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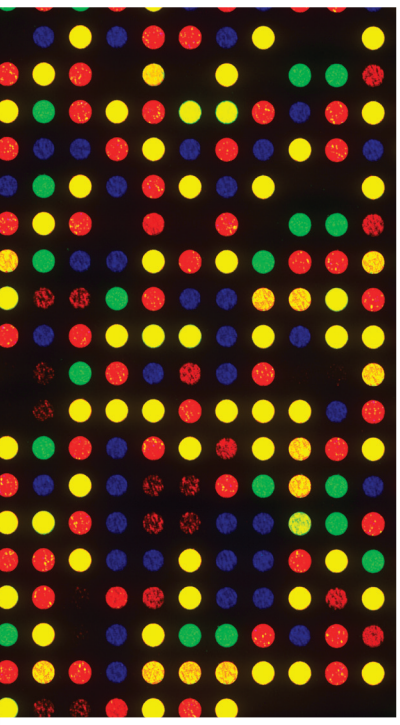
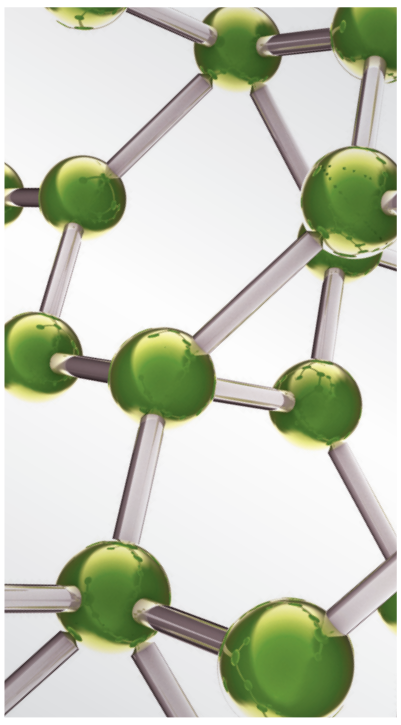
# Complementary and Alternative Medicine for the Treatment of Central Nervous System Disorders

Guest Editors: Ching-Liang Hsieh, Lixing Lao, Yi-Wen Lin, and Gerhard Litscher



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# Editorial Board

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*Editorial*

# Complementary and Alternative Medicine for the Treatment of Central Nervous System Disorders

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Central nervous system (CNS) disorders are difficult and complicated and cause high costs for clinical therapy and basic research due to unknown and puzzling mechanisms. The treatment of CNS disorders needs systematic drugs that can pass through the brain barrier to target specific receptors. Until now, such drugs have severe side effects. Complementary and alternative medicine (CAM) has recently become highly recognized as therapeutic medicine and recommended by the World Health Organization (WHO). Clinical trials, drug development, and basic research of CAM are increased dramatically because of gradual development and knowledge. The current special issue is diversified with several novel and crucial articles concerning CAM.

Cocaine addiction is a major economic, social, and health problem in developed countries that can influence many individuals. Development of new drugs to treat cocaine dependence is urgent and necessary. Cocaine mainly binds to dopamine reuptake transporters resulting in pleasure and addiction. *Rhodiola rosea* L. (RHO) is a well-known CAM with adaptogenic, anxiolytic, antidepressive, and antistress properties. It can reduce nicotine and morphine withdrawal symptoms. F. Titomanlio et al. reported that RHO can potentiate hyperactivity induced by cocaine. RHO also attenuated the acquisition and expression of cocaine-induced conditioned place preference. They concluded that RHO is effective in decreasing the rewarding properties of cocaine but not in cocaine-associated reinstatement.

H.-C. Hsu et al. used epileptic rats to evaluate the antiepileptic effect of *Uncaria rhynchophylla* (UR) and rhynchophylline (RP). They injected kainic acid (KA) to induce seizures in Sprague Dawley rats. They suggested that pretreatment with UR and RP can reliably attenuate seizures accompanied by reduced c-Jun amino-terminal kinase phosphorylation (JNKp) of mitogen-activated protein kinase (MAPK) signal pathways in the cerebral cortex and hippocampus. IL-1$\beta$, IL-6, and TNF-$\alpha$ were unaltered, which means that the therapeutic effects of UR and RP are based on pJNK activation during KA-induced seizure processes. Similarly, T.-W. Guo and colleagues indicated that electroacupuncture (EA) can protect rats from chronic restraint stress. In addition, IL-1$\beta$, IL-6, and TGF-$\beta$ were potentiated in chronic restraint stress rats and can be alleviated by EA pretreatment. The data are crucial that EA can attenuate depression accompanied by altering IL-1$\beta$, IL-6, and TGF-$\beta$ in the hippocampal CA3 region. S.-N. Shi and colleagues reported that valtrate, which is a principle compound isolated from *Valeriana jatamansi* Jones used to treat various mood disorders, can reduce depression and simultaneously reduce the corticosterone level in the rat serum. They conclude that valtrate has an anxiolytic effect in behavioral models through

the hypothalamus-pituitary-adrenal axis. The abovementioned mechanisms implied that herbal medicine and EA can activate similar mechanisms to treat CNS disorders such as depression and epilepsy.

Furthermore, K.-H. Lee et al. wanted to analyze the concurrent use of herbal medicine and hypnotic drugs in Taiwanese insomnia patients. They showed that, among 53,949 insomnia sufferers, 83.6% used hypnotic drugs. Jia-Wei-Xiao-Yao-San and Suan-Zao-Ren-Tang were always used, coadministered with hypnotic drugs. They indicated that the hazard ratio of hip fracture for hypnotic-drug users who used the herbal medicine was lower than hypnotic-drug only. The results are crucial for clinical practice to reduce hip fracture and are beneficial for health and quality of life of patients with insomnia symptoms.

CAM has wide categories to treat many diseases and symptoms. In this special issue, diverse CAM therapies are described to treat different CNS disorders such as epilepsy, depression, insomnia, and addiction. This issue is plentiful and strong in CAM therapy with evidence-based medicine from basic research to clinical results.

We think that the readers of this special issue will get many thought-provoking impulses and information.

*Ching-Liang Hsieh*
*Lixing Lao*
*Yi-Wen Lin*
*Gerhard Litscher*

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*Research Article*

# The Alterations of IL-1Beta, IL-6, and TGF-Beta Levels in Hippocampal CA3 Region of Chronic Restraint Stress Rats after Electroacupuncture (EA) Pretreatment

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Immunological reactions induced by proinflammatory cytokines have been involved in the pathogenesis of depressive disorders. Recent studies showed that Electroacupuncture (EA) was able to reduce depressive symptoms; however, the underlying mechanism and its potential targets remain unknown. In the present study, we used a 21-day chronic restraint stress rats as a model to investigate how EA could alleviate depression. Open field test was carried out to evaluate the depressive symptoms at selected time points. At the end of study, immunohistochemistry (IHC) was performed to detect the expressions of IL-1beta, IL-6, and TGF-beta in hippocampal CA3 region. We found that chronic restraint stress significantly decreased behavioral activities, whereas EA stimulation at points Baihui (GV 20) and Yintang (GV 29) showed protective effect during the test period. In addition, the IL-1beta, IL-6, and TGF-beta increased in rats exposed to chronic restraint stress, while EA downregulated the levels of IL-1beta and IL-6. These findings implied that EA pretreatment could alleviate depression through modulating IL-1beta and IL-6 expression levels in hippocampal CA3 region.

## 1. Introduction

Depression, with a lifetime prevalence of up to 17%, is the leading cause of disability and ranks the 4th among diseases contributing to the global burden [1]. Various medicine treatments including antidepressant medications and psychology therapies play a pivotal role in depression treatment; however, almost one-fourth of patients are unable to achieve favorable effects, especially the improvement of somatic symptoms [2]. Thus, seeking an alternative therapy for depression is an urgent issue which is needed to be addressed. Study has shown that prevention at early stage appears to be the best option to minimize the progression of depression [3]. In clinical practice, electronic acupuncture (EA) has been proved to be an effective therapy in treating mental disorders. Studies have shown that EA can mitigate depression as shown in reduced Hamilton Depression Rating Scale scores in treated patients. In comparison with antidepressants, EA presented comparable therapeutic effects but with faster onset of action and better response rate [4, 5]. Although EA showed promising effects in alleviating the progression of depression, the underlying mechanism is poorly understood.

Over the past decades, large bodies of evidences have suggested that major depression is linked with sign of immunological activation. Specifically, activation of the inflammatory response system (IRS), such as increased production of proinflammatory cytokines, is considered to be the key factor for depression [6]. Both clinical and experimental studies indicated that increased concentration of certain types of cytokine may serve as a leading cause of stress and depression [7]. Dowlati et al. showed that high levels of IL-6 and TNF-alpha were found in depressed patients compared with control subjects [8]. Abbasi showed that antidepressant celecoxib

can reduce HDRS scores as well as IL-6 concentration in patients with major depressive disorders. In animal study, chronic stress-induced depressive mice showed an increased IL-1 level in brain tissue [9]. Hippocampus, as a part of limbic system, plays an important role in the emotion regulation. Repeated stress causes atrophy of dendrites in hippocampal CA3 region [10]. In addition, CA3 neurons are more vulnerable to damages compared with dentate granule and CA1 neurons [11]. Therefore, hippocampal CA3 region is a crucial part for observing the physical changes during chronic stress.

Recent studies implied EA might function via modulation of nerve-endocrine-immune network [12]. The pathogenesis of depressive symptoms is characterized as a complex of network dysfunction in which factors including neurotransmitters, hormones, and cytokines, interact intimately. Therefore, a research strategy focusing on nerve-endocrine-immune network might be applied to identify the key player which involved in EA treatment in depression. In the present study, we hypothesized that EA could modulate proinflammatory cytokine levels and thus reduced depression syndrome.

## 2. Material and Methods

*2.1. Animals.* A total of 30 specific pathogen-free (SPF) Sprague Dawley rats (260~280 g) were supplied by the Institute of Laboratory Animal Sciences, China Academy of Medical Science, animal license number SCXF (Jing)2009-0017. Animals were housed at (22 ± 2)°C, 45% humidity, in 12-hour light/dark cycles (light on at 8:00 am), with free access to food and water. The study was performed 3 days after environment acclimations of the rats. The protocols were conducted in compliance with the Guidance Suggestions for the Care and Use of Laboratory Animals formulated by the National Institute of Health, as well as the 3R principle: Reduction, Replacement, and Refinement. All experiment procedures were approved by the Animal Care and Use Committee at Beijing University of Chinese Medicine.

*2.2. Groups and Treatment.* For control group, no model induction and treatment were performed. For model group, chronic stress was conducted for 21 days on a daily basis with method described as follows: rats were restrained with self-made cylinder-shaped wire net (20 cm in length and 5 cm in diameter) from 9 am to 3 pm. After restraints, they were released for free access to water and food. For EA group, EA pretreatment was conducted daily prior to restraint for 21 days, restraint method was the same as model group.

*2.3. EA Pretreatment.* During acupuncture administration, rats were maintained within a cloth bag. Two points were selected: Baihui (GV20) and Yintang (GV29). GV20 is located above the apex auriculate, on the midline of the head. GV29 is located at the middle point between two eyes [13]. Sterilized disposable stainless steel needles (0.20 ∗ 25 mm, Hua Tuo brand, manufactured by Suzhou medicine Co., Ltd., Suzhou, Jiangsu, China) were inserted obliquely as deep as 3–5 mm for both points. Following the insertions, electrodes were added to the handle of the needles (electric acupuncture apparatus used: Hans-100 A, manufactured by Nanjing Jisheng medicine science Co., Ltd., Nanjing, Jiangsu, China). Electricity simulation parameters were 1 mA, 2 Hz, for 20 minutes.

*2.4. Open Field Test.* At selected time points: day 0, day 7, day 14, and day 21, the open field test was conducted with modifications of previous studies [14]. The apparatus, wood in material, was comprised of a square arena 80 × 80 cm with 40 cm high wall. It was divided into 25 × 25 equal squares which had been drawn in the floor of the arena. A single rat was gently placed in the center of the floor in order to explore the arena for 3 min. The activity of the rat was recorded by a camera installed on top of the lateral high wall. Two observers, blind to the experiment, counted the crossing numbers (defined as at least three paws in a square) and the rearing numbers (defined as the rat standing upright on its hind legs) from a monitor connected to the camera which was set one meter away from the apparatus. After one rat finished the test, alcohol was applied to clean the floor to exclude the intervention of odor signals. The body weight was measured on day 0, day 7, day 14, and day 21 of the experiment.

*2.5. Frozen Section and Immunohistochemistry.* At the end of the study, rats were deeply anesthetized with 10% chloral hydrate (0.3 mL/100 g, i.p.) and perfused with 4°C 4% paraformaldehyde from left apex. After perfusion, hippocampus was harvested and embedded in liquid nitrogen. For sections preparation, chiasma opticum was positioned at first; then the tissue was cut into 20 $\mu$m coronal sections till 2-3 mm posterior to chiasma opticum on a sliding microtome (Leica CM1850, German) and stored at −20°C. Immunohistochemistry was carried on as previously described [15]. The primary antibodies (goat against rat IL-6 IgG, product number: SC-1265R; rabbit against rat IL-1beta IgG, product number: SC-7884; rabbit against rat TGF-beta IgG, product number: SC-146 manufactured by Santa Cruz Biotechnology (Shanghai) Co., Ltd., Shanghai, China) were added and then incubated over night at 4°C. Secondary antibodies conjugated with horseradish peroxidase (HRP) was added afterward and incubated at 37°C for 1 hour. HRP substrates were applied at last for color development. The protein expressions were quantified by Integral Optical Density (IOD) using Image Pro Plus 6.0 software. For each rat, 3 to 4 sections were applied and mean value was obtained to determine the expression level.

*2.6. Statistical Analysis.* Data were presented as means ± S.E.M. SPSS 20.0 (SPSS Inc, Chicago, USA) was deployed for data analysis with one-way ANOVA method after the test of normal distribution and homogeneity of variance, followed by post hoc multiple comparison. Statistical significance was set to $P < 0.05$, while highly statistical significance was set to $P < 0.01$.

## 3. Results

*3.1. Effects of EA Pretreatment on Body Weight.* As shown in Figure 1(a), the body weight increased slowly in model group

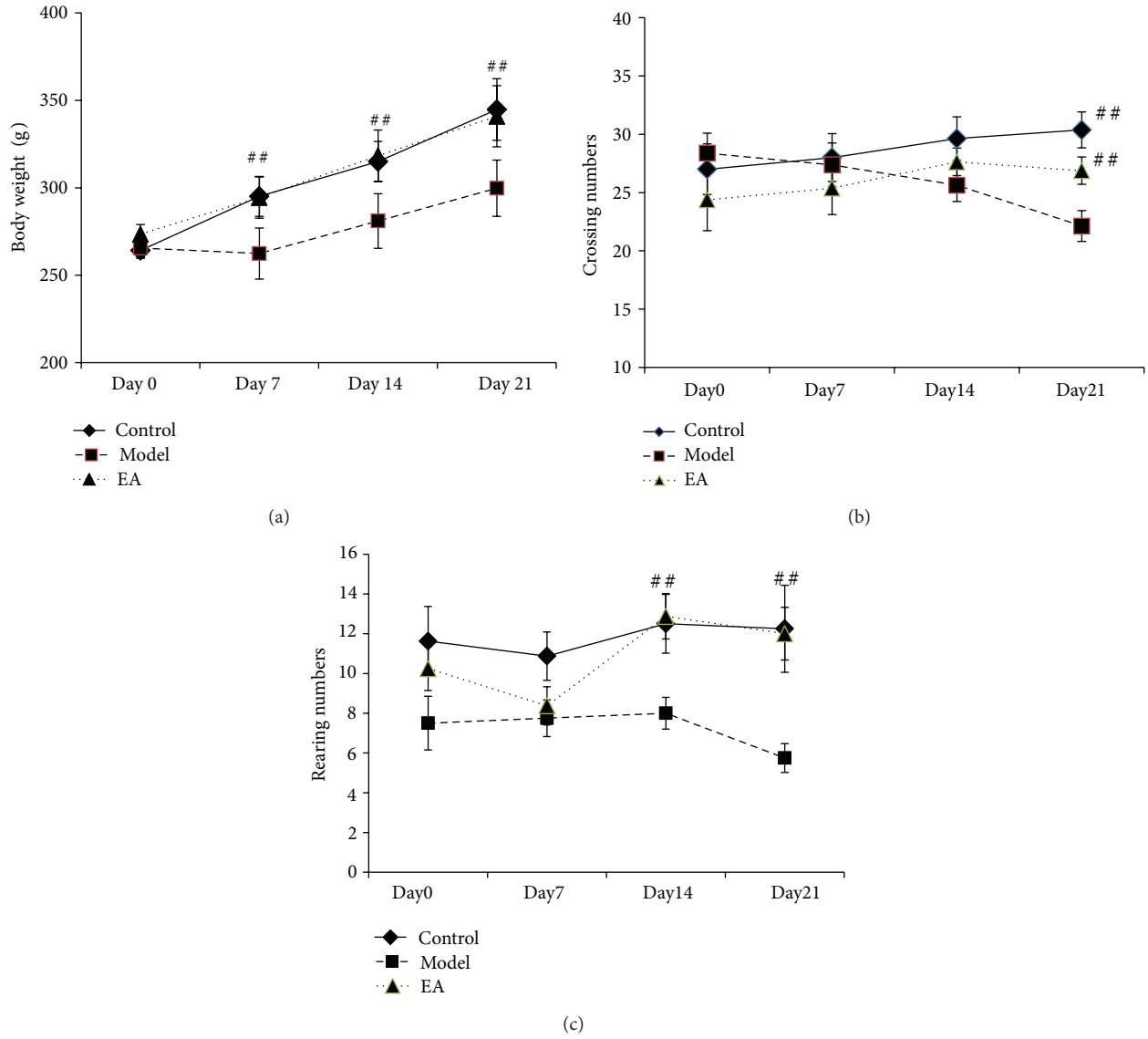



Figure 1: The effect of electric acupuncture (EA) on body weight and locomotor activity in open field test at selected time points in the following groups ($n = 8$ per group): control, model, and EA. (a) Body weight. (b) Crossing numbers in open field test. (c) Rearing numbers in open field test. $^{\#\#}P < 0.01$ as compared with model group.

in contrast with those in control and EA group. 21 days after induction, the body weights in control group and EA group were significantly higher compared with that in model group ($P < 0.01$), whereas no significant differences was found between control group and EA group. This result suggested that EA has a protective effect on body weight.

*3.2. Effects of EA Pretreatment on Open Field Test.* We used the open field test to evaluate the exploratory and locomotor activity [16, 17]. As seen in Figure 1(b), model group presented a decline tendency in crossing numbers during the 21-day restraint stress procedure, while control and EA group showed a rise tendency. 21 days after induction of the crossing numbers in model group was significantly decreased in comparison with those in control and EA group with statistically significant differences ($P < 0.01$). There was no difference between control and model group ($P > 0.05$). In addition, restraint stress stimuli remarkably reduced the rearing numbers in model group compared with that in control and EA group 21 days after induction, whereas no significant difference was found between control and EA group. The results indicated that EA plays a crucial role in ameliorating stress-impaired exploratory and locomotor activities.

*3.3. Effects of EA Pretreatment on IL-1Beta.* As shown in Figure 2, the expression of IL-1beta in model group was significantly increased compared with that in control group (a) and EA group based on IOD value ($P < 0.01$), whereas there was no significantly difference between EA

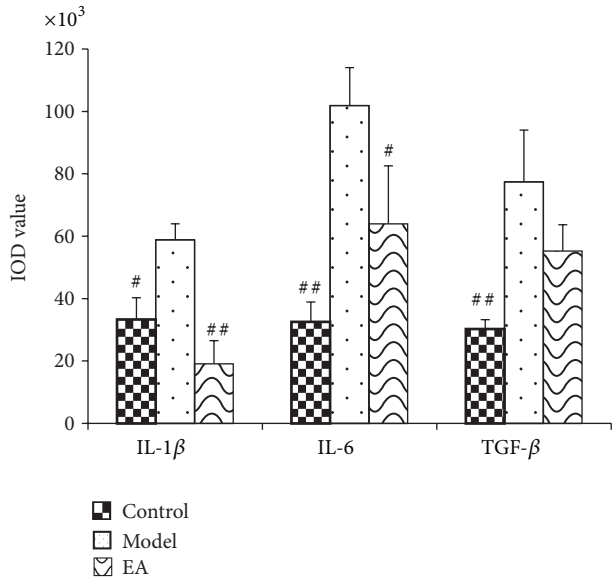



Figure 2: The effect of EA on IL-1beta, IL-6, and TGF-beta protein expression in hippocampus (HP) CA3 region in the following groups ($n = 8$ per group): control, model, and EA. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ as compared with model group.

group and control group ($P > 0.05$). Figures 3(a) and 3(c) immunostaining-positive cells in hippocampal CA3 region in control and EA group were arranged in line with less cytoplasm coloring, whereas Figure 3(b) showed that in model group, large amount of positive cells, in a mass, has deep cytoplasm coloring. These results indicated that EA decreased the expression of IL1beta and showed a protective effect in hippocampal tissue.

*3.4. Effects of EA Pretreatment on IL-6.* Figure 2 showed that after a 21-day procedure, IOD value of IL-6 in model group was significantly increased compared with that in control group ($P < 0.01$) and EA group ($P < 0.05$).

As shown in Figures 4(a) and 4(c) immunostaining-positive cells in hippocampal CA3 region of rats in control and EA group were arranged in line with less cytoplasm coloring. Figure 4(b) showed that in model group, large amount of positive cells, in a mass, has deep cytoplasm coloring. The results suggested that EA suppressed the hypersecretion of IL-6 and therefore protected hippocampus against proinflammatory stress.

