Journal of Advanced Biomedical and Pharmaceutical Sciences

Journal Homepage: http://jabps.journals.ekb.eg

Role of Cystathionine Gamma Lyase in Diabetic Nephropathy

Aliaa F. Anter[1*], Ashraf Taye[1], Mohamed A. El-Moselhy[1,2]

[1] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Minia University, Minia, Egypt .
[2] Department of Clinical Pharmacy and Pharmacology, Ibn Sina National College for Medical Studies, Jeddah, Saudi Arabia.

Received: April 23, 2018; revised: May 11, 2018; accepted: May 12, 2018

Abstract

Diabetic nephropathy is amajor cause of end-stage renal failure. Hydrogen sulfide (H_2S) is an endogenous gasotransmitter with beneficial effects in pathology of many conditions. However, its role in diabetic nephropathy is unclear. This study examined the protective role of H_2S producing enzyme cystathionine gamma lyase (CSE) in kidney impairment induced by diabetes. Diabetes was induced in male Wistar rats by high fat diet for 12 weeks followed by a single i.p. injection of streptozotocin (35mg/kg). Animals were divided into: control (chow fed), diabetic non-treated group (DM), diabetic + L-cysteine(50 mg/kg/day, i.p.) and diabetic+ PAG(37.5mg/kg/day, i.p.) for 4 weeks.

Diabetes decreased CSE expression and activity, increased H_2O_2 and MDA in kidney tissue with impaired SOD and CAT activity. L-cysteine treatment improved kidney function (serum urea and creatinine levels), decreased MDA, H_2O_2 production and increased renal SOD and CAT activity. L-CYS attenuated diabetes-induced expression of NF-κB. Administration of PAG deteriorated the kidney function with significant increase in oxidative stress and inflammation status.

L-CYS rescued kidney dysfunction by reducing oxidative stress, NF-κB expression and decreasing the apoptotic enzyme caspase-3. Recovery of CSE expression mRNA and its activity may also contribute to the beneficial effects of L-CYS.

Key words
L-cysteine, propargylglycine, high fat diet, streptozotocin.

1. Introduction

Hydrogen sulfide (H_2S) is a gaseous transmitter that plays an important role as a protective signaling molecule in mammalian cells. H_2S is produced endogenously by three enzymes:cystathionine-γ-lyase (CSE), cystathionine-β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) (1). Interestingly, the expression of these enzymes is tissues specific. For example, CSE is mainly expressed in the aorta, ileum, liver, heart and kidney, while CBS can be found in the brain and nervous system (2). CSE is an enzyme catalyzing generation of H_2S from cystathionine and cysteine(3). H_2S has many beneficial effects on physiological functions such as lowering blood pressure (4), relaxing blood vessels (5) as well as being an antioxidant (6) and anti-inflammatory (7).

Diabetes complications, which are attributed mainly to increased glucose levels and deranged lipid metabolism, may be microvascular or macrovascular complications. Diabetic nephropathy is one of the microvascular complications of both type 1 and type 2 diabetes mellitus (8). Type 2 diabetes is characterized by hyperglycemia, insulin resistance and hyperlipidemia; all of which are linked to increased oxidative stress and systemic inflammation (9). These metabolic disturbances are common pathways for the progression of diabetic nephropathy(10), while attenuation of such effects is expected to lower the incidence and severity of diabetic nephropathy.

The purpose of this study is to investigate the role of CSE on diabetes and diabetes-induced kidney dysfunction. This was achieved by comparing the role of endogenous H_2S in diabetes by administration of an H_2S donor (L-CYS), with the effects of an inhibitor of CSE enzyme dl-propargylglycine(PAG) being evaluated in a diabetes-induced renal dysfunction model.

2. Materials and methods

2.1 Induction of diabetes

Rats (weighing 100-120 g) were purchased from the Experimental Animal Center of Al-Azhar University of Medical Science, Cairo, Egypt. Diabetes was induced in male Wistar rats as previously described (11). Briefly, animals were fed either normal chow (control non-diabetic group) or a high fat diet (HFD, 40% of calories from fat(12)) for 12 weeks followed by a singlei.p. Injection of 35 mg/kg STZ (Sigma Aldrich, USA) dissolved in isotonic citrate buffer, pH 4 (13, 14). Two days after STZ injection, tail blood glucose level was determined using a commercial glucometer (Accu-Check®, Roche, USA). Rats were considered diabetic when fasting blood glucose exceeded 200 mg/dL.

2.2. Experimental design:

Diabetic rats were randomly divided into three groups; 1: diabetic non-treated group (DM), 2: diabetic treated with L-cysteine (L-CYS; 50mg/kg (15) -Sigma USA, cat. No. 168149),

**** Correspondence: Aliaa F. Anter***
Tel.: *+201001086698* ***; Fax:*** *+20 862369075*
Email Address: *aliaafath@yahoo.com*

3: diabetic treated with dl-propargylglycine (PAG; 37.5 mg/kg (16)-Sigma USA, cat. No. P7888). All drugs were dissolved in saline and given by i.p. injection daily for four weeks after induction of diabetes. At the end of drug treatment period, animals were fasted for 12 hours then anaesthetized with an i.p. injection of thiopental (50 mg/kg). Blood samples were collected by cardiac puncture and centrifuged to collect serum. Kidneys were rapidly harvested and washed with ice-cold saline and blotted on a filter paper. Tissue pieces were flash frozen in liquid nitrogen and stored at -20 ^{o}C for further biochemical analyses, while one portion was fixed in 10% formalin dissolved in phosphate-buffered saline and prepared for histopathological examination.

2.3. Glycemic status and lipid profile:

Fasting blood glucosewas measured by a commercial glucometer (Accu-Check® glucometer, Roche, USA), blood samples were obtained by tail puncture. Serum insulin was assayed using an ELISA kit (Bioscience, USA) and glycemic status was assessed based on the Homeostasis Model Assessment of Insulin Resistance (HOMA IR) (17). Serum total free fatty acids, cholesterol, HDL and TGs were measured colorimetrically using commercially available kits supplied by Biodiagnostic, Egypt.

