

A study of the substance dependence effect of the ethanolic extract and iridoid-rich fraction from *Valeriana jatamansi* Jones in mice

Lin Yu, Xu Ke-ke, Chen Chao-yong, Zhang Rui-tong, Lan Ming, Li Shao-hua, Pan Ling-zhen, Zhang Tian-e¹, Yan Zhi-yong

School of Life Science and Engineering, Southwest Jiaotong University, Chengdu 610031, ¹School of Basic Medicine, Chengdu University of Traditional Chinese Medicine, Chengdu 611137, PR China

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ABSTRACT

Background: Recently we found the ethanolic extract and iridoid-rich fraction from *Valeriana jatamansi* Jones, which is a traditional Chinese medicine exhibited anxiolytic properties. **Objective:** This study aims to the substance dependence effect of the ethanolic extract and iridoid-rich fraction. **Materials and Methods:** The study included two experiments: Mice were given orally with ethanolic extract for 12 weeks or iridoid-rich fraction for 16 weeks in experiment I and experiment II, respectively. Diazepam was used as a control drug and the normal mice groups were administered with 0.5% carboxymethyl cellulose Na in both experiments. All groups were administered twice daily. Natural withdrawal symptoms, withdrawal-induced body weight change, audiogenic tail-erection test (in experiment I), and pentylenetetrazol (PTZ)-induced convulsion test (in experiment II) were measured. **Results:** (1) Compared to normal group in both experiments, the diazepam-treated group exhibited obvious withdrawal symptoms of tail-erection, irritability, teeth chattering, etc; the body weight of them after withdrawal had a period of significant loss ($P < 0.05$ or $P < 0.01$); and the ratios of tail-erection and seizure in two experiments were improved significantly when mice were induced by mixer noise ringtone (experiment I) or PTZ (experiment II) ($P < 0.05$ or $P < 0.01$). (2) In experiment I and II, there were no significant differences between mice that received ethanolic extract or iridoid-rich fraction and normal group in terms of natural withdrawal symptoms and withdrawal-induced body weight change ($P > 0.05$); in audiogenic tail-erection test, it found that the significant difference compared with normal group was just in ethanolic extract 900 mg/kg dose group on week 8 ($P < 0.05$); in PTZ-induced convulsion test, mice in iridoid-rich fraction groups had a slightly tail-erection and seizure, all results of them were with no significant difference compare to normal mice ($P > 0.05$), while significant lower than diazepam group ($P < 0.01$). **Conclusion:** (1) The two experiments successfully established the physical dependence of diazepam by gradually increasing the dose. (2) There were just a few mice received with ethanolic extract for 12 weeks or iridoid-rich fraction for 16 weeks appearing some slight withdrawal symptoms after precipitated withdrawal, but it didn't show significant difference compared to normal mice. Therefore, these indicated that the risks of potential drug dependence about ethanolic extract and iridoid-rich fraction were far lower than that of diazepam.

Key words: Audiogenic tail-erection, dependence, extract, iridoids, pentylenetetrazol-induced convulsion, *Valeriana jatamansi* Jones

Address for correspondence:

Prof. Zhang Tian-e, School of Basic Medicine, Chengdu University of Traditional Chinese Medicine, No. 1166 Liutai Avenue, Wenjiang District, Chengdu 611-137, PR China.
E-mail: 1153615912@qq.com
Prof. Yan Zhi-yong, School of Life Science and Engineering, Southwest Jiaotong University, No. 111, North Section 1, Second Ring Road, Chengdu 610-031, PR China.
E-mail: yzhiy@swjtu.edu.cn

INTRODUCTION

According to the first section of the 2010's edition of "pharmacopoeia of the people's Republic of China",^[1] the dry roots and rhizomes of *Valeriana jatamansi* Jones (*Valerianaceae*) as a traditional Chinese medicine have the efficacies of regulating qi, acting as analgesic, promoting digestion, and alleviating diarrhea, eliminating