*3.5. Effects of EA Pretreatment on TGF-Beta.* Figures 5(a) and 5(c) showed that positive cells in control and EA group were arranged in line with less cytoplasm coloring, while Figure 5(b) showed that in model group, numerous positive cells distributed densely with deep cytoplasm coloring. However, as shown in Figure 2, the IOD value of TGF-beta in model group was significantly increased compared with that in control group, indicating that restraint stress can stimulate the expression of TGF-beta, whereas EA reduced its expression, but the effect was not of statistical significance.

## 4. Discussions

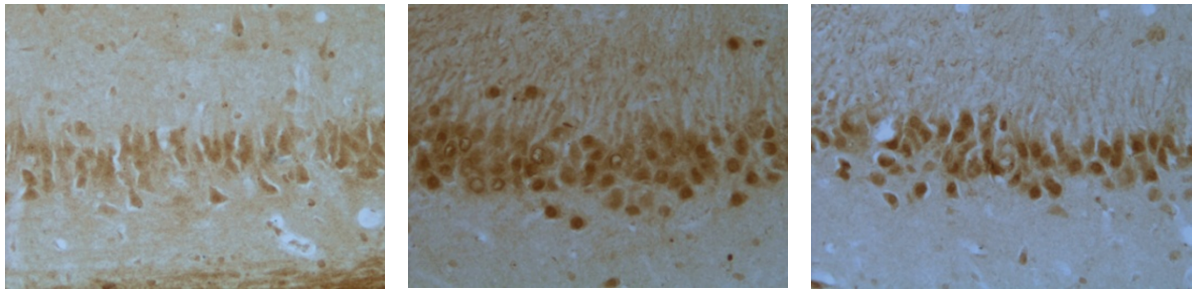
The major finding of the present study is that EA pretreatment modulated the expression of IL-6 and IL-1beta in hippocampal CA3 region in chronic restraint stress rats.

IL-1beta and IL-6 have been intensively investigated for their roles in depressive symptoms. Previous studies indicated that overexpression of IL-6 promotes depressive-like behavior [18, 19]. Lenczowski et al. demonstrated that IL-6 can reduce social investigatory and behavior and locomotor activity in the presence of IL-1beta. Nevertheless, controversial studies offered opposite notions that IL-6 administration failed to elicit sickness behavior [20]. The discrepancy might be explained from differences in stress category, duration, and other experimental procedures.

In parallel to most previous results, we found that elevated secretions of IL-1beta, IL-6, and TGF-beta occurred concomitantly with depressive symptoms, suggesting a hyperactivity of immune function caused by restraint stressor. Cytokines and their receptors such as IL-1, IL-2, IL-6, and TNF-$\alpha$ and some other growth factors are localized in rodent brain with highest densities in the hippocampus and hypothalamus [21, 22]. Therefore, cytokine hyperactivity can stimulate various chain reactions to harm regions related to emotion perception and regulation. The literature suggested that chronic inflammations, shown as overexpression of cytokines, can activate the enzyme degrading tryptophan which leads serotonin depletion and antioxidant defenses impairment [23–25]. In addition, cell-mediated immune cytokines can increase the synthesis of neurotoxic tryptophan catabolites (TRYCATs) which contributes to oxidative stress, impaired mitochondrial metabolism, and apoptosis.

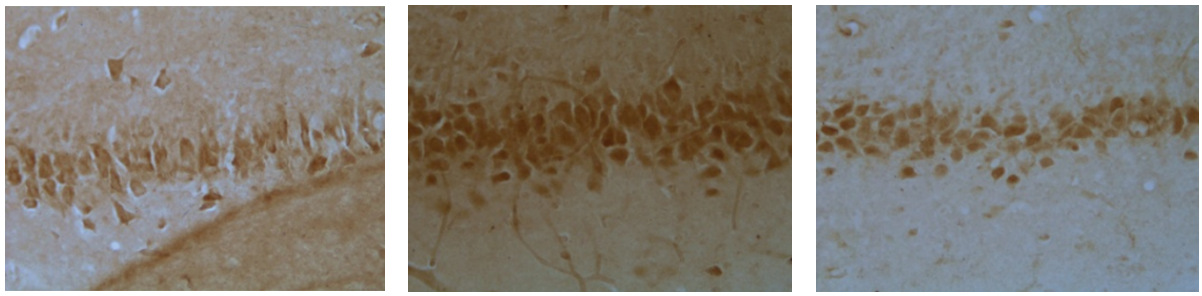
EA has been proved to be capable in reversing excitotoxicity and apoptosis [26]. EA showed protective effects on hippocampal CA3 regions including decreasing presynaptic glutamate synthesis and release, blocking postsynaptic excitatory amino acid receptors, and terminating pathological chain reaction caused by excessive excitatory receptors to inhibit glutamate release. The essential target may be NMDA receptor which can be inhibited to decrease calcium ions influx [27]. In addition, Liang et al. showed that EA can achieve curative effects by involving in the signal pathway of Ras-MKK-JNK; specifically, EA can alleviate apoptosis by decreasing the level of Capase-3 and increasing the ratio of Bcl2 to Bad [28]. In the present study, we found significantly decreased secretion of IL-6 and IL-1beta in hippocampal CA3 region of EA pretreated stressed rats, which might indicate the target cytokines of EA in regulating immune system.

Moreover, IL-6 has been suggested to be associated with brain-derived neurotrophic factor (BDNF) which is highly involved in the physiopathology of depression [29]. It has been suggested that IL-6 activated protein kinase B and then it can phosphorylate the nuclear localization signal on DNA methyltransferase-1 (DNMT1) which hypermethylates BDNF promoter and further reduces BDNF level [30, 31]. BDNF plays a pivotal role in spine formation and synapse plasticity which facilitates the connectivity between different brain regions in limbic system. Similarly, it has also been suggested that EA can boost BDNF level through the modulation of



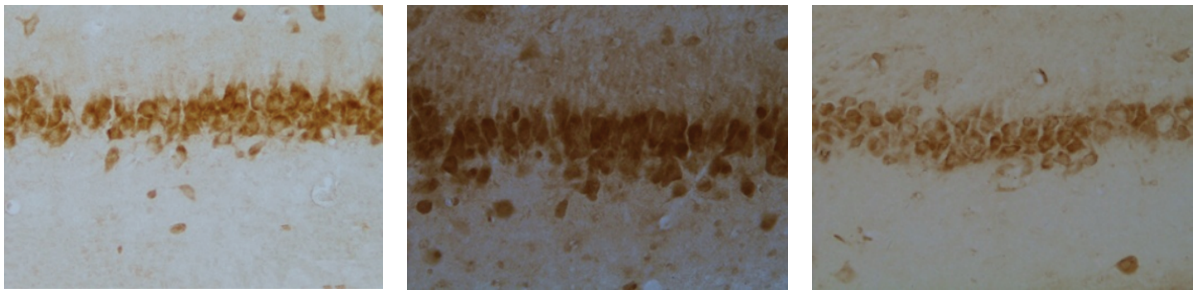
(a) (b) (c)

Figure 3: Representative immunohistochemistry results showing IL-1beta levels and neuron morphology in the hippocampus CA3 region in the following groups ($n$ = 10 per group): (a) control, (b) model, and (c) EA.



(a) (b) (c)

Figure 4: Representative immunohistochemistry results showing IL-6 levels and neuron morphology in the hippocampus CA3 region in the following groups ($n$ = 8 per group): (a) control, (b) model, and (c) EA.



(a) (b) (c)

Figure 5: Representative immunohistochemistry results showing TGF-beta levels and neuron morphology in the hippocampus CA3 region in the following groups ($n$ = 8 per group): (a) control, (b) model, and (c) EA.

Ras-MAPK-ERK pathway to mitigate the phosphorylation of ERK1/2 [32]. Therefore it is speculated that IL-6 serves as a mediator between EA pretreatment and its beneficial effects, specifically, EA could downregulate the expression of IL-6 to restore the level of BDNF.

IL-1beta has been shown to function synergistically with IL-6 to activate HPA axis to reduce social investigatory behavior and locomotor activity [20], indicating the underlying combined mechanism of IL-1beta and IL-6 in depression. Evidence from another acupuncture treatment research for chronic stress model indicated that action of acupuncture may be mediated by an inhibition of HPA axis via attenuated c-fos which symbolizes decreased arginine vasopressin (AVP) and corticotropin releasing hormone (CRH). Based on observed reduced expression of IL-6 and IL-1beta after EA, we think that EA suppress these proinflammatory cytokines to downregulate HPA axis hyperactivity.

TGF-beta, distinguished itself from the above cytokines with a special pathway, performs many cellular functions, including the control of cell growth, proliferation, differentiation, and apoptosis [33]. The literatures reported that TGF-beta is involved in neurodegenerative diseases such as Alzheimer's disease [34], but the correlations between TGF-beta and depression is poorly known. However, based on its pathway in physiopathology, several potential links

could be found between TGF-beta and depressive disorders. The activation of TGF-beta can activate downstream MAPK pathway which has been described as to be implicated in the expression of BDNF [35]. In addition, it has been shown that TGF-beta is also involved in Ras-MKK-JNK pathway which is highly correlated to apoptosis and growth arrest, serving as an underlying mechanism of depressive symptoms. Wu demonstrated that EA can boost the expression of Bcl-2 gene to inhibit apoptosis in brain tissue after chronic stress induction [36]. Thus we hypothesized that EA may exert beneficial effects on depressive symptoms through a mechanism in which TGF-beta activating Erk1/2 pathway as well as JNK pathway. However, according to our results, TGF-beta level declined in EA group without statistical significance in comparison with model group. The role of TGF-beta in immunological activation and EA prevention warrants further investigations.

In the present study, EA pretreatment was administered under a slightly restrained condition. Our previous work (unpublished data) illustrated that no significant difference was observed in serum adrenocorticotropic hormone (ACTH) and corticosterone (CORT) level between the normal rats and normal plus acupuncture rats. It is suggested that the acupuncture administration will not induce stress response.

According to Traditional Chinese Medicine, Baihui and Yintang are points pertaining to Governor Meridian. Based on Meridian and Collateral Theory, Governor Meridian is the convergence of all the Yang meridians; therefore, stimulation on points of Governor Meridian can boost Yang qi of the whole body to reverse the pathogenesis of depression in which it is defined as yang deficiency syndrome. Meanwhile, EA pretreatment design of our study embodies one of the most critical theories in Traditional Chinese Medicine, the principle of "treating diseases prior to its onset" which attaches great significance on disease preventions.

## 5. Conclusions

In summary, the present study demonstrated that the proinflammatory cytokines IL-1beta, IL-6, and TGF-beta in rats' hippocampus mediated the onsets of depressive symptoms after chronic restraint stress inductions. Importantly, our findings suggested that EA can significantly mitigate deficit behavioral activities elicited by chronic restraint stress through a potential mechanism of immunological modulation.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Tianwei Guo and Zhuo Guo contribute equally to this study.

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*Research Article*

# The Anxiolytic Effects of Valtrate in Rats Involves Changes of Corticosterone Levels

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Valtrate is a principle compound isolated from *Valeriana jatamansi* Jones, which is a Traditional Chinese Medicine used to treat various mood disorders. The aim of the present study was to investigate the anxiolytic effects of valtrate in rats. The animals were orally administered valtrate (5, 10, and 20 g/kg daily) for 10 days and exposed to open field test (OFT) and elevated plus-maze (EPM). Then the corticosterone levels in the rat serum were measured by enzyme-linked immunosorbent assay (ELISA). The valtrate (10 mg/kg, p.o.) exhibited the anxiolytic effect in rats by increasing the time and entry percentage into the open arms in the EPM and the number of central entries in the OFT. Valtrate (10 mg/kg, p.o.) significantly reduced the corticosterone level in the rat serum. Taken together, these results suggest that the valtrate has anxiolytic activity in behavioral models that might be mediated via the function of hypothalamus-pituitary-adrenal axis.

## 1. Introduction

Anxiety disorder is a common mental illness on society. Millions of people suffer from a mental or behavioral disorder [1]. Previous studies suggest that benzodiazepines are useful first-line agents for most of the anxiety disorders in the world [2]. However, they may produce fearful side effects; for example, long-term use of benzodiazepine can cause cognitive decline in the elderly [3]. In addition, a lot of patients with anxiety disorders fail to adequately respond to existing pharmacologic treatments [4]. Thus, better antianxiety drugs with greater efficacy and fewer side-effects are needed.

Traditional Chinese prescription has been commonly recognized as a safe and effective prescription in the treatment of various mood disorders in China [5]. *Valeriana jatamansi* Jones was a famous Traditional Chinese Medicine used to treat anxiety disorders in clinical prescription for many years [6]. Recent study has reported that *Valeriana jatamansi* Jones exerts an anxiolytic effect by improving the frequency and time percentage of the open arm in the elevated plus maze [7]. Chemical researches have shown that it includes essential oils, iridoids, and flavonoids compounds [8], but the anxiolytic active components of *Valeriana jatamansi* Jones have not been adequately elucidated. Valtrate is a major component of *Valeriana jatamansi* Jones and has been shown to have antifungal, antitumor, and cytotoxic activities in early studies [9–12]. Currently, valtrate at a high dose has been found to have sedative properties by inhibiting spontaneous motion and increasing the sleeping number induced by pentobarbital sodium in mice [13]. Therefore, these results raises the possibility of the anxiolytic effect of valtrate as the primary antianxiety components in *Valeriana jatamansi* Jones. However, the anxiolytic effect of valtrate and the mechanism have not been reported.

Therefore, in the present study, we investigated the anxiolytic potential of valtrate isolated from *Valeriana jatamansi* Jones in rats. The paradigms we selected here to detect the anxiolytic effect of valtrate are two famous tests of anxiety: the open field test (OFT) and the elevated plus maze test (EPM), which have shown good sensitivity to anxiolytic

drugs. The EPM is a well-established animal model for testing anxiolytic drugs [14] because of its natural stimulus, such as a fear of a new, bright, and open space and the fear of balancing on a relatively narrow raised surface [15]. The OFT has gained popularity as a model of anxiety, which is based on the rodents' natural tendency to stay near the perimeters of a novel environment [16] and the aversion of rodents for open and illuminated spaces [17]. The animals were tested in the OFT and EPM. After the behavior test, we determined whether valtrate altered the serum corticosterone response to stress induced by exposure to the two models.

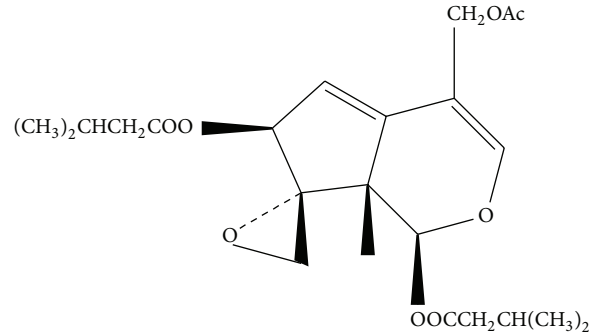


Figure 1: Structure of valtrate.

## 2. Material and Methods

*2.1. Animals.* 60 male 8-week-old Sprague-Dawley rats (150–170 g) were obtained from the Laboratory Animal Center of the Academy of Military Medical Sciences and used for this study. Each animal was housed in individual cages under controlled temperature (22 ± 1°C) and a 12 h/12 h light/dark cycle (lights on at 07:00 AM–19:00 PM) with free access to food and water. The experimenter handled the animals daily to acclimate them to the manipulation. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Psychology of the Chinese Academy of Sciences and in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

*2.2. Plant Material and Isolation of Valtrate.* *Valeriana jatamansi* Rhizoma et Radix was purchased from a commercial source in Yunnan province, China. The identity of the herbal medicine was confirmed by Professor Shi Jin-li, a researcher in the Department of Pharmacognosy, Beijing University of Chinese Medicine. Voucher specimens were deposited at the Herbarium of School of Chinese Materia Medica, Beijing University of Chinese Medicine.

Jatamana Valeriana Rhizome was homogenized to coarse powder (8 kg) and soaked in aqueous ethanol (95%, 12 L, v/v) three times at room temperature, and the combined alcoholic extract was filtered and evaporated under reduced pressure to yield a residue. The concentrated extract was then subjected to chromatographic separation on AB-8 macroporous adsorption resin with 70%, 80%, and 90% EtOH-$H_2O$ to give three fractions. Three fractions were subjected to chromatography on silica gel eluted with petroleum ether-ethyl acetate (20 : 1, 10 : 1, 8 : 1), then The fractions were combined based on the TLC analysis. We got ten compounds; the valtrate (Figure 1) was an oily matter identified by spectroscopic methods (UV, IR, ESI-MS, $^1$H NMR, and $^{13}$C NMR). The purity of valtrate was determined by HPLC analysis, which was identified by comparing with a standard specimen (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China). The sample was chromatographed under the following chromatographic conditions: Chromatographic column: Agilent Extend C18 column, 5 $\mu$m, 250 × 4.6 mm; Mobile phase: gradient elution by acetonitrile-distilled water (68%–32%); Flow rate: 1 mL/min; Column temperature: 30°C with UV detection at 254 nm.

*2.3. Drugs and Treatment.* Diazepam was obtained from Yimin Pharmaceutical Factory of Beijing. All drugs were prepared immediately before use and were given orally in a volume of 1 mL/100 g body weight for 10 days. diazepam at the dose of 1 mg/kg [18] was chosen as a positive control drug. Diazepam and valtrate were both dissolved in 0.5% Tween-80 solution. For vehicle group, distilled water which contained 0.5% Tween-80 was administered at the same volume. In this study, the rats were administered valtrate or diazepam 60 and 30 min before the test, respectively. The Elisa kit was obtained from R&D. All experiments were carried out in quiet room under dim red light between 8:00 a.m. and 14:00 p.m. on the 10th day of treatment.

*2.4. Open Field Test.* The OFT apparatus was a 180 cm diameter cylinder with 60 cm high walls. The center of the bottom of the apparatus had a 52 cm diameter section. As previously described [19], all rats were acclimatized to the test room for 1 h. The rats were placed into the field at the same point against the wall and allowed to freely explore the apparatus for 10 min. The total path length, the number of central entries, and the time spent in the center were recorded by an automatic video tracking system. OFT was performed 60 min after the final treatment of valtrate and 30 min after the diazepam. After each trial, the apparatus was wiped clean with a 10% ethanol solution.

*2.5. Elevated Plus Maze.* Immediately after the OFT, anxiolytic activity was measured using the EPM, which was consisted of two open arms (50.8 cm × 10.2 cm × 1.3 cm) and two closed arms (50.8 cm × 10.2 cm × 40.6 cm) that extended from a central platform (10.2 cm × 10.2 cm). The maze was elevated to a height of 72.4 cm above the floor. The entire maze was constructed of clear Plexiglas [20]. Each rat was placed on the central square facing an open arm and allowed to freely explore the maze for 5 min. Arm entries were defined as the entry of all four paws into an arm. A computer recorded the time spent on and number of entries into the open and closed arms by means of infrared photocells. The apparatus was wiped clean with a 30% ethanol solution and dried after each subject.

*2.6. Determination of Serum Corticosterone.* 10 min after the completion of the two behavioral tests, the rats were sacrificed

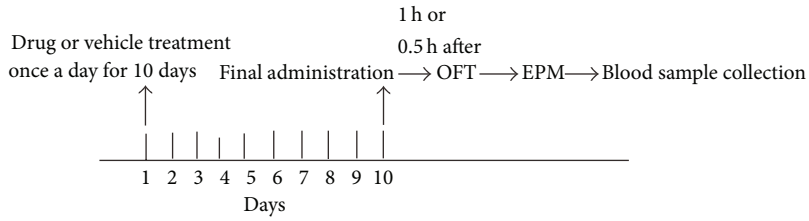


Figure 2: Experimental schedule. Experimental schedule, described in Material and Methods section, involved the OFT, EPM test, and the collection of blood sample.

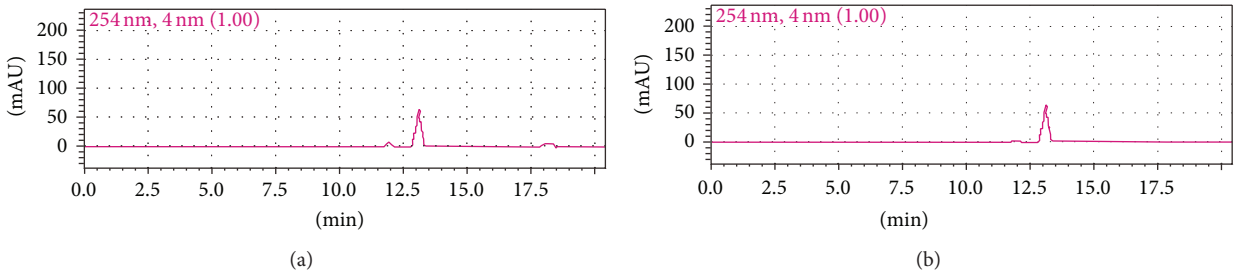


Figure 3: HPLC profile of valtrate using acetonitrile-distilled water (68%–32%) at 1 mL/min on a Agilent Extend C18 column, 5 $\mu$m, 250 × 4.6 mm, 30°C with UV detection at 254 nm. (a) The sample of valtrate. (b) The standard specimen of valtrate.

by decapitation; then trunk blood was collected among the five groups to avoid any substantial time lag in samples collection. Samples were centrifuged at 3000 r·min$^{-1}$ for 15 min at 4°C and supernatants were stored at −20°C until analysis. The content of corticosterone was determined by a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. The absorbance of each sample was measured at a wavelength of 450 nm and the results are presented as ng/mL. All procedures of the experiment were shown in Figure 2.

*2.7. Statistical Analysis.* The data were expressed as mean ± SEM. The statistical analysis was carried out by one-way analysis of variance (ANOVA) following Student-Newman-Keul's post-hoc test using Prism 5.0 (Graphpad Software, Inc). Probability values lower than 0.05 were considered statistically significant.

## 3. Results

*3.1. Assaying of Valtrate by HPLC.* The results suggest that the purity of product can reach 99% (see Figure 3).

