A Pathologic Study on the Inhibitory Effects of a Herbal Medicine against the Glomerular Lesion Induced by *Agkistrodon* Venom in Mice

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**ABSTRACT.** The inhibitory effects of a prescription of herbal medicine, tentatively named P-3, were studied pathologically in an experimental model of the glomerular lesion induced by purified snake *Agkistrodon acutus* venom proteinase (Ac1-P) in mice. Ac1-P was intravenously inoculated at a single LD50 dose. In the treated group, mice were intraperitoneally injected with an extract of P-3 at a designated time once every two days from 2 days before to 1 week after Ac1-P inoculation. The control group mice were injected with saline instead of P-3. In the control group, the main pathologic changes within 48 hrs after inoculation were pulmonary and gastrointestinal tract hemorrhage and renal petchiae with hematuria. The kidney microscopically showed cystic transformation of the glomerular capillary tufts, followed by occlusive thrombosis. One week after inoculation, the glomerular lesions were mostly replaced by proliferative or proliferative-sclerosing changes with occasional crescent formation. Early signs of tubular atrophy accompanying the glomerular changes were observed. In the P-3 treated mice surviving 48 hrs and 1 week, the changes observed in the controls were markedly inhibited, although P-3 treated mice dying earlier than 30 hrs exhibited hemorrhagic changes similar to controls. This indicated that the herbal medicine had efficacy against the tissue injuries induced by Ac1-P as a proteolytic enzyme.—**KEY WORDS:** *Agkistrodon acutus* venom, herbal medicine, renal lesion.

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From the early 1900s, snake venom has been known to injure glomeruli in various animals [1, 2, 5, 7, 9, 12], and we reported the glomerular lesion induced by a purified snake (*Agkistrodon acutus*) venom proteinase (Ac1-P) [8]. The pathologic changes induced by Ac1-P were characterized in the earliest period by hemorrhage in various organs and cystic transformation of glomerular capillary tufts caused by mesangiolysis. The cystic lesions were principally occluded with fibrinous thrombi. The thrombi were replaced by proliferative and sclerosing changes in a few days, followed by a focal segmental glomerulonephritis. This proteinase-mediated glomerular injury may serve as an experimental model of glomerular diseases.

Since ancient times, many prescriptions of herbal medicine in Taiwan, so-called "qingcao" (green grass medicine), have been used as remedy for renal disease in animals and humans, although almost all of them have been transmitted as legend and hardly have any scientific justification. It is hence difficult to find scientific evidence for the efficacy of these traditional medicines, and the evaluation of their therapeutic effects has not been described.

We identified the botanical names of the herbs included in the medicine, and clarified their effect on decreasing mortality in mice with glomerular lesions due to snake venom in a provisional experiment.

The inhibitory effect of the herbal medicine on the glomerular lesion induced by Ac1-P in mice was studied in detail on the basis of the renal pathology.

**MATERIALS AND METHODS**

**Snake venom:** Lyophilized venom (The Japan Snake Institute, Gunma, Japan) from 100-pace snakes (*Agkistrodon acutus*) from Taiwan and mainland China was purified using the method of Nikai et al. [6]. The purified *Agkistrodon* venom, named Ac1-proteinase (Ac1-P), was used to induce the renal lesion in mice.

**Herbal medicine:** The prescription of the herbal medicine, P-3, used in the present study contained the following herbs: *Achyranthes obtusifolia* Lam. (Blending ratio in weight, (1); *Clerodendrum chinense* L. (1), *Desmodium strychnifolium* (Osb.) Merr. (1), leaf of *Eriobotrya japonica* (Thunb.) Lindl. (1), *Glechoma hederacea* L. (1), *Hypericum japonicum* Thunb. (1), *Ludwigia octovalvis* (Jacq.)
Raven (1), Pogonatherum crinitum (Thunb.) Kunth (1), Serissa japonica Thunb. (1), Solanum suratense sensu act. Taiwan (1), hair of Zea mays L. (0.5), and seed of Nasturtium indicum (L.) DC. (0.1). The botanical names of these twelve species of herbs were quoted from elsewhere [3, 4, 10, 13].

All herbs were dried and powdered. In order to simulate the traditional decoction procedure, the mixed herb powder was infused in boiling distilled water of ten-fold net weight for 30 min and centrifuged at 8,000 rpm for 10 min. The separated sediment was again infused in boiling distilled water in the same manner. A mixture of both supernatants was concentrated in an evaporator under reduced pressure, so that 1 g of the mixed powder corresponded to 1 ml of the concentrated extract. The concentrated extract was then centrifuged at 12,000 rpm for 30 min and the supernatant was filtered through a Milipore filter with a pore diameter of 0.45 μ. Finally, the filtrate contained about 100 mg dry weight of the extract in 1 ml.

