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Elevated Serum Hydrogen Sulfide in Age Related Macular Degeneration

Dhivya MA[1], Ovia M[1,2], AnandBabu K[1], Parveen Sen[3], Angayarkanni N[1] and Bharathidevi SR[1]*

[1]RS Mehta Jain Department of Biochemistry and Cell Biology, Vision Research Foundation, India

[2]Sree Sastha Institute of Engineering and Technology, India

[3]Shri Bhagwan Mahavir Vitreoretinal Services, Medical Research foundation, India

Case Report

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***Corresponding author:** Bharathidevi SR, RS Mehta Jain Department of Biochemistry and Cell Biology, Vision Research Foundation, 41, College Road, Nungambakkam, Chennai 600006, India, Tel: 044 4227 1500; Email: drbarathi@snmail.org

Abstract

Introduction: Age related macular degeneration is one of the major causes of global blindness and vision impairment. The study was done to determine the levels of hydrogen sulfide (H2S) in AMD subjects in the study cohort.

Methods: Twenty-six blood samples were collected, 12 control and 14 AMD subjects, the serum obtained from these samples were used for the measurement of H2S by Methylene blue (MB) assay and IL6 using ELISA.

Results: We observed that both the levels of H2S and IL6 to be elevated in AMD subjects when compared to control and H2S and IL6 showed a positive correlation.

Conclusion: We propose H2S to be a marker in both dry and wet AMD.

Keywords: Hydrogen Sulfide; Inflammation; AMD

Abbreviations: AMD: Age Related Macular Degeneration; AREDS: Age Related Eye Disease Study; CBS: Cystathionine β Synthase; CNV: Choroidal Neovascularization; CSE: Cystathionine γ Lyase; GSH: Intraocular Glutathione; DM: Diabetes Mellitus; MST: 3-Mercaptopyruvate Sulfurtransferase; RPE: Retinal Pigment Epithelium; VEGF: Vascular Endothelial Growth Factor

Introduction

Age related Macular degeneration (AMD) is one of the leading causes of blindness globally. The prevalence rate of AMD is estimated to increase to 288 million by the year 2040 [1]. It is divided majorly into two type's dry and wet AMD forms. The most common type, dry AMD is characterized by deposits called drusen in the sub retinal space. Advanced AMD can be classified as geographic atrophy (GA; i.e., dry, or non-exudative, AMD), characterized by a sharply delineated area of Retinal pigment epithelium (RPE) and the other choroidal neovascularization (CNV); i.e., wet, or exudative, AMD), which may involve sub retinal neovascular membranes; sub retinal fluid, exudates, hemorrhage, pigment epithelial detachment and sub retinal/intraretinal scarring [2]. Currently, there is no effective treatment for GA; nutritional antioxidant supplements are widely used as a strategy to prevent the disease progression [3]. On the other hand, in patients with

wet AMD, there is severe vision impairment, intravitreal injection of anti-vascular endothelial growth factor (VEGF) agents such as ranibizumab and aflibercept, have been widely and effectively used worldwide in the clinical treatment of neovascular AMD via targeting CNV [4]. While several risk factors have been suggested including age, smoking and oxidative stress, the exact pathogenesis of the disease is still incompletely understood. Multitude of inflammation-related plasma proteins were detected in the drusen of the AMD patients and establish the involvement of systemic immunological processes in the pathogenesis of AMD. In our earlier study we have reported increased IL6 levels in the serum of AMD patients [5]. Various anti-inflammatory agents are under clinical trial for treating AMD [6]. H2S is produced endogenously in the body predominantly by three enzymes: Cystathionine β synthase (CBS), Cystathionine γ lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (MST) [7]. Since the study of Abe and Kimura in 1996 showed the various enzymatic processes involved in the H2S generation, several studies has been reported on the role of H2S in neuroregulation, inflammation, endocrinologic regulation and vasodilation [8,9]. H2S has been shown to inhibit inflammasome activation in uterine tissues, and inhibits the TLR4/NF-κB signalling pathway [10]. Endogenously produced H2S has been observed in various eye tissues as well with highest production in cornea and retina [11]. H2S is being studied as a therapeutic target in ocular diseases [9]. H2S-producing chemical donor GYY4137, has been reported to stabilize the intraocular pressure, and up regulate the intraocular glutathione (GSH) levels in animal models of glaucoma [12]. A recent study has proposed H2S as a potent molecular target to be explored in AMD [13]. However, there are no studies so far to our knowledge which has measured the serum levels of hydrogen sulfide in AMD where chronic inflammation is reported. We therefore conducted a pilot study to measure the serum levels of H2S in AMD subjects in comparison to control and evaluated the correlation if any with the inflammatory status.

