Original Article

Mizoribine relieves hypertension and kidney injuries in rats after kidney transplantation

Yanqi Mi1, Fuling Wen1, Yunxia Han1, Xuekun Wang2

1Department of Pharmacy, Weifang People’s Hospital, Weifang 261041, Shandong, China; 2Department of Cardiology, Central Hospital of Qingdao, Qingdao 266042, Shandong, China

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Abstract: Hypertension is a common complication with high incidences after kidney transplantation, placing patients into another mortality risk. Mizoribine has been serving as an immunosuppressant in kidney transplant. In order to reveal its influences on hypertension after kidney transplantation, we constructed the kidney-transplanted rat model and treated the rats with mizoribine after the surgery. We measured systolic blood pressure (SBP), and assessed renal fibrosis score and graft-versus-host disease (GVHD) score of the control group and the mizoribine-treated group. We further detected the protein expression of three possible associated factors, the alpha-subunit of epithelial Na+ channel (αENaC), serine/glucocorticoid-regulated kinase (SGK) and with-no-lysine (K) kinase-4 (WNK4). Results showed that SBP of the mizoribine-treated group was significantly lower than the control group (\(P<0.05\)). Renal fibrosis embodied by glomerular fibrosis and tubulointerstitial fibrosis, as well as GVHD, was also significantly mitigated in the mizoribine-treated group (\(P<0.05\)). Expression changes of factors indicated mizoribine inhibited αENaC and SGK, and promoted WNK4 in kidney-transplanted rats (\(P<0.05\)). These results implied that mizoribine was a potent suppressor of hypertension, as well as some symptoms after kidney transplantation, including renal fibrosis and GVHD. Its functions were possibly executed by affecting the expression of factors related to sodium and potassium channels. This study provided the essential basis for application of mizoribine in treating nephropathy.

Keywords: Mizoribine, hypertension, kidney transplantation, renal fibrosis, graft-versus-host disease

Introduction

Hypertension is a frequent complication after kidney transplantation, impairing both adult and children patients [1]. In kidney-transplanted children, the incidence of nocturnal hypertension is 36-71% and masked hypertension 24-45% [2]. Hypertension increases the incidence of cardiovascular diseases and organ rejection syndromes after surgery [3], which endangers the survival of patients seriously. Recent studies have revealed the association between steroids withdrawal and relief of hypertension. One of the primary immunosuppressive drugs, glucocorticoid, participates in the induction of hypertension via its receptor in vascular smooth muscle [4]. The early or late withdrawal or avoidance of steroids significantly decreases cardiovascular risks and improves growth of paediatric kidney-transplanted patients [5, 6]. The mechanisms of steroids in hypertension may be pertinent to their roles of sodium and water retention [7]. So it is of great significance to search for the potential regulatory substances or factors in order to mitigate post-kidney transplantation hypertension.

There are numerous modalities of agents being used to manage post-kidney transplantation hypertension, such as diuretics [8] and calcium channel blockers [9]. Mizoribine is a natural immunosuppressive imidazole nucleoside, commonly used in renal transplantation [10, 11], rheumatic diseases [12] and some other diseases. It exhibits multiple functions in nephropathy treatment. For example, mizoribine prevents the progression of glomerulosclerosis and interstitial fibrosis in non-insulin-dependent diabetic kidneys [13]. A recent study has investigated the aldosterone-salt-induced kidney inflammation and has found mizoribine can ameliorate the renal injury and hypertension of aldosterone-salt-treated rats [14]. But
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In order to reveal the effects of mizoribine in relieving hypertension and renal injury, we constructed the kidney-transplanted rat model and treated the transplanted rats with mizoribine. After the surgery, systolic blood pressure (SBP) and graft-versus-host disease (GVHD) scores of the control group and the mizoribine-treated group were monitored throughout the mizoribine treatment procedure. Also, glomerular fibrosis scores and tubulointerstitial fibrosis scores were assessed. Then we detected the protein expression levels of the alpha-subunit of epithelial Na\(^+\) channel (\(\alpha\)ENaC), serine/glucocorticoid-regulated kinase (SGK) and with-no-lysine (K) kinase-4 (WNK4) to analyze the potential factors involved in the mechanism. Our findings might provide a novel therapy for nephropathy, especially for hypertension after kidney transplantation.

Materials and methods

Animals

Clean grade male Wistar rats (HFK Biotechnology, Beijing, China) of 250-300 g were used as the donors and recipients of kidney transplantation. The rats were fed with standard fodder under warm and airy conditions. All the experiments with animals were approved by a local animal committee for ethics and were performed according to the instructions of our institute.

Kidney transplantation

The rats were deprived from food but free to water for 12 h before surgery. Twenty individuals were randomly selected for surgery perfection and formal transplantation. The surgery was conducted under a surgical microscope (Leica, Wetzlar, Germany). The left kidney of rat was excised together with the artery, vein, and ureter with a bladder cuff of 0.25 cm. These procedures were accomplished within 15 min. The kidney was flushed with 10 mL normal saline solution (4°C) for at least 5 min and then with 3 mL University of Wisconsin (UW) solution (4°C) for over 3 min, and immediately stored at 4°C until transplantation [15]. Before transplantation, the kidney was flushed with 5 mL normal saline solution. The kidney was transplanted to the left renalbed of recipient rat and the artery, vein and bladder cuff were sutured. After the surgery, 6% albumin in normal saline was injected through the tail vein to compensate for the loss of fluids. The success rate of transplantation at 10 d after surgery was 90%.

