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Extract of *Ganoderma lucidum* potentiates pentobarbital-induced sleep via a GABAergic mechanism

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Abstract

Ganoderma lucidum has been used for the treatment of a variety of diseases. For the first time here we report a detailed study on the mechanisms and effects of *G. lucidum* aqueous extract (GLE) on sleep and its sedative activity. GLE showed no effects on sleep architecture in normal rats at doses of 80 and 120 mg/kg. However, GLE significantly decreased sleep latency, increased sleeping time, non-REM sleep time and light sleep time in pentobarbital-treated rats. Suppression of locomotor activity in normal mice induced by GLE was also observed. Flumazenil, a benzodiazepine receptor antagonist, at a dose of 3.5 mg/kg showed a significant antagonistic effect on the shortening in sleep latency, increase in sleeping time, non-REM sleep time or light sleep time in pentobarbital-treated rat induced by GLE. Significant effect was also observed with GLE on delta activity during non-REM sleep and flumazenil did not block this effect. In conclusion, GLE may be a herb having benzodiazepine-like hypnotic activity at least in part.

Keywords: Ganoderma lucidum; Hypnotic effect; Sedative effect; Pentobarbital Sleep; Flumazenil

1. Introduction

Insomnia is an extremely common symptom both de novo and in the context of other medical and psychiatric disorders. It is estimated that more than 27% people in the world suffer from insomnia with difficulty in initiating or maintaining sleep and this figure is expected to grow by the middle of the 21st century and about 3–10% of all people are chronic and frequent users of hypnotics (Quera-Salva et al., 1991;Weyerer and Dilling, 1991; Freeman, 1996). However, it is well known that the most extensively used benzodiazepines show many unpleasant reactions, such as drug dependence, tolerance, rebound insomnia and amnesia. The new type of hypnotics, such as zolpidem, zolpiclone etc, also showed some extent of side effects (Griffiths et al., 1986; Bocca et al., 1999).

Ganoderma lucidum (Leyss. ex Fr.) Karst, one of the most highly ranked herbal medicines by Asian people, has been extensively used in the treatment of a variety of diseases including diabetes, neurasthenia, hypertension, chronic hepatopathy, and cancer (Tang et al., 2005; Gao et al., 2004, 2002). Polysaccharide fraction extracted from *G. lucidum* has been marketed as an over-the-counter product in China. The estimated global production of *G. lucidum* was about 4700 tons in 2002, in which 3800 tons were produced in China (Lai et al., 2004). In China, Ganoderma lucidum has been also used as a tranquilizing agent to treat insomnia for thousands of years.

There exists certain literature on the hypnotic effects of *G. lucidum* extract in human beings (Wang et al., 2001; Tang et al., 2005). In addition, *G. lucidum* components may directly modulate the activity of the central and/or peripheral nervous systems. Animal studies indicated that *G. lucidum* reduced spontaneous motor activity, prolonged barbital-induced sleeping time, prevented nicotine-induced convulsions, and inhibited

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pilocarpine-induced salivary secretion (Honda et al., 1988; Jong and Birmingham, 1992). Wei et al. (2000) reported that the aqueous extract of *G. lucidum* spores has hypnotic and sedative actions on the central nervous system in mice. Shou et al. (2003) also revealed that *G. lucidum* granules significantly decreased the spontaneous motility of normal mice, shortening the sleep latency and increase sleeping time in pentobarbital-treated mice. These findings suggest that *G. lucidum* showed sedative effects. However, little research have been performed that deal with the effects and mechanism of *G. lucidum* on the sleep–wake cycle in animals. In the present study, we studied the effect of *G. lucidum* extract (GLE) on sleep parameters and its sedative activity.

