Increased \( \text{H}_2\text{S} \) synthesizing activity in plasma correlated to fasting blood glucose levels: A study in type 2 diabetes patients

Pinaki Saha\(^1\), Sayantan Dasgupta\(^2\), Sukla Nath\(^2\), Pradipta Ghosh\(^3\), Santanu Sen\(^4\), Tanmay Jyoti Sau\(^2\), Arun Kumar\(^6\), Utpal Kumar Biswas\(^7\)

\(^1\)Junior Resident, \(^2\)Assistant Professor, \(^3\)Demonstrator, Department of Biochemistry, Nilratan Sircar Medical College, \(^4\)Associate Professor, Department of Biochemistry, Calcutta National Medical College, \(^5\)Professor, Department of General Medicine, Calcutta Medical College, \(^6\)Professor, Department of Biochemistry, Gouri Devi Institute of Medical Sciences, Durgapur, \(^7\)Professor, Department of Biochemistry, Institute of Post Graduate Medical Education and Research, Kolkata, West Bengal, India

Submitted: 05-07-2016 Revised: 26-08-2016 Published: 01-11-2016

ABSTRACT

Background: Diabetes mellitus is a major health problem not only in India but worldwide. Our country presently is undergoing an epidemic stage of this non-communicable disease. Though several etiological background of type 2 diabetes has been well explained, yet a number of recent literatures suggested a potential role \( \text{H}_2\text{S} \) producing enzymes in the etiology and management of this metabolic disorder. Aims and Objectives: Our aim was to elucidate the relationship of \( \text{H}_2\text{S} \) synthesizing activity in plasma and fasting blood glucose in type 2 diabetic patients. Materials and Methods: Sixty-two newly diagnosed type 2 diabetes patients and equal number of non-diabetic controls were included in the study. Enzymatic activity of synthesizing \( \text{H}_2\text{S} \) in plasma was estimated following methods described earlier with further modification and standardization in our laboratory. All other parameters were estimated by using standardized kits. Results: FBG, PPBG, HbA\(_1c\), Fasting Insulin, \( \text{H}_2\text{S} \) synthesizing activity in plasma in patients are significantly higher (\( p<0.05 \)) than the corresponding values in healthy controls. \( \text{H}_2\text{S} \) synthesizing activity in plasma is positively correlated with fasting plasma glucose and the correlations is significant (\( p=0.05 \)). Conclusion: Our study though a pilot study with a small sample size, has elucidated that the values of \( \text{H}_2\text{S} \) synthesizing activity in plasma are significantly elevated in type 2 diabetic patients and this may help researchers to develop \( \text{H}_2\text{S} \) modifying agents and enzyme inhibitors which may open up new horizon in the treatment modalities of type 2 diabetes mellitus.

Key words: \( \text{H}_2\text{S} \) synthesizing activity, FBG, Type 2 Diabetes

INTRODUCTION

Type 2 diabetes mellitus has turned out as a multi-factorial epidemic disease. In India, the steady migration of people from rural to urban areas, the economic boom, and corresponding change in life-style are all affecting the level of diabetes.\(^1\) The prevalence of type 2 diabetes mellitus in India was 51 million people in 2010.\(^2\)

In contrast to its role as poison, hydrogen sulfide (\( \text{H}_2\text{S} \)) is considered as the third gasotransmitter after nitric oxide (NO) and carbon monoxide (CO).\(^3\)\(^5\) Though it was first reported in 1982 that it is produced in mammalian tissues, but only now it has emerged as a mediator of important physiologic functions in humans.\(^6\) Experimental evidences have been published implicating \( \text{H}_2\text{S} \) overproduction as a causative factor in the pathogenesis of \( \beta \)-cell death in diabetes.\(^7\)\(^8\)

