Effect of Ligustrazine on Chronic Allograft Nephropathy in Rats
C. Gao, L. Feng, Y.P. Li, and Y. Cheng

ABSTRACT
Objectives. Our previous study demonstrated that Ligustrazine reduced renal dysfunction associated with ischemia-reperfusion injury in mice. In this study, we investigated whether Ligustrazine herbal injection had any preventive and therapeutic effectiveness against chronic allograft nephropathy in rats.

Methods. Male Lewis rats that received left renal grafts from male Fisher 344 rats were randomly divided into five groups: group A only received cyclosporine (CsA; 10 mg · kg⁻¹ · d⁻¹) every day. Groups B, C, and D received low-dose Ligustrazine (50 mg · kg⁻¹ · d⁻¹) + CsA (10 mg · kg⁻¹ · d⁻¹); high-dose Ligustrazine (100 mg · kg⁻¹ · d⁻¹) + CsA (10 mg · kg⁻¹ · d⁻¹); and CsA (10 mg · kg⁻¹) + mycophanolate mofetil (10 mg · kg⁻¹ · d⁻¹), respectively. Group E was an isografted group (Fisher 344 to Fisher 344). Grafts were preserved in 4°C University of Wisconsin solution for 1 hour prolonged cold ischemia. All recipient animals were unilaterally right nephrectomized. Serum creatinine (Scr), blood urea nitrogen (BUN), urinary protein, kidney malondialdehyde (MDA) level, and superoxide dismutase (SOD) were determined, as well as examining kidney histology with immunohistochemistry for Bcl-2, and ICAM-1. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were tested to show whether Ligustrazine has side effects.

Results. Compared with group E, ischemia and rejection produced a ninefold increase in GSR and Banff score, as well as significant increases of Scr, BUN, and urinary protein levels in group A. High doses of Ligustrazine significantly slowed the increase (P < .01). Ligustrazine also decreased MDA levels and ameliorated the down-regulation of SOD activity. Bcl-2 was up-regulated posttransplantation, especially in the Ligustrazine-treated group (P < .01). The up-regulation of ICAM-1 was greatly diminished by Ligustrazine (P < .01). Furthermore, there was little difference in ALT/AST between the Ligustrazine-treated and the isograft group.

Conclusion. These findings suggested that Ligustrazine could postpone chronic renal allograft dysfunction associated with cold ischemia injury and chronic allograft rejection but had no evident hepatic side effects.

ISCHEMIA-REPERFUSION (I/R) injury contributes to acute rejection and chronic renal allograft nephropathy (CAN), which has an important influence on the long-term function of renal allografts. Ligustrazine, a purified and chemical by identified component of a Chinese herbal remedy, scavenges cytotoxic oxygen free radicals, thereby promoting blood flow, antagonizing platelet aggregation, and inhibiting fibrosis.1–3 Furthermore, our previous study also demonstrated that Ligustrazine reduced renal dysfunction associated with kidney I/R by attenuating lipid peroxidation, apoptosis, and ICAM-1 expression.4 However, a protective effect of Ligustrazine has not been verified on solid organ transplantation. I/R injury is unavoidable in solid organ transplantation. Shortening of pretransplanta-
tion ischemia time as far as possible is feasible and effective to protect organ function. In this study, a prolonged cold ischemia CAN model was used to investigate whether Ligustrazine diminished the incidence of CAN.

**MATERIALS AND METHODS**

**Animals and Experimental Design**

The male Lewis rats (200 ± 30 g) and male Fisher 344 rats (200 ± 30 g) were purchased from Experimental Animal Center, Sichuan University (Chengdu, China). All rats were provided with standard laboratory foodstuffs and water and housed in accordance with institutional animal care policies.

All recipient animals were unilaterally right nephrectomized, and procured Fisher 344 left renal grafts were preserved in 4°C. University of Wisconsin (UW) cold storage solution (Du Pont Pharma, USA) before transplantation. The animals were randomly divided into five groups (n = 15 each group): Group A: were only treated with cyclosporine (CsA; 10 mg · kg⁻¹ · d⁻¹) posttransplantation. Group B received CsA (10 mg · kg⁻¹ · d⁻¹) combined with low-dose Ligustrazine (50 mg · kg⁻¹ · d⁻¹) posttransplantation. Group C were prescribed CsA (10 mg · kg⁻¹ · d⁻¹) combined with high-dose Ligustrazine (100 mg · kg⁻¹ · d⁻¹). Group D had CsA (10 mg · kg⁻¹ · d⁻¹) combined with mycophenolate mofetil (MMF) (10 mg · kg⁻¹ · d⁻¹) posttransplantation. Group E was an isograft group (Fisher 344 to Fisher 344). All grafts except group E were preserved in 4°C UW solution for 1-hour prolonged cold ischemia. Harvest of 15 grafts was performed at 4, 8, or 12 weeks.

