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To cite this article: Smeeta Sadar, Dipti Kaspate & Neeraj Vyawahare (2016) Protective effect of L-glutamine against diabetes-induced nephropathy in experimental animal: Role of KIM-1, NGAL, TGF- β 1, and collagen-1, Renal Failure, 38:9, 1483-1495, DOI: [10.1080/0886022X.2016.1227918](https://doi.org/10.1080/0886022X.2016.1227918)

To link to this article: <https://doi.org/10.1080/0886022X.2016.1227918>



Published online: 19 Oct 2016.



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LABORATORY STUDY

Protective effect of L-glutamine against diabetes-induced nephropathy in experimental animal: Role of KIM-1, NGAL, TGF- β 1, and collagen-1

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ABSTRACT

Diabetic nephropathy is a serious microvascular complication and one of the main causes of end-stage renal disease. L-Glutamine (LG) is naturally occurring amino acids with antidiabetic and antioxidant potential. The aim of present investigation was to evaluate the potential of LG against streptozotocin (STZ)-induced diabetic nephropathy (DN) in laboratory rats. DN was induced in male Wistar rats (200–220 g) by intraperitoneal administration of STZ (55 mg/kg). Animals were treated orally with either distilled water (10 mg/kg) or LG (250, 500, and 1000 mg/kg) or Sitagliptin (5 mg/kg). Various biochemical, molecular, and histological (hematoxylin–eosin and Masson's trichrome stain) parameters were assessed. Administration of LG (500 and 1000 mg/kg) significantly inhibited ($p < .05$) STZ-induced alterations in serum and urine biochemistry (urine creatinine, uric acid, albumin, and BUN). It also significantly increased creatinine clearance rate. STZ induced increase in renal oxidative stress was significantly decreased ($p < .05$) by LG (500 and 1000 mg/kg) treatment. Upregulated renal KIM-1, NGAL, TGF- β 1, and collagen-1 mRNA expression after STZ administration was significantly inhibited ($p < .05$) by LG (500 and 1000 mg/kg) treatment. Correlation analysis also revealed that antidiabetic potential of LG attenuates STZ-induced elevated renal KIM-1, NGAL, TGF- β 1, and collagen-1 mRNA expression. Histopathological alteration induced by STZ in renal tissue was ameliorated by LG treatment. In conclusion, results of present investigation suggest that treatment with LG ameliorated STZ-induced DN via the inhibition of oxidative stress as well as downregulation of KIM-1, NGAL, TGF- β 1, and collagen-1 mRNA expressions.

ARTICLE HISTORY

Received 18 April 2016
Revised 20 July 2016
Accepted 18 August 2016
Published online 18 October 2016

KEYWORDS

Diabetic nephropathy;
L-glutamine; oxido-
nitrosative stress; KIM-1;
NGAL; TGF- β 1; collagen-1

Introduction

Diabetic nephropathy (DN) is one of the most serious microvascular complication of type 1 and type 2 diabetes as well as it is one of the main causes of end-stage renal failure and death in diabetic patients.^{1,2} Evidence suggest that more than 30% of all diabetic patients develop DN within 10–20 years after the onset of diabetes.³ In diabetic patients, the incidence of development of microalbuminuria is 20–40% if DN remains untreated. Moreover, within 20–25 years almost 20% of these patients will develop end-stage renal failure⁴ and which further requires renal transplantation or chronic hemodialysis.⁵

The pathogenesis of DN includes hyperglycemia-induced overproduction of free radicals that further activates all pathways involved in the pathogenesis of complications of diabetes.^{1,2} Therefore, elevated oxidative stress played a vital role in induction and

maintenance of DN.⁶ It has been reported that increased blood sugar level caused overproduction of hydrogen peroxide that in turn elevated lipid peroxidation.⁷ It is also responsible for the glycosylation of proteins in blood and tissues, which leads to the production of advanced glycosylated end products (AGEs).^{8,9} Although, factors responsible for DN is not clear, it is argued that increased production of reactive oxygen species, renal polyol formation, advanced gly-cated end-products accumulation, and elevated level of transforming growth factor beta-1 (TGF- β 1) contributes to increased renal albumin permeability and extracellular matrix (ECM) accumulation that leads to elevated proteinuria, glomerulosclerosis, and tubulointerstitial fibrosis.^{10,11}

Available management options for DN are targeted toward glycemic control, but still most of the patients with diabetes prone to developing kidney disease. Consequently, there is an urgent need to identify

additional therapeutic strategies to attenuate the progression of DN in this population. The streptozotocin (STZ)-induced diabetes is well established, widely used, highly reproducible animal model to study diabetic complications including nephropathy, retinopathy, and neuropathy.^{12,13} A single intraperitoneal (ip) administration of STZ leads to the development of DN, which is characterized by changes in both glomerular and tubular structure and function.^{14,15} This model mimics almost all pathobiological features of clinical DN including activation of protein kinase C,¹⁶ deposition of AGEs,¹⁷ mesangial cell activation, and podocyte injury.¹² Thus, STZ-induced DN animal model plays a vital role in the development of therapeutic moieties with antidiabetic potential.

Studies have documented the importance of nonessential amino acids in the management and treatments of various diseases. Glutamine is one of the 20 naturally occurring amino acids in dietary protein, specifically it is a conditionally essential amino acid. It is found very high in dietary meats, eggs, whey protein, and casein protein. Under normal conditions, glutamine serves as a main energy metabolism fuel for intestinal and renal enterocytes. Studies have validated the effectiveness of glutamine supplementation in the reduction of intestinal injuries and improving systemic cell immunity.^{18,19} It is an important precursor for the synthesis of glutathione, which is a non-enzymatic antioxidant. It also plays a vital role in glutathione metabolism as well as maintenance of the cellular redox potential during elevated cellular oxidative stress.²⁰ Accumulating biochemical and clinical evidence support the phenomenon where amino acids exert its antioxidant effects to attenuate oxidative stress-mediated disorders.^{21,22} Furthermore, administration of certain amino acids shown to attenuates DN in experimental animals.^{23–25} Literature lucid with evidence showed that L-glutamine (LG) possesses potent antiulcer, antioxidant, anti-bacterial, cardioprotective, anticancer, hepatoprotective, and anti-apoptotic potential.^{26–29} It has been reported that LG significantly attenuated cisplatin-induced genotoxicity.³⁰ However, its role in DN is yet to unravel. Hence, the aim of the present investigation was to evaluate the potential of LG against STZ-induced DN by assessing various biochemical, molecular, and histological changes in laboratory rats.

Materials and methods

Drugs and chemicals

LG ($\geq 99\%$) and Streptozocin (97%) were purchased from Sigma-Aldrich Co., St Louis, MO.