\( \text{H}_2\text{S} \) is produced \textit{in vivo} from L-cysteine by the action of enzymes, cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE). Both of these enzymes are dependent on pyridoxal-5-phosphate. Recent studies have shown two
other H$_2$S-producing enzymes, 3-mercaptoppyruvate sulfur transferase (3MST), along with cysteine aminotransferase (CAT), which produces H$_2$S in the brain as well as in the vascular endothelium. H$_2$S synthesizing activity in plasma represents the enzyme leakage from the tissues involved in the synthesis of H$_2$S. We have modified and standardized the methods reported earlier. Though several etiological factors of type 2 diabetes mellitus has been well explained, yet a number of recent literatures suggested a potential role of H$_2$S and H$_2$S synthesizing enzymes in the etiology and management of this metabolic disorder.

**AIMS AND OBJECTIVES**

Our aim was to compare H$_2$S synthesizing activity in plasma with fasting blood glucose levels and to find out if there is any correlation among them in type 2 diabetic patients.

**MATERIALS AND METHODS**

62 (Sixty-two) newly diagnosed type 2 diabetes mellitus patients attending O.P.D. of Medicine department at N.R.S. Medical College, Kolkata and 62 non-diabetic control subjects were included in the study after obtaining informed consent with prior approval from institutional ethics committee. 5 ml of 8-10 hours fasting blood samples were collected with heparin and centrifuged at 2500 rpm for 5 minutes. Supernatant plasma were aliquoted and stored in -40˚C refrigerator until use and tests were performed within a week. Reagents used were stored at 2-8˚C and the chromogen (N, N-dimethyl- p- phenylene diamine sulphate) was freshly prepared before the assay.

**Inclusion criteria**

Patients of age ranging from 20 to 50 years suffering from type 2 diabetes mellitus diagnosed and confirmed by clinical and biochemical tests were included in the study.

**Exclusion criteria**

Following patients were excluded:

1. patients having other endocrine disorders,
2. pregnant mothers patients with type-1 diabetes mellitus,
3. polycystic ovarian disease,
4. renal failure,
5. malignant disease and
6. patients receiving plasma H$_2$S level modifying agents and H$_2$S releasing agents like sodium sulfide, sodium hydrogen sulfide and their parent drugs like, sildenafil, mesalagine, naproxen, indomethacin, diclofenac etc.

7. **Assay of H$_2$S synthesizing activity in plasma:** For this, initially we followed the estimation of plasma H$_2$S, by a method modified and standardized in our laboratory and reported earlier. This spectrophotometric method involves the reaction of sulfide with N, N-dimethyl-p-phenylenediamine sulfate in the presence of the oxidising agent Fe$^{3+}$ in hydrochloric acid to form methylene blue which is read at 670 nm.

