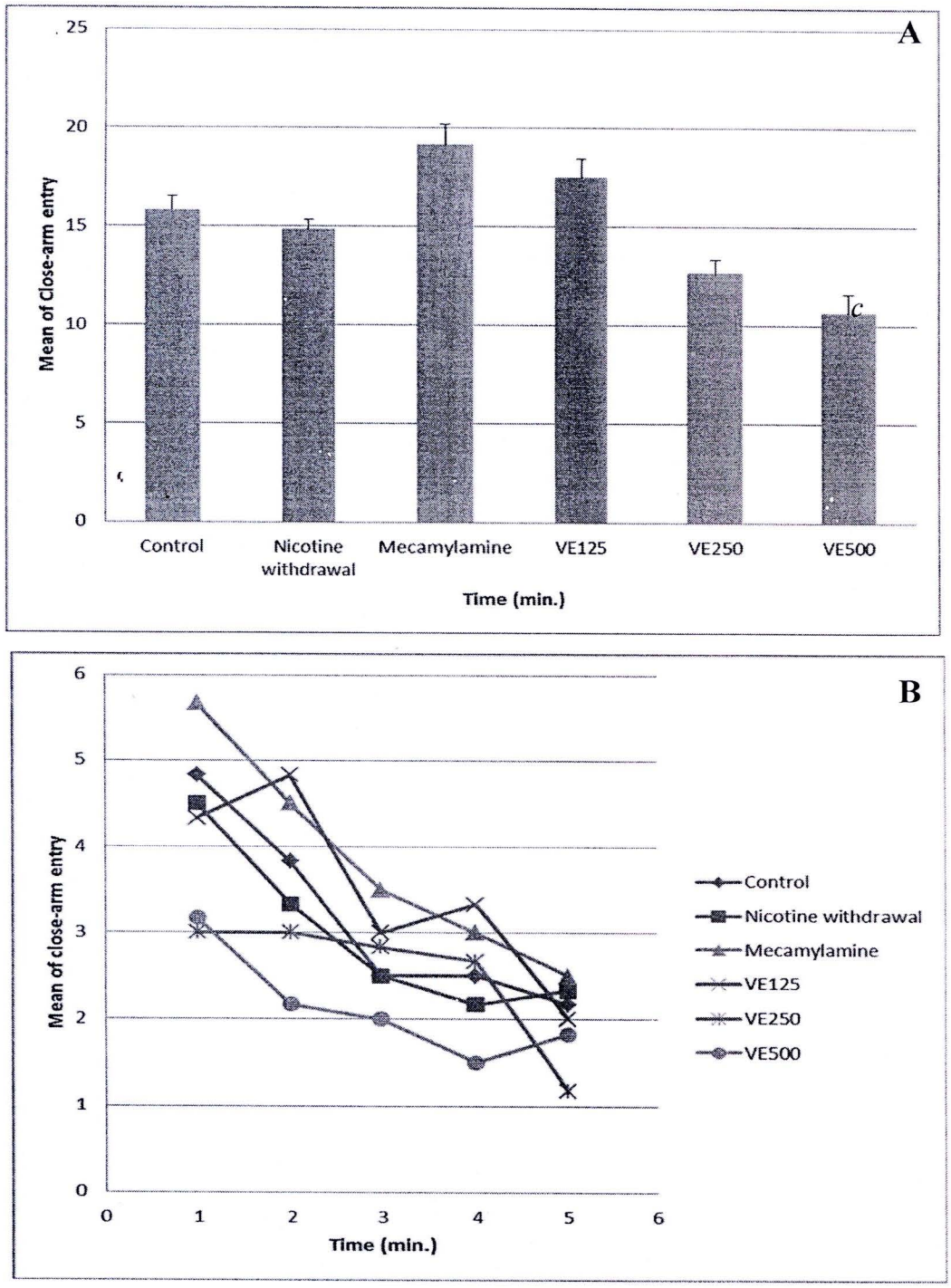


Figure 16 Anxiety-like behaviors of mice at 1 day after nicotine withdrawal. (A) Total close-arm entry. (B) Close-arm entry versus time.

