ORIGINAL ARTICLE

Diabetic Nephropathy Animal Models of Type 2 Diabetes: An Analysis of Kidney Function, Histopathology, and Gene Expression

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ABSTRACT

Introduction: Diabetic nephropathy (DN) is one of the most serious complications of type 2 diabetes mellitus (DM). Existing animal models of DN have several limitations, therefore the development of models that have the characteristics of human chronic kidney disease is still needed. This study aimed to identify DN features based on changes in kidney function, histopathology, and gene expression in the modification of streptozotocin-nicotinamide (STZ-NA) induced-type 2 DM. **Methods:** Male Wistar rats were given double intraperitoneal injections of STZ (50 and 65 mg/kg BW), 15 minutes after NA injection (110 mg/kg BW). Diabetes induction was confirmed on day 15 with random blood glucose ≥ 200 mg/dl and blood glucose was monitored serially. A 24-hour urine was collected from metabolic cages for kidney function analysis at week 8 and the end of week 12, and then the rats were terminated at week 13. **Results**: The blood glucose of DM rats was stable > 200 mg/dl until the end of week 12. Urinary albumin excretion in DN rats was significantly higher than in control rats at week 8 ($p < 0.05$). Urinary creatinine concentration and albumin-creatinine ratio were significantly different between the DN and control rats at each time point. Fibrosis area fraction, glomerulosclerosis, and tubular injury scores ($p < 0.01$) as well as vimentin mRNA expression ($p < 0.05$) were significantly higher in DN rats than in controls. **Conclusion:** Double injection of STZ-NA resulted in a stable DM model and acquired DN characteristics after 8 weeks.
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Keywords: Diabetic nephropathy, Type 2 diabetes mellitus, STZ-NA, Urinary albumin excretion, Glomerulosclerosis

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INTRODUCTION

Diabetes mellitus is a metabolic condition that causes chronic hyperglycemia and glucose intolerance due to disturbances in insulin action or secretion, or both (1). With over 90% of all cases of diabetes globally, type 2 DM is the most common type of diabetes and is characterized by insulin resistance and relative (rather than absolute) insulin deficiency (2, 3). Diabetic nephropathy is a serious complication of chronic diabetes that can progress to end-stage renal failure, leading to high morbidity and mortality worldwide (1, 4-6). Approximately 45 percent of diabetic patients will progress to DN, with the highest incidence after 10-20 years of diabetes, and the incidence is comparable in individuals with type 1 and type 2 DM (1). The clinical features of DN are proteinuria, hypertension, and a progressive decline in kidney function (7).

Hyperglycemia causes an increase of advanced glycation end products (AGEs) coupled with the presence of growth factors, hemodynamic and hormonal disorders that induce the release of reactive oxygen species and inflammatory mediators, then developing into kidney dysfunction (1, 5, 7). This condition induces glomerular hyperfiltration and hypertension, renal hypertrophy, and changes in glomerular composition which then gives rise to signs of albuminuria and hypertension (7). The histopathological alterations of DN include extracellular

matrix (ECM) deposition, especially in the mesangium, thickening of the glomerular basement membrane (GBM), and tubular atrophy, which progresses into tubulointerstitial fibrosis and glomerulosclerosis (6, 7).

Animal models of DM are foundational tools and resources to study the molecular basis, disease pathogenesis, progression of complications, and the utility of novel therapies in diabetes. In general, diabetes animal models are classified into genetic (spontaneous) and experimentally induced (non-spontaneous) (8). Chemically induced diabetic animal models are generally inexpensive, easy to handle, and highly reproducible. The most common diabetogenic chemical is streptozotocin (STZ), an antibiotic that enters B cells by the glucose transporter (GLUT2), causes DNA alkylation and induces activation of poly ADP ribosylation which leads to the formation of superoxide radicals (8-10). Administration of STZ alone induces a type 1 DM; however, when combined with nicotinamide (NA), an amide form of vitamin B3 that attenuates STZ-induced toxicity and partially protects pancreatic β-cells, glucose responsiveness is preserved, resulting in a type 2 diabetes–like phenotype (6, 11, 12).

