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Intracerebral Distribution of α-Pinene and the Anxiolytic-like Effect in Mice Following Inhaled Administration of Essential Oil from *Chamaecyparis obtusa*

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The anxiolytic-like and stress reduction effects following inhaled administration of essential oil from *Chamaecyparis obtusa* (EOCO) have been reported. Volatile components are thought to produce these effects of EOCO by neurological transfer and pharmacological transfer. The regions of the brain in which inhaled compounds are found due to pharmacological transfer of EOCO are not known. This research was undertaken to clarify the relationship between the intracerebral distribution of α-pinene, which is the main component of EOCO, and emotional behavior. α-Pinene was detected as the main component of volatile EOCO. The amount of α-pinene in each region of the brain was measured following inhaled administration of EOCO. The amount of α-pinene was different in each region of the brain. With inhaled administration of 32 μL/L air EOCO, a high concentration of α-pinene was observed. However, no significant differences in the concentration of α-pinene among brain regions were found. A therapeutic concentration of α-pinene (8 μL/L air EOCO) in each region of the brain may induce an anxiolytic-like effect, and a high concentration of α-pinene (32 μL/L air EOCO) in each region of the brain may induce an excitatory-like effect. The increases in the concentration of α-pinene from 8 to 32 μL/L air EOCO in the striatum and the hippocampus were significantly lower compared with the increases in other brain regions. These results indicate that regions besides the striatum and the hippocampus participated in the increase in locomotor activity due to the high concentration of α-pinene in the brain.

Keywords: Volatile compound, Inhalation, Nasal cavity, Aromatherapy, Phytotherapy.

Several reports have described the distribution of drugs in the brain. Shimizu *et al*. reported the internal and intracerebral distribution of vitamin B_1 in rats following peroral administration of dicethiamine hydrochloride or thiamine hydrochloride [1]. Hsieh *et al*. recorded the blood and intracerebral distribution of doxorubicin in rats following intravenous administration of a liposome doxorubicin formulation with cyclosporine A [2]. Takahara *et al*. demonstrated the intracerebral distribution of inaperisone in rats following peroral administration of inaperisone hydrochloride [3]. These are all medicinal drugs and are not volatile. Although humans are frequently exposed to volatile compounds, almost no studies about their distribution, such as those in fragrances, in the various cerebral regions have been reported. In our current study, the distribution in the brain of the volatile compound of the essential oil from *Chamaecyparis obtusa* (EOCO), which is common in Japan, was clarified. We also explored the relationship between EOCO and emotional behavior.

There is an anxiolytic-like activity for inhaled administration of EOCO in mouse [4-6]. When components of EOCO were analyzed in the volatile condition, the most abundant compound was α-pinene. This is thought to be reflected by detection of the amount of this component in the mouse brain. Quantitative analysis of α-pinene in the 12 regions of the brain of the mice was performed after inhaled administration of 8 μL/L air EOCO (Figure 1). The highest concentration of α-pinene was seen in the striatum (6.7 μg/g tissue), and the lowest in the hypothalamus (3.0 μg/g tissue). The differences between the 12 regions of the brain were not statistically significant. Next, quantitative analysis of α-pinene in the 12 regions of the brain of the mice was performed after inhaled administration of 32 μL/L air EOCO (Figure 1). The highest concentration of α-pinene was seen in the medulla (18.7 μg/g tissue), and the lowest in the olfactory bulb (8.6 μg/g tissue). The differences between the 12 regions of the brain were also not statistically significant.

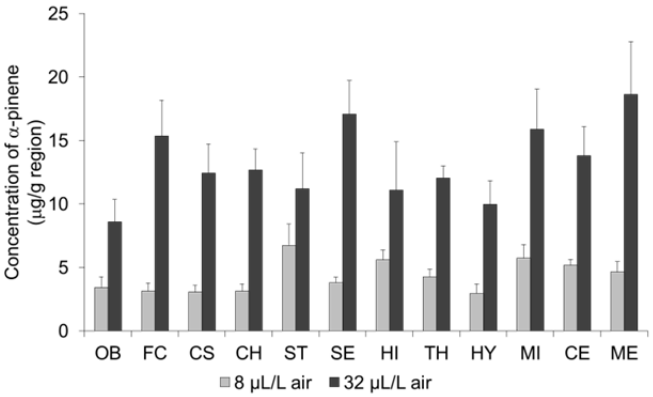

Figure 1: Concentration (μg/g tissue) of α-pinene in 12 regions of the brain of mice after inhaled administration. Mice inhaled EOCO (8 μL/L air or 32 μL/L air) for 90 min. OB: olfactory bulb, FC: frontal cortex, CS: cortex (adjacent to the striatum), CH: cortex (adjacent to the hippocampus), ST: striatum, SE: septum, HI: hippocampus, TH: thalamus, HY: hypothalamus, MI: midbrain, CE: cerebellum, and ME: medulla. Values represent the mean ± SE. $n = 5$.