**Preparation of buffers and reagents for assay of plasma H$_2$S synthesizing activity**

1. 20 molar PBS (pH 7.4): Prepared by mixing 80.4 ml of Solution A (prepared by dissolving 11.47 gm of disodium hydrogen phosphate dissolved in 1000 ml of deionized water) to19.6 ml of Solution B (prepared by dissolving 9.08 gm of potassium dihydrogen phosphate to 1000 ml of deionized water), and pH adjusted to 7.4
2. L-cysteine solution (10 mM/l) prepared with deionized water
3. Pyridoxal -5-phosphate (2 mM/l) prepared with deionized water
4. 1% (w/v) Zinc acetate (MW 219.49)
5. 50% TCA (w/v)
6. 10% Sodium hydroxide-NaOH solution prepared
7. From stock supply of HCl (12N i.e 12M), MW 36.5, 7.2 mM and 1.2 mM solutions were prepared. 10 microliter of 12M stock HCl diluted with deionized water and volume made upto 100 ml to get 1.2 mM HCl, and 60 microliter of 12M stock HCl diluted with deionized water and volume made upto 100 ml to get 7.2 mM HCl
8. 20 milimol N,N-dimethyl- p- phenylenediamine sulphate in 7.2 mM HCl
9. 30 milimol of FeCl$_3$ dissolved in 1.2 mM of HCl
10. **Assay Procedure for H$_2$S synthesizing activity in plasma:** One hundred microliter of plasma was added to 800 microliter of ice-cold PBS buffer (pH 7.4-8.0) in a glass tube. The mixture 900 microliter was incubated at 37˚C for 5 minutes and then cooled on ice for 10 minutes. Next 50 microliter of each of L-cysteine (10 mM/l) and pyridoxal-5-phosphate (2 mM/l) were added to make the final volume of 1 ml. Then 1% zinc acetate 300 microliter added to the glass tube which is then capped or sealed with parafilm. The tubes were then transferred to a shaking water bath and kept incubated there for 90 minutes. Then the cap was removed and 500 microliter of 50% trichloroacetic acid was added and the tubes were again left incubated for 60 minutes, next centrifuged at 2500 for 15 to 20 minutes. The tubes are decapped and 50 microliter of 20 mmol N, N-dimethyl- p- phenylene diamine sulphate and 50 microliter of 30 mmol of FeCl$_3$ were added. Again the resulting solution was kept incubated at room temperature for 20 minutes. Then to all glass tubes 110 microliter of 10% NaOH were added and now the solution appeared turbid and ultimately absorbance was
Saha, et al.: Increased H$_2$S synthesizing activity in type 2 diabetes

Measured values of plasma H$_2$S were significantly higher (p< 0.05)than the corresponding values in healthy controls (Table 1). Figures 2 and 3 elucidates that values of FBG and H$_2$S synthesizing activity in plasma are positively correlated and the correlation is statistically significant (p=0.05).

**DISCUSSION**

H$_2$S can be produced in pancreatic $\beta$-cells by cystathionine $\beta$-synthase (CBS) or cystathionine $\gamma$-lyase (CSE). H$_2$S inhibits insulin release and regulates $\beta$-cell survival. Besides inhibiting insulin release H$_2$S protects $\beta$-cells that were chronically exposed to high glucose from apoptotic cell death. Loss of $\beta$-cell mass and failures of $\beta$-cell function are important in the pathogenesis and/or progression of diabetes mellitus; therefore, molecular analysis of the mechanisms of H$_2$S production and its protective effects on $\beta$-cells may lead to new insights into diabetes mellitus.

In addition, the changes in H$_2$S homeostasis also play a role in the pathogenesis of endothelial injury which developed under the basis of elevated circulation of blood glucose levels in diabetes. Measuring the H$_2$S synthesizing activity in plasma in streptozotocin treated diabetic rats has been undertaken by researchers in recent times. There are some contradictory findings. One study reported that the streptozotocin induced diabetes in the rat is associated with enhanced tissue hydrogen sulfide biosynthesis. Another study suggested that H$_2$S synthesis is progressively reduced as diabetic pathology increased.

Recently, a role for H$_2$S in the etiology of diabetes has been suggested. Pancreatic synthesis of H$_2$S is markedly elevated in the streptozotocin (STZ) induced diabetic rat, in which biphasic effects on beta cells have been observed; at low concentrations, H$_2$S inhibited insulin release through $K_{ATP}$ dependent/Ca$^{2+}$-independent mechanism, whereas higher levels induced beta cell death through endoplasmic-reticular-stress-dependent pathways. In experimental diabetes animal models, both Streptozotocin and Zucker diabetic fatty rats had significantly higher H$_2$S formation in the pancreas.

