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(Citation)

American Journal of Nephrology, 31(4):342-352

(Issue Date)

2010-04

(Resource Type)

journal article

(Version)

Accepted Manuscript

(Rights)

This is the accepted manuscript version of an article published by Karger Publishers in [American Journal of Nephrology/2010/31(4)/342-352 DOI: 10.1159/000297290] and available on <https://doi.org/10.1159/000297290>

(URL)

<https://hdl.handle.net/20.500.14094/0100482894>



Oxidative and nitrosative stress and progression of diabetic nephropathy in type 2 diabetes

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Running title: Nephropathy in type 2 diabetes

Abstract***Background***

The role of nitric oxide (NO) is controversial in diabetes nephropathy progression and the mechanisms remain unknown especially in non-obese type 2 diabetes. To examine mechanisms of nephropathy progression in non-obese type 2 diabetes, we used Spontaneously Diabetic Torii (SDT) rats, a newly established model of non-obese type 2 diabetes.

Methods

Fourteen male Sprague-Dawley (SD) rats were used as a control, and 20-week-old male SDT rats were divided into two groups: diabetic (DM) (n = 8) and DM + insulin (n = 8) groups. Twenty- and 36-week-old rats were sacrificed, and blood, urine, and histomorphometric analyses, mRNA expression analysis of endothelial nitric oxide synthase (eNOS) and NADPH oxidase, and blood pressure measurement were performed.

Results

At 36 weeks, nitric oxide metabolites, and 8-hydroxydeoxyguanosine (8-OHdG) were significantly higher in the diabetic group than the other two groups. Further renal studies showed increased glomerular volume and mesangial area, and intensified eNOS, 8-OHdG, and nitrotyrosine immunostaining in the diabetic group. Oxidative and nitrosative stress were positively associated with increased urinary excretion of albumin, glomerular volume, and mesangial area, which were mostly recovered by insulin therapy.

Conclusions

NO and oxidative stress increased in SDT rats, suggesting that these play key roles in nephropathy progression in non-obese type 2 diabetes.

Key words: diabetic nephropathy, nitric oxide, oxidative stress, asymmetric dimethyl arginine, non-obese type 2 diabetes mellitus, Spontaneously Diabetic Torii rats

Introduction

The number of patients with end-stage renal disease is increasing annually worldwide. The most crucial cause of renal failure is diabetes mellitus. Many reports have investigated the mechanisms of diabetic nephropathy, and many investigators have reported that oxidative stress plays a role in the progression of diabetic nephropathy. On the other hand, the influence of nitric oxide (NO) on progression of diabetic nephropathy is controversial [1, 2]. Some investigators have demonstrated that the synthesis and action of NO are increased in diabetic nephropathy and they greatly contribute to the pathogenesis [3-5]. In contrast, other investigators have reported that endogenous NO synthesis is decreased in diabetes. They demonstrated that not only was the endothelial nitric oxide synthase (eNOS) expression downregulated, but the total renal NO production was also significantly decreased [6-10]. Thus, there are significant controversies regarding the NO system in diabetes, and the mechanism of diabetic nephropathy remains unclear.

Recently, many diabetic animal models have been used for experiments to elucidate the mechanisms of pathogenesis and complications in diabetes. However, there are few reports of non-obese type 2 diabetes. The Spontaneously Diabetic Torii (SDT) rat is a newly established non-obese type 2 diabetic rat model inbred from an outbred colony of

(SD) rats [11]. Male SDT rats spontaneously develop gradual impairment of insulin secretion and hyperglycemia without obesity after 20 weeks of age, with an incidence of 100% until 40 weeks of age. Different from the STZ-induced diabetic model, SDT rats gradually develop insulin secretory defect resulting from gradual pathological damage to β -cells in the pancreatic islets and could survive for a long time even without insulin therapy. Primarily, microvascular events such as congestion and hemorrhage were observed in the pancreatic islet at 8-10 weeks of age. Thereafter, inflammation, fibrosis and atrophy with a loss of β -cells gradually developed in the pancreatic islets until 40 weeks of age. Therefore, to clarify the detailed mechanism of nephropathy progression in non-obese type 2 diabetes, we used this non-obese type 2 diabetic rat model, SDT rat, in the present study.

Materials and methods

Animals

Male SDT rats were obtained from CLEA Japan Inc., Tokyo, Japan. Age-matched male SD rats (CLEA Japan Inc., Tokyo, Japan) were used as a control. The rats were housed with food and water available ad libitum in light- and temperature-controlled environments. Insulin therapy was initiated in the insulin-treated diabetic group at 20

weeks of age. Insulin pellets (LinShin Canada Inc., Scarborough, Ontario, Canada), which release a controlled amount of insulin, were subcutaneously implanted every two weeks. A 24-h urine sample was collected from each rat using a metabolic cage before the rat was sacrificed. At 36 weeks of age, the rats were sacrificed under ether anesthesia. Blood samples for serum measurements were collected from the left ventricle, and both kidneys were removed for RNA extraction and histomorphological analysis. We also collected data from 20-week-old SD and SDT rats, as described above. Animal care and all procedures were approved by the Institutional Animal Care and Use Committee guidelines of Kobe University School of Medicine.

Serum and urine measurements

After centrifugation for 5 min at 3000 rpm, serum samples obtained were stored at -80°C until analysis. Urine samples were also stored at -80°C for later analysis. Serum creatinine and urea nitrogen levels were measured using a FUJI DRI-CHEM 3500 (FUJIFILM Japan, Tokyo, Japan). Urinary creatinine levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA). Urinary excretion of albumin (U-Alb), urinary excretion of nitric oxide metabolites (U-NOx), and 8-hydroxydeoxyguanosine (U-8-OHdG) were

determined using an ELISA kit (Alb: Exocell Inc., Philadelphia, PA; NOx: Dojindo Laboratories, Kumamoto, Japan; 8-OHdG: NOF Corporation, Tokyo, Japan). Serum asymmetric dimethylarginine (ADMA) levels were determined at SRL Inc. (Tokyo, Japan) by a novel high-performance liquid chromatography method.

Blood pressure measurements

Systolic blood pressure measurements were performed in conscious, restrained rats by tail-cuff plethysmography (Model MK-2000; Muromachi Kikai Co. Ltd., Japan). To reduce the possibility of stress artifacts, the rats were allowed to acclimatize sufficiently, and the means of multiple readings were recorded.

Kidney sample preparation and histomorphometric analysis

To evaluate renal changes in diabetes, the kidneys were removed, weighed, and fixed with 10% formol. They were dehydrated at room temperature in an ethanol series and embedded in paraffin. They were then cut into 3- μ m sections. These were stained with hematoxylin–eosin and periodic acid-Schiff stain for routine histology and morphometric studies. Glomerular volume and the mesangial matrix index were calculated as previously reported by Ichinose et al. [12].

Formation of 8-OHdG and nitrotyrosine were assessed using anti-8-OHdG and nitrotyrosine monoclonal antibodies raised in rats (anti-8-OHdG monoclonal antibodies: NOF Corporation, Tokyo, Japan; anti-nitrotyrosine monoclonal antibodies: Upstate Biotechnology Inc., Lake Placid, NY, USA). Expression of (dimethylarginine dimethylaminohydrolase) DDAH-I and DDAH-II, which metabolize ADMA of methylarginine, in the tubules and eNOS in the glomeruli were evaluated with anti-DDAH-I, anti-DDAH-II, and anti-eNOS monoclonal antibodies (anti-DDAH-I and anti-DDAH-II polyclonal antibodies: Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; anti-eNOS polyclonal antibodies: Affinity BioReagents, Golden, CO, USA). In brief, kidney slices were pre-incubated with blocking agents (Simple Stain Rat; Nichirei, Tokyo, Japan) and then incubated with the above-mentioned primary antibodies for 60 min at room temperature. A universal immunoperoxidase polymer (Histofine Simple Stain MAX PO, anti-rat and anti-rabbit; Nichirei, Tokyo, Japan) was used for immunostaining. The 8-OHdG-, nitrotyrosine-, and eNOS-positive cells in the glomeruli, mainly endothelial and mesangial cells, were counted in 20 random microscopic fields to give 8-OHdG-, nitrotyrosine-, and eNOS-positive cell scores. Furthermore, the DDAH-I-positive tubular cells in cortical tissue and DDAH-II-positive tubular cells in the outer medulla were counted in 20 random

microscopic fields to give DDAH-I-positive and DDAH-II-positive scores. All evaluations were performed in a blinded manner by two independent pathologists.

RNA extraction and RT-PCR

Total RNA was extracted from kidneys using an ISOGEN kit (Wako Pure Chemicals Industries, Ltd, Osaka, Japan) according to the manufacturer's instructions. Total RNA from the kidneys was used as the template for cDNA synthesis in a 20- μ L volume with the SuperScript First-Strand Synthesis System (Invitrogen, CA, USA) using the oligo-dT hexamer as per the manufacturer's instructions. The reaction mixture was incubated at 65°C for 5 min and placed on ice for 1 min. Another reaction mixture was then added to it, and the mixture was incubated at 50°C for 50 min, followed by 85°C for 5 min, and then chilled on ice for 1 min. Then, 1 μ L of RNase H was added and the mixture was incubated at 37°C for 20 min. The synthesized cDNA was stored at -20°C until PCR analysis. PCR was performed using a thermal cycler (iCycler, BIO-RAD, CA, USA). For PCR analysis, we used the following primers: rat eNOS (5'-GGTGAGGACTTGTCCAAACACT-3', 5'-GGATTCTGGCAAGACCGATTAC-3'), rat NADPH p22 phox (5'-GGTTGGTAGGTGGCTGCTTGATGG-3',

5'-TGCGGGACGCTTCACGCAGTGG-3'), rat NADPH p47 phox
(5'-GGCAGGACCTGTCTGGAGAAAGGTGG-3',
5'-TGAAGGATGATGGGGCCTGTGGATG-3'), and rat GADPH
(5'-AGACAGCCGCATCTTCTTGT-3, 5'-CCACAGTCTTCTGAGTGGCA-3'). The
PCR products were loaded on a 1.0% TAE-buffered agarose gel and analyzed after gel
electrophoresis by ethidium bromide staining under UV light illumination.
Semiquantitative analysis was performed using NIH image version 1.62.