2.4. Measurement of CSE mRNA expression and its activity in kidney tissue:

CSE mRNA expression was measured in kidney tissue using RT-PCR. Total RNA was isolated from kidney tissues using Trizol reagent (Life Technologies, Rockville, MD, USA), treated with DNase I (Roche Applied Science Mannheim, Germany), and purified using RNA clean-up kit (CW biotech, Beijing, China). One microgram of total RNA was applied for cDNA reverse transcription kit. Primer used for CSE was 5`-CATGGATGAAGTGTATGGAGGC-3`, and the reverse primer sequence was 5`-CGGCAGCAGAGGTAACAATCG-3`(18). The PCR reactions were performed in a total volume of 20 µL using the following thermal cycling parameters: The PCR conditions were 95° C for 30 sec for denaturation, 58 °C for 30 sec for annealing, and extension at 72 °C for 30 sec for 40 cycles. PCR products were analyzed by agarose gel electrophoresis and imaged under a UV lamp. The amount of CSE was determined relative to β-actin cDNA in the same samples. CSE activity was measured by an ELISA assay kit (Mybiosource, USA) according to the manufacturer's instructions.

2.5. Determination of serum urea and creatinine levels:

Creatinine and urea concentrations were measured in serum samples by an enzymatic colorimetric method using commercial kits (Biomed, Egypt) for urea and (Spinreact, Spain) for creatinine.

2.6. Measurement of oxidative stress parameters

Hydrogen peroxide level was measured in tissue homogenates using a colorimetric assay kit according to the manufacturer instructions (Sigma Aldrich, USA, cat. No. MAK311). Malondialdehyde (MDA) level was measured as a marker of lipid peroxidation in kidney homogenates according to a colorimetric method as described by Buege and Aust (19).
Activities of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were measured in kidney homogenates by colorimetric assay kits following the procedures described by the manufacturers (Biodiagnostics, Egypt for catalase and Abcam, United states for SOD).

2.7. Western blot analysis:

The kidney homogenate samples were assayed for total protein concentration using the Lowry assay(20) to ensure equal loading in SDS-PAGE. Protein preparations in equal amounts (20 mg in 10 µl) were run on SDS polyacrylamide gels. The separated protein samples were electro-transferred to polyvinylidene fluoride membranes. Blocking the membrane with 5% skimmed milk for 30 minutes was performed to prevent non-specific background binding of antibodies. Membranes were treated with a primary antibody against NF-κB (Invitrogen, Thermofischer, USA) overnight at 4 °C using a slow rocking shaker. The membranes were then washed 3 times with buffer and treated with HRP-conjugated secondary antibody (Sigma, USA).The chemiluminescence signals generated after addition of the chemiluminescent substrate were captured using a CCD camera-based imager. The results were calculated as the mean ratio of the target protein density to β-actin density.

2.8. Evaluation of caspase-3, transforming growth factor-beta and tumor necrosis factor alpha concentrations:

Renal TNF-α, TGF-β and caspase-3 concentrations were determined using ELISA technique according to instructions of the manufacturers (Thermo fisher, USA, cat. No. KRC3012) for TNF-α, (Invitrogen, USA, cat. No. BMS623-3) for TGF-β measurement and (Biovision, USA, cat. No. K106-25) for determination of caspase-3.

2.9. Histopathological Examination:

Formalin-fixed kidney tissues were cleared in xylene and embedded in paraffin at 56°C in a hot air oven for one day. Paraffin-beeswax blocks were sliced by a microtome into 5 □ m sections. The tissue sections were positioned on glass slides, deparaffinized and stained with hematoxylin and eosin, tissue slides were examined using alight microscope (Olympus CX41)

2.10. Statistical analysis:

All data were expressed as mean ± SEM (standard error of the mean) and were analyzed using one-way ANOVA followed by the Tukey-Kramer post analysis test to compare all groups. pvalues < 0.05 were considered significant. GraphPad Prism software version 6.00 for Windows® (*www.graphpad.com*, SanDiego, CA, USA) was used for analysis.

3. Results:

3.1. Effect of L-CYS and PAG treatmentson glycemic control:

As shown in (**Table 1**), diabetic rats showed a significant increase in fasting BGL, insulin level and HOMA–IR when compared to control non-diabetic rats. On the other hand, treatment with L-CYS for 4 weeks after induction of diabetes significantly attenuated the diabetes effects on fasting glucose and insulin levels compared to non-treated diabetic rats. Inhibition of H_2S production by PAG treatment showed a non-significant elevation in serum insulin level when compared to diabetic animals, while it was significantly different from L-CYS group.

3.2. Effect of L-CYS and PAG treatments on lipid profile

Diabetic rats had significantly greater total cholesterol, LDL and triglycerides levels when compared to control. There was a significant reduction in cholesterol, LDL and triglycerides levels in animals treated with L-CYS. PAG-treated animals showed no significant effect on lipid profile in comparison with diabetic group. Lipid profile results are shown in (**Table 2**).

3.3. Effect of L-CYS and PAG treatments on serum urea and creatinine levels

Data in **(Figure 1)** show that induction of diabetes per seresulted in significant increases in serum urea and creatinine levels when compared with control animals. Treatment with L-CYS attenuated the increase of urea and creatinine level induced by diabetes. However, PAG treatment did not alter the diabetes-induced elevation of urea and creatinine level when compared to diabetic untreated animals.

3.4. Effect of L-CYS and PAG treatments on renal CSE mRNA and activity

The expression of CSE mRNA was significantly diminished in diabetic rats when compared to normal rats as shown in (**Figure 2**) Treatment with L-CYS significantly ameliorated diabetes-induced reduction in CSE mRNA expression. PAG treatment, on the other hand, significantly ($p< 0.05$) decreased CSE mRNA expression when compared to L-CYS-treated animals, while the differences were not significant from diabetic animals.