Mizoribine administration and sampling

After 10 d of recovery from surgery, the rats were randomly grouped into the control group and the mizoribine-treated group, nine individuals in each group. The two groups of rats received standard food and food with mizoribine (3 mg/kg/d AsahiKASEI, Tokyo, Japan) by gavage feeding, respectively. SBP was measured fortnightly by the Tail-Cuff method using BP-2010A (Softron Biotechnology, Beijing, China). The kidney samples were collected at six weeks post treatment.

Histological examination

The kidney samples were fixed in 10% neutral buffered formalin solution, and embedded in paraffin. They were cut into slices of 4 \(\mu\)m, deparaffinized and hydrated for haematoxylin and eosin (HE) staining. The sections were examined by a pathologist blind to group assignments. For each individual, 10 visual fields were randomly selected to count the per-
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Figure 2. Renal fibrosis degrees in rats treated with or without mizoribine. A. HE staining results showing the renal injury of the two groups. Asterisks indicate desquamation of the tubular epithelia. Arrowheads indicate flattened tubular epithelia. B. Glomerular fibrosis score of the control group and the mizoribine-treated group. C. Tubulointerstitial fibrosis score of the control group and the mizoribine-treated group. Control, rats receiving standard food after kidney transplantation. Mizoribine, rats receiving standard food with mizoribine after kidney transplantation. *, Significant differences between the control group and the mizoribine-treated group ($P < 0.05$).

Figure 3. GVHD scores of the control group and the mizoribine-treated group. The scores are assessed at 0, 2, 4, and 6 weeks post treatment. Control, rats receiving standard food after kidney transplantation. Mizoribine, rats receiving standard food with mizoribine after kidney transplantation. GVHD, graft-versus-host disease. *, Significant differences between the control group and the mizoribine-treated group ($P < 0.05$).

to the percent of necrotic cells, namely, 1 (< 5%), 2 (5-25%), 3 (25-50%), 4 (50-75%) and 5 (> 75%).

Analysis of GVHD

The degree of GVHD was assessed based on a scoring system consisting of five parameters, namely, weight loss, posture, activity, fur texture and skin integrity [16]. Each parameter was scored 0-2 according to the severity. Basically, the weight loss percent was measured. The kyphosis or impaired movement, the passive or stationary activity, the flaking or scars in the skin, and the fur ruffling or alopecia were observed and recorded. The five parameters were measured and observed at 0, 2, 4, and 6 weeks post treatment. The
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GVHD degree of each individual was indicated as the sum of the five scores.

Western blot

Protein samples were isolated from kidney of each individual. The samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland). The blot was blocked in 5% skim milk for 2 h at room temperature, and incubated in the specific primary antibody (anti-GAPDH, αENaC, SGK or WNK4, abcam, Cambridge, UK) overnight at 4°C. Then the blot was incubated in the horse reddish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blot was washed three times using Tris-buffered saline Tween-20 (TBST) between steps. Positive signals were detected by chemiluminescence method. The intensity of bands was determined using ImageJ software version 1.49 (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as the internal reference.

Statistical analysis

All data were first tested for the normal distribution using one-sample Kolmogorov-Smirnov (K-S) test. Enumeration data were analyzed with χ² test and measurement data were analyzed with Student’s t test. The analyses were performed using Statistical Product and Service Solutions (SPSS) 19.0 (IBM, New York, USA). Differences were considered significant if P < 0.05.

Results

Mizoribine relieves the hypertension after kidney transplantation

After treatment, SBP of the rats with or without mizoribine treatment were measured fortnightly. At two weeks post treatment, SBP of the control group and mizoribine-treated group showed no significant differences (Figure 1). But after that, SBP of the control group was significantly higher than the mizoribine-treated group (P < 0.05). Generally, SBP of the control group was gradually increased, almost reaching 200 mmHg at six weeks post treatment. When treated with mizoribine after surgery, SBP of the rats remained lower than 150 mmHg throughout the studied process. These results suggested that mizoribine was potent in relieving the hypertension after kidney transplantation.

Mizoribine prevents the progression of renal fibrosis

Then we examined the progression of renal fibrosis in the renal cortex and in the renal medulla. HE staining results indicated that cisplatin treatment induced desquamation of the tubular epithelia and flattened tubular epithelia (Figure 2A), while mizoribine inhibited renal
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Injury, with fewer desquamated or flattened tubular epithelia. In the cortex, the glomerular fibrosis score of the control group was over 2, while that of the mizoribine-treated group was around 1, with significant differences \( (P < 0.05, \text{Figure 2B}) \). Similarly in the medulla, the tubulointerstitial fibrosis score of the mizoribine-treated group was about 2, almost half of the control group \( (P < 0.05, \text{Figure 2C}) \). Based on these results, it could be inferred that mizoribine significantly reduced the percent of renal fibrosis, including the glomerular fibrosis and the tubulointerstitial fibrosis. So it might help to inhibit the progression of renal fibrosis.