Statistical analysis

We used the computer software application StatView 5.0 (SAS Institute Inc., Cary, NC, USA) for all statistical analyses. Values are presented as mean $\pm$ SEM. The significance of differences between two groups was analyzed by Student's t-test. Data among the three groups were assessed by one-way ANOVA followed by the Turkey-Kramer test. Relationships between variables were assessed using linear regression analysis. A p value of less than 0.05 was considered to represent statistical significance.

Results

Animal characteristics and biochemical measurements

Characteristics and biochemical data of the Sprague-Dawley (SD) and SDT rats were comparable at 20 weeks of age (Table 1). At 36 weeks of age, as shown in Table 2, body weight in the diabetic (DM) group decreased significantly compared to that in the control and DM + insulin (INS) groups. Hemoglobin A1c (HbA1c) levels did not change during the study period in the control group. On the other hand, they were significantly higher in the DM group than in the control group. Elevation of HbA1c levels in the DM + INS group were significantly suppressed by insulin therapy. Creatinine clearance (Ccr) and U-Alb were significantly higher in the DM group than in the control and DM + INS groups (Ccr: control group, 5.88 ± 0.89 mL/min; DM group, 11.31 ± 2.35 mL/min; DM + INS group, 6.20 ± 0.72 mL/min; U-Alb: control group, 9.10 ± 2.28 mg/day; DM group, 54.01 ± 7.61 mg/day; DM + INS group, 26.60 ± 4.43 mg/day). Blood pressure measurements during the study period were similar among the three groups (Figure 1).

Assessment of oxidative and nitrosative stress

We measured the urinary excretion of 8-OHdG, which is a sensitive indicator of systemic oxidative stress, NOx, and serum ADMA levels, which are endogenous

inhibitors of NOS. Twenty-four-hour U-8-OHdG was remarkably higher in the DM group than in the other two groups (Figure 2A; control group, 64.6 ± 11.1 ng/day; DM group, 632.1 ± 77.1 ng/day; DM + INS group, 164.2 ± 36.4 ng/day). Urinary excretion of 8-OHdG was significantly correlated with HbA1c levels and U-Alb (Figure 3). Despite elevation of serum ADMA levels, 24-h U-NOx was highest in the DM group (Figure 2B and C; ADMA: control group, 0.47 ± 0.13 nmol/mL; DM group, 0.66 ± 0.02 nmol/mL; DM + INS group, 0.54 ± 0.04 nmol/mL; NOx: control group, 638.3 ± 149.8 ng/day; DM group, 3086.5 ± 834.4 ng/day; DM + INS group, 1007.7 ± 383.9 ng/day).

Furthermore, we evaluated oxidative and nitrosative stress in kidney tissues. Formation of 8-OHdG and nitrotyrosine in glomeruli significantly increased in the DM group compared to the control and DM + INS groups (Figure 4).

Evaluation of histomorphometric glomerular abnormalities and their relationship with oxidative and nitrosative stress

As shown in Table 3, kidney and relative kidney weight were significantly greater in the DM group than the other two groups. Histomorphometric analysis revealed glomerular hypertrophy and expansion of the mesangial area in the DM group. Insulin therapy reduced glomerular volume and mesangial matrix proliferation (Table 3).

Furthermore, we evaluated the relationship between these glomerular abnormalities and oxidative and nitrosative stress at 36 weeks of age. Both glomerular volume and mesangial matrix proliferation were significantly correlated with increased oxidative and nitrosative stress in the kidney tissues (Figure 5).

eNOS, dimethylarginine dimethylaminohydrolase (DDAH)-I, and DDAH-II expressions in kidney

We performed immunohistological analysis to assess eNOS as well as DDAH-I and DDAH-II expressions in renal tissue. As previously reported by Onozato et al. [13], DDAH-I was mainly observed in the proximal tubule, while DDAH-II was mainly observed in the distal convoluted tubule and collecting duct. The eNOS-positive cell score was significantly higher in the DM group than in the control and DM + INS groups (Figure 6A; eNOS-positive cell score: control group, 0.9 ± 0.1 ; DM group, 16.4 ± 2.8 ; DM + INS group, 12.1 ± 2.0). On the other hand, DDAH-I-positive and DDAH-II-positive cell scores were significantly lower in the DM group than in the control and DM + INS groups (Figure 6B and C; DDAH-I-positive cell score: control group, 920.8 ± 39.9 ; DM group, 284.5 ± 35.4 ; DM + INS group, 662.3 ± 53.1 ; DDAH-II-positive cell score: control group, 1949.3 ± 57.9 ; DM group, 470.0 ± 44.2 ;

DM + INS group, 1442.8 ± 106.0).

mRNA expression of eNOS, NADPH p22, and NADPH p47

To assess gene expression of eNOS and NADPH oxidase, mRNA expression of eNOS as well as NADPH p22 phox and NADPH p47 phox, which are subunits of NADPH oxidase, were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). At 20 weeks of age, the mRNA expression of eNOS and NADPH p22 phox in the kidney did not differ between the SD and SDT rats (data not shown). However, at 36 weeks of age, the mRNA expression of eNOS, NADPH p22 phox, and NADPH p47 phox was significantly higher in the DM group than in the control and DM + INS groups, and these increased expressions were reduced by insulin therapy (Figure 7).

Discussion

Our study showed the following: (1) NO production as well as oxidative and nitrosative stress were significantly increased, (2) glomerular volume and mesangial matrix expansion were significantly increased, and these were significantly correlated with both oxidative and nitrosative stress, and (3) glomerular abnormalities and increased oxidative and nitrosative stress were improved after insulin therapy in

non-obese type 2 diabetes.

In most studies, the STZ-induced diabetic animal model was commonly used. However, this is a model of type 1 diabetes, and hence, inappropriate for analysis of type 2 diabetes because pathophysiological status is far different. Several rat models of type 2 diabetes are available, such as the Wistar fatty rat, Otsuka Long-Evans Tokushima Fatty rat, Goto-Kakizaki rat, Zucker Diabetic Fatty (ZDF) rat, and ZSF1 rat [6, 14-17]. However, these are obese type 2 diabetic model rats. These rats mostly show hyperinsulinemia, hyperlipidemia, and hypertension, and these factors greatly influence progression of nephropathy. Nephropathy in these models is associated with the so-called metabolic syndrome. On the other hand, as shown in Figure 1, blood pressure did not differ among the three groups during the study period in the present study. In addition, despite increased diet and water intake, body weight was rather reduced in non-treated SDT rats at 36 weeks compared with SD and treated SDT rats. It is considered that weight loss may be a result of increased catabolism and hypovolemia. Insulin deficiency impairs glucose utilization in skeletal muscle and increases fat and muscle breakdown. Glycosuria also causes an osmotic diuresis, leading to extracellular fluid volume depletion and further loss of weight. We speculated that these may be mechanisms of weight loss in SDT rats. Furthermore, this model manifests no

significant increase in cholesterol levels but only mild triglyceridemia at 35 weeks [18]. Therefore, we considered that the influence on progression of nephropathy is relatively small. Thus, the SDT rat differs from other type 2 diabetic rat models in not showing hyperinsulinemia, hypertension, and obesity. Therefore, it enables us to assess the mechanisms of diabetic nephropathy progression without the influence of such factors. In Asian countries, including Japan, most type 2 diabetic patients are non-obese and have impaired insulin secretion [19, 20]. Therefore, it is important that these pathological types be divided to elucidate the detailed mechanisms of diabetic nephropathy. We believe that it is appropriate to use SDT rats in Asian type 2 diabetic nephropathy analysis.

