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Beneficial Effect of Rhein on the Treatment of Diabetic Nephropathy in Nonobese Diabetic (NOD) Mice

Zhonghui Jia[1], Xiaohua Guo[2], Jianping Wang[2] and Zhihong Liu[2]*

[1]*The General Hospital of Tianjin Medical University, Tianjin, 300052, China*
[2]*Research Institute of Nephrology, Jinling Hospital, Nanjing University School of Medicine, Nanjing, 210002, China*

Abstract

Objective: Rhein has been discovered early to have the function of inhibiting both electron transfer and ADP-driven H^+ uptake in mitochondria. It has been also found that rhein can inhibit the up regulation of GLUT1 (glucose transporting protein-1) function and the activity of hexosamine biosynthetic pathway in mesangial cells in vitro. Futhermore, we have showed the renal protective effect of rhein on STZ-induced diabetic rats. So we try to explore the therapeutic effects of rhein on diabetic nephropathy (DN) in another diabetic model of NOD mice.

Methods: In our study, NOD mice of eight weeks of age were injected intraperitoneally four times with STZ (50 mg/kg) at 7-day interval. Mice with glycosuria and plasma glucose level above 14.0 mmol/L were verified for diabetes. Diabetic NOD mice were subdivided into control and rhein treatment groups. Mice in rhein treatment group were continuously administraed with rhein (150 mg/kg/d) for 15 weeks after diabetes was developed. Plasma parameters (including plasma glucose and lipid level), urinary protein level and histopathology of kidneys were all observed at the end of the experiment.

Results: We demonstrate that rhein not only reduced the urinary protein excretion (0.37 ± 0.17 vs 3.32 ± 0.68 mg/24h, P<0.05), but also prevent the elevation of serum creatinine in diabetic mice after treatment of 15 weeks. In addition, rhein led to a marked decrease of plasma glucose level in experimental model, this effect reached its peak at 15 weeks (7.8 ± 3.80 vs 31.9 ± 4.77 mmol/L, P<0.01), accompanied with decrease of plasma triglyceride (0.74 ± 0.13 vs 2.16 ± 0.73 mmol/L, P<0.01) and cholesterols (1.84 ± 0.55 vs 6.53 ± 5.27 mmol/L, P<0.01). The morphologic studies showed the diabetic NOD mice present glomerular hypertrophy, mesangial expansion and diffuse sclerosis. The accumulation of fibronectin and deposition of immunoglobulin examined by immunostaining in glomeruli were found decreased in rhein-treated NOD mice. Examinition of pancreatic islet sections from NOD mice revealed peri-islet and intraislet mononuclear cells filtration. Treatment with rhein ameliorated cellular aggregation. Stain with Gomori aldehyde fuchsine method showed that loss of β-cells was reduced in rhein-treated mice.

Conclusions: Taken together, these results indicate that rhein is able to ameliorate hyperglycemia and halt the progression of diabetic nephropathy in NOD mice, which possesses potential foreground on the management of human diabetes and its complications.

Keywords: Rhein; Diabetic nephropathy; NOD mouse

Introduction

Rhein (4,5-dihydroxyanthraquinone-2-carbxylic acid) is one of the most important active components of rhubara (*Rheum officinale*), a traditional Chinese herb showing broad pharmacological effects. Rhein was early reported to affect oxidative phosphorylation by inhibiting both electron transfer and ADP-driven H^+ uptake in mitochondria, which is responsible for the formation of lipid peroxides [1,2]. Several of our past studies have shown clearly that rhein can regulate expression of GLUT1 and protect renal injury in STZ-induced diabetic rat [3,4,5]. However, the Reno protective and metabolic effects on congenital type 1 models is unclear. The NOD mouse is a hypoinsulinemic type 1 model of diabetes during immunological injury of β-cells in islet and develops renal abnormalities which are more according to the process of type 1 diabetic nephropathy in human beings [6]. Early studies of renal histopathology revealed some glomerular basement membrane thickening and diffuse glomerulosclerosis in NOD mouse [7]. The renal complication of NOD mouse is much more severe and a more suitable diabetic model for study of diabetic nephropathy than STZ-induced diabetic rat. So we try to explore the potential theraputic effect of rhein on diabetic nephropathy in NOD mouse of type 1 diabetes.

Materials and Methods

Reagents

Rhein was provided by the China Pharmaceutical University (purity>98%) and dissolved in sodium carboxymethyl cellulose (15%). Streptozotocin(STZ) was purchased from Sigma and antibody to fibronectin, IgG, IgM and IgA from Dako company. The glucose-testing kit was got from Ke Xing Biotechnical Institute (Shanghai, China).

