Antithrombotic effects in rats of Germidine, an alkaloid from *Veratrum Nigrum* L. var. *ussuriense* Nakai

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Abstract  
Germidine is one of ester type steroidal alkaloids extracted from *Veratrum Nigrum* L. var. *ussuriense* Nakai (“Wusuli Lilu” in Chinese), a herbal medicine that has long been used as an anti-hypertensive remedy in Chinese traditional medicine. Although the antithrombotic effects of the *Veratrum nigrum* alkaloids (VnA) have been reported, the effective chemical constituent of VnA is still unclear. In view of this fact, we investigated the inhibitory activity of germidine on thrombosis in rats. In rat carotid artery thrombosis model, germidine (iv 1.25-20.0 µg·kg⁻¹) significantly and dose-dependently prolonged the occlusion time (OT) of carotid artery injured by electrical stimulation. In rat inferior vena thrombosis model, germidine (iv 2.5-20.0 µg·kg⁻¹) decreased the thrombus dry weight in a significant and dose-dependent manner. LD₅₀(iv) in mice of germidine was found to be 6.6619 mg·kg⁻¹, a value much greater than that of VnA. We conclude that germidine has powerful inhibitory effects against both arterial and venous thrombosis in rats and plays an important role in the antithrombotic effects of VnA.

Key words  
Germidine, *Veratrum nigrum* L. var. *ussuriense* Nakai, alkaloids, thrombosis, rat

Introduction  
Over the past two decades our lab addressed themselves to the phytochemical and pharmacological researches of *Veratrum nigrum* L. var. *ussuriense* Nakai (“Wusuli Lilu” in Chinese), a well-known Chinese traditional herbal medicine widely distributed in the Northeast region of China[1-7]. So far, eleven ester type steroidal alkaloids have been isolated from the crude drug and chemically identified by means of spectrophotometry, IR spectrum, mass spectrum, NMR and other modern analytical techniques[1-4]. It was discovered by us for the first time that the *Veratrum nigrum* alkaloids (VnA), namely, the total alkaloids isolated from the above stated herb, also had powerful antithrombotic and blood viscosity-lowering effects apart from its well-known and extensively studied antihypertensive effect[5-7]. However, which constituent of VnA acts is still unclear.

To date, no information is available regarding the pharmacological and toxicological effects of single complement contained in VnA. The present study was undertaken to investigate the antithrombotic effects of germidine (Fig 1), a purified ester type steroidal alkaloid isolated from VnA, with *in vivo* rat thrombosis models as well as the acute toxicity in mice of germidine was also examined.
Materials and Methods

Chemicals and instruments

Gimidine hydrochloride injection (500.0 µg·mL⁻¹) was a gift from the Department of Pharmaceutical Engineering, Dalian University of Science and Technology, China. It was diluted to the required concentration with sterile saline solution prior to use. Heparin sodium was purchased from Beijing Ao Bo Xing Biotechnology Co., Ltd, Beijing, lot No. 20050819; d l-lysine-acetylsalicylate (LAS) was a product of Anbao Pharmaceutical Factory, Anhui Provience, lot No.950327. BT 87-3model experimental in vivo thrombosis instrument was purchased from Cardiovascular Research Department, Baotou Medical College, Baotou, Inner Mongolia Provience.

Animals

Male Sprague-Dawley rats weighing 180-220g were supplied by Animal Center of Dalian Medical University. Male ICR mice (Experimental Animal Center of Dalian Medical University) weighing 18-22g were used. They were randomly assigned to be group-housed in an animal laboratory which was maintained at constant temperature (22±2°C) and relative humidity (55±10%) and with a 12-h light/dark cycle. Food and water were freely available.

Toxicity of germidine and LD₅₀ determination

A total of 40 male mice (18-22g) were randomly divided into 4 groups (10 mice each group) receiving different doses of germidine by i.v. route. The animals were kept in plastic cages (5 animals per cage) and mortality, physiological and behavioural signs of toxicity were recorded for a consecutive7 day period. The LD₅₀ was calculated by means of NDST program edited by Professor Sun Rui-yuan.

Rat carotid artery thrombosis model

The carotid artery thrombosis in rats was induced by the method developed by Hladovec[8] with minor modification. Rats were randomly divided into groups and anesthetized by ip 20% urethane (1g·kg⁻¹). After unilateral carotid artery was isolated surgically, a direct current stimulating electrode and a temperature electrode were placed at heart-proximal and heart-remote ends of carotid artery, respectively. Rats received a single bolus iv administration of germidine in drug-treated groups, LAS in positive control group and equal volume of saline solution in negative control group, respectively. The continual electric stimulation (1.6mA, 7min) was started in 20 minutes postinjection. The time elapsing from the beginning of stimulation to abrupt fall in temperature of carotid artery surface was recorded by means of the temperature electrode of the instrument. This time represented the occlusion time (OT) of carotid artery injured electrically, namely, thrombus formation time. Then, the percent increase of OT of germidine-treated groups vs saline control group served as an index to evaluate anti-arterial thrombosis effects of the drug.