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wind and dampness, as well as tranquilizing the mind. In recent years, *V. jatamansi* has been reported to have a lot of pharmacological activities, such as anti-depressants,^[2,3] anticonvulsants,^[4,5] sedative and analgesic,^[4,6,7] and neuroprotective,^[8,9] etc., Recently, we found a new application of *V. jatamansi* as an anxiety therapy based on its tranquilizing effect.^[10]

Our previous studies found that ethanolic extract^[11-13] and iridoid-rich fraction^[14] showed obvious anxiolytic effects on the anxiety models of the elevated plus maze, light-dark box; Vogel conflict drinking, and open box drinking. Besides, the studies proved the anxiolytic mechanism of *V. jatamansi* might be related to regulate the level of brain neurotransmitter such as 5-hydroxytryptamine (5-HT), noradrenaline, dopamine,^[12] γ -aminobutyric acid,^[14] affect excited/inhibited equilibrium by regulating glycine, catecholamine, regulate hypothalamic-pituitary-adrenal axis by β -endorphins, and corticosterone,^[11,13] and the content of 5-HT in blood.^[14]

Drug dependence as an adverse reaction to some psychotropic drugs is a serious health hazard, and a medical and social problem. Improper use of anxiolytic drugs can cause side effects of drug dependence. We confirmed iridoid-rich fraction was safe in the clinical dose, and found the maximum tolerance dose in mice and nontoxic safety dose in rats were 3,200 mg/kg and 120 mg/kg/d, respectively, the latter was the 100 times of clinical dose.^[15] Cao^[4] also proved that animals received the aqueous extract of *V. jatamansi* in experimental doses for 7 days were observed without toxicity, and Xiao^[6] reported rats administered with extract of *V. jatamansi* for 90 days did not show significantly abnormal of hematologic and biochemical indices and viscous tissues. Thus, *V. jatamansi* may be able to become a new potential anxiolytic drug, but its drug dependence has not been reported. This study based on the above was intended to evaluate the drug dependence of ethanolic extract and an iridoid-rich fraction.

MATERIAL AND METHODS

Valeriana jatamansi Jones were collected from a commercial source from Lou mountain, Zunyi city, China. The samples consisted of roots and rhizomes were identified by Prof. Cheng Yong-xian, (20110328), and has been deposited in the herbarium of the institute. The methods of evaluating drug dependence in animals referred to the paper of "The technical guidelines of studies on nonclinical drug dependence (2007) No. (ZH) GPT 3-1" issued by the State Food and Drug Administration in China.

Preparation of ethanolic extract and iridoid-rich fraction

The method of preparation of ethanolic extract and iridoid-rich fraction was referred to Yan^[12] and Li^[14] papers, respectively. As follows: The total plant powder was extracted twice at 30°C for 30 min, on each occasion with 6 times 95% ethanol (W/V), and the combined extract was concentrated until dry in a rotary evaporator to obtain ethanolic extract.

The ethanolic extract were dispersed with water by ultrasonic, then separated by means of D101 macroporous absorbent column: Absorption ratio was 72 mg/g, adsorption rate was 1 bed volume/1 h (BV/h), elution rate 2 BV/h, then was eluted successively with 6 BV water, 4 BV 60% ethanol, and 4 BV 95% ethanol, collected 95% ethanolic eluent to evaporate under reduced pressure, and 40°C vacuum drying to obtain iridoid-rich fraction with 2.1% yield. High-performance liquid chromatography detected the total iridoids content with 71.5% which was calculated by Chloroaltrate.

Animals

Kunming mice weighing 22–28 g were purchased from Chengdu Dashuo Biotechnology Ltd., (Approval No. SCXK 2008-24). The animals were housed in five cages in a quiet room with controlled temperature ($22 \pm 3^\circ\text{C}$) and humidity ($60\% \pm 5$) with a 12 h light/dark cycle (07:00–19:00 h light). The mice were given food and tap water *ad libitum*. Before the treatments, the animals were allowed 3 days to acclimatize to the lodging conditions.

