ORIGINAL ARTICLE

Islet transplantation restores the damage of glomerulus filtration membrane in a rat model of streptozotocin-induced diabetic nephropathy

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Abstract

Objective: To evaluate the effects on filtration membrane of glomerulus after islet transplantation in a rat model of streptozotocin-induced diabetic nephropathy.

Methods: The experimental case-control study was conducted at Wenzhou Medical University, Wenzhou, China from January to May 2015, and comprised male Sprague Dawley rats obtained from the Laboratory Animal Centre of Wenzhou Medical University. The rats were intraperitoneally injected with streptozotocin to induce diabetic nephropathy. Diabetic rats were divided into two groups; the islets group received islets transplantation under the kidney capsule; and the diabetic nephropathy (DN) group consisted of untreated diabetic nephropathy rats. The control group consisted of non-diabetic rats. Islets were surgically transplanted under the kidney capsule. Kidney function and blood glucose were measured and pathological changes in the kidney were observed by electron microscope, while the expressions of Wilms' tumour-1, caspase-3 and transforming growth factor-beta 1 were tested by immunohistochemical method and Western blot analysis.

Results: Each of the three groups had 6 rats each with body weights ranging from 180g to 220g. Reduced urinary protein excretion and alleviated damage of podocytes and glomerular basement membrane were seen in the islet-transplanted rats. The alleviation of podocyte damage was related to alteration in the synthesis of caspase-3, transforming growth factor-beta 1, and Wilms' tumour-1 protein in the glomerulus.

Conclusion: Diabetic nephropathy rats after islet transplantation can ameliorate the damage of podocytes and basement membrane by inhibiting the pathway of transforming growth factor-beta 1.

Keywords: Diabetic nephropathy; Islet transplantation; Podocytes; Glomerular basement membrane, TGF-β1.
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Introduction

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease (ESRD).[1] Reportedly, 40% diabetic patients develop nephropathy irrespective of glycaemic control.[2] Glucose-dependent pathways, such as advanced glycation, play an important role in the development of diabetic renal disease. In early-stage, hyperglycaemia-induced impairment of the glomerulus filtration membrane, including podocytes and the glomeruli basement membrane (GBM), could lead to the occurrence of proteinuria.[3,4] Furthermore, persistent proteinuria would lead to the damage of kidney structure and promote the fibrosis of kidney.

Islet transplantation is the most effective measure for type 1 diabetes. It is reported that islet transplantation can ameliorate albuminuria and alleviate the damage of kidney.[5] But few researches have addressed the effect of islet transplantation on glomerulus filtration membrane and its precise mechanism. The current study was planned to evaluate the beneficial effects of islet transplantation on glomerular filtration structure in DN rats.

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Subjects and Methods

The experimental case-control study was conducted at Wenzhou Medical University, Wenzhou, China from January to May 2015, and comprised male Sprague Dawley rats obtained from the Laboratory Animal Centre of Wenzhou Medical University. All rats were freely fed with water and rodent chow. All animal procedures were based on international guidelines and were approved by the Wenzhou Medical University Animal Policy and Welfare Committee.

Twelve rats received a single dose of streptozotocin injection (STZ; Sigma Aldrich,USA) 55mg/kg intraperitoneally to induce the DN model. One week later, blood samples from tail vein were collected to measure the blood glucose. Rats were considered diabetic if the blood glucose level was between 288mg/dl and 540mg/dl for more than two consecutive days without fasting. At week 8 after the modelling, diabetic rats were divided into two

groups. The first group (Islets group) received islets transplantation under the kidney capsule; and the second group (DN group) consisted of untreated DN rats. The control group consisted of six non-diabetic rats.

Twelve male Sprague Dawley rats (body weight: 200-250g) were used as donors. Islets from two donor rats were supplied for each recipient. Islet isolation was performed according to the method described in literature.[6] The islets were harvested by reversely perfusion of collagenase V into common bile duct and purification by Histopaque (Sigma-Aldrich, USA) density gradient followed by manual picking. A small incision was performed on the right flank of the recipient rats and the right kidney was exposed. About 800-1000 islet equivalent (IEQ) islets were transplanted under the kidney capsule of the diabetic rats.

After transplantation, blood glucose levels of all groups were measured once a week until the rats recovered euglycaemic state (defined as blood-glucose <10 mmol/L). Also, 24-h urine specimen was collected by metabolic cages and measured by Fully Automatic Biochemistry Analyser (HITACHI 7600, Japan).

