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Increased plasma H_2S levels well correlated with total oxidative stress and total antioxidant defense in type 2 diabetes patients

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ABSTRACT

Background: Diabetes mellitus, a worldwide health problem, is associated with increased oxidative stress and reduced antioxidant defense in the body. The role of hydrogen sulfide and the H_2S synthesizing enzymes in relation to type 2 diabetes is a recently unveiled field which needs further research work. **Aims and Objectives:** Aim of the study was to find out the relationship of plasma H_2S levels and H_2S synthesizing activity in plasma with oxidative stress conditions in the patients suffering from type 2 diabetes mellitus. **Materials and Methods:** Sixty two patients suffering from type 2 diabetes as well as similar number of healthy volunteers were enrolled for the study. Plasma H_2S levels and H_2S synthesizing activity in plasma as well as total oxidative stress and total antioxidant levels were measured by earlier methods already published from our laboratory. **Results:** The values of FBG, PPBG, HbA_{1C}, fasting Insulin, plasma H_2S and H_2S synthesizing activity in plasma and TOS values in patients are significantly higher ($p< 0.05$) than the corresponding values in healthy controls. The median and inter quartile range of TAD values in controls is significantly higher than those of the patients (Mann Whitney U, $Z<0.001$). Plasma H_2S levels show significant positive correlation with TOS values ($r= 0.793$, $P<0.001$), and significant negative correlation with TAD values ($r= -0.753$, $P<0.001$). **Conclusion:** The current study elucidated that there is significant increase in both plasma H_2S levels and the activity of H_2S synthesizing enzymes in type2 diabetics and well correlated with the total oxidative stress and antioxidant defense.

Key words: H_2S, diabetes, oxidative stress, antioxidant

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INTRODUCTION

Diabetes mellitus is a worldwide major health problem with India presently undergoing an epidemic stage of this non-communicable disease. In India, the population migration from rural to urban areas, the economic growth, and corresponding change in life-style are all affecting the level of diabetes.[1] Though modern treatment is available with insulin and several hypoglycemic drugs along with dietary and lifestyle modifications, the disease seems to be spreading heavily in the community.

Hydrogen sulfide (H_2S), besides its poisonous effects mainly on mitochondrial electron transport chain, is considered as the third gasotransmitter after (NO) and carbon monoxide (CO).[2-4] Though it was first reported in 1982 that it is produced in mammalian tissues, but now recognized as a mediator with important physiologic functions in humans.[5] H_2S is formed in vivo from L-cysteine by enzymes, cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE), both require pyridoxal-5- phosphate as cofactor. Recent studies have shown two other H_2S-producing enzymes, 3-mercaptopyruvate sulfur transferase

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(3MST) and cysteine aminotransferase (CAT), which produces H_2S in the brain as well as in the vascular endothelium.[6,7]

Recent reports suggest that the changes in the equilibrium of hydrogen sulfide (H_2S) level play an important role in the pathogenesis of β-cell dysfunction. In addition, 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. Experimental evidences have been published implicating H_2S overproduction as a causative factor in the pathogenesis of β-cell death in diabetes.[6,7] Some other experiments have suggested H_2S deficiency due to increased H_2S consumption by hyperglycemic cells, in the pathogenesis of diabetic endothelial dysfunction, diabetic nephropathy, and cardiomyopathy.[6] Since the modulation of H_2S production may be a potential therapeutic strategy for diabetes mellitus,[7] this possibility has led researchers to investigate H_2S related substances for treatment of diabetes.[8,9]

As per the current concept, the pathogenesis and the complications of diabetes are mainly as a result of imbalance between oxidative stress and antioxidant defense mechanisms.[10-12] Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications.[13,14] Free radicals and oxidative stress may act as a common pathway to diabetes itself, as well as to its complications.[13,15] However, the relationship of H_2S levels with oxidative stress conditions in the type-2 diabetes patients has not yet been clearly understood. So there is a need of further study to address this problem.

