THE STUDY OF REACTIVE OXYGEN SPECIES (ROS) PRODUCTION IN TYPE II DIABETES MELLITUS AND ITS RELATION WITH GLYCEMIC CONTROL.

Swati Kurdekar*, Sushma S **

*Post Graduate, **Assistant Professor, Department of Physiology, Bangalore Medical College and Research Institute, Bengaluru- 560002.

ABSTRACT : BACKGROUND & OBJECTIVES: Type 2 Diabetes Mellitus (DM) is a chronic, metabolic disease, which leads over time to serious damage to the heart, blood vessels, eyes, kidneys, and nerves, with a prevalence of 8.8% in India .Chronic hyperglycemia induced diabetic complications are due to oxidative stress, increased production of reactive oxygen species (ROS), and cellular death. Hyperglycemia induced signaling involve NADPH-oxidase mediated Polymorpho nuclear Neutrophilic (PMNs) production of ROS.The study was taken up with objective to study the relationship between ROS and Glycemic control in Type 2 DM. **MATERIALS AND METHODS:** The study was conducted on Type 2 DM patients & healthy controls, in the age group of 35-60yrs from Victoria Hospital, BMCRI. Informed written consent was taken. The Neutrophilic production of ROS was tested using NitroBlue Tetrazolium (NBT) assay. The number of cells with formazan crystals were counted. Statistical analysis was done using SPSS version 24.0. The data was expressed using Mean, Median $\pm$SD. Student's t-test and Pearson's co-relation co-efficient was used and $p<0.05$ was taken as statistically significant. **RESULTS:** ROS production in Diabetics was significantly increased ($p= 0.041$). Positive co-relation was found between ROS production & glycemic control($6.6\pm0.5\%$) among the Diabetics($r =0.323$).**CONCLUSION:** Hyperglycemia induced ROS may be responsible for progression and complications. Effective glycemic control may be one of the method to reduce the oxidative stress and delay the complications of DM.

KEYWORDS: PMN, Diabetes Mellitus, ROS, Glycemic control.

Author of Correspondence: Dr Sushma S, Assistant Professor, Department of physiology, Bangalore Medical College and Research Institute, Bengaluru-560002. e-mail: suvina76@gmail.com.

INTRODUCTION: Type 2 Diabetes Mellitus(DM) is a metabolic syndrome characterized by insulin insensitivity as a result of insulin resistance, declining insulin production, and eventual beta cell failure.[1] The prevalence of diabetes mellitus, a major public health problem is predicted to double globally from 171 million in 2000 to 366 million on 2030 with maximum increase in India. By 2030, diabetes mellitus may afflict up to 79.4 million individuals in India.[2] DM has been associated with reduced T cell response, disorders of complement factor 4, decreased cytokine response and altered Neutrophil functions.[3] Hyperglycemia of DM activates a particular metabolic route that involves diacylglycerol (DAG)—protein kinase C (PKC)—and NADPH-oxidase—culminating in Production of Reactive Oxygen Species(ROS). ROS is induced by hyperglycemia in diabetic patients through mitochondrial respiratory chain enzymes, xanthine oxidases, lipoxygenases, cyclooxygenases, nitric oxide synthases, and peroxidases.[4] Studies have clearly demonstrated that efforts to reach normoglycaemia and optimal metabolic control significantly reduce the complications of diabetes, especially those involving microangiopathy.[5] Glycemic control, in conjunction with modulation of PKC and/or NADPH-oxidase, downregulated the proinflammatory cytokines, leading to a reduced amount of ROS, and, consequently, decreased cellular death.[4] However, there are no specific studies addressed to relate Neutrophilic ROS production with Glycemic Control in Type 2 Diabetes Mellitus patients of Indian population. The present research evaluates these associations using NitroBlue Tetrazolium (NBT) assay to measure immune-modulatory role of glycemic status on Neutrophil ROS production.

MATERIALS AND METHODS: This research is an Cross-sectional study done in Hematology Lab, Department of Physiology, Bangalore Medical College & Research Institute. The study population included Type 2 Diabetics and Healthy controls. The period of study was from May-July 2019. Written and informed consent was taken from all the participants before the start of the study. Ethical Clearance was taken from Institutional Ethics

Committee. Participants recruited for the study were grouped into Diabetic group and the healthy control group with 30 subjects in each respectively.

