Regular Article

Anxiolytic Effect of Cedrol on Behavior and Brain Neurotransmitter Levels of Female Mice

Kai Zhang and Lei Yao*

School of Agriculture and Biology, Shanghai Jiao Tong University; 800 Dongchuan Rd. Minhang District, Shanghai 200240, China.

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Cedrol has been reported to be effective in reducing anxiety of male mice. The limited application of females in animal models of anxiety makes it difficult to systematically investigate new drug substitutes with potential anxiolytic activity. In the present study, we investigated the behavioral response of female ICR mice to cedrol after intraperitoneal (i.p.) administration using the elevated plus maze (EPM) and the light–dark box (LDB) test, followed by determination of neurochemical changes in brain. The data suggested that cedrol at dose of 1200–1600 mg·kg^{−1} exhibited anxiolytic activity on the female mice, as reflected by greater percentage of entries into the open arms and time spent in the open arms in the EPM, and greater transitions between chambers and percentage of time spent in the light chamber in the LDB. Cedrol increased the level of 5-hydroxytryptamine (5-HT), decreased the level of dopamine (DA), reduced the ratio of 5-hydroxyindoleacetic acid (5-HIAA)/5-HT and increased the ratio of 3,4-dihydroxyphenyl acetic acid (DOPAC)/DA, compared with the control group, indicative of an anxiolytic-like effect on female mice *via* the 5-HTnergic or DANergic pathways.

Key words anxiolytic effect; cedrol; 5-hydroxytryptamine; elevated plus maze; light–dark box

INTRODUCTION

Essential oils (EOs) are traditionally used in aromatherapy, an important complementary therapy, to treat mental disorders such as epilepsy, insomnia or anxiety.^{1–3)} Compared with some psychotropic medicines extensively in use, EOs have attracted more attention owing to their fewer adverse effects.^{4,5)} Lots of references have indicated that the anxiolytic effect of EOs could be attributed to their main active components, such as citronellol and 2-phenethyl alcohol in rose oil,^{6,7)} linalool in lavender oil,^{8,9)} and limonene in orange oil,^{5,10)} nevertheless, specific mechanisms of EOs seem to be complicated due to the characteristic components in EOs, which may exhibit synergistic or antagonistic effects.

EOs or their main active components not only modify the contents of neurotransmitters, but also modulate their uptake or release. *Hypericum perforatum* L. (St. John's wort) oil was reported to increase 5-hydroxytryptamine (5-HT) concentration of basal nuclei.¹¹⁾ Lemon oil was proved to reduce the dopamine (DA) level, which is associated with the serotonergic (5-HTnergic) neurons activity.¹²⁾ Linalool, the major component of lavender oil, was demonstrated to have an inhibitory effect on the glutamatergic transmission in the rat cerebral cortex.¹³⁾

Cedrol, primarily derived from eastern redcedar (*Juniperus virginiana* L.) essential oil (ERO), has a light woody aroma. Several references have highlighted its impact on the behavioral and autonomic activities of mammals, by suppressing sympathetic outflow and increasing parasympathetic outflow.^{14–16)} In human experiments, cedrol inhalation increased the parasympathetic activity and decreased the sympathetic activity.¹⁴⁾ In animal experiments, exposure to cedrol appeared to induce a sedative effect regardless of the animal species.^{17,18)}

Therefore, we surmised that cedrol might have relaxant and

anxiolytic properties. This hypothesis was supported by our previous study, in which we demonstrated, in animal models of anxiety, that intraperitoneal (i.p.) administration of ERO and cedrol exhibited an anxiolytic effect on male ICR mice, *via* the 5-HTnergic and dopaminergic (DANergic) pathways.¹⁹⁾ However, the anxiolytic effect of cedrol on female mice is still unknown.

In preclinical experiments or commonly-used behavioral tests, especially in the field of brain and behavior, female animals have often been omitted deliberately due to their own estrous cycle.^{20–22)} This limited application of females in behavioral experiments, the study on the anxiolytic activity of EOs, for instance, thus results in the presence of sexually dimorphic patterns of prevalence and/or severity,²²⁾ as well as the lack of systematic studies on the underlying mechanism of anxiety behaviors.^{20,23,24)}

The main objective of the present study was to evaluate the anxiolytic effect of cedrol on female ICR mice *via* the elevated plus maze (EPM) and light–dark box (LDB) paradigm. Moreover, we evaluated neurochemical changes, which are associated with the anxiolytic effect, by measuring the contents of 5-HT, DA and their metabolites in brains.