**Mice:** Four weeks old albino ddY mice were used.

**Experimental design:** AC5-P was inoculated into the lateral tail vein of the mice at a single LD50 dose, 0.2 ml containing 6.5 to 7.2 mg per kg body weight.

In herb group, mice received the P-3 extract in a dose of 0.1 ml intraperitoneally at a consistent time once every two days, from two days before to one week after inoculation with AC5-P. The dose used was about 10 to 25 times greater than the oral dose used for treating renal disease in a domestic animal.

In control group, mice were injected with saline in the same manner instead of the extract.

The two groups consisting of a total of 191 mice were subdivided and sacrificed at 48 hrs and 1 week after inoculation (Tables 1 and 2). The survival rate, hematuria, kidney and body weights, the degree of hemorrhage in the lungs, stomach, and kidneys, and concentration of blood urea nitrogen (BUN) were determined at time of sacrifice. Occult hematuria was checked using commercial testing paper (Multistix-III; Miles-Sankyo Co., Ltd., Tokyo).

**Preparation of tissues for light and electron microscopy:** For light microscopy, the kidneys of 14 control and 21 herb-treated mice at 48 hrs and 20 mice from both groups at 1 week after inoculation were fixed in 10% neutral-buffered formalin. Paraffin sections were stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS), and the total glomeruli in a section of one kidney were counted and observed. For transmission and scanning microscopy (TEM and SEM), unilateral kidneys of 6 mice per subgroup were processed 1 week after inoculation. Tissues for TEM were fixed in phosphate-buffered 2% glutaraldehyde and in phosphate-buffered 1% osmium tetroxide. The specimens were then dehydrated in ethanol and embedded in epon. Ultrathin sections were stained with uranyl acetate and lead citrate and then observed using a JEM-100CX electron microscope (JEOL, Tokyo). For SEM, after fixation and dehydration, the specimens were dried using the critical point method, coated with gold-palladium and observed using a JSM-35 electron microscope (JEOL, Tokyo).

The other organs were routinely processed for HE-staining.

**Microangiography:** The kidneys of 10 mice per subgroup were examined using our method described previously [11].

**Statistical analyses:** Nonparametric data were analyzed using the Mann-Whitney U-test, normal data using the Student's t-test and proportional data using the X2-test.

**RESULTS**

**General changes:** The survival rates were higher and the BUN values lower in the herb-treated group than in the control group both at 48 hrs and 1 week.

<p>| Table 1. Survival rate and BUN value 48 hours after injection with AC5-P |
|--------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of Mice tested</th>
<th>Died</th>
<th>Survived</th>
<th>Survival rate</th>
<th>BUN (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26</td>
<td>12</td>
<td>14</td>
<td>53.8(%)</td>
<td>80.4±58.6</td>
</tr>
<tr>
<td>Herb(P&lt;0.3)</td>
<td>25</td>
<td>4</td>
<td>21</td>
<td>84.0*</td>
<td>33.2±30.2*</td>
</tr>
</tbody>
</table>

* P<0.05.  
a) Analyzed by X2 test.  
b) Blood Urea Nitrogen, represents mean±standard deviation and analyzed by U-test.
after venom inoculation (Tables 1 and 2). The absolute and relative kidney weights of the survivors at 1 week were higher in the herb group (Table 3).

**Macroscopic findings:** On dissection, mice dying earlier than 12 hrs after inoculation in both groups showed intense hemorrhage in the lungs, stomach and duodenum. Renal hemorrhages were inconspicuous at this time interval. Then, while the hemorrhage in the lungs, stomach and duodenum became less, the kidneys became increasingly scattered with petechiae. The renal petechiae were most conspicuous between 20 and 30 hrs and subsequently tapered rapidly. The mice dying less than 30 hrs after inoculation showed no statistically significant differences between the two groups at the same time intervals, although the lesions appeared less in herb-treated mice.

In the mice dying around 48 hrs or killed 48 hrs after inoculation, the hemorrhages and hematuria were more intense and in much higher incidence in the controls (Table 4). One week after inoculation, the kidneys in controls were anemic and smaller, occasionally exhibiting a granular surface. These renal changes were less intense and lower in incidence in the herb group (Figs. 1a, b and 2a, b; Table 4). The other organs showed no significant macroscopic changes 1 week after venom inoculation.