Healthy subjects in the age group of 35-65yrs and subjects diagnosed with Type II Diabetes Mellitus as per American Diabetes Association (ADA) were included in the study. Subjects with h/o Hypertension and cardiac disease, Obesity and h/o any endocrinological disorder, h/o any hematological disorder or any carcinoma, h/o hepatic cirrhosis, h/o acute and chronic infections, smokers and known alcoholics, h/o drug intake like steroids, antipyschotics, antidepressants were excluded from the study.

The study was started after the subjects fulfilled the inclusion criteria and were enrolled after obtaining consent .The study group includes a total of 60 participants in the age group of 35-65 years out of which 30 of them were Type 2 Diabetes Mellitus patients (12 males and 18 females) and 30 of them were healthy controls (13 males and 17 females). Collection of data and blood samples from the Diabetic group were taken from patients visiting OPD clinic of Victoria Hospital, BMCRI, Bengaluru. History and General Physical Examination along with anthropometry was done. The subject's demographic details (Age, Sex, BMI) were taken . Subjects were matched for Age , Sex and BMI.

BIOCHEMICAL PARAMETERS: HbA1c levels was assessed with 2 ml venous blood sample after 8 hours of fasting.The Hba1c levels were estimated by latex agglutination assay using BIORAD D-10 machine at Infosys Lab, Victoria Hospital, Bangalore.

NITROBLUE TETRAZOLIUM (NBT) ASSAY:

The production of superoxide can be tested by reduction of cytochrome c and the extracellular release of H_2O_2 can be measured by horse radish peroxidase induced oxidation of phenol red.

The cells are exposed to yellow dye NBT. Unstimulated neutrophils do not ingest the dye,but if cells are stimulated to phagocytic activity, then they take the dye into phagosomes and intracellular reduction of dye converts it to an insoluble blue crystalline form (formazan crystals). These blue crystals are visible in the light microscope. This test gives information about the phagocytic function since the dye is not taken into cells except by phagocytosis and also about the metabolic function since the intracellular reduction depend upon the production of ROS.

In NBT test two aliquots of whole blood are mixed separately with Hanks's salt solution and 0.34% NBT dye. Esch. Coli endotoxin is added to one aliquot to stimulate the neutrophils and the other is left unstimulated to act as control. After incubation for about 4minutes, smears are prepared from both test and control samples, stained with Giemsa and the number of cells with formazan crystals are noted. In normal sample, only few cells in control(unstimulated) and $\geq$ 50% should show presence of crystals.[6]

STATISTICAL ANALYSIS :

The data was analysed using descriptive statistics to match the subjects based on Age, Sex and BMI. Students 't' test was done to compare the differences between the 2 subject groups .Pearson's co-relation co-efficient was used to see co-relation between qualitative variables. The statistical analysis was done in Microsoft Excel version 2010 and analysed using SPSS version 24.0. Data is expressed as mean ± SD. p value <0.05 is considered statistically significant.

RESULTS:

Table 1

	Diabetics	**Non Diabetics**	**p value**
Participants	30	30	
Male	12	13	
Female	18	17	
NBT assay	57.5±8.3	48.6±2.8	0.04*
Glycemic control (HbA1c)	6.6%±0.5	5.4%±0.21	0.0001*

Table 1 shows the gender distribution of participants in Group-I (Diabetics) and Group II (Healthy controls), mean NBT assay and mean Glycemic control. p < 0.05* is statistically significant. The mean±SD value for NBT assay in Group I were **57.5±8.3** and in Group 2 were **48.6±2.8** and the p value was 0.04 which is statistically significant. Likewise, the mean±SD value for HbA1c levels in Group I was 6.6±0.5 and the p value was 0.0001 which is statistically significant.

