Zinc supplementation alleviates the progression of diabetic nephropathy by inhibiting the overexpression of oxidative-stress-mediated molecular markers in streptozotocin-induced experimental rats

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Abstract

Zinc deficiency during diabetes projects a role for zinc nutrition in the management of diabetic nephropathy. The current study explored whether zinc supplementation protects against diabetic nephropathy through modulation of kidney oxidative stress and stress-induced expression related to the inflammatory process in streptozotocin-induced diabetic rats. Groups of hyperglycemic rats were exposed to dietary interventions for 6 weeks with zinc supplementation (5 times and 10 times the normal level). Supplemental-zinc-fed diabetic groups showed a significant reversal of increased kidney weight and creatinine clearance. There was a significant reduction in hyperlipidemic condition along with improved PUFA:SFA ratio in the renal tissue. Expression of the lipid oxidative marker and expression of inflammatory markers, cytokines, fibrosis factors and apoptotic regulatory proteins observed in diabetic kidney were beneficially modulated by zinc supplementation, the ameliorative effect being concomitant with elevated antiapoptosis. There was a significant reduction in advanced glycation, expression of the receptor of the glycated products and oxidative stress markers. Zinc supplementation countered the higher activity and expression of polyol pathway enzymes in the kidney. Overexpression of the glucose transporters, as an adaptation to the increased need for glucose transport in diabetic condition, was minimized by zinc treatment. The pathological abnormalities in the renal architecture of diabetic animals were corrected by zinc intervention. Thus, dietary zinc supplementation has a significant beneficial effect in the control of diabetic nephropathy. This was exerted through a protective influence on oxidative-stress-induced cytokines, inflammatory proliferation and consequent renal injury.

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Keywords: Diabetic nephropathy; Zinc supplementation; Oxidative stress; Polyol pathway; Inflammatory cytokines; Glucose transporters; Fibrosis

1. Introduction

In recent years, metabolic abnormalities and tissue damages in diabetes have been widely attributed to interplay between oxidative stress and induced inflammatory processes. There is considerable experimental evidence that the proinflammatory and adhesion molecules are important in the development of diabetic complications [1]. Oxidative stress and inflammatory mediators activate a series of receptors, leading to β-cell dysfunction and apoptosis, impairment of insulin signaling, systemic endothelial dysfunction and altered vascular flow, which constitute final common pathways to the vascular complications of diabetes [2,3]. Several studies have shown that inflammation and oxidative stress directly damage kidney in streptozotocin (STZ)-induced diabetic animals leading to diabetic nephropathy (DN). The manifestations of this inflammatory state comprise overproduction of nuclear factor-κB (NF-κB)-dependent inflammatory mediators such as tumor necrosis factor-α (TNF-α), cyclooxygenase (COX-2) and transforming growth factor-β (TGF-β) in the kidneys of diabetic rats [4]. Thus, inhibition of these oxidative stress and inflammatory response inducer NF-κB may be useful in the management of patients with DN.

Distinctly, several mechanisms for the pathogenesis of DN have been proposed [5], which include enhanced oxidative stress and induced inflammatory pathway [6], increased production of advanced glycation end products (AGEs) [7], hyperfiltration [8] and enhanced polyol pathway [9]. Moreover, disturbing the balance between antioxidative defense system and overproduced proinflammatory cytokines not only aggravates systemic inflammatory stress in diabetic entities but also endorses the progression of diabetes-associated renal injury. Therefore, potentiating systemic defense via boosting antioxidant or antiglycation capacity, enhancing anti-inflammatory cytokines production, regulating convoluted polyol or inflammatory pathways and annihilation of transforming growth factors can be the mainstay of prevention of renal hypertrophy induced by diabetes.

Food-based compounds can be profoundly relied upon to treat diabetes-induced disorders given the lack of adversative effects. Zinc as a trace element is an imperative cofactor for many proteins attendant with cell proliferation, differentiation and apoptosis [10], as well as gene expression [11]. There is an intricate relationship between zinc and diabetes because zinc is a module of insulin crystals [12]. Zinc plays a significant role against the immune-mediated free
radical consequences in the β-cells of the pancreas, although the intricate mechanism of zinc is yet to be elucidated. Zinc possesses an antioxidant role as it protects sulfhydryl groups from oxidation subsequently inhibiting the production of reactive oxygen species and also has anti-inflammatory potentiality as it acts as a negative feedback to the inflammatory pathway [13]. Zinc moreover stimulates the metallothionein protein which is a potent antioxidant, and exalted expression of metallothionein may be reasonably protective in diabetic rodents [14,15]. Diabetes endorses zinc dyshomeostasis; conversely, zinc deficiency also was found to increase the risk of diabetes and its complications [16]. We have recently reported that exogenous zinc supplementation could significantly ameliorate hypoinsulinemia, hyperglycemia with its attendant metabolic abnormalities [17] and oxidative stress in STZ-induced diabetic rats [18]. Hence, with the virtue of zinc supplementation in the previous observation [17,18], it could be relevant to evaluate the beneficial influence of supplemental zinc on the progression of diabetic nephropathy. While there are several studies reporting the beneficial effect of zinc supplementation on diabetic kidney [19–21], an exhaustive and focused investigation on this subject is merited. The present study was thus undertaken to systematically examine the effects of zinc supplementation on diabetes-induced morphological and biochemical alterations in the kidney. Particular focus was also made on the oxidative stress markers and the modulatory influence of supplemental zinc on the expression of inflammatory cytokines and apoptotic regulatory proteins in the kidney of diabetic animals. Two supplemental doses of zinc salt corresponding to 5 times and 10 times the normal requirement are particularly explored here to compensate for the loss of body zinc in diabetic condition induced by streptozotocin in rats.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin, 1-butyl hydroperoxide (TBHP), cholesterol, 1-chloro-2,4-dinitrobenzene (CDNB), cytochrome-C, o-dianisidine, dipalmitoyl phosphatidylcholine, 2,4-dinitrophenylhydrazine, 5,5′-dithiothreitol (2-nitrobenzoic acid) (DTMB), ethylene diamine tetraacetic acid (disodium salt; EDTA), glucose oxidase, glutathione reductase, glutathione reduced (GSH), glutathione oxidized (GS(G), hepatoxenise peroxidase (HPR), nicotinamide adenine dinucleotide phosphate reduced (NADPH), streptozotocin (STZ), thiobarbituric acid, triolein, xanthin, xanthine oxidase and zinc carbonate were procured from Sigma-Aldrich (St. Louis, MO, USA). TSQ (N-(6-methoxy-2-methyl-4H-one) was obtained from Nimesh Corporation (Mumbai, India). Maize starch, cane sugar powder and refined groundnut oil were purchased from the local market.