2. Materials and methods

2.1. Drugs and drug administration

The fruiting bodies of *G. lucidum* (2 kg) collected from southern China were extracted twice with water at 70 °C for 3 h and the water extracts were precipitated by addition of ethanol (final concentration is 75%, vol/vol) and obtained crude precipitation (216 g, yield: 10.8%). The polysaccharide content in the crude precipitation was 26.7% determined by the phenol-sulfuric acid method. For enriching the polysaccharide, the crude extract was further purified by resolving in water and precipitated with ethanol and washing with acetone three times, respectively. The polysaccharide content in final *G. lucidum* extract (GLE, 86.46 g, yield: 4.32%) was 66.7% and the concentration of proteins was 0.35% as determined by the bicinchoninic acid method. Triterpenes were not detected in the final extracts by silica gel thin-layer chromatography or visualized by UV light shadowing. GLE was suspended in 0.5% carboxymethyl cellulose solution while diazepam and pentobarbital sodium (Sigma-Aldrich, St. Louis, MO) in distilled water, flumazenil (4th Changzhou Pharmaceutical Co. Ltd., China) in 10% dimethyl sulfoxide.and administered orally at 9:00.

In pentobarbital-hypnosis test, mice or rats were treated with GLE (40, 80 and 120 mg/kg, p.o.) or distilled water for 3 days. On the 3rd day, 1 h after the last administration of GLE, pentobarbital sodium (50 mg/kg for mice or 45 mg/kg for rats, i.p.) was given. In the antagonism experiments, flumazenil (3.5 mg/kg, i.p.) was administered 20 min prior to the last administration of GLE (80 mg/kg, p.o.) or diazepam (2.5 mg/kg, p.o.). In normal sleep analysis test, electroencephalogram (EEG) and electromyogram (EMG were recorded from 9:00 to 15:00. In pentobarbital–sleep analysis, EEG and EMG were recorded until the rat was awake.

2.2. Animals

Male ICR mouse (20–24 g) and male Sprague Dawley rat (180–220 g, Grade I, purchased from Animal Center of Peking University, Beijing) were used in this study. Mice were housed in acrylfiber cages and rats were housed in individual cages and maintained in an air-conditioned room with controlled temperature (24±2 °C) and humidity (55±15%) and were kept on a 12 hr light/dark cycle. They were fed with standard diet and water ad libitum and acclimated 7 days before they were used. The animals were allowed free access to food and water during the experiments. All procedures involving animals were conducted in accordance with the European Community guidelines for the use of experimental animals and approved by the Peking University Committee on Animal Care and Use.

2.3. Surgery

Sterile surgery was performed under general anesthesia induced by intraperitoneal pentobarbital (50 mg/kg). To fix body position, the rats were placed in a stereotaxic apparatus with blunt ear bars. Two stainless steel screws attached to insulated wire were implanted in the skull over the frontal-parietal cortex to record the EEG. One of these electrode was placed ~2 mm anterior and 2 mm to the right of bregma, and the other ~3 mm posterior and 2 mm to the left of bregma. Two insulated stainless steel wires bared at the tips were sutured onto the dorsal cervical neck muscles to record the EMG. All of these electrodes were attached to a miniature connector. The implant assembly was affixed to the skull with dental acrylic. After the surgical implantation, rats were injected with antibiotics and housed individually in cages at least 7 days for recovery before the onset of experiments.

2.4. EEG and EMG recordings and analysis

EEG and EMG were recorded with an electroencephalograph (Model MP 150, BIOPAC, CA, USA) from 09:00 to 15:00 in normal rats, otherwise, in pentobarbital–hypnosis test recording was carried out 5 min before pentobarbital injections for each rat, and finished when the rat waked. The recording was carried out according to the method described by Shinomiya et al. (2004). The signals were amplified and filtered (EEG, 0.5–30 Hz; EMG, 16–128 Hz), then digitized at a sampling rate of 128 Hz and recorded using AcqKnowledge software (BIOPAC systems, Inc. USA). The sleep–wake states were automatically classified by 10-s epochs as wake, rapid eye movement (REM) sleep or non-rapid eye movement (NREM) by SleepSign ver.2.0 (BIOPAC, CA, USA). As a final step, the defined sleep–wake stages were examined visually, and corrected, if necessary. Each state was characterized as follows: wake, low-amplitude EEG and high-voltage EMG activities; NREM sleep, high-amplitude slow or spindle EEG and low-EMG activities; SWS, high-amplitude, low-frequency synchronous pattern, and decreased EMG activity; light sleep, equals NREM sleep subtracts SWS; REM sleep, low-voltage EEG and EMG activities. The delta activity during NREM sleep was determined using a program of SleepSign ver. 2.0. The power spectrum densities, integrated and averaged, could be divided into the four frequency areas: delta wave (0.5–4 Hz), theta wave (4–8 Hz), alpha wave (8–13 Hz) and beta wave (13–30 Hz). The data of delta power in NREM sleep were expressed as percentage of the average delta activity in NREM sleep during the entire recording period of each group.