Aims and objectives

The aim of the study is to elucidate the relationship of plasma H_2S levels and H_2S synthesizing activity in plasma with oxidative stress conditions in the patients suffering from type-2 diabetes mellitus with the following objectives:

1. Assay of H_2S level, H_2S synthesizing activity in plasma, and oxidative stress parameters, in patients suffering from type2 diabetes mellitus.
2. To find out if there is any correlation among the above and other clinico-biochemical parameters in patients compared to healthy controls.

MATERIALS AND METHODS

Sixty-two diabetic patients (30 males and 32 females) were enrolled for this case-control study, with their ages ranging from 20 to 50 years, and similar number of healthy voluntary control subjects (37 males and 25 females) of the same age group was recruited for the study. The study was preapproved by the Institutional Ethics Committee of N.R.S. Medical College, Kolkata, India. Patients with other endocrine disorders like type1 diabetes mellitus, thyroid disorders, pregnant mothers, patients suffering from polycystic ovarian disease, renal failure, any malignant disease and patients receiving antioxidant or plasma H_2S level modifying agents are excluded from the study.

Sample collection, sample and reagent storage

8-10 ml of whole blood (after 8 to 10 hours calorie deprivation) was drawn aseptically from superficial vein, in heparin containing vials, centrifuged for 5 minutes at 2500 r.p.m. and the plasma stored at -40°C and used weekly for estimation of different biochemical parameters. The reagents used for different assays were stored in 2-8°C except the chromogen and ferric chloride were freshly prepared every day before the assay.

Measurement of H_2S concentration in plasma

The estimation of plasma H_2S levels was done following the methods reported earlier[7,16-18] which is further modified and standardized in our laboratory.[19]

Principle

Zn^{2+} was added to plasma sample to deposit H_2S, H^{2-} and S^{2-}, as well as plasma protein, then used NaOH to re-dissolve plasma protein. ZnS deposition was re-dissolved by the addition of N, N -dimethyl-p-phenylenediamine, and the remnant protein was deposited by trichloroacetic acid. After centrifugation, ferric chloride was added to the supernatant fluid to generate methylene blue, which was analyzed by spectrophotometer at 670 nm.

Assay procedure

Four twenty-five microliters of PBS (phosphate buffered saline) was taken (for standard curve NaHS was diluted in deionized water) in a glass tube and 75 microliters of plasma added along with 250 microliters of 10% tri-chloroacetic acid and the tube capped. Next the tube was centrifuged at 3000 rpm for 30 mins and the supernatant decanted in another glass tube and 250 microliters of 1% zinc acetate added and capped again(with rubber cap or parafilm). 133 microliters of 20 mmol N, N-dimethyl- p- phenylenediaminesulphate and 133 microliters of 30 milimol of $FeCl_3$ added and the tube recapped. 60 microliters of 10% NaOH was added and the resulting solution was incubated for 10 minutes at room temperature. All samples were assayed in triplicate and concentration in the solution was calculated against a calibration curve of sodium sulfide. Results of plasma H_2S concentration were expressed in micromol/l.

Standardization of plasma H_2S level assay

For measurement of plasma H_2S level, sodium sulfide (NaHS) has been used to construct the calibration curve. When NaHS is dissolved in water, HS^- is released and forms H_2S, with H^+ ions present in water. Different dilution of NaHS from a stock solution of 250 micromol/l were made in different test tubes, and under the same conditions of the method of estimation of H_2S in plasma and following the same technique absorbance was obtained at 670 nm and plot on a graph (Figure 1). The maximum intra-assay variation was 7.576 and inter-assay variation was 3.944. The linearity limit is 25-200 micromol/l of NaHS. The plasma concentration of H_2S of each sample has been extrapolated from this calibration curve.

Assay procedure for H_2S synthesizing activity in plasma[7,19,20,21]

100 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 37^0C 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 microliters 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 mmolN, N-dimethyl- p- phenylenediaminesulphate and 50 microliter of 30 mmol of $FeCl_3$ 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 taken at 670 nm. H_2S synthesizing activity of the plasma expressed as micromol per 100 g of protein.

