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A study of CBS gene polymorphism, plasma H_2S levels and their association in type-2 diabetes mellitus

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ABSTRACT

Background: Hydrogen Sulphide (H_2S), in recent years, is getting significant attention, as more evidences are emerging about its diverse biological roles. There are evidences of H_2S having anti-inflammatory, neuro-modulator, vasodilator, anti-apoptotic and mitochondrial protective roles in various tissues. Among different tissues, β-cells of pancreas, according to some recent studies, get significantly affected by the imbalance in H_2S homeostasis, leading to β-cell dysfunction and Diabetes Mellitus (DM). Cystathionine- β-synthase (CBS) enzyme is involved in the synthesis of H_2S from cysteine in various tissues. Among various possible mutations in the CBS gene, a particular 833T-C mutation, has been found to be associated with various diseases. **Aims and Objectives:** The present study was aimed to determine the extent of abnormality of H_2S homeostasis in type-2 DM patients, and to find out presence and association (if any) of 833T-C mutation in CBS gene, in the patients of type-2 DM, in comparison to healthy control subjects, in the Indian population. **Materials and Methods:** A cross sectional study was done with 40 clinically and biochemically diagnosed DM type II patients attending OPD of Endocrinology department of NRS Medical College & Hospital, Kolkata, and 40 age and gender matched non-diabetic control subjects. DNA was isolated from EDTA blood of all the study subjects, PCR done and results compared. Plasma H2S was measured by the N,N-dimethyl-p-phenylene-diamine method. Plasma glucose and serum insulin were measured by standardized commercial kits. **Results:** Our study found the plasma H_2S levels in the patients of type II DM to be significantly higher($P<0.001$) than the control subjects. The results also found significant positive correlation between plasma H_2S level with fasting serum Insulin level ($P<0.001$) and fasting plasma glucose level ($P<0.001$) in the diabetic patients. Among 40 DM patients, only two were heterozygous for the mutation, and had both mutated allele (242bp) and normal allele (174bp). Rest of the patients and all the control subjects were homozygous for the normal allele (174bp). This marginal difference in the incidence of mutated allele was not found to be statistically significant. **Conclusion:** our study shows significant association of H_2S dys-regulation with the type-2 Diabetes Mellitus in Indian population. The marginal but insignificantly higher incidence of 833T-C mutation in CBS gene, found in our study, warrants further research with higher number of study population, to more conclusively infer about the role of this mutation in the pathogenesis of type-2 DM.

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INTRODUCTION

Hydrogen sulphide (H_2S) was traditionally considered as a toxic pollutant gas, devoid of any physiological or biological role, until 1989, when H_2S was eventually measured in the brain, and it quickly emerged as a critically important signalling molecule with widespread physiological actions.[1,2] H_2S is now considered as the third gaso-transmitter, after

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nitric oxide (NO) and carbon monoxide (CO). Reports suggest physiological role of hydrogen sulphide as a potent anti-inflammatory molecule with modest vasodilator action.[3] H_2S is also considered as a neuro-modulator. Activation of ATP-dependant potassium (K^+_{ATP}) channels, especially in vascular smooth muscles, is another well-established role. It has also been shown to protect mitochondria and ultimately improve cellular respiration and promote biogenesis which is also supported by some studies that showed enhanced mitochondrial electron transport and cellular bioenergetics on stimulation of endogenous production of H_2S(10-100nmol/L).[4] However, at high concentrations, H_2S becomes toxic, causing inhibition of mitochondrial respiration via direct inhibition of Cytochrome C Oxidase enzyme.[5,6] H_2S is also able to reduce oxidative stress.[7,8] Many of in vitro and in vivo studies done to examine the potential therapeutic role of H_2S in the setting of ischemia/reperfusion (I/R) injury in the heart, brain, lungs, and liver, have reported beneficial actions of H_2S when administered at physiological or pharmacological concentrations. The protective actions are thought to result from anti-apoptotic, anti-inflammatory, antioxidant, and mitochondrial actions of H_2S.[9,10]