Rat inferior vena cava thrombosis model

The inferior vena cava thrombosis was induced by venous blood stasis in accordance with Reyer’s method[9]. Briefly, rats were randomly divided into groups and anesthetized as above. The middle abdominal wall of rats was incised, then inferior vena cava was separated surgically; different groups of rats were treated with iv varying doses of germidine (1.25-20.00 µg·kg⁻¹), heparin (400 µg·kg⁻¹) as positive control and equal volume saline as negative control, respectively. 20 min later, the inferior vena cava was ligated to induce stasis. Then, abdominial cavity was closed, and reopened 4 hours after ligation. The blood vessel of inferior vena cava was clamped at 2 cm below the ligation. The thrombus clot in the blood vessel was removed (if any) and weighed after drying at 60°C for 20 min. The animal numbers of thrombus formation and thrombus drying weight, which were used to evaluate the anti-venous thrombotic effects of the drug, were recorded so as to calculate thrombus formation rate (animal number of thrombosis / test animal number) and percent inhibition of thrombus weight, respectively.

Statistical analysis
Data were analyzed by one-way ANOVA. Whenever ANOVA was significant, further comparisons between vehicle- and drug-treatment groups were made using Dunnett’s t-test. All analysis was performed using the software SPSS V11.5 for windows. The level of statistical significance adopted was $P < 0.05$.

**Results**

**Acute toxicity of germidine and LD$_{50}$ determination**

The 24 hr mortality of mice were 0.20, 0.40, 0.60 and 0.80 at doses of 5.22, 6.14, 7.23 and 8.50 mg·kg$^{-1}$, respectively. The LD$_{50}$ of germidine in mice was estimated to be 6.6619 mg·kg$^{-1}$ (i.v.) (with a 95% confidence limit of 6.4272 mg·kg$^{-1}$ ~ 6.9052 mg·kg$^{-1}$). The observed toxic symptoms include sialorrhea, palpebral closure, shudder, ecphysisis, respiratory distress, convulsion. At last, the mice died from respiratory depression. Quick gastrointestinal motility could be seen when abdominal cavity was opened. All deaths occurred during the first 10 minutes postdose. During the following six days, no death was detectable in the rest of animals.

**Effects of germidine on rat carotid artery thrombosis**

As shown in Fig 2, iv injection of germidine resulted in a dose-dependent increase in the OT. The OTs in the germidine-treated groups (n=10, each group) were (794.9±15.6) (p<0.05), (869.7±18.6), (913.6±10.3), (982.9±15.7) and (1133.7±62.8) (P<0.01) seconds at doses of 1.25, 2.50, 5.00, 10.00 and 20.00 µg·kg$^{-1}$, respectively, compared with (683.0±12.9) seconds in negative control group and (1040.2±18.5) seconds in positive control group. The percent increases of OT in germidine-treated groups relative to the negative control group were 16.4%, 27.3%, 33.8%, 43.9% and 66.0% Table 1, respectively, indicating that germidine had significant effects against experimental arterial thrombosis.

**Table 1. Effect of germidine (iv) on carotid artery thrombosis in rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>OT (s)</th>
<th>OT Increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td>683.0±12.9</td>
<td></td>
</tr>
<tr>
<td>Germidine</td>
<td>1.25 µg·kg$^{-1}$</td>
<td>794.9±15.6*</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td>2.50 µg·kg$^{-1}$</td>
<td>869.7±18.6**</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td>5.00 µg·kg$^{-1}$</td>
<td>913.6±10.3**</td>
<td>33.8</td>
</tr>
<tr>
<td></td>
<td>10.00 µg·kg$^{-1}$</td>
<td>982.9±15.7**</td>
<td>43.9</td>
</tr>
<tr>
<td></td>
<td>20.00 µg·kg$^{-1}$</td>
<td>1133.7±62.8**</td>
<td>66.0</td>
</tr>
<tr>
<td>LAS</td>
<td>18.0 mg·kg$^{-1}$</td>
<td>1040.2±18.5**</td>
<td>52.3</td>
</tr>
</tbody>
</table>

Values represent means±S.E.M. from 10 rats. Significance of difference: * $P < 0.05$, ** $P < 0.01$ compared with vehicle condition; Dunnett’s t-test after one-way ANOVA (df=6, 69 F=29.95, $P < 0.01$).

