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EXOGENOUS HYDROGEN SULFIDE (H_2S) IMPROVES THE ENDOTHELIAL AND RENAL EXCRETORY FUNCTIONS IN STREPTOZOTOCIN INDUCED WKY DIABETIC RATS

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ABSTRACT

Nephropathy is one of the most common microvascular complications of diabetes. Hydrogen sulfide (H_2S) has been implicated in controlling the renal glomerular (vascular) and tubular functions. This study investigates the metabolism of H_2S and its effect on the progression of diabetic nephropathy. Diabetes was induced in WKY rats by streptozotocin in two groups. One diabetic group received NaHS, a H_2S donor. While a vehicle treated group served as a control. Blood pressure was measured in conscious rats and at the end of the treatment period in anesthetized rats. In addition, pulse wave velocity (PWV) was also observed. Plasma and urine H_2S levels and creatinine concentration and electrolytes were measured weekly throughout the 34-day period. Diabetic rats had lower ($p<0.05$) plasma and urine levels of H_2S and lower urinary sodium to potassium ratio. Moreover, diabetic group had higher plasma sodium, higher absolute urinary sodium excretion, higher plasma creatinine and higher PWV (all $p<0.05$) but with similar mean arterial pressure compared to control ($p>0.05$). Treatment with the H_2S donor restored H_2S, plasma creatinine, plasma sodium and urinary sodium to potassium ratio significantly in diabetic-NaHS treated group and also reduced the PWV (all $p<0.05$). Moreover, the treated diabetic group had higher ($p<0.05$) creatinine clearance compared to diabetic group. The results suggested that exogenously administered H_2S improves the renal excretory functions and vascular endothelium impairment in experimental diabetes in rats.

INTRODUCTION: Diabetes is a chronic metabolic disorder that affects the metabolism of carbohydrates and other nutrients as a result of impaired insulin release and/or insulin resistance resulting in hyperglycemia [1]. Being a chronic disease, Diabetes mellitus is well known for its complications. Endothelial dysfunction is considered as the first step in the pathogenesis of micro and macro vascular complications of diabetes [2].

Hyperglycemia associated with diabetes modifies the endothelial function through a numbers of complex mechanisms including oxidative stress [3], glycation of protein and lipids [4] and activation of protein kinase C [5].

Nephropathy is the hallmark of microvascular complications of diabetes. Glomerular haemodynamic changes that includes hyperfiltration and hyperperfusion have been implicated as key factors in

the development of diabetic nephropathy and can be detected at the early stage of the disease [6].

Hydrogen sulfide (H_2S) can be generated in many types of mammalian cells [7] and is recognized as a novel gaseous transmitter [8], with a concentration of ~46µM in the rat's serum [9]. In addition to the circulating H_2S, a significant amount of H_2S is produced in various tissues such as the brain [10] heart and blood vessels [9]. Recent studies have shown that vascular tissues are capable of generating the measurable amounts of H_2S [9].

The two pyridoxal phosphate-dependent enzymes, cystathionine β synthase (CBS) and cystathionine γ lyase (CSE) are responsible for the majority of the endogenous production of H_2S in mammalian tissues that use l-cysteine as the main substrate [7]. The expression of CBS and CSE has been identified in the liver, heart, blood vessels, kidney and brain [11]. The expression of both the enzymes, concerned with the production of H_2S are reported to present in kidney and generated H_2S have been linked in controlling the renal glomerular (vascular) and tubular functions [12].

Since the discovery of H_2S as a valuable molecule, several physiological function have been characterized such as it has been shown to relax vascular smooth muscle cells, induce vasodilatation of isolated blood vessels, reduce blood pressure [13], inhibit leukocyte–endothelial cell interactions in *vivo* [14], is a potent anti-inflammatory molecule, a potent antioxidant (under chronic conditions such as diabetes and hypertension), effectively inhibits apoptosis of a number of cell types [15].

So in the light of above suggestions, we hypothesized that exogenous administration of H_2S may have the ability to ameliorate renal and vascular functions in diabetes. Hence, the present study was undertaken to investigate the metabolism of H_2S in experimental diabetes and further explores its renoprotective role in the progression of diabetic nephropathy.

MATERIALS AND METHODS:

Subjects of Study: WKY rats weighing 235 to 250g were obtained from animal housing and breeding facility of Universiti Sains Malaysia and were divided into three groups namely control, diabetic and diabetic treated with NaHS (n=6 in each group).

