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Research Paper

ROLE OF REACTIVE NITROGEN SPECIES IN CARDIAC ASSOCIATED COMPLICATION AMONG TYPE II DIABETIC MELLITUS PATIENTS

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Abstract

Overwhelming mass of evidence demonstrating the development of Endothelial dysfunction among diabetic patients. Evidence has accumulated Indicating that the generation of Reactive Nitrogen Intermediate plays an important role in the etiology of diabetes associated Cardiac Complication. Study attempts to quantify the level of Reactive Nitrogen Species and its associated vascular injury leading to Cardio Vascular Disease among type II Diabetic patient. The study involves fifty patients with Type II Diabetes Mellitus (25 Male and 25 Female) attending Diabetology Unit of Government Rajaji Hospital, Madurai, subjected for the present study. Serum sample were collected and Reactive nitrogen species was quantified using Griess Reagent method and Serum Glutamate Oxaloacetate Transaminase (SGOT) using Auto analyzer. The study documents highest percentage of deviation of Reactive Nitrogen Species and SGOT level from the control observed in Male 23.7%, 78.1% and Female 19.6%, 73.8% respectively .Study documents the elevated level of Reactive Nitrogen Species and SGOT from the control samples which emphasize the clinical incidence of Cardiac associated complication among Diabetes. Endothelial dysfunction as a first step in the pathogenesis of diabetes promotes arteriosclerosis. Mechanically uncoupling of endothelial nitric oxide synthase in blood vessels lead to nitrosative stress. Antioxidant supplementation might reduce the nitrosative stress thereby minimizing the Incidence of vascular injury associated cardiac complication among diabetic patients.

Key words: *Nitrosative Stress, Vascular Injury, SGOT, Atherosclerosis, Diabetes*.

INTRODUCTION

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. The effects of diabetes mellitus include long-term damage, dysfunction and failure of various organs. Diabetes mellitus may present with characteristics symptoms such as thirst, polyuria, blurring of vision, and weight loss. The abnormalities of carbohydrate, fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (1).

The increased incidence of vascular disease in patients with diabetes only partially explains the greater incidence of heart failure that they exhibit. In diabetes the heart is exposed to an increased supply of fatty acids and of glucose. The increase in substrate supply is accompanied

by systemic and myocardial insulin resistance. This extreme metabolic environment influences energy substrate metabolism and directly affects myocyte survival, together with cardiac structure and contractility (2). Cardiac efficiency is decreased, because complete oxidation of fatty acids yields less ATP per molecule of oxygen consumed than oxidation of glucose, and because fatty acids promote mitochondrial uncoupling (3).High rates of uptake of long-chain fatty acid by mitochondria enhance the production of reducing equivalents in the mitochondrial electron transport chain, and cause an overproduction of reactive oxygen species and reactive nitrogen species (4). In addition, mitochondrial dysfunction in diabetes is characterized by reduced expression of components of the electron transport chain, and impaired mitochondrial respiration.

Excessive generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) may contribute to endothelial dysfunction and play a critical role in the progressive deterioration of vessel structure and function. Hyperglycemia may impair endothelial NO production and alter the intracellular reduction-oxidation state in endothelial cells, which is believed to associate with endothelial dysfunction and vascular complications in diabetes (5**)**. Endothelial dysfunction is a hallmark of vascular injury in diabetes and is preceded by the development of overt cardiovascular diseases. Cardiovascular disease is a common complication of diabetes. Endothelial dysfunction as a first step in the pathogenesis of diabetes promotes arteriosclerosis. Mechanistically, uncoupling of the endothelial nitric oxide synthase (eNOS) in blood vessels of diabetic patients leads to excessive superoxide anion (O_2^-) production and diminishes nitric oxide (NO) availability. Endothelial progenitor cells (EPCs) are bone marrow– born cells with the potential to differentiate into functional mature endothelial cells, which can substitute diseased endothelium. Decreased levels of circulating EPCs are correlated with increased risk for coronary artery disease and myocardial infarction. Reduced levels of EPCs have been described in both type 1 and type 2 diabetic patients (6).

Atherosclerosis is regarded as a progressive disease arising from the combination of endothelial dysfunction and inflammation. Indeed, inflammation has been recognized as a key point in the cluster of the insulin resistance syndrome, including diabetes, obesity, hypertension and hyperlipemia. Much of the inflammatory reactions are mediated by the endothelium, located at the interface of blood and tissue, which maintains a balance between these two compartments. (7).