To model type 2 DM, the optimal range of NA dosage in combination with STZ has been investigated (11). Various dosages of STZ (45 to 65 mg/kg body weight (BW) and NA (60 to 290 mg/kg BW) were used to induce this animal model (13). The streptozotocin-nicotinamide animal creates stable and moderate hyperglycemia due to a 40-60% reduction in beta cells and insulin secretion but does not cause insulin resistance and glucose intolerance (11, 13-15). Compared with other type 2 DM animal models, such as the combination of high fat diet-STZ model, the STZ-NA model is not obese and consumes less time and cost (8, 12).

Various experimental animal models have been developed to study the course of DN and to analyze the potential for new therapies (1). An ideal DN animal model should demonstrate a 50% reduction in renal function, a greater than ten-fold rise in proteinuria, and features of pathological injury (6). In various animal models of DN, serum creatinine, blood urea nitrogen (BUN) and urinary albumin excretion rate (UAE) are usually chosen as the renal function indicators (6, 16). The streptozotocin-nicotinamide model is valuable for investigating the long-term complication of diabetes including DN and evaluating the potential antidiabetic and renoprotective effect of a substance (8, 12, 16).

Among several experimental animal models of DM, none has been able to display all parameters of DN in humans, especially those related to kidney dysfunction and nephropathy. The success rate of a single injection of STZ-NA to model type 2 DM remains uncertain. Previous studies have shown that STZ (60 mg/kg BW) and NA (200–230 mg/kg BW) injections induced stable non-fasting hyperglycemia (150–180 mg/dL) in approximately 75–80% of rats, while 20–25% develop blood glucose levels above 180 mg/dL and the others remained normoglycemic (<150 mg/dL); importantly, no rats exhibit stable hyperglycemia in the 200–300 mg/dL range for two to three weeks under these dosing conditions (17,18). Hence, the development of a type 2 diabetes–associated DN model using STZ–NA that exhibits high reproducibility and sustained long-term hyperglycemia remains necessary. Therefore, the aim of this study was to develop a DN model and to characterize the features of DN in the commonly used STZ–NA–induced type 2 diabetes model by analyzing functional, structural, and molecular aspects of the kidney. In this study, we employed a double-injection STZ–NA protocol as a modification of previously reported single-injection STZ-NA induction methods (16), designed to induce sustained hyperglycemia, a key initiator of DN progression.

MATERIALS AND METHODS

Induction of diabetic nephropathy

The experimental protocol was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia with number KE/FK/1127/EC/2022. Adult male Wistar rats (150-200 g, 7-10 weeks) were purchased from the Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada and acclimatized for 7 days before the initiation of the experiment. The animals were kept under controlled-environmental conditions with lighting (12 h-12 h). Rats were randomly divided into control and DN groups. Five animals were used in each group studied. Induction of DM was performed in overnight fasted rats by a double injection of STZ (Cayman Chemical®) and NA (Sigma-Aldrich®) as shown in Fig. 1. The STZ-NA or diluent was injected twice with an interval of 15 days. The doses of STZ were 50 mg/kg BW for the first injection and 65 mg/kg BW for the second injection in a cold citrate buffer with a pH of 4.5. The aged-matched control rats were given an equivalent volume of citrate buffer. Fifteen minutes prior to the first and the second STZ injection, the rats were injected with NA (110 mg/kg BW) intraperitoneally, and the control rats were injected with NaCl 0.9 %. The experimental rats were given standard rat feed (Rat-Bio), water ad libitum and routine checks immediately after the STZ-NA induction. Random blood glucose was measured serially at day 3 and day 15 following both the first and the second STZ-NA injection using a glucometer (Easy Touch®) to monitor changes in blood glucose levels. Blood glucose measurements are carried out on the blood drained from the tail vein. Rats were considered DM if their random blood glucose levels at day 15 post the second STZ-NA injection were greater than 200 mg/dL. The rats were kept until 13 weeks post the second injection of STZ-NA

and then sacrificed. The rat's body weight and blood glucose were monitored serially.