Rats were acclimatized for one week before any experimental procedure. All the animals were housed in the same environmental conditions with free access to food (Gold Coin Sdn. Bhd., Penang, Malaysia) and drinking water *ad libitum*. The entire animal's procedures and experiments used in this study have prior approval from Universiti Sains Malaysia Animal Ethics Committee.

Induction of Diabetes: After 12 h fasting, rats were injected with streptozotocin (Nova Laboratories, Sdn. Bhd., Selangor, Malaysia) at a dose of 40 mg/kg. Streptozotocin was dissolved in freshly prepared ice cold sodium citrate buffer (0.1 mol/L, pH 4.5) and injected intraperitoneally [16]. The control rats received an equal amount of buffer intraperitoneally. After 3 days, a drop of blood was obtained by nicking the tail of overnight fasted rats and glucose concentrations were obtained using glucometer (GlucoSure plus, Apex Biotechnology Corp., Hsinchu, Taiwan) to determine the extent of hyperglycemia. Only animals exhibiting fasting blood glucose above or equal to 350 mg/dl were included in the study.

Treatment with NaHS: Treated group of animals were administered with NaHS (Sodium hydrosulfide) as a donor of exogenous H_2S [17-19]. Rats were injected with NaHS (Sigma Aldrich, Malaysia) at dose of 56µmol/kg intraperitoneally in saline at the same time daily for 5 weeks [18]. NaHS was freshly prepared every day by dissolving the NaHS into saline.

Blood Pressure Measurement: The arterial blood pressure was determined indirectly by the tail cuff method using the Model 29 pulse amplifier and Model 20NW cuff pump coupled to a computerized data acquisition system (PowerLab®, ADInstruments, Sydney, Australia) on days 0 and 21. On day 34th of the study, an invasive blood pressure measurement was obtained under ketamine and xylazine anesthesia in surgically prepared animals.

Collection of Metabolic Data: Metabolic data was collected in all the groups of animals involved in present study on days 0, 21 and 34 of 35 days study duration. Rats were placed in metabolic cages for 24 hours after which water intake and urine output was measured. Similarly, blood sample was obtained from the rat tail on days 0, 21 and 34 into a pre-cooled

heparinized eppendroff tube and plasma was obtained by centrifugation of the blood sample. Collected samples were stored at −30°C for further analysis of H_2S, creatinine, sodium and potassium.

Surgical procedure for Invasive Blood Pressure and Pulse Wave Velocity: All the rats were fasted overnight before the acute experiment and anaesthetized with a mixture of ketamine (Ilium, Australia) 80 mg/kg and xylazine (Ilium, Australia) 10 mg/kg i.p. [20], and were supplemented intravenously with ketamine at a dose of 50 mg/kg if required. Tracheotomy was then performed to maintain a clear air way by using the endotracheal cannula (PP 240, Portex Ltd. Kent, UK). Left jugular vein was catheterized with PP 50 tubing (Portex Ltd. Kent, UK) to permit the infusion of supplementary anesthesia and drugs if any. The right carotid artery was cannulated and the cannula was advanced up to the aortic arch.

Following this, a midline abdominal incision was made and the left kidney and iliac artery were exposed. Left iliac artery was catheterized and the cannula was pushed up to the abdominal aorta just proximal to the point where iliac bifurcation starts. Both cannulas were connected to pressure transducer (P23 ID Gould, Statham Instruments, UK) linked to a data acquisition system (PowerLab®, ADInstruments, Sydney, Australia) through a Quad Amp (ADInstruments, Australia) using chart Pro (V.5.5) software.

The animals were allowed to stabilize for one hour upon completion of above surgical procedure. After the stabilization period mean arterial pressure, systolic blood pressure, diastolic blood pressure, pulse pressure and heart rate were recorded for 30 minutes continuously.

Measurement of Pulse Wave Velocity (PWV): PWV was measured by the previously described method [21, 22]. Proximal and distal pressure waves were recorded at the same time and displayed on data acquisition system. PWV was calculated by dividing the propagation distance (d) by the propagation time (t) and measured in meters per second. After the euthanization of the animal, the full length of aorta was exposed and tip of the two cannulas of the carotid and iliac arteries was identified. The distance between these two points was determined by using a wet cotton thread. The thread was laid straight for the measurement of distance between the two cannulas. The time for the propagation of pulse wave from the aortic arch to the abdominal aorta was measured by the time delay between the upstrokes (foot) of each pressure wave front (foot to foot technique). The average of 10 normal consecutive cardiac cycles was used to calculate the propagation time.