Biochemical markers of myocardial injury in serum / plasma include Aspartate aminotransferase, Lactate dehydrogenase and its isoenzymes LD1, Creatinine kinase and its isoenzyme MB, Myoglobin, Cardiac Troponins Glycogen phosphorylase isoenzyme BB and Heart fatty acid binding protein. The levels of serum AST activity begin to rise 3-8 hours after the onset of the myocardial injury with peak levels on an average at 24 hours and finally it returns to normal levels in 3- 6 days (8). It was considered as a very good marker of cardiac injury as it was found to be normal in pulmonary embolism, acute abdominal conditions and other heart conditions such as angina and pericarditis (9).

MATERIALS AND METHODS

Collection of Samples

Study subject: 50 volunteer Diabetic patients, who were undergoing treatment in Government Rajaji hospital, Madurai were subjected to the present study. The study population involves 25 males and 25 females. The samples were collected with concerned of the patient.

Blood collection: 3 ml of whole blood was collected from the patients with a sterile syringe. The tubes devoid of anti coagulant were involved for the separation of serum. The serum was used for quantification of reactive nitrogen species, serum glutamate oxaloacetate transaminase, in the diabetic patients.

Quantification of SGOT

1mL of working reagent was mixed with 50µl of serum sample and after for each 1 minute initial absorbance was recorded for 3 minutes. Then the difference between consecutive absorbance, and the average absorbance difference per minute was calculated (Δ A/min).

The AST/SGOT concentration in the serum sample is calculated using the formula:

$$\Delta A/\min \times \frac{Vt \times 10^6}{\varepsilon \times 1 \times Vs}$$

Δ A/min – Absorbance difference per minute**,** Vt – Total reaction volume**,** ε – Molar absorbance of NADH at 340nm (6300)**,** Vs – Sample volume**,** l –Light path (1 cm).

Quantification of reactive nitrogen species in serum.

1gm of powdered zinc was washed with HCL(1mol/L)three times, and then placed in 100ml of distilled water .A saturated solution of cadmium acetate was added drop by drop. Having stringently washed slowly added on to the treated zinc, it was then washed once more with distilled water .catalyst was stored in an NH_4Cl borate buffer (Merck) adjusted to Ph 8.5 at 4C.The catalyst was always used within one week of preparation. Serum nitrite concentration was determined employing Griess reagent using the diazotization reaction as a calorimetric method. 100 µl of serum was diluted to two fold with deionised water (200µl) and 200 µl of zinc cadmium catalyst was added and allowed to react for one hour at room temperature .it was then centrifuged at 15,000rpm for 2 minutes and 100 µl of supernatant was mixed with Griess reagent and the absorbance was read at 630 nm after the formation of purple color. Nitrate concentration in serum was measured after reducing it in to nitrite using the catalyst after incubation of 1 hr RT. The absorbance was read on a micro plate reader, using a reference wave length of 630nm.Different concentration (10µM - 100µM) of sodium nitrate was prepared for the standard.

RESULTS & DISCUSSION

The incidence and prevalence of diabetes (10, 11, 12) is increasing in general public. In 2007 the number of people with diabetes in adult population was estimated to be 246 million. Based on demographic changes like urbanization by the year 2025 the number of individuals with diabetes is estimated to rise to 380 million (13). The worldwide prevalence of diabetes is similar in men and women, being somewhat higher in men under the age of 60 years and in women at advanced age. Majority of individuals with diabetes in the developed countries are over the age of 64 years and in the developing countries between 45 to 64 years (11). Individuals with diabetes generally have more CVD risk factors compared with non-diabetic individuals. For example obesity, increased blood pressure, and dyslipidemia are more common in individuals with diabetes. [14, 15]. People with diabetes are at increased risk for cardiovascular, peripheral vascular and cerebrovascular disease (16).

Diabetes includes multiple clinical complications, one such is cardiovascular disease. Diabetes plays a very silent role in cardiac arrest, Diabetes mellitus may be considered as second cause of Acute Myocardial Infarction (AMI).Diabetes is associated with micro vascular complications and diabetic individuals are at high risk of developing macrovascular diseases. CVDs account for circa two thirds of deaths in diabetic patients (17). It is estimated that most individuals with type 2 diabetes have a manifest CVD (Cardio Vascular Disease) or have a greatly increased risk of a CVD event in the future (17). Diabetes is a well-known risk factor for CHD (Coronary Heart Disease) development in both genders. It is because in diabetes the heart is exposed to an increased supply of fatty acids and of glucose.