Animals and experimental design

NOD/Wehi mice of 20 weeks old were purchased from the experimentai animal center of Chinese Academy of Sciences (Shanghai, China). All animals were given peritoneal injection with STZ at dose of 50 mg/kg every week for four times. Then the diabetic mice (Plasma glucose>14 mmol/L) were subdivided randomly into diabetic control and rhein-treatment groups: Rhein (suspended in saline) was given once daily in the morning by oral gavage at a dosage of 150 mg/kg

***Corresponding author:** Dr. Zhihong Liu, Research Institute of Nephrology, Jinling Hospital, Nanjing University School of Medicine, Nanjing, 210002, China, E-mail: ycjjzfttff@yahoo.com.cn

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body weight for 15 consecutive weeks, while untreated mice were given saline as control. The mice were fed in a suitable environment free of bacteria.

Measurement of plasma parameters

Blood samples from the tail vein were collected at approximately in hematocrit tubes coated with 15% EDTA. Plasma glucose, total cholesterol, triglycerides, creatinin concentrations were assayed by using a commercially available kit (Auto Biochemistry analyzer, HITACHI 7080, Japan).

Evaluation of proteinuria

24-h urine samples were collected using metabolic cages. Proteinuria detection was carried out with method of Bradford assay of Coomassie brilliant blue G-250.

Renal tissue preparation and histological examinations

Mice were sacrificed at the end of the study. The kidneys were harvested and weighed rapidly. Half of the kidney was snap-frozen and stored at -70˚C for immunohistochemical analysis. Portions of the renal cortex were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (3 μm), and stained with periodic acid/Schiff reagent (PAS). Coronal sections of renal tissue are examined by light microscopy as a "blind" experiment (Figure 1). Fifteen glomeruli were randomly selected from each section. The area of each glomerular and mesangial matrix was measured manually by tracing the glomerular outline on a computer screen and calculating that area by computerized morphometry using SPI analysis software (Compix Inc., Cranberry Township, USA).

Pathology of pancreases

Pancrease was fixed in Bouin agent (made up with 36% aldehyde 25 ml, acetate acid 5 ml and picric acid 75 ml) for 24 hours after exoriated, then was HE-stained for observing infiltrating cells and Gomori aldehyde stain was performed for observation of βcells in islet .

Immunofluorescence staining for fibronectin and immunoglobin

For immunofluorescence, renal tissues in OCT compound were snap-frozen in liquid nitrogen, cut into 4 μm thick sections, 10 blocked for 10 minutes and stained with FITC conjugated to antimouse antibody (1:100 dilution) for 45 minutes at room temperature in the dark.

Statistics

Statistical calculations were performed with SPSS10.0 software. Data are presented as mean ± SE, with *n* the number of animals. Groups were compared by ANOVA, P<0.05 was considered significant.

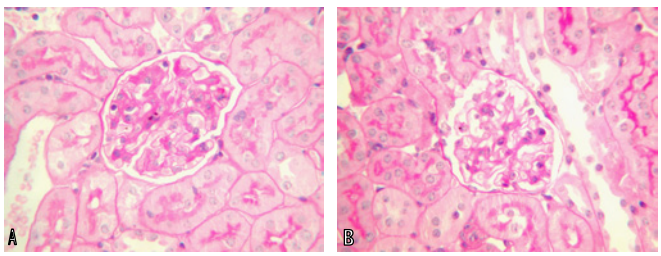

Figure 1: A: Light microscopy of diabetic NOD mice. Showing large glomerulus increased mesangial matrix and cellularity B:Light microscopy of rhein-treated diabetic NOD mice. Showing only mild mesangial sclerosis (PAS stain, ×400).

Results

Evaluation of diabetic model preparations

The plasma glucose level of NOD mice increased evidently after injection of STZ (6.9 ± 0.87 vs 21.5 ± 8.7) which means the feasibility of our protocal.

Proteinuria and plasma creatinine level

Proteinuria and plasma creatinine level, reflection of renal injury, was observed in diabetic mice during the experiment. After treatment for 5 weeks, the rhein treatment group showed a clear decrease in proteinuria excretion compared with control group (1.09 ± 0.51 vs 2.74 ± 1.27 mg/24h, P< 0.05). There was more significant drop of proteinuria seen in rhein-treatments group compared with the diabetic NOD group after 10 weeks and reach its peak after 15 weeks. The plasma Scr level of rhein-treated diabetic mice decreased significantly compared with the untreated control group at 10 weeks of experiment (50.7 ± 6.6 vs 67 ± 10.2 mmol/l, P<0.01) (Table 1).