*3.2. Effects of Valtrate on Open Field Test in Rats.* The results for the OFT are shown in Figure 4. Analyses demonstrated significant effects on number of center entries ($F(4, 55) = 3.541$, $P < 0.05$) and time spent in central area ($F(4, 55) = 3.127$, $P < 0.05$); further analyses showed that valtrate at dose of 10 mg/kg significantly increased the entries in central area ($P < 0.05$). Valtrate at the dose of 20 mg/kg did not significantly increase the entries in central area ($P > 0.05$). Diazepam significantly increased the number of center entries ($P < 0.05$) and the time spent in central area ($P < 0.05$). All of the doses of valtrate did not significantly increase the time spent in central area ($P > 0.05$). No difference in total path length was observed among the five groups ($F(4, 55) = 1.207$, $P > 0.05$). Locus diagram of open field test of every group is shown in Figure 5.

*3.3. Effects of Valtrate on Elevated Plus Maze in Rats.* As shown in Table 1 and Figure 6, the ANOVA indicated significant effects on percentage of time spent on the open arm ($F(4, 55) = 7.755$, $P < 0.01$) and open arm entries ($F(4, 55) = 6.054$, $P < 0.01$). Compared to vehicle group, valtrate at the dose of 10 mg/kg significantly increased the percentage of time spent in the open arms and entry percentage into the open arms in the elevated plus maze ($P < 0.01$; $P < 0.01$), and valtrate at the dose of 20 mg/kg increased the percentage of time spent in the open arms of the maze ($P < 0.01$) but did not increase percentage of open arm entries ($P > 0.05$). Diazepam also significantly increased the percentage of time spent on open arms ($P < 0.01$) and percentage into the open arms ($P < 0.05$). No difference was observed in total arm entries among groups ($F(4, 55) = 1.042$, $P > 0.05$).

*3.4. The Level of Serum Corticosterone.* As seen in Figure 7, the data show that administration of valtrate at the dose of 10 mg/kg and 20 mg/kg dose reduced the corticosterone level ($P < 0.01$, $P < 0.05$). Similarly, serum corticosterone levels of rats treated with diazepam were lower than those of the vehicle group ($P < 0.01$).

## 4. Discussion

The present study was performed to analyze the behavioral effects of anxiolytic valtrate isolated from *Valeriana jatamansi* Jones, using two behavioural measurements of

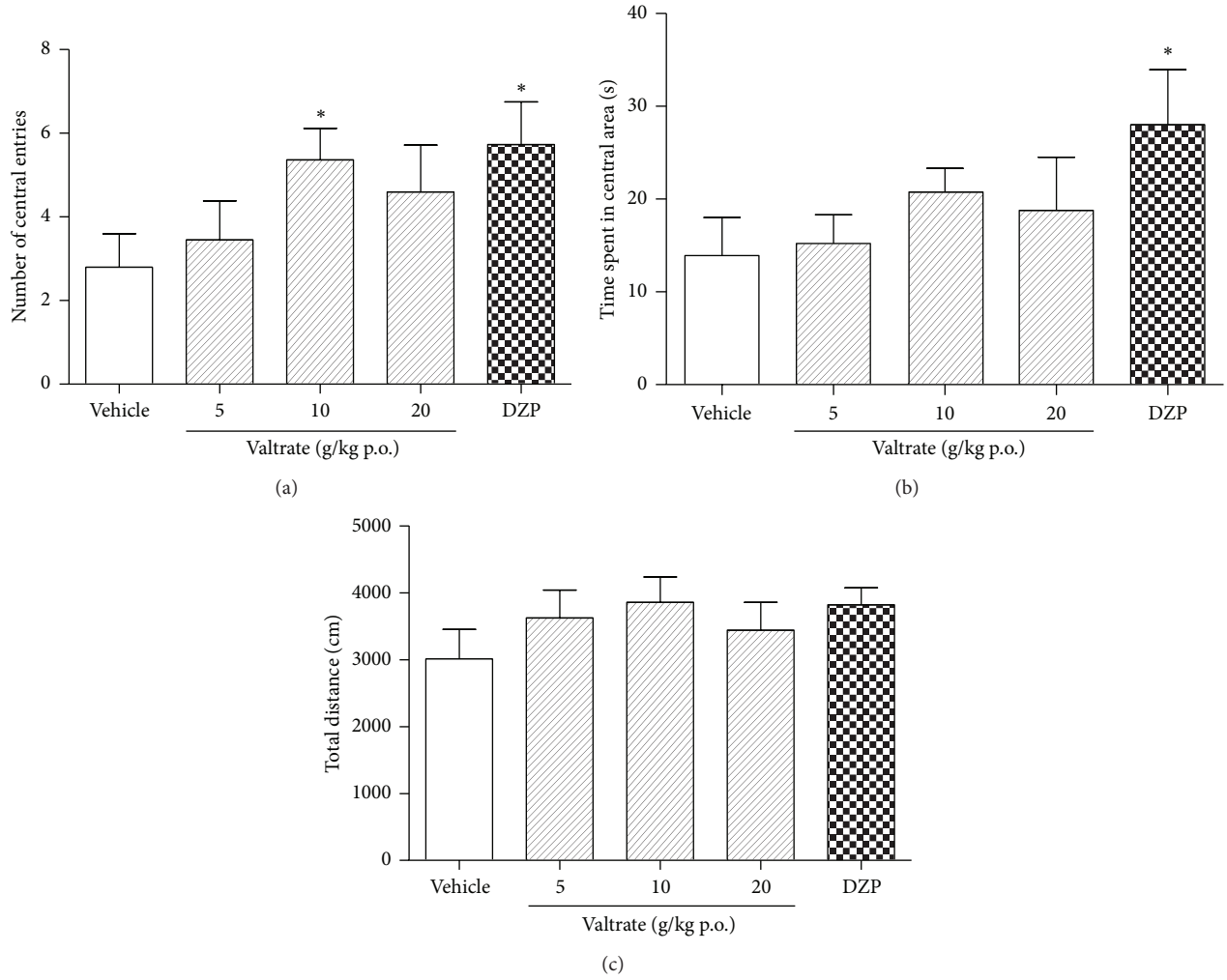


Figure 4: Effect of valtrate on the behavior of rat in the OFT in a 10 min session in the open field performed 1 h after the administration of vehicle (p.o.), valtrate (5, 10, and 20 mg/kg, p.o.), and 0.5 h after the administration of diazepam (1 mg/kg, p.o.). (a) Number of central entries, (b) time spent in central area, and (c) total distance. Columns represent the means ± SEM, $n = 12$ rats. $^{*}P < 0.05$ compared to the vehicle group.

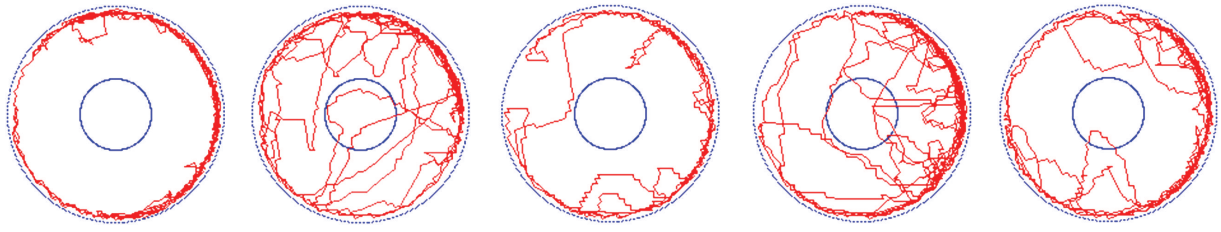


Figure 5: Locus diagram of OFT. (a) Vehicle, (b) DZP, and (c) Valtrate 5 mg/kg, (d) valtrate 10 mg/kg, and (e) valtrate 20 mg/kg.

Table 1: Effect of diazepam and valtrate on the behavior of rats in the elevated plus-maze test.

| Group | Dose (mg/kg) | Open arm entries | Closed arm entries | Total arm entries | Time in open arms (s) | Time in closed arms (s) |
|---|---|---|---|---|---|---|
| Vehicle | — | 3.07 ± 0.59 | 9.16 ± 0.95 | 10.07 ± 1.45 | 48.41 ± 4.36 | 189.94 ± 10.57 |
| Diazepam | 1 | 4.80 ± 0.73 | 9.70 ± 0.76 | 12.20 ± 0.97 | 98.92 ± 10.07 | 138.55 ± 9.54 |
| Valtrate | 5 | 2.00 ± 0.29 | 10.71 ± 0.97 | 9.50 ± 1.59 | 52.21 ± 8.09 | 183.31 ± 15.45 |
| | 10 | 6.36 ± 1.16 | 10.55 ± 1.23 | 13.33 ± 1.41 | 87.20 ± 10.20 | 143.13 ± 8.92 |
| | 20 | 4.22 ± 0.47 | 8.78 ± 0.70 | 11.75 ± 1.11 | 82.01 ± 8.69 | 147.51 ± 8.68 |

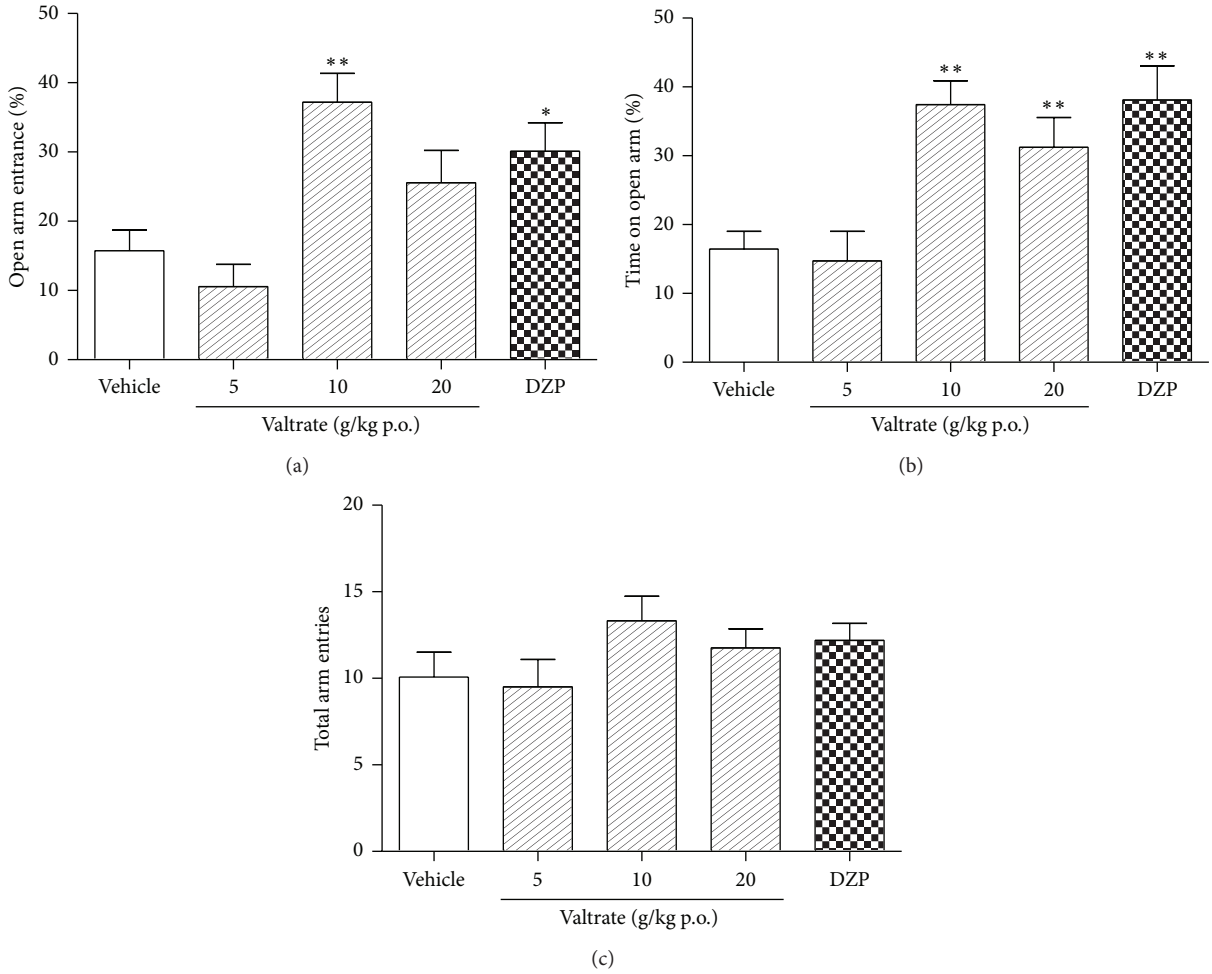


Figure 6: Behavioural performance of rat registered in a 5 min session in the EPM performed 1 h after the administration of vehicle (p.o.), valtrate (5, 10, and 20 mg/kg, p.o.), and 0.5 h after the administration of diazepam (1 mg/kg, p.o.). (a) Percentage of number of entries into the open arm, (b) percentage of time spent into the open arms, and (c) total arm entries. Columns represent the means ± SEM, $n = 12$ rats. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the vehicle group.

anxiety, OFT, and EPM. The results showed that valtrate exhibited anxiolytic-like activity and did not induce sedative side effects. We also found that valtrate could attenuate HPA axis activity by reducing the corticosterone level.

Valtrate was successfully isolated from subterranean parts of subterranean parts of various Valeriana species for the first time by Thies [21]. Our laboratory developed a high efficiency and practicality method for purifying Valtrate from *Valeriana jatamansi* Jones with AB-8 macroporous adsorption resin. The resin yielded the best efficiency when the concentration of the extraction was 3.5 mg/mL, the 70% ethanol acted as the eluant, and the eluting speed was two column volumes per hour. AB-8 macroporous adsorption resin significantly increased the purity of valtrate (99%), with advantage of high absorption, high elution rate, and low expense.

Hall originally described the OFT for the study of emotionality in rats [22], which is one of the most popular procedures in animal psychology and has been widely used to assess anxiety, emotionality, or responses to stress in animals [23, 24]; the test is based on the rodents' natural tendency to stay near the perimeters of a novel environment and the aversion of rodents for open and illuminated spaces. The number of central entries or the time spent in the center area served as indices of anxiety and the distance was considered an index of locomotor activity [25, 26]. Rats treated with valtrate at the dose of 10 mg/kg significantly increased the number of center entries and the total distance was not significantly affected. Therefore, valtrate (10 mg/kg) has a significant anxiolytic-like effect in this paradigm.

To further strength these data, we tested the anxiolytic-like effects of these treatments in EPM, Which is a classical animal analog for anxiolytic drugs and can play a key role in the screening of anxiolytic drugs on the central nervous system currently [27, 28]. Normally, rodents tend to avoid open areas of the maze and a preference for sections enclosed by protective walls. Anxiolytic drugs shift the behavioral response toward exploration of the open arms [29]. The percentage entries into the open arms and time spent in the open arms are generally used as indices of anxiety and drugs increasing these measures show anxiolytic properties.

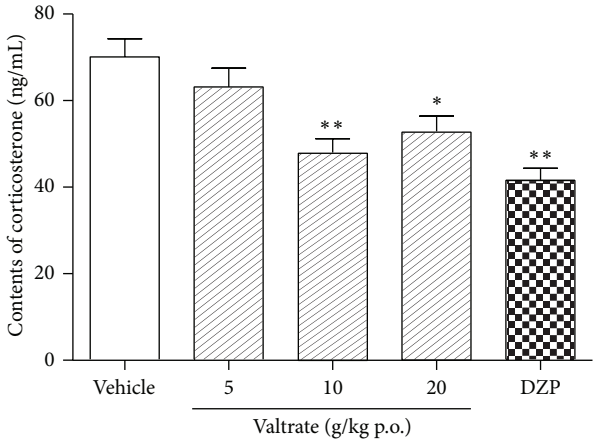


FIGURE 7: Effects of bergamot valtrate (5, 10, and 20 mg/kg, p.o.) and diazepam (1 mg/kg, p.o.) compared with vehicle groups on serum corticosterone after the behavior test. Columns represent the means ± SEM, $n = 12$. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the vehicle group.

The number of entries into the total arms was considered an index of locomotor activity. In the present study, the rats were treated with the higher doses of valtrate (10 mg/kg and 20 mg/kg) for 10 days, and anxiety-like behavior in the EPM was significantly attenuated, without altering the number of total arm entries, suggesting that valtrate induces specific anxiolytic-like effects.

The alcohol extract from *Valeriana jatamansi* Jones (0.3, 0.6, 0.9 g/kg) could increase the open entries percent and open time percent in the EPM [30]. As the content of valtrate in alcohol extract from *Valeriana jatamansi* Jones is about 2.87%, 5 mg/mL valtrate in rat is equivalent to 0.3 g/mL alcohol extract of *Valeriana jatamansi* Jones in mice in terms of equal valtrate efficacy. Thus, the doses of valtrate (5, 10, and 20 mg/kg) were chosen in this study. We also performed an acute experiment to study the anxiolytic effect of valtrate, and valtrate did not affect the behavior tests in EPM and OFT (data not shown). Therefore, the valtrate was administered with ten days. Diazepam is a classical drug to treat anxiety and was chosen as a positive drug in this study. As expected, diazepam had a significant anxiolytic-like effect in both EPM and OFT. The anxiolytic effect of the valtrate (10 mg/kg) was almost equivalent to that of diazepam (1 mg/kg) in EPM and the number of central entries in OFT. Although valtrate did not increase the time spent on the center of the open field compared to the vehicle group, there were marginal significant differences between the valtrate and the vehicle group on these tests. In addition, both valtrate (10 mg/kg) and diazepam (1 mg/kg) could reduce the corticosterone levels after behavior tests. Therefore, we thought that the anxiolytic effect of valtrate (10 mg/kg) was almost equivalent to diazepam (1 mg/kg).

The drugs (valtrate or diazepam) in this study were administered 60 and 30 min before the test; there should be a control group for each drug treatment. We compared these two vehicle groups (administrated 30 min and 60 min, resp.). The rats of these groups were exposed to the same procedure. The results suggested that there was no difference between the two groups (data not shown). To avoid too much groups in the present study, one vehicle group (60 min before test) was used as control.

It should be noted that the intermediate dose of valtrate was the most effective in decreasing anxiety-like behavior tests and corticosterone concentrations. One possible reason may be that the highest dose of valtrate or its metabolites may act as an inducer for hepatic microsomal enzyme, which can increase metabolism of the drug and result in reducing curative effect. In addition, valtrate (10 mg/kg and 20 mg/kg) was able to decrease corticosterone concentration ($P < 0.01$ and $P < 0.05$, resp.), but this effect was not observed in the behavior tests. Although valtrate at the dose of 20 mg/kg did not increase percentage of open arm entries ($P = 0.052$) and the number of central entries ($P = 0.058$), there were marginal significant differences between the valtrate (20 mg/kg) and the vehicle group on these two tests. Moreover, individual differences among rats in the highest dose valtrate (20 mg/kg) group are larger than these in intermediate dose valtrate (10 mg/kg) group. The same situation was observed in the open field test. However, valtrate at the dose of 10 mg/kg and 20 mg/kg did not increase the time spent on the center of the open field compared to the vehicle group ($P = 0.057$ and $P = 0.063$, resp.). There were marginal significant differences between the valtrate group (10 mg/kg or 20 mg/kg) and the vehicle group in the time spent in the center area.

Neuroendocrine system plays a key role in the stability of the body environment. Hormones change these neurons' network by making change in information and altering the neurotransmitter between cells in cell level, thus affecting the central nervous system function [31]. It is well known that hypothalamic-pituitary-adrenocortical (HPA) axis activation is a key component of the physiological response to stress and anxiety. The HPA axis is activated by stress; then corticosterone is released from the adrenal gland. The stress hormone corticosterone was measured to investigate the response of the HPA axis to valtrate. Research suggests that the exposure of rodents to the standard elevated plus maze activates the HPA axis, leading to an enhancement of plasma corticosterone [32]. In addition, there was a peak in corticosterone secretion which occurs 5 to 10 min after exposure to two different anxiety/fear tests [33]. It has been reported that *Valeriana jatamansi* Jones extract played a role in antianxiety via regulation of the HPA axis [30]. In the present study, as the stress hormone corticosterone was measured as the rats were subjected to both EPM and OF tests. These two tests were performed in two adjacent rooms, the delay time was less than one minute, and we thought that these procedures might not influence the effectiveness of the drug or plasma corticosterone concentrations, which were also used by other researchers [34, 35]. Our data showed that valtrate (10 mg/kg), a dose which produced anxiolytic activity in the behavioural experiments, attenuated the activity of HPA axis by reducing the corticosterone response to the stress of exposure to the elevated plus maze. These findings indicate that the decreased anxiety-related behaviours may be related to the attenuation of HPA axis activity.

You et al. had reported the anxiolytic-like effects of compound *Valeriana jatamansi* Jones in mice [36]. Compound *Valeriana jatamansi* Jones is composed of Valerianae Jatamansi Rhizoma et Radix, Ziziphi Spinosae Semen, and Albiziae Cortex and Junci Medulla (in a ratio of 12 : 9 : 9 : 1). They reported that the compound Valerianae Jatamansi Jone has anxiolytic effects but no sedative effect at dose of 2.4 and 4.8 g/kg. As the content of valtrate in *Valeriana jatamansi* Jones is about 1.8%, 2.4 g and 4.8 g valtrate compound might contain valtrate 16.74 mg and 33.48 mg, respectively. In our study, valtrate at the dose of 10 mg/kg and 20 mg/kg has anxiolytic-like effect in rats. Thus, valtrate might be the main component to possess the anxiolytic-like effect of compound *Valeriana jatamansi* Jones. However, it is very interesting to contrast some pharmacological property of valtrate and *Valeriana jatamansi* Jones (or compound Valeriana jatamansi Jones) on behavior and plasma corticosterone. In addition, we did not measure the corticosterone levels in treated nonstressed rats. As the present procedure is characterized by two factors (stress and treatment), the corticosterone levels in treated nonstressed rats could strongly improve our present results and will be added in the future study.

