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dissertation entitled

QUANTITATIVE DETERMINATION OF
GLUCOSE-6-PHOSPHATE DEHYDROGENASE
AND
ITS POTENTIAL IN DIABETIC COMPLICATIONS

presented by

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has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in CHEMISTRY

Major Professor's Signature

12-09-08

Date

QUANTITATIVE DETERMINATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND ITS POTENTIAL IN DIABETIC COMPLICATIONS

BY

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Chemistry

2008

ABSTRACT

QUANTITATIVE DETERMINATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND ITS POTENTIAL IN DIABETIC COMPLICATIONS

By

C. Wasanthi Subasinghe

Diabetes mellitus has become an epidemic during the last few decades without a cure and with more than 170 million people diagnosed worldwide. In addition, oxidative stress arising from hyperglycemia facilitates cardiovascular disease in diabetes, increasing the risk factor by threefold in comparison to healthy controls. Appropriate treatment of disease is dependent on the knowledge of the patho-physiology of the disease, the mechanism underlying hyperglycemia and efficacy of exogenous insulin and various pharmaceutical agents to improve postprandial hyperglycemia.

This work describes an attempt to evaluate the degree of damage caused by oxidative stress and hyperglycemia to the red blood cells (RBCs) by investigating molecular level changes in structure and certain metabolites within the RBC. Deformation induced RBC-derived ATP release from erythrocytes is related to cellular antioxidant status. Simultaneous monitoring of glucose-6-phosphate dehydrogenase G6PD activity (via fluorescence determination of cellular NADPH) and ATP release from erythrocytes in a microflow system is described. To confirm the relationship between these analytes, a determination of NADPH and ATP release was performed while inhibiting G6PD. Upon inhibition, NADPH concentrations decreased to 8.26 ±

0.29 μM from an original value of 12.73 ± 0.50 μM, while ATP release decreased from 0.21 ± 0.07 μM to 0.06 ± 0.02 μM. Results confirm that decreasing cellular NADPH levels coincide with a decrease in ATP release from the RBCs. A similar trend was observed using erythrocytes from type 2 diabetes patients.

In support of this hypothesis, the effect of proinsulin C-peptide (activated with Zn^{2+}) on G6PD-inhibited RBCs was investigated. G6PD inhibition leads to the inhibited glucose transport into the RBC, lowered RBC-derived ATP release and lowered incorporation of C-peptide in to the RBCs. In an attempt to investigate the mechanism of metformin in type 2 diabetes, it was revealed that metformin (in combination with C-peptide) exerts a "sensitizing" effect improving the ability of C-peptide to facilitate glucose uptake into RBCs.

The sorbitol pathway was up-regulated under hyperglycemia. A further investigation of G6PD inhibition on sorbitol pathway lead to inconclusive results. However, despite the belief that hyperglycemia increases sorbitol levels, a relative reduction in sorbitol level was observed upon changing the glucose concentration from 5.5 mM to 7.5 mM, although further increase in glucose concentration up to 17.5 mM increased the sorbitol concentration.

In conclusion, G6PD enzyme activity is of extreme importance in maintaining proper cellular antioxidant defense systems. Inhibition of G6PD resulted from an exposure to hyperglycemia, leads to facilitate observed cardiovascular complications in diabetes individuals.

~ To my Family ~

ACKNOWLEDGEMENTS

First and foremost I offer my sincerest gratitude to my supervisor, Dr Dana Spence, who has supported me throughout my PhD work with his knowledge and guidance whilst allowing me the room to work in my own way. His enthusiasm, his inspiration, and his great efforts to explain things clearly and simply gave me determination and dedication needed to continue my work especially at times when the things are not moving as they should. One simply could not wish for a better or friendlier supervisor.

Also I would like to thank Dr. Garry Blanchard, Dr. John McCracken and Dr. Gregory Baker for serving on my committee as well their helpful reviews on my dissertation. Note of thanks goes to the Department of Chemistry, Michigan State University for providing me this opportunity. Also my gratitude goes to NIH and various funding agencies that funded my studies. I would like to thanks many people who have taught me Chemistry: my high school teachers and professors at University of Colombo, Sri Lanka and Wayne State University, Detroit, Michigan.

