Basic Study on Anti-stress Effect of Essential Oils

（エッセンシャルオイルの抗ストレス効果に関する基礎的研究）

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PREFACE

CHAPTER 1

Behavioral changes by acute inhalation of essential oils on group housed and socially isolated mice

PREFACE

1. Background of essential oils

Pure essential oils have been said to possess various effects on humans and other mammalian species when inhaled, applied, or ingested, and have been traditionally used folk medicine for a long time. In these years, essential oils have attracted lots of attention as a practical alternative treatment and many studies have been conducted. Some of those effects, such as the control of emotion and mood, (e.g. sedative [1-3], anxiolytic [4-8], antidepressant [9, 10], hypnotic [6, 11, 12], alert [6, 12]), antispasmodic [13], control of the autonomic nervous system activity [14, 15] and endocrine system [16, 17], strengthening of the immune system by stimulating the production of white blood cells [18], pain mitigation [16], anti-tumor [19], increase in lipolysis [20] antibacterial action [21], anti-inflammatory effect [22] etc., have been documented.

Among the properties of pure essential oils, the emotional and behavioral modulations, although there are many anecdotal or empirically speculated efficacious effects, are often difficult to examine and demonstrate in a scientifically controlled condition. Furthermore, the mechanisms of the effects of each essential oil have not yet been made clear and seem to differ for each essential oil. On the other hand, the efficacy of essential oils may differ considerably among mammalian species, as the absorption and metabolic capacity of chemicals and their mechanism differ from among mammalian species.

2. Hypothesis of how to essential oils penetration in a body by inhalation or application

When humans inhale or apply the essential oils, two routes are hypothesized for the penetration of the oil components into the body [23-25]. At first, the essential oil stimulates

the olfactory system chemically, and the signals are converted to electrical impulses, which are sent to the olfactory bulb, olfactory tract, frontal piliform cortex and amygdaloid complex. Finally, the fragrant can affect on the limbic system, performing various pharmacological actions (indirect effect via the olfactory nerve pathways). Olfaction is the only perception that affects visceromotor activity in an effective manner as regards emotional behavior, i.e., the autonomic nervous system, endocrine system, and immune system [26]. The second route is absorption of molecules into the bloodstream through the lungs via respiration, and/or through skin by application immediately (direct effect via the blood to the brain) [24]. Some components of the essential oil may be carried via the bloodstream to the brain through the blood-brain barrier and infiltrated into respective constitutional tissues or organs, and then might perform multiple functions physically, emotionally, and mentally [24, 25]. Since the components of the essential oil are soluble in lipids, they are quickly distributed and metabolized in each structure. In the liver, they are easily polarized, converted into water-soluble substances, and eliminated by urination or defecation [27-29].

When laboratory animals, i.e., rats and mice and dogs are exposed or applied to the essential oil, the oils are presumed to act the same way as humans do.

3. Difference in the efficacies of essential oils among animal species

On the other hand, the absorption and metabolic capacity of chemicals in essential oils and their mechanism differ among animal species, i.e., humans, mice, and dogs. In particular, since the abilities of chemical detoxification in the dog's liver are different from those in humans, since some chemicals innocuous to humans might cause a severe side effect in dogs. For example, xylitol, which is almost harmless to humans, causes hypoglycemia and hepatic failure in dogs after ingestion [30, 31]. The amount of theobromine found in chocolate is

small enough that chocolate can be safely consumed by humans in large quantities, but dogs that metabolize theobromine more slowly can easily consume enough chocolate to cause chocolate poisoning. The first signs of theobromine poisoning are nausea, vomiting, diarrhea, and increased urination. These can progress to cardiac arrhythmias, epileptic seizures, internal bleeding, heart attacks, and eventually death [32].

As a consequence, we believe that the efficacy of essential oils may differ considerably among humans, mice, and dogs, certain essential oils may actually cause opposite properties for between dogs and humans. Nonetheless, there are slim and none about the data to directly determine how to clinical use of essential oil in dogs (dose, administration time, precautions, toxicity and safety, the result of skin sensitization tests, etc.). Therefore, most of veterinarians have used essential oil extrapolating the data that verified the safety and effects in humans as it is now, it must be dangerous.

4. Socially isolated mouse model

Social isolation induces a complex set of neurobehavioral abnormalities in primates [33], in rats [34] or mice [35]. The behavioral alteration is particularly conspicuous within social contexts involving an unfamiliar conspecific [36, 37]. The isolation-induced alteration on the behavioral and physiological responses in laboratory animals have been studied, and already the usefulness of socially isolated (SI) mouse as a pathological model for emotional disorders, schizophrenia and incontinentia including phenotypes of anxiety, depression, and aggression has been established. Furthermore, the exaggeration of the responses to a novel stimulus or to stimuli predictive of danger and locomotor hyperactivity [38, 39] has been reported on the SI mice. It is well known that the mouse brain is not matured histologically or functionally immediately after weaning. Since then, various social events can influence the maturation of

the synaptic composition and brain development. Breaking off contact with the littermates after weaning may induce abnormal development of the neurotransmitter systems in the brain.

Dopamine (DA) plays an important role in mediating the effects of isolated housing on the social behavior of mice. Gendreau *et al.* [40] reported that DA agonists potentiate defensive behavior and/or social fearfulness in the SI mice. They further suggested that D_3 and D_2 DA receptors differentially modulate the expression of social-emotional reactivity. Reduction of brain $GABA_A$ receptor function in the SI mice also was reported, as evaluated by measuring GABA-evoked Cl^- currents in xenopus oocytes [41]. As to serotonin (5-HT), Schiller *et al.* [42] analyzed by in vitro autoradiography, and they reported that 5-HT_{1A} and 5-HT_{2A} receptor densities in the brains of SI mice were strongly reduced. Ago and Matsuda *et al.* [43, 44] examined the modulation of 5-HT, DA, and noradorenaline (NA) release by selective 5-HT_{1A} receptor agonists in the cortex of the SI mice by microdialysis. In this study, the basal level of extracellular DA in the frontal cortex was higher in the SI mice than in the group-housed (GH) mice. The 5-HT_{1A} receptor agonist, MKC-242 induced increases in the cortical DA release was less pronounced in the SI mice than the GH mice. These findings suggest that the socially isolation-induced distress enhances the activity of cortical DAnergic neurons and reduces the responses of DAnergic terminals to 5-HT_{1A} receptor stimulation.

5. Objectives

Among the many efficacies of essential oil, anxiolytic, antidepressant, and anti-stress effects are very helpful for psychiatry and psychopharmacology, since combining the medicine and essential oil can reduce the dose of those medicines and essential oil may help prevent the side effects of the anxiolytic and antidepressant medicines. All clinically available anxiolytics and antidepressants have limited clinical efficacy because of their adverse side

effects, such as the amnestic effect of benzodiazepine.

The anxiolytic, antidepressant, and anti-stress effects of essential oils and their precise mechanisms have not yet been made clear. Some studies have suggested that essential oils may affect the modulation of the glutamatergic system [45], the strength of the GABA neuron system [23], or the stimulation of 5-HT secretion and inhibition of selective 5-HT reuptake [46].

The present studies were designed to find the oils possessing anxiolytic, antidepressant, and anti-stress effects and to investigate the mechanisms. At first, the behavioral effects of inhaling lavender (*Lavandula angustifolia*), rose (*Rosa damascene*), and lemon (*Citrus limon*) oil vapor were estimated by three tests of anxiety such as elevated plus-maze (EPM), open field test (OFT), and forced swimming test (FST) on mice. Concerning lemon oil, which neurotransmitter activity was related to the effects was investigated by pre-treatment with neurotransmitter's agonists, antagonists and reuptake inhibitor in behavioral tests, and both acute and chronic inhalations were examined. Furthermore the contents of monoamines and their metabolites in the prefrontal cortex, the hippocampus, and the striatum and the levels of plasma corticosterone were measured.

On subject animal, the GH mice at 6-week-old were used as the model in normal and the SI mice for 3 weeks after wearing were used as the pathological model for emotional disorders and whether the lemon oil inhalation was effective to emotional disease was demonstrated.

In addition, we assessed the relaxing efficacy of lavender oil (*Lavandula angustifolia*) quantitatively in dogs via dermal application. To be more precise, we evaluated the autonomic nervous system activities of dogs to which lavender oil had been applied in succession. From the results and the other literatures, we assessed the properties of the lavender oil in making

dogs more relaxed. If lavender oil had those properties, its application might become practical alternative treatments for the dogs that have some behavioral problems. There behaviors could include hyperexciting, aggressiveness, separation anxiety disorder, travel-induced excitement, and sleep disorder in aged dogs. Veterinarians often witness hyperexciting dogs in clinics.

CHAPTER 1

Behavioral changes by acute inhalation of essential oils on group housed and socially isolated mice

INTRODUCTION

Some essential oils, lavender, rose, and lemon have been generally said to possess anxiolytic and antidepressant effects. Thus the behavioral tests were examined on the GH mice that inhaled those oil vapors for 90 min (acute inhalation). Among the behavioral tests, the EPM and the OFT are established animal model of psychotical anxiety and the FST is a pharmacologically useful screening test for antidepressant properties.

Since the result proved lemon oil vapor to possess the most anxiolytic and antidepressant effects of the three, lemon oil alone was examined in the following examination on the SI mice. The SI mouse model are exposed long-term isolation stress, though under the more distress condition than the normal GH mice, and are considered to be a pathological model of mood disorders. Social isolation stress causes anxiety, antidepressant, and aggressive behavior. Therefore lemon oil may be available in such a case.

MATERIALS AND METHODS

1. Animals

ICR strain mice were obtained from Tokyo Laboratory Animal Co., Ltd. (Tokyo, Japan). They were mated, and then newborn mice were weaned at 20 days of age. Immediately after the weaning, male mice were randomly separated into two groups: GH and SI. In the GH group, five mice were housed in the same cage (35.5 x 30.5 x 16.5 cm) for three weeks. In the SI group, all mice were housed individually in cages (22 x 14.5 x 12 cm) for three weeks, and were not allowed physical or visual contact with other mice (Fig.1-1). Male mice were used, because female rats showed a reduced aversion to the open arms compared to male rats in the EPM [47] and we observed similar results in mice.

The mice were maintained at a controlled temperature (23±2°C) and on a regular light/dark cycle (7:00 to 19:00 h, light), and all animals had free access to food and water. The animals were native to essential oils, and each mouse was studied once only. And in every experiment, the numbers of animals per group were 5-10. All experiments were conducted in accordance with the guidelines regarding the care of experimental animals, as approved by the Animal Research Committee at Tottori University.

2. Inhalation of essential oil vapor

The essential oils of lavender, rose, and lemon were supplied by Soda Aromatic Co., Ltd. (Tokyo, Japan). The component analyses of essential oils used in this study are shown in Table 1-1, which was submitted by Soda Aromatic Co., Ltd.

To be efficiently vaporized, the essential oil was mixed with the same volume of ethanol and was then soaked up by cotton set on the upper side of an inhalation box. In all experiments, 1-2 ml of the essential oil was vaporized in the container (30 x 23 x 15 cm). Ethanol was applied as the control treatment. The inhalation of essential oil was started 90 min before the behavioral tests (acute experiment). In case of OFT, the inhalation was started 90 min before the test, and maintained throughout the test (Fig.1-2, acute experiment).

3. Behavioral analyses

3.1. Elevated plus-maze test

The EPM was performed according to the protocol used in the previous report [48] with slight modifications (Fig.1-3). In brief, the mice were placed in the experimental room to facilitate adaptation at least 1 h before the test. All tests were carried out during the light

period (11:00-15:00), in a counterbalanced random order. Each mouse was placed at the center of crossed arms, and then its behavior was recorded for 5 min using a video camera (CCD-TR 313, Sony, Tokyo, Japan). Between subjects, the maze floor was thoroughly cleaned with cotton and ethanol. Parameters consisted of the frequency of the open and closed arm entries, the percentage of entries into the open arms, and the amount of time spent on the open arms, which were determined by replaying the videotape.

3.2. Forced swimming test

Forced swimming test (FST) was carried out according to the method of Porsolt *et al.* [49]. Mice were dropped individually into glass cylinders (height 25 cm, diameter 10 cm) containing 10 cm of water, maintained at 23-25°C (Fig.1-4). Mice were left in the cylinder for 6 min. After the first 2 min, the total duration of immobility in mice was measured during a 4 min test. The mouse was judged to be immobile when it remained floating passively in the water.

3.3. Open field test

The open field test apparatus consisted of a clear acrylic box (30 x 30 x 35 cm) with a lid (Fig.1-5). The floor was divided into thirty-six 5 cm squares by drawing. The test was performed in a light- and sound-attenuated shield box with a dim light.

Each mouse's behavior was then recorded by video camera for 45 min. We evaluated the following behavior with 5 min intervals: the locomotor activity counts (the frequency across the squares) and the rearing counts (the frequency of rearing around the wall). Between subjects, the box was thoroughly cleaned with cotton and ethanol.

4. Statistical analysis

All results are expressed as means ± SE. Differences between the GH and SI mice groups were assessed by unpaired Student's *t*-test. Differences between treatment groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A probability level of $P<0.05$ was taken to be statistically significant in the analyses.

RESULTS

1. Behavioral changes by inhalation of essential oils

Total numbers of entries into the open and closed arms in the EPM were not significantly changed by inhalation of ethanol, lavender, rose, or lemon oil (Fig.1-6A). On the other hand, the percentage of entries into the open arms was significantly increased in the lemon oil-treated mice ($F(2, 30)=18.29$; $P<0.01$) (Fig.1-6B). The time spent on the open arms was also significantly increased in the lemon oil-treated mice ($F(2, 30)=8.024$; $P<0.01$) (Fig.1-6C).

An antidepressant effect of lemon oil was more apparent in the FST ($F(2, 15)=5.841$; $P<0.05$) (Fig.1-7). Lavender and rose oil had no effect on the immobility time in the FST.

In the OFT (Fig.1-8A, B), both the locomotor activity and the rearing counts for 45 min were lower in the mice treated with lavender or lemon oil inhalation than in mice in other groups, and a significant effect was determined only in the lemon oil-treated group ($F(2, 15)=6.936$; $P<0.05$ and $F(2, 15)=11.53$; $P<0.01$). Lavender oil had tendencies to decrease in the locomotion and rearing counts, however those were not significant from ethanol-treated mice.

These results show that lemon oil vapor has anxiolytic, antidepressant, and sedative

effects and suggest that lemon oil vapor has a suppressive effect on distress in mice.

2. Behavioral characteristics of the SI mice

Body weight was measured on every eighth day throughout the experimental period and until 90 days old, and there was no significant difference between the GH and SI groups. The wet weights of the thymus, liver, pancreas, spleen, testis, epididymal adipose, kidneys, and adrenal glands in the SI group were also similar to those in the GH group (data not shown).

After the treatments for three weeks were completed, the mice were used for the behavioral tests. As shown in Fig.1-9, the total number of open and closed arm entries, the percentage of entries into the open arms, and the time spent on the open arms in the SI mice tended to reduce compared with those in the GH mice, respectively in the EPM. But there were no significant differences between the two groups by t-test. In the FST, the immobility duration did not show any alternation with isolation treatment (Fig.1-10).

In the OFT, the mice in both groups revealed a high degree of locomotor activity and rearing counts immediately following the start of the test (Fig.1-11A, B). While the locomotor activity and rearing counts in the GH mice gradually calmed down as the time course progressed, those in the SI mice kept higher levels. The total locomotor activity and rearing counts for 45 min was significantly higher in the SI group than in the GH group by t-test ($P<0.01$). These results suggest that the social isolation-induced stress may induce abnormal behaviors such as hyperactivity.

3. Behavioral changes by the inhalation of lemon oil vapor on the SI mice

The total numbers of open and closed arm entries ($F(2,23)=5.218$; $P=0.0150$), the percentage of entries into the open arms ($F(2,23)=4.221$; $P=0.0296$), and the time spent on the

open arms ($F(2,23)=25.92$; $P<0.0001$) significantly increased with lemon oil treatment in the EPM (Fig.1-9).

The immobility duration also significantly reduced with lemon oil treatment in the FST ($F(2,23)=15.29$; $P<0.0001$) (Fig.1-10). On the other hand, locomotor activity and rearing counts showed a tendency to decrease due to acute lemon oil inhalation but not significantly in the OFT (Fig.1-11A, B).

DISCUSSION AND CONCLUSION

The present study showed that the 90 min inhalation of lemon oil vapor induced anxiolytic and antidepressant effects in the normal mice housed in group. These results suggest that lemon oil vapor has a suppressive effect on distress in mice. There are many reports showing the anxiolytic (p.o.) [4], sedative (i.p.) [50], antispasmodic (i.p. and p.o.) [51], and antidepressant (inhale) [9] effects of lemon, lemon odor, or limonene in mice or rats. Limonene is one of the major components of lemon oil. In humans, it has been shown that ambient lemon odor can improve creativity, mood, and perceived health [52]. Heart rate changes have also been reported after exposure to lemon essential odor [53]. However, the precise mechanisms of these psychological or pharmaceutical effects induced by lemon oil vapor are still unknown.

The social isolation-induced stress increased aversion to the open arms in the EPM, did not show any effect to immobility duration in the FST, and kept high levels of behavioral activity under novel situation in the OFT. The result of the OFT suggested that the SI mice fell into hyperactivity, therefore the immobility duration in the FST reduced even though the SI mice were under depressant. The social isolation distress induced an anxiogenic and disturbance state easily, neophobia.

On the other hand, lemon oil vapor were also effective in such as psychologically distressed model mice, i.e. the SI mice as well as on the mice in normal. 90 min inhalation of lemon oil vapor also induced anxiolytic and antidepressant effects in the SI mice. However, lemon oil vapor did not suppress hyperlocomotion on the SI mice better than on the GH mice. This reason is also that the social isolation distress may induce the mice under a condition of "hyperactivity" strongly.

In conclusion, the acute inhalation of lemon oil vapor proved to possess anxiolytic, antidepressant, and sedative effects and those effects were obtained on both the mice in normal and the psychologically distressed model mice. These results suggest that lemon oil vapor has a suppressive effect on distress in mice.

1. The model in normal
... group housed mice at 6-week-old

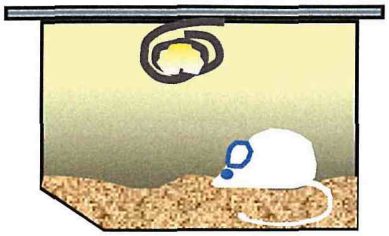
2. The pathological model for emotional disorders
... socially isolated mice for 3 weeks after wearing

Fig.1-1. Housing conditions of the animal (♂)

Table 1-1 Main components of the lavender, lemon, rose oil analyzed by GC-MS

Lavender oil Compound name	GC area%
Linalyl acetate	39.07
Linalool	28.15
Caryophyllene	5.46
4-Terpineol	3.53
1,8-Cineole	3.50
Lavandulyl acetate	2.42
Trans-β-Ocimene	2.23
cis-β-Ocimene	1.20
β-Farnesene	1.13
Borneol	1.09
α-Terpineol	0.86
1-Octen-3-yl acetate	0.82
Lavandulol	0.64
Lemon oil Compound name	GC area%
Limonene	54.48
β-Pinene	15.82
γ-Terpinene	11.33
α-Pinene	3.29
Geranial	2.31
β-Myrcene	2.22
Neral	1.62
p-Cymene	1.16
Rose oil Compound name	GC area%
Rose P	47.73
Citronellol	10.42
Geraniol	5.28
Eugenol	2.32
Nerol	2.02
Methyl eugenol	1.02
Farnesol	0.92
Benzyl alcohol	0.73
Phenylethyl acetate	0.67
Geranyl acetate	0.59

1. Acute experiment
... inhalation just 90 min
before the behavioral tests



2. Chronic experiment
... inhalation for 120 min during the dark period
every day until the day before the behavioral tests

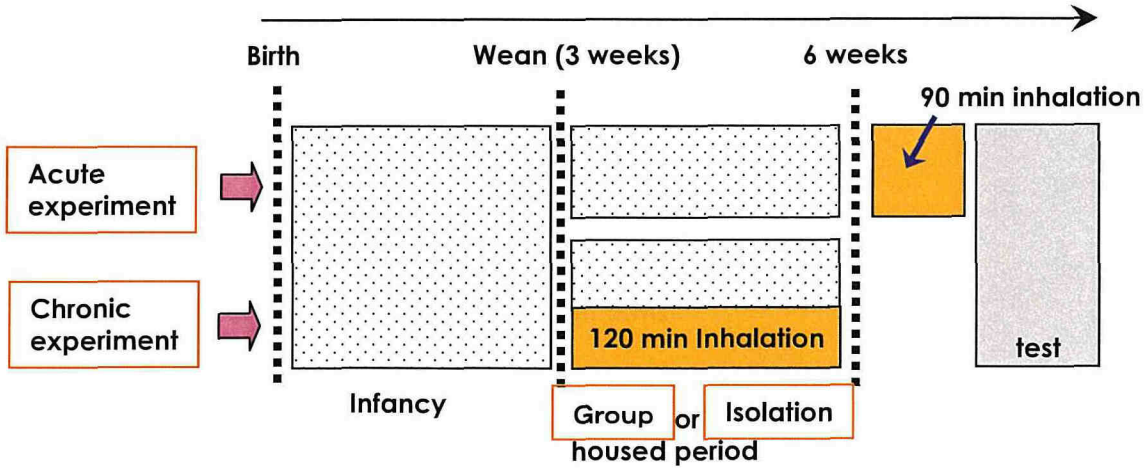
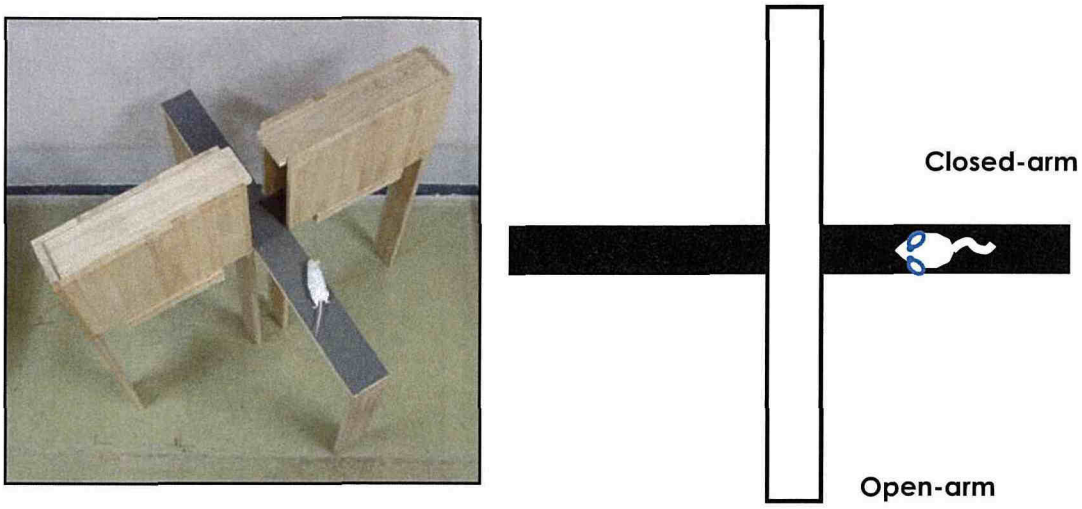