Measurement of Plasma and Urine H_2S Level: H_2S levels in plasma and urine were measured spectrophotometrically according to a previously described method [18]. Briefly, 100µl of aliquots of the samples were mixed with 50µl of distilled water in micro-centrifuge tubes containing 300µl of zinc acetate (1% w/v) to trap H_2S. The reaction was terminated after 5 min by adding 200µl of *N, N*-2dimethyl-p-phenylenediamine sulfate (20mM in 7.2 M HCl) and immediately followed by addition of 200µl of $FeCl_3$ (30mM in 1.2 M HCl).

The mixture was kept in the dark for 20 minutes. In order to precipitate protein from the samples 150µl of trichloroacetic acid (10% w/v) was added. The mixture was centrifuged at 10,000 rpm for 10 minutes. The absorbance of the resulting supernatant was measured at 670 nm using a 96-well plate reader (Bio-Tek instruments, INC, USA). All samples were assayed in duplicates. Finally, H_2S concentration in the plasma or urine was calculated against the calibration curve of standard H_2S solutions (NaHS: 3.125-100 µM). All chemicals used were obtained in pure form from Sigma (Sigma Aldrich, Malaysia).

Measurement of Plasma and Urine Creatinine, Sodium and Potassium: Plasma and urinary creatinine concentrations were measured spectrophotometrically (Jaffe's reaction). Sodium and potassium concentrations in plasma and urine were measured by using the flame photometer (Jenway Ltd., Felsted, UK).

Calculation of Renal Functional Parameters: Urine flow rate, creatinine clearance, absolute sodium excretion ($U_{Na}V$), and urinary sodium to potassium ratio (Na: K) were calculated from the plasma and urinary creatinine, sodium and potassium values.

Statistical Analysis: All the data were expressed as mean ± SEM. Statistical significance was set at $p<0.05$. Statistical analysis was performed by one way analysis

of variance followed by Bonferroni's /Dunn all means *post hoc* test using the statistical package, Superanova (Abacus Inc., CA, USA).

RESULTS:

Blood Glucose, Body Weight, Water Intake and Urine Flow Rate: Observations were made on three different occasions during the study period (day 0, 21 and 34). It was observed that diabetic and diabetic treated groups were hyperglycemic throughout the study period regardless of the exogenous H_2S. As the study progresses, the body weight of diabetic or diabetic treated groups significantly decreased as compared to the control in respective days (**Table 1**). In contrast, the body weight increased in control group with time (all $p<0.05$). Furthermore, diabetic and diabetic treated rats exhibited polydepsia and interestingly at the end of the treatment period, treated rats had higher water intake when compared to untreated diabetic rats ($p<0.05$). Finally, both diabetic treated and untreated groups had higher urine excretion (all $p<0.05$) as compared to control. However, at the end of the treatment period, NaHS treatment increased the urine flow rate in Diabetic treated group in comparison to diabetic group ($p<0.05$).

TABLE 1: BLOOD GLUCOSE, BODY WEIGHT, WATER INTAKE AND URINE FLOW RATE OF CONTROL, DIABETIC AND TREATED GROUPS OF RATS

		Days of observation		
Parameters	**Groups**	**Day 0**	**Day 21**	**Day 34**
Blood glucose (mg/dl)	Control	86.69±4.31	85.04±6.90	89.65±8.68
	Diabetic	388.09±24.98*	378.45±21.17*	372.18±10.80*
	Diabetic Treated	384.48±19.22*	387.64±18.23*	385.46±15.45*
Body weight (g)	Control	241.83±2.78	276.66±6.25ж	299.33±3.50ж
	Diabetic	243.50±3.88	224.83±2.92*ж	215.66±2.06*ж
	Diabetic Treated	246.83±4.07	223.83±4.35*ж	207.66±0.66*ж
Water intake (ml/24 h)	Control	54.00±3.79	51.33±7.07	55.34±3.44
	Diabetic	78.83±5.49*	73.33±8.75*	64.00±5.09*
	Diabetic Treated	82.50±5.24*	77.50±9.35*	100.00±7.07*#
UFR(µl/min /100g Bw)	Control	2.55±0.14	2.38±0.24	2.45±0.08
	Diabetic	13.33±1.46*	19.01±1.74*	23.83±0.96*
	Diabetic Treated	14.34±1.01*	20.33±1.09*	27.09±1.72*#

The values are given as mean ±S.E.M. (n=6 in each group). * indicates $p<0.05$ i.e. Diabetic and Diabetic treated vs. Control rats in respective days. ж indicates $p<0.05$ at day 21 and day 34 as compared to day 0 of respective group of rats. # indicates significant difference, comparison made between Diabetic vs. Diabetic treated in respective days.