Four weeks after islet transplantation, a midline laparotomy was performed on experimental animals. The kidney was in situ perfused with saline by aortic cannulation. A part of right kidney, including transplanted islets, was immersed into 10% neutral formalin solution and 2.5% glutaraldehyde for histopathological analysis. The other part of right kidney and the left kidney was divided into pieces and immediately frozen in liquid nitrogen for Western blot assays.

Portions of renal cortical tissue from four rats per group were prepared according to the method described by other investigators[7] and detected by electron microscope (Philips 301).

The kidney tissues were embedded in paraffin and cut into 4-μm for next measure. Haematoxylin and eosin (H&E) staining and immunofluorescence analysis with rabbit monoclonal antibody against insulin (Santa Cruz, USA) were used to observe the survival and secretion function of transplant islets under the kidney capsule.

For immunohistochemical (IHC) staining, the expression of Wilms' tumour-1 (WT-1), caspase-3 and transforming growth factor-beta 1 (TGF-β1) was detected by immunoperoxidase staining with antibodies, including rabbit monoclonal WT-1 (Santa Cruz, USA), rabbit polyclonal active-caspase-3 (Santa Cruz, USA) and rabbit polyclonal TGF-β1 (Santa Cruz, USA). Briefly, renal tissues were sliced into 4μm sections. After removal of the paraffin by xylene and dehydration by graded alcohol, slides were transferred into a 10mmol/L citrate buffer solution and then heated at 80°C for 5 minutes for antigen retrieval. After washing, 3% peroxide was applied for 20 minutes to block the activity of endogenous peroxidase. To avoid non-specific staining, slides were incubated with normal goat serum at room temperature for 20 minutes. The primary anti-rat WT-1, caspase-3 or TGF-β1 monoclonal antibody (MoAb) was added respectively at 1:150 dilution and kept at 4°C overnight. Negative control sections were stained under identical conditions by substituting the primary antibody with equivalent concentrations of normal rabbit immunoglobulin G (IgG). Slides were detected using horseradish peroxidase-conjugated streptavidin, and peroxidase activity was identified by reaction with 3, 3'-diaminobenzidine tetrahydrochloride substrate. Sections were then counterstained with Mayer's haematoxylin, dehydrated and mounted. To evaluate WT-1, caspase-3 and TGF-β1 staining, glomerular grid field was scored semi-quantitatively by image-ProPlus system (Media Cybernernetics, USA). The results of cell immune-staining were scored (no staining -, weak staining +, moderate staining ++ and strong staining +++).

The expression of TGF-β1 proteins was examined by Western blot using total lysate as previously described.[8] Kidney cortex tissues were homogenised in lysis buffer and protein concentrations were determined using a Micro bicinchoninic acid (BCA) Protein Assay kit. Equal amount of protein was separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, blotted onto nitrocellulose membranes, and probed with specific antibodies against TGF-β1 (Santa Cruz, USA), and β-Actin (Santa Cruz Biotechnology) was used as control. The antigen-antibody complex was developed by enhanced chemiluminescence (Amersham, Piscataway, NJ, USA), exposed in the dark room and analysed for integral absorbance of the protein bands using Quantity One software Version 4.6.2 (Bio-Rad, USA).

Data was presented as means ± standard error of the mean (SEM). Statistical analyses were performed using one-way analysis of variance (ANOVA), and $p<0.05$ was considered significant. All statistical analyses were performed using SPSS 13.

Results

Each of the three groups had 6 rats each with body weights ranging from 180gto 220g. The levels of blood glucose (BG) in DN rats decreased obviously significantly at 24h after islet transplantation ($p<0.05$). At week 4 after transplantation, the levels of BG and 24-hour urine

