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Research Article

The Potential Role of Gasotransmitters in the Protective Effect of Sitagliptin Against Diabetic Nephropathy in Rats: Mechanistic Insights

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ABSTRACT

Sitagliptin has pleiotropic benefits in addition to its ability to lower blood sugar levels. In order to understand how sitagliptin protects against Diabetic Nephropathy (DN), this study examined the role of gasotransmitters and their mechanism (s) in sitagliptin protective impact on DN. Based on measurements of serum renal function markers and histopathological examination, sitagliptin provided protection against DN-induced renal impairment. In addition to reducing blood sugar levels, sitagliptin prevented renal oxidative stress, nitrosative stress, inflammation, and apoptosis brought on by DN. Additionally, it prevented DN-induced decline in renal heme oxygenase-1 (HO-1) and hydrogen sulphide (H_2S) levels as well as the rise in nitric oxide (NO) production. Furthermore, sitagliptin prevented DN-induced decrease in the protein expressions of endothelial NO synthase, HO-1, and cystathionine-γ-lyase and increase in the protein expression of inducible NO synthase in the kidney tissue. All of sitagliptin effects were increased by co-administration of the NO biosynthesis inhibitor L-NAME, carbon monoxide (CO)-releasing molecule-A_1 (CORM-A_1) or the H_2S donor NaHS. The NO donor, L-arginine, the CO biosynthesis inhibitor, zinc protoporphyrin or the H_2S biosynthesis inhibitor, DL-propargylglycine antagonized sitagliptin` impacts. These findings show, for the first time, that sitagliptin protective impact against DN is significantly influenced by rising CO and H_2S levels and subsequently falling NO levels. The ability of CORM-A_1, NaHS, and L- NAME to reduce renal oxidative stress, nitrosative stress, inflammation, and apoptosis may be favourably correlated with their ability to increase the protective effect of sitagliptin in DN.

INTRODUCTION

In both type 1 and type 2 diabetes, Diabetic Nephropathy (DN) is a chronic microvascular complication [1]. Oxidative stress, one of the molecular mechanisms involved in the development [2] and progression [3,4] of DN is produced by persistent hyperglycemia. Additionally, it causes inflammation which is involved in the development and progression of DN [2,4,5]. Furthermore, oxidative stress and inflammation brought on by prolonged hyperglycemia contribute to apoptosis in renal cells [6-8] which causes DN progression. The gasotransmitter, Nitric Oxide (NO) is produced endogenously by neuronal NO synthase (nNOS) and endothelial NO

synthase (eNOS) which under normal conditions are in charge of the continual basal release of small amounts of NO. Additionally, it produced by inducible NO synthase (iNOS) which is activated during pathogenic processes and generates large levels of NO for lengthy periods of time [9]. Deficiency of eNOS in diabetic animals hastens nephropathy progression [10] while increased eNOS expression and activity produces renoprotective effect [11]. Furthermore, iNOS activity and expression were elevated in diabetics and DN animals, [12,13] which causes renal injury. The enzyme Heme Oxygenase (HO) catalyses a process that produces the majority of the endogenous gasotransmitter, CO. The main source of endogenous CO in higher organisms is the stress inducible enzyme, HO-1 [14]. Enhancement of endogenous CO production or direct delivery of exogenous CO have been applied in many health research fields and clinical settings. In numerous types of cellular injury inhalation of low concentration of CO exerts anti-inflammatory, antiapoptotic and antioxidant activities [15,16]. However, the use of gaseous CO to inhibit cellular injury is restricted because it is harmful to cellular respiration. The ability to more thoroughly research CO-mediated impacts is made possible by the development of novel CO-releasing molecules (CORMs) which release CO in biological systems in a controlled manner without affecting cellular respiration [15]. The gasotransmitter, Hydrogen sulfide (H_2S) is endogenously produced by cystathionine- γ-lyase (CSE) and Cystathionine-β-Synthase (CBS) [17]. While CBS can be identified in the brain and nervous system, CSE is mostly expressed in the liver, heart and kidney [18]. It has been found that physiological H2S concentrations produce antioxidant, anti- inflammatory and antiapoptotic properties [19]. Patients and animals with chronic kidney diseases have been shown to have decreased H2S levels [20,21]. Furthermore, Lodhi et al. [22] observed that chronic hyperglycemia causes H2S to be depleted, which results in DN. Additionally, H2S decreased the protein expression of apoptotic Bax and caspase-3 but increased that of antiapoptotic Bcl-2 in rat kidney [23]. The antidiabetic medications, dipeptidyl peptidase-4 (DPP-4) inhibitors, such as sitagliptin, in addition to their glucose reducing effect, exert pleiotropic actions [24]. DPP-4 inhibitors have been shown in numerous studies reduce oxidative stress in diabetic patients and diabetic rats [25] as well as renal oxidative stress, inflammation, and apoptosis in diabetic animals [24,26,27]. In light of these findings, the current study was designed to investigate the interrelationship between gasotransmitters and the protective effect of sitagliptin against DN in rats. The role of oxidative stress, nitrosative stress, inflammation and apoptosis in mediating sitagliptin and gasotransmitter effects was also monitored in this study.

MATERIALS AND METHODS

Animals

Male adult Wistar rats weighing 180-220 g, purchased from animal house of Faculty of Medicine, Assiut University were used in all experiments. The animals were housed in stainless steel cages under a 12 h light dark cycle at 25 C. Rats had free access to water and chow (laboratory chow). This study was conducted in accordance with the internationally accepted principles for Guide for the Care and Use of Laboratory Animal. Experiments reported here were approved by our institutional ethics committee (Ethical approval NO. 17200153, 2018).

Induction of Type 2 diabetes mellitus

Type 2 diabetes was induced in overnight fasted rats by an intraperitoneal (i.p.) injection of 110 mg/kg nicotinamide (NA, 4% solution in saline) followed after 15 min by a single i.p. injection of 60 mg/kg Streptozotocin (STZ) freshly prepared in 0.1 mol cold citrate buffer (pH 4.5) [26,28]. After the STZ+NA challenge, the animals were allowed to drink 5% glucose solution instead of drinking water for 24 h to overcome the initial STZ- induced hypoglycemic mortality. Blood samples were taken from tail vein of rats before and 3 and 7 days after STZ+NA injection. Blood glucose levels were measured using a hand-held glucometer. Rats with random blood glucose levels greater than 250 mg/dl on both days were considered as diabetic and were used for diabetic nephropathy studies [26,29]. Control animals were treated with the citrate buffer.

Experimental design

In preliminary experiments, two groups of diabetic animals, 8 rats each were used. Blood and urine samples were taken from the first group 4 weeks after STZ+NA injection and from the second group 8 weeks after STZ+NA injection. Blood and urine samples were also collected from two groups of control animals 4 and 8 weeks after citrate buffer injection. In another set of

experiments, 7 days after administration of STZ+NA, diabetic animals were divided into 8 groups of 8 rats each. Group-I were orally administered sitagliptin (0.5% solution in saline) at dose level of 10 mg/kg/day for seven weeks. Group-II and III rats were treated for seven weeks with 10 mg/kg/day sitagliptin orally along with 100 mg/kg/day L-arginine (L-A, 4% solution in saline) and 10 mg/kg/day L-N(G)-nitro arginine methyl ester (L-NAME, 1% solution in saline) i.p., respectively. For seven weeks, animals in groups IV and V received 10 mg/kg/day sitagliptin orally along with 0.1 mg/kg/day carbon monoxide-releasing molecule-A1 (CORM-A1, 0.01% solution in saline) and 0.25 mg/kg/day zinc protoporphyrin (ZnPP, 0.05% solution in saline) i.p., respectively. Rats of group-VI and VII were treated for seven weeks with 10 mg/kg/day sitagliptin orally in combination with 3 mg/kg/day sodium hydrosulfide (NaHS, 0.3% solution in saline) and 5 mg/kg/day DL-propargylglycine (DL-PAG, 0.5% solution in saline) i.p., respectively. Animals used as control were treated with normal saline. Doses of sitagliptin and gasotransmitter modulators were selected according to earlier studies [30,31].

Biochemical measurements

One day before the end of each study period, each rat was housed individually in a metabolic cage for 24 h in order to collect urine samples from the control and diabetic animals. At the end of each experimental period, the overnight fasted rats were sacrificed by decapitation. Blood and kidney tissues were taken from each animal for biochemical measurements. Blood and urine samples were centrifuged for 10 min. Following centrifugation, serum and urine samples were collected in order to estimate of urea, Blood Urea Nitrogen (BUN), creatinine, creatinine clearance and albumin levels. The serum and urine samples might be used right away or they could be stored at -80^{o}C until assay. The isolated kidneys from each animal were rinsed in ice-cold saline. One kidney was kept in 10% formalin for histopathological and immunohistochemical studies and the other kidney was rinsed in ice-cold saline, dissected, cleaned from the fat and other tissues and blotted carefully. An appropriate weight of the kidney tissue was broken into small pieces and homogenized in 10% w/v phosphate buffer (pH 7.4) or saline. The homogenate was divided into 2 parts. The first part was centrifuged for 10 min at 10,000 rpm and the supernatant was either used immediately for estimation of the levels of malondialdehyde (MDA), tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), caspase-3, nitrite, heme oxygenase-1 (HO-1) and hydrogen sulfide (H2S) or it was stored at -80^{o}C until assay. To the second portion of homogenate a similar volume of perchloric acid (1 mol/l) was added and mixed by vortexing. 5 min at room temperature were given for the mixture to stand. After centrifugation for 10 min, the supernatant was collected carefully and used immediately for estimation of intracellular reduced glutathione (GSH) level or it was stored at -80^{o}C until assay. According to the manufacturer's instructions, the urea levels in the serum and urine were measured by urease-colometric method using the commercially available SPECTRUM Diagnostics Urea/BUN-Liquizyme Kits (Egyptian Company for Biotechnology, Cairo, Egypt). In order to convert the results from serum urea to blood urea nitrogen, they were multiplied by 0.467.