We recently reported the mechanisms of bone abnormalities using SDT rats [21]. We demonstrated that oxidative stress was remarkably increased and may play a key role in the diabetic SDT rat. In the present study, we found that nitrosative stress, and not only oxidative stress, was significantly increased in non-obese type 2 diabetes. Hyperglycemia is thought to be closely associated with increased oxidative and nitrosative stress, which can trigger the development of diabetic nephropathy. Hyperglycemia stimulates the production of advanced glycosylated end products, activates NADPH oxidase through protein kinase C, and enhances the polyol pathway,

thus leading to increased superoxide anion formation [22-24]. Certainly, mRNA expression of the NADPH subunit and the urinary excretion of 8-OHdG, which is a marker of oxidative DNA damage, were significantly increased in our diabetic model. Furthermore, oxidative stress in renal tissue was also increased in the diabetic group. Oxidative stress was significantly and positively associated with HbA1c, proteinuria, and glomerular change. These results partly corresponded with those of a previous study using other diabetic models [6, 25]. Thioredoxin-1 is a small protein with antioxidant property and expressed in the various organs. Thioredoxin-1 transgenic mice have been reported to have a protective property against oxidative stress-related organ damage, such as ischemic injury in the brain, diabetic osteopenia and adriamycin-induced cardiomyopathy [26-28]. In a previous study, our group demonstrated a crucial role of oxidative stress in development of diabetic nephropathy using the thioredoxin-1 transgenic mice [25]. Despite comparable HbA1c levels, urinary excretion of Alb and glomerular abnormalities in that previous study were significantly suppressed in the diabetic thioredoxin-1 transgenic mice compared to the diabetic wild-type mice. In the present study, increased oxidative stress was reduced through the control of blood glucose levels using insulin, and these abnormalities were ameliorated. In addition, immunostaining for 8-OHdG and nitrotyrosine was markedly intensified in the tubular

cells in the diabetic group compared with other two groups in the present study. It has been reported that altered microcirculation and increase in tubular cell oxygen consumption due to the excessive formation of ROS may lead to renal parenchymal hypoxia [29]. Nuclear factor- κ B (NF- κ B), which is an oxidative stress-related tissue factor and an inflammatory marker, also suggested to be activated in the tubular cells rather than the glomerular cells in diabetes [30]. Recent studies have suggested the inflammatory processes play a crucial role in the pathogenesis of diabetic nephropathy. Thus, the results of our study might reflect a potential link between tubular 8-OHdG and nitrotyrosine with diabetic tubulointerstitial disease in the non-obese type 2 diabetic model.

It is well known that the NO system is an important modulator of diabetic nephropathy. However, many studies in various experimental and clinical settings have often reported confusing data and contradictory findings. Keynan et al. reported that the levels of eNOS activity and protein did not change, but the production of NO was significantly reduced in early diabetes using STZ-induced diabetic rats [31]. Prabhakar et al. demonstrated that the renal lesions were correlated with increased oxidative stress and decreased NO in obese type 2 diabetes [6]. They also showed that expression of eNOS was decreased in the glomeruli and tubules and that decrease in NO levels is

further augmented through consumption of NO for formation of peroxynitrite in renal tissues. On the other hand, Komers et al. demonstrated that the production of NO was increased and associated with glomerular hyperfiltration [32]. In fact, NO production and Ccr were significantly increased in the diabetic group compared to the other two groups in our study. These results agree with the study by Komers et al. A number of studies reported that eNOS expression is increased in diabetes. The clinical study by Hohenstein et al. also demonstrated that expression of cortical and glomerular eNOS was increased in patients with type 2 diabetes [33]. To clarify the change in NO and its contribution to diabetic nephropathy progression, we assessed the NO system in detail. Our research revealed that the production of NO and expression of eNOS in the kidney were significantly increased in non-obese type 2 diabetes rats.

ADMA is an endogenous competitive inhibitor of NOS, and a number of clinical and experimental studies indicated that serum ADMA levels are increased in diabetes [34, 35]. Therefore, we also examined serum ADMA levels in this study. Similarly, the results of our study showed that serum ADMA levels were significantly increased in the diabetic group and reduced by insulin therapy. In the light of the property of ADMA, this result did not agree with our findings of the NO system. To further investigate the molecular mechanism for this ADMA elevation, we examined the renal expression of

DDAH-I and DDAH-II. The previous study by Onozato et al. showed that DDAH I expression was decreased while DDAH II expression was increased in diabetic kidneys [13]. By contrast, immunohistological analysis showed that both DDAH-I and DDAH-II expressions in renal tissue were significantly decreased in the diabetic group compared to the other two groups in the present study. The result of increase in serum ADMA suggests several possibilities. First, though we could not elucidate the detailed mechanism, an enhanced NO system may be superior to the effect of ADMA. Second, as Druhan LJ et al. reported [36], ADMA may increase superoxide generation by uncoupled NOS while it behaves as an inhibitor of coupled NOS. Clinical study by Antoniadis C et al. also demonstrated an association between ADMA and vascular O₂-production and eNOS uncoupling in human vessels [37]. Moreover, as Satoh et al. suggested, uncoupled eNOS and total eNOS protein levels may be increased, while coupled eNOS levels may be decreased in diabetes [38]. Thus, increased uncoupling of eNOS may lead to production of both ROS and nitrosative stress. Third, ADMA may enhance tubulointerstitial ischemia and accelerate the progression of diabetic nephropathy. In fact, Shibata et al. demonstrated that reduction of ADMA ameliorated renal interstitial ischemia and albuminuria in diabetic animal [39].

Chander et al. eliminated a crucial role of oxidative and nitrosative stress in the

progression of diabetic nephropathy using ZDF rats [40]. Increased oxidative stress reacts with NO, resulting in formation of peroxynitrite (ONOO^-). Some studies found increased peroxynitrite in diabetic kidneys, indicating enhanced interaction between NO and O_2^- [41, 42]. Taken together, we hypothesized that this may be a crucial mechanism of nephropathy progression in non-obese type 2 diabetes. Therefore, we evaluated nitrosative stress in this rat model. A hallmark of nitrosative stress was noticed by immunohistochemical staining in glomeruli and chemical analysis of kidneys for accumulated nitrotyrosine. Our study revealed that eNOS, NOx, nitrotyrosine, and 8-OHdG were significantly increased in the diabetic group and reduced by insulin therapy. These observations may imply that hyperglycemia causes both coupling and uncoupling of NOS with generation of both NO and superoxide anions, resulting in the formation of peroxynitrite. Furthermore, our study demonstrated that glomerular abnormalities, such as glomerular volume and mesangial matrix proliferation, were significantly correlated with increased oxidative and nitrosative stress in kidney tissues. Thus, our findings suggest that increased oxidative and nitrosative stress may play key roles in the progression of nephropathy in SDT rats. Moreover, from these findings, we speculated that correction of hyperglycemia normalizes NADPH and eNOS expression and thereby reduces oxidative and nitrosative stress in the kidney. Therefore, we

considered that insulin therapy could ameliorate the kidney damage.

As a limitation of our study, we could not demonstrate a role of elevated ADMA. Kidney lesions in this model were relatively slight. Therefore, we speculate that ADMA levels may be relatively low, and its effect may be inferior to that of increased eNOS at an early stage of diabetic nephropathy. If we could observe an advanced stage of diabetic nephropathy, we may have found a more important role for ADMA. Moreover, we could not inhibit production of oxidative stress and/or NO using antioxidative drugs and/or L-NAME. These interventions will enable us to shed more light on the role of uncoupled-NOS contribution to $O_2^{\cdot-}/ONOO^-$ generation. If we perform a study by pharmacological intervention, we could demonstrate the detailed mechanisms of diabetic nephropathy in non-obese type 2 diabetes. Further study to resolve these issues is now underway in our laboratory.

In conclusion, our results indicate that increased oxidative and nitrosative stress is associated with glomerular abnormalities in non-obese type 2 diabetes and that these abnormalities are ameliorated by insulin. In addition, we conclude that the SDT rat is a useful animal model for elucidating the mechanisms of diabetic nephropathy in non-obese type 2 diabetes.

Conflict of interest: None

For Peer Review

References

1. Komers R, Anderson S. Paradoxes of nitric oxide in the diabetic kidney. *Am J Physiol Renal Physiol*. 2003; 284: F1121-1137.
2. Prabhakar SS. Role of nitric oxide in diabetic nephropathy. *Semin Nephrol*. 2004; 24: 333-344.
3. Veelken R, Hilgers KF, Hartner A, Haas A, Böhrer KP, Sterzel RB. Nitric oxide synthesis isoforms and glomerular hyperfiltration in early diabetic nephropathy. *J Am Soc Nephrol*. 2000; 11: 71-79.
4. Hiragushi K, Sugimoto H, Shikata K, Yamashita T, Miyatake N, Shikata Y, Wada J, Kumagai I, Fukushima M, Makino H. Nitric oxide system is involved in glomerular hyperfiltration in Japanese normo-and micro-albuminuric patients with type2 diabetes. *Diabetes Res Clin Pract*. 2001; 53: 149-159.
5. Sugimoto H, Shikata K, Matsuda M, Kushiro M, Hayashi Y, Hiragushi K, Wada J, Makino H. Increased expression of endothelial cell nitric oxide synthase (ecNOS) in afferent and glomerular endothelial cells is involved in glomerular hyperfiltration of diabetic nephropathy. *Diabetologia*. 1998; 41: 1426-1434.
6. Prabhakar S, Starnes J, Shi S, Lonis B, Tran R. Diabetic nephropathy is associated with oxidative stress and decreased renal nitric oxide production. *J Am Soc Nephrol*.

2007; 18: 2945-52.

7. Li Z, Rodriguez-Iturbe B, Ni Z, Shahkarami A, Sepassi L, Vaziri ND. Effect of hereditary obesity on renal expressions of NO synthase, caveolin-1, Akt, guanylate cyclase, and calmodulin. *Kidney Int.* 2005; 68: 2766–2772.
8. Erdely A, Freshour G, Maddox DA, Olson JL, Samsell L, Baylis C. Renal disease in rats with type 2 diabetes is associated with decreased renal nitric oxide production. *Diabetologia.* 2004; 47: 1672–1676.
9. Koo JR, Vaziri ND. Effects of diabetes, insulin and antioxidants on NO synthase abundance and NO interaction with reactive oxygen species. *Kidney Int.* 2003; 63: 195–201.
10. Khamaisi M, Keynan S, Bursztyn M, Dahan R, Reinhartz E, Ovadia H, Raz I. Role of renal nitric oxide synthase in diabetic kidney disease during the chronic phase of diabetes. *Nephron Physiol.* 2006; 102: 72-80.
11. Masuyama T, Komeda K, Hara A, Noda M, Shinohara M, Oikawa T, Kanazawa Y, Taniguchi K. Chronological characterization of diabetes development in male Spontaneously Diabetic Torii rats. *Biochem Biophys Res Commun.* 2004; 13: 870–877.