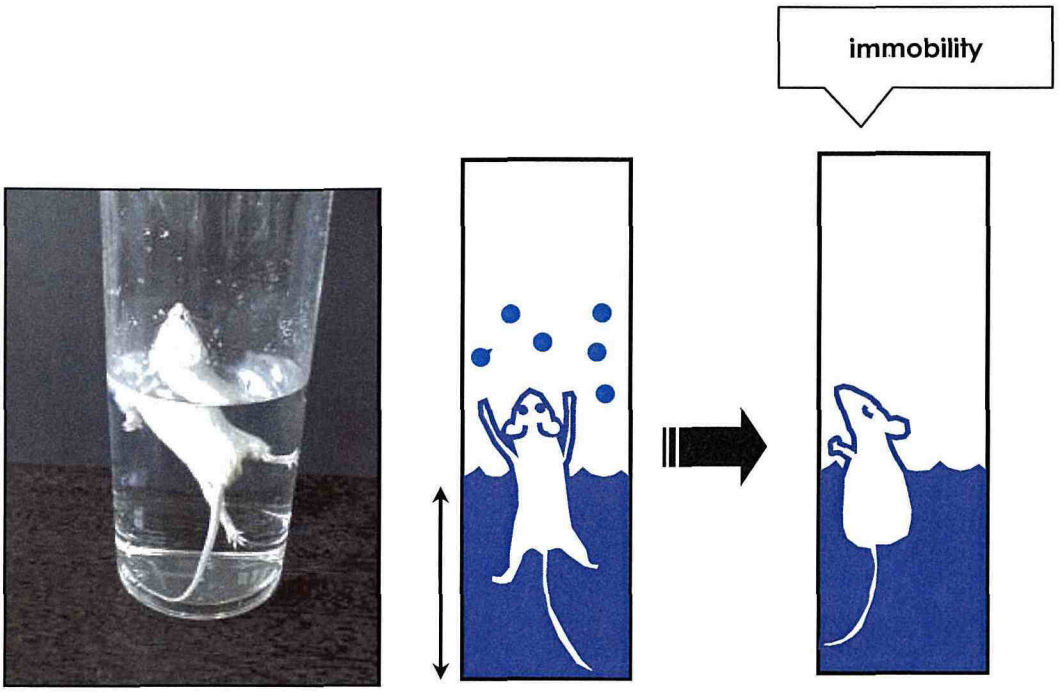
Fig.1-2. Schedule of inhaling lemon oil



Parameters

1. Frequency of arm entries
2. Percentage of entry into open arms
3. Time spent in the open arms

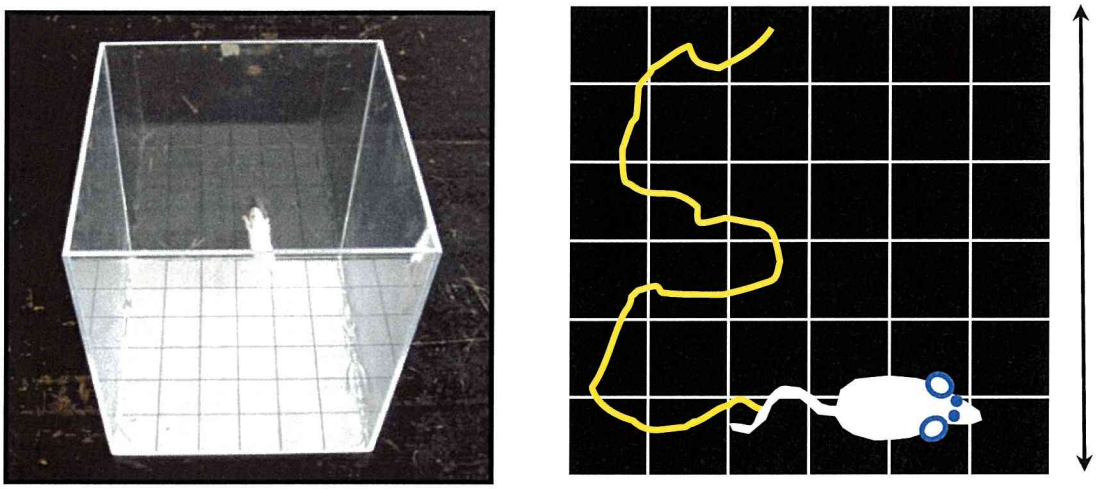
Fig.1-3. Outline of Elevated plus-maze test



Procedure:

After the first 2 min, the total duration of immobility was measured during a 4 min period.
an increase of Immobility time = depression or anxiety

Fig.1-4. Outline of Forced swimming test



Parameters:

1. Frequency across the squares
2. Frequency of rearing around the wall

Fig.1-5. Outline of Open field test

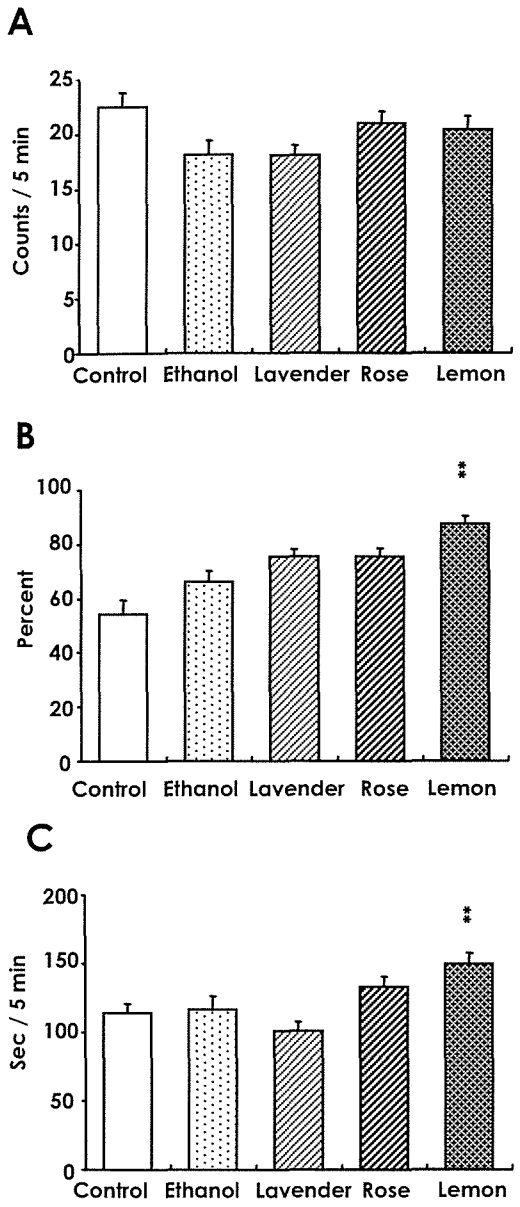


Fig. 1-6. Effects of the inhalation of essential oil vapor on the elevated plus-maze test in GH mice.
A: The total number of open arm entries,
B: The percentage of entries into the open arms,
C: The time spent on the open arms during a 5-min period.
Each value represents the mean±SE of 5-10 mice.
* $P<0.05$, ** $P<0.01$ compared to the ethanol group by one-way ANOVA with Tukey's multiple comparison test.

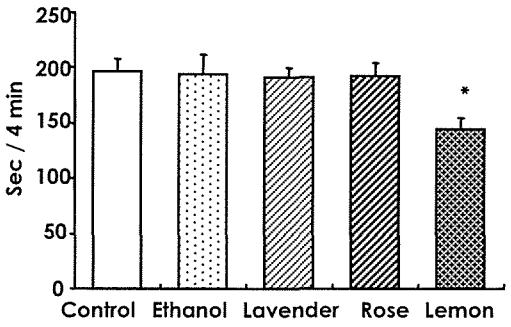


Fig. 1-7. Effects of the inhalation of essential oil vapor on the forced swim test in GH mice.
The graph represents the immobility duration time during a 4-min period. Each value represents the mean±SE of 5-10 mice.
* $P<0.05$ compared to the ethanol group by one-way ANOVA with Tukey's multiple comparison test.

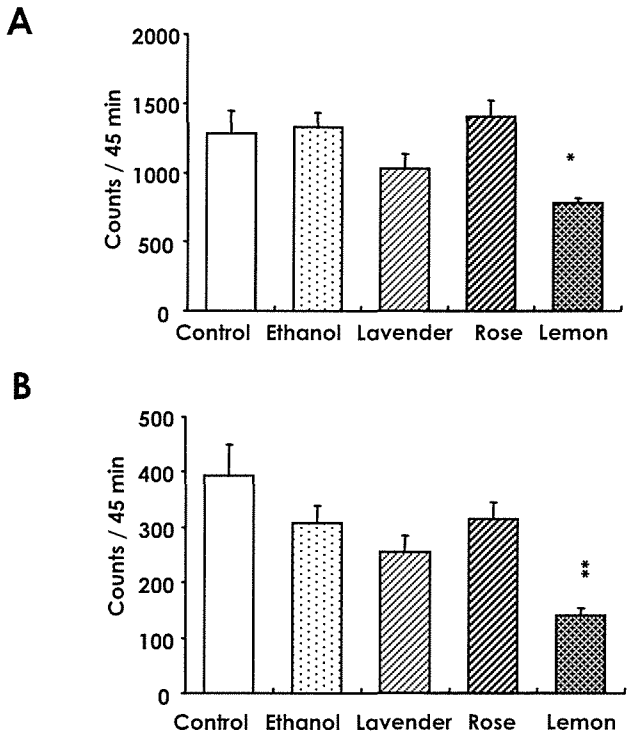


Fig. 1-8. Effects of the inhalation of essential oil vapor on the open field test in GH mice.

A: The total locomotor activity counts, B: The rearing counts for 45 min. Each value represents the mean±SE of 5-10 mice.

* $P<0.05$, ** $P<0.01$ compared to the ethanol group by one-way ANOVA with Tukey's multiple comparison test.

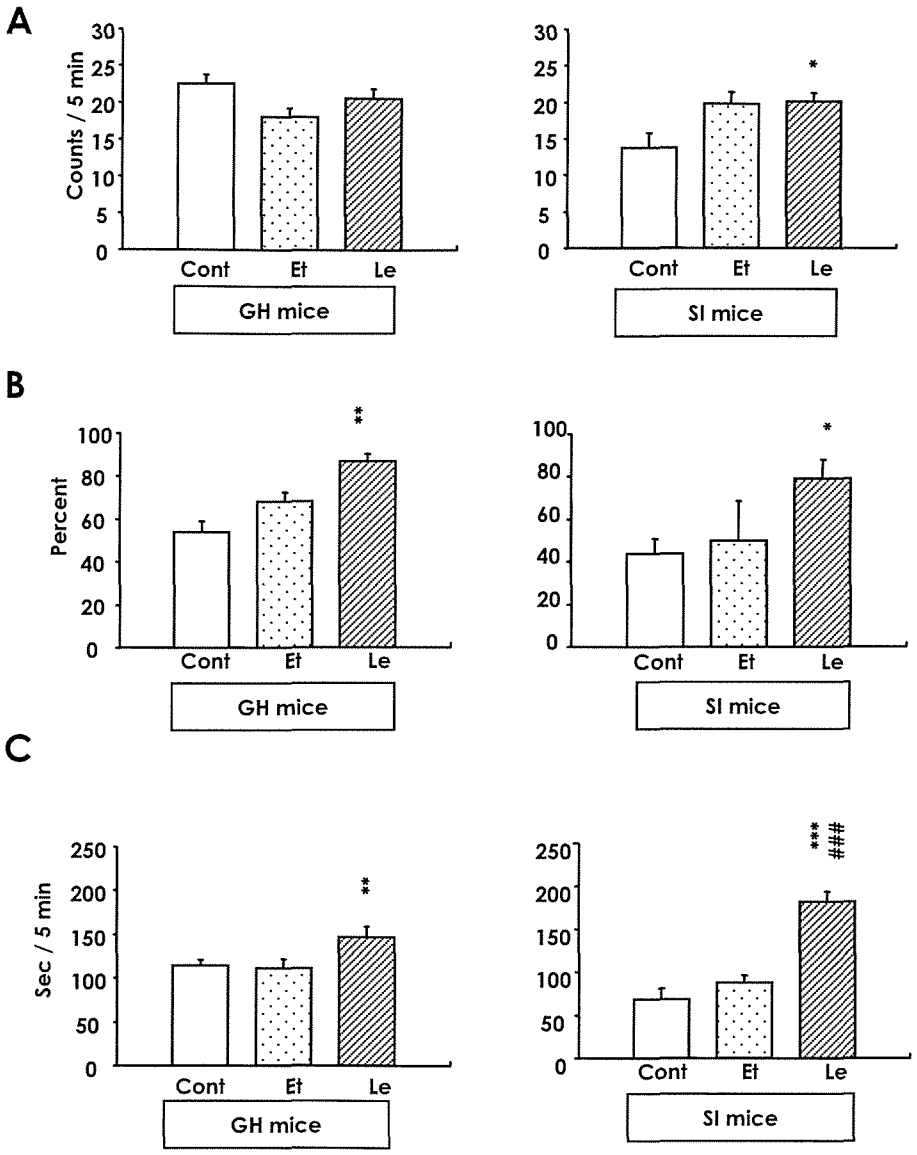


Fig. 1-9. Effects of the inhalation of lemon oil vapor on elevated plus-maze test in GH and SI mice.

A: The total number of open and closed arm entries,
B: The percentage of entries into the open arms,
C: The time spent on the open arms during a 5-min period.
Each value represents the mean±SE of 5-11 mice. The data are assessed by one-way ANOVA with Tukey's multiple comparison test.
* P<0.05, ** P<0.01, *** P<0.001; significantly different from GH or SI control group, respectively. ### P<0.001; significantly different from ethanol inhalation group.
Cont: control, Et: ethanol inhalation, Le: lemon oil vapor inhalation.
GH mice: group-housed mice, SI mice: socially isolated mice.

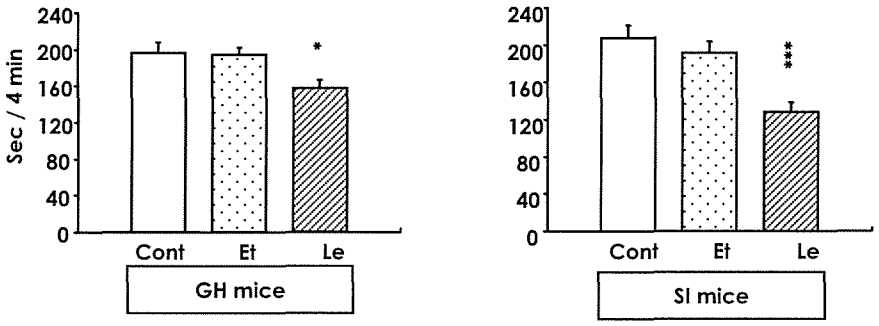


Fig. 1-10. Effects of the inhalation of essential oil vapor on the forced swim test.
The graph represents the immobility duration time during a 4-min period. Each value represents the mean±SE of 5-11 mice.
*P<0.05, *** P<0.001 compared to the ethanol group by one-way ANOVA with Tukey's multiple comparison test.
GH mice: group-housed mice, SI mice: socially isolated mice.

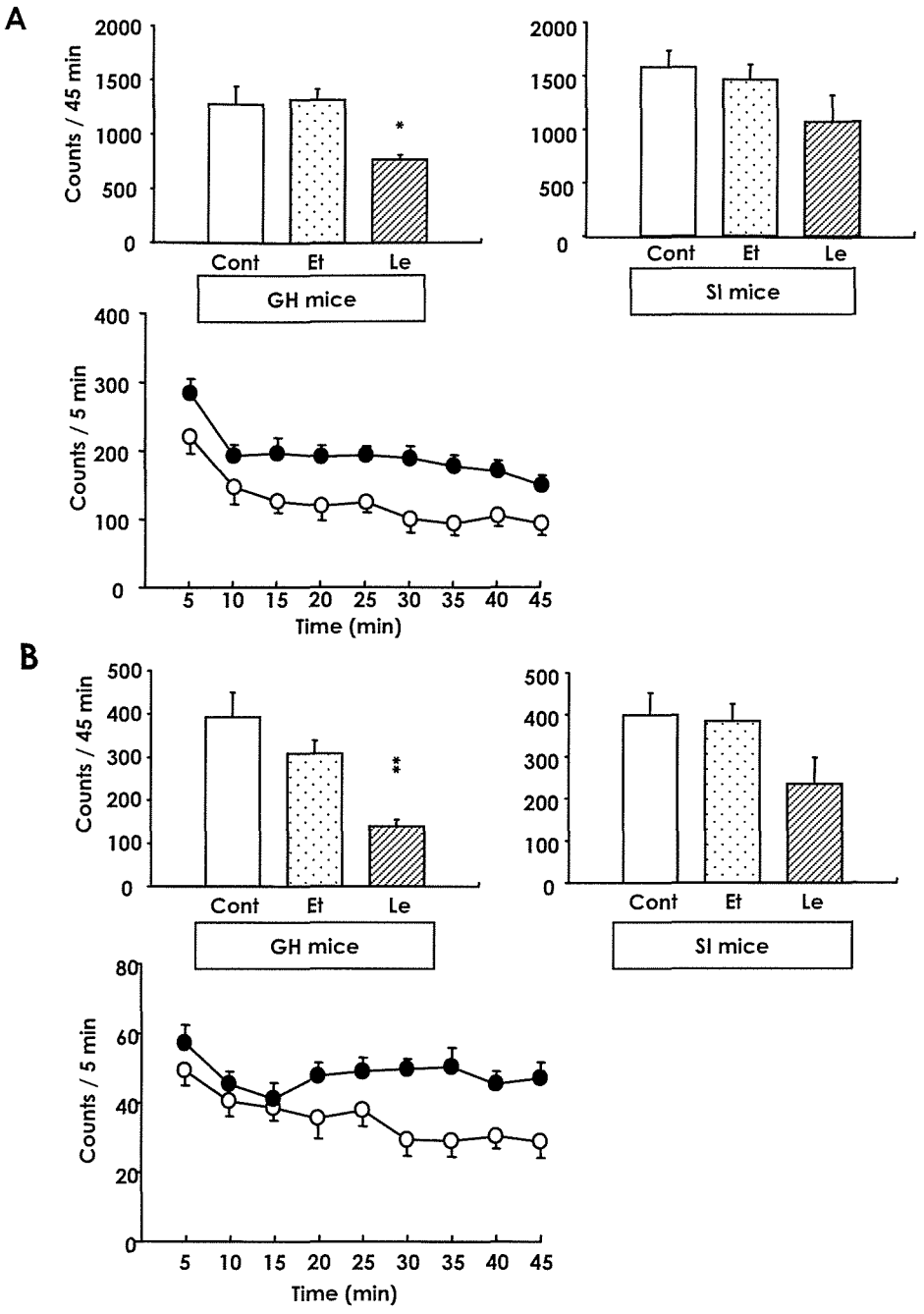


Fig. 1-11. Effects of the inhalation of essential oil vapor on the open field test in mice.

A: The total locomotor activity counts, B: The rearing counts for 45 min.
The open circle points indicate GH control mice, the filled circle points indicate SI control mice, respectively. Each value represents the mean±SE of 5-10 mice.
* $P<0.05$, ** $P<0.01$ compared to the ethanol group by one-way ANOVA with Tukey's multiple comparison test.
GH mice: group-housed mice, SI mice: socially isolated mice.

CHAPTER 2

Behavioral characteristics on the socially isolated mice following chronic inhalation of lemon oil vapor

INTRODUCTION

The present study was addressed to investigate whether the chronic inhalation of lemon oil change the behavioral reaction on the SI mice. Since breaking off contact with the littermates after weaning for 3 weeks must induce abnormal development of the neurotransmitter systems in the brain of mice, the SI mice are thought to modulate DAnergic neuron, 5-HTergic neurons, and any other neurons in the brain regions.

On the other hand, Fujiwara *et al.* [18] reported that the animal easily became accustomed to some fragrances through long-term exposure. They observed that immunological disorders were restored when mice were exposed to lemon and oakmoss over 3 weeks, but not when exposed to tuberose and labdanum. They also tested the use of lavender oil for the same disorder, and they confirmed that lavender had no effect when mice were exposed to it over 3 weeks (unpublished). Furthermore, the results of EPM in group housed rats following long-term exposure of lemon oil odor by Ceccarelli *et al.* [16] showed aversion to the open arms.

Then, whether lemon oil recovered those neuron modulations by social isolation-induced stress following chronic inhalation of lemon oil vapor during isolation term for 3 weeks were investigated.

MATERIALS AND METHODS

1. Animals

The mice were described in Chapter 1.

2. Inhalation of lemon oil

How to vaporize lemon oil was described in Chapter 1. The inhalation of lemon oil vapor

was administered for 120 min during the dark period (22:00-24:00) every day from 3 to 6 weeks of age, during 3 weeks of isolation (chronic experiment) (Fig.1-2). The next day after the completion of the treatments, the mice were tested by EPM, FST and OFT. Ethanol was applied as the control treatment.

3. Behavioral analyses

Refer to chapter 1.

4. Statistical analysis

Refer to chapter 1.

RESULTS

Interestingly, in the EPM, the chronic inhalation of lemon oil vapor significantly decreased all the parameter, the total numbers of entries into the open and closed arms ($F(2,15)=7.679$; $P=0.0071$), the percentage of entries into the open arms ($F(2,19)=5.234$; $P=0.0178$), and the time spent on the open arms ($F(2,19)=27.54$; $P<0.0001$) compared with no inhaled SI control group, although ethanol inhaled control itself also showed an decrease of the three parameters ($F(2,15)=5.970$; $P=0.0159$) (Fig.2-1).