1,1',3,3'-Tetraethoxypropane, crystalline beef liver catalase, 5,5'-dithiobis (2-nitrobenzoic acid) were purchased from SD Fine Chemicals, Mumbai, India. Sulfanilamides, naphthylamine diamine HCl, and phosphoric acid were obtained from Loba Chemie Pvt. Ltd., Mumbai, India. Creatinine and BUN kits were purchased from Accurex Biomedical Pvt. Ltd., Mumbai, India. Total RNA Extraction kit and One-step RT-PCR kit was purchased from MP Biomedicals India Private Limited, India.

Experimental animals

Adult male Wistar rats (200–220 g) were purchased from the National Institute of Biosciences, Pune (India). They were maintained at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with a relative humidity of 45–55% and 12:12 h dark/Light cycle. The animals had free access to standard pellet chow (Nutrivet Life Sciences, Manikbag, Sinhagad Road, Pune) and water throughout the experimental protocol. All experiments were carried out between 09:00 and 17:00 h. The experimental protocol (DYPCOP/IAEC/2016/2) was approved by the Institutional Animal Ethics Committee (IAEC) of Pad. Dr D. Y. Patil College of Pharmacy, Akurdi, Pune and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India on animal experimentation.

Induction and assessment of diabetes

A single dose of 55 mg/kg streptozotocin (STZ) prepared in citrate buffer (pH 4.4, 0.1 M) was injected intraperitoneally to induce diabetes.³¹ The age-matched control rats received an equal volume of citrate buffer and were used along with diabetic animals. Diabetes was confirmed 48 h after streptozotocin injection; the blood samples were collected via retro-orbital plexus technique using heparinized capillary glass tubes, and plasma glucose levels were estimated by the enzymatic GOD-POD (glucose oxidase-peroxidase) diagnostic kit method. The rats having plasma glucose levels more than 250 mg/dL were selected and used for the present study. The body weight and plasma glucose levels were measured before and at the end of the experiment. Food intake and water intake were measured with by placing the animals in a metabolic cage (Metabolic cage, Biomedical, India) before and at the end of the experiment.

Experimental design

After a basal recording of nociceptive reaction at week four after streptozotocin injection, the control and

diabetic rats were randomly selected and divided into seven groups of 8–10 animals each as follows:

(A) Non-diabetic animals

Group I: Normal non-diabetic (N): animals received a single injection of citrate buffer (vehicle) and oral gavage of double distilled water (10 mg/kg).

Group II: *Per se*: animals received LG (1000 mg/kg) in double distilled water by oral gavage.

(B) Diabetic animals

Group I: Diabetic control (DC): Diabetic animals received double-distilled water by oral gavage (10 mg/kg).

Group II: Diabetic (STZ) + Sita (5): Diabetic animals received Sitagliptin (5 mg/kg) in double-distilled water by oral gavage.

Group III: Diabetic (STZ) + LG (250): Diabetic animals received LG (250 mg/kg) in double-distilled water by oral gavage.

Group IV: Diabetic (STZ) + LG (500): Diabetic animals received LG (500 mg/kg) in double-distilled water by oral gavage.

Group V: Diabetic (STZ) + LG (1000): Diabetic animals received LG (1000 mg/kg) in double-distilled water by oral gavage.

Freshly prepared LG was administered daily in three different dosages (250, 500, and 1000 mg/kg).³² The dose of Sitagliptin (5 mg/kg) was selected based on the previous study.³³ At the end of the study, whole blood samples were collected from retro-orbital plexus to obtain serum for renal function parameters. Body weights and kidney weights of all animals were recorded, and animals were sacrificed by cervical dislocation. Kidney tissues were harvested, fatty and connective tissue layer were removed, rinsed in normal saline and stored in -80°C freezer for further biochemicals and RT-PCR studies. A kidney of rat from each group was isolated and fixed in 10% formalin solution for histopathological examination.

Serum and urine biochemistry

The serum was separated by centrifugation using an Eppendorf Cyto centrifuge, maintained at 4°C and run at a speed of 7000 rpm for 15 min. Serum as well as urine creatinine, uric acid, albumin, and BUN were measured by a spectrophotometer using reagent kits according to the procedure provided by the manufacturer (Accurex Biomedical Pvt. Ltd., Mumbai, India).

Biochemical estimation

Kidney tissue homogenate preparation, antioxidants, lipid peroxidation (MDA), NO, and hydroxyproline estimation

A known weight of the kidney tissue homogenates was prepared with 0.1 M tris-HCl buffer (pH 7.4), and supernatant of homogenates was employed to estimate superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation (MDA), nitric oxide (NO), and hydroxyproline level as described previously.^{34–41}

Determination of KIM-1, NGAL, TGF- β , and collagen-1 by reverse transcriptase-PCR in kidney

The levels of mRNA were analyzed in renal tissue using a reverse transcription (RT)-PCR approach as described previously.^{34,42–44} Briefly, single-stranded cDNA was synthesized from 5 μg of total cellular RNA using reverse transcriptase (MP Biomedicals India Private Limited, India) as described previously.³⁴ Amplification of β -actin served as a control for sample loading and integrity. The primer sequences for KIM-1, NGAL, TGF- β , collagen-1, and β -actin were selected according to the previously reported method.⁴⁵ (Table 1) PCR products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. The size of amplicons was confirmed using a 100-bp ladder as a standard size marker. The amplicons were visualized, and images were captured using a gel documentation system (Alpha Innotech Inc., San Leandro, CA). Gene expression was assessed by generating densitometry data for band intensities in different sets of experiments, by analyzing the gel images on the Image J program (Version 1.33, Wayne Rasband, National Institutes of Health, Bethesda, MD) semi-quantitatively. The band intensities were compared with constitutively expressed β -actin. The intensity of mRNAs was standardized against that of the β -actin mRNA from each sample, and the results were expressed as PCR-product/ β -actin mRNA ratio.

Table 1. Primer sequences for KIM-1, NGAL, TGF- β , collagen-1, and β -actin.

Gene	Primer sequences (5'–3')	Length (bp)
KIM-1	5'-ACTCCTGCAGACTGGAATGG-3'	212
	5'-CAAAGCTCAGAGAGCCCATC-3'	
NGAL	5'-GATGTTGTTATCCTTGAGGCC-3'	230
	5'-CACTGACTCAGACCAAGTTGCC-3'	
TGF- β	5'-GTTCTTCAATACGTGACAGATTGCG-3'	309
	5'-CATTATCTTGCTGTCAAGAGC-3'	
Collagen-1	5'-GAGCGGAGAGTACTGGATCG-3'	218
	5'-GGTTCGGGCTGATGACCAAG-3'	
β -actin	5'-AGGCATCCTGACCTGAAGTAC-3'	764
	5'-TCTTCATGAGGTAGTCTGTCAAG-3'	

Table 2. Effect of LG on STZ-induced alterations in body weight, blood glucose level, food intake, water intake, urine output, and relative kidney weight in diabetic rats.