12. Ichinose K, Maeshima Y, Yamamoto Y, Kitayama H, Takazawa Y, Hirokoshi K, Sugiyama H, Yamasaki Y, Eguchi K, Makino H. Antiangiogenic endostatin peptide ameliorates renal alterations in the early stage of a type 1 diabetic nephropathy model. *Diabetes*. 2005; 54: 2891-2903.
13. Onozato ML, Tojo A, Leiper J, Fujita T, Palm F, Wilcox CS. Expression of NG,NG-dimethylarginine dimethylaminohydrolase and protein arginine N-methyltransferase isoforms in diabetic rat kidney: effects of angiotensin II receptor blockers. *Diabetes*. 2008; 57: 172-180.
14. Koh EH, Kim MS, Park JY, Kim HS, Youn JY, Park HS, Youn JH, Lee KU. Peroxisome proliferator-activated receptor (PPAR)-alpha activation prevents diabetes in OLETF rats: comparison with PPAR-gamma activation. *Diabetes*. 2003; 52: 2331–2337.
15. Ahmad T, Ugarph-Morawski A, Li J, Bileviciute-Ljungar I, Finn A, Ostenson CG, Kreichbergs A. Bone and joint neuropathy in rats with type-2 diabetes. *Regul Pept*. 2004; 15; 119:61–67.
16. Ahmad T, Ohlsson C, Saaf M, Ostenson CG, Kreichbergs A. Skeletal changes in type-2 diabetic Goto-Kakizaki rats. *J Endocrinol*. 2003; 178: 111–116.

17. Ostenson CG, Fiere V, Ahmed M, Lindstrom P, Brismar K, Brismar T, Kreicbergs A. Decreased cortical bone thickness in spontaneously non-insulin-dependent diabetic GK rats. *J Diabetes Complications*. 1997; 11: 319–322.
18. Shinohara M, Masuyama T, Shoda T, Takahashi T, Katsuda Y, Komeda K, Kuroki M, Kakehashi A, Kanazawa Y. A new spontaneously diabetic non-obese Torii rat strain with severe ocular complications. *Int J Exp Diabetes Res*. 2000; 1: 89-100.
19. Kim DJ, Song KE, Park JW, Cho HK, Lee KW, Huh KB. Clinical characteristics of Korean type 2 diabetic patients in 2005. *Diabetes Res Clin Pract*. 2007; 77 Suppl 1: S252-7.
20. Takeshita N, Ishida H, Yamamoto T, Koh G, Kurose T, Tsuji K, Okamoto Y, Ikeda H, Seino Y. Circulating levels and bone contents of bone gamma-carboxyglutamic acid-containing protein in rat models of non-insulin-dependent diabetes mellitus. *Acta Endocrinol (Copenh)*. 1993; 128: 69–73.
21. Fujii H, Hamada Y, Fukagawa M. Bone formation in spontaneously diabetic Torii-newly established model of non-obese type 2 diabetes rats. *Bone*. 2008; 42: 372-379.
22. Yamagishi S, Inagaki Y, Okamoto T, Amano S, Koga K, Takeuchi M. Advanced glycation end products inhibit de novo protein synthesis and induce TGF-beta

- overexpression in proximal tubular cells. *Kidney Int.* 2003; 63: 464-473.
23. Xia L, Wang H, Munk S, Kwan J, Goldberg HJ, Fantus IG, Whiteside CI. High glucose activates PKC-zeta and NADPH oxidase through autocrine TGF-beta1 signaling in mesangial cells. *Am J Physiol Renal Physiol.* 2008; 295: F1705-1714.
24. Chung SS, Ho EC, Lam KS, Chung SK. Contribution of polyol pathway to diabetes-induced oxidative stress. *J Am Soc Nephrol.* 2003; 14(8 Suppl 3): S233-236.
25. Hamada Y, Miyata S, Nii-Kono T, Kitazawa R, Kitazawa S, Higo S, Fukunaga M, Ueyama S, Nakamura H, Yodoi J, Fukagawa M, Kasuga M. Overexpression of thioredoxin1 in transgenic mice suppresses development of diabetic nephropathy. *Nephrol Dial Transplant.* 2007; 22: 1547-1557.
26. Takagi Y, Mitsui A, Nishiyama A, Nozaki K, Sono H, Gon Y, Hashimoto N, Yodoi J. Overexpression of thioredoxin in transgenic mice attenuates focal ischemic brain damage. *Proc Natl Acad Sci USA.* 1999; 96: 4131-4136.
27. Hamada Y, Fujii H, Kitazawa R, Yodoi J, Kitazawa S, Fukagawa M. Thioredoxin-1 overexpression in transgenic mice attenuates streptozotocin-induced diabetic osteopenia: a novel role of oxidative stress and therapeutic implications. *Bone.* 2009; 44: 936-941.
28. Shioji K, Kishimoto C, Nakamura H, Masutani H, Yuan Z, Oka S, Yodoi J.

- Overexpression of thioredoxin-1 in transgenic mice attenuates adriamycin-induced cardiotoxicity. *Circulation*. 2002; 106: 1403–1409.
29. Palm F, Cederberg J, Hansell P, Liss P, Carlsson PO. Reactive oxygen species cause diabetes-induced decrease in renal oxygen tension *Diabetologia*. 2003; 46: 1153-1160.
30. Morcos M, Sayed AA, Bierhaus A, Yard B, Waldherr R, Merz W, Kloeting I, Schleicher E, Mentz S, Abd el Baki RF, Tritschler H, Kasper M, Schwenger V, Hamann A, Dugi KA, Schmidt AM, Stern D, Ziegler R, Haering HU, Andrassy M, van der Woude F, Nawroth PP. Activation of tubular epithelial cells in diabetic nephropathy. *Diabetes*. 2002; 51: 3532-3544.
31. Keynan S, Hirshberg B, Levin-Iaina N, Wexler ID, Dahan R, Reinhartz E, Ovadia H, Wollman Y, Chernihovskey T, Iaina A, Raz I. Renal nitric oxide production during the early phase of experimental diabetes mellitus. *Kidney Int*. 2000; 58: 740-747.
32. Komers R, Allen TJ, Cooper ME. Role of endothelium-derived nitric oxide in the pathogenesis of the renal hemodynamic changes of experimental diabetes. *Diabetes*. 1994; 43: 1190-1197.
33. Hohenstein B, Hugo CP, Hausknecht B, Boehmer KP, Riess RH, Schmieder RE. Analysis of NO-synthase expression and clinical risk factors in human diabetic

- nephropathy. *Nephrol Dial Transplant*. 2008; 23: 1346-1354.
34. Fard A, Tuck CH, Donis JA, Sciacca R, Di Tullio MR, Wu HD, Bryant TA, Chen NT, Torres-Tamayo M, Ramasamy R, Berglund L, Ginsberg HN, Homma S, Cannon PJ. Acute elevations of plasma asymmetric dimethylarginine and impaired endothelial function in response to a high-fat meal in patients with type 2 diabetes. *Arterioscler. Thromb. Vasc. Biol*. 2000; 20: 2039-2044.
35. Ito A, Egashira K, Narishige T, Muramatsu K, Takeshita A. Angiotensin-converting enzyme activity is involved in the mechanism of increased endogenous nitric oxide synthase inhibitor in patients with type 2 diabetes mellitus. *Circ. J*. 2002; 66: 811-815.
36. Druhan LJ, Forbes SP, Pope AJ, Chen CA, Zweier JL, Cardounel AJ. Regulation of eNOS-derived superoxide by endogenous methylarginines. *Biochemistry*. 2008; 47: 7256-7263.
37. Antoniadou C, Shirodaria C, Leeson P, Antonopoulos A, Warrick N, Van-Assche T, Cunningham C, Tousoulis D, Pillai R, Ratnatunga C, Stefanadis C, Channon KM. Association of plasma asymmetrical dimethylarginine (ADMA) with elevated vascular superoxide production and endothelial nitric oxide synthase uncoupling:

- implications for endothelial function in human atherosclerosis. *Eur Heart J.* 2009; 30: 1142-1150.
38. Satoh M, Fujimoto S, Arakawa S, Yada T, Namikoshi T, Haruna Y, Horike H, Sasaki T, Kashihara N. Angiotensin II type 1 receptor blocker ameliorates uncoupled endothelial nitric oxide synthase in rats with experimental diabetic nephropathy. *Nephrol Dial. Transplant.* 2008; 23: 3806 - 3813.
39. Shibata R, Ueda S, Yamagishi S, Kaida Y, Matsumoto Y, Fukami K, Hayashida A, Matsuoka H, Kato S, Kimoto M, Okuda S. Involvement of asymmetric dimethylarginine (ADMA) in tubulointerstitial ischaemia in the early phase of diabetic nephropathy. *Nephrol. Dial. Transplant.* 2009; 24: 1162 - 1169.
40. Chander PN, Gealekman O, Brodsky SV, Elitok S, Tojo A, Crabtree M, Gross SS, Goligorsky MS. Nephropathy in Zucker diabetic fat rat is associated with oxidative and nitrosative stress: prevention by chronic therapy with a peroxynitrite scavenger ebselen. *J Am Soc Nephrol.* 2004; 15: 2391-2403.
41. Ishii N, Patel KP, Lane PH, Taylor T, Bian K, Murad F, Pollock JS, Carmines PK. Nitric oxide synthesis and oxidative stress in the renal cortex of rats with diabetes mellitus. *J Am Soc Nephrol.* 2001; 12: 1630-1639.
42. Onozato ML, Tojo A, Goto A, Fujita T, Wilcox CS. Oxidative stress and nitric

oxide synthase in rat diabetic nephropathy: effects of ACEI and ARB. *Kidney Int.* 2002; 61: 186-194.