Shimizu *et al*. reported the internal and intracerebral distribution of vitamin B1 in rats following peroral administration of either dicethiamine hydrochloride (100 mg/kg p.o.) or thiamine hydrochloride (70.1 mg/kg p.o.) [1]. They reported that the concentrations of free vitamin B1 in the cortex, hippocampus, striatum, thalamus, midbrain, and medulla were 0.44-2.32 nmol/g tissue (dicethiamine hydrochloride) and 0.10–0.86 nmol/g tissue (thiamine hydrochloride). The highest concentration was seen in the midbrain. Hsieh *et al*. reported the blood and intracerebral

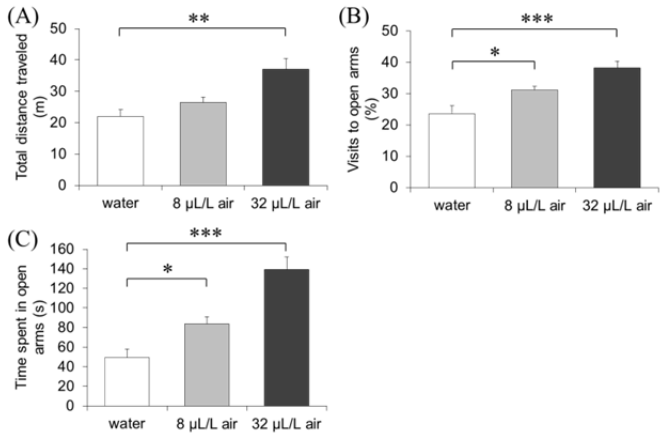

Figure 2: Total distance traveled (m) in the elevated plus maze (EPM) after 90 min of inhalation (A). Visits to open arms (%) in the EPM after 90 min of inhalation (B). Time spent in open arms (s) in the EPM after 90 min of inhalation (C). Mice inhaled either water vapor (10 μL/L air) or EOCO (8 μL/L air or 32 μL/L air). Values represent the mean ± SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Dunnett's test. $n = 7$.

distribution of doxorubicin in rats following intravenous administration of a liposome doxorubicin formulation (5 mg/kg i.v.) with cyclosporine A (10 mg/kg i.v.) [2]. The concentrations of doxorubicin in the cortex, cerebellum, hippocampus, brainstem, and striatum were 8–220 ng/g tissue (liposome doxorubicin formulation) and 30-280 ng/g tissue (liposome doxorubicin formulation with cyclosporine A). The highest concentration was seen in the cerebellum. Takahara *et al*. studied the intracerebral distribution of inaperisone in rats following peroral administration of inaperisone hydrochloride (50 mg/kg p.o.) [3]. They reported that the concentrations of inaperisone in 38 regions of the brain were 36.5-57.5 μg/g tissue at 10 min after and 20.0–31.1 μg/g tissue at 30 min after administration. These concentrations were almost the same.

Our experiment was not made following either peroral or intravenous administration, but rather inhaled administration. The pharmacological route following inhaled administration involves a route via blood from the lung and a direct route from the nasal mucosa to the brain. Inhaled administration of 8 and 32 μL/L air EOCO resulted in similar intracerebral distribution as that of inaperisone. Thus, the characteristics of the compound and the administration route influenced the distribution of the compound in the brain.

Next, we studied the relationship between the cerebral concentration and emotional behavior (Figure 2). Significant increases in the number of visits to open arms and the time spent in open arms were observed following inhaled administration of either 8 or 32 μL/L air EOCO. On the other hand, a significant increase in locomotor activity was only observed following inhaled administration of 32 μL/L air EOCO. We also observed an increase in the concentration of α-pinene in most parts of the brain following inhaled administration of 32 μL/L air EOCO. This may have led to the increase in the locomotor activity. The anxiolytic-like effect without an increase in locomotor activity following inhaled administration of 8 μL/L EOCO may have been caused by a low, constant concentration of α-pinene in certain parts of the brain.