Methodology

Sample Collection

Twenty-six blood samples were collected as part of the study. Out of the 26, 14 were AMD patients (7 Wet AMD and 7 Dry AMD; 6F and 8M; Mean age: 71±7 years) and 12 control (6M and 6F, Mean age: 54±10). The study was conducted in adherence to the principles of the Helsinki declaration and approved by the Institutional Ethics committee. Written consent was obtained from the participants. Detailed ophthalmic evaluation was done. Subjects with history of Smoking, Diabetes Mellitus (DM), renal dysfunction, hepatic disease were excluded and ocular disease other than AMD such as high myopia, retinal dystrophies, central serous retinopathy, vein occlusion, diabetic retinopathy, and uveitis or similar outer retinal diseases which have been present prior to the age of 50 years were excluded. AMD diagnosis was based on the Age-Related Eye Disease Study (AREDS) guidelines [14].

Hydrogen Sulphide (H_2S) Estimation

The methylene blue method is the most reported method used in the literature to measure hydrogen sulfide in biological samples. The method is based on spectrophotometry of the acidic conversion of hydrogen sulphide to methylene blue. We have previously measured the levels of H_2S in lymphocytes in healthy controls [15]. To perform the assay initially 250 µL of 1% (wt/vol) Zinc acetate was added with 50 µL of sample followed by 450 µL of distilled water. Then 133 µL of 20 mM N, N-dimethyl-p-phenylenediamine sulfate solution prepared in 7.2 M of HCl was added followed by 133 µL of 30 mM Ferric chloride solution prepared in 1.2 M of HCl. The resulting mixture was incubated at room temperature for 15 minutes and then centrifuged for 5 minutes at 12,000 rpm. Absorbance of the aliquots of the supernatant was then determined at 670 nm in spectrophotometer. H_2S was calculated against a calibration curve of NaHS (10-100 µm) [16].

IL6 ELISA

Serum IL6 was measured using R&D ELISA kit based on the manufacturer's protocol. Briefly 100 µl of the serum sample was used for the assay. A standard graph in the range of 9.375 - 600 pg/ml was used for calibration.

Statistical Analysis

Statistical analysis was performed by Mann Whitney U test using GraphPad Prism. Data is represented as Mean ± SEM. $p < 0.05$ was considered as significant. Binary logistic regression was performed to determine the risk factor for the outcome variable. Simple linear regression analysis was done to correlate the variables H_2S and IL6.

Results

The clinical details and the medication taken by the AMD and control cases are given in Table 1 & 2.

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Clinical details of AMD subjects								
Patient ID	Age (y)	Gender	AMD type	Duration of Hyper-tension (y)	Smoking status	Alcohol status	Anti-hyper-tensive medication	Anti-VEGF status
A1	74	M	Wet	Nil	Nil	Nil	Nil	Nil
A2	83	F	Wet	30	Nil	Nil	Yes	OD: Avastin once, one month before
A3	70	F	Dry	Nil	Nil	Nil	Nil	Nil
A4	70	F	Dry	Nil	Nil	Nil	Nil	Nil
A5	60	F	Dry	Nil	Nil	Nil	Nil	Nil
A6	60	F	Dry	Nil	Nil	Nil	Nil	Nil
A7	76	M	Atrophy	10	Nil	Nil	Yes	Nil
A8	78	M	Atrophy	10	Nil	Nil	Yes	OD: Avastin 7 times
A9	75	F	Dry	Nil	Nil	Nil	Nil	Nil
A10	79	M	Wet	3	Past smoker	Chronic Drinker	Yes	OD: Avastin once
A11	70	M	Wet	2	Nil	Nil	Yes	OD: Intra Vitreal inj 4 times in 2011
A12	65	M	Wet	Nil	Nil	Nil	Nil	OD: Avastin inj 2 times (latest one month before)
A13	55	M	Dry	Nil	Past smoker	Chronic drinker	Nil	Nil
A14	84	M	Dry	Nil	Nil	Nil	Nil	Nil

Table 1: Clinical details and medication taken by the AMD patients.