Treatment	Body weight (g)	Blood glucose level (mg/dL)	Food intake (g)	Water intake (mL)	Urine output (mL)	Kidney/body weight
N	239.00 ± 6.25	115.8 ± 6.42	22.00 ± 3.33	35.83 ± 3.07	9.17 ± 1.19	2.39 ± 0.41
DC	200.7 ± 7.54#	261.2 ± 4.9#	68.00 ± 3.37#	111.3 ± 2.47#	44.85 ± 0.68#	5.47 ± 0.33#
Sita (5)	229.2 ± 3.2* $\S$	157.2 ± 16.31* $\S$	31.67 ± 3.42* $\S$	47.83 ± 2.44* $\S$	16.99 ± 1.17* $\S$	2.91 ± 0.23* $\S$
LG (250)	203.2 ± 4.67	241.2 ± 12.94	58.17 ± 2.4	99.33 ± 3.55	41.15 ± 0.74	4.65 ± 0.3
LG (500)	217.2 ± 6.23* $\S$	206.4 ± 8.15* $\S$	43.83 ± 3.03* $\S$	69.33 ± 2.23* $\S$	30.69 ± 1.51*	4.07 ± 0.22*
LG (1000)	226.2 ± 7.66* $\S$	179.6 ± 15.5* $\S$	36.00 ± 2.97* $\S$	57.67 ± 2.5* $\S$	23.86 ± 0.71* $\S$	3.18 ± 0.17* $\S$
Per se	235.2 ± 3.89	139.8 ± 14.15	22.83 ± 2.7	41.00 ± 2.32	13.96 ± 1.35	2.38 ± 0.24

Results are represented as mean ± SEM, ($n = 5$) data were analyzed by One-way ANOVA followed by Tukey's multiple range test for each parameter separately.

$p < .05$ as compared with normal group.

* $p < .05$ as compared with diabetic control (DC) group.

$\S p < .05$ as compared with each other. N: normal group; DC: diabetic control group; Sita (5): sitagliptin (5 mg/kg, p.o.)-treated group; LG (250): L-glutamine (250 mg/kg, p.o.)-treated group; LG (500): L-glutamine (500 mg/kg, p.o.)-treated group; LG (1000): L-glutamine (1000 mg/kg, p.o.)-treated group.

Histological examination

The dissected kidney tissue specimens were fixed in 10% formaldehyde, processed routinely for embedding in paraffin. Sections were stained with hematoxylin–eosin stain and Masson's trichrome stain as described previously.⁴⁶ Kidney sections were analyzed qualitatively under a light microscope (40× and 100×) for various histopathological alterations.

Statistical analysis

Data were expressed as mean ± standard error mean (SEM). Data analysis was performed using Graph Pad Prism 5.0 software (Graph Pad, San Diego, CA). Data of biochemical parameters were analyzed using one-way analysis of variance (ANOVA), and Tukey's multiple range test was applied for *post hoc* analysis. A value of $p < .05$ was considered to be statistically significant.

Results

Effect of L-glutamine on STZ-induced alterations in body weight and relative kidney weight in diabetic rats

There was significant decrease ($p < .05$) in body weight and significant increase ($p < .05$) in relative kidney weight of STZ control rats as compared to normal rats. These STZ-induced decrease in body weight was significantly halted by LG (500 and 1000 mg/kg) administration. Whereas, relative kidney weight was decrease significantly ($p < .05$) after LG (500 and 1000 mg/kg) treatment as compared to STZ control rats. Sitagliptin (5 mg/kg) treatment also significantly inhibited ($p < .05$) STZ induced an alteration in body weight and relative kidney weight as compared to STZ control rats. However, increase in relative kidney weight was more significantly attenuated ($p < .05$) by LG (1000 mg/kg) and sitagliptin (5 mg/kg) administration than LG (500 mg/kg) (Table 2).

Effect of L-glutamine on STZ-induced alterations in blood glucose level, food intake, water intake, and urine output in diabetic rats

There was a significant increase ($p < .05$) in plasma glucose level as well as food intake, water intake, and urine output in STZ control rats as compared to normal rats after four weeks of intraperitoneal STZ administration. These increased plasma glucose level, food intake, water intake, and urine output were significantly inhibited ($p < .05$) by LG (500 and 1000 mg/kg) treatment as compared to STZ control rats (Table 2).

Effect of L-glutamine on STZ induced alterations in serum and urine biochemical parameters in diabetic rats

When compared with normal rats, intraperitoneal administration of STZ caused significant elevated ($p < .05$) in serum BUN, creatinine, and uric acid levels in STZ control rats. Administration of LG (500 and 1000 mg/kg) significantly reduced ($p < .05$) these elevated levels of serum BUN, creatinine, and uric acid as compared to STZ control rats. Sitagliptin (5 mg/kg) treatment also showed a significant reduction ($p < .05$) in serum BUN, creatinine, and uric acid as compared to STZ control rats (Table 3).

Effect of L-glutamine on STZ-induced alterations in creatinine clearance in diabetic rats

There was significant decreased ($p < .05$) in creatinine clearance rate in STZ control rats as compared to normal rats after four weeks of post-STZ administration. Whereas, treatment with LG (500 and 1000 mg/kg) significantly attenuated ($p < .05$) STZ induced an alteration in creatinine clearance rate as compared to STZ control rats. Administration of sitagliptin (5 mg/kg) also significantly increase ($p < .05$) the rate of creatinine clearance as compared to STZ control rats (Table 3).

Effect of L-glutamine on STZ induced alterations in renal SOD and GSH levels in diabetic rats

Intraperitoneal administration of STZ caused significant decrease ($p < .05$) in renal SOD and GSH levels in STZ control rats as compared to normal rats. Administration of LG (500 and 1000 mg/kg) significantly increased ($p < .05$) renal SOD and GSH levels as compared to STZ control rats. When compared with STZ control rats, sitagliptin (5 mg/kg) treatment also showed significant ($p < .05$) inhibition in STZ-induced decreased renal SOD and GSH levels (Table 4).

Effect of L-glutamine on STZ induced alterations in renal MDA and NO levels in diabetic rats

There was a significant increase ($p < .05$) in renal MDA and NO levels in STZ control rats as compared to normal rats after four weeks of post-STZ administration. Whereas, treatment with LG (500 and 1000 mg/kg) significantly inhibited ($p < .05$) STZ induced increased in renal MDA and NO levels as compared to STZ control rats. Administration of sitagliptin (5 mg/kg) also

significantly decreased ($p < .05$) renal MDA and NO levels as compared to STZ control rats (Table 4).