Moreover, in comparison with control rats, kidney CSE activity was significantly lower in the diabetic group (10.25± 0.60 vs. 23.00 ± 1.61pg/ml, $p< 0.05$). Interestingly, treatment with L-CYS almostrestored the activity of CSE enzyme (20.7 ± 1.16pg/ml). PAG-treated animals showed a non-significant suppression of CSE activity when compared to diabetic rats. However, these animals showed significantly lower CSE activity when compared to animals that received L-CYS treatment (**Figure 2C**).

3.5. Effect of L-CYS and PAG treatments on renal NF-κB protein expression

Figure 3 (A & B) demonstrate the anti-inflammatory effect of chronic treatment with L-CYS on diabetic rats, which resulted in attenuation of diabetes-induced increase in protein expression of NF-κB. However, administration of the CSE inhibitor, PAG, resulted in a significant increase in NF-κB protein expression when compared to L-CYS and diabetic group.

3.6. Effect of L-CYS and PAG treatments on renal caspase-3, TNF-α and TGF-β concentrations:

Diabetic rats showed a significant elevation of caspase-3, TNF-α and TGF-β concentrations in kidney homogenate when compared to control normal rats (**Figure 3**). However, L-CYS treatment significantly suppressed the diabetes-induced apoptotic enzyme caspase-3 as well as the inflammatory markers (TNF-α and TGF-β) levels. On the other hand, PAG treatment significantly increased the renal concentrations of caspase-3, TGF-β and TNF-α when compared to the diabetic and L-CYS groups.

3.7. Effect of L-CYS and PAG treatments on renal MDA and hydrogen peroxide production:

Induction of type 2 diabetes led to a significant elevation of H_2O_2 production and the MDA level in the kidney tissues compared to control rats (data in **Figure 4 (A&B)**). Rats administered L-CYS for four weeks showed a significant attenuation of diabetes-induced increase in H_2O_2 production and MDA levels when compared to diabetic animals. On the other hand, MDA level and H_2O_2 production were significantly increased in diabetic animals that received PAG when compared to diabetic untreated animals.

3.8. Effect of L-CYS and PAG treatments on the levels of renal antioxidant enzymes

Data in **Figure 4 (C & D)** show that renal levels of CAT and SOD, which were measured as markers of antioxidant capacity, have significantly declined in diabetic as well as PAG-treated diabetic rats when compared to controls. On the other hand, treatment of diabetic animals with L-CYS mitigated the effect of diabetes on these antioxidant enzyme levels in kidney tissue.

3.9. Histopathological changes

Normal histological structure of the glomeruli and tubules at the cortex were recorded in **photomicrograph 5A** of kidney of control animals. Focal areas of coagulative necrosis in some individual tubules were detected in the cortex as shown in **photomicrograph 5B** of kidneys sections of diabetic rats.

Photomicrograph 5C of kidney section of rat from the group that received L-CYS showed little congestion in the blood vessels at the cortex. However, **photomicrograph 5D** showed focal inflammatory cells infiltration observed between the tubules and glomeruli as well as between the tubules and in the perivascular tissue. There was congestion in the glomerular tufts associated with fatty change in the epithelial cells of the tubules.

Table 1: The effect of diabetes induction on fastinf blood glucose level, insulin level and HOMA-IR. HDF/STZ model resulted in increased the level of fasing BGL, insulin level and HOMA-IR. L-CYS treatment abolished the effect of diabetes on glycemic status.
DM: diabetes mellitus, L-CYS: L-cystiene, PAG: dl-propagylglycerol.
Data represent the mean ± SEM of 6 observations; # significant difference from the control group at $p<0.05$, * Significant difference from DM group at $p<0.05$ and ○ significant difference from DM+ L-CYS group at $p<0.05$.

Group	Fasting BGL (mg\dl)	Insulin level (µU\ml)	HOMA-IR
Control	84.00 ± 2.75	2.03 ± 0.15	0.40 ± 0.03
Diabetic group	212.70 ± 7.14 #	3.95 ± 0.38#	2.07 ± 0.07#
Diabetes +L-CYS	99.67 ± 6.29 *	2.51 ± 0.21*	0.56 ± 0.04*
Diabetes +PAG	214.00 ± 20.69 ○	4.26 ±0.40 ○	2.96 ± 0.28 *○

Table 2: The effect of different treatments on lipid profile listed in the table. Results showed that diabetes significantly increases cholesterol, TGs and LDL level. HDL level decreased in diabetic rats. PAG treatment had no effect on diabetes effect on lipid profile. L-CYS treatment attenuated diabetes induced hyperlipidemia with improvement in HDL level.
DM: diabetes mellitus, L-CYS: L-cystiene, PAG: dl-propagylglycerol.
Data represent the mean ± SEM of 6 observations; # significant difference from the control group at $p<0.05$, * Significant difference from DM group at $p<0.05$ and ○ significant difference from DM+ L-CYS group at $p<0.05$.