Figure 1

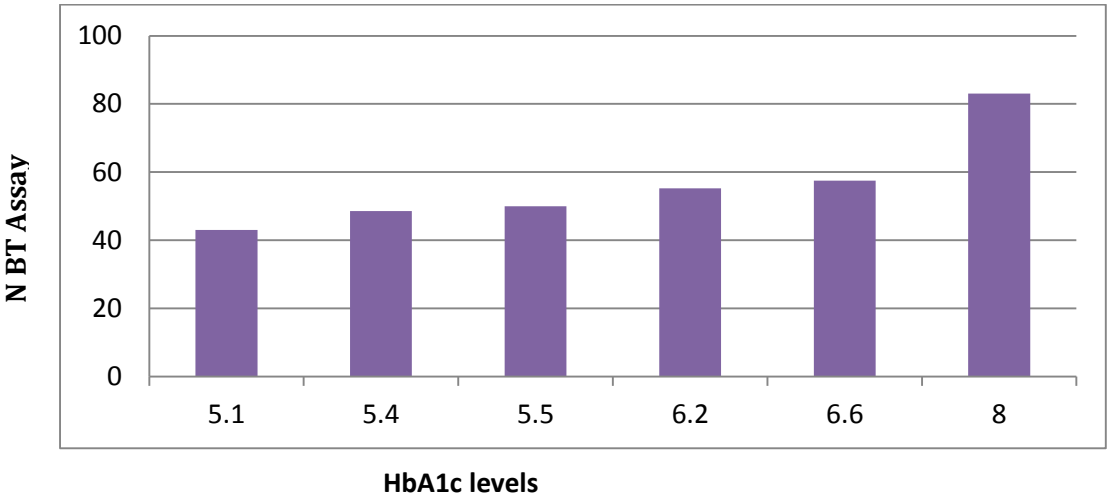

Figure 1 shows NBT assay at different levels of HbA1c among both groups. With increasing trend in HbA1c levels increase in Neutrophilic ROS production via NBT assay was found when subjects were grouped on basis of Glycemic control.

Figure 2

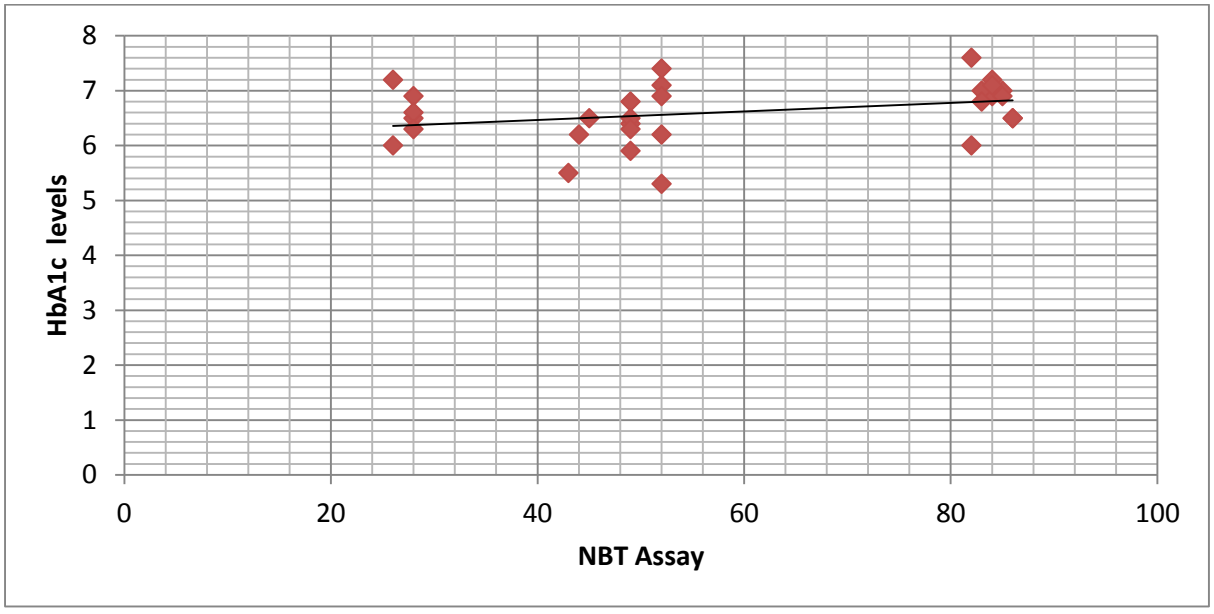

Figure 2 shows a positive co -relation between Glycemic control and NBT assay with r=0.323.

DISCUSSION:

It is believed that oxidative stress plays important role in the development of vascular complications in diabetes particularly type 2 diabetes.[7] Free radical formation in diabetes by non-enzymatic glycation of proteins, glucose oxidation and increased lipid peroxidation leads to damage of enzymes, cellular machinery and also increased insulin resistance due to oxidative stress.[8] In this study increase in neutrophilic ROS production in the Diabetic group was seen compared to the non diabetics.