**Assessment of Renal Grafts Morphology**

Each graft, transected at the renal pedicle level, was split into two sections: one for 20 g/L glutaraldehyde fixation and ultramicroslicing for transmission electron microscopy; the another for 100 g/L neutral for malin fixation, paraffin-embedding, and sectioning with hematoxylin–eosin and Masson staining to detect glomerulosclerosis by a double-blind method. We defined glomerulosclerosis as containing glomerular changes, Bowman’s capsule adhesion, and glassy degeneration. Five fields with 200 glomeruli in each specimen were randomly chosen to calculate the percentage of sclerotic glomeruli.

**Immunohistochemistry**

Fifteen 6-μm serial sections were obtained from formalin-fixed, paraffin-embedded rat renal graft specimens from every recipient. To enhance the immunostaining, the antigen was preadsorbed in citrate buffer using cooking in a microwave oven for 5 minutes twice. Then the sections were treated with 3% hydrogen peroxide to eliminate native peroxidase activity. Immunohistochemistry staining was performed using the avidin-biotin peroxidase complex method and DAKO Liquid DAB+ Substrate-Chromogen System (DAKO Co, Carpinteria, USA), which led to the development of a brown reaction product at sites of Bcl-2 and ICAM-1 expression. Immunohistochemistry staining was performed using the avidin-biotin peroxidase complex method and DAKO Liquid DAB+ Substrate-Chromogen System (DAKO Co, Carpinteria, USA), which led to the development of a brown reaction product at sites of Bcl-2 and ICAM-1 expression. Immunohistochemistry staining was performed using the avidin-biotin peroxidase complex method and DAKO Liquid DAB+ Substrate-Chromogen System (DAKO Co, Carpinteria, USA), which led to the development of a brown reaction product at sites of Bcl-2 and ICAM-1 expression. Immunohistochemistry staining was performed using the avidin-biotin peroxidase complex method and DAKO Liquid DAB+ Substrate-Chromogen System (DAKO Co, Carpinteria, USA), which led to the development of a brown reaction product at sites of Bcl-2 and ICAM-1 expression.

**Determination of Malondialdehyde Levels and Total Superoxide Dismutase Activity**

Trichloroacetic acid was added to kidney homogenates. Then TBA-water solution was added to the supernate and boiled for 60 minutes. After cooling, the optical density at 532 nm was measured. Total superoxide dismutase (SOD) activity was determined by monitoring the rate of reduction of nitroblue tetrazolium, utilizing a xanthine-xanthine oxidase system as the source of O₂. One SOD enzyme unit was defined as the amount that produced 50% inhibition of the formation of formazan. Data are expressed as mean values ± SD (n = 15/group).

**Table 1. BUN and Scr of Recipients Posttransplantation**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>4 wk (mmol/L)</th>
<th>8 wk (mmol/L)</th>
<th>12 wk (mmol/L)</th>
<th>4 wk (μmol/L)</th>
<th>8 wk (μmol/L)</th>
<th>12 wk (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15</td>
<td>13.29 ± 1.77</td>
<td>21.36 ± 3.22</td>
<td>19.72 ± 3.31</td>
<td>109.17 ± 8.57</td>
<td>106.96 ± 12.35</td>
<td>149.34 ± 14.33</td>
</tr>
<tr>
<td>B</td>
<td>15</td>
<td>8.56 ± 0.39†</td>
<td>14.57 ± 0.83‡</td>
<td>13.61 ± 0.42‡</td>
<td>88.54 ± 3.52*</td>
<td>103.56 ± 14.31</td>
<td>133.58 ± 10.2†</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>8.11 ± 0.49†</td>
<td>11.78 ± 0.53‡</td>
<td>12.51 ± 0.78‡</td>
<td>70.70 ± 6.23†</td>
<td>84.32 ± 4.13‡</td>
<td>99.27 ± 9.11†</td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>9.12 ± 0.73‡</td>
<td>10.39 ± 1.03†</td>
<td>11.82 ± 0.69†</td>
<td>56.38 ± 5.73†</td>
<td>80.85 ± 6.15†</td>
<td>87.38 ± 6.05†</td>
</tr>
<tr>
<td>E</td>
<td>15</td>
<td>8.63 ± 0.63*</td>
<td>7.59 ± 0.82‡</td>
<td>7.97 ± 0.52‡</td>
<td>58.67 ± 7.93†</td>
<td>31.32 ± 2.98†</td>
<td>29.65 ± 4.25†</td>
</tr>
</tbody>
</table>

*P < .05 versus group A; †P < .01 versus group A.

