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Urine-derived stem cells for the therapy of diabetic nephropathy mouse model

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Abstract. – OBJECTIVE: Diabetic nephropathy (DN) is one of the most representative diabetic microangiopathy complications. So far, there have been no satisfactory therapeutic strategies, and the injection of stem cells provides a target for DN therapy.

PATIENTS AND METHODS: Urine-derived stem cells (USCs) were obtained from 9 healthy men. 24 mice were randomly and equally divided into control group, DN model group, DN+hUSC group (treated with USCs for 3 times). Hematoxylin-eosin (HE) and Masson staining were used to detect histological changes of kidney injury. Creatinine and blood urea nitrogen (BUN) were measured to assess renal function. Besides, myofibroblast accumulation, macrophage infiltration, cell proliferation, and oxidative stress were detected by immunohistochemical analysis.

RESULTS: Compared with DN model group, DN+hUSC group showed lower function loss, cell infiltration, and oxidative stress, as well as less renal fibrosis, histological damage, and cell proliferation.

CONCLUSIONS: USC can alleviate inflammation and oxidative stress, reduce renal interstitial fibrosis, improve renal tissue structure and protect renal function through paracrine effect.

Key Words:
Diabetic nephropathy, Urine-derived stem cells, NAPDH, Alpha-SMA, PEDF, NADPH.

Abbreviations
Diabetic nephropathy (DN); Urine-derived stem cells (USCs); End-stage renal disease (ESRD); Pigment epithelium-derived factor (PEDF).

Introduction

Diabetic nephropathy (DN), a diabetic glomerulosclerosis, is a well-known diabetic microangiopathy complication[1]. At present, there are about 200 million diabetic patients around the world, and the number of DN patients will reach 3 billion in 2025. In addition, DN is the major risk factor of end-stage renal disease (ESRD) in Western populations, and it occurs in individuals aged sixty-five and older, whose prevalence is increasing due to the acceleration of population aging. Additionally, ESRD unquestionably leads to the high rates of hospitalization and death[2].

The chief renal replacement therapy of ESRD is hemodialysis, peritoneal dialysis, and renal transplantation[3,4]. Each of them has their own advantages and disadvantages. Hemodialysis and peritoneal dialysis, as methods for palliative care, are accompanied by many complications[5,6]. Renal transplantation is applied in the treatment of ESRD in hospitals, and the limited number of kidneys makes it more important to find the right kidney for patients. Therefore, early diagnosis and treatment are vital for delaying the development of DK and improving the prognosis of the patients.

Stem cell research offers us a novel approach to improving healthcare for human beings[7]. Clones of USCs are easily cultured and high-efficiently propagated into mature functional cells, such as urothelial, smooth muscle, endothelial and interstitial cell markers with relatively expense[8-11]. USCs also can secrete a variety of nutritional factors to promote cell expansion and cell proliferation through para-

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crine[12]. These stem cells share many characteristics with normal stem cells, including self-renewal and differentiation without tumorigenicity[13]. The application of stem cell transplantation is archived as an effective and safe therapy by delaying the aggravation of DN[14], but the study on the effects of USC treatment on DN progression is rare. Herein, a mouse model of DR was established to simulate the pathophysiological features of DN to evaluate the therapeutic effects of USCs to DN, and the preliminary mechanism of USCs to DN was detected by immunochemistry.

Patients and Methods

Collection of Sterile Urine Samples

Sterile urine samples were collected from nine healthy donors (5 males and 4 females). The isolation process was the concrete step in Guan et al[2] (2015). This study was approved by the Ethics Committee of Children's Hospital of Chongqing Medical University. Written informed consent was obtained from all study participants.

Cell-Surface Antigen Assay

USCs at passage 4 were stained with fluorochrome-conjugated monoclonal antibodies against CD29, CD34, CD44, CD45, CD73, CD90, CD133, and HLA-DR (all from BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Then, the cell culture and analysis were operated.

