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Anxiolytic Effects of Woohwangcheongsimwon in Mice

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Abstract – Woohwangcheongsimwon (WHCSW) is a traditional oriental medicinal fomula which has been clinically used for treating strokes, palpitation, loss of consciousness and anxiety. The purpose of this study was to characterize the putative anxiolytic properties of WHCSW using an elevated plus-maze (EPM) and hole-board test. Control mice were orally treated with an equal volume of vehicle (10% Tween 80 solution), and positive control mice were treated with diazepam (1 mg/kg, i.p.). In the EPM test, WHCSW significantly increased the percentage of time-spent in the open arms (200 mg/kg, $P < 0.05$) and the percentage of open arm entries (200 and 400 mg/kg, $P < 0.05$). WHCSW also significantly increased the number of head-dips in the hole-board test (200 mg/kg, $P < 0.05$). In addition, the anxiolytic properties of WHCSW examined in the EPM test were inhibited by flumazenil (10 mg/kg, i.p.), a $GABA_A$ antagonist. However, no changes in spontaneous locomotor activity or myorelaxant effects were observed versus 10% Tween 80 controls. These results suggested that WHCSW is an effective anxiolytic agent, and that its anxiolytic effects are mediated via $GABA_A$ receptors.
Keywords – Woohwangcheongsimwon (WHCSW), Anxiolytic effect, $GABA_A$ receptor, Elevated plus-maze test, Hole-board test

Introduction

Woohwangcheongsimwon (WHCSW) is an herbal medicine as a pill which is composed of 27 components including Benzoar Bovis, Moschus, Dioscoreae Rhizoma, and Glycyrrhizae Radix (Table 1). This prescription is based on the ancient Korean medicinal literature, 'Dong-eui-bo-gam'. According to 'Dong-eui-bo-gam', WHCSW supplies or regulates the lack of heart-Qi, instability of kidney-intelligence and is useful for bipolar disorder, insanity, and stupor of mind. In traditional medical practices in Korea, WHCSW has been used empirically for a long time to ameliorate or relieve the states of stroke, hypertension, cardiopalmus, respiratory distress syndrome, anxiety, acute and chronic convulsion, autonomic imbalance, insensibility, and etc. It can be assumed that various components in WHCSW may act individually either positively or negatively, and may affect multiple neuronal, metabolic, and hormonal systems in concert (Scholey *et al.*, 2005). However, the role of WHCSW on the anxiety-related behavioral properties has not yet been investigated in detail.

Anxiety is one of the most prevalent psychiatric disorders (Lepine, 2002). In the clinical treatment for anxiety disorder, benzodiazepines, $GABA_A$ receptor agonist, and buspirone, $5\text{-}HT_{1A}$ receptor agonist, are mainly prescribed as the first choice treatment. Chronic administration of benzodiazepines, however, results in physical dependence, such as sedation, myorelaxation, ataxia, amnesia, and pharmacological dependence (Lader and Morton, 1991; O'brien, 2005; Whiting, 2006). Moreover, buspirone also results in dizziness, headache, nervousness, light-headedness, diarrhea, paresthesia, excitation, and sweating as adverse effects (Newton *et al.*, 1986). Therefore, research has been conducted to identify safer, more specific, and perhaps lower cost therapies

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Table 1. Composition of Woohwangcheongsimwon

Composition	Amount (mg) in 1 pill
Dioscoreae Rhizoma	450
Glycyrrhizae Radix	320
Ginseng Radix	160
Typhae Pollen	160
Massa Medicata Fermentata	160
Glycine Semen Germinatum	130
Cinnamomi Ramulus	130
Asini Gelatinum	130
Paeoniae Radix	110
Liriopis Tuber	110
Scutellariae Radix	110
Angelicae Gigantis Radix	110
Ledebouriellae Radix	120
Atractylodis Macrocephalae Rhizoma	110
Bupleuri Radix	80
Platycodi Radix	80
Poria	84
Cnidii Rhizoma	180
Bovis Calculus	30
Saigae Tataricae Cornu	215
Borneolum Syntheticum	90
Ampelopsis Radix	46
Zingiberis Rhizoma Recens	46
Jujubae Fructus	150
Moschus	5
Mel	215
Aurum	3
Total	3534

(Carlini, 2003). Because WHCSW has been used for generations, it is unlikely that it has side effects unless it is administered in excessive doses. It would, therefore, be interesting to investigate the anxiolytic-like activity of WHCSW.

The purpose of this study was to characterize the anxiolytic effect of the WHCSW using several behavioral tasks in mice. Elevated plus maze (EPM) test and hole-board test were employed to identify the anxiolytic-like effects of WHCSW. In addition, myorelaxant effect was also investigated for examining the adverse effect of WHCSW using a horizontal wire task.

