Research Article

The mechanisms of hydrogen sulfide in Ameliorating the development of diabetic nephropathy in adult male albino rats

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Abstract
Aim of work: This research aimed to study the reno-protective effect of hydrogen sulfide (H_2S), in the experimental induced diabetic nephropathy, as well as its cross-talk with the other gasotransmitters like (nitric oxide) NO. **Materials and Methods:** thirty-two adult male albino rats were randomly divided into four equal groups: control, STZ-induced diabetic (DN), DN+NaHS, and DN+L-NAME/NaHS groups. NaHS is a water soluble H_2S inducer administrated intra-peritoneally once daily for 7 days (56 mmol/ kg/day) after induction of diabetes by single streptozotocin dose (50 mg/kg). L-NAME is an inhibitor of nitric oxide synthase (NOS), and hence inhibiting NO synthesis *in vivo* given in a dose of 5 mg/kg/day for 1 week for STZ-induced diabetic treated by NaHS. **Results:** the present study showed improvement of kidney functions in the diabetic group by NaHS evidenced by the significantly lower levels of renal injury markers: serum urea, creatinine, uric acid, urinary albumin excretion (UAE) and urinary albumin/creatinine (A/C) as compared with diabetic group. In addition, oxidative stress and inflammation are incriminated, as the levels of total antioxidant (T-AOC), Nitric oxide (NO) and Tumor necrosis factor- α (TNF-α) were lowered by NaHS treatment. However, this improvement in kidney functions produced by NaHS was reduced by combination with L-NAME. **In conclusion:** The beneficial effects of H_2S are partially antagonized by L-NAME co-administration, indicating that NO partially mediates H_2S effects.
Key words: hydrogen sulfide, Nitric oxide, L-NAME, renal injury markers and Tumor necrosis factor- α.

Introduction
Recently, the incidence of diabetes mellitus (DM) has grown significantly throughout the world, and it becomes the most common cause of renal injury. It is supposed that 25% to 40% of patients with type 1 DM (DM1) and 5% to 40% of those with type 2 DM (DM2) will suffer from diabetic nephropathy (DN)[1].

The patho-physiology of DN is multifactorial, involving both genetic and environmental components. DN develops as a result of interactions between hemo-dynamic and metabolic factors. Hyperglycemia plays a central role in the initiation of renal structural injury, as subjects without diabetes do not develop the same type of nephropathy[2].

Hydrogen sulfide (H_2S) is one of the gasotransmitters which are lipid-soluble, endogenously produced gaseous signaling molecules that freely permeate the cell membrane leading to activation of intracellular targets without the need for membrane-bound receptors. According to their order of identification; gasotran-smitters include nitric oxide (NO), carbon monoxide (CO), and H_2S respectively[3].

Recently, it has emerged as an important mediator of numerous cellular signal transduction and pathophysiological responses including neuro-modulation, blood vessel relaxation, cardio-protection, insulin release, angiogenesis, energy production, as well as amelioration of the cytotoxic cellular responses, e.g. necrosis,

oxidative stress, inflammation, and apoptosis[4].

Unlike NO and CO, H_2S does not appear to stimulate guanylyl cyclase. H_2S signals inside the cells through posttranslational modification of proteins, known as sulfhydration[5]. Therefore, the present study was conducted to evaluate the different oxidative and inflammatory mechanisms of the renoprotective effects of H_2S in experimental induced diabetic nephropathy, as well as its cross-talk with the other gasotransmitters like NO.

Material and methods

I- Animals

Thirty-two adult male albino rats (Sprague-Dawley strain), of average weight 150-200 g, about 4 months old were used in the present study. They were purchased from the National Research Center, Cairo; Egypt. They were housed in groups of six in stainless steel cages that offered adequate space for free movement and wandering (40 cm x 40 cm x 25 cm) at room temperature with natural dark/light cycles, and allowed free access to water and commercial rat's diet (Nile Company, Egypt) for two weeks for acclimatization. All experimental protocols were approved by the animal care committee of Minia University which coincides with international guidelines. Rats were classified randomly into the following four groups (eight rats each):