**Table 2. ALT and AST of Recipients Posttransplantation**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>4 wk (U/L)</th>
<th>8 wk (U/L)</th>
<th>12 wk (U/L)</th>
<th>4 wk (U/L)</th>
<th>8 wk (U/L)</th>
<th>12 wk (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>15</td>
<td>13.29 ± 1.35</td>
<td>15.77 ± 6.53</td>
<td>17.86 ± 2.20</td>
<td>23.29 ± 1.35</td>
<td>25.77 ± 6.11</td>
<td>27.86 ± 2.20</td>
</tr>
<tr>
<td>E</td>
<td>15</td>
<td>17.22 ± 2.34</td>
<td>25.33 ± 2.79*</td>
<td>18.99 ± 3.21</td>
<td>26.22 ± 2.34</td>
<td>25.33 ± 3.79</td>
<td>28.99 ± 3.21</td>
</tr>
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*P < .05 versus group A.
Statistical Analysis
All results are presented as mean value ± standard error of the mean. Differences among categorical variables were analyzed with analysis of variance. A P value < .05 was considered significant.

RESULTS
Blood Urea Nitrogen, Serum Creatinine, Urinary Protein, Alanine Aminotransferase and Aspartate Aminotransferase Posttransplantation. Compared with the other four groups, group A showed a significantly greater mean blood urea nitrogen (BUN), Serum Creatinine (Scr), and urinary protein values posttransplantation (P < .05). Group C and D displayed little evident variation from 4 to 12 weeks posttransplantation (P > .05). Furthermore, there was little difference between these two groups (P > .05). Group C showed fewer mean Scr values than group B (P < .05) at all times posttransplantation. Little difference was observed in alanine aminotransferase (ALT) or aspartate aminotransferase (AST) among the four allograft groups (P > .05), and the ALT of the allograft group was lower than that of the isograft group (P < .05; (Tables 1 and 2, and Fig. 1).

Renal Histological Assessment
Four weeks posttransplantation, intimal thickening in renal interstitial small vessels with glomerulosclerosis in group A was observed by microscopy, while glomerular endothelial cell fusion was seen under electron microscopy. Four weeks later, pathological changes of interstitial fibroplasias, hyaline degeneration of capillary walls, and tubular atrophy with protein casts. Electron microscope revealed three-layered structures of endothelial cells, basement membranes, and epithelial cells merged into a unclear structure. The glomerular capillary lumen was obstructed by the hyperplastic basement membrane of endothelial cells. But in groups B, C, and D, arteriolar intimal thickening and CAN appeared later, and progressed more slowly. At 8 weeks posttransplantation, glomerular sclerosis ratio and Banff score in group B were much higher compared with groups C and D (P < .05). After another 4 weeks, focal glomerular sclerosis, arteriolar intimal hyperplasia, glomerular basement membrane (GBM) thickening, and disappearance of the endothelial cell gap were frequently observed with similar pathological changes in groups C and D, albeit less than group B. The predominant changes were tubulopathy, such as atrophied tubules with “lace patterns.” GBM and endothelial cells gaps were not abnormal. Evaluation of the pathological changes by light and electron microscopy revealed little difference between groups C and D according to Banff standards (P > .05; Table 3).

Immunohistochemical Analyses
Rat renal allografts showed increased ICAM-1 staining on tubular cells, which was significantly decreased in the kidneys of animals with Ligustrazine treatment posttransplantation. Expression of Bcl-2 was much greater in the kidneys of the Ligustrazine-treated group than in other groups at the same times posttransplantation (Table 4).

Effects of Ligustrazine on the Kidney Malondialdehyde Level and SOD Activity
Rat renal grafts showed significantly increased malondialdehyde (MDA) levels from 1.6 ± 0.3 (group E, 4 week) to 4.1 ± 0.4 (group A, 12 weeks) U/g wet tissue (P < .05), suggesting increased lipid peroxidation. Ligustrazine produced a substantial reduction in MDA levels from 4.1 ± 0.4 (group A, 12 week) to 1.9 ± 0.3 (group C, 12 week) U/g wet tissue (P < .01).

Renal SOD activity decreased in all allotransplanted groups compared to group E. The decrease was more prominent in group A compared with group C (106.2 ± 7.9 U/mg protein vs 51.3 ± 5.7 U/mg protein, P < .01) at 12 weeks posttransplantation. Ligustrazine also ameliorated the decrease of SOD in group C compared with group D (41.2 ± 7.9 U/mg protein vs 25.7 ± 5.2 U/mg protein, P < .01) at 12 weeks posttransplantation.