Creatinine levels in the serum and urine as well as creatinine clearance were measured by buffered kinetic jaffe reaction without deproteinization method using a commercially available SPECTRUM Creatinine-Jaffe kits (Egyptian Company for Biotechnology, Cairo, Egypt) in accordance with the manufacturer's instructions. According to the manufacturer's instructions, a commercially available SPECTRUM Albumin-BCG (Acetate Buffer) kit (Egyptian Company for Biotechnology, Cairo, Egypt) was used to measure the serum level of albumin Using Modified bromocresol green colorimetric method. A commercially available BioSystems ALBUMIN (MICROALBUMINURIA) LATEX kit [BioSystems S.A., Barcelona, Spain) was used in accordance with the manufacturer's instructions to measure the urine level of albumin. The thiobarbituric acid reactive substances method previously described by Ohkawa et al. [32] was used to determine Lipid peroxidation in the supernatant. The neutralized supernatant`s intracellular GSH content was assayed using Ellman's reagent and the method of Ellman [33]. By the enzyme-linked immunosorbent assay, the kidney levels of TNF-α, IL-1ß and caspase-3 were determined in the supernatant using the commercially available Rat TNF- α, IL-1ß and CASP3 ELISA kits, respectively (Elabscience Biotechnology Inc., U.S.A) in line

with the guidelines provided by the manufacturer's. Using Griess reagents (Griess, 1879), nitrite level in in the supernatant was assayed as described by Miranda et al. [34]. The kidney level of H2S was determined in the supernatant in accordance with the method of Wilinski et al. [35].

Examination of the histopathology

At the end of the 4 and 8-week treatments, the rats of each group were sacrificed. Phosphate-Buffered Saline (PBS) was used to wash the kidneys after they had been carefully removed. One kidney from each animal was cut into longitudinal sections, which stored in 10% neutral buffered formalin and embedded in paraffin for evaluation of histopathology. Micron sections of 4 to 5 µm thickness were cut and stained with hematoxylin and eosin stain (H&E; Tianjin Runtai, Co., Ltd., Tianjin, China) and periodic acid Schiff base (PAS; Tianjin Runtai, Co., Ltd.) for histopathological observations using a HMIAS-2000 Image Analysis system (Guangzhou Longest Technology, Guangzhou, China). The extent of tubular and glomerular damage was then determined by analyzing the sections. The degree of glomerulosclerosis, mesangiolysis and mesangial expansion were used to calculate the Glomerular Damage Index (GDI) which ranged from 0 to 4. For each section, approximately 80-100 glomerular cells from the renal cortex were observed. GDI was obtained by averaging the scores from the counted glomerular cells. Glomerulosclerosis was semi-quantitatively evaluated in non- HE-stained paraffin sections as follows: Grade 0 represents a normal glomerulus, Grade

1 represents the beginning of mesangial expansion, thickening of the basement membrane and/or irregular lumina of capillaries, Grade 2 represents mild to moderate segmental hyalinosis/sclerosis involving less than 50% of the glomerular tuft, Grade 3 represents diffuse glomerular hyalinosis/ sclerosis involving more than 50% of the tuft and Grade 4 represents diffuse glomerulosclerosis with total tuft obliteration and collapse.

Analysis of the immunohistochemistry

From the previous paraffin blocks, additional sections with a thickness of 3 to 5 µm were cut and mounted on Aminopropyltriethxysilane (APSE) coated slides. After mounting, the slides were dried to get rid of any water that may be trapped under the section. This was accomplished by leaving the slides at 60oC oven overnight. Utilizing the standardized commercially available UltraVision Detection System anti-polyvalent HRP/DAB kit (Scy Tek, U.S.A), immunohistochemical examination of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), heme oxygenase-1 (HO-1) and cystathionine –γ-lyase (CSE) was carried out as instructed by the manufacturer. By omitting the primary antibody, negative control slides were created. Sections from lung carcinoma were stained as a positive control for eNOS, iNOS, while sections from hepatic carcinoma were stained as a positive control for HO-1 and CSE. Slides were examined using an Olympus CX21 optical microscope outfitted with an Olympus E330-AD 41.2X digital camera connected to a computer. The stained slides were assessed and immunoreactivity score was determined for both intensity (-, +, ++, and +++) and proportion (<5%, 6–25%, 26–50%, 51–75%, and > 75%) of cells. The extent of staining was scored as 0: <5%; 1: 6-25%; 2: 26-50%; 3: 51-75% and 4: >75% of cells are stained. Staining intensity was scored from 0 to 3. The final staining score (immunoscore) which was used for statistical analysis was created by multiplying or adding these values together.

Chemicals

Sitagliptin, Streptozotocin, Nicotinamide, N,N-Dimethyl-p-phenylenediamine sulfate, Carbon monoxide-releasing molecule-A_1 and L-N (G)-nitroarginine methyl ester were purchased from Sigma-Aldrich Co (USA). Glutathione reduced was obtained from ICN Biomedicals INC., (USA), Malondialdehyde bis-(dimethylacetal) was obtained from Merck (Germany). Thiobarbituric acid was purchased from MP Biomedicals INC. (France). All other chemicals were of analytical grade.

Statistical analysis of results

The results were presented as the mean ± standard error of the mean (X ± S.E.M.). The one-way analysis of variance (ANOVA) followed by Bonferroni's test as post hoc analysis was used to statistically analyze the difference between the GraphPad Prism software (GraphPad; San Diego CA, USA) was used for all statistical calculations.

RESULTS

Effect of Treatment of Rats with Streptozotocin (STZ) and Nicotinamide (NA) for 4 and 8 Weeks on the Blood Glucose

Level, Kidney Function Markers and Renal Levels of Biochemical Parameters

Results presented in table 1 show that treatment of rats with 110 mg/kg NA i.p. followed after 15 min by i.p. injection of 60 mg/kg STZ produced a significant increase in the blood glucose levels after 4 and 8 weeks when compared to control rats. It is evident from the same table that after 4 weeks of treatment of animals with NA and STZ levels of serum urea, blood urea nitrogen (BUN), urine urea, serum creatinine, urine creatinine, creatinine clearance, serum albumin and urine albumin (microalbuminuria) did not change significantly. After 8 weeks of treatment of animals with the same doses of NA and STZ i.p. the serum BUN, serum creatinine and urine albumin levels were significantly increased. The urine urea, urine creatinine, creatinine clearance and serum albumin levels were significantly decreased by this treatment when compared to the control animals (Table 1). After 4 weeks of treatment of rats with 110 mg/kg NA and 60 mg/kg STZ i.p., the kidney MDA and GSH levels were not changed. In comparison to the control animals, similar treatment of rats produced after 8 weeks a significant increase in the kidney MDA level and a significant decrease in the kidney GSH level (Table 1). Data presented in table 1 also show that i.p. treatment of animals with 110 mg/kg NA followed by 60 mg/kg STZ produced , after 4 weeks, no changes in the renal TNF-α, IL-1ß and caspase-3 levels in comparison to the control rats. A significant increase in the renal TNF-α, IL-1ß and caspase-3 levels was observed after 8 weeks of similar treatment of animals with NA and STZ. It is noteworthy that the mean arterial blood pressure of rats was not increased significantly after 4 and 8 weeks of treatment of animals with NA and STZ.

Effect of Treatment of Rats with Streptozotocin (STZ) and Nicotinamide (NA) on the Renal Nitrite, Heme Oxygenase-1 (HO-1) and Hydrogen Sulfide (H_2S) Levels

Administration of 110 mg/kg NA followed by 60 mg/kg STZ i.p. produced after 4 weeks no changes in the renal nitrite, HO-1 and H2S levels. Treatment of animals with NA and STZ produced, after 8 weeks a significant decrease in the renal HO-1 and H2S levels and a significant increase in the renal nitrite level in comparison to the control animals (Table 1).

Table 1: Effect of treatment of rats with 60 mg/kg Streptozotocin (STZ) and 110 mg/kg Nicotinamide (NA) Intraperitoneally (i.p) on the blood glucose level,kidney function markers, renal biochemical parameters, nitrite, HO-1 and H_2S levels after 4 and 8 weeks.