Some recent studies suggest that an imbalance of hydrogen sulfide (H_2S) homeostasis can play an important role in the pathogenesis of β-cell dysfunction that occurs in response to type 1 and type-2 diabetes. Changes in H_2S homeostasis also play a role in the pathogenesis of endothelial injury, which develop on the basis of chronically or intermittently elevated circulating glucose levels in diabetes. H_2S may also inhibit insulin release and regulate β-cell survival.[11] H_2S overproduction has been found to be a causative factor in the pathogenesis of β-cell death in diabetes.[12,13] Pancreatic synthesis of H_2S is seen to be markedly elevated in the streptozotocin (STZ) induced diabetic rat,[14] where biphasic effects on beta cells have been observed. At low concentrations, H_2S inhibited insulin release through K_{ATP} dependent/Ca2+-independent mechanism,[15] whereas higher levels of H_2S induced beta cell death through endoplasmic-reticular-stress-dependent pathways.[14]

In mammalian tissues, H_2S is synthesized from L-cysteine by different enzymes, principally by cystathionine *β*-synthase (CBS) and cystathionine *γ*-lyase (CSE). CBS and CSE are expressed in many tissues, including kidney and liver. In the human brain, CBS is the main producer of H_2S, while in thoracic aorta, ileum, portal vein, and uterus, CSE is predominant.[16-18]

The CBS gene (chromosome 21q) encodes cystathionine beta-synthase enzyme (EC 4.2.1.22), which catalyzes the first irreversible step of trans-sulfuration reaction between serine and homocysteine to produce cystathionine. The CBS locus contains a number of DNA sequence repeats and single base variations that are polymorphic in Caucasians.[19] Kraus (1994) tabulated 14 mutations in the CBS gene that he and his colleagues had demonstrated in homocystinuria.[20] In a case-control study involving patients with premature coronary artery disease, a 833T-C mutation in CBS gene has been found in slightly higher frequency in patients than in controls.[21] A Chinese study has reported that CBS gene polymorphism (844 ins 68bp) is also associated with increased risk of type 2 diabetes mellitus.[22] In another study in Chinese population, results indicated CBS T833C polymorphism is associated with increased incidence of stroke. Despite various limitations, results of a meta-analysis of different studies suggest that there was a significant association between the CBS T833C genetic polymorphism and risk of stroke.

As of today, there is very little data available about the prevalence of CBS gene polymorphism and its association with plasma levels of H_2S, in patients of Diabetes Mellitus (type-2), especially in the Indian population. This study was started with an intension to gain some knowledge in this very little-known aspect of cellular biochemistry.

MATERIALS AND METHODS

40 clinically and biochemically diagnosed DM type II patients attending OPD of Endocrinology department of NRS Medical College & Hospital, Kolkata, and 40 age and gender matched non-diabetic control subjects were included in the study. Informed consent from all the participants and prior approval from institutional ethics committee (NMC/6804 Dated 13.11.2014) were obtained beforehand.

Inclusion criteria

Patients with type-2 diabetes mellitus diagnosed and confirmed by clinico-biochemical parameters.

Exclusion criteria

1) Patients of Type-1 diabetes mellitus
2) Patients having other endocrine disorders.
3) Pregnant women.
4) Patients of Renal failure.
6) Patients taking H_2S donor/inhibitor drugs.

Sample collection and preservation: 10 ml of EDTA blood was collected from each subject for DNA isolation. DNA isolation was done in fresh blood sample. 3 ml of clotted blood sample was collected from each subject, was centrifuged, serum was separated and then stored for other biochemical parameters. Serum was stored at -40°C. Fresh heparinised plasma was used for H_2S estimation.

DNA isolation

DNA isolation was done by proteinase K digestion and ethanol extraction method. Fresh 10 ml EDTA blood sample was mixed with 30 ml red cell lysis buffer and

centrifuged at 4°C at 4000 rpm for 10 minutes and the process repeated, until buffy coat was separated, which was mixed with 10% SDS and 500µl of Proteinase-K solution and incubated overnight. Next day, after centrifugation of the incubated sample with 1660µl of NaCl, ice-cold 96% ethanol was mixed to the supernatant slowly. DNA was separated and stored at -20°C.

Detection of alleles carrying mutations

To detect presence of 833T > C mutation, first ARMS-PCR of the DNA samples was performed.[23] The PCR reaction was carried out in total volume of 40µl reaction mixture containing 150-200ng of DNA, 10 pmols of each of the reconstituted primers, 1U of Taq polymerase, 4 pmols of each of dNTP, 4µl of Taq buffer and rest of milipore water in a micro-centrifuge tube. To detect presence of 833T > C mutation, 3 allele-specific primers were used, with an artificially introduced mismatch(underlined): forward normal allele(5'-CCTGA AGCCGCGCCCTCTGCAGATAAT-3'), forward-mutant allele (5'-CCTGAAGCCGCGCCCTCTGCAGATAAC-3') and reverse primer (5'GTGGCCGGGCTCTGGAC TCGACCTACC-3'). PCR were performed in thermocycler in the following protocol: 5 minutes at 95°C, then 40 cycles of [30 sec at 94°C, 30 secs at 68°C, 30 sec at 72°C], then 7 minutes at 72°C. After completion of all these procedures, the amplicons were stored at 4°C.