Karyotyping

The chromosomal stability of USCs at passage 3, 5, and 7 was assessed by karyotyping. 15 cells were treated and chosen for analysis, and the band resolution was 600. CytoVision 3.7 visualized chromosome image capture and karyotyping. The same experiment was repeated 3 times, independently.

DN Mice Model Establishment

NOD/SCID mice (18-26 g) were offered by the Animal Experimental Center of Chongqing Medical University. All the experiments were conducted in accordance with the Animal Experimentation Guide of the Chongqing Medical University Animal Experimental Ethics Committee. This investigation was approved by the Ethical Committee of Children's Hospital of Chongqing Medical University, China. The animals were apportioned into G1: an unconditional control group (n = 8), G2: a DN model group (n = 8), and G3: DN+USCs mouse group (n = 8). DN model group was induced by intraperitoneal injection of STZ 40mg/kg for four consecutive days. All groups of mice received a standard diet. After 7 days, the blood sugar and weight were recorded on the seventh day and the tenth day. Subsequently, the blood sugar and weight exhibited a continuous strong rising trend compared the G1 and reached up to 19.4 g and 21.67 mmol/L. This trend confirmed the successful modeling of DN. Finally, the weight and blood sugar were monitored continuously every 7 days until the mice were sacrificed for experiments.

Cell Implantation In Vivo

DN treatment group was injected with 2×10^6 USCs cells (suspended in 200 μL PBS) three times every one week. Ventricular injection was applied in the first two times, while caudal vein injection in the third time. All operations were performed under anesthesia with chloralhydrate (10% in PBS, 0.3 mL/kg).

Renal Function Changes

Creatinine and blood urea nitrogen (BUN) were measured using a creatinine analyzer model 2 and a BUN analyzer 2 (Beckman Instruments, Fullerton, CA, USA), respectively[5].

Histopathology of Mice Kidneys

All animals were sacrificed on the 32nd day. All surgeries were performed under sodium pentobarbital anesthesia to minimize suffering. After fixed in a 4% paraformaldehyde solution for 2 days, the tissues subjected to paraffin-embedding, and kidney tissue sections were stained with HE and Masson trichrome solutions and observed under a light microscope.

Differentiation of renal myofibroblasts, infiltration of macrophage, and cell multiplication were identified on processed tissues incubated with a primary mouse anti-α-SMA/ED1 antibody or a primary mouse anti-PCNA-antibody and a secondary goat anti-mouse antibody.

Pigment Epithelium-Derived Factor (PEDF) Immunocytochemistry

Pigment epithelium-derived factor (PEDF), a 50 kDa glycoprotein, belongs to the serine protease inhibitor superfamily. After fixed and blocked nonspecific binding sites, kidney samples were processed with (1:50) polyclonal antibodies PEDF and (1:50) β-actin for 1 h. After extensively washed, the

prepared sections were inculcated with the secondary antibody (1:1,000) before DAB developer was applied to immunolabeled tissues for 10 min. Ultimately, labled samples were visualized and evaluated under a confocal microscope.

NADPH Oxidase Activity Assay

Enriched mitochondrial fractions of the renal cortex were measured by NADPH oxidase activity assay by utilizing Immunohistochemical technique.

Statistical Analysis

Statistical analysis was conducted using the Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA). Data were represented as mean ± SD (Standard Deviation). The *t*-test was used for analyzing measurement data, and differences between the two groups were examined by the Student's *t*-test. In addition, comparison between multiple groups was done using one-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ indicated a statistically significant difference.

Results

Changes in Physical Parameters

Before cell implantation, body weight was similar in control group (21.11±2.99 g), DN model group (21.38±3.076 g), and DN+hUSC group (21.375±2.78 g). After fed for 32 days, all the mice in control group (24.85±3.511 g) gained weight. By contrast, the mice in DN model group (18.27±4.11 g) and DN+hUSC group (18.471± 3.50 g) lost weight. No significant difference existed between G2 and G3, but loss weight of the DN+hUSC group was less compared with that of the DN group. Figure 1 presents no significant difference in the body weight between the DN model group and DN+hUSC group ($p=0.51$). All the mice survived.