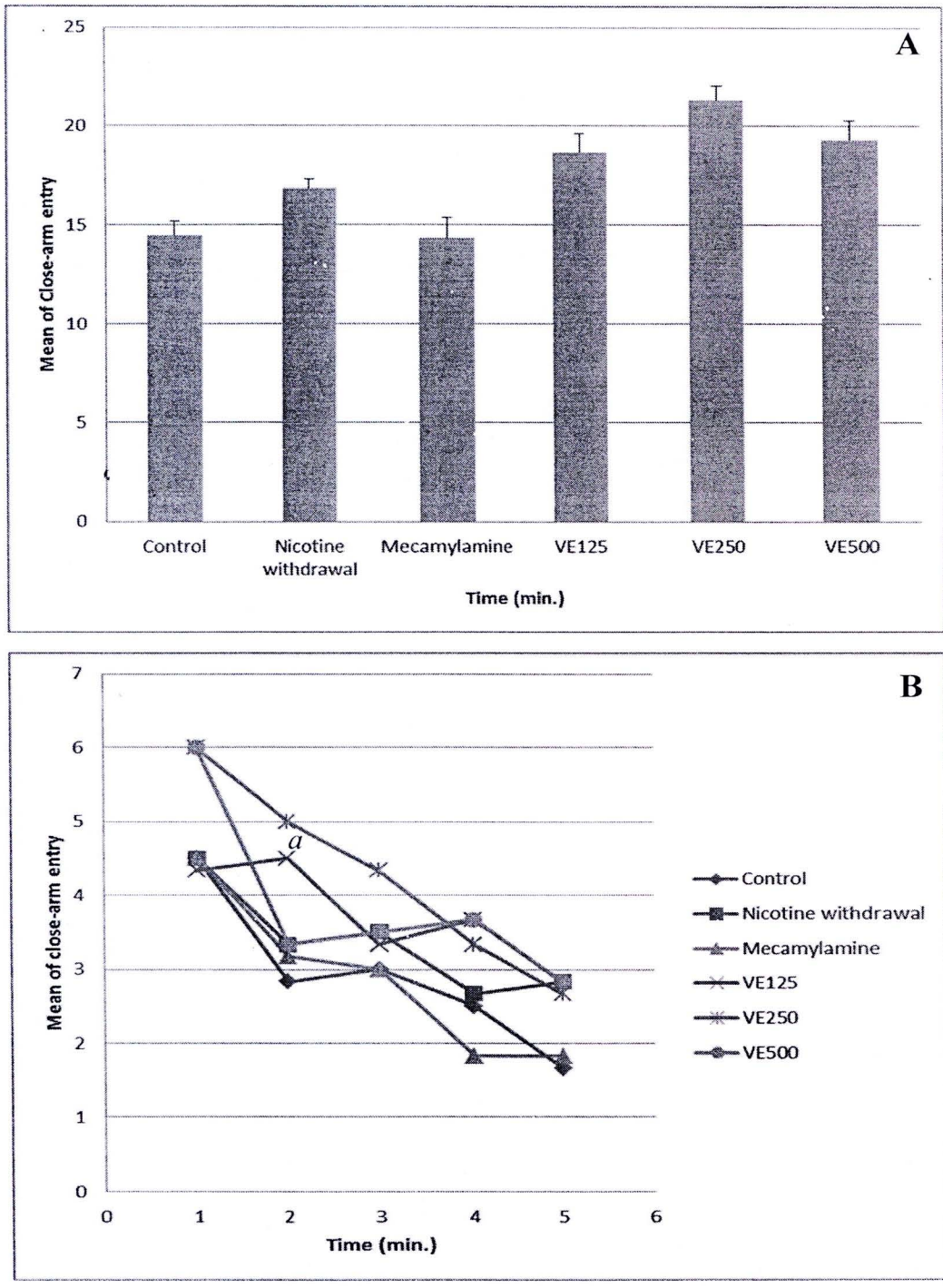
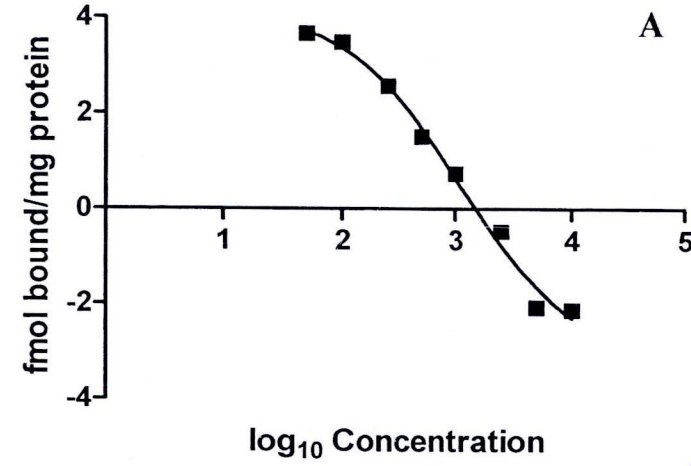