2.2. Experimental animals

All the experimental procedures in this animal study were approved by the Institutional Animal Ethics Committee (CSIR-CFTRI, Mysore), and all precautions were taken to reduce pain and discomfort to the animals. Adult 12-week-old female Wistar rats (140–150 g) were procured from the Experimental Animal Production Facility of this Institute. Rats were individually housed in stainless steel cages at controlled temperature (25°C±2°C) and humidity (40%–60%) with 12-h/12-h light–dark cycles. Rats were fed with ad libitum diet and water. Diabetes was persuaded by a single intraperitoneal injection of STZ (40 mg kg−1 body weight in freshly prepared solution in 0.1 M (pH 4.5) citrate buffer). The animals were given 5% glucose water for initial 48 h to prevent STZ-induced hypoglycemia and mortality. 1 week of STZ administration fasting blood glucose levels were determined by glucose oxidase method [22] in blood samples drawn from retro-orbital plexus. Rats having fasting blood glucose levels >15 mmol L−1 were considered as diabetic rats.

2.3. Diets and animal treatment

Normal basal diet (AIN-76) was modified with the addition of zinc carbonate to make experimental diets. Zinc supplementation was such that it conformed to 5 times (0.32 g kg−1 diet; Zn-dose 1) and 10 times (0.64 g kg−1 diet; Zn-dose 2) of normal level. Rats were divided into six groups out of which three groups were diabetic (12 rats in each group) and the remaining three groups were normal (8 rats in each group). These groups of rats were maintained ad libitum on basal control diet and two experimental diets for 6 weeks (Supplementary Fig. 1). Thus, the six animal groups were (1) normal control (N), (2) normal+Zn-dose 1 (N+Zn-1), (3) normal+Zn-dose 2 (N+Zn-2), (4) diabetic control (D), (5) diabetic+Zn-dose 1 (D+Zn-1) and (6) diabetic+Zn-dose 2 (D+Zn-2).

During the study period, body weight and blood sugar were observed at regular intervals. The 24-h urine samples were collected at the end of the study period for the determination of urinary metabolites. The urine samples were filtered; the volume was noted and stored frozen (−80°C) until further analysis. At the end of the experimental period of 6 weeks, the rats were refrained overnight and sacrificed under euthanasia by exsanguination from the heart. Blood was collected in heparinized tubes by puncturing the heart, and the plasma was aliquoted by centrifugation and preserved at −80°C until further analysis. The kidney was quickly excised, weighed and processed for various extractions and stored at −80°C until analysis. Ice-cold 50 mM phosphate buffer (pH 7.4) was used to prepare a 10% (w/v) tissue homogenate followed by centrifugation to isolate cytosolic supernatant for the determination of all biochemical parameters. Lysis buffer was used for protein analysis (Western blot). A portion of the kidney was stored in RNAlater for reverse-transcription polymerase chain reaction (RT-PCR) and 4% paraformaldehyde for histopathology and immunohistochemistry study.

2.4. Creatinine clearance

Creatinine clearance was calculated using the formula:

\[
\text{Creatinine clearance (mL/min) } = \frac{\text{Urinary creatinine (mg/dL)} \times \text{urine volume (mL)}}{\text{body weight (g)} \times 1000}\]

2.5. Oxidative stress markers

ROS formation in kidney homogenate was quantified from a standard DCF-standard curve as described by LeBel et al. [23], and data are expressed as pmol DCF formed min−1 mg protein−1. Lipid peroxides in the renal tissue were assessed as malondialdehyde (MDA) concentration spectrophotometrically using thiobarbituric acid reactive substances (TBARS) (Shimadzu UV-1800; Shimadzu Corporation, Kyoto, Japan) employing the method of Okawa et al. [24]. Protein carbonyl levels were quantified as described by Reznick and Packer [25], and results are expressed in nmol carbonyls/mg protein (×22,000/M/cm).

2.6. Activities of antioxidant enzymes and concentration of antioxidant molecules

A 10% (w/v) kidney homogenate was assayed for the activities of various antioxidant enzymes. Superoxide dismutase (SOD) activity was evaluated by quantitating the inhibition of cytochrome-C reduction in the xanthine–xanthine oxidase system according to Flohe and Ottig [26]. Catalase (CAT) activity was assayed following the rate of decomposition of hydrogen peroxide according to the method of Aebi [27]. Glutathione peroxidase (Gpx) was evaluated using NADPH oxidation as described by Flohe and Gunzler [28]. Glutathione reductase (GR) activity was measured by the method of Carberg and Mannervik following the oxidation of NADPH by glutathione [29]. Glutathione-S-transferase (GST) activity was analyzed by determining the chlorodinitrobenzene–glutathione conjugate formed using 1-chloro-2,4-dinitrobenzene as the substrate as described by Warholm et al. [30]. Glutathione was estimated using Ellman’s reagent by the protocol of Beutler et al. [31]. Ascorbic acid was assessed by spectrophotometrically measuring the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid according to Omaye et al. [32].

2.7. Activities of polyol pathway enzymes and concentration of molecules

Aldehyde reductase (AR) activity was measured spectrophotometrically following the method of Ahn and Oh [33]. Sorbitol dehydrogenase (SDH) activity was measured using fructose as substrate according to the method of Gerlach and Hilly [34]. The polyol pathway metabolites sorbitol [35], fructose [36] and glucose [22] were estimated by employing standard procedures.

2.8. Activities of Na+,K+-ATPase

The activity of Na+,K+-ATPase in the renal tissue was assayed according to Kaplay [37] after isolating the microsomal fraction and measuring the released inorganic phosphate with Tris-ATP as substrate in both the presence and absence of ouabain. Activity of Ca2+–ATPase was independently measured by the method of Ramanadham and Kaplay [38].