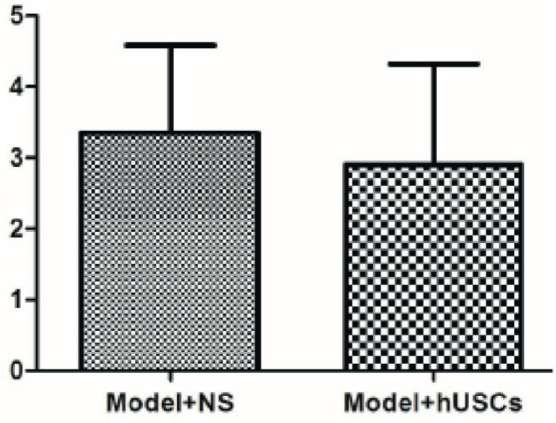

Figure 1. Comparison of weight loss between DN model group and DN+hUSC group.

In the first 3 weeks, the level of glucose in mice fed with the high fat diet showed a high growth speed. After the injection of USCs, blood sugar in G3 dropped significantly compared with that in G2. No significant difference was showed on the 17th day ($p=0.16$), but in comparison with control group, DN+hUSC group exhibited a markedly reduction of blood sugar ($p<0.01$, Figure 2) after 24 days. The differences were enlarged in the following days (Figure 2A). Therefore, USC treatment significantly reduced the DN progression.

Effect of hUSCs on Renal Function Changes

Serum creatinine and BUN levels, as two important of renal function markers, were evaluated. The creatinine and BUN values in DN model group and DN+hUSC group were three times those of control group ($p<0.01$). However, they were significantly lower in DN+hUSC group than those in DN model group ($p<0.01$, Figure 3). These results suggest that hUSC treatment is effective in relieving the DN.

Effect of hUSCs on Histological Abnormalities in DN Model Kidneys

The kidneys of mice in control group showed a normal shape with a notably smooth surface. This was in stark contrast to the appearance of kidneys from the DN mice. The most distinctive characteristic was that kidneys were dilated, and dramatic thinning of the renal parenchyma and hydronephrosis occurred.

In the H&E-stained sections, control group exhibited normal tubules with proper morphology and organization. However, dramatic morphological and histological differences were observed in DN mice. At 32 days after post-transplantation, kidneys in DN groups showed vacuolar degeneration in the renal tubular epithelium, less glomerulus, visible capillary lobule, enlargement of renal tubular lumen, cell exfoliation in the cavity, and fibrous hyperplasia in renal tissues. Otherwise, DN+hUSC group had a significantly less histological injury compared with DN model group (Figure 4). It was demonstrated that pathological changes of DN progressively slowed down in mice kidney allografts.