Experimental

Animals – Male ICR mice (25 - 30 g) were purchased from the Orient Co., Ltd. a branch of the Charles River Laboratories (Seoul, Korea). Mice were housed 10 per cage and were provided with food and water ad libitum and kept under a 12-h light/ dark cycle (light on 07:00-19:00) at constant temperature (23 ± 1 °C) and humidity ($60 \pm 10\%$). Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85 - 23, revised 1985) and the Animal Care and Use Guidelines issued by Kyung Hee University, Korea.

Materials – WHCSW was provided by Kwang Dong Pharm. Co., Ltd (Table 1). WAY 100635, flumazenil was purchased from the Sigma Chemical Co. (USA). Diazepam was purchased from DaeWon Pharmaceutical Co., Ltd. (Korea). All other materials were of the highest grade commercially available.

Sample preparation – WHCSW (100, 200, 400, and 800 mg/kg) and flumazenil (10 mg/kg) were suspended in a 10% Tween 80 solution. WHCSW was orally administered and flumazenil was administered intraperitoneally (i.p.). Diazepam and WAY 100635 were dissolved in saline and administered i.p.

Spontaneous locomotor behavior test – Spontaneous locomotor behavior was measured as described previously (Jung *et al*., 2006). Mice were placed in the center of a horizontal locomotor activity box ($40 \times 40 \times 40$ cm) and locomotor activity was measured for 10 min after administrating WHCSW (100, 200, 400, and 800 mg/kg, p.o.) or diazepam (1 mg/kg, i.p.) and video-recorded. Locomotor activity was converted as total ambulatory distance.

Elevated plus Maze test – The EPM for mice consisted of two vertical open arms (30×7 cm) and two closed arms (30×7 cm) with 20 cm-height walls, extending from the central platform (7×7 cm). The open and closed arms were joined by a central square to give an implement of a plus symbol appearance. The maze was raised to height of 50 cm above the floor in a soundproof room with dimly light (20 lux) and a video camera was suspended above the maze to a record for the movements for analysis (Jung *et al*., 2006a; Handley and Tricklebank, 1995; Pellow *et al*., 1985). Mice were placed at the center of the maze platform with its head facing an open arm. Animals were tested individually for 5 min, and the maze was cleaned after each trial to remove any residue or bad smell. The following measurements were taken and analyzed using the video-based Ethovision System (Noldus, Wageningen, The Netherlands): the percentage of open and closed arms entries, the percentage of time spent in each arm covered in the EPM. One hour after WHCSW treatment (100, 200, 400, and 800 mg/kg, p.o.),

mice were placed in the EPM. Mice in the control group were given 10% Tween 80 only.

In a separated antagonism study, mice were co-administrated of WHCSW (200 mg/kg, p.o.) and WAY 100635 (0.3 mg/kg, i.p.) or flumazenil (10 mg/kg, i.p.) 1 h and 30 min prior to testing, respectively. Diazepam (1 mg/kg, i.p.) was used as a positive control and was administered 30 min before the EPM test. All experiments were carried out between 10:00 and 16:00 o'clock.

Hole-board test – This test was performed as described elsewhere (Jung *et al*., 2006b). The hole-board apparatus (Ugo Basile, Italy) consisted of gray Perspex panels (40 × 40 cm, 2.2 cm thick) containing 16 equidistant holes (3 cm diameter) in its floor. The board was positioned 15 cm above a table. The number of head-dips was measured by placing photocells below the holes. Mice were transported in a soundproof room with dimly light (20 lux) used for this test at least 1 h before testing. Mice were treated with WHCSW (100, 200, 400, or 800 mg/kg, p.o.) 1 h prior to testing. Diazepam (1 mg/kg, i.p.) was used as a positive control and was administered 30 min before the hole board test. Animals were placed individually in the center of the board facing away from the observer, and head-dip numbers were recorded for 5 min.

Horizontal wire test – Horizontal wire test was carried out by treating the mice with WHCSW (100, 200, 400 or 800 mg/kg, p.o.) or diazepam (1 mg/kg, i.p.) using a slight modification of a previously described method (Bonnetti *et al*., 1982). Briefly, mice were lifted by the tail and allowed to grasp a horizontally strung wire (1 mm diameter, 15 cm long, and 20 cm above a table surface) with their forepaws, and then released. The number of mice in each treatment group that did not grasp the wire with forepaws or actively grasped the wire with at least one hind paw within 10 sec period was recorded.