2.9. Lipid profile and fatty acid composition

Renal lipid was extracted by the procedure of Folch et al. [39]. Cholesterol was quantitated by the method of Searcy and Bergquist [40]. Phospholipid was estimated by the method of Stewart [41] and triglycerides according to Fletcher [42]. Fatty acids of kidney lipid extracts were methylated [43], and the fatty methyl esters were analyzed by gas chromatography (Perkin-Elmer) using Carbowax column GC-C580. The column temperature was maintained to increase from 170 to 240 at 6°C/min. Individual fatty
2.1. Advanced glycation end products (AGEs)-related and tropphoblast fluorescence
deposition of collagen (FITC) and 365 nm (TSQ) to observe the images. The
microscopic (Carl Zeiss, LSM 700, Jena, Germany; ZEN 2009 software) study,
spectral increment 1 a.u., through 30.5-
2.11. Western blotting analysis
Samples were homogenized using ice-cold lysis buffer (pH 7.5). Each lysate (50 μg
protein) was resolved on 10%-12% sodium dodecyl sulfate polyacrylamide gel
electrophoresis (Native-PAGE for Type I collagen), and the separated proteins
were transferred onto PVDF membranes and immunoblotted with the specified polyclonal
antibody. The protein bands were blocked with 5% skim milk solution for 2 h
and incubated with primary antibodies for RAGE (Cat. #R5278, Sigma Aldrich, St. Louis, MO,
USA), GLUT1 (Cat. #ab14683), GLUT2 (Cat. #ab54460), SDH (Cat. #ab185705), 4-
hydroxynonenal (4-HNE) (Cat. #ab46545), ACR181 (Cat. #ab153897), Bax (Cat.
#ab32503), Bcl-2 (Cat. #ab82120), β-actin (Cat. #ab25894; Abcam, Cambridge, MA,
USA) and Type I collagen (Cat. #AB4500362) (Sigma Aldrich, St. Louis, MO, USA) as per
instructions given by the manufacturer, followed by an appropriate HER-CONJUGATED
secondary antibody. Western blot signal was developed according to standard
procedures using ECL chemiluminescence reagents (Sigma Aldrich, St. Louis, MO,
USA). Band intensity was quantified (GeneTools image analysis software) considering
the ratio to β-actin.

2.12. Western blotting analysis
Oxidative stress markers in kidney

Diabetic renal membranes were manifested by a significant decrease in the
activities of ouabain-sensitive Na+ -K+ -ATPase as compared with normal control animals (Table 2). The decreases in
these ATPase activities were 33% and 42%, respectively. Intervention with zinc supplement to diabetic animals produced a pronounced
reversal in this amendment in renal ATPase activities. Diabetic rats maintained on the zinc supplement had elevated ouabain-sensitive
Na+ -K+ -ATPase and Ca2+ -ATPase activities (by 25% and 26% in the
D+Zn-1 group; by 31% and 36% in the D+Zn-2 group).

3.4. Influence of dietary zinc supplementation on oxidative stress markers in kidney

Fig. 2 depicts increased oxidative stress in renal tissue in diabetic condition as reflected in the titres of reactive oxygen species, lipid
peroxides and protein carbonyl content and the influence of dietary zinc supplementation on the same. ROS, lipid peroxides and protein
carbonyl levels in the diabetic kidney were significantly (P<.05) higher
(by 50%, 58% and 89%, respectively) compared to those of normal control animals. Dietary zinc supplementation produced noticeable
decrease (P<.05) in ROS, lipid peroxides and protein carbonyl content
(by 22%, 16% and 38% in D+Zn-1; by 15%, 9% and 45% in D+Zn-2).
3.5. Beneficial influence of dietary zinc supplementation on kidney antioxidant status

Activities of most of the antioxidant enzymes (SOD, GPx, GR and GST) were significantly increased in diabetic animals ($P < 0.05$) concomitant with a diminished level of antioxidant molecules (ascorbic acid and GSH) compared to normal control animals (Table 3). Increased activity of these antioxidant enzymes may be an adaptive mechanism. Elevated activity was restricted in diabetic rats treated with zinc supplementation with increased level of antioxidant molecules. The activity of SOD was increased by 66% ($P < 0.05$) as a result of diabetes compared to normal control, whereas the same was significantly ($P < 0.05$) diminished by zinc supplementation (by 30% and 37%, respectively). The activities of GPx, GR and GST in kidney also were elevated by 2- and 3.4-fold in diabetic group compared to normal control, while dietary zinc supplementation partially restored the same (26%, 17% and 50% decreases, respectively, in D+Zn-1; 22%, 11% and 49% decreases, respectively, in D+Zn-2). Among the antioxidant enzymes, only the activity of CAT was reduced by 58% in diabetic condition, while zinc supplementation restored the enzyme activity by 86% and 38%, respectively, compared to diabetic control. The concentration of antioxidant molecules ascorbic acid and GSH decreased by

Table 1
Influence of zinc supplementation on polyol pathway enzymes and metabolites in the kidney of diabetic rats

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Kidney</th>
<th>AR (1)</th>
<th>SDH (1)</th>
<th>Glucose (2)</th>
<th>Sorbitol (3)</th>
<th>Fructose (3)</th>
<th>Urinary sorbitol (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (N)</td>
<td>14.3±0.5 246.2±6.8 0.56±0.04 1.03±0.09 1.84±0.11</td>
<td>1.45±0.27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N+Zn-1</td>
<td>14.2±0.2 231.7±5.1 0.61±0.14 1.32±0.03 2.30±0.48</td>
<td>1.23±0.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N+Zn-2</td>
<td>14.0±0.7 230.8±8.9 0.50±0.02 1.46±0.07 2.56±0.36</td>
<td>1.14±0.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic control (D)</td>
<td>24.9±1.0a 397.9±9.8a 4.18±0.17a 4.70±0.89a 8.49±0.83a</td>
<td>48.07±3.35a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D+Zn-1</td>
<td>16.3±0.8b 277.1±8.4b 3.11±0.12b 2.50±0.11b 4.47±0.69b</td>
<td>28.80±4.14b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D+Zn-2</td>
<td>14.9±0.7b 279.1±7.2b 3.43±0.03b 2.72±0.22b 4.89±0.63b</td>
<td>25.54±4.47b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. of eight animals in each group.
Units: 1: activity/h/100 mg protein; 2: mg/g tissue; 3: μmol/g tissue; 4: μmol excreted/24 h.