For Peer Review

Acknowledgments: This study was partly presented at the annual meeting of American Society of Nephrology, 2008.

Source of Funding: This work was supported by a Grant-in-Aid for Young Scientist (B) (No. 21790810 to H.F) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan.

Table 1. Animal characteristics at 20 weeks of age

	SD	SDT	
	(n = 6)	(n = 6)	<i>p</i>
Body weight (g)	565.2 ± 25.7	539.2 ± 10.9	0.373
Serum creatinine (mg/dL)	0.53 ± 0.07	0.55 ± 0.04	0.846
Blood urea nitrogen (mg/dL)	23.3 ± 1.2	25.5 ± 1.7	0.298
HbA1c (%)	2.7 ± 0.1	3.0 ± 0.4	0.071
ADMA (ng/mL)	0.55 ± 0.01	0.58 ± 0.01	0.103
U-8-OHdG (ng/day)	124.2 ± 20.6	133.3 ± 40.9	0.886
U-NOx (nmol/day)	467.8 ± 264.1	508.9 ± 54.6	0.835

HbA1c, hemoglobin A1c; ADMA, asymmetric dimethylarginine; U-8-OHdG, urinary excretion of 8-hydroxydeoxyguanosine; U-NOx, urinary excretion of nitrate and nitrite oxide metabolites

Values are mean ± SEM.

Table 2. Animal characteristics at 36 weeks of age

	Control	DM	DM + INS
	(n = 8)	(n = 8)	(n = 8)
Body weight (g)	671.8 ± 75.8	434.8 ± 33.4 †*	558.8 ± 48.9 #
Serum creatinine (mg/dL)	0.51 ± 0.03	0.63 ± 0.12 †*	0.53 ± 0.10 #
Blood urea nitrogen (mg/dL)	21.5 ± 2.0	36.4 ± 7.8 †*	26.8 ± 5.4 #
HbA1c (%)	2.8 ± 0.4	11.3 ± 1.0 †*	6.7 ± 1.9 #

DM, diabetes mellitus; INS, insulin; HbA1c, hemoglobin A1c

Values are mean ± SEM.

†, control vs. DM, $p < 0.05$; *, DM vs. DM + INS, $p < 0.05$; #, control vs. DM + INS, $p < 0.05$

Table 3. Kidney weight and histomorphometric glomerular abnormalities at 36 weeks of age

	Control	DM	DM + INS
	(n = 8)	(n = 8)	(n = 8)
Kidney weight (g)	2.52 ± 0.19	3.20 ± 0.03 ^{†*}	2.71 ± 0.09
Relative kidney weight (kidney weight/body weight 100g)	0.32 ± 0.03	0.76 ± 0.03 ^{†*}	0.49 ± 0.01 [#]
Glomerular volume (μm ³)	3843.9 ± 205.8	4047.4 ± 120.3 ^{†*}	3661.0 ± 131.2
Mesangial matrix index	0.133 ± 0.003	0.173 ± 0.006 ^{†*}	0.149 ± 0.005

Values are mean ± SEM.

†, control vs. DM, $p < 0.05$; *, DM vs. DM + INS, $p < 0.05$; #, control vs. DM + INS, p

< 0.05

Figure legends

Figure 1. Change in blood pressure among control, DM, and DM + INS groups during the study period

There was no significant difference in blood pressure among the control, DM, and DM + INS groups during the study period.

Figure 2. Urinary 8-hydroxydeoxyguanosine (8-OHdG), nitrate, and nitrite oxide metabolites (NOx) excretion and serum asymmetric dimethylarginine (ADMA) levels

A, Urinary 8-OHdG excretion.

B, Urinary NOx excretion.

C, serum ADMA levels.

†, control vs. DM, $p < 0.05$; *, DM vs. DM + INS, $p < 0.05$.

Values are means $\pm$ SEM.

Figure 3. Correlation of urinary 8-OHdG excretion with hemoglobin A1c (HbA1c) levels and U-Alb at 36 weeks of age.

A, Relationship between HbA1c levels and urinary 8-OHdG

HbA1c levels were significantly associated with urinary 8-OHdG excretion. ($r = 0.562$, $p < 0.005$).

B, Relationship between U-Alb and 8-OHdG excretion

U-Alb excretion was significantly associated with urinary 8-OHdG excretion. ($r = 0.500$, $p < 0.05$).

Figure 4. Oxidative and nitrosative stress in kidney tissues among the control, DM, and DM + INS groups at 36 weeks of age

A, 8-OHdG-positive cell score

B, Nitrotyrosine-positive cell score

†, control vs. DM, $p < 0.05$; *, DM vs. DM + INS, $p < 0.05$.

Values are means $\pm$ SEM.

Figure 5. Correlation between glomerular abnormalities as well as oxidative and nitrosative stress in kidney tissue

A, Relationship between 8-OHdG-positive cell score and glomerular volume ($r = 0.667$, $p < 0.005$).

B, Relationship between 8-OHdG-positive cell score and mesangial matrix index

($r = 0.770, p < 0.001$).

C, Relationship between nitrotyrosine-positive cell score and glomerular volume

($r = 0.602, p < 0.01$).

D, Relationship between nitrotyrosine-positive cell score and mesangial matrix index.

8-OHdG-positive and nitrotyrosine-positive cell scores were significantly associated with both glomerular volume and mesangial matrix index ($r = 0.865, p < 0.0001$).

Figure 6. eNOS and DDAH-I and DDAH-II expressions in kidney

A, eNOS-positive cell score in glomeruli.

B, DDAH-I-positive cell score in the cortical medulla.

C, DDAH-II-positive cell score in the outer medulla.

†, control vs. DM, $p < 0.05$; *, DM vs. DM + INS, $p < 0.05$; #, control vs. DM + INS, $p < 0.05$.

Values are means $\pm$ SEM.

Figure 7. mRNA expression of *eNOS*, *NADPH p22*, and *NADPH p47* among control, DM, and DM + INS groups at 36 weeks of age.

