STUDY ON SOD1 GENE +35A>C POLYMORPHISM IN IMPAIRED GLUCOSE REGULATION PATIENTS OF BANGLADESHI ORIGIN

A DISSERTATION SUBMITTED TO THE BRAC UNIVEVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTERS OF SCIENCE IN BIOTECHNOLOGY

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DECLARATION OF ORIGINALITY OF THE WORK

This is to confirm that the thesis entitled 'Study on SOD1 gene +35a>c polymorphism in impaired glucose regulation patients of Bangladeshi origin' is submitted by the undersigned in partial fulfillment for the degree of Masters in Biotechnology, Department of Mathematics and Natural Sciences, BRAC University, Dhaka and the work was carried out under joint supervision of **Professor Naiyyum Choudhury**, Coordinator, Biotechnology and Microbiology Programmes, BRAC University and **Professor Liaquat Ali**, Vice Chancellor, Bangladesh University of Health Science Dhaka, Bangladesh in the Laboratory of BUHS during the period of December, 2012 to December, 2013.

No part of the work has been submitted for any other degree or diploma in any other institute at home or abroad.

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DEDICATED

TO

MY BELOVED PARENTS

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
ADP	Adenosine Di Phosphate
ATP	Adenosine Tri Phosphate
AGE	Advanced Glycation End Products
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
BMI	Body Mass Index
BMRG	Biomedical Research Group
CI	Confidence interval
DBP	Diastolic Blood Pressure
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DN	Diabetic Nephropathy
DR	Diabetic Retinopathy
EDTA	Ethylene Diamine Tetracetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ESRD	End-Stage Renal Disease
FG	Fasting Glucose
GDM	Gestational Diabetes Mellitus
GFR	Glomerular Filtration Rate
HDL-c	High Density Lipoprotein Cholesterol
HOMA %B	Homeostasis Model Assessment B-cell Function
HOMA %S	Homeostasis Model Assessment Insulin Sensitivity
Ht	Heterozygous
Hz	Homozygous
IDDM	Insulin Dependent Diabetes Mellitus
IDF	International Diabetes Federation
IFG	Impaired Fasting Glucose
IGT	Impaired glucose tolerance
LDL-c	Low Density Lipoprotein Cholesterol
MODY	Maturity Onset Diabetes in Young

mRNA	Messenger Ribonucleic Acid
MAP	Mitogen-Activated Protein Kinase
NIDDM	Non-insulin Dependent Diabetes Mellitus
NIH	National Institute of Health
OGTT	Oral Glucose Tolerance Test
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PKC	Protein Kinase C
ROS	Reactive Oxygen Species
RFLP	Restriction Fragment Length Polymorphism
SBP	Systolic Blood Pressure
SD	Standard Deviation
SGPT	Serum Glutamic Pyruvic Transaminase
SNP	Single Nucleotide Polymorphism
SOD 1	Superoxide Dismutase 1
SOD 2	Superoxide Dismutase 2
SOD 3	Superoxide Dismutase 3
SPSS	Statistical Package for Social Sciences
T Chol	Total Cholesterol
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglyceride
VLDL	Very Low Density Lipoprotein
VLDL-c	Very Low Density Lipoprotein Cholesterol
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

LIST OF SYMBOLS

%	Percentage
>	Greater than
<	Less than
^{o}C	Degree Centigrade
µg	Microgram
µl	Microliter
$\bullet O_2^-$	Superoxide Radical
H_2O_2	Hydrogen Peroxide
ONOO−	Peroxynitrite

ABSTRACT

Superoxide dismutase 1 (SOD1), located at the cytosol has been postulated to represent between 50%-80% of the total SOD activity. The SOD gene is located at 21q22.1. Polymorphisms in the genes encoding these enzymes found to be significantly affecting its antioxidant role. As oxidative stress is a common pathogenic factor for the dysfunction of beta and endothelial cells, polymorphism of SOD genes and oxidative stress has become a subject of intense scrutiny for their association with different disease conditions including T2DM. The study was aimed to assess the possible association of SOD1 gene +35A/C polymorphism in the pathogenesis of IGR subjects of Bangladeshi origin. A total number of 54 middle aged (age range: 30-65year) subjects with Impaired Glucose Regulation (IGR) were consecutively recruited. Total number of healthy control was 55. IGR was diagnosed following WHO guideline. Blood glucose was measured by glucose-oxidase and, triglyceride, total cholesterol and HDL-cholesterol by enzymatic colorimetric method. Insulin was estimated by enzyme linked immunosorbent assay (ELISA). DNA was extracted using QIAGEN Blood DNA Kit which utilizes silica gel DNA separation. SOD1 gene +35A/C variant was determined by PCR-RFLP using restriction endonuclease HhaI. Data were managed using Statistical Program for Social Science (SPSS). Unpaired Student's -`t' test and Chi-squared tests, as appropriate, were performed. The number of study subjects of wild AA genotype in control and IGR group was 52 and 54 respectively whereas in case of heterozygous (Ht) variant AC genotype the number was 0 and 2 respectively. SOD1 +35A>C genotype frequency analysis has not shown statistical significant association ($\chi^2 = 2.075$ and $p= 0.150$). For control group, the genotype frequencies of AA and AC were 1.0 and 0 respectively. For IGR group, the frequencies were 0.963 and 0.037 respectively. The data showed that SOD1 gene +35A>C variant is substantially different from other population and the polymorphic allele is not associated with insulin secretory defect of IGR subjects of Bangladeshi origin.

Chapter 1

Introduction

1. INTRODUCTION

Diabetes is one of the most prevalent and devastating chronic non-communicable diseases having serious health, economic and social consequences (IDF 2006). Diabetes mellitus has, long been considered as a disease of minor significance to world health, however, it is now treated as one of the main threats to human health in the 21" century (Zimmet *et al*., 2000). In the past two decades there has been an explosive increase in the number of people diagnosed with diabetes all over the world (Amos *et al*., 1997; King *et al*., 1998). Therefore, it may seem strange that the developing world, which is associated with hunger and malnutrition, is now experiencing an epidemic in Type 2 diabetes. 80% of people with diabetes live in low- and middle-income countries (IDF, 2012). Subsequent study estimated the global prevalence of diabetes for all age - groups to rise from 2.8% in 2000 to 4.4% in 2030 and also demonstrated the total number of diabetics to rise from 171 million in 2000 to 366 million in 2030. It was also shown that about two third of the rise in total diabetic patients would be constituted from those of the developing countries particularly China, India, Indonesia, Pakistan and Bangladesh (Wild *et at.,* 2004). Based on recent community based study, prevalence of diabetes in Bangladesh was found to be about 7%.Studies involving rural and urban population revealed the prevalence of diabetes 4% and11.4% respectively (Sayeed *et at*., 2007).

The term diabetes mellitus describes a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both (WHO, 1999; ADA, 2005). The effects of diabetes mellitus include long term damage, dysfunction and failure of various organs. Diabetes mellitus may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss (WHO, 1999). In its most severe forms, ketoacidosis or a non–ketotic hyperosmolar state may develop and lead to stupor, coma and, in absence of effective treatment, death (WHO, 1999).

The World Health Organization (WHO) published estimates for the years 2000 and 2030, using data from 40 countries but extrapolated to the 191 WHO member states (Wild *et al*., 2004). Diabetes mellitus and its complications contribute significantly to ill health, disability, poor quality of life and premature death. Additionally, diabetes mellitus is a contributing factor to

several other causes of morbidity and mortality. It also increases the risk of a variety of complications including end-stage kidney disease, heart disease, stroke and other vascular diseases. According to WHO 346 million people worldwide have diabetes. More than 80% of diabetes deaths occur in low and middle-income countries. WHO projects that diabetes death will double between 2005 and 2030 (WHO 2011).

1.1 Classification of Diabetes Mellitus

Diabetes mellitus has long been classified based on its clinical presentation; age of onset and special feature, and need for insulin of the individual to control blood glucose. Lawrence (1951) was first to introduce the nomenclature type I and type 2 to describe two distinct forms of diabetes mellitus. In late seventies the National Institute of Health (NIH), United States of America (USA) set up the National Diabetes Data Group (NDDG) that formulates the classification and diagnostic criteria. The NDDG introduced the term insulin dependent diabetes mellitus (IDDM, Type 1) and noninsulin dependent diabetes mellitus (NIDDM, Type 2) to describe two main classes of diabetes (NDDG 1979).

Table 1.1: Etiological Classification of Diabetes Mellitus (WHO, 1999)

A. Type 1 (B-cell destruction, usually leading to absolute insulin deficiency)

Type I A- Autoimmune mediated type

Type 1 B- Non-immune mediated idiopathic type

B. Type 2 (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance)

C. Other Specific Types of Diabetes

- Genetic defects of B-cell function
- Genetic defects in insulin action
- Diseases of the exocrine pancreas
- Endocrinopathies
- Drug- or chemical-induced
- Infections (Congenital rubella, Cytomegalovirus, Others)
- Uncommon forms of immune-mediated diabetes
- Other Genetic Syndromes Sometimes Associated with Diabetes

(Down's syndrome, Friedreich's ataxia, Turner's syndrome, etc)

D. Gestational diabetes

According to the present classification of diabetes four main classes of the disease are type 1, type 2, Other Specific types and Gestational Diabetes Mellitus (Table 1.1) (WI 10 1999 and ADA 1997).The other subclass was protein deficient diabetes mellitus (PDDM). Among the young diabetes FCPD was found to be 13% and PDDM 42% (Azad Khan and Ali 1997). The others with BMI>19 and diabetes diagnosed at younger age were termed as non insulin dependent diabetes mellitus in young (NIDDY).

1.1.1 Type 1 Diabetes Mellitus

T1DM is characterized by sudden onset of symptoms, proneness to kitoacidosis and need of insulin for survival. The hallmark of the T1DM is pancreatic B cell damage resulting in very low to absolute loss of insulin secretion. T1DM mainly occur in children and young adults and accounts for about 10% of all diabetic patients (ADA 1997). The incidence of T1DM was found to be lowest in Asia (0.1 per 1000,000 in China) and highest in Scandinavia (35.5 per 1000,000 in Finland). However, there are notable exceptions, for instance the incidence is low in Iceland but in Sardinia, Italy the incidence is similar to that of Finland (Karvonen *et al.,* 2000). A report from Southern India has shown that these the incidence of T1DM is similar (10.5/1000,000) to many European populations (Rainachandran *et al.,* 1986). T1DM is believed to be less prevalent in Bangladesh, however, population based studies are still lacking.

T1DM is sub-classified on the basis of type of damage of B-cells; immune mediated type 1 and non-immune mediated type 1 (idiopathic type 1, T1DM) (ADA 1997; WHO 1999). Autoimmune T1DM is characterized by immune mediated damage, targeted against self antigens, resulting in the destruction of the B-cells of the pancreas. A number of autoantibodies have been detected against different islet proteins. The rate of B-cell destruction is found to vary widely. It has been seen to be usually faster in children and slower in adults. A number of T1DM patients have a typical sudden onset of the disease and are ketosis prone. But they lack classical autoantibodies directed to islets and/or islet cell proteins and are called idiopathic T1DM. This form of diabetes is thought to be most common in African and Asian countries (McLarty *et at.,* 1990). Idiopathic T1DM usually occurs to obese teens. Once their blood glucose is controlled by insulin oral hypoglycemic agents can be subsequently used before the onset of complete insulin dependence as in T1DM. While the exact etiological factor(s) are still unknown, multiple genetic and environmental factors are thought to be involved (ADA 1997; WHO 1999).

In another in depth study, autoantibody and candidate gene markers were investigated in young onset diabetic subjects. T1DM diabetes related antibodies; anti Glutamic acid decarboxylase (GAD) antibody and anti 1A2 antibodies were found to be positive around 25% of the study subjects (Hassan *et al.*, 2005). INS-VNTR polymorphism did not show any association with diabetes. T1DM related auto antibodies were found to be positive in 20% of study subjects (Hassan 2006). This suggested diabetes in the young subjects of Bangladesh may represent a group of atypical T1DM.

1.1.2 Type 2 Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is characterized by the presence of disorders of insulin action and/or insulin secretion (Reaven 1988). Both defects are usually present in a T2DM patient; however, the primacy of the two factors for the development of diabetes still remains to be clearly understood.

In American Indians and South Pacific Islanders, T2DM is the only form of the disorder. Overall prevalence of diabetes varies between 15-20%. The highest prevalence of T2DM (50%) was found among Pima Indians (49.4% in male and 51.1 % in female) in USA and Nauru (41 %; male 40.0% and female 42%) and a very low (0-1.4%; male 0% and female 1.4%) was observed among the Mapuches population in Chile and the prevalence was almost nil in rural and peri-rural population of Papua New Guinea (WHO 1994). In the non-Euripides population the prevalence has been shown to vary from <1% in rural Bantu of Tanzania and Mainland China to 40-50% in Pima Indians (USA) and Nauru (King and Rewers, 1993). Age-standardized prevalence of T2DM among native Asians and non-Indian Asians demonstrated an increased trend from rural to urban and markedly increased trend among the migrants to the west and affluent countries. A similar trend also has been observed among native and migrant Pacific Island populations (Coughlan et al., 1997). In multiethnic US society the prevalence of diabetes has shown to vary among different ethnic groups; 5% in the Caucasian origin, 10% in African-Americans, 16% in Cuban origin and 26% in Puerto Ricans (Harris 1991; Flegal *et al*., 1991). Studies involving rural and suburban population in Bangladesh revealed the prevalence of T2D to be 4.3% and 4.1% respectively (Sayeed *et al*., 1997 and 2003).

1.1.3 Gestational diabetes

Gestational diabetes is a type of diabetes that arises during pregnancy (usually during the second or third trimester). In some women, it occurs because the body cannot produce enough insulin to meet the extra needs of pregnancy. In other women, it may be found during the first trimester of pregnancy, and in these women, the condition most likely existed before the pregnancy (Claire 2008). Gestational diabetes affects up to 5% of all pregnancies (Lancet 2008). Women who are overweight or obese are at a higher risk of gestational diabetes. The lifetime risk of developing T2DM after gestational diabetes is 30% (Girling *et al*., 2004).

1.2 prevalence of diabetes mellitus

1.2.1 Global prevalence of diabetes mellitus

Diabetes mellitus (DM) is one of the most common non-communicable diseases (NCDs) globally. Diabetes is undoubtedly one of the most challenging health problems in the 21st century. The IDF Diabetes Atlas (5th edition) estimates that diabetes caused 4.6 million deaths in 2011 and 183 million people (50%) with diabetes are undiagnosed (IDF, 2012). The number of people with diabetes is increasing in every country. Recent global feature of DM is summarized in the table 1.2

1.2.2 Diabetes in Bangladesh

Bangladesh, which is still fighting with communicable diseases, is already overburdened with non-communicable diseases. The total number of people with diabetes in Bangladesh is projected to rise from 8.4 million in 2011 to 16.8 million in 2030 (IDF, 2012). Several small surveys in Bangladesh have shown an increasing trend (Mahtab *et al*., 1983; Ali *et al*., 1985; Sayeed *et al*., 1985; Sayeed *et al*., 1995). Over the past 30 years, the increase in prevalence is rising exponentially in South Asian region; data suggest a threefold increase (from 2.0 to 7.0%) in the urban population of Bangladesh within 5 years (Rahim *et al*., 2010). Although the rural population constitutes >85% of the country's total population, almost 65% of the registered diabetic subjects of the Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) were from urban areas (Sayeed *et al*., 1993; Sayeed *et al*., 1994).

According to the finding of Bangladesh Non-Communicable Diseases (NCD) risk factor survey 2010, the prevalence of diabetes was 3.9%. Bangladesh Urban Health Survey 2006 suggests that in urban area the prevalence is just double (10%) the rural area (5%). In 2010, the IDF estimated that 5.7 million (6.1%) and 6.7 million (7.1%) of people living in Bangladesh is suffering from diabetes and impaired glucose tolerance (IGT) respectively. By 2030, that number of diabetic population is expected to rise to 11.1 million. This explosion in diabetes prevalence will place Bangladesh among the top seven countries in terms of the number of people living with diabetes in 2030. According to the latest WHO data published in April 2011 diabetes mellitus deaths in Bangladesh reached 19,598 or 2.05% of total deaths. The death rate is 23.80 per 100,000 of population, which ranked Bangladesh in 109th position in the world (World health rankings, 2010).

Table 1.2 Prevalence of DM and IGT estimated for the years 2011 and 2030 among Asian population (IDF, 2012):

Country	Prevalence (%) adjusted to				Numbers of adults with diabetes (000s)		Mean annual increment (000s)
	World Population		National population		2010	2030	
	2010	2030	2010	2030			
Bangladesh	6.6	7.9	6.1	7.4	5681	10423	237
Cambodia	5.2	7.5	4.3	5.8	354	724	19
China	4.2	5.0	4.5	5.8	43157	62553	970
Dem. Rep of Korea	5.3	6.2	5.7	6.8	943	1256	16
India	7.8	9.3	7.1	8.6	507568	78036	1813
Indonesia	4.8	5.9	4.6	6.0	6964	11980	251
Japan	5.0	5.9	7.3	8.0	7089	6879	-11
Malaysia	11.6	13.8	10.9	13.4	1846	3245	70
Myanmar	3.2	4.3	2.8	4.3	922	1755	42
Nepal	3.9	5.2	3.3	4.2	511	1070	28
Philippines	7.7	8.9	6.7	7.8	3398	6164	138
Republic of Korea	7.9	9.0	9.0	11.4	3292	4323	52
Sri Lanka	10.9	13.5	11.5	14.9	1529	2158	31
Taiwan	7.5	8.5	5.7	6.8	816	1232	21
Thailand	7.1	8.4	7.7	9.8	3538	4956	71
Viet Nam	3.5	4.4	2.9	4.4	1647	3415	88

In Bangladesh, the percentage of diabetes was 5.2% (rural 4.3%, urban 6.9%) in 1994-95 and increased to 11.2% (urban) and 6.8% (rural) in 2003-O4 (Mahbubur *et al*., 2011). A recent

population based study showed a significant increase in the prevalence of DM in rural Bangladesh from 2.3% to 6.8% over 5 years (Afroza 2011). Table 1.2 summarizes the 2006 WHO recommendations for the diagnostic criteria for diabetes and intermediate hyperglycaemia.

