Linanol (LIN), a monoterpenic alcohol, is a major volatile component of coriander seeds, which is traditionally known to possess antidiabetic potential. The present study investigated whether LIN can attenuate diabetes and prevent diabetes-induced changes in rat kidney. Diabetic rats were treated with LIN (25 mg/kg b.w.) for 45 days, after which plasma chemistry, proinflammatory cytokines, oxidative stress parameters, kidney function and the expression levels of transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α) and nuclear factor kappa B (NF-κB) were measured. The ability of LIN to improve glucose utilization in the normal rat diaphragm in the presence and absence of insulin and its antiglycation property were monitored in vitro. LIN-treated diabetic rats showed decreased cytokine levels and oxidative changes, improved renal function and reduced lipid levels in kidney as compared to untreated diabetic rats. LIN also rescued the kidney from inflammation by decreasing the expression of TGF-β1, TNF-α and NF-κB mRNA levels. Moreover, the histologic observations evidenced that LIN effectively improved structural integrity of the kidney. LIN enhanced the utilization of glucose in the rat diaphragm and prevented glycation of the model protein bovine serum albumin in vitro in a dose-dependent manner. These findings demonstrate that LIN can attenuate the risk of diabetic nephropathy secondary to reduction in hyperglycemia.

INTRODUCTION

Hyperglycemia, the hallmark of diabetes, is believed to generate reactive oxygen species (ROS) through the activation of polyol pathway, hexosamine pathway, advanced glycation endproduct (AGE) formation and activation of protein kinase C signaling that eventually lead to oxidative stress and microvascular complications in several organs (1). Supraphysiological levels of glucose are notorious to provoke these changes in the kidney causing diabetic nephropathy (DN), since the entry of glucose in the kidney is not regulated by insulin (2). Besides, growing evidence suggest that elevation in circulating lipids may also
contribute to renal disease progression. A correlation between the progression of renal disease and dyslipidemia in diabetic subjects has been noted (3).

The development of DN involves various cell types of kidney such as podocytes of glomeruli, mesangial and endothelial cells, tubular epithelial cells, interstitial cells, and interstitial fibroblasts. DN is a gradual process that starts with hyperfiltration and microalbuminuria followed by deterioration of renal function and changes in the structure of the glomeruli and tubulointerstitial compartments (4). The changes include hyperplasia and hypertrophy of various cell types of the glomeruli and tubules, thickening of glomerular and tubular basement membrane, and tubulointerstitial and mesangial expansion (5). The mechanisms responsible are suggested to be hyperglycemia-induced ROS generation of AGEs, activation of proinflammatory cascades and stimulation of mesangial cells to produce extracellular matrix proteins (ECM) (6).

Recently, cytokines have attracted most attention among all pathogenic factors related to kidney disease. Proinflammatory cytokines are a group of pharmacologically active, low molecular weight polypeptides that act as mediators of diverse inflammatory reactions contributing to DN. Among the cytokines, tumor necrosis factor-α (TNF-α) can promote local ROS generation resulting in alterations in glomerular permeability leading to albuminuria (7). There is enough evidence that serum and urinary TNF-α levels are significantly upregulated in various renal diseases including DN (8). More recent studies in diabetic patients demonstrate significant association between DN and interleukin-6 (IL-6), another proinflammatory cytokine. IL-6 mediates glomerular basement membrane thickening, a crucial lesion of DN, and is a strong predictor of renal disease progression (9).

Transforming growth factor β1 (TGF-β1), a pro-sclerotic cytokine, is an important player in the progression of DN. Stimulation of TGF-β1 and upregulation of type II TGF-β receptor promote renal deposition of extracellular matrix components like collagen I, collagen IV and fibronectin, and stimulate hypertrophy of mesangial cells inducing renal fibrosis and glomerulosclerosis (10). TGF-β1 level is shown to be elevated in animal models of diabetic kidney disease (11).

Nuclear factor κB (NF-κB), an ubiquitous transcription factor, is one of the cross-talk points of multiple signal transduction pathways. Activated NF-κB plays a key role in the regulation of transcription and expression of many genes involved in inflammatory responses (12) including TNF-α and IL-6. An increase in the nuclear translocation of NF-κB has been demonstrated in human and experimental renal diseases (13).

(-)-Linalool (LIN), a monoterpen, is a major volatile component of the essential oils of several aromatic plant species including Coriandrum sativum Linn. It is used as an additive for processed foods and beverages and as a fragrance ingredient in cosmetics, toiletries and household detergents (14). LIN-containing essential oils possess antimicrobial, antibacterial and antiviral effects, as well as anti-inflammatory, analgesic and local anesthetic activities (15-19). LIN is suggested to be responsible for the antidiabetic activity of coriander seeds, which are used traditionally to control diabetes (20). The hypoglycemic effect of LIN in diabetic rats has already been reported (21). Although LIN has been subjected to such extensive research, the antioxidant, hypolipidemic and antiglycation effects of LIN have not been investigated in diabetic rats. Further, the effect of LIN on cytokines is not found in the literature either. These facts prompted us to investigate the effect of LIN supplementation in a chronic model of DN.