Figure 17 Anxiety-like behaviors of mice at 7 days after nicotine withdrawal. (A) Total close-arm entry. (B) Close-arm entry versus time. (*a* significant difference compared to control group, $p \leq 0.05$, n = 6)

The affinity of VE on nicotinic and muscarinic receptors

Receptor displacement assays are generally used for study of binding affinity or selectivity of any substances to known receptor [89] and report as number of mole bound per weight of protein.

Nicotinic receptor activity of the extract was presented by binding of [^{3}H]-nicotine. The result showed binding of the extract to nicotinic receptors in mouse brain in dose-dependent manner (Figure 18). The similar pattern of binding was also seen with muscarinic receptor, which has affinity to [^{3}H]-scopolamine. IC_{50} values of binding of nicotinic and muscarinic receptor were 1.145 mg/ml and 2.487 mg/ml, respectively (Figure 18). VE have lower IC_{50} values for nicotinic receptor than muscarinic receptor, thus VE has higher affinity to nicotinic receptor than muscarinic receptor.



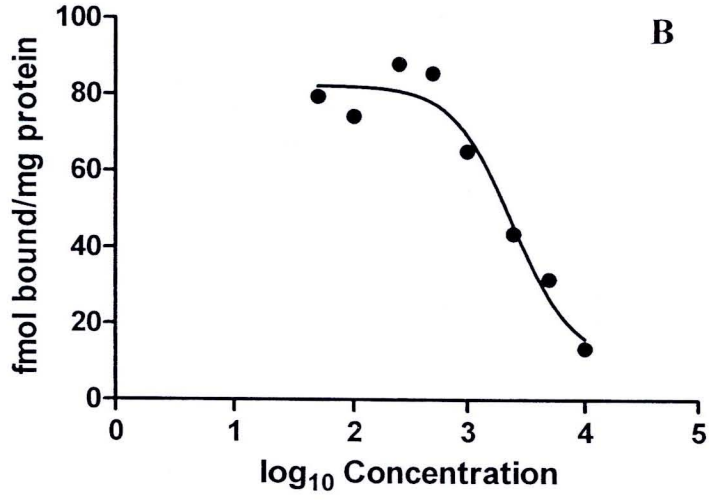


Figure 18 Receptor displacement curve for VE in mouse brains, (■) [^{3}H]-nicotine and (●) [^{3}H]-scopolamine binding.

Effect of VE on nicotinic and muscarinic receptors expression in mouse brain

1. Immunoblotting study

1.1 The effect of VE on the expression of α7 nicotinic receptor.

The bands of α7 nicotinic receptor expression with molecular weights of 56 kDa were detected. No significant differences in α7 nicotinic receptor expressions were found in all groups of mice (Figure 19).