Mean Arterial Pressure and Systolic Blood Pressure: It was observed that there was no significant difference of mean arterial blood pressure and systolic blood pressure between the three groups throughout the study period ($p>0.05$) (**Table 2**).

TABLE 2: MEAN ARTERIAL PRESSURE (MAP) AND SYSTOLIC BLOOD PRESSURE OF CONTROL, DIABETIC AND TREATED GROUPS OF RATS

Parameters	**Groups**	**Days of observation**		
		Day 0	**Day 21**	**Day 34**
MAP (mmHg)	Control	107.99±2.35	106.27±1.46	103.46±1.35
	Diabetic	105.58±1.81	103.59±1.86	102.92±1.84
	Diabetic Treated	104.64±2.73	106.18±3.13	101.32±2.09
SBP (mmHg)	Control	125.99±3.35	125.02±1.26	121.95±2.84
	Diabetic	126.49±2.36	126.74±2.52	122.52±1.50
	Diabetic Treated	123.21±2.07	127.19± 2.72	119.77±1.69

The values are given as mean ±S.E.M. (n=6 in each group). None were significantly different.

Plasma and urinary H_2S: It was observed that there was no significant difference in the plasma and urinary values of H_2S between the three groups at days 0 and day 21 (all $p>0.05$). On the other hand, plasma and urinary concentrations of H_2S in diabetic group was lower than the control on day 34 (all $p<0.05$). With NaHS treatment, the plasma and urinary concentrations of H_2S of diabetic treated group increased significantly when compared to diabetic group on day 34 (all $p<0.05$) (**Figs. 1 and 2**).

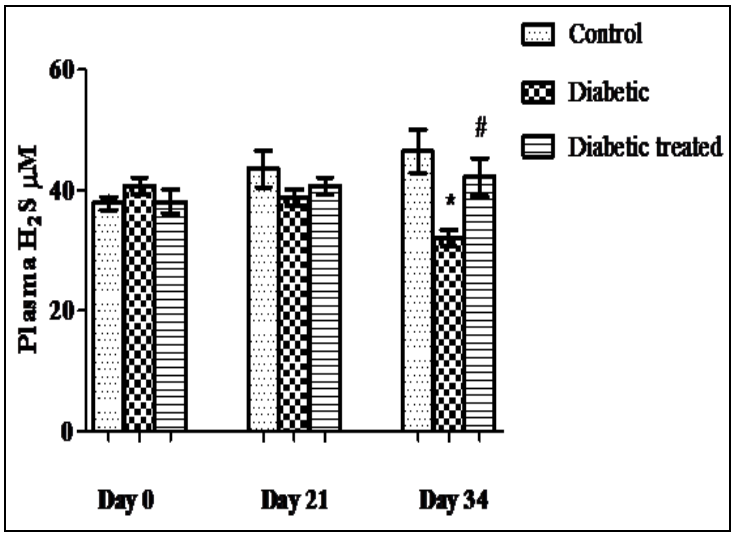

FIG. 1: PLASMA H_2S OF CONTROL, DIABETIC AND TREATED GROUPS OF RATS

The values are given as mean ±S.E.M. (n=6 in each group).* indicates p<0.05 i.e. Diabetic and Diabetic treated vs. Control rats in respective days. # indicates significant difference, comparison made between Diabetic vs. Diabetic treated in respective days.