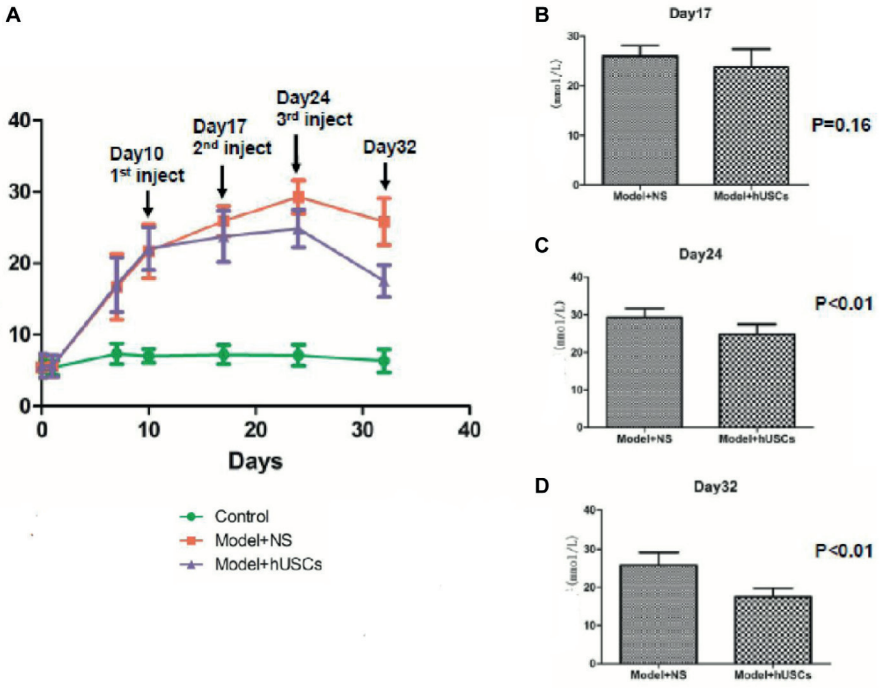

Figure 2. Effect of hUSCs on the chronic kidney disease. **A,** The curve of blood sugar in three groups at 32 days. **B-D,** Differences between DN model group and DN+hUSC group on the 17th, 24th, and 32nd day.

In control group, Masson trichrome staining was mainly present in the basement membrane, mesangial areas, and surrounding capillaries, but rarely visible in the interstitium surrounding the tubules (Figure 5). Collagen distribution area was markedly expanded in DN model group compared with control group, and its expression was unregulated in the renal tubular basement membrane and kidney interstitium on the 32nd day in G2 while, by contrast, the collagen distribution area of treatment group was decreased (Figure 5).

Renal Myofibroblast Differentiation

Mice in DN group showed significant upregulation in glomerular and tubulointerstitial α-SMA expression, while the protein expression was rarely observed in control group. Notably, DN+hUSC group exhibited a downregulated expression of the targeted protein (Figure 6).

Renal Macrophage Infiltration and Cell Proliferation

As shown in Figure 7, there is no signal of ED1-positive cells indicating macrophages in

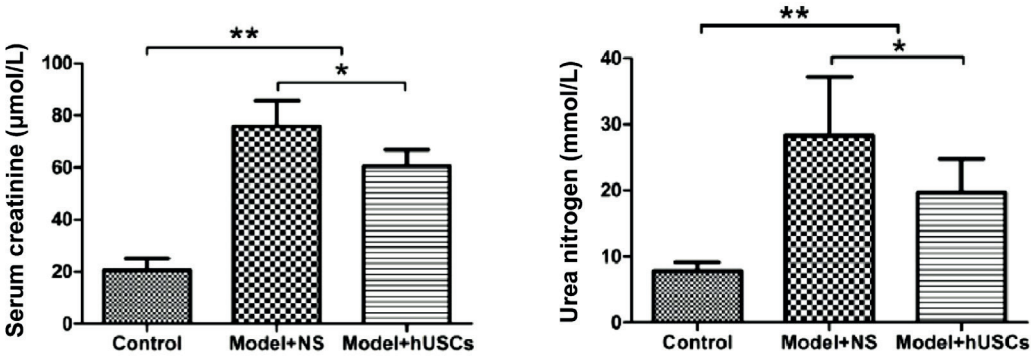

Figure 3. Comparison of serum creatinine and BUN levels in the three groups on the 32nd day.