Metabolic cage for urine collection
Rats were placed individually in a metabolic cage and given food and drink in measured quantities. The rats were removed after a 24-hour period, and the volume of urine from the metabolic cage was measured. Urine was subjected to a centrifugation process for five minutes at 10,000g and 40 C, and then separated into sediment and supernatant. The supernatant was collected and stored in the -200 C freezer for further examination.

Blood collection
The blood for measurement of creatinine and albumin concentrations was carried out via the retro-orbital vein using hematocrit tube at week 5 after induction and before the rats were sacrificed. The blood was then centrifuged at 10,000 rpm, 100 C, for 10 minutes and the serum was collected and kept in the -200 C freezer for further analysis.

Termination of rats
Rats were euthanized under deep anesthesia using an injection of a cocktail of ketamine, xylazine, and acepromazine intraperitoneally. The rat kidneys were harvested for further analysis, with one part fixed in Normal Buffer Formalin for paraffin block processing and the other part was stored in RNA preservation solution for RNA extraction.

Biochemical assays
The serum and urine supernatants were handed over to the Integrated Research and Testing Laboratory (LPPT) Universitas Gadjah Mada for analysis of creatinine and albumin serum and urine creatinine measurement. Serum insulin concentration was measured using an ELISA assay (Elabscience, E-EL-R3034), while urinary albumin concentration was determined using an ELISA kit according to the manufacturer's instructions (Bethyl Laboratories, E111-125). The concentration of albumin was multiplied by urine volume to obtain UAE that expressed as mg per 24 hours (19). The urinary albumin creatinine ratio was calculated at week 8 and week 12. Estimated Glomerular Filtration Rate (eGFR) was calculated by the creatinine clearance as previously described (20, 21).

Histological Analysis
The paraffin sections were stained with the Periodic Acid Schiff (PAS) stain to assess the glomerulosclerosis and tubular injury scores. Glomerular injury was evaluated in 20 glomeruli across 20 fields of view per sample at 400x magnification. Tubular injury was assessed in 15 fields of view per sample on Sirius Red–stained sections at 400x magnification. Histopathological scoring was performed using predefined criteria by three observers to ensure consistency and minimize observer bias. Glomerulosclerosis was scored semi quantitatively based on the method described previously, with the following criteria: 0 (no sclerosis); 1 (sclerosis less than 25%); 2 (affecting 25-50%); 3 (affecting 50-75%); and 4 (affecting more than 75%) of the glomerular tuft area (22). Scores were assigned to each glomerulus based on the extent of sclerotic lesions, which include deposition of matrix, capillary occlusion, and adhesion of capillary tuft to the Bowman's capsule (22). Tubular injury score was measured by semi quantitative calculation of the percentage of structural lesion of tubules that include tubular dilatation, loss of the brush border, intra-luminal cast formation, tubular atrophy (23–25). Tubular injury score was classified into 0 (no tubular injury); 1 (tubular injury <25%); 2 (tubular injury between 25 –50%); 3 (tubular injury between 50 – 75%); and 4 (tubular injury >75%) (23, 24). Tubulointerstitial fibrosis was evaluated by measuring the percentage of fibrosis area on Sirius red stain sections using ImageJ software in 15 randomly selected fields (400x magnification) for each sample.

RNA isolation, cDNA synthesis and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)
The RNA from kidney tissues were isolated using Tri-RNA reagent (FavorPrep™) and RNA concentrations were quantified using the nanodrop spectrophotometer. The synthesis of cDNA was conducted using ExcelRT™ Reverse Transcription Kit II (SMOBIO Technology) based on the manufacturer's instructions. The sequence of the primers of the tested genes was listed in Table I. The reverse transcription-polymerase chain reaction (RT-PCR) was performed using GoTaq Green Master Mix (Cat No. M7122). The DNA ladder (AccuBandTM, SMOBIO Technology) and PCR products were loaded on 2% agarose gel electrophoresis. Using ImageJ software, densitometric analysis was done to quantify the mRNA expression, and expression was normalized using the ß actin mRNA expression.