Effect of L-glutamine on STZ induced alterations in renal hydroxyproline level in diabetic rats

When compared with normal rats, intraperitoneal administration of STZ caused a significant increase ($p < .05$) in renal hydroxyproline level in STZ control rats. Administration of LG (500 and 1000 mg/kg) significantly reduced ($p < .05$) these elevated levels of renal hydroxyproline as compared to STZ control rats. Sitagliptin (5 mg/kg) treatment also showed a significant reduction ($p < .05$) in renal hydroxyproline level as compared to STZ control rats (Table 4).

Effect of L-glutamine on STZ induced alterations in renal KIM-1, NGAL, TGF- β 1, and collagen-1 mRNA expressions in diabetic rats

Intraperitoneal administration of STZ caused significant upregulation ($p < .05$) in renal KIM-1, NGAL, TGF- β 1,

Table 3. Effect of LG on STZ-induced alterations in serum and urine biochemical parameters in diabetic rats.

Treatment		BUN (mg/dL)	Uric Acid (mg/dL)	Creatinine (mg/dL)	Albumin (g/dL)	Creatinine clearance (mL/min)
N	Serum	10.37 $\pm$ 1.67	1.99 $\pm$ 0.24	0.2 $\pm$ 0.02	4.01 $\pm$ 0.41	1.57 $\pm$ 0.17
	Urine	34.65 $\pm$ 1.50	3.51 $\pm$ 0.16	48.48 $\pm$ 1.32	0.57 $\pm$ 0.06	
DC	Serum	63.02 $\pm$ 1.99#	5.46 $\pm$ 0.2#	0.75 $\pm$ 0.02#	11.97 $\pm$ 0.69#	0.44 $\pm$ 0.05#
	Urine	5.42 $\pm$ 0.86#	1.71 $\pm$ 0.16#	10.68 $\pm$ 1.28#	2.00 $\pm$ 0.14#	
Sita (5)	Serum	16.46 $\pm$ 2.81* $\$$	2.41 $\pm$ 0.19* $\$$	0.34 $\pm$ 0.02* $\$$	7.01 $\pm$ 0.46* $\$$	1.53 $\pm$ 0.14* $\$$
	Urine	31.01 $\pm$ 1.99* $\$$	3.21 $\pm$ 0.19* $\$$	43.02 $\pm$ 1.03* $\$$	0.96 $\pm$ 0.04* $\$$	
LG (250)	Serum	59.49 $\pm$ 1.53	5.24 $\pm$ 0.18	0.68 $\pm$ 0.03	10.84 $\pm$ 0.85	0.57 $\pm$ 0.07
	Urine	10.47 $\pm$ 2.81	2.07 $\pm$ 0.15	13.35 $\pm$ 1.18	1.85 $\pm$ 0.08	
LG (500)	Serum	40.66 $\pm$ 1.5* $\$$	4.72 $\pm$ 0.21*	0.51 $\pm$ 0.02* $\$$	8.81 $\pm$ 0.51*	1.18 $\pm$ 0.14* $\$$
	Urine	17.46 $\pm$ 1.66* $\$$	2.69 $\pm$ 0.24* $\$$	27.45 $\pm$ 1.29* $\$$	1.76 $\pm$ 0.06*	
LG (1000)	Serum	23.46 $\pm$ 1.66* $\$$	3.41 $\pm$ 0.25* $\$$	0.36 $\pm$ 0.01* $\$$	7.59 $\pm$ 0.7* $\$$	1.62 $\pm$ 0.04* $\$$
	Urine	27.47 $\pm$ 1.53* $\$$	3.14 $\pm$ 0.2* $\$$	35.49 $\pm$ 1.48* $\$$	1.28 $\pm$ 0.07* $\$$	
Per se	Serum	9.66 $\pm$ 2.34	2.23 $\pm$ 0.16	0.26 $\pm$ 0.02	5.37 $\pm$ 0.78	1.91 $\pm$ 0.25
	Urine	33.92 $\pm$ 1.48	3.43 $\pm$ 0.17	48.79 $\pm$ 1.37	0.54 $\pm$ 0.05	

Results are represented as mean $\pm$ SEM, ($n = 5$) data were analyzed by One-way ANOVA followed by Tukey's multiple range test for each parameter separately.

$p < .05$ as compared with normal group.

* $p < .05$ as compared with diabetic control (DC) group.

$\$p < .05$ as compared with each other. N: normal group; DC: diabetic control group; Sita (5): sitagliptin (5 mg/kg, p.o.)-treated group; LG (250): L-glutamine (250 mg/kg, p.o.)-treated group; LG (500): L-glutamine (500 mg/kg, p.o.)-treated group and LG (1000): L-glutamine (1000 mg/kg, p.o.)-treated group.

Table 4. Effect of LG on STZ-induced alterations in renal oxidant stress and hydroxyproline level in diabetic rats.

Treatment	SOD (U/mg of protein)	GSH (μ g/mg of protein)	LPO (nM/mg of protein)	NO (μ g/mL)	Hydroxyproline (μ g/mg protein)
N	15.21 $\pm$ 1.42	11.76 $\pm$ 0.63	5 $\pm$ 0.52	229.6 $\pm$ 25.05	0.79 $\pm$ 0.05
DC	3.87 $\pm$ 0.5#	2.54 $\pm$ 0.58#	24.73 $\pm$ 0.64#	655.3 $\pm$ 24.16#	2.09 $\pm$ 0.17#
Sita (5)	15.38 $\pm$ 0.66* $\$$	10.07 $\pm$ 0.62* $\$$	10.32 $\pm$ 0.71* $\$$	294.7 $\pm$ 30.26* $\$$	1.12 $\pm$ 0.07* $\$$
LG (250)	6.84 $\pm$ 0.72	2.82 $\pm$ 0.41	22.01 $\pm$ 0.45	607.9 $\pm$ 23.57	1.92 $\pm$ 0.06
LG (500)	11.65 $\pm$ 0.53* $\$$	5.42 $\pm$ 0.85* $\$$	16.75 $\pm$ 0.98* $\$$	524.1 $\pm$ 29.99*	1.61 $\pm$ 0.06*
LG (1000)	14.12 $\pm$ 1.51* $\$$	9.35 $\pm$ 0.81* $\$$	13.19 $\pm$ 1.57* $\$$	445.6 $\pm$ 25.72* $\$$	1.2 $\pm$ 0.07* $\$$
Per se	15.76 $\pm$ 1.14	11.12 $\pm$ 0.57	5.414 $\pm$ 0.9	246.3 $\pm$ 30.85	0.73 $\pm$ 0.08

Results are represented as mean $\pm$ SEM, ($n = 5$) data were analyzed by One-way ANOVA followed by Tukey's multiple range test for each parameter separately.