1.3 Natural history of diabetes mellitus

Patients with IFG and/Or IGT are now referred to as having 'prediabetes' by ADA or IGR by WHO (Figure 1.1). IFG and IGF refer to a metabolic state intermediate between normal glucose homeostasis and diabetes. They are not interchangeable and represent different abnormalities of glucose regulation, one in the fasting state and one postprandial (WHO consultation, 1999).

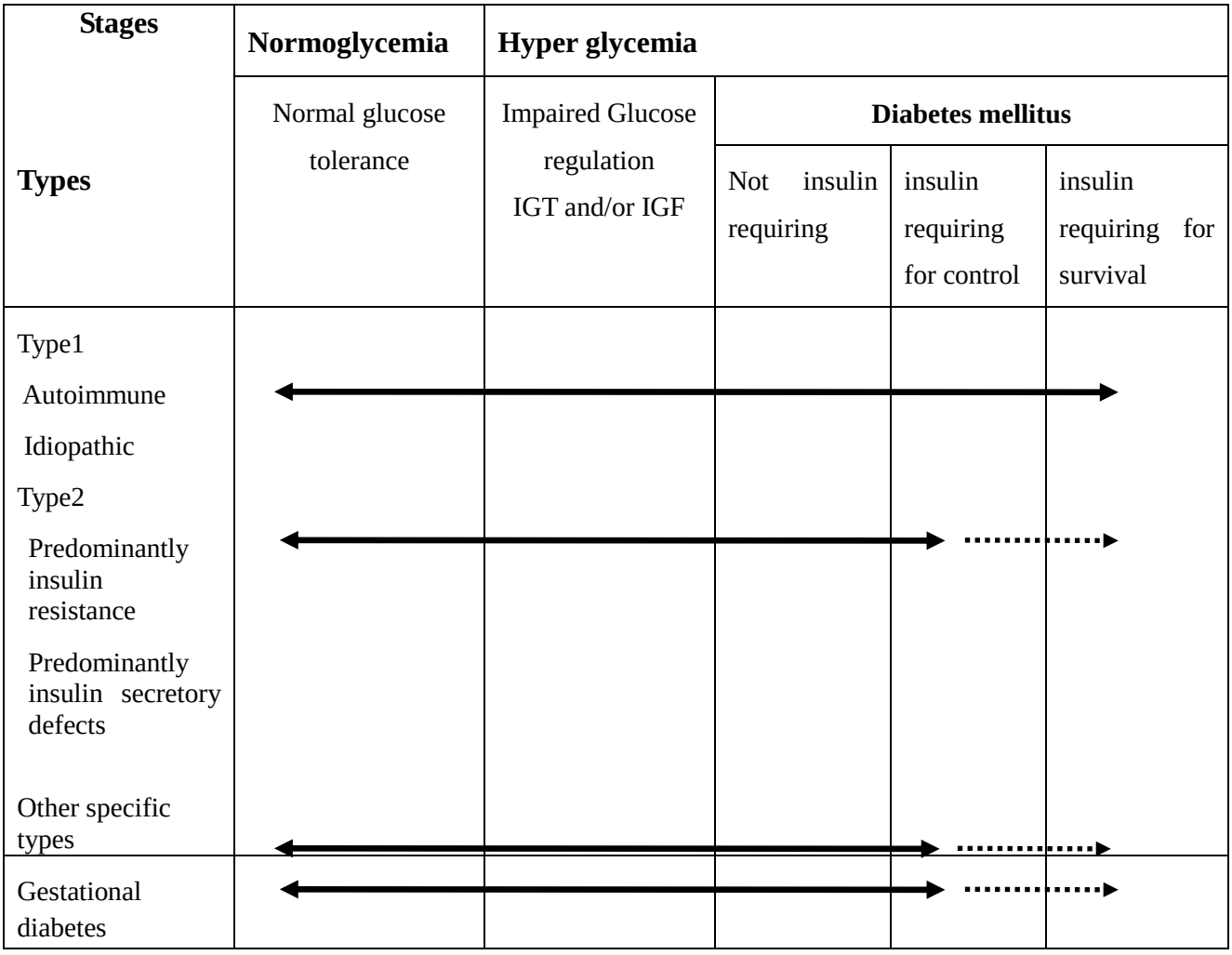

Figure 1.1: The stages of glucose regulation in different types of DM (Balkan and Eschwege 2003).

All forms of diabetes can pass through a stage of IFG and/or IGT. These categories are a part of the natural history of diabetes and not a type of diabetes. That is why they are not included in the classification of diabetes mellitus by ADA. The additional study, conducted in Denmark was to see progression from impaired fasting glucose and impaired glucose tolerance to diabetes in a high-risk screening program in general practice. IFG and IGT identified in general practice during a stepwise high-risk screening program for type 2 diabetes has high 1-year progression rates to diabetes. The progression rate for IFG was 17.6 and for IGT 18.8 cases per100 persons-year. When analyzing IGT as two separate categories, i.e. isolated IGT and combined IFG - IGT, the progression r were 12.0 and 28.1 diabetes cases per 100 person-years respectively. (Rasmusseatesn *et al*, 2007). Interestingly, there is a tendency for the prevalence rates of IGT to decline as those of type 2 diabetes mellitus (DM) rise, perhaps suggesting that areas with a high ratio of IGT, Type 2 DM are at an earlier stage of type 2 DM epidemic and thus a particular target for preventive strategies (Tony and Cockram, 2003).

In Taiwan, during the 3-year follow-up, 4.3% of the total population developed diabetes. Of those with IFG at baseline, 9.6% progressed to diabetes, but only 2.5% of normoglycemic people did so. The multivariate-adjusted odds ratio of developing diabetes was 4.4% for persons with IFG compared with those who were normoglycemic at baseline. Other Significant predictors' pf progression to diabetes was higher waist-hip ratio (WHR), triglyceride and apolipoprotein B (apo B) levels. In these Asian Chinese populations, IFG is a strong predictor of diabetes. The high rate of conversion from lFG to diabetes, combined with the previously observed high IFG prevalence, suggests future high prevalence rates of diabetes (Chen *et al*, 2003).

1.4 Prediabetes

Two intermediate groups at risk for diabetes are impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). IFG and IGT refers a metabolic stage intermediate between normal glucose homeostasis and diabetes; these are now termed as prediabetes by ADA and impaired glucose regulation by (IGR) WHO. They are not interchangeable and represent different abnormalities of glucose regulation, one in fasting state and one postprandial (WHO, 1999). According to fasting and post load glucose concentration, at present patients with IGR or prediabetes may be stratified into three subcategories - (1) Isolated IGT; (2) Isolated IFG; and (3) Combination of the two 'IFG-IGT' (WHO and ADA, 2002).

1.4.1 Impaired fasting glucose

IFG is defined, as fasting plasma glucose between 6.1 and 6.9 mmol/l and 2h plasma glucose <7.8 mmol/l. Recently ADA has lowered the cut off value of fasting plasma glucose in IFG from 6.1 mmol/l to 5.6 mmol/l (ADA, 2005).

1.4.2 Impaired glucose tolerance

IGT IS defined as fasting plasma glucose <6.1 mmol/l and 2h plasma glucose between 7.8 and 11.0 mmol/l (ADA. 2005). Historically the term IGT was first introduced by the National diabetes Data Group in 1979 and later re-established by WHO. In this stage blood glucose values are higher than the defined normal levels but not high enough to meet the diagnostic criteria for diabetes (Rao er al., 2004).

1.4.3 Combined IFG-IGT

Studies involving Bangladeshi as well as other population demonstrated some subjects have feature of both IFG and IGT, they are termed as combined IFG-IGT. They have fasting plasma glucose 6.1- 6.9 mmol/1 and 2h plasma glucose 7.8 - 11.0 mmol/l (ADA, 2005). In one particular study in Denmark the progression of IFG-IGT to diabetes found to be 28% per year (Rasmussen *et al*., 2007). IFG and IGT are asymptomatic and unassociated with any manifested morbidity, but their sole significance lies in the fact that they predict future diabetes or cardiovascular diseases (Stern and Burke, 2000).

1.4.4 Features of IFG-IGT

The main features of IFG/IGT are: 1) a stage in the natural history of disordered glucose metabolism, 2) can lead to any type of diabetes, 3) increased risk of progression to diabetes, 4) increased risk of cardiovascular diseases 5) little or no risk of micro vascular diseases, and 6) some patient may revert to normoglycemic state (Balkau and Eschwege, 2003). Both IFG and IGT are similarly associated with an increased risk of diabetes mellitus. Risk is higher where IGT and IFG coexists (Unwin, 2002).

1.4.5 Prevalence of prediabetes

The prevalence of IFG and IGT found to increase with age (Shaw *et al*., 1999). The prevalence of IFG tends to plateau in middle age whereas the prevalence of IGT rises into old age (Unwin, 2002). IGT is more prevalent than IFG, less than or equal to 50% of people with IFG has IGT and 20-30% with IGT also has IFG. The rising prevalence of IGT is assumed to increase from 8.2 to 9.0% worldwide and 7.1 to 7.8% in Bangladesh from 2003 to 2005 in adults (20-79 yrs age groups). The 40-59 age group currently has the greatest number of persons with IGT (Sicree *et al*., 2003). On the other hand, the crude the prevalence of IFG was 12.4% in rural population of Bangladesh (Sayeed *et al*., 2003).

Table 1.3: Prevalence of IGF and IGT among different population (Abdul-ghani *et al*., 2006)

Population	Prevalence of isolated IGT	Prevalence of isolated IFG	Prevalence of IGT with IGT	Prevalence of IFG with IGT
European	8.8	6.9	26.0	31.0
Australian	8	5.7	24.5	31.3
Mauritius	13.8	4.2	19.4	44.4
Pima Indian	10.7	1.9	18.9	56.8
Swedish	20.3	9.7	27.2	43.9
Chinese	6.3	0.9	14.7	53.0
American	11	4.4	26.3	46.9
Korean	20.1	2.7	13.4	53.0

The prevalence of IFG found to be similar in men and women, but IGT is more frequent in women (Shaw *et al*., 1999). Study involves Dutch population, reported the prevalence of IFG and IGT among men to be 9.7% and 13.8%, respectively and in women is 6.1% and 14.6% (Corpeleijn *et al*., 2002). In the AusDiab study, a cross-sectional survey of adults aged 25 years and older IGT was present in 10.6% of subjects, being more common in women (11.9% v 9.2% in men), and IFG was present in 5.8%, being more prevalent in men (8.1% v 3.4% in women). This represents an overall prediabetes prevalence of 16.4% in Australian adults (≥25 years) (Dunstan *et al*., 2002). The prevalence of both IFG and IGT varies considerably based on ethnicity, ranging from a low of 6.3% in Chinese (Ko *et al*., 1998) to a high of 20.3% in a

Swedish (Larsson *et al*., 1998) *(Table 1.4)*. From DECODA (Diabetes Epidemiology: Collaborative of Diagnostic criteria in Asia) study, it was found that IGT is more prevalent than IFG in all Asian populations studied for all age-2003). The rising prevalence rate of IGT may be mainly due to diabetogenic lifestyle factors that lead to obesity and increasing life expectancy.

1.5 Pathogenesis of prediabetes and type 2 diabetes mellitus

1.5.1 Pathogenesis of prediabetes

Even though IFG and IGT represent intermediate stages of glucose intolerance, epidemiological studies demonstrated that they are likely to be distinct conditions with different pathological etiologies (Abdul-ghani *et al*., 2006). Individuals with isolated IFG manifest mainly of hepatic insulin resistance, but have relatively normal skeletal muscle insulin sensitivity. In contrast, those with isolated IGT are characterized by more severe muscle insulin resistance and less severe hepatic insulin resistance. Differences in insulin secretory abnormalities are also apparent between subjects with isolated IFG and isolated IGT whereas those with isolated IFG have defects in first phase or early insulin secretion, individuals with isolated IGT have more severe defects in second-phase or late insulin secretion. As might be expected, individuals with combined IFG-IGT manifest hepatic and muscle insulin resistance as well as impairments in both first and second phase insulin secretion. Among subjects with prediabetes, those with combined IFG-IGT most closely resemble subjects with T2DM (Weyer *et al*., 1999; Abdul-Ghani *et al*., 2006).

1.5.2 Pathogenesis of type 2 diabetes mellitus

The maintenance of normal glucose homeostasis depends on a precisely balanced and dynamic interaction between tissue sensitivity to insulin (especially in muscle and liver) and insulin secretion (Defronzo, 1988). T2DM is postulated to results from defects in both insulin secretion and/or action; both of these defects can have a genetic as well as an acquired component (Defronzo, 1998; YKI-Jarvinen, 1994; Ferrannini, 1998).

1.5.2.1 Insulin resistance

Insulin resistance believed to the early features in the pathogenesis of T2DM and plausibly results from a genetically determined reduction in insulin sensitivity, compounded by exposure

to the environmental factors (Olefsky *et al*., 1982). Important sites of insulin resistance include liver and the peripheral tissues, skeletal muscle and fat. In muscle, insulin resistance is manifested by decreased glucose uptake and impaired utilization of glucose by non-oxidative pathway, primarily glycogen formation, as well as a slight decrease glucose oxidation. In the liver, insulin resistance leads to failure of insulin to suppress hepatic glucose production, which is fuelled by glycogen breakdown and particularly by gluconeogenesis (Defronzo, 1998).

1.5.2.2 Defect in insulin secretion

Defect in insulin secretion assume to mean lower insulin secretion. However, there used to prevail some confusion by the findings of higher blood insulin concentrations compatible to or even higher than in normal subjects in newly diagnosed T2DM patients (Olefsky *et al*., 1982; Defronzo, 1998). This does not, however, is the indication of normally functioning B cells activity since non-diabetic subjects would generate much higher insulin levels if their blood glucose would have artificially risen into the diabetic range (Defronzo, 1998; Porte, 1991). Thus insulin secretion is already defective at the time of diagnosis and declines steadily thereafter. Other feature of B cell dysfunction in the disease includes defective cleavage of the insulin precursor, leading to variably increased concentrations of proinsulin and its early 'split product' derivatives (Gray *et al*., 1984).

It is generally accepted that insulin resistance precedes the failure of insulin secretion and exuberates this by imposing an increased secretory demand on the B cells. However subtle abnormalities in B cell function have been demonstrated early in the course of T2DM (Pratley and Weyer, 2001), and even in their first degree relatives (Pimenta *et al*., 1995) suggesting a possible basis for an inherited component of B cell failure, Moreover a prospective study among Pima Indians showed that passage from normal to impaired glucose tolerance (IGT) and finally to T2DM was accompanied by a progressive decline in B-cell secretory capacity (Weyer *et al*., 1999). Finally, extrapolation of the observed rate of B-cell decline observed in the UKIPDS study suggests that the loss of B-cell function begins some 10-12 years before diabetes is diagnosed (Holman, 1998).

1.6 Risk factors for T2DM

1.6.1 Environmental factors

A number of environmental factors implicated in the pathogenesis of type 2 varieties, which include ethnicity, age, diet, and viral infection and stress (Knip and Akerblom 1999; Dahlquist 1998).

Ethnicity: T2DM is up to six times more common in people of South Asian descent and upto three times among people of Africa and Africa-Caribbean origin (David, 1999). According to the Health Survey for England 2004, doctor diagnosed diabetes is almost four times as prevalent in Bangladeshi men, and almost three times as prevalent in Pakistani and Indian men compared with men in the general population. Among women, diabetes is more than five times as likely among Pakistani women, at least three times as likely in Bangladeshi and Black Caribbean women, and two and a half times as likely in Indian women, compared with women in the general population. Diabetes found to be generally rare among those aged 16 to 34 years but was highest among Indian men (2%), Black African men (1.7%) and Irish women (1.7%) (Elizabeth *et al*, 2006).

Age: Glucose tolerance has been shown to decrease with age (Shimokata *et al.,* 1991).
However, whether the deterioration is caused by increased chronological age or secondary age related factors, such as decreased physical activity, is still to be clearly understood.

Diet: Diets with high-fat, low-carbohydrate impair the action of insulin on endogenous glucose production, glucose oxidation, and probably lipolysis but it does not clearly affect the action of insulin on total glucose disposal and tend to enhance the action of insulin on non-oxidative glucose disposal (Bisschop *et al.,* 2001). Intake of excessive amounts of saturated fats have lead to obesity in individuals, who were found to be more susceptible to develop insulin resistance (Lovejoy *et al.,* 2001).