	Time of measurement		
	Control	After 4 weeks	After 8 weeks
Blood glucose level (mg/dl)	113.80 ±2.37	532.94 **±14.99	561.67 **±19.29
Serum urea (mg/dl)	24.33±0.86	23.33±0.47	99.58 **±4.44
Blood urea nitrogen (mg/dl)	11.30±0.39	10.93±0.26	46.50 **±2.08
Urine urea (g/24 h)	34.01±2.02	33.26±1.79	15.15 **±0.75
Seum creatinine (mg/dl)	0.96±0.07	0.95±0.08	3.93 **±0.23
Urine creatinine (mg/dl)	2.10±0.10	2.05±0.15	0.43 **±0.03
Creatinine clearance ml/min)	1.80±0.09	1.71±0.06	0.43 **±0.03
Serum albumin (g/dl)	4.03±0.33	3.65±0.18	1.45 **±0.09
Microalbuminuria (mg/dl)	13.15±0.62	13.47±0.50	1.45 **±0.09
MDA (nmol/g w. wt.)	513.40±26.04	520.4±25.94	1678.00 **±66.94
GSH (µmol/g w. wt.)	15.80±0.91	16.18±0.77	8.46 **±0.35
TNF-α (ng/g w. wt.)	19.05±1.27	19.55±1.15	228.10 **±7.58
IL-1ß (pg/g w. wt.)	40.85±2.013	41.47±1.79	117.00 **±4.38
Caspase-3 (ng/g w. wt.)	465.30±18.88	471.30±19.49	1102.00 **±34.14
Nitrite (nmol/g w. wt.)	387.60±20.00	393.60±19.38	1343.00**±55.44
HO-1 (ng/g w. wt.)	202.91±8.03	207.40±8.12	63.94**±3.15
H2S (nmol/g w. wt.)	17.57±1.05	18.07±1.8	6.89 **±0.19

Blood samples and kidney tissue were collected for biochemical measurements after 4 and 8 weeks of induction of diabetes. Each value represents the mean ± S.E.M. of 8 observations.** $p < 0.01$ vs.; control values.

Effect of Sitagliptin and Sitagliptin Combined with Nitric Oxide, Carbon Monoxide and Hydrogen Sulphide Modulators on Blood Glucose Level and Kidney Function of Diabetic Rats

Daily administration of 10 mg/kg/day sitagliptin orally for seven weeks to diabetic rats produced a significant decrease in the blood glucose levels after 4 and 8 weeks of induction of diabetes (Table 2). Co-administration of 100 mg/kg/day L-A i.p with sitagliptin to diabetic rats for 7 weeks significantly increased the blood glucose level after 4 and 8 weeks of induction of diabetes. Concomitant administration of 10 mg/kg /day L-NAME i.p. with sitagliptin to diabetic rats for 7 weeks significantly decreased the blood glucose level after 4 and 8 weeks of induction of diabetes in comparison to the rats treated with sitagliptin only for a similar duration (Table 2). Concomitant administration of either 0.1 mg/kg/day CORM-A1 or 3 mg/kg/day NaHS i.p. with sitagliptin to diabetic rats significantly decreased the blood glucose level after 4 and 8 weeks of induction of diabetes (Table 2). The same table shows that administration of either 0.25 mg/kg/day ZnPP or 5 mg/kg/day DL-PAG i.p. concurrently with sitagliptin to diabetic rats significantly increased the blood

glucose level after 4 and 8 weeks of induction of diabetes in comparison to sitagliptin-treated diabetic animals for the same periods. Administration of 10 mg/kg/day sitagliptin orally for 7 weeks to diabetic rats significantly decreased the serum urea (Figure 1A) and BUN (Figure1B) levels and significantly increased the urine urea level (Figure1C) in comparison to STZ+NA-treated animals. Daily co-administration of 100 mg/kg L-A i.p. with sitagliptin for 7 weeks to diabetic rats significantly increased the serum urea (Figure1A) and BUN (Figure1B) levels and significantly decreased the urine urea level (Figure1C). Concomitant administration of 10 mg/kg/day L-NAME i.p. with sitagliptin to diabetic rats for 7 weeks significantly decreased the serum uea (Figure1A) and BUN (Figure1B) levels and significantly increased the urine urea level (Figure1C) as compared to the diabetic animals treated with sitagliptin only for the same duration. Concomitant administration of either 0.1 mg/kg/day CORM-A1 or 3 mg/kg/day NaHS i.p. with sitagliptin to diabetic rats for 7 weeks significantly decreased the serum urea (Figure 1 A) and BUN (Figure1B) levels and significantly increased the urine urea level (Figure 1 C). Daily administration of either 0.25 mg/kg ZnPP or 5 mg/kg DL-PAG i.p with sitagliptin to diabetic rats for 7 weeks produces a significant increase in the serum urea (Figure 1 A) and BUN (Figure1 B) levels and a significant decrease in the urine urea level (Figure 1C) in comparison to the sitagliptin-treated diabetic animals for the same duration.

Table 2: Effect of concomitant administration of sitagliptin with Nitric Oxide (NO), Carbon Monoxide (CO) or Hydrogen Sulfide (H2S) modulators on the blood glucose level of diabetic rats.

Treatment	Time of measurement	
	After 4 weeks	After 8 weeks
Control	117.71±2.40	119.89±2.40
STZ+NA	532.90 *±14.99	561.60 ** ±19.29
Sita+STZ+NA	239.90 ##±9.45	235.30 ##±9.45
Sita+L-A+STZ+NA	367.10 ++ ±15.96	370.60 ++±15.28
Sita+L-NAME+STZ+NA	195.30 +±2.43	121.01 ++±2.35
Sita+CORM-A_1 +STZ+NA	96.31+ ±6.08	119.9++ ±2.33
Sita+ZnPP+STZ+NA	394.10 ++ ±17.57	423.33++ ±13.53
Sita+NaHS+STZ+NA	202.10 +±16.48	121.98 ++±3.89
Sita+DL-PAG+STZ+NA	394.40 ++±18.19	395.50 ++±23.75

Blood samples were collected from the tail vein after 4 and 8 weeks of induction of diabetes. Each value represents the mean ± S.E.M. of 8 observations.** p < 0.01 vs. control values; ##p<0.01 vs.; STZ+NA values; + p<0.05 vs.; Sita+STZ+NA values; ++p < 0.01 vs. Sita+STZ+NA values.

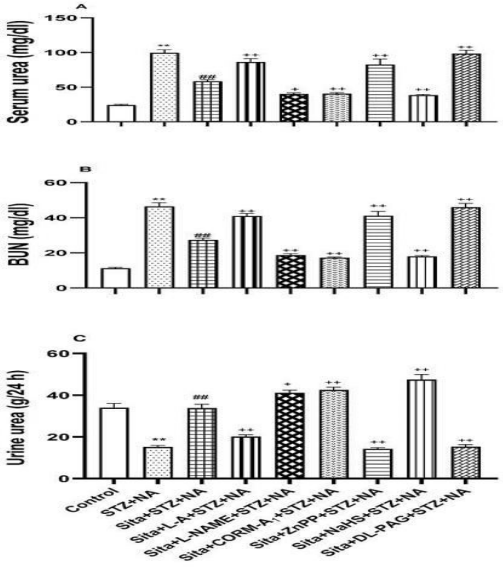

Figure 1: The Effects of concurrent administration of Nitric Oxide (NO), Carbon Monoxide (CO) or hydrogen sulphide (H_2S) modulators with 10 mg/kg/day sitagliptin (Sita) orally to diabetic rats for seven weeks on the levels of (A) serum urea, (B) blood urea nitrogen (BUN) and (C) urine urea. 100 mg/kg /day L-arginine (L-A), 10 mg/kg/day L-N(G)-nitroarginine methyl ester (L-NAME), 0.1 mg/kg/day Carbon Monoxide-releasing molecule-A1 (CORM-A1), 0.25 mg/kg/day zinc protoporphyrin (ZnPP), 3 mg/kg/day sodium hydrosulfide (NaHS) or 5 mg/kg/day DL-propargylglycine (DL-PAG) were given i.p. to the animals concurrently with Sita. At the end of the treatment duration, blood and 24h urine samples were collected for biochemical analysis. Each value is the mean ± S.E.M. of 8 observations. ** p<0.01 vs. control values; ## p<0.01 vs. STZ+NA values; + p<0.05 vs. Sita+STZ+NA values; ++ p<0.01 vs. Sita+STZ+NA values.

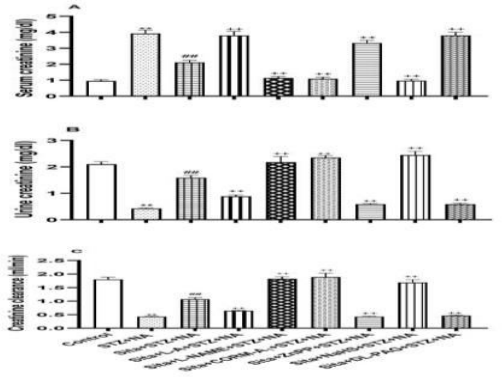

Figure 2: The effects of concurrent administration of Nitric Oxide (NO), Carbon Monoxide (CO) or hydrogen sulphide (H_2S) modulators with 10 mg/kg/day sitagliptin (Sita) orally to diabetic rats for seven weeks on the levels of (A) serum creatinine, (B) urine creatinine and (C) creatinine clearance. 100 mg/kg /day L-arginine (L-A), 10 mg/kg/day L-N(G)- nitroarginine methyl ester (L-NAME), 0.1 mg/kg/day Carbon Monoxide-releasing molecule-A1 (CORM-A1), 0.25 mg/kg/day zinc protoporphyrin (ZnPP), 3 mg/kg/day sodium hydrosulfide (NaHS) or 5 mg/kg/day DL-propargylglycine (DL-PAG) were given i.p. to the animals concurrently with Sita. At the end of the treatment duration blood and 24h urine samples were collected for biochemical analysis. Each value is the mean ± S.E.M. of 8 observations. ** p<0.01 vs. control values; ## p<0.01 vs. STZ+NA values; ++ p<0.01 vs. Sita+STZ+NA values.

