# Effect of zinc deficiency on mouse renal interstitial fibrosis in diabetic nephropathy

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Abstract. There is emerging evidence that tubulointerstitial fibrosis is the final common pathway of the majority of chronic progressive renal diseases, including diabetic nephropathy (DN). Zinc, an essential dietary element, has been suggested to be important for a number of protein functions during fibrosis in vivo and in vitro. However, the effect of zinc deficiency (ZnD) on renal interstitial fibrosis in DN remains unclear. The present study investigated the effect and the underlying mechanisms of ZnD on renal interstitial fibrosis during DN using an streptozotocin-induced model of diabetes with immunofluorescence staining and western blot analysis. The present study identified that dietary zinc restriction significantly decreased zinc concentrations in the plasma and mouse kidney. ZnD enhanced albuminuria and extracellular matrix protein expression, associated with diabetic renal interstitial fibrosis by activation of renal interstitial fibroblasts and regulation of the expression of fibrosis-associated factors, which may be mediated by the activation of fibroblasts via the TGF-β/Smad signaling pathway. The data indicates that ZnD serves an important role in the pathogenic mechanisms of renal interstitial fibrosis during the development of DN.

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*Abbreviations:* FITC, fluorescein isothiocyanate; TGF-β1, transforming growth factor-β1; ECM, extracellular matrix; DN, diabetic nephropathy; ZnD, zinc deficiency

#### Introduction

It is estimated that >285 million people worldwide have diabetes mellitus, and that this figure will reach 439 million by 2030 (1). Diabetes mellitus is a leading cause of end-stage renal disease and chronic kidney failure that has become a worldwide problem (2-4). Growing evidence suggests that tubulointerstitial fibrosis is the final common pathway of almost all forms of chronic progressive renal disease, including diabetic nephropathy (DN) (5-7). Clinical evidence has confirmed that early tubular injury, including fibrosis, has been reported in patients with diabetes mellitus (8-10). Renal interstitial fibrosis involves expansion of interstitial fibroblasts, myofibroblast activation and extracellular matrix (ECM) accumulation, leading to the loss of normal kidney function and, ultimately, renal failure (11). The specific therapeutic options to inhibit the progression of DN are not available in the clinic. A major reason for this relentless progression is potentially associated with the incomplete understanding of the pathogenic mechanisms of DN, which is fundamental for the development of more effective preventive or therapeutic strategies.

Transforming growth factor (TGF)-β1, a strong profibrotic cytokine, has been consistently implicated in the pathogenesis of ECM accumulation in DN (12,13). Factors that are associated with the pathogenesis of DN can increase renal TGF-\beta1 expression in vivo in experimentally induced-diabetes, and in diabetic humans (14,15). A previous study reported that chronic treatment of db/db mice with a neutralizing anti-TGF- $\beta$ 1 antibody successfully prevented mesangial matrix expansion and renal insufficiency, indicating that the TGF-\u00b31 system is important in the development of DN (13). In addition, in cell culture experiments, cultured proximal tubular cells exposed to media containing increasing concentrations of D-glucose synthesize more TGF- $\beta$ 1 than control cells cultured in normal-glucose medium (16,17). Furthermore, in vivo study demonstrated that repeated administration of a neutralizing anti-TGF-\beta1 antibody ameliorates certain early changes observed in the kidneys of streptozotocin (STZ)-induced diabetic mice, including increased mRNA levels of collagen and fibronectin, and renal and glomerular hypertrophy (18). The Smad protein family is an important signaling pathway by which TGF-\beta1 activates

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the transcription of several well-established TGF- $\beta$ 1-induced genes with various functions. TGF- $\beta$ 1 receptor activation triggers phosphorylation of receptor-regulated Smad2 and 3, which bind to Smad4 and accumulate in the nucleus, where they activate transcription (19,20).

Zinc is an essential element. Intracellular zinc is associated with proteins, primarily via complex interactions with cysteines, acting as an integral component of numerous metalloenzymes, structural proteins and transcription factors (21,22). Zinc deficiency (ZnD) is associated with multiple disorders. In particular, hyperzincuria and low intestinal absorption of zinc in diabetic patients suggests that they are more susceptible to ZnD, which may result from hyperglycemia, impaired intestinal absorption or increased urinary zinc loss (23-25). In addition, low-dietary zinc intake and low levels of serum zinc are associated with a high prevalence of cardiovascular diseases, diabetes and glucose intolerance, and low zinc status may to contribute to diabetes-associated renal injury (26,27). Furthermore, a recent clinical study demonstrated that advancing DN, indicated by decreased glomerular filtration rate, and increasing microalbuminuria is associated with lower serum zinc levels (27). Indeed, numerous studies have indicated that zinc supplementation inhibits fibrosis, including myocardial, liver, perivascular and cystic fibrosis (28-30). A previous report demonstrated that zinc is involved in high glucose-induced epithelial-mesenchymal transition (EMT) in normal rat tubular epithelial cells by modulating TGF-B1 and reactive oxygen species production, and phosphoinositide 3-kinase and mitogen-activated protein kinase activation (31). However, the effect of zinc on DN, particularly in renal interstitial fibrosis, in vivo has not been previously studied.