MATERIALS AND METHODS

Experimental Animals Female ICR mice, aged 5–6 weeks, obtained from Laboratory Animal Center of Shanghai Jiao Tong University were used. Prior to experiments, mice were maintained for one week (5 mice per cage), under controlled temperature (25 ± 1°C), light (12 h/12 h light/dark cycle) and humidity (with 50 ± 5%) conditions, with food and water *ad libitum*. Females were randomly selected to submit to behavioral tests. All the behavioral tests were carried out between 11:00 a.m. and 4:00 p.m. to minimize the influence of

* To whom correspondence should be addressed. e-mail: yaolei@sjtu.edu.cn

dynamic change in hormone.

All the experimental procedures were manipulated in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International and approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University.

Drugs and Treatments Cedrol was obtained from Jiangxi Global Natural Spices Co., Ltd. (Jiangxi, China). Diazepam (DZP) was purchased from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. (China). DZP or cedrol were dissolved in olive oil (OO), which did not alter the anxiolytic-like parameters (data not published). Thirty minutes prior to the test sessions, olive oil (control), DZP ($2\text{ mg}\cdot\text{kg}^{-1}$, positive control) and cedrol (200, 400, 800, 1200 and $1600\text{ mg}\cdot\text{kg}^{-1}$) were i.p. administrated at a dosage of $0.1\text{ mL}/10\text{ g}$ body weight, with which we could avoid animal's preferences and sensitivity to odorous components, according to the previous reports.^{25–27} The drug doses were selected based on pilot data in our laboratory. All efforts were made to minimize the number of animals and their suffering.

EPM The EPM, utilized extensively to examine the anti-anxiety effect of both traditional and non-traditional anxiolytic agents,²⁸ is based on rodent's conflict between the innate fear of open areas and the drive to explore the novel places.²⁹ The maze was designed as described with several structural modifications.³⁰ The apparatus consisted of two open arms ($30\times 6\text{ cm}$, length $\times$ width) and two closed arms ($30\times 6\times 15\text{ cm}$, length $\times$ width $\times$ height) connected by an open central platform ($6\times 6\text{ cm}$, length $\times$ width). The maze was elevated 55 cm above the floor and placed inside a sound-attenuated room. 30 min after the administration, mouse was individually placed on the platform. Behavior of each animal was recorded for 5 min *via* a video camera above the EPM.

The anxiety parameters were determined as described in the previous study.⁵ The number of entries and the time spent in open or closed arms were evaluated. The percentage of entries into the open arms (OAE%) and the percentage of time spent in the open arms (OAT%) were calculated as anxiety parameters. Subsequently, the distance travelled in the model was recorded as a measure of locomotor activity. An entry was defined when the animal stepped into an arm with its four paws at a given time.³¹

LDB The LDB, used to test unconditioned anxiety and exploratory behavior, is based on the innate aversion of mice to bright light in novel areas.³² The box was designed as described with several structural modifications.³³ The apparatus consisted of a plywood box divided by a connecting door ($5\times 5\text{ cm}$, length $\times$ height) into a light chamber

($27\times 18\times 18\text{ cm}$, length $\times$ width $\times$ height) and a dark chamber ($18\times 18\times 18\text{ cm}$, length $\times$ width $\times$ height). The light chamber was entirely painted white and illuminated with 400 lx . After the EPM test, the animal was immediately placed in the center of the light chamber. Behavior was monitored for 5 min *via* the micro-camera in the top of chamber cover.

The anxiety parameters were defined according to the previous study.⁵ The time spent in each chamber and the number of transitions between chambers was registered, respectively. The percentage of time spent in the light chamber (LCT%) was calculated. The distance travelled in the model was also recorded.