Plasma glucose, lipid levels

The plasma glucose level of rhein-treated group had decreased from the fifth week and more clearly at times of completion of the experiment (7.80 ± 3.80 vs 31.9 ± 4.77 mmol/L, P<0 01). Dyslipidemia was gradually developed in NOD mice. There was a marked reduction in the cholesterol and TG level after 5 weeks of treatment with rhein compared with diabetic control group which was more obvious after treatment of 15 weeks (Table 2).

Renal histology

NOD mice present severe renal injury, which was manifested by expansion of mesangial area, proliferation of mesangial cells, thicking of basement membrane and some with nodular sclerosis. For rhein-treatment groups, glomerular hypertrophy, mesangial expansion and proliferation was markedly exacerbated compared with diabetic control group (Table 3).

Immunohistochemistry findings

Fibronectin, as one of extracellular matrix protein, the deposition in glomerulus are strong (3+) and diffuse For diabetic control group, after rhein- treatment, the intensity and scope of fibronectin and immunoglobin in glomeruli was much more lessened(1+). In this experiment, diffuse depositions of immunoglobulins (IgG, IgM, and IgA) were found in NOD mice. However, the intensity of immunoglobulin staining was markedly reduced, and the distribution of glomerular staining became focal and segmental I after rhein-treatment (Figure 2).

Effect of rhein treatment on islet morphology

Early stage of progression of diabetes in NOD mice was associated with the development of pancreatic-cell loss and dysfunction, and in this study we demonstrated that rhein prevented morphological damage in the pancreatic islets. Gomori aldehyde fuchsin method showed that β cells in islet of NOD mice were decreased, deregulated and replaced by fibrous tissue and the islet architecture was grossly distorted, at the same time, pancreatic tissue was infiltrated by large mount of monocyte. By contrast the normal distribution and morphology of β

	Group	5w	10w	15w
Serum creatinine(umol/L)	Control group(n=5)	49.2±1.9	67±10.2	61.8±6.6
	Rhein-treated group(n=7)	44.4±3.5*	50.7±6.6**	45.6±9.0**
Proteinuria(mg/24h)	Control group(n=5)	2.74±1.27	3.25±1.07	3.32±0.68
	Rhein-treated group(n=7)	1.09±0.51*	0.41±0.29**	0.37±0.17**

*P<0.05*vs* control group; **P<0.01 *vs* control group

Table1: Change of proteinuria and serum creatinine in diabetic NODmice treated with rhein.

	Group	5w	10w	15w
Plasma glucose(mmol/L)	Control group(n=5)	34.4±6.94	35.4±8.18	31.9±4.77
	Rhein-treated group(n=7)	20.2±8.44*	14.7±5.29**	7.80±3.80**
Plasma triglyceride(mmol/L)	Control group(n=5)	1.76±1.03*	2.42±0.72*	2.16±0.73**
	Rhein-treated group(n=7)	0.83±0.18	1.54±0.48	0.74±0.13
Plasma cholesterol(mmol/L)	Control group(n=5)	5.21±1.85	6.92±1.53	6.53±5.27
	Rhein-treated group(n=7)	3.31±0.83*	2.39±0.58**	1.84±0.55*

*P<0.05*vs* control group; **P<0.01 *vs* control group

Table2: Evaluation of metabolism index in diabetic NOD mice treated with rhein.

	Control group(n=5)	Rhein-treated group(n=7)
Glomerular area(μm^2)	6507±133	5173±1092**
Capillary loop area(μm^2)	5667±1110	3993±1038**
Mesangial area(μm^2)	1327±407	657±353**
Capillary loop area/ Glomerular area	0.87±0.04	0.77±0.05**
Mesangial area/ Capillary loop area	0.23±0.37	0.16±0.34**
Kidney weight(g)	0.32±0.05	0.25±0.03**
Kidney weight/body weight(×10^{-3})	14.6±1.34	10.5±1.25**

**P<0.01 *vs* control group

Table 3: Renal morphological change in diabetic NOD mice treated with rhein.

cells was preserved and infiltration of monocyte was lessened in islet of rhein-treated mice (Figures 3,4).

Discussion

Type 1 diabetes mellitus (T1DM) is an autoimmune disease leading to near complete pancreatic beta-cell destruction. So the commonly used model of STZ–induced pancreas injury is not able to sophisticatedly mimick the pathogenesis and progression of type 1 diabetes mellitus in human beings because the self immunoreaction contribute to the pathogenesis of type 1 diabetes [8,9]. We have showed before that rhein can inhibit the up regulation of GLUT1 function, the activity of hexosamine biosynthetic pathway in mesangial cells in vitro and the renal protective effect of rhein on STZ-induced diabetic rats .We use the NOD mouse which is an established model of autoimmune diabetes mellitus to prove the therapeutic effect of rhein on DN.