The immobility duration did not alter in the FST treated with lemon oil vapor inhalation (Fig.2-2), compared with the acute inhalation of lemon, significantly increased ($P<0.001$). The counts of the locomotor activity in the OFT showed no significant difference among no inhaled control group, ethanol inhaled group, and lemon oil inhaled group. The rearing counts also revealed the same tendency (Fig.2-3).

DISCUSSION AND CONCLUSION

The chronic inhalation of lemon oil vapor on the SI mice showed opposite results in all of tests from the acute inhalation, then, those findings suggested that the chronic inhalation significantly induced anxiogenic and did not possess antidepressant effects. The study by Ceccarelli *et al.* suggest that long-term exposure of lemon oil vapor changes in neuronal circuits involved in anxiety and pain similar result on behavioral test was obtained in group housed rats [16].

The long-term successive inhalation of essential oil might produce "tolerance" possibly. Fujiwara *et al.* [18] reported that the animal easily became accustomed to some fragrances through long-term exposure. They observed that immunological disorders were restored when mice were exposed to lemon and oakmoss over 3 weeks, but not when exposed to tuberose and labdanum. About anxiolytic effect of lemon oil in this study, the mice might be apt to accustom to lemon, or might relate with the metabolic capacity of chemicals in mice.

The SI mice are thought to modulate DAnergic neuron, 5-HTergic neurons, and any other neurons in the brain regions due to a long-term exposure under isolation stress. Because those organic modulations were strong, that chronic inhalation of lemon oil vapor failed to recover those modulations could be conjectured. However acute inhalation of lemon oil vapor induced anxiolytic and antidepressant effects on the behavioral tests and the data of chronic inhalation of lemon oil vapor were inferior to those of SI control. Acute inhalation of lemon oil, in other word, inhalation of lemon oil right before the behavioral tests might make the SI mice interim anxiolytic and antidepressant mood.

In conclusion, although the acute inhalation of lemon oil vapor induced anxiolytic and antidepressant effects on the behavioral tests, chronic inhalation of lemon oil vapor failed to recover the abnormal behaviors in the SI mice.

To clear these mechanism, the behavioral tests were carried out following pre-treatment with GABA, 5-HT, and DA neurotransmitter's agonists, antagonists and reuptake inhibitor in addition to acute inhalation of lemon oil vapor on GH mice, and the contents of monoamines and their metabolites in the prefrontal cortex, the hippocampus, and the striatum of GH and SI mice following the chronic inhalation of lemon oil vapor were measured in Chapter 3, 4.

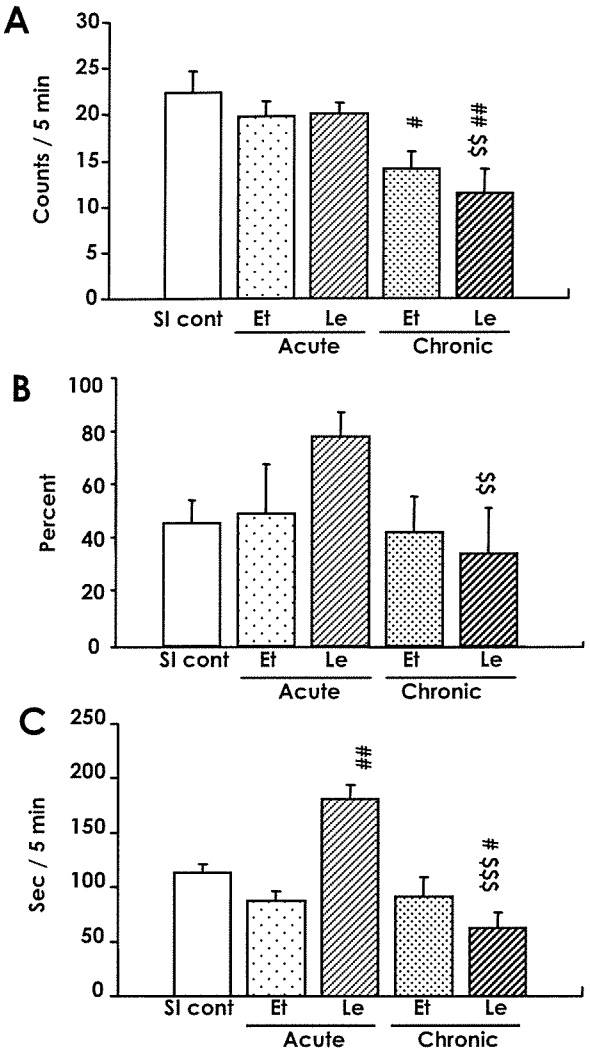


Fig. 2-1. Effects of the acute and chronic inhalation of lemon oil on elevated plus-maze test in SI mice.
A: The total number of open and closed arm entries,
B: The percentage of entries into the open arms, and
C: The time spent on the open arms during a 5-min period.
Each value represents the mean±SE of 5-9 mice. The data are assessed by one-way ANOVA with Tukey's multiple comparison test.
# $P<0.05$, ## $P<0.01$ compared to the isolation control group,
$$ $P<0.01$, $$$ $P<0.001$ compared the acute inhalation with chronic inhalation of lemon oil. SI cont: socially isolated mice that inhaled nothing as control, Et: ethanol inhalation, Le: lemon oil inhalation.

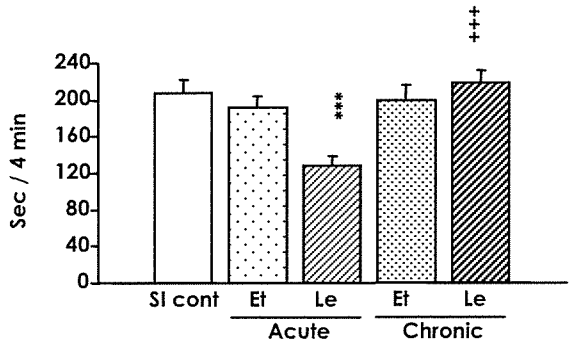


Fig. 2-2. Effects of the acute and chronic inhalation of lemon oil on the forced swimming test in SI mice.

The graph represents the immobility duration time during a 4 min period. Each value mean±SE of 5-11 mice. The data are assessed by one-way ANOVA with Tukey's multiple comparison test.

*** P<0.001, compared to the isolation control group.

+++ P<0.001 compared the acute inhalation with the chronic inhalation of lemon. SI cont: socially isolated mice that inhaled nothing as control, Et: ethanol inhalation, Le: lemon oil vapor inhalation.

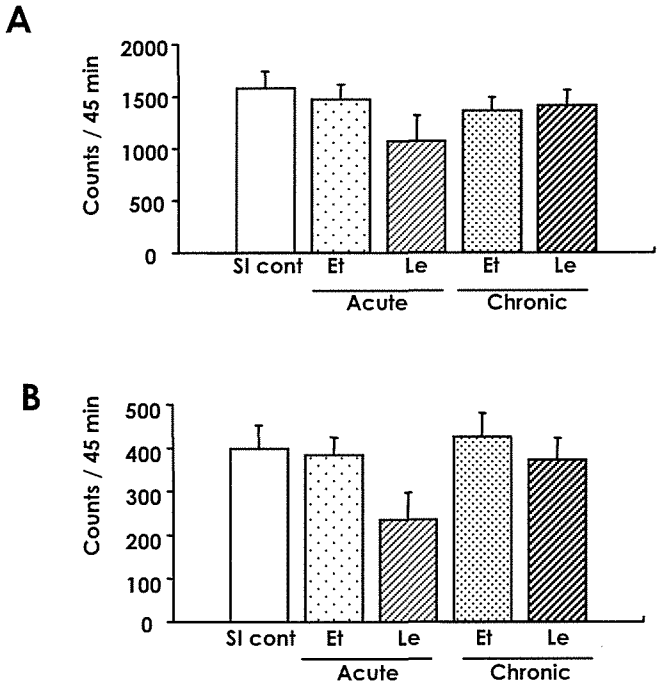


Fig. 2-3. Effects of the acute and chronic inhalation of lemon oil on the open field test in SI mice.
A: The locomotor activity counts, C: The total rearing counts over 45 min.
Each value represents the mean±SE of 5 mice.
The data are assessed by one-way ANOVA with Tukey's multiple comparison test.
SI cont: socially isolated mice that inhaled nothing as control
Et: ethanol inhalation, Le: lemon oil vapor inhalation.

CHAPTER 3

Possible mechanisms of anti-stress effect by the lemon oil vapor

INTRODUCTION

Some studies have suggested that essential oils affect the modulation of the central neurotransmitter system. Linalool, a major component of lavender oil, is reported to have an effect on glutamate receptors in vitro [45]. Pinene, which is one of the components of lemon oil, and lavender, hinokitiol, eugenol, citronellol, and citronellal are reported to potentiate the responses in the presence of GABA at low concentrations and inhibit the responses in the presence of GABA at high concentrations in vitro [23]. *Hypericum perforatum L* (St. John's wort) is supposed to inhibit the synaptosomal uptake of 5-HT in rats [46], and therefore the 5-HT concentration of basal nuclei may increase. In addition, *Hypericum perforatum L* is also reported to increase extracellular dopamine levels in the rat prefrontal cortex [54].

In this chapter, to investigate whether the anti-stress effects of acute lemon oil inhalation were related to activities of GABA, 5-HT, and DA neurons, behavioral tests were carried out by pre-treatment with neurotransmitter's agonists, antagonists and reuptake inhibitor on GH mice.

MATERIALS AND METHODS

1. Animals

Refer to Chapter 1.

2. Inhalation of lemon oil

Refer to Chapter 1.

3. Drug application

Diazepam (0.5 mg/kg, 1.0 mg/kg)(Takeda Pharmaceutical Co. Ltd, Osaka, Japan), a

benzodiazepine (BZP) receptor agonist, was diluted with 0.9% saline. Flumazenil (1.0 mg/kg, 5.0 mg/kg), a BZP receptor competitive antagonist, buspirone (2.0 mg/kg, 6.0 mg/kg), a 5-HT_{1A} receptor agonist, WAY100,635 (0.1mg/kg, 0.3 mg/kg, 1.0 mg/kg), a 5-HT_{1A} receptor antagonist, (±)-2-5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) (0.3 mg/kg, 1.0 mg/kg), a 5-HT_{2A} receptor agonist, mianserin (1.0 mg/kg, 3.0 mg/kg), a 5-$HT_{2A/C}$ receptor agonist, fluoxetine (1.8 mg/kg, 3.0 mg/kg, 3.5 mg/kg, 10.0 mg/kg), a selective serotonin reuptake inhibitor (SSRI), imipramine (30 mg/kg, 50 mg/kg), a tricyclic antidepressant and a 5-HT and NA uptake inhibitor, apomorphine (0.5 mg/kg, 1.5 mg/kg, 3.0 mg/kg), a nonselective DA receptor agonist, clonidine (0.05 mg/kg, 0.1 mg/kg), a selective α2 adrenergic receptor agonist, and yohimbine (0.5 mg/kg, 1.5 mg/kg, 2.5 mg/kg, 4.0 mg/kg), a selective α2 adrenergic receptor antagonist (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in 0.9% saline. Haloperidol (0.5 mg/kg, 1.0 mg/kg), a D_2, D_3, D_4 receptor antagonist, was dissolved in acidic 0.9% saline under heated condition. All drugs and vehicle were injected intraperitoneally and an injection volume was 0.2 ml/30g.

4. Behavioral analyses

4.1. Elevated plus-maze test

The EPM was described in Chapter 1. The mice received an injection of saline or vehicle or drugs 30 min before the test, except fluoxetine and imipramine. Fluoxetine was injected 120 min before the test, and imipramine was injected 60 min before the test.

4.2. Forced swimming test

The FST was described in Chapter 1. The mice received an injection in a way similar to that used in the EPM.

5. Statistical analysis

All results are expressed as means ± SE. Differences between treatment groups were assessed by one-way ANOVA followed by Tukey's multiple comparison test. A probability level of $P<0.05$ was taken to be statistically significant in the analyses.

RESULTS

1. Role of GABA receptor in the anti-stress effect of lemon oil on GH mice

Diazepam, a BZP receptor agonist, and flumazenil, a BZP receptor competitive antagonist, were used to estimate the role of GABA receptor in the lemon oil-induced anti-stress reaction. Diazepam and flumazenil were injected 30 min before the tests. The percentage of entries ($F(2, 22)=18.56$; $P<0.0001$) and the time spent on the open arms ($F(2, 22)=12.93$; $P=0.0003$) in the EPM showed an increase in mice treated with diazepam (Fig. 3-1A, B). However, the combination of lemon oil and diazepam did not show any effect on these two parameters ($F(2, 22)=1.438$; $P=0.2620$ and $F(2, 22)=0.7271$; $P=0.4970$). In contrast, flumazenil significantly blocked the anti-stress effect of lemon oil in both percent of entry ($F(2, 22)=10.90$;$P=0.0009$) and time spent on the open arms ($F(2, 22)=4.916$; $P=0.0145$).

The immobility duration in the FST significantly increased following a treatment with diazepam (1.0 mg/kg) ($F(2, 22)=4.817$; $P=0.0272$). The antidepressant effect of lemon oil was significantly reversed by treatments with diazepam ($F(2, 22)=39.72$; $P<0.0001$) or flumazenil ($F(2, 22)=28.81$; $P<0.0001$) (Fig.3-2).

2. Role of 5-HT receptor activity under lemon oil vapor inhalation on GH mice

Buspirone, a 5-HT_{1A} receptor agonist, WAY 100,635, a 5-HT_{1A} receptor antagonist, DOI, a 5-HT_{2A} receptor agonist, mianserin, a 5-$HT_{2A/C}$ receptor agonist, fluoxetine, a SSRI, and imipramine, a tricyclic antidepressant and a 5-HT and NA uptake inhibitor, were used to estimate the role of 5-HT receptor in the lemon oil-induced anti-stress reaction.

The percentage of entries into open arms showed a significant increase in the EPM in mice treated with buspirone ($F(2, 22)=5.729$; P=0.0113), though the time spent on the open arms did not show any effects (Fig.3-3A, B). The combination of lemon oil and buspirone had a tendency to enhance an increase in the percentage of entries into open arms, however it was not potentiative ($F(2, 22)=3.151$; P=0.0658).

WAY 100,635 showed a tendency to decrease the percentage of entries and the time spent on the open arms with all doses, however there were no significant differences ($F(3, 28)=0.7462$; P=0.5352 and $F(3, 28)=1.853$; P=0.1718). The combination of lemon oil and WAY 100,635 did not show any potentiative effects.

DOI ($F(2, 22)=0.720$; P=0.5010) and mianserin ($F(2, 22)=2.710$; P=0.0951) did not have apparent effects in the percentage of entries into open arms in the EPM. DOI and mianserin showed a tendency to increase time spent on the open arm; however, there were no significant differences from the control group ($F(2, 22)=0.1446$; P=0.8668 and $F(2, 22)=0.0706$; P=0.9321).

The immobility duration in the FST did not reduce following a treatment with not DOI nor mianserin (Fig.3-4). The combination of lemon oil and DOI (0.3 mg/kg) or mianserin (1.0 mg/kg) significantly erased the lemon effect.

Fluoxetine, a SSRI, caused a significant increase in both percentage of entries ($F(2, 22)=9.257$; P=0.0016) and time spent on the open arms ($F(2, 22)=12.10$; P=0.0004) (Fig.3-5A,

B). However, the lemon oil did not enhance the anti-stress effect of fluoxetine. In contrast, imipramine, a tricyclic antidepressant and a 5-HT and NA uptake inhibitor, showed a significant increase in percentage of entries into open arms in the EPM ($F(2, 22)=5.282$; $P=0.0164$). Moreover, there were no significant differences between lemon oil alone and the combination of imipramine and lemon oil.

Interestingly, buspirone ($F(2, 22)=9.209$; $P=0.0028$), DOI ($F(2, 22)=36.46$; $P<0.0001$), and mianserine ($F(2, 22)=49.16$; $P<0.0001$) blocked the antidepressant effect of lemon oil in the FST (Fig.3-4), but WAY100,635 did not ($F(2, 22)=0.610$; $P=0.5563$). In addition, imipramine had an apparent antidepressant effect at a dose of 50 mg/kg ($F(2, 22)=6.908$; $P=0.0082$), and fluoxetine did not show any antidepressant effect at the dosages we used in this study (3 or 10 mg/kg) ($F(2, 22)=0.8449$; $P=0.4504$), even though fluuoxetine blocked the antidepressant effect of lemon oil ($F(2, 22)=9.636$; $P=0.0020$) (Fig.3-6).

3. Role of DA receptor under lemon oil vapor inhalation on GH mice

Apomorphine, a nonselective DA receptor agonist, and haloperidol, a D_2, D_3, D_4 receptor antagonist, were used to estimate the role of DA receptor in the lemon oil-induced anti-stress reaction.

Apomorphine showed an anti-stress effect in both the percentage of entries into open arms ($F(2, 22)=4.393$; $P=0.0302$) and the time spent on the open arms ($F(2, 22)=0.5170$; $P=0.6090$) in the EPM (Fig.3-7A, B). These effects of apomorphine were erased by treatment with lemon oil ($F(2, 22)=6.665$; $P=0.0073$). In contrast, lemon oil did not affect the haroperidol-treated mice ($F(2, 22)=2.063$; $P=0.1595$).

The highest dose of apomorphine (3.0 mg/kg) significantly reduced the immobility duration in the FST ($F(2, 22)=3.811$; $P=0.0443$) (Fig.3-8). Moreover, the combination of

lemon oil and apomorphine (0.5 mg/kg) or haloperidol (0.5 mg/kg) did not show any significant effect in the FST.

4. Role of α2 adrenergic receptor with lemon oil vapor inhalation on GH mice

Clonidine, a selective α2 adrenergic receptor agonist, and yohimbine, a selective α2 adrenergic receptor antagonist, were used to estimate the role of α2 adrenergic receptor in the lemon oil-induced anti-stress reaction.

Yohimbine showed a significant increase in the percentage of entries into open arms EPM ($F(2, 22)=5.363$; P=0.0175) and the time spent on the open arms in the EPM (F(2, 22)=5.120; P=0.0202), although clonidine had no effect (Fig.3-9A, B). The combination of lemon oil and clonidine or yohimbine did not show any alteration on the effect of lemon oil alone.

A low dose of clonidine (0.05 mg/kg) or yohimbine (1.5 mg/kg) blocked the antidepressant effect of lemon oil in the FST ($F(2, 22)=7.096$; P=0.0083 and $F(2, 22)=11.29$; P=0.0014) (Fig.3-10).

DISCUSSION AND CONCLUSION

The present study showed that lemon oil vapor enhanced entries into the open arm in the EPM under pretreatment with buspirone, a 5-HT_{1A} receptor agonist. Lemon oil had a similar effect under the pretreatment with haloperidol, a D_2, D_3, D_4 receptor antagonist. In addition, flumazenil, a BZP receptor antagonist, blocked the anti-stress effect of lemon oil. These results suggest that lemon oil vapor possibly modulates the response to the 5-HTnergic, DAnergic, and GABA-BZP receptor systems.

In the FST, the progesterone $GABA_A$-modulatory metabolite 5α-pregnan-3α-ol-20-one (allopregnanolone) is released and rapidly metabolized after an acute stress session [55]. Briones-Aranda *et al.* [56] reported that either forced swimming or a high dose of allopregnanolone can block the anxiolytic effect of diazepam. In the present study, diazepam had the anti-stress effect, and flumazenil caused a stressful reaction in the EPM. On the other hand, neither diazepam nor flumazenil showed an antidepressant effect in the FST, and in fact these two drugs blocked the antidepressant effect of lemon oil in the FST. Briones-Aranda *et al.* [56] suggested that forced swimming produces conformational changes in the GABA-BZP complex that alter the pharmacological profile of BZP. The results of these previous reports together with our present results suggest that the lemon oil-induced antidepressant effect does not occur via a direct GABA-BZP pathway, because lemon oil caused the antidepressant effect even in the forced swimming condition that was not suited to the agonist to BZP receptor.

On the other hand, lemon oil vapor did not enhance the pharmaceutical effects of antidepressant drugs including fluoxetine and imipramine in the FST, although these drugs blocked the antidepressant effect of lemon oil vapor. Imipramine is widely used as a positive control in the FST. Interestingly, imipramine completely blocked the antidepressant effect of lemon oil, although imipramine showed a strong effect when used alone. Exploring this reversible effect of imipramine on the function of lemon oil may give us useful information for undersanding the mechanism of the antidepressant effect of lemon oil.

A prominent participation of 5-HT in depression and anxiety is generally recognized [57, 58], although the complex of emotional states cannot be reduced to imbalances of a single neurotransmitter. In the present study, buspirone, DOI, and mianserine blocked the antidepressant effect of lemon oil in the FST, but WAY100,635 did not (Fig.3-3B).

In conclusion, these findings suggest that the antidepressant effect of lemon oil is closely related with the 5-HTnergic pathway, especially via 5-HT_{1A} receptor. However, further investigations are necessary to clarify the precise signal transduction induced by lemon oil.

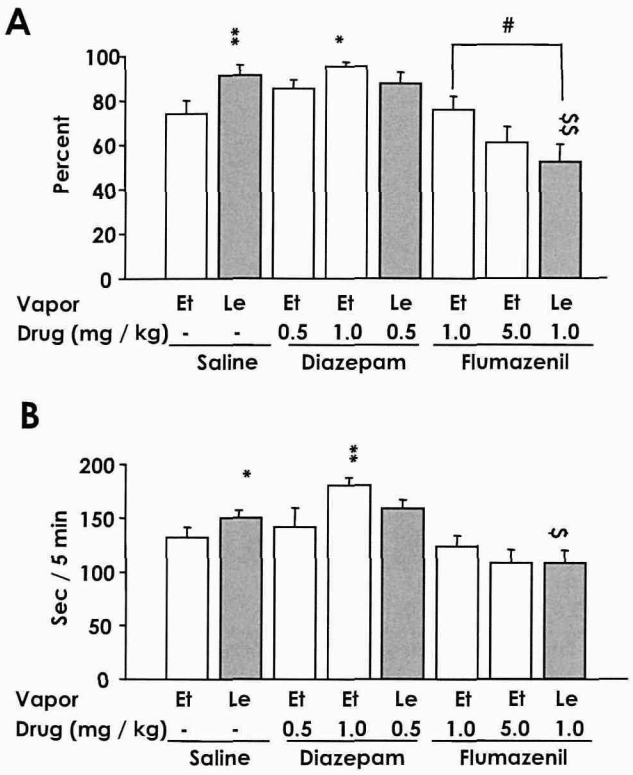


Fig. 3-1 Effects of BZP receptor agonist and antagonist on the elevated plus-maze test in the mice that inhaled lemon oil vapor.
A: The percentage of entries into open arms,
B: The time spent on the open arms during a 5-min period.
Each value represents the mean±SE of 5-10 mice.
* P<0.05, ** P<0.01compared to the ethanol+saline group,
$ P<0.05, $$ P<0.01 compared to the lemon oil+saline group,
# P<0.05 compared between flumazenil (1.0 mg/kg) alone and the combination of lemon oil inhalation and flumazenil injection. All data are assessed by one-way ANOVA with Tukey's multiple comparison test.
Et: ethanol inhalation, Le: lemon oil vapor inhalation.