1- **Control group:** in which 6 rats were fed a commercial rat's diet and received citrate buffer only.
2- **STZ-induced diabetic group (DN):** rats were injected IP with streptozotocin, STZ (***Sigma, USA***) at a dose level of 50 mg/kg, dissolved in citrate buffer, pH (4.5) after an overnight fast (Uil et al., 2018). The STZ-treated animals were allowed to drink 20% glucose solution for 24h to overcome initial drug-induced hypoglycemic mortality. Diabetes was verified 3 days later by evaluating blood glucose levels with the use of glucose-oxidase reagent strips (Accu-Chek, Roche Inc., Indiana-polis, IN). Rats having blood glucose level of 200 mg/dl or greater were considered to be diabetic and selected for the study[6].
3- **STZ-induced diabetic+NaHS group (DN+NaHS):** rats were given NaHS (water soluble H_2S donor) (Honeywell Fluka Co., China) intraperitoneally 50 µmol/kg twice daily for 7 day[7].
4- **STZ-induced diabetic+NaHS+L-NAME group (DN+L-NAME/ NaHS):** L-NAME is one of the L-arginine analogues which are widely used as inhibitors of nitric oxide synthase (NOS), and hence inhibiting NO synthesis *in vivo*. STZ-induced diabetic rats were given NaHS by the same previous dose and L-NAME by IP injection in a dose of 5 mg/kg/day, dissolved in H_2O, for 1 week[8].

II- Experimental protocol

One day before the end of the study, each rat was housed separately in a metabolic cage for collection of 24h urine to determine creatinine and albumin by colorimetric assay kits (Spinreact SAU, Spain) according to the manufacturer's instructions. Measured creatinine with albumin used to determine albumin/ creatinine (A/C) ratio.

After that, the rats were sacrificed by decapitation. The blood samples were immediately collected from the jugular veins in 10 ml Eppendorf tubes, allowed to clot, and then centrifuged at 3000 rpm for 20 minutes. The serum samples were separated in 2 ml Eppendorf tubes, and stored at -20°C until used for estimation of the following parameters, urea (BioSystems SA, Spain), creatinine (Spinreact SAU, Spain), and uric acid (Stanbio Lab., USA) by using an enzymatic colorimetric assay kits according to the manufacturer's instructions.

The kidneys were also removed from each rat, and from each organ were weighed and homogenized in potassium phosphate buffer 10 mM. The ratio of tissue weight to homogenization buffer was 1:10. The homogenates were centrifuged at 5000 rpm for 10 min at 4°C. The resulting supernatant was used for determination of total antioxidant (T-AOC) by colorimetric assay

kit (LC Diagnostics Ltd, Korea), Nitric oxide by colorimetric assay kit (Bio-diagnostic, Egypt) and Tumor necrosis factor- α (TNF-α) by ELISA kit (Boditech Med. Inc., Korea) according to the manufacturer's instructions

Statistical analysis

Data were represented as means ± standard errors of the mean (SEM). Statistical analysis was performed using Graph pad Prism 5 software and significant difference between groups was done by one-way ANOVA followed by Tukey-Kramar post hoc test for multiple comparisons with a value of $P \leq 0.05$ considered statistically significant.

Results

A- Evaluation of the renal injury markers:

The data presented in table (1) show that serum urea, creatinine and uric acid were significantly higher in the DN group than in the control group. NaHS significantly lowered these parameters in the DN+NaHS group than in the DN group indicating improvement of kidney functions in the diabetic group by NaHS. However, this improvement in kidney functions produced by NaHS was deteriorated by combination with L-NAME as indicated by the significantly higher parameters levels than in the DN and DN+NaHS groups.

The data of the present study clearly demonstrated that urinary albumin excretion (UAE) and urinary albumin/ creatinine (A/C) ratio were significantly higher in the DN group than in the control group. NaHS significantly lowered these parameters in the DN+NaHS group than in the DN group. However, the DN+NaHS/L-NAME group showed significant higher parameters levels than in the DN and DN+NaHS groups (table 2).

B- Evaluation of the oxidative and inflammatory parameters:

The present data show that renal tissue total antioxidant capacity (T-AOC) and TNF-α level were significantly higher in the control group than in the DN group. NaHS significantly increased T-AOC in the DN+NaHS group than in the DN group. However, this improvement in kidney functions produced by NaHS was reduced by combination with L-NAME than in the DN+NaHS group (table 3).

