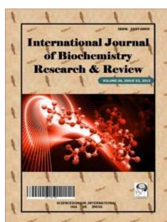

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Mechanistic Insight of Transient Receptor Potential Melastatin 8 Role in Streptozotocin Induced Diabetic Nephropathy

Doaa Hussein Zineldeen[1*], Naglaa Ibrahim Sarhan[2], Nahid Mohamed Tahoon[3] and Rania El-Sayed Wasfy[4]

[1]*Department of Medical Biochemistry, Faculty of Medicine, Tanta University, Tanta, Egypt.*
[2]*Department of Histology, Faculty of Medicine, Tanta University, Tanta, Egypt.*
[3]*Department of Physiology, Faculty of Medicine, Tanta University, Tanta, Egypt.*
[4]*Department of Pathology, Faculty of Medicine, Tanta University, Tanta, Egypt.*

Authors' contributions

This work was carried out in collaboration between all authors. Author DHZ designed the study, performed biochemical and molecular biology experiments, drafted and revised the paper. Author NIS performed electron microscopy. Author NMT was involved in animal work and data analysis. Author RESW performed the immunohistochemistry and light microscopy. All authors have read the final paper and approved the submission.

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ABSTRACT

Background: The transient receptor potential melastatin 8 (TRPM8) channel is a cold-sensing non-selective cation channel involved in cellular proliferation and signaling, yet its role in diabetic nephropathy (DN) remains poorly characterized. The TRPM8 agonist, geraniol (GE) is a dietary acyclic monoterpene alcohol known for its anti-oxidant, hypoglycemic and renal chemoprotective potentials. This study aims at defining the role of TRPM8 via the use of its agonist GE in an animal model of diabetic nephropathy.
Methods: A total of 80 male Wistar rats were equally divided into 4 equal groups: control, GE sham, diabetic rats received a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg)

**Corresponding author: E-mail: zineldeen@gmail.com, Doaa.eldeen@med.tanta.edu.eg;*

and STZ+GE diabetic rats received GE orally at a dose of (100 mg/kg/day). Indices of DNA damage, 8- Hydroxyguanosine (8-OHdG), nephrotoxicity and metabolic derangement parameters were measured eight weeks after diabetes induction. Real-time PCR was performed to detect mRNA expression levels of renal TRPM8 and podocyte marker. Renal histopathological and ultrastructural changes were recorded. Western blotting and immunohistochemistry were performed to determine TRPM8 protein expression.

Results: Diabetic rats displayed downregulation of TRPM8 mRNA and protein expression levels in renal tissues. Upon administration of GE, biochemical, ultrastructural and oxidative stress findings were significantly improved in treated diabetic animals compared to control groups and coincided with upregulation of renal TRPM8 expression as well as enhancement of podocytopathy.

Conclusion: The present study revealed that the ameliorative effect of GE in DN is TRPM8 mediated and highlights a mechanistic role of TRPM8 and its agonists in management of diabetic renal complications.

Keywords: Geraniol; streptozotocin; TRPM8; oxidative stress; diabetic nephropathy.

ABBREVIATIONS

STZ : Streptozotocin
DN : Diabetic Nephropathy
TRPM8 : Transient Receptor Potential Melastatin 8
VEGF : Vascular Endothelial Growth Factor
ESRD : End Stage Renal Disease
8-OHdG : 8- Hydroxyguanosine

1. INTRODUCTION

Diabetic nephropathy (DN) is considered one of the most common and serious complication of diabetes and a major cause of end-stage renal disease (ESRD) [1]. DN is recognized as one of the major podocyte-associated diseases [2]. Its hallmarks comprise glomerular hypertrophy, glomerular basement membrane (GBM) thickening and eventually proteinuria [3]. Intracellular calcium signaling plays a robust pathogenic mechanisms in podocytopathy and DN [4]. Vascular endothelial growth factor (VEGF) is mainly secreted by podocytes and endothelial cells and is considered as a fundamental regulator of normal and abnormal angiogenesis [5]. Various VEGF signaling pathways have critical effects on podocyte physiology and integrity of its cytoskeleton [6]. The transient receptor potential melastatin 8 (TRPM8) is a cold-sensing non-selective calcium permeable cation channel that plays a pivotal role in regulating the cell cycle and Ca^{2+} signalling [7]. Evidence from other systems, however, has shown that TRPM8 channels are also localized to intracellular compartments, where they may act as calcium release channels [8]. TRPM8 is expressed in a subset of normal tissues including urinary bladder, urogenital tract and temperature-sensing neurons. Moreover, TRPM8 is overexpressed in a variety of tumors particularly prostate and pancreatic ones and is considered crucial for cell cycle progression [9]. Some TRP channels are expressed in kidney and are involved in various types of nephropathies [10]. TRPM6, (TRPM family member) which is localized to the apical membrane of distal tubules has been reported to be downregulated in experimental diabetic nephropathy [11]. TRPM8 role has been well characterized in the context of neuropathy, where spatiotemporal plasma membrane dynamics of TRPM8 control the electrical activity of cold sensitive neurons [12]. However, a role for TRPM8 in diabetic nephropathy has yet to be clarified [13]. TRPM8 knockout mice have increased rates of insulin clearance and degradation compared to wild type ones, revealing a novel role of TRPM8 as a regulator of insulin homeostasis [13]. Additionally, TRPM8 is expressed in brown adipose tissue and its activation can prevent obesity [14].

Different plant-derived natural products with variable potencies have been identified as TRPM8 agonists, including menthol, eugenol, neferine, eucalyptol and geraniol [15]. Geraniol (GE) (trans-3,7-Dimethyl-2,6-octadien-1-ol) is an acyclic monoterpene alcohol normally isolated from *Cymbopogon flexuosus* and also presents in essential oils of various herbs and food additives [16]. Geraniol is as a TRPM8 agonist with structural similarity to menthol in a dose relevant and cell type specific manner [17,18]. Geraniol has been reported to have a plethora of therapeutic potentials as an antioxidant, hypoglycemic, hypolipidemic, anti-inflammatory, antiproliferative and antiangiogeneic [19,20]. Geraniol has been implicated to control multiple signaling pathways that are involved in diverse

biological processes, such as cellular proliferation, apoptosis, autophagy and metabolism [21].

However, its role in alleviating diabetic nephropathy has been reported in terms of hypoglycemic and antioxidant potentials [22]. TRPM8 expression has been well characterized in the context of diabetic neuropathy, [12]. Nonetheless, a role for TRPM8 in DN has yet to be elucidated. Therefore, it is intriguing to investigate the potential role of TRPM8 in an animal model of diabetic nephropathy before and after administration of its agonist geraniol.

2. MATERIALS AND METHODS

2.1 Chemicals

Streptozotocin (STZ) (PubChem CID: 29327), Geraniol (GE) 98% purity (PubChem CID: 637566) and all other chemicals and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of high analytical grade.

2.2 Animals

Adult male Wistar rats weighing approximately 160- 180 g were obtained by the Faculty of Medicine Tanta University and acclimatized under laboratory conditions for 1 week prior to experiments. Rats were housed in polyplastic cages with steel wire tops and maintained under standard conditions of temperature (23±2℃) and humidity (50±10%) with (12/12 h light/dark cycle) and allowed access to commercial rodent chow and tap water *ad-libitum*.

All animal protocols were approved by the Ethical Committee of Faculty of Medicine, Tanta University, Egypt with the number 31210/11/16.