The EPM test is considered one of the most widely validated tests for assaying new benzodiazepine-like anxiolytic agents [37]. GABA is the most important inhibitory neurotransmitter in the human central nervous system. Most of GABA receptors have separate modulatory sites sensitive to benzodiazepines. It is well known that the GABA mediated inhibition of the HPA axis at the level of the paraventricular nucleus of the hypothalamus [38]. As a consequence, we hypothesized that the decreased corticosterone levels by valtrate may also be related to the GABAergic neurotransmission.

## 5. Conclusions

In conclusion, the present study indicates that valtrate exhibits anxiolytic-like profiles in the elevated plus maze test and the open field test. Valtrate also attenuated HPA axis activity by reducing the corticosterone level.

## Conflict of Interests

No conflict of financial interests exists.

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*Research Article*

# Antiepileptic Effect of *Uncaria rhynchophylla* and *Rhynchophylline* Involved in the Initiation of c-Jun N-Terminal Kinase Phosphorylation of MAPK Signal Pathways in Acute Seizures of Kainic Acid-Treated Rats

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Seizures cause inflammation of the central nervous system. The extent of the inflammation is related to the severity and recurrence of the seizures. Cell surface receptors are stimulated by stimulators such as kainic acid (KA), which causes intracellular mitogen-activated protein kinase (MAPK) signal pathway transmission to coordinate a response. It is known that *Uncaria rhynchophylla* (UR) and *rhynchophylline* (RP) have anticonvulsive effects, although the mechanisms remain unclear. Therefore, the purpose of this study is to develop a novel strategy for treating epilepsy by investigating how UR and RP initiate their anticonvulsive mechanisms. Sprague-Dawley rats were administered KA (12 mg/kg, i.p.) to induce seizure before being sacrificed. The brain was removed 3 h after KA administration. The results indicate that pretreatment with UR (1.0 g/kg), RP (0.25 mg/kg), and valproic acid (VA, 250 mg/kg) for 3 d could reduce epileptic seizures and could also reduce the expression of c-Jun aminoterminal kinase phosphorylation (JNKp) of MAPK signal pathways in the cerebral cortex and hippocampus brain tissues. Proinflammatory cytokines interleukin (IL)-1$\beta$, IL-6, and tumor necrosis factor-$\alpha$ remain unchanged, indicating that the anticonvulsive effect of UR and RP is initially involved in the JNKp MAPK signal pathway during the KA-induced acute seizure period.

## 1. Introduction

Inflammatory processes play a critical role in neurodegenerative disorders such as stroke, epilepsy, and Alzheimer's disease [1]. Seizure can cause inflammation of the central nervous system, which can affect the severity and frequency of recurring seizures [2]; moreover, anti-inflammatory effects such as those caused by a ketogenic diet, can produce anticonvulsant effects [2]. Previous research has reported that seizure induces the generation of cytokines, such as interleukin (IL)-1$\beta$, IL-6, and tumor necrosis factor (TNF)-$\alpha$, and that the interactions among these cytokines are related to the development of epilepsy and play a crucial role in epileptogenesis [3]. Nuclear factor (NF)-$\kappa$B is a transcription factor that can be activated by TNF-$\alpha$ and the Fas ligand. NF-$\kappa$B presents in the cytosol in an inactive form as a 3-subunit complex comprising p65/p50 dimers and I$\kappa$B [4]. NF-$\kappa$B is activated by phosphorylation of I$\kappa$B$\alpha$ and subsequently translocates into the nucleus where it binds to form NF-$\kappa$B-DNA [4]. Long-lasting seizures can also cause NF-$\kappa$B expression and translocation [5]. Furthermore, the activation of NF-$\kappa$B can contribute to susceptibility to seizures [6]. The glial NF-$\kappa$B activation can induce the generation of proinflammatory cytokines and neurotoxic reactive oxygen

species, resulting in neuronal apoptosis [4]. Therefore, NF-$\kappa$B plays multiple roles in inflammation.

Cells can transmit intracellular signals to coordinate a response to external stimuli [7]. Cell surface receptors such as the N-methyl-D-asparate (NMDA) and kainate (non-NMDA) receptors of glutamate play a crucial role in epilepsy because they can cause activation of mitogen-activated protein kinase (MAPK), which plays a critical role in the development of epilepsy [8]. The MAPK signal pathways comprise the following 3 main subfamilies that are activated by phosphorylation [7]: (1) extracellular signal-regulated protein kinase (ERK); (2) c-Jun aminoterminal kinase (JNK)/stress-activated protein kinase (SAPK); and (3) p38.

*Uncaria rhynchophylla* (UR) is a traditional Chinese herb that has been used to treat epileptic seizures for centuries. According to the recording of Bencoogongmu, the UR can treat vertigo and epileptic seizure [9]. Our previous study showed that UR can reduce epileptic seizures [10]. It can also reduce microglial activation, neuronal and inducible nitric oxide synthase immunoreactive cells of the hippocampus in kainic acid (KA)-treated rats, and also acts as a neuroprotector [11]. In addition, our previous research indicated that UR and its *rhynchophylline* (RP) component can attenuate JNKp expression and NF-$\kappa$B activation at 24 hr following KA-induced epileptic seizures [12]. However, following our previous studies [12], further clarification is required to identify the initiating event during the seizures and whether this initiator was possibly related to the development of epilepsy (epileptogenesis). Therefore, to develop a novel strategy for treating epilepsy, the purpose of this study is to investigate the anticonvulsive mechanisms of UR and its RP component during acute seizures. In this study, Sprague-Dawley (SD) rats were pretreated with UR and RP. Because the findings of our previous studies [10, 12] showed that peritoneal administration of KA can cause acute seizures at 3 h, in this study the concentrations of the proinflammatory cytokines IL-1$\beta$, IL-6, and TNF-$\alpha$, as well as the MAPKs and NF-$\kappa$B-activation of brain tissues, were measured at 3 h following KA administration.

## 2. Material and Methods

*2.1. Preparation of UR Extract.* Crude UR [*Rubiaceae, Uncaria rhyncholhylla* (Miq.) jack] was extracted by following our previous study [12]. The crude UR was extracted twice (500 g each) with 3.5 and 2.5 L of distilled water by boiling for 1 h. The extract was filtered twice and freeze-dried, and the total yield was 63.55 g (12.71%). The freeze-dried extract was stored in a refrigerator at 4°C and was analyzed using a high-performance liquid chromatography system (Hitachi Instruments Service Co. Ltd., Interface D-700, Pump L-7100, UV-Vis Dector L-7420, Ibarki-Ken, Japan) with RP (Matsumura Yakugyo Co., Ltd, Japan) as a standard from Koda Pharmaceutical Company. Because 1 g of the freeze-dried UR extract contained 0.22 mg of RP, the dosage of RP employed in this study was 0.25 mg/kg.

*2.2. Animal Models.* The adult male SD rats (200–300 g) employed in this study were purchased from Biol-Asco Taiwan Co. Ltd. The rats were housed in the animal center of China Medical University, under a 12-h light-dark cycle (25°C ± 1°C), and provided with food and water. All of the procedures in the experiments were conducted in accordance with the *Guideline Principles for the Care and Use of Laboratory Animals.*

*2.3. Electrode Preparation.* The electrodes were prepared 1 wk prior to electroencephalogram (EEG) and electromyogram (EMG) recordings, in accordance with our previous research [12]. The head of the rats was fixed in a stereotactic apparatus under an anesthetic state (chloral hydrate 400 mg/kg, i.p.). The hair of the rat head was cut using scissors. Using a surgical knife, an incision was made on the scalp from the midline and the skull was exposed. The stainless screw electrodes were implanted in the bilateral sensorimotor cortices just as locate epidural service as the recordings, and another electrode was located in the frontal sinus as a reference for the EEG recordings. For the EMG recordings, bipolar electrode wires were placed around the neck muscle through the subcutaneous tissues. Finally, all of the electrodes were plugged into a relay and then connected to an EEG and EMG recording machine (MP100WSW, BIOPAC System, Inc. Goleta, CA, USA).

*2.4. Experimental Procedure.* The 36 SD rats examined in this study were randomly divided into 6 groups (6 rats/group), detailed as follows: (1) normal group (Group N), phosphate-buffered saline (PBS) solution 1.0 mL/kg i.p only; (2) control group (Group KA), KA (12 mg/kg; King Don Co., Taiwan) i.p. only; (3) PK (Group PK), PBS (1.0 mL/kg/day, i.p.) for 2 d, as well as 15 min prior to KA administration; (4) UR (Group UR), UR (1.0 g/kg/day, i.p.) for 2 d, as well as 15 min prior to KA administration; (5) RP (Group RP), RP (0.25 mg/kg/d, i.p.) for 2 d, as well as 15 min prior to KA administration; and (6) VA (Group VA), VA (250 mg/kg/d, i.p.; Sigma USA) for 2 d, as well as 15 min prior to KA administration. The epileptic seizures were confirmed by behavioral observation and EEG and EMG recordings. The EEG and EMG were recorded 15 min prior to drug administration until 3 h following KA administration, and the rats were sacrificed under anesthesia (chloral hydrate, 400 mg/kg, i.p.). The rat brain was removed and separated into the cortex and hippocampus regions to measure the IL-1$\beta$, IL-6, and TNF-$\alpha$, as well as the JNK, ERK, and p38 of MAPK and NF-$\kappa$B.

*2.5. Rat Brain Tissue Preparation.* The brain tissue was washed twice under cold PBS solution and then dried. Subsequently, 200 $\mu$L 0.25 × buffer H (HEPES-KOH pH 7.9, 100 mM; KCl, 250 mM; Spermine, 1.5 mM; Spermine, 5 mM; EGTA, 10 mM; EDTA, 10 mM), 0.5 mM DTT, 1 × protease inhibitor mixture (PMSE, 50 mM; Benzamidine, 0.1 M; leupeptin, 50 $\mu$g/mL; pepstatin, 100 $\mu$g/mL), and 1 × phosphatase inhibitor mixture (okadaic acid, 200 nM; sodium orthovanadate, 20 mM; cypermethrin, 8 nM) were added. The sample was transferred to a new centrifuge tube,

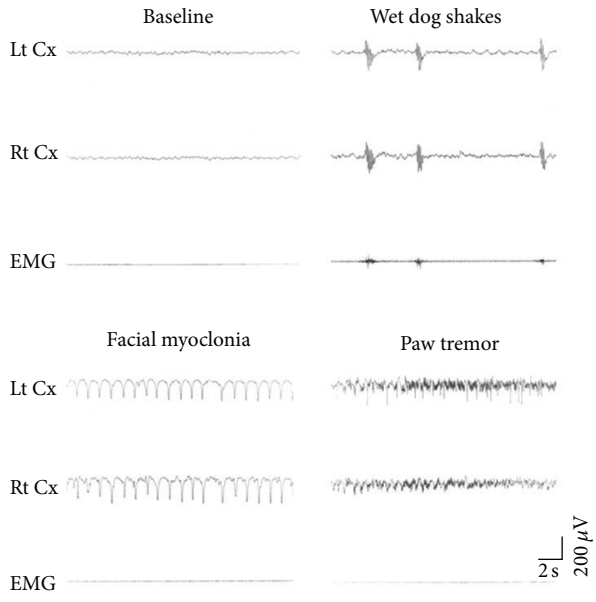


Figure 1: KA-induced epileptic seizure and EEG and EMG changes. KA (i.p.) in rats caused 4 types of epileptic seizures: wet dog shakes with polyspike-like artifacts on the EEG and EMG, facial myoclonia with continuous sharp waves on the EEG, and paw tremor with continuous spikes on the EEG. Lt Cx: EEG recorded at left cerebral cortex. Rt Cx: EEG recorded at right cerebral cortex. Baseline: EEG and EMG recordings prior to KA administration.

and 2 × buffer H, 20% glycerol, 0.5 mM DTT, 1 × protein inhibitor mixture, and 1 × phosphatase inhibitor were added, the volume of which was identical to that of the brain tissue. The sample mixture was homogenised and centrifuged (2500 rpm) at 4°C for 10 min to dislodge the supernatants. The sample was added into an identical volume of low-salt buffer (1 × buffer H, 20% glycerol, 1 mM DTT, 1 × protease inhibitor mixture, 1 × phosphate inhibitor mixture) and subsequently added to a high-salt buffer (1 × buffer H, 1 M KCl, 20% glycerol, 1 mM DTT, 1 × protease inhibitor mixture, 1 × phosphate inhibitor mixture) that was equal to 0.72 × of the sample. Next, the sample was extracted at 4°C for 45 min. Finally, the sample was centrifuged (12 000 rpm) at 4°C for 5 min, and the supernatants were stored in a refrigerator at −70°C. The concentration of protein was determined using a Bradford assay.

*2.6. Enzyme-Linked Immunosorbent Assay (ELISA).* The IL-1$\beta$ (Bender MedSystems, USA), IL-6 (Bender MedSystems, USA), and TNF-$\alpha$ (Bender MedSystems, USA) kits were added to various concentrations of supernatants for the protein marker. Subsequently, a colorimetric method (optical density) was employed using the ELISA reader (Dynex MRX, Virginia, USA) to determine the concentration of IL-1$\beta$, IL-6, and TNF-$\alpha$.

*2.7. Western Blotting Analysis.* Western blotting analysis was performed by following our previous study [12]. Briefly, the brain tissue was homogenized in lysis buffer solution (pH 7.2 Tris-HCl, 50 mM; 5% Triton X-100; 1 × protease inhibitors). The homogenous brain tissue was centrifuged (12 000 rpm) at 4°C for 4 min, and the supernatants were then stored in a refrigerator at −70°C until the AP-1 activity was determined. The protein extracts (10 $\mu$g) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein bands were subsequently transferred electrophoretically to nitrocellulose membranes. The membranes were blocked in blocking buffer solution (pH 7.6 Tris-HCl, 20 mM; NaCl, 140 mM; Tween-20, 0.1%; skim milk powder, 5%) and probed with antibodies that recognized phosphorylated ERK (ERKp), JNKp, phosphorylated p38 (p38p), ERK, JNK, or p38. The bound antibody was detected using peroxidase-conjugated anti-rabbit antibody followed by chemiluminescence (ECL system, Amersham, Buckinghamshire, UK) and exposure to film. We used the software (AlphaEase FC) to calculate the density of protein expression from Western blot assay and then JNK as a denominator and JNKp as numerator. Similarly, ERK and p38 are the denominator, and ERKp and p38p are numerator to quantitate the protein express.

*2.8. Electrophoretic Mobility Shift Assay.* Electrophoretic mobility shift assay (EMSA) was conducted in accordance with our previous study [12]. The nuclear extract (10 $\mu$g for protein) was incubated in a binding buffer solution with double-stranded biotin-labeled oligonucleotide probes at 25°C for 30 min and then separated using 6% polyacrylamide gel (40% bis/acryl amide (19 : 1), 1.5 mL; 10 × TBE, 250 $\mu$L; 10% APS, 50 $\mu$L; TEMED, 5 $\mu$L; $DDH_2O$, 8.25 mL) electrophoresis (30 V) for 2.5–3.0 h. The protein bands were then transferred to nylon membranes for 1 h, which were irradiated in a UV cross-linking apparatus. Subsequently, the membranes were blocked using a blocking buffer solution (10 × maleic acid buffer, 13 mL; 10 × blocking solution, 13 mL; $DDH_2O$, 100 mL) and probed using alkaline phosphate-conjugated streptavidin. The membrane was washed twice with washing buffer solutions. The oligonucleotides were detected using CSPD and exposure to film.

*2.9. Statistical Analysis.* The data are represented as mean (± standard deviation, SD). The groups were compared by oneway analysis of variance (ANOVA), followed by Tukey's test. Any $P$ values < .05 were considered statistically significant.

## 3. Results

*3.1. Effect of UR, RP, and VA on the Animal Models of KA-Induced Epileptic Seizures in SD Rats.* Peritoneal administration of KA in the SD rats caused epileptic seizure, and their main behaviors included wet dog shakes (WDS) with polyspike-like artifacts observed on the EEG and EMG, facial myoclonia (FM) with continuous sharp waves on the EEG, and paw tremor (PT) with continuous EEG spikes (Figure 1).

The WDS counts for Groups KA and PK increased significantly compared with those for Group N (both $P$ < .001; Figure 2). These increases were lower in Groups UR, RP,

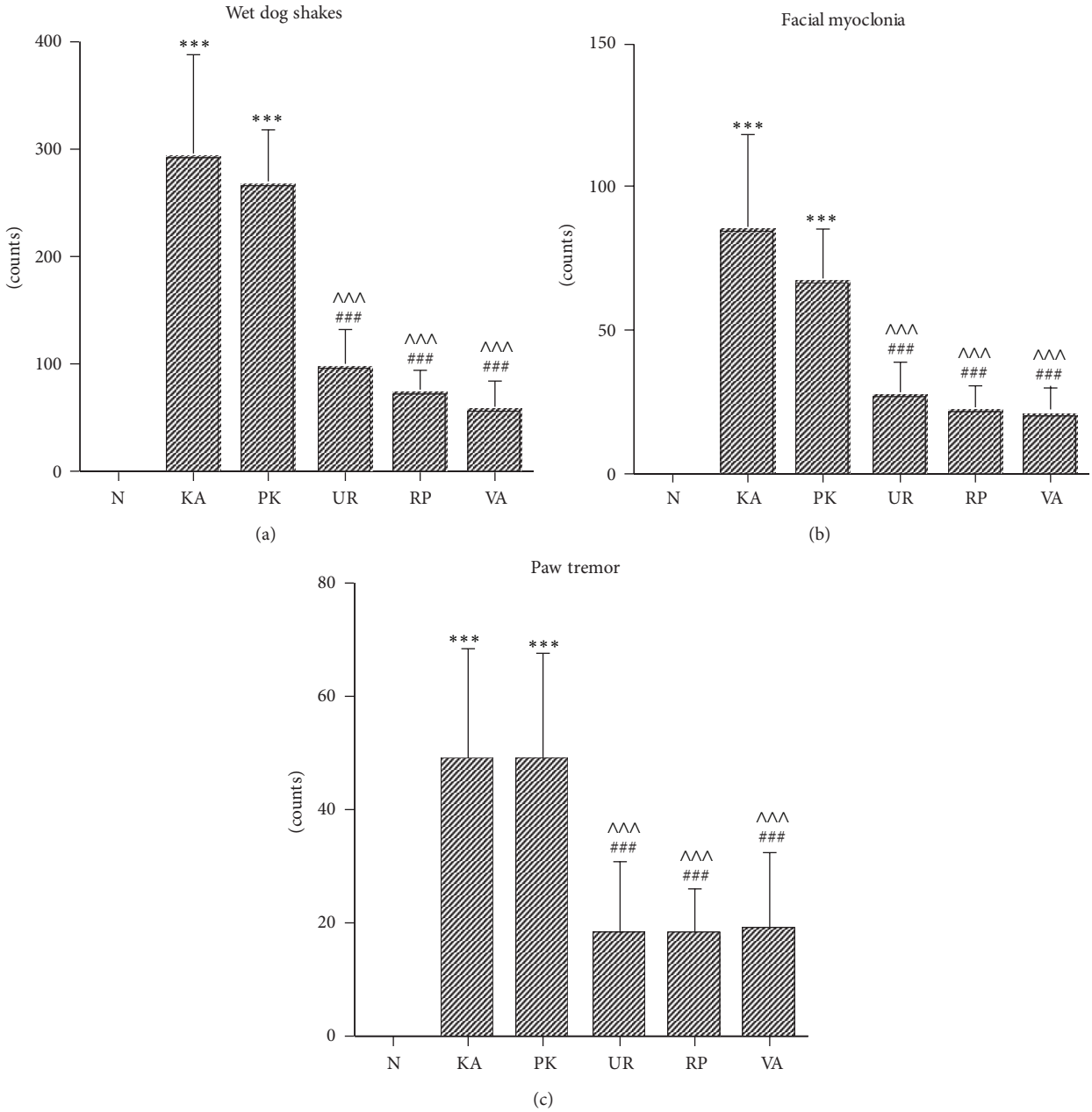


Figure 2: Effect of UR and RP on animal models of KA-induced epileptic seizures in rats. KA (i.p.) increased the frequency of wet dog shakes, facial myoclonias, and paw tremors; these increases were lower in Groups UR, RP, and VA (N, normal group; KA, KA/control group; PK, PK group; UR, UR group; RP, RP group; VA, VA group). $^{***}P < .001$ compared with N; $^{\#\#\#}P < .001$ compared with KA; $^{\wedge\wedge\wedge}P < .001$ compared with PK.

and VA (all $P < .001$; Figure 2). Similar WDS counts were observed between Groups KA and PK, UR and RP, and RP and VA (all $P > .05$; Figure 2).

The FM counts for Groups KA and PK increased significantly compared with those for Group N (both $P < .001$; Figure 2). These increases were lower in Groups UR, RP, and VA (all $P < .001$; Figure 2). The FM counts were similar between Groups KA and PK, UR and RP, and RP and VA (all $P > .05$; Figure 2).

The PT counts for Groups KA and PK were significantly higher than those for Group N (both $P < .001$; Figure 2). These increases were lower in Groups UR, RP, and VA (all $P < .001$; Figure 2). The PT counts for Groups KA and PK were similar, as well as UR and RP, and RP and VA (all $P > .05$; Figure 2).

*3.2. Effect of UR, RP, and VA on IL-1β, IL-6, and TNF-α in the Animal Models of KA-Induced Epileptic Seizures in SD Rats.* The IL-1$\beta$ levels in the cerebral cortex and hippocampus brain tissues were similar between two groups in Groups N, KA, PK, UR, RP, and VA at 3 h following KA administration (all $P > .05$; Figure 3).