Evaluation of Monoamines and Their Metabolites in Brain After the LDB test, mice were immediately euthanized to extract the whole brain. Tissues were homogenized in a solution containing 0.2 M perchloric acid, 0.2 mM sodium pyrosulfite and 0.01% ethylenediamine tetraacetic acid disodium salt ($\text{EDTA}\cdot\text{Na}_2$) together with $0.3\text{ }\mu\text{M}$ 3,4-dihydroxybenzylamine hydrobromide (DHBA) as an internal standard, and then centrifuged at $10000\times g$ for 20 min at 4°C . The supernatants were collected and stored at -80°C .

The procedure of determination was carried out as described in the literatures with several modifications.^{19,34–36} DA, 5-HT, norepinephrine (NE), 3,4-dihydroxyphenyl acetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) were determined by HPLC with an electrochemical detector (LC-4C, BAS, U.S.A.). Separation was performed on a TMRP18 column ($4.6\times 250\text{ mm}$, $5.0\text{ }\mu\text{m}$, GL Science, Japan). The mobile phase (methanol-water, 16:84, v/v), mixed with sodium 1-octanesulfonate, sodium acetate and citric acid, was delivered at rate of $1\text{ mL}\cdot\text{min}^{-1}$. The concentrations of neurotransmitters were calculated by the calibration curve of external standards.

Statistical Analysis Animals' behaviors in the EPM and LDB tests were recorded and analyzed using the SuperMaze video tracking system (XR-Xmaze, Xinruan information technology Co., Ltd., China). Overall differences in behavioral procedures and contents of neurotransmitters were examined by one-way ANOVA followed by Tukey's *post hoc* test to evaluate the anxiolytic effect of cedrol. $p<0.05$ was established as the significant level.

RESULTS

Effect of Cedrol on Female Mice Behavior in the EPM

Test The ANOVA for OAE% (Fig. 1A) revealed significant differences among the treatment groups ($F(6, 49)=10.567$, $p<0.001$). Female mice treated with DZP ($p<0.001$), and

Fig. 1. The Anxiolytic Effect of Cedrol on Female Mice in the EPM Test

Effect of cedrol (200, 400, 800, 1200 and $1600\text{ mg}\cdot\text{kg}^{-1}$, i.p.) on the percentage of entries into the open arms (A), the percentage of time spent in the open arms (B), distance travelled (C). DZP: diazepam, $2\text{ mg}\cdot\text{kg}^{-1}$ (i.p.); OO: olive oil (i.p.). Results are expressed as mean $\pm$ standard error of the mean (S.E.M.) ($n=8$ per group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. the olive oil group.

Fig. 2. The Anxiolytic Effect of Cedrol on the Female Mice in the LDB Test

Effect of cedrol (200, 400, 800, 1200 and 1600 mg·kg⁻¹, i.p.) on the transitions between chambers (A), the percentage of time spent in the light chamber (B), distance travelled (C). DZP: diazepam, 2 mg·kg⁻¹ (i.p.); OO: olive oil (i.p.). Results are expressed as mean ± S.E.M. (*n* = 8 per group). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. the olive oil group.

Fig. 3. Changes of Monoamines and Their Metabolites in the Brains of Female Mice

Concentrations of NE (A), 5-HIAA (B), 5-HT (C), the ratio of 5-HIAA/5-HT (D), HVA (E), DOPAC (F), DA (G) and the ratio of DOPAC/DA (H). DZP: diazepam, 2 mg·kg⁻¹ (i.p.); OO: olive oil (i.p.). Results are expressed as mean ± S.E.M. (*n* = 8 per group). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. the olive oil group.

cedrol at dose of 1200 mg·kg⁻¹ (*p* < 0.01) and 1600 mg·kg⁻¹ (*p* < 0.001) entered more into the open arms when compared with the OO group. Same analysis for the parameter OAT% showed differences among groups ($F(6, 49) = 10.992$, *p* < 0.001), namely DZP (*p* < 0.001), 1200 mg·kg⁻¹ (*p* < 0.05) and 1600 mg·kg⁻¹ (*p* < 0.01) groups spent more time in the open arms than OO group (Fig. 1B). DZP significantly increased the total exploration distance in the EPM (*p* < 0.01), whereas no significant differences were observed among cedrol treatments (Fig. 1C). It was assumed that cedrol (1200 and 1600 mg·kg⁻¹) exhibited an obvious anxiolytic effect in the EPM test, with exclusion of the influence on locomotor activity.