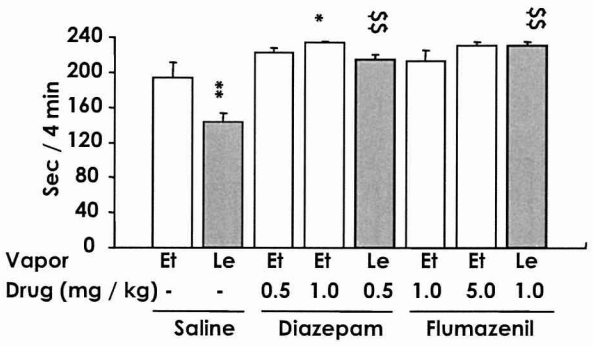


Fig. 3-2 Effects of BZP receptor agonist and antagonist on the forced swim test in the mice that inhaled lemon oil vapor.
The graph represents the immobility duration time during a 4-min period. Each value represents the mean±SE of 5-10 mice.
* P<0.05, ** P<0.01 compared to the ethanol+saline group,
$$ P<0.01 compared to the lemon oil+saline group.
All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Le: lemon oil vapor inhalation.

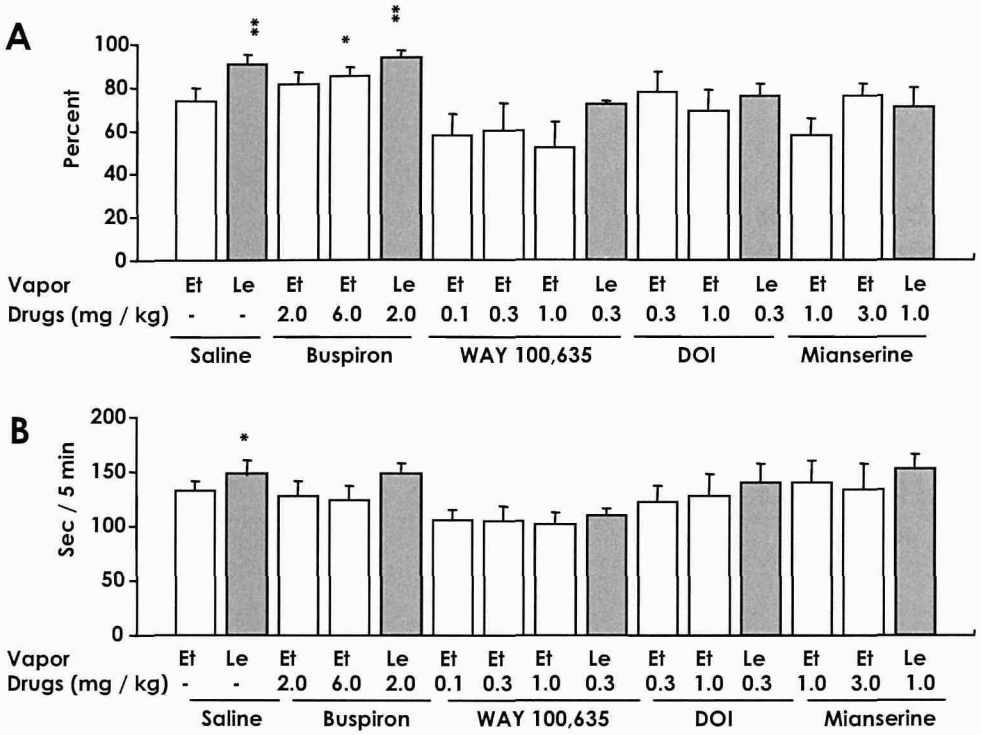


Fig. 3-3. Effects of 5-HT agonist and antagonist on the elevated plus-maze test in the mice that inhaled lemon oil vapor.
A: The percentage of entries into open arms,
B: The time spent on the open arms during a 5-min period.
Each value represents the mean±SE of 5-10 mice.
* $P<0.05$, ** $P<0.01$ compared to mice that ethanol+saline group.
All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Le: lemon oil vapor inhalation.

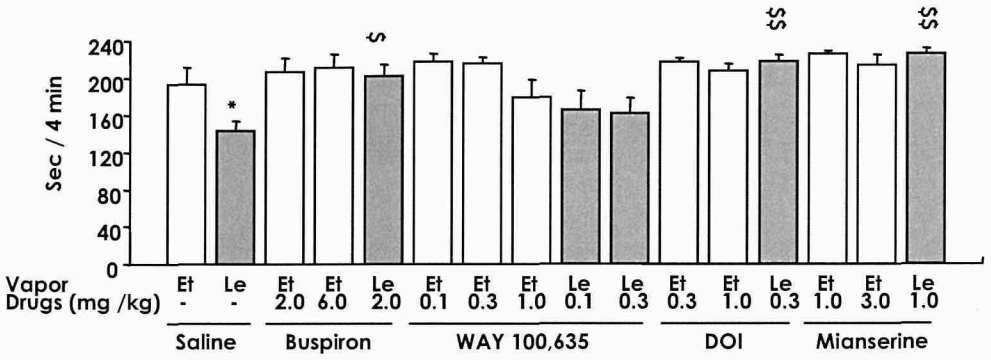


Fig. 3-4. Effects of 5-HT agonist and antagonist on the forced swim test in the mice that inhaled lemon oil vapor.
The graph represents the immobility duration time during a 4-min period. Each value represents the mean±SE of 5-10 mice. * P<0.05 compared to mice that ethanol+saline group,
$ P<0.05, $$ P<0.01 compared to lemon oil+saline group.
All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Le: lemon oil vapor inhalation.

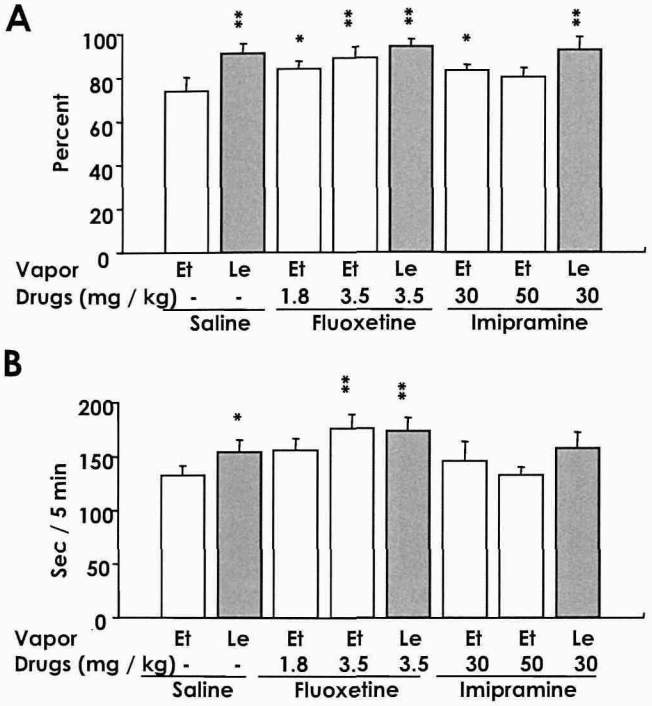


Fig. 3-5. Effects of a SSRI and a tricyclic antidepressant on the elevated plus-maze test in the mice that inhaled lemon oil vapor.
A: The percentage of entries into open arms, B: The time spent on the open arms during a 5-min period. Each value represents the mean±SE of 5-10 mice.
* P<0.05, ** P<0.01 compared to the ethanol+saline group.
All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Le: lemon oil vapor inhalation.

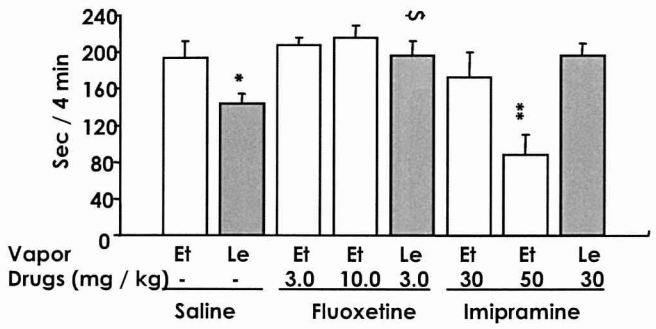


Fig. 3-6. Effects of a SSRI and a tricyclic antidepressant on the forced swim test in the mice that inhaled lemon oil vapor.
The graph represent the immobility duration time during a 4-min period. Each value represents the mean±SE of 5-10 mice.
* $P<0.05$, ** $P<0.01$ compared to the ethanol+saline group,
\$ $P<0.05$ compared to the lemon oil+saline group.
All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Le: lemon oil vapor inhalation.

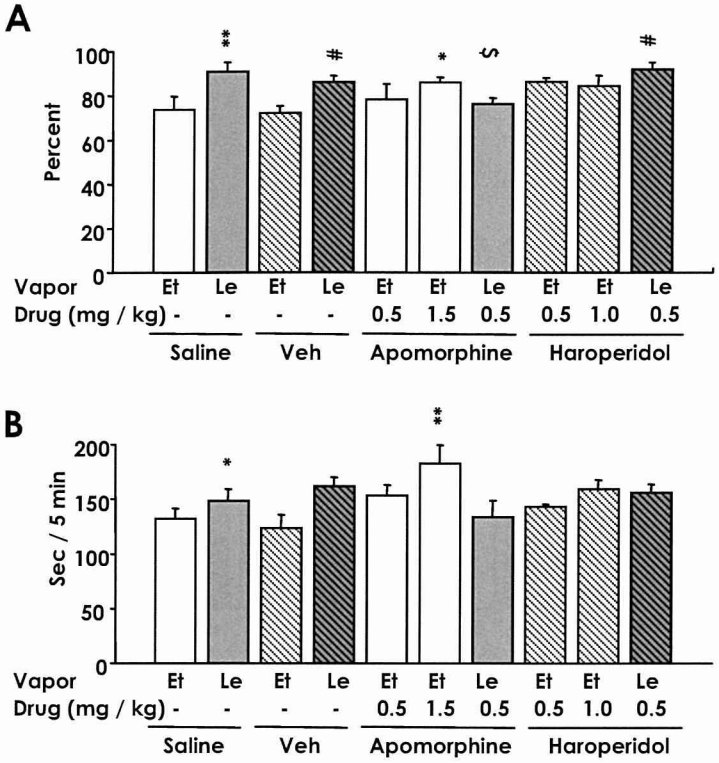


Fig. 3-7. Effects of DA agonist and antagonist on the elevated plus-maze test in the mice that inhaled lemon oil vapor.

A: The percentage of entries into open arms,
B: The time spent on the open arms during a 5-min period.
Each value represents the mean±SE of 5-10 mice.
* P<0.05, ** P<0.01 compared to the ethanol+saline group,
# P<0.05 compared to the ethanol+vehicle group,
$ P<0.05 compared to the lemon oil+saline group.
All data are assessed by one-way ANOVA with Tukey's multiple comparison test.
Et: ethanol inhalation, Le: lemon oil vapor inhalation,
Veh: injection of saline with a few drops of hydrochloric acid 1N (vehicle).

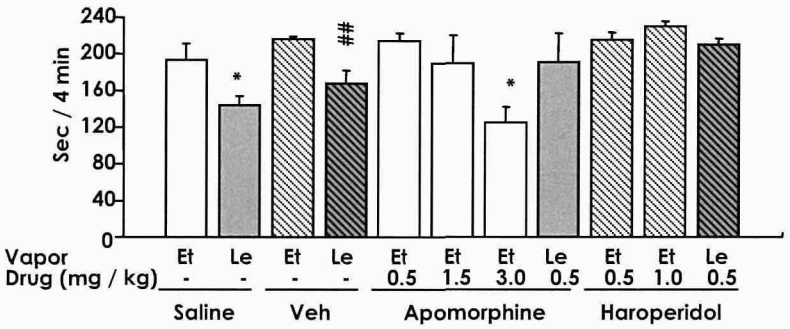


Fig. 3-8. Effects of DA agonist and antagonist on the forced swim test in the mice that inhaled lemon oil vapor.

The graph represents the represents the immobility duration time during a 4-min period. Each value represents the mean±SE of 5-10 mice.
* P<0.05 compared to the ethanol+saline group,
P<0.01 compared to the ethanol+vehicle group.
All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Le: lemon oil vapor inhalation, Veh: injection of saline with a few drops of hydrochloric acid 1N (vehicle).

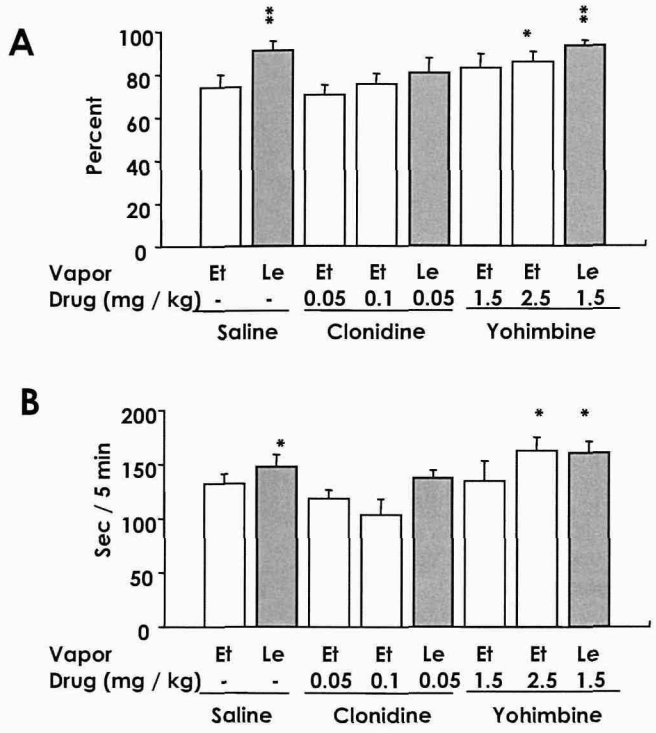


Fig. 3-9. Effects of adrenaline α2 agonist and antagonist on the elevated plus-maze test in the mice that inhaled lemon oil vapor.
A: The percentage of entries into open arms,
B: The time spent on the open arms during a 5-min period.
Each value represents the mean±SE of 5-10 mice.
* P<0.05, ** P<0.01 compared to the ethanol+saline group.
All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Le: lemon oil vapor inhalation.

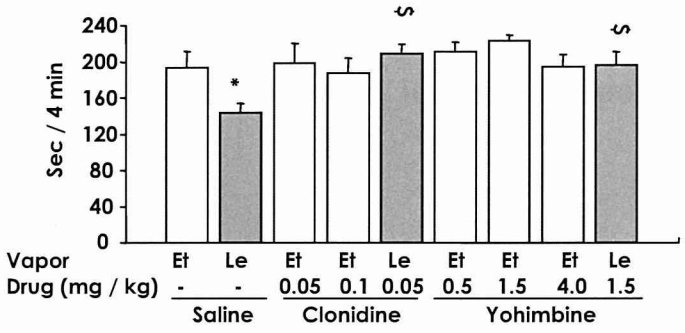


Fig. 3-10. Effects of adrenaline α2 agonist and antagonist on the forced swim test in the mice that inhaled lemon oil vapor.
The graph represents the immobility duration time during a 4-min period. Each value represents the mean±SE of 5-10 mice.
* P<0.05 compared to the ethanol+saline group,
$ P<0.05 compared to the lemon oil+saline group.
All data are assessed by one-way ANOVA with Tukey's multiple comparison test. Et: ethanol inhalation, Le: lemon oil vapor inhalation.

CHAPTER 4

Changes in the contents of monoamines and their metabolites in the brain of mice following lemon oil vapor inhalation

INTRODUCTION

DA plays an important role in mediating the effects of isolated housing on the social behavior of mice. Gendreau *et al.* [40] reported that DA agonists potentiate defensive behavior and/or social fearfulness in the SI mice. They further suggested that D_3 and D_2 DA receptors differentially modulate the expression of social-emotional reactivity. Reduction of brain $GABA_A$ receptor function in the SI mice also was reported, as evaluated by measuring GABA-evoked Cl^- currents in xenopus oocytes [41]. As to 5-HT, Schiller *et al.* [42] analyzed by in vitro autoradiography, and they reported that 5-HT_{1A} and 5-HT_{2A} receptor densities in the brains of SI mice were strongly reduced. Ago and Matsuda *et al.* [43, 44] examined the modulation of 5-HT, DA, and noradorenaline (NA) release by selective 5-HT_{1A} receptor agonists in the cortex of SI mice by microdialysis. In this study, the basal level of extracellular DA in the frontal cortex was higher in the SI mice than in the GH mice. The 5-HT_{1A} receptor agonist, MKC-242 induced increases in the cortical DA release was less pronounced in the SI mice than the GH mice. These findings suggest that the socially isolation-induced distress enhances the activity of cortical DAnergic neurons and reduces the responses of DAnergic terminals to 5-HT_{1A} receptor stimulation.

On the other hand, above-mentioned results in Chapter 1-3 suggest that the anxiolytic and antidepressant effects of lemon oil inhalation might be caused by a suppression of DA activity via enhanced 5-HTnergic neurons. Therefore it is easy to think that the lemon oil vapor inhalation is effective to emotional disease.

The present experiments were addressed to clear the possible regulatory mechanism of lemon oil vapor effects. The levels of plasma corticosterone and the contents of monoamines and their metabolites in the prefrontal cortex, the hippocampus, the striatum in mice were measured to investigate whether lemon oil vapor modulate the hypothalamic-pituitary-

adrenocortical cortex (HPA) axis and accelerate the turnover or activity of monoamines or not and the reason that the chronic inhalation of lemon oil showed opposite results in all behavioral tests from the acute inhalation on the SI mice were discussed.

MATERIALS AND METHODS

1. Animals

Refer to Chapter 1 and 2.

2. Inhalation of lemon oil vapor

Refer to Chapter 1.

3. Assay of plasma Corticosterone

Blood samples were collected from the mice after three-week isolated housing period by heart puncture. During three-week period, the chronic experiment group inhaled lemon oil vapor from 3 to 6 weeks of age, the acute experiment group inhaled lemon oil vapor for 90 min before collected blood sample. These samples were centrifuged at 1,000 g at 4°C for 15 min, the plasma separated from the red blood cells and transferred to individual tubes. Plasma corticosterone levels were determined by means of a RIA kit (Amarsham Pharmacia Biotech, UK).

4. Quantification of monoamines and their metabolites by HPLC

In the acute experiment, ninety minutes after mice were treated with the inhalation of lemon oil, they were decapitated and the whole brain of each animal was removed and

immediately stored at -80°C until monoaminergic determination.

In the chronic experiment, three weeks after mice were treated with the inhalation of lemon oil during the dark period (22:00-24:00) every day, they were decapitated and the whole brain of each animal was removed and immediately stored at -80°C until monoaminergic determination.

Separation of monoamines was performed according to the procedure described previously [59]. The samples were homogenized in 1 ml of 0.1 M potassium perchlorate containing 0.2 mM sodium bisulfite and 0.2 mM EDTA2Na, and 10 ng isoproterenol was added to each sample as an internal standard to control for procedural losses. The homogenates were centrifuged (20,000 x g, 15 min at 0°C), and supernatants were used for monoamine determination using high performance liquid chromatography with electrochemical detection (HPLC-ECD).

Monoamines NE, DA and 5-HT as well as their main metabolites, homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxy-3-indoleacetic acid (5-HIAA), were measured. Standards were run concurrently, and concentrations of unknowns were determined by comparison to peak areas of standards after correction for recovery of the internal standard (Fig.4-1).

5. Statistical analysis

Refer to Chapter 1.

RESULTS

1. Changes in the levels of plasma corticosterone in the GH and SI mice that inhale acute and chronic lemon oil vapor

The social isolation significantly increased the levels of plasma corticosterone in mice ($P<0.05$) by t-test (Fig.4-2). Statistical analysis revealed a significant decrease in the levels by the chronic inhalation of both ethanol and lemon oil ($F(2,20)=6.166$; $P=0.0097$). The chronic inhalation of ethanol alone ($F(2,20)=4.375$; $P=0.0293$) and lemon oil ($F(2,20)=4.280$; $P=0.0312$) significantly reduced the levels compared to the acute inhalation, respectively. The reduction of the levels of plasma corticosterone by acute inhalation was slight.

2. Changes in the contents of monoamines and their metabolites in the brain following lemon oil inhalation on GH mice

Ethanol inhalation did not have any significant effect on the NE, DA, and 5-HT contents and those ratios in the brain (Fig.4-3, 4-4, 4-5). The inhalation of lemon oil vapor for 180 min significantly increased the DA content in the hippocampus ($F(3,20)=4.162$, $P=0.0234$) and the DOPAC/DA ratio in the prefrontal cortex ($F(3,20)=3.964$, $P=0.0274$). Furthermore, the inhalation of lemon oil vapor for 90-180 min resulted in a significant increase of 5-HT in the prefrontal cortex ($F(3,20)=9.411$, $P=0.008$) and 180 min inhalation significantly increased the 5-HIAA/5-HT ratio in the striatum ($F(3,20)=14.72$, $P=0.0001$). The 5-HT content and the 5-HIAA/5-HT ratio in the hippocampus tended to be enhanced by lemon oil inhalation for 90 or 180 min.

3. Changes in the contents of monoamines and their metabolites induced by social isolation distress in mice

The social isolation significantly decreased the contents of HVA in the prefrontal cortex, the hippocampus, and the striatum ($P<0.01$) by t-test (Fig.4-6). In all three regions, as the contents of NE did not alter between two groups, the HVA/NE ratio in the prefrontal cortex and the hippocampus significantly decreased ($P<0.01$) by t-test and in the striatum tended to decrease but not significantly. The contents of DA significantly declined in the prefrontal cortex ($P<0.05$) and increased in the hippocampus ($P<0.01$) by t-test (Fig.4-7). The synthesis of 5-HT in the prefrontal cortex was enhanced ($P<0.01$) and the contents of 5-HIAA in the prefrontal cortex and the hippocampus significantly increased ($P<0.05$) by t-test (Fig.4-8). The 5-HT/5-HIAA ratio in the prefrontal cortex was restrained significantly ($P<0.01$) by t-test.