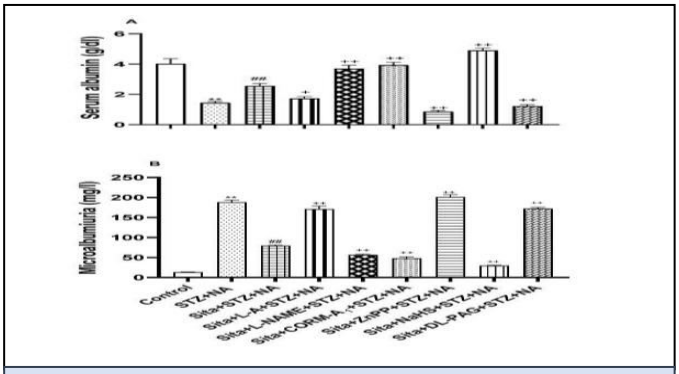

Figure 3: The effects of concurrent administration of Nitric Oxide (NO), Carbon Monoxide (CO) or hydrogen sulphide (H_2S) modulators with 10 mg/kg/day sitagliptin (Sita) orally to diabetic rats for seven weeks on the levels of (A) serum albumin and (B) urine albumin (microalbuminuria). 100 mg/kg /day L-arginine (L-A), 10 mg/kg/day L-N(G)- nitroarginine methyl ester (L-NAME), 0.1 mg/kg/day Carbon Monoxide-releasing molecule-A1 (CORM-A1), 0.25 mg/kg/day zinc protoporphyrin (ZnPP), 3 mg/kg/day sodium hydrosulfide (NaHS) or 5 mg/kg/day DL-propargylglycine (DL-PAG) were given i.p. to the animals concurrently with Sita. At the end of the treatment duration blood and 24h urine samples were collected for biochemical analysis. Each value is the mean ± S.E.M. of 8 observations. ** $p<0.01$ vs. control values; ## $p<0.01$ vs. STZ+NA values; + $p<0.05$ vs. Sita+STZ+NA values; ++ $p<0.01$ vs. Sita+STZ+NA values.

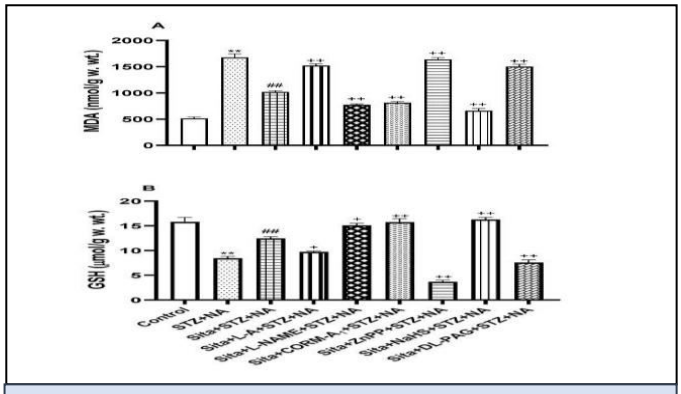

Figure 4: The effects of concurrent administration of Nitric Oxide (NO), Carbon Monoxide (CO) or hydrogen sulphide (H_2S) modulators with 10 mg/kg/day sitagliptin (Sita) orally to diabetic rats for seven weeks on the renal levels of (A) malondialdehyde (MDA) and (B) intracellular reduced glutathione (GSH). 100 mg/kg /day L-arginine (L-A), 10 mg/kg/day L-N(G)-nitroarginine methyl ester (L-NAME), 0.1 mg/kg/day Carbon Monoxide-releasing molecule-A1 (CORM-A1), 0.25 mg/kg/day zinc protoporphyrin (ZnPP), 3 mg/kg/day sodium hydrosulfide (NaHS) or 5 mg/kg/day DL-propargylglycine (DL-PAG) given i.p. to the animas concurrently with Sita. At the end of the treatment duration, kidney tissue was collected for biochemical analysis. Each value is the mean ± S.E.M. of 8 observations. ** $p<0.01$ vs. control values; ## $p<0.01$ vs. STZ+NA values; + $p<0.05$ vs. Sita+STZ+NA values; ++ $p<0.01$ vs. Sita+STZ+NA values.

As shown in figure 2, administration of 10 mg/kg/day sitagliptin orally for 7 weeks to diabetic rats significantly decreased the serum creatinine level (Figure 2A) and increased the urine creatinine (Figure 2B) and creatinine clearance (Figure 2C) levels as compared to the animals treated with STZ+NA. Daily administration of 0.1 mg/kg CORM-A1 or 3 mg/kg NaHS i.p. concurrently with sitagliptin for 7 weeks to diabetic animals produced a significant decrease in the serum creatinine level (Figure 2A) and a significant increase in the urine creatinine (Figure 2B) and creatinine clearance (Figure 2C) levels in comparison to diabetic treated with sitagliptin only for the same duration. Co-administration of 0.25 mg/kg/day ZnPP or 5 mg/kg/day DL-PAG i.p. with sitagliptin for 7 weeks to diabetic rats significantly increased the serum creatinine level (Figure 2A) and significantly decreased the urine creatinine (Figure 2B) and creatinine clearance (Figure 2C) levels.

Figure 2, also shows that administration of 100 mg/kg/day L-A i.p concurrently with sitagliptin to diabetic rats for 7 weeks significantly increased the serum creatinine level (Figure 2A) and significantly decreased the urine creatinine (Figure 2B) and creatinine clearance (Figure 2C) levels as compared to diabetic animals treated with sitagliptin only. Treatment of diabetic rats with 10 mg/kg/day L-NAME i.p. in combination with sitagliptin for 7 weeks significantly decreased the serum creatinine level (Figure2A) and significantly increased the urine creatinine (Figure 2B) and the creatinine clearance (Figure 2C) levels in comparison to sitagliptin-treated diabetic animals for a similar period. Data presented in figure 3 show that administration of 10 mg/kg/day sitagliptin orally to diabetic rats for 7 weeks significantly increased the serum albumin level (Figure 3A) and significantly decreased the urine albumin (Microalbuminurea) level (Figure 3B) as compared to STZ+NA treated animals. Also, it can be seen in figure 3 that daily administration of 100 mg/kg L-A i.p. in combination with sitagliptin to diabetic rats for 7 weeks produced a significant decrease in the serum albumin level (Figure 3A) and a significant increase in the microalbuminuea level (Figure 3B) as compared to sitagliptin-treated diabetic animals for the same duration. Administration of 10 mg/kg/day L- NAME i.p. concomitantly with sitagliptin to diabetic rats for 7 weeks significantly increased the serum albumin level (Figure 3A) and significantly decreased the

microalbuminuea level (Figure 3B). Simultaneous administration of 0.1 mg/kg/day CORM-A1 or 3 mg/kg/day NaHS i.p. with sitagliptin to diabetic rats for 7 weeks significantly increased the serum albumin level (Figure 3A) and significantly decreased the urine albumin level (Figure 3B) in comparison to the animals treated with sitagliptin only for the same period. Daily co-administration of 0.25 mg/kg ZnPP or 5 mg/kg DL- PAG i.p. with sitagliptin to diabetic rats for 7 weeks produced a significant decrease in the serum albumin level (Figure 3A) and a significant increase in the urine albumin level (Figure 3B).

Effect of Sitagliptin and Sitagliptin Combined with Nitric Oxide, Carbon Monoxide or Hydrogen Sulphide Modulators on the Renal Levels of Biochemical Parameters of Diabetic Rats

Administration of 10 mg/kg/day sitagliptin orally for 7 weeks to diabetic rats significantly decreased the renal MDA level (Figure 4A) and significantly increased the renal intracellular GSH level (Figure 4B) as compared to STZ+NA-treated animals. In comparison to sitagliptin-treated diabetic animals, daily co- administration of 100 mg/kg L-A. and sitagliptin to diabetic rats for 7 weeks produced a significant increase in the renal MDA level (Figure 4A) and a significant decrease in the renal intracellular GSH level (Figure4 B). Administration of 10 mg/kg/day L-NAME i.p. in combination with sitagliptin to diabetic rats for 7 weeks significantly decreased the renal MDA level (Figure 4A) and significantly increased the renal intracellular GSH level (Figure 4B). As seen in figure 4 that co-administration of either 0.1 mg/kg/day CORM-A1 or 3 mg/kg/day NaHS i.p. with sitagliptin to diabetic rats for 7 weeks produced a significant decrease in the renal MDA level (Figure4A) and a significant increase in the intracellular renal GSH level (Figure4B). Concurrent administration of 0.25 mg/kg/day ZnPP or 5 mg/kg DL-PAG i.p. with sitagliptin to diabetic rats for 7 weeks significantly increased the renal MDA level (Figure4A) and significantly decreased the renal intracellular GSH level (Figure 4B) in comparison to sitagliptin-treated diabetic animals.