Statistical analysis
Some data were analysed using IBM® SPSS® Statistics Version 25 program, and others were analysed using JASP 0.18.1 software, with a probability value (p)<0.05 considered statistically significant. Two independent groups were analysed using the independent t-test, or Mann-Whitney for non-normally distributed data. Meanwhile, for dependent data, they were analysed using a paired t-test or Wilcoxon rank sum test for non-

Table I: List of primers

Gene		Sequences	Target genes (bp)
Vimentin	F:	5′- ACCAGAGACGGACAGGTGAT -3′	111
	R:	5′- CTTGCGCTCCTGAAAACTGC -3′	
Nephrin	F:	5′- ACTCAGGCTGACATCTGGGAT-3′	299
	R:	5′- AGAGCTGGAATGACAGTGATGG -3′	
B Actin	F:	5′- GCAGATGTGGATCAGCAAGC -3′	100
	R:	5′- GGTGTAAAACGCAGCTCAGTAA -3′	

normally distributed data. Data are presented in Mean ± SD.

RESULTS

Characterization of Streptozotocin Nicotinamide-diabetic rats

Diabetes induction with double injections of STZ-NA showed a significant increase in blood glucose levels (> 200 mg/dL) in DN compared to control groups at each observation time point, the blood sugar levels were stable above 200 mg/dL after the 15th day post the second induction with STZ-NA until the 13th week (Fig. 1A). In DN rats, the average body weight tended to be lower than in control rats (Fig. 1B). The average 24-hour urine volume was significantly higher in DN rats, especially in week 12 following the second STZ-NA induction (p<0.05) (Fig. 1C).

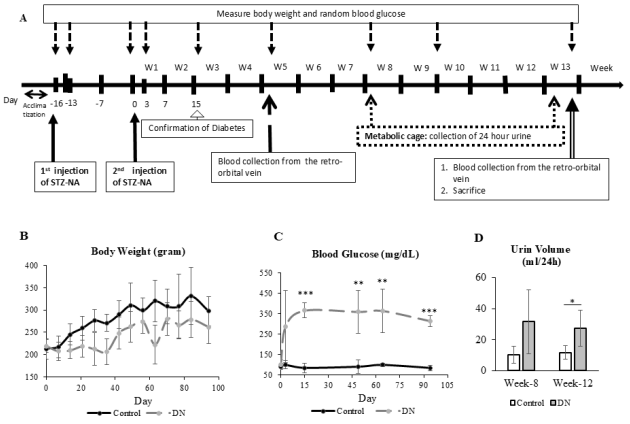

Figure 1: Diabetic nephropathy induction protocol and characteristics of diabetic Rats. A. Type 2 diabetes induction protocol with double injection of STZ-NA. B-D. Body weight, blood glucose and 24- hour urine volume measurement. * p<0.05; ** p<0.01; *** p<0.001.

Biochemical parameters of diabetes and renal function

The average serum insulin level in DN rats at week five was around 40% of normal rats' levels, which is significantly lower than normal rats' levels (p<0.05) (Fig. 2A). Serum creatinine levels at weeks 8 or 12 and serum albumin levels at week 12 did not differ significantly between DN and control rats (Fig. 2B and 2C). At week 8, the UAE and albumin creatinine ratio were significantly higher in DN compared to control rats (p<0.05 and p<0.01; respectively), and at week 12, the albumin creatinine ratio was also significantly higher in DN rats (p<0.05) (Fig. 2D and 2F). Urinary creatinine concentration was significantly lower in DN rats at both weeks 8 and 12 (p<0.05) (Fig. 2E). At week 12, there was a trend for DN rats to have lower eGFR than control rats, although this difference was not statistically significant (Fig. 2G).