Apart from the hyperglycemic condition of diabetes the Oxidative and Nitrosative stress also play an important role in coronary heart damage in diabetes. As they are likely to have high oxidative and nitrosative stress which damage the cells and affect the oxidation of biomolecule thereby increase the risk of heart failure. Hong Nie, *et al.,* worked on endothelial nitric oxide synthase dependent tyrosine Nitration of prostacyclin synthase in diabetes in vivo and reported that there is evidence that reactive nitrogen species are implicated in diabetic vascular complications, but their sources and targets remain largely unidentified**.** They reported that exposure of human aortic endothelial cells to high glucose leads to augmented production of superoxide anion ($O2^{\cdot-}$), which may quench NO, thereby reducing the efficacy of this potent endothelium-derived vasodilator system and generating toxic oxidant species, such as peroxynitrite ($ONOO^{-}$) .$ONOO^{-}$ is a highly reactive species and can initiate both nitrosative and

oxidative reactions in vitro and in vivo. A characteristic reaction of $ONOO^{--}$ is the nitration of protein-bound tyrosine residues to generate 3-nitrotyrosine–positive proteins (19)
Igor Afanas'ev also suggested that physiological free radicals superoxide and nitric oxide together with their derivatives hydrogen peroxide and peroxynitrite (all are named reactive oxygen species (ROS) and reactive nitrogen species (RNS)) play a more important role in heart diseases through their signaling functions. Overproduction of ROS (oxidative stress) is a main origin of the transformation of normal physiological signaling processes into the damaging ones. Nitric oxide synthases (NOS) catalyze conversion of L- arginine to L-citrulline and nitric oxide but under uncoupling conditions these enzymes also produce superoxide:

$$\text{NOS} + \text{L-arginine} + O2 + \text{NADPH} \longrightarrow \text{NO} + \text{citrulline} + \text{NADP}+$$
$$\text{NOS (Fe (II) heme)} + O2 - \rightarrow \text{NOS (Fe(III)heme)} + O_2^{\bullet -}$$

Two NOS isoforms neuronal NOS (nNOS, NOS1) and endothelial NOS (eNOS, NOS3) are constitutively expressed in cardiomyocytes while inducible NOS (iNOS, NOS2) is absent in the healthy heart but its expression might be stimulated by prooxidants (21). It was showed that hypertrophied myocytes exhibited the elevated level of iNOS. Chen et al. found the expression of all genes regulated NO synthases was reduced in the hearts of patients with coronary heart disease (CHD). As NOSs are able to produce both RNS and ROS, the effects of these enzymes on cardiovascular system can be very complicated—they can enhance or diminish heart damage. Nitric oxide is the endothelium-derived relaxing factor (EDRF); therefore its function must be mainly favorable at the heart. However, the diffusion-controlled reaction of nitric oxide with superoxide produces the highly reactive peroxynitrite, a really harmful agent.
Long-term presence of high glucose may enhance cellular senescence and decrease cell number/ proliferation of EPCs. Furthermore, impaired NO- rather than activated oxidative stress–related mechanisms could be the main contributor to high glucose–induced EPC dysfunction.(5)
Mutual existence between host and therapy is the only possible tool to reduce the intensity of diabetes associated complication, but as age proceeds the mutual balance gets altered, thereby the intensity of clinical complications increases as age proceeds. Proper nutritional interventions, physical and mental involvement of the individual plays a key role in fighting against diabetes apart from therapeutic management.
As men are already at the high risk of cardiac attack, this stress intensifies the risk of the same. Our study documents the relation between cardiac dysfunction and RNI levels in which both increases accordingly. Fig1, 2 and 3 infers the association of SGOT and RNI with glucose and SGOT with RNI and documents positive correlation.

Statistical Analyses

Table: 1

Variable	**Mean ± SD**	
	Controls (n=30)	Diabetic (n=50)
Blood Glucose (mg/dl)	90.1±6.467	240.44±85.31
SGOT (units/L)	35.44±2.501	43.36±18.02
Serum RNI (µg/ml)	120±4.03	160.02±31.09

*All Variables are Significant at P<0.001
SD: Standard Deviation, SGOT: Serum Glutamate Oxaloacetate Transaminase, RNI: Reactive Nitrogen Intermediates.