2.3 Experimental Design

Animals were allocated into 4 equal groups (n= 20); group I (normal control group) rats received neither STZ nor GE, only vehicles; group II (GE treated sham group) rats received only GE by oral gavage at a dose of (100 mg/kg body weight/day) in vegetable oil (where GE was dissolved in corn oil at a dose of 2.5 ml/kg body weight/day); group III (STZ-induced diabetic Group) overnight fasted rats received a single dose of streptozotocin (STZ), 65 mg/kg body weight, intraperitonally) in 0.1 M cold citrate buffer (pH 4.5); group 4 (GE treated diabetic group abbreviated as STZ+GE) rats received GE orally at a dose of (100 mg/kg body weight/day) in vegetable oil for 8 weeks starting from the 4th day after STZ injection. After 3 days of induction, rats with marked hyperglycemia (fasting blood glucose >250 mg/dl) were selected as diabetic group that was left for 8 weeks untreated to induce early diabetic nephropathy. Other groups were injected with equivalent amounts of citrate buffer as a vehicle. Blood glucose levels were monitored every other day from the tail vein.

At the end of the experimental period, animals were placed in individual metabolic cages for 24h to collect urine samples that were purged of air and stored at -80°C.

After an overnight fast, all animals were weighted, and sacrificed. Plasma/serum samples were obtained by centrifugation at 3000 *g* for 10 min, aliquoted and stored at -80°C for further assays. Tissues were weighted, snap frozen in liquid nitrogen and stored at -80°C till analysis.

For microscopy, tissues were fixed in 10% neutral-buffered formalin, 2.5% gluteraldehyde and/or 4% paraformaldehyde (PFA).

2.4 RNA Extraction, cDNA Synthesis and Real Time PCR

Total RNA was extracted from renal tissues using Isogene (Nippon Gene, Toyama, Japan) according to manufacturer's instructions. RNA was stored at -80℃ until use. 5 µg of total RNA was subjected to first strand cDNA synthesis in using the random hexamers and M-MuLV reverse transcriptase of Viva 2-Steps RT-PCR kit (#RTPL12, Vivantis, USA) according to manufacturer's instructions. The resulting cDNA was amplified using Power SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA) and sequence specific primers for TRPM8 and VEGF-A. PCR condition was set as follows: a single cycle of DNA polymerase activation for a 10 min hold at 95℃ followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min for annealing and 60℃ for 1 min for extension, followed by a melting curve analysis to confirm the amplicon specificity. Real-time PCR assays were run on a Rotor-Gene Q 6plex machine (Qiagen, Valencia, CA, USA). Relative gene expression was calculated by the Rotor-Gene Q series software

2.0.3 (Qiagen, Valencia, CA, USA) using the comparative threshold (Ct) method [23] normalized against the housekeeping gene GAPDH that was used as endogenous control in all experiments.

Sequences of primers used for Real time-PCR are detailed in (Table 1). Sequence specific primers were designed by Primer3 software [24].

2.5 Biochemical Assays

Enzyme linked immunosorbent assay (ELISA) was used to detect plasma Insulin levels by RayBio® Rat Insulin ELISA Kit (Ray RayBiotech, Inc., Norcross, GA). Fasting Blood Sugar (FBS) was measured by the oxidase method (Biodiagnostic, Egypt), total lipid profile including total cholesterol (TC), triglycerides (TG) and HDL-cholesterol (HDL-Ch) were measured by enzymatic-colorimetric methods (Biodiagnostic, Egypt). LDL cholesterol (LDL-Ch) concentration was calculated according to Friedewald equation [25].

Urinary albumin, plasma protein, blood urea nitrogen (BUN), uric acid and creatinine were estimated using commercial kits (HUMAN Diagnostics, Wiesbaden, Germany) according to supplier's instructions.

2.6 Pro-oxidant/ Antioxidant Status Assessment

For determination of 8-OHdG (8-Hydroxyguanosine), urine samples were centrifuged at 2,000 g for 20 min, and the supernatant was used for the by a standard sandwich ELISA kit (Chongqing Biospes Co., Ltd, Chongqing, China) according to supplier's instructions. Levels of Malondialdehyde (MDA) and superoxide dismutase (SOD) enzyme activity in renal tissues were assayed by available commercial kits (Biodiagnostic, Egypt).

2.7 Antibodies, Homogenization and Western Blotting

Antibodies used in this study were as follow: anti-TRPM8 (#KM060, TransGenic Inc., Kobe, Japan), anti-TRPM8 (#ab109308, Abcam, Cambridge, UK), and anti- β actin (#8226; Abcam). Total protein extracts were prepared by homogenization of tissues into a lysis buffer containing (50 mM Tris-HCl pH7.6, 250 mM NaCl,1% Triton X-100, 0.5% Triton X100, 3 mM EDTA, 3 mM EGTA, 10% glycerol, 2mM DTT, 1mM PMSF and 1mM sodium orthovanadate), supplemented with mini complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by Bradford method [26] with bovine serum albumin as a standard. Total proteins of 25 µg were used after boiling it with Laemmli buffer (4% SDS, 20% glycerol, 120 mM Tris [pH 6.8]) at 95℃ for 5 min. Proteins were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane (GE Healthcare, Piscataway, NJ, USA). Blots were blocked with 5% skim milk in 0.1% TBST buffer (20 mM Tris HCl [pH 7.5]), 48 mM NaCl, 0.1% (v/v) Tween 20) for 1 h at room temperature prior to incubation with anti-TRPM8 and anti- β-actin primary antibodies, at a 1:1000; 1:5000 dilutions in blocking buffer; respectively at 4℃ for an overnight. Following three washes in TBST, blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies (MBL, Nagoya, Japan; diluted in blocking buffer), as appropriate, for 1 h at room temperature. Protein bands were developed using chemiluminescence (GE Healthcare, Piscataway, NJ, USA). Western blot bands were scanned and band intensities were identified by densitometric analysis using the image analysis software package; ImageJ version 1.50i. (NIH, Bethesda, MD, USA).

Table 1. Sequences of primers used in the study

Gene	Accession number	Nucleotide sequence	Amplicon (base pairs)
Rat TRPM8	NM_134371.2	F 5` -GAAGCCCATTGACAAGCACAAG-3` R 5`-ACGAAGACCAGGGCATAGAG-3`	189
Rat VEGF-A	NM_031836.3	F 5`-AGGAAAGGGAAAGGGTCA-3` R 5`-ACAAATGCTTTCTCCGCT-3`	98
Rat GAPDH	NM_017008.4	F 5`-TCAACTACATGGTCTACATGTTCCAG-3` R 5`-TCCCATTCTCAGCCTTGACTG-3`	113

2.8 Immunohistochemistry and Electron Microscopy

Hematoxylin and Eosin (H&E) and electron microscopy (EM) preparation was performed as previously described [27].

For TRPM8 staining 4 µm thick sections were incubated with anti-TRPM8 antibody at (1:100 dilution). Peroxidase anti-peroxidase was used as a secondary reagent (DakoCytomation, Cambridge, UK) and 3,3-diaminobenzidine/ H_2O_2 as a substrate. Slides were viewed by (Leica Imaging System LTD., Cambridge, UK). Immunoreactivity of TRPM8 appeared as brown staining of varying degrees of intensity. EM was performed by a transmission electron microscope (JEOL, Tokyo, Japan). Morphometric analysis was carried out to evaluate the foot process effacement on 80kV electron micrographs by using ImageJ version 1.50i. The width of the foot process was calculated according to [28].