Clinical details of control subjects					
Control ID	Age (Y)	Gender	Medical history	Smoking status	Alcohol status
C1	48	M	Nil	Past smoker	Once a year
C2	55	F	NIL	Nil	Nil
C3	48	M	Hypertension	Nil	Nil
C4	88	M	Nil	Nil	Nil
C5	50	F	Nil	Nil	Nil
C6	48	F	Nil	Nil	Nil
C7	51	F	Nil	Nil	Nil
C8	52	M	Nil	Nil	Nil
C9	51	M	Nil	35 Y	Social drinker
C10	50	F	Nil	Nil	Nil
C11	61	M	Nil	Nil	Nil
C12	47	F	Nil	Nil	Nil

Table 2: Clinical details of the control cases.

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Estimation of Hydrogen Sulfide (H_2S) and IL6 in Serum

H_2S in the AMD plasma samples was measured using MB assay. There was a significant increase in the levels of serum H_2S with a mean of 60.08 ± 4.83 µM when compared to the control samples that showed a mean of 45.93 ± 3.92 µM (p = 0.04) (Figure 1a). The mean serum H_2S levels in wet AMD was 62.8 ± 25.46 µM, (n = 5) when compared to dry AMD that showed 52.01 ± 4.77 µM, (n = 9).The AMD patients who had their anti-VEGF injections (64.06 ± 10.43 µM; n = 5) showed no statistical variations in the serum H_2S levels when compared to those cases who had no such intra-vitreous injections (51.31 ± 5.139 µM; n = 9).There was a significant increase in the levels of serum IL6 in AMD patients (17.38 ± 2.87 pg/ml) when compared to controls (14.9 ± 0.87 pg/ml, p = 0.002) (Figure 1b), whereas there was no significant difference between dry (18.13 ± 3.28 pg/ml) and wet AMD cases (16.03 ± 1.306 pg/ml).

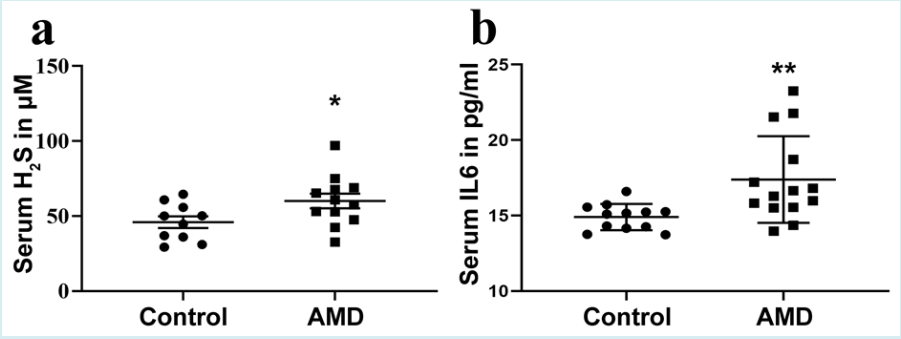

Figure 1: Estimation of serum H_2S and IL6 in AMD cases: **a)** Serum H_2S by methylene blue method. **b)** Serum IL6 levels measured using ELISA.

Correlation between Serum H_2S and IL6

Simple linear regression correlation between serum H_2S and IL6 showed a significant positive correlation (r = 0.4569; p = 0.0325) in the total study cohort of AMD and control. Correlation between the parameters in the controls alone showed no significant correlation (r = -0.1132; p = 0.7261), whereas in AMD patients there was a significant positive correlation between the serum H_2S and IL6 (r = 0.7163; p = 0.0299) (Figure 2).