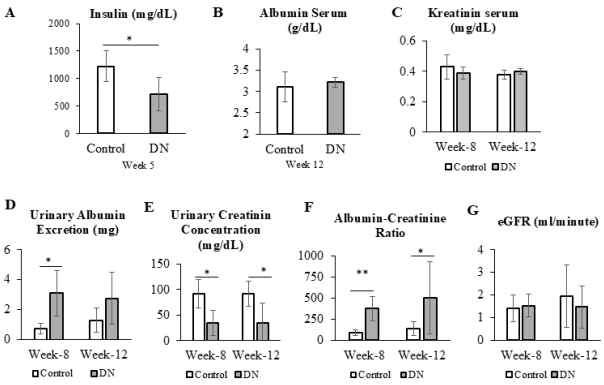

Figure 2: Biochemical characteristics of diabetes and diabetic nephropathy. The data are presented as mean ± SD. * p<0.05; ** p<0.01

Histopathological features of the kidney in the Streptozotocin Nicotinamide induced diabetic nephropathy model

Sirius red staining showed that a significant amount of fibrosis had developed in the DN group compared to the control group at week 13 (p<0.001) and collagen accumulation occurs in the tubulointerstitial area (Fig. 3A; D). In diabetic rats, the scores for glomerulosclerosis and tubular injury were also significantly higher compared to controls (p<0.001) (Fig. 3). In the DN group, there was an increased glomerular mesangial matrix deposition and occlusion of the glomerular capillary lumen, as well as lesions in the tubular structure.

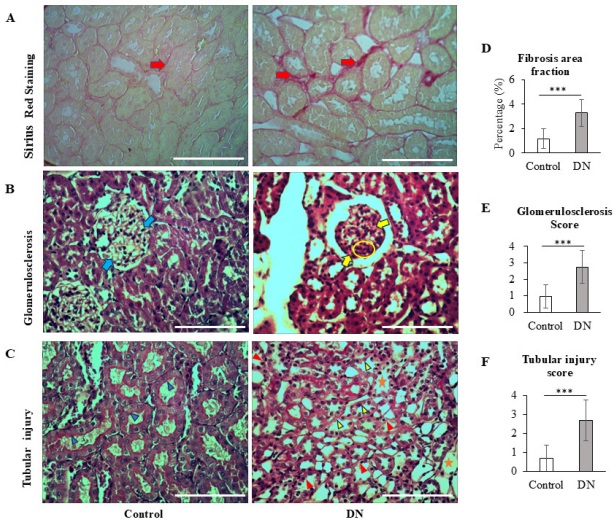

Figure 3: Histopathological alteration in diabetic nephropathy rats. A. The DN group showed increased collagen deposition in the tubulointerstitial area (red arrow). B. The DN group showed glomerulosclerosis, characterized by mesangial matrix expansion (yellow circle), and occlusion of the glomerular capillary lumen (yellow arrow) accompanied by glomerular shrinkage. In contrast, the control group exhibited open and well-preserved glomerular capillaries (blue arrow) with normal glomerular size. C. The DN group showed loss of the brush border (yellow arrowhead), tubular atrophy with epithelial thinning (red arrowhead) and tubular dilation (orange asterisk), whereas the control group exhibited an intact brush border (blue arrowhead) with normal tubular architecture. Scale bar 100µm. *** p<0.001.

Increased expression of mesenchymal marker in the kidney of diabetic rats

We demonstrated that the mRNA expression of vimentin was significantly higher in the kidneys of STZ-NA model rats compared with the control group at week 13 (Fig. 4). The mRNA expression of the podocyte marker nephrin in DN rats tended to be higher in DN rats than control rats, but the difference between the two groups was not statistically significant (p=0.236).

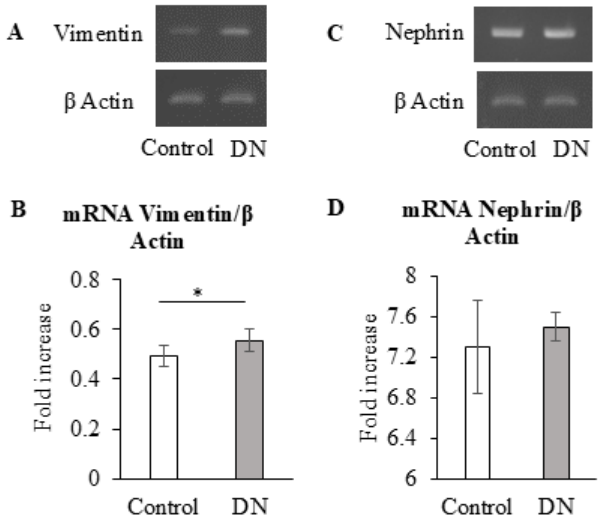

Figure 4: mRNA expression of vimentin, and nephrin in diabetic nephropathy rats. A, C. Representative images of gel electrophoresis from RT-PCR products of vimentin, and nephrin and its corresponding β actin, respectively. B, D. Relative quantification of the mRNA expressions of vimentin, and nephrin to the β actin. The data are presented as mean ± SD. *p< 0.05