In my daily work I have been blessed with a friendly, fun and cheerful group of fellow students. Special note of thanks goes to Luiza Genes and Madushi Raththagala for being true companions who shared good and bad times from the fist day I met them almost five years ago. Also I would like to thank Ajith Karunarathne for his help with labVIEW and other computer troubles which I seem to run in to more often than others.

My appreciation goes to Andrea, Chia, Jen and Nicole for their contribution in rabbit surgery. Finally rest of the Spence group: wathsala (for good times in and out of the lab), Teresa, Steve and Adam (may be I should say steve....?) need some recognition too. It was a true pleasure to be a part of Spence group.

I wish to thank all my friend specially Niro, Amila, Thilini, Aruni and Rhazes for helping me get through the difficult times, and for all the emotional support, camaraderie, entertainment, and caring they provided. Last but not least my special gratitude goes to every single person who helped me even in a simplest possible way during last four and half years.

Finally, I would like to thank my parents and my sister for their support, encouragements and unconditional love and standing by me regardless of them approving my decisions. To them I dedicate this thesis.

TABLE OF CONTENTS

CHAPTER **PAGE**

List of tables

LIST OF FIGURES

FIGURE **PAGE**

FIGURE **PAGE**

FIGURE **PAGE**

FIGURE **PAGE**

Chapter one

INTRODUCTION TO DIABETES MELLITUS

1.1 Diabetes mellitus

Diabetes mellitus is metabolic syndrome often characterized by disordered metabolism, followed by increased blood sugar levels (hyperglycemia) resulting from hereditary and environmental causes.[1,2] Under normal conditions blood glucose level is tightly regulated in the human body between about 4 and 6 mM (mmol/ L) with normal average of about 5 mM.[3] The homeostasis of blood glucose is achieved via the two closely interacting, mutually antagonistic hormones, insulin and glucagon.[4] Under normal conditions the hormone insulin, secreted by pancreatic beta (β) cells, promotes glucose uptake into the liver, muscle cells and peripheral tissues in order to maintain normal fasting blood glucose level.[5,6] Insulin is a small peptide hormone consists of the two peptide chains (total 51 amino acids) linked together by disulfide linkages. In mammalian species, insulin is synthesized in the pancreas within the β- cells of the islets of Langerhans in the endocrine part of the pancreas.[7] Upon dietary intake of carbohydrate, resulting elevation of blood glucose triggers a series of signal transduction pathways that trigger insulin secretion.[4]

Development of the diabetes mellitus can be characterized by impaired insulin secretion from pancrease, insulin resistance and increased hepatic glucose production resulting from both increased glyconeogenesis and glyconeolysis.[8-10] Gluconeogenesis is the process that synthesizes glycogen from dietary glucose in the liver and glyconeolysis cleaves glycogen into glucose, addressing glucose demand of the body in-between the meals.[4,11] Acute symptoms of diabetes include excessive urine production, resulting compensatory thirst and increased fluid intake, blurred vision, unexplained weight loss, lethargy, and changes in energy metabolism.[12,13]

Over the last two decades diabetes mellitus has become an epidemic with about 180 million people diagnosed with the disease worldwide.[14] According to the American Diabetes Association website, 23.6 million children and adults in the United States, alone are diabetic. In other words 7-8 % of the American population are diabetic individuals. Of the 23.6 million, 17.9 million are diagnosed with the disease while another 5.7 million remains unaware of the disease.[15,16] Annual economic cost for direct and indirect diabetes care in 2007 was estimated at $174 billion in the United States alone.[15,16] With the increasing adult population due to advances in health care, and obesity among younger generations due to high calorie diets and lack of exercise, the United States has witnessed an almost epidemic progression in diabetic cases.