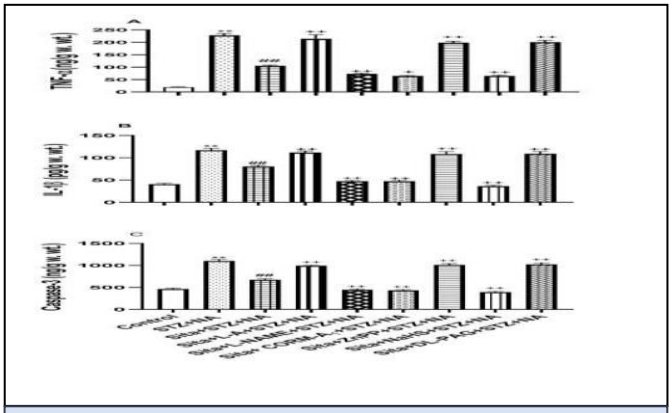

Figure 5: The effects of concurrent administration of Nitric Oxide (NO), Carbon Monoxide (CO) or hydrogen sulphide (H_2S) modulators with 10 mg/kg/day sitagliptin (Sita) orally to diabetic rats for seven weeks on the renal levels of (A) tumor necrosis factor-alpha (TNF-α) and (B) interleukin-1 beta (IL-1ß) and (C) caspase-3. 100 mg/kg /day L-arginine (L-A), 10 mg/kg/day L-N(G)-nitroarginine methyl ester (L-NAME), 0.1 mg/kg/day Carbon Monoxide-releasing molecule-A1 (CORM-A1), 0.25 mg/kg/day zinc protoporphyrin (ZnPP), 3 mg/kg/day sodium hydrosulfide (NaHS) or 5 mg/kg/day DL-propargylglycine (DL-PAG) were given i.p. to the animals concurrently with Sita. At the end of the treatment duration, kidney tissue was collected for biochemical analysis. Each value is the mean ± S.E.M. of 8 observations. ** $p<0.01$ vs. control values; ## $p<0.01$ vs. STZ+NA values; + $p<0.05$ vs. Sita+STZ+NA values; ++ $p<0.01$ vs. Sita+STZ+NA values.

Data presented in figure 5 show that administration of 10 mg/kg/day sitagliptin orally to diabetic rats for 7 weeks significantly decreased the renal TNF-α (Figure 5A), IL-1ß (Figure 5B) and caspase-3 (Figure 5C) levels as compared to STZ+NA-treated animals. It is also evident from figure 5 that daily administration of 100 mg/kg L-A i.p. concurrently with sitagliptin to diabetic rats for 7 weeks produced a significant increase in the renal TNF- α (Figure 5A), IL-1ß (Figure 5B) and caspase-3 (Figure5C) levels as compared to diabetic animals treated only with sitagliptin for a similar duration. Administration of 10 mg/kg/day L-NAME i.p. concurrently with sitagliptin to diabetic rats for 7 weeks significantly decreased the renal TNF-α (Figure 5A), IL-1ß (Figure 5B) and caspase-3 (Figure 5) levels. Daily administration of 0.1 mg/kg CORM-A1 or 3 mg/kg NaHS i.p. concurrently with sitagliptin to diabetic rats for 7 weeks produced a significant decrease in the renal TNF-α (Figure 5A), IL-1ß (Figure 5B) and caspase-3 (Figure 5C) levels. Co- administration of 0.25 mg/kg/day ZnPP or 5 mg/kg/day DL-PAG i.p. with sitagliptin to diabetic rats for 7 weeks significantly increased the renal TNF-α (Figure 5A), IL-

1ß (Figure 5B) and caspase-3 (Figure 5C) levels as compared to sitagliptin-treated diabetic animals for a similar period.

Effect of Sitagliptin and Sitagliptin combined with Nitric Oxide, Carbon Monoxide or Hydrogen Sulphide Modulators on the Renal Nitrite, Heme Oxygenase-1(HO-1) and Hydrogen Sulphide (H_2S) Levels of Diabetic Rats

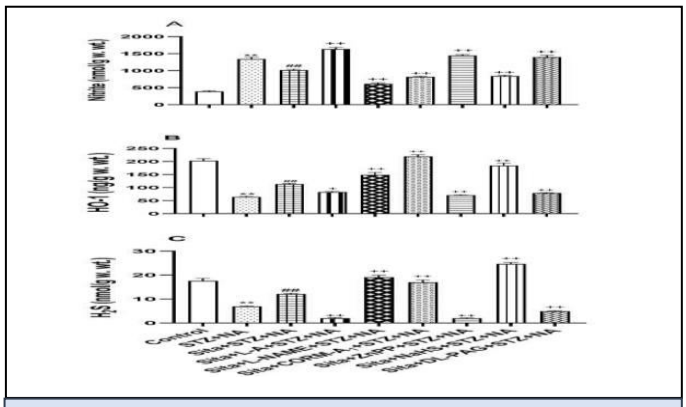

Figure 6: The effects of concurrent administration of Nitric Oxide (NO), Carbon Monoxide (CO) or hydrogen sulphide (H_2S) modulators with 10 mg/kg/day sitagliptin (Sita) orally to diabetic rats for seven weeks on the renal levels of (A) nitrite (B), heme oxygenase- 1(HO-1) and hydrogen sulfide (H_2S). 100 mg/kg /day L-arginine (L-A), 10 mg/kg/day L- N(G)-nitroarginine methyl ester (L-NAME), 0.1 mg/kg/day Carbon Monoxide-releasing molecule-A1 (CORM-A1), 0.25 mg/kg/day zinc protoporphyrin (ZnPP), 3 mg/kg/day sodium hydrosulfide (NaHS) or 5 mg/kg/day DL-propargylglycine (DL-PAG) were given i.p. to the animals concurrently with Sita. At the end of the treatment duration, kidney tissue was collected for biochemical analysis. Each value is the mean ± S.E.M. of 8 observations. ** $p<0.01$ vs. control values; ## $p<0.01$ vs. STZ+NA values; ++ $p<0.01$ vs. Sita+STZ+NA values.

Figure 6 shows that administration of 10 mg/kg/day sitagliptin orally to diabetic rats for 7 weeks produced a significant decrease in the renal nitrite level (Figure 6A) and a significant increase in the renal HO-1 (Figure 6B) and H2S (Figure 6C) levels in comparison to the animals treated with STZ+NA. Co-administration of 100 mg/kg/day L-A i.p. with sitagliptin to diabetic rats for 7 weeks produced a significant increase in the renal nitrite level (Figure 6A) and a significant decrease in the renal HO-1 (Figure 6B) and H2S (Figure 6C) levels in comparison to sitagliptin-treated diabetic animals for the same period. Administration of 10 mg/kg/day L-NAME i.p concurrently with sitagliptin to diabetic rats for 7 weeks significantly decreased the renal nitrite level (Figure 6A) and significantly increased the renal HO-1 (Figure 6B) and H2S (Figure 6C) levels. Co-administration of 0.1 mg/kg/day CORM-A1 or 3 mg/kg NaHS i.p. with sitagliptin to diabetic rats for 7 weeks produced a significant decrease in the renal nitrite level (Figure 6A) and a significant increase in the renal HO-1 (Figure 6B) and H2S (Figure 6C) levels. Concomitant administration of 0.25 mg/kg/day ZnPP i.p. or 5 mg/kg/day DL-PAG with sitagliptin to diabetic rats for 7 weeks significantly increased the renal nitrite level (Figure 6A) and significantly decreased the renal HO-1 (Figure 6B) and H2S (Figure 6C) levels in comparison to sitagliptin-treated diabetic animals for a similar duration.

Analysis of the Kidney Tissue`s Histopathology

The kidney tissues obtained after 4 weeks from animals treated with 110 mg/kg NA and 60 mg/kg STZ i.p. showed no histopathological changes. The histopathological examination of the kidney tissue obtained after 8 weeks from animals treated similarly with NA and STZ, showed thickening of the glomerular basement membrane, diffuse mesangial expansion, hypercellularity with formation of mesangial nodules, adhesions between glomerular tuft and parietal cells and lobulation of glomerular tuft (Figure 7C&D) in comparison to the control animals (Figure 7A&B). As seen in figure 7 (E&F) administration of 10 mg/kg/day sitagliptin orally to diabetic rats for 7 weeks showed mild thickening of the glomerular basement membrane and mild mesangial expansion. Daily administration of 100 mg/kg L-A i.p. concurrently with sitagliptin for 7 weeks to diabetic rats produced thickening of the glomerular basement membrane and marked mesangial matrix expansion and mild hydropic change of the tubules (Figure 7 G&H). Administration of 10 mg/kg/day L-NAME i.p simultaneously with 10 mg/kg/day sitagliptin to diabetic rats for 7 weeks showed mild thickening of glomerular basement membrane, mild mesangial matrix and mild hydropic change of the tubules (Figure 7 I&J). Concomitant administration of 0.1 mg/kg/day CORM-A1 i.p with sitagliptin. to diabetic rats for 7 weeks produced normal glomerular structure and normal mesangial matrix (Figure 7 K&L). Daily administration of 0.25 mg/kg ZnPP i.p. combined with sitagliptin to diabetic rats for 7 weeks produced mesangial matrix expansion, hypercellularity and lobulation of the glomerular tuft (Figure 7 M&N) Co-administration of 3 mg/kg/day NaHS i. p. with sitagliptin to diabetic rats for 7 weeks showed normal glomerulus and normal mesangial matrix (Figure7 O&P). Daily administration of 5 mg/kg DL-PAG i.p. concurrently with sitagliptin to diabetic

animals for 7 weeks produced thickening of the glomerular basement membrane with diffuse mesangial expansion and lobulation of the glomerular tuft (Figure7 Q&R).