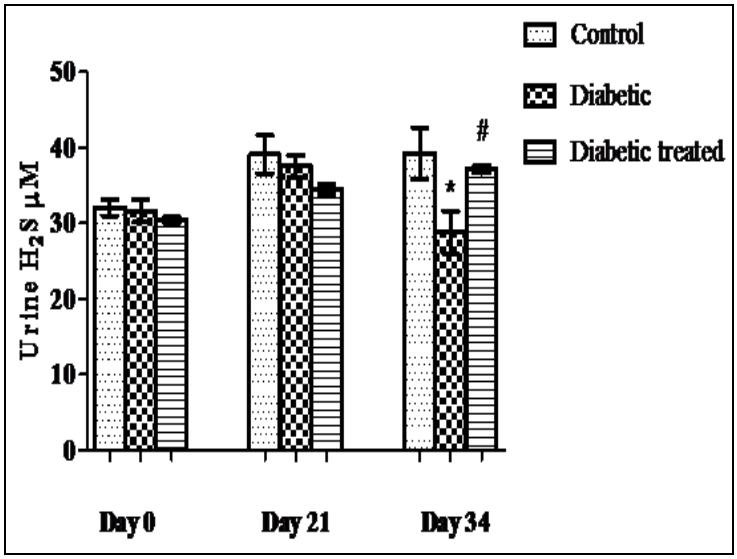

FIG. 2: URINARY H_2S OF CONTROL, DIABETIC AND TREATED GROUPS OF RATS

The values are given as mean ±S.E.M. (n=6 in each group). * indicates p<0.05 i.e. Diabetic and Diabetic treated vs. Control rats in respective days. # indicates significant difference, comparison made between Diabetic vs. Diabetic treated in respective days.

Pulse Wave Velocity: Pulse wave velocity of diabetic group was significantly higher as compared to control (p<0.05) NaHS had decreased pulse wave velocity significantly (p<0.05) but it did not reach to that of the control (**Fig. 3**). Renal cortical blood perfusion of the three groups was not significantly different (all p>0.05) (Fig. 3).

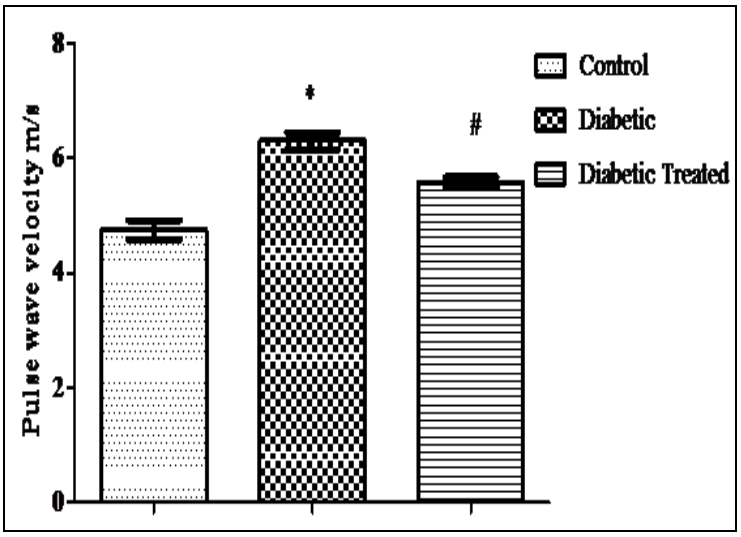

FIG. 3: PULSE WAVE VELOCITY OF CONTROL, DIABETIC AND TREATED GROUPS OF RATS

The values are given as mean ±S.E.M. (n=6 in each group).* indicates p<0.05 i.e. Diabetic and Diabetic treated vs. Control rats in respective days. # indicates significant difference, comparison made between Diabetic vs. Diabetic treated in respective days.

Plasma Creatinine and Creatinine Clearance: It was noticed that diabetic rats had a higher plasma creatinine level in comparison to control throughout the study period (p<0.05). Treatment with H_2S decreased plasma creatinine level which reached to significant level on day 34 (**Table 3**). Furthermore, a higher creatinine clearance was observed in the diabetic rats when compared to control at days 21 and 34 (all p<0.05). Moreover with the treatment, creatinine clearance had increased in diabetic treated rats as compared to the untreated counterparts (all p<0.05) (Table 3).

Plasma Sodium Concentration, Absolute Sodium Excretion and Urinary Sodium to Potassium Ratio: Plasma sodium concentration was not significantly different between the three groups on day 0. As the study progress the diabetic and diabetic treated groups exhibited with higher plasma sodium level as compared to control at days 21 and 34 (all p<0.05).

The treatment with exogenous H_2S had decreased plasma sodium concentration significantly as compared to untreated diabetic group on days 21 and 34 (all p<0.05). It was noticed that diabetic and diabetic treated groups had higher values of absolute sodium excretion in comparison to the control on all the three points of observations (all p<0.05).

Moreover, it was also observed that with the treatment, absolute sodium excretion of diabetic treated group had increased as compared to diabetic group at days 21 and 34 (all $p<0.05$). Moreover, it was observed that there was a significant decrease of urinary sodium potassium ratio of diabetic treated and untreated groups as compared to control on all the three points of observations (all $p<0.05$). It was further noticed that with NaHS treatment, sodium potassium ratio treated group increased on days 21 and 34 (all $p<0.05$) as compared to diabetic group (Table 3).

