Effect of single-use versus combined-use moschus and diazepam on expression of amino acid neurotransmitters in the rat corpus striatum

Na Zhang1,2, Ping Liu1, Xinrong He1

1 Traditional Chinese Medicine Pharmacy, Department of Pharmaceutical Care, Chinese PLA General Hospital, Beijing 100853, China
2 Department of Pharmacy, Shijiazhuang Fourth Hospital, Shijiazhuang 050011, Hebei Province, China

Abstract
The present study analyzed experssional changes of excitatory neurotransmitters and inhibitory neurotransmitters in the rat corpus striatum after single-use and combined-use diazepam and Chinese herb moschus. The influence of moschus on the central nervous system was analyzed, in particular whether moschus increased penetration of other drugs into the brain. Reverse-phase high-performance liquid chromatography, which included pre-column derivation with orthophthaldehyde detection, showed varied increased levels of excitatory neurotransmitters, including aspartate and glutamate, and inhibitory neurotransmitters, including glycine and γ-aminobutyric acid, in the corpus striatum after treatment with moschus alone, diazepam alone, or a combination of both. Compared with the diazepam group, aspartate levels significantly decreased at 30 and 60-105 minutes after combined treatment with moschus, while glutamate significantly increased at 30 minutes, and γ-aminobutyric acid increased at 75-105 minutes. These findings suggested that moschus increased the inhibition effects of diazepam on the brain.

Key Words: aspartate; diazepam; glutamate; glycine; high-performance liquid chromatography; microdialysis; moschus; neurotransmitter; γ-aminobutyric acid

INTRODUCTION
Diazepam is sedative, hypnotic, and anti-anxiety drug that has been used in clinical treatment. The underlying mechanisms of diazepam depend on binding of γ-aminobutyric acid (GABA) and the GABA_A receptor, which enhances GABA facilitation. However, patients are prone to adverse reactions, such as fatigue and dizziness, and high doses lead to ataxia, motor disorders, delirium, coma, and respiratory depression; long-term use results in tolerance, dependence, and even addiction[1]. These side effects also contribute to GABA_A receptors[1]. However, little is known regarding the efficacy and effect on adverse reactions with diazepam. Modern pharmacology studies and clinical trials have shown that resuscitation-inducing drugs act on the brain and also facilitate the crossing of other drugs through the blood-brain barrier, thereby increasing efficacy[2-3].

Moschus is a combination of dried secretions from mature male musk deer of the following species: Moschus berezovskii Flerov, M. sifanicus Przewalski, or M. moschiferus Linnaeus. Moschus has been shown to induce resuscitation and restore consciousness, promote blood circulation, clear obstructions in channels, subdue swelling, and alleviate pain[2-4]. The present study analyzed whether moschus could also promote diazepam efficacy and reduce adverse reactions.

Microdialysis and high-performance liquid chromatography detection were utilized to observe concentration changes of excitatory neurotransmitters aspartate (Asp) and glutamate (Glu), as well as inhibitory neurotransmitters glycine (Gly) and GABA, in the rat corpus striatum after treatment with Chinese herb moschus, diazepam alone, and in combination.

RESULTS
Quantitative analysis of experimental animals
A total of 24 rats were equally and randomly assigned to four groups: blank control, moschus, diazepam, and combination. At 60 minutes after probes implantation, the corpus striatum was sampled by brain microdialysis, respectively, at 15, 30, 45, 60, 75, 90, and 105 minutes after oral administration. All 24 rats met criteria for dialysis probe collection positions through...
histological verification and were included in the final analysis.

**Linear range of Asp, Glu, Gly, and GABA determinations**

Agilent 1200 high-performance liquid chromatography (HPLC) was used to measure concentrations of the amino acid neurotransmitters Asp, Glu, Gly, and GABA. The plotted standard curve (0.031 25-2.000 mg/L) of the four amino acids had a good linear relationship (Table 1, Figure 1).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Asp, Glu, Gly, and GABA standard curves and linear coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acids</td>
<td>Standard curve</td>
</tr>
<tr>
<td>Asp</td>
<td>Y=370.59X+2.052 7</td>
</tr>
<tr>
<td>Glu</td>
<td>Y=360.37X+3.963 6</td>
</tr>
<tr>
<td>Gly</td>
<td>Y=846.10X+68.794 0</td>
</tr>
<tr>
<td>GABA</td>
<td>Y=817.64X+3.328 8</td>
</tr>
</tbody>
</table>

Asp: Aspartate; Glu: glutamate; Gly: glycine; GABA: γ-aminobutyric acid.