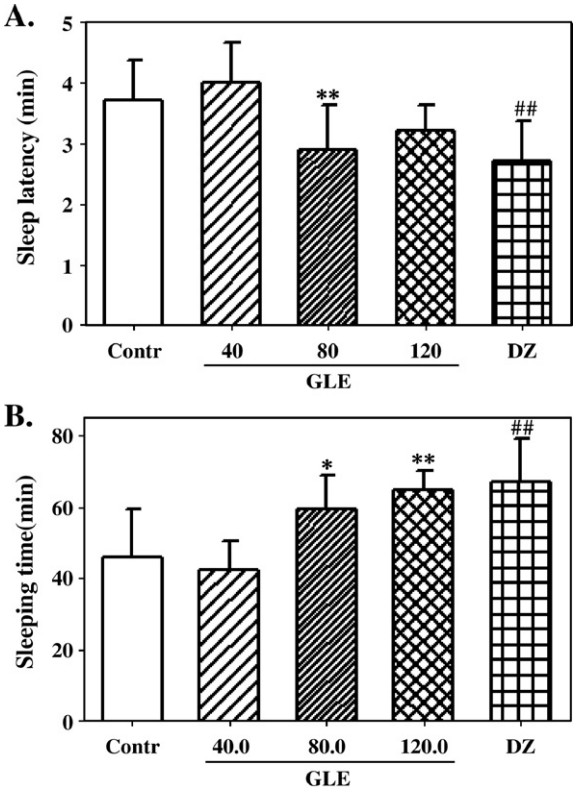

Fig. 1. Effect of GLE on pentobarbital-induced sleep in mice. The sleep latency (A) [$F(3, 36)=6.00$, $P=0.002$] and sleeping time (B) [$F(3, 34)=11.56$, $P<0.000$] were assessed. Data represent mean±S.E.M ($n=9$–10). Contr: control; DZ: diazepam (2.5 mg/kg, ip); GLE: 80 mg/kg (po). $^{*}P<0.05$ and $^{**}P<0.01$ vs control (Student–Newman–Keuls test); $^{\#\#}P<0.01$ vs. control (Student's *t*-test).

2.5. Evaluation of sleep latency and sleeping time in mice

Observers were blind to the drug treatment. Following the pentobarbital injection, the mice were observed for the onset of sleep with the criterion for sleep onset being placed on its back and loss of righting reflex over 5 min. The mice lost the righting reflex less than 5 min were considered to be awake. The sleep latency time was recorded from the injection of pentobarbital to 1 min after lost the righting reflex and the sleeping time was recorded from 1 min after the loss of righting reflex to recovery.

2.6. Locomotor test in mice

Locomotor activity was measured by an ambulometer with five activity chambers (JZZ98, Institute of Materia Medica, Chinese Academy of Medical Sciences, China). Each activity chamber of 26 cm × 14 cm × 15 cm (length × width × height) was consisted of white opaque perspex walls, a transparent perspex lid and a floor. The floors were composed of 25 parallel copper bars with a fixed interval of 1 cm between the adjacent bars. The odd bars were grounded and the even bars were active and connected with micro-amper energy sources. The paws of the mice contacted or disconnected the active bars producing random configurations that were converted into pulses. The pulses, which were proportional to the locomotor activity of the mice, were recorded as the cumulative total counts of motor activity for a predetermined period of minutes.

2.7. Statistical analysis

All values obtained are represented as mean±S.E.M. Student's *t*-test was used to evaluate the difference between positive control and vehicle groups at the same time. For multiple comparisons, data were analyzed by one way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. Significant *F* scores were performed using ANOVA and differences were considered significant at $P<0.05$.