The plasma levels of glucose, insulin, lipids, oxidant-antioxidant balance, proinflammatory changes, and TGF-β1, TNF-α and NF-κB expression in kidney were analyzed to verify the protective effects of LIN on DN. In addition, the antiglycating property and glucose disposal effect of LIN in vitro were studied.
MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ) and LIN were purchased from Sigma-Aldrich Chemicals Pvt. Ltd., MO, USA. The kits for glucose, insulin, TNF-α and IL-6 assay were purchased from Qualigen Diagnostics, Mumbai, India, Boehringer Manheim, Germany, BD Biosciences, San Jose, CA, USA and Koma Biotech, Seoul, South Korea, respectively. Bovine serum albumin (BSA) was purchased from Merck Pvt. Ltd, Mumbai, India. Horse radish peroxidase (HRP) detection system was purchased from Biogenex Laboratories, Chennai, India. Antibodies against TGF-β1 (rabbit polyclonal) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, and anti-rabbit secondary antibody from Genei, Bangalore, India. All other chemicals and solvents used were of analytical grade and were purchased from Sisco Research Laboratories, Mumbai, India.

Treatment and maintenance of animals

Male adult Wistar rats of body weight 150-180 g were obtained from Department of Experimental Medicine, Central Animal House, Rajah Muthiah Medical College (RMMCH), and the experiments were carried out at Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar, Tamil Nadu, India. The rats were housed under controlled conditions (22-25 °C) at 12 h light/12 h dark cycles and received standard pellet diet (Karnataka State Agro Corporation Ltd., Agro Feeds Division, Bangalore, India) and water ad libitum. The study protocol was approved by the institutional Animal Ethics Committee, RMMCH, Annamalai Nagar.

Induction of experimental diabetes

Rats were rendered diabetic by administering STZ (45 mg/kg i.p.) dissolved in freshly prepared 0.01M citrate buffer, pH 4.5. Control rats received the vehicle alone. Fasting blood glucose concentration was measured 72 h later. Rats that showed blood glucose levels above 200 mg/dL were used in the study.

Experimental groups

The following groups of rats were maintained for a total period of 45 days:

Group 1 (CON) – control rats receiving normal diet
Group 2 (DIA) – diabetic rats administered STZ (45 mg/kg, dissolved in 0.1 M citrate buffer, pH4.5)
Group 3 (DIA+LIN) – diabetic rats treated with LIN (25 mg/kg b.w./day dissolved in carboxy methyl cellulose p.o.)
Group 4 (CON+LIN) – control rats treated with LIN.

The dosage of LIN was adopted from the literature (17). Food intake, body weight and fluid intake were determined at regular intervals. Urinary variables were measured in 24-h samples. For this, the animals were placed individually in metabolic cages and urine was collected. The sample volume was measured and preserved by adding 0.2 mL 10 N hydrochloric acid until analysis.

Oral glucose tolerance test

On day 44, the rats were fasted overnight. Blood samples were collected by sino-ocular puncture. Samples were again collected at 60 and 120 min after the administration of glucose (2 g/kg). The oral glucose tolerance test (OGTT) curves were drawn by plotting blood glucose (mmol/L) against time (min). The integrated area under the curve for glucose (AUCglucose) (mmol.L⁻¹ min⁻¹) was calculated using GraphPad Prism version 5.1.

At the end of the experimental period, the animals were put on overnight fast and anesthesized with an intramuscular injection of ketamine hydrochloride (35 mg/kg, i.m.) and sacrificed by cervical dislocation. Kidneys were removed, cleaned, dried and homogenized in 0.1 M Tris-HCl buffer, pH 7.4. Blood, plasma, and kidney homogenate were used for the following investigations.

Biochemical assays

Glucose and insulin levels in plasma were quantified using kits. Glycated hemoglobin (22) and fructoseamine (23) were estimated.
The levels of TNF-α, IL-6 were determined by ELISA according to the manufacturer’s instructions provided in the kit. Thiobarbituric acid reactive substances (TBARS) (24) and lipid hydroperoxides (LHP) (25) were determined as a measure of lipid peroxidation. In brief, the concentration of TBARS was estimated by measuring the pink-colored chromophore formed upon heating the sample with thiobarbituric acid (0.375% in distilled water) at 535 nm. The standard curve was prepared using 1,1’,3,3’-tetramethoxy propane. Lipid hydroperoxides were measured in methanol-extracted tissue homogenates. An aliquot of lipid sample (0.2 mL) was mixed with 1.8 mL of the reagent, which contained 90 mL of methanol, 10 mL of 250 mM sulfuric acid, 88 mg butylated hydroxyl toluene, 7.6 mg xylene orange and 9.8 mg ferrous ammonium sulfate. The color developed was read at 560 nm. Protein carbonyl (PC) (26) and nitrosothiol (27) were determined.

Renal function markers such as urea, uric acid and creatinine in plasma and urine were assayed on an autoanalyzer (Technicon-RA-XT, Boehringer, Germany). Urea and creatinine clearance rates were calculated from the equation:

\[ C = \frac{(U \times V)}{P} \]

where \( C \) is clearance rate, \( U \) is concentration of the substance in urine, \( V \) is the mL of urine excreted per minute, and \( P \) represents concentration of the substance in plasma. The rates were expressed in mL/s. Urinary albumin was determined using a kit obtained from Bayer Diagnostics, Germany.

Enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S transferase (GST) and non-enzymatic antioxidants, namely vitamin E, ascorbic acid and glutathione (GSH) were assayed by standard procedures described elsewhere (28). Briefly, SOD was assayed by the inhibition of the formation of NADH-phenazine methosulfate nitroblue tetrazolium formazan. CAT and GPx activities were assayed by measuring the amount of the substrate consumed (hydrogen peroxide and glutathione, respectively) after carrying out the reactions for a specified period of time. The hydrogen peroxide remaining was measured by dichromate color reaction at 620 nm, while the GSH remaining was measured using color reaction with dithiobis nitrobenzoic acid (DTNB) reagent, which produced a yellow color that was measured at 412 nm. The activity of GST was measured by following the increase in absorbance at 340 nm using 1-chloro-2,4, dinitro benzene (CDNB) as the substrate. GR activity in kidney was assayed by measuring the rate of NADPH oxidation. Vitamin E was estimated by the reduction of ferric ions to ferrous ions by the sample followed by the formation of a red-colored complex with 2,2 dipyridyl. Ascorbic acid was measured by the conversion to dehydroascorbic acid in the presence of thiourea, a mild reducing agent followed by reacting with dinitro phenyl hydrazine (DNPH)-thiourea-copper sulfate reagent (DTC). The product formed was converted to a red-colored complex by treating with \( \text{H}_2\text{SO}_4 \), which was read at 520 nm.

**Lipid extraction and analysis**

Lipids from kidney were extracted by the method of Folch et al. (29). The contents of cholesterol, triglycerides (TG) and free fatty acids (FFA) in plasma and kidney were measured (30-32).

**Western blot analysis of TGF-β1 in kidney**

Kidney samples were homogenized in an ice-cold buffer (20 mM Tris-HCl, pH 7.4, containing 150 mM sodium chloride, 1 mM ethylene diamine tetra-acetic acid (EDTA), 0.5% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1mM phenyl methosulfonyl fluoride and 10 µL of protease inhibitor cocktail) and then centrifuged at 10,000 xg for 15 min at 4°C. The solubilized protein (50 µg) was electrophoresed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% gel) and then blotted onto a nitrocellulose membrane. After 1 h, the membrane was incubated in a blocking buffer (3% BSA in Tris-buffered saline, pH 7.5) containing 0.1% Tween-20 (TBST) and then probed with the primary antibody (rabbit polyclonal anti-TGF-β1, 1:1000 diluted in blocking buffer) at 4 °C overnight. The membrane was washed three times
with 0.1% TBST for 5 min and then incubated with HRP-conjugated anti-rabbit antibody (1:1500 dilution) for 2 h at room temperature. β-actin was used as the house-keeping control (1:1000 dilution). The bands were detected by color reaction with H$_2$O$_2$ and then scanned. Intensity was measured using densitometry software (AlphaEaseFC Software, Alphatech Innotech Co., Johannesburg, South Africa).

**RNA preparation and RT-PCR analysis of TGF-β₁, TNF-α and NF-κB**

Total cellular RNA was extracted from kidney tissue with TriZol reagent, which is based on the guanidium thiocyanate method (33). Two µg of total RNA was mixed with oligo DT primer (0.5 µg/µL), dNTP mix (deoxy nucleotide triphosphate mix) (10mM each, 2 µL), RNase free water (2 µL) and heated at 65 °C for 5 min. Reverse transcription was carried out with 5X Moloney-Murine Leukaemia virus (M-MuLV) reverse transcription buffer (4 µL), M-MuLV reverse transcriptase enzyme (1.5 U, 1 µL ) and RNase inhibitor solution (4 U, 0.5µL), incubated at 42 ºC for 90 min and the reaction was terminated at 85 °C for 5 min. PCR amplification was performed in a mixture containing 2.5 µL Thermus aquaticus buffer (Taq buffer), 3.5 µL of RNase free water, 1 µL of each sense and antisense primers (Sigma-Aldrich, Bangalore), 1 µL dNTPs, 5 µL reverse transcribed template solution. Nucleotide sequence of the primers used is shown in Table 1. Thermocycling conditions for TGF-β₁ were denaturation at 95 °C for 30 sec, annealing at 53 °C for 30 sec and 72 °C for 40 sec for primer extension (final extension at 72 °C for 10 min) for a total of 30 cycles; PCR conditions for TNF-α were denaturation at 94 °C for 30 sec, annealing at 52 °C for 3 min and 45 sec for primer extension (final extension at 72 °C for 10 min) for a total of 30 cycles; for NF-κB the conditions were denaturation at 95 °C for 3 min, annealing at 51 °C for 30 sec and 40 °C for 45 sec for primer extension (final extension at 72 °C for 10 min) for a total of 30 cycles; and for β-actin the conditions were denaturation at 55 °C for 45 sec, annealing at 72 °C for 40 sec and for 45 sec for primer extension (final extension at 72 °C for 10 min) for 30 cycles. Five µL of PCR products were applied to 1.5% agarose gels and electrophoresed for 20 min, visualized and photographed. The photodensitometry analysis was done with the Image J software system. All reactions were run in triplicate for each sample. The relative quantity was represented by the ratio of band intensities of TNF-α, TGF-β₁ and NF-κB to that of β-actin.

**Renal histology**

A portion of kidney tissue removed from each group was fixed in 8% neutral formalin, dried and embedded in paraffin wax. Sections of 3-5 µm were cut, processed, stained with hematoxylin and eosin, and examined under light microscope.

**Glycation of BSA in vitro**

The ability of LIN to prevent glycation in vitro was studied using BSA as the standard protein and a mixture of glucose and fructose as the glycating agent. The incubation mixture contained 14 mg BSA, 25 mM fructose, 25 mM glucose and penicillin-VK 100 U mL-1. The tubes were incubated for 30 days at 37 °C after adding either water (control), LIN at 5, 10 and 20 mM, or AG at 10 mM (reference standard). After 30 days, the samples were dialyzed and then measured for glycation (34). The values obtained were expressed as percentage with reference to control tube that contained only water.