Five measurements were taken for each ultrathin section of each group.

2.9 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism 6.0 software (GraphPad Inc, San Diego, California, USA). Results are presented as means ± s.e.m (standard error of the mean). Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test. Nonparametric data were analyzed using Kruskal-Wallis test followed by Dunn's post-hoc test. Correlations were analyzed using Pearson's correlation coefficients. Statistical significance was considered when the *P* value was ≤ 0.05.

3. RESULTS

3.1 Effects of Geraniol on Body and Kidney Weight and Glycemic Indices

Rats injected with STZ developed type 1 diabetes characterized by the significant increase in plasma glucose level and decrease in plasma insulin level (Fig. 1 A, B). Diabetic rats experienced a significant decrease in body weight as well as a significant gain in kidney weight (as a marker of diabetic nephropathy) (Fig. 1 C, D) when compared to normal rats. However, GE supplementation significantly decreased plasma glucose levels, improved plasma insulin and decreased renal hypertrophy (Fig. 1 A-D) strengthening the described hypoglycemic effect of GE.

3.2 Effects of GE on STZ-induced Nephrotoxicity

STZ-induced diabetic rats exhibited significant alterations in nephrotoxicity markers where, urinary albumin, plasma uric acid, creatinine and plasma BUN showed significant elevation (Fig. 1 E-H). However, GE treatment efficiently reduced those alterations and appeared to act as a renoprotective agent in DN.

3.3 Effects of GE on Lipid Profiling and DNA Damage Indices

STZ- induced diabetic rats exhibited significant dyslipidemia, 8 weeks GE treatment weeks significantly restored lipid profiling to near control levels (Fig. 2 A-D). In our present study, nephrotoxicity was associated with imbalance between kidney antioxidant/pro-oxidant status with heightened oxidative stress that was evident by significant increase in lipid peroxidation product MDA, urinary oxidative stress marker 8-OHdG excretion levels and decrease the antioxidant activity of SOD (Fig. 2 E-G). Treatment with GE effectively decreased the alterations in these oxidative stress related parameters revealing GE as a good antioxidant tool that protects rat kidney from diabetes-induced oxidative damage.

3.4 Geraniol protects from STZ-induced Renal Injury

Histological examination of H&E stained sections from diabetic kidney showed characteristic diabetic nephropathy in the form of increased glomerular size, lobulation, degenerative changes in the proximal tubules, cellular infiltration and areas of hemorrhage, (Fig. 3c) relative to the preserved renal structures from the non-diabetic groups (Fig. 3 a, b). These alterations were effectively decreased post treatment with GE (Fig. 3d). This observed results conferred a protective action of GE in diabetic renal injury.

3.5 Analysis of TRPM8 Expression in STZ-induced Diabetic Rats

In order to explore that GE observed effects were TRPM8 mediated we investigated the expression

of TRPM8 mRNA in kidney of diabetic rats at 2 and 8 weeks intervals. Following 2 weeks of STZ- injection, renal TRPM8 mRNA expression was downregulated as compared to other groups. However, that reduction was not of statistical significance (Fig. 4 A).

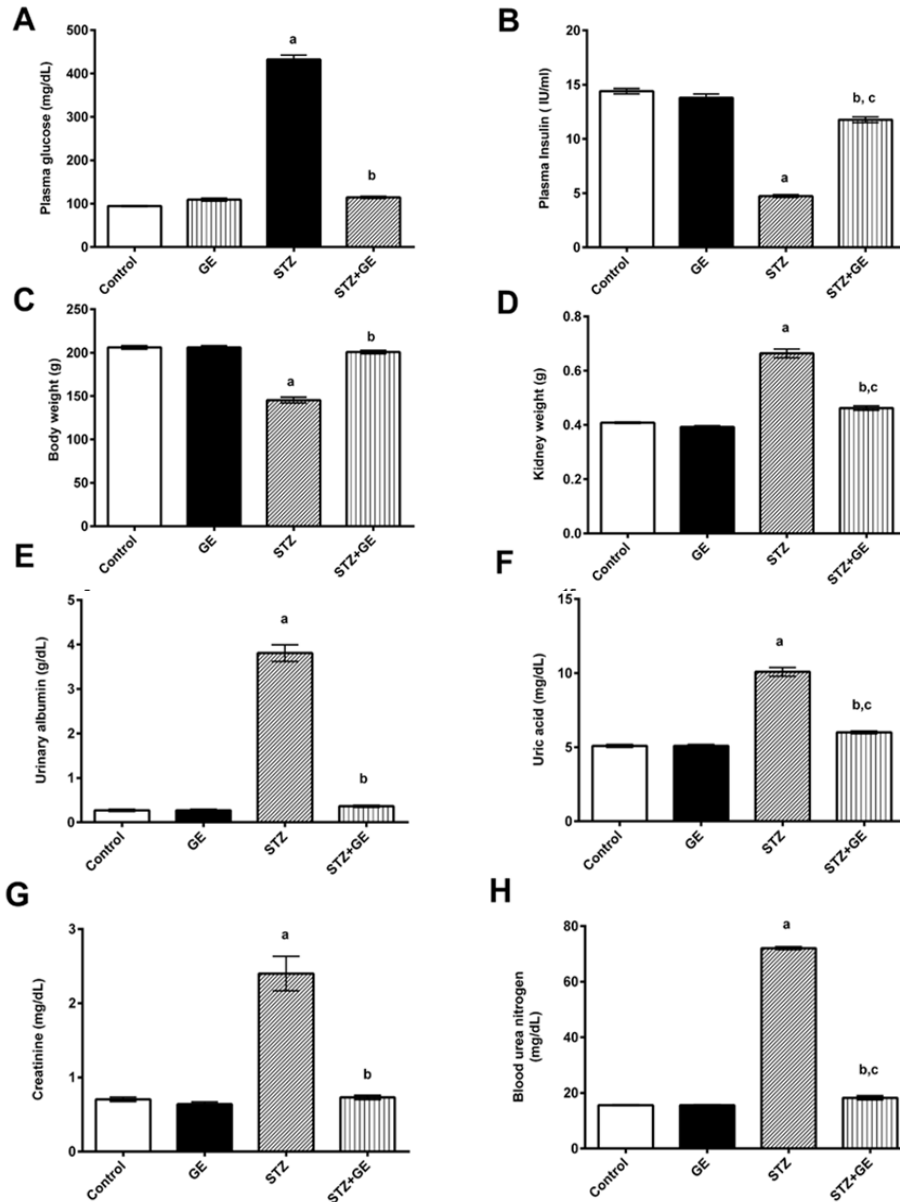

Fig. 1. Effects of geraniol on glycemic and nephrotoxic indices of STZ-induced diabetic rats

Control: normal control; GE: treated with geraniol; STZ: STZ-induced (diabetic); STZ+GE: GE treated post to STZ induction (A) plasma glucose, (B) Plasma Insulin (C) body weight, (D) kidney weight, (E) urinary albumin, (F) uric acid, (G) creatinine, (H) blood urea nitrogen. Data present mean ± s.e.m., n= 20. "a" indicates significant difference between the normal control and STZ-induced groups, "b" indicates the significant difference between STZ-induced and GE treated groups and "c" indicates the significant difference between the STZ+GE group and normal control group using ANOVA followed by Tukey's post-hoc test. ($P^a < 0.0001$, $P^b < 0.0001$, $P^c < 0.0001$)