Statistical analysis – The values are expressed as means ± S.E.M. Data were analyzed by one way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. Differences were considered significant at the $P < 0.05$ level.

Results

Effect of WHCSW on the spontaneous locomotor activity and horizontal wire test – Locomotor activity was measured to differentiate the possible stimulatory or inhibitory activities of WHCSW on the modulation of exploratory behavior. In the present study, no significant changes in spontaneous locomotor activity at any doses of WHCSW or diazepam (1 mg/kg) were observed compared with the vehicle-treated control group (Table 2). Myorelaxant impairs the ability of mice to grasp the wire, and muscle relaxation is commonly associated with sedation. WHCSW (50, 100, 200, or 400 mg/kg) did not compromise the mice grasping the wire compared with vehicle-treated control group (data not shown), indicating a lack of myorelaxation at these doses.

Table 2. The effects of Woohwangcheongsimwon (WHCSW) on the locomotor activity in the open field test

Drugs	Dose (mg/kg)	Locomotor activity (cm/10 min)
Control		3569.40 ± 430.17
Diazepam	1	3337.85 ± 551.06
WHCSW	100	3621.74 ± 169.26
	200	2996.88 ± 285.82
	400	3013.32 ± 278.73
	800	3356.11 ± 781.93

The exploratory behaviors of mice in the open field test were observed for 10 min one hour after drug or vehicle treatment. Data are expressed as means ± S.E.M (n = 20 per group).

Effect of WHCSW in the EPM test – We confirmed the percentage of time spent in open arms and the percentage of frequency of entering into open arms through in EPM test. Vehicle-treated control mice spent much time in the closed arms and avoided entering the open arms. The percentage of time spent in open arms and the percentage of frequency of entering into open arms were 24.70 ± 2.13% and 41.15 ± 1.83%, respectively, in the vehicle-treated control group. In the WHCSW-treated group (200 mg/kg), the percentage of time spent in open arms was significantly higher than that of vehicle-treated control group (Fig. 1; $P < 0.05$). Moreover, the percentage of open arm entries was significantly increased in the WHCSW-treated group (200 and 400 mg/kg) compared with vehicle-treated control group (Fig. 1; $P < 0.05$). At other doses, those parameters were slightly increased but not significant. In the diazepam-treated positive control group, the percentage of time spent in the open arms and the percentage of frequency of entering into open arms were significantly increased (Fig. 1; $P < 0.05$).

To investigate which neurotransmitter system, e.g., GABAergic or serotonergic nervous system, is involved in the anxiolytic effect of WHCSW, WHCSW-treated mice were subjected to co-treatment with either WAY 100635 (a 5-HT_{1A} receptor antagonist) or with flumazenil (a $GABA_A$ receptor antagonist). The percentage of time spent in open arms and the percentage of open arm entries were not changed by WAY 100635 or flumazenil alone. As shown in Fig. 2, the increase of the percentage of time

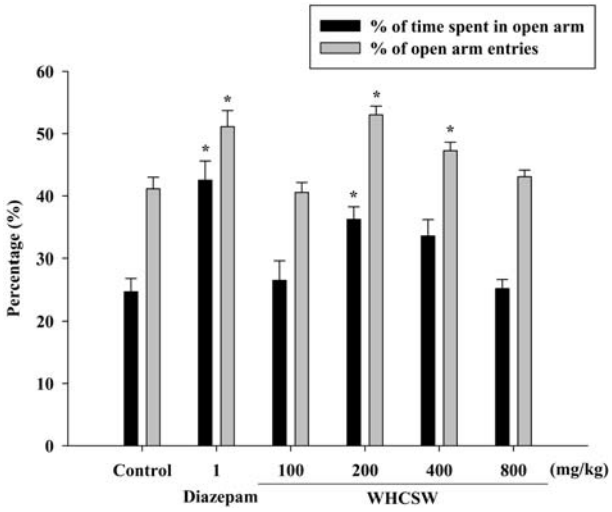

Fig. 1. Effect of a single treatment of WHCSW on the percentage time spent in and the number of entries into open arms of the elevated plus-maze over a 5 min test period in mice. Bar represents mean ± S.E.M. (n = 20). *P* values for group comparisons were made using one way ANOVA followed by the Student-Newman-Keuls test (**P* < 0.05 versus the vehicle-treated control group).