Table 2
Influence of zinc supplementation on the activities of kidney Na⁺,K⁺-ATPase and Ca²⁺-ATPase in diabetic rats

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Na⁺,K⁺-ATPase</th>
<th>Ca²⁺-ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity</td>
<td>Ouabain-sensitive</td>
</tr>
<tr>
<td>N</td>
<td>0.466±0.04</td>
<td>0.432±0.04</td>
</tr>
<tr>
<td>N+Zn-1</td>
<td>0.477±0.03</td>
<td>0.435±0.02</td>
</tr>
<tr>
<td>N+Zn-2</td>
<td>0.472±0.02</td>
<td>0.439±0.04</td>
</tr>
<tr>
<td>D</td>
<td>0.320±0.02a</td>
<td>0.290±0.02a</td>
</tr>
<tr>
<td>D+Zn-1</td>
<td>0.394±0.03b</td>
<td>0.362±0.02b</td>
</tr>
<tr>
<td>D+Zn-2</td>
<td>0.411±0.03b</td>
<td>0.379±0.02b</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. of eight animals in each group. Enzyme activities were expressed in terms of μmol/h/mg protein.

a Significantly different from N group ($P < 0.05$).
b Significantly different from D group ($P < 0.05$).
44% and 56%, respectively, \((P<.05)\) in diabetic rats compared to the normal group. This was nullified by dietary interventions with D+Zn-1 (76% and 2-fold) and with D+Zn-2 (40% and 57%, respectively). Zinc supplementation did not alter significantly in the kidney antioxidant defense system of normal rats.

3.6. Beneficial influence of dietary zinc supplementation on AGEs and tryptophan fluorescence

Kidney AGEs-related protein fluorescence and tryptophan spectra of different groups are presented in Fig. 3A/B and Fig. 3C/D. The data

Table 3
Influence of zinc supplementation on kidney antioxidant status in diabetic rats

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Antioxidant enzymes</th>
<th>Antioxidant molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOD (unit/min/mg protein)</td>
<td>CAT (mmol/min/mg protein)</td>
</tr>
<tr>
<td>N</td>
<td>25.2±0.9</td>
<td>107.6±5.4</td>
</tr>
<tr>
<td>N+Zn-1</td>
<td>24.4±1.7</td>
<td>110.9±8.7</td>
</tr>
<tr>
<td>N+Zn-2</td>
<td>26.8±0.8</td>
<td>118.8±5.1</td>
</tr>
<tr>
<td>D</td>
<td>41.8±1.6a</td>
<td>168.0±8.2a</td>
</tr>
<tr>
<td>D+Zn-1</td>
<td>28.3±0.8b</td>
<td>125.6±7.7b</td>
</tr>
<tr>
<td>D+Zn-2</td>
<td>26.5±1.2b</td>
<td>132.1±5.1b</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. of eight animals in each group.

a Significantly different from N group \((P<.05)\).

b Significantly different from D group \((P<.05)\).
specify that diabetic rats have elevated AGEs levels (by 98%) and lower tryptophan level (by 36%) as compared with normal control rats ($P < 0.05$). With Zn supplementation to diabetic animals, AGEs level was normalized by 41% (D+Zn-1) and 37% (D+Zn-2) as compared to diabetic control rats, and tryptophan level was increased by 27% and 33%, respectively.

### 3.7. Beneficial impact of dietary zinc supplementation on renal lipid profile and fatty acid composition

Zinc supplementation had a beneficial influence on the increased lipid concentration in the kidney tissue under diabetic condition (Table 4). The renal cholesterol, phospholipid, and triglyceride contents were increased by 2.0-, 1.6-, and 2.2-fold, respectively, compared to normal control rat. Dietary intervention with supplemental zinc showed a favorable effect on the same with pronounced decreases by 38%, 20% and 41% in D+Zn-1 and 29% (in cholesterol) and 54% (in triglyceride) in D+Zn-2, respectively. The higher ratio of cholesterol:phospholipid was effectively normalized by feeding supplemental zinc ($P < 0.05$). Diabetic rats demonstrated a prominent decrease in the MUFA and PUFA — palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4) — in the renal tissue (53%, 39%, 32% and 27%, respectively) as compared to normal controls (Table 5). Zinc treatment significantly reversed the decrease.

### Table 4

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Cholesterol (mg/g tissue)</th>
<th>Phospholipid (mg/g tissue)</th>
<th>Triglyceride (mg/g tissue)</th>
<th>Cholesterol:phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>3.0±0.2</td>
<td>34.8±3.3</td>
<td>23.5±1.7</td>
<td>0.09±0.002</td>
</tr>
<tr>
<td>N+Zn-1</td>
<td>3.4±0.3</td>
<td>41.7±2.7</td>
<td>27.2±3.2</td>
<td>0.08±0.008</td>
</tr>
<tr>
<td>N+Zn-2</td>
<td>3.6±0.2</td>
<td>43.1±0.7</td>
<td>21.1±2.5</td>
<td>0.08±0.001</td>
</tr>
<tr>
<td>D</td>
<td>6.5±0.2</td>
<td>55.4±4.5</td>
<td>52.4±5.4</td>
<td>0.12±0.007</td>
</tr>
<tr>
<td>D+Zn-1</td>
<td>4.0±0.3</td>
<td>44.3±3.2</td>
<td>30.7±2.2</td>
<td>0.09±0.002</td>
</tr>
<tr>
<td>D+Zn-2</td>
<td>4.6±0.3</td>
<td>56.7±1.9</td>
<td>24.3±2.3</td>
<td>0.08±0.001</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. of eight animals in each group. *Significantly different from normal control group ($P < 0.05$). bSignificantly different from diabetic control group ($P < 0.05$).
Table 5