**Glucose utilization assay**

Utilization of glucose in rat diaphragm in vitro was carried out (35). Hemidiaphragms were removed from control rats and placed in a cold buffer solution

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Nucleotide sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β₁</td>
<td>Sense</td>
<td>TGAAGCTGCTGTCTTTTGACG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ACTTCCAAACCACATCTTTC</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Sense</td>
<td>CTCAGGTGACAAGGCGGTAG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TTGACCTCAGCGCTGACGAG</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Sense</td>
<td>AAGATCAATGGCTACACGGG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CCTCAATGGCTCTTCTCTGCC</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense</td>
<td>TGTGATGGGCTTGACGAGG</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TTTGATGTCACGCAGATTGC</td>
</tr>
</tbody>
</table>
containing 4x10^{-2} M sodium phosphate, pH 7.2, 5x10^{-3} M KCl, 4x10^{-3} M MgCl_2, and 8.3x10^{-2} M NaCl at 37 °C. The pieces of diaphragm were blotted in filter paper, weighed and incubated with the medium containing 4x10^{-2} M sodium phosphate, pH 7.2, 5x10^{-3} M KCl, 4x10^{-3} M MgCl_2, 8x10^{-2} M NaCl, and 6x10^{-3} M glucose at 37 °C for 24 h. LIN was added at 1 and 2 mM concentrations, and insulin at 0.2 U/mL concentration in separate tubes. Control tube did not contain LIN or insulin. The levels of glucose in the medium were measured at the start (0 h) and after incubation (2 h). The values were given as glucose h^{-1}.

### Statistical analysis

Values are expressed as means ± SD. Data within the groups were analyzed by ANOVA followed by Duncan’s multiple range test (DMRT). For in vitro studies, Student’s t-test for unpaired comparisons was used. A value of \( P < 0.05 \) was considered statistically significant.
RESULTS

Effect of LIN on the symptoms of diabetes

Body weight (initial and final), kidney weight, and food and fluid intake/day of animals are given in Table 2. Increased food and water intake and failure to gain body weight were observed in diabetic rats. These symptoms were present throughout the experimental period. The data shown in Table 2 also indicate that LIN treatment of diabetic rats resulted in a significant reduction ($P<0.05$) of food intake, fluid intake and kidney weight as compared with untreated diabetic animals.

Effect of LIN on oral glucose challenge

Figure 1 shows results of OGTT in experimental animals. Diabetic rats exhibited fasting hyperglycemia and significant elevation of glucose at 60 and 120 min ($P<0.05$) after oral glucose load. On the other hand, both LIN-treated control and diabetic rats showed normal response. $AUC_{\text{glucose}}$ (mmol/L/min)) for experimental animals was as follows: $\text{CON}=8.73\pm0.42$, $\text{DIA}=20.33\pm1.02$, $\text{DIA+LIN}=8.76\pm0.42$ and $\text{CON+LIN}=8.81\pm0.46$. $AUC_{\text{glucose}}$ was significantly higher in diabetic rats as compared to that of control rats. LIN supplementation significantly reduced the $AUC_{\text{glucose}}$ values.

Table 5. Renal function markers in plasma and 24-hour urinary excretion of control and experimental animals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>DIA</th>
<th>DIA+LIN</th>
<th>CON+LIN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>1.66±0.08</td>
<td>2.68±0.06a</td>
<td>2.08±0.05b</td>
<td>1.59±0.07</td>
</tr>
<tr>
<td>Uric acid (mmol/L)</td>
<td>488.92±23.6</td>
<td>347.36±16.4</td>
<td>492.49±23.6</td>
<td>213.04±10.5</td>
</tr>
<tr>
<td>Creatinine (mmol/L)</td>
<td>237.80±10.1</td>
<td>184.76±8.53</td>
<td>234.26±10.2</td>
<td>213.04±10.3</td>
</tr>
<tr>
<td>Total protein (μmol/L)</td>
<td>12.66±0.1</td>
<td>6.92±0.31a</td>
<td>13.01±0.83b</td>
<td>13.10±0.45</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>44.0±3.33</td>
<td>32.3±1.25a</td>
<td>40.6±1.37b</td>
<td>43.4±1.26</td>
</tr>
<tr>
<td><strong>24-hour urinary excretion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (mmol/day)</td>
<td>2.69±0.08</td>
<td>2.20±0.10a</td>
<td>2.44±0.09b</td>
<td>2.57±0.11</td>
</tr>
<tr>
<td>Uric acid (mmol/day)</td>
<td>44.6±1.08</td>
<td>27.36±1.04a</td>
<td>38.06±1.06b</td>
<td>37.8±1.2</td>
</tr>
<tr>
<td>Creatinine (mmol/day)</td>
<td>28.2±1.03</td>
<td>22.1±1.06a</td>
<td>24.7±1.04b</td>
<td>25.6±1.05</td>
</tr>
<tr>
<td>Albumin (g/day)</td>
<td>0.84±0.07</td>
<td>1.56±0.14a</td>
<td>0.72±0.06b</td>
<td>0.60±0.05</td>
</tr>
<tr>
<td>Urea clearance (mL/s)</td>
<td>0.02±0.0014</td>
<td>0.023±0.0008</td>
<td>0.027±0.001b</td>
<td>0.029±0.006</td>
</tr>
<tr>
<td>Creatinine clearance (mL/s)</td>
<td>0.023±0.004</td>
<td>0.001±0.000a</td>
<td>0.002±0.000b</td>
<td>0.023±0.004</td>
</tr>
</tbody>
</table>

Values are means ± SD of 6 rats from each group: CON, control rats; DIA, diabetic rats; DIA+LIN, diabetic rats treated with linalool (25 mg/kg b.w./day); CON+LIN, control rats treated with linalool (25 mg/kg b.w./day); *significant as compared with CON group; ** significant as compared to DIA group [one-way ANOVA followed by DMRT ($P<0.05$)].
Lipid profile

Lipid levels in plasma and kidney of experimental animals are shown in Table 4. Elevated levels of total cholesterol, TG and FFA were observed in diabetic rats. LIN brought back these levels to near-control levels.