**Light microscopic findings in the dead animals:** The hemorrhagic changes described above were considerably uniform in quality: alveolar hemorrhage in the lungs with occasional thickening of alveolar walls by slight leukocytic infiltration, and mucosal and submucosal hemorrhage in the stomach and duodenum. The grossly observable peptichiae in the kidneys were the earliest glomerular lesions, namely segmental cystic transformation of the capillary tufts, which we described in detail previously [8]. The cystic transformation in the mice dying between 20 and 30 hrs after inoculation occurred in

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**Table 2. Survival rate and BUN value 1 week after injection with Ac1-P**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of mice tested</th>
<th>Died</th>
<th>Survived</th>
<th>Survival rate a)</th>
<th>BUN b) (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>75</td>
<td>38</td>
<td>37</td>
<td>49.3(%)</td>
<td>31.5±8.0</td>
</tr>
<tr>
<td>Herb(P-3)</td>
<td>65</td>
<td>19</td>
<td>46</td>
<td>70.8**</td>
<td>26.2±3.1*</td>
</tr>
</tbody>
</table>

* P<0.05, **P<0.01.
a) Analyzed by X² test.
b) Blood urea nitrogen, represents mean±standard deviation and analyzed by U-test.

**Table 3. Body and kidney weights of mice sacrificed 1 week after injection with Ac1-P**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of mice tested</th>
<th>B.W. before a)</th>
<th>B.W. after a)</th>
<th>K.W. b)</th>
<th>K.W./B.W. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>18.2±1.2 b)</td>
<td>23.5±2.8</td>
<td>0.29±0.05</td>
<td>1.23±0.15</td>
</tr>
<tr>
<td>Herb(P-3)</td>
<td>36</td>
<td>18.1±1.1</td>
<td>24.8±1.7</td>
<td>0.32±0.03*</td>
<td>1.34±0.13*</td>
</tr>
</tbody>
</table>

* P<0.05, analyzed by U-test.
a) B.W. before and after, represent body weight in gram on the 2 days before and 7 days after injection with Ac1-P, respectively.
b) Kidney weight in gram.
c) Mean±S.D.

**Table 4. Incidences of hemorrhage in organs and hematuria within 48 hours after injection with Ac1-P**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of mice tested</th>
<th>Hemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stomach (%)</td>
</tr>
<tr>
<td>Control</td>
<td>26</td>
<td>15(57.7)</td>
</tr>
<tr>
<td>Herb (P-3)</td>
<td>25</td>
<td>6(24.0)*</td>
</tr>
</tbody>
</table>

* P<0.05, **P<0.01, analyzed by X² test.
more than 90% of the glomeruli and was filled with blood with or without mural thrombosis. Fibrin clots and erythrocytes were frequently found in tubular lumina. Proximal tubular epithelia, interstitial capillaries and small veins were injured. The liver and heart were usually congested without parenchymal changes.

Light and electron microscopic findings in the survivors: At 48 hrs after inoculation, the incidence of the cystic lesions was much lower in the survivors in both groups than in the dead mice. In the survivors, herb-treated mice showed about one half the incidence of the cystic lesions observed in controls (Table 5; Figs. 3a, b). In controls, most of the cystic lesions were occluded with fibrinous thrombi. Fibrin clots were found in tubular lumina near affected glomeruli. Mitoses were frequently observed in tubular epithelia. In herb-treated mice, cystic lesions were inconspicuous and the main glomerular change was a slight increase in cells and/or matrix in the mesangium. The mesangial change also occurred in the controls, independent of the cystic lesions.

One week after inoculation, the thrombi in the cystic lesions had mostly been replaced by hypercellularity, resulting in segmental proliferative or proliferative-sclerosing lesions. Crescent formation occurred more frequently in controls (Fig. 4a) and intact glomeruli were found slightly more often in herb-treated mice (Table 5). In herb-treated mice,
Table 5. Glomerular changes of mice sacrificed 48 hours and 1 week after injection with Ac₁-P

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of mice examined</th>
<th>Mean No. of glomeruli observed</th>
<th>Increase in mesangial cells and/or matrix*</th>
<th>Cystic lesion</th>
<th>Crescents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thrombosed (§)</td>
<td>Proliferative (%)</td>
<td>Sclerotic (%)</td>
</tr>
<tr>
<td>(48 hours)</td>
<td></td>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>Control</td>
<td>14</td>
<td>186</td>
<td>7.2</td>
<td>45.1</td>
<td>47.7</td>
</tr>
<tr>
<td>Herb(P-3)</td>
<td>21</td>
<td>210</td>
<td>8.5</td>
<td>65.5**</td>
<td>26.0**</td>
</tr>
<tr>
<td>(1 week)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>165</td>
<td>5.7</td>
<td>54.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Herb(P-3)</td>
<td>20</td>
<td>182</td>
<td>7.6**</td>
<td>68.0*</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* P<0.05, **P<0.01, analyzed by t-test after the arcsin transformation. 
a) Glomeruli with cystic lesion are excluded.