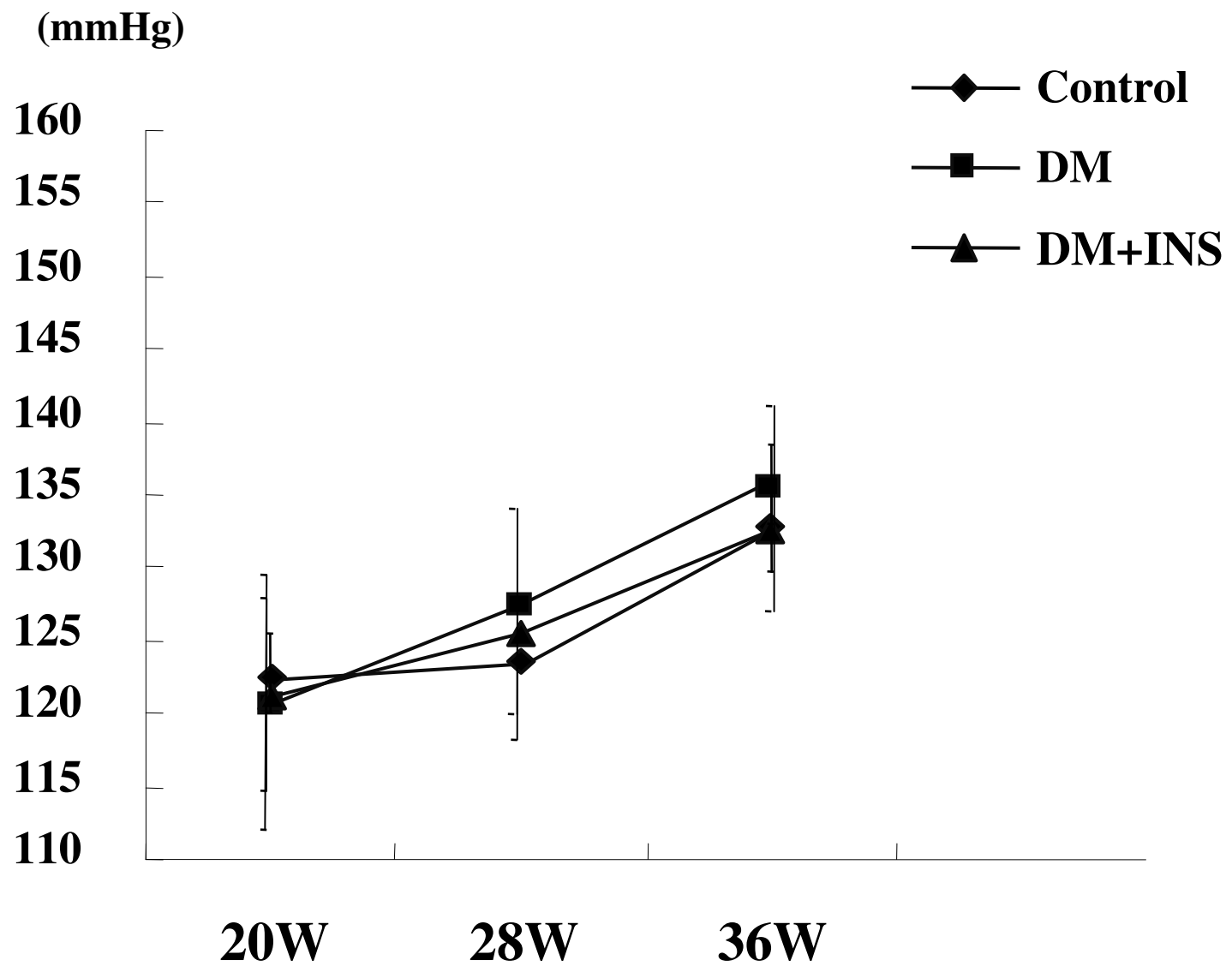
A, mRNA expression of *eNOS*

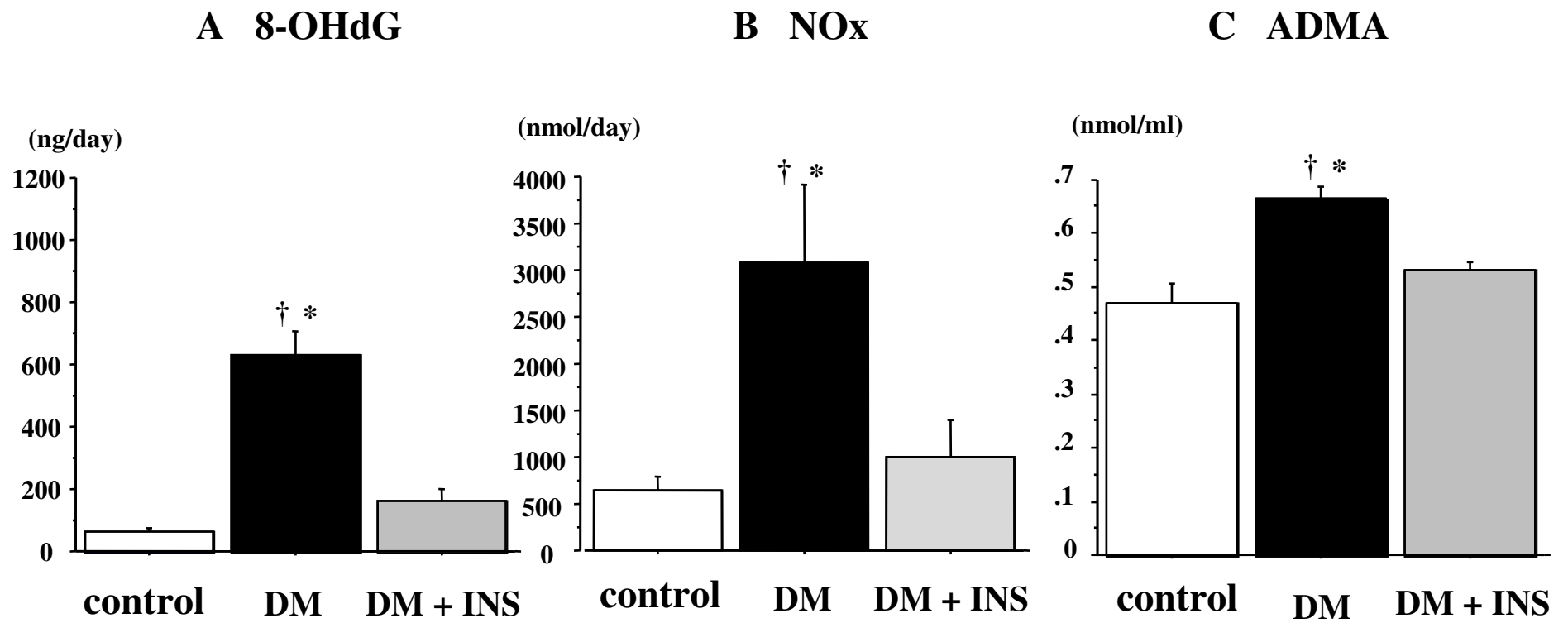
B, mRNA expression of *NADPH p22*

C, mRNA expression of *NADPH p47*

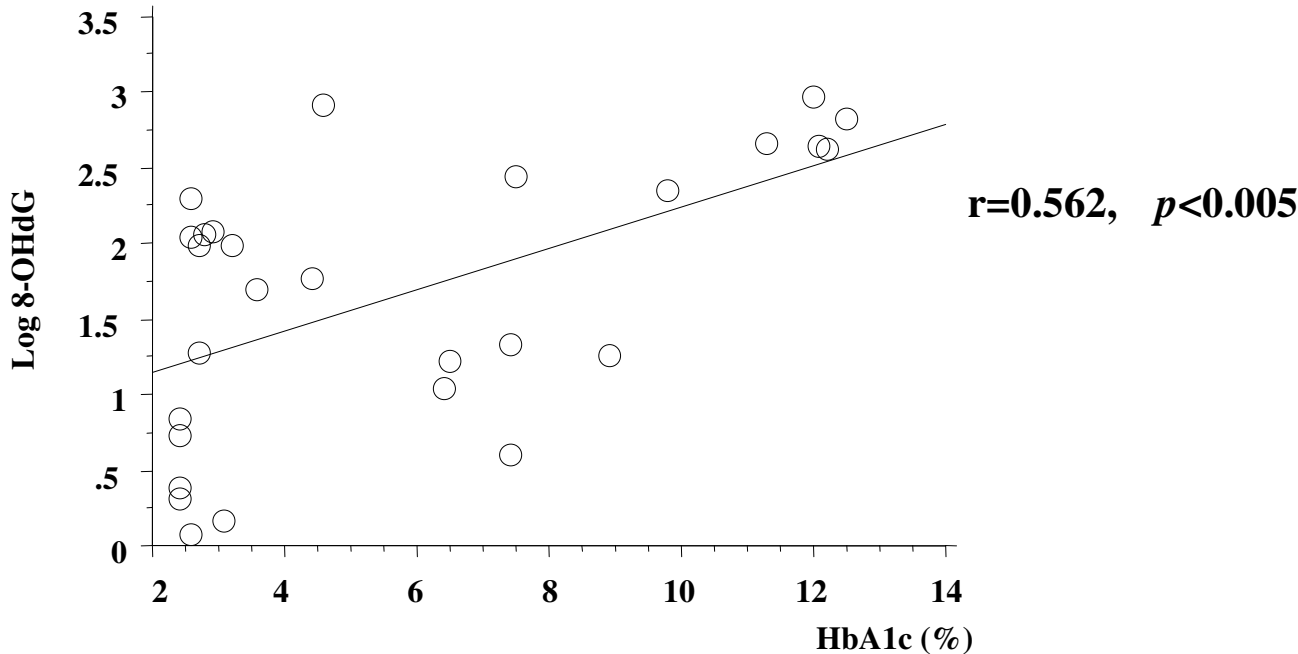
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Values are means $\pm$ SEM.