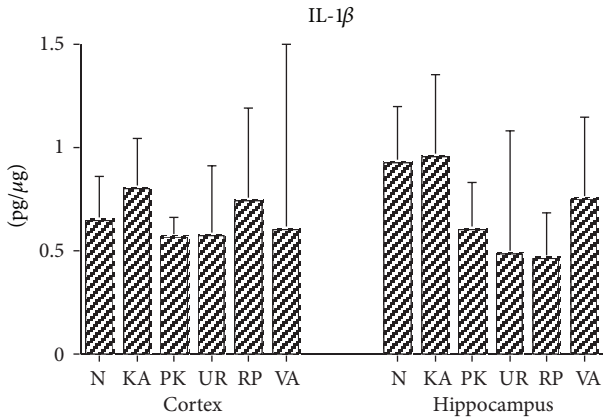


Figure 3: Effect of UR and RP on IL-1$\beta$ in KA-induced epileptic seizures in rats. The IL-1$\beta$ levels in the cerebral cortex (Cortex) and hippocampus (Hippocampus) brain tissues were similar in Groups N, KA, PK, UR, RP, and VA (N: normal group; KA: KA/control group; PK: PK group; UR: UR group; RP: RP group; VA: VA group).

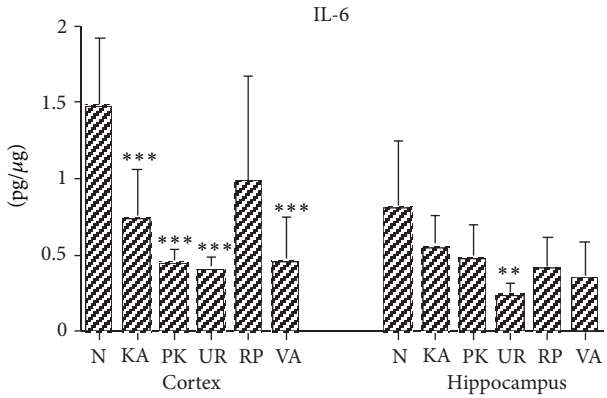


Figure 4: Effect of UR and RP on IL-6 in KA-induced epileptic seizures in rats. The IL-6 levels were lower in the cerebral cortex (Cortex) brain tissue for Groups KA, PK, UR, and VA than those in Group N. The IL-6 levels in the hippocampus (Hippocampus) brain tissue for Group UR were lower than those in Group N (N, normal group; KA, KA/control group; PK, PK group; UR, UR group; RP, RP group; VA, VA group). JNK: $^{*}P < .05$, $^{***}P < .001$ compared with N.

The IL-6 levels in Group N were significantly higher than those in Groups KA, PK, UR, and VA (all $P < .001$; Figure 4). However, the IL-6 levels for Groups N and RP were similar ($P > .05$; Figure 4). The IL-6 levels in the cerebral cortex brain tissues were similar between two groups in Groups KA, PK, UR, RP, and VA at 3 h following KA administration (all $P > .05$; Figure 4).

The IL-6 levels in Group N were significantly higher than those in Group UR ($P < .05$; Figure 4). The IL-6 levels in the hippocampus of brain tissue were similar between two groups in Groups N, KA, PK, RP, and VA at 3 h after KA administration (all $P > .05$; Figure 4).

The TNF-$\alpha$ levels in Group N were significantly higher than those in Groups PK, UR, and VA (all $P < .001$; Figure 5). The TNF-$\alpha$ levels in Groups N, KA, and RP were similar (all

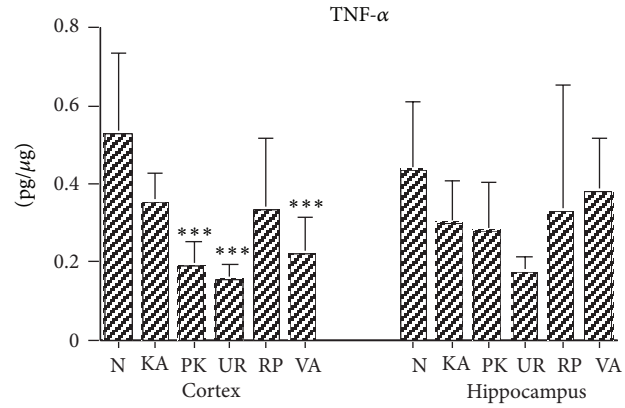


Figure 5: Effect of UR and RP on TNF-$\alpha$ in KA-induced epileptic seizures in rats. The TNF-$\alpha$ level in the cerebral cortex (Cortex) brain tissue for Groups PK, UR, and VA were lower than those in Group N. The TNF-$\alpha$ levels in the hippocampus (Hippocampus) brain tissue were similar in Groups N, KA, PK, UR, RP, and VA (N: normal group; KA: KA/control group; PK: PK group; UR: UR group; RP: RP group; VA: VA group). $^{***}P < .001$ compared with N.

$P > .05$; Figure 5). The TNF-$\alpha$ levels in the cerebral cortex brain tissues were similar between two groups in Groups PK, UR, and VA at 3 h after KA administration (all $P > .05$; Figure 5).

The TNF-$\alpha$ levels in the hippocampus brain tissues were similar between two groups in Groups N, KA, PK, UR, RP, and VA at 3 h following KA administration (all $P > .05$; Figure 5).

*3.3. Effect of UR, RP, and VA on JNK, ERK, p38 MAPK, and NF-κB in the Animal Models of KA-Induced Epileptic Seizures in SD Rats.* In the cerebral cortex brain tissues, an increase in the JNKp expression of MAPK signal pathways was observed in Groups KA and PK, whereas those increases were lower in Groups UR, RP, and VA at 3 h following KA administration (Figure 6).

In the hippocampus brain tissues, an increase in the JNKp expression of MAPK signal pathways was observed in Groups KA and PK, whereas these increases were lower in Groups UR, RP, and VA at 3 h following KA administration (Figure 6).

In both the cerebral cortex and hippocampus brain tissues, the ERKp and p38p expressions of MAPK signal pathways were similar in Groups N, KA, PK, UR, RP, and VA at 3 h following KA administration (Figure 6).

Regarding the NF-κB activity, no conclusive evidence was obtained because of the inconsistent results following 3 repeated experiments (Figure 7).

## 4. Discussion

The results showed that pretreatment using UR, RP, and VA reduced the WDS, FM, and PW counts during the epileptic seizures; indicating that UR, RP, and VA have anticonvulsive effects on animal models of KA-induced epileptic seizures in SD rats; furthermore, these results are in good agreement

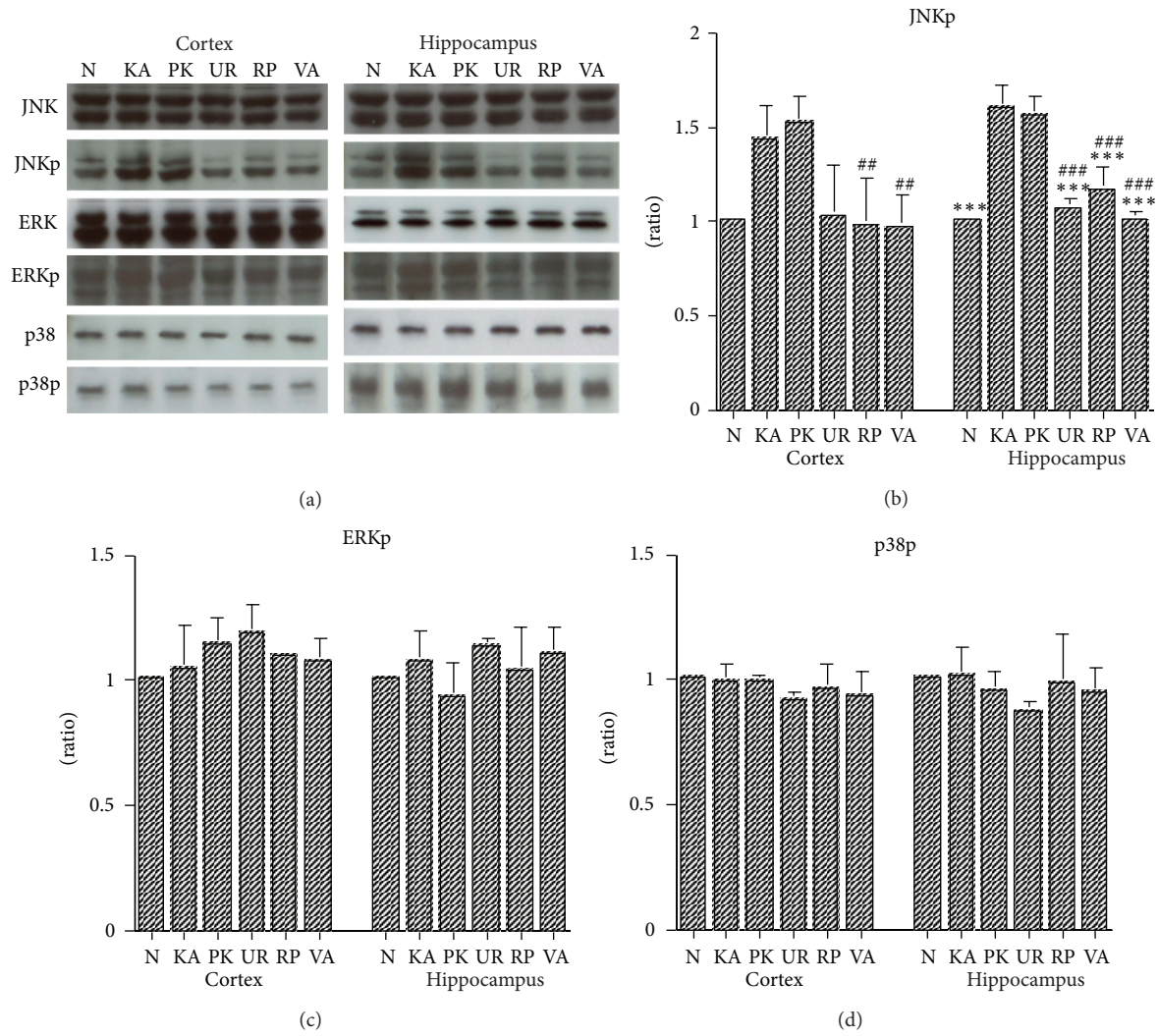


Figure 6: Effect of UR and RP on MAPK in KA-induced epileptic seizures in rats. The JNKp expression of MAPK in the cerebral cortex (Cortex) and hippocampus (Hippocampus) brain tissues increased for Groups KA and PK; these increases were lower in Groups UR, RP and VA. The JNK, ERK, ERKp, p38, p38p expression of MAPk in the cerebral cortex and hippocampus brain tissues were similar in Groups N, KA, PK, UR, RP, and VA (p: phosphorylation; N: normal group; KA, KA/control group; PK: PK group; UR: UR group; RP: RP group; VA: VA group). $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ compared to PK; $^{***}P < .001$ compared to KA.

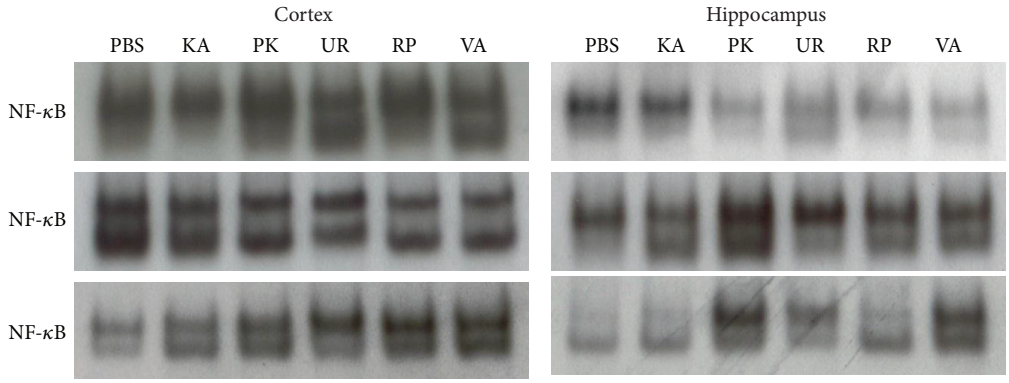


Figure 7: Effect of UR and RP on NF-$\kappa$B activity in KA-induced epileptic seizures in rats. The NF-$\kappa$B activity was no conclusive because of inconsistent results following 3 repeated experiments from EMSA. N: normal group; KA: KA/control group; PK: PK group; UR: UR group; RP: RP group; VA: VA group.

with those from our previous study [12]. Our previous proteomic study showed that under expression of macrophage migration inhibitory factor (MIF) and cylcophilin proteins in either the cerebral cortex or hippocampus brain tissues of KA-treated rats, whereas those under expression could be reversed by administering UR or RP as a pretreatment [13]. Treatment with UR can reduce population spikes in hippocampal neuronal cells, and it can also reduce glial proliferation and S100 protein expression [14]; in addition, it can attenuate mossy fiber sprouting in animal models of KA-induced epileptic seizures in rats [15]. These findings indicate that pretreatment using UR, RP, and VA can suppress the development of epilepsy.

Brain damage such as that caused by encephalitis induces inflammatory responses that can promote neuronal excitability, which contributes to the generation of seizures. Consequently, the inflammation becomes exacerbated and results in epileptogenesis [2]. IL-1$\beta$ is produced in injured brain tissue, and the bonding of IL-1$\beta$ to the IL-1 receptor can initiate the MAPK signaling pathways and NF-$\kappa$B activation [16]. Our results show that the IL-1$\beta$ levels in the cerebral cortex and hippocampus brain tissues were similar between two groups in Groups N, KA, PK, UR, RP, and VA at 3 h after KA administration. Pernot et al. [17] reported that the levels of IL-1$\beta$ mRNA increased between 5 and 24 h following KA intrahippocampal injection.

The results of this study show that the IL-6 levels were greater in Group N than those in Groups KA, PK, UR, and VA, whereas the IL-6 levels in the cerebral cortex brain tissues were similar between two groups in Groups KA, PK, UR, RP, and VA; however, the IL-6 levels in Group N were greater than those in Group UR, whereas the IL-6 levels in the hippocampus brain tissues were similar between two groups in Groups N, KA, PK, RP, and VA at 3 h following KA administration. Based on these results, we considered whether the IL-6 levels in the cerebral cortex and hippocampus brain tissues were unchanged at 3 h following KA administration. Uludag et al. [18] showed that the serum levels of IL-6 and IL-1 receptor antagonist (IL-1Ra) increased at 12 h following seizures in 23 epilepsy patients. Lehtimäki et al. [19] reported that the increased IL-6 levels in the cerebrospinal fluid (CSF) and serum following seizures were greater in recurrent generalized tonic-clonic seizure (GTS) patients than those in either single GTS or prolonged partial seizure patients; therefore, they concluded that seizures could induce cytokine production and that the CSF and serum IL-6 levels might correlate with the severity of a seizure. A recurrent seizure can induce IL-6 production, which can cause changes in neuronal tissue, resulting in the development of refractory seizures.

The results of this study indicated that the TNF-$\alpha$ levels in the cerebral cortex brain tissues for Group N were greater than those in Groups PK, UR, and VA, although the TNF-$\alpha$ levels were similar between two groups in Groups N, KA, and RP, as well as between two groups in Groups PK, UR, and VA; however, the TNF-$\alpha$ levels in the hippocampus brain tissues were similar between two groups in Groups N, KA, PK, UR, RP, and VA at 3 h following KA administration. Accordingly, we considered whether the TNF-$\alpha$ levels in the cerebral cortex and hippocampus brain tissues had not changed at 3 h following KA-induced epileptic seizures. Previous research reported that the TNF-$\alpha$ mRNA peaked at 6 h following limbic seizure with epilepticus [20]. TNF-$\alpha$ plays dual roles of anti- and proconvulsion, which is mediated by its neuronal p75 receptor, which can inhibit seizures and exert an anticonvulsive effect. In contrast, another study reported that TNF-$\alpha$ was mediated by the p55 receptor, which acted as a proconvulsant [21]. This discussion shows that the levels of IL-1$\beta$, IL-6, and TNF-$\alpha$ did not increase at 3 h following KA administration in this study, indicating that timing is a critical factor in the production of IL-1$\beta$, IL-6, and TNF-$\alpha$. Our results indicated that UR, RP, and VA could reduce epileptic seizures, although they did not change the levels of proinflammatory cytokines IL-1$\beta$, IL-6, and TNF-$\alpha$. Therefore, we infer that the anticonvulsive effect of UR, RP, and VA—at least for proinflammatory cytokines IL-1$\beta$, IL-6, and TNF-$\alpha$—did not play a key role in the KA-induced acute seizure.

Our results also indicated that the JNKp expression of the MAPK signal pathway increased in the cerebral cortex and hippocampus in Groups KA and PK. These increases can be attenuated by pretreatment using UR, RP, and VA for 3 d. Previous research showed that MAPKs play a critical role in cell differentiation, proliferation, and death and is activated by phosphorylation cascades [22]. KA is an analogue of glutamate, and can induce limbic seizures. Furthermore, previous research has shown that KA can induce the activation of JNK and p38, which are both involved in cell death [23]. The MAPK protein and its active form increase between 30 and 60 min following pilocarpine-induced epilepticus. Previous research hypothesized that this activation could contribute to the mechanisms of acute epileptogenesis and long-lasting changes of neuropathology [24]. Che et al. [25] reported that p38 MAPK of brain tissue increased at 4 d following KA-induced seizures in mice and that this p38 MAPK signal pathway plays a crucial role in either neuronal death or reactive gliosis. Previous studies have shown that ERK MAPK plays a critical role in cell death [26] and that the MAPK signal pathway plays a critical role in the physiological and biochemical regulation of NMDA receptors [27]. Sokka et al. [28] reported that KA activates non-NMDA receptor and that induced seizures can cause stress of the endoplasmic reticulum (ER), resulting in the accumulation of unfolded protein. Furthermore, this stress of the ER has been shown to cause the activation of JNK, which is similar to cell surface receptor reactions to extracellular signal stimulation [29]. KA can induce the release of glutamate from neuronal cells and can also induce the ER stress of astrocyte. Chihara et al. [30] reported that the old astrocyte specifically induced substance (OASIS) in astrocyte can respond to the ER stress and that it played a protective role in KA-induced ER stress. Lee et al. [31] showed that pretreatment using an alkaloid fraction of UR can block NMDA-induced cytotoxicity, which acts as a neuroprotector by inhibiting apoptosis. In addition, in this study, the results for the NF-$\kappa$B levels were inconclusive because of the inconsistent results following the 3 experiments; the levels of NF-$\kappa$B were the upstream of the pro-inflammatory cytokines IL-1$\beta$, IL-6, and TNF-$\alpha$. Previous studies have reported that the DNA-binding activity of NF-$\kappa$B increased

at 24 h following KA-treatment in rat models [10, 32]. In summary, we assert that the JNKp MAPK signal pathway plays a critical role in epileptogenesis at 3 h following KA administration; therefore, UR, RP, and VA suppression of the expression of JNKp MAPk inhibit the development of epilepsy. Thus, we suggest that the present study is more advantage to develop the novel antiepileptic drug than our previous study [12]. Because the results indicated that JNKp MAPK signal pathway involve to acute seizure.

Some limitation in the present study is as follows: (1) the dose of UR and RP is difficult to determine due to high dose possible reduce the effect of UR; (2) although UR is most commonly used for the treatment of vertigo and epilepsy in Taiwan and in our clinic and according to our knowledge, the severe side effect and herb-drug interaction were not found. The further observation and study still remain to be needed; (3) the present study limits in animal model level. Therefore, how to design a randomized, double blind, placebo-controlled clinical trial in epileptic patient is an import issue in the future.

In conclusion, UR, RP, and VA reduce epileptic seizures and also reduce the JNKp expression of the MAPK signal pathway at 3 h following KA administration, indicating that the antiepileptic effect of UR and RP is involved in initiating the JNKp MAPK signal pathway.

## Conflict of Interests

The authors declare there is no financial or commercial conflict of interests related to this study.

## Acknowledgments

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*Research Article*

# Concurrent Use of Hypnotic Drugs and Chinese Herbal Medicine Therapies among Taiwanese Adults with Insomnia Symptoms: A Population-Based Study

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*Background*. The increased practice of traditional Chinese medicine (TCM) worldwide has raised concerns regarding herb-drug interactions. The purpose of our study is to analyze the concurrent use of Chinese herbal products (CHPs) among Taiwanese insomnia patients taking hypnotic drugs. *Methods*. The usage, frequency of services, and CHP prescribed among 53,949 insomnia sufferers were evaluated from a random sample of 1 million beneficiaries in the National Health Insurance Research Database. A logistic regression method was used to identify the factors that were associated with the coprescription of a CHP and a hypnotic drug. Cox proportional hazards regressions were performed to calculate the hazard ratios (HRs) of hip fracture between the two groups. *Results*. More than 1 of every 3 hypnotic users also used a CHP concurrently. *Jia-Wei-Xiao-Yao-San* (Augmented Rambling Powder) and *Suan-Zao-Ren-Tang* (Zizyphus Combination) were the 2 most commonly used CHPs that were coadministered with hypnotic drugs. The HR of hip fracture for hypnotic-drug users who used a CHP concurrently was 0.57-fold (95% CI = 0.47–0.69) that of hypnotic-drug users who did not use a CHP. *Conclusion*. Exploring potential CHP-drug interactions and integrating both healthcare approaches might be beneficial for the overall health and quality of life of insomnia sufferers.

## 1. Introduction

Inadequate sleep and sleep disorders are common, and are associated with an increased risk of poor health, diminished work and academic performance, and negative safety outcomes that have critical clinical and economic ramifications [1]. Although substantial progress has recently been made in the pharmacologic treatment of insomnia, including the use of benzodiazepines and Z-drugs (zaleplon, zolpidem, and zopiclone), adverse effects and the potential for abuse, addiction, and the development of tolerance have led to poor compliance for hypnotic regimens among insomniac patients [2, 3]. Many poor sleepers have turned to traditional Chinese medicine (TCM) remedies to manage their symptoms because they believe that such treatments exert fewer subjective residual effects [4].