Obesity: The major environmental risk factor for T2DM is obesity and a sedentary lifestyle (Bleich *et at.,* 2007). Thus, the tremendous increase in the rates of T2DM in recent years has been attributed, primarily, to the dramatic rise in obesity worldwide (Zimmet *et al.,* 2001). It has been estimated that approximately 80% of all new T2DM cases are due to obesity (Lean, 2000). This is true for adults and children. In the Pima Indians, 85% of the T2DM children were either

overweight or obese (Fagot-Campana *et al.,* 2000). Although excess weight and obesity (BMI>25 kg/m2) account for 64% cases of diabetes in men and *74%* in women, many cases of diabetes occur in lean subjects (Chan *et al.,* 1994; Colditz *et al.,* 1995).

Sex: Some studies had showed a higher age-adjusted prevalence in women (Eriksson et. *al.,* 1993), where as some investigators demonstrated male preponderance (Lintott *et al.,* 1992). Variation in T2DM prevalence between men and women also has been shown to vary in different age group in the same community.

Stress: Stress has long been suspected as having important effects on the development of diabetes. Slawson *et al* (1963) showed that 80% of a group of 25 adult diabetes patients gave a history of antecedent stress mainly in terms of losses, 1-48 months prior to the onset of diabetes. These were mostly patient of depression. Metabolic stress may develop due to disproportionate amount of free radical productions in diabetes by glucose autoxidation, polyol pathway and non-enzymatic glycation of proteins. In a study, oxidative stress pathway markers in the diabetic patients, type 1 and type 2, was compared to normoglycemic subjects. The increased level of glycosylated hemoglobin was observed in the diabetic patients and this increase is directly proportional to the blood glucose level (Strain *et al.* 1991). This suggests the increase in oxidative stress due to hyperglycemia. Hypoinsulinaemia in diabetes increases the activity of the enzyme fatty acyl coenzyme A oxidase, which initiates R-oxidation of fatty acids , resulting in lipid peroxidation, which impairs membrane function changing the activity of membrane-bound enzymes and receptors (Halliwell *et al.,* 1997).

1.6.1.1 Oxidative stress

Oxidative stress is defined in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Turko *et al.*,2001; Maritim *et al.*,2003). ROS include free radicals such as superoxide ($\bullet O_2^-$), hydroxyl (•OH), peroxyl ($\bullet RO_2$), hydroperoxyl ($\bullet HRO_2$-) as well as nonradical species such as hydrogen peroxide (H_2O_2), and hydrochlorous acid (HOCl) (Turko *et al.*,2001; Evans *et al.*,2002). Overproduction of free radicals can cause oxidative damage to biomolecules, (lipids, proteins, DNA), eventually leading to many chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other

degenerative diseases in humans (Freidovich, 1999; Yun-Zhong *et al.*,2002). Excess vascular $\bullet O_2^-$ production could contribute to hypertension and vasospasm (Huie *et al.*,1993; Lepoivre *et al.*,1994; Moncada and Higgs, 1993).

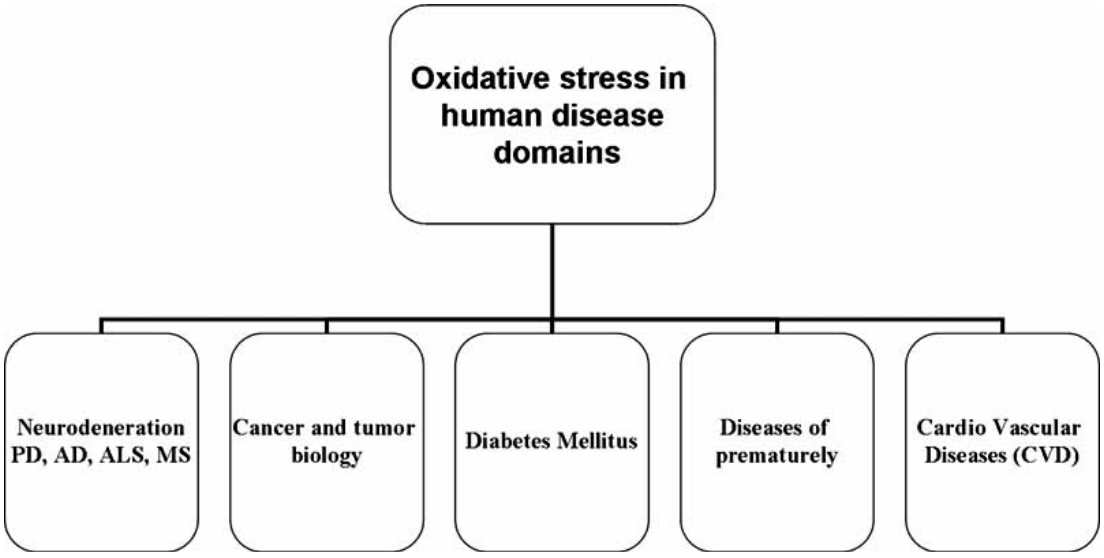

Figure 1.2: Human diseases where oxidative stress plays direct or indirect role in pathophysiology of disease (Uttara *et al.*, 2009)

1.6.1.2 Mechanism of ROS Generation

ROS generation is prerequisite of metabolic system in order to interact with organic molecules *in vivo* as interaction of organic molecules with oxygen is energetically unfavorable. In all forms of ROS generation, molecular oxygen needs to be activated and cellular system have evolved range of metallo-enzymes those facilitates ROS generation upon interaction of redox metals with O_2 using various catalytic pathways. Since free radicals are toxic to cells, under normal circumstances, cells have efficient regulating system for O_2 and metal ion interaction leading to free radicals and ROS generation (Bush, 2000).

$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$ (Step I)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$ (Step II) Known as Fenton reaction (Roger *et al.*,1997)

Combining step I&II

$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + HO^- + O_2$

Apart from direct ROS generation, there are different *in vivo* pathways that contribute substantively ROS generation by calcium activation with metallo-enzymes. Calcium is an important signaling molecule and it is required for many cellular responses and cell-cell

communication. Thus, any disturbance in stimulus and regulation of calcium pathway may disrupt the cellular physiology (Angelo *et al.,* 2005).

1.6.1.3 Sources of oxidative stress in diabetes

Direct evidence of oxidative stress in diabetes is based on studies that focused on the measurement of oxidative stress markers such as plasma and urinary F_2-isoprostane as well as plasma and tissue levels of nitrotyrosine and $\bullet O_2^-$ (Vega-Lopez *et al.*,2004; Oberg *et al.*,2004; Guzik *et al.*,2000; Ceriello *et al.*,2001; Guzik *et al.*,2002). There are multiple sources of oxidative stress in diabetes including nonenzymatic, enzymatic and mitochondrial pathways.

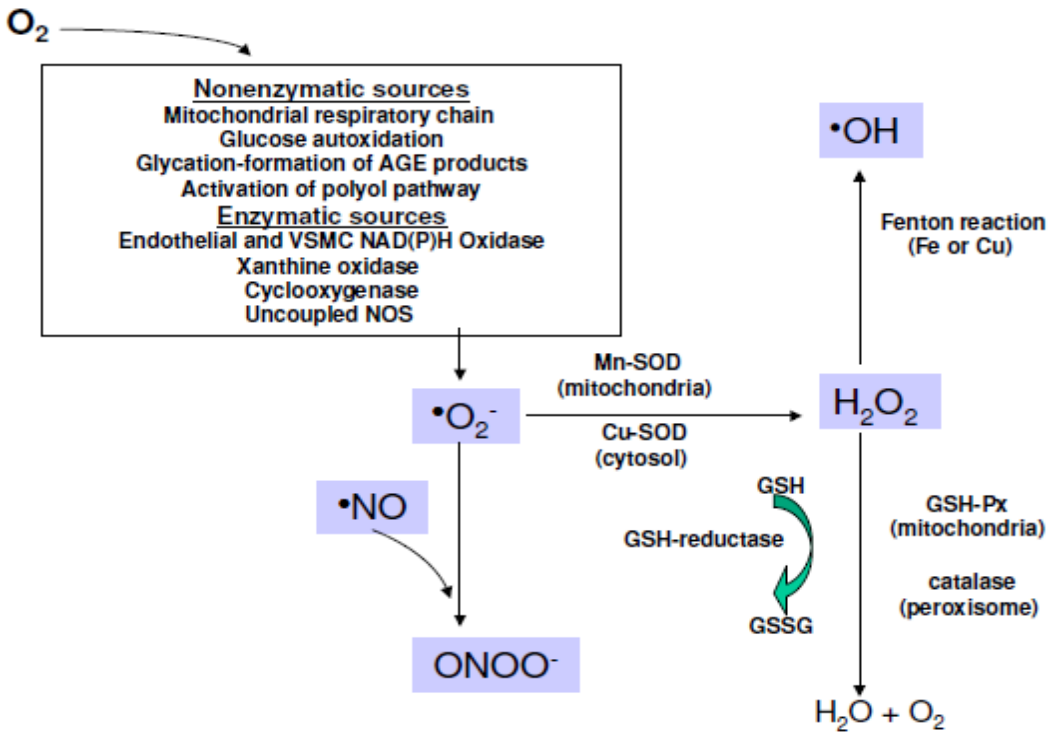

Figure 1.3: Generation of reactive species in diabetes (Johansen *et al.*, 2005). Highlighted in gray are some of the most important ROS and RNS in vascular cells. Oxygen is converted to $\bullet O_2^-$ via the activation of enzymatic and nonenzymatic pathways, which is then dismutated to H_2O_2 by SOD. H_2O_2 can be converted to H_2O by catalase or glutathione peroxidase (GSH-Px) or to •OH after reaction with Cu or Fe.

Nonenzymatic sources of oxidative stress originate from the oxidative biochemistry of glucose. Hyperglycemia can directly cause increased ROS generation. Glucose can undergo autoxidation and generate •OH radicals (Turko *et al.,* 2001). In addition, glucose reacts with proteins in a nonenzymatic manner leading to the development of Amadori products followed by formation of

AGEs. ROS is generated at multiple steps during this process. In hyperglycemia, there is enhanced metabolism of glucose through the polyol (sorbitol) pathway, which also results in enhanced production of $\bullet O_2^-$.

Enzymatic sources of augmented generation of reactive species in diabetes include NOS, NAD(P)H oxidase and xanthine oxidase (Guzik *et al.,* 2000; Aliciguzel *et al*., 2003; Guzik *et al.,*2002). All isoforms of NOS require five cofactors/prosthetic groups such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, BH4 and Ca^{2+}– calmodulin. If NOS lacks its substrate L-arginine or one of its cofactors, NOS may produce $\bullet O_2^-$ instead of •NO and this is referred to as the uncoupled state of NOS (Maritim *et al.,*2003; Guzik *et al.,*2000; Aliciguzel *et al.,*2003; Guzik *et al.,*2002).

NAD(P)H oxidase is a membrane associated enzyme that consists of five subunits and is a major source of $\bullet O_2^-$ production (Guzik *et al.,*2000 and 2002; Kitada *et al.,*2003; Etoh *et al.,*2003). Guzik *et al*. investigated $\bullet O_2^-$ levels in vascular specimens from diabetic patients and probed sources of $\bullet O_2^-$ using inhibitors of NOS, NAD(P)H oxidase, xanthine oxidase and mitochondrial electron transport chain. The study demonstrated that there is enhanced production of $\bullet O_2^-$ in diabetes and this is predominantly mediated by NAD(P)H oxidase. Furthermore, the NOS-mediated component is greater in patients with diabetes than in patients who do not have diabetes (Guzik *et al.,*2000). NAD(P)H oxidase activity is significantly higher in vascular tissue (saphenous vein and internal mammary artery) obtained from diabetic patients (Ergul *et al.,* 2004). There is plausible evidence that PKC, which is stimulated in diabetes via multiple mechanisms, i.e. polyol pathway and Ang II, activates NAD(P)H oxidase (Amiri *et al.,* 2002).

1.6.1.4 Hyperglycemia-induced mitochondrial superoxide production in diabetes

Under normal conditions, $\bullet O_2^-$ is immediately eliminated by natural defense mechanisms. A recent study demonstrated that hyperglycemia-induced generation of $\bullet O_2^-$ at the mitochondrial level is the initial trigger of vicious cycle of oxidative stress in diabetes (Nishikawa *et al.,*2000; Brownlee *et al.,*2001). When endothelial cells are exposed to hyperglycemia at the levels relevant to clinical diabetes, there is increased generation of ROS and especially $\bullet O_2^-$, which precedes the activation of four major pathways involved in the development of diabetic complications. Nishikawa and colleagues elegantly demonstrated that generation of excess

pyruvate via accelerated glycolysis under hyperglycemic conditions floods the mitochondria and causes $\bullet O_2^-$ generation at the level of Complex II in the respiratory chain. What is more important is that blockade of $\bullet O_2^-$ radicals by three different approaches using either a small molecule uncoupler of mitochondrial oxidative phosphorylation (CCCP), overexpression of uncoupling protein-1 (UCP1) or overexpression of Mn-SOD, prevented changes in NF-κB as well as polyol pathway, AGE formation and PKC activity. Based on this information, it has been postulated by several groups that mitochondrial $\bullet O_2^-$ is the initiating snowball that turns oxidative stress into an avalanche in diabetes by stimulating more ROS and RNS production via downstream activation of NF-κB-mediated cytokine production, PKC and NAD(P)H oxidase (Figure 1.4). Thus, inhibition of intracellular free radical formation would provide a causal therapy approach in the prevention of oxidative stress and related vascular complications in diabetes.

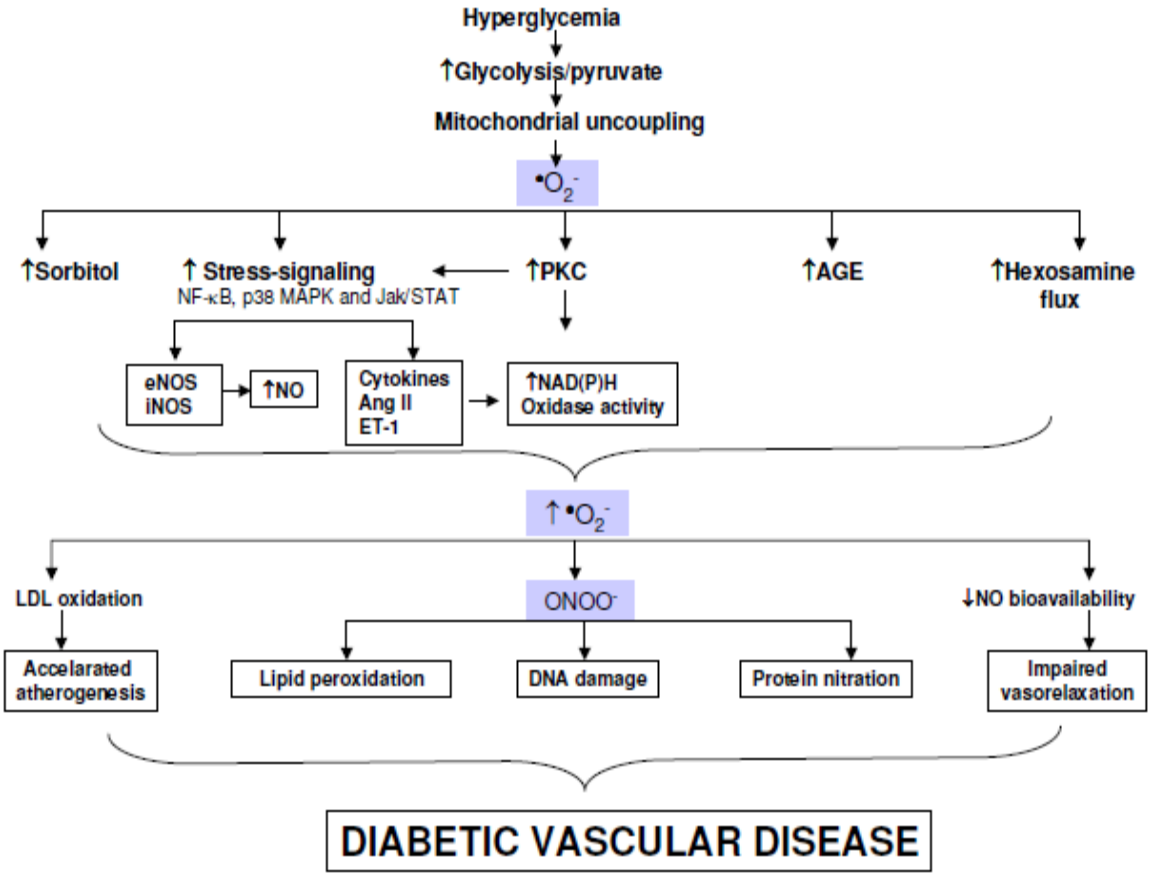

Figure 1.4: Current working model for the generation of reactive species and downstream targets in diabetes (Johansen *et al.*, 2005)

ROS can stimulate oxidation of low-density lipoprotein (LDL), and ox-LDL, which is not recognized by the LDL receptor, can be taken up by scavenger receptors in macrophages

leading to foam cell formation and atherosclerotic plaques (Boullier e*t al.*,2001).•O_2^- and H_2O_2 stimulate stress-related signaling mechanisms such as NF-κB, p38-MAPK and STAT-JAK resulting in VSMC migration and proliferation. In endothelial cells, H_2O_2 mediates apoptosis and pathological angiogenesis (Taniyama *et al.*,2003). Furthermore, •O_2^- immediately reacts with •NO generating cytotoxic $ONOO^-$ and this reaction itself has several consequences. First, $ONOO^-$ alters function of biomolecules by protein nitration as well as causing lipid peroxidation (Turko *et al.*,2001).