The American Diabetes Association classifies five classes within the disorders that represent diabetic symptoms. Those types are shown in the table 1.1 and discussed in detail below. Out of those five classes, types 1, 2 and gestational diabetes are the most commonly occurring forms and attempts to classify others remain somewhat controversial.[17]

Type	**Characteristics**
Type 1	– No insulin production due to β-cell destruction – Developed during early years – Can not be cured but can be controlled by daily lifelong insulin administration – If untreated, can be fetal due to brain damage and coma
Type 2	– Insulin resistance of the target cells/tissues – Eventually leads to reduced insulin secretion – Visible symptoms are mild during development – No ketoacedotic episodes – Leads to development of retinopathy, neuropathy and cardiovascular diseases
Gestational diabetes	– Resembles type 2 diabetes – Developed during 24-28 weeks of pregnancy – If untreated can be lethal to the infant
Diabetes associated with contributed clinical states, diseases, drugs and/or chemicals	– Resembles type 2 – Arise due to other disease conditions, chemical agents or drugs used
Malnutrition-associated diabetes	– Resembles type 2 – Requires insulin – No ketoacidosis

Table 1.1: Different types of diabetes and their characteristics.

1.1.1 Type 1 diabetes

Type 1 diabetes, also commonly known as insulin dependent diabetes or juvenile diabetes, is thought to be an autoimmune disease resulting from a genetic or environmental trigger. As indicated by the name, type 1 diabetes usually develops during childhood or the early stages of life even though the disease can develop during latter stages as well.[18,19] Type 1 patients demonstrate autoimmune destruction of pancreatic β-cells which in turn, results in little to no insulin production in the body.[20] As a result, individuals suffering from type 1 diabetes are either totally or almost totally deprived of insulin.[18,19] In addition, various antibodies to islet antigens such as insulin and glutamic acid decarboxylase are found in these individuals.[18,19]

Conditions associated with type 1 diabetes include hyperglycemia, hypoglycemia, ketoacidosis which acidosis results from increased ketone body formation due to deamination of amino acids and celiac disease, an autoimmune disorder of the small intestine.[6] Current treatments for type 1 diabetes include lifelong intravenous insulin administration upon intake of a carbohydrate rich meal. In fact, treatment does not disrupt a normal life style if sufficient care awareness and timely testing and insulin administration is taken care of.[21,22] But if untreated glucose depravation or hypoglycemia of cells specially the brain cells can lead to the comma and decease.[21,22]

1.1.2 Type 2 diabetes

Type 2 diabetes, also known as adult onset diabetes mellitus is insulin independent and develops mostly during latter stages of life and contributes to 90 – 95 % of the diagnosed patients in United States.[15] In fact, according to the Center of Disease

Control, one fifth of the United States population over 60 years of age is diagnosed with type 2 diabetes. Type 2 diabetes is often characterized by a resistance to insulin function by target tissues followed by impaired insulin secretion.[20,23] Insulin resistance is the first sign of the development of type 2 diabetes. Usually an insulin resistance develops 5 -10 years prior to elevation of fasting blood glucose levels up to a diabetic range of above 7 mM[24]. As a result of this resistance, the redistribution of the glucose transporter GLUT 4 into the muscle cell membrane fails, reducing the glucose uptake into the muscle cells.[4,25] This insulin resistance can be characterized by abnormally high levels of insulin and C-peptide in the circulatory system. In response to this insulin resistance, insulin synthesis by pancreatic β-cells is up regulated, leading to extensive insulin production and eventual β-cell death due to over exhaustion.[25] Therefore as the disease progress replacement of insulin become a necessity for survival.[26]

Although there are numerous explanations for the pathogenesis and disease progression of type 2 diabetes, to date, an exact explanation is unavailable. Obesity is believed to be one of the major contributors for the development of type 2 diabetes. In fact statistics indicate 55 % of the type 2 diabetes patients in United States are obese. In facts there are reports suggesting the central obesity influence individuals to be insulin resistance, by secreting group of hormones called adipokinases that could impair glucose tolerance. Other major contributors include aging and family history.[26]

Treatment options to type 2 diabetes include an inclined physical activity, decreased carbohydrate intake. Weight loss, especially from abdominal fat deposits also improves the insulin sensitivity significantly.[26] The oral medications and insulin injection will follow if the above options were unsuccessful in controlling the disease progression.[26] Though insulin resistance is one of the major causes of type 2 diabetes it is not the only cause. Recent studies indicate C-peptide, a small peptide secreted during insulin biosynthesis, may play a major role in blood glucose level control and complications associated with diabetes specially in cardiovascular system. The effect of C-peptide in diabetes pathogenesis will be discussed in-detail below and in chapter 5.