Although TCM remedies are promoted as natural, and therefore harmless, complementary, and alternative medicines that can also be used in Western countries [5, 6], data are limited regarding their safety when used in combination with hypnotic drugs, particularly regarding the risk of herb-drug interactions, such as that resulting from interference with the clearance of either of the drugs. Studies on the prevalence of hypnotic-drug use and the prescription patterns of TCM remedies among hypnotic-drug users are scant.

Comprising unique traditional therapies for various ailments, TCM has been used in Taiwan for hundreds of years, and its popularity remains unabated, despite the present availability of modern medical care in Taiwan. In addition, one distinguishing feature of the national healthcare system in Taiwan is the coexistence of modern Western medicine (WM) and TCM, including acupuncture and manipulative

therapies and Chinese herbal products (CHPs), claims for which have been covered by the National Health Insurance (NHI) system since 1995 [7].

People in Taiwan are free to choose from care offered by WM clinics or TCM clinics. With an insured rate of 98% to 99%, the random sample that comprises the NHI research database (NHIRD) is representative of the general population of Taiwan and should allow a reasonably accurate assessment of the concurrent usage of TCM and modern medical resources in Taiwan; therefore, the NHIRD provides an ideal platform for pharmacoepidemiological studies [8]. Our study aimed to describe the demographics and patterns of CHP usage among hypnotic-drug users and, by using a large population-based retrospective database, explore the risk of hip fracture among a cohort of hypnotic-drug users prescribed with a CHP concurrently compared with hypnotic-drug users who did not use Chinese herbs. Our findings provide evidence-based information for formulating appropriate management strategies for drug safety and integrative medicine.

## 2. Materials and Methods

*2.1. Data Source and Participants.* Our study protocols were approved by the Institutional Review Board of the Committee on Chinese Medicine and Pharmacy (CCMP), Department of Health, Taiwan. Our population-based study retrospectively analyzed the reimbursement records of 1 million NHI beneficiaries in the NHIRD that had been previously selected at random from the 22 million beneficiaries of the NHI to determine the prevalence of concurrent CHP and hypnotic use between January 1, 2002, and December 31, 2008, in Taiwan. The electronic records of the NHIRD use beneficiary identification numbers that are encrypted and maintained by the National Health Research Institutes (NHRI) of Taiwan [9, 10].

The NHIRD records contain demographic information, including age and sex, and clinical data, including all records of clinical visits and hospitalizations, and all information regarding prescribed drugs and dosages, including those for CHPs. The diagnoses used in the NHIRD are coded according to the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) [11].

Because the cost of all prescribed hypnotic drugs is reimbursed by the NHI, they cannot be dispensed at a pharmacy without a physician's prescription. To construct a fixed cohort, all outpatients with insomnia who used hypnotic drugs, including benzodiazepines, zolpidem, and zopiclone, were reviewed. For the purpose of studying the use of CHPs, we downloaded the claims forms for reimbursed CHPs from the website of the Bureau of National Health Insurance. The corresponding information regarding the CHP was then obtained from the CCMP website, including the name of each herb, the proportion of each constituent of the mixture, the date and period of approval for the drug, the CCMP manufacturer code, and the name of the CHP manufacturer. All CHPs with the same CCMP standard formula are classified in the same category, regardless of slight variations in the products among different CHP manufacturers [12]. For our analysis of the demographic and clinical variables, a coprescription of a CHP and a hypnotic drug (defined as prescriptions for both issued simultaneously or issued separately with overlapping treatment periods) was used to determine the use of a CHP and a hypnotic drug on the same day.

The participant selection from the NHIRD was performed as shown in Figure 1. We reviewed all beneficiaries with at least three outpatient visits with any nonorganic insomnia (ICD-9-CM codes 307.40-42, 307.45-49, 780.50-52, and 780.55-59) and excluded patients who were admitted to hospital and received a hip fracture diagnosis between 1999 and 2001 ($n = 138$). Patients with hyperinsomnia (ICD-9-CM codes 327, 307.43, 307.44, 780.53, and 780.54) for the calendar year in which it occurred were excluded. The prevalent insomnia cases diagnosed before the end of 2001 ($n = 9,242$) and patients under 20 years of age ($n = 4,691$) were excluded to ensure that all participants had been newly diagnosed with adult insomnia. Patients with incomplete data for age or sex ($n = 21$) and insomniac patients not taking hypnotic drugs ($n = 10,628$) were also excluded.

*2.2. Study Variables.* To identify the key factors associated with the coadministration of hypnotics and CHP among insomnia sufferers (CAHCHP as an acronym for this population), we selected the demographic factors according to previous studies [1, 7, 8]. Patients were classified, based on age, into one of seven groups as follows: 20–29 years, 30–39 years, 40–49 years, 50–59 years, 60–69 years, 70–79 years, and ≥80 years. The geographic areas of Taiwan in which patients resided were classified as one of the following seven regions: Taipei city, Kaohsiung city, Northern region, Central region, Southern region, Eastern region, and Outlying islands. Patients' monthly income in New Taiwan Dollars (NT$) was categorized as one of the following four levels: $0, $1–$19,999, $20,000–$39,999, and ≥$40,000.

The variables for hypnotic-drug use included in our analyses were defined according to the specific proprietary hypnotic preparation used during the study period. We categorized the types of preparation used as follows: long-half-life benzodiazepine; short-half-life benzodiazepine; zolpidem; zopiclone; and mixed regimens, including two, three, or more of the aforementioned preparations.

To estimate the impact of CHP use on the rate of hip fracture, we selected subjects who were first recorded with a diagnosis of hip fracture (ICD-9-CM codes 820.0–820.9) between January 1, 2002, and December 31, 2008. Furthermore, we also analyzed the risk of hip fracture according to the period of time patients were administered hypnotic drugs (≥30 days and <30 days).

*2.3. Statistical Analysis.* Data analysis was conducted using descriptive statistics, including the prescription rates of patients' concurrent use of a CHP and a sedative hypnotic drug stratified by age and sex, indications for the prescribed CHP, and the most frequently coprescribed herbal formulas for treating insomnia. The indications were coded according to the ICD-9-CM and grouped into different broader disease categories. The ICD-9-CM codes 460–519 were classified as diseases of the respiratory system. Codes 780–799 were grouped as symptoms, signs, and ill-defined conditions, and

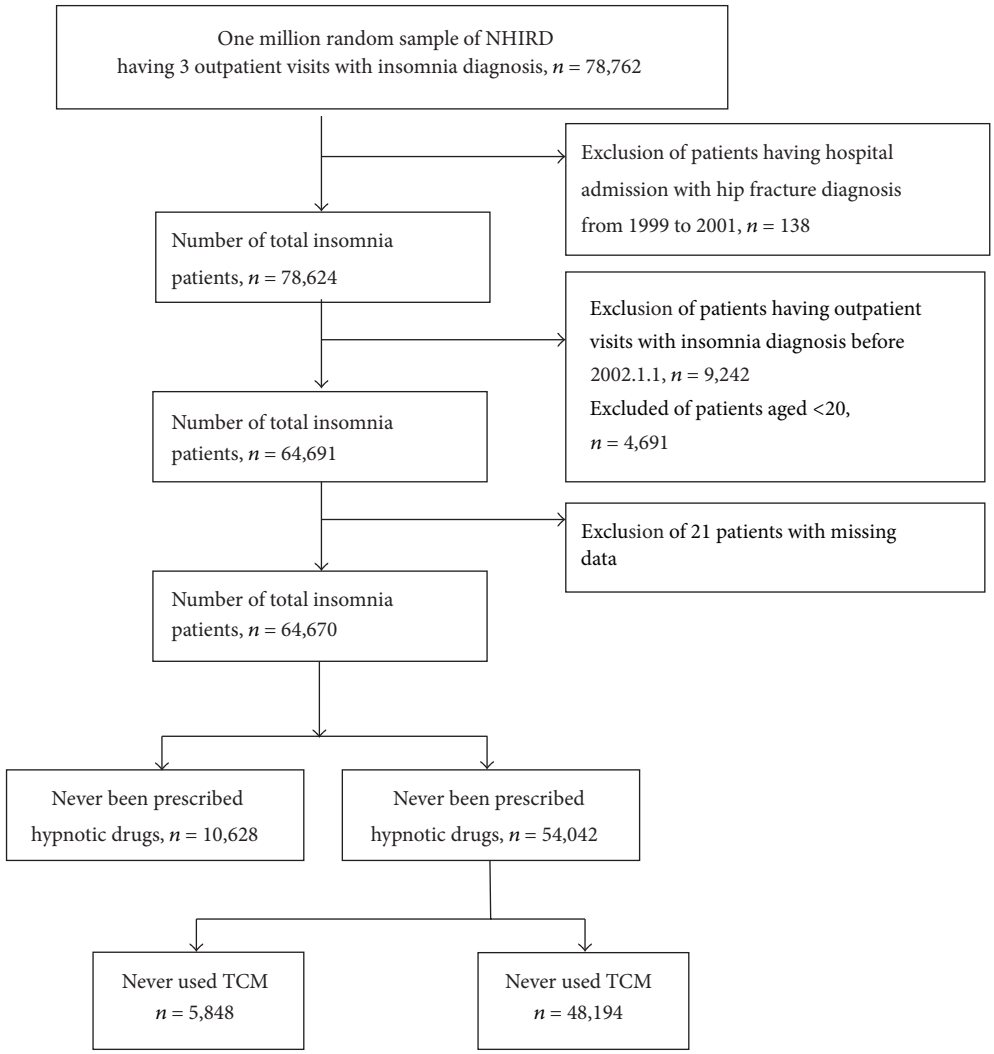


Figure 1: Flowchart of recruitment of subjects with insomnia from the 1 million random sample of the National Health Insurance Research Database (NHIRD) from 2002 to 2008 in Taiwan.

codes 520–579 were classified as diseases of the digestive system. Multiple logistic regression was conducted to evaluate factors that correlated with CHP use based on the odds ratio (OR) and the 95% confidence interval (CI). Cox proportional hazards regressions were performed to calculate the hazard ratio (HR) of hip fracture among hypnotic-drug users who used a CHP concurrently and hypnotic-drug users who did not use Chinese herbs. A significance level of $\alpha = 0.05$ was selected. The SAS statistical software, version 9.3 (SAS Institute, Cary, NC, USA), was used for data management and analyses.

## 3. Results

The database of outpatient claims contained information for 64,670 sleep-disturbance sufferers during the 2002–2008 period. A total of 54,042 (83.6%) insomnia patients treated with sedative-hypnotic drugs were included in our study. Most of these patients used short-acting benzodiazepines. Most of the sedative-hypnotic users (89.2%) also sought care from TCM practitioners. Up to 37.0% ($n = 19,994$) of the sedative-hypnotic users received a coprescription for a CHP, resulting in the coadministration of a sedative-hypnotic drug with a CHP. Such patients who were coadministered the two types of drug are hereafter referred to as CAHCHP patients. A total of 19,994 CAHCHP patients were identified in our insomnia cohort.

The hypnotic users who did not use a CHP were significantly older and more likely to be male than were the CAHCHP patients. More CAHCHP patients had income levels of \$20,000 to \$39,999 and above, resided in Central Taiwan, and used two or more types of sedative-hypnotic drugs than the sedative-hypnotic users who did not use a CHP. The adjusted ORs (aORs) and 95% CIs calculated using the logistic regression model are displayed in Table 1. After adjusting for other factors, the aORs of CAHCHP patients (aORs = 2.49) were higher for women than for men (aORs = 1.00) except patients aged 80 years and older. Compared with the patients aged 40–49 years (aORs = 1.00), the aORs of the CAHCHP patients decreased with age. Patients who used at

Table 1: Demographic characteristics and results of multiple logistic regression showing the adjusted odds ratio (aOR) and 95% confidence interval (CI) of patients with newly diagnosed insomnia ever been prescribed hypnotic drugs from the 1 million random sample of the National Health Insurance Research Database (NHIRD) from 2002 to 2008 in Taiwan.

| Characteristics | Total no. | Hypnotic users without using Chinese medicine, no. (%) | Hypnotic users coprescribed Chinese medicine, no. (%) | Hypnotic users coprescribed Chinese medicine/hypnotic users without using Chinese medicine | |
|---|---|---|---|---|---|
| | | | | Odds ratio | (95% CI) |
| Numbers of hypnotic users among insomnia population | 54,042 | 5,848 (10.8) | 19,994 (37.0) | | |
| Sex | | | | | |
| Male | 19,354 | 3,234 (55.3) | 6,350 (31.8) | 1.00 | (2.34–2.66) |
| Female | 34,688 | 2,614 (44.7) | 13,644 (68.2) | 2.49 | |
| Mean (SD) age at inclusion, years | 48.8 (15.4) | 55.1 (17.2) | 49.9 (14.9) | | |
| *Age groups at inclusion, years no. (%)* | | | | | |
| 20~29 | 5,616 | 362 (6.2) | 1,631 (8.2) | 1.06 | (0.93–1.22) |
| Male | | 184 (3.0) | 477 (5.8) | | |
| Female | | 178 (3.2) | 1,154 (2.4) | | |
| 30~39 | 11,105 | 891 (15.2) | 3,820 (19.1) | 1.03 | (0.94–1.14) |
| Male | | 506 (6.6) | 1,184 (13.2) | | |
| Female | | 385 (8.6) | 2,636 (5.9) | | |
| 40~49 | 13,531 | 1,193 (20.4) | 5,074 (25.4) | 1.00 | |
| Male | | 675 (8.9) | 1,536 (17.7) | | |
| Female | | 518 (11.5) | 3,538 (7.7) | | |
| 50~59 | 10,637 | 1,072 (18.3) | 4,184 (20.9) | 0.85 | (0.77–0.93) |
| Male | | 550 (8.9) | 1,118 (15.3) | | |
| Female | | 522 (9.4) | 3,066 (5.6) | | |
| 60~69 | 6,657 | 865 (14.8) | 2,798 (14.0) | 0.76 | (0.68–0.84) |
| Male | | 488 (6.5) | 962 (9.2) | | |
| Female | | 377 (8.3) | 1,836 (4.8) | | |
| 70~79 | 4,787 | 922 (15.8) | 1,951 (9.8) | 0.52 | (0.46–0.58) |
| Male | | 545 (6.5) | 821 (5.7) | | |
| Female | | 377 (9.3) | 1,130 (4.1) | | |
| Over 80 | 1,709 | 543 (9.3) | 536 (2.7) | 0.24 | (0.21–0.28) |
| Male | | 286 (4.4) | 252 (1.4) | | |
| Female | | 257 (4.9) | 284 (1.3) | | |
| *$NT/month (Premiums)* | | | | | |
| 0 | 11,542 | 1,279 (21.9) | 4,735 (23.7) | 1.00 | |
| 1–19,999 | 27,391 | 3,268 (55.9) | 10,123 (50.6) | 0.85 | (0.79–0.92) |
| 20,000–39,999 | 9,601 | 729 (12.5) | 3,303 (16.5) | 1.13 | (1.01–1.26) |
| ≥40,000 | 5,508 | 572 (9.8) | 1,833 (9.2) | 1.03 | (0.91–1.17) |
| *Insured area* | | | | | |
| Taipei city | 9,495 | 1,159 (19.8) | 3,239 (16.2) | 1.00 | |
| Kaohsiung city | 3,735 | 364 (6.2) | 1,392 (7.0) | 1.46 | (1.27–1.68) |
| Northern Taiwan | 14,900 | 1,848 (31.6) | 4,929 (24.7) | 0.98 | (0.90–1.08) |
| Central Taiwan | 12,554 | 824 (14.1) | 5,490 (27.5) | 2.49 | (2.24–2.77) |
| Southern Taiwan | 11,910 | 1,401 (24.0) | 4,407 (22.0) | 1.28 | (1.16–1.41) |
| Eastern Taiwan | 1,078 | 155 (2.7) | 439 (2.2) | 1.01 | (0.82–1.24) |
| Outlying islands | 370 | 97 (1.7) | 98 (0.5) | 0.36 | (0.27–0.49) |

Table 1: Continued.

| Characteristics | Total no. | Hypnotic users without using Chinese medicine, no. (%) | Hypnotic users coprescribed Chinese medicine, no. (%) | Hypnotic users coprescribed Chinese medicine/hypnotic users without using Chinese medicine | |
|---|---|---|---|---|---|
| | | | | Odds ratio | (95% CI) |
| *Types and prescription patterns of hypnotics* | | | | | |
| Usual treatment regimens | 16,182 | 1,352 (23.1) | 3,310 (16.6) | 1.00 | |
| Zolpidem | 1,623 | 350 (6.0) | 265 (1.3) | | |
| Zopiclone | 109 | 16 (0.3) | 20 (0.1) | | |
| BZD-long | 8,657 | 293 (5.0) | 1,703 (8.5) | | |
| BZD-short | 5,793 | 693 (11.9) | 1,322 (6.6) | | |
| Mixed regimens | | | | | |
| Co-prescribed two types of hypnotics | 18,466 | 2,168 (37.1) | 6,734 (33.6) | 1.30 | (1.20–1.42) |
| Zolpidem + Zopiclone | 79 | 20 (0.3) | 18 (0.1) | | |
| Zolpidem + BZD-long | 1,796 | 275 (4.7) | 486 (2.4) | | |
| Zolpidem + BZD-short | 4,418 | 849 (14.5) | 1,499 (7.5) | | |
| Zopiclone + BZD-long | 136 | 25 (0.4) | 40 (0.2) | | |
| Zopiclone + BZD-short | 308 | 63 (1.1) | 99 (0.5) | | |
| BZD-long + BZD-short | 11,729 | 936 (16.0) | 4,592 (23.0) | | |
| Coprescribed more than two types of hypnotics | 19,394 | 2,328 (39.8) | 9,950 (49.8) | 1.90 | (1.75–2.06) |

NT$ refers to new Taiwan dollars, of which 1 US$ = 30 NT$.

least two types of sedative hypnotic drugs (two types: OR = 1.30; 95% CI: 1.20–1.42 and ≥ three types: OR = 1.90; 95% CI: 1.75–2.06) were more likely to be CHP users than were the patients who used only one sedative hypnotic drug.

By analyzing the percentage distribution of the 19,994 CAHCHP patients, we observed that sleep disturbance (1,450,671 TCM visits) was the most common major disease category for coprescription events, followed by diseases of the digestive system (1,349,092 TCM visits) and the diseases of the respiratory system (1,319,859 TCM visits), as summarized in Table 2. As shown in Table 3, zolpidem, alprazolam, lorazepam, estazolam, and fludiazepam were the five sedative hypnotic drugs that were most frequently coprescribed with a CHP. The details of the most frequently used CHP formulas are shown in Table 3. *Suan-Zao-Ren-Tang* (Zizyphus Combination) was the most frequently used CHP, followed by *Jia-Wei-Xiao-Yao-San* (Augmented Rambling Powder) and *Tian-Wang-Bu-Xin-Dan* (Ginseng and Zizyphus Combination). The average number of days of concurrent use of a sedative hypnotic drug and a CHP was approximately 30 days per CAHCHP patient during the 7-year study period.

The HRs of hip fracture for CAHCHP patients and hypnotic-drug users who did not use Chinese herbs are presented in Table 4. After adjusting for potential confounders, the HR of hip fracture for CAHCHP patient was 0.57-fold (95% CI = 0.47–0.69) that of comparison subjects. We further analyzed the risk of hip fracture according to the length of hypnotic treatment. Table 4 shows that, compared with hypnotic-drug users who did not use Chinese herbs, the HR of hip fracture for CAHCHP patients who were prescribed hypnotics for ≥30 days was 0.62 (95% CI = 0.50–0.77) and as low as 0.33 (95% CI = 0.20–0.54) for CAHCHP patient who were prescribed hypnotics for <30 days. The incidence rate of hip fracture among the CAHCHP patients was no higher than that of hypnotic-drug users who did not use Chinese herbs (≥30-day group: 3.4 versus 6.5 per 100 person-years; <30-day group: 2.9 versus 11.1 per 100 person-years).

## 4. Discussion

According to our review of the literature, this study is the first to use a random population-based cohort to document the coprescription of hypnotic drugs and CHPs in insomnia patients in Taiwan. We observed that hypnotic use is common among insomnia patients in Taiwan and that benzodiazepines were the most frequently prescribed category of sedative hypnotic, as shown in Table 1. Previous studies have reported that patients treated with benzodiazepines may be at higher risk for falls and have been associated with an increased risk of hip fracture. Both physicians and patients should be aware of the association between hypnotic use and the potential risk for injury in Taiwan. The possibility of recall or selection bias can be excluded because we included patients who were newly diagnosed with insomnia by qualified conventional physicians during the 2002–2008 period from a random sample of the population-based NHI database.