DISCUSSION
CAN, with the typical pathological changes of severe structure or obstruction of arteriolar lumen and renal graft

| Table 3. Renal Histological Evaluation by Glomerulus Sclerosis Ratio and Banff Score |
|-----------------|-----------------|-----------------|-----------------|
|                 | Glomerulus Sclerosis Ratio (%) |                 | Banff Score |
|                 | 4 wk | 8 wk | 12 wk | 4 wk | 8 wk | 12 wk |
| A               | 2.30 ± 1.29 | 25.62 ± 3.23 | 55.13 ± 4.97 | 0.69 ± 0.34 | 4.75 ± 0.46 | 8.57 ± 0.81 |
| B               | 1.78 ± 0.95† | 18.93 ± 2.91* | 35.36 ± 2.32* | 0.59 ± 0.27 | 3.22 ± 0.34* | 7.29 ± 0.67 |
| C               | 1.64 ± 0.33* | 13.77 ± 0.56† | 27.93 ± 3.51† | 0.52 ± 0.31* | 2.99 ± 0.50† | 6.51 ± 0.53* |
| D               | 1.54 ± 0.75† | 11.81 ± 1.73† | 28.96 ± 4.02† | 0.57 ± 0.41* | 2.69 ± 0.34* | 5.84 ± 0.78* |
| E               | 1.28 ± 0.49† | 3.52 ± 0.79† | 5.88 ± 1.37† | 0.43 ± 0.19* | 0.92 ± 0.25† | 0.88 ± 0.031* |

*P < .05 versus group A; †P < .01 versus group A.
fibrosis, causes interference with long-term grafts survival. Dennis et al first introduced a CAN model of Lewis to Fisher 344 rat kidney transplantation.5 In this study, prolonged cold ischemia was used to demonstrate the protective effects on functional and histopathologic status of renal allografts. Ligustrazine, an active Chinese herb.

There has been increasing evidence that free radicals generated by I/R and rejection contribute to functional organ injury.6,7 Reactive oxygen species (ROS) attack a variety of critical biological molecules, including membrane lipids, essential cellular proteins, and DNA.8–10 We studied the effects of Ligustrazine on lipid peroxidation, which was measured in terms of MDA. Ligustrazine reversed the increase in MDA levels to a considerable extent, confirming its antioxidant role. Furthermore, we showed that SOD levels increased following Ligustrazine treatment. SOD is the first line of defense against free radical generation. It has been reported that total SOD is down-regulated following renal I/R,11 rendering a tissue susceptible to oxidant injury. Therefore, the elevated SOD levels induced by Ligustrazine may reduce superoxide radicals following transplantation.

In our study, the histology of the allografts confirmed the clinical observations. In general, the severity of injury correlated with the treatment. The animals in group A showed significant functional impairment of both BUN and Scr. However, Ligustrazine increased the proportion of animals that had normal renal function. In these animals, the Banff score was decreased and more normal glomeruli were preserved. This improvement of renal function and histopathologic findings revealed the protective effect of Ligustrazine against chronic graft dysfunction (CGD).

Another principal finding of this work is that Ligustrazine increased Bcl-2 expression in renal allografts. In addition, Ligustrazine significantly reduced the number of sclerosed glomeruli, and the Bcl-2 expression appeared to correlate with the antiscerotic effects. The major mechanism was probably attributed to the interference of Ligustrazine with ROS release, which induces apoptosis by causing DNA damage, oxidation of lipid membranes, and activation of the...
proteins responsible for apoptosis. Among these apoptosis regulatory proteins, Bcl-2 acts as a cell death preventer.\textsuperscript{12,13} Indeed, the role of the Bcl-2 in regulating apoptosis has been characterized in kidney transplantation. In addition, overexpression of Bcl-2 may play a protective role against CGD posttransplantation. Recent studies have also demonstrated Ligustrazine to attenuate oxidative damage and apoptosis both in vitro and in vivo. In our study, treatment with Ligustrazine was related to an up-regulated level of the antiapoptotic protein Bcl-2, suggesting that Ligustrazine exhibited an inhibitory effect on CGD through modulation of Bcl-2.

Furthermore, we examined another important factor, the expression of adhesion molecules. After transplantation, a destructive inflammatory reaction develops that may involve cytokine production, overexpression of adhesion molecules, and infiltration of neutrophils.\textsuperscript{14,15} Overexpression of adhesion molecules has been implicated as a critical mediator in renal transplantation. ICAM-1 is a neutrophil adhesion molecule. Ligustrazine down-regulated the expression of ICAM-1, preventing neutrophil infiltration into allografts. One primary source of ROS is activated neutrophils, and ROS participate in neutrophil recruitment by up-regulating adhesion molecules and chemotactic factor. Thus, the antioxidant ability of Ligustrazine may protect tissues from CGD after transplantation.

Finally, AST and ALT were examined to test whether Ligustrazine had any hepatic side effects. The results suggested that high doses of Ligustrazine in combination with CsA equaled MMF in protecting renal allograft function with no hepatic side effects. Ligustrazine may offer a new strategy to decrease the frequency of CGD. (Fig. 2).

REFERENCES


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