1.6.2 Genetic factors

Genetic susceptibility has been implicated in the pathogenesis of both T1DM and T2DM diabetes. Tendency of aggregation of T1DM in families has been observed by different researchers (Risch *et al.,* 1993; Todd and Farrall 1996). The concordance rate for T1DM among monozygotic twins found to be higher between 20-71% compared to that of among dizygotic twins (Kyvik *et al.,* 1995; Redondo *et al.,* 1999) suggesting strong genetic role in its pathogenesis. The human histocompatibility antigen locus has been found to be in linkage disequilibrium in substantial number of T1DM patients (Wolfer *al.,* 1983). Later on variation in HLA Class II gene, particularly DQB I and DR4 allele was found to be strongly associated with T1DM (Donner *et al.,* 2000; Park *et al.,* 1998). Among other gene loci found to be implicated with T1DM are in chromosome 4p15 (IDDM2 and IDDM4) (Sawicki *et al.,* 1997; Hayes *et al.,* 1998) chromosome 2q13 (IDDM 12), chromosome 2q3-2q34 (IDDM13) (Morahan *et al.,* 1996; Larsen *et al.,* 1999), chromosome 6g21(I DDM 15) (Temple *et al.,* 2000).

Table 1.4: Several Type 2 Diabetic Susceptibility Genes (Cox et al., 2004)

Gene	Locus	Variant	Estimated RR
PPARy	3p25	Pro12Ala	1 – 3
ABCC8	11p15.1	Ser1369Ala	2 – 4
KCNJ11	11p15.1	Glu23Lys	1 – 2
CALPN10	2q37.3	A43G	1 – 4

PPARy gene is an important regulator of adiposite development and lipid metabolism (Tontonoz *et al.,* 1994) has become a potential therapeutic target for the treatment of a diverse array of disorders, including T2DM, dyslipidacmia, inflammation and malignancy. One form of the *PPARy* gene (Pro) decreases insulin sensitivity and increases T2DM risk several fold.

INS gene is located in the IGF2-INS region on short arm of human chromosome 11, locus (Powell *et al.,* 2005). The INS gene has a variable number of tandem repeats (VNTR) immediate adjacent to the 5' promoter region.

The VNTR polymorphism can be classified into two main groups: small class I alleles (28-44 repeats) and large class Ill allele (138-159 repeats) at frequencies of about 70 and 30%, respectively, and class II alleles of intermediate size are rare (Stead and Jeffreys, 2000). Different degrees of association have been reported between the INS VNTR class I/Ill genotype and insulin-related traits. The class I allele is associated with increased expression of insulin mRNA and insulin levels (Lucassen *et al.,* 1995; Bennett *et al.,* 1996; Le Stunff *et al.,* 2000). The allelic variation of VNTR is also associated with the risk of diabetes. It has been found consistently that the class I allele increases the risk of TIDM (Julicr *et al.,* 1991; Lucassen *et al.,* 1993).

CTLA-4 (cytotoxic T lymphocyte-antigen 4) gene is located on chromosome 2g31-35, where multiple TIDM genes may be located. CTLA-4 gene variants have been associated with TIDM, as well as other autoimmune disease. CTLA-4 is a protein that plays a key role in T cell activation (Barbara Angel *et al.,* 2009) by recognizing its specific antigen which is presented by an antigen presenting cell in a peripheral lymphoid organ, the T cell receives co-stimulatory signals to differentiate and proliferate into armed effectors T cells but the principal co-stimulatory molecule for activation and clonal expansion expressed by T cells are the CD^{28} receptors. CD^{28} proteins bind to B7 molecules, which are expressed by antigen presenting cells (Janeway *et al.,* 2005).

Also, subsequent to T cell activation, the lymphocyte secretes the cytokine interleukin-2 (IL-2), which drives proliferation and differentiation of the cell. Activated T cells express a high affinity for IL-2 receptors, which binds to the cytokine IL-2. However, it was discovered that when CTLA-4 is induced, it decreases IL-2 production (Alegre *el al.,* 1998). The extra cellular domain

of CTLA-4 can be fused with the antibody IgG (Freeman *el al.*, 1998). CTLA-4 Ig is a protein that is induced to block the production IL-2 and CD^{28}/B7 co-stimulation (Alegre *et al.*, 1998).

A meta-analysis was done by Fotini (2005) of 33 studies examining the association of TIDM mellitus with polymorphisms in the cytotoxic T-lymphocyte-associated antigen-4 *(CTLA-4)* gene, including the *A49G* (29 comparisons), *C (-318) T* (three comparisons) and *(AT)* n niicrosatcllite (six comparisons) polymorphisms.

CAPN 10 encodes an intracellular calcium-dependent cysteine protease that is ubiquitously expressed (Cox *et cil.*, 2001). A haplotype that was initially allowed to
T2DM included an intronic A to G mutation at position 43, which appears to be involved *in CAPN10* transcription.

1.7 Superoxide dismutases (SODs)

Mammalian cells have evolved a variety of antioxidant mechanisms to control reactive oxygen species (ROS) production and propagation (Fridovich, 1999). The superoxide dismutases (SODs) are the first and most important line of antioxidant enzyme defense systems against ROS and particularly superoxide anion radicals (Igor *et al*., 2002). SODs are metalloenzymes that catalyze the dismutation of superoxide radical ($\bullet O_2^-$) into hydrogen peroxide (H_2O_2).
Dismutation is a term that refers to a special type of reaction, where two equal but opposite reactions occur on two separate molecules. SOD takes two molecules of superoxide, strips the extra electron off of one, and places it on the other. So, one ends up with an electron less, forming normal oxygen, and the other ends up with an extra electron. The one with the extra electron then rapidly picks up two hydrogen ions to form hydrogen peroxide.

Dismutation constitute the first enzymatic step that plays a vital role in the control of cellular $\bullet O_2^-$, production. Of course, hydrogen peroxide is also a dangerous compound, so the cell must use the enzyme catalase to detoxify it. Glutathione (GSH) peroxidases (GPXs) are located within the mitochondrial matrix and the cytoplasm, and catalase (CAT) found primarily within peroxisomes, both belong to the secondary defence mechanism by catalyzing the conversion of H_2O_2 to H_2O. GSH reductase (GSR) catalyzes GSH disulphide to reduced GSH, with NADPH as the reducing agent. GPX reduces H_2O_2 or other hydro peroxides using GSH as donor substrate.

It follows that SOD1, SOD2, CAT, GPX and GSR represent co-ordinately operating defences against ROS propagation (Igor *et al*.,2002) (Figure 1.5).

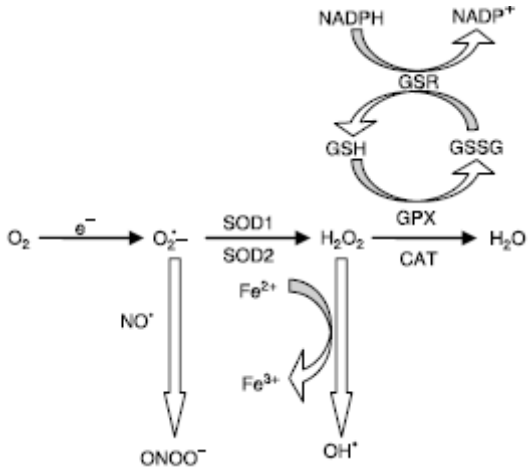

Figure 1.5: Schematic representation of the pathways producing ROS and key cellular antioxidant enzymatic systems controlling ROS production (Igor *et al.*, 2002). Superoxide radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), nitric oxide (NO•), peroxynitrite (ONOO−), hydroxyl radical (OH•), copper/zinc-superoxide dismutase (SOD1), manganese-SOD (SOD2), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GSR), reduced glutathione (GSH), oxidized glutathione (GSSG), NADPH and oxidized $NADP^+$.

1.7.1 Isoforms of SOD

At present, three distinct isoforms of SOD have been identified in mammals, and their genomic structure, cDNA, and proteins have been described.

1. SOD1, or CuZn-SOD (EC 1.15.1.1), was the first enzyme to be characterized and is a copper and zinc-containing homodimer that is found almost exclusively in intracellular cytoplasmic spaces.
2. SOD2, or Mn-SOD (EC 1.15.1.1), exists as a tetramer and is initially synthesized containing a leader peptide, which targets this manganese-containing enzyme exclusively to the mitochondrial spaces.
3. SOD3, or EC-SOD (EC 1.15.1.1), is the most recently characterized SOD, exists as a copper and zinc-containing tetramer, and is synthesized containing a signal peptide that directs this enzyme exclusively to extracellular spaces.

   Following figure 1.7 represents genomic organization human SOD isoforms.

Two isoforms of SOD have Cu and Zn in their catalytic center and are localized to either intracellular cytoplasmic compartments (CuZn-SOD or SOD1) or to extracellular elements (EC-

SOD or SOD3). The copper and zinc ions are bridged by anionic form of imidazole ring of histidine. SOD1 has a molecular mass of about 32,000 Da and has been found in the cytoplasm, nuclear compartments, and lysosomes of mammalian cells (Chang *et al.*, 1988; Keller *et al*., 1991; Crapo *et al.*, 1992; Liou *et al*., 1993).

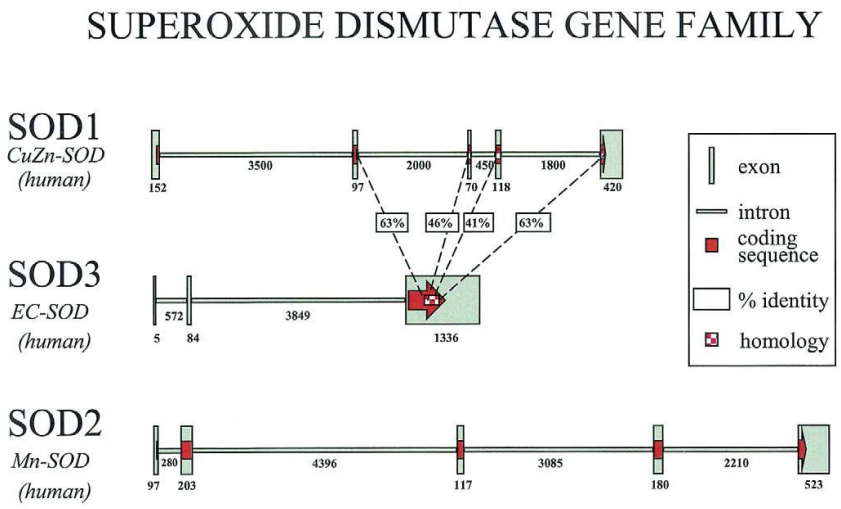

Figure 1.6: Genomic organization of the three known members of the human SOD enzyme family (Igor *et al*., 2002). SOD3 was placed in the middle in order to demonstrate areas of amino acid sequence homology between SOD1 and SOD3. SOD2 has no significant amino acid sequence homology with either SOD1 or SOD3. The size of each exon and intron, in base pairs, is shown in association with that fragment. Data for this figure was extracted from the following sources: SOD1 (Levanon *et al*.,1985), SOD2 (Ho *et al*., 1991), and SOD3(Folz and Crapo, 1994).

SOD3 is the most recently discovered and least characterized member of the SOD family. The enzyme exists as a homotetramer of molecular weight 135,000 Da with high affinity for heparin (Marklund *et al.*, 1982). SOD3 was first detected in human plasma, lymph, ascites, and cerebrospinal fluids (Marklund *et al.*, 1982 and 1986). The expression pattern of SOD3 is highly restricted to the specific cell type and tissues where its activity can exceed that of SOD1 and SOD2. A third isoform of SODs has manganese (Mn) as a cofactor and has been localized to mitochondria of aerobic cells (Mn-SOD or SOD2) (Weisiger *et al.*,1973). It exists as a homotetramer with an individual subunit molecular weight of about 23,000 Da (Barra *et al.*,1984). SOD2 has been shown to play a major role in promoting cellular differentiation and tumorgenesis (Clair *et al.*,1994) and in protecting against hyperoxia-induced pulmonary toxicity (Wispe *et al.*,1992). However, the available information related to SOD3 has not been reviewed in a comparative perspective along with the other two isoforms. The following figure 1.6

represents cellular localization and antioxidant role of SOD1, SOD2 and SOD3 isoforms in vascular smooth muscle cells.

1.7.2 Gene Structure and Organization of SOD1

When SOD1 was isolated for the first time, it was thought to be a copper storage protein (Mann and Kleinin, 1938); the catalytic function of SOD1 was discovered in 1969 by McCord and Fridovich (McCord and Fridovich, 1969), and it was clear that SOD1 acts as a scavenger of superoxide, through a two-step reaction involving reduction and reoxidation of the copper ion in its active site (Fridovich *et al.*,1975).

The human *SOD1* gene (Entrez Gene ID 6647) is located on chromosome 21q22.11 (long (q) arm of chromosome 21 at position 22.11) and it codes for the monomeric SOD1 polypeptide (153 amino acids, molecular weight 16 kDa). More precisely, this gene is located from base pair 33,031,935 to base pair 33,041,241 with a genomic size of 9307 bp, according to UCSC Genome Browser (GRCh37/hg19).

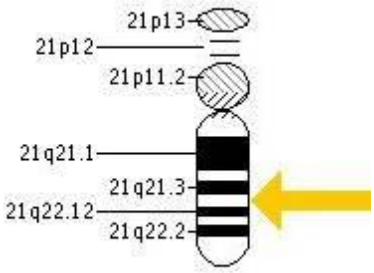

Figure 1.7: Molecular Location of SOD1 gene on chromosome (http://genome. ucsc.edu/***)***

The coding region of SOD1 gene consists of five exons interrupted by four introns (Figure 1.7). Several polymorphisms have been identified in *SOD1* gene, mainly distributed in the regulatory regions, including promoter UTRs, and introns. With regards to SOD1 mRNA splicing, it has been demonstrated that the donor sequence at the first intron presents a T to C variant and, consequently, it deviates from the highly conserved 5' GT... AG 3' consensus sites. Nevertheless, it has been demonstrated that this unconventional splice junction (5' GC... AG 3') is functional (Levanon *et al.*,1985).The proximal promoter of human SOD1 gene, involved in the basal transcription, has been well studied, and it contains not only the TATA box, but also a CCAAT box and a GC-rich region, which are recognized by

CCAAT/enhancer binding proteins (C/EBPs) and specificity protein 1 (Sp1), respectively (Seo *et al.*,1996).

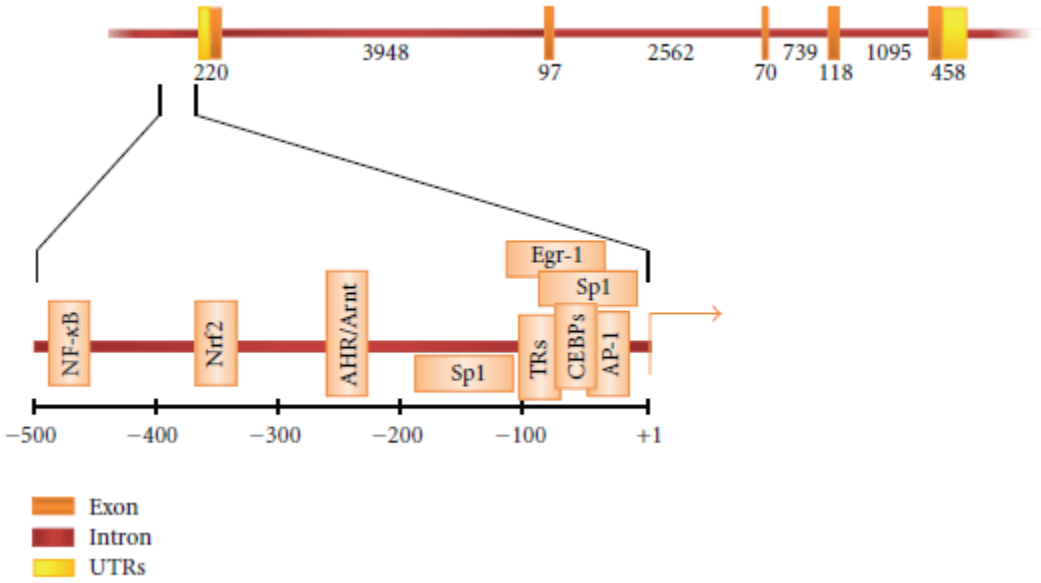

Figure 1.8: Genomic organization of human *SOD1* gene (http://genome.ucsc.edu/). The size of exons and introns, in base pairs, is shown in association with each fragment. The 5' flanking region is expanded, and the transcription factors, interacting with the corresponding DNA regulatory elements, are shown at the bottom. The transcription start site is depicted as an arrow at position +1.

Other binding sites for Egr1, AP1, AHR, Nrf2, NF-*κ*B, and TR transcription factors have been also identified and verified by functional studies (Figure 1.8). These transcription factors are involved in the regulation of *SOD1* inducible gene expression under specific extra- and intracellular conditions. With regard to the 5' untranslated region (5'UTR), Sherman and colleagues (Sherman *et al.*,1983) demonstrated that SOD1 mRNA posseses various 5' termini, which are mapped by both primer extension and Sl mapping. The authors showed that the vast majority of the mRNA species has a 5' start site located 23 bp downstream the TATA box (TATAAA), while the other mRNA molecules have 5' termini 30, 50, and 65bp upstream from the major transcription start site. Consequently, these mRNA species are produced through TATA-independent transcription. It would be interesting to perform in-depth investigations about the functional relevance of these multiple transcription start sites, mainly to determine the potential cell and tissue specificity of the different mRNA species.