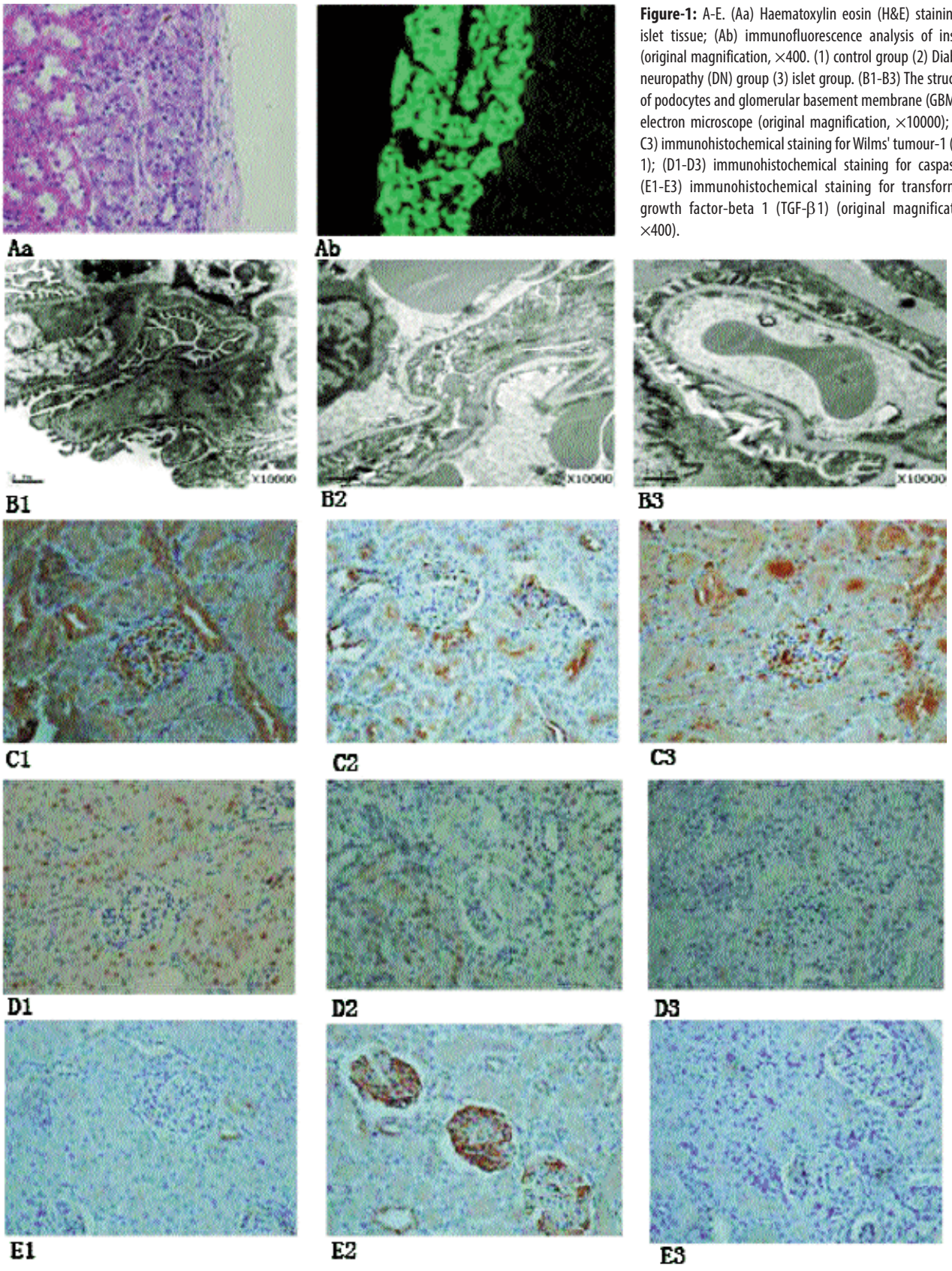

Figure-1: A-E. (Aa) Haematoxylin eosin (H&E) staining of islet tissue; (Ab) immunofluorescence analysis of insulin (original magnification, ×400. (1) control group (2) Diabetic neuropathy (DN) group (3) islet group. (B1-B3) The structure of podocytes and glomerular basement membrane (GBM) by electron microscope (original magnification, ×10000); (C1-C3) immunohistochemical staining for Wilms' tumour-1 (WT-1); (D1-D3) immunohistochemical staining for caspase-3; (E1-E3) immunohistochemical staining for transforming growth factor-beta 1 (TGF-β1) (original magnification, ×400).