PCR products were analysed by submarine agarose gel electrophoresis using 3% agarose and staining done by ethidium bromide.

Amplification signal obtained with the mutant allele-specific primer signifies presence of C in 833 position---indicated by PCR product of 242 bp length. Amplicon obtained with the normal allele-specific primer demonstrates presence of T in position 833 --- indicated by PCR product of 174 bp.

Plasma hydrogen sulphide (H_2S) estimation

Estimation of plasma H2S levels were done following the methods reported earlier,[25,26] which is further modified and standardized in our laboratory.[27]

425 µl of PBS taken in a glass tube, 75µl of plasma added along with 250µl of.10% tri-chloroacetic acid and then centrifuged at 3000 rpm for 15 mins and the supernatant decanted in another glass tube and then 250µl of 1% zinc acetate added to the supernatant. Next 133µl of 20mM N,N-dimethyl-p-phenylene-diamine sulphate and 133µl of 30mM FeCl3 and 60µl of 10% NaOH was added. The resulting solution was incubated for 10 minutes at room temperature. The absorbance was taken in spectrophotometer at 670 nm. All samples were assayed in triplicate and the concentration of the solution was calculated against a calibration curve of sodium sulfide. Results of plasma H2S concentration were expressed in micromol/L.

Estimation of serum insulin: serum insulin was measured by ELISA method; using Calbiotech ELISA kit, based on sandwich ELISA principle.

Fasting blood sugar estimtion: We measured FBS by commercial kit based on GOD-POD method.

RESULTS

When compared by independent t test, the plasma H_2S level in diabetic patients, (69.12±7.09 µmol/ml) were found to be significantly (P< 0.001) higher than controls subjects(40.28 ± 6.074 µ mol/ml) (figure. 1).

Plasma H_2S level showed significant positive correlation with fasting Plasma glucose levels in the diabetic patients(r= 0.475, P<0.001), as shown in figure. 2.

Plasma H_2S level was also found to be significantly positively correlated with fasting serum Insulin levels (r= 0.655, P<0.001) of the diabetic patients, as shown in Fig. 3.

Results of ARMS-PCR of CBS gene c.833 T>C Polymorphism in Type 2 Diabetic Patients & Healthy Controls

PCR product of 174 bp demonstrates the presence of T in position 833, which is normal allele. PCR product of 242bp length signifies the presence of C in position 833. It is the mutant allele. Figure 4 shows the gel doc images of the PCR products.

In all the control subjects and 38 of the DM patients, the normal gene product of 174bp was found (TT). Mutant gene product of 242 bp was found in only 2 diabetic patients. The two diabetic individuals having the 833T>C mutation were heterozygous (TC) for the mutation, showing both 242bp as well as 174bp gene products.

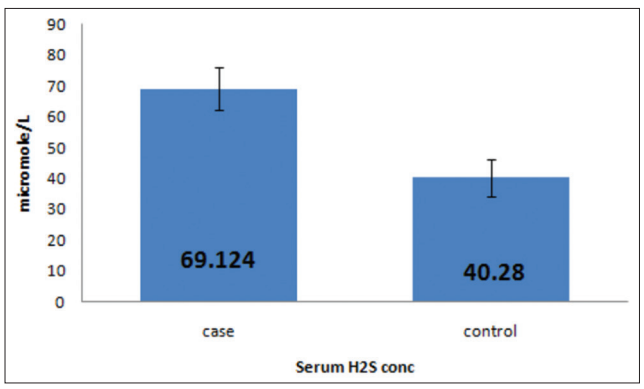

Figure 1: Comparison of plasma H_2S level in study subjects

Association of CBS Gene c.833 T→C Polymorphism with Type 2 Diabetes Mellitus

Frequency of C (Thr) allele in 833 position was found to be marginally (p>0.05) higher (0.25 vs. 0.0) in the diabetic patients as compared to the controls (Table 1). Overall, 95 present of the patients carried T allele (TT+TC) as against 100% of the controls having this allele (TT).