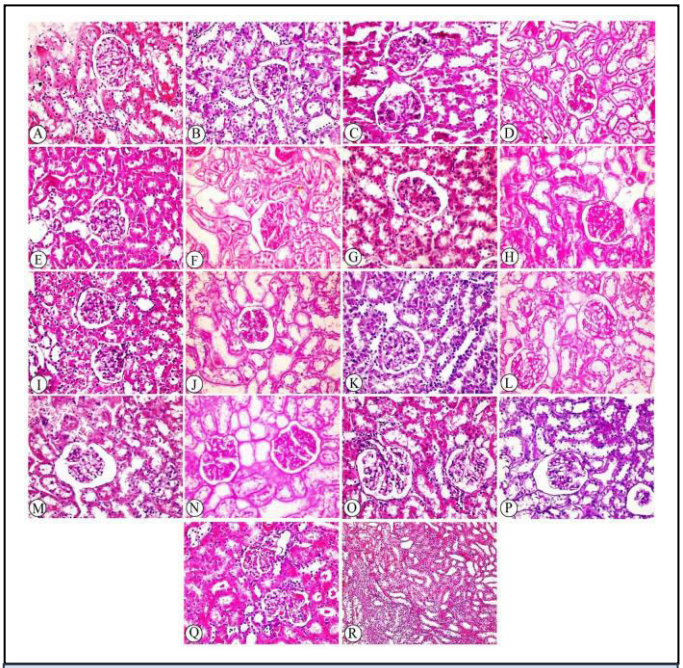

Figure 7: Photomicrographs of representative kidney slices from A&B: control rats demonstrating normal kidney tissue composed of normal glomeruli, tubules and interstitial tissues (A: H&E stain x200, B: PAS stain X400); C&D: DN (STZ+NA) rats demonstrating thickening of glomerular basement membranes, mesangial expansion and hypercellularity with formation of mesangial nodules and adhesions between glomerular tuft and parietal cells (C: H&E stain, x400), D: lobulation of glomerular tuft and mesangial matrix ecxpanssion (PAS stain, x400); E&F: DN rats treated with Sita demonstrating mild thickening of glomerular basement membrane and mild mesangial expansion (E: H&E stain, x400, F: PAS stain, x400); G&H: DN rats treated with Sita+L- A demonstrating G: thickening of glomerular basement membrane and marked mesangial expansion (H&E stain, x400), H: mesangial matrix expansion and mild hydropic change of the tubules (PAS stain, x400); I&J: DN rats treated with Sita+L-NAME demonstrating mild thickening of glomerular basement membrane, mesangial expansion and hydropic change of the tubules, I: H&E stain x400; J: PAS stain, x400 (; K&L: DN rats treated with Sita+ CORM-A1 demonstrating K: normal glomerular structure (H&E stain x400), L: normal mesangial matrix (PAS stain x 400); M&N: DN rats treated with Sita+ ZnPP demonstrating M: mesangial expansion and hypercellularity (H&E stain x400), N: lobulation of the glomerular tuft and mesangial matrix expansion (PAS stain x400); O&P: DN rats treated with Sita+NaHS showing normal glomerulus structure (O: H&E stain, x400; P: PAS stain, x 400), Q&R: DN rats treated with Sita+DL-PAG demonstrating Q: thickening of glomerular basement membrane with glomerular congestion and thrombosis (H&E, x400), R: Hydropic change of tubules, interstitial inflammation and fibrosis, (PAS x 400).

Analysis of immunohistochemistry

In the present study, the immunohistochemical analysis of the kidney tissue obtained from DN (STZ+NA) rats showed a decrease in the protein expressions of eNOS (Figure8 aB), and HO-1(Figure8 cB) and CSE (Figure8 dB) within kidney tissue. The protein expression of iNOS (Figure8 bB) was elevated in the kidney tissue of these diabetic rats.

The protein expressions of eNOS (Figure8 aC), HO-1 (Figure8 cC) and CSE (Figure8 dC) were increased after administration of 10 mg/kg/day sitagliptin orally to diabetic rats for 7 weeks. The same treatment decreased the protein expression of iNOS (Figure8 bC) in the kidney tissue. Daily administration of 100 mg/kg L-A i.p. concurrently with sitagliptin to diabetic rats for 7 weeks increased the protein expressions of eNOS (Figure8 aD) and iNOS (Figure8 bD) in the kidney tissue. However, the protein expressions of HO-1 (Figure8 cD) and CSE (Figure8 dD) were decreased in the kidney tissue. Concomitant administration of 10 mg/kg L-NAME i.p. with sitagliptin to diabetic rats for 7 weeks produced a decrease in the protein expressions of eNOS (Figure8 aE) and iNOS (Figure8 bE) in the kidney tissue. On the other hand, this combination enhanced the stimulatory effect of sitagliptin on the protein expressions of HO-1 (Figure8 cE) and CSE (Figure8 dE).

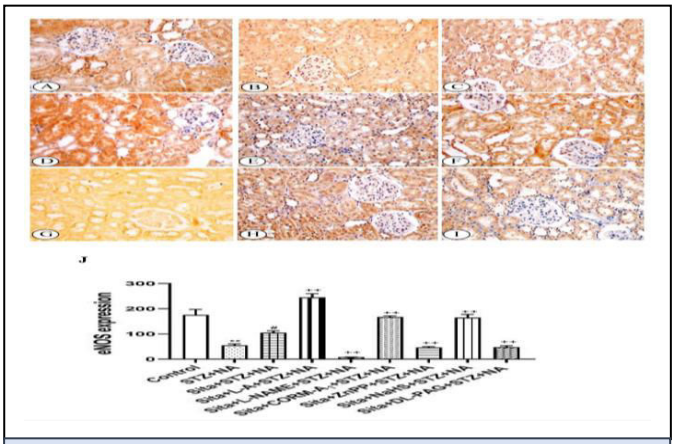

Figure 8a: Endothelial Nitric Oxide Synthase (eNOS) immunohistochemistry in kidney slices from A: Control rats exhibiting strong expression of eNOS, B: DN (STZ+NA) rats exhibiting weak expression of eNOS , C: DN rats treated with Sita showing moderate expression of eNOS,D: DN rats treated with Sita+L-A exhibiting strong expression of eNOS, E: DN rats treated with Sita+L-NAME exhibiting weak expression of eNOS, F: DN rats treated with Sita+ CORM-A1 showing strong expression of eNOS, G: DN rats treated with Sita+ ZnPP exhibiting week expression of eNOS, H: DN rats treated with Sita+NaHS exhibiting strong expression of eNOS and I: DN rats treated with Sita+DL- PAG showing weak expression of eNOS.
J: Protein expression of eNOS in the renal tissue of DN rats and DN rats treated with Sita, Sita+ L-A, Sita+ L-NAME, Sita+ CORM-A1, Sita+ ZnPP, Sita+ NaHS or Sita+ DL- PAG. Each value is the mean ± S.E.M. of 6 observations. ** $p<0.01$ vs. control values; # $p<0.01$ vs. STZ+NA values; ++ $p<0.01$ vs. Sita+STZ+NA values.

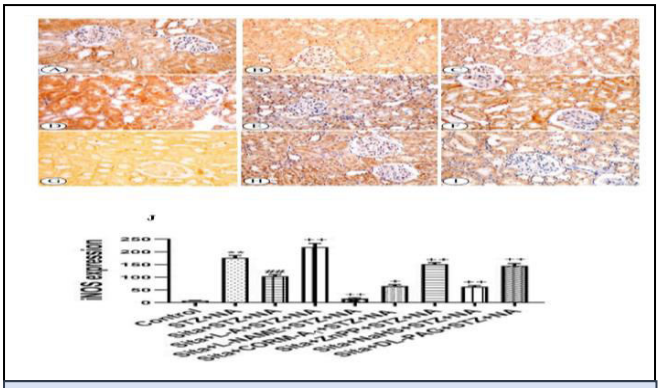

Figure 8b: Inucible Nitric Oxide synthase (iNOS) immunohistochemistry in kidney slices from A: Control rats demonstrating weak expression of iNOS , B: DN (STZ+NA) rats demonstrating strong expression of iNOS , C: DN rats treated with Sita demonstrating moderate expression of iNOS, D: DN rats treated with Sita+ L-A demonstrating strong expression of iNOS, E: DN rats treated with Sita+ L-NAME demonstrating weak expression of iNOS, F: DN rats treated with Sita+ CORM-A1 demonstrating week expression of iNOS, G: DN rats treated with Sita+ ZnPP demonstrating strong expression of iNOS, H: DN rats treated with Sita+NaHS demonstrating weak expression of iNOS and I: DN rats treated with Sita+ DL-PAG demonstrating strong expression of iNOS.