Influence of zinc supplementation on kidney fatty acid composition (mol/100 mol) in diabetic rats

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>N</th>
<th>N+Zn-1</th>
<th>N+Zn-2</th>
<th>D</th>
<th>D+Zn-1</th>
<th>D+Zn-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.90±0.08</td>
<td>0.79±0.07</td>
<td>0.73±0.03</td>
<td>2.48±0.07</td>
<td>0.54±0.06</td>
<td>0.53±0.07</td>
</tr>
<tr>
<td>14:1</td>
<td>0.59±0.07</td>
<td>0.59±0.04</td>
<td>0.58±0.06</td>
<td>1.82±0.06</td>
<td>0.59±0.02</td>
<td>0.55±0.06</td>
</tr>
<tr>
<td>16:0</td>
<td>21.34±1.2</td>
<td>20.57±1.04</td>
<td>21.02±0.93</td>
<td>25.96±1.2</td>
<td>20.59±1.29</td>
<td>20.56±0.82</td>
</tr>
<tr>
<td>16:1</td>
<td>1.46±0.1</td>
<td>1.56±0.12</td>
<td>1.25±0.05</td>
<td>0.69±0.06</td>
<td>0.72±0.02</td>
<td>0.40±0.03</td>
</tr>
<tr>
<td>18:0</td>
<td>10.15±0.8</td>
<td>12.52±0.5</td>
<td>12.72±0.2</td>
<td>22.64±0.59</td>
<td>14.14±0.84</td>
<td>15.09±0.71</td>
</tr>
<tr>
<td>18:1</td>
<td>22.88±1.4</td>
<td>22.35±0.91</td>
<td>22.14±0.8</td>
<td>14.05±0.94</td>
<td>19.94±0.91</td>
<td>18.33±0.6</td>
</tr>
<tr>
<td>18:2</td>
<td>19.42±0.6</td>
<td>17.59±0.64</td>
<td>16.24±0.5</td>
<td>13.24±0.63</td>
<td>16.00±0.72</td>
<td>15.82±0.7</td>
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<td>18:3</td>
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<td>0.31±0.06</td>
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<td>0.42±0.01</td>
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Values are mean±S.E.M. of eight animals in each group.

<sup>a</sup> Significantly different from N group (P<.05).

<sup>b</sup> Significantly different from D group (P<.05).

Fig. 4. Effect of zinc supplementation on the expression profile of RAGE mRNA and protein in diabetic kidney. (A) Relative amount of RAGE mRNA (folds) (relative mRNA expressions were normalized to β-actin level and expressed in arbitrary units as fold change compared with normal control). (B) Western blot analysis of RAGE protein abundance in whole kidney lysates in diabetic rats. (C) Relative protein expressions (normalized to β-actin level and expressed as fold change compared with normal control). Values are mean±S.E.M. of eight animals in each group. *Significantly different from normal control group (P<.05). **Significantly different from diabetic control group (P<.05).
Fig. 5. Effect of zinc supplementation on the expression profile of mRNA in diabetic kidney: (A) TGF-β1, (B) NF-κB, (C) TNF-α and (D) COX-2. Values are mean±S.E.M. of eight animals in each group. Relative gene expressions were normalized to β-actin level and expressed in arbitrary units as fold change compared with normal control. *Significantly different from normal control group (P<.05). †Significantly different from diabetic control group (P<.05).

Fig. 6. Effect of zinc supplementation on the protein expression of glucose transporters (GLUT-1 and GLUT-2) in diabetic kidney. (A) Immunohistochemistry (representative figures at 20×; a–d). (B) Immunofluorescence (representative figures at 20×; e–h). (C) Western blotting analysis of GLUT-1 and GLUT-2 protein abundance in lysates of kidney tissue in diabetic rats. Relative protein expressions of (D) GLUT-1 and (E) GLUT-2 (normalized to β-actin level and expressed as fold change compared with normal control). Values are mean±S.E.M. of eight animals in each group. *Significantly different from normal control group (P<.05). †Significantly different from diabetic control group (P<.05). N, Normal control (a & e); D, diabetic control (b & f); Zn-1, zinc supplemented at dose 1 (c & g); Zn-2, zinc supplemented at dose 2 (d & h).
of oleic acid, linoleic acid and arachidonic acid by increasing the concentration of the same (42%, 21% and 56% in D+Zn-1; 31%, 20% and 66% in D+Zn-2). This was accompanied by a decrease in elevated level of myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0) and arachidic acid (20:0) in diabetic rats (2.75-, 1.25-, 2.2- and 2.8-fold increases, respectively). The decreases in these fatty acids resulting from zinc supplementation were 78%, 21%, 38% and 41% in D+Zn-1 and 79%, 21%, 33% and 66% in D+Zn-2, respectively. The PUFA/SFA ratio was thus significantly declined in the diabetic situation (approx. 49%) as compared to normal control animals. Zinc supplementation to diabetic animals resulted in the normalization of the altered PUFA/SFA ratio.

3.8. Influence of dietary zinc supplementation on diabetes-induced renal tissue mRNA transcriptional profile and renal protein expression profile

The favorable effect of zinc supplementation was accompanied by the inhibition of diabetes-induced overexpression of genes for inflammatory and oxidative-stress-mediated molecular markers in the kidney. Renal tissue mRNA transcriptions of the receptor (RAGE), polyol pathway enzymes (SDH and AR), inflammatory markers (COX-2), cytokines (TNF-α, NF-κB, and TGF-β1), fibrosis (fibronectin and collagen IV), and proapoptotic (Bax) and antiapoptosis (Bcl-2) protein marker were analyzed in parallel by RT-qPCR.