$p < .05$ as compared with normal group.

* $p < .05$ as compared with diabetic control (DC) group.

$\$p < .05$ as compared with each other. N: normal group; DC: diabetic control group; Sita (5): sitagliptin (5 mg/kg, p.o.)-treated group; LG (250): L-glutamine (250 mg/kg, p.o.)-treated group; LG (500): L-glutamine (500 mg/kg, p.o.)-treated group; LG (1000): L-glutamine (1000 mg/kg, p.o.)-treated group.

and collagen-1 mRNA expressions in STZ control rats as compared to normal rats. Administration of LG (500 and 1000 mg/kg) significantly inhibited ($p < .05$) STZ-induced upregulated renal KIM-1, NGAL, TGF- β 1, and collagen-1 mRNA expressions as compared to STZ control rats. When compared with STZ control rats, sitagliptin (5 mg/kg) treatment also showed significant downregulation ($p < .05$) in KIM-1, NGAL, TGF- β 1, and collagen-1 mRNA expressions (Figure 1).

Correlation analyses of blood glucose level and KIM-1, NGAL, TGF- β 1 as well as collagen-1 mRNA expressions in diabetic rats

Correlation coefficient by linear regression analyses was carried out between various parameters. Direct correlation was observed between blood glucose level and renal KIM-1, NGAL, TGF- β 1 as well as collagen-1 mRNA expressions indicating that increased dose of LG significantly inhibited blood glucose as well as downregulated renal KIM-1 ($r = 0.878523$, $p < .05$), NGAL ($r = 0.86497$, $p < .05$), TGF- β 1 ($r = 0.919845$, $p < .05$) and collagen-1 ($r = 0.856981$, $p < .05$) mRNA expression. Thus, all renal marker tested with respect to kidney showed high degree of correlation (Figure 2).

Effect of L-glutamine on STZ-induced alterations in renal histopathology in diabetic rats

Renal tissue from normal rats showed the well-organized architecture of renal histology. It showed normal glomerulus surrounded by the Bowman's capsule, proximal, and distal convoluted tubules without any inflammatory infiltration, fibrosis, and necrosis (Figures 3(A) and 4(A)). Whereas, different grades of various pathological lesions were observed in the renal tissue of diabetic rats (Figure 3(B)). It showed glomerular necrosis with distorted glomerular symmetry, thickening of the basement membrane, mesangial cells proliferation and widened mesangial regions. It showed the presence of inflammatory infiltration and congestion. MT staining showed the presence of fibrosis in the renal tissue from STZ control rats (Figure 4(B)). All these changes were attenuated by the treatments with LG (500 and 1000 mg/kg) (Figures 3(E,F) and 4(E,F)) and sitagliptin (5 mg/kg) (Figures 3(C) and 4(C)). However, LG (250 mg/kg) treatment failed to do so (Figures 3(D) and 4(D)).

Discussion

DN is one of the major microvascular complications of diabetes mellitus and leading cause of end-stage renal disease.⁴⁷ Current treatments have been shown to attenuate the functional and structural abnormalities

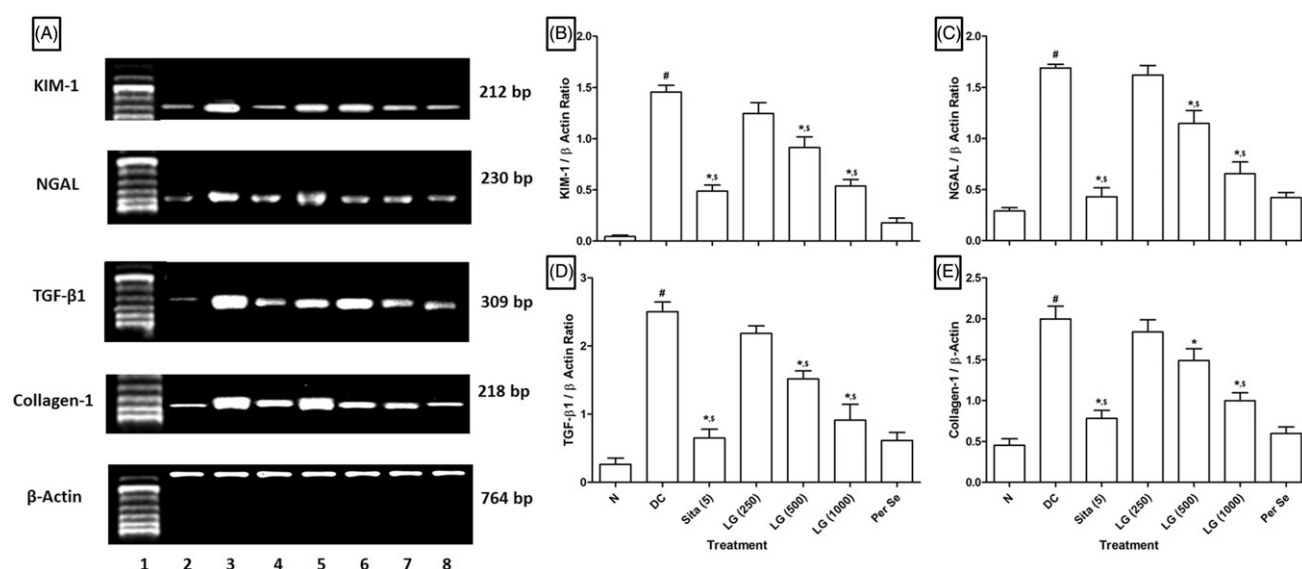


Figure 1. (A) Effect of LG on STZ-induced alterations in renal KIM-1, NGAL, TGF- β , and Collagen-1 mRNA expression in diabetic rats. (B) Quantitative representation of the mRNA expression of KIM-1, (C) NGAL, (D) TGF- β , and (E) Collagen-1. Results are represented as mean $\pm$ SEM, ($n = 4$) data were analyzed by One-way ANOVA followed by Tukey's multiple range test for each parameter separately. $\#p < .05$ as compared with normal group, $*p < .05$ as compared with diabetic control (DC) group and $\$p < .05$ as compared with each other. N: normal group; DC: diabetic control group; Sita (5): sitagliptin (5 mg/kg, p.o.)-treated group; LG (250): L-glutamine (250 mg/kg, p.o.)-treated group; LG (500): L-glutamine (500 mg/kg, p.o.)-treated group; LG (1000): L-glutamine (1000 mg/kg, p.o.)-treated group. Lane 1: Ladder 1000 bp; Lane 2: mRNA expression of normal group; Lane 3: mRNA expression of diabetic control group; Lane 4: mRNA expression of sitagliptin (5 mg/kg, p.o.) treated group; Lane 5: mRNA expression of L-glutamine (250 mg/kg, p.o.) treated group; Lane 5: mRNA expression of L-glutamine (500 mg/kg, p.o.) treated group; Lane 7: mRNA expression of L-glutamine (1000 mg/kg, p.o.) treated group and Lane 8: mRNA expression of *per se* group.