---

**Determination of precision and probe recovery rate**

According to chromatographic conditions, the *in vitro* probe recovery rate of each amino acid was calculated (6.67% Asp, 6.09% Gly, 12.5% Glu, and 13.7% GABA) and subsequently converted into amino acid concentrations, respectively. The relative standard deviations of measured peak area and peak migration time are shown in Table 2.

**Effect of moschus and/or diazepam on levels of Asp, Glu, Gly, and GABA in the corpus striatum**

Compared to a standard HPLC plot, the four amino acids (Asp, Glu, Gly, and GABA) were separated from the brain dialysis samples (Figure 2).

**Asp concentration**

Compared with the control group, Asp concentration significantly increased in the moschus, diazepam, and combination groups (*P* < 0.05 or *P* < 0.01). Compared with the diazepam group, Asp concentration significantly decreased in the combination group at 30 minutes and 60-105 minutes (*P* < 0.05; Table 3).

**Glu concentration**

Compared with the control group, Glu concentration significantly increased in the moschus group (*P* < 0.05 or *P* < 0.01), significantly increased in the combination group at 45 minutes and 75-105 minutes (*P* < 0.05 or *P* < 0.01), and significantly increased in the diazepam group at 30 minutes (*P* < 0.05).
Concentrations also increased at the remaining time points, but the differences were not significant. Compared to the diazepam alone group, Glu levels significantly increased in the combination group at 45 and 75–105 minutes ($P < 0.05$ or $P < 0.01$; Table 4).

### Table 4 High-performance liquid chromatography detection of glutamate concentration (×10^−3 μmol/L) changes in rat brains after treatment with moschus and/or diazepam

<table>
<thead>
<tr>
<th>Group</th>
<th>Sampling time (minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15 30 45 60</td>
</tr>
<tr>
<td>Moschus</td>
<td>18.74±2.74</td>
</tr>
<tr>
<td>Diazepam</td>
<td>10.06±2.45</td>
</tr>
<tr>
<td>Combination</td>
<td>8.77±0.86</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD from six rats in each group. *P < 0.05 vs. control group; **P < 0.01 vs. control group; ***P < 0.001 vs. diazepam group (one-way analysis of variance).

**Gly concentration**

Compared with the control group, Gly concentration significantly increased in the moschus group at 15 and 45–90 minutes ($P < 0.05$ or $P < 0.01$), and significantly increased in the diazepam and combination groups at each time point ($P < 0.01$). Compared with the diazepam alone group, Gly levels significantly increased in the combination group at 105 minutes ($P < 0.05$). However, there was no significant difference in levels at other time points between groups (Table 5).

### Table 5 High-performance liquid chromatography detection of glycine concentration (×10^−3 μmol/L) changes in rat brains after treatment with moschus and/or diazepam

<table>
<thead>
<tr>
<th>Group</th>
<th>Sampling time (minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15 30 45 60</td>
</tr>
<tr>
<td>Moschus</td>
<td>2.07±0.38</td>
</tr>
<tr>
<td>Diazepam</td>
<td>2.33±0.50</td>
</tr>
<tr>
<td>Combination</td>
<td>2.97±0.40</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD from six rats in each group. *P < 0.05 vs. control group; **P < 0.01 vs. control group; ***P < 0.001 vs. diazepam group (one-way analysis of variance).

**GABA concentration**

Compared to the control group, GABA levels significantly increased in the moschus, diazepam, and combination groups ($P < 0.01$). Compared to the diazepam alone group, GABA levels significantly increased in the combination group at 30 and 75–105 minutes ($P < 0.05$ or $P < 0.01$). There was no significant difference at the remaining time points (Table 6).