3.8.1. Influence on RAGE, transforming growth factor and inflammatory markers

Transcriptions of mRNA, conforming to quantitative analysis by Western blotting of the expression of the kidney for RAGE (receptor for AGEs), showed an elevated level in diabetic condition (>6- and 1.3-fold, respectively) compared with the normal kidney (Fig. 4). Dietary zinc supplementation produced down-regulation of the same by 47% and 82% and by 35% and 23%, respectively, in D+Zn-1 and D+Zn-2 groups. The transforming growth factor (TGF-β1), inflammatory cytokines (NF-κB and TNF-α) and COX-2 were significantly increased in the diabetic renal tissue (by >6.2-, 2.15-, 6.84- and 3.05-fold) compared to normal control, signifying NF-κB activation in diabetic renal tissue (Fig. 5). In the case of diabetic rats maintained on zinc supplementation, significant down-regulation of the transforming and inflammatory mRNA expression (79%, 69%, 46% and 64% in D+Zn-1; 71%, 71%, 42% and 58% in D+Zn-2, correspondingly) was seen, indicating inhibition of this signaling inflammatory pathway (Fig. 5).

3.8.2. Influence on kidney glucose transporters and polyol pathway enzymes (AR and SDH)

Quantitative analysis by Western blotting of the expression of kidney glucose transporters GLUT-1 and GLUT-2 in diabetic rats showed that diabetic rats were associated with a highly significant...
increase in protein expressions of GLUT-1 and GLUT-2 (1.94- and 2.87-fold, respectively) (Fig. 6). While GLUT-2 expression was significantly ameliorated by D+Zn-1 (71%) and D+Zn-2 (36%), GLUT-1 protein was down-regulated mainly by D+Zn-1 (68%). This was also further supported by immunohistochemical and immunofluorescence studies. To understand at a molecular level the polyol pathway enzymes in diabetic condition as well as the effects of zinc treatment, mRNA and protein expressions of renal AR and SDH were evaluated (Fig. 7). In zinc-fed diabetic rats, there was a down-regulation of mRNA expression for AR and SDH along with quantitative protein fold by Western blot (25% and 26% in D+Zn-1; 73% and 37% in D+Zn-2, respectively). This was also reflected in the same pattern by immunohistochemical and confocal microscopic observation (Fig. 8).

### 3.8.3. Influence on renal 4-HNE content

To better understand the molecular mechanism underlying alleviation of oxidative stress in diabetic renal tissue by zinc supplementation, protein expression of 4-HNE was evaluated (Fig. 9). The kidney of diabetic animals showed augmented 4-HNE protein expression which was >10-fold compared with that of normal control group. Zinc supplementation of these diabetic rats pronoucedly countered expression of 4-HNE (P<0.05) (by 67% in D+Zn-1 and by 47% in D+Zn-2 group).

### 3.8.4. Influence on renal fibrosis markers

Increased transcription of mRNA for markers (fibronectin and type IV collagen) and concomitant augmented protein expression of type I collagen were evident in diabetic condition as seen by >8.9-, 27- and 7.9-fold expression, respectively, compared with the kidney of normal animals (Fig. 10). Zinc treatment inhibited the up-regulation of fibronectin and type IV collagen by 84%/73% and 91%/68%, respectively, in D+Zn-1 and D+Zn-2 groups. Diabetic animal group showed progressive renal fibrosis with increased type I collagen deposition in the renal tissue which was markedly attenuated in zinc supplementation, mainly by D+Zn-1 (86%). This tubulointerstitial fibrosis was also supported by further histopathological evaluation with Masson’s trichrome staining, suggesting that zinc supplementation could attenuate diabetes-induced fibroblast expression in rat kidneys.

### 3.8.5. Influence on renal Bax and Bcl-2 protein expressions in diabetic rats

Diabetic hyperglycemia elevated the expression of Bax mRNA and down-regulated the expression of Bcl-2 mRNA. This was abrogated by dietary zinc treatment in a dose-dependent manner (Fig. 11). Both immunohistochemical study and confocal microscopic observation were consistent with these effects of supplemental zinc (Fig. 12). Thus,
the ratio of Bax/Bcl-2 which was dramatically elevated in diabetic rats (P<.05), due to overexpression of Bax and down-regulation of Bcl-2, was significantly modulated by zinc treatment, leading to the suppression of Bax/Bcl-2 ratio. Quantitative analysis by Western blotting of the expression of the same in diabetic rats showed that diabetic rats were associated with a highly significant increase in protein expressions of Bax (9.5-fold) and significant decrease in Bcl-2 (64%) (Fig. 11). While Bcl-2 expression was significantly ameliorated by D+Zn-1 (1.9-fold) and D+Zn-2 (2.3-fold), Bax protein was down-regulated in D+Zn-1 (80%) and D+Zn-2 (72%) groups. These findings suggested that the antiapoptotic effect of zinc was concomitant with regulation of the balance of Bax and Bcl-2 mRNA and protein expression.

3.9. Influence of zinc supplementation on zinc status of diabetic renal tissue in experimental rats

Persistent hyperglycaemia in diabetes leads to reduction in cellular free zinc in the kidney. Fluorescent staining for zinc by TSQ revealed pronouncedly lesser fluorescence in the diabetic kidney compared to normal rats in confocal laser scanning microscopy (Fig. 13). The intense fluorescent staining manifested in diabetic animals which were supplemented with zinc, suggesting that the exogenous zinc can combat the decreasing effect of hyperglycaemia on cellular free zinc in renal tissue.

3.10. Influence of zinc supplementation on histopathology of renal tissue in diabetic rats

Effects of Zn supplementation on diabetic nephropathy were examined with respect to morphological changes and development of fibrosis. H&E-stained renal sections revealed shrunken glomeruli in the diabetic group of rats, while glomeruli were nearly normal in the D+Zn-1 and D+Zn-2 groups (Fig. 14). Moreover, mesangial matrix expansion, inflammatory cell infiltration, mucopolysaccharide deposition and higher degree of tubular clarifications or vacuolization were detected in the diabetic group, while these diabetes-induced histopathological alterations were not evident in the normal control (Fig. 14a and b) and nearly normalized in zinc-supplemented diabetic rats (Fig. 14c and d). Similarly, PAS and MT staining showed a significant increase in PAS-positive (purple plaques) materials indicative of glycogen accumulation and in MT-stained substances indicative of collagen fibers deposition in diabetic glomeruli (Fig. 14f and j) being absent in zinc-supplement-fed diabetic animals (Fig. 14g, h and k, l).