The data in Table 1 show the increase in α-pinene from 8 to 32 μL/L air EOCO in each brain region. The increase in the striatum was significantly lower than that in the frontal cortex, cortex (adjacent to the striatum), septum, cortex (adjacent to the hippocampus), hypothalamus, and medulla. The increase in the hippocampus was significantly lower than that in the frontal cortex, cortex (adjacent to the striatum), septum, cortex (adjacent to the hippocampus), hypothalamus, and medulla, similar to what was seen for the striatum. These results indicated that increased locomotor activity due to a high concentration of α-pinene involved brain regions besides the striatum and the hippocampus. A role for the striatum and hippocampus in the anxiolytic-like effect by EOCO has been suggested. This result is consistent with the mainly serotoninergic projections of the striatum and hippocampus from the dorsal raphe nucleus [7].

Table 1: Change in α-pinene in each region of mouse brain between 8 μL/L air and 32 μL/L air EOCO. The same letter indicates a significant difference ($p < 0.05$). OB: olfactory bulb, FC: frontal cortex, CS: cortex (adjacent to the striatum), CH: cortex (adjacent to the hippocampus), ST: striatum, SE: septum, HI: hippocampus, TH: thalamus, HY: hypothalamus, MI: midbrain, CE: cerebellum, and ME: medulla. Values represent the mean ± SE. $n = 5$.

	Change in the concentration of α-pinene from 8 μL/L air to 32 μL/L air EOCO	Significant difference
OB	2.6 ± 0.2	a,b
FC	5.1 ± 0.7	a,c,d,h,i,j
CS	4.2 ± 0.4	k,l
CH	4.2 ± 0.3	m,n
ST	1.7 ± 0.1	f,h,k,m,o,p
SE	4.4 ± 0.3	b,e,o,q
HI	1.8 ± 0.4	g,i,l,n,q,r
TH	2.9 ± 0.2	j
HY	3.9 ± 0.6	p,r
MI	2.8 ± 0.1	c
CE	2.6 ± 0.2	d,e
ME	3.9 ± 0.4	f,g

This result suggests a relationship between the unequal distribution of α-pinene in the various brain regions and emotional behavior.

Experimental

Gas chromatography (GC) analysis: A Clarus 500 (Perkin Elmer Inc., Waltham, MA, USA) was used for GC–mass spectrometry (GC-MS) analysis, and a GC-2010 Plus (Shimadzu Corporation, Kyoto, Japan) for GC-flame ionization detection (FID) analysis. The details of the GC analysis have been described elsewhere [8]. Briefly, EOCO was diluted with *n*-hexane and injected into the GC-MS, which was operated under the electron impact ionization mode. A DB-5ms capillary column (30 m × 0.25 mm ID, 0.25 μm, non-polar column; Agilent Technologies Inc., Tokyo, Japan) was used. GC conditions were as follows: carrier gas, helium (99.99995%, 1.82 mL/min); splitless; inlet line temperature, 230°C; source temperature, 230°C; column temperature, 40°C for 5 min, 40°C to 280°C at 5°C/min, and then 280°C for 20 min; mass spectra, electron impact at 70 eV. Individual components were identified by comparison with authentic standards and/or the GC-MS NIST library (version 1.7) and linear retention indices [9]. Briefly, EOCO was diluted with *n*-hexane to produce a standard curve. One μL of individual standard solutions and samples was injected into the GC device. Hydrogen gas (H2, 99.9999%) was used for the GC-FID. A DB-5ms non-polar capillary column (30 m × 0.25 mm ID, 0.25 μm) was used for GC analysis. The conditions were as follows: carrier gas, helium (1.82 mL/min); splitless; inlet line temperature, 230°C; source temperature, 230°C; column temperature, 40°C for 5 min, 40°C to 280°C at 5°C/min, and then 280°C for 20 min.

Essential oil from* C. obtusa *(EOCO): In October 2010, Hinoki Seiko Co., Ltd. (Nagano, Japan) collected branches from trees of *C. obtusa* (Siebold et Zucc.) Endl., family Cupressaceae, growing in Nagano, Japan. After cutting the branches into small pieces, the

essential oil was extracted by steam distillation. The main components of EOCO have been reported elsewhere [4] and include δ-cadinene (152.9 g/L, 17.5%), α-pinene (134.0 g/L, 15.3%), γ-cadinene (90.8 g/L, 10.4%), α-muurolene (59.8 g/L, 6.8%), τ-muurolene (52.6 g/L, 6.0%), α-cadinol (40.5 g/L, 4.6%), and γ-muurolene (36.1 g/L, 4.1%). Volatile components of EOCO, which were inhaled by mice from a 5 L glass container, were analyzed. α-Pinene (24.9 μg/L, 58.6%), limonene (2.4 μg/L, 5.7%), myrcene (2.3 μg/L, 5.3%), terpinolene (1.5 μg/L, 3.5%), and β-pinene (1.5 μg/L, 3.4%) were detected as the main components of volatile EOCO.