As regards renal total NO level, the data presented in table (3) show that it was significantly higher in the DN group than in the control group. NaHS significantly lowered total NO level in the DN+NaHS group than in the DN group indicating improvement of kidney functions in the diabetic group by NaHS. Combination of NaHS with L-NAME significantly decreased total NO level compared to treatment with NaHS alone.

Table (1): Changes in the serum parameters in the diabetic and the diabetic-treated groups:

Groups (n = 8) / Parameters	Control	DN	DN+NaHS	DN +NaHS/L-NAME
Serum urea (mg%)	30.9± 1.58	86.8± 1.06 [a]	41.96± 0.74 [ab]	94.1± 1.38 [abc]
Serum creatinine (mg%)	0.29 ± 0.02	1.83± 0.03 [a]	0.71± 0.02 [ab]	2.1± 0.07 [abc]
Serum uric acid (mg%)	2.28± 0.22	8.26± 0.13 [a]	3.64± 0.2 [ab]	9.05± 0.08 [abc]

Data represent mean ± S.E. ***n*****:** number of rats in each group. **DN:** diabetic nephropathy. **DN+NaHS:** diabetic nephropathy treated with sodium hydrosulfide. **DN+NaHS/L-NAME:** diabetic nephropathy treated with sodium hydrosulfide and N_{ω}-Nitro-L-arginine methyl ester.
[a]: significant difference from the control group. **[b]:** significant difference from the DN group.
[c]: significant difference from the DN+NaHS group, $P < 0.05$.

Table (2): Changes in the urinary parameters in the diabetic and the diabetic-treated groups:

Group (n = 8) Parameters	Control	DN	DN+NaHS	DN +NaHS/L-NAME
UAE (mg/24 h)	2.4± 0.06	81.6± 4.4 [a]	34.2± 2.5 [ab]	96.5± 1.52 [abc]
Urinary A/C ratio (µg/mg)	6.67± 0.22	539.4± 6.1 [a]	279.3± 15 [ab]	607.1± 17.8 [abc]

Data represent mean ± S.E. ***n*:** number of rats in each group. **DN:** diabetic nephropathy. **DN+NaHS:** diabetic nephropathy treated with sodium hydrosulfide. **DN+NaHS/L-NAME:** diabetic nephropathy treated with sodium hydrosulfide and N_{ω}-Nitro-L-arginine methyl ester. **[a]:** significant difference from the control group. **[b]:** significant difference from the DN group. **[c]:** significant difference from the DN+NaHS group, $P < 0.05$.

Table (3): Changes in the renal tissue parameters in the diabetic and the diabetic-treated groups:

Group (n = 8) Parameters	Control	DN	DN+NaHS	DN +NaHS/L-NAME
Renal T-AOC (mmol/L)	0.24± 0.02	0.07± 0.01 [a]	0.17± 0.01 [ab]	0.12± 0.01 [abc]
Renal total NO (nmol/g tissue)	4.81± 0.03	36.11± 1.43 [a]	13.95± 0.82 [ab]	2.42± 0.17 [bc]
Renal TNF-α (pg/g tissue)	298.4± 3.1	623.5± 3.8 [a]	343.9± 1.8 [ab]	421.7± 12.5 [abc]

Data represent mean ± S.E. ***n*:** number of rats in each group. **DN:** diabetic nephropathy. **DN+NaHS:** diabetic nephropathy treated with sodium hydrosulfide. **DN+NaHS/L-NAME:** diabetic nephropathy treated with sodium hydrosulfide and N_{ω}-Nitro-L-arginine methyl ester. **[a]:** significant difference from the control group. **[b]:** significant difference from the DN group. **[c]:** significant difference from the DN+NaHS group, $P < 0.05$.

Discussion

Hydrogen sulfide (H_2S), along with nitric oxide (NO) and carbon monoxide (CO), is proved to have significant renoprotective effects in various renal diseases including DN[9].