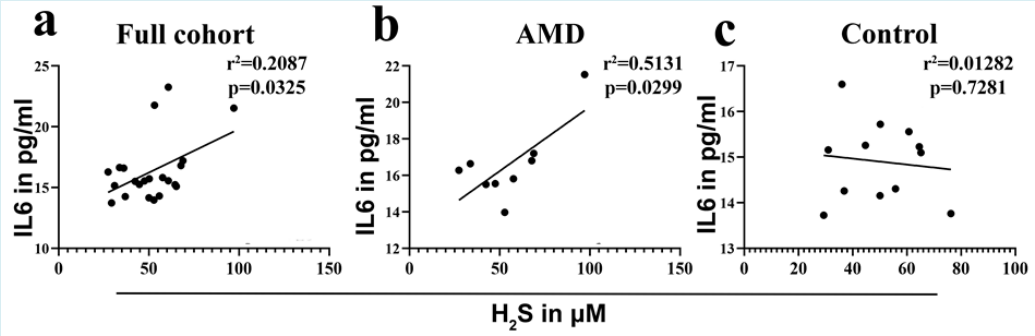

Figure 2: Correlation between serum H_2S and IL6: a) Correlation between the parameters in Control group; b) Correlation between the parameters in the AMD group.

Discussion

H_2S plays an essential role as a gaso-transmitter similar to nitrous oxide and acts as a signaling molecule in mammals [8,17]. Hydrogen sulphide (H_2S), a potent neuromodulator, was initially thought to be a toxic gas. In humans, the concentration of H_2S in serum is reported to be from 70 to 125 µM [18] and regulates various physiological cellular functions like synaptic transmission, vascular tone, inflammation, transcription, and angiogenesis and protects cells from oxidative stress. The level of H_2S is crucial for the regulation of inflammatory response, wherein low

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dose decreases inflammation while high doses of H_2S donor show controversial results. Therefore, H_2S dosage is a switch to control the biphasic regulation of H_2S donors on inflammation, and the generation of H_2S can also be augmented by the appearance of inflammation [11]. In severe acute pancreatitis, TNFα and IL6 have been shown to induce the expression of CSE and CBS, thereby enhancing the production of H_2S. The increased H_2S inhibited intestinal mobility and enhanced the inflammatory response caused due to pancreatitis [19]. H_2S has been reported to act as a pro-inflammatory mediator in rheumatoid arthritis and was found to be correlated with the disease activity score and tender joint count [20]. In AMD, H_2S has been hypothesized to be a therapeutic target for disease management [13].In this pilot study, we find serum H_2S level is significantly increased in AMD patients compared to control which is reported for the first time that needs to be validated in larger sample size. Serum IL-6 levels were also significantly increased in AMD patient samples in comparison to control. We show a positive correlation between H_2S and IL6 in AMD patients and not in control. There are not many studies that associate H_2S and pro-inflammatory cytokines and we speculate that H_2S might induce the expression of IL-6 [19]. Also, since there was no significant difference between wet and dry AMD, H_2S may be a common factor in the initiation of AMD in general and maybe a disease marker for both dry and wet AMD. Future extensive studies in larger sample size are needed to evaluate this hypothesis.

Limitations

The analysis of the H_2S and IL6 were done in stored samples of the previous study. The samples were stored at -800C until analysis (no freeze thaw).

Acknowledgement

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Statement of Ethics

Study Approval Statement

This study protocol was reviewed and approved by Institutional Review Board (ethics committee), and the approval number is 149-2009P.

Consent to Participate Statement

Informed consent was obtained from the participants to collect blood and participate in the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

OM: H_2S analysis, ADM: H_2S analysis verification and manuscript preparation, KAB: Sample Collection, AK: Funding, discussion for manuscript preparation and patient recruitment and sample documentation, PK: patient recruitment and clinical detail documentation SRB: Study design, funding, and manuscript editing.

Data Availability Statement

The data that support the findings of this study are not publicly available as they contain information that could compromise the privacy of research participants but are available from the corresponding author [SRB] upon reasonable request.

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