DISCUSSION

In this study, we demonstrated that random blood sugar levels of rats with double STZ-NA injections were above 200 mg/dl until the end of week 13. It suggested that double administration of STZ-NA results in stable and lasting hyperglycemia condition. Furthermore, the STZ-NA model rats also exhibited diabetic signs, including a slight weight loss and a significant increase in urine volume. Serum insulin levels in these DN model rats were also shown to be lower, declining by almost 40% when compared to controls. This is in accordance with previous studies which reported that the STZ-NA model results in type 2 DM that is insulin-deficient but not insulin-resistant (8, 9). Double STZ-NA injections better mimic the pathogenesis of type 2 DM and low mortality rate (unpublished data). To strengthen the validation of the experimental animal model of type 2 DM, further research is needed to examine insulin resistance, such as measuring the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR).

Blood glucose measurements for the determination of DM in several STZ-NA models varied from 72 hours to 8 weeks (8). Based on our observations, blood glucose levels that reached more than 200 mg/dl on the 15th day post the second STZ-NA administration tended to be stable (> 200 mg/dl) and persisted until the 13th week. Prior to the fifteenth day following the first NA/STZ injection, blood sugar levels tended to fluctuate. This is in line with previous studies, which also determined the presence of DM, with a glucose level value of 250 mg/dl as the limit, 15 days after STZ administration to ensure stable hyperglycemia (26). Based on observations of blood sugar levels in this study, it appears that a single injection of STZ-NA is insufficient to induce DM. There are several differences among the STZ-NA animal model, including the strain and age of the experimental animals, the doses of NA and STZ used, the methods of administration, and the aim of the study of either inducing a DM animal model with a short observation period or with a long observation period (8, 16). The survival of DM experimental animals using each of these methods in previous studies is also unknown. Repeated STZ-NA exposure may increase the risk of more severe pancreatic damage, potentially shifting the phenotype toward type 1 DM. In this study, pancreatic histopathological or molecular evaluations were not performed; therefore, we cannot definitively confirm that the model exclusively represents type 2 DM. In this study, the observed diabetic profile is consistent with a type 2 DM–like phenotype. Nevertheless, partial overlap with more extensive β-cell injury cannot be fully excluded. Further studies are warranted to define a safe dosing regimen for the double-injection STZ–NA protocol to ensure consistent induction of a type 2 DM while minimizing pancreatic injury and animal mortality.

The present study showed that DN model rats given double STZ-NA injections at week 8 exhibited the main features of impaired kidney function in DN, including increased UAE, decreased urine creatinine concentration, and increased albumin creatinine ratio. Previous study reported that STZ-induced type 1 DM and STZ-NA-induced type 2 DM models developed DN in 4 weeks, as evidenced by altered renal function and tissue architecture, increased renal hypertrophy index and oxidative stress (16). We are unable to determine when the decline in renal function started in this model since we did not observe changes in kidney function from week to week in this study. However, we can suggest that in week 8, several markers of DN have developed in this DN rat model. At the end of the 13th week, there was a slight decrease in the average eGFR in the DN model rats, but it was not statistically significant.

The development of human DN is commonly preceded by lower grade proteinuria, which is also known as 'microalbuminuria' and is defined as albumin excretion of 30-299 mg/24 hours (28). Without proper diabetes treatment, microalbuminuria will progress to proteinuria and DN (27). The main feature of the transition from diabetes to DN is albuminuria accompanied by a

decrease in GFR (6). Proteinuria serves as important diagnostic marker of DN and reflects disease severity and progression (5,7). Decreased renal function is characterized by increased serum creatinine concentrations or decreased eGFR (7). A previous study reported that the onset of proteinuria can be identified at 8 weeks post induction of STZ-NA (13). Prolonged hyperglycemia is the main factor that triggers DN and is closely related to its onset and development (28, 29).