Standardization of assay of H_2S synthesizing activity in plasma

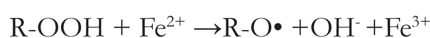
Initially absorbance values obtained from each sample in the technique of assay of H_2S synthesizing activity, were extrapolated from the standard curve of H_2S (Figure 1) and then 30% of the determined value was considered as the synthesizing activity of H_2S in plasma.[19]

Estimation of total oxidative stress (TOS) in plasma

It is a simple colorimetric test based on the principle of Free Oxygen Radical Test (FORT) modified and standardized in our laboratory.[22-25] Hence we have used a different chromogen (N, N-dimethyl- p- phenylenediaminesulphate) since the crude non-lyophilized form of the chromogen [lyophilized stable form originally used in FORT test] in solution turned out very unstable.

Principle

It is based on the ability of transition metals, such as iron, to catalyze the breakdown of hydroperoxides into derivative radicals, according to Fenton's reaction. Once they are formed in cells, ROOH maintain their chemical reactivity and oxidative capacity to generate proportional amounts of alkoxyl (RO·) and peroxyl (ROO·) radicals. These derivative radicals are then preferentially trapped by the same suitably buffered chromogen (N,N-dimethyl- p- phenylenediaminesulphate) and develop, in a linear kinetic based reaction at 37°C, a colored fairly long-lived radical cation photometrically detectable. The intensity of the color correlates directly with the quantity of radical compounds, according to the Lambert-Beer's law, and it can be related to the oxidative status of the sample.



$$R\text{-}OOH + Fe^{2+} \rightarrow R\text{-}O\bullet + OH^- + Fe^{3+}$$

$$R\text{-}OOH + Fe^{3+} \rightarrow R\text{-}OO\bullet + H^+ + Fe^{2+}$$

$$RO\cdot + ROO\cdot + 2CrNH_2 \rightarrow R\text{-}O\bullet + R\text{-}OO\bullet + [Cr\text{-}NH_2^{+\cdot}]$$

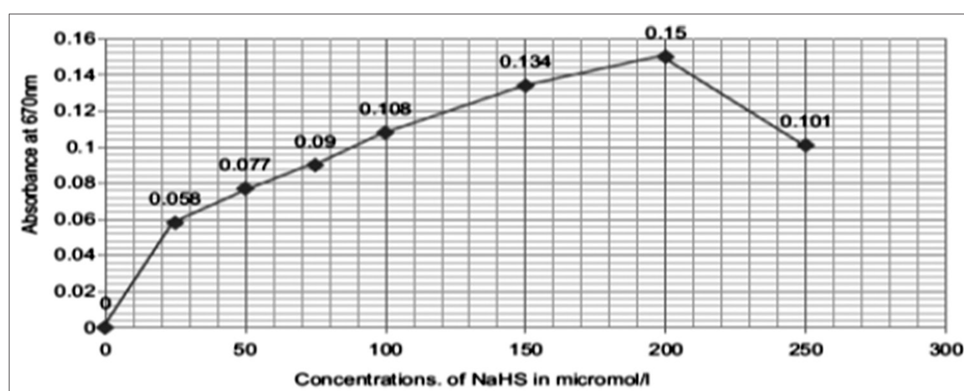


Figure 1: Standard curve of plasma H_2S assay

The visible spectrum of the $ChNH_2$ radical cation, shows two peaks of absorbance at 505 and 550 nm. The overall spectral intensity increases with time.

Assay procedure

When 100 microliters of 20 times diluted plasma/serum was dissolved in one ml of acetate buffer in a test tube, the hydroperoxides reacted with the transition metal ions liberated from the proteins in the acidic medium and were converted to alkoxy (RO·) and peroxy (ROO·) radicals. Twenty-five microliters of working chromogen solution added to the mixture. The radical species produced by the reaction interact with the chromogen that forms a colored, fairly long-lived radical cation evaluable by spectrophotometer at 505 nm (linear kinetic-based reaction, 37°C). The intensity of the color correlates directly to the quantity of radical compounds and to the hydroperoxides concentration and, consequently, to the oxidative status of the sample according to the Lambert-Beer law. The absorbance values obtained at 4 to 6 minutes for each sample against blank, were compared to the curve obtained using H_2O_2.