3. Results

3.1. Effect of GLE on pentobarbital-induced sleep in mice

Sleep latency and sleeping time in pentobarbital (45 mg/kg, ip) treated mice were 3.7±0.2 and 45.8±4.5 min, respectively. In a preliminary study, GLE showed no hypnotic activity in normal mice even at 120 mg/kg (data not shown). In pentobarbital (45 mg/kg)-treated mice, GLE not only decreased sleep latency, but also prolonged the duration of sleep significantly at doses of 80 and 120 mg/kg (Fig. 1A and B, $P<0.05$) and these activities were significantly antagonized by

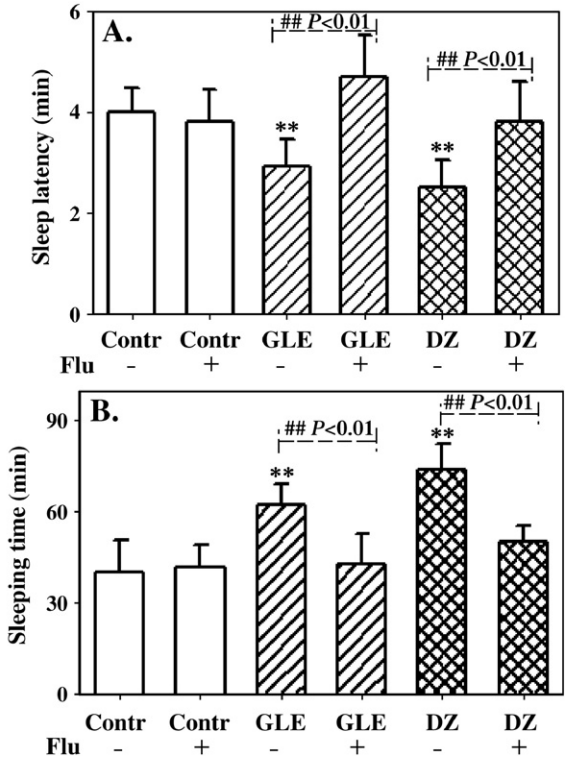

Fig. 2. Effects of flumazenil on the changes in sleep latency and sleeping time induced by GLE in pentobarbital-treated mice. The sleep latency (A.) [$F(5, 54)=14.98$, $P<0.000$] and sleeping time (B.) [$F(5, 53)=26.51$, $P<0.000$] were assessed. Data are presented mean±S.E.M ($n=10$). $^{**}P<0.01$ vs Contr (Student–Newman–Keuls test). $^{\#\#}P<0.05$ vs the group treated with GLE or DZ alone, respectively. Contr: control; DZ: diazepam (2.5 mg/kg, ip); GLE: 80 mg/kg (po); Flu: flumazenyl (3.5 mg/kg, ip).

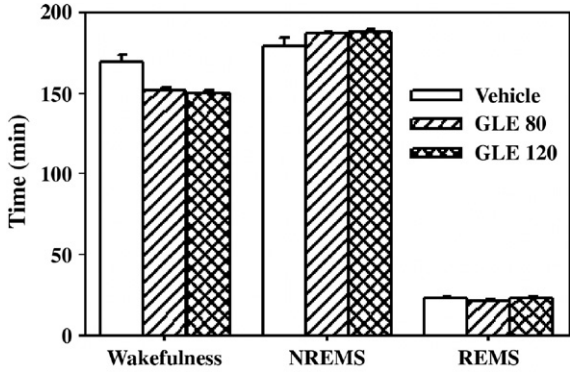

Fig. 3. Effects of GLE on total waking and NREM sleep and REM sleep in normal rats. Data are presented mean±S.E.M (n=5).

flumazenil, a benzodiazepine receptor antagonist, at a dose of 3.5 mg/kg (Fig. 2A and B). This antagonistic effect was also observed with diazepam, the positive control (Fig. 2A and B). On the other hand, no significant effects were observed with flumazenil even at 14.0 mg/kg on pentobarbital (50 mg/kg)-induced sleep in mice (data not shown).