### Table 1: Clinico-biochemical parameters of patients & controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean±SD</th>
<th>Control (N=62)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>44.03±5.27</td>
<td>43.63±5.56</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>30/32</td>
<td>37/25</td>
<td></td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>24.15±4.09</td>
<td>24.81±2.63</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting blood glucose (mg/dl)</td>
<td>134.14±26.88</td>
<td>79.06±12.64</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Post prandial blood glucose (mg/dl)</td>
<td>176.08±46.26</td>
<td>108.76±11.16</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Glycosylated haemoglobin (%)</td>
<td>6.85±1.14</td>
<td>3.8±0.34</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Fasting plasma insulin (micro IU/l)</td>
<td>11.02±7.59</td>
<td>7.67±3.1</td>
<td>0.002*</td>
</tr>
<tr>
<td>Plasma H$_2$S synthesizing activity (micromol/100 g of protein)</td>
<td>11.40±5.38</td>
<td>4.62±1.91</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>6.4±0.44</td>
<td>6.6±0.41</td>
<td>0.008*</td>
</tr>
</tbody>
</table>

Student’s t-test was done, * are significant (P<0.05), with 95% confidence level: NS: Not significant
In our study, the $H_2S$ synthesizing activity in plasma of patients is $11.40 \pm 5.38 \mu \text{mol}/100 \text{ g}$ of protein which is significantly ($p<0.001$) higher than that in plasma of controls equivalent to $4.62 \pm 1.91 \mu \text{mol}/100 \text{ g}$ of protein. The fasting blood glucose levels of the patients ($134.14 \pm 26.88 \text{ mg/dl}$) are significantly ($P<0.001$) higher than the controls ($79.06 \pm 12.64 \text{ mg/dl}$) and the values of FBG and $H_2S$ synthesizing activity in plasma are positively correlated and the correlation is significant ($p=0.05$).

M. Yusuf et al. has reported that streptozotocin-induced diabetes in rat is associated with enhanced tissue hydrogen sulfide biosynthesis. The activities of $H_2S$ producing enzymes are known to increase under diabetic conditions as reported by Kaneko Y. et al. Elevated hydrogen sulfide levels in plasma were reported in patients with proliferative diabetic retinopathy. In streptozotocin induced type 2 diabetic experimental rats in the plasma it has been earlier reported from our laboratory that along with reduced plasma $H_2S$ levels, there is reduction in the $H_2S$ synthesizing activity. Some other studies have reported reduced $H_2S$ levels in the plasma as well as in the tissues in this condition like the one reported by Sushil K. Jain which demonstrated lower circulatory levels of $H_2S$ in type 2 diabetic patients, but associated levels of pro-inflammatory cytokines may have affected the outcome. But elsewhere it has been reported that biosynthesis of $H_2S$ at the tissue level in diabetic animal model has shown both increase of $H_2S$ concentrations and increment in the activity and expression of the enzymes related with its synthesis.

### CONCLUSION

Though our study consisted of less sample size, it was able to demonstrate the link between $H_2S$ and diabetes, furthermore elucidating significantly increased synthesis of $H_2S$ in type 2 diabetic patients. This is an eye-opener which might pave way for researchers to develop $H_2S$ modifying agents and enzyme inhibitors opening up new horizon in the treatment modalities of type 2 diabetes mellitus.

### REFERENCES

2. Gupta V. Type 2 Diabetes Mellitus In India. South Asia Network For Chronic Disease. Report submitted to South Asia Network for Chronic Disease (www. sancd. org), 2012.

---

**Figure 2:** Comparison between patients and controls with respect to fasting blood glucose (FBG) and plasma $H_2S$ synthesizing activity

**Figure 3:** Scatter plot showing correlation between fasting blood glucose and plasma $H_2S$ synthesizing activity

Authors Contribution:
UKB - Concept and design of the study, statistical analysis, helped manuscript preparation and critical revision of the manuscript; PS, SN, PG - Concept, collected data, literature search, statistically analyzed and interpreted, prepared first draft of manuscript; SDG & SS - Helped in standardization and statistical analysis and helped in preparing first draft of manuscript. TJS - Conceptualized study, helped in patient selection; AK - Edited the manuscript, critical revision of the manuscript, helped in final draft of manuscript.

Source of Support: Nil. Conflict of Interest: None.