Standardization and assay of total oxidative stress (TOS)

Standard curve of TOS was prepared using different dilutions of hydrogen peroxide (H_2O_2) in millimolar concentrations per liter and difference in absorbance values taken at 505 nm in a six-minute time-scan between 6th and 4th minute (increase in absorbance noted maximum in this period) as depicted in Figure 2. Each data is the mean of three different values by the same procedure performed in four different occasions. The assay was completed in 6 minutes.

Intraassay and interassay coefficients of variation

The maximum intraassay variation at 4min was 4.762 and that at 6 min was 4.145 and interassay variations at 4 and 6 minutes were 2.717 and 2.105 respectively. The maximum sensitivity of the assay was 1.22 mmol/l H_2O_2 and the linearity up to 120 mmol/l.

Estimation of total antioxidant defense (TAD) in plasma

It is also a simple colorimetric test based on the principle of Free Oxygen Radical Defense (FORD) test, modified and standardized in our laboratory.[22,24,25]

Principle

The test uses preformed stable and colored radicals and determines the decrease in absorbance that is proportional to the antioxidant concentration of the sample according to the Lambert-Beer law. In the presence of an acidic buffer (pH = 5.2) and a suitable oxidant ($FeCl_3$), the chromogen (N, N-dimethyl- p- phenylenediaminesulphate) forms a stable and colored radical cation that is photometrically detectable at 505 nm. Antioxidant compounds in the sample reduce the radical cation of the chromogen, quenching the color and producing a discoloration of the solution, which is proportional to their concentration. The absorbance values obtained for the samples are compared with a standard curve obtained using Trolox (6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid), a permeable cell derivative of vitamin E commonly used as an antioxidant.

Chromogen (no color) + Fe2+ + H+ → chromogen·+ (purple)

Chromogen·+ (purple) + AOH → chromogen (no color) + AO

The UV-visible spectrum of the FORD chromogen radical cation shows maximum of absorbance at approximately 330 nm, 510 nm and 550 nm. Hence, an end-point analysis of the colorimetric reaction at 505 nm and at 37°C was selected.

Assay procedure

One ml of acetate buffer (pH = 5.2) taken in a test tube. Twenty-five µl chromogen reagent that contains N, N-dimethyl- p- phenylenediaminesulphate and 10 µl $FeCl_3$ solutions are added. The chromogen forms a stable and colored radical cation photometrically detectable at 505 nm. When 10 µl of 20 times diluted serum/plasma added to the mixture, the antioxidant compounds in the sample reduce the radical cation of the chromogen, quenching the color and producing a discoloration of the solution, which is proportional to their concentration. The absorbance values (at six minute in 37°C) obtained for the samples against blank are compared with a standard curve obtained using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a permeable cell derivative of vitamin E commonly used as an antioxidant.

Standardization of total antioxidant defense (TAD) assay[24,25]

Standard curve of TAD by modified FORD assay was prepared by using different dilutions of trolox, a potent antioxidant and it quenches the color of the FORD chromogen maximally between 4th and 6th minute. With different concentrations of trolox, the difference in absorbance between the absorbance values at 505 nm at 4th and 6th minute of the 6 minutes time scan was plot and a calibration curve was constructed (Figure 3).