Plasma and urinary levels of non-protein nitrogen constituents, urine albumin and clearance rates

Table 5 shows plasma and 24-h urinary excretion levels of urea, uric acid, creatinine and albumin, and clearance rates of creatinine and urea in experimental animals. The level of urea was significantly increased ($P<$0.05), while the levels of uric acid, creatinine, total protein and albumin were significantly decreased ($P<$0.05) in plasma of diabetic rats as compared with control rats. Urinary urea, uric acid and creatinine clearance rates were lower in diabetic rats. LIN administration prevented these alterations and brought back these parameters to near-normal levels. Improved glomerular function in LIN-treated diabetic rats is indicated by enhanced clearance rates as compared to those of normal rats.

Oxidative stress markers and nitrosothiol

Table 6 gives the levels of oxidative stress markers such as LHP, TBARS, PC and nitrosothiol in kidney. Diabetic animals showed significantly ($P<$0.05) higher levels of these parameters as compared with control animals. In DIA+LIN group, the levels of these substances were significantly lowered ($P<$0.05) as compared with DIA group.

Enzymatic and nonenzymatic antioxidants

Table 7 shows the activities of enzymatic antioxidants in the kidney of experimental animals. Significant decrease in the activities of SOD (31%), CAT (23%), GPX (22%), GST (27%) and GR (26%) was seen in diabetic group. The administration of LIN to diabetic rats significantly upheld the activities of these enzymes. However, there were no significant differences in the activities of these enzymes between CON and CON+LIN.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Plasma</th>
<th>Kidney</th>
<th>Plasma</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHP$^A$</td>
<td>0.92 ± 0.07</td>
<td>1.72 ± 0.13</td>
<td>1.31 ± 0.10$^a$</td>
<td>2.38 ± 0.18$^a$</td>
</tr>
<tr>
<td>TBARS$^A$</td>
<td>2.80 ± 0.21</td>
<td>1.66 ± 0.13</td>
<td>5.28 ± 0.40$^a$</td>
<td>2.76 ± 0.21$^a$</td>
</tr>
<tr>
<td>LHP$^B$</td>
<td>34.10 ± 2.3</td>
<td>58.30 ± 4.8$^a$</td>
<td>1.12 ± 0.08$^b$</td>
<td>1.97 ± 0.22$^b$</td>
</tr>
<tr>
<td>TBARS$^B$</td>
<td>36.0 ± 2.8$^a$</td>
<td>33.10 ± 3.0</td>
<td>0.96 ± 0.07</td>
<td>1.74 ± 0.14</td>
</tr>
<tr>
<td>PC$^B$</td>
<td>1.90 ± 0.14</td>
<td>1.97 ± 0.08$^b$</td>
<td>2.10 ± 0.16$^b$</td>
<td>1.64 ± 0.12</td>
</tr>
<tr>
<td>Nitrosothiol$^B$</td>
<td>34.10 ± 2.3</td>
<td>58.30 ± 4.8$^a$</td>
<td>1.12 ± 0.08$^b$</td>
<td>1.97 ± 0.22$^b$</td>
</tr>
</tbody>
</table>

Values are means ± SD of 6 rats from each group. CON, control rats; DIA, diabetic rats; DIA+LIN, diabetic rats treated with linalool (25 mg/kg b.w./day); CON+LIN, control rats treated with linalool (25 mg/kg b.w./day); $^a$significant as compared with CON group; $^b$significant as compared to DIA group [one-way ANOVA followed by DMRT ($P<$0.05)].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Activity</th>
<th>CON</th>
<th>DIA</th>
<th>DIA+LIN</th>
<th>CON+LIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD$^A$</td>
<td>4.01 ± 0.312</td>
<td>2.74 ± 0.21$^a$</td>
<td>3.6 ± 0.12$^b$</td>
<td>3.98 ± 0.38</td>
<td></td>
</tr>
<tr>
<td>CAT$^B$</td>
<td>58.99 ± 4.80</td>
<td>44.89 ± 3.43$^a$</td>
<td>50.29 ± 3.85$^b$</td>
<td>56.55 ± 4.33</td>
<td></td>
</tr>
<tr>
<td>GPx$^C$</td>
<td>5.98 ± 6.46</td>
<td>4.67 ± 0.35$^a$</td>
<td>5.89 ± 0.45$^b$</td>
<td>6.00 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>GST$^D$</td>
<td>5.80 ± 0.44</td>
<td>4.22 ± 0.35$^a$</td>
<td>5.68 ± 0.43$^b$</td>
<td>5.88 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>GR$^E$</td>
<td>23.4 ± 1.6</td>
<td>17.4 ± 1.0$^a$</td>
<td>22.2 ± 1.3$^b$</td>
<td>23.1 ± 2.0</td>
<td></td>
</tr>
</tbody>
</table>

$^A$units/mg protein; $^B$μmol of H2O2.mg/protein/min; $^C$μmol of GSH consumed/min/mg protein; $^D$mmol of chloro dimethyl nitrobenzene-glutathione conjugate formed/min/mg protein; values are means ± SD of 6 rats from each group. CON, control rats; DIA, diabetic rats; DIA+LIN, diabetic rats treated with linalool (25 mg/kg b.w./day); CON+LIN, control rats treated with linalool (25 mg/kg b.w./day); $^a$significant as compared with CON group; $^b$significant as compared to DIA group [one-way ANOVA followed by DMRT ($P<$0.05)].
The levels of nonenzymatic antioxidants in plasma and kidney of experimental animals are listed in Table 8. The levels were significantly lower \((P<0.05)\) in diabetic group than in control animals. On the other hand, LIN-treated diabetic rats displayed significantly higher levels of nonenzymatic antioxidants.