1.1.3 Gestational Diabetes

Gestational diabetes is usually developed during the pregnancy specifically around 24th to 28th weeks.[27] Occurrence rate for this disease is about 2 – 5 % among pregnant women. Prevalence of the gestational diabetes is high among the older, obese, women with family history of diabetes and women from diabetes prone ethnic groups.[28,29] Symptoms of the disease are more or less similar to that of type 2 diabetes and these women either fully recover from the disease or develop in to type 2 diabetes at the end of their pregnancy.[30] Statistics suggests that about 20 – 50 % of these women develop types 2 diabetes later in life.[17]

Even though gestational diabetes is temporary, if untreated it can damage both mother and the fetus or even can be fetal to the fetus. Untreated elevated blood glucose can cause macrosomia which is overweight of the fetus at the birth, congenital cardiac and central nervous system anomalies, respiratory distress syndrome and hyperbilirubinemia resulting from RBC destruction.[31]

1.1.4 Diabetes associated with contributed clinical states, diseases, drugs and/or chemicals

This is a type 2 diabetes condition associated with other clinical states, drugs administrated or chemical agents. Pancreatic diseases such as chronic pancreatitis, cystic fibrosis, genetic syndromes such as muscular dystrophy and Huntington's chorea can cause development of type 2 diabetes. Also administration of certain drugs such as beta blockers, glucocorticoids, some antidepressant medications and conditions that that requires pancreatectomy leads to development of diabetes mellitus. Usually this can be cured or controlled by either eliminating the drugs that cause the distress or treating the clinical condition.

1.1.5 Malnutrition – associated diabetes

Malnutrition – associated diabetes is a condition somewhat similar to type 2 diabetes. In this case patients require insulin for glycemic control but they do not develop diabetic ketoacidosis. Malnutrition – associated diabetes is usually developed in individuals of age range between 10 and 40.

Both untreated or severe type 1 and type 2 diabetes can lead to the secondary complications such as retinopathy, neuropathy, nephropathy and complications in the cardiovascular system such as high blood pressure, angina and heart attack. Diabetic retinopathy is retinopathy or damage to the retina of the eye, caused by elevated blood glucose and resulting changes in microvasculature in diabetes mellitus, which can eventually lead to blindness.[32] In fact, there are reports suggesting that eight out of the 10 individuals diagnosed and liveing with diabetes, for 10 or more years develop diabetes retinopathy.[32] Diabetic neuropathy is a deranged function and structure of peripheral nervous system resulting from injuries to the vasa nervorum (small blood vessel feeding the nerves) and diabetic nephropathy is a progressive kidney disease caused by angiopathy (disease) of capillaries in the kidney. It is the prime cause for dialysis in diabetes patients.

In fact, the elevated blood glucose believed to be the major cause of structural and rheological alternations in red blood cells (RBCs) and microvasculature which leads to the complications. Therefore the firm understanding of the underlying glucose metabolism mechanism, metabolic pathways involved and the energy status of the cells specially, in RBCs is beneficial in disease control of diabetes and associated cardiovascular complications. In-fact, there are reports suggesting the impact of altered activity of different metabolic pathways such as pentose monophosphate shunt and sorbitol pathway on development of the cardiovascular complications in diabetic individuals.

1.2 Glucose-6-phosphate dehydrogenase and the pentose phosphate pathway

The pentose phosphate pathway (PPP) also known as the phosphogluconate pathway or the hexose monophosphate shunt (HMP shunt) is an exclusively cytosolic process that generates NADPH and synthesizes pentose (5-carbon sugars) during the process (Figure 1.1).[4,33] There are two major phases in the PPP, namely the oxidative phase and the non-oxidative phase.[4] During the oxidative phase, reducing equivalents are generated in the form of nicotinamide adenine dinucleotide phosphate (NADPH) (Fig 1.2