### Table 6 High-performance liquid chromatography detection of γ-aminobutyric acid concentration (×10^−3 μmol/L) changes in rat brains after treatment with moschus and/or diazepam

<table>
<thead>
<tr>
<th>Group</th>
<th>Sampling time (minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15 30 45 60</td>
</tr>
<tr>
<td>Moschus</td>
<td>1.37±0.20</td>
</tr>
<tr>
<td>Diazepam</td>
<td>2.01±0.62</td>
</tr>
<tr>
<td>Combination</td>
<td>2.41±0.36</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD from six rats in each group. *P < 0.05 vs. control group; **P < 0.01 vs. control group; ***P < 0.001 vs. diazepam group (one-way analysis of variance).

**DISCUSSION**

Asp, Glu, Gly, and GABA are widely distributed in the central nervous system of mammals and plays an important role in inhibition of neuronal excitation. The present study was the first to show the function of moschus by quantifying concentrations of Asp, Gly, Glu, and GABA after treatment with the central inhibitory drug diazepam and/or moschus. As shown in Figure 2, four amino acids were separated from the brain dialysis samples, and the process was not affected by other impurities in the samples. Asp, Gly, GABA, and Glu levels increased at 30 minutes after treatment with diazepam alone, which was consistent with a previous study[6]. Compared to diazepam alone, Asp levels significantly decreased at 30 and 60–105 minutes after combination therapy, but Gly levels significantly increased at 105 minutes and GABA levels significantly increased at 30 and 75–105 minutes. These results suggested that moschus promoted the inhibitory effects of diazepam in the brain, and an equal dose could induce greater sedation. Therefore, it is suggested to decrease the diazepam dose to reduce the toxic side effects and addiction. After combined therapy, Glu levels significantly increased at 45 minutes and 75–105 minutes. These results could be due to the combination of drugs. Further studies are needed to determine the underlying mechanisms associated with these drug combinations. A recent pharmacological study showed that[6] moschus...
rapidly absorbs and acts on lesions, directly targets the central nervous system, prolongs survival time in mice under normal pressure ischemic conditions, and also alleviates nerve cell damage\[6\]. Very little is known about the role of moschus as an adjuvant and messenger drug for inducing other resuscitation drugs. The present study showed that moschus promoted the inhibition effect of diazepam in the brain.

### MATERIALS AND METHODS

#### Design

A randomized, controlled, pharmacological, animal experiment.

#### Time and setting

Experiments were performed from March 2010 to March 2011 at the Pharmacy of Traditional Chinese Medicine, Chinese PLA General Hospital, China.

#### Materials

**Animals**

A total of 24 healthy, male, specific pathogen-free, Sprague Dawley rats, aged 2 months and weighing 250 ± 10 g, were provided by the Experimental Animal Center of Chinese PLA General Hospital, China [license No. SCXX (Beijing) 2009-0007]. All animal experimental protocols were in strict accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of China\[7\].

**Drugs**

Moschus was purchased from Beijing Zhirigeng Factory, China, lot No. 200080301, and identified by Ping Liu, senior pharmacist from Pharmacy of Traditional Chinese Medicine, Chinese PLA General Hospital, China.

#### Methods

**Drug intervention**

Rats were equally and randomly assigned to four groups (n = 6). Control group: rats were intragastrically administered with 1 mL purified water per day; moschus group: rats were intragastrically administered with a moschus water suspension at a dose of 90 mg/kg per day\[8\]; diazepam group: rats were intragastrically administered with diazepam (Tianjin Lisheng Pharmaceutical, China) water suspension at a dose of 2.5 mg/d; combination group: rats were intragastrically administered with a suspension of moschus (90 mg/kg) and diazepam (2.5 mg) per day. All interventions were given for 7 successive days\[8\].

**Preparation of artificial cerebrospinal fluid**

Artificial cerebrospinal fluid comprised 7.363 g NaCl, 0.122 g CaCl\(_2\), 2.31 g NaHCO\(_3\), 0.172 g MgCl\(_2\), 0.179 g KCl, 0.071 g Na\(_2\)SO\(_4\), and 0.068 g KH\(_2\)PO\(_4\) dissolved in 1 L distilled water at pH 7.38, followed by filtration through a 0.2 μm microporous membrane. The prepared artificial cerebrospinal fluid was stored at 4°C.