4. Changes in the concentration of monoamines and their metabolites in the brain of SI mice following acute and chronic inhalation of lemon oil

The acute ethanol inhalation did not have any significant alteration in the brain regions except the contents of DA ($F(2,10)=6.862$; $P=0.0224$), and HVA/NE ($F(2,15)=4.768$; $P=0.0299$) in the hippocampus (Fig.4-9, 4-10). Both of them increased with chronic treatment. The contents of 5-HT in the striatum significantly increased ($F(2,14)=5.122$;$P=0.0268$) following acute inhalation of lemon oil, also showed a significant increase compared with acute ethanol inhalation ($F(2,15)=6.126$; $P=0.0147$) (Fig.4-11). In the acute experiments on the SI mice, this was all the significant alteration.

Following chronic experiments, the contents of HVA significantly increased in the prefrontal cortex ($F(2,13)=8.446$; $P=0.0071$) and the striatum($F(2,14)=4.918$; P=0,0298) (data

not shown). As the contents of NE did not altered, the HVA/NE ratio in the prefrontal cortex significantly enhanced ($F(2,13)=5.676$; P=0.0225). The contents of DA in the hippocampus significantly decreased by both acute and chronic inhalation of lemon oil ($F(2,11)=12.34$; P=0.0036), though even ethanol alone also reduced DA contents ($F(2,10)=6.862$; P=0.0224) (Fig.4-10). On the other hand, the DA contents in the prefrontal cortex also significantly decreased ($F(2,14)=4.112$; P=0.0464). The DOPAC/DA ratio in the prefrontal cortex significantly increased compared with both chronic ethanol inhalation ($F(2,13)=4.488$; P=0.0406) and acute lemon inhalation ($F(2,14)=5.185$; P=0.0259) and those in the striatum also significantly increased ($F(2,14)=5.185$, P=0.0259). The 5-HIAA/5-HT ratio in the prefrontal cortex increased significantly compared with the acute inhalation of lemon oil ($F(2,14)=4.566$; P=0.036)(Fig.4-11).

DISCUSSION AND CONCLUSION

HPLC determination of monoamines and their metabolites in the brain of GH mice showed that the acute inhalation of lemon oil vapor for 90-180 min significantly accelerated the metabolic turnover of DA in the prefrontal cortex and the hippocampus, and that of 5-HT in the prefrontal cortex and the striatum. The metabolic turnover of 5-HT in the hippocampus also tended to be enhanced. Thus 5-HTnergic activity in all of three regions was enhanced following inhalation of lemon oil. Furthermore, the inhalation of lemon oil significantly accelerated the DA synthesis in the hippocampus.

It has been reported that DA and 5-HT fibers normally compete for striatal target sites during postnatal development, and DA might tonically suppress the release of neurotrophic factors that promote 5-HT axon growth [60]. Although the precise modulating mechanisms of lemon oil for the 5-HT and DA neurons are still unknown, the hypotheses that activated

5-HTnergic neurons suppress the DAnergic neurons under the lemon oil inhalation condition is thought. Furthermore, Renard *et al.* [61] have reported that DA concentration in the whole brain of mice increased from the fifth minute of the FST and returned to the basal level after 20 min. DOPAC concentration increased after a 20-min test period. On the other hand, 5-HT concentration increased after an 8-min test period. NE was not modified during the FST. This report suggests that DA activity is more rapidly induced by a higher stressor like the FST than by a mild stressor. Interestingly, lemon oil vapor induced anti-stress effects under both mild and higher stressors in my study. Considering that, it could be said that lemon oil may enhance 5-HT activity at first, and then 5-HTnergic neurons modulate the DAnergic system.

While, some reports have shown that 5-HT has a stimulatory effect in hyperlocomotion. For example, systemic administration of 5-HT_2 receptor antagonists prevented hyperactivity, whereas intrastriatal injection of 5-$HT_{2A/2C}$ agonists increased locomotion [62]. In contrast, nonselective 5-HT receptor agonists or 5-HT transporter inhibitors possibly reduced hyperactivity in neonatally DA-depleted rats [63, 64]. These reports suggest that 5-HT acts as an inhibitory locomotor in the DA-depleted model. In the present study, lemon oil inhalation increased 5-HT content in the prefrontal cortex. This result may be related to the idea that locomotion is normally under DAnergic control and to a previous observation showing that partial 5-HT depletion did not affect spontaneous locomotion [65]. Haloperidol pretreatment, however, enhanced the anxiolytic effect of lemon oil vapor. It is, therefore, possible that the enhanced 5-HTnergic system inhibits stressful behavior under the lemon oil inhalation without DA activity. It is also well known that 5-HT can exert both stimulatory and inhibitory effects on psychological behaviors, probably depending on complex interactions with other neurotransmitters systems.

In the SI mice, the HVA/NE ratios in the prefrontal cortex and the hippocampus were

significantly lower than those in the GH mice. This result suggested that the SI mice suppressed the metabolic turnover of NE and attenuated the NEnergic activity. In contrast, the DA content in the hippocampus and the 5-HT content in the prefrontal cortex significantly increased, and the DA content in the prefrontal cortex significantly decreased.

On the other hand, the levels in plasma corticosterone were enhanced by social isolation. Jacobson *et al.* deduced that the hippocampus inhibits most aspects of HPA activity [66]. In consideration of this point, social isolation-induced distress must modulate DAnergic neurons in the hippocampus. The synthesis of 5-HT in the prefrontal cortex was suggested to enhance and exceed the capacity to metabolize 5-HT. These findings suggested that the SI mice were not near so much a depression as a schizophrenia, considering the results of the SI mice in the FST were similar to that of GH mice and the anti-immobility effect of antidepressant medicines may be mediated by facilitating central dopamine neurotransmission [67]. Besides amphetamine is widely known to reduce the immobility duration in the FST and to cause hyperactivity, and then amphetamine is normally thought to act by increasing dopamine concentrations in the brain. On the other hand, under a schizophrenia, it is said that the turnover of monoamines in the frontal lobe and the activity of nigrostriatal DA pathway diminish while the activity of the mesolimbic pathway enhance [68]. Lapiz *et al.* also [39] concluded that isolation-induced behavioral changes seemed to parallel to a certain degree those seen in human schizophrenia.

Acute inhalation of lemon oil recovered the changes of DA content in the prefrontal cortex and the hippocampus by social isolation distress and enhanced the 5-HT content in the striatum. This alteration of DA contents in the hippocampus on the SI mice is opposite to those on the GH mice. Acute inhalation of lemon oil might normalize the DA content in the hippocampus. On the other hand, the alterations of 5-HT contents in the SI mice that inhaled

acute lemon oil were not observed, except in the striatum, while the synthesis of 5-HT were observed in the prefrontal cortex and the hippocampus of the GH mice.

On the other hand, following chronic inhalation of lemon oil on the SI mice, the metabolic turnover of NE in the prefrontal cortex, those of DA in all of three regions, and that of 5-HT in the prefrontal cortex were accelerated. To take the contents of monoamines and their metabolites in the prefrontal cortex into consideration, the results suggest that chronic inhalation of lemon oil enhanced the metabolic turnover and activity of monoamines, especially NE in the prefrontal cortex. The excess DA synthesis in the hippocampus was suppressed and the DAnergic activity in the striatum was enhanced by chronic inhalation of lemon oil. The alteration of 5-HT activity was not observed such as acute inhalation of lemon oil on the GH mice. Thus chronic inhalation must fail to activate 5-HTnergic neurons and it is assumed that the manifestations of anxiolytic and antidepressant effects need to activate 5-HTnergic neurons. This finding may be the cause that the behavioral tests on chronic inhalation were rather poor as compared with those on acute inhalation. Even so, chronic inhalation may reduce distress because of the result of the plasma corticosterone levels and the alteration of the contents of monoamines in the brain regions, especially the recover of the DA content in the hippocampus.

In conclusion, lemon oil possibly reduces distress by modulating GABAnergic, 5-HTnergic, and DAnergic systems in the brain of the GH and SI mice. This effect might be caused by a suppression of DA activity via enhanced 5-HTnergic neurons under the lemon oil inhalation condition.

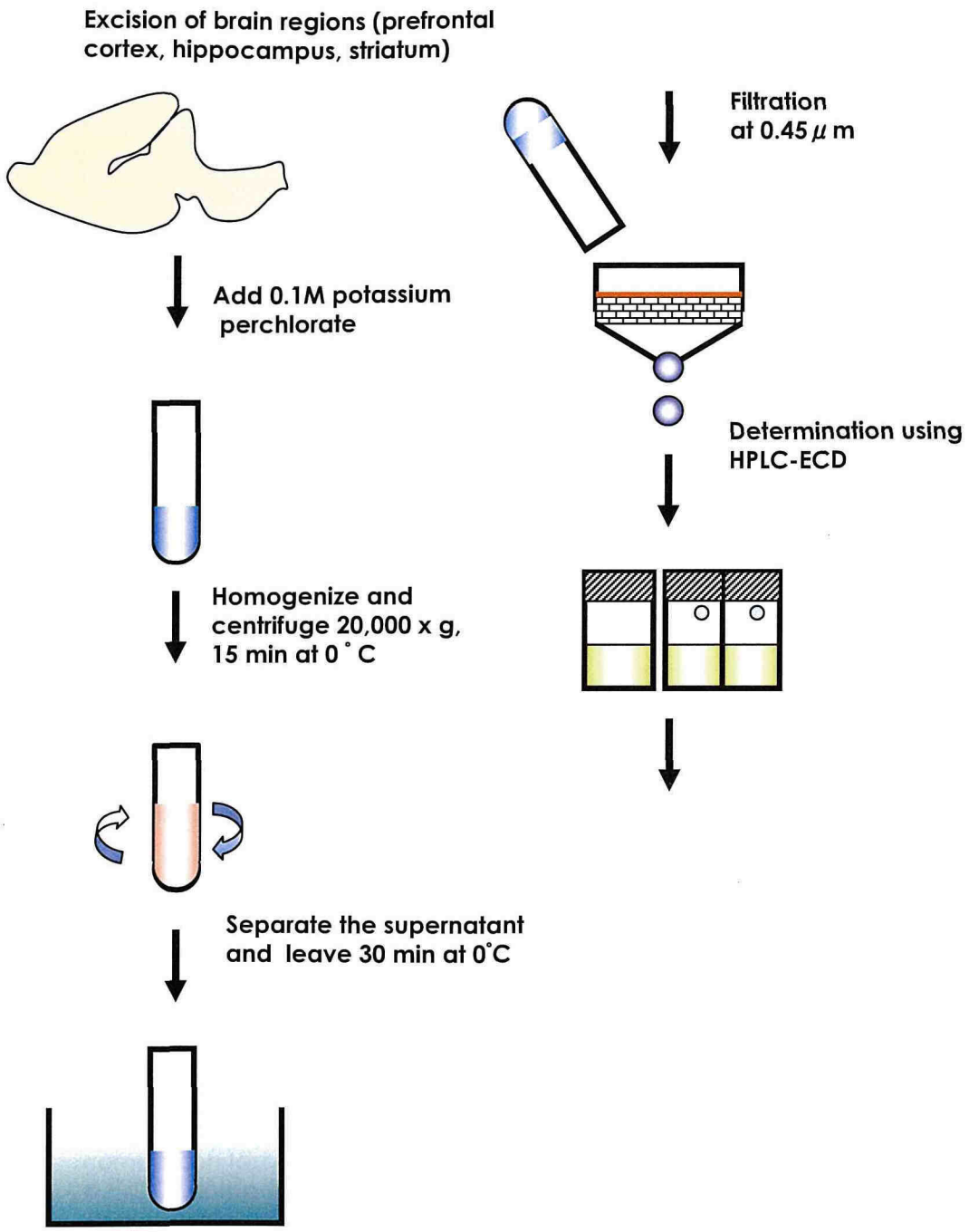


Fig.4-1. Quantification of monoamines and their metabolites by HPLC

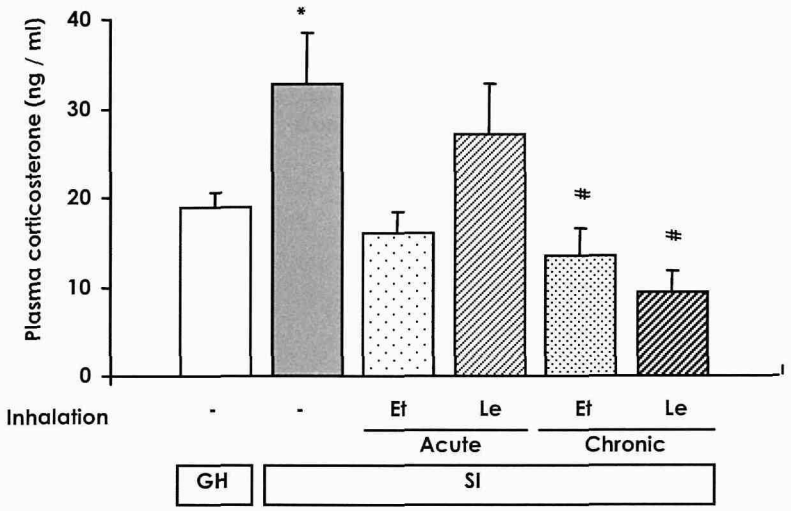


Fig. 4-2. Effects of the acute and chronic inhalation of lemon oil vapor on the level of plasma corticosteroid in SI mice.
Each value represents the mean±SE of 5 mice, except the non-inhaled control groups (n=10).
* P<0.05 compared to group control by unpaired Student's t-test.
SI mice's data are assessed by one-way ANOVA with Tukey's multiple comparison test.
# P<0.05 compared to the isolation control group.
Et: ethanol inhalation, Le: lemon oil vapor inhalation,
GH: group-housed mice, SI: socially isolated mice.

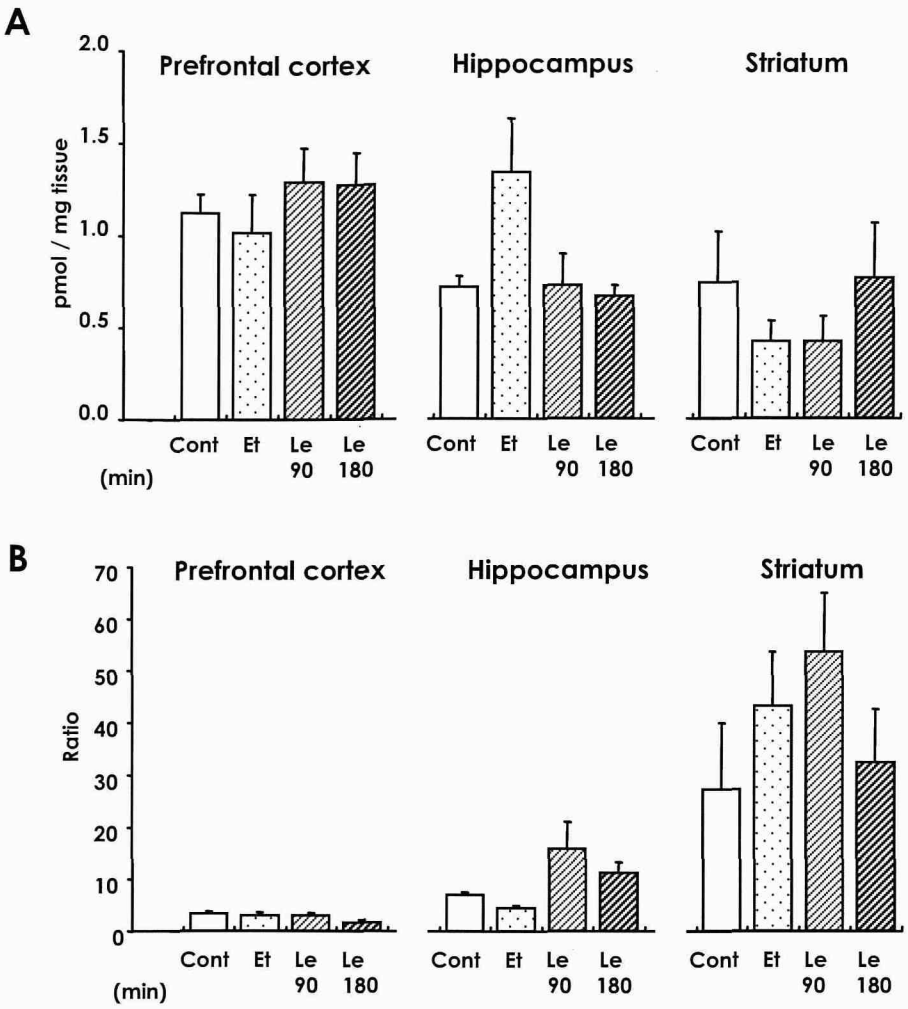


Fig. 4-3. HPLC determination of norepinephrine (NE), homovanillic acid (HVA) in the prefrontal cortex, hippocampus and striatum in lemon oil inhaled GH mice.
A: NE, B: HVA/NE ratio.
Each value represents the mean±SE of 5 mice.
The data are assessed by one-way ANOVA with Tukey's multiple comparison test, respectively.
Cont: control, Et: ethanol inhalation, Le: lemon oil vapor inhalation

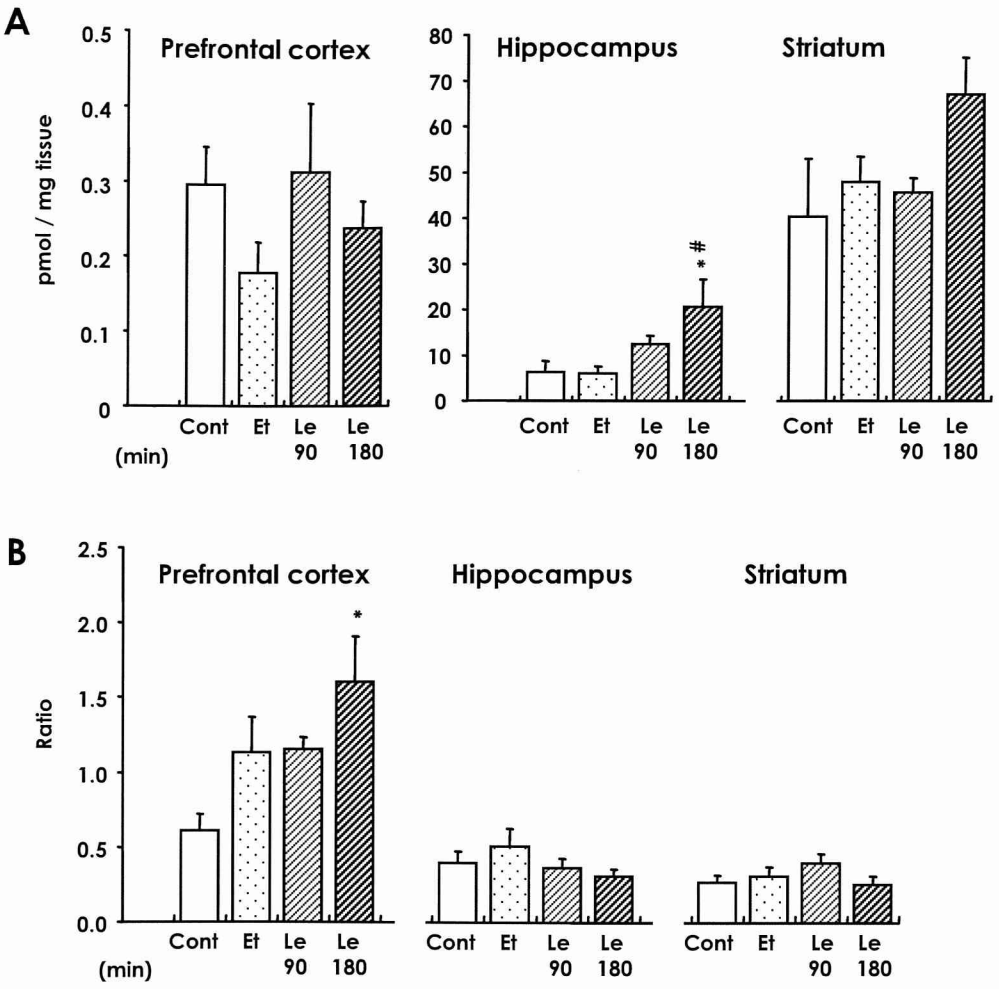


Fig. 4-4. HPLC determination of dopamine (DA), dihydroxyphenylacetic acid (DOPAC) in the prefrontal cortex, hippocampus and striatum in lemon oil inhaled mice.

A: DA, B: DOPAC/DA ratio.

Each value represents the mean±SE of 5 mice. The data are assessed by one-way ANOVA with Tukey's multiple comparison test, respectively.

* $P<0.05$ compared to control group,

# $P<0.05$ compared to ethanol inhaled group, respectively.

Cont: control, Et: ethanol inhalation, Le: lemon oil vapor inhalation.