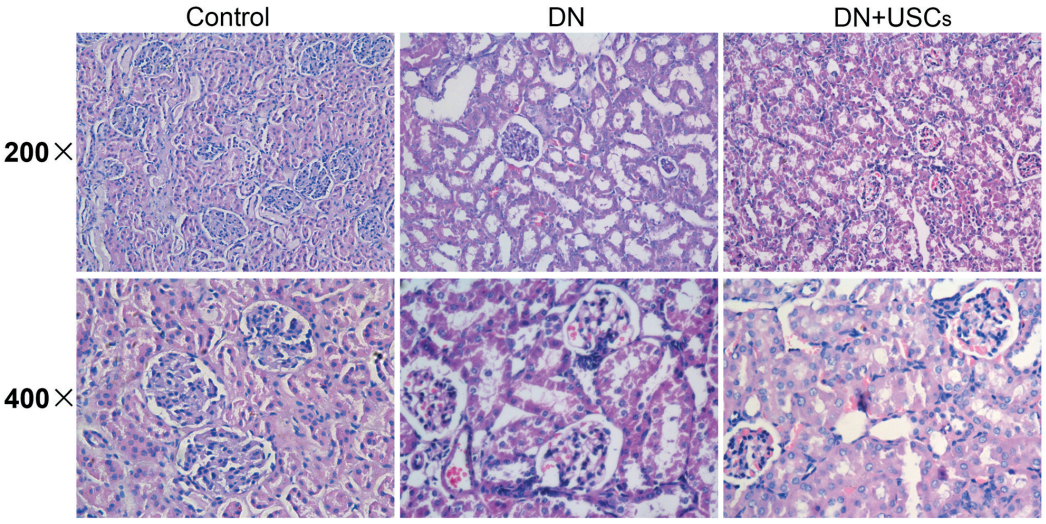

Figure 4. Histopathological changes detected by HE staining. Original magnification: ×200, ×400).

control group. However, ED1-positive cells were accumulated near the glomerulus in DN model group, and treatment with hUSCs decreased tubulointerstitial and glomerular infiltration with macrophages.

Effects of hUSCs on Oxidative Stress Detection of Renal Tissues in DN Mice

PEDF immunocytochemistry assay showed that PEDF protein was located (Figure 8) mainly in the vasculature, interstitial spaces, glomeruli, medulla, and tubular epithelial cells. After 32 days, PEDF expression was significantly decreased in mice tissues.

PEDF expression in DN+hUSC group was observed to be significantly more than that of untreated DN group. In spite of PEDF expression differentia in diabetic mice, the distribution within the kidney tissues was parallel to that in control group.

In addition, Figure 9 shows that NADPH oxidase activity is remarkably greater in the DN mice compared to that in control mice due to the DN progression. Of note, DN+hUSC group exhibited lower oxidase activity than DN model group. In consequence, the NADPH oxidase activity was gradually inhibited by hUSC treatment.

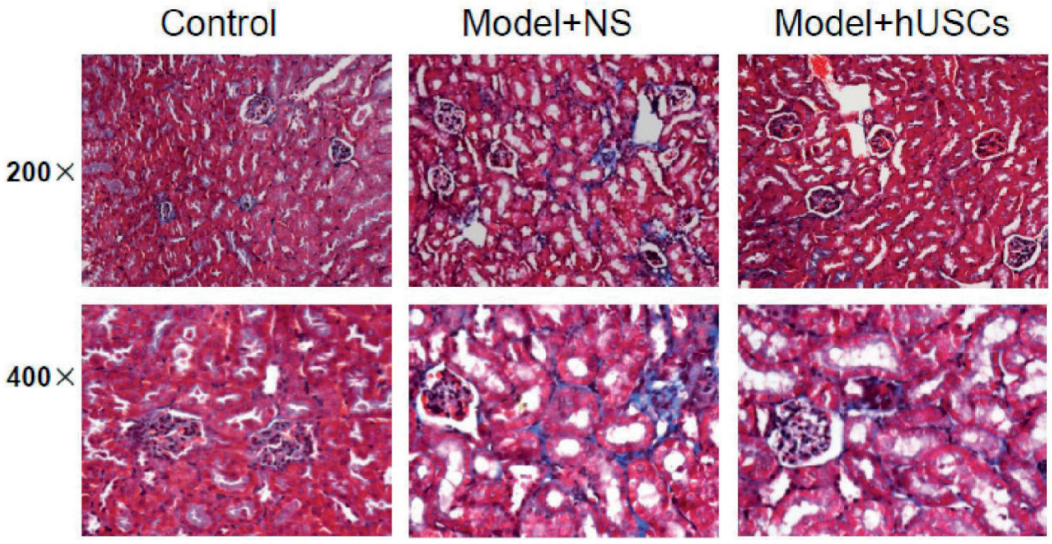

Figure 5. Masson's trichrome staining after transplantation (magnifications ×200, ×400).