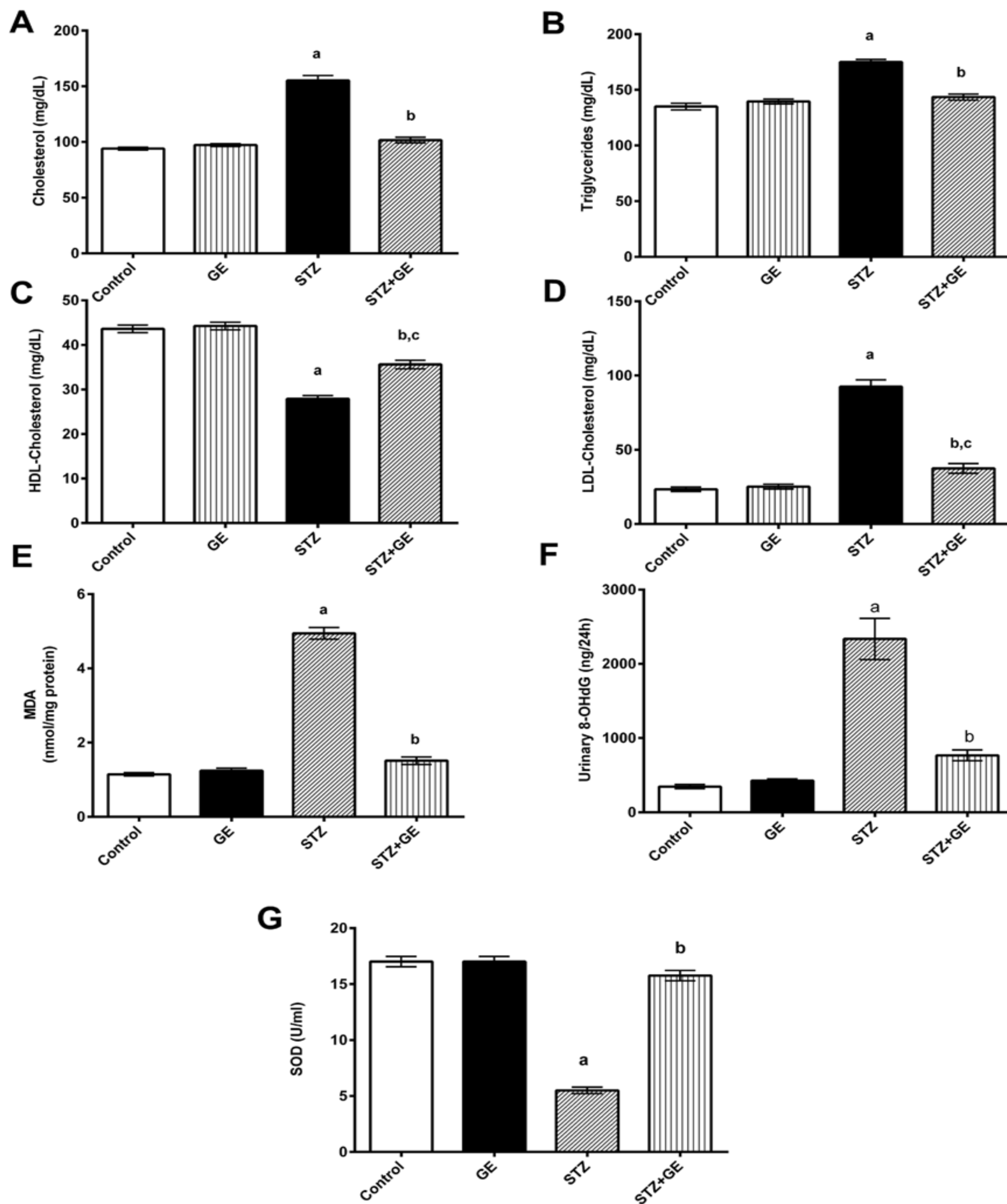

Fig. 2. Effects of geraniol on lipid profiling and DNA damage indices of STZ-induced diabetic rats

Control: normal control; GE: treated with geraniol; STZ: STZ-induced (diabetic); STZ+GE: GE treated post to STZ induction (A) Cholesterol, (B) Triglycerides, (C) HDL-Cholesterol, (D) LDL-Cholesterol, (E) MDA, (F) urinary 8-OHdG, (G) SOD. Data present mean ± s.e.m., n= 20. "a" indicates significant difference between the normal control and STZ-induced groups, "b" indicates the significant difference between STZ-induced and GE treated groups and "c" indicates the significant difference between the STZ+GE group and normal control group using ANOVA followed by Tukey's post-hoc test. ($P^a < 0.0001$, $P^b < 0.0001$, $P^c < 0.05$). HDL: high density lipoproteins; LDL: low density lipoproteins; MDA: malondialdehyde; 8-OHdG: 8- Hydroxyguanosine; SOD: superoxide dismutase