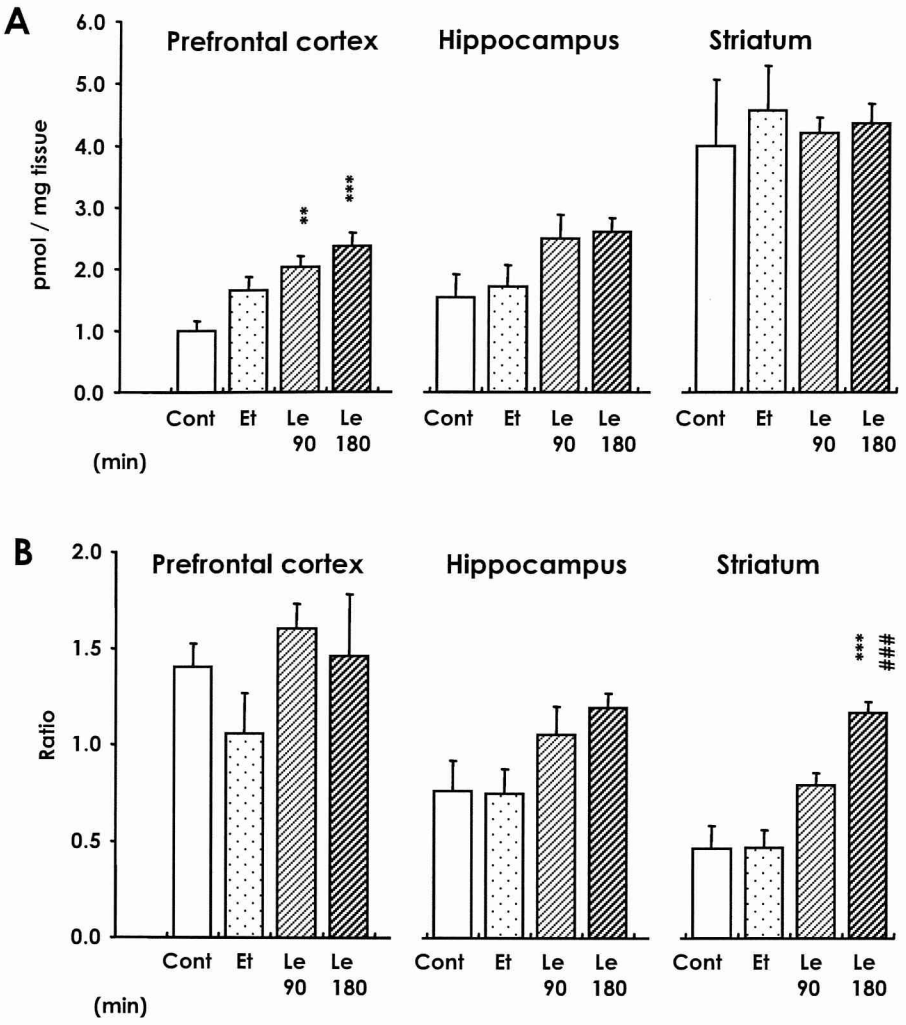


Fig. 4-5. HPLC determination of serotonin (5-HT), 5-hydroxyindole acetic acid (5-HIAA) in the prefrontal cortex, hippocampus and striatum in lemon oil inhaled GH mice.

A: 5-HT, B: 5-HIAA/5-HT ratio. Each value represents the mean±SE of 5 mice. The data are assessed by one-way ANOVA with Tukey's multiple comparison test. **P<0.01, *** P<0.001 compared to the control group, #### P<0.001 compared to the ethanol inhaled group, respectively.
Cont: control, Et: ethanol inhalation, Le: lemon oil vapor inhalation.

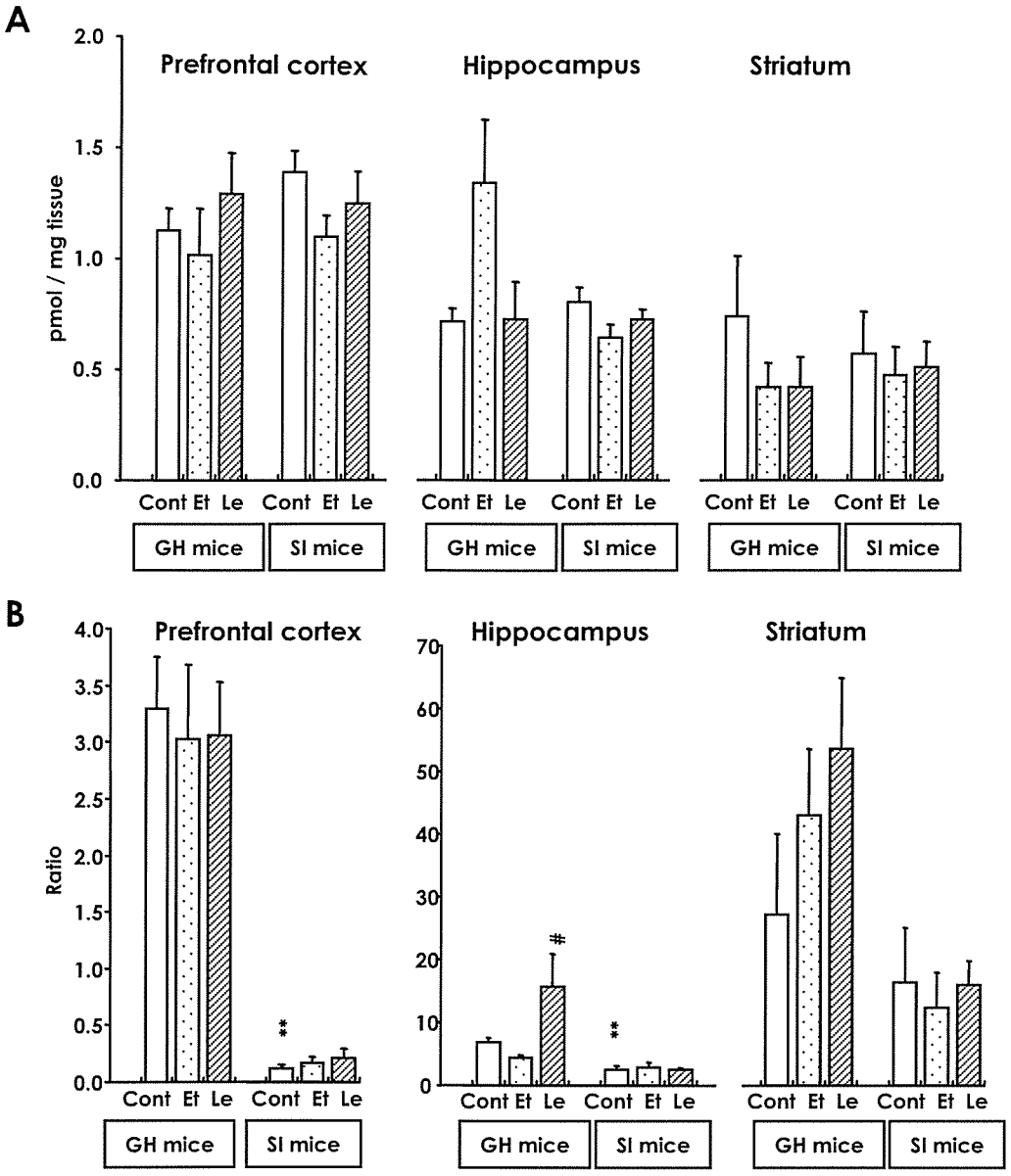


Fig. 4-6. HPLC determination of norepinephrine (NE), homovanillic acid (HVA) in the prefrontal cortex, hippocampus and striatum in lemon oil inhaled mice.
A: NE, B: HVA/NE ratio. Each value represents the mean±SE of 5 mice.
The GH and SI mice's data are assessed by one-way ANOVA with Tukey's multiple comparison test,
# $P<0.05$ compared to ethanol inhaled mice, respectively.
** $P<0.01$; significantly different from GH control mice by unpaired Student's *t*-test. Cont: control, Et: ethanol inhalation, Le: lemon oil vapor inhalation.
GH mice: group-housed mice, SI mice: socially isolated mice.

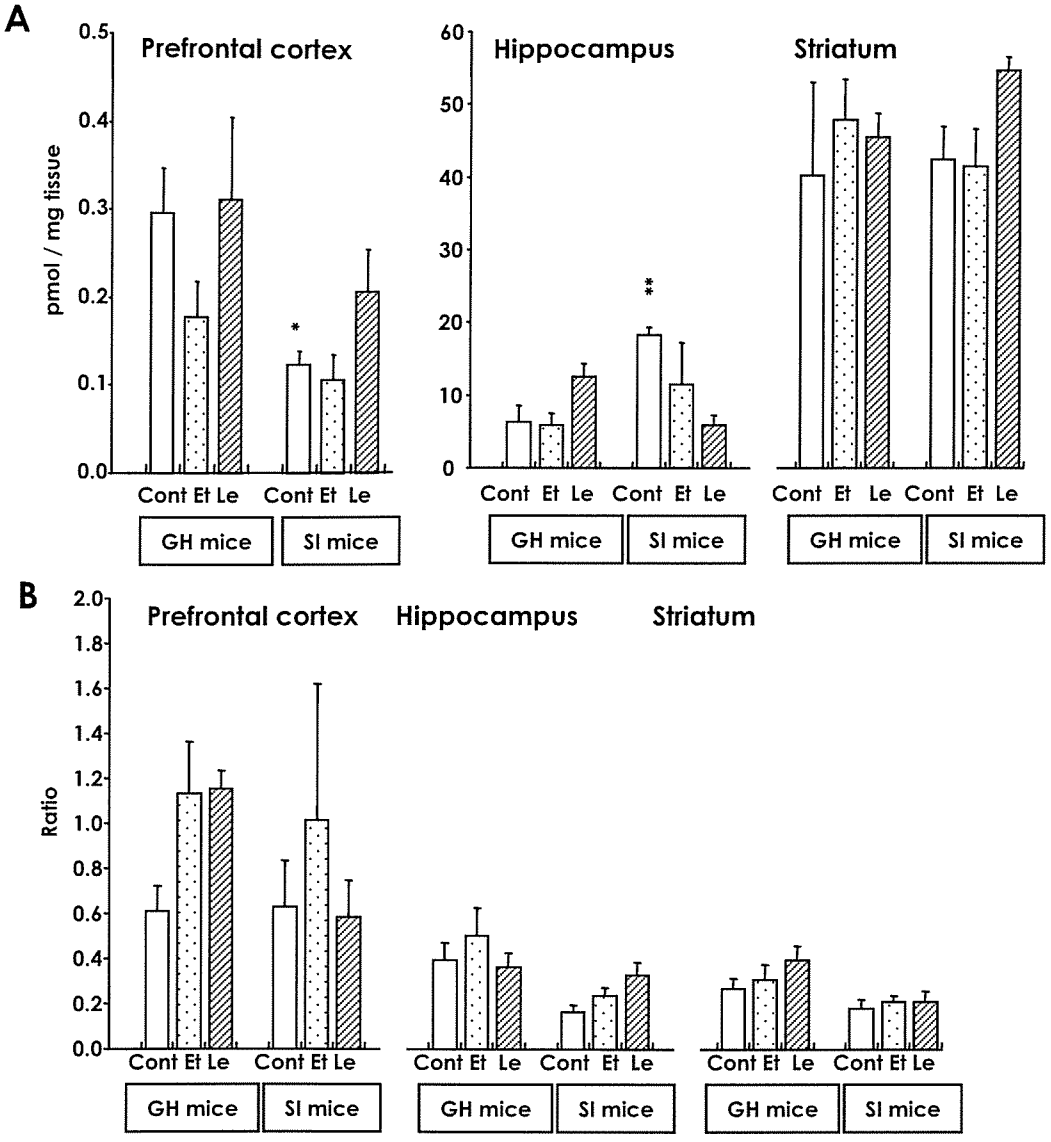


Fig. 4-7. HPLC determination of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), in the prefrontal cortex, hippocampus and striatum in mice that inhaled lemon oil.
A: DA, B: DOPAC/DA ratio.
Each value represents the mean±SE of 5 mice. The GH and SI mice's data are assessed by one-way ANOVA with Tukey's multiple comparison test, respectively.
* $P<0.05$, ** $P<0.01$; significantly different from GH control mice by unpaired Student's *t*-test. Cont: control, Et: ethanol inhalation, Le: lemon oil vapor inhalation.
GH mice: group-housed mice, SI mice: socially isolated mice.

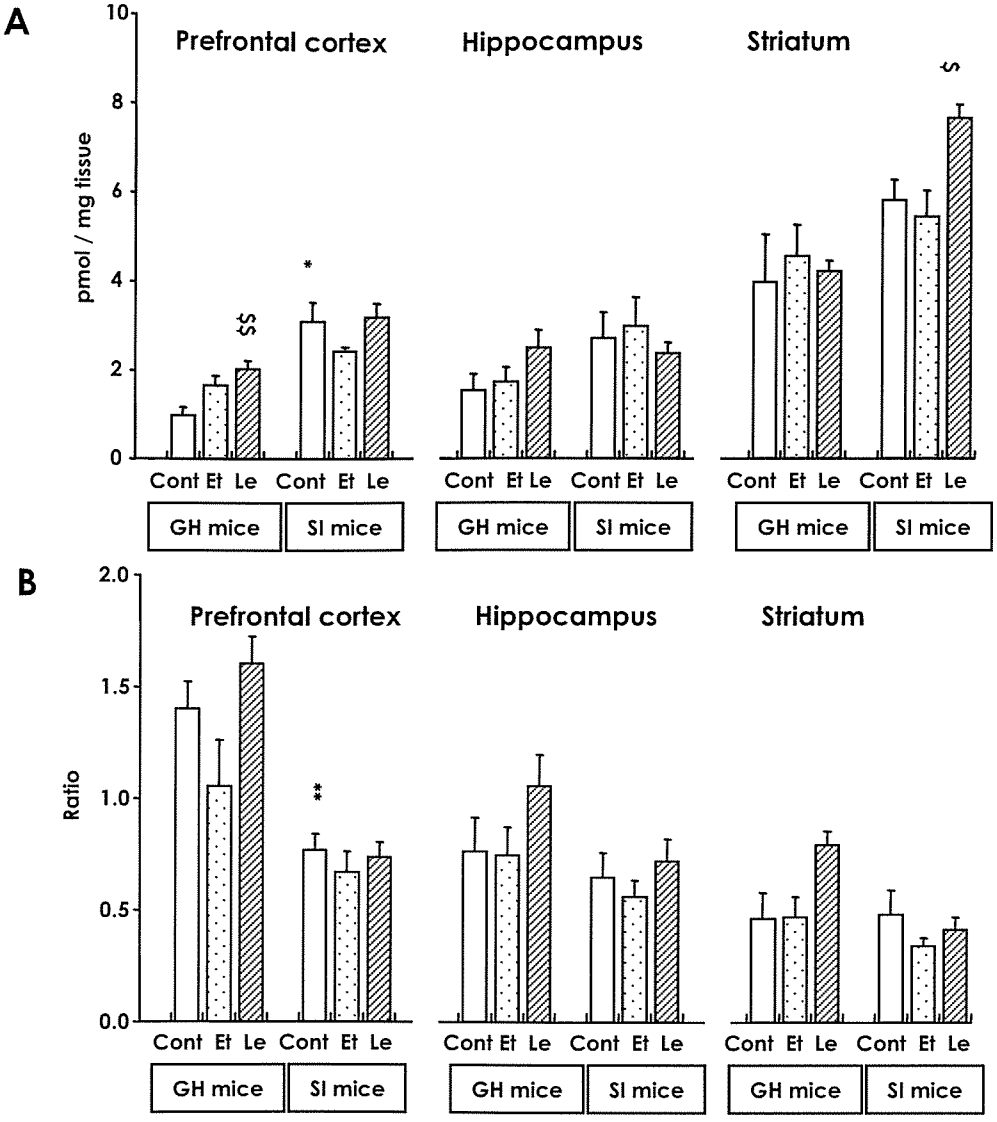


Fig. 4-8. HPLC determination of serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) in the prefrontal cortex, hippocampus and striatum in mice that inhaled lemon oil.

A: 5-HT, B: 5-HIAA/5-HT ratio.

Each value represents the mean±SE of 5 mice. The mice's data are assessed by one-way ANOVA with Tukey's multiple comparison test,

$ $P<0.05$, $$ $P<0.01$ compared to GH or SI control mice, respectively.

** $P<0.01$; significantly different from GH control mice by unpaired Student's *t*-test. Cont: control, Et: ethanol inhalation, Le: lemon oil vapor inhalation. GH mice: group-housed mice, SI mice: socially isolated mice.

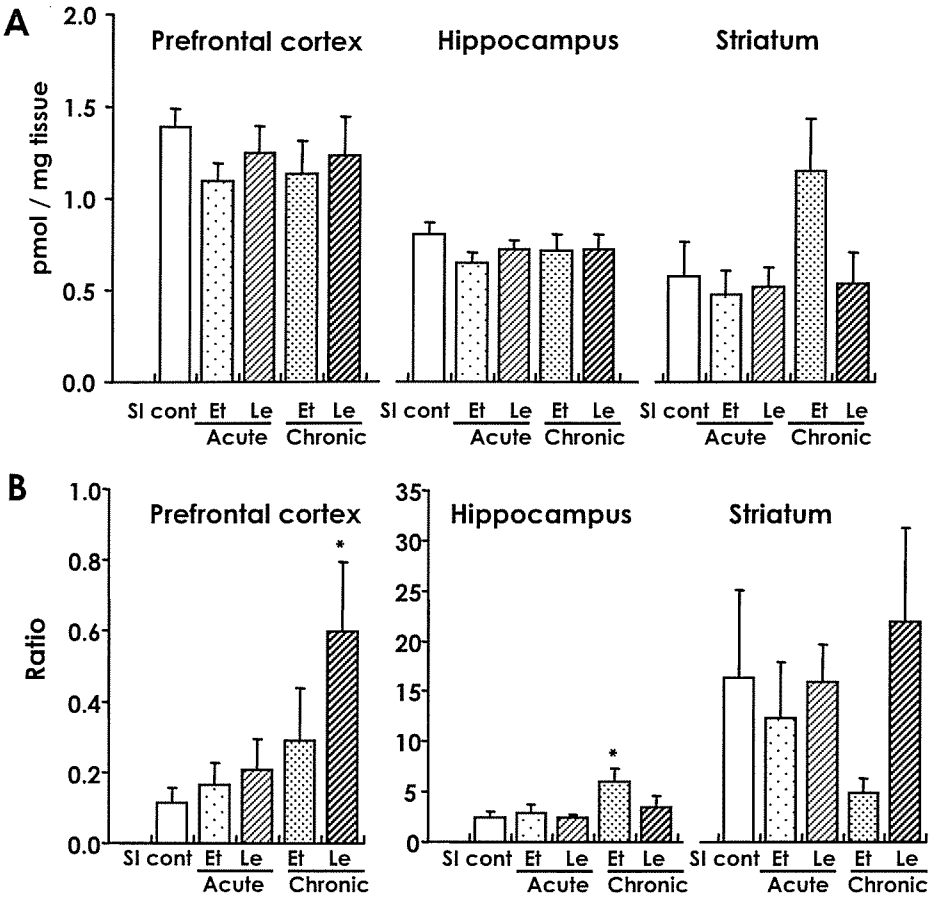


Fig. 4-9. HPLC determination of (NE) and 5-hydroxyindole acetic acid (5-HIAA) in the prefrontal cortex, hippocampus and striatum in Sl mice that inhaled lemon oil.

A: NE, B: HVA/NE ratio. Each value represents the mean±SE of 5 mice. All data are assessed by one-way ANOVA with Tukey's multiple comparison test,

* $P<0.05$ compared to the control group.

Sl cont: socially isolated control, Et: ethanol inhalation,

Le: lemon oil vapor inhalation.

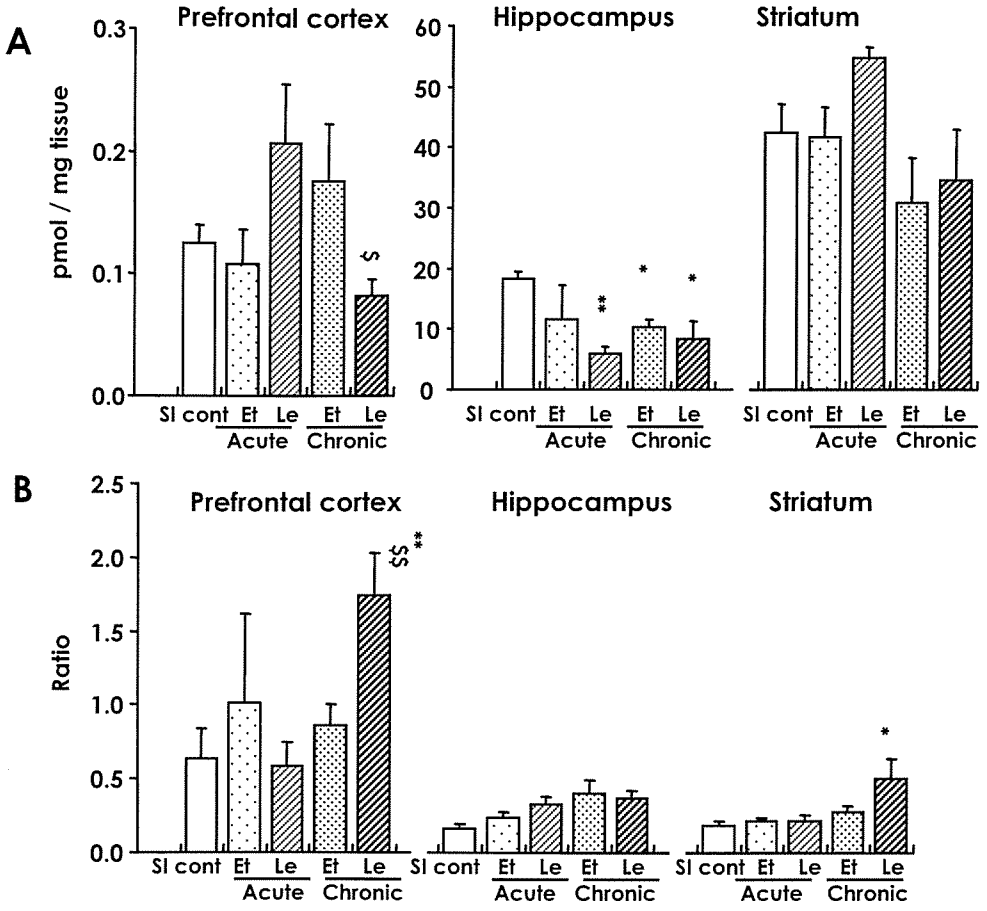


Fig. 4-10. HPLC determination of dopamine (DA) and dihydroxyphenylacetic acid (DOPAC) in the prefrontal cortex, hippocampus and striatum in Sl mice that inhaled lemon oil.
A: DA, B: DOPAC/DA ratio.
Each value represents the mean±SE of 5 mice. All data are assessed by one-way ANOVA with Tukey's multiple comparison test,
* $P<0.05$, ** $P<0.01$ compared to the Sl control group,
\$ $P<0.05$, \$\$ $P<0.01$ compared the acute inhalation of lemon oil group, respectively. Sl cont: socially isolated control, Et: ethanol inhalation, Le: lemon oil vapor inhalation.