4. Discussion

Body Zn store is precisely maintained by the efflux and influx mechanisms intricately regulated through different Zn transporters. Uncompromised diabetes disrupts this equilibrium, which in turn leads to Zn dyshomeostasis [15]. Zn supplementation to STZ-diabetic rats has been previously reported to alleviate hyperglycaemia and...
attendant metabolic abnormalities [17] and so also the diabetes-mediated oxidative stress [18]. Numerous studies have demonstrated that oxidative stress mediated mainly by hyperglycemia-induced generation of free radicals and formation of AGEs contributes to the development and progression of diabetes [45]. An extensive evidence now indicates that oxidative-stress-induced inflammatory mechanism may further contribute to the pathogenesis of diabetic nephropathy [1]. Therefore, investigations into the antioxidative as well as anti-inflammatory strategies may offer a new approach for mitigating diabetes-induced nephropathy. In this context, the present study was intended to examine whether zinc supplementation can significantly protect the kidney during diabetes. The present study has demonstrated that Zn treatment can significantly improve diabetes-induced renal functional and pathological changes in STZ-induced diabetic rats. Zn supplementation particularly alleviated hyperglycemia-mediated kidney damage by inhibition of glucose translocation, oxidant stress and hyperglycemia-mediated upsurge in polyol pathway in the renal tissue. The anti-inflammatory property of Zn was associated with suppression of NF-κB signaling. While Zn supplementation at 5 times the normal requirement was found to be beneficial in this context, the higher dose (10 times the normal requirement) did not produce any further additional benefit.

In a recent study on diabetic mice [46], high Zn supplement (150 mg kg\(^{-1}\) diet) showed beneficial effect on kidney weight than low Zn (30 mg kg\(^{-1}\) diet) supplement which was not enough to wipe off Zn loss during diabetes. In our present study, feeding diabetic rats with diets supplemented with zinc at 5 and 10 times than the normal requirement showed significant countering of increased kidney weight and reversal of increased creatinine clearance. Induction of diabetes intensified ROS generation in the kidney, which subsequently increased auto-oxidation of PUFA in lipids and amino acids of proteins, leading to the increased formation of lipid peroxidation and protein carbonyl. These elevated levels of ROS, MDA and protein carbonyl were amended by dietary zinc supplementation, which are indicative of the antioxidant and free radical scavenging properties of Zn. The antioxidant role of Zn can also be due to the probable inhibition of the NADPH-oxidase enzyme and induction of metallothionein, which is involved in the reduction of hydroxyl radicals and in the sequestration of the reactive oxygen species produced under stress conditions [47]. In our study, SOD and all the GSH-linked antioxidant enzymes were increased, with concomitant decrease in catalase, ascorbic acid and GSH in diabetic state. This contradictory trend observed in antioxidant defense might be specific for the time point at which we examined them during the disease progression. The activity of SOD is increased at the onset of diabetes as the body’s first level of defense to neutralize the ROS generated. The increased activity of SOD would result in H\(_2\)O\(_2\) generation, which also results in coordinated increase in the activities of GSH-linked enzymes to combat oxidative stress. These results were concomitant with augmented oxidative stress markers (ROS, MDA, and protein carbonyl) and reduced level of antioxidant scavengers ascorbic acid and GSH, a substrate for GSH-linked enzymes trying to obliterate the oxidants in the diabetic situation. Supplementation of Zn significantly restored the activity of antioxidant enzymes and levels of SOD and GSH-linked antioxidant enzymes.

![Fig. 10. Effect of zinc supplementation on the expression profile of kidney fibrosis factors in diabetic rats: (A) Relative amount of fibronectin mRNA (folds). (B) Relative amount of type IV collagen mRNA (folds). (C) Western blot analysis of type I collagen. (D) Relative expressions of type I collagen (normalized to β-actin level and expressed as fold change compared with normal control). Values are mean±S.E.M. of eight animals in each group. *Significantly different from normal control group (P<.05). **Significantly different from diabetic control group (P<.05).](image-url)
of antioxidant molecules which was substantiated with diminished oxidative stress markers. The role of Zn in the antioxidant defense system has been widely investigated; studies have highlighted its role in the regulation of glutathione peroxidise, in the expression of metallothionein and as a co-factor for superoxide dismutase. Another mechanism by which Zn acts as an antioxidant is by affecting the expression of glutamate–cysteine ligase, which is the rate-limiting enzyme of glutathione de novo synthesis, thus being responsible for the modulation of the total cellular glutathione concentration [48].

The present study has shown that dietary Zn supplementation significantly reduced the elevated level of cholesterol and triglycerides, resulting in a beneficial lowering of cholesterol: phospholipid ratio in the renal tissue. The ROS can attack the PUFA of the phospholipid bilayer on renal cell membranes leading to an overproduction of lipid peroxidative bioactive product such as 4-HNE [49]. Thus, diminished PUFA:SFA ratio under diabetic condition is due to subsequent lipid peroxidative loss of monounsaturated fatty acids (mainly oleic acid, 18:1) and polyunsaturated fatty acids (mainly linoleic acid, 18:2 and arachidonic acid, 20:4). Fatty acid profile evaluated in this study revealed that the content of linoleic acid (18:2) and arachidonic acid (20:4) in the renal tissue of Zn-fed diabetic animals was significantly higher. The present study revealed that zinc supplementation significantly countered diminished PUFA:SFA ratio and down-regulated the expression of 4-HNE in diabetic kidney. This might be due to the antioxidant property of Zn, which could ameliorate the accumulation of 4-HNE by hindering lipid oxidation and ROS generation. Zn supplement at a dose of 5 mg kg\(^{-1}\) body weight given for a 3-month duration has been reported to significantly down-regulate the expression of 4-HNE in type 1 diabetic mouse model [50].

Kidney plays an important role in glucose homeostasis as they ensure its reabsorption through glucose transporters. The modulation of glucose transporters is demonstrated to be important in the development of diabetic nephropathy. Our study indicated that up-
regulation of glucose transporters (GLUT1 and GLUT2) under diabetic condition was significantly alleviated by Zn supplementation, suggesting the reduction of renal glucose reabsorption, which could contribute to improving glycemic control.