The prevalence of the concurrent use of sedative hypnotics and CHPs was 37% (Figure 1). The abuse of benzodiazepines has become a serious problem in North America [13]. The fear of sedative hypnotic side effects or dependency may motivate patients to seek TCM therapies [2, 3, 14]. Although barbiturates have been reported to be the most prescribed hypnotic in Taiwan, our findings indicate a change

Table 2: Frequency distribution of traditional Chinese medicine (TCM) visits by major disease categories (according to 9th ICD codes) in subjects with insomnia ever been prescribed hypnotic drugs from 2002 to 2008 in Taiwan.

| Diagnosis | ICD-9-CM codes | Treatment days (people) | |
|---|---|---|---|
| | | Chinese herbal remedies | Acupuncture and manipulative therapies |
| Infectious and parasitic diseases | 001–139 | 46,426 (2,027) | 47 (14) |
| Neoplasms | 140–239 | 63,080 (904) | 777 (32) |
| Endocrine, nutritional, and metabolic diseases and immunity disorders | 240–279 | 213,461 (4,425) | 890 (90) |
| Psychotic diseases | 290–319 | 164,603 (4,417) | 1,781 (135) |
| Somnambulism | 307.4 | 57,639 (2,073) | 268 (34) |
| Others | | 106,964 (2,542) | 1,513 (105) |
| Disease of nervous system and sense organs | 320–389 | 332,372 (10,391) | 6,057 (1,200) |
| Disease of circulation system | 390–459 | 257,825 (6,088) | 4,384 (288) |
| Disease of respiratory system | 460–519 | 1,319,859 (26,741) | 2,334 (253) |
| Disease of digestive system | 520–579 | 1,349,092 (25,113) | 3,501 (275) |
| Disease of genitourinary system | 580–629 | 824,093 (15,653) | 4,102 (189) |
| Disease of the skin and subcutaneous tissues | 680–709 | 238,578 (8,315) | 857 (72) |
| Disease of musculoskeletal system and connective tissue | 710–739 | 647,207 (17,991) | 135,465 (23,473) |
| Symptom, signs, and ill-defined conditions | 780–799 | 3,036,297 (40,342) | 11,642 (1,348) |
| Sleep disturbance | 780.5 | 1,450,671 (35,386) | 6,733 (590) |
| Others | | 1,585,626 (29,955) | 4,909 (845) |
| Injury and poisoning | 800–999 | 46,331 (3,324) | 138,053 (26,466) |
| Supplementary classification | V01–V82, E800–E999 | 375 (30)<br>0 (0) | 2 (2)<br>4 (1) |
| Others* | | 67,793 (2,570) | 1,053 (353) |
| Total | | 8,607,392 (45,847) | 310,943 (34,454) |

*Include ranges of 280–289, 630–677, 740–759, and 760–779 ICD-9-CM code and missing data.

Table 3: The top five coprescribed Chinese formulas and sedative and hypnotic drugs for treating insomnia (ICD9: 307.4 or 780.5) between 2002 and 2008.

| Chinese Medicine-sedative and hypnotic drugs | Total days of coprescribing | Total people of coprescribing | Average days of coprescribing Chinese and Western medicine (days/people) |
|---|---|---|---|
| Total | | | |
| Formulae | 1,507,601 | 18,837 | |
| *Jia-Wei-Xiao-Yao-San* (Augmented Rambling Powder) | 70,708 | 4,029 | 17.5 |
| *Suan-Zao-Ren-Tang* (Zizyphus Combination) | 58,115 | 3,646 | 15.9 |
| *Tian-Wang-Bu-Xin-Dan* (Ginseng and Zizyphus Combination) | 46,040 | 2,744 | 16.8 |
| *Chai-Hu-Jia-Long-Gu-Mu-Li-Tang* (Bupleurum and Mu Li Combination) | 39,494 | 2,409 | 16.4 |
| *Gan-Mai-Da-Zao-Tang* (Licorice and Jujube Combination) | 38,158 | 2,097 | 18.2 |
| Sedative and hypnotic drugs | 2,041,718 | 19,994 | |
| Zolpidem[1] | 462,395 | 6,415 | 30.6 |
| Alprazolam[1] | 313,833 | 4,990 | 27.0 |
| Lorazepam[1] | 276,865 | 5,053 | 24.4 |
| Estazolam[1] | 180,646 | 2,400 | 31.3 |
| Fludiazepam[1] | 171,842 | 2,229 | 34.0 |

[1]Short-acting BZD (elimination half time ≤ 24 hours).

Table 4: Number (no.) of new cases, population-at-risk, and incidence rates and hazard ratios (HR); 95% confidence intervals (CI) for hip fracture estimated from multivariate Cox regression model on a random sample of the National Health Insurance Research Database among sample subjects and followed from 2002 to 2008.

| Presence of hip fracture during the follow-up period | Hypnotic users without using Chinese medicine, no. cases/population | Hypnotic users coprescribed Chinese medicine, no. cases/population | Hypnotic users coprescribed Chinese medicine/hypnotic users without using Chinese medicine | |
|---|---|---|---|---|
| | | | HR | (95% CI) |
| *Numbers of hip fractures among hypnotic users* | 152/5,848 | 294/19,994 | 0.57 | (0.47–0.69) |
| ≥30 days<br>Incidence rate per 1,000 person-years | 123/5,045<br>6.5 | 260/17,149<br>3.4 | 0.62 | (0.50–0.77) |
| <30 days<br>Incidence rate per 1,000 person-years | 29/803<br>11.1 | 34/2,845<br>2.9 | 0.33 | (0.20–0.54) |

in the prescribing habits of physicians, with nonbarbiturates, particularly zolpidem, being coprescribed more frequently than barbiturates. We also observed that poor sleepers who were prescribed multiple types of hypnotics were more likely to use a CHP concurrently compared with patients who did not seek TCM treatment.

In Taiwan, the prescribing of hypnotic drugs must be accompanied by a standard ICD-9-CM diagnosis code [9, 10] to adhere to the requirements for NHI claims reimbursement. The change in prescription patterns that we observed might represent an attempt by physicians to achieve a greater therapeutic effect for patients with a history of tolerance, poor response, or dependence in previous hypnotic-drug treatment [15] or it might reflect an increasing concern among physicians regarding the potential abuse of sedative hypnotic drugs. Considering the increasing incidence of hypnotic-CHP coprescriptions, we suggest that a more critical attitude toward the use of hypnotics and CHPs in combination is required among both physicians and hypnotic-drug users. We observed that hypnotic-drug users who use a CHP concurrently were not more strongly associated with an increased risk of hip fracture than were hypnotic users who did not use Chinese herbs. Little is known regarding the potential interactions of CHPs with hypnotic drugs; therefore, healthcare providers and public-health policy analysts should focus greater attention on the potential long-term impact of this particular healthcare-seeking behavior on health outcomes [16].

Our previous clinical trials demonstrated that *Jia-Wei-Xiao-Yao-San* (Augmented Rambling Powder) and *Suan-Zao-Ren-Tang* (Zizyphus Combination), the two CHPs that were most commonly coprescribed with a hypnotic drug, may be an efficacious therapy for improving sleep quality [5, 6], as shown in Table 3. Among the five most frequently coprescribed formulas for treating insomnia, *Tian-Wang-Bu-Xin-Dan* (Ginseng and Zizyphus Combination), *Gan-Mai-Da-Zao-Tang* (Licorice and Jujube Combination), and *Chai-Hu-Jia-Long-Gu-Mu-Li-Tang* (Bupleurumand Mu Li Combination) all have a long history of use in Taiwan. They are said to nourish the blood and calm the nerves and are frequently prescribed by TCM practitioners to alleviate sleep disturbances [17].

The concomitant use of benzodiazepines and Z-drugs was the most common combination of hypnotics that were used by patients who used multiple types of hypnotics. Because of a lack of sufficient evidence supporting this less judicious prescription pattern, we suggest that such patients may be more likely to "doctor shop," resulting in the high prevalence of the concurrent use of CHPs and hypnotic drugs. Our findings indicate that the lower incidence rate and HR of hip fracture in CAHCHP patients than that in hypnotic-drug users who did not use Chinese herbs might imply that TCM physicians in Taiwan encouraged insomnia patients to coadminister CHPs and hypnotic drugs to improve sleep quality, which might allow lower hypnotic dosages and reduce the risk of hypnotic adverse effects and dependence. However, insufficient evidence exists to support such a conclusion.

Although previous studies have demonstrated that acupuncture might be an alternative therapy for insomnia [18, 19], our data indicated that insomnia patients typically sought acupuncture treatment for injury, poisoning, and diseases of the musculoskeletal system and connective tissue. Following insomnia, diseases of the digestive system were the second most frequent disease category for TCM visits by CAHCHP patients. These results indicate that health care providers should also address the general health condition of insomnia patients, particularly gastroenterological symptoms, to identify possible causes of insomnia and provide appropriate treatment for other such medical needs that do not require hypnotic treatment. Further studies are warranted to investigate the cost effectiveness of the coadministration of hypnotic drugs with CHPs and comprehensive gastroenterological care for insomnia patients, particularly those with a history of hypnotic dependence.

Our study has three limitations: first, the NHI only reimburses the cost of the CHP; the cost of decoction is not reimbursed, which may have affected patients' decisions to use CHPs. Thus, the frequency of the concurrent use of hypnotic drugs and CHPs may be underestimated in our results. However, because the NHI provides comprehensive coverage and the copayment for prescriptions is always $50 (approximately equal to US $1.50), which is less than the typical cost of herbs sold in Taiwan's markets, the likelihood that patients purchased herbs outside of the NHI system is low. Second, we were unable to draw any conclusions regarding the relationship between the severity of the insomnia and TCM usage because such clinical data are not included in the NHIRD. Third, the retrospective design of our study

and the lack of a randomized placebo group might diminish the statistical power of our findings. Thus, our results must be interpreted cautiously because we cannot exclude the possibility of placebo effects.

## 5. Conclusion

Our findings may have implications for the treatment of insomnia patients. Our results suggest that, with equal availability of conventional medical and TCM care, more than one-third of the hypnotic-drug users used CHPs concurrently for the relief of sleep disturbance and gastroenterological symptoms. Recognizing the benefits of TCM, exploring potential interactions and adverse effects, and integrating both healthcare approaches might be beneficial to the overall health and quality of life of insomnia patients, particularly those with a history of hypnotic dependence. Thus, health care providers should proactively explore personalized, optimal detoxification for hypnotic dependence while attending to patients' psychosocial and physical needs.

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*Research Article*

# *Rhodiola rosea* Impairs Acquisition and Expression of Conditioned Place Preference Induced by Cocaine

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A novel approach to the treatment of adverse effects of drugs of abuse is one which makes use of natural products. The present study investigated the effect of *Rhodiola rosea* L. hydroalcoholic extract (RHO) on cocaine-induced hyperactivity and conditioned place preference (CPP) in mice. In a first experiment, mice received RHO (15, 20 or 25 mg/kg, IG), cocaine (25 mg/kg, i.p.) (COC), or a combination of both drugs (COC + RHO15, COC + RHO20, and COC + RHO25), and their locomotor activity was evaluated. In a second experiment, the effects of RHO on the acquisition, expression, and reinstatement of cocaine CPP (induced by drug priming or social defeat stress) were evaluated. RHO alone did not increase activity but potentiated the hyperactivity induced by cocaine. *Rhodiola* did not induce motivational effects by itself but attenuated the acquisition and expression of cocaine-induced CPP. Moreover, it was found that RHO did not block reinstatement. The results indicate that RHO is effective in reducing the rewarding properties of cocaine but is ineffective in preventing priming or stress-induced cocaine reinstatement. In light of these findings, the benefits of *Rhodiola rosea* L. as a treatment of cocaine addiction would seem to be limited.

## 1. Introduction

Cocaine addiction has become one of the most serious economic and health problems of developed societies, as it affects a great number of individuals [1]. Development of effective treatments for cocaine dependence is necessary to reduce its impact upon both individual and society. However, currently, there are no approved medications for cocaine dependence, despite numerous studies of pharmacologic agents in both animal and human models [2, 3]. A novel approach to the treatment of adverse effects of drugs of abuse is one which makes use of natural products. Recently, there has been an increase in the number of preclinical models and clinical trials addressing the effectiveness of such agents and their active constituents [4]. *Rhodiola rosea* L. (fam. Crassulaceae) is a well-known traditional oriental medicine with adaptogenic, anxiolytic, antidepressive, and antistress properties [5–10]. The positive effects of a *Rhodiola rosea* L. extract (RHO) in preventing nicotine and morphine withdrawal symptoms and countering the development of dependence on these drugs have recently been demonstrated in animal models [11, 12]. A recent study demonstrated that the acquisition, expression, and reinstatement of morphine-induced conditioned place preference (CPP) are blocked by RHO [13]. These results suggest that RHO is capable of reducing craving and vulnerability to relapse and might be an effective natural remedy for the treatment of opioid addiction [13].

The CPP paradigm has been used to evaluate the rewarding action of drugs of abuse, including cocaine [14–16], and it can be used as an animal model of relapse to drug-seeking behaviour when, after extinction of CPP, exposure to drug priming or stress induces the reinstatement of CPP [17]. In previous studies, we have demonstrated that cocaine induces CPP in mice at doses between 1 and 50 mg/kg [18–22], and that cocaine priming produces reinstatement of cocaine-induced CPP [18, 19, 21, 22]. Several stressful events also induce reinstatement of cocaine CPP [17, 23–25], including social defeat stress [26].

The present study aimed to evaluate the effects of RHO on the motor and rewarding effects of cocaine in the CPP paradigm because RHO appears to modulate the levels and activities of biogenic monoamines, such as serotonin (5-HT), dopamine (DA), and noradrenaline (NA), in the neural pathways involved in the regulation of addiction [6, 27–29]. We hypothesised that the effects of cocaine can be altered by RHO administration but in a different way to those observed with morphine, since behavioural and neurobiological differences between opiates and psychostimulants have been reported [30]. Therefore, the objective of the present work was to test the effectiveness of RHO as a treatment for cocaine addiction in an animal model. We evaluated whether treatment with RHO altered the hyperactivity and rewarding effects of cocaine (acquisition, expression, and priming- or stress-induced reinstatement of CPP) in mice.

## 2. Materials and Methods

*Subjects*. OF1 male mice (Charles River, Barcelona, Spain) arrived at the laboratory at 42 days of age and were housed in groups of four in plastic cages (28 cm × 28 cm × 14.5 cm) for 10 days prior to the initiation of experiments, under the following conditions: constant temperature (21 ± 2°C), a light schedule (white lights on: 07.30–19.30 h), and food and water available ad libitum. Each animal was handled briefly on the 3 days preceding the initiation of experiments and acclimatised to intragastric administration. Procedures involving mice and their care were conducted in compliance with national, regional, and local laws and regulations, which are in accordance with the European Communities Council Directives of 24 November 1986 (86/609/EEC).

*Apparatus*. Locomotor activity was automatically measured by an actimeter (CIBERTEC S.A., Spain) consisting of eight cages (33 × 15 × 13 cm), each with eight infrared lights located in a frame around the cage (body level of mice). The different frames are separated from each other by a distance of 4 cm and, since they are opaque, prevent animals from seeing cospecifics. The conditioning place preference apparatus consists of four identical Plexiglas place-conditioning boxes. Each box consists of two equally sized compartments (30.7 cm × 31.5 cm × 34.5 cm) separated by a gray central area (13.8 cm × 31.5 cm × 34.5 cm). The compartments have different coloured walls (black versus white) and distinct floor textures (smooth in the black compartment and rough in the white). Four infrared light beams in each compartment of the box and six in the central area allow the recording of the position of the animal and its crossings from one compartment to the other. The equipment was controlled by an IBM PC computer using MONPRE 2Z software (CIBERTEC, SA, Spain).

*Drugs*. A dried hydroalcoholic extract from the roots of *Rhodiola rosea* L. (RHO) was employed for the experiments (EPO S.r.l., Milan, Italy; lot number 601252). The HPLC analysis showed a content of 3% total rosavins, expressed as rosavin, 1% salidroside, and 0.8% tyrosol (for more details see [13]). The extract was dissolved in 1% v/v ethanol solution and administered by gavage (IG) at doses of 15, 20, and 25 mg/kg/10 mL. The same vehicle (1% v/v ethanol solution) was administered IG to the control groups. The doses of RHO were selected based on previous studies in which 10, 15, and 20 mg/kg blocked the effects of nicotine and morphine [11–13]. In the present experiments given that a different drug of abuse was being evaluated, we employed a higher dose of RHO (25 mg/kg) and did not employ 10 mg/kg (the less effective dose in previous studies [11–13]). Cocaine chlorhydrate (Laboratorios Alcaliber, Madrid, Spain) was dissolved in saline (NaCl 0.9%) in a volume of 0.01 mL/g and was administered intraperitoneally (i.p.) at a dose of 25 mg/kg. This dose was selected based on a previous study in which it consistently induced CPP [18]. Half of this dose was used for cocaine priming (12.5 mg/kg), also due to results obtained in the aforementioned study [18].

*Experiment 1.* Effects of RHO on cocaine-induced hyperactivity.

*2.1. Procedure and Experimental Design.* In this experiment, eight groups of mice were used ($n = 8$ per group). Spontaneous motor activity was recorded for 30 minutes (habituation, 0–30 min). Afterwards, two groups received a vehicle, two groups received 15 mg/kg of RHO, two groups received 20 mg/kg of RHO, and the last two groups received 25 mg/kg of RHO, and their activity was recorded for 60 minutes in blocks of 30 min (31–60, 61–90 min). Next, each of the two previously named groups was given either saline (groups Veh-Sal, RHO15, RHO20, RHO25) or 25 mg/kg of cocaine (Veh-Coc, RHO15-Coc, RHO20-Coc, and RHO25-Coc), and their activity was registered for a further 60 minutes (91–120, 121–150 min).

*2.2. Statistical Analysis.* Motor activity data registered during the total 150 min register were analyzed using a mixed analysis of variance (ANOVA) with one "between-subjects" variable "treatment" with eight levels (Veh-Sal, RHO15, RHO20, RHO25, Veh-Coc, RHO15 + Coc, RHO20 + Coc, and RHO25 + Coc) and a "within-subjects" variable "time" with five levels (0–30, 31–60, 61–90, 91–120, and 121–150). All post hoc comparisons were performed with the Bonferroni multiple comparisons test (corrected "alpha" 0.05/40). Calculations were made using the SPSS statistical package 17.0. A $P$ value of less than 0.05 was considered statistically significant.

*Experiment 2.* Effects of RHO on the acquisition, expression, and reinstatement of cocaine-induced CPP.

*2.3. Procedure and Experimental Design.* The CPP procedure, unbiased in terms of initial spontaneous preference, was performed as described previously [18]. In short, in the first phase (preconditioning/pre-C), mice were allowed access to both compartments of the apparatus for 15 min (900 s) per day over 2 days. On day 3, the time spent in each compartment during a 900 s-period was recorded. Animals showed strong unconditioned aversion (less than 27% of the session time, i.e., 250 s) or preference (more than 73%, i.e., 650 s) for any

compartment and were therefore discarded from the rest of the experimental procedure. In each group, half the animals received the drug or physiological saline in one compartment, and the other half received it in the other. After assigning animals to the compartments, an analysis of variance (ANOVA) revealed no significant differences between the time spent in the drug-paired and vehicle-paired compartments during the preconditioning phase. This is an important step in the experimental procedure that avoids any preference bias prior to conditioning. In a second phase (conditioning or acquisition) of 4 days, animals received an injection of physiological saline before being confined to the saline-paired compartment for 30 minutes, and after an interval of 4 h received an injection of 25 mg/kg of cocaine immediately before being confined to the drug-paired compartment for 30 minutes. In this phase, RHO or the vehicle was administered 60 min prior to initiation of conditioning (i.e., 60 min before the cocaine injection in the corresponding groups). Confinement was imposed in both cases by closing the guillotine door that separated the two compartments. During the third phase (postconditioning/post-C), which took place on day 8, the guillotine door separating the two compartments was removed and the time spent by the untreated mice in each compartment during a 900 s observation period was recorded. The difference in seconds between the time spent in the drug-paired compartment in the post-C test and that spent in the pre-C phase is a measure of the degree of reward induced by the drug.

In order to evaluate the role of RHO in the acquisition of cocaine-induced CPP, eight groups of mice ($n$ = 11–13 per group) were assessed. The control group received IG vehicle and one IP injection of saline (Veh + Sal), three groups received RHO 15, 20 or 25 mg/kg plus saline (RHO15, RHO 20, and RHO 25), one group received vehicle plus 25 mg/kg of cocaine (Veh + Coc), and the last three groups received RHO 15, 20, or 25 mg/kg plus 25 mg/kg of cocaine (RHO15 + Coc, RHO20 + Coc, and RHO25 + Coc). The vehicle or RHO was administered IG 60 min before cocaine or saline, which was administered IP immediately prior to four conditioning sessions. The interval of 60 min between RHO and cocaine was based on experience gained in previous studies [13], as was the absence of any interval between cocaine injection and exposure to the drug-paired compartment [18].

In order to evaluate the role of RHO in the expression of cocaine-induced CPP, four groups of mice ($n$ = 9-10 per group) were conditioned with 25 mg/kg of cocaine and received the vehicle (Veh) or RHO 15, 20, or 25 mg/kg (RHO15, RHO20, and RHO25) IG 60 minutes before post-conditioning test in the colony room.

To evaluate the effects of RHO on reinstatement of the CPP induced by re-exposure to cocaine or social defeat, we followed the procedures described previously ([18, 31], resp.). Six groups of mice ($n$ = 10–13) were conditioned with 25 mg/kg of cocaine and after the post-C test, animals underwent an extinction session every 72 hours which consisted of placing the animals in the apparatus (without the guillotine doors separating the compartments) for 15 min. No drugs were administered during these sessions. The criterion for considering the preference extinguished was lack of statistical significance (according to the Student's $t$-test) between the time spent by the animals of a given group in the drug-paired compartment in the extinction session and in the pre-C session. This measure was repeated 24 h later in order to confirm the extinction. After 24 hours of confirmation to extinction, the reinstating effects of cocaine or social stress (alone or with RHO) were evaluated. Reinstatement tests were the same as those for post-C (free ambulation for 15 min), except that the animals were tested after administrating 12.5 mg/kg of cocaine or inflicting social defeat. Three groups received 12.5 mg/kg of cocaine in the colony room (a neutral place not previously associated with cocaine) 15 min before the reinstatement tests. The first group received cocaine plus vehicle (Coc), and the other two groups received cocaine plus 15 or 20 mg/kg of RHO (Coc + RHO15, Coc + RHO20), respectively (vehicle or RHO was given IG 60 min before the reinstatement test, i.e., 45 min prior to the cocaine IP injection). The other three groups of mice, exposed to social defeat (SD), received vehicle, 15, or 20 mg/kg of RHO (SD, SD + RHO15 and SD + RHO20) IG 60 min before the reinstatement test, performed immediately after SD. The social stress was performed following the procedure described by Ribeiro Do Couto et al. [31]. It took place in a different room and consisted of a 10-min agonistic encounter (and 1 min of exploration) in a neutral transparent plastic cage (23 × 13.5 × 13 cm) with a defeat result for the experimental mouse. Each experimental mouse was confronted with an aggressive opponent (of equal age and body weight) with previous fighting experience and had been shown to have a high level of aggression in previous screening. This procedure can be considered a type of social stress (see [31, 32]). Experimental mice presented avoidance/flee and defensive/submissive behaviours after suffering aggression (threat and attack) from an opponent. The criterion used to define an animal as defeated was the adopting of a specific posture of defeat, characterized by an upright submissive position, limp forepaws, upwardly angled head, and retracted ears [32, 33]. Some animals excluded from the CPP procedure ($n$ = 24) were used as aggressive opponents. They were housed individually, in isolation, in plastic cages (23 × 13.5 × 13 cm) for a month before experiments to induce heightened aggression [32]. All defeated mice experienced similar levels of aggression as the opponent displayed attack behaviour as soon as it saw the experimental mouse (latency < 30 s).