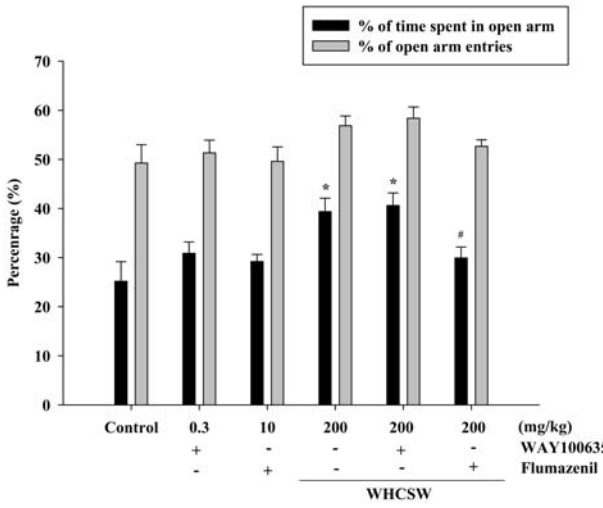

Fig. 2. Anxiolytic effects of WHCSW were blocked by flumazenil but not by WAY100635. These blockers (flumazenil, 10 mg/kg; WAY100635, 0.3 mg/kg) were intraperitoneally administered 30 min after administrating WHCSW (200 mg/kg). Mice were placed singly in the EPM 30 min after blocker treatment. Data are expressed as mean ± S.E.M. of the percentage of time spent in and the number of entries into open arms of the EPM over a 5 min test period. Control group animals were treated with vehicle (a 10% aqueous solution of Tween 80) instead of blocker. Bars represent means ± S.E.M. (n = 10). *P* values for group comparisons were made by one way ANOVA followed by the Student-Newman-Keuls test (**P* < 0.05 versus vehicle-treated controls, #*P* < 0.05 versus the WHCSW-treated group).

spent in open arms by WHCSW (200 mg/kg) was significantly decreased to the vehicle-treated control level

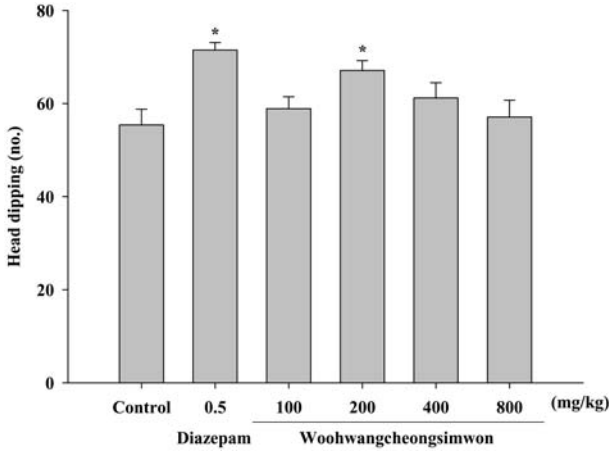

Fig. 3. Effect of WHCSW on changes in head-dipping behavior during the hole-board test. Mice were administered WHCSW (100, 200, 400 and 800 mg/kg, p.o.). Bars represent mean ± S.E.M. (n = 20). *P* values for group comparisons were obtained by one way ANOVA followed by the Student-Newman-Keuls test (**P* < 0.05 versus the vehicle-treated control group).

by flumazenil (10 mg/kg) but not by WAY 100635 (0.3 mg/kg) ($P < 0.05$).

Effect of WHCSW by the hole-board test – The effect of WHCSW on head-dipping behavior in mice was shown in Fig. 3. WHCSW-treated mice manifested significant increase in the number of head-dips in the hole-board system at 200 mg/kg (Fig. 3; $P < 0.05$). However, no significant changes in the head-dip behaviors were observed in any other doses of WHCSW except for 200 mg/kg. In the diazepam-treated positive control group, the frequency of head dipping were significantly increased (Fig. 3; $P < 0.05$).

Discussion

We investigated whether WHCSW had anxiolytic effect through EPM test and hole-board test in mice. The percentage of time spent in open arm and that of open arm entries in EPM were increased in the WHCSW-treated group. Moreover, those parameters were reversed by flumazenil, a $GABA_A$ receptor antagonist, not by WAY 100635, a $5HT_{1A}$ receptor antagonist. WHCSW-treated group also exhibited the increase of the frequency of head-dipping in hole-board test. These results showed that WHCSW had anxiolytic effect and its effect was mediated by GABAergic signaling.