Group	Cholesterol level (mg/dL)	TGs level (mg/dL)	HDL level (mg/dL)	LDL Level (mg/dL)
Control	83.65 ± 0.73	94.22 ± 1.09	52.56 ± 0.69	13.77 ± 1.23
Diabetic group	277.0 ± 0.86 #	324.4 ± 2.51 #	15.97 ± 0.12 #	197.9 ± 1.67 #
Diabetes +L-CYS	134.7 ± 1.52 *	187.8 ± 9.28 *	29.58 ± 0.21 *	69.2 ± 1.86 *
Diabetes +PAG	260.5 ± 2.99 ○	316.0 ± 5.81 ○	15.72 ± 0.31 ○	184.8 ± 3.33 ○

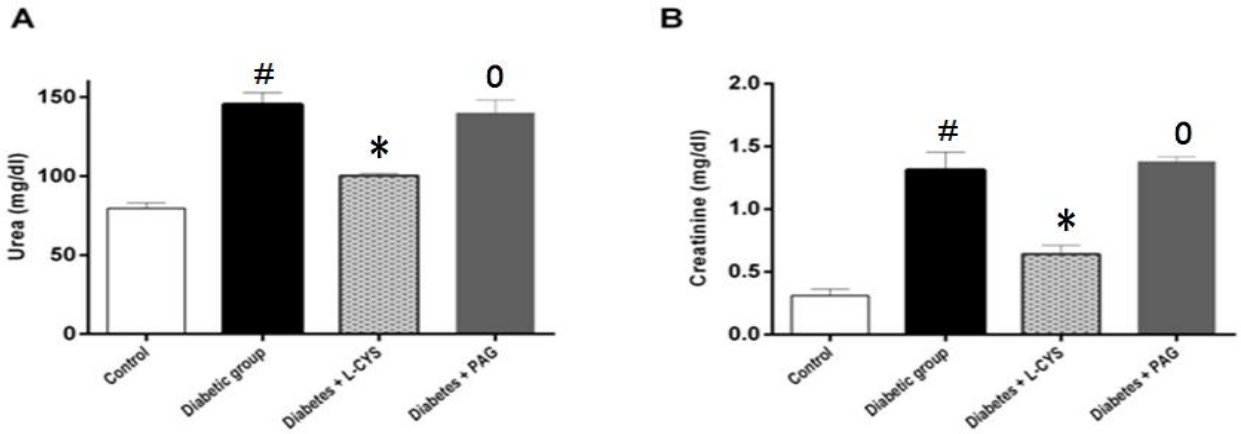

Figure 1: Effect of diabetes induction and chronic administration of different treatments on serum urea and creatinine level (A,B). **A:** H_2S donor prevents elevated serum urea level caused by diabetes, PAG treatment failed to improve that. **B:** L-CYS treatment prevents diabetes induced increase in serum creatinine level.
L-CYS: L-cystiene, PAG: dl-propargylglycerol.
Data represent the mean ± SEM of 6 observations; # significant difference from the control group at $p<0.05$, * Significant difference from diabetic group at $p<0.05$, and ○ significant difference from diabetes + L-CYS group at $p<0.05$.

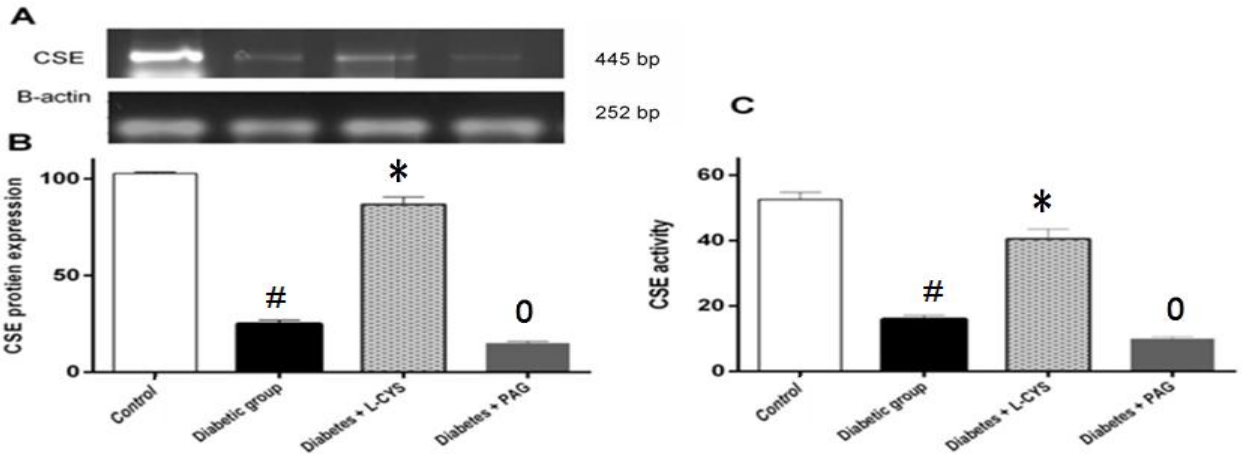

Figure 2: A: RT PCR blots and B: bar chart representing the CSE mRNA expression in different groups. **C:** CSE activity in renal tissue represented by a bar chart. Diabetes model showed a significant decrease in CSE mRNA expression and activity. Endogenous H_2S significantly prevented effects of diabetes on these two parameters, while PAG showed no difference on diabetes effects.
CSE: cystathionine gamma layse, L-CYS: L-cystiene, PAG: dl-propargylglycerol.
Data represent the mean ± SEM of 6 observations; # significant difference from the control group at $p<0.05$, * Significant difference from diabetic group at $p<0.05$, and○ significant difference from diabetes + L-CYS group at $p<0.05$.