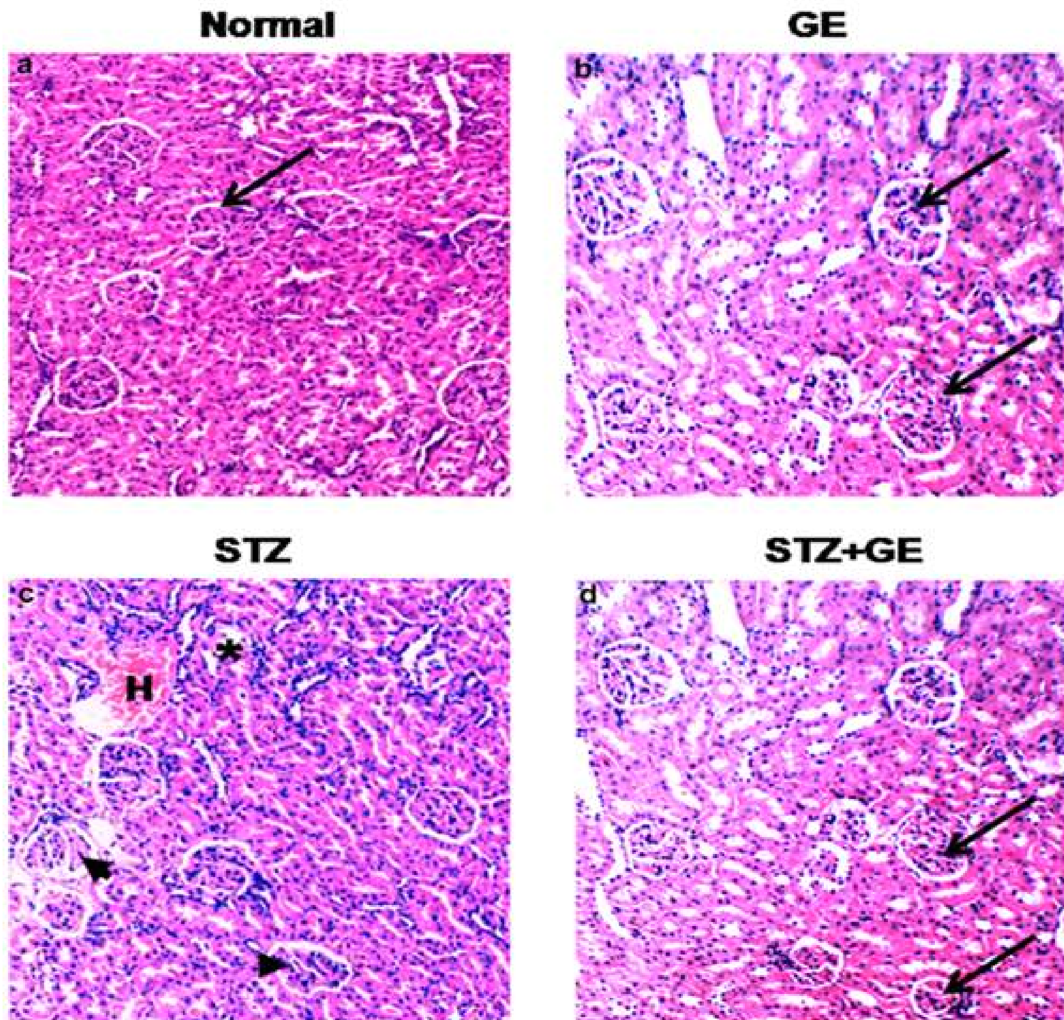

Fig. 3. Effects of geraniol on STZ-induced diabetic nephropathy in rats

Photomicrographs of rat kidney stained with H&E (X100) showing (a) normal control group with normal renal architecture (b) GE only treated group with preserved renal architecture, (c) STZ-induced diabetic group showing massive cellular infiltration (*), areas of interstitial hemorrhage (H) and lobulated glomeruli (double arrows) (d) GE treated diabetic group with improved renal architecture. Black arrows represent normal sized glomeruli

At 8 weeks after diabetes induction there was a significant decrease of TRPM8 mRNA expression of the kidney relative to the control group (Fig. 4B). Meanwhile, GE treatment for 8 weeks significantly increased the altered renal TRPM8 mRNA expression, when compared to diabetic rats (Fig. 4B). Additionally, TRPM mRNA expression in pancreatic tissues from diabetic and non diabetic groups were examined by RT-PCR, where diabetic animals showed decreased pancreatic TRPM8 mRNA expression levels when compared to non-diabetic ad treated groups ($P < 0.0001$). Geraniol treatment significantly upregulated the pancreatic TRPM8 expression levels (Fig. 4D). Renal TRPM8 mRNA expression correlated negatively with the glycemic status and renal VEGF mRNA levels. It exhibited significant positive correlations with plasma insulin levels and the antioxidant SOD enzyme levels (Table 2). We furtherly confirmed renal TRPM8 protein expression levels by western blotting followed by densitometry, that showed significant reduction of TRPM8 levels in renal tissues from diabetic rats relative to control ones.

TRPM8 protein expression levels were increased 8 weeks following GE treatment to reach about 90% of controls (Fig. 5 A, B). Immunohistochemistry of kidney sections

demonstrated decreased immunoreactivity of TRPM8 in sections from diabetic rats relative to control ones (Fig. 5 C, a). GE administration for 8 weeks efficiently increased protein expression of TRPM8 with intense immunoreactivity noted at both distal and proximal renal tubules (Fig. 5C, b).

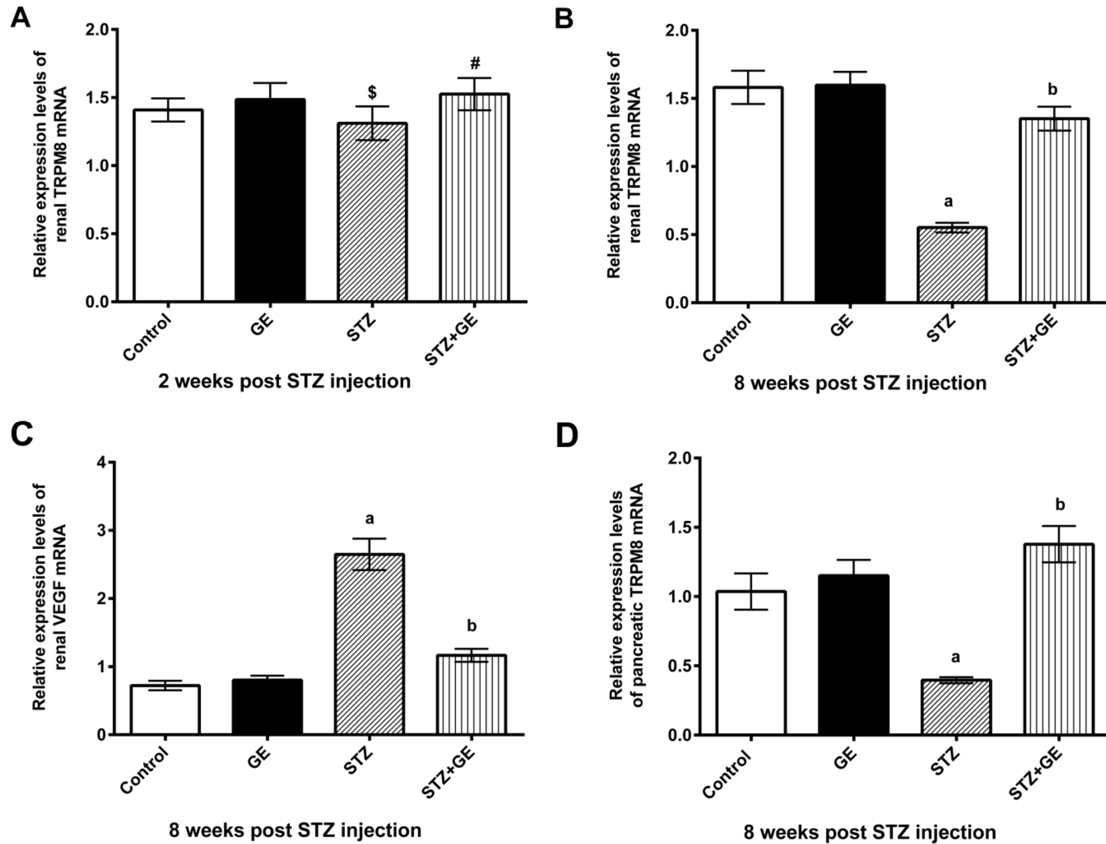

Fig. 4. Effect of geraniol on TRPM8 and VEGF mRNA expression in STZ-induced diabetic rats

Total RNA was extracted from kidney tissues from; control: normal control; GE: treated with geraniol; STZ: STZ-induced (diabetic); STZ+GE: GE treated post to STZ induction, followed by real time PCR. Histogram showing (A) TRPM8 relative mRNA expression at 2 weeks, $ denotes STZ VS control, P= 0.928 and # denotes STZ VS STZ+GE, P= 0.544 (non-significant). (B) TRPM8 relative mRNA expression at 8 weeks (C) VEGF relative mRNA expression at 8 weeks. (D) Pancreatic TRPM8 relative mRNA expression at 8 weeks. GAPDH was used as internal control. Data present mean ± s.e.m., n= 20. "a" indicates significant difference between the normal control and STZ-induced groups, "b" indicates the significant difference between STZ-induced and GE treated groups using ANOVA followed by Tukey's post-hoc test. (P^a< 0.0001, P^b< 0.0001)

Table 2. Correlation of renal TRPM8 mRNA expression with glycemic, angiogenic and antioxidant markers among the studied groups