3.2. Effect of GLE on the sleep parameters in pentobarbital treated rats

Even though GLE showed no effects on sleep architecture in normal rats at doses of 80 and 120 mg/kg (Fig. 3), GLE (80 mg/kg) decreased sleep latency (Fig. 4) and increased both total sleep and NREM sleep in pentobarbital- (45 mg/kg) treated rats, but revealed no significant influence on REM sleep (Fig. 5). During NREM sleep, GLE mainly prolonged light sleep (Fig. 5).

3.3. Effect of flumazenil on the changes in sleep parameters and delta activity induced by GLE in pentobarbital treated rats

Flumazenil alone showed no significant effects on sleep latency, total waking time and NREM sleeping time (Fig. 4). The shortening of sleep latency, increase in total sleeping time

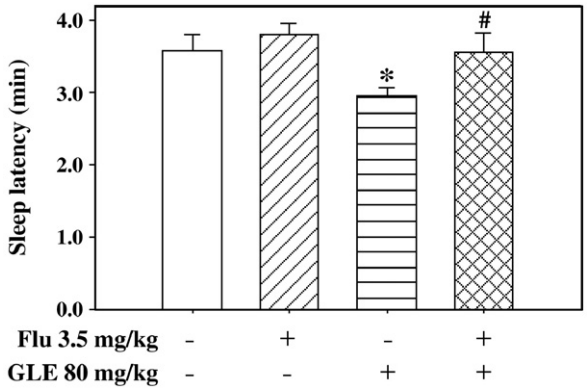

Fig. 4. Effect of flumazenil on the changes in sleep latency induced by GLE in pentobarbital-treated rats. The sleep latency [F(5, 54)=14.98, P<0.000] was assessed. Data are presented mean±S.E.M (n=10). *P<0.01 vs Contr and $^{\#}P$<0.05 vs the group treated with GLE or DZ alone, respectively (Student–Newman–Keuls test). Contr: control; DZ: diazepam (2.5 mg/kg, ip); GLE: 80 mg/kg (po); Flu: flumazenyl (3.5 mg/kg, ip).

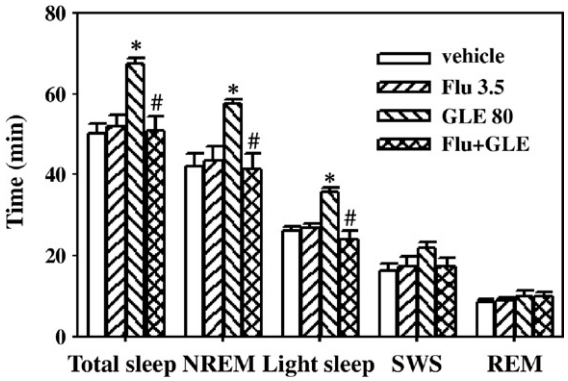

Fig. 5. Effects of flumazenil on the changes in sleep parameters induced by GLE in pentobarbital treated rats. Total sleep [F(3, 15)=9.00, P=.001], REM [F(3, 15)=0.57, P=0.646], NREM [F(3, 15)=6.18, P=0.006], SWS [F(3, 15)=1.85, P=0.182] and light sleep [F(3, 15)=10.94, P=0.000]] and were assessed. Data are presented mean±S.E.M (n=4–5). *P<0.05 and **P<0.01 vs. vehicle and $^{\#}P$<0.05 and $^{\#\#}P$<0.01 vs. group treated with GLE alone. Rats were administered GLE (80 mg/kg, p.o.) consecutively for 3 days and Flu (3.5 mg/kg, i.p.) was administered 20 min prior to the last administration of GLE. 60 min after GLE administration, rats were treated with pentobarbital (35 mg/kg, i.p.) and the EEG and EMG were recorded immediately until rats were awake.

and total NREM sleep time induced by GLE at a dose of 80 mg/kg was significantly antagonized by flumazenil at a dose of 3.5 mg/kg.