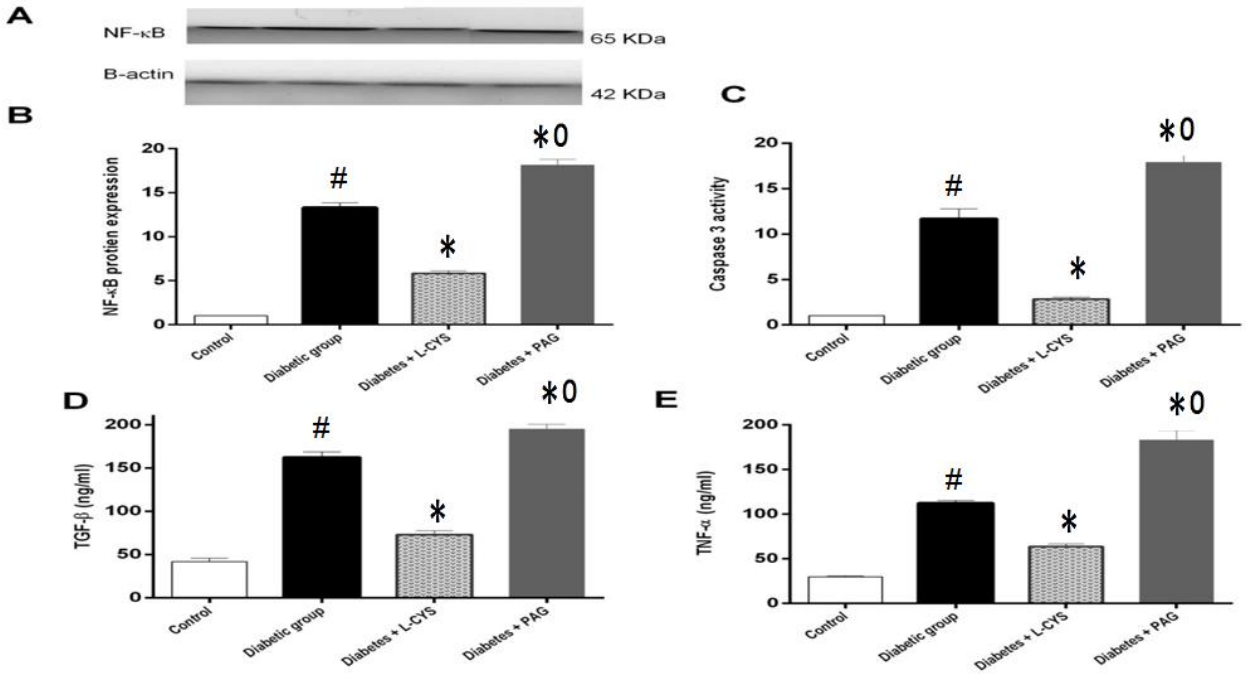

Figure 3: A: representative western blots and **B:** bar chat. Both represent the protein expression of NF-κB in kidney tissue and its alteration by diabetes induction and chronic administration of different drugs. **C:** Bar chart showing the effect of diabetes and H_2S donor and inhibitor on caspase-3 activity in kidney tissue. **D & E** the two bar charts showing the level of renal TGF-β and serum level of TNF-α in different animal groups respectively. Diabetes significantly increases levels of TGF-β and TNF-α. While treatment with L-CYS diminishes this increase, animals received PAG showed a significant elevation of TGF-β level in kidney tissue and TNF-α level in serum.
NF-κB: nuclear factor kappa B. TGF-β: transforming growth factor β, TNF-α: tumor necrosis factor alpha, L-CYS: L-cystiene, PAG: dl-propargylglycerol.
Data represent the mean ± SEM of 6 observations; # significant difference from the control group at $p<0.05$, * Significant difference from diabetic group at $p<0.05$, and ○ significant difference from diabetes + L-CYS group at $p<0.05$.