Effect of Cedrol on Female Mice Behavior in the LDB Test The ANOVA for the transitions between chambers (Fig. 2A) presented significant differences among the treatment groups ($F(6, 49) = 6.452$, *p* < 0.001). Female mice treated with DZP (*p* < 0.05) and cedrol at dose of 1200 mg·kg⁻¹ (*p* < 0.001) performed more transitions than OO group. The same analysis for the parameter LCT% (Fig. 2B) also showed differences among groups ($F(6, 49) = 3.665$, *p* < 0.01), namely DZP (*p* < 0.01), 1200 mg·kg⁻¹ (*p* < 0.05) and 1600 mg·kg⁻¹ (*p* < 0.01) groups spent more time in the light chamber than the OO group. Figure 2C revealed that DZP markedly increased the total exploration distance in the LDB (*p* < 0.05),

in addition, cedrol at dose of 1200 and 1600 mg·kg⁻¹ produced a similar profile with DZP (*p* < 0.05, *p* < 0.05, respectively). These findings showed that cedrol (1200 and 1600 mg·kg⁻¹) exhibited a significant anxiolytic effect in the LDB test, with an increase on locomotor activity.

Changes of Monoamines and Their Metabolites in Brain

Results from the evaluation of monoamine neurotransmitters in the brains of female mice were shown in Fig. 3. Cedrol at dose of 1200 and 1600 mg·kg⁻¹ (Figs. 3B–E) significantly reduced the level of 5-HIAA ($F(3, 28) = 7.727$, *p* < 0.01), the ratio of 5-HIAA/5-HT ($F(3, 28) = 6.829$, *p* < 0.001), the level of HVA ($F(3, 28) = 7.879$, *p* < 0.01) and elevated the level of 5-HT ($F(3, 28) = 5.703$, *p* < 0.01), when compared with the control group. Meanwhile, cedrol at dose of 1600 mg·kg⁻¹ significantly decreased the level of DA ($F(3, 28) = 1.835$, *p* < 0.05), and increased the ratio of DOPAC/DA ($F(3, 28) = 6.186$, *p* < 0.01), when compared with the control group (Figs. 3G, H). No significant changes were seen in the levels of NE ($F(3, 28) = 0.827$, *p* > 0.05) and DOPAC ($F(3, 28) = 1.216$, *p* > 0.05) (Figs. 3A, F).

DISCUSSION

The present work has clearly shown the anxiolytic effect of cedrol on female mice through the EPM and LDB tests.

Though no significant anxiolytic effects were observed at 200–800 mg·kg⁻¹ on the ethological measures, cedrol at dose of 1200–1600 mg·kg⁻¹ significantly increased the parameters of anxiolytic-like activity evaluated in both EPM and LDB tests. According to the hypothesis that the biological properties of EOs could be attributed to their main active constituents,³⁴⁾ the results above further strengthened the fact that *Juniperus virginiana* L. essential oil or cedrol possesses anxiolytic effect.

Many animal anxiety models are based on behaviors that depend on body activity and locomotor activity in apparatus, which partially illustrates that it is difficult to dissociate a pure measure of emotionality from non-emotional confounding factors (e.g., motor activity-distance travelled).³⁷⁾ A number of studies have employed distance travelled in models to assess locomotor activity.^{5,35,38)}

We employed the distance travelled in EPM and LDB as a covariate for accurate and reliable statistical analysis of anxiolytic parameters. Cedrol failed to increase the distance travelled in the EPM, whereas 1200 and 1600 mg·kg⁻¹ of cedrol significantly increased the distance parameter in LDB test ($p < 0.05$), displaying a slightly greater locomotor activity. The discrepancy might be caused by the difference in the application of animal models of anxiety.

The correlation between neurotransmitters and anxiety has been well documented in many references, in which monoamines and their metabolites in the central nervous system are associated with the modulation of anxiety disorder.^{34,39,40)} Decreased DA level⁴¹⁾ and increased γ -aminobutyric acid (GABA) level^{42,43)} or 5-HT level³⁴⁾ indicate an anxiolytic-like effect. Strikingly, changes in monoamines and their metabolites in the present study tended to follow the same trend in the previous research.