As there are few reports that have investigated the effects of ZnD in renal interstitial fibrosis, it is necessary to understand the matrix synthesis and degradation, and the mechanisms by which ZnD influences the functional and pathological consequences of DN in mouse models of DN.

### Materials and methods

Animals.C57BL/6J mice (4 weeks old; ~13±2g) were purchased from the Experimental Animal Center, China Medical University (Shenyang, China). C57BL/6 J mice were administered with STZ (n=16; 150 mg/kg; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) diluted in 0.1 M citrate buffer (pH 4.5) or citrate buffer alone (n=8) by intraperitoneal injection, as described previously (32). Mice serving as vehicle controls (nondiabetic mice) were administered with the same volume of citrate buffer. Mice were weighed and their blood glucose levels were measured using an Accu-chek glucometer (Roche Diagnostics, Basel, Switzerland) and only diabetic animals with blood glucose >16 mmol/l were considered diabetic. When diabetic mice were diagnosed on day 3 following STZ treatment, the mice were then randomized into two groups, receiving treatment with zinc-deficient (n=8) or zinc-adequate (n=8) diets for 5 weeks prior to euthanasia at 12 weeks. The zinc-deficient mice received egg white-based diet (0.85 ppm; cat. no. AIN-76A; Research Diets Company, New Brunswick, NJ, USA) and were provided with deionized water; the zinc-adequate mice (30 ppm) and had ad libitum access to water. The zinc-adequate mice received a diet with normal zinc levels produced by the Experimental Animal Center of China Medical University. The mice were housed in cages in a controlled environment (22-25°C; 50% humidity; and 12-h light/dark cycle). All animal experimental procedures were approved by the Experimental Animal Ethical Committee of China Medical University, in accordance with the criteria described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. At the time of sacrifice, 24-h urines were collected in metabolic cages. Urine albumin levels were determined using the murine microalbuminuria ELISA kit (cat. no. 1011; Exocell, Inc., Philadelphia, PA, USA). The mice were terminally anesthetized with sodium pentobarbital (4%; Merck Millipore) and perfused with isotonic saline, followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The kidney was removed and postfixed with 4% paraformaldehyde overnight at 4°C for use in immunofluorescence. To obtain tissues for western blot analysis, the mice anaesthetized with 4% sodium pentobarbital and subsequently decapitated.

Zinc analysis. The zinc concentration in the plasma and kidney was measured by atomic absorption spectrophotometry (AAS) at the Experimental Center, China Medical University as described previously (33). Total zinc in the tissues, including free zinc and protein-bound zinc, were measured and expressed as  $\mu$ g/g of dry tissue. The results are expressed as mg/g of wet weight and as averages of at least two determinations per sample.

Immunofluorescence staining. Serial paraffin sections (5  $\mu$ m) of kidney tissues were prepared, dewaxed in xylene, and rehydrated using gradient alcohol solutions. Subsequently, the cryostat sections were pre-incubated with 5% normal donkey serum (1:20; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h and incubated overnight at room temperature with anti-lectin (1:100; polyclonal antibody; cat. no. HPA000646; Sigma-Aldrich; Merck Millipore), anti-collagen-I (1:100; cat. no. sc-59772; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-a-smooth muscle actin (α-SMA; 1:100; cat. no. sc-324317; Santa Cruz Biotechnology, Inc.). Secondary antibodies used included fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit (FITC-DAR) immunoglobulin G (IgG; cat. no. 705 07 003) and Texas Red-conjugated donkey anti-goat (Texas Red-DAG) IgG (cat. no. 711 542 152). They were purchased from Jackson ImmunoResearch Laboratories, Inc. Following rinsing with PBS, the sections were incubated for 2 h with DAR-FITC (1:50) and Texas Red-DAM (1:50) at room temperature. The sections were mounted and examined using a confocal laser scanning microscope (SP2; Leica Microsystems GmbH, Wetzlar, Germany).