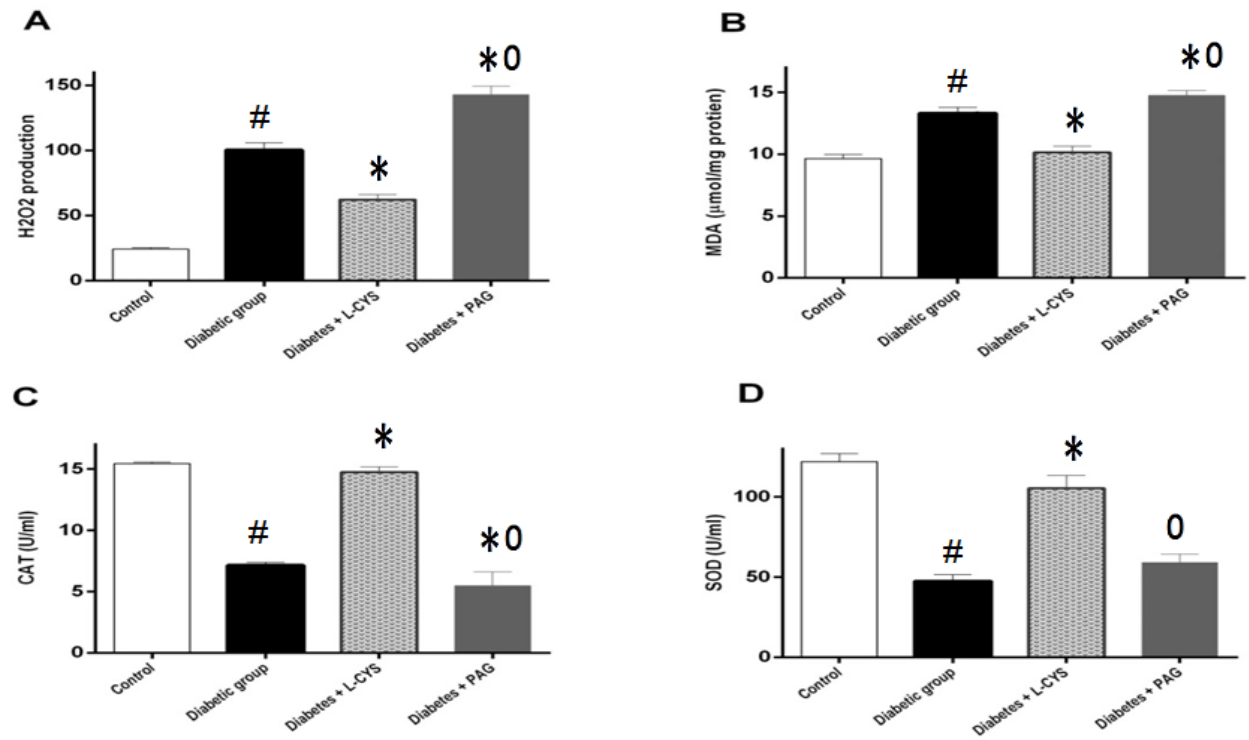

Figure 4: Bar charts represent **A:** H_2O_2 production, **B:** MDA level **C:** catalase activity and **D:** SOD activity in kidney tissue. Model of diabetes showed elevated levels of H_2O_2 and MDA and has a reduced effect on antioxidant enzyme activity (CAT and SOD). H_2S donor diminished the effects observed in model of diabetes.
H_2O_2: hydrogen peroxide, MDA: malondialdhyide CAT: catalase, SOD: superoxide dismutase, L-CYS: L-cystiene, PAG: dl-propargylglycerol.
Data represent the mean ± SEM of 6 observations; # significant difference from the control group at $p<0.05$, * Significant difference from diabetic group at $p<0.05$ and ○ significant difference from diabetes + L-CYS group at $p<0.05$.

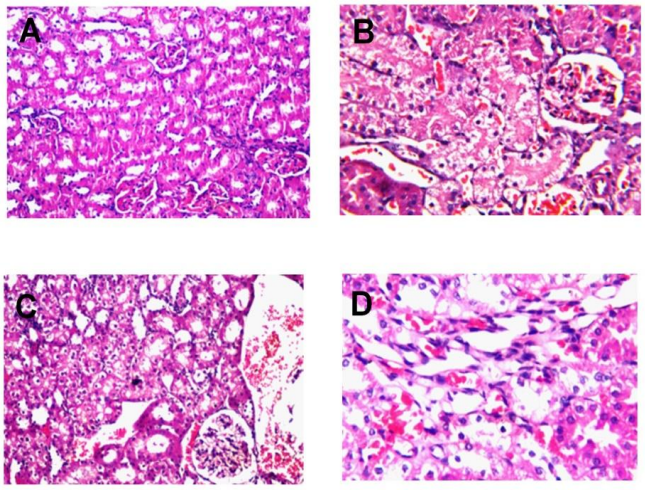

Figure 5: Control: Normal histological structure of the glomeruli and tubules at the cortex of kidney **6A**
Diabetic group: Focal areas of coagulative necrosis in some individual tubules were detected in the cortex **6B**.
Diabetes + L-CYS: little congestion in the blood vessels at the cortex of kidney **6C**.
Diabetes + PAG: Focal inflammatory cells infiltration was observed in between the tubules and glomeruli as well as in between the tubules and in the perivascular tissue. There was congestion in the glomerular tufts associated with fatty change in the epithelial cells of the tubules **6D.**

4. Discussion

In the current study, T2DM was induced by feeding high fat diet (40% fats) to rats for 12 weeks followed by a single injection of STZ, which resulted in impaired glucose tolerance, insulin resistance and hyperlipidemia with low level of HDL. Moreover, diabetes induction was accompanied with inflammation evident as NF-κB over expression in kidney tissue, which presumably resulted in increased activity of the apoptotic enzyme caspase-3, and possibly other inflammatory mediators. Not surprisingly, induction of diabetes was accompanied with increased renal H_2O_2 production and malondialdhyde (MDA) level, both of which are well-documented markers of oxidative stress and lipid peroxidation. Altogether, these factors lead to deteriorated kidney function as evidenced by increased serum urea and creatinine levels. Furthermore, histopathological examination of diabetic kidneys revealed pathological findings that were consistent with the aforementioned diabetes-induced biochemical and functional changes.

The aim of the current work was to examine the role of CSE in diabetes-related kidney dysfunction and the possible contribution of endogenous H_2S to renal protection. Thus, our experimental design involved supplying a donor of H_2S (L-CYS) as well as blocking one of the endogenous sources of the gasotransmitter (PAG for CSE). When the endogenous H_2S donor was administered to diabetic rats, significant improvement of hyperglycemia and hyperlipidemia was achieved. In addition, L-CYS attenuated diabetes-induced inflammation and oxidative stress, and expectedly recovered kidney dysfunction induced by diabetes. On the other hand, chronic administration of the CSE inhibitor, PAG, resulted in worsening of glycemic, inflammatory and oxidative stress status. PAG has been used in several studies to test the biological effects of inhibiting endogenous H_2S production (21). Importantly, induction of diabetes per se resulted in decreased expression and activity of endogenous cystathionine gamma-lyase (CSE). These findings are consistent with previously reported results showing that either diabetes orglucose intolerance greatly affects the level of H_2S indifferent models of diabetes (22-24). CSE is amajor H_2S-producing enzyme that utilizes L-cysteine as the main substrate in mammalian cells (25). Animals treated with L-CYS for four weeks after diabetes induction showed an obvious increase in CSE mRNA expression and activity in kidney tissue. These effects might be partially attributed to the cytoprotective effects of L-CYS, or due to increased production of H_2S.

Hyperlipidemia is an important factor that contributes to kidney disease. The relationship between hyperlipidemia and progression of chronic kidney disease is well established (26). Patients with elevated cholesterol levels are at higher risk of developing kidney disease (27). In the current study, lipid profile evaluation revealed the direct impact of high fat diet and different treatments on lipid metabolism. Rats on HFD showed increased plasma total cholesterol, LDL and triglycerides levels, while treatment of diabetic animals with L-CYS resulted in an improvement in lipid profile. Decreasing cholesterol and triglycerides may be one of L-CYS-mediated mechanisms implicated in protecting the kidney from diabetes induced nephropathy. Conversely, chronic inhibition of CSE by PAG further contributed to diabetes-induced hyperlipidemia as evident by high serum cholesterol and triglycerides.