TABLE 3: RENAL FUNCTIONAL PARAMETERS OF CONTROL, DIABETIC AND DIABETIC TREATED GROUPS OF RATS

		Days of observation		
Parameters	**Groups**	**Day 0**	**Day 21**	**Day 34**
Plasma creatinine (mg/dl)	Control	0.841±0.065	0.895±0.078	0.966±0.023
	Diabetic	1.086±0.107*	1.039±0.093*	1.052±0.084*
	Diabetic Treated	1.047±0.101*	1.029±0.069	0.905±0.074#
Cr. clearance (ml/min/100gBw)	Control	0.461±0.044	0.416±0.040	0.399±0.019
	Diabetic	0.431±0.092	0.771±0.042*	0.856±0.058*
	Diabetic Treated	0.456±0.084	0.969±0.070#	1.785±0.162#
Plasma sodium (mmol/liter)	Control	141.00±4.31	139.50±1.67	141.41±5.70
	Diabetic	142.01±3.46	167.66±4.41*	175.33±3.59*
	Diabetic Treated	145.25±5.22	156.66±4.08*#	151.75±3.31*#
$U_{Na}V$ (mmol/hour/100g Bw	Control	0.02±0.01	0.020±0.02	0.02±0.01
	Diabetic	0.041±0.01*	0.074±0.01*	0.111±0.01*
	Diabetic Treated	0.04±0.01*	0.11±0.01*#	0.220±0.01*#
Urinary Na:K ratio	Control	3.89±0.58	3.21±0.35	2.46±0.14
	Diabetic	0.26±0.03*	0.302±0.02*	0.300±0.02*
	Diabetic Treated	0.28±0.02*	0.867±0.04*#	1.89±0.15*#

The values are given as mean ±S.E.M. (n=6 in each group).* indicates $p<0.05$ i.e. Diabetic and Diabetic treated vs. Control rats in respective days. # indicates significant difference, comparison made between Diabetic vs. Diabetic treated groups in respective days.

DISCUSSION: The major findings of present study are that the streptozotocin (STZ) induced diabetes is associated with lowers plasma and urinary H_2S levels along with reduced renal excretory and vascular endothelial functions. Consequently, supplementation of exogenous H_2S has improved H_2S levels, renal excretory and vascular functions in diabetes.

In the present study, STZ was used to produce the diabetes. STZ has been used for many decades to produce the experimental diabetes. It was reported that a single injection of STZ could cause the increase in blood glucose level and decrease in body weight [16]. We observed that post-STZ injection the rats exhibited the classical trait of diabetes mellitus i.e., hyperglycemia, polyurea and polydepsia.

Hyperglycemia produced by STZ was about the same magnitude in all the groups. This can be explained on the basis of mechanism of STZ destroying the insulin producing β cells of pancreas [23]. Exogenous H_2S in the form of NaHS did not have any effect on hyperglycemia. Polyurea and polydepsia observed can be explained on the basis of hyperglycemia, defective reabsorption of glucose by proximal renal tubules, glucosuria and increase in the osmotic pressure resulting in increased urine production. Subsequently lost in blood volume will be replaced by intracellular water causing dehydration and increased thirst [24].

Moreover, the above findings are reinforced by the significant weight loss in diabetic groups of rats which is a characteristics feature of insulin dependent diabetes mellitus (IDDM). These findings are consistent with the several earlier studies that the weight loss is one of the common happening in STZ induced diabetes in rats [25, 26]. The weight loss in STZ induced diabetes can be elucidated in term of basic aspect of carbohydrate metabolism due lack of insulin [27].

Moreover, the diminution in body weight may be due to loss of calories in urine, dehydration and muscle wastage [28]. NaHS treatment did not alter the body weight suggesting that exogenous H_2S had no effect body weight. On the contrary, it was noticed that in the diabetic rats treated with exogenous H_2S, the urine flow rate increased significantly as compared to their non treated counterparts.

One possible explanation that can be offered is that the administration of H_2S resulted in natriuresis as observed in this study and hence the water followed passively with the sodium that resulted in an increase in urine output.