**Glucose disposal effect of LIN**

Table 9 presents the glucose disposal effect of LIN and insulin. A significant increase \((P<0.05)\) in glucose utilization was seen in tubes containing LIN as compared with control. The glucose utilization effect of LIN was more pronounced when insulin was also present. The rate of glucose disposal effect in isolated rat diaphragm is depicted in Fig. 2A. Significant increase \((P<0.05)\) in glucose disposal was exerted by LIN at both concentrations (1mM and 2mM). The effect of LIN (INS+LIN 1mM and INS+LIN2mM) was higher when insulin (0.2 U/mL) was also present.

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**Table 8. Non-enzymatic antioxidants in plasma and kidney of control and experimental animals**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CON</th>
<th>DIA</th>
<th>DIA+LIN</th>
<th>CON+LIN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH (μmol/L)</td>
<td>936.80±71.17</td>
<td>648.55±49.65a</td>
<td>842.64±65.38b</td>
<td>934.42±71.54</td>
</tr>
<tr>
<td>Vitamin C (μmol/L)</td>
<td>164.15±12.56</td>
<td>102.86±7.87a</td>
<td>139.88±9.93a</td>
<td>163.01±12.48</td>
</tr>
<tr>
<td>Vitamin E (μmol/L)</td>
<td>24.43±1.87</td>
<td>15.98±1.23a</td>
<td>18.48±1.42a</td>
<td>24.98±1.91</td>
</tr>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH (μmol/mg protein)</td>
<td>5.32±0.41</td>
<td>4.64±0.35a</td>
<td>4.02±0.31b</td>
<td>5.28±0.40</td>
</tr>
<tr>
<td>Vitamin C (μmol/mg protein)</td>
<td>40.99±2.57</td>
<td>24.02±1.84a</td>
<td>30.84±1.57a</td>
<td>39.13±3.03</td>
</tr>
<tr>
<td>Vitamin E (μmol/mg protein)</td>
<td>20.22±1.78</td>
<td>12.96±0.99a</td>
<td>15.38±1.49b</td>
<td>19.48±2.07</td>
</tr>
</tbody>
</table>

Values are means ± SD of 6 rats from each group; CON, control rats; DIA, diabetic rats; DIA+LIN, diabetic rats treated with linalool (25 mg/kg b.w./day); CON+LIN, control rats treated with linalool (25 mg/kg b.w./day); asignificant as compared with CON group; bsignificant as compared to DIA group [one-way ANOVA followed by DMRT \((P<0.05)\)].

**Table 9. Effect of LIN on glucose utilization in rat hemi diaphragm incubated with glucose in vitro**

<table>
<thead>
<tr>
<th>Group</th>
<th>LIN (mM)</th>
<th>Insulin (U/mL)</th>
<th>Glucose utilization (µmol/g/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>-</td>
<td>-</td>
<td>8.09±0.468</td>
</tr>
<tr>
<td>LIN 1</td>
<td>1</td>
<td></td>
<td>12.83±0.95a</td>
</tr>
<tr>
<td>LIN 2</td>
<td>2</td>
<td>-</td>
<td>16.14±1.22a</td>
</tr>
<tr>
<td>INS</td>
<td>-</td>
<td>0.2</td>
<td>17.63±1.21</td>
</tr>
<tr>
<td>LIN 1 + INS</td>
<td>1</td>
<td>0.2</td>
<td>25.32±1.77b</td>
</tr>
<tr>
<td>LIN 2 + INS</td>
<td>2</td>
<td>0.2</td>
<td>33.48±1.93b</td>
</tr>
</tbody>
</table>

The incubation medium contained 0.04 M sodium phosphate (pH 7.2), 0.005 M potassium chloride, 0.004 M magnesium chloride, 0.006 M glucose and 0.08 M sodium chloride; LIN1, 1 mM LIN; LIN 2, 2mM LIN; INS, 0.2 U/mL insulin; acomparred with CON; bcompared with INS; significant at \(P<0.05\) (Student’s t-test); values are mean ± SD of 6 experiments.

The levels of nonenzymatic antioxidants in plasma and kidney of experimental animals are listed in Table 8. The levels were significantly lower \((P<0.05)\) in diabetic group than in control animals. On the other hand, LIN-treated diabetic rats displayed significantly higher levels of nonenzymatic antioxidants.

**Glucose disposal effect of LIN**

Table 9 presents the glucose disposal effect of LIN and insulin. A significant increase \((P<0.05)\) in glucose utilization was seen in tubes containing LIN as compared with control. The glucose utilization effect of LIN was more pronounced when insulin was also present. The rate of glucose disposal effect in isolated rat diaphragm is depicted in Fig. 2A. Significant increase \((P<0.05)\) in glucose disposal was exerted by LIN at both concentrations (1mM and 2mM). The effect of LIN (INS+LIN 1mM and INS+LIN2mM) was higher when insulin (0.2 U/mL) was also present.