Another contributing factor to diabetic nephropathy is increased oxidative stress. Oxidative stress is defined as an imbalance between production of free radicals and the activity of scavenging enzymes (28). In our experiment we measured peroxide anion production and MDA level in the kidney tissues. Moreover, renal CAT and SOD as defensive enzymes were also evaluated. Diabetic model showed an increase in free radical production with a concomitant reduction in the activity of CAT and SOD enzymes. Whether or not the increase in oxidative stress is a direct effect of decreased renal CAT and SOD cannot be ruled out by the results of this study. Nonetheless, increased oxidative stress is a characteristic diabetes-related deleterious factor that is linked to renal dysfunction (29, 30).In the current work, L-CYS treatment attenuated the diabetes-induced oxidative stress. Furthermore, renal SOD and CAT activities were restored to normal levels when animals were treated with L-CYS for four weeks, with subsequent reduction in H_2O_2 and MDA levels. Inhibition of CSE enzyme aggravated the oxidative stress status induced by diabetes. These data suggest that H_2S plays an important role in cellular redox signaling regulation and that it might be a potential renoprotective molecule possibly via attenuation of diabetes-induced oxidative stress and decrease in the activity of CAT and SOD.

Diabetic nephropathy is considered as an inflammatory process, and the involvement of immune cells in its pathogenesis is well-documented (31). For example, hyperglycemia induces macrophage production and obesity accompanied by hyperglycemia activate nuclear factor kappa B (NF-κB).

Activation of NF-κB leads to stimulation of cytokines such as transforming growth factor beta TGF-B and tumor necrosis factor alpha TNF-α (32), as well as increased ROS production. All of these signals can converge into activated inflammatory status and apoptosis (33). In the current study, diabetic animals showed an increase in NF-κB expression with elevation in renal caspase-3, TGF-β and TNF-α. Administration of L-CYS for four weeks after diabetes induction attenuated the elevation of the previously-mentioned inflammatory markers induced in diabetic model. Inhibition of CSE enzyme by PAG chronic treatment resulted in a significant increase of renal NF-κB expression associated with high levels of TGF-β, TNF-α and caspase-3. These results provide further evidence on the protective role of H_2S against diabetes-induced renal inflammatory changes. These findings are further accentuated by the negative effect of inhibiting H_2S production by PAG, which resulted in deteriorated inflammatory status leading to worsening of kidney function.

In summary, L-CYS treatment resulted in improvement of glucose metabolism, lipid profile, oxidative stress and inflammation induced by diabetes. Animals treated with L-CYS showed a significant decrease in serum urea and creatinine levels. Thus, administration of L-CYS as an endogenous substrate for producing H_2S may be a novel agent for delaying the progression of kidney impairment induced in type 2 diabetes mellitus. These findings beside the worsening effects resulted with PAG treatment (CSE enzyme inhibitor, which is one of main enzymes responsible for H_2S production in kidney) proved that L-CYS exerts its protective effects, at least in part, through increasing H_2S generation. To sum up, the results of this study highlighted the importance of the enzyme CSE, and its product H_2S as important renoprotective agents in diabetic nephropathy.

References

[1] Hou C-L, Wang M-J, Sun C, Huang Y, Jin S, Mu X-P, Chen Y, Zhu Y-C. Protective Effects of Hydrogen Sulfide in the Ageing Kidney. Oxidative Medicine and Cellular Longevity. 2016;2016:7570489.

[2] Jenkins TA, Nguyen JC, Hart JL. Decreased vascular H_2S production is associated with vascular oxidative stress in rats fed a high-fat western diet. *Naunyn-Schmiedeberg's archives of pharmacology.* 2016;389(7):783-90.

[3] Fujii K, Sakuragawa T, Kashiba M, Sugiura Y, Kondo M, Maruyama K, Goda N, Nimura Y, Suematsu M. Hydrogen sulfide as an endogenous modulator of biliary bicarbonate excretion in the rat liver. *Antioxidants & redox signaling*. 2005;7(5-6):788-94.

[4] Sun Y, Huang Y, Zhang R, Chen Q, Chen J, Zong Y, Liu J, Feng S, Liu AD, Holmberg L, Liu D, Tang C, Du J, Jin H. Hydrogen sulfide upregulates KATP channel expression in vascular smooth muscle cells of spontaneously hypertensive rats. *Journal of molecular medicine (Berlin, Germany).* 2015;93(4):439-55.

[5] Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, Wang R. H_2S as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science (New York, NY).* 2008;322(5901):587-90.

[6] Zheng J, Zhao T, Yuan Y, Hu N, Tang X. Hydrogen sulfide (H_2S) attenuates uranium-induced acute nephrotoxicity through oxidative stress and inflammatory response via Nrf2-NF-kappaB pathways. *Chemico-biological interactions*. 2015;242:353-62.

[7] Du J, Huang Y, Yan H, Zhang Q, Zhao M, Zhu M, Liu J, Chen SX, Bu D, Tang C, Jin H. Hydrogen sulfide suppresses oxidized low-density lipoprotein (ox-LDL)-stimulated monocyte chemoattractant protein 1 generation from macrophages via the nuclear factor kappaB (NF-kappaB) pathway. *The Journal of biological chemistry*. 2014;289(14):9741-53.

[8] Kong LL, Wu H, Cui WP, Zhou WH, Luo P, Sun J, Yuan H, Miao LN. Advances in murine models of diabetic nephropathy. *Journal of diabetes research*. 2013;2013:797548.

[9] Kong L-l, Wu H, Cui W-p, Zhou W-h, Luo P, Sun J, Yuan H, Miao L-n. Advances in Murine Models of Diabetic Nephropathy. *Journal of diabetes research*. 2013;2013:797548.

[10] Wada J, Makino H. Inflammation and the pathogenesis of diabetic nephropathy. *Clinical science (London, England : 1979).* 2013;124(3):139-52.

[11] Boric M, Jelicic Kadic A, Puljak L. The expression of calcium/calmodulin-dependent protein kinase II in the dorsal horns of rats with type 1 and type 2 diabetes. *Neuroscience letters.* 2014;579:151-6.