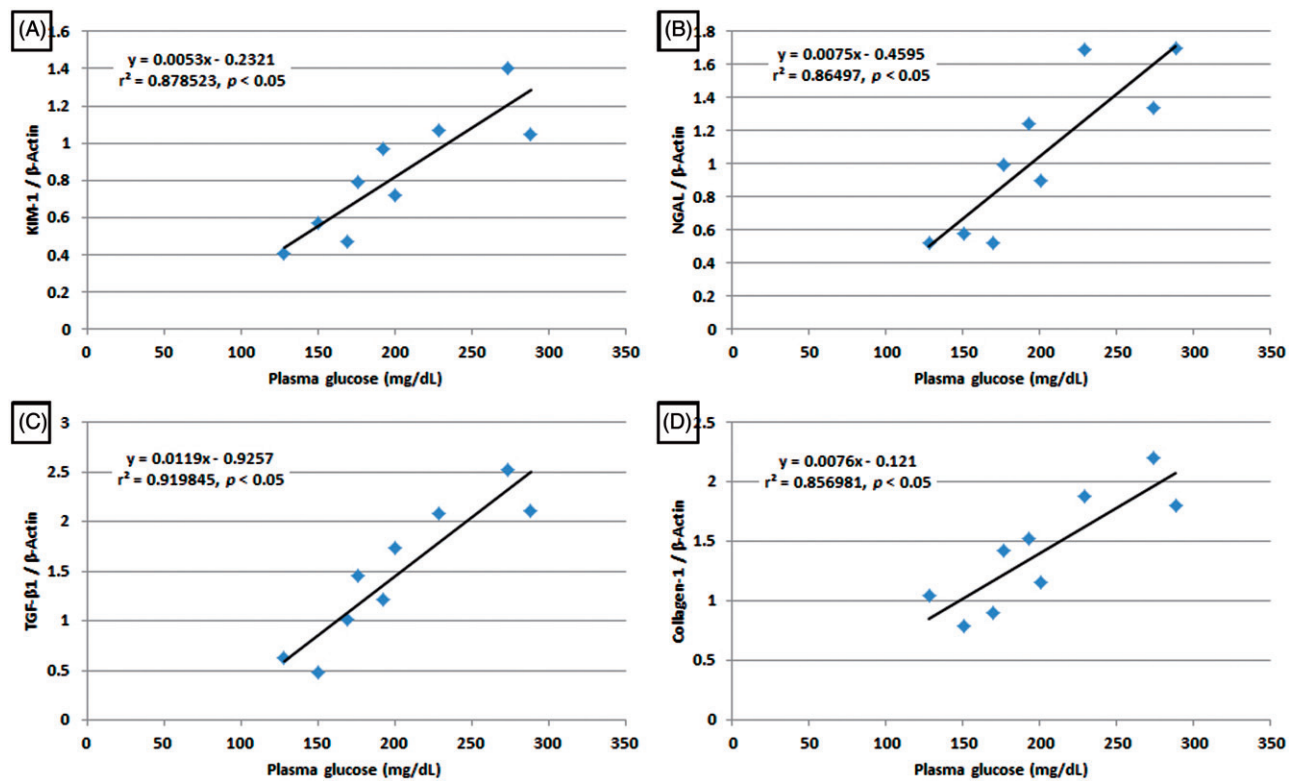


Figure 2. (A) Correlation analysis between blood glucose level and KIM-1, (B) NGAL, (C) TGF- β as well as (D) Collagen-1 mRNA expression in diabetic rats.

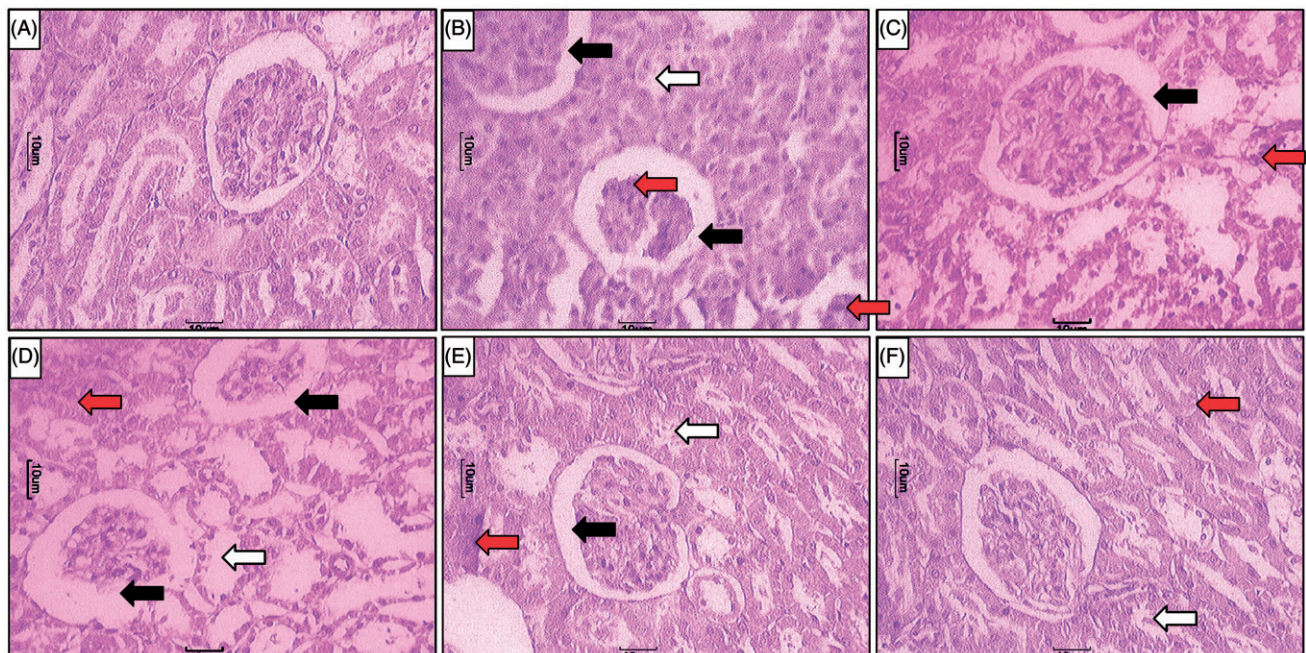


Figure 3. Effect of LG on STZ-induced alterations in kidney histology. Photomicrograph of sections of kidney of (A) normal, (B) diabetic control rats, (C) Sitagliptin (5 mg/kg, p.o.) treated rats (C), LG (250 mg/kg, p.o.) treated rats (D), LG (500 mg/kg, p.o.) treated rats (E) and LG (1000 mg/kg, p.o.) treated rats. H & E staining at 100 $\times$. Increase in thickening of the basement membrane (black arrow), necrosis (white arrow) and inflammatory infiltration (red arrow).