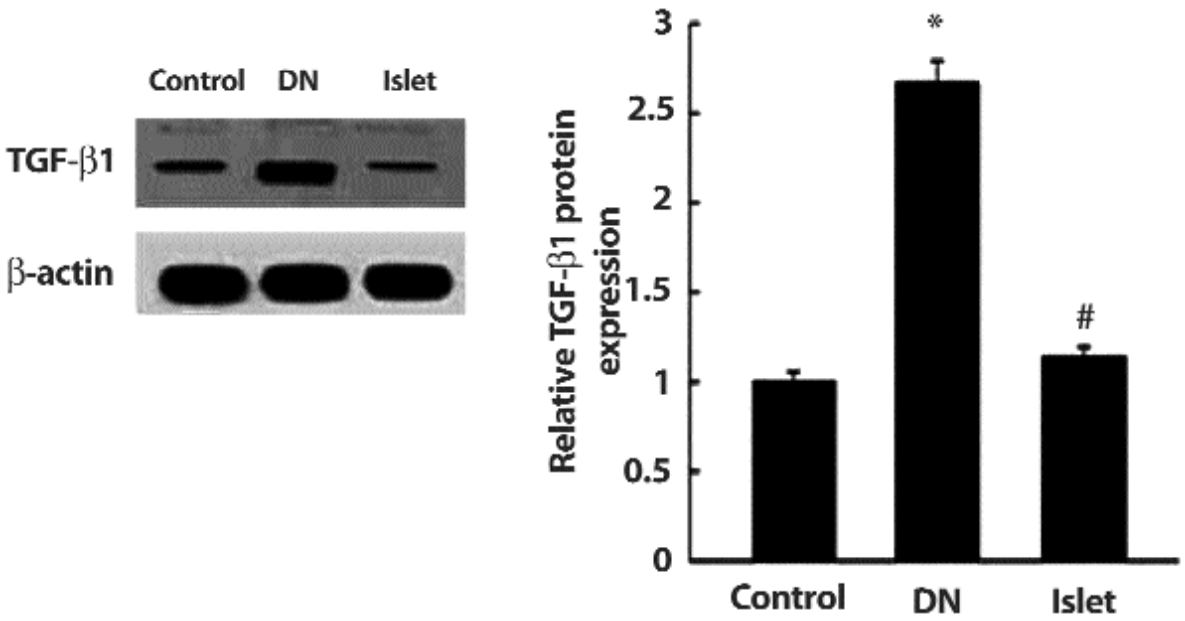

Figure-2: Protein expression of transforming growth factor-beta 1 (TGF-β1) in kidney of all groups was analysed by Western blot with β-actin as the internal control. *P<0.01, compared with the control group; #P <0.01, compared with the Diabetic neuropathy (DN) group.

protein from all groups were tested. All rats in the Islets group were normoglycaemic. The level of BG in DN group was higher than that in Islets group (28.6±0.58 versus 9.48±0.31mmol/L; p<0.01). Islets were adjacent to the kidney capsule, and some red blood cells were buried into transplant islets. Also, the transplant islets produced insulin by immunofluorescence image (Figure-1A). This suggested that the blood glucose level in DN rats returned to normal level after islet transplantation.

There was a marked decrease in 24-hour urine protein in the Islets group compared to the DN group (54.07±2.00 versus 9.45±1.38 mg;p<0.01). Irregular thickening of glomerular basement membrane (GBM)and the fusion of podocytes was observed in the DN group (Figure-1-B). However, the fusion of podocytes' foot process was rare and the damage of GBM was modified in the Islets group (Figure-1. B3). WT-1 protein is an optimal marker of podocytes.[9] WT-1 immunostaining was observed in all groups (Figure-1-C). The scores of WT-1-positive podocytes were the lowest in DN group compared to the Islets group and control groups (17.17±0.51 versus 33.28±0.57;p<0.01 and 17.17±0.51 versus 26.33±0.95; p<0.01). It suggested that islet transplantation could attenuate the level of proteinuria and ameliorate GBM damage and podocytes in DN rats.

Table: Immunostaining of caspase-3 and TGF-?1 in the kidney tissue of all group.

Group(n=6)	Caspase-3		TGF-β1	
	Glomeruli	Tubuli	Glomeruli	Tubuli
Control group	-	++	-	+
DN group	+	+++	+++	++
Islet group	+	++	+	+

No staining = (-), weak staining = (+), moderate staining = (++) and strong staining = (+++)
DN: Diabetic neuropathy.
TGF-β1: Transforming growth factor-beta 1.