Relationship between CBS 833 T-C polymorphism and plasma H_2S

Plasma levels of H_2S in individual genotypes (TT and TC) in patients are shown in Fig. 5. However no significant difference was found between the levels of H_2S in patients with TC (n=2) genotypes compared and the patients with wild TT (n=38) alleles (P > 0.05).

DISCUSSION

India is presently having several fold increase in the prevalence of type-2 diabetes mellitus over last two decades.[28] A number of recent literature have suggested a potential role of H_2S and H_2S modifying agents in the aetiology and management of this metabolic disorder.[29] Hydrogen sulphide has now been proposed as a mediator of important physiological functions in human. It has been well established that H_2S plays a very important role in maintaining the insulin levels towards homeostasis as it prevents excess exhaustion of the beta cells.

The current study was aimed to find out whether there is any involvement of H_2S in type II DM and if association exists between the plasma levels of H_2S, and c.833T>C polymorphism of CBS gene in the patients of type-2 diabetes mellitus in Indian population.

Our study found the serum H2S levels in the patients of type II DM to be significantly higher(P<0.001) [69.12 ± 7.09μ mol/l with the values ranging from 60.45 to 87.49 μ mol/l] than the control subjects. [40.28 ± 6.07μ mol/l, with values ranging from 29.12 to 55.25μ mol/l] (figure 1). The results also found significant positive correlation between plasma H2S level with fasting serum Insulin level (P<0.001) and fasting plasma glucose level (P<0.001) in the diabetic patients (fig. 3 & 2).

To understand the role of H_2S in pathogenesis of Type-2 DM, it is important to remember, that ATP-sensitive K^+-channel regulates the resting membrane potential in β cells of pancreas. Increased intracellular ATP blocks K^+_{ATP} channel, causing membrane depolarization and

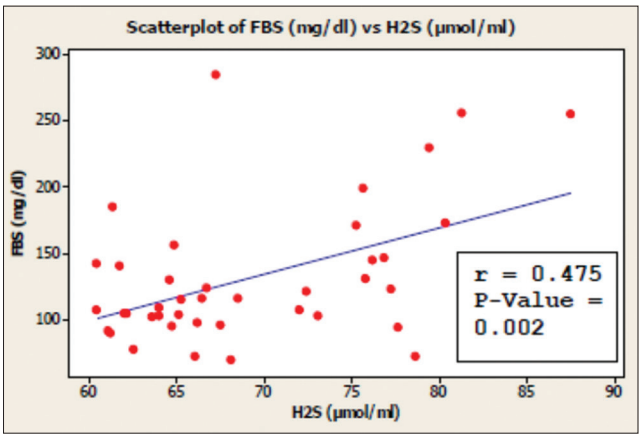

Figure 2: Correlation between H_2S and fasting blood sugar levels in type-2 diabetes patients

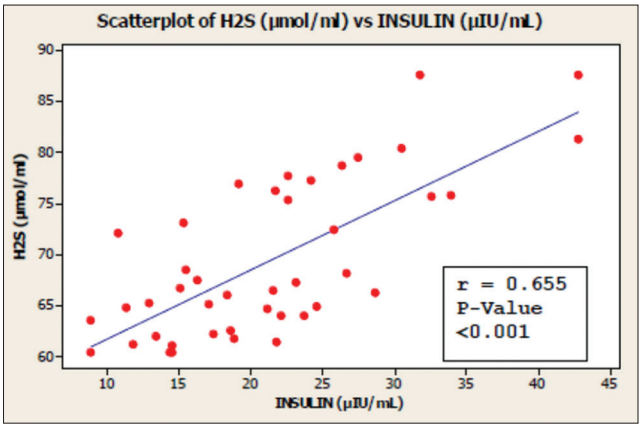

Figure 3: Correlation between H_2S and fasting insulin Levels in Type 2 Diabetic patients

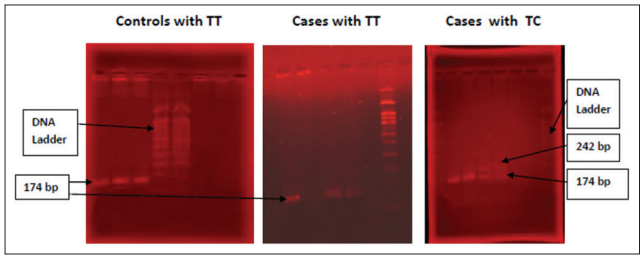

Figure 4: Gel doc image of controls and cases with wild (TT) and mutant allele (TC) of CBS Gene c.833 >C polymorphysim