Drugs

The following drugs were used: Diazepam powder (Henan Qianfeng Pharmaceutical Co., Ltd., China) was suspended in 0.5% sodium carboxymethyl cellulose (CMC Na). Fresh pentylenetetrazol (PTZ) (Sigma, German) was dissolved in physiological saline to 2.5 mg/mL. The ethanolic extract and iridoid-rich fraction were prepared with 0.5% CMC Na. All animals received drug treatment with twice daily by gavage from 8 am to 10 am and from 5 pm to 7 pm. The drugs were stored in dark at 4°C and were shaken prior to use.

Experiment I

The mice were randomly divided by weight into four groups of 20 animals each (10 females and 10 males). The following treatments were given to per group, respectively: Ethanolic extract at 0.3 g/kg; ethanolic extract at 0.9 g/kg; diazepam initially at 8 mg/kg, increasing dose/week with 2 mg/kg; the normal animals received 0.5% CMC Na. The treatments were administered for 6 consecutive days every week for 12 consecutive weeks.

Natural withdrawal symptoms

Withdrawal symptoms were recorded on the withdrawal day of every week.

Withdrawal-induced weight change

The weight was recorded after time of drug administration on week 12: Zero, 4, 8, 12, 16, 20, and 24 h. Their weight prior to withdrawal was used as the baseline, and the percentage of the animal withdrawal-induced weight change was calculated.

Audiogenic tail-erection test

From week 4, all groups were subjected to audiogenic tail-erection test using a mixer noise ringtone (8–12 kHz, 105 db \pm 10 db)^[17] for 15 min, at 17:00 pm of the withdrawal day of every week. The animals were transferred into clean individual cages (43 cm \times 22 cm \times 20 cm) before testing. Audiogenic tail-erections were scored by personnel who were blinded to the treatment conditions.

Experiment II

The mice were randomly divided by weight into five groups of 30 animals each (15 females and 15 males). Three groups were received with the iridoid-rich fraction starting at dose of 6 mg/kg, then increasing their dose weekly with 2.18 mg/kg (low dose), 4.45 mg/kg (middle dose), and 9.90 mg/kg (high dose). The administrations of diazepam and the normal group were same as these of experiment I. The treatments were stopped 1-day/week for 16 consecutive weeks.

Natural withdrawal symptoms

Method was a reference to natural withdrawal symptoms test in experiment I.

Withdrawal-induced weight change

The weight was recorded at 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 h after the final drug administration. The data processing was reference to the treatment in experiment I.

Pentylentetrazol-induced convulsion test

Animals were intraperitoneally injected with PTZ solution after 24 h of the final administration. The numbers of tail-erecting and seizure were immediately recorded after the injection for 15 min.

Statistical analysis

The data of withdrawal-induced weight change were expressed as mean \pm standard error of the mean. One-way analysis of variance followed by Student's *t*-test was performed. Chi-square test was used for comparing the ratio of tail-erecting and seizure. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

Withdrawal symptoms of mice in experiments I and II

In experiments I and II, the diazepam-treated mice were subjected to tail-erection, irritability, combative behavior, mutual bite, teeth chattering tests from week 7 to 8, and these symptoms got worse over longer period of treatment. While there were no significant differences between ethanolic extract-treated or iridoid-rich fraction-treated mice and normal mice in appearance, the locomotor activity, food intake, general activity, and others.

Withdrawal-induced body weight loss in experiments I and II

As shown in Table 1, the body weight of diazepam-treated animals in experiments I and II significantly decreased from time of withdrawal 12–20 h ($P < 0.05$ or $P < 0.01$) [Table 1], and from 16–28 h ($P < 0.01$) [Table 2], respectively. However, animals administered with ethanolic extract or iridoid-rich fraction showed negligible statistical significance compared with the normal group ($P > 0.05$) [Tables 1 and 2].