The streptozotocin (STZ)-induced diabetic rats represent a good experimental diabetic model. Therefore, this animal model was selected for the study[10] to assess the underlying mechanisms of the renoprotective effects of H_2S in diabetic nephropathy, as well as its cross-talk with the other gasotransmitters like NO.

On studying the effect of diabetic induction on the kidney the results obtained in the present study showed that the renal injury markers (RIM); serum urea, creatinine and uric acid, were significantly higher in the STZ-induced diabetic group than in the control group. These results are in line with Koothappan et al., (2018) who reported that serum urea, creatinine and uric acid levels were significantly increased in the STZ-induced diabetic rats[11].

The results obtained in the present study showed also that the urinary albumin excretion (UAE) and urinary albumin/ creatinine (A/C) ratio were significantly higher in the STZ-induced diabetic group than in the control group. These results are in line with Sehnine et al., (2018) who reported that urinary albumin was significantly increased in STZ-induced hyperglycemic mice[12].

The development of DN can be explained by the hyperglycemia-induced increased sensitivity of efferent arterioles to angiotensin II (Ang II). This effect leads to increased glomerular capillary pressure,

hyperperfusion, hyperfiltration, and micro-albuminuria[1].

On an attempt to explain the mechanisms beyond diabetic-induced renal injury; total nitric oxide (NO), oxidative stress markers, and inflammatory markers were measured in the renal tissue.

Regarding the renal tissue antioxidants, the results obtained in the present study showed that total antioxidant capacity (T-AOC) was significantly lower in the STZ-induced diabetic group than in the control group. These results are in line with Thomson et al., (2017) who reported that renal tissue T-AOC level and renal tissue catalase activity were significantly decreased in STZ-induced diabetic rats[13].

Regarding renal tissue total (NO) level, the results obtained in the present study showed that it was significantly higher in the STZ-induced diabetic group than in the control group. These results are in line with Morsy et al., (2015) who reported that hyperglycemia has been shown to increase iNOS-induced NO production, and promote extracellular matrix accumulation in rat mesangial cells[14]. At toxic concentrations produced by inducible NO synthase (iNOS), its 'bad face' appears as being involved in the renal inflammatory conditions through the formation of peroxynitrite ($ONOO^-$), a pro-inflammatory cytokine, by reacting with superoxide anion ($O_2^{\bullet -}$). NO is the only biological molecule produced in high concentrations enough to out-compete SOD for $O_2^{\bullet}$ [15].

As regards the renal tissue pro-inflammatory cytokines, the results obtained in the present study showed that renal tissue TNF-α level was significantly higher in the STZ-induced diabetic group than in the control group. These results are in line with Sun and Kanwar (2015) who reported that renal TNF-α level was significantly increased in the STZ-induced diabetic rats by different signaling cascades[16].

Collectively, the results of the present study verified that STZ-diabetic induction produced renal injury, proved by increasing the renal injury markers in the diabetic group, through increasing renal tissue NO, inflammatory mechanism, and oxidative mechanism.

On studying the effect of H_2S on the diabetic induced renal injury, the results obtained in this study showed that the treatment of diabetic rats with NaHS led to a significant decrease in the measured RIM; serum urea, creatinine, and uric acid levels compared to the diabetic group. These results are in line with Qian et al., (2016) who reported that S-propargyl-cysteine, a novel H_2S-releasing compound, significantly reduced the serum creatinine level in the STZ-induced diabetic rats[17].

Moreover, the present study showed also that the treatment of diabetic rats with NaHS led to a significant decrease in UAE and urinary A/C ratio compared to the diabetic group. These results are in line with Qian et al., (2016) who reported that the H_2S donor, S-propargyl-cysteine, significantly reduced the albuminuria in the STZ-induced diabetic rats[17].

Regarding the renal tissue antioxidants, the results obtained in the present study showed that the treatment of diabetic rats with NaHS led to a significant increase in renal tissue of T-AOC compared to the diabetic group. These results are in line with Zhou et al., (2014) who reported that NaHS treatment of STZ-induced diabetic rats was associated with decreased ROS and malondialdehyde (MDA) levels and increased SOD and GPx activities[18].