TAD values of patients as well as controls have been calculated from this calibration curve (Figure 3). The

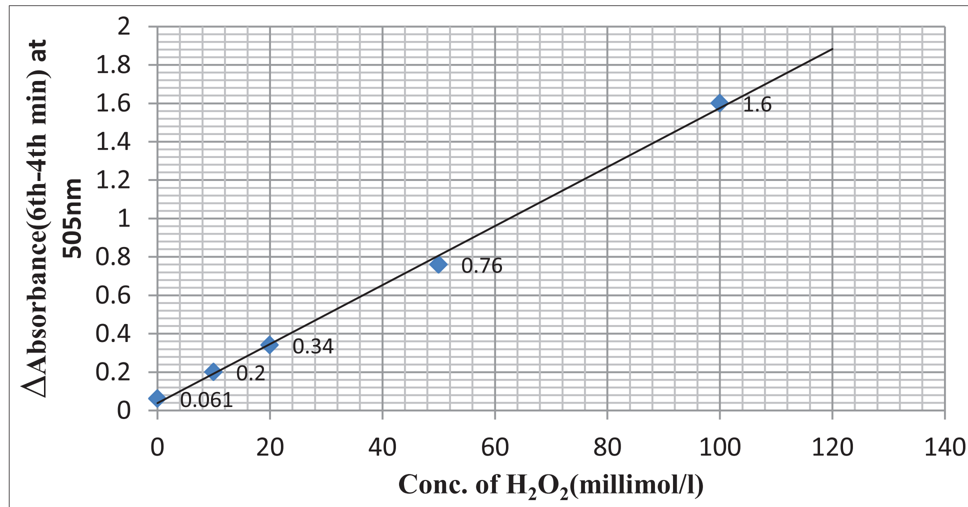


Figure 2: Standard curve for estimation of total oxidation stress (TOS)

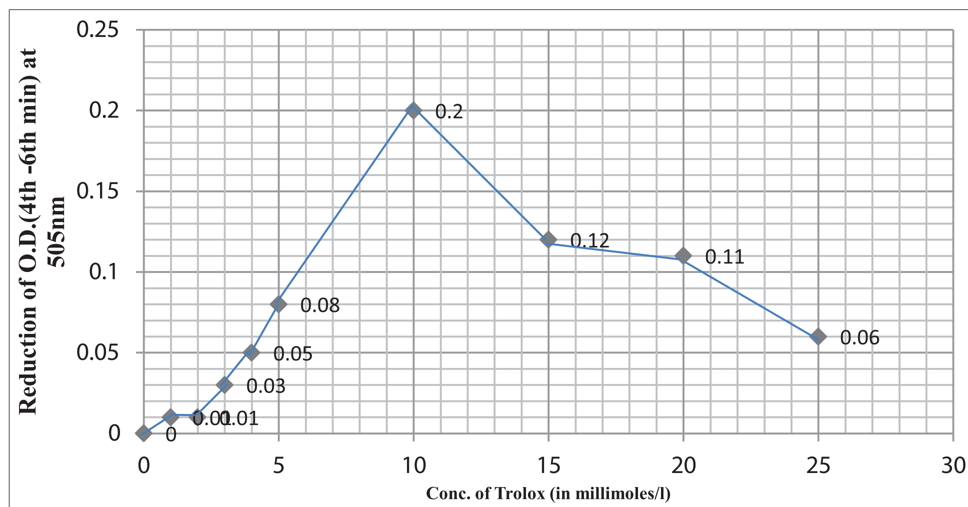


Figure 3: Standard curve for estimation of total antioxidant defence (TAD)

results are expressed as Trolox equivalents (mmol/l) using the calibration curve plotted with different amounts of standard Trolox as shown in Figure 3. Each data is the mean of three determinations performed in four different occasions.

Intra-assay and inter-assay coefficients of variation

The maximum intra-assay variations at 4 and 6 minutes were 7.914 and 9.009 respectively and inter-assay variation at 4minutes and 6minutes were 2.173 and 4.717 respectively, whereas the linearity ranged from 0.25 to 10.0 mmol/l trolox.

Other biochemical parameters are measured using standardized kits, FBG and PPBG by GOD-POD, HbA_{1C} by ion-exchange chromatography, fasting Insulin by ELISA, total protein by Biuret method.