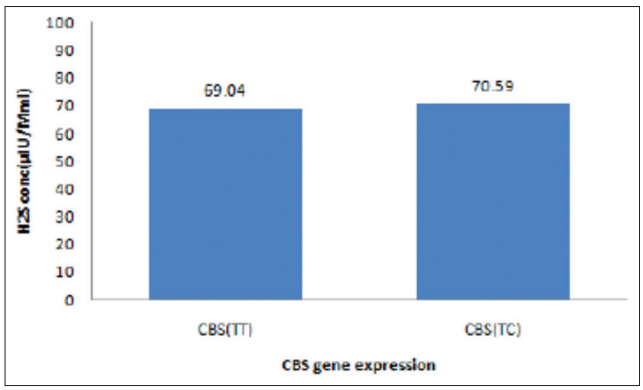

Figure 5: Levels of H_2S in type-2 Diabetic patients with homozygous (TT) and heterozygous(TC) alleles.

Table 1: Distribution of c.833 T>C genotypes and frequency of alleles in controls and type 2 DM patients

Genotypes and Alleles	Controls n = 40 (80)	Patients n = 40 (80)	p value
Genotypes			
TT, n, (%)	40 (100)	38(95)	NS
TC, n (%)	0(0.0)	2 (5.0)	NS
CC, n (%)	0(0.0)	0(0.0)	-----
TC+CC	0(0.0)	2 (5.0)	------
Alleles			
T (Ile) Frequency,	1	0.975	------
C (Thr) Frequency,	0.0	0.025	------

Controls indicate healthy subjects; Patients: Type2 DM subjects; Genotype frequencies are indicated in absolute values and percentages in parenthesis. T and C represent each allele of the polymorphisms; n: number of subjects (number of alleles)
Fischer's Exact Test was done.

opening of voltage dependant calcium channels, leading to Ca^{2+} signal induced insulin secretion. Thus, the insulin secretion is dependent upon K^+_{ATP} channel opening and closing. H2S is K^+_{ATP} channel opener; but, it has no effect of ATP concentration.[30] In experimental animal models, both streptozotocin and Zucker diabetic fatty rats had significantly higher H_2S formation in the pancreas.[31] H2S concentration was reported to be higher in Zuker diabetic rats as compared to that in and Zuker lean rats. Expressions of both the enzymes responsible for H_2S production, CSE and CBS, were also high in streptozotocin-induced diabetic rats.[32]

Although, H_2S is not responsible for development of diabetes, but high H_2S level in diabetes is due to over-expression of H_2S producing enzyme. This over-expression of enzymes may be due to metabolic irregulation in diabetes.

According to another study, high-glucose–induced CSE expression increases H_2S production to protect β-cells, which thereby inhibit insulin release and protect themselves. H_2S may function as an 'intrinsic brake' in pancreatic β-cells in this regard.[33] Increases in CBS and CSE expression and H_2S production in these diabetic tissues are reversed by insulin treatment, suggesting that this may be a secondary result from hyperglycemia or hypoinsulinemia.[14]

The human CBS gene is reported to have several kinds of polymorphism. 833T>C polymorphism, is characterized by a T to C mutation at 833 position, causing an Isoleucine to Threonine amino acid substitution.[34] In the current study, ARMS-PCR of the DNA samples for detection of CBS gene c.833T > C mutation were performed as per the method developed by J. Sokolova, & B. Janosikova with a few modifications,[24] using standardized reagents. Among the forty DNA samples of diabetic patients, in 38 cases we found the 174 bp PCR product indicating homozygous normal allele. In only 2 DNA samples we found one 174 bp band and another 242 bp band, signifying heterozygosity of the CBS gene with c.833T>C mutation of one allele. In the DNA samples of healthy control subjects, PCR revealed that all the samples were homozygous with the 174 bp product of normal (TT) allele of CBS gene. No mutant band was found in control (fig. 4).

In our study, we could not find any significantly higher incidence of CBS gene c.833 T>C polymorphism in type 2 Diabetes mellitus patients. (table 1) The frequency of (C) allele was only marginally higher in the diabetic patients, in comparison to controls. Most of the study subjects (every healthy control subjects and even 95% of the diabetic patients) have wild (TT) alleles.