Variables	Control		GE		STZ		STZ+GE	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Renal VEGF mRNA	-0.7313	0.0002*	-0.7243	0.0003*	-0.7338	0.0002*	-0.7113	0.0004*
Renal SOD (U/mg protein)	0.8319	<0.0001*	-0.4614	0.0406*	0.7206	0.0003*	0.7108	0.0004*
Plasma Insulin (µIU/ml)	0.9027	<0.0001*	0.7083	0.0005*	0.6436	0.0022*	0.7103	0.0004*
FBG (mg/dL)	-0.9266	<0.0001*	-0.5247	0.0175*	-0.7338	0.0002*	-0.7028	0.0005*

Values are Pearson correlation coefficients. Asterisk ;(*) marks significance at P< 0.05
FBG: fasting blood glucose; SOD: superoxide dismutase

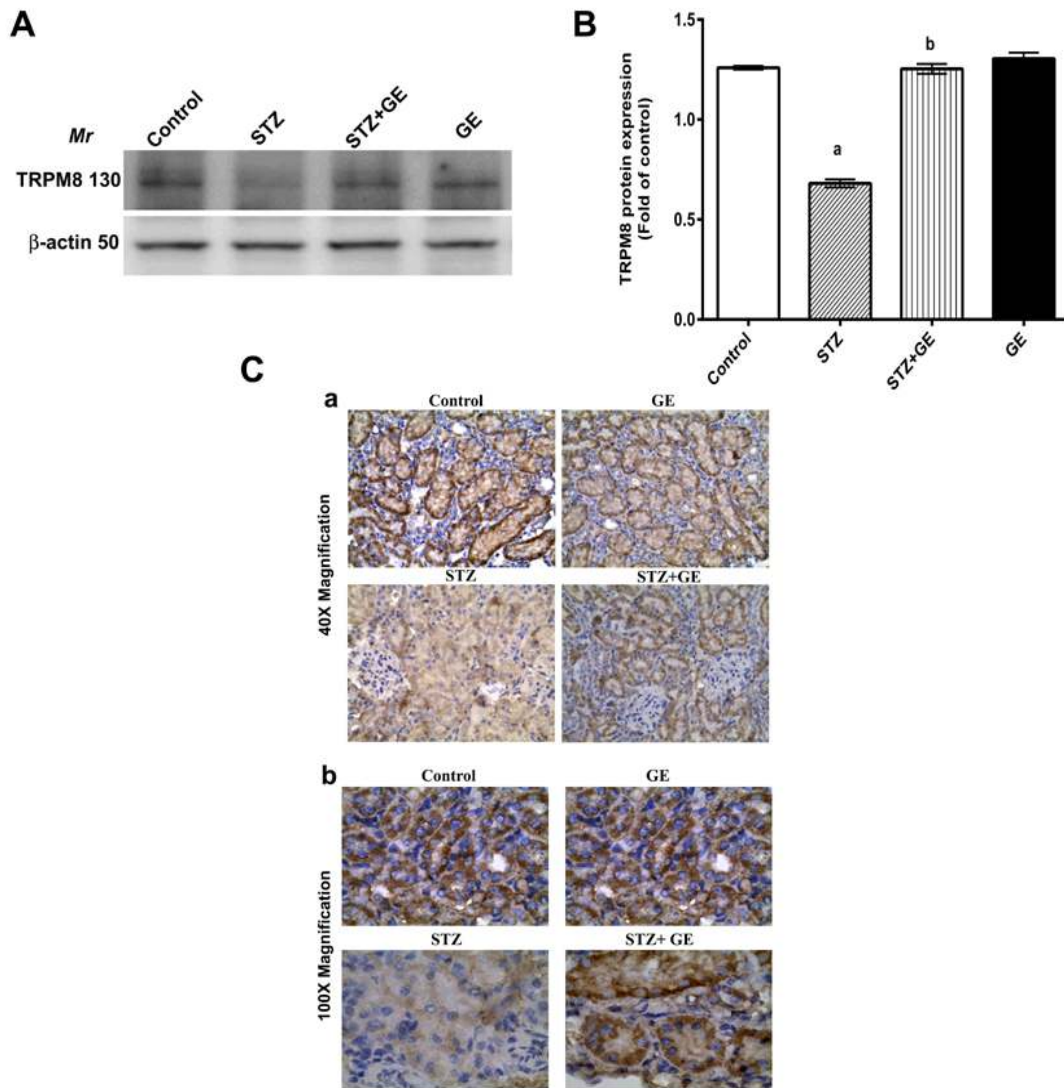

Fig. 5. Decreased TRPM8 protein expression in STZ-induced diabetic rats

(A) Lysates from kidney tissues from control: normal control; STZ: STZ-induced (diabetic); STZ+GE: GE treated post to STZ induction; GE: treated with geraniol, were analyzed by western blotting for TRPM8 protein levels; β-actin was used as a loading control. (B) Bands from (A) were quantified by densitometry, and expressed as a value relative to control (β- actin). Mr; Molecular weight marker in kDA (kilodaltons). Values are presented as means± s.e.m., n= 6, "a" indicates significant difference between the normal control and STZ-induced groups, "b" indicates the significant difference between STZ-induced and GE treated groups using Kruskal-Wallis test followed by Dunn's multiple comparisons test.
($P^a < 0.0001$, $P^b < 0.0001$). (C) Photomicrographs showing immunohistochemistry of rat kidney cortical sections of groups as in (A), stained with anti-TRPM8 (brown), counterstained with Hematoxylin (blue), (a) 40X magnification (b) High power 100x magnification, showing decreased TRPM8 immunoreactivity at renal tubules of diabetic rats, GE treated tubules showing intense TRPM8 immunostaining

3.6 Effects of Geraniol Treatment on Angiogenesis and Podocytes in Diabetic Rats

Next we assessed, whether GE could mitigate the diabetic podocytopathy. Renal vascular endothelial growths factor (VEGF), an angiogenic and podocyte marker, mRNA expression was significantly increased in diabetic rats compared to control ones, (Fig. 4C). Ultrastructural studies by transmission electron microscopic examination of kidney from diabetic animals

displayed podocytopathy, effacement, swelling and/or fusion of foot processes with narrowing of the subpodocytic space (Fig. 6A) as well as significant increase of podocyte foot processes width (FPW) in nm (Fig. 6B) confirming that decreased renal TRPM8 expression was coupled with cytoskeletal derangement and podocytopathy during DN. Eight weeks of GE administration significantly reduced VEGF mRNA expression levels to control levels. Meanwhile, at the ultrastructural level GE+ STZ treated animals revealed restoring of normal structure with preserved podocyte morphology and significant lowering of foot processes width (FPW) in nm (Fig. 6 A, B). Collectively these findings convey an effective role of GE in alleviating diabetic nephropathy via modifying TRPM8 gene expression and remodeling podocytopathy.