The protective effect of H_2S against oxidative stress is thought to be mediated by its ability to directly scavenge ROS, degrade lipid peroxides, up-regulate GSH synthesis, and potentiate the antioxidant effects[19]. This ability depends on activation of Nrf2 pathway which is the key element in the regulation of the cellular response to the oxidative stress[20].

The results obtained in the present study showed also that the treatment of diabetic rats with NaHS led to a significant decrease in renal tissue total NO level compared to the diabetic group. These results are in line with Yang et al., (2017) who reported that

NaHS treatment of STZ-induced diabetic rats led to decreased total NO level in both serum and renal tissues via decreasing the protein expression of iNOS[21]. Furthermore, this study showed that renal tissue NO level is still significantly higher in the NaHS-treated diabetic group than in the control group. This can be explained by the upregulation of eNOS under the effect of H_2S [22].

As regards the renal tissue pro-inflammatory cytokines, the results obtained in the present study showed the treatment of diabetic rats with NaHS led to a significant decrease in renal tissue TNF-α level compared to the diabetic group. These results are in line with Zhou et al., (2014) who reported that NaHS treatment of STZ-induced diabetic rats was associated with decreased renal tissue TNF-α level via decreasing renal tissue MCP-1, ICAM, and VCAM levels, as well as decreasing the protein expression of TNF-α[18].

It is becoming increasingly clear that there are important interactions among the gasotransmitters: NO, CO, and H_2S.
Previous studies reported that there is a possible crosstalk between them[23]. In order to investigate whether the renoprotective effect of H_2S in case of DN is dependent on NO pathway or not, we treated the diabetic rats with NaHS and L-NAME (NO inhibitor).

The results obtained in this study showed that the treatment of diabetic rats with NaHS and L-NAME led to a significant increase in the measured RIM; serum urea, creatinine, and uric acid levels compared to both the diabetic group and the diabetic group treated with NaHS alone. These results are in line with Nakagawa et al., (2007) who reported that diabetic eNOS knockout mice developed hypertension and renal insufficiency with increased serum urea level[24].

The results obtained in the present study showed also that the treatment of diabetic rats with NaHS and L-NAME led to a significant increase in UAE and urinary A/C ratio compared to both the diabetic group and the diabetic group treated with NaHS alone. These results are in line with Nakagawa et al., (2007) who reported that diabetic eNOS knockout mice developed albuminuria and increased urinary A/C ratio[24].

Regarding the renal tissue antioxidants, the results obtained in the present study showed that the treatment of diabetic rats with NaHS and L-NAME led to a significant decrease in renal tissue T-AOC compared to the diabetic group treated with NaHS alone. These results are in line with Calabró et al., (2018) who reported that L-NAME increased NADPH oxidase-dependent $O_2^{\bullet-}$ production[25].

The results obtained in the present study showed also that the treatment of diabetic rats with NaHS and L-NAME led to a significant decrease in renal tissue total NO level compared to both the diabetic group and the diabetic group treated with NaHS alone. These results can be explained by inhibition of NOS under the effect of L-NAME[25].

As regards the renal tissue pro-inflammatory cytokines, the results obtained in the present study showed the treatment of diabetic rats with NaHS and L-NAME led to a significant increase in renal tissue TNF-α level compared to the diabetic group treated with NaHS alone. These results are in line with Ozkurt et al., (2018) who reported that the plasma and kidney mRNA levels of TNF-α increased in the L-NAME-treated rats[26].

In addition to the above mentioned oxidative stress and pro-inflammatory effects produced by L-NAME, the deleterious effects of L-NAME in the NaHS-treated diabetic group may be explained by inhibition of eNOS and nNOS, whose expression was increased by NaHS treatment. Inhibition of eNOS in DM predisposes to the classic DN. The mechanism likely is due to uncoupling of the vascular endothelial growth factor (VEGF)-eNOS axis, resulting in increased levels of VEGF and excessive endothelial cell proliferation with excessive endothelial cell proliferation coupled with altered autoregulation consequent to the develop-

ment of pre-glomerular arteriolar disease [24].

In conclusion, the results obtained from the present study revealed that the renoprotective effects of H_2S through its effects on renal tissue antioxidants and pro-inflammatory cytokines can be partially dependent of NO pathway.

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