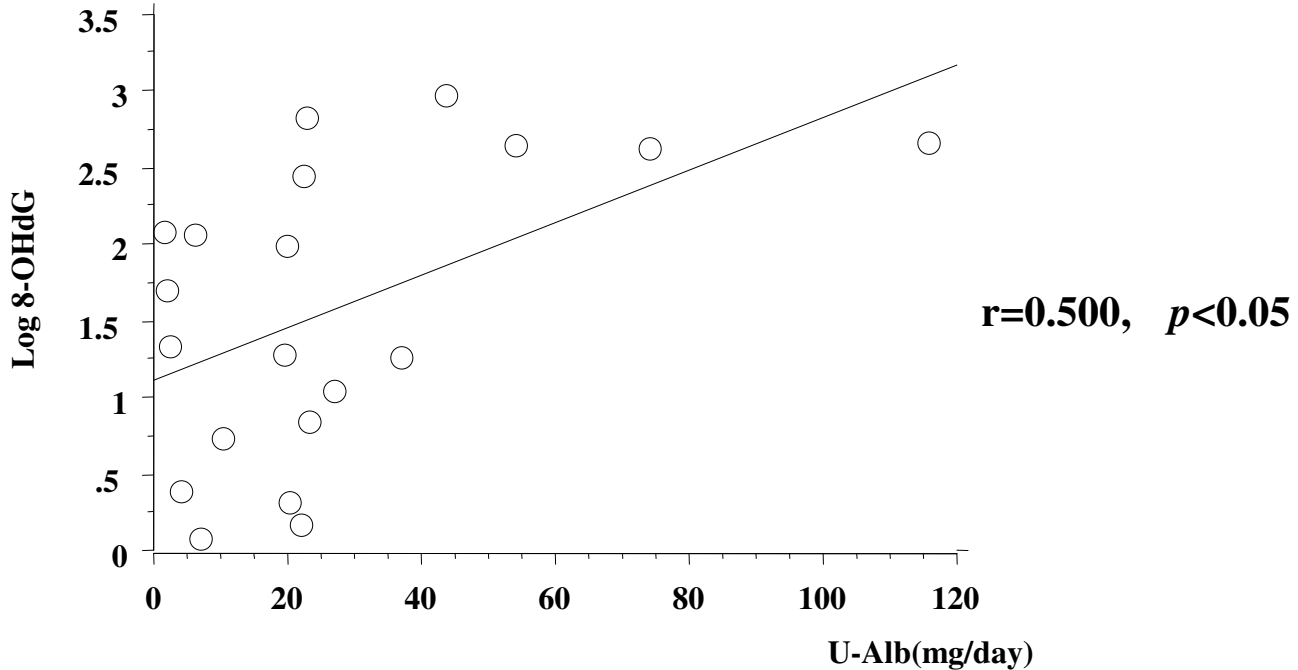




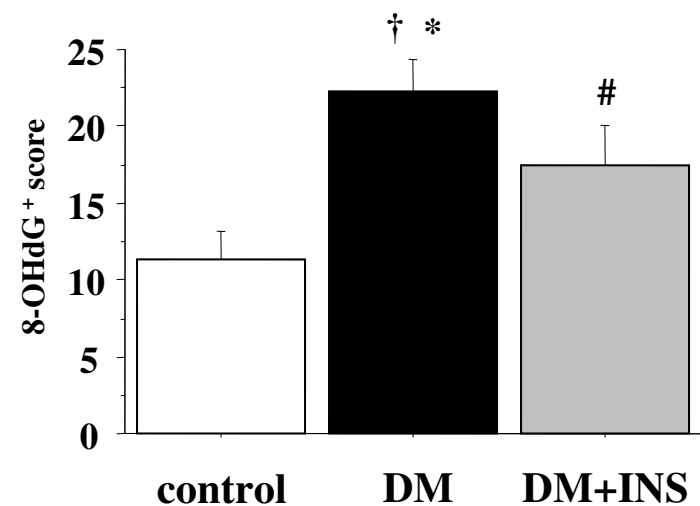
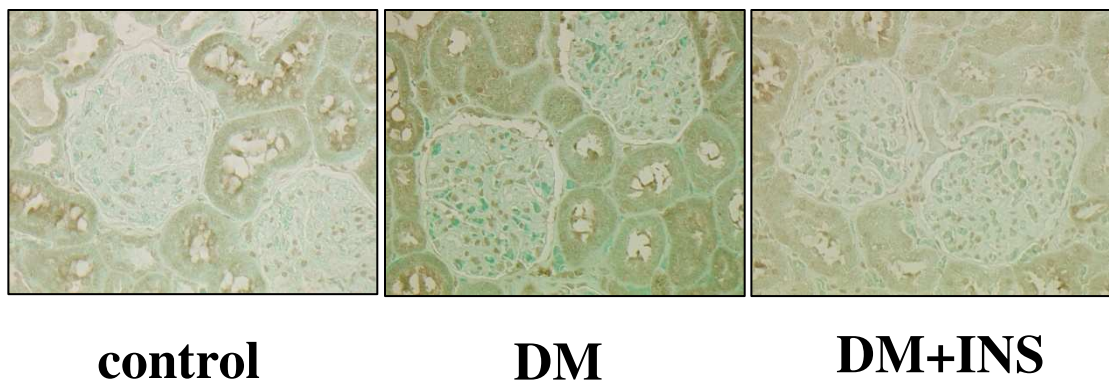
A



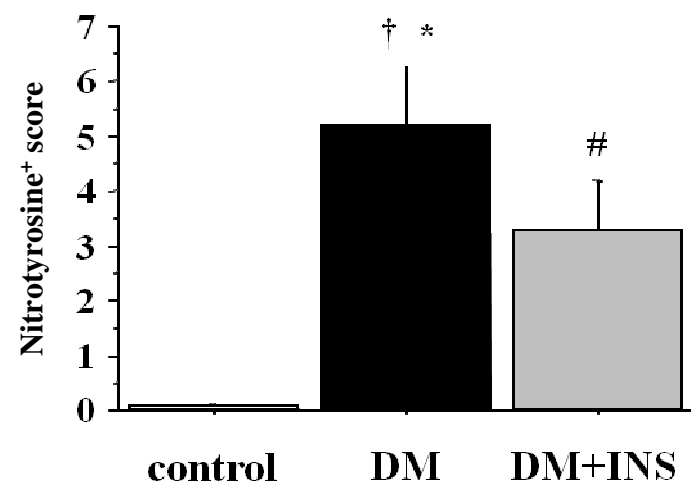
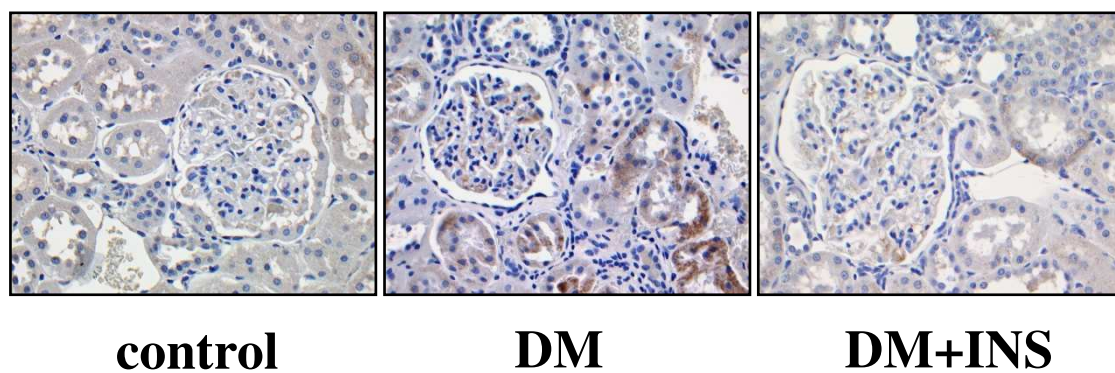
B



A 8-OHdG

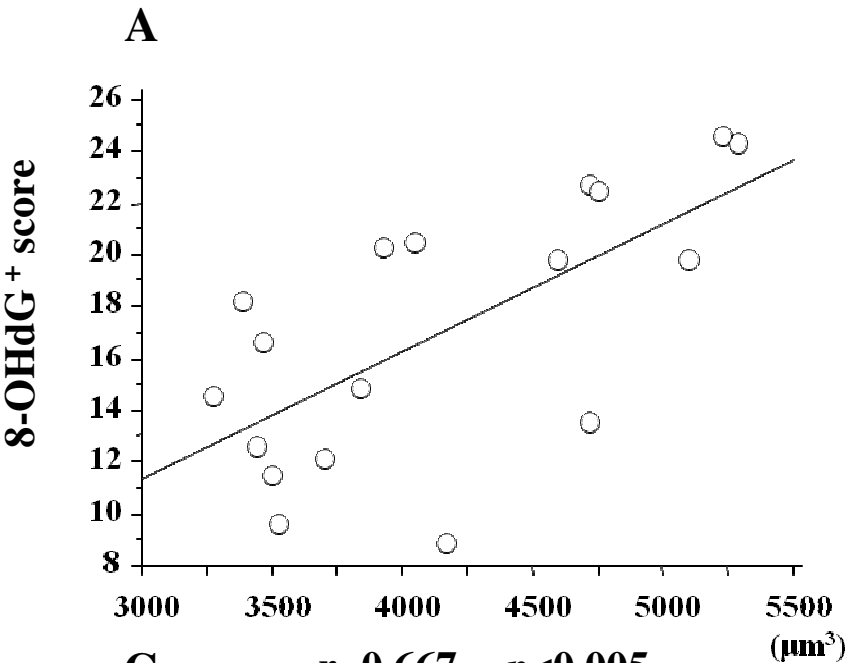


B Nitrotyrosine

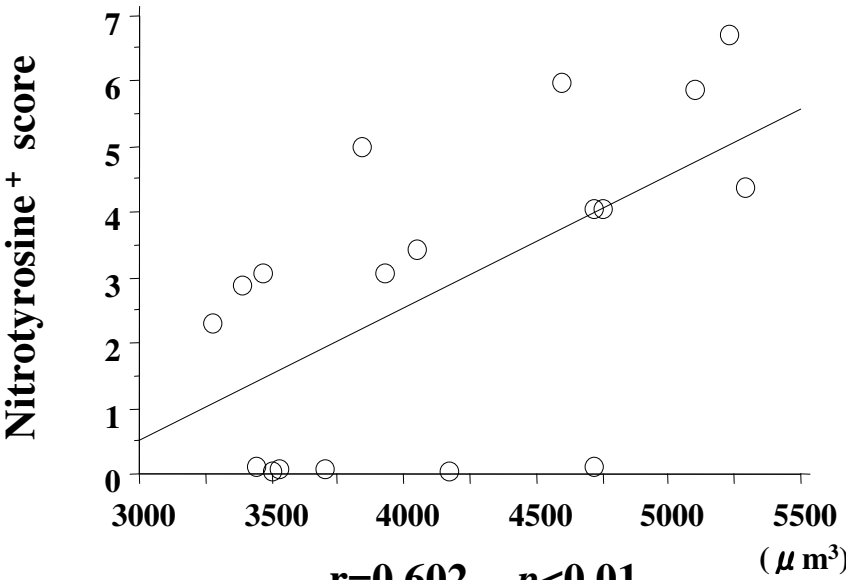


[†] ; control v.s DM, $p < 0.05$, * ; DM v.s DM + INS, $p < 0.05$, # ; control v.s DM + INS, $p < 0.05$