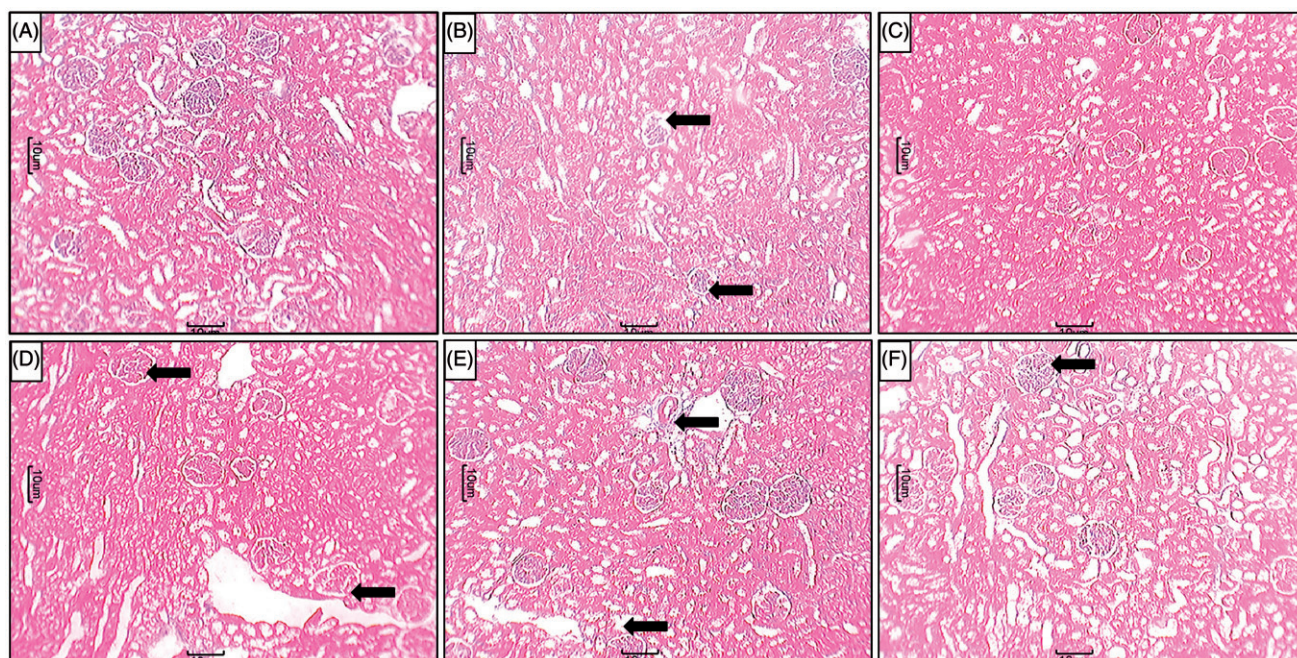


Figure 4. Effect of LG on STZ-induced alterations in kidney histology. Photomicrograph of sections of kidney of (A) normal, (B) diabetic control rats, (C) Sitagliptin (5 mg/kg, p.o.) treated rats (C), LG (250 mg/kg, p.o.)-treated rats (D), LG (500 mg/kg, p.o.)-treated rats (E) and LG (1000 mg/kg, p.o.)-treated rats. MT staining at 40 $\times$. Fibrosis (Black arrow).

seen in DN disease. However, the associated side effect of these agents limits their use in the treatment of DN. Thus, in the present investigation, we have evaluated the effect of oral administration of LG in STZ-induced diabetic nephropathic rat as it exhibits clinicopathological features including biochemical, oxidative, and metabolic changes that were also presented in human.

Literature is punctuated with evidence that diabetes is associated with features like polydipsia, and polyphagia which may be due to decrease in the availability of glucose and amino acid to cell.⁴⁸ Impaired cellular biosynthesis and metabolism has been reported to be the underlying cause of diabetes-induced polydipsia, polyphagia, polyuria, and bodyweight loss. In the present investigation, rats administrated with STZ exhibited the features including a loss in body weight, increased food intake as well as water intake and urine output. These features have been investigated by other researchers also, and results of the present investigation are in line with these researchers.^{48,49} However, this STZ-induced polydipsia, polyphagia, polyuria, and loss in body weight was halted in LG-treated animals when compared with diabetic control animals. The result of present investigation is in accordance with findings of the previous investigation where the administration of glutamine reduced urine output in nephropathic animals.⁵⁰

It has been well documented that DN is associated with increased urinary albumin excretion thus

microalbuminuria is served as a hallmark of DN. Furthermore, kidneys play vital role in the excretion of metabolic wastes including urea, uric acid, and creatinine. Hence, alteration in the levels of BUN, creatinine, and uric acid in serum, as well as urine, reflected renal dysfunction.^{2,51} Glucosuria induces an osmotic diuresis and dehydration that resulted in a condition of prerenal acute renal failure (ARF). The similar results were noted in a present investigation where 4-week post STZ administration resulted in significantly increased serum albumin, BUN, creatinine, and uric acid as well as urinary albumin excretion. These cumulative alterations in the biochemical parameter of serum and urine reflected in decreased glomerular filtration rate (GFR) which was assessed in terms of creatinine clearance. A study carried out by the previous researcher also showed alteration in these hallmarks of renal dysfunction in STZ-induced diabetic rats.⁵² However, administration of LG resulted in significant attenuation in BUN, uric acid, creatinine, albumin, and GFR. The previous researcher also showed substantial attenuation in serum creatinine and creatinine clearance after the administration of LG in cisplatin-induced nephropathy.⁵⁰ Thus LG treatment attenuated this serum and urinary biochemical alteration suggesting it's direct or indirect role in providing protection against STZ-induced DN or delay in its development.