Our observations reveal a significant association of serum H2S levels, with type-2 diabetes mellitus. In our study, only a marginally higher incidence of c833T > C polymorphism of the gene synthesizing the CBS enzyme was found, which did not suggest any significant role of this polymorphism in the pathogenesis of the disease. To find more conclusive evidences about any possible role of this polymorphism in relation to the imbalance in H_2S homeostasis, a further large scale study with a larger sample size is warranted.

REFERENCES

1. Goodwin LR, Francom D, Dieken FP, Taylor JD, Warenycia MW, Reiffenstein RJ, et al. Determination of sulfide in brain tissue by gas dialysis/ion chromatography: postmortem studies and two case reports. J Anal Toxicol 1989;13:105–109.
2. Savage JC and Gould DH. Determination of sulfide in brain tissue and rumen fluid by ion-interaction reversed-phase high-performance liquid Chromatography. J Chromatogr 1990;526:540–545.
3. Wang ZT, Lau CW, Chan FL, Yao X, Chen ZY, He ZD, et al. Vasorelaxant effects of cardamonin and alpinetin from Alpinia henryi K. Schum. J Cardiovasc Pharmacol 2001; 37:596-606.
4. Módis K, Coletta C, Erdélyi K, Papapetropoulos A and Szabo C. Intramitochondrial hydrogen sulfide production by 3-mercaptopyruvate sulfurtransferase maintains mitochondrial electron flow and supports cellular bioenergetics. FASEB J 2013;27:601–611.
5. Hill BC, Woon TC, Nicholls P, Peterson J, Greenwood C and Thomson AJ. Interactions of sulphide and other ligands with cytochrome c oxidase. An electron-paramagnetic-resonance study. Biochem J 1984;224:591–597.
6. Nicholls P and Kim JK. Sulphide as an inhibitor and electron donor for the cytochrome c oxidase system. Can J Biochem 1982;60:613–623.
7. Vacek TP, Gillespie W, Tyagi N, Vacek JC and Tyagi SC. Hydrogen sulfide protects against vascular remodeling from endothelial damage. Amino Acids 2010; 39: 1161–1169.
8. Calvert JW, Jha S, Gundewar S, Elrod JW, Ramachandran A,

Pattillo CB, et al. Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. Circ Res 2009; 105: 365–374.