[12] Khalifa MMA, Mangoura SA, El-Moselhy MA, El-Daly ME. The effectiveness of beta adrenoceptor antagonists on progression of insulin resistance and type 2 diabetes in high fat diet-fed rats. *Saudi Pharmaceutical Journal*. 2009;17(1):19-28.

[13] Shao M, Lu X, Cong W, Xing X, Tan Y, Li Y, Li X, Jin L, Wang X, Dong J, Jin S, Zhang C, Cai L. Multiple low-dose radiation prevents type 2 diabetes-induced renal damage through attenuation of dyslipidemia and insulin resistance and subsequent renal inflammation and oxidative stress. *PloS one*. 2014;9(3):e92574.

[14] Li L, Liao G, Yang G, Lu Y, Du X, Liu J, Li L, Wang C, Li L, Ren Y, Zhong Z, Cheng J, Chen Y. High-fat diet combined with low-dose streptozotocin injections induces metabolic syndrome in Macaca mulatta. *Endocrine*. 2015;49(3):659-68.

[15] Medeiros JV, Bezerra VH, Gomes AS, Barbosa AL, Lima-Junior RC, Soares PM, Brito GA, Ribeiro RA, Cunha FQ, Souza MH. Hydrogen sulfide prevents ethanol-induced gastric damage in mice: role of ATP-sensitive potassium channels and capsaicin-sensitive primary afferent neurons. *The Journal of pharmacology and experimental therapeutics.* 2009;330(3):764-70.

[16] Rong-na L, Xiang-jun Z, Yu-han C, Ling-qiao L, Gang H. Interaction between hydrogen sulfide and nitric oxide on cardiac protection in rats with metabolic syndrome. *Zhongguo yi xue ke xue yuan xue bao Acta Academiae Medicinae Sinicae*. 2011;33(1):25-32.

[17] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28(7):412-9.

[18] Collin M, Anuar FB, Murch O, Bhatia M, Moore PK, Thiemermann C. Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. *British journal of pharmacology*. 2005;146(4):498-505.

[19] Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods in enzymology*. 1978;52:302-10.

[20] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *The Journal of biological chemistry*. 1951;193(1):265-75.

[21] Mok YY, Atan MS, Yoke Ping C, Zhong Jing W, Bhatia M, Moochhala S, Moore PK. Role of hydrogen sulphide in haemorrhagic shock in the rat: protective effect of inhibitors of hydrogen sulphide biosynthesis. *British journal of pharmacology.* 2004;143(7):881-9.

[22] Zhong X, Wang Y, Wu J, Sun A, Yang F, Zheng D, Li T, Dong S, Zhao Y, Yang G, Xu C, Sun D, Lu F, Zhang W. Calcium sensing receptor regulating smooth muscle cells proliferation through initiating cystathionine-gamma-lyase/hydrogen sulfide pathway in diabetic rat. Cellular physiology and biochemistry : *international journal of experimental cellular physiology, biochemistry, and pharmacology.* 2015;35(4):1582-98.

[23] Brancaleone V, Roviezzo F, Vellecco V, De Gruttola L, Bucci M, Cirino G. Biosynthesis of H_2S is impaired in non-obese diabetic (NOD) mice. *British journal of pharmacology*. 2008;155(5):673-80.

[24] El-Sayed SS, Zakaria MN, Abdel-Ghany RH, Abdel-Rahman AA. Cystathionine-gamma lyase-derived hydrogen sulfide mediates the cardiovascular protective effects of moxonidine in diabetic rats. *European journal of pharmacology*. 2016;783:73-84.

[25] Zhao K, Li H, Li S, Yang G. Regulation of cystathionine gamma-lyase/H(2)S system and its pathological implication. Frontiers in bioscience (*Landmark edition*). 2014;19:1355-69.

[26] Samuelsson O, Mulec H, Knight-Gibson C, Attman PO, Kron B, Larsson R, Weiss L, Wedel H, Alaupovic P. Lipoprotein abnormalities are associated with increased rate of progression of human chronic renal insufficiency. Nephrology, dialysis, transplantation : *official publication of the European*

Dialysis and Transplant Association - European Renal Association. 1997;12(9):1908-15.
[27] Schaeffner ES, Kurth T, Curhan GC, Glynn RJ, Rexrode KM, Baigent C, Buring JE, Gaziano JM. Cholesterol and the risk of renal dysfunction in apparently healthy men. *Journal of the American Society of Nephrology : JASN.* 2003;14(8):2084-91.
[28] Betteridge DJ. What is oxidative stress? Metabolism: *clinical and experimental.* 2000;49(2 Suppl 1):3-8.
[29] Hong YA, Lim JH, Kim MY, Kim Y, Park HS, Kim HW, Choi BS, Chang YS, Kim HW, Kim T-Y. Extracellular Superoxide Dismutase Attenuates Renal Oxidative Stress Through the Activation of Adenosine Monophosphate-Activated Protein Kinase in Diabetic Nephropathy. *Antioxidants & redox signaling*. 2017.
[30] Kachhawa K, Agrawal D, Rath B, Kumar S. Association of lipid abnormalities and oxidative stress with diabetic nephropathy. *Journal of Integrative Nephrology and Andrology*. 2017;4(1):3.
[31] Rivero A, Mora C, Muros M, Garcia J, Herrera H, Navarro-Gonzalez JF. Pathogenic perspectives for the role of inflammation in diabetic nephropathy. *Clinical science (London, England : 1979).* 2009;116(6):479-92.
[32] Ha H, Yu MR, Choi YJ, Kitamura M, Lee HB. Role of high glucose-induced nuclear factor-kappaB activation in monocyte chemoattractant protein-1 expression by mesangial cells. *Journal of the American Society of Nephrology : JASN*. 2002;13(4):894-902.
[33] Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, Shah AM. NADPH oxidases in cardiovascular health and disease. *Antioxidants & redox signaling*. 2006;8(5-6):691-728.