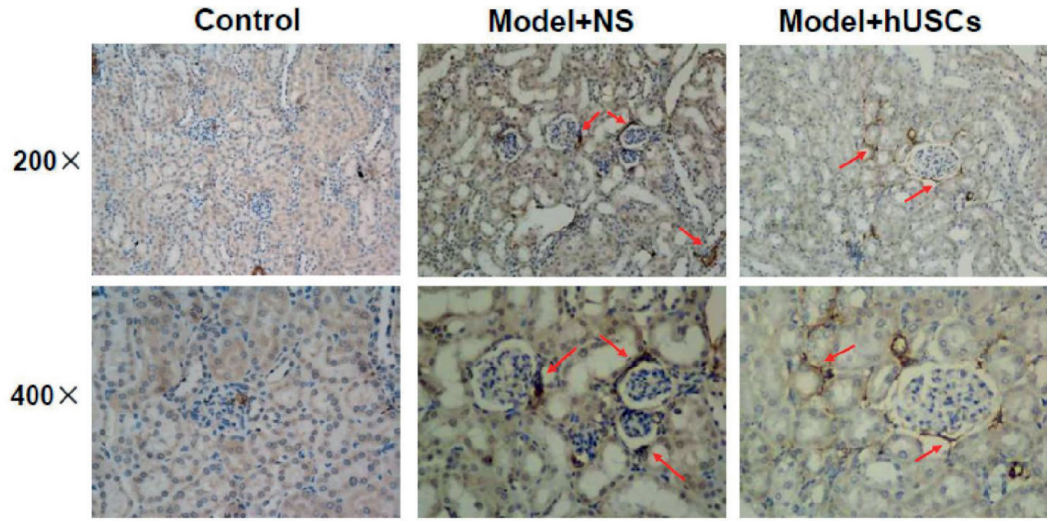

Figure 6. α-SMA expression examined by immunohistochemical staining (magnifications ×200, ×400.

Discussion

DN is a typical pathological change in the kidney such as glomerular hypertrophy, renal tubular distension, and tubulointerstitial fibrosis, which is the main reason of end-stage renal disease. To control blood pressure and plasma glucose is generally considered as the essential step to postpone the progress of DN, but these methods cannot prevent the disease. In some studies[10-12], researchers have demonstrated that USCs may promote kidney repair and improve function, which may be considered a potential therapeutic strategy to human kidney diseases. This study was designed to explore the renoprotective potential of USCs in a DN mouse model and offer theoretical basis for the application of USCs.

Decreases were observed in serum creatinine and BUN levels in DN+USC group in comparison with DN model group. Notably, analysis of DN+USC group indicated less characteristic morphological changes in tubular dilation in addition to tubulointerstitial lymphocyte and monocyte infiltration compare with DN model group. It was also found that interstitial collagen accumulation was significantly decreased in DN+USC group. In addition, injection with USCs restricted progres-