**Preparation of derivative reagent**

Orthophthaldehyde (Sigma, St. Louis, MO, USA; 12.5 mg) was precisely weighed and dissolved in 0.25 mL methanol, mixed with 2.5 mL borate buffer (0.4 M, pH 9.5), and then added to 30 μL β-mercaptoethanol (Sigma). The resultant reagent was stored at 4°C.

#### Chromatographic conditions

HPLC device (Agilent 1200), G1311A series quaternary gradient pump, G1329A automatic sampler, G1329A ALS fluorescence detector, HP Rev.A.0501 ChemStation, and Agilent TC-C\(_18\) (5 μm, 250 mm × 4.6 mm) chromatographic column were provided by Agilent, Santa Clara, CA, USA. Prior to derivatization, the column was tested with G1329A ALS fluorescence detector at excitation wavelength 340 nm and emission wavelength 45 nm. Mobile phase: phosphoric acid buffer solution (0.1 M, prepared with Na\(_2\)HPO\(_4\), pH 6.86) → methanol (70: 30), gradient elution; velocity 1.0 mL/min; column temperature 30°C; injection volume 10 μL.

**Plotting standard curve**

Asp, Glu, Gly (China's National Institute for the Control of Pharmaceutical and Biological Products, China, lot number: 624-200104), and GABA (Sigma) standards were precisely weighed and prepared into amino acid standard solutions at different concentrations of 2.000, 1.000, 0.500 0, 0.250 0, 0.125 0, 0.062 5, 0.031 25, and 0.015 63 mg/L, respectively. A 10-μL sample solution at each concentration was detected to plot the standard curve, with the concentration representing abscissa and peak area for the vertical axis.

**Determination of precision**

Standard solution of mixed amino acids at concentrations of 0.125 0, 0.250 0, and 0.031 25 mg/L were continuously sampled three times using the above chromatographic conditions. The relative standard deviation from four amino acid peak areas and peak migration times were measured.

**Determination of probe recovery**

Prior to sampling, dialysis probes were placed in a 2 mg/L mixed solution to collect in vitro dialysis solution with the same perfusion fluid, perfusion speed, and time interval. According to the above-described chromatographic conditions, the in vitro probe recovery rate of each amino acid was measured to convert into in vivo amino acid concentrations.

**Microdialysis probe implantation**

Rats were intraperitoneally anesthetized with 10% chlororhydrate (3.45 mL/kg) and fixed in a WDT-stereotaxic instrument (State-operated Northwest Optical Instrument Factory, Xi’an City, Shaanxi Province, China). Rats were fixed with ear rods and tooth ring, and were placed on a thermal blanket. The scalp was cut open and the exposed skull was drilled according to coordinates described in the Rat Brain Stereotaxic Coordinates\[9\]. Caution was taken to avoid damage to the cerebral dura mater. Using the spinning vertical arm of the stereotaxic instrument, the BR-4 brain microdialysis probe (CMA, Stockholm, Sweden) was implanted into the
Sample collection

Microdialysis perfusion system (CMA) was used to perfuse artificial cerebrospinal fluid. Perfusion velocity was maintained at 2.0 μL/min by controlling the micro-syringe pump (CMA-400 type). At 60 minutes after the probe was implanted, dialysis solution was collected every 15 minutes for a total of seven samples, and immediately stored at −80°C. After dialysis was implemented, the rats were sacrificed by decapitation, and the microdialysis sampling location was verified using histological methods. If the probe membrane was dislocated or brain damage was severe, the results were not included in the final analysis[10].

HPLC detection of Asp, Glu, Gly, and GABA concentrations in the corpus striatum

Based on above-described analytical methods, Asp, Glu, Gly, and GABA concentrations in the rat striatum were measured at different sampling time points.

Statistical analysis

Data are expressed as mean ± SD and were analyzed using SPSS 9.0 statistical software (SPSS, Chicago, IL, USA). Comparisons between groups were performed using analysis of variance, and pairwised comparison was performed using the SNK-q test. P < 0.05 was considered statistically significant.

Author contributions: Na Zhang and Ping Liu had full access to all data and participated in data integrity and data analysis accuracy. All authors were responsible for data collection, interpretation, and study design.

Conflicts of interest: None declared.

Ethical approval: The project received full ethical approval from the Animal Committee of the Chinese PLA General Hospital in China.

REFERENCES


(Edited by Wang RG, Su YH/Yang Y/Wang L)