In this study, several pathological changes also occurred in the kidneys of STZ-NA-induced DN rat models, including an increased glomerulosclerosis and tubular injury scores, and increased tubulointerstitial fibrosis. Apart from a reduction in renal function, an optimal model of DN should exhibit pathologic lesions, including GBM thickening, mesangial stromal dilatation, and tubulointerstitial fibrosis (6). In human DN, glomerular and tubulointerstitial lesions play a major role in the reduction of renal function, which is linked to an increase in albuminuria (30). The double injections of STZ-NA induced prolonged and stable hyperglycemia leading to decreased renal function and altered renal histological structure. Repeated injections of STZ may not only increase pancreatic β-cell toxicity but also potentially cause direct acute injury to the kidney, liver, and intestine (31). Consequently, the renal changes observed in STZ-NA models are difficult to distinguish between long-term diabetic effects and direct STZ-related toxicity. Therefore, further research is needed to assess renal function periodically after STZ-NA induction.

The development of DN in diabetic patients can be classified into five stages: the early stage of DN begins with thickened GBM, hypertension, normal GFR, and lack of albuminuria (12, 32). The second stage of DN is characterized by the development of mesangial expansion and normal GFR; the third stage is identified by glomerular injury, nodular sclerosis and increased microalbuminuria. The fourth stage of DN, advanced diabetic glomerulosclerosis, is characterized by vascular and tubulointerstitial abnormalities and the fifth stage is recognized by a GFR less than 15 mL min^{-1} per 1.73 m^2 which indicates the end-stage renal failure (12, 32). Several studies mostly use biochemical and histopathological parameters to confirm the presence of DN in animal model (12). In this study, the STZ–NA–induced DN animal model exhibited renal dysfunction and pathological features that mimic early- to mid-stage DN (stages 2–3).

In the present study, the DN rats showed an increase in expression of a mesenchymal marker vimentin. In diabetes, vimentin expression is significantly increased in the glomerular endothelium as a hemodynamic response to hyperglycemia and is also observed in tubular cells, indicating transdifferentiation toward a fibroblastic lineage and reflecting tubular cell dedifferentiation during hyperglycemic injury and repair processes (33). Epithelial-to-mesenchymal transition (EMT) is involved in the development of fibrosis characterized by loss of epithelium and high production of ECM and contributes to the development of DN resulting in tubulointerstitial fibrosis (34). Renal fibrosis is a crucial DN feature and characterized by excessive collagen deposition in the matrix, activation of myofibroblasts, loss of characteristic epithelial cells, and accumulation of mesenchymal cells in the kidney (35).

Nephrin, a podocyte-specific protein, plays a crucial role in maintaining the normal structure of the glomerular slit diaphragm, and its expression temporarily increases during the first eight weeks of diabetes in several animal models, potentially due to protein kinase C activation in the diabetic kidney (36). However, other studies have reported downregulation of nephrin at eight weeks in type 1 diabetic mouse models of DN (37). There is increasing evidence that other proteins also play an important role in podocyte pathology, including podocin and synaptopodin, and their role as biomarkers of renal disorders such as in DN. Therefore, further studies are required to investigate long-term changes in nephrin expression and distribution, as well as to evaluate nephrin protein levels in urine samples.

In this double-injection STZ–NA DN model, we acknowledge several limitations, including the relatively small sample size, the absence of insulin resistance data, the lack of week-by-week renal functional assessments, and the absence of pancreatic histopathological or molecular evaluations. Therefore, further studies incorporating larger sample sizes, insulin resistance measurements, longitudinal renal function assessments, and comprehensive pancreatic analyses are warranted to better characterize pancreatic β-cell integrity and further validate the model.

CONCLUSION

The double-injection STZ–NA DN model exhibits biochemical evidence of renal dysfunction, characterized by increased UAE and albumin creatinine ratio, and is supported by renal histopathological changes reflecting early- to mid-stage diabetic nephropathy.

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