1.8 Rationale of the present study

Like many other countries, T2DM constitutes the bulk of the diabetic patients in Bangladesh. Series of studies have been undertaken to explore the pathophysiological basis of T2DM and its complications in Bangladeshi population. Both insulin secretory dysfunction and insulin resistance are found to be present, but compromised insulin secretory capacity is found to be the major feature both in nonobese and obese type 2 diabetic subjects of Bangladeshi origin (Junaid, 2000; Zinnat *et al*., 2003; Al-Mahmud, 2000; Roy *et al*., 2007). Some of the established polymorphic genetic markers, implicated in the pathogenesis of T2DM, have been studied in this population for the understanding of its pathogenic mechanism. These include INS-VNTR, CAPN10, and VDR gene common polymorphisms. It understood that T2DM is an extremely heterogeneous disorder. Available data on genetic analyses also have shown huge variations among different populations.

Superoxide dismutase 1 (SOD1) is located at the cytosol level (Marchler-Bauer et al., 2007) and represents between 50% and 80% of the total SOD activity (Faraci et al.,2004; Fukai et al.,1998). Polymorphisms in the genes encoding these enzymes is found to be significantly affecting its antioxidant role. As oxidative stress is a common pathogenic factor for the dysfunction of beta and endothelial cells, polymorphisms of SOD genes and oxidative stress has become subject of intense scrutiny for their association with different disease conditions including T2DM.

The purpose of this study is to assess the possible association of +35A/C (rs2234694) polymorphism in the pathogenesis of IGR in a Bangladeshi population.

Hypothesis

It is understood that T2DM is an extremely heterogeneous disorder. Available data on genetic analyses also have shown huge variations among different populations. Oxidative stress is a common pathogenic factor for the dysfunction of beta and endothelial cells, polymorphisms of SOD genes and oxidative stress is suggested to cause pancreatic B cell damage resulting in its insulin secreting dysfunction and thus becomes a subject of intense scrutiny for its association with diabetes mellitus. Thus, it was hypothesized that Superoxide dismutase 1 (SOD1) gene +35 A>C variant is associated in the pathogenesis of Impaired Glucose Regulation in Bangladeshi population.

Objectives

General objective

The general objective of the present study was to determine genotype of SOD1 gene +35A>C variant in a group of Bangladeshi patient with IGR and explore its role in its pathogenesis.

Specific objectives

The specific objectives of the study were to:

- determine the frequency of SOD1 gene +35A>C polymorphic markers in a group of IGR and compare with a group of healthy subjects of Bangladeshi origin
- explore the relationship between SOD1 gene +35A>C and insulinemic status of the study subjects and
- explore the association, if any, between SOD1 gene +35A>C polymorphic allele and anthropometric indices.

CHAPTER 2

SUBJECTS AND METHODS

2. SUBJECTS AND METHODS

2.1 Place of the study

The study was conducted in the Department of Physiology and Molecular Biology, Bangladesh University of Health Science (BUHS), Dhaka, Bangladesh.

2.2 Study period

This study was done during the period of December, 2012 to December, 2013.

2.3 Study design

It was a cross-sectional observational study.

2.4 Subjects

A total number of 54 middle aged (age range: 30 - 65year) subjects with Impaired Glucose Regulation (IGR) was consecutively recruited from Out-patient department of Bangladesh Institute of Health Science (BIHS). Impaired Glucose Regulation (IGR) was diagnosed following WHO guideline (WHO, 1999). Age and Sex matched healthy subjects (n=55) recruited as controls.

2.5 Recruitment of the subjects

Subjects consecutively attending the Out-patient department (OPD), BIHS Hospital, an enterprise of Bangladesh Diabetic Samity (BADAS) for their regular check up were recruited. Healthy control subjects were recruited through personal communication from the community.

Purpose and method of the study were briefed to each individual and informed consent (Appendix I) was obtained. A predesigned case record form (Appendix II) was used to record age, hereditary, anthropometric and clinical data of the consenting subjects.

Subjects were requested to report on a prescheduled morning after overnight fasting (8-10 hours).

2.6 Selection criteria

2.6.1 Inclusion criteria

- Adult subjects with age range 30 to 60 years.
- Voluntarily agreed to take part in this study by providing informed consent.
- Age-matched healthy subjects.

2.6.2 Exclusion criteria

- Subjects with co-morbid diseases (infection, stroke, myocardial infarction, major surgery, essential hypertension, malabsorption etc.).
- Subjects with history of medication, which may significantly affect glucose metabolism (glucocorticoids, oral contraceptives containing levonorgestral or high-dose estrogen, phenytoin, high-dose thiazide diuretics etc.).
- Pregnant women and lactating mother.
- Subjects had recent blood transfusion

2.7 Ethical issues

Precaution was taken at the time of collection to address the ethical aspect. Consent was obtained from the volunteers for the future studies involving molecular analysis in these samples.

It was informed that the data a will only be used for scientific purpose. Identity of the individual would not be disclosed. The volunteers under no circumstances would be communicated involving any further analysis.

2.8 Anthropometric measurements

Height

Standing height was measured using appropriate scales (Detect-Medic, Detect scales INC, USA) without shoes. The patient was positioned fully erect, with the head in the Frankfurt plane (with the line connecting the outer canthus of the eyes and the external auditory meatus perpendicular to the long axis of the trunk); the back of the head, thoracic spine, buttocks, and heels touched the vertical axis of the anthrop meter and the heels were together. Height was recorded to the nearest 5 mm.

Weight

The balance was placed on a hard flat surface and checked for zero balance before measurement. The subjects were in the center of the platform wearing light cloths without shoes. Weight was recorded to the nearest 0.5 Kg.

Calculation of BMI (Kg/m^2)

Body mass index (BMI) of the subjects was calculated using following formula:

$$BMI = \frac{Weight\ (kg)}{[Height\ (m)]^2}$$

Waist circumference

Waist circumference was measured to the nearest 0.5 cm with a soft non-elastic measuring tape. The tape was snug, but not so tight as to cause skin indentation or pinching. The waist circumference was taken to the nearest standing horizontal circumference between the lower border of the 12^{th} rib and the highest point of the iliac crest on the mid-axillary line at the end of normal expiration.

Hip circumference (cm)

Hip circumference was measured at the maximum circumference over the buttocks using soft non-elastic measuring tape and reading was taken to the nearest 0.5 cm.

Calculation of WHR

Waist to hip ratio (WHR) of the study subjects was calculated as the ratio of waist circumference divided by hip circumference.

2.9 Measurement of blood pressure

Blood pressure was measured using Barometric Sphygmomanometer. Standard protocol was followed to record blood pressure data. Blood pressure was measured in sitting position, with calf at the level of the heart. After 10 minutes of rest a second reading was taken and average was recorded. Recorded Korotkoff sound I (the first sound) and V (the disappearance of sound) denoted the systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively (according to WHO-IHS).

2.10 Methods

2.10.1 Collection and storage of blood samples

Overnight fasting (8-10 hours) blood was collected between 8.00-9.30 am. Venous blood (10 ml) was obtained by venipuncture following standard procedure. Subjects were then given glucose to drink (75 g in 300 ml drinking water). They were requested not to take any food and be rested for two hours. The second blood sample was then obtained at two hours.

A portion of blood (5 ml) sample was taken into a tube containing EDTA (1 mg/ml), mixed properly and preserved at $-30^{o}C$ for future DNA extraction and subsequent experimentation. Fasting and postprandial blood samples were taken into plain tube allowed to clot and serum

was separated by centrifugation for 10 min at 3000 rpm using refrigerated centrifuge and separated serum was preserved at -25^{o}C for further biochemical analyses.

2.10.2 Biochemical analyses

The following biochemical parameters were analyzed for the study.

2.10.2.1 Estimation of glucose

Glucose was estimated by enzymatic colorimetric (GOD-PAP) method in the Hitachi 704 Automatic Analyzer, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

Principle

Glucose was determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts with phenol and 4-aminophenazone under catalysis of peroxidase to form a red violet quinoneimine dye as indicator (Trinder, 1969).

$$\text{Glucose} + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxi}} \text{Quinoneimine} + 4H_2O$$

Reagents

Contents	Initial concentration of solution
Phosphate Buffer	0.1 mol/L, pH 7.0
Phenol	11 mol/L
4-aminophenazone	0.77 mmol/L
Glucose oxidase	□ 1.5 kU/L
Peroxidase	□ 1.5 kU/L
Glucose Standard	5.55 mmol/L (100 mg/dL)
Uranyl Acetate	0.16%

Materials

- Micro-centrifuge tube
- Micropipettes and pipettes
- Disposable tips
- Automatic Analyzer (Boehringer Mannheim, 704; HITACHI)

Procedure

The method determines glucose without deproteinization. The instrument was calibrated before estimation. Serum and reagent were taken in specific cup. They were arranged serially into the Auto analyzer. The Auto analyzer was programmed for the estimation of glucose and allowed to run with following procedure:

5µl sample and 500µl reagent were mixed and incubated at 37° C for 10 minutes. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of the result

Optical densities or absorbances were fed into a computer and calculation was done using the software program. Values for the unknown samples were calculated by extrapolating the absorbance for the standard using following formula.

$$\text{Glucose concentration (mmol/L)} = \frac{A_{Sample}}{A_{Standard}} \times 5.55$$

2.10.2.2 Estimation of total cholesterol

Total cholesterol was measured by enzymatic endpoint method (Cholesterol Oxidase/Peroxidase) method in the Hitachi 704 Automatic Analyzer, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

Principle

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantiphyrine in the presence of phenol and peroxidase (Richmond, 1973).

Reagents

Contents	**Initial concentration of solution**
Phosphate Buffer	0.1 mol/L, pH 7.0
Phenol	11 mol/L
4-aminophenazone	0.77 mmol/L
Glucose oxidase	≥1.5 kU/L
Peroxidase	≥1.5 kU/L
Glucose Standard	5.55 mmol/L (100 mg/dL)

Materials

- Micro-centrifuge tube
- Micropipettes and pipettes
- Disposable tips
- Automatic Analyzer (Boehringer Mannheim, 704; HITACHI)

Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the Auto analyzer. 5 µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the Auto lab. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

Calculation of the result

Concentration of cholesterol in the sample was calculated by using software program with the following formula.

Cholesterol concentration (mg/dL) = $\frac{A_{Sample}}{A_{Standard}} \times$ concentration of standard.

2.10.2.3 Estimation of triglyceride (TG)

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

Principle

Sample triglycerides incubated with a lipoprotein lipase liberate glycerol and fatty acids. Glycerol is converted to glycerol-3-phosphate by glycerol kinase and ATP.Glycerol-3-phosphate oxidase (GPO) oxidizes glycerol-3-phosphate into dihydroxy acetone phosphate and H_2O_2. In the presence of peroxidase, hydrogen peroxide oxidizes the chromogen-4-aminoantipyrine and 4-chlorophenol to a violet colored complex. The quinone formed is proportional to the amount of triglycerides present in the sample. The principle is based on the following reaction system (Fossati and Prencipe, 1982).

Triglyceride $\xrightarrow{Lipase}$ glycerol + Fatty acids

Glycerol + ATP $\xrightarrow{Glycer\ Kinase}$ glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 $\xrightarrow{Glycer\ Phospl\ Oxidas}$ dihydroxy acetone phosphate + H_2O_2

$2H_2O_2$ + 4-aminophenazone + 4- chlorophenol $\xrightarrow{Peroxi}$ quinoneimine + HCl + $4H_2O$

Reagents

Content	Concentrations in the Test
Buffer	
Pipes Buffer	40 mmol/L, pH 7.6
4-choloro-phenol	5.5 mmol/L
Magnesium-ions	17.5 mmol/L
Enzyme Reagent	
4-aminophenazone	
ATP	1.0 mmol/L
Lipases	>150 U/ml
Glycerol-3-phosphate oxidase	1.5 U/ml
Peroxidase	0.5 U/ml
Standard	2.29 mmol/L (200 mg/dL)

Materials

- Micropipettes and pipettes
- Disposable tips
- Auto analyzer (Boehringer Mannheim, 704; HITACHI)

Procedure

Serum and reagents were taken in specific cup. They were arranged serially. Then ID number for test was entered in the analyzer. Five (5) µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the cell. Reading was taken at 500 nm.

Calculation of result

Triglyceride concentration was calculated by following formula:

$$\text{Triglyceride concentration (mg/dL)} = \frac{A_{Sample}}{A_{Standard}} \times \text{Concentration of standard.}$$

2.10.2.4 Estimation of high density lipoprotein (HDL) cholesterol

High density lipoprotein cholesterol (HDL-C) was measured by enzymatic colorimetric (CHOD- PAP) method using reagent of RANDOX laboratories Ltd., UK.

Principle

HDL (High Density Lipoprotein) is separated from chylomicrons, VLDL (very low density lipoprotein) and LDL (Low density lipoprotein) by precipitating reagent (phosphotungstic acid-magnesium chloride). After centrifugation, the cholesterol content of HDL fraction, which remains in the supernatant, was determined by the enzymatic colorimetric method using CHOD- PAP (Friedwald *et al*., 1972).

Materials and reagents

1. Precipitant Buffer
2. Lipid Controls
3. Randox aqueous Cholesterol Standard: 200 mg/dL
4. Reagent solution for cholesterol CHOP-PAP assay.
5. Pipettes (5 µl –50 µl, 100 µl-1000 µl) and Pipette Tips.
6. Multi-Channel Pipettes and Pipette Tips: 50-300 µl
7. Buffer and Reagent Reservoirs
8. Vortex Mixture
9. Deionized Water
10. Microtiter Plate Reader capable of reading absorbency at 450 nm 590 nm
11. Orbital Microtiter Plate Shaker
12. Absorbant Paper

Reagents composition

Phosphotungstic Acid: 0.55 mmol/L

Magnesium Chloride: 25 mmol/L

Standard Preparation

Dilute Randox aqueous cholesterol standard (200 mg/dl) with deionized water by volume of 0, 20, 40, 50, and 100 µl. The final volume was 200 µl.

Assay Procedure

1. 100 ml serum sample was taken in microcentrifuge tube.
2. 250 ml HDL-C precipitant was added.
3. Mixed well and allowed to sit for 10 minutes

4. The mix components were vortexed and centrifuged for 15 minutes at 4000 rpm.
5. 30 µl of each Standard was transferred in first six wells.
6. 30 µl of clear supernatant was transferred into the other wells.
7. 250 µl of cholesterol reagent was then added into all the 96 wells quickly using multi-channel pipettes.
8. Incubated for 5 minutes at 37^0C on orbital microtiter plate shaker.
9. Absorbance was read at 490 nm.

Calculation of the result

The HDL-C value of each sample was obtained as follows:

The net absorbance value for each level, obtained by subtracting the value for the HDLC concentration (mg/dl) from the value of individual. The smooth linear curve was drawn and the results of unknown samples were calculated using logistic function.

2.10.2.5 Estimation of low density lipoprotein (LDL) cholesterol

The LDL-Cholesterol level in serum was calculated by using by Friedewald formula (Friedwald *et al.,* 1972).

Formula

$$\text{LDL cholesterol} = \{\text{Total cholesterol} - (\text{HDL cholesterol} + \frac{1}{5} \times \text{Triglyceride})\}$$

2.10.2.6 Estimation of serum glutamate-pyruvate transaminase (SGPT)

Serum glutamate-pyruvate transaminase (GPT) or alanine aminotransferase (ALT) was estimated by UV method using ALT (GPT) opt. kit (RANDOX) (IFCC, 1980).

Principle

$$\alpha\text{- oxogluterate + L – alanine} \xrightarrow{\text{SGPT}} \text{L-glutamate + pyruvate}$$

$$\text{Pyruvate + NADH + H}^+ \xrightarrow{\text{LDH}} \text{L-lactate + NAD}^+$$

Reagents

Contents	Concentration in the test
1. Buffer/Substrate	
Tris biffer	100 mmol/L, pH 7.5
L-alanine	0.6 mol/L
2. Enzyme/Coenzyme/α-oxoglutarate	
α-oxoglutarate	15 mmol/L
LD	≥1.2 U/ml
NADH	0.18 mmol/L

Preparation of Solutions

1. Buffer/Substrate: Buffer/Substrate supplied in the kit was used as it is.
2. Enzyme/Coenzyme/α-oxoglutarate: One vial of Enzyme/Coenzyme/α-oxoglutarate2 was reconstituted with the appropriate volume of Buffer/Substrate 1:

2 ml	for the	20 x	2 ml	kit (AL 1200)
10 ml	for the	20 x	10 ml	kit (AL 1205)
20 ml	for the	5 x	20 ml	kit (AL 1268)

Cat No AL 2360 5 x 100 ml

One vial of Enzyme/Coenzyme/α-oxoglutarate 2 was reconstituted with a portion of Buffer/Substrate 1 and then the entire content was transferred to bottle 1 rinsing bottle 2 several times.

Procedure

Wavelength:	340 nm (Hg 334 nm or Hg 365 nm)	
Cuvette:	1 cm light path	
Temperature:	25/30/37^{o}C	
Measurement:	against air	
Pipetted into cuvette:	Macro	Micro
Serum	0.2 ml	0.1 ml
Enzyme/Coenzyme/α-oxoglu-tarate 2	2.0 ml	1.0 ml

Mixed and initial absorbance was read after 1 minute. Again after 1, 2 and 3 minutes the absorbance was read. The absorbance change per minute was noted and if the value is between

0.11 and 0.16 at 340 nm/Hg 340 nm

0.06 and 0.08 at Hg 365 nm

Only then the values for the first 2 minutes were used for the calculation.