Western blot analysis. Kidney tissues were homogenized in cold radioimmunoprecipitation assay lysis buffer, incubated on ice for 1 h, centrifuged at 12,000 x g for 20 min at 4°C and then the supernatants were transferred to a clean tube. Protein concentrations were quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total protein from each sample (50  $\mu$ g) was subjected to SDS-PAGE using 10% gradient Tris/glycine gels. Then, the proteins were transferred to polyvinylidene difluoride membranes (EMD



Millipore, Billerica, MA, USA). Following blocking with 5% fat-free milk for 1 h, the blots were incubated with the following primary antibodies at 4°C overnight: Type I collagen (1:800; cat. no. sc-59772; Santa Cruz Biotechnology, Inc.); rabbit polyclonal anti-vimentin (1:800; cat. no. sc-5565; Santa Cruz Biotechnology, Inc.); goat polyclonal anti- $\alpha$ -SMA (1:800; cat. no. sc-324317; Santa Cruz Biotechnology, Inc.); anti-TGF-β1 (1:1,000; cat. no. 3711; Cell Signaling Technology, Inc., Danvers, MA, USA); anti-Smad2 (1:1,000; Cell Signaling Technology, Inc.); anti-Smad3 (1:1,000; Cell Signaling Technology, Inc.); and mouse monoclonal anti-β-actin (1:2,000; sc-8432; Santa Cruz Biotechnology, Inc.). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, including rabbit anti-goat IgG (1:1,000; cat. no. sc-2922; Santa Cruz Biotechnology, Inc.) and rabbit anti-mouse IgG (1:1,000; cat. no. sc-358920; Santa Cruz Biotechnology, Inc.) for 2 h at 4°C. Subsequently, the membranes were visualized using an enhanced chemiluminescence kit (Santa Cruz Biotechnology, Inc.) using the ChemiDoc<sup>™</sup> XRS system with Quantity One software (version 4.6; Bio-Rad Laboratories, Inc.) and the G-BOX EF Chemi HR16 gel imaging system (Syngene, Frederick MD, USA). Following development, the band intensities were quantified using Image Pro Plus 6.0 analysis software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Results from at least three independent experiments were expressed as the mean  $\pm$  standard error. Statistical analysis of the data for multiple groups was performed by one-way analysis of variance and the Tukey's multiple comparison test was used. P<0.05 was considered to indicate a statistically significant difference.

#### Results

ZnD enhances renal injury in an STZ-induced diabetic model. The effect of ZnD on DN was determined using an *in vivo* model of DN. Urinary albumin excretion over 24 h was significantly increased in the diabetic mice (113.6+12.53 mg/24 h) compared with the non-diabetic control ( $27.63\pm6.31$  mg/24 h; P<0.01), and this increase was significantly enhanced by ZnD (156.2+16.27 mg/24 h) compared with the diabetic mice (P<0.01; Fig. 1).

*Effect of ZnD on the concentration of zinc ions.* In an *in vivo* experiment, AAS technology was used to examine the concentration of zinc ions in the mouse kidney and plasma. As reported previously (34), diabetic mice fed ZnD diet exhibited significantly decreased levels of zinc in the kidney and plasma compared with the control and diabetic mice (all P<0.01), indicating that the diet successfully induced ZnD (Fig. 2).

ZnD improves ECM gene expression and increases renal interstitial fibrosis in diabetic mice. Increased ECM protein expression is a key characteristic of renal fibrosis in DN (35,36). Double-immunofluorescence staining for lectin (a tubular marker) and type I collagen was performed to analyze the distribution and localization of type I collagen protein in the renal interstitium. Diabetes increased the expression of type I collagen localized to the interstitial areas of diabetic kidneys compared with control mice, and ZnD enhanced this



Figure 1. ZnD enhances renal injury in an STZ-induced diabetic model. Kidney function was assessed by measuring the 24-h urinary albumin excretion. The 24-h urinary albumin excretion was significantly increased in DM mice treated with ZnD. Results are presented as the mean ± standard error. \*\*P<0.01 vs. control group. ##P<0.01 vs. DM. n=8. DM, diabetes mellitus; ZnD, zinc definciency.