Table:2 Regression analysis of SGOT and Serum RNI on Blood glucose level of diabetic patients

Variables	Controls (n=30)	Patients (n=50)
SGOT	R square =0.06773	R square=0.2881
Serum RNI	R square =0.06889	R square =0.4017

*p< 0.0001

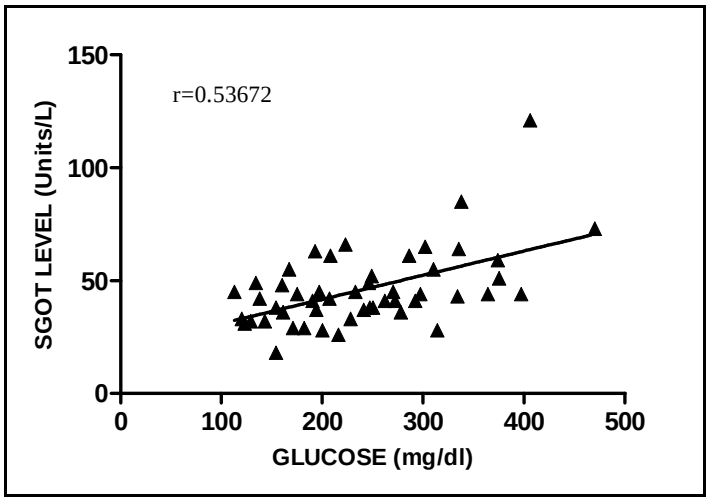

Fig: 1 Graph shows correlation between blood glucose and SGOT level in the patient group

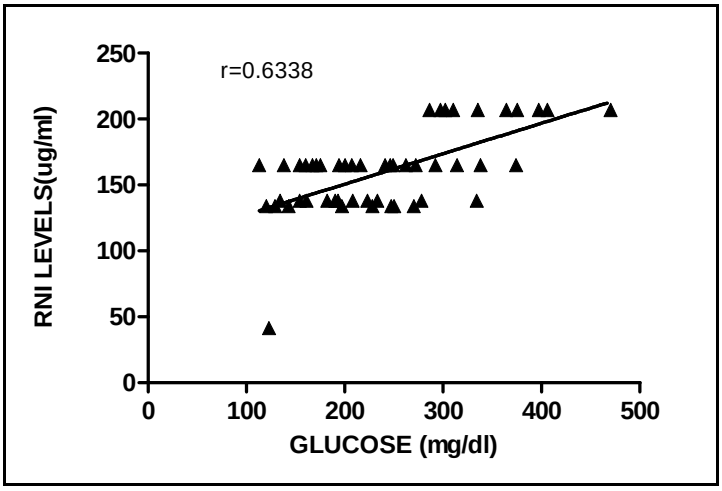

Fig: 2 Graph shows correlation between blood glucose and Serum RNI level in the patient group

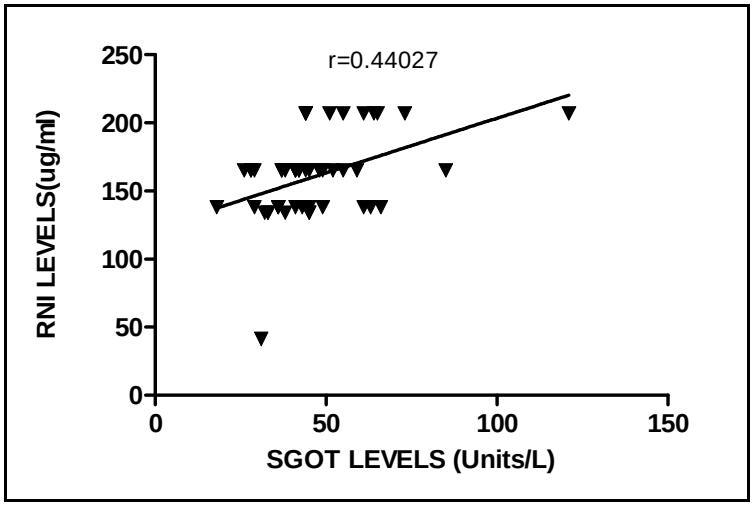

Fig: 3 Graph shows correlation between SGOT and Serum RNI level in the patient group.

CONCLUSION

Reactive Nitrogen Intermediates (RNI) is non-specific immune defenses of the system. During the oxidative stress / deficient anti-oxidant potential may lead to nitrosative stress (accumulation of RNI). These toxic moieties bind on the receptors of the vascular tissue leading to the aggressive biological disturbances of cardiac function. Proper anti-oxidant supplement need to be emphasized to minimize the cardiac associated complication among diabetic.

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