The EPM test has been used for anxiety-related behavior testing in rodents (Pellow *et al*., 1985; Dawson and Tricklebank, 1995; Lister, 1987) and usually applied to confirm the anxiolytic effect of $GABA_A$ receptor-

mediated agents. However, drugs via 5-HT_{1A} receptor could not always exhibit anxiolytic effect through EPM test. For example, buspirone has been used for treatment of anxiety disorder, but its effect in the EPM test is not obvious, i.e. anxiolytic (Jung *et al*, 2006a; Dunn *et al*, 1989), no effective (Wada and Fukuda, 1991) or anxiogenic (Klint, 1991; Moser, 1989). In the present study, we used diazepam as a positive control and observed that the percentages of time-spent in the open arms and of open arm entries were increased by diazepam treatment. Therefore, the EPM test was effective for evaluating anxiolytic effect in the present study. WHCSW significantly increased the percentage of time-spent in the open arms and the percentage of open arm entries. Compared with diazepam-treated group, the results of WHCSW were similar to those of diazepam in terms of open arm entries and time spent in open arms. These results suggested that WHCSW could be used as an anxiolytic agent.

The hole-board test has been used to test emotionality, anxiety and/or responses to stress in animal (Rodríguez Echandía *et al*, 1987). The anxiolytic agents increased the frequency of head-dipping in the hole (Takeda *et al*, 1998). WHCSW (200 mg/kg) significantly increased the frequency of head-dipping compared with that of vehicle-treated control group. The dose-response relationships were inverted U-shape both in the EPM test and in the hole-board test. Until now, we do not know why the dose-effect curve shows an inverted U-shape. It is likely that the effective dose window is narrow. In locomotor activity test, no sedative or stimulating activities were observed, suggesting that WHCSH exhibits anxiolytic effect both in the EPM test and in the hole-board test without general behavioral changes (Cabib *et al*, 1990).

Although various neurotransmitter systems are related to anxiety, $GABA_A$ agonist and 5-HT_{1A} agonist have mainly used for treating anxiety disorders in clinic. Therefore, it is important to clarify which neurotransmitter systems are involved in the anxiolytic effects of WHCSW, and we performed antagonism study using $GABA_A$ receptor antagonist or 5-HT_{1A} receptor antagonist. Co-administration of flumazenil ($GABA_A$ receptor antagonist) or WAY 100635 (5-HT_{1A} receptor antagonist) with WHCSW (200 mg/kg) was conducted. We confirmed that the anxiolytic-like effects of WHCSW was antagonized by co-administration of flumazenil, not by that of WAY100635. Therefore, we could identify that anxiolytic effect of WHCSW was exerted via GABAergic neurotransmitter system. However, because WHCSW contained various herbs, the possibility that anxiolytic effect of WHCSW was exerted via other neurotransmitter systems could not exclude.

WHCSW has used for recovering placidity in Korea for long time. Because these usages have not confirmed experimentally until now, the present study will be helpful to understand its traditional usage. Through some experiments, we tried to investigate which component(s) plays an important role in the anxiolytic effects of WHCSW. However, we could not know which component (s) manifested anxiolytic effect or which component(s) acted on GABAergic neurotransmitter system. Of components of WHCSW, *Scutellaria baicalensis* (Hui *et al*, 2002; Wang *et al*, 2002; Jung *et al*., 2004), *Glycyrrhiza glabra* (Ambawade *et al*., 2001), *Panax ginseng* (Bhattacharya and Mitra, 1991), *Glycine max* (Trent and Edwin, 2001), *Angelica sinenesis* (Chen *et al*., 2004; Min *et al*., 2005), *Zingiber officinale* (Vishwakarma *et al*., 2002), and *Ziziphus jujube* (Peng *et al*., 2000) had been reported to have anxiolytic effect. The anxiolytic effect of *Scutellaria baicalensis* (Hui *et al*, 2002), *Glycine max* (Trent and Edwin, 2001), and *Angelica sinenesis* (Chen *et al*., 2004) was exhibited via GABAergic neurotransmitter system. That of *Zingiber officinale* was manifested via serotonergic neurotransmitter system. In addition, *Ziziphus jujuba* was manifested its anxiolytic effect by decrease of monoaminergic activity in the brain. Furthermore, those modes of action of *Glycyrrhiza glabra* and *Panax ginseng* were not known until now. As mentioned above, there are many constituents showing anxiolytic-like effects through GABAergic, serotonergic, or some other monoaminergic neurotransmissions. Although these herbs had been known to have anxiolytic effect, exact constituents that exhibited anxiolytic effect of WHCSW are not determined in this study. It is likely that WHCSW could reveal anxiolytic effect through interacting some of constituents. Further studies are needed to clarify these issues.

In conclusion, the present study suggested that WHCSW would be a good anxiolytic agent without a generally stimulating locomotor activity due to exposure to a novel environment (Cabib *et al*, 1990), and that the anxiolytic effect of WHCSW may be mediated by the GABAergic neurotransmission, especially, the $GABA_A$ receptor.

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