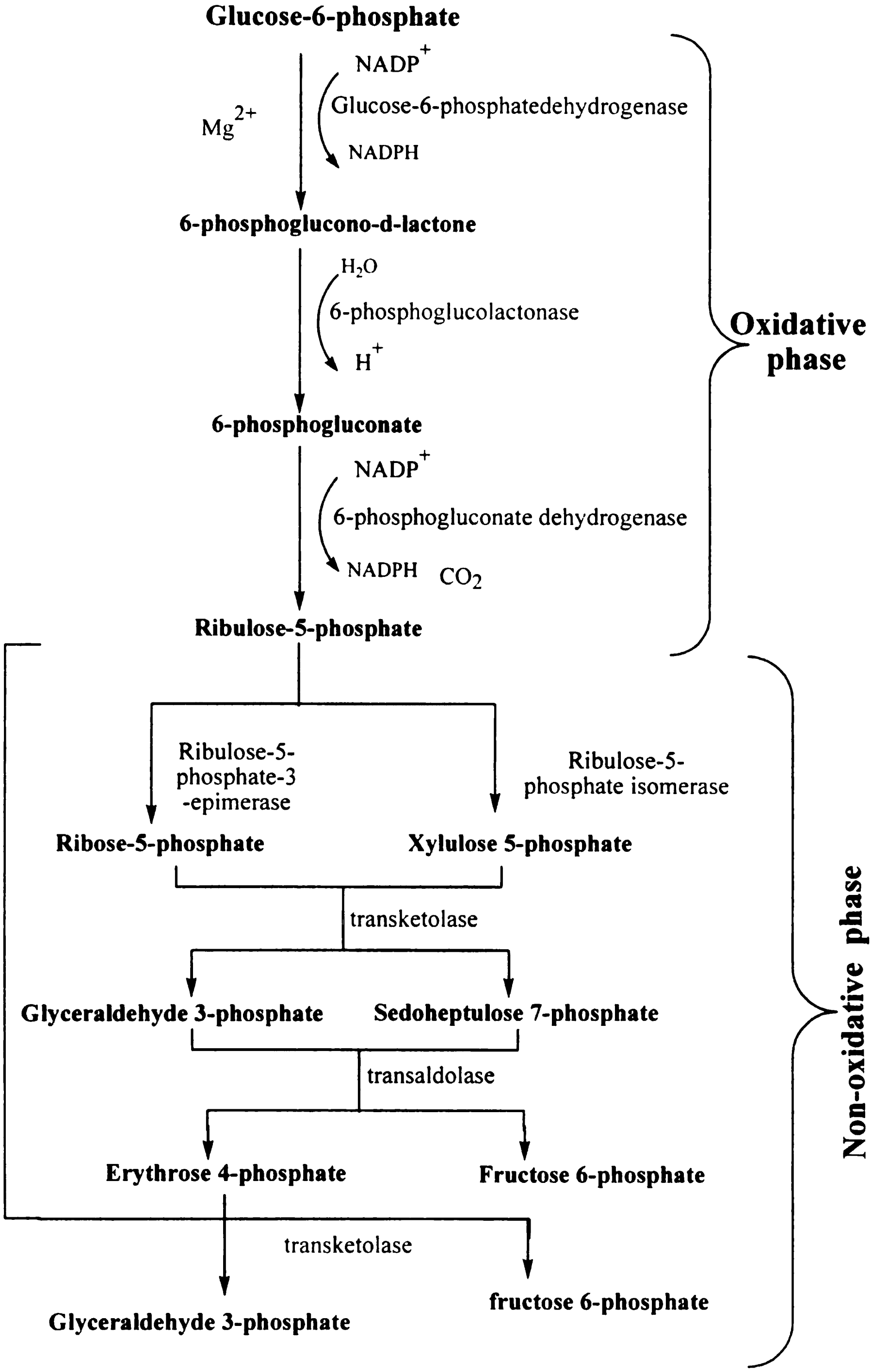

Figure 1.1: Schematic representation of the pentose phosphate pathway. During oxidative phase G6P is converted to ribulose-5-phosphate. During the nonoxidative phase pentose sugars are formed.

a.) for reductive biosynthesis reactions within cells, and the synthesis of 5-carbon sugars, which are essential for DNA and RNA synthesis, occur during the non-oxidative phase.[4] The PPP is an alternative to the glycolysis, which is the primary method of glucose oxidation in the body.[4] Even though the PPP does involve in the oxidation of glucose, its primary role is anabolic rather than catabolic.[4]

Upon entering the cell, glucose is converted to glucose-6-phosphate (G6P) by enzyme hexokinase.[4] This G6P can either be channeled to glycolysis for energy production or the PPP. During the oxidative phase, the G6P molecule is subjected to series of oxidative reactions resulting in two NADPH molecules and ribulose 5-phosphate (Fig 1.1).[4]