Figure 2. Effects of ZnD on the concentration of zinc ions. Atomic absorption spectroscopy technology was used to examine the zinc ion concentration in the kidney and plasma. The zinc ion concentrations in kidney and plasma were significantly decreased in the DM + ZnD group compared with the control group or DM group. All the experiments were repeated three times, and the results are depicted as the mean  $\pm$  standard error. \*\*P<0.01 vs. control, \*\*P<0.01 vs. DM. DM, diabetes mellitus; ZnD, zinc definciency.

response (Fig. 3A). Furthermore, western blot analysis also demonstrated that the expression of type I collagen (Fig. 3B) and fibronectin (Fig. 3C) in ZnD-diabetic kidneys was significantly increased compared with control and diabetic mice. These observations were consistent with the results of



Figure 3. ZnD improves matrix gene expression and increase renal interstitial fibrosis of diabetic mice. (A) Immunofluorescence staining reveals the localization of type I collagen (red) and the tubular cell marker lectin (green) in the control, DM and DM + ZnD kidneys. Original magnification, x400. Representative western blots of protein expression of (B) type I collagen and (C) fibronectin in the kidneys (n=8). Each value represents the mean  $\pm$  standard error. All experiments were performed three times with similar results.  $\beta$ -actin was used as loading control. \*P<0.05, \*\*P<0.01 vs. control group; ##P<0.01 vs. DM group. DM, diabetes mellitus; ZnD, zinc definciency.

immunofluorescence staining. It was clear that ZnD increases interstitial matrix production and enhances renal fibrosis in the model of DN *in vivo*.

ZnD enhances the activation of renal fibroblasts in the kidneys of diabetic mice. Subsequently, the effects of ZnD on the activation of renal fibroblasts were examined.

Double-immunofluorescence staining for lectin and  $\alpha$ -SMA demonstrated that  $\alpha$ -SMA expression was in the interstitial areas of diabetic kidneys compared with non-diabetic controls, and that ZnD enhanced  $\alpha$ -SMA expression in the kidneys of diabetic mice compared with the diabetic controls (Fig. 4A). Consistently, western blot analyses of kidney tissues demonstrated that the expression levels of  $\alpha$ -SMA and vimentin



Figure 4. ZnD enhances the activation of renal fibroblasts in kidneys of diabetic mice. (A) Representative images of  $\alpha$ -SMA immunofluorescence staining in the renal cortex from control, DM and DM + ZnD mice. Original magnification, x400. (B) Representative western blots and quantification of the protein expression of  $\alpha$ -SMA and vimentin in the kidneys (n=8). Each value represents the mean  $\pm$  standard error. All experiments were performed three times with similar results.  $\beta$ -actin was used as a loading control. \*P<0.05, \*\*P<0.01 vs. control group; \*P<0.05, \*\*P<0.01 vs. DM group.  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; DM, diabetes mellitus; ZnD, zinc definciency.

were significantly increased in the kidneys of diabetic mice compared with non-diabetic controls (P<0.05 and P<0.01, respectively), and the levels were significantly enhanced by the administration of ZnD compared with the levels in diabetic mice (P<0.01 and P<0.05, respectively; Fig. 4B). Collectively, these data suggest that ZnD is mechanistically involved in the activation of fibroblasts in DN *in vivo*.

ZnD enhances the activation of TGF- $\beta$ 1 and Smad2/3 expression in the kidneys of diabetic mice. TGF- $\beta$ 1, and the TGF- $\beta$ /Smad signaling pathway, has been previously reported to serve a critical role in kidney fibrosis (19,37). Thus, the effect of ZnD on the expression of the TGF- $\beta$ /Smad signaling pathway was examined. As indicated in Fig. 5, the protein expression level of TGF- $\beta$ 1 and phosphorylation of Smad2/3 were significantly increased in diabetic mice compared with the control group (all P<0.01). Additionally, TGF- $\beta$ 1 expression and Smad2/3 phosphorylation were significantly increased in ZnD-diabetic mice compared with diabetic mice (P<0.05, P<0.01 and P<0.01, respectively; Fig. 5). Considering the above findings, these data clearly indicate that ZnD is involved in mediating the expression or activation of several important proteins known to be relevant in the TGF- $\beta$ /Smad signaling pathway that in stimulated in interstitial fibroblasts to regulate ECM accumulation *in vivo*.

## Discussion

Several studies in animal models and certain clinical studies have demonstrated that zinc deficiency may be associated with fibrosis in chronic inflammatory diseases, including in liver, myocardial and cystic fibrosis (38-41). Although renal interstitial fibrosis was previously demonstrated in rat and mouse models of progressive DN, and in kidneys from patients with long-standing type 1 diabetes, the majority of



Figure 5. ZnD enhances the activation of TGF- $\beta$ 1 and Smad2/3 expression in kidneys of diabetic mice. Representative western blots demonstrating the protein expression of TGF- $\beta$ 1 and phosphorylation of Smad2/3 in the kidneys are presented (n=8). Each value represents the mean ± standard error. All experiments were performed three times with virtually identical results.  $\beta$ -actin was used as a loading control, \*\*P<0.01 vs. control group; \*P<0.05, \*\*P<0.01 vs. DM group. TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; p-, phosphorylated; DM, diabetes mellitus; ZnD, zinc deficiency.