NOD mouse spontaneously develops T-cell-mediated autoimmune pancreatic beta destruction that is similar to type-1 diabetes in humans.

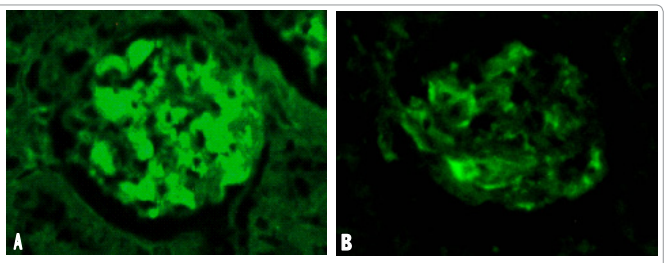

Figure 2: A: Immunostain with antibody against FN showed strongly mesangial fibronectin deposits in glomerulus from diabetic NOD mice. B: Immunostaining with antibody against fibronectin showed mild mesangial fibronectin deposits in glomerulus of rhein-treated diabetic NOD mice (×400).

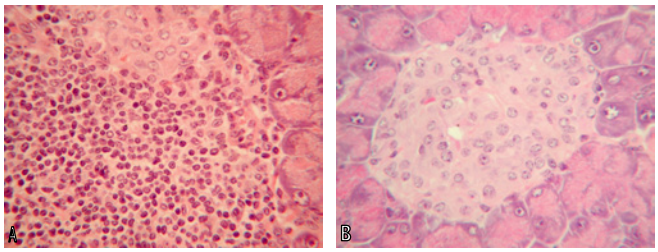

Figure 3: A: Light microscopy of diabetic NOD mice. Showing increased infiltrating inflammatory cell in islet B: Light microscopy of rhein-treated diabetic NOD mice. Showing only mild infiltrating inflammatory cell in islet (HE stain, ×400).

Various lines of NOD mice differ in their incidence of spontaneous diabetes, e.g. 93% of female NOD/Lt mice compared with 46% of female NOD/Wehi mice develop diabetes by 250 days. Proinsulin is a primary autoantigen of the NOD mouse, and are speculated that organ-restricted autoimmune disorders with marked major histocompatibility complex (MHC) restriction of disease are likely to have specific primary autoantigens. However, not all the NOD mouse are destinated to developed the diabetes. Some laboratory try to increase the by using cytotoxic agent [10,11]. So, in our experiment, we attenuate the injury of pancreas by way of injection of STZ intermittently, which obviously accentuated the immune injury of pancrease. STZ injection aggravate self-immune reaction in islet mainly due to the toxity on target cells, exposing renascent antigen and releasing the existing antigen itself.

The interesting fingding in our study was documented by the significant attenuation of 24-h urinary protein excretion and renal function preservation after invention of rhein as well as showed in the rats of STZ-induced diabetic model before model. In addition of the

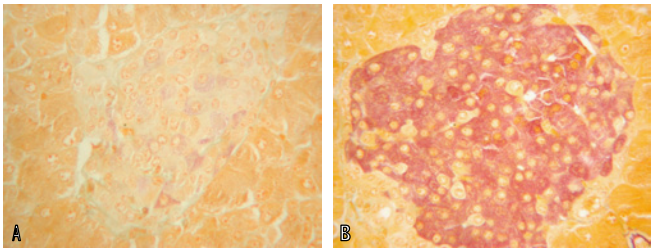

Figure 4: A: Light microscopy of diabetic NOD mice. Showing decreased βcell in islet B: Showing the preservation of βcell in islet after rhein-treatment in diabetic NOD mice. (Stain with Gomori aldehyde fuchsin , ×400)