Activation of AR and the subsequent accumulation of intracellular sorbitol consequent to uncontrolled hyperglycemia are suggested to contribute to the pathogenesis of chronic diabetic tissue damage, including nephropathy (By causing osmotic stress). During hyperglycemia, consumption of NADPH by AR could obstruct antioxidant capacity by the diminution of GSH and glutathione peroxidase activity. With increased sorbitol concentration, SDH gets upregulated, channelizing to fructose facilitated by NAD+ as co-factor getting reduced to NADH (a substrate for NADH oxidase that further generates reactive oxygen species) [51]. Zn supplementation in the present study has beneficially impeded the up-regulated expression of polyol cycle probably by ameliorating the hyperglycemia [17], hindering the ROS generation and oxidant stress in the renal tissue.

Fructose, the ultimate metabolite of polyol pathway, is further transformed to fructose-3-phosphate and subsequently degraded to 3-deoxyglucose; the latter is a more potent nonenzymatic glyating agent [51]. The AGEs which are formed during persistent hyperglycemia interact with their receptor (RAGE) induce oxidative stress and evoke subsequent inflammatory reactions resulting in increased membrane permeability, loss of integrity, progressive alteration in renal architecture and finally leading to the loss of renal function [52]. Binding of AGE with RAGE induces the expression of proinflammatory cytokines (interleukin-1α, interleukin-6 and TNF-α) through NF-κB, which in turn leads to enhanced ROS production.

NF-κB is proposed to play an important role in mesangial cell instigation leading to renal injury, and its overexpression as observed here is advocated in diabetic renal tissue damage [53]. Up-regulated expression of TNF-α persuades a local inflammatory response through the instigation of a cascade of cytokines and aggregates vascular permeability, thereby directing macrophage and neutrophils to the site of infection [54]. The renal TNF-α level in diabetic nephropathy is correlated with urinary albumin excretion in rats [3]. Moreover, the increase in renal TNF-α level precedes the elevation in albuminuria in diabetes concomitant with diabetic nephropathy [17]. Glycation also results in increased synthesis of type III collagen, α3(IV) collagen, type V collagen, type VI collagen, laminin and fibronectin in the extracellular matrix (ECM), most likely via up-regulation of TGF-β [55]. The present study has demonstrated the augmented expression...
of Bax and declined expression of Bcl-2 in diabetic renal tissue. This dissimilar amendment in Bax and Bcl-2 expression could be due to the response to hyperglycemia-induced oxidative stress or TGF-β1 stimuli [56]. The increased expression of COX-2 in podocytes has been reported in various experimental models of progressive glomerular injury and in cultured podocytes stimulated by mechanical stress [57]. In the present investigation, additional Zn supplementation exerts beneficial influence not only on AGE-RAGE pathway but also on cascade of signaling pathways via NF-κB inhibition, leading to an attenuation of renal damage. Also, Zn-supplemented diabetic rat exhibited a reduction in TGF-β1 signaling pathway as well as down-regulation of mRNA of inflammatory markers TNF-α and COX-2 in kidneys, suggesting that Zn supplementation attenuates inflammatory factors in diabetic rat, which suggests that zinc functions both as an antioxidant and as an anti-inflammatory agent.

Diminished ATPase activity in the kidney of diabetic rat could be due to excessive nonenzymatic glycation of the enzyme itself or could be due to lipid peroxidation and loss of membrane fluidity. The reduced renal membrane-bound Na⁺,K⁺-ATPase and Ca²⁺-ATPase activities were alleviated by dietary interventions with Zn, which could be due to its antioxidative properties or due to conserving the integrity of cell membranes.

Excess matrix synthesis as manifested by overexpression of mRNAs for type IV collagen, and fibronectin instigates to appear in the kidney of diabetic animal consequent to renal enlargement and connotation with the onset of kidney hypertrophy. Our study has demonstrated that matrix mRNA expression is increased in association with a significant upsurge in the size of the proximal tubular cell in diabetic renal hypertrophy and increased size of the tubular basement membrane by acute induction of the synthesis of matrix molecules such as type IV collagen [58]. Upsurge in collagen I could be due to the initiation of cell proliferation or increased by the process of protein synthesis due to wound in diabetic condition. Zn supplementation reduced the expression of ECM component, i.e., fibronectin and type I and type IV collagen (Masson’s trichrome staining). This might be a result of down-regulating the TGF-β1/Smad signaling followed by NF-κB negative feedback which subsequently reduces the glomerular basement membrane thickening by abnormal deposition of ECM in diabetic nephropathy. The H&E staining further indicated that Zn treatment pronouncedly countered the renal infiltration of leukocytes, including macrophages and other architectural alteration. Morphometric analysis of PAS-stained diabetic kidney section exhibited induced glycogen deposition indicating that the alteration is compounded as a result of constant exposure to hyperglycemia which has been ameliorated by Zn intervention.

5. Conclusion

This animal study envisaged and explored the propensity of supplemental Zn in alleviating renal damage in DN. The pathological abnormalities in the renal architecture of diabetic animals were corrected by external Zn intervention. Dietary Zn supplementation showed a significant beneficial effect in alleviating diabetic nephropathy which was achieved in addition to exerting a better glycemic control [17] by modulation of glucose transporters; a protective influence on oxidative stress induced cytokines and inflammatory markers specifically NF-κB proliferation, reducing the overproduction of polyol pathway metabolites and modifying the renal lipid profile. Zn supplementation corresponding to five times the normal requirement produced this renoprotective effect, while the dose two times higher than this did not produce any higher degree of protection. Thus, our study revealed that Zn supplementation may offer a potential for effectively addressing diabetic nephropathy. Clinical trials in validating this benefit for optimizing the zinc nutrition are however warranted.
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Disclosure

The authors disclose no competing financial interests.

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Reference


Fig. 14. Influence of Zn supplementation on kidney histopathology in diabetic rats. Representative figures (40× objective) of H&E (a–d; shrunken glomerulus, mesangial matrix expansion, inflammatory cell infiltration, mucopolysaccharide depositions and higher degree of tubular clarifications or vacuolization), periodic acid and Schiff solution staining (e–h) for glycogen (purple colored) and Masson’s trichrome staining (i–l) for type IV collagen (blue colored).

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