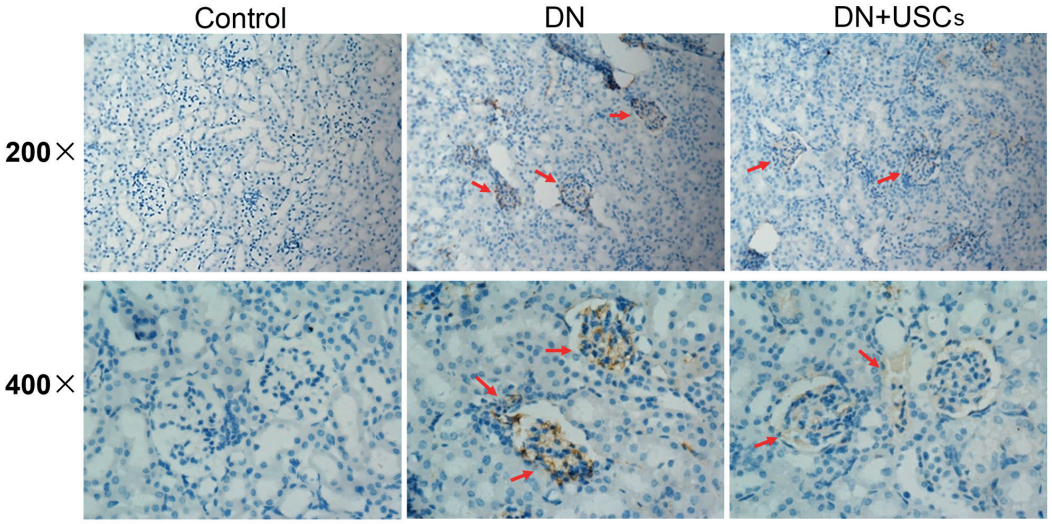

Figure 7. Expression of ED1 in nephrology tissues in all groups detected by immunohistochemistry (magnifications ×200, ×400.)

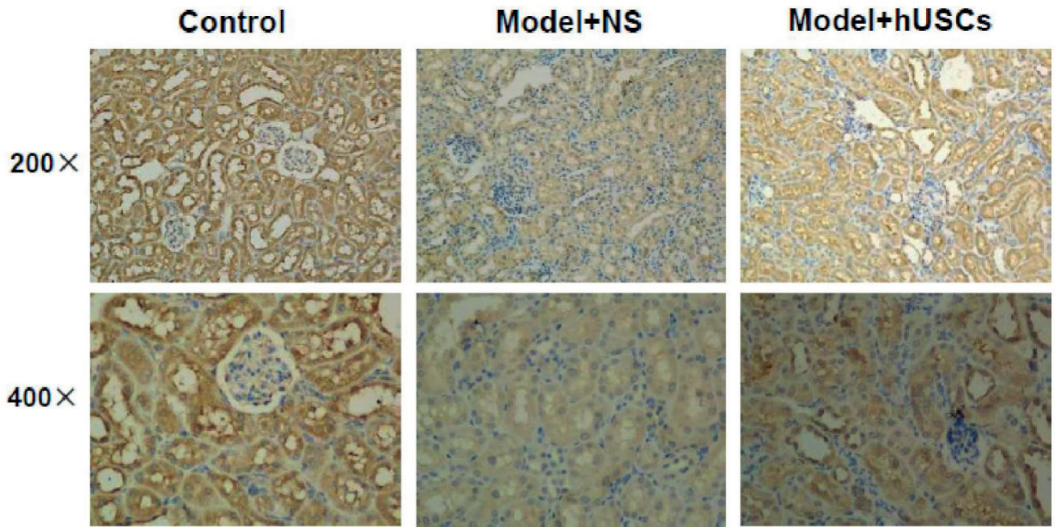

Figure 8. Localization and distribution of PEDF (magnifications ×200, ×400).

sive course of DN and might significantly suppress deterioration of renal function.

α-SMA activation is responsible for the fibrotic pathogenesis. Strutz and Zeisberg[13] have demonstrated that α-SMA-positive fibroblasts and myofibroblasts are present in both acute and chronic renal injury. In this study, α-SMA expression was increased rapidly and persistently in DN model group. Contrary to these changes, α-SMA expression was brought down following treatment with USCs. Taken together, the decrease in α-SMA expression observed in this study further reinforced the conclusion that USCs treatment could help to prevent development of fibrosis and restore renal function. Macrophage infiltration into renal tissue showed in macrophagic cells that it was activated and played a role in inflammatory reaction which will further induce renal fibrosis[14]. So, macrophage as a target for the treatment of nephrotic fibrosis has been widely considered as an effective treatment of renal fibrosis. In this research, immunohistochemical detection of ED1 revealed that ED1 expression was decreased after the injection of USCs. This result enriched the USC treatment to alleviate the nephrotic fibrosis.