It has been well documented that hyperglycemia caused abnormalities in ECM which intern caused

increase in vascular permeability, abnormalities in blood flow, and inhibition in the formation of endothelial trophic factors.^{53,54} Elevated plasma glucose stimulates the production of reactive oxygen species (ROS) which in turn generates oxidative stress, these together cause the formation of edema, ischemia, and hypoxia that lead to DN.^{55,56} In glomerulus; hyperglycemia induces production of hydrogen peroxide that increases lipid peroxidation. Furthermore, cellular death causes generation of ROS, which ultimately lead to the generation of SOD and catalase. SOD indulge in the dismutation of superoxide into oxygen and hydrogen peroxide whereas, catalase involve in the cleavage of hydrogen peroxide into water and oxygen. These hydroxyl radicals pierce the membrane barriers and react mutagenically with DNA in the cell nucleus and show the toxic effect to the cells.⁵⁷ Intraperitoneal administration of STZ resulting in ROS generation causes lipid peroxidation followed by deterioration of membrane lipid bilayer arrangement and increased tissue permeability through the inactivation of membrane-bound enzymes and receptors which are an essential feature of oxidative stress.⁵⁸ GSH is a potent ubiquitous, non-enzymatic biological antioxidant^{48,59,60} plays a vital role in the detoxification of free radical species such as hydrogen peroxide, superoxide, and alkoxy radicals to maintain the cell metabolism and integrity.⁴⁰ The level of glutamine is successfully reduced in renal tissue of rat under stressful conditions.⁶¹ However, glutamine plays an important role in glutathione metabolism.⁶² Thus, oral glutamine administration may provide ideal conditions for the synthesis of glutathione, which may induce a positive effect on diabetic metabolic abnormalities.

Previously it has been believed that focal segmental glomerulosclerosis is a main manifestation in DN followed by a similar fibrosing process in the tubulointerstitial region.^{63,64} However, recent studies showed that tubulointerstitial fibrosis could also occur in the early stage of DN and directly induce deterioration of renal function, independent of the glomerular lesions. It has been well documented that renal tissue of STZ-administered animals is associated with aberration of renal histology including thickening of capillary basement membrane, mesangial proliferation, and nodular glomerulosclerosis.⁶⁵ Researcher showed that hyperglycemia directly induces mesangial expansion via increased matrix production in renal tissue of STZ-treated rats.⁶⁶ Moreover, extracellular membrane (ECM) protein activation and matrix degradation subdue is correlated with TGF- β -induced fibrogenesis.^{67,68} Interestingly, TGF- β signal transduction is greatly connected with the Smads.^{67,68} TGF- β on binding with two specific type I and type II serine/threonine kinase

receptor convey its signal across the plasma membrane. Clinical study also showed elevated serum and urinary TGF- β 1 level in DN patients.⁶⁹ In the present study, the expression of TGF- β 1 was upregulated in renal tissue of STZ control rats, which is in line with findings of the previous investigator, demonstrating the possible contribution of TGF- β 1 to DN.^{70,71} Treatment with glutamine showed significantly downregulated renal TGF- β 1 expression. This notion was further supported by histological findings of renal tissue from glutamine treated rats where thickening of the GBM and tubular necrosis markedly reduced which might be due to the inhibition of TGF- β 1 expression.

In the past, the researchers have underlined the correlation between iNOS and DN.^{72,73} The peroxynitrite, which is a potent and aggressive cellular oxidant formed by the reaction of NO with O₂⁻.⁷⁴ Nitrite/nitrate levels, as the end products of nitric oxide conversion that caused generation of vicious cycles leads to cell death.⁷⁵ It has been well documented that streptozotocin-induced diabetes caused an increase in the activity and expression of iNOS.⁷⁶ In the present investigation, intraperitoneal administration of STZ demonstrated the involvement of iNOS in the inflammatory process reflected by the elevated level of NO in renal tissue. LG treatment in results in inhibition of STZ-induced elevated NO level, and this result is in line with findings of the previous researcher.²⁶

Kidney Injury Molecule-1 (KIM-1), an extracellular protein which is the hallmark of acute kidney injury (AKI) and its expression is elevated during DN in patients with T1DM with or without albuminuria.^{1,77} Human neutrophil gelatinase-associated lipocalin (NGAL) is a ubiquitous lipocalin iron-carrying protein which bound to gelatinase in specific granules of the neutrophil⁷⁸ has been identified as an early biomarker involved in ischemic renal injury and repair processes.⁷⁹ Previously it has been reported that KIM-1 correlate positively with the high glucose level in diabetic patients.⁸⁰ Expression of KIM-1 and NGAL reported to be increased in renal tissue of rats during DN which is consistent with the results of our present investigation.⁸¹ Administration of LG showed significant inhibition in upregulation of diabetes-induced KIM-1 and NGAL mRNA expression, which might be due to its antihyperglycemic potential.

Transforming growth factor- β 1 (TGF- β 1) is a multifunctional cytokine circulating in a biologically inactive form in human plasma.^{82,83} It is also known as key mediator of the sclerosing process in kidney, liver, skin, and other organs and its elevated production have been implicated in DN in both animal models^{84,85} and humans.⁸⁶ Research carried out over past decades showed that high glucose levels caused activation of

TGF- β 1 that leads to initial structural damage to glomeruli.^{87,88} Thus, the elevated expression of TGF- β 1 serve as a predictor of early diabetic nephropathy. Furthermore, elevated levels of TGF- β 1 is related to glomerulosclerosis and interstitial fibrosis in various renal diseases via increases in the synthesis of ECM components, including collagens, fibronectin, and laminin.⁸⁹ The similar findings were noted in the present investigation where elevated TGF- β 1 is associated with upregulated collagen-1 mRNA expression as well as hydroxyproline level in renal tissue. LG treatment significantly inhibited elevated levels of TGF- β 1, collagen-1 mRNA, and hydroxyproline which might attribute to its antidiabetic property. Correlation analysis also revealed that antidiabetic potential of LG might play an important role in the attenuation of STZ-induced elevated renal TGF- β 1 and collagen-1 mRNA expressions.

Clinically efficacy of glutamine has been proven where administration of oral glutamine (15 g twice a day for seven consecutive days) significantly reduces oxaliplatin-induced neuropathy in colorectal cancer patients.⁹⁰ In conclusion, results of present investigation suggest that treatment with LG ameliorated STZ-induced DN via the inhibition of oxidonitrosative stress as well as downregulation of KIM-1, NGAL, TGF- β 1, and collagen-1 mRNA expressions. This finding may open novel vistas in therapeutic option with amino acids like LG to treat diabetes-induced nephropathy.

Acknowledgements

The authors would like to acknowledge Col. S. K. Joshi, Director and Dr N. S. Vyawahare, Principal, Pad. Dr D. Y. Patil College of Pharmacy Akurdi, Pune, India, for providing necessary facilities to carry out the study. The authors also acknowledge Invaluesys Research Group to carry out statistical analysis of the study.

Disclosure statement

The Authors declare that they have no conflicts of interest to disclose.

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