DN research has generally focused on the glomerular lesions in this condition (42). However, it is increasingly appreciated that tubular injury has an important role in the progression of DN resulting in several of the molecular and cellular changes. Thus, the present study was undertaken to define the effect and underlying mechanism of ZnD on renal interstitial fibrosis in DN. The results in the present study demonstrated that ZnD enhances diabetic renal interstitial fibrosis, as indicated by an increase in levels of type I collagen, fibronectin,  $\alpha$ -SMA and vimentin, which is may occur via the TGF- $\beta$ /Smad2/3 pathway. To the best of our knowledge, these results are the first to demonstrate the effect of ZnD on the pathogenic mechanisms of renal interstitial fibrosis during the development of DN.

Clinical studies have previously demonstrated that patients with diabetes with low serum zinc levels have a high risk of developing kidney injury compared with those with normal serum zinc levels, and zinc supplementation may potentially be used clinically to prevent diabetes-induced complications in multiple organs (43,44). A previous study confirmed that the protective effects of zinc supplementation on renal pathological changes, fibrosis and oxidative damage were more significant compared with the effects on 24 h urinary protein increase, partially because urinary protein level is a reflection of both tubular and glomerular function (43).

Increased ECM protein synthesis and/or decreased ECM degradation ultimately contributes to the development of diabetes-associated tubulointerstitial fibrosis (19,45). The

activation of interstitial fibroblasts to become  $\alpha$ -SMA-positive myofibroblasts is recognized as a crucial step in the development of chronic kidney disease, including DN. In addition to activated resident interstitial fibroblasts, myofibroblasts may also be derived from tubular epithelial cells via EMT, which is considered to be primarily responsible for interstitial matrix accumulation and deposition (33,46). The present study observed that STZ induction of diabetes increased the number of α-SMA-positive renal fibroblasts, and ZnD significantly increased the activation of kidney fibroblasts, parallel with a substantial increase in ECM synthesis, which is in accordance with a previous report (47). Collectively, previous studies and the current study have demonstrated that ZnD induced an increase in renal tubular collagen synthesis and may be also responsible for collagen upregulation and renal tubular injury during diabetes in vivo.

TGF- $\beta$ 1, a strong profibrotic cytokine, and the TGF- $\beta$ /Smad pathway has been consistently implicated to be critical during the pathogenic ECM accumulation in DN (3,5,19). A previous study has demonstrated that TGF- $\beta$ 1 upregulation was induced by high glucose in the p38 mitogen-activated protein kinase signaling pathway (48). In addition, there is evidence that high glucose-induced TGF- $\beta$ 1 production is involved in the extracellular matrix metabolism in DN (12,49). Furthermore, a previous study demonstrated that a ZnD lead to TGF- $\beta$ 1 induction during neurogenesis, impairs neuronal precursor cell proliferation and induces apoptosis via regulating p53-dependent molecular mechanisms (50). Another study indicated that TGF- $\beta$ 1 has



stimulatory and inhibitory effects on osteoclast-like cell formation in mouse marrow cultures, and that zinc can inhibit the stimulatory effect of TGF- $\beta$ 1 (51). A recent study reported that zinc can inhibit human lens epithelial cell migration and proliferation by decreasing the expression of TGF- $\beta$ 1 and increasing the expression of tumor necrosis factor- $\alpha$ , and subsequently induce apoptosis/necrosis (52). In the present study, expression of TGF- $\beta$ 1 and phosphorylation of Smad2/3 were increased in diabetic mice, and further increased by ZnD, suggesting that ZnD can aggravate renal tubular interstitial fibrosis in DN, and may affect key fibrotic factors and signaling pathways.

In conclusion, the present study provides novel evidence regarding the association between ZnD and renal interstitial fibrosis in STZ-induced diabetic mice. ZnD enhanced albuminuria and ECM protein expression associated with diabetic renal interstitial fibrosis through activation of renal interstitial fibroblasts and regulation of the expression of fibrosis-associated factors expressed by fibroblasts, which may occur via the TGF- $\beta$ /Smad2/3 pathway. These findings suggest that suboptimal zinc status induces renal tubulointerstitial injury associated with the development of DN. These findings indicate that suboptimal zinc status induces renal tubulointerstitial injury associated with the development of DN and suggest that zinc supplementation may benefit in the medication and prevention of the disease.

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