Recent animal research has also elucidated that PEDF is a key factor in the early onset of DN[15],

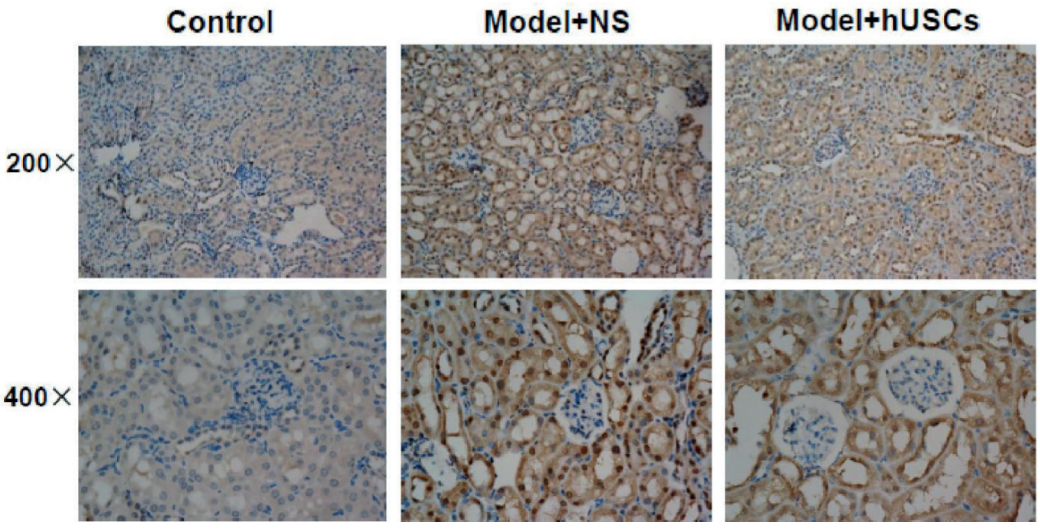

Figure 9. NADPH oxidase activity assay (magnifications ×200, ×400).

Recent animal research has also elucidated that PEDF is a key factor in the early onset of DN15. It is a protective factor of DN. In addition to reducing glomerular capillary permeability, PEDF has anti-inflammatory and antioxidation effects. Its expression in normal mice is significantly higher than that in diabetic mice16, which are consistent with this study results. In control group, kidney tissues were mostly PEDF-positive, which was shown in dramatic PEDF staining in the cytoplasm of renal tubular epithelial cells. PEDF staining decreased in DN model group. Interestingly, PEDF-positive staining in DN+hUSC group was augmented compared with DN model group. The above findings suggest that USCs may promote the expression of PEDF to recover the function.

Oxidative stress is another key pathological process in the progression of renal fibrosis. NO, SOD, and MDA are very important indexes, which can reflect the severity of oxidative stress indirectly in coronary renal disease. As it is known, the SOD is mainly from NAPDH oxidase. Consequently, increased NAPDH oxidase is perhaps considered as one of the markers for DN progression. These results are consistent with the findings in this study. In control group, NAPDH oxidase expression was hardly negative. Inversely, its expression was unregulated enormously in DN model group. After treatment with USCs, its expression was reduced to some extent.

Mounting evidence[17] prove that stem cell therapies are safe and feasible in several kidney injury models. USCs are characterized by more homology with the urinary system, compared with other stem cells. This fact demonstrates that USCs are more suitable for urinary regenerative engineering, since there has been no data about humans so far. In addition, USCs have the same characteristics with stem cells, such as a highly proliferative capacity. They can produce osteocytes, chondrocytes, myocytes, and adipocytes. Most importantly, USCs can be obtained easily and expanded for utilization by a noninvasive approach[18]. USCs have paved the way for novel therapeutic strategies in regenerative medicine, with lower intensity of rejection, multi-directional differentiation potential, and a more steady and sufficient supply.

Conclusions

In summary, USCs are capable of lessening renal injury in DN mice. USC can alleviate inflammation and oxidative stress, reduce renal interstitial fibrosis, improve renal tissue structure and protect renal function through paracrine effect. In a word, USCs offer a new method to treat DN.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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