![Figure 2. Effect of LIN on glucose utilization by the diaphragm in the presence and absence of insulin with time. Both LIN and insulin individually increased glucose utilization. When present together, the effect was greater. CON, control; LIN, linalool; INS, insulin. LIN1- 1 mM LIN; LIN 2- 2mM LIN, INS- 0.2 U/mL insulin; values are mean ± SD of six experiments (Student's t-test; \(P<0.001\)); *significant compared with CON (Student’s t-test ); #significant at \(P<0.05\) when compared with INS (Student’s t-test).](image-url)
Inhibition of sugar-induced glycation of BSA by LIN

LIN inhibited the glycation of BSA in a concentration-dependent manner (Fig. 3). At a concentration of 20 mM, LIN produced maximum inhibition of glycation (85%). The strongest (95.22%) inhibitory effect on glycation was observed at a concentration of 20 mM, LIN+10 mM AG. In the presence of both LIN and AG, the glycation inhibitory effect was greater than with LIN or AG alone.

Figure 3. Glycation inhibitory effect of LIN and AG; maximal glycation inhibitory concentration of LIN was 20 mM; GF, glucose + fructose; LIN 1 (10 mM); LIN 2 (15 mM); LIN 3 (20 mM); AG1, aminoguanidine (10 mM) and LIN+AG1, (20 mM) + aminoguanidine (10 mM). Composition of reaction mixture and the concentrations of additives are described in Materials and methods section. Values are mean ± SD of six experiments.

Figure 4 illustrates the results of fluorescence analysis of samples. The formation of nonenzymatic glycation endproducts was observed after both 15 and 30 days of incubation. The level of fluorescent products formed was higher in 30-day sample than in 15-day sample. LIN inhibited this process in a dose-dependent manner. The percentage inhibition for 30-day samples was 24% (5 mM LIN), 54% (10 mM LIN) and 72% (20 mM LIN). Furthermore, AG (10mM) also showed inhibition at 64.8%. Interestingly, the presence of both LIN (20mM) and AG (10mM) produced maximum inhibition (92%) of fluorescent products.

Figure 4. Fluorescence of the samples after 15 days and 30 days of incubation. Reaction mixtures contained glucose (25 mM, G), fructose (25 mM, F) and LIN 5 mM, 10 mM and 20 mM; LIN1, LIN2, LIN3), AG1, aminoguanidine (10mM). Relative fluorescence was recorded at 450 nm upon excitation of 350 nm. Values are mean ± SD of six experiments; *compared to GF; #compared to LIN 3 and AG (P<0.05).

TNF-α, TGF-β1 and NF-κB

Representative blots showing the expression of TGF-β1 in kidney and densitometry data analysis are shown in Figs. 5A and 5B, respectively. TGF-β1 expression was higher in diabetic rats, while the administration of LIN significantly suppressed (P<0.05) the expression. Control and control rats treated with LIN showed no significant changes in the expression.

Increased mRNA levels of TGF-β1, TNF-α and NF-κB were seen in diabetic rats as compared with control. The results of densitometry data analysis are given in Figs. 6B, 6C and 6D for TGF-β1, TNF-α and NF-κB, respectively. A noticeable and significant decline (P<0.05) in the expression levels of TGF-β1, TNF-α and NF-κB was observed when LIN was co-administered. There was no significant difference in the expression between control and control rats treated with LIN.

Renal histology

Under light microscopy (100X magnification), kidney sections from CON and CON+LIN showed normal glomerular and tubular histology (Figs. 7a and 7d). In diabetic rats, interstitial inflammatory cell
infiltrate and necrosis were observed in a part of the glomeruli (Fig. 7B). However, LIN treatment protected the kidney by reducing glomerular and tubular changes (Fig. 7C).

Figure 5. Representative immunoblots for TGF-β1 in kidney total cell lysate and densitometry analysis of western blots (Fig. B). Data are expressed as relative intensity after normalizing with β-actin and are mean ± SD of three independent experiments. CON, control rats; DIA, diabetic rats; DIA+LIN, diabetic rats treated with LIN (25 mg/kg b.w./day); CON+LIN, control rats treated with LIN; *compared to CON group; #compared to DIA group (P<0.05).

Figure 6. Reverse transcriptase-PCR analysis of TGF-β1, TNF-α and NF-κB mRNA levels normalized with a housekeeping gene (β-actin). The values are expressed as intensity to β-actin ratio and are means ± SD of three independent experiments. Lanes 1: CON; 2: DIA; 3: DIA + LIN; 4: CON+LIN; *compared with control rats; #compared with diabetic rats (P<0.05).
Figure 7. Histology of kidney. Sections from control rat show normal glomerular and tubular histology (Fig. 7A). Fig. 7B1, Fig. 7B2, Fig. 7B3 are kidney sections of diabetic rat. The arrows show pathologic changes such as capillary size reduction (B1), mesangial expansion (B2), hyaline tubular casts in the lumen of the tubules, segmental glomerulonephritis and necrosis (B3). Administration of LIN reduced these changes in the glomeruli (Fig. C) as compared to LIN-unsupplemented diabetic rats. Fig. 7D is a section from LIN-treated control animals showing normal histology.
DISCUSSION

STZ-induced diabetic rodent model is considered a good one for screening antidiabetic agents (36) and is widely used by researchers. In the present study, STZ-induced rats exhibited hypoinsulinemia and hyperglycemia. These changes persisted throughout the experimental period of 4 weeks. LIN reduced the plasma glucose level by increasing insulin levels. The antihyperglycemic effect of LIN could be due to the stimulation of β-cells and subsequent release of insulin and activation of insulin receptors. Potentiating of pancreatic secretion of insulin was clearly evidenced by the increased level of insulin in diabetic rats treated with LIN. In addition, we observed that LIN could promote glucose utilization by cells since LIN promoted glucose uptake in the diaphragm. This property of LIN could be a contributing mechanism for its antidiabetic activity.