**Mizoribine relieves GVHD**

The observation of the five parameters reflected significant differences between the control group and the mizoribine-treated group (Figure 3). GVHD of the control group was increasingly severe after the surgery. But in the mizoribine-treated group, the GVHD score was significantly lower than the control group \( (P < 0.05) \), indicating the GVHD in the mizoribine-treated group was obviously relieved. Based on these results, mizoribine could play roles in relieving GVHD after kidney transplantation.

**Mizoribine influences expression of αENaC, SGK and WNK4**

To analyze the functional mechanisms of mizoribine, we detected the expression changes of αENaC, SGK and WNK4 in the kidney protein samples of rats after kidney transplantation. Western blot results showed that the protein levels of αENaC were significantly down-regulated in the mizoribine-treated group compared to the control group \( (P < 0.05, \text{Figure 4A}) \). SGK protein levels possessed similar change patterns, also with significant differences \( (P < 0.05, \text{Figure 4B}) \). But the expression of WNK4 protein was promoted in the mizoribine-treated group \( (P < 0.05, \text{Figure 4C}) \). These results indicated mizoribine, SGK and WNK4 participated in the functional mechanisms of mizoribine. Mizoribine might influence the expression of the three factors, and consequently relieve the hypertension after kidney transplantation.

**Discussion**

In this study, we verified the roles of mizoribine in relieving hypertension and kidney injury after kidney transplantation using the kidney-transplanted rat model. Mizoribine was found to be capable of mitigating hypertension, inhibiting renal fibrosis and relieving GVHD after kidney transplantation. Besides, mizoribine was proved to down-regulate αENaC and SGK, and up-regulate WNK4, which might relate to the functional mechanisms of mizoribine in relieving hypertension.

αENaC, SGK and WNK4 are some of the crucial factors that have been intensively studied in maintaining Na⁺ and K⁺ balance. αENaC mediates the activation of electrogenic Na⁺ transport [17, 18] and maintains Na⁺ and K⁺ balance [19]. Promoted Na⁺ absorption mediated by ENaC results in Na⁺ retention and hypertension [20]. SGK stimulates ENaC [21] and contributes to Na⁺ retention and K⁺ elimination in kidney [22]. However, WNK4 inhibits ENaC [23] and also regulates Na⁺ reabsorption and K⁺ secretion, thus controlling blood pressure [24]. In the present study, mizoribine could significantly down-regulate αENaC and SGK, and up-regulate WNK4 in kidney-transplanted rats, which was consistent with the former studies that the three factors played roles in regulating hypertension. It could be deduced that the functions of mizoribine was possibly executed through affecting αENaC/SGK/WNK4-mediated Na⁺ retention and K⁺ secretion, which contributed to the mitigation of hypertension after kidney transplantation.

In addition to the relief of hypertension, mizoribine also ameliorated the severities of renal fibrosis and GVHD. As was shown in this study, the degrees of glomerular fibrosis and tubulointerstitial fibrosis were significantly inhibited when using mizoribine after kidney transplantation. Renal fibrosis involves multiple and complicated cellular events, such as epithelial to mesenchymal transition (EMT) and cell apoptosis [25]. Studies have detected the accelerated apoptosis of tubular epithelial cells in renal fibrosis [26]. Together with our results, it might be conjectured that mizoribine affected the aberrant cell apoptosis or EMT, thus inhibiting the process of renal fibrosis after kidney transplantation. GVHD is a complication usually occurs after transplantation of liver, small intestine and pancreas [27-29], which is lethal in many reported cases [30], albeit the low incidence. GVHD after kidney transplantation has been investigated, and the patients have devel-
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Based on our findings, mizoribine had significant efficacy in reducing GVDH score and was a possible agent for attenuating GVHD after kidney transplantation.

To sum up, our study in the kidney-transplanted rat model indicates that mizoribine relieves the hypertension after kidney transplantation, as well as some post-transplantation symptoms, including renal fibrosis and GVHD. These results uncover new application values of mizoribine and provide fundamental information for using mizoribine in treatment of nephropathy.

Disclosure of conflict of interest

None.

Address correspondence to: Xuekun Wang, Department of Cardiology, Central Hospital of Qingdao, 127 Siliunan Road, Shibei District, Qingdao 266042, Shandong, China E-mail: wangxuekun36_13@126.com

References

[18] Tan CD, Selvanathar IA and Baines DL. Cleavage of endogenous gammaENaC and elevated abundance of alphaENaC are associated with increased Na(+) transport in response to api-
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[21] Rauh R, Dinudom A, Fotia AB, Paulides M, Kumar S, Korbmacher C and Cook DI. Stimulation of the epithelial sodium channel (ENaC) by the serum- and glucocorticoid-inducible kinase (Sgk) involves the PY motifs of the channel but is independent of sodium feedback inhibition. Pflugers Arch 2006; 452: 290-299.