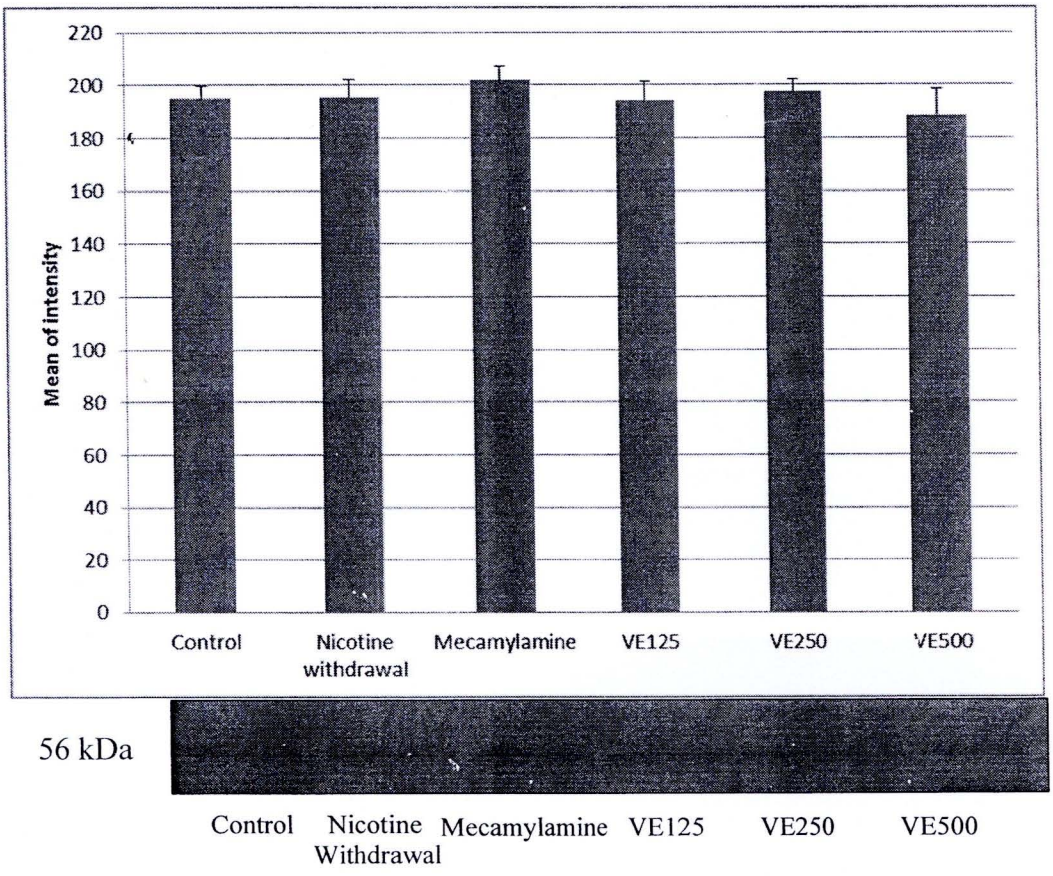


Figure 19 α7 nicotinic receptor expressions probed with nAChRα7 antibody were visualized using the enhanced chemiluminescence (ECL) detection kit and exposed to x-ray film.

1.2 The effect of VE on the expression of M5 muscarinic receptors

The bands of M5 muscarinic receptor expression with molecular weights of 60 kDa were detected. M5 muscarinic receptor expressions investigated by immunoblotting technique was found no significant differences between groups (Figure 20).