In the present study, diabetes was associated with polydipsia, loss of body weight and hypertrophy of the kidney. LIN administration gave rise to a decrease in daily water consumption and food intake and final kidney weight. This could be due to better control of the hyperglycemic state by LIN.

Elevation of lipids plays a pivotal role in the induction and progression of DN. This contention is supported by the marked increase in cholesterol, TG and FFA in plasma and kidney of diabetic rats observed in this study. There is evidence that circulating lipids bind to and become trapped by ECM where they undergo oxidation, increasing the formation of ROS, which may deteriorate the structure and function of diabetic kidney (37). Treatment with LIN prevented lipid changes and this could be one mechanism for its renoprotective action. Diabetic rats showed decreased kidney function and glomerular filtration as shown by albuminuria and alterations in the renal function parameters. However, LIN supplementation reduced the levels of non-protein nitrogen constituents and glomerular filtration.

The present findings also suggest oxidative changes in the kidney of diabetic rats. This can damage the renal architecture and create an environment for the induction of nephropathic changes. However, co-administration of LIN reduced the oxidative injury by markedly reducing the levels of TBARS and LHP with concomitant elevation in renal antioxidant levels. Çelik and Özkaya (38) report that intraperitoneal administration of LIN reduces H2O2 induced changes in guinea pig brain by its antioxidant property.

Increase in protein carbonyl and nitrosothiol content in STZ-induced rats suggest protein modification by oxidation and nitration. Peroxynitrite could cause nitration of free tyrosine residues and cellular proteins, with a subsequent loss of protein structure resulting in reduction of the kidney function (39).

Consistent with other studies (40,41), we also found increased expression of TNF-α in rat kidney. LIN treatment reduced the rise in TNF-α levels. TNF-α, by binding to its receptor, activates the inflammatory response by influencing the expression of transcription factors and an array of inflammatory mediators (42). Moreover, TNF-α has been shown to contribute to sodium retention and renal hypertrophy in diabetic rats (41).

IL-6 mediates the development of renal injury by inducing mesangial cell proliferation and fibronectin expression (43). Kidney biopsies from patients with nephropathy stain positive for IL-6 mRNA and there is positive correlation of IL-6 mRNA with albuminuria and mesangial expansion (44). Although we did not quantitate the mRNA abundance, we noted a rise in plasma levels of IL-6 in diabetic rats, which were reduced in LIN-treated diabetic rats. In this regard, the anti-inflammatory activity of LIN has been evidenced by its ability to reduce the carrageenan-induced paw edema in rats (17).

LIN reduced TGF-β1 levels significantly compared to diabetic rats. The observed beneficial LIN effect on renal function can therefore be related to suppression of augmented TGF-β1 signaling, which in turn may be attributed to its ability to reduce hyperglycemia. The increase in TGF-β1 mRNA in diabetic kidney could be mediated by prolonged hyperglycemia, since glucose induces TGF-β1 production in cultured mesangial cells (45). Further, TNF-α could promote transcription of TGF-β1. TGF-β1 binds to type II receptor and activates the Smad pathway that stimulates the genes encoding...
several matrix molecules (46). Activation of TGF-β₁ in kidney has been implicated in renal injury and mesangial expansion (47). Several clinical studies have demonstrated a rise in glomerular TGF-β₁ expression (48) and urinary levels that correlate with the severity of DN (49) and glycemic control (50). One limitation of this study is that the PCR results are semi-quantitative and data have to be verified with real-time PCR.

We also measured the expression of NF-κB in kidney since growing evidence suggests that NF-κB is involved in diabetic disease progression and an increase in nuclear translocation of NF-κB has been demonstrated (51). NF-κB gets activated in diabetic state by glucose-induced ROS production resulting in the expression of a number of genes that encode inflammatory factors such as cytokines and adhesion molecules (52). These products have been documented to play a vital role in the pathogenesis of renal diseases. In the present study, LIN reduced the overexpression of NF-κB, which in turn reduced the expression of other cytokines. The mechanism by which LIN prevents the overexpression of NF-κB is not known and needs to be investigated. One possible explanation could be the reduction of elevated blood glucose and subsequent oxidative stress by LIN.

The renoprotective nature of LIN is also supported by histologic studies. The major pathologic alteration observed in diabetic kidney was the interstitial chronic inflammatory infiltrate in the interstitial cells. Diabetic kidney sections also revealed hyaline tubular casts in the lumen of the tubules and necrosis in the glomeruli. Concurrent administration of LIN markedly reduced the glomerular and tubular changes in diabetic rats.

AG is suggested to have higher chemical reactivity than proteins and thus spare proteins from glycation. Delay in development of diabetic complications by AG through the interaction of its nucleophilic hydrazine group with carbonyl groups of reducing sugars or dicarbonyl intermediates has been shown in STZ-treated animals (53). Interestingly, we also found LIN to possess antiglycating efficacy, which was higher than AG.

Thus, the present investigation imparts new information on the renoprotective action of LIN in diabetic rats, associated with reduction of oxidative stress, lipid levels, protein glycation and inflammation. These effects might stem from the antidiabetic potential of LIN, since hyperglycemia is the causative factor for the pathogenesis and progression of DN. Further studies are necessary to examine the molecular interactions of LIN in vivo to establish remission of DN by LIN. Overall, LIN holds promise as an adjunct for diabetic therapy.

Acknowledgment

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