Statistical methods

Data were expressed as mean ± standard deviation(SD) or mean and interquartile range, Comparison of data was done using unpaired two-tailed student's t-test and Pearson's correlation for Gaussian distribution and Mann Whitney U test and Spearman's correlation for non- Gaussian distribution and <0.05 was considered as significant. Statistical analysis was done using Microsoft Office Excel-2007 and SPSS Statistics version 2020.

RESULTS

In our study the values of FBG, PPBG, HbA_{1C}, fasting Insulin, plasma H_2S and H_2S synthesizing activity in plasma and TOS values in patients are significantly higher($p< 0.05$) than the corresponding values in healthy controls (Table 1 and Figure 4).

The median and inter quartile range of TAD values in controls is significantly higher than those of the patients (Mann Whitney U, Z<0.001) (Table 1a and Figure 5).

Plasma H_2S levels show significant positive correlation with TOS values (r= 0.793, P<0.001), as shown in Figure 6 and significant negative correlation with TAD values (r= -0.753, P<0.001) as shown in Figure 7.

Plasma H_2S synthesizing activity level shows significantly positive correlation with the plasma H_2S level (r= 0.547, P<0.001), as shown in Figure 8.

DISCUSSION

Plasma H_2S levels in the patients in this study is significantly increased than the controls but both are comparable with earlier studies which is within the range of 10 to100 micromol/l in human subjects reported by most of the laboratories.[26] M. Yusuf et al has reported that streptozotocin-induced diabetes in rat is associated with enhanced tissue hydrogen sulfide biosynthesis.[20] Elevated hydrogen sulfide levels in plasma were reported in patients with proliferative diabetic retinopathy,[27] as well as patients with cardiovascular disease,[28]cerebral ischemic damage,[29] and septic shock.[30] The 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,[31] but associated levels of pro-inflammatory cytokines may have affected the outcome. A recent study report suggested that the

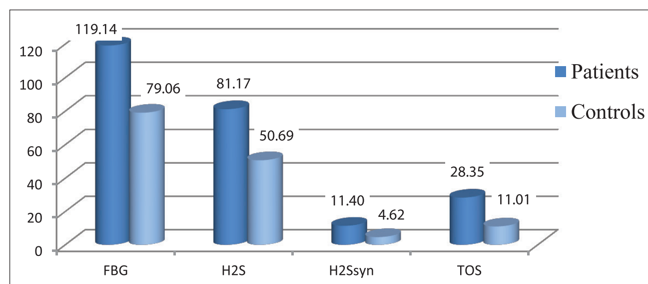


Figure 4: Comparison of means of patients and controls regarding FBG, H_2S, H_2S synthesizing activity and TOS

Table 1: Clinical & biochemical parameters of patients and controls (Gaussian parameters)

Variables (Gaussian distribution)	Mean±SD		p-value
	Patient	Control	
Age (years)	44.03±5.27	43.63±5.56	
Sex (M/F)	30/32	37/25	
Body mass index (BMI)	24.15±4.09	24.81±2.63	NS
Fasting blood glucose (mg/dl)	119.14±26.88	79.06±12.64	<0.001*
Post prandial blood glucose (mg/dl)	176.08±46.26	108.76±11.16	<0.001*
HbA_{1c}(%)	6.85±1.14	3.8±0.34	<0.001*
Fasting plasma insulin (micro IU/l)	11.02±7.59	7.67±3.1	0.002*
Total oxidative stress or TOS (milimolof H_2O_2/l)	28.35±11.21	11.01±4.55	<0.001*
Plasma H_2S level (micromol/l)	81.17±16.40	50.69±8.69	<0.001*
Plasma H_2S synthesizing activity (micromol/100 g of protein)	11.40±5.38	4.62±1.91	<0.001*
Total protein (g/dl)	6.4±0.44	6.6±0.41	0.008*

Student's t-test was done, *are significant (p<0.05), with 95% confidence level, NS=Not significant

Table 1a: Clinical & biochemical parameters of patients and controls (non-Gaussian parameters)