Audiogenic tail-erection test in experiment I

As shown in Table 3, animals treated with diazepam or ethanolic extract were induced to produce tail-erection from withdrawal of week 4. Tail-erection ratios of diazepam group had been higher than that of ethanolic extract groups, and had a significant difference from week 9 ($P < 0.01$). Moreover, with prolonged treatment, the tail-erection ratio of diazepam group showed substantial increase (up to 100% at the last test), while that of ethanolic extract groups declined significantly (<10% in the final two tests). Moreover compared to normal group, tail-erection ratio of diazepam group had exhibited statistical differences from week 6 ($P < 0.05$ or $P < 0.01$), while only on week 8 that of 900 mg/kg ethanolic extract group was significantly increased ($P < 0.05$), the values of the other ethanolic extract groups didn't reached statistical significance.

Table 1: Withdrawal-induced weight change rate in experiment I ($n=10$)

	Normal group	Diazepam group	High-dose ethanolic extract group	Low-dose ethanolic extract group
H 4	1.00 \pm 0.18	0.99 \pm 0.26	1.00 \pm 0.46	1.00 \pm 0.13
H 8	0.99 \pm 0.15	0.98 \pm 0.33	0.98 \pm 0.67	0.99 \pm 0.15
H 12	1.03 \pm 0.13	0.97 \pm 0.29*	1.01 \pm 0.85	1.01 \pm 0.41
H 16	1.02 \pm 0.11	0.96 \pm 0.35**	1.00 \pm 0.83	1.00 \pm 0.35
H 20	1.01 \pm 0.10	0.95 \pm 0.34**	0.98 \pm 0.77	0.99 \pm 0.49
H 24	1.01 \pm 0.34	0.97 \pm 0.29	1.00 \pm 0.47	1.01 \pm 0.63

* $P < 0.05$, compared with normal group; ** $P < 0.01$, compared with normal group. The data was measured by electronic balance ($d=0.01$ g)

Table 2: Withdrawal-induced weight change rate in experiment II (n=25)

	Normal group	Diazepam group	High-dose iridoid-rich fraction group	High-dose iridoid-rich fraction group	Low-dose iridoid-rich fraction group
H 4	0.9962±0.0041	1.0238±0.0038**	0.9977±0.0038	0.9960±0.0047	0.9953±0.0044
H 8	0.9894±0.0053	1.0311±0.0701**	0.9923±0.0047	0.9898±0.0059	0.9963±0.0061
H 12	0.9994±0.0051	1.0192±0.0058**	1.0001±0.0052	1.0005±0.0069	0.9985±0.0042
H 16	1.0034±0.0059	0.9973±0.0041**	1.0053±0.0052	1.0032±0.0085	1.0052±0.0054
H 20	1.0063±0.0055	0.9910±0.0076**	1.0063±0.0064	1.0066±0.0074	1.0099±0.0073
H 24	1.0015±0.0028	0.9844±0.0063**	1.0035±0.0051	1.0030±0.0092	1.0045±0.0081
H 28	0.9993±0.0034	0.9768±0.0070**	0.9994±0.0038	0.9981±0.0077	0.9992±0.0055
H 32	0.9969±0.0040	0.9827±0.0084	0.9967±0.0058	1.0020±0.0067	0.9961±0.0043
H 36	1.0026±0.0039	0.9903±0.0069	1.0058±0.0049	1.0055±0.0085	1.0026±0.0045

**P<0.01, compared with normal group. The data was measured by precision electronic balance (d=0.0001g)

Table 3: The ratios of tail-erection in audiogenic tail-erection test of experiment I (n=10)

	Week 4	Week 5	Week 6	Week 8	Week 9	Week 10	Week 11	Week 12
Normal group (%)	0	0	0	0	0	0	0	0
Diazepam group (%)	40	40	50*	80**	80**	70**	90**	100**
High-dose ethanolic extract group (%)	20	30	20	50*	20 ^{△△}	10	0 ^{△△}	10 ^{△△}
Low-dose ethanolic extract group (%)	10	10	10	30	40	40	10 ^{△△}	10 ^{△△}