GLE at a dose of 80 mg/kg showed a significant increase in delta activity during non-REM sleep at 10–20, 20–30 and 30–40 min (Table 1) and flumazenil did not reveal significant effect on it.

3.4. Effect of flumazenil on the changes in autonomic activities induced by GLE in normal mice

The locomotor activity was measured for 5 min after 60 min of last treatment of GLE. Acute treatment with GLE (80 mg/kg) had no significant effect on locomotor activity in the mice (Fig. 6). However, after 3 days of treatment with GLE (80 mg/kg), the locomotion of the mice was significantly suppressed [F(3, 28)=13.29, P<0.000] (Fig. 6) and this effect was reversed by the pretreatment of flumazenil (3.5 mg/kg, i.p.) for 20 min prior to the last treatment of GLE. These data indicate that GLE is less effective as sedatives in the presence of benzodiazepine receptor antagonist and is probably exerting its sedative effect, at least in part, through the benzodiazepines receptor.

4. Discussion

The present study showed that GLE significantly potentiated the hypnotic activity of pentobarbital both in mice and rats by shortening the sleep latency and increasing the sleep time. Sleep analysis test showed that GLE markedly modified sleep parameters in pentobarbital treated rats with a prolongation of NREM sleep time or light sleep time without the influence on SWS and REM sleep. In addition, GLE also showed sedative effect by suppression the locomotor activity of normal mice.

In general, the barbiturates enzyme induction should be considered when evaluating the potentiation of barbiturates-

Table 1
Effect of flumazenil on the increase in delta activity during NREMs induced by GLE in pentobarbital treated rats.

Groups	Delta activity during NREMs (volts$^2 \times 10^3$)					
	0–10 min	10–20 min	20–30 min	30–40 min	40–50 min	50–60 min
Control	6.43±0.88	7.98±0.95	8.32±1.12	7.18±1.08	6.63±0.85	4.22±0.80
Flu	5.63±0.71	7.64±1.69	8.88±1.93	7.76±1.64	8.13±0.94	5.18±1.11
GLE	7.72±0.96	10.54±0.56 *	11.32±0.35 *	13.53±1.52 *	9.77±1.51	8.01±1.44
GLE±FLU	7.30±0.74	9.59±0.87	10.96±1.22	9.98±2.95	6.45±0.70	5.66±0.51

Each value represents the mean±S.E.M. for 5 rats.
* $P<0.05$ vs control (Student–Newman–Keuls test).

induced sleep by other drugs. In the primary research it was confirmed that 3 days treatment of GLE (80 and 160 mg/kg) did not affect the cytochrome P-450 and b5 content (data not shown).

To investigate the detailed mechanisms involved in the hypnotic and sedative potencies caused by GLE, the effects of flumazenil (benzodiazepine antagonist) on the sedative and hypnotic activities of GLE were studied. Flumazenil at a dose that caused no obvious effect when used alone showed a significant antagonistic effect on the decrease in sleep latency, increase in sleeping time, non-REM sleep time or light sleep time induced by GLE in pentobarbital-treated rat and suppression of locomotor activity by GLE in normal mice.

It is well known that benzodiazepine receptor agonists, such as diazepam, triazolam and flunitrazepam, caused the decrease in sleep latency, total waking time on the sleep–wake cycle in humans and animals. Gaillard and Blois (1989) and Borbély et al. (1991) reported that the flumazenil was capable of reversing several effects of benzodiazepines on sleep, such as increased total sleep time, decreased waking after sleep onset and shortened sleep latency. From the present results it should be presumed that the sedative and hypnotic effects induced by GLE may be mediated through the benzodiazepine receptors.