Glucose-6-phosphate dehydrogenase (G6PD) is the first and the key enzyme of the PPP.[33] This X-linked enzyme show in figure 1.2.b, in its active enzyme form, is made up of either two or four identical subunits, each having a molecular mass of about 59 kD; this is more than three times as large as the hemoglobin. G6PD catalyzes the oxidation of G6P into 5-glucono-1,5-lactone-6-phosphate, while reducing one $NADP^+$ molecule to its reduced form (NADPH) transferring an electron from one molecule to another. Being the rate determining step of the PPP, G6PD is responsible for maintaining adequate levels of NADPH within the cell.[33]

NADPH is a required cofactor in many biosynthetic reactions and extremely important in maintaining/regenerating glutathione (GSH), a small tri-peptide, in its reduced form (Figure 1.2.C).[34] Reduced GSH acts as a scavenger for dangerous oxidative metabolites in the cell such as hydrogen peroxide and other free radical generating species.[34] Therefore, the function of G6PD is critical to human survival. The activity of

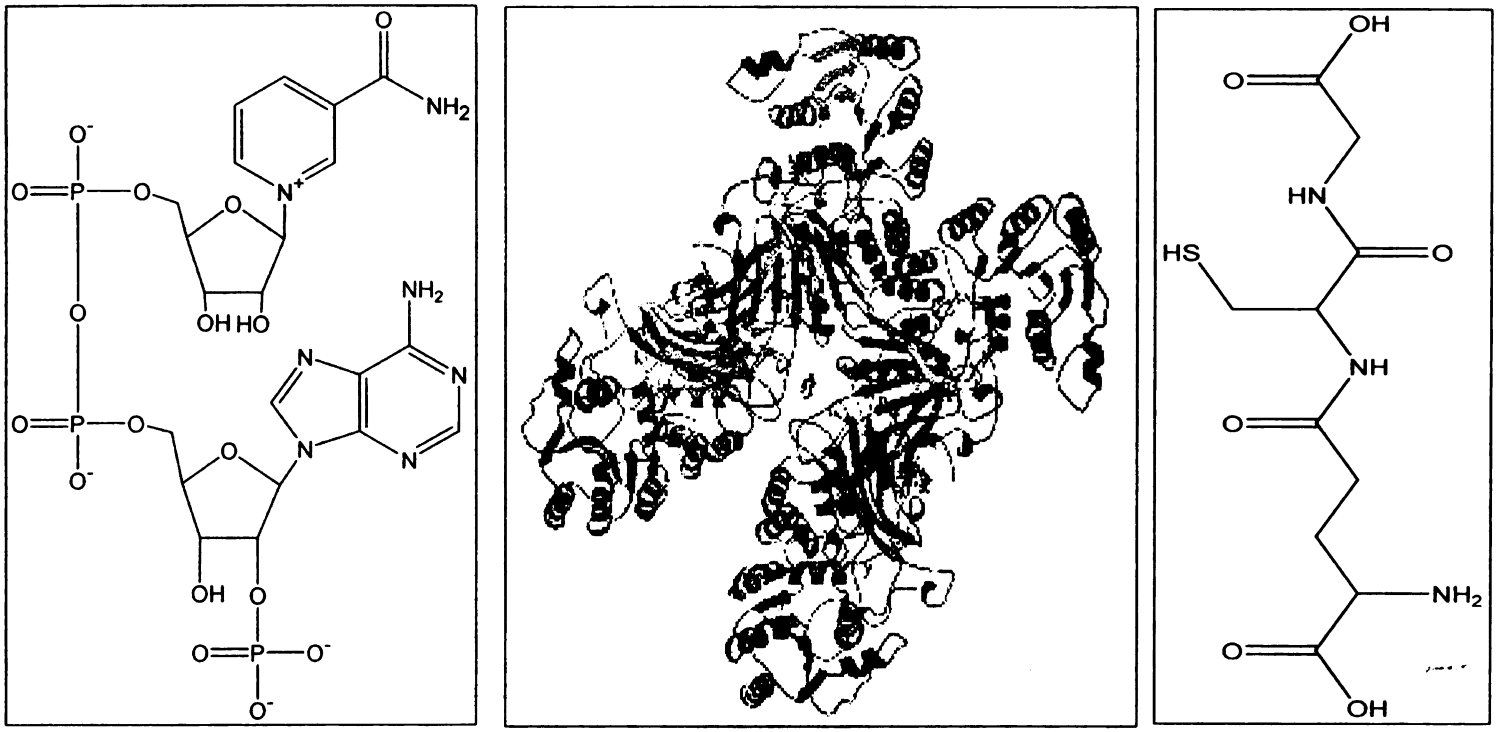

Figure 1.2: (a) Structure of NADPH, NADPH (b) protein structure of G6PD. G6PD consists of two or four identical subunits each weighing about 59 kD (c) structure of glutathione is a small tripeptide of cysteine, glutamate and lysine with unusual peptide bond between the amine group of cysteine and the carboxyl group of the glutamate side chain. GSH is the most common non enzymatic antioxidant present in human RBCs.

G6PD has a profound effect on the stability of red blood cells (RBCs) because they are especially sensitive to oxidative stresses and G6PD is the only NADPH-producing enzyme present in RBCs. As a result, G6PD deficient individuals are not capable of handling oxidative stress and consequently, hemolysis ensues.[35]

It is well established that G6PD deficient individuals are subjected to high oxidative stress due to the lack of NADPH production, and hence a weakened antioxidant defense system.[36-39] Though G6P levels appear to be normal in RBCs obtained from people with diabetes, it is well known that G6PD activity in the RBCs from patients with diabetes is deficient compared to healthy individuals[40-42], thus resulting in decreased NADPH levels in the diabetic.

In addition to its importance in the RBC as a major determinant in oxidative stress, G6PD activity is also employed as an indicator of cell age.[43] As the cell ages G6PD activity decreases, making cell age inversely related to G6PD activity.[44] Therefore by understanding the behavior of PPP and G6PD in aged RBCs may exert beneficial effect in understanding its activity under hyperglycemia and its contribution to the development of cardiovascular complications.