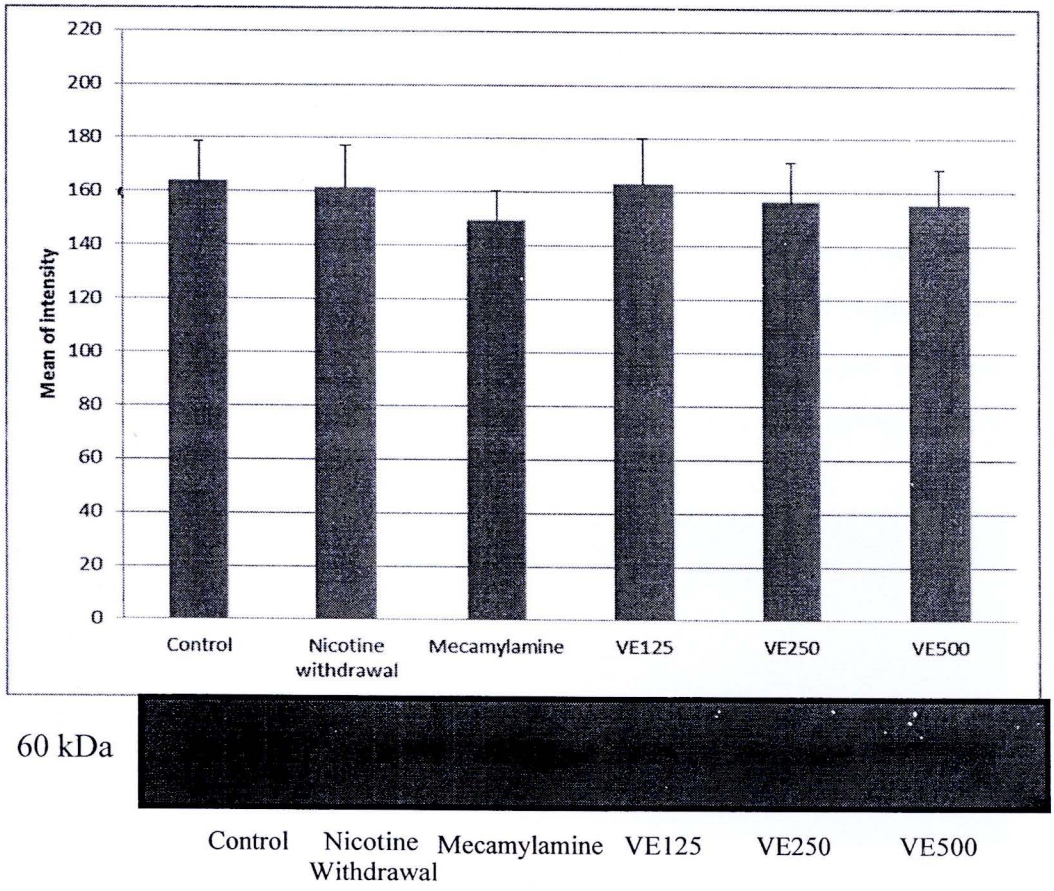


Figure 20 M5 muscarinic receptor expressions probed with mAChRm5 antibody were visualized using the enhanced chemiluminescence (ECL) detection kit and exposed to x-ray film.

2. Autoradiographic study

2.1 [^{3}H]-nicotine binding

Any binding for nicotinic receptor was failed to detect after incubation of the brain sections with tritium sensitive film for 30 days. The loss of nicotinic receptor binding might be less nicotinic receptor expression in the brain than muscarinic receptor. Low level of nicotinic receptor is confirmed with a previous study which reported low density of nicotinic receptor in NAcc, hippocampus, and VTA in mouse brain [90].

2.2 [^{3}H]-scopolamine binding

Autoradiographs of [^{3}H]-scopolamine binding in the mouse brains was shown in Figure 21. The result of autoradiographic study showed the same direction as seen in immunoblotting study for muscarinic receptor expression. There were no significant differences in muscarinic receptor level in NAcc, hippocampus, and VTA of the mouse brains between VE treated, NW, and MEC groups (Table 3).

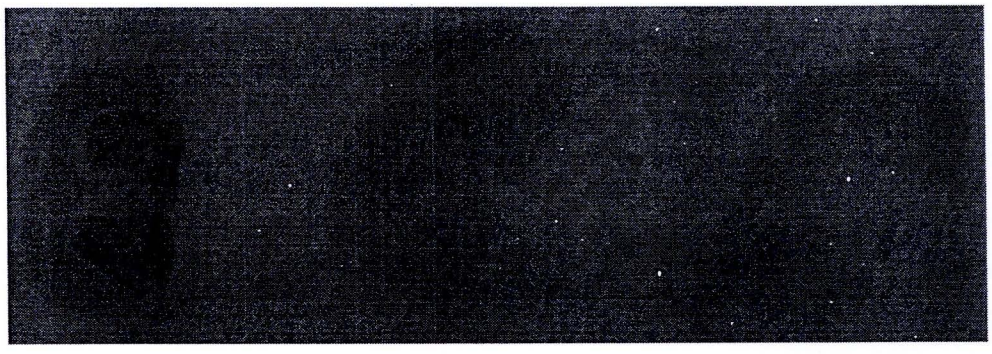


Figure 21 Autoradiographs of [^{3}H]-scopolamine binding in the nucleus accumbens (left), hippocampus (center), ventral tegmental area (right)

Table 3 Muscarinic receptor level in mouse brains measured by [^{3}H]-scopolamine binding

Treatment Group	Muscarinic Receptor Binding (fMol/mg)		
	NAcc	Hippocampus	VTA
Control	537.52±30.45	494.75±23.3	303.27±55.21
Nicotine withdrawal	584.10±12.93	405.77±30.75	302.53±14.05
Mecamylamine	511.84±80.97	429.92±32.68	291.77±39.38
VE125	555.42±25.17	413.43±34.73	182.93±15.77
VE250	591.94±48.9	444.17±12.54	206.68±8.15
VE500	572.30±28.5	413.07±19.58	173.16±19.24

Note: Values are shown as Mean±SEM with N=3.

Specific detection of α7 nicotinic and M5 muscarinic receptors in mouse brains using western blot showed no significant differences in all treatment groups. Muscarinic receptor levels in NAcc, hippocampus, and VTA of mice measured by the autoradiographic study was also showed no alteration with VE treatment as well as other treatments. These results suggest that administration of VE has no effect on nicotinic and muscarinic receptors in order to decrease nicotine withdrawal symptoms. However, other mechanisms which are considered to involve with nicotine dependence occur through major neuronal receptors such as dopaminergic and glutamatergic receptors [91, 92]. Therefore, VE may be involved with reduction of nicotine withdrawal symptoms by alteration in dopaminergic or glutamatergic activities.