*2.4. Statistical Analysis.* Data of the acquisition (the time spent in the drug-paired compartment) were analysed with a mixed ANOVA, with "treatment" as a "between-subjects" variable with eight levels (Veh + Sal, RHO15, RHO20, RHO25, RHO15 + Coc, RHO20 + Coc, RHO25 + Coc) and "days" as a "within-subjects" variable with two levels (pre-C and post-C). Data of the expression were analysed with the same ANOVA, but "treatment" variable has four levels (Veh, RHO15, RHO20, RHO25). Data of the reinstatement were analysed with the same ANOVA but, "treatment" has six levels (Coc, Coc + RHO15, Coc + RHO20, SD, SD + RHO15 and SD + RHO20) and "days" four levels (pre-C, post-C, extinction and reinstatement).

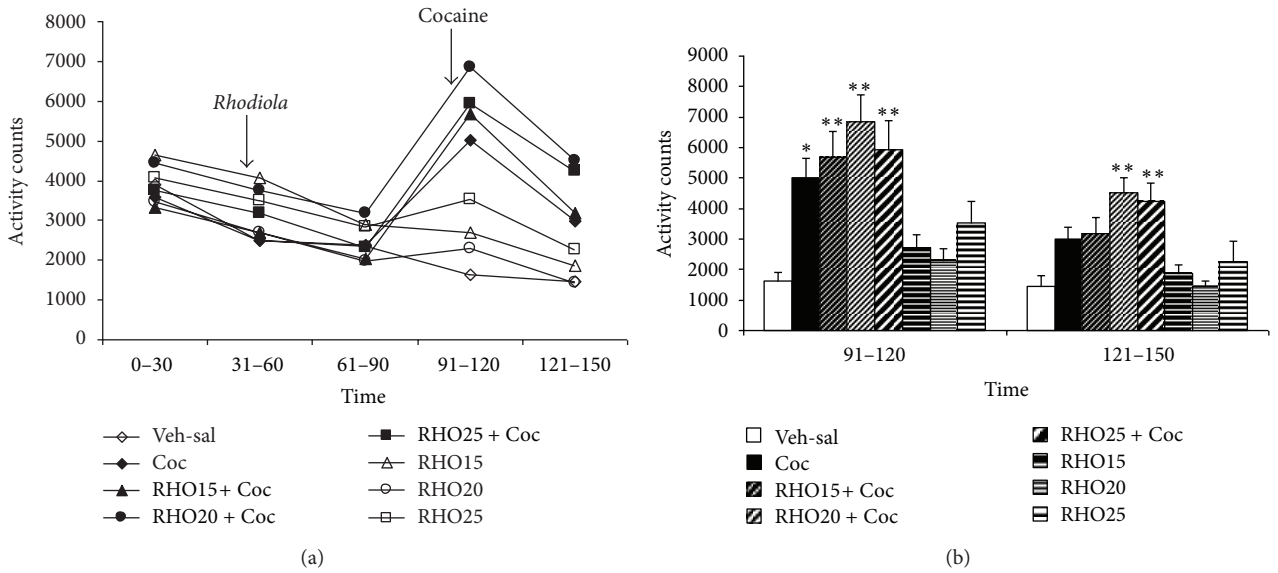


Figure 1: Effects of RHO on cocaine-induced hyperactivity. Mice ($n = 8$ per group) were placed in the actimeter for a 30-min adaptation period (0–30 min). Afterwards, two groups received IG vehicle (Veh-sal and Coc), two groups received IG RHO 15 mg/kg (RHO15 + Coc, RHO15), two groups RHO 20 mg/kg (RHO20 + Coc, RHO20), and two groups RHO 25 mg/kg (RHO25 + Coc, RHO25), and their motor activity was registered over another hour (31–60, 61–90 min). Finally, the first two groups received a IP injection of saline (Veh-Sal) or cocaine 25 mg/kg (Coc), and the groups treated with RHO received an IP injection of cocaine 25 mg/kg (RHO15 + Coc, RHO20 + Coc, and RHO25 + Coc) or saline (RHO 15, RHO 20, and RHO 25), and their motor activity was registered over a further hour (91–120, 121–150 min). (a) represents the motor activity of all groups over the complete time of testing (0–150 min) and shows the time of RHO and cocaine administration in the corresponding groups. (b) represents the data for the last hour of the test (after cocaine administration to the corresponding groups). Values are means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; significant difference with respect to the values of the control (Veh-sal) group at the same time test.

All post hoc comparisons were performed with the Bonferroni multiple comparisons test (corrected "alpha" of 0.05/16 for acquisition data, 0.05/6 for expression data, and 0.05/24 for reinstatement data). Calculations were performed using the SPSS statistical package 17.0. A $P$ value of less than 0.05 was considered statistically significant.

## 3. Results

*Experiment 1.* The results regarding the effect of RHO on cocaine-induced hyperactivity are represented in Figure 1. The ANOVA showed a significant effect of the variable time ($F(4, 224) = 43.358$; $P < 0.001$), treatment ($F(7, 56) = 3.674$; $P < 0.002$), and the interaction time × treatment ($F(28, 224) = 8.145$; $P < 0.001$). The Bonferroni post hoc comparison showed that cocaine increased motor activity in comparison with the vehicle during the 30 min after its administration ($P < 0.05$). The groups treated with cocaine plus RHO also showed an increase in activity with respect to controls ($P < 0.001$), and the groups RHO15 + Coc and RHO20 + Coc presented more activity than the groups RHO15 and RHO20, respectively ($P < 0.05$ and $P < 0.001$). Between 31 and 60 min after cocaine administration, only the groups treated with RHO20 + Coc and RHO25 + Coc showed an increase in activity in comparison to controls ($P < 0.001$), and the group RHO20 + Coc also displayed more activity than RHO20 group ($P < 0.001$).

*Experiment 2.* The results regarding the effect of RHO on acquisition of cocaine-induced CPP are represented in Figure 2. The ANOVA revealed a significant effect of the variable days ($F(1, 88) = 7.240$; $P < 0.01$) and the interaction treatment × days ($F(7, 88) = 4.210$; $P < 0.001$). Bonferroni post hoc comparisons revealed that the groups Veh + Coc, RHO15 + Coc, and RHO25 + Coc spent more time in the drug-paired compartment in post-C than in pre-C ($P < 0.05$, $P < 0.001$ and $P < 0.01$, resp.). Thus, at the doses used, RHO did not exert motivational effects, and only that of 20 mg/kg was capable of impairing the acquisition of cocaine-induced CPP.

The effects of RHO on the expression of cocaine-induced CPP are represented in Figure 3. The ANOVA revealed a significant effect of the variable days ($F(1, 35) = 33.264$; $P < 0.001$) and the interaction treatment × days ($F(3, 35) = 3.378$; $P < 0.05$). Bonferroni post hoc comparisons revealed that the groups receiving vehicle or RHO 20 or 25 mg/kg spent more time in the drug-paired compartment in post-C than in pre-C ($P < 0.001$, $P < 0.001$, and $P < 0.005$, resp.). In this way, only the dose of 15 mg/kg of RHO blocked the expression of cocaine-induced CPP.

The effects of RHO on the priming- and social defeat-induced reinstatement of cocaine CPP are represented in Figure 4. The ANOVA revealed a significant effect of the variable days ($F(3, 59) = 26.101$; $P < 0.001$). Bonferroni post hoc comparisons revealed that more time was spent in the drug-paired compartment during post-C and the reinstatement test than during pre-C or extinction. The variables treatment and interaction were not significant. These results show that RHO does not block the reinstatement of cocaine-CPP induced by priming or stress.

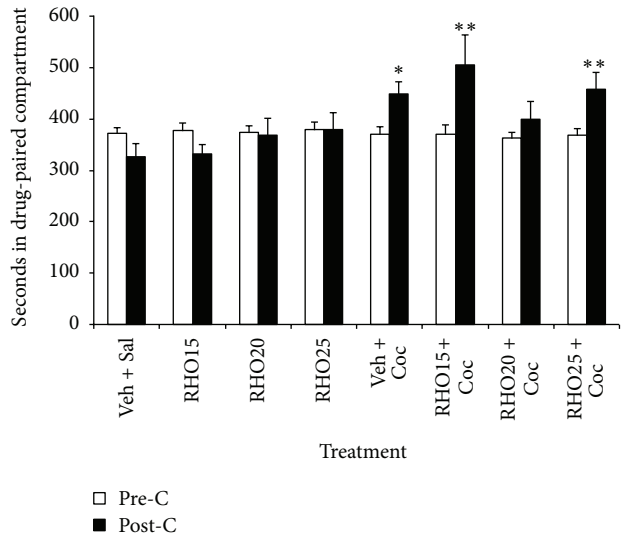


Figure 2: Effects of RHO on the acquisition of cocaine-induced CPP. Mice ($n$ = 11–13 per group) were treated with a vehicle IG, RHO (15, 20 or 25 mg/kg, IG), cocaine (Coc 25 mg/kg, IP), or RHO 15, 20 or 25 plus Coc. RHO was administered 60 min before each saline or cocaine injection. Immediately after this second injection mice were confined to the drug-paired compartment for the conditioning phase. Bars represent the time in seconds spent in the drug-paired compartment during preconditioning (white) and postconditioning (black). Values are means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; significant difference in the time spent in preconditioning versus postconditioning sessions.

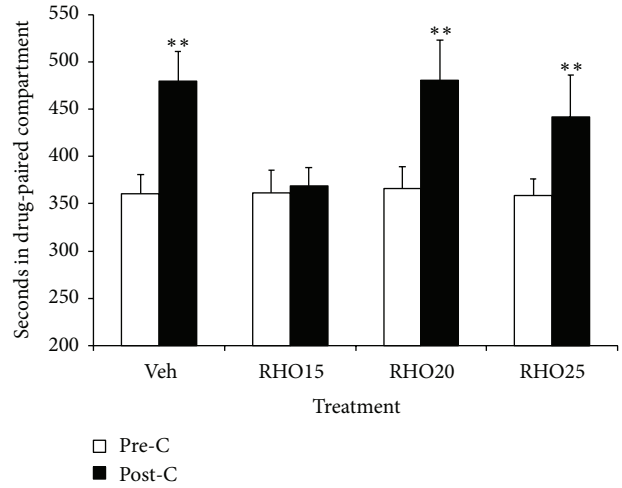


Figure 3: Effects of RHO on the expression of cocaine-induced CPP. Mice ($n$ = 9-10 per group) were conditioned with Cocaine (Coc 25 mg/kg, IP) and received IG Veh or RHO (15, 20 or 25 mg/kg) 60 min before the post-conditioning test. Bars represent the time in seconds spent in the drug-paired compartment during preconditioning (white) and post-conditioning (black). Values are means ± SEM. $^{**}P < 0.01$; significant difference in the time spent in preconditioning versus post-conditioning sessions.

## 4. Discussion

The results obtained in this study demonstrate that RHO is capable of decreasing the rewarding effects of cocaine, since both acquisition and expression of a cocaine-induced CPP were impaired by RHO, though only with specific doses of this compound. However, cocaine-induced hyperactivity and the reinstatement of cocaine CPP induced by priming or stress were not blocked by RHO. These results provide evidence that RHO does not block all the behavioural effects of cocaine and that the impairing effects of RHO on the rewarding actions of cocaine are in function of the dose used and the conditioning process studied.

First, we have seen that RHO does not block the hyperactivity induced by cocaine and even seems to increase the duration of this effect, since animals treated with cocaine plus the intermediate and high doses of RHO showed hyperactivity when the stimulant motor effects of cocaine were insignificant (between 30 and 60 minutes after administration). Many of the behavioural effects of cocaine, including its locomotor-activating properties, have been attributed to the ability of this drug to block DA transporters and enhance DA activity [34]. Our results suggest that RHO increases DA levels slightly. This effect was not sufficient to significantly modify motor activity in the animals treated with RHO alone but together with the stimulatory effects of cocaine induced a marked hyperactivity.

The CPP paradigm has been used to evaluate the rewarding action of drugs of abuse, including morphine and cocaine [14–16]. We observed that cocaine induced rewarding effects, in accordance with previous studies in our laboratory [18]. Conversely, the administration of RHO did not induce motivational effects, which is also in line with previous reports [13]. The administration of RHO impaired the acquisition and expression of cocaine-induced CPP, although these effects were observed only with specific doses of this compound (20 mg/kg blocked acquisition and 15 mg/kg blocked expression). Thus, these partial effects of RHO on cocaine-induced CPP could be related to the dose employed. The effect of *Rhodiola* on the CNS and other body systems did not vary in a consistent manner with the dose. The dose-dependent curve has a bell shape, *Rhodiola* is inactive at small doses, is active at intermediate doses, and becomes inactive again at high doses [10]. The fact that RHO did not induce aversive or motor effects by itself suggested that it prevents cocaine CPP selectively, thus undermining the rewarding effects of this drug. The possibility that RHO impairs learning and memory of cocaine conditioning is improbable, since RHO is known to exert a positive influence on the development of conditioned reflexes and learning [35, 36]. Our results are only partially in agreement with those of Mattioli et al. [13], since they demonstrated that different doses of RHO (10, 15 and 20 mg/kg) impaired the acquisition and expression of morphine CPP. Thus, RHO seems to have more effectiveness in decrease opiate than cocaine reward. The precise mechanism underlying the efficacy of RHO in blocking morphine-induced CPP is unknown, but Mattioli et al. [13] speculate that RHO could exert this effect by enhancing the functional tone of endogenous opioids and 5-HT, DA, and NA in brain areas related to reward [6, 27]. Differences in the mechanism of action of morphine and cocaine and

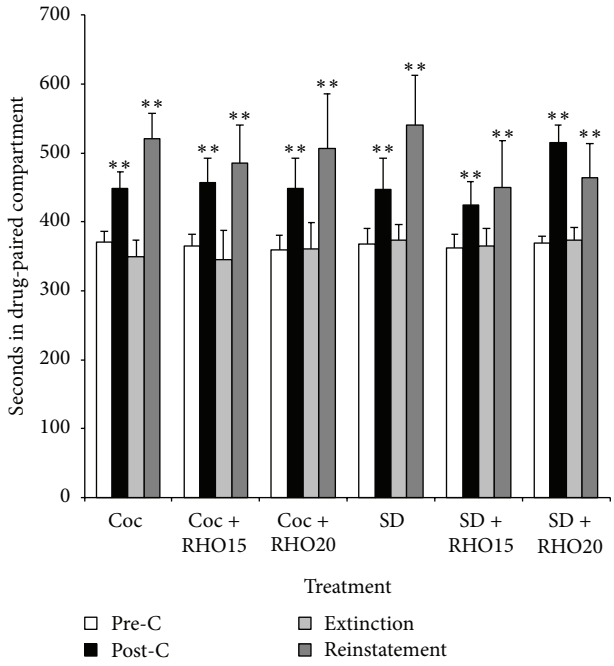


FIGURE 4: Effects of RHO on the priming- and stress-induced reinstatement of cocaine-induced CPP. Six groups of mice ($n$ = 10–13 for group) were conditioned with cocaine (Coc 25 mg/kg, IP), underwent daily extinction sessions until the CPP was extinguished, and received the following treatments before the reinstatement test: vehicle, RHO 15, or 20 mg/kg 45 min before 12.5 mg/kg IP cocaine priming (Coc, Coc + RHO15, Coc + RHO20); vehicle, RHO 15, or 20 mg/kg 45 min before social defeat exposure (SD, SD + RHO15, SD + RHO20). Bars represent the time in seconds spent in the drug-paired compartment before conditioning sessions in the pre-C test (white bars), after conditioning sessions in the post-C test (black bars), in the last extinction session (light gray bars), and in the reinstatement test (dark gray bars). $^{**}P < 0.001$, significant difference in the time spent in the drug-paired compartment in preconditioning versus post-conditioning or reinstatement test.

in the behavioural effects of these drugs [30] can explain why RHO is less effective to reduce cocaine reward. Cocaine is a psychomotor stimulant that facilitates monoaminergic neurotransmission by binding to DA, 5-HT, and NA transporters (DAT, SERT, and NAT) and inhibiting the reuptake of these monoamines. Cocaine produces reward through simultaneous actions at more than one protein site [34, 37]. For example, only DAT/SERT knockout mice exhibit impairment of cocaine CPP [37] and the contribution of DA, 5-HT, and NA transporters to the rewarding, and aversive effects of cocaine seem to vary [34]. Activation of the mesolimbic dopaminergic reward circuitry has been proposed as a modality in the long-term treatment of reward deficiency syndrome and drug addiction disorders [38]. In addition to the abovementioned dose problem, the fact that RHO impairs acquisition or expression of cocaine CPP only at specific doses could be related to the effects that this compound induces monoamines (DA, 5-HT, and NA) and different kinds of monoaminergic precursors, receptors, transporters, or degradation enzymes. In such circumstances, impairment of acquisition/expression of cocaine CPP would be induced only by doses of RHO that produce an effective combination of alterations in the various monoamine proteins involved in the acquisition and/or expression of the rewarding effects of cocaine.

Although the positive reinforcing is the main factor in the acquisition of a drug habit, relapse is the overriding characteristic of addiction and the foremost challenge to the treatment of drug addiction. The CPP paradigm can be used to model relapse in rodents, and re-exposure to drug or stress after extinction can trigger reinstatement of CPP [17]. We observed that cocaine CPP is reinstated by cocaine priming and social defeat stress in accordance with previous studies [18, 26]. However, the administration of RHO did not block priming- or stress-induced reinstatement. These results contrast with those obtained by Mattioli et al. [13] who reported that RHO prevents the reinstatement of morphine CPP induced by drug re-exposure and restraint stress. Again, these divergent results could be related to the different mechanisms underlying the reinstatement of CPP induced by morphine or cocaine. It is improbable that the increase in monoaminergic activity induced by RHO blocks priming-induced reinstatement of cocaine CPP since it has been reported that administration of the DA D1 agonist SKF 81297 [39] and the DA enhancer modafinil [40] induces the reinstatement of cocaine CPP. On the other hand, the lack of effects of RHO on stress-induced reinstatement of cocaine CPP is surprising, since this compound exerts antistress effects and interacts with the hypothalamus-pituitary-adrenal (HPA) system, reducing cortisol levels [7–9]. However, it has been reported that corticosterone plays at most a permissive role in the facilitating effects of social stress on cocaine self-administration in the rat [41] and that stress-induced reinstatement of cocaine self-administration appears to be independent of corticosterone [42]. Alternatively, it is possible that a single administration of an acute dose of RHO prior to reinstatement tests is insufficient to decrease to attenuate the reinstatement of cocaine CPP.

The doses employed in the present study are in the range of those administered to humans for therapeutic purposes, although clinical studies have tended to use higher doses [43, 44]. According to the calculating method described by Reagan-Shaw et al. [45], in a human weighing 60 kg, 15 mg/kg of RHO corresponds with 72.9 mg, 20 mg/kg corresponds with 97.2 mg, and 25 mg/kg corresponds with 120 mg. Extracts of RHO have already been used in humans without severe adverse consequences, which endorses the safety of the preparations in question [5, 9, 35, 43, 44, 46–48]. Only a few mild adverse events have been reported, including headache or hypersalivation [43]. Moreover, a lack of interaction with other drugs (warfarin and theophylline) has been observed [49]. However, it remains to be determined whether or not RHO interacts with cocaine or other drugs in clinical settings.

Presently, there are no FDA-approved therapies or medications for treating cocaine addiction [2, 3] that can be used as a positive control in the evaluation of RHO's effects. However, there are recent reports of the positive effects of derivatives of genera Stephania and Corydalis in the treatment of cocaine addiction in animal models. Levo-tetrahydropalmatine has

been shown to attenuate cocaine self-administration [50] and reinstatement of extinguished cocaine seeking by cocaine, stress, or drug-associated cues in rats [50, 51]. In light of this evidence, levo-tetrahydropalmatine has been suggested as a potential new medication for the treatment of cocaine addiction [52].

The main limitation of our work lies in the fact that RHO was administered over a short period of a few days (in four acquisition sessions) or acutely (in expression and reinstatement tests). It is likely that more pronounced effects of RHO would be observed with a longer period of administration (e.g., during the extinction period). Another limitation of our approach is the use of a single animal model to evaluate the rewarding effects of cocaine. The CPP paradigm used in the present study measures the conditioned rewarding effects of the drug while the drug self-administration paradigm measures the primary hedonic properties of the drug [17]. Future research should evaluate the efficacy of RHO in reducing the acquisition of cocaine self-administration and reinstatement of drug seeking after exposure to cocaine priming, drug-conditioned cues, or stress.

In conclusion, we can report that RHO impairs the rewarding effects of cocaine, although its effects on acquisition and expression of CPP depend on the dose used. The fact that RHO does not affect priming- or drug-induced reinstatement of cocaine CPP limits its possible usefulness as a natural treatment for cocaine dependence. Nevertheless, though the discovery of an effective therapy that addresses all aspects of cocaine addiction continues to elude researchers, the anti-craving effect of RHO shows potential as a component of combined therapy.

## Conflict of Interests

The authors declare that there is no conflict of interests.

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