Reno protective effect of rhein in the study, the results of the experiment showed that rhein can ameliorate hyperglycemia of diabetic NOD mice. Hyperglycemia are key factors in initiation of DN. Reducing glucose level was parallely with coming down of renal complications in diabetes [12]. Important participating elements include activation of hexosamini pathway, oxide stress, and all kinds of growth factors and so on [13]. We found that rhein effectively prevent beta-cell of islet from destruction in NOD mouse model and even maintained euglycemia up to 145 days after treatment withdrawal. The therapeutic effect of rhein was associated with improved glucose metabolism. To determine whether the antidiabetic action of rhein, heretofore attributed to insulin sensitization, also involves protection of β cells, we observed the morphemical change of islet after rhein treatment. Whatever the molecular mechanism, this study in NOD mouse showed that rhein protects β cells from loss implies part of the therapeutic action of rhein in diabetic models. We suppose that rhein may play its role through reducing the inflammation or immune reaction in pancreases. Studies in vitro showed that rhein inhibits interleukin-1 beta-induced activation of MEK/ERK pathway and DNA binding of NF-kappa B and AP-1 in chondrocytes cultured in hypoxia [14]. Recent study showed that the major ultrastructural changes of microvessel in NOD mice are indicated by the swelling and vacuolization of the endothelial cell. Swollen cells are the first noticeable lesion of the cell response in reversible degeneration that is caused by the failure of homeostatic control. Loss in endothelial cell homeostasis is primarily a marker of endothelial dysfunction that plays a key role in the pathogenesis of diabetic vascular disease by losing the control of vascular tone [15]. Our study has present that rhein inhibits transforming growth factor beta1 induced plasminogen activator inhibitor-1 in endothelial cells and suggest rhein may preserve the islet morphology ang function through protecting endothelial cells [16].

Dyslipidemia are one of complications in DN and play an important role in the injury of kidney [17-19]. Morphologically, the renal tissue of dyslipidemic patients shows hyperperfusion and massive lipid infiltration in glomerular cells, and modified lipoprotein has been observed in the glomeruli or interstitium of DN patients, processes thought to accelerate sclerosis and fibrosis [20]. We have found that rhein can regulated the dyslipidemia in type 2 and STZ- induced diabetes models, we proved again in this study that rhein can coordinate the lipid level to relatively nomal state in NOD mouse, which partake in the protection of renal injury in DN. Although the mechanisms of weight and lipid control by rhein remain unknown so far, it has been shown that mitochondria might be involved in this process. Rhein was reported to affect oxidative phosphorylation by inhibiting both electron transfer and ADP-driven H^+ uptake in mitochondria, which is responsible for the formation of lipid peroxides. Our findings of protection of rhein on injury of mitochondrion in DN may give explanation to the adjustment of abnormal lipid level in NOD mouse [21].

Pathological change in kidney of diabetic nephropathy is characterized by excessive glomerular matrix accumulation, basement membrane thickening and sclerosis. Although it is clear that systemic metabolic disturbances precipitate such renal changes, the signals and pathways involved in this process are not fully elucidated. Amelioration in hyperglycemia and dyslipidemia of rhein may contribute to the recuperation of renal pathological change in NOD mice. Recent evidence suggests that growth factors/cytokines are intimately involved in the pathogenesis of diabetic nephropathy. Recent invetigation indicate that susceptibility to diabetic glomerulopathy in MNS rats is associated with increased GLUT1-dependent glucose transport activity in response to hyperglycaemia and/or TGF-beta, which may amplify ECM overproduction [22]. Our previous study showed that rhein was able to reverse cell hypertrophy and inhibit ECM overproduction in GLUT1-transfected mesangial cells, TGF-β1 stimulated mesangial cells. Recent investigations discovered positive-feedback regulation of the mesangial cell GLUT1 transporter by glucose, and a regulatory role for GLUT1 in glucose metabolism and extracellular matrix synthesis. Futher investigations of glucose transporters contribute to the pathogenesis of diabetic renal disease in multiple directions [23,24]. It has been shown in our study that rhein can ameliorate glomerular hypertrophy and the deposition of mesangial matrix. We have shown previously that rhein can inhibit cell hypertrophy and reduce accumulation of the extracellular matrix in renal tubular epithelial cells induced by TGF-β1 [25], which may underly the mechanism of the effect of rhein on DN in NOD mouse in vivo. Some study implicates TGF-β1 as an aetiologic mediator of DN and the ubiquitous GLUT1 as an important permissive factor for tissue injury caused by hyperglycemia, our previous study also suggest that rhein can inhibit the up regulation of GLUT1 in vitro, which are very important pathway rhein step in to avoid injury of hyperglycemia. Deposition of immunoglobin in kidney are thought to exacerbate the renal injury and also contribute to the progression of DN in NOD mouse [26]. We revealed that rhein can relieve the local immunoglobin in renal tissue of NOD mouse. We speculated that rhein may abate the release of antigen from pancrease to prevent the formation of immunocompex which was dued to its effect on immune system [27].

In conclusion, the present study demonstrated that rhein markedly attenuated renal injury in addition of the amelioration of metabolic disorders, including hyperglycemia n NOD mice, supplying a novel route for the treatment of diabetic nephropathy.

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