9. Predmore BL, Kondo K, Bhushan S, Zlatopolsky MA, King AL, Aragon JP, et al. The polysulfide diallyl trisulfide protects the ischemic myocardium by preservation of endogenous hydrogen sulfide and increasing nitric oxide bioavailability. Am J Physiol Heart Circ Physiol 2012;302:H2410–H2418.
10. Peake BF, Nicholson CK, Lambert JP, Hood RL, Amin H, Amin S, et al. Hydrogen sulfide preconditions the db/db diabetic mouse heart against ischemia-reperfusion injury by activating Nrf2 signaling in an Erk-dependent manner. Am J Physiol Heart Circ Physiol 2013;304:H1215–H1224.
11. Taniguchi S and Niki I. Significance of Hydrogen Sulfide Production in the Pancreatic β-Cell. Journal of Pharmacological Sciences 2011; 116.
12. Szabo C. Roles of Hydrogen Sulfide in the Pathogenesis of Diabetes Mellitus & its complications. Antioxidants & Redox Signaling. Antioxid Redox Signal 2012; 17(1): 68–80.
13. Dutta M, Biswas UK, Chakraborty R, Banerjee P, Roychaudhuri U and Kumar A. Evaluation of plasma H_2S levels and H_2S synthesis in streptozotocin induced Type 2 diabetes. Asian Pac J Trop Biomed 2014; 4(Suppl 1): S483–S487.
14. Yusuf M, Kwong HB, Whiteman M, Bhatia M and Moore PK. Streptozotocin-induced diabetes in the rat is associated with enhanced tissue hydrogen sulfide biosynthesis. Biochem Biophys Res Commun 2005;333(4):1146-1152.
15. Ali MY, Whiteman M, Low CM and Moore PK. Hydrogen sulphide reduces insulin secretion from HIT-T15 cells by a K-ATP channel dependent pathway. Journal of Endocrinology 2007; 195.
16. Jain SK, Bull R, Rains JL, Bass PF, Levine SN and Reddy S. Low Levels of Hydrogen Sulfide in the Blood of Diabetes Patients and Streptozotocin-Treated Rats Causes Vascular Inflammation? Antioxidants and Redox Signalling 2010; 12.
17. Abe K and Kimura H. The possible role of Hydrogen Sulfide as endogenous neurotransmodulator. The Journal of Neuroscience 1996; 16(3).
18. Hosoki R, Matsuki N and Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. Biochem Biophys Res Commun 1997; 237.
19. Kraus JP, Janosik M, Kozich V, Mandell R, Shih V, Sperandeo MP, et al. Cystathionine beta-synthase mutations in homocystinuria. Hum Mutat 1999; 13: 362-375.
20. Kraus JP. Molecular basis of phenotype expression in homocystinuria. J Inherit Metab Dis 1994; 17: 383-390.
21. Tsai MY, Bignell M, Schwichtenberg K and Hanson NQ. High prevalence of a mutation in the cystathionine beta-synthase gene. Am J Hum Genet 1996; 59: 1262-1267.
22. Luo D, Yan S, Cheng X and Song Y. Level of homocysteine and polymorphism of homocysteine metabolism-related enzymes in patients with type-2 diabetes mellitus and coronary heart disease. Wei Sheng Yan Jiu 2009; 38(1): 39-42.
23. Sokolova J, Janosikova B,Terwilliger JD, Freiberger, Kraus JP and Kozich V. Cystathionine beta synthase deficiency in Central Europe: Discrepency between biochemical and Molecular genetic screeninig for homocystinuric alleles. Human Mutation. Mutation in brief. 2001
24. Sebastio G, Sperandeo MP, Panico M, de Franchis R, Kraus JP and Andria G. The molecular basis of homocystinuria due to cystathionine beta-synthase deficiency in Italian families, and report of four novel mutations. Am J Hum Genet 1995; 56: 1324-1333.
25. Zheng Y, Liao F, Du JB, Tang CS, Xu GH and Geng B. Modified methylene blue method for measurement of hydrogen sulfide level in plasma. Sheng Li Xue Bao 2012;64(6):681-686.
26. Ali MY, Whiteman M, Low CM and Moore PK. Hydrogen sulphide reduces insulin secretion from HIT-T15 cells by a $K-_{ATP}$ channel dependent pathway. Journal of Endocrinology 2007; 195.
27. Saha P, Banerjee P, Pal P, Auddya L, Sen S, Sau TJ, et al. Enhanced plasma H_2S levels associated with fasting blood glucosein type-2 diabetes mellitus. Asian Journal of Medical Sciences 2015; 6(6): 11-15.
28. Ramachandran A, Snehalatha C, Kapur A, Vijay V, Mohan V, Das AK, et al. High Prevalence of Diabetes and Impaired Glucose tolerance in India: National Urban Diabetes Survey. Diabetologia 2001;44(9):1094-1101.
29. Taniguchi S and Niki I. Significance of Hydrogen Sulfide Production in the Pancreatic β-Cell. Journal of Pharmacological Sciences 2011; 116.
30. Weimin Z, Jing Z, Yanjie L and Wang R. The vasorelaxant effect of H_2S as a novel endogenous gaseous K_{ATP} channel opener**.** EMBO J 2001; 20:6008-6016.
31. Jia X, Yang W, Jakic Z, Wang R and Wu L. Role of H2S in insulin resistance. Canadian Journal of Cardiology 2004; 20 (Suppl D) 56D.
32. Marathe PA, Parekar RR, Shinde SP and Rege N. A split dose regimen of streptozotocin to induce diabetes in a neonatal rat model. Indian J Pharmacol 2006; 38(6): 432-433.
33. Shigeki T and Ichiro N. Significance of Hydrogen Sulfide Production in the Pancreatic β-Cell. Journal of Pharmacological Sciences 2011; 116: 1-5.
34. Robert K, Vialard F, Thiery E, Toyama K, Sinet PM, Janel N, et al. Expression of the cystathionine beta synthase (CBS) gene during mouse development and immunolocalization in adult brain. J Histochem Cytochem 2003; 51: 363-371.

Authors Contribution:
SDG- Concept and design of the study, review of literature, statistical analysis, manuscript preparation, critical revision of manuscript; **IM**- Sample collection, Data organisation, review of literature, preparation of first draft of manuscript; **UKB**- Conceptualized and designed the study, critical revision of the manuscript.

Work attributed to: Department of Biochemistry, NRS Medical College, 138 AJC Bose Road, Kolkata-14.

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