Compared with DN group, the expression of caspase-3 protein was significantly decreased in the islets group. Immunostaining of caspase-3 was markedly increased in glomeruli of DN rats (Figure-1-D), but very limited numbers of positive cells were found in the control and Islets groups (Table).

The expression of TGF-β1 in kidney tissues were examined in all groups. An increased expression of TGF-β1 in the DN group was observed by IHC analysis and Western blot assays compared with the control group (p<0.01). The expression of TGF-β1 in the Islets group decreased significantly after islet transplantation (Figure-2).

Discussion

The present study found that the damage in kidney from early-stage DN rats was ameliorated after islet transplantation. The possible mechanism was related to the alleviation of the damages of podocyte and GBM, and the reduction in urine protein by inhibiting the pathway

of TGF-β1.

Recently, some clinical studies discovered that islet transplantation could reverse the early kidney lesion for diabetic patients, whereas the specific mechanism is still unclear.[5,10] Clinically, diabetic nephropathy is mainly characterised by increased proteinuria resulting from irregular thickening of basement membrane and damages to podocytes in the early stage.[11] Thus, a rat model in our study was established for early-stage DN with a single dose of STZ. At 12 weeks after modelling, the amount of proteinuria increased significantly. Electron microscopy showed that foot processes of podocytes were partially effaced and basement membranes were irregularly thickened. Immunohistochemically, there was a decrease of WT-1 (podocytes markers). These changes in rats used in our study were consistent with the clinical changes of early-stage diabetic nephropathy. The rats were used as recipients of islet transplantation.

Impairment of glomerular filtration barrier is a major cause of proteinuria in the procedure of early-stage diabetic nephropathy. The filtration barrier is mainly composed of endothelial cells, basement membrane and epithelial cells (podocytes), among which podocytes were the most critical for the composition of the filtration membrane and their impairment would lead to the formation of proteinuria.[12] It was reported that the podocytes were discovered to have a higher level of apoptosis,[9] decrease, detach from GBM[13] and be partially effaced at foot processes[14] in the pathogenesis of DN. However, the remaining podocytes failed to fill up the impaired areas. As a consequence, the filtration membranes became more permeable, where proteinuria was produced. As another important structure of the filtration barrier, the GBM was selectively permeable. As an extracellular matrix component of the glomerular filtration barrier, the GBM acts as a size-selective and charge-selective physical filter barrier.[15] In the early stage of diabetic nephropathy, it is irregularly thickened, and the structure is thus impaired through which macromolecular substances pass.[16] In our study, the damages to of podocyte and GBM in DN rats were ameliorated after islet transplantation.

As a pleiotropic cytokine, TGF-β1 plays an important role in the progression of DN. The secretion of TGF-β1 may be enhanced in the kidney in case of sustained hyperglycaemia. TGF-β1 was found to induce the podocytes to experience the epithelial-to-mesenchymal transition (EMT) and cause glomerularsclerosis.[17] Besides, TGF-β1 was discovered to activate the Notch signal pathway to facilitate damage and apoptosis of podocytes.[18] Following the application of anti-TGF-β1 antibody, the apoptosis of podocytes was apparently inhibited.[19] Krag et al[20] discovered that the high filtration could not promote the thickening of GBM in the early stage of diabetic nephropathy. However, GBM got significantly thicker due to high expression of TGF-β1 and high filtration. Wang et al[21] discovered that the thickening of GBM was apparently lessened by inhibiting the expression of downstream signals of the TGF-β1 pathway. All of these studies have suggested that it is possible to inhibit the impairment of glomerular filtration barrier and delay the progression of glomerular sclerosis by regulating the synthesis of TGF-β1.

In our study, it was demonstrated that sustained hyperglycaemia in early-stage DN rats activated the pathway of TGF-β1 and led to the damage of filtration membranes, which is in line with previous studies.[22] As an effective way for treating diabetic nephropathy, islet transplantation can reverse the recipients' sustained hyperglycaemia. The blood glucose level was found to drop significantly in rats after islet transplantation and can be kept in the normal range. So, the activation of TGF-β1 pathway was inhibited after islet transplantation.

Conclusion

The study demonstrated that a novel mechanism by which proteinuria in early stage DN rats after islet transplantation can be ameliorated is related to modifying the barrier of glomerular filtration by inhibiting the pathway of TGF-β1. Besides, islet transplantation for late-stage DN rats is worthy of further research from the perspective of treatment timing and mechanism.

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