Animals: Male ICR mice (Clea Japan, Tokyo, Japan), which were 5 weeks of age at the start of each experiment, were individually housed in cages for 1 week. The cages were placed in a room that was artificially illuminated by fluorescent lamps on a 12 h light/dark schedule (light period: 08:00–20:00) and maintained at 24 ± 2°C. The mice were given free access to food (Clea Rodent Diet CD-7, Clea Japan) and water. The animals had not previously been exposed to essential oil, and each animal was used only once during the experiment. A total of 31 mice were used. It was thought that the mouse used by the experiment is in a few stress states more than a usual mouse for independent housing, inhaled administration and the elevated plus maze (EPM) test. It was thought that the evaluation of anxiolytic-like behavior becomes clear by using the state of this mouse. All experiments were conducted in accordance with the guidelines regarding the care of experimental animals as approved by the Animal Research Committee at Toho University.

Drug administration: Mice inhaled either EOCO or water vapor as a negative control for 90 min, according to the following protocol. An individual mouse was placed in a glass container (5 L, L100 × W250 × H200 mm). A piece of filter paper (GE Healthcare Japan, Tokyo, Japan) soaked in either EOCO (8 or 32 μL/L air) or water (8 μL/L air) was placed on the upper side of the glass container, and the mouse was exposed to the sample at room temperature (24 ± 2°C) for 90 min. EPM test and quantitative analysis of α-pinene in the brain were begun immediately at the end of the 90 min of inhalation. We assumed that the entire sample was volatilized for the calculation of sample concentrations.

Dissection of the brain for quantitative analysis of EOCO components: Main components of EOCO are δ-cadinene and α-pinene, but only α-pinene was detected by inhaled administration of EOCO in the brain. Therefore, α-pinene was made a measurement index. Immediately after inhalation, the mice were decapitated (n = 5 per group) after anesthesia with 0.3 mg/kg (i.p.) medetomidine hydrochloride (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), 4.0 mg/kg (i.p.) midazolam (Astellas Pharma Inc., Tokyo, Japan), and 5.0 mg/kg (i.p.) butorphanol tartrate (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and whole brains were removed. The brain was dissected immediately into 12 regions: olfactory bulb, frontal cortex, striatum, cortex (adjacent to the striatum), septum, hippocampus, cortex (adjacent to the hippocampus), thalamus, hypothalamus, midbrain, cerebellum, and medulla. Collected samples were immediately flash frozen in liquid nitrogen and stored at –80°C until use in subsequent analyses. These regions of the brain were homogenized in 1 mL *n*-hexane with an ultrasonic disruptor (UD-201, Tomy Seiko Co., Ltd., Tokyo, Japan).

EPM test: The EPM [10] test consists of two open arms (200 × 50 mm, length × width) and two closed arms (200 × 50 mm, length × width) with clear walls that cross each other at right angles. The maze was located 600 mm above the floor, and the apparatus was illuminated with a 200-lx light at floor level. Mice (n = 7 per group) were randomly assigned to the above-mentioned experimental groups (including a negative control). Each mouse was placed at the crossing point of the maze, and its behavior was subsequently recorded for 10 min using an attached camera (CMS-V26SETSV, Sanwa Supply Inc., Okayama, Japan). Data were analyzed using ANY-maze software (Stoelting Co., Wood Dale, IL, USA). The details of this test have been described elsewhere [11,12]. In the EPM test, total distance traveled (m), visits to open arms (% of total number of times), and time in open arms (s) were measured. Total distance traveled reflects the locomotor activity. Time spent in open arms and visits to open arms reflect the anxiolytic-like effect of the treatment, because when anxious, mice tend to stay in closed arms. The apparatus was cleaned thoroughly between trials.

Statistical analysis: Results are expressed as the means ± standard error (SE) (n = 5 or 7). All results were initially analyzed using one-way ANOVA, and then analyzed using Tukey's test using the statistical package R (http://www.r-project.org/). Differences with $p < 0.05$ were considered significant. The effect sizes for the model, as well as the power, were calculated using the program G*Power 3 [13,14].

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