Calculation

To calculate the ALT activity the following formulae was used:

U/L	=	1746	x	ΔA 340 nm/min
U/L	=	1780	x	ΔA Hg 334 nm/min
U/L	=	3235	x	ΔA Hg 365 nm/min

Normal values in serum

	25°C	**30°C**	**37°C**
Men	Up to 22 U/L	Up to 29 U/L	Up to 40 U/L
Women	Up to 17 U/L	Up to 22 U/L	Up to 31 U/L

Linearity

If the absorbance change per minute exceeds

0.16 at 340 nm/Hg 334 nm

0.08 at Hg 365 nm

0.1 ml of sample was diluted with 0.9 ml pf 0.9% NaCl solution and reassessed. The result was multiplied by 10.

2.10.2.7 Estimation of insulin

Insulin was measured by enzyme linked immunosorbent assay (ELISA) method using kit from Linco Research Inc., USA.

Principle

This assay is a sandwich ELISA based, sequentially, on: 1) concurrent capture of human insulin molecules from samples to the wells of a microtiter plate coated by a pre-titered amount of monoclonal anti-human insulin antibodies and the binding of a second biotinylated monoclonal mouse anti- antibody to the captured insulin, 2) washing of unbound materials, 3) binding of horseradish peroxidase (HRP) to the immobilized biotinylated antibodies, 4)

washing of free enzyme conjugates, and 5) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase (HRP) activities in the presence of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB). The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm after acidification of formed products. Since the increase in absorbance is directly proportional to the amount of captured human insulin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human insulin.

Reagents supplied

Each kit was sufficient to run one 96-well plate and contains the following reagents:

1. Microtiter plate: Coated with mouse monoclonal anti-human insulin antibodies.

2. Adhesive plate sealer

3. 10X concentrate horseradish peroxidase (HRP) wash buffer (2□ 50ml)

4. Standards: Human insulin in buffer 2, 5, 10, 20, 50, 100 and 200 µU/ml; 0.5 ml/bottle, ready to use.

5. Quality Controls 1 and 2: Purified recombinant human insulin in assay buffer, ready to use.

6. Matrix Solution: Heat-treated charcoal stripped off the clot human insulin serum (2□ 1 ml), ready to use.

7. Assay Buffer: 0.05 M Phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% Sodium azide, 1% BSA, 2x9 ml, ready for use.

8. Human insulin detection antibody: Pre-titered biotinylated monoclonal mouse anti-human insulin antibody (2x12 ml), ready to use.

9. Enzyme solution: Pre-titered streptavidin-horseradish peroxidase. Conjugation Buffer (2x12 ml), ready to use.

10. Substrate: 3,3',5,5'-tetramethylbenzidine (TMB) in buffer (12 ml), ready to use. Minimize exposure to light.

11. Stop solution: 0.3 M HCl (12 ml), ready to use.

Materials required

1. Pipettes and pipette tips: 10 µl –20 µl or 20 µl-100 µl

2. Multi-channel pipettes: 5~50 µl and 50~300 µl
3. Reagent reservoirs
4. Vortex mixture
5. Deionized water
6. Microtiter plate reader capable of reading absorbency at 450 nm 590 nm
7. Orbital microtiter plate shaker
8. Absorbant paper
9. Standard refrigerator

Reagent preparation

HRP Wash Buffer: Diluted 10X HRP washing buffer concentrate 10 fold by mixing the entire content of each bottle with 450 ml deionized water.

Storage and Stability

All components of the kit were stored at 2-8º C. Multiple freeze/thaw cycles of the insulin standards and Matrix solution were avoided.

Assay Procedure

1. The reagents were allowed to come to room temperature. Then each well filled with 300 µl of diluted HRP wash buffer and incubated at room temperature for 5 minutes. Then wash buffer was decanted and the residual amount was removed from all wells by inverting the plate and tapping it smartly onto the absorbent towels several times.
2. The microtitre plate was marked. A layout of the plate for blank, standards, QCs and unknown samples were made and recorded on a paper.
3. 20 µl assay buffer was added to the NSB (Non Specific Binding) wells and each of the sample wells.
4. 20 µl matrix solution was added to the NSB, Standard and Control wells.
5. 20 µl human insulin standards in the order of ascending concentration was added to the appropriate wells.
6. 20 µl of QC 1 and QC 2 was added to the appropriate wells.
7. Then 20 µl of unknown samples were added sequentially in wells according to the plate layout.
8. After that 20 µl of detection antibody was added to all wells. All procedures were performed within 30 minutes. Plates were covered using plate sealer and incubated at

room temperature for 1 hour on an orbital microtitre plate shaker to rotate at moderate speed approximately 400 rpm.

9. At the end of incubation, plate sealer was removed and solutions were decanted. Residual solutions were removed by tapping as before. Each wells was then washed for 3 times with 300 µl diluted HRP wash buffer. After each wash the plate was tapped on a pad of tissue to dry it off.

10. 100 µl of enzyme solution was added to each well using a multichannel pipette. Plates were covered with sealer and incubated again with moderate shaking at room temperature for 30 minutes on microtitre plate shaker.

11. Then sealer was removed and solutions were decanted from the plate and tapped to remove the residual fluid. Then wells were washed for 5 times using 300 µl HRP wash buffer per well per wash and after each wash the plate was tapped smartly to remove the residual buffer.

12. 100 µl of substrate solution was added to each well and covered with sealer and incubated with moderate shaking at room temperature for 10 minutes on microtitre plate shaker. Blue color was formed in the wells of insulin standards with intensity proportional to increasing concentrations of insulin.

13. Finally sealer was removed and 100µl of stop solution was added. Plate was placed on a microtitre plate shaker for gentle shaking (400 rpm).

14. Reading was taken using a microplate reader at 450 nm.

Calculation of results for unknown samples

Optical densities of standard and unknown samples were fed in to a computer programme. Results of unknown samples were calculated extrapolating standard four parameter logistic curves using a Software Kinetical 3.

2.10.3 Genetic analyses

Superoxide dismutase 1 (SOD1) genotype was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

2.10.3.1 DNA extraction

Extraction of DNA was performed using FavorPrep™ Blood/Cultured Cell Genomic DNA Extraction Kit (FAVORGEN®, Taiwan). The kit uses the principal of silica gel DNA isolation from whole blood adapted in spin column.

Equipment, reagent and accessories

- Water bath
- Vortex mixer
- Centrifuge
- Microcentrifuge tubes (1.5 ml)
- Pipette tips at different capacities (10 µl, 20 µl, 100 µl, 200 µl and 1000 µl)
- Micropipettes (2µl, 20µl, 100µl, 200µl, 100-1000 µl)
- Absolute ethanol (95-100 %)

Content of DNA kit

- Extraction Column (FABG Column)
- Collection tubes
- Proteinase K
- RNase-A solution
- Lysis Buffer FABG
- Wash Buffer W1
- Wash Buffer
- Elution Buffer

Preparation of reagents

Wash buffer W1: 45 ml absolute ethanol (96-100%) was added to 124 ml Wash Buffer W1.

Wash buffer: 200 ml absolute ethanol (96-100%) was added to 50ml Wash buffer.

Extraction procedure

1. Frozen blood was brought to room temperature and made homogenous by brief vortexing. Aliquot of blood (200 µl) was transferred into 1.5 ml microcentrifuge tube.
2. 2 µl of an RNase-A stock solution (100 mg/ml) was added to the sample and incubated for 2 minutes at room temperature.
3. 20 µl FAVORGEN proteinase K and 200 µl Lysis Buffer FABG were added to the sample and mixed thoroughly by pulse-vortexing.
4. Incubated at 60°C for 15 minutes to lyse the sample. During incubation, in order to ensure efficient lyses, the sample pulse-vortexed every 5 minutes.
5. To remove drops from the inside of the lid at the tube briefly centrifuged.

6. 200 µl ethanol (96-100%) was added to the sample, and mixed by pulse-vortexing for 30 seconds.
7. To remove drops from the inside of the lid at the tube briefly centrifuged.
8. FABG Mini spin Column was placed to a 2 ml collection tube. The sample mixture from the step 7 (including any precipitate) was transferred carefully to the FABG Mini spin Column without wetting the rim. Centrifuged at 6000 x g for 1 min and the flow-through were discarded. Then FABG Mini spin Column was placed on a fresh 2 ml collection tube.
9. FABG Mini spin Column was opened carefully and 500 µl Wash Buffer W1 was added without wetting the rim. Centrifuged at 6000 x g for 1 min. The collection tube was discarded and the spin column was placed on fresh collection tube.
10. The FABG Mini spin Column was opened carefully and 750 µl Wash Buffer was added without wetting the rim. The cap was screwed carefully and centrifuged at 20000 x g for 1 minute. At this stage the column was appeared to be clean.
11. Centrifuged at 6000 x g for an additional 3 minutes to dry the column.
12. The FABG Mini spin Column was placed to Elution tube.
13. 100 µl Elution Buffer was added to the membrane center of FABG Mini spin column. Incubated at room temperature for 15 minutes. Centrifuged at 6000 x g for 2 minutes. This elute supposed to contain DNA.
14. For effective elution, the elution solution must dispend onto the membrane center and absorbed completely. Additional 100 µl Elution Buffer was added to the membrane center of FABG Mini spin column. Incubated at room temperature for 30 minutes. Centrifuged at 6000 x g for 2 minutes. This step elutes any residual DNA that may not be eluted in the first elution step.
15. The DNA fragment was stored at 4^{o}C or -20^{o}C.

2.10.3.2 Check for DNA yield

DNA yield for each sample was checked by agarose gel (1%) electrophoresis.

Reagents

i) Agarose (Invitrogen, USA and VIOGENE, Taiwan).
ii) TBE (Tris-borate EDTA) buffer.
iii) Gel loading buffer (5X concentration).

iv) Ethidium bromide (0.5µg /ml).

Composition of TBE (Tris-borate EDTA) buffer

1. Tris Base (54.0 g)
2. Boric acid (27.5g)
3. EDTA (20 ml of 0.5M)

Deionized H_2O added to make the total volume 1L.

Procedure

To prepare agarose gel, appropriate amount of agarose was taken into polypropylene conical flasks containing required volume of working tris-borate EDTA (TBE) buffer. Agarose and buffer solution were mixed by swirling of the flasks. It was then heated to boiling point in a microwave oven with intermittent mixing until agarose was properly boiled. The gel was cooled nearer to the gelling point and ethidium bromide (0.5 µg/ml) was added. The gel was then poured into horizontal gel mould, combs inserted, and allowed to polymerize. The gel was subsequently placed in horizontal electrophoresis tank filled with working TBE buffer.

To resolve DNA extract, 3 µl of DNA elute was mixed with appropriate amount of loading buffer and then the mixer was loaded in agarose gel prepared earlier. The gel was run for at medium voltage for required time. DNA presence was visualized under UV light and gel image was captured.

The intensity of the bands obtained for the extracted DNA samples were visually compared with that of known concentration of DNA and then the extracted DNA was diluted according to the intensity of the bands to make the concentration of all DNA samples between 10-50 ng/ml, optimum for PCR amplification.

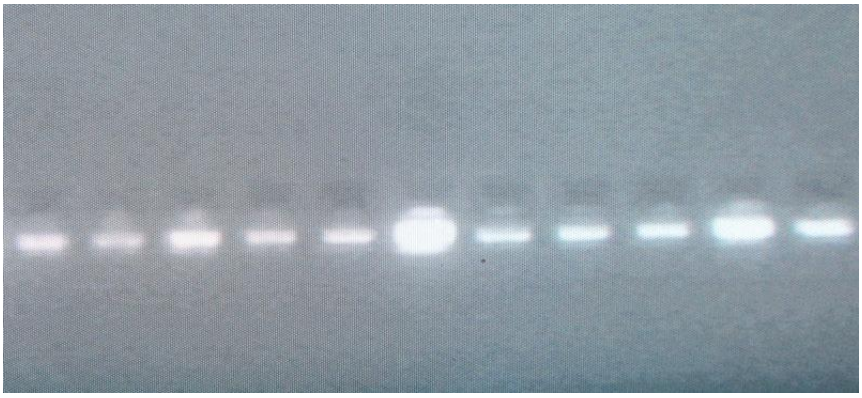

Figure 2.1: Gel image of DNA electrophoresis to check the extraction yield

2.10.3.3 PCR amplification of the SOD1 gene

The segment of the SOD1 gene containing the polymorphic marker +35A>C was amplified by polymerase chain reaction (PCR) and the amplified product was checked by agarose gel electrophoresis.

Reagents

- HotStart Taq Polymerase (QIAGEN, USA)
- Buffer (QIAGEN, USA)
- dNTPs (BIONEER, South Korea)
- Primers (Bioserve, India)
- Agarose (Invitrogen, USA)
- Ethidium bromide (Sigma, USA)
- TBE (Tris-borate EDTA) buffer
- 100 bp DNA ladder (BIONEER, South Korea. Email:sales@bioneer.com)

PCR of the SOD1 gene

Polymerase chain reaction (PCR) was carried out in 10 µl reaction volume. PCR product size for the primer set is 278bp. PCR was carried out using the following primer set:

Forward 5′-CTA TCC AGA AAA CAC GGT GGG CC- 3′

Reverse 5′- TCT ATA TTC AAT CAA ATG CTA CAA AAC -3′

PCR primer was selected according to the protocol followed in Flekac *et al.*, 2008.

PCR condition

PCR was carried out using HotStar Taq polymerase. Conditions for the amplification included initial step of denaturation 95^{o}C for 15 minutes followed by 35 cycles of denaturation at 94^{o}C for 45 seconds, annealing at 55^{o}C for 30 seconds and elongation at 72^{o}C for 30 seconds and finally a step of final elongation at 72^{o}C for 10 minutes. PCR assays were performed in a DNA Thermal Cycler (Biometra, USA). A negative control (reagent blank), which contained all components of the reaction mixture with the exception of the sample DNA, was included in every PCR procedure.

Concentration of PCR component

Composition of reaction mix for a PCR of 10 µl contained the following:

Name of the component	Volume (µl)	Concentration
DNA	3.0	10-50 ng/ml
Buffer	1.0	10x
dNTPs	0.2	200 µmol/l
Forward Primer (F)	0.5	100 pmol/µl
Reverse Primer (R)	0.5	100 pmol/µl
HotStart Taq Polymerase	0.07	5 U/µl
ddH_2O	4.73	
Total	10.00 µl	

Evaluation of PCR

3 µl of PCR product was checked for yield in a 2% agarose gel. The optimum size of the product was acertained compairing it with ladder DNA. The amplified DNA was visualized using ethidium-bromide under UV light and gel image captured and documented.

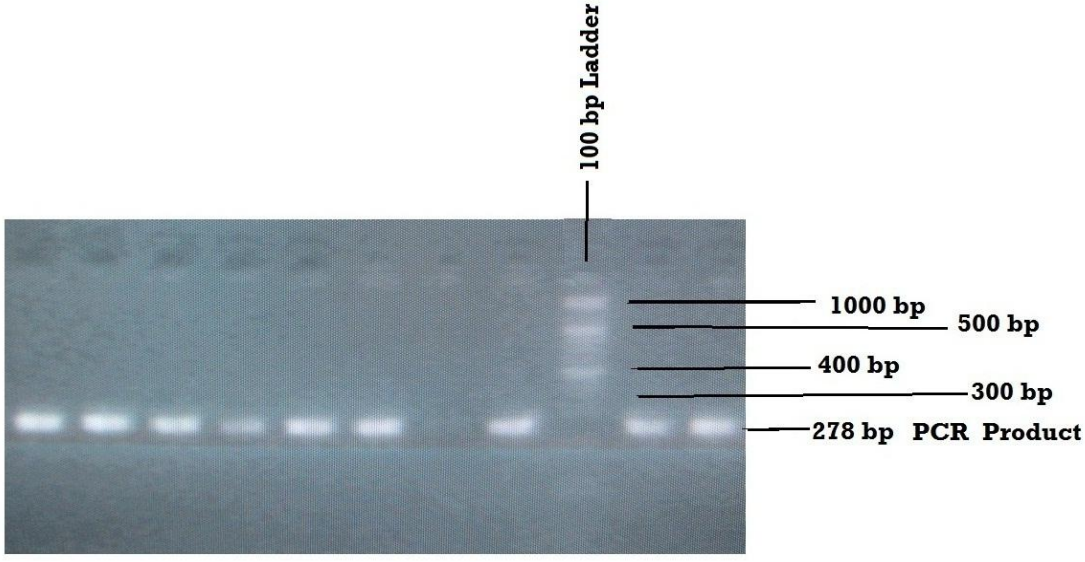

Figure 2.2: Image of gel analysis of SOD1 gene PCR products in a 2% agarose gel

2.10.3.4 RFLP analysis of SOD1 gene candidate markers

SOD1 gene candidate polymorphic marker +35A>C was analyzed using site-specific HhaI restriction enzyme. Restriction enzyme digestion was performed following standard digestion

protocol. Candidate SOD1 gene +35A>C varient disrupt restriction enzyme site and were detected by restriction fragment length polymorphism (RFLP) assay.