Dorota P et al[5] studied the influence of meal & post prandial glycemia on neutrophilic ROS production , by cytochrome c reduction and phenol red oxidation in type 2 DM . Significant co-relation was seen between an increase in glycemia and ROS production ($r=0.2$, $p<0.05$) suggesting an increment in post prandial glycemia, that stimulates ROS production. This study showed increase in neutrophilic ROS production was seen with increase in HbA1c levels, and poor Glycemic control had higher ROS production. This indicates consitent Hyperglycemia of Type 2 Diabetes is associated with increased ROS production. Diabetic complications that are induced by hyperglycemia appear to be due to an imbalance between the oxidizing species (ROS), leading to oxidative stress and cellular death[9]. Increased ROS production activates apoptosis [10]. Both apoptosis and necroptosis have important roles in the progression of diabetic complications and they may culminate in tissue injuries in the heart, retina, kidneys, and nervous system[11,12]. Shichiri M et al[13] conducted a 8yr prospective study on the effects of intensive glycemic control on the severity of diabetic microvascular complications. It was observed that the values of HbA1c < 6.5%, FBS < 110 mg/dl, and 2-h postprandial blood glucose concentration < 180 mg/dl prevents the onset and progression of diabetic microvascular complications.

LIMITATIONS: The study would be more effective if the sample size was more. For more accurate analysis effective methods like Flow Cytometry could have been used. More detailed population based studies with larger sample size are required to arrive at conclusive results.

CONCLUSION:

Hyperglycemia induced ROS may be responsible for progression and complications. Effective glycemic control may be one of the method to reduce the oxidative stress and delay the complications of DM. As we have obtained statistically significant findings with our small sample size, the NBT test can be applied as screening test in larger studies. Although we have attempted to exclude all patients with pre-existing infections at baseline, we cannot fully exclude the possibility of underlying subclinical infections, which were not documented. This might have precipitated a high resting oxidative burst in some subjects. Targeting neutrophil related dysfunctions with molecular or biochemical techniques may reduce the morbidity due to ROS induced microvascular complications of type 2 diabetes mellitus.

ACKNOWLEDGEMENT: I m grateful to all the participants of the study for their kind cooperation and to technical professionals of Infosys Lab,Victoria Hospital for conducting the biochemiccal laboratory investigations. Source of Funding is self.

REFRENCES:

1. Kahn CR, Lecture B.Insulin action, diabetogenes, and the cause of type II diabetes. Diabetes. 1994;43(8):1066-1084.
2. Wild S, Roglic G, Green A, Sicree R, King H.Global prevalence of diabetes –estimates for the year 2000and projections for 2013.Diabetes care.2004;27(3):1047-53.
3. Casqueiro J, Casqueiro J, Alves C.Infections in patients with diabetes mellitus:A review of pathogenesis.J Endocrinol and Metabol.2012;16:27-36.
4. Volpe CM, Delfino PH, Anjos PM, Majado JA.Cell death and Disease.J Cell Death Diff Ass.2018;9:119.
5. Dorota P, Dorota Z, Henry W, Wysocka B.Increase in glycaemia stimulates reactive oxygen species (ROS) production by polymorphonuclear neutrophils in type 2 diabetic patients. J Pre Clin Clin Res. 2011;5(1):22–27.
6. Wilkins PC. Neutrophils leukocyte function tests. In :Thompson RA,Techniques in Clinical Immunology. 2nd ed. USA : Blackwell Scientific Publication ;1982.273-93

7. Pham-Huy LA, He H, Pham-Huy C.Free Radicals, Antioxiidants in Disease and Health. Int J Biomed Sci. 2008 Jun; 4(2): 89–96.
8. Maritim AC, Sanders RA, Watkins JB 3rd.Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol. 2003;17(1):24-38.
9. Gonzalez CD, Lee MS, Marchetti P, Pietropaolo M, Towns R, Vaccaro MI, Watada H, Wiley JW.The emerging role of autophagy in the pathophysiology of diabetes mellitus. Autophagy. 2011 Jan;7(1):2-11.
10. Kohnert KD, Freyse EJ, Salzsieder E.Glycemic variability and pancreatic β cell dysfunction. Curr Diabetes Rev. 2012 Sep;8(5):345-54.
11. Liang W, Chen M, Zheng D, He J, Song M, Mo L.A novel damage mechanism:Contribution of the interaction between necroptosis and ROS to high glucose induced injury and inflammation in H9c2 cardiac cells. Int J Mol Med. 2017 Jul;40(1):201-208. doi: 10.3892/ijmm.2017.
12. Rosa MD, Distefano G, Gagliano C, Rusciano D, Malaguarnera L.Autophagy in Diabetic Retinopathy. Curr Neuropharmacol. 2016;14(8):810-825.
13. Shichiri M, Kishikawa H, Okhubo Y, Wake N.Long-Term Results of the Kumamoto Study on Optimal Diabetes Control in Type 2 diabetic Patients. Diabetes Care 23 (Suppl. 2):B21–B29, 2000.

Disclosure: There was no conflict of interest