J: Protein expression of iNOS in renal tissue of DN rats and DN rats treated with Sita, Sita+L-A, Sita+L-NAME, Sita+ CORM-A1, Sita+ ZnPP, Sita+NaHS or Sita+DL-PAG. Each value is the mean ± S.E.M. of 6 observations ** p<0.01 vs. control values; ## p<0.01 vs. STZ+NA values; + p<0.01 vs. Sita+STZ+NA; ++ p<0.01 vs. Sita+STZ+NA values.

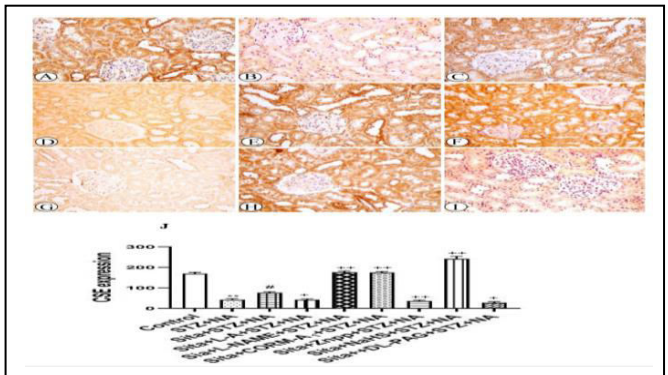

Figure 8d: Cystathionine-γ-lyase (CSE) immunohistochemistry in kidney slices from. A: Control rats exhibiting strong expression of CSE, B: DN (STZ+NA) rats exhibiting weak expression of CSE, C: DN rats treated with Sita exhibiting moderate expression of CSE, D: DN rats treated with Sita+ L-A exhibiting weak expression of CSE, E: DN rats treated with Sita+ L-NAME exhibiting strong expression of CSE, F: DN rats treated with Sita+ CORM-A1 exhibiting strong expression of CSE, G: DN rats treated with Sita+ ZnPP exhibiting weak expression of CSE, H: DN rats treated with Sita+ NaHS exhibiting strong expression of CSE, I: DN rats treated with Sita+ DL-PAG exhibiting weak expression of CSE.

J: Protein expression of CSE in renal tissue of DN rats and DN rats treated with Sita, Sita+ L-A, Sita+ L-NAME, Sita+ CORM-A1, Sita+ ZnPP, Sita+ NaHS or Sita+ DL-PAG. Each value is the mean ± S.E.M. of 6 observations. ** p<0.01 vs. control values; # p<0.01 vs. STZ+NA values; + p<0.01 vs. Sita+STZ+NA values; ++ p<0.01 vs. Sita+STZ+NA values.

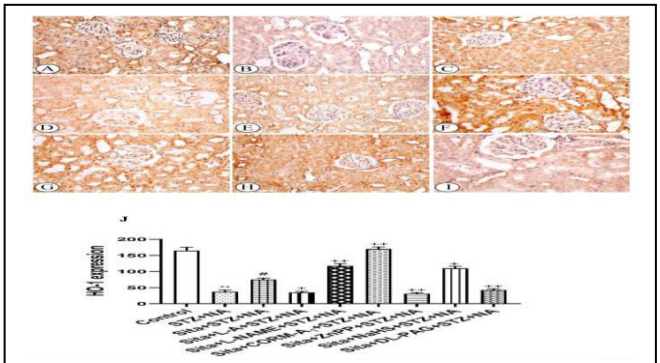

Figure 8c: Heme oxygenase-1(HO-1) immunohistochemistry in kidney slices from. A: Control rats exhibiting strong expression of HO-1, B: DN (STZ+NA) rats exhibiting weak expression of HO-1, C: DN rats treated with Sita exhibiting moderate expression of HO-1, D: DN rats treated with Sita+ L-A exhibiting weak expression of HO-1,E: DN rats treated with Sita+ L-NAME exhibiting strong expression of HO-1, F: DN rats treated with Sita+ CORM-A1 exhibiting strong expression of HO-1, G: DN rats treated with Sita+ ZnPP exhibiting weak expression of HO-1, H: DN rats treated with Sita+NaHS exhibiting strong expression of HO-1 and I: DN rats treated with Sita+DL-PAG showing weak expression of HO-1.

J: Protein expression of HO-1 in renal tissue of DN rats and DN rats treated with Sita, Sita+ L-A, Sita+ L-NAME, Sita+ CORM-A1, Sita+ ZnPP, Sita+ NaHS or Sita+ DL-PAG. Each value is the mean ± S.E.M. of 6 observations. ** p<0.01 vs. control values; # p<0.01 vs. STZ+NA values; + p<0.01 vs. Sita+STZ+NA values; ++ p<0.01 vs. Sita+STZ+NA values.

Table 3: Summary of the experimental protocol.

Groups	Treatment
Group-I: Control animals	Vehicle for 8 weeks.
Group-II: Diabetic animals	A single intraperitoneal (i.p.) injection of 110 mg/kg nicotinamide (NA, 4% solution in saline) followed after 15 min by a single i.p. injection of 60 mg/kg streptozotocin (STZ) and left for 8 weeks.
Group-III: Diabetic animals treated with Sita	10 mg/kg/day sitagliptin (0.5% solution in saline) orally for seven weeks starting from the 7th day after STZ+NA injection
Group-IV: Diabetic animals treated withSita+LA	10 mg/kg/day sitagliptin (0.5% solution in saline) orally in combination with100 mg/kg/day L-arginine (L-A, 4% solution in saline) i.p. for seven weeks starting from the 7th day after STZ+NA injection.
Group-V: Diabetic animalstreated with Sita+L-NAME	10 mg/kg/day L-N(G)-nitro arginine methyl ester (L-NAME, 1% solution in saline) i.p. for seven weeks starting from the 7th day after STZ+NA injection
Group-VI: Diabetic animals treated withSita+CORM-A$_1$	10 mg/kg/day sitagliptin orally in combination with 0.1 mg/kg/day carbon monoxide-releasing molecule-A$_1$ (CORM-A$_1$, 0.01% solution in saline) i.p. forseven weeks starting from the 7th day after STZ+NA injection.
Group-VII: Diabetic animals treated with Sita+ ZnPP	10 mg/kg/day sitagliptin orally in combination with 0.25 mg/kg/day zinc protoporphyrin (ZnPP, 0.05% solution in saline) i.p. for seven weeks starting from the 7 th day after STZ+NA injection.
Group-VIII: Diabetic animals treated with Sita+ NaHS	10 mg/kg/day sitagliptin orally in combination with 3 mg/kg/day sodium hydrosulfide (NaHS, 0.3% solution in saline) i.p for seven weeks starting from the 7th day after STZ+NA injection.
Group-IX: Diabetic animals treated withSita+DL-PAG	10 mg/kg/day sitagliptin orally in combination with 5 mg/kg/day DL- propargylglycine (DL-PAG, 0.5% solution in saline) i.p. i.p. for seven weeks starting from the 7th day after STZ+NA injection.

Concurrent administration of 0.1 mg/kg/day CORM-A1 i.p with sitagliptin to diabetic rats for 7 weeks increased the stimulatory effect of sitagliptin on the protein expressions of eNOS (Figure8 aF), HO-1 (Figure8 cF) and CSE (Figure8 dF) and enhance its inhibitory effect on the protein expression of iNOS (Figure8 bF) in the kidney tissue. Daily co- administration of 0.25 mg/kg ZnPP i.p. with sitagliptin to diabetic animals for 7 weeks showed a decrease in the protein expressions of renal eNOS (Figure 8aG), HO-1 (Figure8 cG) and CSE (Figure8 dG) and an increase in the protein expression of iNOS (Figure8 bG). Simultaneous administration of 3 mg/kg/day NaHS i.p. with sitagliptin to diabetic rats for 7 weeks increased the stimulatory effect of sitagliptin on the protein expressions of eNOS (Figure8 aH), HO-1 (Figure8 cH) and CSE (Figure8 dH) and enhanced its inhibitory effect on the protein expression of iNOS (Figure8 bH) in the kidney tissue. Daily co- administration of 5 mg/kg DL-PAG i.p with sitagliptin to diabetic animals for 7 weeks showed a decrease in the protein expressions of renal eNOS (Figure8 aI), HO-1 (Figure8 cI) and CSE (Figure8 dI). The same combination increased the protein expression of iNOS (Figure8bI) in the kidney tissue.

Administration of sitagliptin and sitagliptin combined with NO, CO and H2S modulators resulted in appreciable modifications in the protein expressions of eNOS (Figure8 aJ), iNOS (Figure8 bJ), HO-1 (Figure8 cJ) and CSE (Figure8 dJ) in the kidney tissue of diabetic rats.