To confirm whether or not GLE enhances sleep quality, the changes in delta activities during NREM sleep that are thought to reflect sleep quality were studied. In this experiment, GLE showed a significant increase in delta power during non-REM sleep in pentobarbital-treated rat and flumazenil showed no significant antagonistic effects on it. By potentiating the GABAergic neurotransmission through an allosteric modulation of GABA type A (GABAA) receptor, benzodiazepines and analogs modify sleep and waking EEG patterns (Lancel et al., 1994; Aeschbach et al., 1994; Parrino and Terzano, 1996). In humans and rodents, these compounds typically reduce EEG delta activity in NREM sleep (Kopp et al., 2003; Landolt and Gillin, 2000; Tobler et al., 2001). However, flumazenil caused no antagonistic effect on the benzodiazepine-induced decrease in delta power activity (Shinomiya et al., 2004). These results demonstrated that the decrease in delta power activity by benzodiazepine hypnotics may not be mediated by benzodiazepine receptors. These findings taken together with our results suggested that an activation of benzodiazepines receptor induced by GLE may cause the hypnotic potencies and sedative activity. However, the enhancement of delta activity during NREM sleep caused by GLE may be related to the unknown pathway other than the modulation of benzodiazepines receptor.

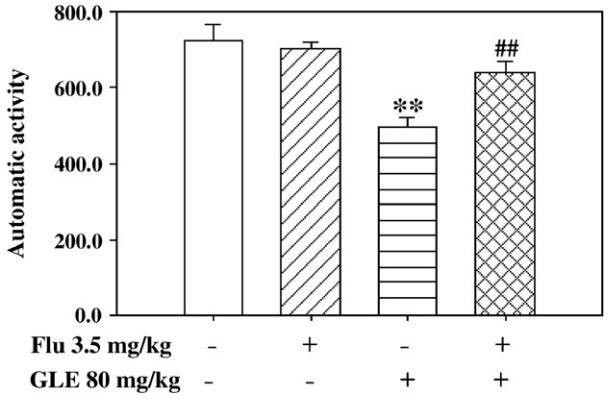

Fig. 6. Effect of flumazenil on the change in autonomic activities induced by GLE in normal mice. Mice were treated with GLE for 3 days. On 3rd day, mice were pretreated with Flu (3.5 mg/kg, i.p.) for 20 min prior to last administration of GLE (80 mg/kg,p.o.). Distilled water was given as control. 60 min after GLE administration, autonomic activities were tested [$F(3, 28)=13.29$, $P<0.000$]. Data are presented mean±S.E.M ($n=8$). **$P<0.01$ vs. vehicle and $^{\#\#}$ $P<0.05$ vs. group treated GLE alone.

Tang et al. (2005) reported that Ganopoly, the polysaccharide extract of *G. lucidum*, improved the insomnia severity scores in patients with neurasthenia. The mechanism for the beneficial effect of *G. lucidum* on insomnia is unknown. Several possible contributing factors may be involved.

Many polysaccharides (in particular β-D-glucans and glycoproteins) and triterpenoids, which are considered as the major active components, have been isolated and identified from *G. lucidum*. In our primary research glycoprotein with a molecular weight of 584,900, which has 17 amino acids from *G. lucidum* (Zhang et al., 2003), did not show any effect on pentobarbital sleep (data not shown). As described above, GLE used in this study does not contain triterpenoids. For these reasons, it should be presumed that the sedative and hypnotic activities of GLE observed in this study were not contributed by glycoproteins and triterpenoids of *G. lucidum*. The mycelium extract of *G. lucidum* inhibited sympathetic nerve activity in anesthetized rabbits and rats (Lee and Rhee, 1990). Moreover, adenosine isolated from the fruiting body of *G. lucidum* inhibited central inhibitory-reduced spontaneous motor activity, elevated pain threshold, prolonged the death time induced by caffeine, and relaxed skeletal muscle in mice (Kasahara and Hikino, 1987). However, it is unknown what component of *G. lucidum* contributes to its sedative and hypnotic activity and mechanistic studies are required to identify the molecular targets of *G. lucidum* in the brain.

These results indicated that caution should be taken when *G. lucidum* is used at higher doses or combined with other drugs such as benzodiazepines or barbiturates. Thus, monitoring of adverse events and/or plasma drug concentrations when *G. lucidum* is co-administered with drugs such as benzodiazepines should be systematically carried out, and potential drug interactions should be identified. This would enable a safer use of *G. lucidum*. In conclusion, *G. lucidum* is a herbal medicine having not only hypnotic effects but also sleep quality-enhancement effects.

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