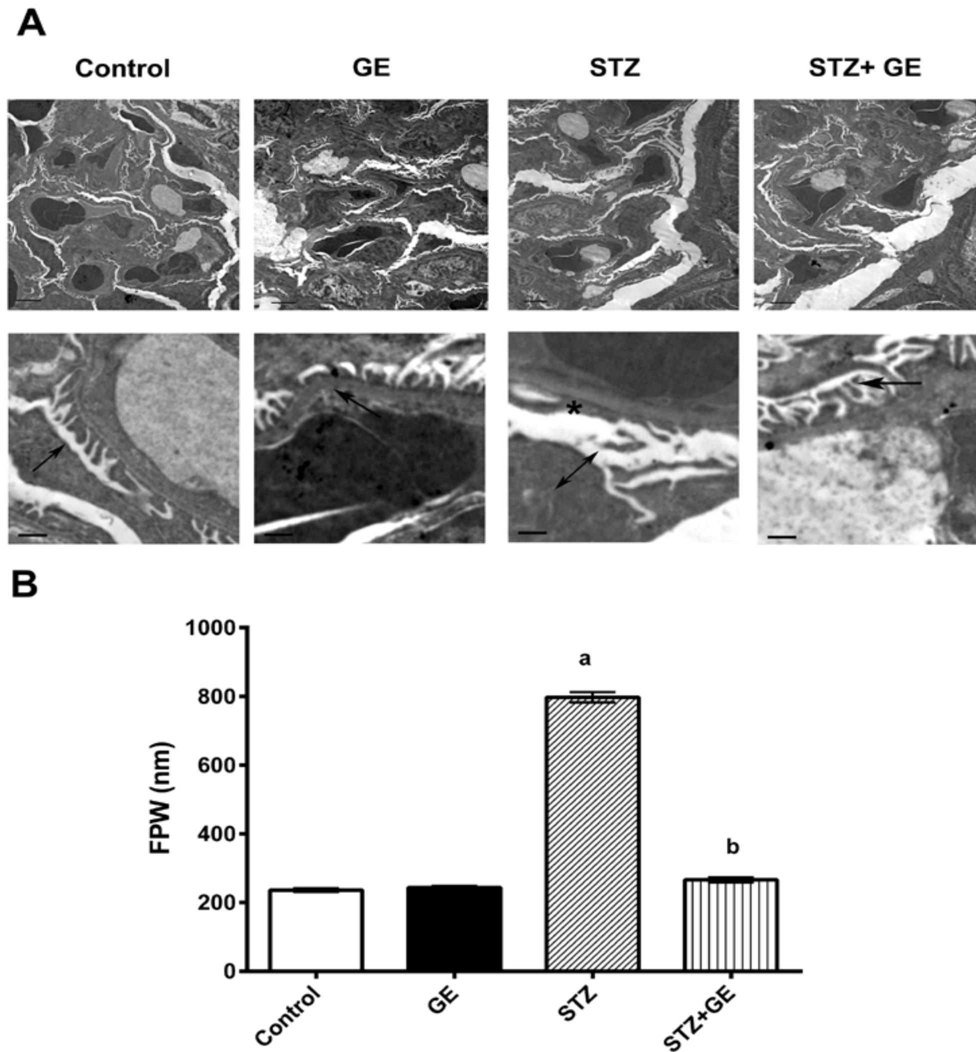

Fig. 6. Effect of geraniol on ultrastructural features of diabetic nephropathy

(A) Photomicrographs of kidney samples from the four experimental groups were examined under transmission electron microscope as indicated. Upper panel; X1000 magnification (scale bar 2 μm), lower panel; X6000 magnification (scale bar 5 μm). STZ group revealed part of Malpighian corpuscle with many blood capillaries (BC) with their covering podocytes. Narrowing and obliteration of subpodocytic (*) space can be seen with focal thickening of the glomerular basement membrane and fusion, effacement and swelling of foot processes (double arrow). Other non-diabetic and treated groups displayed fenestrated blood capillary with its basement membrane of normal thickness covered by regularly spaced foot processes of podocytes (arrow). (B) Quantification of foot process width (FPW) from all indicated groups in (A), data present mean ± s.e.m., (n= 5). "a" indicates significant difference between the normal control and STZ-induced groups, "b" indicates the significant difference between STZ-induced and GE treated groups using ANOVA followed by Tukey's post-hoc test. ($P^a < 0.0001$, $P^b < 0.0001$)

4. DISCUSSION

The molecular mechanism (s) underlying the development of DN is complex and remains ill defined that accounts for the ineffectiveness of currently available interventions [29]. Although geraniol has been reported to ameliorate hyperglycemia, dyslipidemia and oxidative stress in rodent models of T1DM and metabolic syndrome [22,30], its effect on DN and the underlying mechanisms remained largely uncharacterized.

The present study provides novel evidence on the renoprotective potential of geraniol via modulation of TRPM8 expression as documented by improvement of DN biochemical markers and restoring of normal renal architecture. Furthermore, GE mitigates diabetic podocytopathy via downregulation of VEGF and alleviation of the observed heightened oxidative stress. Persistent hyperglycemia drives reactive oxygen species (ROS) generation which is considered as one of the dominant mechanisms driving DN development [31]. Several studies have illustrated the increased lipid peroxidation in clinical and experimental diabetes [22]. This is in agreement with current results that showed increased levels of lipid peroxidation product (MDA), urinary 8-OHdG, as well as decreased SOD activity at diabetic kidney. GE treatment effectively improved glycemic status and insulin levels as well as reduced oxidative stress indices and restored renal SOD antioxidant activity which is attributed to its potent hypoglycemic, renoprotective and antioxidant effect. These findings are coincident with previous reports [22, 32]. It is of note that GE dose used in the present study (100 mg/kg BW) was efficient to induce antioxidant effects, where a prior report had implicated a pro-oxidant potential of GE at higher doses [16]. TRPM8 channel is a cation selective one, with special preference for calcium ions [33]. Although the expression of TRPM8 in many tissues has been reported, to the best of our knowledge, this study is the first to report a role of TRPM8 expression in DN. The results of the present study demonstrated TRPM8 mRNA and protein levels to be downregulated in renal tissues of diabetic rats as compared to controls, 8 weeks following T1DM induction. This is in line with a recent study describing that the reactive metabolite methylglyoxal, a byproduct of glycolysis that is increased in the plasma of diabetic patients, inhibits the activity and reduces mRNA expression of TRPM8 in dorsal root ganglion cells [34].

The mRNA and protein expression of renal TRPM8 was up-regulated after GE administration in diabetic treated animals. Renal TRPM8 mRNA expression positively correlated with the renal antioxidant enzyme SOD. Interestingly, the observed herein oxidative stress did not elicit increased TRPM8 expression in diabetic animals. Consequently, cells treated with H_2O_2 failed to increase TRPM8 activity [35]. In disagreement with our current findings, Nocchi et al. [36] stated that H_2O_2 treatment upregulates TRPM8 expression in aged mouse urothelium. This discrepancy may be tissue specific due to different redox potentials in aging animals. Similarly, increased TRPM8 expression was detected at dorsal root ganglion as well as in bladder tissues from diabetic animals [37,38]. On the other hand Facer et al. [39] did not find any change in TRPM8 expression in skin from diabetic patients; indicating that TRPM8 expression is tissue and cell specific. It is of note that in these contradictory studies TRPM8 expression was not assessed beyond 2 weeks of diabetic induction. In the current study we also could not retrieve a significant downregulation of TRPM8 expression at 2 weeks. Only 8 weeks post-induction and establishment of DN, TRPM8 expression displayed a significant reduction when compared to other studied groups. Therefore, it is seemingly that renal TRPM8 downregulation is linked to the pathogenesis and structural changes of DN.