Fig. 4. Glomerular changes 1 week after inoculation.

a. Control. Early segmental sclerotic change (arrowheads) with a global crescent. Capillaries in other segments are collapsed. PAS. x480.
b. Herb-treated. Cells and matrix are moderately increased in the mesangium. PAS. x480.

the incidence of proliferative mesangial changes remained unchanged from 48 hrs after inoculation (Table 5; Fig. 4b). Urinary tubules of smaller than normal diameter were grouped in patches near sclerotic glomeruli in the cortex. These tubules were lined with cuboidal epithelial cells and accompanied by a slight thickening of the basement membrane. These changes occurred much more frequently in controls and seemed to be early signs of tubular atrophy.

Scanning electron microscopy of normal appearing glomeruli in controls revealed atrophy of podocytes 1 week after inoculation, accompanied by flattening and fusion of their interdigitating processes (Fig. 5a). In addition, transmission electron microscopy showed glomerular changes such as occasional swelling of the podocyte cell body, narrowing of the capillary lumen with a wrinkled basement membrane, and mesangial interposition circling the capillary (Fig. 6a). These scanning and transmission microscopic changes were slight in the herb-treated mice (Figs. 5b, 6b).

Microangiographic findings: Marked tortuosity with attenuation of arcuate and interlobular arteries, pruning and reduced number of interlobular arteries, and increased flow of contrast medium into the vasa rectae were observed in controls 1 week after inoculation (Fig. 7a). In herb-treated mice, these changes were much less and almost normal, except for slight attenuation of the interlobular arteries (Fig. 7b).

DISCUSSION

There are a few reports of hemorrhage in the lungs, digestive tract, heart, and kidneys with cystic transformation of glomeruli after inoculation with Agkistrodon acutus venom [2, 5]. It is obvious that these hemorrhagic lesions were caused by injuries to the microvasculature. According to our previous observations using Ac₁-P at LD₅₀ in mice [8], mesangiolysis resulting in the cystic transformation
Fig. 5. Scanning electron micrographs of glomeruli 1 week after inoculation.

a. Control. The cell body and secondary foot processes of podocytes are atrophic and there are numerous long trabeculae (arrowheads). Bar: 10 μm. ×8,000.

b. Herb-treated. Podocytes are somewhat atrophic but secondary foot processes are closely interdigitated. Bar: 10 μm. ×7,000.

Fig. 6. Transmission electron micrographs of glomeruli 1 week after inoculation.

a. Control. Swelling of the cell body of a podocyte (p) and extensive fusion of foot processes are seen. A narrowed capillary lumen (asterisk) with marked wrinkling of the basement membrane and mesangial interposition circling the capillary. Bar: 10 μm. ×6,000.

b. Herb-treated. The mesangium shows slight increase of cells (m) and matrix, and podocyte foot processes are partially fused. Bar: 10 μm. ×6,000.
was preceded by local deposition of the venom. Mesangiolyis began within minutes and finished within hours after inoculation; the venom became hardly detectable by immunohistochemistry after 1 hr and overt contraction of the kidneys was observed after 3 weeks. In the present study tubular atrophy secondary to the glomerular lesion appeared to begin 1 week after inoculation, as seen in the kidney weight and histologic findings.

The survival rate and absolute and relative kidney weights were higher, and the BUN value was lower in the herb group. While hemorrhage in the various organs was intense in controls, it was slight in herb-treated mice. Histologic observation also revealed clear differences between the two groups. The cystic transformation of glomeruli, including the subsequent proliferative-sclerosing changes and associated renal atrophy, was the characteristic features in controls. Conversely, in herb mice, the incidence of cystic transformation was nearly halved and the increases in cells and matrix in the mesangium was rather profound. A half LD_{50} dose of ACl-P result in only mesangial cell proliferation (unpublished data). It is therefore evident that P-3 inhibited hemorrhage in vital organs and lowered the incidence of the glomerular cystic lesion in the earliest stage.

Scanning and transmission electron microscopy of normal appearing glomeruli indicated a suppressive effect by P-3 on the changes with possible relation to functional impairment of the glomeruli. Microangiographic findings in control kidneys revealed contraction of the cortex and increased resistance in the cortical vasculature, as recognized by tortuous, poorly contrasted cortical arteries and conversely, well contrasted vasa vasorum. The inhibition of these changes in herb-treated mice seems to be consistent with the histologic findings.

Most of the differences between controls and herb-treated mice may thus be attributable to the inhibition of microvascular injuries which are induced shortly after venom inoculation and can cause acute death or chronic renal failure secondary to the acute glomerular injury. It may be concluded that P-3 has efficacy against tissue injuries induced by ACl-P as a proteolytic enzyme.

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