The increase in plasma creatinine [29] and creatinine clearance is related to the decline in renal functions. The creatinine clearance can be widely used as a marker of glomerular filtration rate (GFR) and had an edge on the approximation of GFR than plasma creatinine [30]. Interestingly, the present study has also demonstrated increased plasma creatinine and creatinine clearance in diabetic groups of rats. The increased in plasma creatinine with the onset of diabetes observed in present study is in accord with a number of earlier reports [31, 32].

As far as, the glomerular filtration rate (GFR) is concern its regulation is not well understood, however it is widely accepted that there is considerable increase in the early stage of diabetes [32]. Moreover increased creatinine clearance is a risk factor and one of the earliest sign in the development of diabetic nephropathy [33]. These earlier reports therefore, are a support to our present finding (increased creatinine clearance with the onset of diabetes) on the presence of diabetic nephropathy in diabetic rats of present study. The most acceptable interpretation of present finding is hyperfiltration [34].

Primarily, increase in glomerular capillary pressure and glomerular plasma flow have been linked to hyperfiltration [35]. These changes could be elucidated as a defect in autoregulation mechanism as the enhanced perfusion pressure is associated with preglomerular vasoconstriction in order to keep the GFR at constant rate [6]. Alternatively augmented reabsorption of sodium evident in the present study by the renal tubules, also contribute to an increase in GFR by the intact macula densa mechanism [36].

In the present study, we demonstrated that treatment with NaHS significantly decreased the plasma creatinine. On the contrary, it was found that treating the rats with exogenous H_2S significantly increased creatinine clearance. The findings are in line with the previously reported study where the exogenous administered H_2S per se produced the greater vasodilatation of preglomerular arterioles than the postglomerular arterioles [12], however non diabetic rats were used. Increased in creatinine clearance with NaHS treatment in the present setting can be explained, taking into account of the vasodilator effect [9] of administered H_2S, possibly causing the dilatation of afferent arterioles.

From the background and the observations obtained from this study it is strongly proposed that the exogenous H_2S causes the relaxation of renal blood vessels in a setting of increased renovascular resistance, a common phenomenon in diabetes. It was previously reported that the abnormalities in sodium reabsorption has been linked to diabetic nephropathy [36].

In the present study, it was noticed that with the progression of diabetes, diabetic group was presented with higher plasma sodium level and absolute sodium excretion. The abnormal alteration in the plasma and urinary sodium levels indicated a possible nephropathy in these animals [37]. The increased in the plasma sodium levels in diabetic rats may be due to the diabetic induced hypertrophy of renal tubules causing the amplified reabsorption of sodium [38].

Moreover, the increase in the GFR, documented in the present study, is also been linked to the net increase in filtered and reabsorbed sodium probably due to the over activity of renal $Na^{+/}K^{+}$-ATPase [39]. Recently it was shown that H_2S plays an important role regulation of sodium metabolism by affecting the renal sodium transporter mechanisms. In the present it was observed that with the administration of exogenous H_2S, the plasma sodium concentration of treated rats decreased and absolute sodium excretion increased.

These findings can be explained on the basis of an earlier report that the exogenous H_2S, in the form of NaHS, more likely inhibits the renal Na^{+}/K^{+}-2Cl co-transport mechanism and renal $Na^{+/}K^{+}$-ATPase activity [12]. However, Xia *et al.* conducted the experiments in non diabetic rats. Decrease in plasma sodium levels with NaHS treatment in this experimental study is strongly suggestive that exogenous H_2S helps in reducing the sodium retention in diabetic by increasing the urinary sodium excretion. Urinary sodium to potassium ratio is a vital marker of renal functions, the

value of which is inversely proportional to the plasma aldosterone level [40, 41]. Aldosterone is secreted from the adrenal cortex as consequence of direct stimulation of angiotensin II [42]. So in the light of above fact urinary sodium potassium ratio can be taken as a surrogate marker of angiotensin II level. The higher the sodium potassium ratio the lower is the angiotensin II level and vice versa. The hyperglycemia associated with diabetes stimulates the production of ANG II [38].

Interestingly in the present study it was shown that urinary sodium to potassium ratio of diabetic rats was significantly decreased as compared to control starting with onset of diabetes remained low till the end of study indicating the high level of ANG II. The present finding concurs with previous studies and possibly can be explained that the persistent hyperglycemia stimulates expression of rennin and angiotensinogen in mesangial and tubular cells [43]. This stimulation results in an increase in local ANG II concentrations. Furthermore it can be explained in terms of increased production of reactive oxygen species (ROS), a characteristic feature of diabetes, which thought to be responsible for the upregulation of angiotensinogen in proximal tubular cells [44].