Glomerular volume

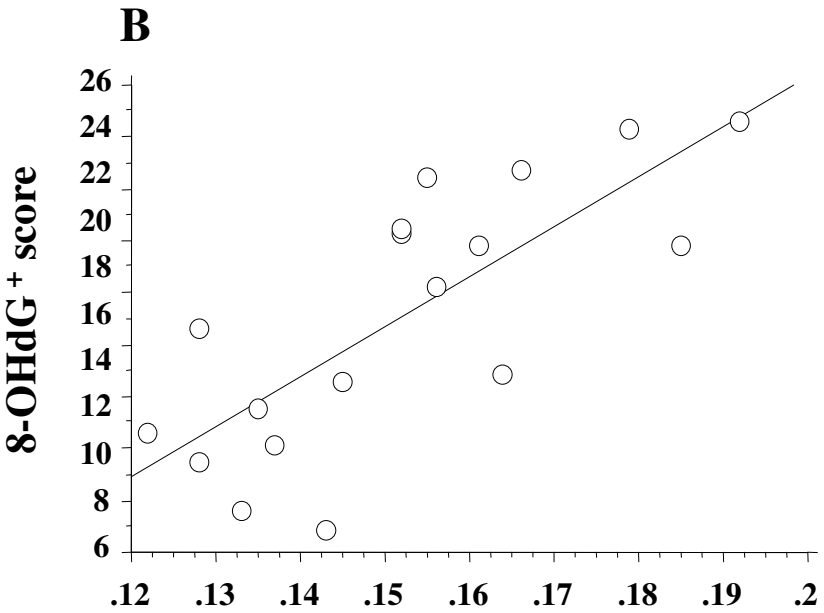


C $r=0.667, p<0.005$

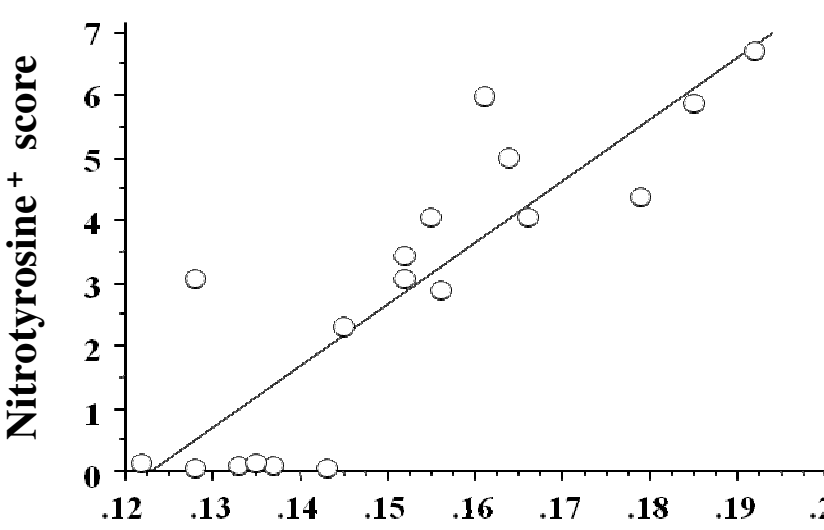


$r=0.602, p<0.01$

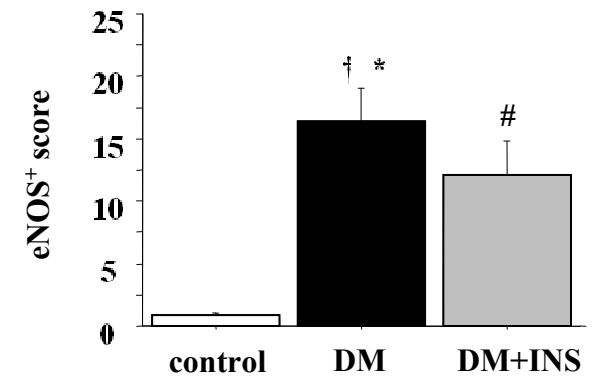
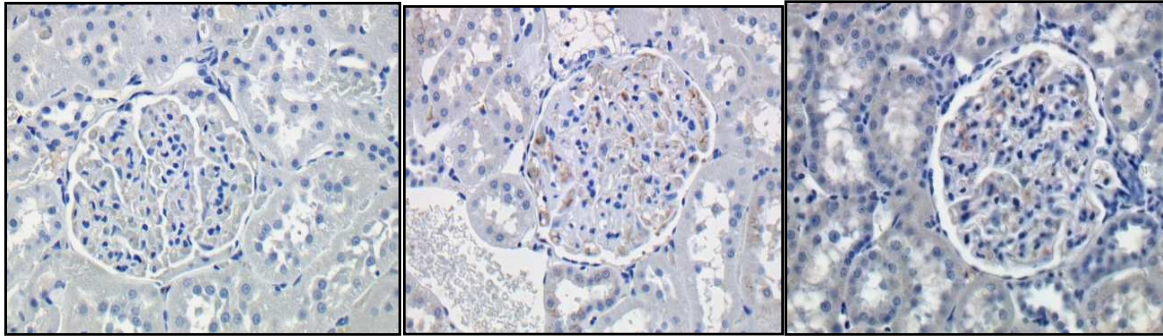
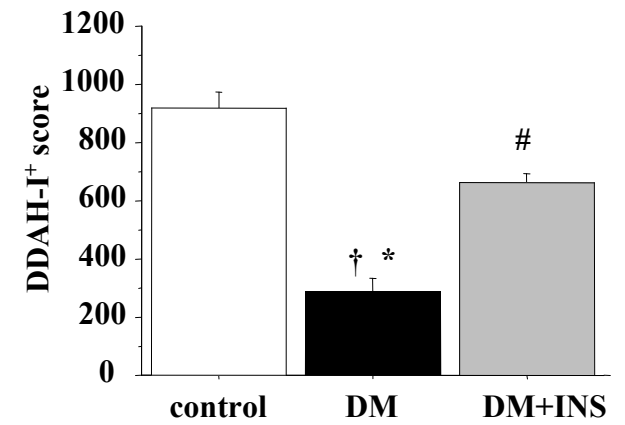
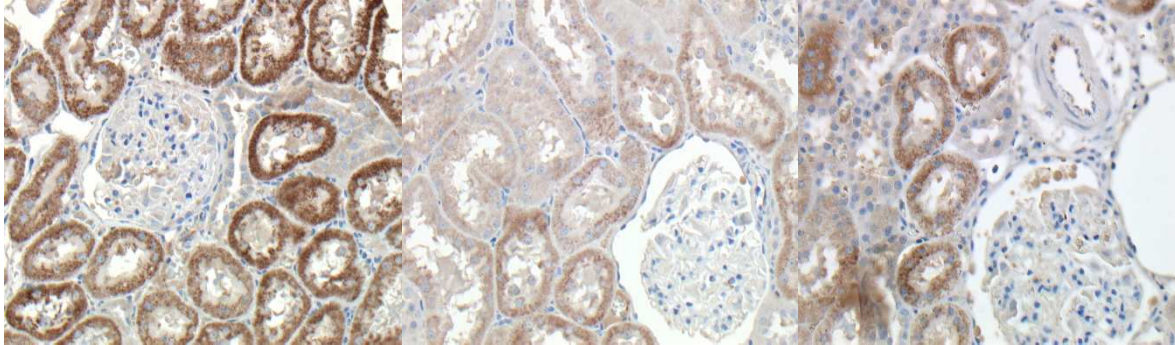
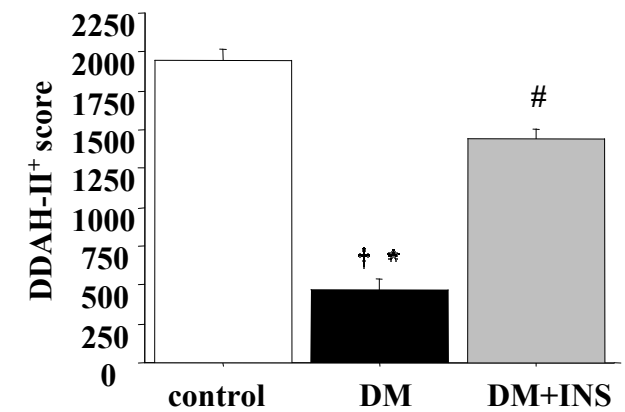
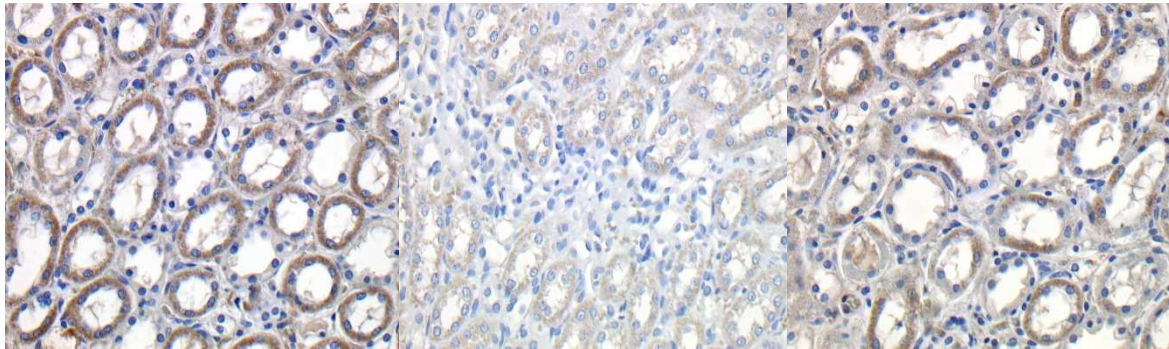
Mesangial matrix index



D $r=0.770, p<0.001$



$r=0.865, p<0.0001$

A eNOS**B DDAH-I****C DDAH-II**

†; control v.s DM, $p < 0.05$, *; DM v.s DM + INS, $p < 0.05$, #; control v.s DM + INS, $p < 0.05$