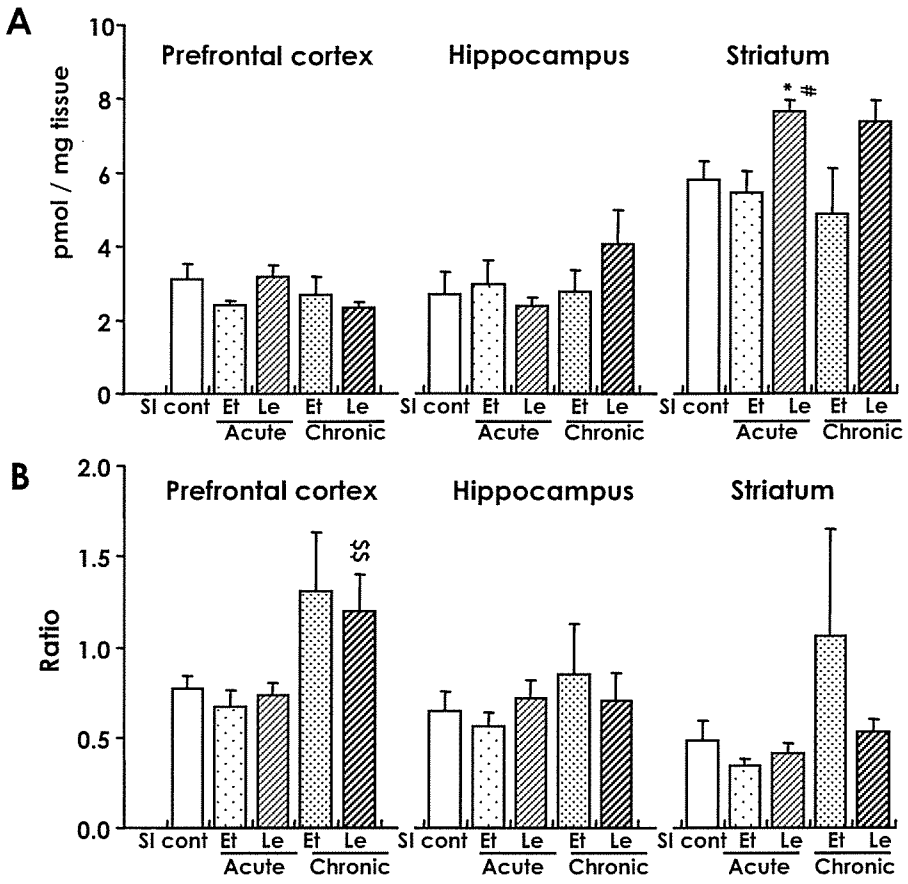


Fig. 4-11. HPLC determination of serotonin (5-HT) and 5-hydroxyindole acetic acid (5-HIAA) in the prefrontal cortex, hippocampus and striatum in SI mice that inhaled lemon oil.

A: 5-HT, B: 5-HIAA/5-HT ratio

Each value represents the mean±SE of 5 mice. All data are assessed by one-way ANOVA with Tukey's multiple comparison test,

* $P<0.05$ compared to the SI control,

# $P<0.05$ compared the acute Inhalation of ethanol group,

$$ $P<0.01$ compared to the acute inhalation of lemon oil group, respectively.

SI cont: socially isolated control, Et: ethanol inhalation,

Le: lemon oil vapor inhalation.

CHAPTER 5

The effect of dermal application of lavender oil on 24-hour autonomic nerve activity in dogs

INTRODUCTION

Essential oils such as valerian, lavender, or German chamomile have been said to possess sedative, anxiolytic, and hypnotic effects. Lavender oil is one of the most popular essential oils for human inhaling. Several studies of both humans and laboratory animals, i.e., rats and mice, have demonstrated that lavender oil acts as a central nervous system (CNS) depressant with effective sedative properties, thus improving sleep quality [1, 69-73]. However, similar investigations in dogs are limited, because the quantification of either emotional and mood modulations or neurotransmitters such as monoamines in the dog brain are difficult. It would be nearly impossible to get the experimental data in dogs as already stated in Chapter 1-4, the evaluation of anxiolytic, antidepressant, and anti-stress effects on essential oil. On the other hand, though there are few evidences and slim and none about the data to directly determine how to clinical use of essential oil in dogs (dose, administration time, precautions, toxicity and safety, the result of skin sensitization tests, etc.), lavender oil sometimes has been used as a practical alternative treatment for hyperexciting dogs, some veterinarians have used lavender oil extrapolating the data in humans as it is now. However, the absorption and metabolic capacity of chemicals and their mechanisms in dogs differ from those of humans, and the baselines of autonomic nerve activity in the dog are also different. As some chemicals innocuous to humans might cause a severe side effect in dogs, the extrapolating is dangerous.

This chapter was designed to assess the relaxing efficacy of lavender oil (*Lavandula angustifolia*) "quantitatively" in dogs via dermal application. We thought it was able to evaluate in dogs a relaxing effect of lavender oil by analysing the autonomic nervous system activities of the dogs in succession. Lemon and citrus oil have been said to cause poisoning in dogs, that addictive components are limonene and linalool, and as a sedative effect, lavender would surpass in lemon, then we used lavender in dogs. From the results and the other

literatures, we assessed the properties of the lavender oil in making dogs more relaxed.

MATERIALS AND METHODS

1. Animals

Five healthy male Beagles (31.4 ± 8.8 months old, 13.17 ± 1.4 kg) were used. These dogs were confirmed to be healthy based on the results of physical examinations, complete blood counts and serum chemistry analyses. The dogs were for laboratory use, housed in individual stainless steel cages (570 (W) × 570(D) × 700 (H) mm) under controlled temperature and entry to the room was unlimited (8:00-19:00). Lights were turned on at 7:00 and turned off at 19:00 daily. The dogs were given food and took light exercise outside the cages for about 30 min, during which time the cages were cleaned. The inside of the cages were cleaned twice daily at approximately 8:00 and 17:00. Water was given *ad libitum*. The use of these animals, as well as the procedures performed, was approved by the Animal Research Committee at Tottori University.

2. Holter ECG recording and computer analysis

A Digital Holter recorder (QR2100, Fukuda ME Kogyo, Tokyo, Japan) was used to record electrocardiograms (ECG) of the dogs. The dogs' fur was shaved in the chest region, where adhesive electrodes were attached and connected on 2 channels with M-X lead and L-R lead (A-B lead) methods to the Holter recorder [74]. This appliance was held in a side pocket of a Holter jacket (Fukuda ME Kogyo, Tokyo, Japan) worn by the dogs. All dogs had previously been made familiar with wearing the jacket with appliances to eliminate any fright of the test, and any influence on the results.

The recorded ECG data was averaged for 5-min periods by using an ECG analyzing

system (HS1000V, Fukuda ME Kogyo, Tokyo, Japan). Spectral indices of the heart rate (HR) variability were computed. The high-frequency (HF) power is considered to reflect modulation solely of parasympathetic tone by breathing, whereas the low-frequency / high-frequency ratio (LF/HF) is considered as an index of the cardiac sympathovagal balance [75]. These values were calculated, to evaluate the functioning of the autonomic nervous system. Frequencies ranging from 0.01 to 0.10 Hz were regarded as LF, and those ranging from 0.10 to 0.60 Hz were regarded as HF. The HR variability, HF power, and LF/HF ratio are well known to be useful parameters for evaluation of the effect of various drugs such as blocking or activating the autonomic nervous system as reported by Matsunaga *et al.* [76].

3. Essential oil

The lavender oil (country of origin: USA) was provided by The Japan Animal Aromatherapy Association (Chiba, Japan). The component analysis of the batch of lavender oil used in this study is shown in Table 5-1, which was determined by gas chromatography and mass spectrometry (GC-MS). The specific gravity and the optical rotation that we determined were 0.883 (22/20°C) and -7.824° (20°C), respectively. We contracted out these analyses to outside company, Essential Oil University (New Albany, IN, USA).

All dogs were given a simple lavender oil patch test on the abdomen prior to the experiment. We applied a drop of oil on the skin and repeatedly checked the skin for allergic sensitivity reaction for 36 hours. Following a period of 10-14 days rest, the area was again challenged with a repeated test.

During the experiment, the dogs received topically 0.18 ml of the lavender oil inside the pinna of both ears (0.36 ml in total, approximately 6 drops), respectively, at 8:30, 12:00, 15:30, and 19:00 per day as the lavender treatment (Lav.). Apart from this, as a control

treatment (Cont.), 0.36 ml of saline was applied to the same animals in different weeks.

In addition, we checked the dogs' skin after an appropriate successive application of the lavender oil to determine if there were any dermatologic lesions caused by irritation or sensitivity reactions.

4. Experimental protocol

As shown in Fig.5-1, all dogs were studied for three consecutive days, following the placement of the Holter recorder and treatment with lavender oil (Lav.) or saline (Cont.). The three consecutive day treatment was repeated 4 times in each dog, in alternate shifts of lavender and saline treatment with an interval of 3-4 days between treatment sessions.

The treatment for three consecutive days was as follows: one day prior to the day when the recording ECG was started, the dogs were fitted with Holter recorders and the jackets were put on for familiarization purposes. On the next day, i.e., Day 1, at 8:00, ECG recording was started in the dogs, which were unrestrained over the subsequent 48 hours. Throughout that period at 8:30, 12:00, 15:30, and 19:00, the dogs were checked to see that their Holter recorders were working properly. The small memory card and battery in the Holter recorder were replaced with new ones, respectively, on Day 2 at 8:00, as the data capacity of one small memory card permits recording of data for approximately 24 hours. On the same day, the dogs received 0.36 ml of the lavender oil or saline inside the pinna of both ears 4 times, at the same times when the Holter recorders in their pockets were checked, i.e., 8:30, 12:00, 15:30, and 19:00. On Day 3 at 8:00, the electrodes, the Holter recorder, and the jacket were removed from the dogs and the ECG recording was finished.

5. Statistical analysis

All statistical analysis was performed by using JMP software (ver. 5.1.1., SAS Institute Japan Inc., Tokyo, Japan). R-R intervals before and after an artifact were excluded from the analysis. The values of HR, HF power, and LF/HF ratio were expressed as 12-segment moving-averages for changes in the means obtained at 5-min intervals. Serial changes of HR, HF power, and LF/HF ratio for 23.5 hours and for 3.5 hours after each oil administration i.e., 8:30, 12:00, 15:30, 19:00, and for 9.5 hours from 22:30 to 8:00 in the data were assessed by a repeated measured MANOVA (multiple analysis of variance) test. P values less than 5% were regarded as significant.

RESULTS

The serial changes of HR, HF power, and LF/HF ratio had obvious rhythmicity in the 5 dogs of Cont., on both Day 1 and Day 2. Similar data having a circadian rhythm was reported in healthy adult Beagles by Matsunaga *et al.* [76].

There were similar in the serial changes of HR ($F_{(1, 18)} = 0.093$, P = 0.21), HF power, ($F_{(1, 18)} = 0.015$, P = 0.60), and LF/HF ratio ($F_{(1, 18)} = 0.003$, P = 0.81) for 24 hours on Day 1, as can be seen in Fig.5-2 and Table 5-2 between Lav. and Cont. Comparing the differences in the serial changes on Day 2 between the Lav. and Cont. (Fig.5-3 and Table 5-2), we found that the HR values in the Lav. declined significantly in the period of 19:00-22:30 more than that in Cont. ($F_{(1, 18)} = 0.269$, P = 0.04). The HF power in the Lav. increased significantly ($F_{(1, 18)} = 0.293$, P = 0.03) in the period of 15:30-19:00 more than that in the Cont. However, there were no significant differences in the serial changes of LF/HF ratio between the two groups ($F_{(1, 18)} = 0.006$, P = 0.74).

In addition to the above results, we found that an appropriate successive application of the

lavender oil to dogs' skin did not cause any clinical dermatologic lesions.

CLINICAL APPLICATION

Using results from described above, we recorded ECG on a dog maintained in a domestic environment during cooping her up in a hotel cage. She was excited, wallows, barking, and eats nothing, every time when she was cooped up in a hotel cage. The experiment protocol was in much the same way as Beagles described previously were done. After she was made familiar with a jacket and appliances, under the three situations as follows, ECG was recorded for three consecutive days: At first situation, she was fitted with Holter recorders and put on the jackets, and ECG was recorded for three consecutive days. In the meanwhile, she was at home, went on with her day ("at home"). At second situation, she was taken a hotel cage from Day 2 at 8:30 and received the saline treatment ("saline treatment in hotel"). At third situation, she was in a hotel cage from Day 2 at 8:30 and received the lavender oil treatment ("lavender oil treatment in hotel"). Treatment was conducted 4 times on Day 2. Those three consecutive days treatment was repeated 6 times, in alternate shifts of "at home", "saline treatment in hotel", and "lavender oil treatment in hotel" with an interval of 3-4 days between treatment sessions.

The results were represented raw data, not calculated the moving average. All results were assessed by paired t-test. As shown in Fig.5-4, during her up in a hotel cage, the serial changes in HR of "saline treatment in hotel" significantly rose than those of "at home" in the period of 9:00-12:00 ($P<0.0001$), and 15:30-19:00 ($P<0.0001$). In the period of 12:00-15:30 ($P=0.5831$) and 19:00-22:30 ($P=0.0753$), the serial changes in HR of "saline treatment in hotel" tended to rise. In the period of 22:30-08:00, the serial changes in HR of "saline treatment in hotel" significantly declined than those of "at home" ($P<0.0001$). The serial

changes in HF power of "saline treatment in hotel" significantly declined except the period of 22:30-08:00 (9:00-12:00 $P<0.0001$, 12:00-15:30 $P=0.0008$, 15:30-19:00 $P<0.0001$, 19:00-22:30 $P=0.0002$). The period of 22:30-08:00, the serial changes in HF power of "saline treatment in hotel" significantly rose than those of "at home" ($P<0.0001$). The serial changes in LF/HF ratio of "saline treatment in hotel" significantly increased in the entire periods (9:00-12:00 $P<0.0001$, 12:00-15:30 $P<0.0001$, 15:30-19:00 $P<0.0001$, 19:00-22:30 $P<0.0001$, 22:30-08:00 $P<0.0001$).

As shown in Fig.5-5, when she received the lavender oil treatment in a hotel cage, the serial changes in HR of "lavender oil treatment in hotel" significantly declined than those of "saline treatment oil in hotel", in the entire periods except the period of 9:00-12:00 ($P=0.063$) by paired t-test (12:00-15:30 $P=0.0094$, 15:30-19:00 $P<0.0001$, 19:00-22:30 $P<0.0001$, 22:30-08:00 $P=0.0017$). The serial changes in LF/HF ratio and HF power of "lavender oil treatment in hotel" also significantly declined than those of "saline treatment oil" in the entire periods by paired t-test (LF/HF ratio: 9:00-12:00 $P=0.0162$, 12:00-15:30 $P=0.0138$,15:30-19:00 $P<0.0001$, 19:00-22:30 $P<0.0001$, 22:30-08:00 $P<0.0001$, HF power: 9:00-12:00 $P=0.0441$,12:00-15:30 $P<0.0001$, 15:30-19:00 $P<0.0001$, 19:00-22:30 $P<0.0001$, 22:30-08:00 $P=0.0145$). At the day we applied the lavender oil to her, she was not excited, barking, and was resting, eating.

DISCUSSION AND CONCLUSION

Dogs have been reported so far to display behaviors suggestive of relaxing [77] and suppressing travel-induced excitement [78] by exposure to lavender odors. Our study may become the most compelling evidence in support of these behavioral findings. Because our study is a report to "quantify" physiologically the properties of the whole lavender oil *in vivo*

in dogs using Holter recorders to assess the HR variability.

The use of HR variability is widely accepted for the evaluation of cardiac autonomic modulation [79, 80]. Frequency-domain analysis allows the HR variability to be dissected into its specific frequency components. For example, the HF component is synchronous with respiratory sinus arrhythmia and is mainly supported by vagal activity [81], while the LF component is largely correlated with sympathetic efferent activity [81]. On the other hand, the LF/HF ratio is considered to mirror sympathovagal balance or to reflect sympathetic modulation [82]. For these evidences, we selected frequency-domain analysis due to assess autonomic modulation.

The serial changes for 24 hours on Day 1 and Day 2 in the Cont. were similar in the HR, HF power, and LF/HF ratio (data not shown). These results indicated that none of the dogs were excited, stressed, or strained by the fixing of electrodes or wearing a jacket. If they had been excited, these values might have been somewhat different between Day 1 and Day 2 because of acclimation to the apparatus as time advanced.

The HR variability and autonomic activity in dogs were observed as following circadian rhythm in both Lav. and Cont. The HR values of Lav. began to decrease from evening and significantly decreased after the 4th administration of the lavender oil in the period of 19:00-22:30 more than those of Cont. Furthermore, vagal activities during the entire treatment of Lav. tended to increase ($F_{(1, 18)} = 0.194$, P = 0.077) and significantly increased in the period during 15:30-19:00, which is after the 3rd administration of the lavender oil. That also had a tendency to increase in the following consecutive periods of 19:00-22:30 ($F_{(1, 18)} = 0.221$, P = 0.06) and 22:30-07:00 ($F_{(1, 18)} = 0.224$, P = 0.06) as compared with that of Cont. As decrease of the HR and enhancing the vagal nervous activities are assumed to cause calming and relaxing state by appearances under behavioral reaction, these findings suggest that the

lavender oil via dermal application enhanced vagal activity, and it may have made dogs more relaxed. Similar experiments which lavender oil enhances vagal activity have already been reported in humans [72, 83, 84].

We obtained only two significantly different aspects between the Cont. and Lav. in our study. These results may have been attributable to an insufficient number of dogs. However, the subject dogs must have almost the same background; sex, age, the life environment, and life pattern. If these had differed the results would have indicated larger deviations of the values, especially as far as the emotion, mood, and behavior are concerned. It is truly very difficult to evaluate these parameters.

Other possible reasons may include that the action of lavender oil to enhance the vagal activity in dogs might not have been strong enough in the morning or daytime. Arguments for the deficiency of lavender oil effectiveness during the daytime can be hypothesized as follows. One reason is that lavender oil might have suppressed excess vagal activities and corrected the deficit. This suggests that lavender oil might be able to regulate sympathovagal balance corresponding to sympathovagal activity depending on the circadian rhythm. The assumption resembles the report that isoflavone, which is a phytoestrogen, metabolites can act as an estrogenic agonist or antagonist depending on the estrogen concentration [85]. Interestingly, Takeuchi *et al.* reported that aged dogs showed increases in the total amount of time spent in slow wave sleep during the daytime followed by an increasing of time spent in wakefulness during the night, and the LF/HF ratio showed a very low degree of variance throughout the day [86]. This indicates that lavender oil might be effective for aged dogs suffering from a sleep disorder.

Another possible specifically considered is that a cumulative effect of the lavender oil applied 4 times during the daytime might affect the results. It is inferred that the absorption

and metabolism take a few hours (human [87, 88], mice [89]), the complete clearance may take a few days [24]. We assumed that the metabolites might have remained in the blood for a period, and possibly reacted with the additionally applied oils at the same time.

All dogs received lavender oil inside the pinna of both ears, and then two routes were hypothesized for the penetration of the oil components into the body [23, 24, 25] as already stated in PREFACE. Both an indirect effect via the olfactory nerve pathways and a direct effect via the blood to the brain are likely to relate to the modulation of the neurotransmitter production and release into the brain. Several studies in human and laboratory animals, i.e., rats and mice, have demonstrated that lavender oil has CNS depressant activity with sedative effectiveness in improving sleep quality [1, 69-73]. There are few reports about the mechanism, however Aoshima *et al.* conjectured from *in vitro* examinations that the intake of lavender oil modulates the neural transmission in the brain through ionotropic GABA(A) receptors [23]. Yamada *et al.* reported that the increase in plasma adrenocorticotropic hormone and decrease in adrenaline, noradorenaline, and DA levels induced by ether inhalation tended to recover by the inhalation of *Lavandula burnetii super* in menopausal model rats [90] . An increase of vagal activities by the administration of lavender oil in dogs was observed in this study. This finding also may relate to the modulation of DA activities and/or GABA receptor activities.

In addition, let us point out the concentration of essential oil applied the dogs' skin. We usually use the concentration of 4% for various blend oils for clinical use, according to the safety data published in a survey in humans by Martin Watt [91, 92], and this concentration caused no problems such as irritation or sensitization in dogs in our clinical practice. However, we decided very consciously to use undiluted lavender oil to investigate the pure efficacy of the oil without using any base oils for dilution. We have been obtaining very positive effects

without any skin irritation or sensitization problems since the essential oil was genuine and high quality. That said, it must be avoided misleading that clinicians try to apply undiluted oil according to this study. Furthermore, even if the essential oil is genuine, a successive application for a week must be prohibited. We have found out no report that skin sensitization test of lavender oil was performed on "dog" until now. It is necessary to directly determine how to clinical use of lavender oil (dose, administration time, precautions, toxicity and safety, the result of skin sensitization test in dogs, etc.) with further experiments and we would like to report that.

In conclusion, the lavender oil via dermal application enhanced vagal activity, and it may have made dogs more relaxed. This study helps to verify the sedative effect of lavender oil for dogs quantitatively.