Recently, the aldosterone working autonomously from ANG II has been linked to the development of nephropathy [45]. Decreased urinary sodium potassium ratio in diabetic groups of rats is suggestive of increased aldosterone activity. The drugs blocking the actions of renin angiotensin aldosterone system (RAAS) still are the core basis of treatment in the prevention of diabetic nephropathy progression [46]. It has been shown that H_2S is a potent inhibitor of ACE (angiotensin converting enzyme) [47].

Interestingly, it was found that treating the rats with exogenous H_2S reversed the sodium potassium ratio significantly in treated group as compared to their non treated control. This observation indicated that the exogenous H_2S decreased the angiotensin II and aldosterone activity. With this back ground and data obtained in this work, it is proposed that exogenous H_2S inhibits aldosterone either indirectly via the inhibition of angiotensin converting enzyme (ACE) or by the direct antagonism of aldosterone. However the possible direct antagonism of aldosterone by H_2S needs to research further.

To the support of above renal abnormalities, it was found that the plasma and urinary concentrations of H_2S in diabetic group significantly decreased as compared to the control at the end of study period. The suggestions of an earlier studies support our present finding [48, 49].

The most good enough explanation for the present finding is the possible endothelial dysfunction that is a common occurrence in diabetes [2, 50]. The possible mechanism of reduced plasma and urinary H_2S in diabetic rats include that the persistent hyperglycemia as found in the present study may increased the levels of reactive oxygen species (ROS) due to enhanced formation of free radicals. The augmented production of ROS may results in oxidative stress and compromised endothelial function [50].

Arterial stiffness is also linked to endothelial dysfunction and the pulse wave velocity was taken as marker of arterial stiffness [22]. The stiffer the artery, the faster is the pulse wave velocity and vice versa. In this study, it was found that all the diabetic groups of rats had increased pulse wave velocity as compared to their respective controls.

The present finding is in accord with several other previously reported studies [21, 51] indicating that the marked reduction was found in the extensibility of blood vessels in diabetes which resulted in increased arterial stiffness. This can possibly be elucidated on the basis that persistent hyperglycemia leads to depletion of the antioxidant defence mechanism thus promotes the generation of free radicals [50] resulting in an endothelial dysfunction and reduced vascular elasticity.

The therapeutic entities targeting toward the enhancement of endothelial functions are now a days in the lime light of research and several earlier studies reported the role of antioxidants in fighting against oxidative stress and improvement of endothelial function [21, 52] in diabetes. Cells can be salvaged from oxidative stress by means of either dependent on or independent of glutathione metabolism. It was observed that the treatment with exogenous H_2S increased the plasma and urinary H_2S and decreased the pulse velocity significantly as compared to diabetic rats.

The present finding can possibly be explained on the basis of a previously reported study that stated H_2S is a reducing agent that readily reacts with hydrogen peroxide and possibly scavenge oxygen species [15]. The possible antioxidant mechanism of exogenous H_2S is that it induces the production of glutathione, a major and potent antioxidant [15], thereby augmenting the oxidative resistance mechanism and resulting in improvement of vascular endothelial function. This possibly causes the improvement of plasma and urinary H_2S levels and pulse wave velocity in present setting.

The improvement in the pulse wave velocity by the exogenous H_2S in the form of NaHS is supportive evidence to the above finding. In the present study the compromised endothelial function is possibly responsible for the low levels of plasma and urinary H_2S in diabetic rats but without any significant changes in arterial blood pressure.

CONCLUSION: In the present study, it was demonstrated that STZ treated rats exhibited the typical changes of diabetic nephropathy. These alterations are manifested by increased in plasma creatinine, hyperfiltration, resulting in increased creatinine clearance, abnormalities in sodium handling, and indirect evidence of activation of renin-angiotensin aldosterone system. Moreover, endothelial dysfunction evident by decreased plasma and urinary H_2S and pulse wave velocity.

Treatment with NaHS, a donor exogenous H_2S, decreased the plasma creatinine, plasma sodium, pulse wave velocity and reversed the sodium to potassium ratio. Moreover exogenous applied H_2S significantly improved the plasma and urinary H_2S levels. These observations imply the significance of normal level of endogenous H_2S in preserving the renal functions in experiment diabetes mellitus and pharmacological augmentation of H_2S may be considered for improving the endothelial and renal excretory function in diabetes.

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