In addition, our TRPM8 immunostaining results revealed maximum intensity at distal and proximal tubules, inferring a structural and functional role of TRPM8 in normal kidney. The ultrastructural findings of the current study detected characteristic diabetic podocyte dysfunction as previously stated [40]. VEGF has been known to be involved in podocytopathy of DN due to high glucose levels, oxidative stress, dysregulation of podocyte signaling and cytoskeletal remodeling [2,41]. The present study and other ones have detected upregulation of renal VEGF mRNA expression in diabetic rats as compared to control groups [42,5]. On the other hand GE treated diabetic rats showed decreased mRNA expression of VEGF, decreased FPW and alleviation of podocytopathy. In agreement with present data, Carnesecchi et al. [43] and others [44] have reported the ability of dietary monoterpenes to inhibit angiogenesis in animal models and cancer cell lines through apoptosis induction and increasing the expression of the pro-apoptotic proteins. In the present study TRPM8 mRNA levels exhibited a statistically

significant negative correlation with VEGF mRNA levels in kidneys of the studied groups. This is in line with Zhu et al. [45] who described a downregulation of VEGF in prostate cancer cells in response to TRPM8 overexpression. Therefore, one can speculate a disturbed TRPM8/VEGF signaling in DN particularly at podocytes. The current proposed mechanism of GE clarifies the role of TRPM8 in normal renal physiology that is downregulated by hyperglycemia in DN. Mechanistically GE observed action herein is multifactorial, first by, activation of TRPM8 receptors that in turn can regulate their function by modulating their own expression at the plasma membrane via rapid vesicular translocation and fusion [15] particularly, at the renal and pancreatic tissues that can modulate hypoglycemic effects, controlling insulin homeostasis and renal structural integrity. Whether TRPM8 itself can modify other genes in renal transcriptome requires further studies. Second, GE acts through direct transcriptional activation and decrease of DNA synthesis, which influences S phase progression of the cell cycle, thus decreasing renal cell proliferation [46]. Meanwhile, GE can produce evident changes in the resting potential and cell membrane polarization that trigger modifications of membrane-bound proteins activities and alter the intracellular signaling transduction pathways in the corticomedullary tubular cells [19,47,43]. The observed dyslipidemia in the current work can trigger signaling pathways that are involved in the development of DN [48].

GE significantly attenuated the observed hyperlipidaemia in diabetic rats, mostly by modifying the expression of genes involved in lipid metabolism and via TRPM8 activation [49]. Intriguingly, an anti-inflammatory role for TRPM8 has been reported, where TRPM8-deficient mice exhibited aggravated inflammatory conditions [50]. Dyslipidemia and inflammatory markers such as tumor necrosis factor-α (TNF-α) associated well with renal impairment in diabetes [51].

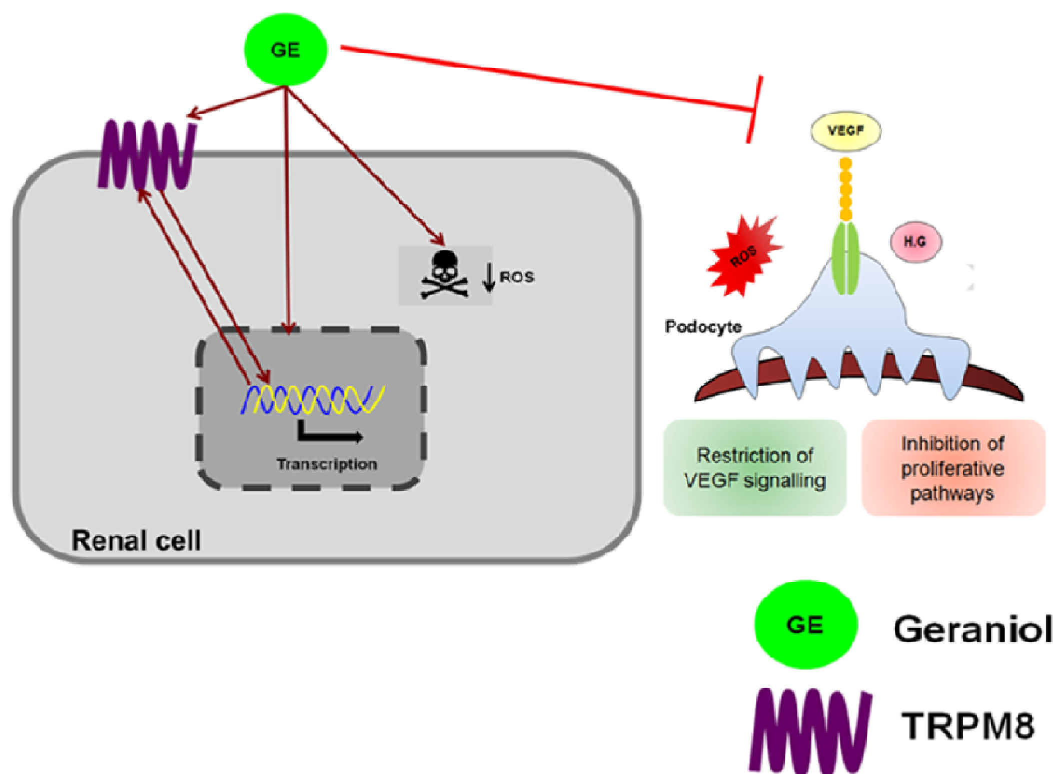

Fig. 7. Proposed model of TRPM8 in diabetic nephropathy

Under normal condition there is restriction of growth factor signaling. Hyperglycemia, ROS, inflammation, VEGF activation and podocytopathy lead to alteration of TRPM8 expression. GE treatment activated and increased TRPM8 expression that in turn increases its own expression as well as decreased VEGF signalling

Based on the ability of TRPM8 to modulate cytokine expression by increasing the expression of the anti-inflammatory cytokine; interleukin-10 and decreasing that of TNF-α [33], a TRPM8 mediated role of GE in immunoinflammation can be concluded.

Other member of the TRPM family, TRPM5 is expressed within the pancreatic islets of Langerhans, where it regulates the frequency of Ca2+ oscillations and contributes to insulin release by β-cells [52]. The present work showed decreased TRPM8 expression in pancreata of diabetic rats that was modulated by GE treatment revealing that the observed hypoglycaemic effect of GE might be TRPM8-mediated.

Furtherly, TRPM8 knockout mice have increased rates of insulin clearance and degradation compared to wild type ones; revealing a novel role of TRPM8 as a regulator of serum insulin [13]. A role of TRPM8 in insulin signaling can't be omitted, since insulin rapidly and directly signals to podocyte and dynamically remodels its cytoskeleton [53]. In the current study renal TRPM8 expression correlated negatively with glucose levels and positively with plasma insulin. Coinciding with this, TRPM8 has been expressed in brown adipocytes and associated with glucose homeostasis; TRPM8 knockout mice developed obesity on high fat diet and failed to respond to menthol induced weight loss suggesting an important role of TRPM8 in molecular signaling of obesity.

Collectively, and as depicted in Fig. 7 diabetic podocytopathy may act on translational or posttranslational pathways to modify renal transcriptome and to determine the progression of nephropathy [54]. Therefore, persistent hyperglycemia, ROS, inflammatory cytokines as well as altered insulin homeostasis would end in activated VEGF signaling, decreased expression of TRPM8, cytoskeletal remodeling and ultimately ESRD.

5. CONCLUSION

The present study demonstrates that GE treatment ameliorated STZ-induced DN in rats via multifaceted molecular mechanisms. Our salient finding is the novel role of TRPM8 in DN. GE increased renal TRPM8 expression, abrogated oxidative stress, decreased VEGF expression and improved podocytopathy.

The exact mechanism of TRPM8 at various types of renal cells warrants future studies to open new avenues for developing new therapeutic targets of DN.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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