Variables (non-Gaussian distribution)	Patient median & InterQuartile Range	Control median & InterQuartile Range	Mann Whitney UZ value
Total Antioxidant Defense or TAD (milimol of Trolox/l)	80 & 142	300 & 293	<0.001*

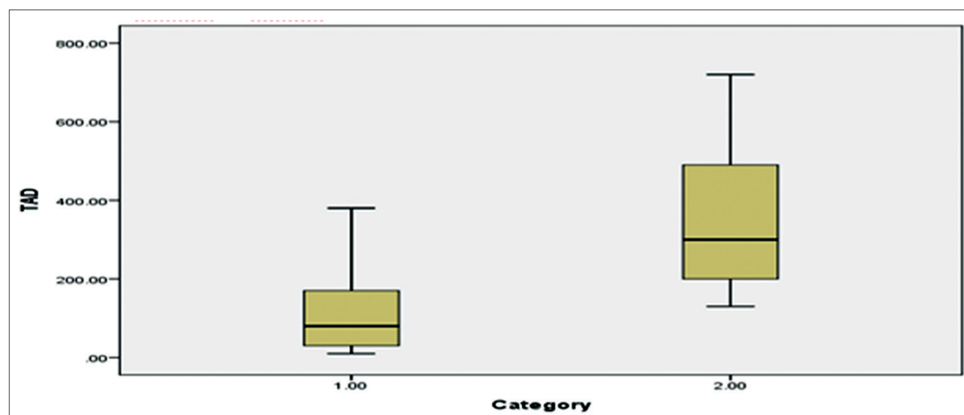


Figure 5: Comparison of Medians and Inter Quartile Range of Total Antioxidant Defense or TAD values (milimol of Trolox/l) of patients and controls expressed as box-whisker plot. Category-1.00- TAD values of patients, category-2.00-TAD values of controls

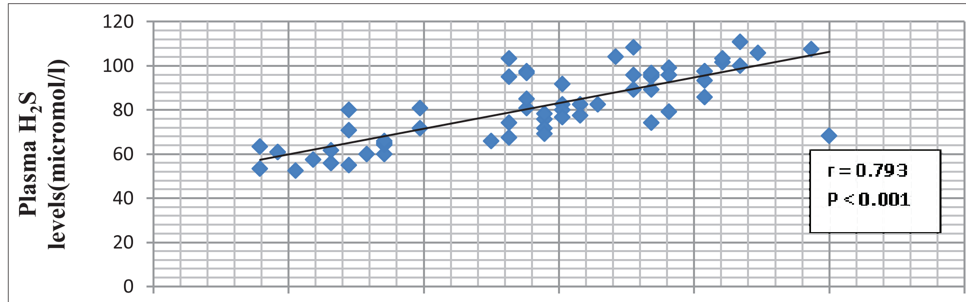


Figure 6: Scatter plot showing correlation between plasma H_2S and TOS values in patients

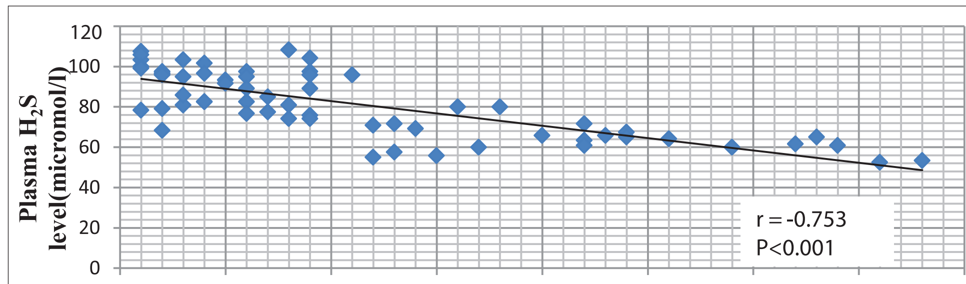


Figure 7: Scatter plot showing correlation between plasma H_2S and TAD values in patients