Table 5-1 Main components of the lavender oil analized by GC-MS

RT	Compound name	Area %
21.69	alpha-Thujene	0.10
22.14	alpha-Pinene	0.24
23.26	Camphene	0.18
25.97	1-Octen-3-ol	0.17
26.51	3-Octanone	1.04
26.86	Myrcene	0.61
27.29	Butyl butyrate	0.13
28.72	N-Hexyl acetate	0.43
29.42	p-Cymene	0.19
29.73	delta-3-Carene	0.54
29.90	1,8-Cineole	0.69
30.70	(Z)-beta-Ocimene	3.27
31.47	(E)-beta-Ocimene	1.14
32.19	gamma-Terpinene	0.19
33.30	cis-Linalool oxide	0.14
34.53	alpha-Terpinolene	0.23
35.86	Linalool	32.82
36.58	Octen-3-yl acetate	0.78
37.48	Octanol acetate	0.07
37.78	Ocimene	0.30
38.93	Camphor	0.33
39.34	Hexyl isobutanoate	0.08
40.61	Borneol	0.76
40.80	Lavandulol	0.74
41.57	Terpinen-4-ol	1.84
42.54	alpha-Terpineol	1.02
45.37	Nerol	0.30
46.17	Cumin aldehyde	0.11
47.70	Linalyl acetate	41.63
49.59	Bornyl acetate	0.35
49.96	Lavandulyl acetate	2.01
56.33	Geranyl acetate	1.04
58.53	alpha-cis-Bergamotene	0.07
58.94	beta-Caryophyllene	3.25
59.86	cis-alpha-Bergamotene	0.26
61.22	(E)-beta-Farnesene	2.01
62.92	d-Germacrene	0.60
65.00	gamma-Cadinene	0.15
69.35	Caryophyllene oxide	0.16

RT: retention time

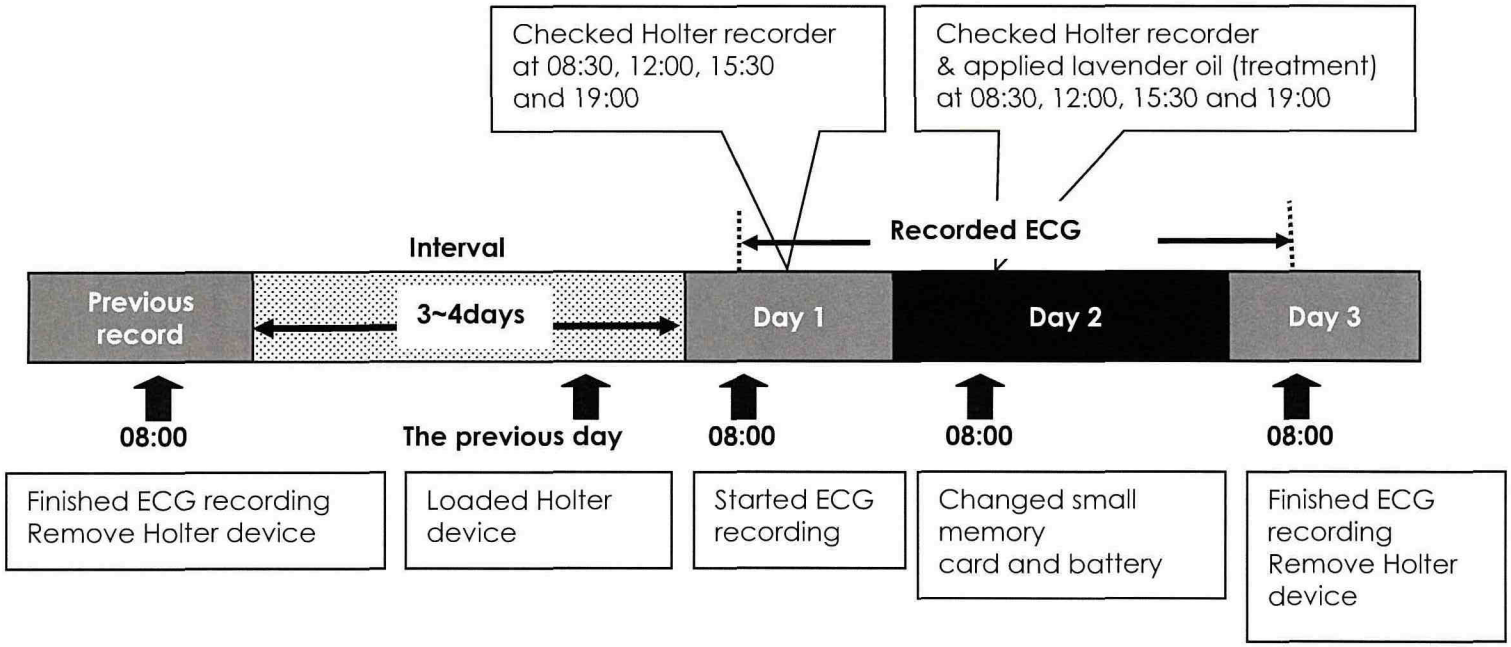


Fig.5-1. Schedule for ECG recording in dogs

Treatments are lavender oil for the Lav. treatment and saline for the Cont. treatment, respectively. Treatment was conducted 4 times for each dog on Day 2 of 3 consecutive experiment days, alternating the two types with an interval of 3-4 days in between treatment sessions.

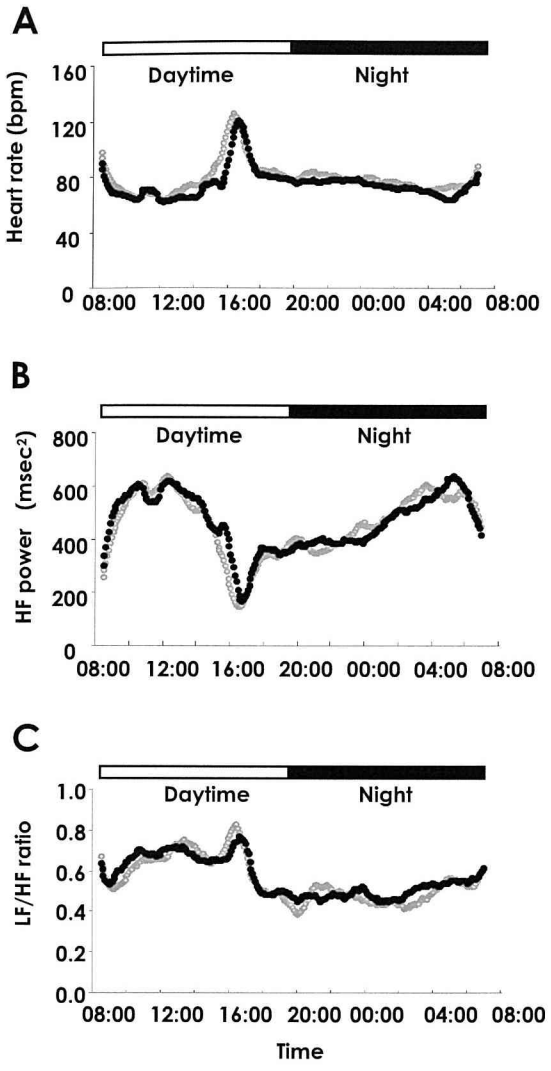


Fig.5-2 The serial changes of HR (A), HF power (B), and LF/HF ratio (C) for 24 hours on Day in 5 dogs receiving the control treatment (open circles and a gray line) and lavender treatment (solid circles and black line).
Dots represent 12-segment moving-averages of the means obtained at 5-min intervals. bpm = Beats per minute. * $P<0.05$ compared to the Cont.

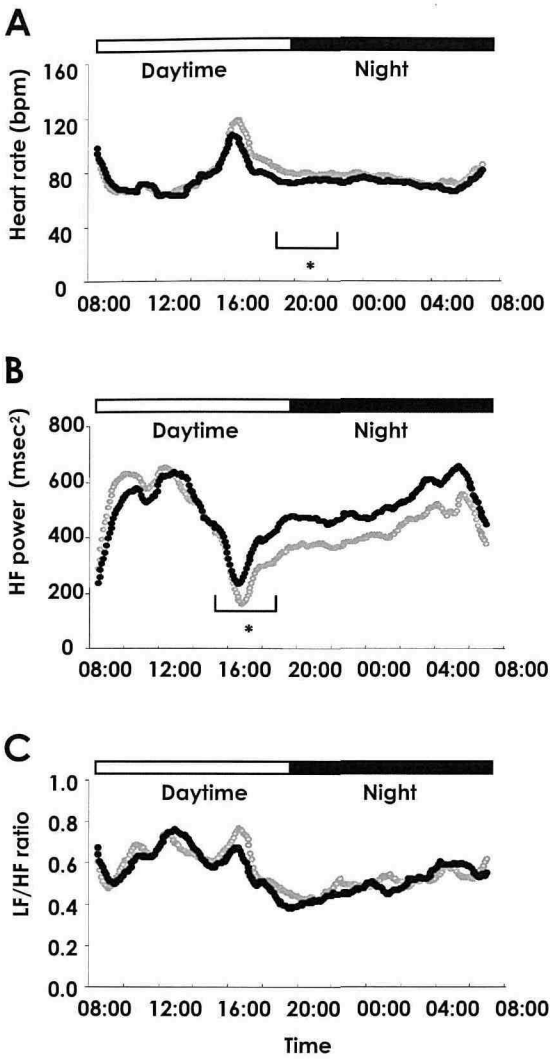


Fig.5-3 The serial changes of HR (A), HF power (B), and LF/HF ratio (C) for 24 hours on Day 2 in 5 dogs receiving the control treatment (open circles and a gray line) and lavender treatment (solid circles and black line).
Dots represent 12-segment moving-averages of the means obtained at 5-min intervals. bpm = Beats per minute. * $P<0.05$ compared to the Cont.

Table 5-2 Statistical results from the serial changes in HR, HF power and LF/HF ratio between control and lavender oil treatment in dogs

Parameter	Lavender oil application period					All period
	08:30 | 12:00	12:00 | 15:30	15:30 | 19:00	19:00 | 22:30	22:30 | 07:00	
HR						
Day 1	0.40	0.35	0.10	0.19	0.45	0.21
Day 2	0.60	0.89	0.12	0.04 *	0.15	0.23
HF power						
Day 1	0.55	0.51	0.09	0.75	0.90	0.60
Day 2	0.26	0.79	0.03 *	0.06	0.06	0.08
LF/HF ratio						
Day 1	0.70	0.90	0.81	0.85	0.61	0.81
Day 2	0.94	0.69	0.17	0.31	0.87	0.74

Lavender oil was administered at 8:30, 12:00, 15:30 and 19:00. Data shows P value between control and lavender oil treatment in each inter-period (n=5). * $P<0.05$.

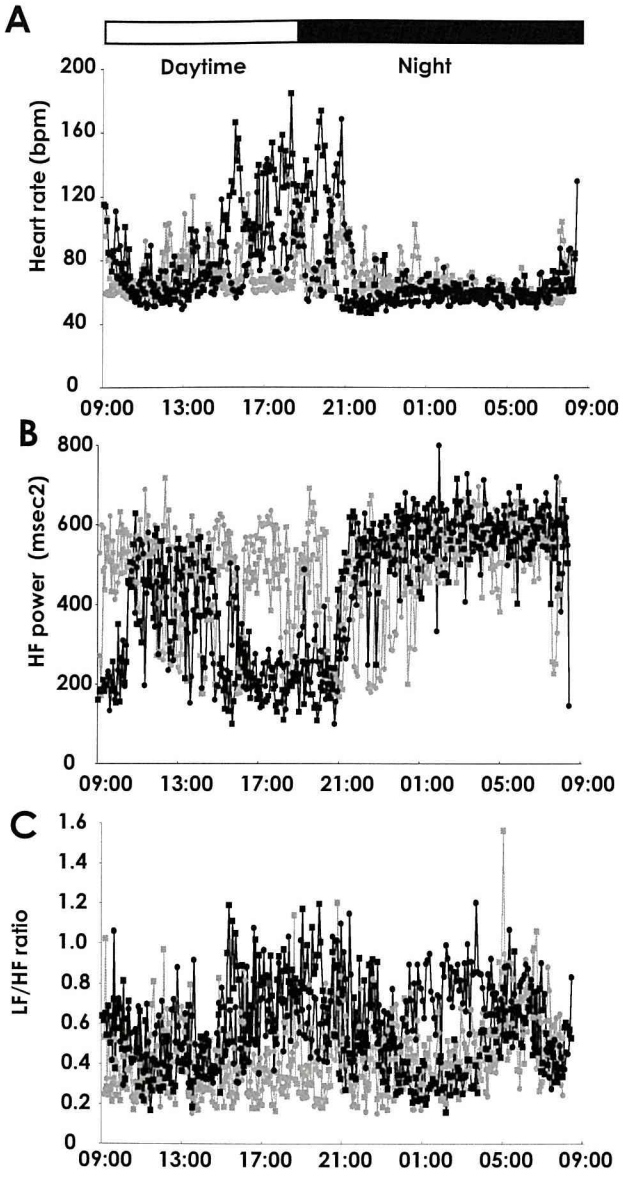


Fig. 5-4 The serial changes of HR (A), HF power (B), and LF/HF ratio (C) for 24 hours on Day 2 in a dog maintained in a domestic environment, during in her home (gray line), during cooping her up in a hotel cage receiving the control treatment (black line).

Dots represent the means obtained at 5-min intervals.

bpm = Beats per minute.

* P<0.05 compared to the Cont.

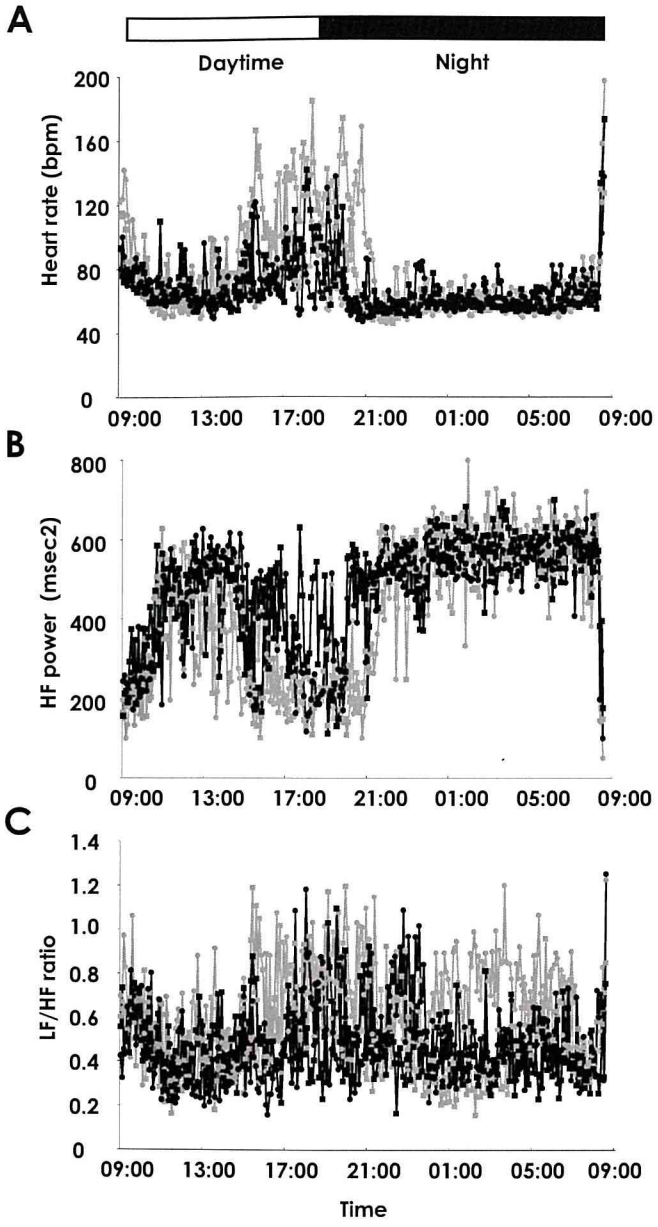


Fig. 5-5 The serial changes of HR (A), HF power (B), and LF/HF ratio (C) for 24 hours on Day 2 in a dog maintained in a domestic environment, during cooping her up in a hotel cage, receiving the control treatment (gray line) and lavender treatment (black line).
Dots represent the means obtained at 5-min intervals.
bpm = Beats per minute.
* P<0.05 compared to the Cont.

GENERAL DISCUSSION

The present study showed that the inhalation of lemon oil vapor caused significant anxiolytic, antidepressant, anti-stress and sedative effects on both the GH mice as the model in normal and the SI mice as the pathological model for emotional disorders. These effects of lemon oil might be closely related with the 5-HTnergic pathway, especially via 5-HT_{1A} receptor. To tell more explain, lemon oil vapor would act via the suppression of DA activity related to enhanced 5-HTnergic neurons.

Furthermore, to get the evidence of essential oil effects more suitable for practical use, we carried out experiments using beagle dogs. In this experiment, we used not lemon oil but lavender oil, since it would be proper for the dogs to assess a sedative effect via analyzing autonomic nerve activity. Lavender oil is one of the most popular essential oils as having sedative properties for humans. We could verify that dermal application of lavender oil tended to enhance vagal activity from evening to night time, in partial periods of time, significantly enhanced. Our findings suggest that the lavender oil has possibilities to make dogs more relaxed and its application might be a practical alternative treatment for hyperexciting dogs in some circumstances. This study helps to verify the effect of lavender oil for dogs quantitatively. However, further experiments are needed to clarify the properties of lavender oil especially regarding the circadian rhythms of automatic nerve activities in animals.

Anxiolytic, antidepressant, and sedative effects of essential oils are very helpful for psychiatry and psychopharmacology, since combining the medicine and essential oil can reduce the dose of those medicines and essential oil may help prevent the side effects of the anxiolytic and antidepressant medicines. In companion animals case, if only an application of essential oil causes sufficient effects, the owners could remove the burden of dosing the

domestic animal with medicine. If we, veterinarians, could get the strong evidence that lavender oils make dogs sedative and relaxing, and essential oils possibly affect dog's emotion, we would use the oils for dogs willingly as a medical attendance, in case a dog has some behavioral problems, namely aggressiveness, separation anxiety disorder, travel-induced excitement, and sleep disorder for aged dogs.

Unfortunately, all the results so far contained both our study and any other studies on lavender oil are controversial. It is necessary to directly determine how to clinical use of lavender oil (dose, administration time, precautions, toxicity and safety, the result of skin sensitization test in dogs, etc.) with further experiments and we would like to report that.

In this way, there has been little evidence about the alternative therapy using essential oils. Nonetheless, if we would wait to do aromatherapy until we would get sufficient evidences, we could not use essential oil as a practical alternative treatment for a long time. So we advice veterinarians to use essential oils treatment carefully as follows: (1) Do not treat for cats, ferrets, exotic pets, small birds, reptiles, amphibians with essential oils. (2) Use high quality of essential oils. (3) Be sure to give a simple essential oil patch test on the abdomen prior to the application. (4) Reduce the usage of essential oils at a minimum frequency. (5) Blend in the essential oils used to animal patients in lesser concentration than 4 percentages. (6) Avoid long-term successive application of essential oils. (7) avoid using essential oils to the individual during young age and pregnancy. (8) Use essential oils to aging individuals with the possible care.

To provide safety, high-quality, and stable essential oils is the most important. But in fact, major components of an essential oil can differ greatly in quality from one batch to another as it is now. Although every plant has its own makeup with almost identical appearance, there are great differences even in the same species. The essential oils produced in a plant vary in

chemical composition, major components, and hence toxicity depending on how and where the plant grows, and depending on the conditions present when the plant is harvested [93, 94]. Furthermore, as a consequence of the present upsurge in demand for essential oils due to the boom in aromatherapy, essential oils in the market not infrequently contain synthetic chemical substances produced by advanced techniques and/or are adulterated illegally [93,94]. Once neatly synthesized impurities are mixed into an essential oil, it is extremely difficult to distinguish genuine oil from adulterated ones. To make matters more complicated, genuine essential oils do not always have strong therapeutic properties.

Nevertheless, a careful and appropriate use of genuine essential oils that have sufficient scientific safety data is normally free of complications, or toxic effects [91, 92, 95]. However synthetic essential oils sometimes can even be harmful, as these compounds are considered to be linked to an increase in asthma, allergies, and sensitization [91, 92, 94, 95]. Even a drop of genuine essential oil consists of dozens of trace components, which may work synergistically or inhibitorally [93]. This is, if any show sensitization potential by itself, the other pure components may inhibit such effects. This kind of inhibition is called "quenching". For example, cinnamic aldehyde, which is a strong sensitizer, when formulated with an equal weight of eugenol (quencher), prevents sensitization [96]. If someone added synthetic chemicals in the essential oil, the composition of the oil would alter, and such an exquisite balance provided by nature might be destroyed, and the effect of "quenching" might be dislocated. Prashar *et al.* reported that cytotoxicity of linalyl acetate, which is a major component of lavender oil, was higher than that of the lavender oil itself, suggesting suppression of its activity by an unknown factor in the oil in their research of cytotoxicity of the lavender oil and its major componemts [97].

Many studies on essential oils have been performed on oils with synthetic chemicals, and

researches are apt to extrapolate the results obtained from synthetic chemicals and/or one of the main components as being the action of the whole oil. We consider this a very bad assumption. We therefore would like to suggest that a componential analysis should be carried out in every study involving essential oils in order to verify the quality of materials used in the study, especially in studies assessing the whole essential oil efficacy. This might contribute to guaranteeing that aromatherapy research is based on more scientific and informative evidence as regards essential oils.

We have thought that aromatherapy could have basic evidence for becoming a practical alternative treatment.

Most of part in this study was published in the articles [98, 99].

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SUMMARY

Anxiolytic, antidepressant, and sedative effects of essential oils are very helpful for psychiatry and psychopharmacology, since combining the medicine and essential oil can reduce the dose of those medicines, and essential oil may help prevent the side effects of the anxiolytic and antidepressant medicines. However, the evidences of the effects of each essential oil have been few, and the mechanisms of the effects have not yet been made clear and seem to differ for each essential oil. So I examined the anti-stress (anxiolytic, antidepressant) and sedative action of some essential oils, including lavender, rose, and lemon oils. I obtained several evidences as follows;

1. Lemon oil vapor for 90 min before the tests (acute inhalation) had the strongest anti-stress effect in an elevated plus-maze test (EPM), a forced swimming test (FST), and an open field test (OFT) in group-housed (GH) mice as the model in normal.

2. The acute inhalation of lemon oil vapor also suppressed the characteristic behaviors such as a tendency to aversion to the open arms in the EPM, increased levels of the locomotor activity and rearing in the OFT in socially isolated (SI) mice. However, the chronic inhalation of lemon oil vapor for 2 hours a day during the three-week isolation term in the SI mice did not show any significant effects.

3. I further investigated a regulatory mechanism of lemon oil by pre-treatments with agonists or antagonists to benzodiazepine, 5-HT, DA, and adrenaline receptors by the EPM and the FST in the acute inhalation. The results suggest that the antidepressant effect of lemon oil is closely related with the 5-HTnergic pathway.

4. The level of plasma corticosterone was elevated by social isolation. The reduction of the level by the acute inhalation of lemon oil was slight. The chronic inhalation of both ethanol alone and lemon oil significantly reduced the level, respectively.

5. I quantified the contents of monoamines and their metabolites in the prefrontal cortex, the hippocampus, and the striatum of mice after inhalation of lemon oil. The acute inhalation of lemon oil accelerated the metabolic turnover of monoamines in some regions in both the GH and the SI mice. In the chronic inhalation of lemon oil, the metabolic turnover of NE in the prefrontal cortex, those of DA in all of three regions, and that of 5-HT in the prefrontal cortex were accelerated in SI mice.

6. I carried out the experiment using beagle dogs for practical use. Electrocardiogram (ECG) was recorded for three consecutive days by Holter recording system. Heart rate (HR) variability, the high-frequency (HF) power, and the low-frequency/high-frequency ratio (LF/HF), were calculated to evaluate the state of relaxation of the dogs via straining of the autonomic nervous system. Dermal application of lavender oil, at 8:30, 12:00, 15:30, and 19:00 on the second day caused a reduction of HR values in the period of 19:00-22:30 and an increasing of the HF power in the period of 15:30-19:00.

7. In conclusion, I demonstrated an anti-stress efficacy of lemon oil in mice and relaxing efficacy of lavender oil in dogs. These mechanisms might relate to modulating the 5-HT and DA activities. I have thought that aromatherapy could have basic evidence for becoming a practical alternative treatment.

END