Equipments

- Water bath (JENCONS, USA)
- Electric balance (Scientech, SA 210, USA)
- Vortex mixer (Gallenkamp, UK)
- Refrigerated microcentrifuge (Hettich, USA)
- Centrifuge (Hanil, South Korea)
- Microwave oven
- Gel electrophoresis system (Biometra, USA)
- Gel documentation system (Bio-Rad, USA)
- Restriction enzyme kit (Thermo Scientific, USA)

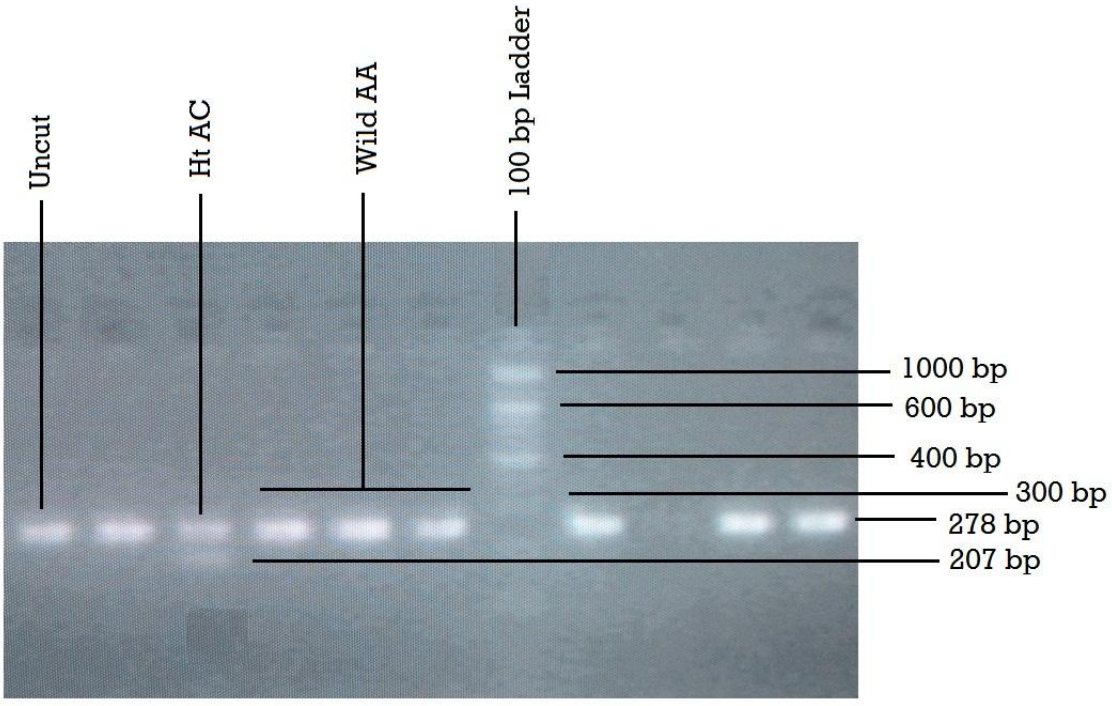

Figure 2.3: 3% agarose gel image of SOD1 gene +35A>C candidate marker analysis by Hha I restriction enzyme digestion.

Hha I digestion

SOD1 +35A>C variant polymorphic marker was analyzed using Hha I restriction enzyme. The digestion was carried out in a final reaction volume of 10 µl. The enzyme digestion protocol was as follows:

Hha I restriction enzyme digestion protocol:

Name of the component	Volume (µl)
PCR product	3.0
10x Buffer Tango	1.0
Restriction enzyme (Hha I)	0.4 (10U/µl)
H_2O	5.6
Total reaction volume	10.00 µl

Hha I restriction enzyme digestion was carried at 37^{o}C for 16 hours in a water bath. Enzyme digestion product was resolved in 3% agarose gel and digested product was visualized using gel documentation system following ethidium bromide staining.

The +35A>C polymorphism restricts the Hha I restriction enzyme site as follows:

5′…G C G↓C…3′
3′… C↑G C G…5′

Fragments produced by the Hha I restriction enzyme digestion of the PCR products was as follows: Homozygous wild (AA) genotype 278 bp; heterozygous (Ht) variant (AC) genotype 78bp, 207 bp and 71 bp. Fragment 71 bp could not be resolved.

2.10.4 Statistical analyses

Statistical analysis was performed using Statistical Package for Social Science (SPSS) software for Windows version 17 (SPSS Inc., Chicago, Illinois, USA) and AcaStat Software. Data were expressed as mean±SD, number (percentage) as appropriate. The statistical difference between two groups was assessed by unpaired Student's't' test. Chi-squared test and Z test were also performed in appropriate situation. A two-tailed p value of <0.05 was considered statistically significant.

CHAPTER 3

RESULTS

3. RESULTS

Superoxide dismutase 1 (SOD-1) gene +35A/C polymorphism was analyzed in total number of 109 unrelated subjects of Bangladeshi origin where 55 were healthy controls and 54 IGR subjects.

3.1 Age, BMI and blood pressure of the study subjects

3.1.1 Age (years)

Mean (±SD) age (years) of the control and IGT subjects was 41.56±11.13 and 45.31±9.46 respectively which did not show statistical significant difference (p=0. 061) (Table 3.1).

3.1.2 Body Mass Index (BMI, Kg/m^2)

Mean (±SD) BMI of the control and IGT subjects was 23.44±4.39 and 24.75±4.17 respectively which did not show any statistical significant difference (p=0. 116) (Table 3.1).

3.1.3 Waist Hip Ratio (WHR)

Mean (±SD) WHR of the control and IGT subjects was 0.90±0.06 and 0.94±0.08 respectively which did not show any statistical significant difference (p=0. 010) (Table 3.1).

Table 3.1: Age, BMI and blood pressure of the study subjects

Variables	Wild AA (n=55)	Ht AC (n=54)	t/p value
Age	41.56±11.13	45.31±9.46	-1.892/0.061
BMI	23.44±4.39	24.75±4.17	-1.583/0.116
WHR	0.90±0.06	0.94±0.08	-2.610±0.010
SBP	113.51±14.06	116.66±14.13	-1.161/0.248
DBP	75.98±9.45	78.43±11.41	-1.218/0.226

3.1.4 Blood pressure (mmHg)

3.1.4.1 Systolic Blood Pressure (SBP, mmHg**):** Mean (±SD) SBP of the control and IGT subjects was 113.51±14.06 and 116.66±14.13 respectively which did not show statistical significant difference (p=0. 248) (Table 3.1)

3.1.4.2 Diastolic Blood Pressure (DBP, mmHg**):** Mean (±SD) of the control and IGT subjects was 75.98±9.45 and 78.43±11.41 respectively which did not show statistical

significant difference (p=0. 226) (Table 3.1).

3.2 SOD1 +35A>C Variant Marker Analysis

3.2.1 SOD1 +35A>C genotype frequencies of the study subjects

The number of study subjects of wild (AA) genotype in control and IGR group was 55 (100%) and 52 (96.3%) respectively and in case of heterozygous (Ht) variant AC genotype was 0 and 2 (3.7%) respectively. SOD +35A/C polymorphic wild genotype was 1.00 and 0.963 in the controls and IGR subjects respectively. Variant genotype (A/C and C/C) was 0 and 0.037 respectively in the two groups. These frequency distribution among the groups did not exhibit statistical significant association (χ^2 = 2.075 and p= 0.150) (Table 3.2).

In the control the frequency was all 'A' allele and in the IGR group 'A' allele was 0.981 and 'C' allele was 0.018. This allele frequency distribution among the groups did not show statistical significant association (χ^2=*1.441*; p=*0.152*) (Table 3.2).

Table 3.2: SOD1 +35A>C genotype and allele frequencies of variants of the study subjects

Variables	**Control (n=55)**	**IGR (n=54)**
Genotype		
Wild AA [%(n)**]**	1.0(55)	0.963(52)
Ht AC [% (n)**]**	0	0.037 (2)
χ2 / p value= 2.075/0.150		
Allele frequency		
A	1. 0	0.981
C	0	0.18
χ^2 / p value=1.441/0.152		

Data presented as frequency (number of subjects). Chi square test was performed to calculate statistical association. A two-tailed p value of <0.05 was considered statistically significant.

SOD1, Superoxide dismutase; AA,Wild type; AC, Heterozygous variant (Ht);

3.3.2 SOD1 +35 A>C genotype frequencies among control and IGR subjects on the basis of gender

The number of male and female subjects in the study was 51 and 58 respectively.

In male the genotype frequencies for AA and AC were 0.980 and 0.020 respectively and for female the frequencies were 0.983 and 0.017 respectively. These frequency distribution did not show statistical significant association ($\chi 2$ = .008 and p= 0.927) (Table 3.3).

Table 3.3: SOD1 +35A>C genotype frequencies of the study subjects on the basis of gender

Variables	Wild AA (n)	Ht AC (n)	$\chi 2$ / p value
Gender			
Male (n=51)	0.980 (50)	0.020 (1)	0.008/0.927
Female (n=58)	0.983 (57)	0.017 (1)	

Data presented as frequency (number). Chi square test was performed to calculate statistical association. A two-tailed p value of <0.05 was considered statistically significant.

SOD1,Superoxide dismutase; AA,Wild type; AC,Heterozygous variant (Ht).

3.3 Characteristics of the total study subjects on the basis of SOD1+35A>C genotype

The data were analyzed according to SOD1+35A>C genotypes AA and AC in total study subjects. AA denotes homozygous wild type and AC denotes heterozygous variant type.

3.3.1 Anthropometric characteristics of the total study subjects on the basis of SOD1+35A>C genotype

Age (years)

Mean (±SD) age (years) is subjects with AA and AC genotype was 43.5±10.5 and 40.5±3.5 respectively which did not show statistical significant difference (p=0.690) (Table 3.3).

Body Mass Index (BMI, Kg/m^2)

Mean (±SD) BMI in subjects with AA and AC genotype was 24.03±4.34 and 26.74±1.26 respectively which did not show statistical significant difference (p=0.381) (Table 3.3).

Waist Hip Ratio (WHR)

Mean (±SD) WHR in subjects with AA and AC genotype was 0.92±0.07 and 26.74±1.26 respectively. WHR value in subjects with AA and AC genotype did not show statistical significant difference (p=0.381) (Table 3.3).

Blood pressure (mmHg)

Systolic Blood Pressure (SBP, mmHg**):** Mean (±SD) SBP in subjects with AA and AC subjects was 115.15±14.17 and 110.00±14.14 respectively which did not show statistical significant difference in AA compared to AC genotype subjects (p=0.612) (Table 3.4).

Diastolic Blood Pressure (DBP, mmHg**):** Mean (±SD) DBP of the AA and AC subjects was 77.08±10.55 and 82.50±3.54 respectively which did not show statistical significant difference in AA compared to AC genotype subjects (p=0.472) (Table 3.4).

Table 3.4: Anthropometric characteristics of the total study subjects distributed according to their SOD1 +35A>C genotype

Variables	Wild AA (n=107)	Ht AC (n=2)	t/p value
Age	43.5±10.5	40.5±3.5	0.399/0.690
BMI	24.03±4.34	26.74±1.26	-0.880/0.381
WHR	0.92±0.07	0.91±0.09	0.032/0.974
SBP	115.15±14.17	110.00±14.14	0.509/0.612
DBP	77.08±10.55	82.50±3.54	0.722/0.472

Data presented as mean±SD. Statistical comparison between groups was performed using unpaired Student's 't' test. A two-tailed p value of <0.05 was considered statistically significant.
AA, Wild type; AC, Heterozygous variant (Ht); BMI, Body Mass Index; WHR, waist Hip Ratio; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure.

3.3.2 Glycemic status, lipid profile, liver function status and insulinemic status of the total study subjects on the basis of SOD1+35A>C genotype

Glycemic and insulinemic status

Fasting glucose (FG, mmol/l**):** Mean (±SD) FG in subject with AA and AC genotype was 5.33±.89 and 5.55±1.06 respectively which did not show statistical significant difference

(p=0.731) (Table 3.5).

2h after glucose (2h AG, mmol/l): Mean (±SD) AG in subject with AA and AC was 6.69±1.76 and 6.35±2.47 respectively which did not show statistical significant difference (p=0.786) (Table 3.9).

Insulin (µIU/ml**):** Mean (±SD) insulin in subject with AA and AC was 13.80±8.65 and 22.56±14.24 respectively which did not show statistical significant difference (p=0.163 (Table 3.5).

Table 3.5: Biochemical characteristics of the study subjects according to their SOD1 35A>C genotype

Variables	Wild AA (n=107)	Ht AC (n=2)	t/p value
Glycemic status			
FG	5.33±0.89	5.55±1.06	-0.344/0.731
AG	6.69±1.76	6.35±2.47	0.273/0.786
Insulinemic status			
Insulin	13.80±8.65	22.56±14.24	-1.405/0.163
Lipid profile			
TG	159 ±8.19	190.00±88.5	-0.489/0.626
T chol	188 ±41	146.00±8.51	1.465/0.146
HDL-c	36.31±7.41	35.00±12.71	0.241/0.810
LDL-c	119±36	73.00±24.04	1.815/0.072

Data presented as mean±SD. Statistical comparison between groups was performed using unpaired Student's 't' test. A two-tailed p value of <0.05 was considered statistically significant.

AA, Wild type; AC, Heterozygous variant (Ht); FG, Fasting glucose (mmol/L); AG, After Glucose (2 hours after 70g oral glucose load)(mmol/l); TG, Triglyceride (mg/dl); T chol, Total cholesterol (mg/dl); HDL-c, High density lipoprotein cholesterol (mg/dL); LDL-c, Low Density Lipoprotein Cholesterol (mg/dl); CREAT, Serum creatinine (mg/dl); SGPT, Serum glutamate pyruvate transaminase (U/L).

Lipid Profile

Triglyceride (TG, mg/dl**)**: Mean (±SD) serum TG value in subject with AA and AC genotype was 159±8.19 and 190±88.5 respectively which did not show statistical significant difference (p=0.626) (Table 3.5).

Total cholesterol (T chol, mg/dl**):** Mean (±SD) serum T chol value in subject with AA and AC was 188±41 and 146±8.51 respectively. Difference of cholesterol between AA and AC subjects was not statistically significant (p=0.146) (Table 3.5).

High density lipoprotein cholesterol (HDL-c, mg/dl**):** Mean (±SD) HDL-c value in subject with AA and AC was 36.31±7.41 and 35±12.71 respectively which did not show statistical significant difference (p=0.146) (Table 3.5).

Low density lipoprotein cholesterol (LDL-c, mg/dl**):** Mean (±SD) LDL-c value in subject with AA and AC was 119±36 and 73±24.01 respectively which did not show statistical significant difference (p=0.072) (Table 3.5).

3.4 Analysis of the association of SOD1 polymorphic variants AA and AC genotype

3.4.1 Distribution of the SOD1+35A>C genotype on the basis of BMI

On the basis of Body Mass Index (BMI, Kg/m^2) the study population was divided into three groups as Normal, Overweight and Obese. In normal group, the frequencies were 1.0 and 0, in overweight group, the frequencies were 0.977 and 0.023 respectively, and in obese group, the frequencies were 0.955 and 0.045 respectively. These frequency distribution among the groups did not show any statistical significant association ($\chi 2 = 1.714$ and p= 0.425) (Table 3.6).

Table 3.6: SOD1 +35A>C genotype frequencies of the study subjects on the basis of BMI

Variables	Wild AA (n=107)	Ht AC (n=2)	χ^2 / p value
BMI Group			
Normal	1.0 (42)	0	1.714/0.425
Overweight	0.977 (43)	0.023 (1)	
Obese	0.955 (21)	0.045 (1)	

Data presented as frequency (number of subjects). Chi square test was performed to calculate statistical association. A two-tailed p value of <0.05 was considered statistically significant. SOD1, Superoxide dismutase; AA, Wild type; AC, Heterozygous variant (Ht);
BMI, Body Mass Index

Cut of point of BMI for Asian population: (WHO, 2004)
Normal range, 18.60 - 24.9; Overweight, 23.00-27.5; Obese, ≥27.5

3.4.2 Distribution of the SOD1+35A>C genotype on the basis of blood pressure

Systolic Blood Pressure (SBP, mmHg**)**

On the basis of Systolic Blood Pressure (SBP, mmHg) the study population was divided into two groups as Normal and High. For normal group the genotype frequencies of AA and AC were 0.978 and 0.022 respectively. For high group, the frequencies were 1.0 and 0 respectively. These frequency distribution among the groups exhibit statistical highly significant association (χ^2 = 0.408 and p= 0.523) (Table 3.7).

Diastolic Blood Pressure (DBP, mmHg**)**

On the basis of Diastolic Blood Pressure (DBP, mmHg) the study population was divided into two groups as Normal and High. For normal group the genotype frequencies of AA and AC were 0.989 and 0.011 respectively. For high group the frequencies were 0.947 and 0.053 respectively. These frequency distribution among the groups did not show any statistical significant association (χ2 = 1.476 and p= 0.224) (Table 3.7).

Table 3.7: SOD1 +35A>C genotype frequencies of the study subjects on the basis of blood pressure

Variables	Wild AA (n=107)	Ht AC (n=2)	χ^2 / *p value*
Systolic Blood Pressure			
Normal	0.978 (88)	0.022 (2)	0.408/0.523
High	1.0 (18)	0	
Diastolic Blood Pressure			
Normal	0.989 (88)	0.011 (1)	1.476/0.224
High	0.947 (18)	0.053 (1)	

Data presented as frequency (number of subjects). Chi square test was performed to calculate statistical association. A two-tailed p value of <0.05 was considered statistically significant. SOD1,Superoxide dismutase; AA, Wild type; AC, Heterozygous variant (Ht).