1.3 Diabetes induced oxidative stress, antioxidant status of the red blood cell and its effect on cardiovascular complications

Understanding the injuries at the cellular level induced by hyperglycemia is critical in both damage control and treatment of diabetes. At the cellular level, various metabolic pathways can be activated or up-regulated by either the elevated glucose levels or metabolic derivatives of glucose. These pathways and products that are accelerated as a result of hyperglycemia, includes increase in reactive oxidative species such as peroxides, superoxides and other reactive free radicals, increase in formation of advanced glycation end-products (AGE), activation of polyol pathway and enhanced aldose reductase activity and activation of protein kinase C isoforms.

Activation of the above mentioned pathways/enzymes results in the accumulation of products that lead to an oxidatively-stressed cellular environment, thereby damaging important cellular components. In human RBCs that lack oxidative protection due to a lowered G6PD activity and hence weakened anti-oxidant defense under hyperglycemia, this effect seems profound.

RBCs are biconcave disk shape structures that lack nuclei and many other cellular organelles such as mitochondria and golgi apparatus.[45] RBCs that are synthesized in the bone marrow contribute to about 45 % of the whole blood and are also the main cellular component in blood. RBCs are about 6 – 10 μm in diameter with about 80 fL volume and have a distinct red color as a result of the abundant oxygen carrier hemoglobin.[45,46] Hemoglobin is an iron-containing metalloprotein that contributes up to 35 % of the volume of the mammalian RBCs.

Though RBCs major purpose is to transport oxygen and carbon dioxide in and out of the tissues, recent findings suggest RBCs are not mere "sacks of hemoglobin" but they actively participate in controlling vascular tone.[45] Therefore it is important to understand the effects of diabetes relative oxidative stress on RBCs structure and function and its consequences in ensuing cardiovascular complications that arise from diabetes [40,47].

Commonly known as glutathione, 2-amino-5-{[2-[(carboxymethyl)amino]-1-(mercaptomethyl)-2-oxoethyl]amino}-5-oxopentanoic acid is small tripeptide of cysteine, glutamate and lysine (Figure 1.2.C). GSH is the most common non enzymatic antioxidant present in human RBCs.[48] It contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. In cells thiol groups are kept in a reduced state and the reduced thiol group concentration is about 5 mM within the cell.[48] In its reduced form GSH, readily undergoes oxidation to the dimer (GSSG) forming a disulfide linkage, to protect much important cellular components such as proteins from oxidant insults (Fig 1.3)[49].