*P<0.05, **P<0.01, compared with normal group; ^{△△}P < 0.01, compared with diazepam group

Pentylentetrazol -induced convulsion test in experiment II

With sub-threshold dose of 25 mg/kg intraperitoneal injection of PTZ, the ratios of tail-erection and seizure of diazepam group were 77.78%, 44.44%, respectively, and one mouse was dead because of severe seizure. The iridoid-rich fraction-treated group showed tail-erection ratios of 14.28% (high dose), 12.00% (medium dose), and 11.54% (low dose), which were significantly lower than that of diazepam group (P < 0.01, P < 0.01, P < 0.01), respectively, and only one mice in high dose of iridoid-rich fraction group presented seizure with 3.57%, which was significantly lower than that of diazepam group (P < 0.01) [Table 4].

DISCUSSION

In natural withdrawal trials, changes in autonomic nerve function in mice are difficult to quantitative observation, and withdrawal signs and behavioral changes occur later, as well as they are of long duration. To save time and avoid the long experimental period causing the death of animal, the induction methods can be adopted to promote the withdrawal symptoms, moreover the produce of tail-erection, seizure, and the weight loss.^[18-20] After withdrawal can be used as good indicators to evaluate the potential physical dependence. And the sub-threshold dose of central stimulant (for instance, PTZ) and mixer noise ringtone are common induction methods.^[21] Therefore, this study evaluated the risk of drug dependence about ethanolic extract and iridoid-rich fraction, respectively

Table 4: The ratios of tail erection and seizures in PTZ-induced convulsion test of experiment II

	Tail-erection (ratio)	seizure (ratio)
Normal group (%)	0/27 (0.00)	0/27 (0.00)
Diazepam group (%)	14/18 (77.78**)	8/18 (44.44**)
High-dose iridoid-rich fraction group (%)	4/28 (14.28 ^{△△})	1/28 (3.57 ^{△△})
Medium-dose iridoid-rich fraction group (%)	3/25 (12.00 ^{△△})	0/25 (0.00 ^{△△})
Low-dose iridoid-rich fraction group (%)	3/26 (11.54 ^{△△})	0/26 (0.00 ^{△△})

**P<0.01, compared with normal group; ^{△△}P < 0.01, compared with diazepam group

given to mice for 12 weeks and 16 weeks from four aspects: Observation of natural withdrawal symptoms, weight loss after withdrawal, tail-erection, and seizure induced by mixer noise ringtone and PTZ.

In experiments I and II, different natural withdrawal symptoms in diazepam groups were observed, such as tail-erection, combative behavior, mutual bite, teeth chattering, etc., And they got a significant weight loss after withdrawal. Moreover, the obvious withdrawal symptoms of tail-erection and seizure were stimulated to produce in the two induced experiments. According to the above experimental results, these indicated that the diazepam-treated mice in experiments I and II were successfully established the model of diazepam dependence. During long-term administration of ethanolic extract or iridoid-rich fraction, animals did not appear obvious natural withdrawal symptoms, significant weight

loss, and tail-erection or seizure induced by stimulating. Based on the all above results and “the technical guidelines of studies on nonclinical drug dependence (2007) No. (ZH) GPT 3–1”, we could conclude that mice administrated with ethanolic extract for 12 weeks or iridoid-rich fraction for 16 weeks did not appear obvious physical dependence.

CONCLUSION

In the present study, there were no noticeable withdrawal symptoms and body weight change when mice were given ethanolic extract for 12 weeks or iridoid-rich fraction for 16 weeks. They would show the phenomenon of a little slight tail-erection and seizure induced by mixer noise ringtone or PTZ, but which was no significant difference compared to the normal group. Therefore, these suggest that the present risks of the potential drug dependence about ethanolic extract and iridoid-rich fraction were far lower than that of diazepam.

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