DISCUSSION

Streptozotocin+NA is the most popular and appropriate animal model for induction of type 2 diabetes mellitus and DN [26]. The levels of blood glucose, urinary albumin excretion, serum creatinine and blood urea nitrogen were increased in DN [5,11,36]. Furthermore, it is well known that microalbuminuria is one of the earliest clinical diagnostic markers for DN [29]. In line with these findings, the levels of blood glucose, serum urea, blood urea nitrogen and serum creatinine markedly increased after STZ+NA injection into animals in our study. Additionally, STZ+NA treatment decreased urine urea, urine creatinine, creatinine clearance, and serum albumin levels but increased urine albumin level (microalbuminuria). These renal functional traits point to the DN`s development [5].

The main pathological changes in DN patient and animals include glomerular hypertrophy, excessive accumulation of the extracellular matrix, glomerular matrix expansion, glomerular basement membrane thickening, mesangial expansion, mesangial hypercellularity, tubular injury, inflammatory cells infiltration [5,36,37]. In our study, the histopathological analysis of kidney tissue from DN rats, revealed similar obvious structural damage and changes.

Hyperglycemia, oxidative stress, and diabetic complications are thought to be closely related in many studies [36]. Thus, it was proposed that one of the main processes in the pathogenesis of micro-and macrovascular complications of diabetes was oxidative stress [3,2,22]. Also, the development and progression of DN and its pathological mechanism

are influenced by the release and expression of the inflammatory cytokines such as TNF- α, IL-6 and IL-1β [36-38]. Furthermore, it has been reported that apoptosis in kidney tissues can be attributed to hyperglycemia-induced oxidative stress [6,7], which can result in DN development and progression. Abd EL Motteleb and Abd El Aleem [39] found that STZ+NA-induced DN in rats increased the renal caspase-3 level which is an essential mediator of programmed apoptosis [2]. Because these findings suggested a connection between oxidative stress, inflammation, apoptosis and DN, we investigated this hypothesis in our study. By raising the renal MDA level and loweing the intracellular GSH level this study found that the induction of DN in rats was linked to oxidative stress. Additionally, rats with DN had higher levels of TNF-α, IL-1ß and caspase-3 in their kidney tissue. Thus, it can be hypothesized that oxidative stress, inflammation and apoptosis may play the crucial role in STZ+NA- induced DN in rats.

According to studies DN development and progression cannot be stopped by strict blood sugar control and conventional therapy [40]. Therefore, in addition to maintaining good glycemic control, inhibition of oxidative stress, inflammation and apoptosis in type 2 diabetes may produce protective effect against DN [2,41]. Recently, it has been found that sitagliptin also has antiapoptotic, antioxidant and antiinflammatory effects in addition to its ability to lower blood glucose [24,25]. Marques et al. [27] reported that sitagliptin produced glycaemic control, improved kidney function, and decreased oxidative stress, IL-1ß and TNF-α levels in the kidney of diabetic rats. Additionally, sitagliptin was found to reduce

albuminuria in many type 2 diabetic patients with nephropathy [42]. Our results show that treatment of diabetic rats with sitagliptin inhibited the development of the kidney function and structure impairments. In addition, to its blood glucose-lowering effect it inhibited the development of oxidative stress, nitosative stress, inflammation and apoptosis in the kidney tissue of diabetic rats.

The question of whether DN causes an increase or decrease in NO production is still up for debate. Yang et al. [11] found that eNOS expression and activity were downregulated in STZ-induced DN in mice. In addition, it has been found that in DN rats, there is an increase in the renal NO level and in iNOS protein expression [13,39]. In our study, an elevation of the nitrite level in the kidney tissues was linked to the impairment of the renal function and structure in rats with DN. iNOS protein expression increased while eNOS protein expression decreased in the kidney tissues after immunostaining. The renoprotective effect of sitagliptin in DN in this study was linked to a reduction in NO overproduction in renal tissue. Immmunostaining of the kidney tissue showed that the protein expression of iNOS was decreased and that of eNOS was increased. Thus, NO may play a role in the development and progression of DN and in the renoprotective effect of sitagliptin. The effects of NO modulators ; L-A and L-NAME, on sitagliptin effect were investigated to further clarify the potential role of NO in the protective effect of sitagliptin against DN. According to our findings the sitagliptin`s inhibitory effect on DN-induced renal functional and structural impairments, oxidative stress, nitrosative stress, inflammation and apoptosis was enhanced by concomitant administration of L- NAME, a non-selective NO synthase inhibitor [43]. The ameliorated effect of sitagliptin on DN-induced reduction of the renal HO-1 and H2S levels, inhibition of the protein expression of eNOS, HO-1 and CSE and increase in the protein expression of iNOS was potentiated by concomitant administration of L-NAME. The metabolic precursor for NO biosynthesis, L-A [44] produced the opposite effects of L-NAME. As a result, the sitagliptin`s ability to protect against DN can be attributed to its ability to inhibit NO overproduction and renal expression of iNOS.

By ensuring sustained release of CO in biological systems, carbon monoxide-releasing molecules (CORMs), are compounds that have been developed to mimic the antioxidant, antiinflammatory and cytoprotective properties of CO (Motterlini et al. [45]). Slowly releasing CO, CORM-A_1 may be more effective in treating of certain chronic diseases where the continuous effect of CO may be needed. The natural role of HO-1 which is anticipated to produce endogenous CO from heme in a sustained manner may be more closely mimicked by CORM-A_1 [46, 47]. The potential role of CO in DN was examined in this study because of its antioxidant, antiinflammatory and antiapoptotic effects. According to the results of the current study, the renal HO-1 level and protein expression in rats with DN were decreased. The inhibitory effect induced by DN on renal HO-1 level and protein expression was ameliorated in animals treated with sitagliptin. Co-administration of CORM-A1, enhanced the inhibitory effects of sitagliptin on DN-induced functional and structural impairmrnts, oxidative stress, nitrostive stress, inflammation and apoptosis. It improved the ameliorated effect of sitagliptin on DN-induced reduction in the renal HO-1 and H2S levels, inhibition of the protein expression of renal eNOS, HO-1 and CSE and increase in the protein expression of iNOS. On the other hand, ZnPP, which is an HO-1 inhibitor [48] had the opposite effects. As a result, the protective effects of sitagliptin and sitagliptin in combination with CORM-A_1 in DN in rats can be linked to increased HO-1 level and expression and consequently CO production. In agreement with Kim et al. [49] increasing CO production resulted in an increase in eNOS expression, an increase in H2S production and an increase in CSE expression while decreasing NO production and iNOS activity and expression.

It was demonstrated that H2S plays a role in both diabetes and in DN. Lower levels of H2S were found to be responsible for the development and progression of DN in diabetic patients [50]. Moreover, Lodhi et al. [22] have reported that depletion of H2S brought on by persistent hyperglycemia may contribute to DN. According to numerous studies, H2S has antioxidant, antiinflammatory, and antiapoptotic properties [51]. Accumulating evidence has shown that upregulation of H2S protects aganist diabetes-induced glomerular podocyte injury [25]. Glomerular podocyte injury was brought on by H2S biosynthesis inhibitor, DL-PAG, through Inhibition of CSE [52,53]. The H2S exogenous donor NaHS [54] produced

antioxidant effect in the kidney of diabetic rats and prevented the progression of DN [55]. Furthermore, NaHS ameliorated tissue injury, kidney dysfunction, inflammation and apoptosis and lowered renal levels of NO, TNF-α., protein expression of the renal iNOS and increased protein expression of eNOS in renal I/R [56] and gentamycin-induced renal injuries [57]. In light of these effects of H_2S, we investigated its potential role in DN. Our findings demonstrate that DN was linked to a reduction in H_2S level along with reduction of protein expression of CSE in the rat kidney tissue. Treatment of these animals with sitagliptin inhibited DN-induced decline in the renal H_2S level and protein expression of CSE in the rat renal tissue. Co-administration of NaHS enhanced the inhibitory effects of sitagliptin on DN-induced functional and structural impairments, oxidative stress, nitrostive stress, inflammation and apoptosis. NaHS enhanced the inhibitory effect of sitagliptin on DN-induced reduction in the HO-1 and H_2S levels, eNOS, HO-1 and CSE protein expressions and increase in the protein expression of iNOS. DL-PAG, produced the opposite effects.

Thus, the beneficial effects of sitagliptin in DN can be linked to a decrease in NO production and an increase in CO and H2S levels. Increase in CO and H2S levels prevented NO overproduction which in turn increases CO and H2S levels. This suggested a crosstalk between these gasotransmitters. In agreement with many authors [30,31,58,59] oxidative stress, nitrosative stress, inflammation and apoptosis were prevented in the kidney tissue of DN rats by increasing CO and H2S levels and decreasing NO overproduction. Collectively, our results suggest that sitagliptin inhibited renal function and structure impairment brought on by DN. Along with its ability to lower blood sugar, sitagliptin also inhibited DN-induced oxidative stress, nitrosative stress, inflammation, apoptosis, NO overproduction and decrease in CO and H2S levels in the kidney tissue, all of which contributed to its protective effects. L-NAME, CORM-A1 and NaHS enhanced the protective effect of sitagliptin by decreasing NO overproduction and increasing CO and H2S levels. On the other hand, NO donor and CO and H2S biosynthesis inhibitors countered the renoprotective effect of sitagliptin. As a result, there is an interrelationship between DN, sitagliptin and gasotransmitters. Additionally, gasotransmitters seem to play a crucial role in the development and progression of DN and in the renoprotective effect of sitagliptin against it.

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