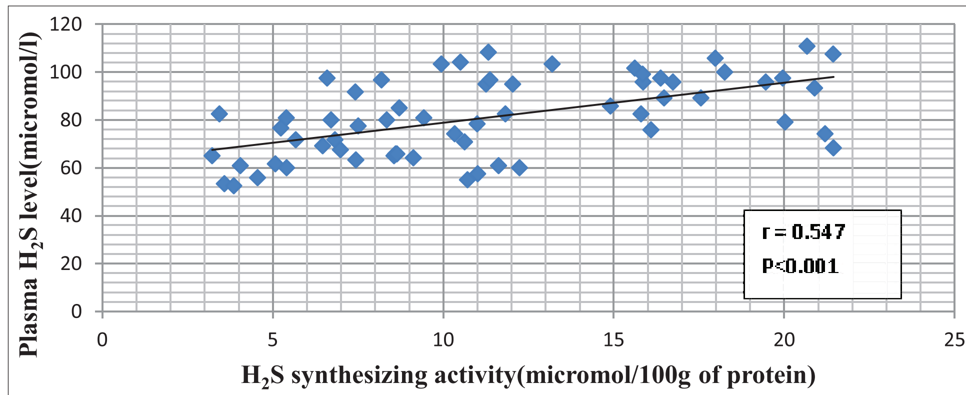


Figure 8: Scatter plot showing correlation between plasma H_2S and H_2S synthesizing activity in patients

lipid constituents of biological membranes release nitric oxide from nitrosoglutathione induced by H_2S/HS^-.[32] So increased H_2S levels in our patients might have a beneficial effect by acting as an antioxidant.

The activity of H_2S synthesizing enzymes in our study is significantly enhanced in type 2 diabetic patients than the healthy controls. The activities of H_2S producing enzymes and the tissue H_2S contents are known to increase under diabetic conditions as reported by Kaneko Y. et al.[33] Measurement of the H_2S synthesizing activity in plasma in streptozotocin treated diabetic rats has been undertaken by researchers in recent times.

The plasma total oxidative stress (TOS) in our study is significantly elevated in patients whereas plasma total antioxidant level (TAD) is significantly reduced in the study subjects than the healthy controls in this study, and the findings are quite consistent to previous studies.[34] In the TOS levels and a significant negative correlation with the plasma TAD values. The activities of H2S synthesizing enzymes also have a significant positive correlation with the plasma H_2S levels. Previous studies in this direction suggest that oxidative stress results in periodic depletion of glutathione and cysteine, which in turn causes stimulation of the assimilation pathway and H_2S production[35] in Saccharomyces cerevisiae model. Wei-Ning Niu et al. demonstrated the potential for increased H_2S production under oxidative stress conditions in tissues where cystathione beta synthase activity is increased by s-glutathionylation.[36] Although a few earlier studies advocated protective role of H_2S in oxidative stress situations.[37] Yet further evaluation is needed whether elevation of plasma H_2S level is playing a protective or harmful role in this condition.

CONCLUSION

The current study elucidated that there is significant increase in both plasma H_2S levels and the activity of H_2S synthesizing enzymes in type 2 diabetics and well correlated with the total oxidative stress and antioxidant defense. Further study of these parameters in other tissue fluids may open up a new horizon of the role of H_2S modifiers as well antioxidants in the treatment of diabetes.

LIMITATIONS OF THE STUDY

The sample size is small since it is a pilot study and the change of H_2S and the activity of H_2S synthesizing enzymes at tissue level have not been estimated. Due to shortage of funding original lyophilized FORT reagent could not be procured from Italy, instead we used the crude form of chromogen in solution in our modified methods for oxidative stress and antioxidant defense and this form is not as stable as the lyophilized stable form of chromogen in the original reagent kits available from Italy.

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Authors Contribution:
UKB - Concept and design of the study, reviewed the literature, statistical analysis, helped manuscript preparation and critical revision of the manuscript; **PS -** Concept, collected data, literature search, statistically analyzed and interpreted, prepared first draft of manuscript; **SS and SDG -** 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.

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