As expected, cedrol functioned through the 5-HTnergic system, since it enhanced the level of 5-HT, accompanied with the decreased 5-HIAA level. Likewise, the lower ratio of 5-HIAA/5-HT suggested that cedrol could accelerate the synthesis of 5-HT in female mice. However, we cannot discard the possibility that cedrol also acted *via* DAnergic system. The decreased DA level and increased DOPAC/DA ratio were observed at 1600 mg·kg⁻¹ group. It has been reported that 5-HT and DA fibers normally compete for target sites in striatum, and DA might suppress the 5-HT axon growth.^{41,44,45)} We can infer that cedrol might affect the DAergic neuron by activating 5-HTergic neuron. A previous study proposed a similar mechanism, in which lemon oil vapor possibly affected the response to DAnergic activity by modulating the 5-HTnergic system.¹²⁾

Female mice intrinsically were less responsive to cedrol, when compared with male ICR mice in our previous study, in which cedrol administration (i.p.) produced significant anxiolytic effect in both EPM and LDB tests (400–1600 and 800–1600 mg·kg⁻¹, respectively), as well as increased the distance travelled in apparatuses at 200–1600 mg·kg⁻¹.¹⁹⁾

Though the basic neurobiology of anxiety may be similar between males and females, sex differences in sensitivity to certain anxiolytic drugs or substitutes have been acknowledged in many preclinical experiments.^{46,47)} For example, DZP (1 mg·kg⁻¹) was more effective in males than females,⁴⁸⁾ suggesting that the therapeutic effects of benzodiazepines are closely related with sex and hormone cycle stage.⁴⁹⁾ Zhang

*et al.*³⁵⁾ demonstrated that ylang-ylang (*Cananga odorata* (LAM.) HOOK. f. & THOMSON) essential oil showed significant anxiolytic effect on the male ICR mice. Sex difference was also observed in the EPM test in gerbils after rose essential oil exposure.⁷⁾

The gonadal hormone contributed to the gender-specific anxiety behaviors. For example, testosterone exhibited anxiolytic effect through androgen receptor,⁵⁰⁾ and estrogen decreased the anxiety level in females.⁵¹⁾ Previous researches also proved that estradiol eliminated difference in OAT% between proestrous and diestrous rats.⁵²⁾ In addition, estradiol administration elicited anxiolytic and antidepressant-like effects on ovariectomized rats,⁵¹⁾ or enhanced the oxytocin-induced anxiolytic effect in mice.⁵³⁾

The effect of estrogen action on anxiety might associated with the estrous cycle stage. Females seemed to be more anxious during the metestrous and diestrous phases, compared with proestrous and estrous phases.^{52,54)} Similarly, the activation effect of estrogen augments the hypothalamic–pituitary–adrenal (HPA) axis response to increase the level of estrogen.⁵⁵⁾ Aoki *et al.*⁵⁶⁾ hypothesized that ovary-derived factors (but not 17 β -estradiol) controlled the HPA axis response in female ICR mice.

Some investigations also indicated that estrogen, but not testicular hormones, significantly modulated DA level of females over the estrous cycle, instead of castrated males.^{41,57–59)} Shams *et al.*⁶⁰⁾ and Becker⁶¹⁾ proposed that 17 β -estradiol increased DA transmission in the dorsal striatum of ovariectomized rats. In addition, when 5-HT_{1A} receptor antagonists were co-administered with estradiol, the behavioral effects of estradiol treatment were attenuated.⁶²⁾ These results provide some possible mechanism basis for our further hypothesis.

In view of this, we proposed the possible mechanism of anxiolytic effect of cedrol on female mice: cedrol was absorbed into blood, then carried to the brain area, acted on the HPA axis, with which cedrol facilitated the release of estrogen, that induced an increased 5-HT level or decreased DA level in brain. Synergism or antagonism between hormone and cedrol administration on mice should be considered in the next experiment.

CONCLUSION

All these things considered, cedrol possesses a significant anxiolytic effect on female mice in the EPM and LDB tests, without significant suppression on locomotor activity. The anxiolytic effect might be caused by accelerating the synthesis of 5-HT and suppressing the synthesis of DA.

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Conflict of Interest The authors declare no conflict of interest.

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