![Fig 2. Effect of germidine (iv) on carotid artery thrombosis in rats](image-url) Values represent means±S.E.M. from 10 rats. Significance of difference: * $P<0.05$, ** $P<0.01$ compared with vehicle condition; Dunnett’s t-test after one-way ANOVA(df=6, 69 F=29.95, $P<0.01$).
Table 2. Effect of germidine (iv) on stasis-induced venous thrombosis in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose(µg/kg)</th>
<th>Thrombus formation rate</th>
<th>Dry weight of thrombus mg</th>
<th>Inhibition of dry weight(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>10/10</td>
<td>5.75±0.40</td>
<td>——</td>
<td>——</td>
</tr>
<tr>
<td>Germidine</td>
<td>1.25</td>
<td>10/10</td>
<td>5.35±0.37</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>2.50</td>
<td>10/10</td>
<td>2.37±0.31**</td>
<td>58.8</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>10/10</td>
<td>1.70±0.33**</td>
<td>70.4</td>
</tr>
<tr>
<td></td>
<td>10.00</td>
<td>10/10</td>
<td>1.56±0.24**</td>
<td>72.9</td>
</tr>
<tr>
<td></td>
<td>20.00</td>
<td>9/10</td>
<td>0.98±0.36**</td>
<td>83.0</td>
</tr>
<tr>
<td>Heparin</td>
<td>400.0</td>
<td>8/10</td>
<td>0.86±0.27**</td>
<td>85.0</td>
</tr>
</tbody>
</table>

Values represent means±S.E.M. from 10 rats. Significance of difference: ** P<0.01 compared with vehicle condition; Dunnett’s t-test after one-way ANOVA. (df=6, 69  F=41.77, P<0.01)

Effects of germidine on rat inferior vena cava thrombosis:

From Table 2, it could be found that at doses of 2.50, 5.00, 10.00 and 20.00 µg·kg\(^{-1}\) germidine significantly and dose-dependently inhibited venous thrombosis, as evidenced by the decreased dry weight of thrombus in germidine-treated groups compared with saline-treated group (2.37, 1.70, 1.56 and 0.98 mg vs 5.75 in dry weight), with percent inhibition being 58.8%, 70.4%, 72.9% and 83.0%, respectively. It was evident that the antithrombotic activity produced by high dose (20.00 µg·kg\(^{-1}\)) was close to that by iv heparin 400 µg·kg\(^{-1}\) (83.0% vs 85.0%). It was also apparent that there was no significant difference (p>0.05) in thrombus formation rate (animal number of thrombosis / test animal number) between germidine- and saline-treated groups.

Discussion

In this study the antithrombotic effects of germidine were investigated using rat carotid artery thrombosis model and rat inferior vena cava thrombosis model. In the former model, electric stimulation of carotid artery led to endothelia injury, followed by platelet adhesion, aggregation and formation of a platelet-rich thrombus which caused occlusion of the artery. So, the OT was used to examine the inhibitory effect of germidine against arterial thrombosis. In the latter model inferior vena cava was ligated for four hours and thus rendered blood stasis, consequently leading to activation of blood clotting system and formation of thrombus. Above stated models are simple, reproducible and reliable for evaluating antithrombotic effects of drugs, including LAS and heparin, which were used as positive controls, and germidine, which was examined in the present experiment.

To date, no information is available regarding the pharmacological and toxicological effects of germidine. The present study showed for the first time that germidine had powerful anti-venous and anti-arterial thrombosis effects at doses of 1.25~20.00 µg·kg\(^{-1}\), a value much smaller than the effective doses (3.0~45.0 µg·kg\(^{-1}\)) required for VnA to inhibit the thrombosis in the same rat models\(^{[7]}\). The above findings strongly demonstrate that germidine has more potent inhibitory activity against thrombosis than VnA.

In this study LD\(_{50(IV)}\) of germidine was estimated to be 6.6619 mg·kg\(^{-1}\) with a 95% confidence limit of 6.4272 mg·kg\(^{-1}\) ~ 6.9052 mg·kg\(^{-1}\), whereas in a previous study carried out in our lab VnA was found to have a LD\(_{50(IV)}\) of 0.1734±0.0179 mg·kg\(^{-1}\) \(^{[10]}\). By calculation, LD\(_{50}\) of germidine is 38 times as much as that of VnA, suggesting that germidine has much lower toxicity than VnA.

In summary, germidine has powerful inhibitory effects against both arterial and venous thrombosis in rats and acts in a dose-dependent manner. Germidine plays an important role in the antithrombotic effects of VnA, and has lower toxicity than VnA.

References

1. Zhao WJ, Chen J, Guo YT, Xu LS, Sun NJ. Chemical


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