Normal value of systolic blood pressure 120 mmHg and diastolic blood pressure 80 mmHg.

3.4.3 Distribution of the SOD1+35A>C genotype on the basis of lipid profile

Triglyceride (TG, mg/dl**)**

On the basis of TG the study population was divided into two groups as Normal and High. For normal group the genotype frequencies of AA and AC were 1.0 and 0 respectively. For

high group the frequencies were 0.952 and 0.048 respectively. These frequency distribution among the groups did not show any statistical significant association ($\chi 2$ = 3.202 and p= 0.074) (Table 3.8).

Total cholesterol (mg/dl**)**

On the basis of total cholesterol the study population was divided into two groups as normal and high. For normal group the genotype frequencies of AA and AC were 0.974 and 0.026 respectively. For high group the frequencies were 1.0 and 0 respectively. These frequency distribution among the groups did not show any statistical significant association ($\chi 2$ = 0.810a and p= 0.368) (Table 3.8).

Table 3.8: SOD1 +35A>C genotype frequencies of the study subjects on the basis of lipid profile

Variables	Wild AA (n=107)	Ht AC (n=2)	$\chi 2$ / p value
TG			
Normal	1(67)	0	3.202/0.074
High	0.952 (40)	0.048 (2)	
T chol			
Normal	0.974 (76)	0.026 (2)	0.810/0.368
High	1.0 (31)	0	
HDL-c			
Normal	1.0 (13)	0	0.276/0.599
Low	0.979 (94)	0.021 (2)	
LDL-c			
Normal	0.979 (94)	0.021 (2)	0.276/0.599
High	1.0 (13)	0	

Data presented as frequency (number of subjects). Chi square test was performed to calculate statistical association. A two-tailed p value of <0.05 was considered statistically significant. SOD1, Superoxide dismutase; AA, Wild type; AC, Heterozygous variant (Ht). TG, Triglyceride; T chol, Total cholesterol; HDL-c, High-density lipoprotein cholesterol; LDL-c, Low density lipoprotein cholesterol.

Cut-off values of lipid profile

TG (50-150 mg/dl); T Cholesterol (150-200 mg/dl); HDL-c (Male: >55; Female: >65 mg/dl) LDL-c (Upto150 mg/dl)

High density lipoprotein cholesterol (HDL-c, mg/d**)**

On the basis of HDL-C the study population was divided into two groups as normal and low. For normal group the genotype frequencies of AA and AC were 1.0 and 0 respectively. For low group the frequencies were 0.979 and 0.021 respectively. These frequency distribution among the groups did not show any statistical significant association ($\chi 2$ = 0.276 and p= 0.599) (Table 3.8).

Low density lipoprotein cholesterol (LDL-c, mg/dl**)**

On the basis of LDL-C the study population was divided into two groups as normal and high. For normal group the genotype frequencies of AA and AC were 0.979 and 0.021 respectively. For high group the frequencies were 1.0 and 0 respectively. These frequency distribution among the groups did not show any statistical significant association ($\chi 2$ = 0.276 and p= 0.0.599) (Table 3.8).

3.4.4 Distribution of the study subjects on the basis of family history of chronic diseases

The study subjects were distributed on the basis of the family history of chronic diseases such as diabetes mellitus (DM) and hypertension (HTN).

Table 3.9: Distribution of subjects with SOD1 +35A>C genotype frequencies of the study subjects on the basis of family history diabetes mellitus and blood pressure

Variables	Wild AA (n=95)	Ht AC (n=2)	χ^2 / *p value*
DM family history			
Absent	0.980 (49)	0.020 (1)	0.002/0.965
Present	0.979 (46)	0.021 (1)	
HTN family history			
Absent	0.984 (60)	0.016 (1)	0.145/0.703
Present	0.972 (35)	0.028 (1)	

Data presented as frequency (number of subjects). Chi square test was performed to calculate statistical association. A two-tailed p value of <0.05 was considered statistically significant.

SOD1,Superoxide dismutase; AA,Wild type; AC,Heterozygous variant (Ht).
HTN, Hypertension; DM, Diabetes Mellitus.

Family history of Diabetes mellitus (DM)

The genotype frequencies of AA and AC in subjects without family history of diabetes were 0.980 and 0.020 respectively. In subjects with family history of diabetes, the frequencies were 0.979 and 0.021 respectively. These frequency distribution among the groups did not show any statistical significant association ($\chi 2 = 0.002a$ and $p= 0.965$) (Table 3.9).

Family history of hypertension (HTN)

The genotype frequencies of AA and AC in subjects without family history of hypertension were 0.984 and 0.016 respectively. In subjects with family history of hypertension, the frequencies were 0.972 and 0.028 respectively. These frequency distribution among the groups did not show any statistical significant association ($\chi 2 = 0.145$ a and $p= 0.703$) (Table 3.9).

3.4.5 Distribution of SOD1 +35A>C genotype on the basis of addictive habits

History of Smoking

SOD1+35A>C genotype AA and AC frequency in subjects without smoking habit were 0.968 and 0.032 respectively and in subjects with smoking habit the frequencies were 1.0 and 0 respectively. These frequency distribution among the groups did not show any statistical significant association ($\chi 2 = 1.055/$ and $p= 0.304$) (Table 3.10).

Table 3.10: SOD1 +35A>C genotype frequencies of the study subjects on the basis of smoking and tobacco abuse

Variables	Wild AA (n=92)	Ht AC (n=2)	χ^2 / p value
Smoking			
Absent	0.968 (60)	0.032 (2)	1.055/0.304
Present	1.0 (32)	0	
Tobacco			
Absent	0.976 (83)	0.024 (2)	0.216/0.642
Present	1.0 (9)	0	

Data presented as frequency (number of subjects). Chi square test was performed to calculate statistical association. A two-tailed p value of <0.05 was considered statistically significant.

SOD1,Superoxide dismutase; AA,Wild type; AC,Heterozygous variant (Ht).

History of Tobacco abuse

SOD1+35A>C genotype AA and AC frequencies in subjects without history of tobacco abuse were 0.976 and 0.024 respectively. In those with history of tobacco abuse the frequencies were 1.0 and 0 respectively. These frequency distribution among the groups did not show any statistical significant association ($\chi 2 = 0.216$ and $p= 0.642$) (Table 3.10).

CHAPTER 4

DISCUSSION

4. DISCUSSION

Free radicals has been hypothesized to cause oxidative damage of biomolecules, (lipids, proteins, DNA), eventually leading to many chronic diseases such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, cardiovascular diseases, chronic inflammation, stroke and septic shock, aging and other degenerative diseases in humans (Freidovich, 1999; Yun-Zhong *et al*., 2002). Reactive oxygen species (ROS) has been shown to act as a common mediator of pathophysiological effects of hyperglycaemia, which is hypothesized to lead to increased mitochondrial ROS formation (Brownlee *et al*., 2001). A recent study demonstrated that hyperglycemia-induced generation of superoxide radical ($\bullet O_2^-$) at the mitochondrial level is the initial trigger of a vicious cycle of oxidative stress in type 2 diabetes (T2DM) (Nishikawa *et al*., 2000; Brownlee *et al*., 2001).

Mammalian cells have evolved with a variety of antioxidant mechanisms to control ROS production and propagation (Fridovich 1999). The superoxide dismutases (SODs) are the first and most important line of antioxidant enzyme defense systems against the ROS and particularly superoxide anion radicals (Igor *et al*., 2002). SODs are metalloenzymes that catalyze the dismutation of superoxide radical ($\bullet O_2^-$) into hydrogen peroxide (H_2O_2). Superoxide dismutase 1 (SOD1) is located at the cytosol (Marchler-Bauer *et al*., 2007) and represents between 50% and 80% of the total SOD activity (Faraci *et al*., 2004; Fukai *et al*., 1998). SOD1-gene has five exons and four introns and the +35A/C polymorphism (21q22.1) is located adjacent to the splicing point being related to the SOD1-activity – AA-genotype having the higher SOD1-activity (Flekac *et al*., 2008).

It is understood that single nucleotide polymorphisms (SNPs) in DNA sequence are present at 1% or more within a population. The frequency of each SNP is highly variable between different populations within a single species. Polymorphism in the gene encoding the enzyme has been found to significantly affect its antioxidant role. As oxidative stress is a common pathogenic mechanism for the beta and endothelial cell damage, polymorphisms of SOD1 gene has become a subject of intense scrutiny for their association with metabolic disorder like diabetes.

A number of gene variants have been identified in the SOD1 gene (Flakar *et al.*, 2008). These variants have been found to be linked with functional difference of the enzyme. SOD1 gene

35A>C polymorphism adjacent to spicing site and reported to show lowering of its phenotypic activity (Flakar *et al.*, 2008)

Attempt was made to compare this genotype pattern with different ethnic population. Ghattas and Abo-Elmatty (2012) in their study demonstrated that healthy control subjects had wild 'AA' genotype 71.3% of the Egyptian population and in healthy Czech population control subjects had 27%, 50% and 25% AA, AC and CC genotype respectively. This finding suggest that SOD1 gene +35A>C polymorphic genotype are substantially different in Bangladeshi subjects from those of other ethnic background. However, it may be pointed out that only a relatively small number of subjects was included in study of both the healthy controls and impaired glucose regulation (IGR) group. Thus the finding of the present study needs to be substantiated by recruiting adequate number of subjects to achieve the optimum statistical power.

The main aim of the study was to determine whether genetic varieties of superoxide dismutase1 (SOD1) gene +35A>C (21q22.1) is associated with IGR patients of Bangladeshi origin. It has been mentioned that all the controls had wild 'AA' genotype and only two out of 54 IGR subjects were heterozygous. Owing to this fact that variant genotype sub-classified and analyzed the study variables for wild and variant genotype were inappropriate. It has been already mentioned about the smaller number of study subjects and inclusion of appropriate number of subjects will allow analyzing the target variable on the basis of wild and variant genotype and explore its relationship in the pathogenesis of insulin secretory capacity and ultimately understand the pathophysiology of diabetic

In the present study a total of 109 unrelated subjects (55 healthy controls and 54 IGR) were screened for glycemic status and SOD1 gene common polymorphic variants were investigated to explore its association with IGR and their biochemical traits. Among the subjects 51 (46.8%) were male and 58 (53.2%) were female.

It was observed that the all control subject had 'AA' genotype. The trend was similar in the IGR subjects as well. Out of 54 IGR subjects 52 had wild 'AA' genotype and the rest 2 (two) were heterozygous. The frequencies of the SOD1 gene +35A>C polymorphism did not show any association with IGR. The wild genotype was predominantly present in this study subject [100% is control and 96.3% is IGR]. This observation is supported by previous study carried

out on T2DM. In that study 96.9% of TDM subject had wild 'AA' genotype. It appears that the candidate SOD1 +35A>C polymorphism is relatively lower in frequency.

SOD1 +35A>C genotype frequency analysis did not shown statistical significant association (χ^2 = 2.075 and p= 0.150). The number of study subjects of wild AA genotype in control and IGR group was 52 and 54 respectively where in case of heterozygous (Ht) variant AC genotype the number was 0 and 2 respectively. For control group the genotype frequencies of AA and AC were 1.0 and 0 respectively. For IGR group, the frequencies were 0.963 and 0.037 respectively. Frequencies of the SOD1 +35A>C allele were 0.514 and 0 for A and C alleles respectively in the control group. In the IGR group the frequencies were 0.986 and 0.5 respectively. This allele frequency distribution among the groups have not shown statistically significant association (χ^2=*1.441*; p=*0.152*)

Thus it may be concluded that SOD1 gene +35A>C variant is substantially different from other population and the polymorphic allele is not associated with insulin secretory defect of IGR subjects of Bangladeshi origin.

Chapter 5

Conclusions

5. CONCLUSION

The observations made in the present study indicate that SOD1 +35A>C polymorphism may not be associated with the pathogenesis of Impaired Glucose Regulation (IGR) and insulin secretory mechanism in Bangladeshi subjects.

CHAPTER 6

LIMITATIONS AND RECOMMENDATIONS

6. LIMITATIONS AND RECOMMENDATIONS

Limitations

- In the present study a small number of subjects was recruited.
- Measurement of blood superoxide dismutase level was not done; this would have strengthened the data substantially.
- Measurement of Insulin secretory capacity (HOMA%B) and insulin sensitivity (HOMA%S) were not done; this would have given the opportunity to explore its relationship with marker allele.

Recommendations

- The study needs to be expanded involving adequate number of patients to provide optimum statistical significance.
- Further study with larger sample size should be designed to explore the effect of SOD1 gene +35A>C polymorphism with insulin secretory capacity of the IGR subjects of Bangladeshi origin.

Chapter 7

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7. REFERENCES

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Appendix I

– সম্মতি পত্র –

শিরোনাম: বাংলাদেশী জনগণ এর ক্ষেত্রে সুপারঅক্সাইড ডিসমিউটেজ জিন পলিমরফিসম এবং ইম্পেয়ারড গ্লুকোস রেগুলেশন এর সাথে সম্পর্ক নির্ণয় করা।

আমি গবেষণার বিষয়ে বিস্তারিত জেনেছি। জিজ্ঞাস্য সকল বিষয়ে সম্পূর্ণভাবে অবগত। সজ্ঞানে আমি এই গবেষণায় অংশ গ্রহণের সম্মতি জ্ঞাপন করছি। আমি জানি যে গবেষণায় অংশ নিলে আমার নিজের কোন স্বাস্থ্যঝুঁকি নাই। আমি এটাও জেনেছি যে এই প্রকল্পে অংশগ্রহণ করা না করা বা যেকোন সময় নাম প্রত্যাহার করার স্বাধীনতা আমার আছে।

গবেষকের নাম :-.....সাক্ষর/আঙ্গুলের ছাপ।

গবেষণায় অংশগ্রহনকারীর নাম:-.....সাক্ষর/আঙ্গুলের ছাপ।

সাক্ষীর নাম:-. .....সাক্ষর/আঙ্গুলের ছাপ।

তারিখ:

Appendix II

Data collection Form
Dept. of Physiology and Molecular Biology
BIHS

Project title: Superoxide Dismutase 1 (SOD-1) gene +35A/C (intron3/exon3) Polymorphism in Bangladeshi type 2 diabetic subjects

Code no: **Date:** **Gender: M/F**

Name:		Age (yr)	

Address & Phone no.	

Anthropometry:

Height (cm)	Weight(kg)	Waist circum(cm)	Hip circum (cm)	Mid-arm Circum(cm)	Body fat mass (%)

Systolic blood pressure		Diastolic blood pressure	

Blood pressure (mmHg):

Marital Status:

Married	1	unmarried	2

Residence :

Rural	Semiurban	Urban
1	2	3

Religion :

Muslim	Hindu	Christian	Buddhist	Other
1	2	3	4	5

Education:

Illiterate	Primary	Secondary	Graduate	Above
1	2	3	4	5

Occupation:

Student	Professionals	Office job	Skilled worker	Unskilled worker
1	2	3	4	5

Annual income:

<5000	<10,000	10,000-20,000	20,000-30,000	>30,000
1	2	3	4	5

Obstetrical history:

Menopause	yes	1	No	0
Menstrual cycle	Regular	1	Irregular	2
OCP	Yes	1	No	0

Physical activity:

Physical work	1	Sedentary work	2

Type of activity:

walking	Free hand	Gym
1	2	3

Duration of exercise:

<1 hr	1	>1 hr	2

Habits:

Smoking : Present:

No	0	Yes	1	Duration		No. of sticks	

Previous :

No	0	Yes	1	duration		No. of sticks	

Tobacco leaf chewing/ using powder:

Previous user	No	0	Yes	1	Duration	
Present user	No	0	Yes	1	Duration	

Betel leaf chewing :

Present user	No	0	Yes	1	Duration	
Previous user	No	0	Yes	1	Duration	

Tobacco powder sniffing:

Present user	No	0	Yes	1	Duration	
Previous user	No	0	Yes	1	Duration	

History of beverage drinking:

Alcohol :

Present user	No	0	Yes	1	Duration	
Previous user	No	0	Yes	1	Duration	

Tea / coffee:

Present user	No	0	Yes	1	Duration	
Previous user	No	0	Yes	1	Duration	

Soft drinks (habitual=regular basis):

Present user	No	0	Yes	1	Duration	
Previous user	No	0	Yes	1	Duration	

Energy drinks:

Present user	No	0	Yes	1	Duration	
Previous user	No	0	Yes	1	Duration	

Time of supper:

Consumption of meat per week:

once	1	twice	2	thrice	3	more	4

Medical history:

Present:

Previous:

Family history of chronic diseases:

Diabetes:

Father	Mother	Brother	Sister	M uncle Aunt	P uncle aunt	M GP	P GP
1	2	3	4	5	6	7	8

Hypertension:

Father	Mother	Brother	Sister	M uncle Aunt	P uncle aunt	M GP	P GP
1	2	3	4	5	6	7	8

Heart disease:

Father	Mother	Brother	Sister	M uncle Aunt	P uncle aunt	M GP	P GP
1	2	3	4	5	6	7	8

Stroke:

Father	Mother	Brother	Sister	M uncle Aunt	P uncle aunt	M GP	P GP
1	2	3	4	5	6	7	8