Under normal conditions, enzyme glutathione reductase regenerates GSH at the expense of the reducing cofactor NADPH. Therefore, a steady supply of NADPH is vital for maintenance of healthy antioxidant defense in cells specially in RBCs where GSH is the main non enzymatic antioxidant. Though other cells have several methods to produce NADPH, RBCs rely solely on the enzyme G6PD for regeneration of NADPH.

In addition to having highly oxidative environment resulting from hyperglycemia, having weakened antioxidant defense makes RBCs of diabetes individuals more prone to oxidative damage. It is well established that spectrin a common membrane skeletal protein of these diabetes RBCs are readily subjected to oxidation thus changing their specific three dimensional structure, hence their function ensuing a stiffened RBC membrane. [50] Stiffened RBC membrane will not only hinder the shape change they need to undergo upon travelling through microvasculature but will result in decrease in degree of deformation, resulting decrease in release of adenosine tripeptide (ATP) upon mechanical deformation.[40]

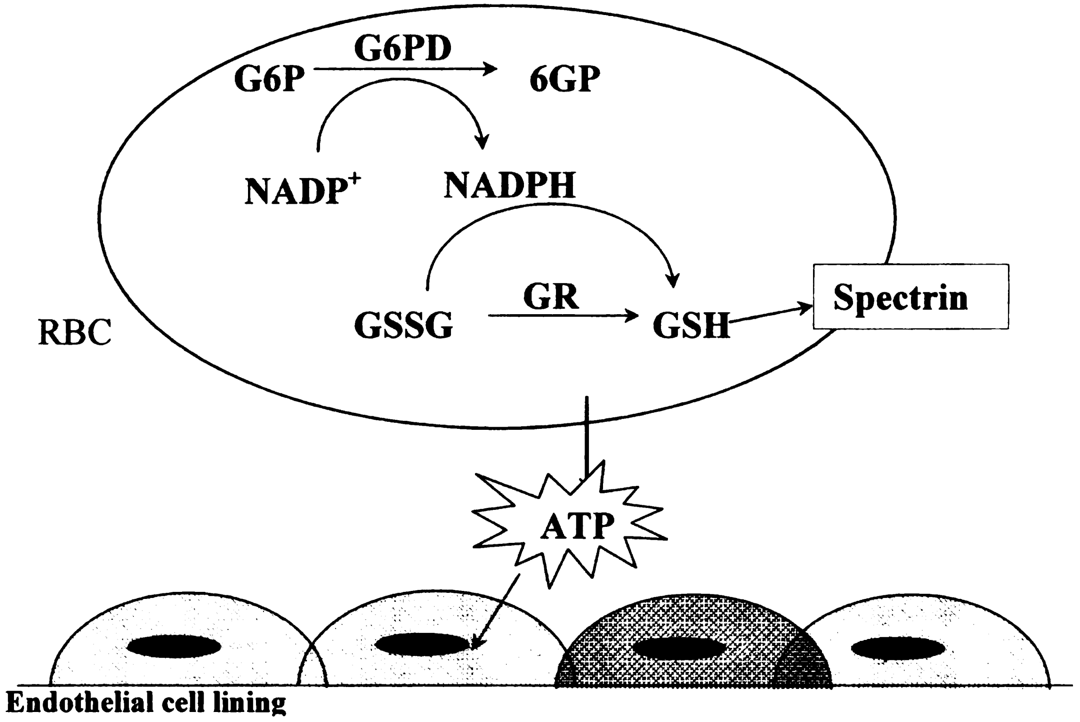

Figure 1.3: Schematic representation of antioxidant defence mechanism in RBCs. GSH undergoes oxidation to form GSSG protecting such important components like spectrin. Glutathione reductase (GR) regenerates GSH with the expense of NADPH. G6PD supply the required NADPH in the process oxidizing G6P to 6GP. ATP released when subjected mechanical deformation acts as no stimulator to the endothelial cells.

1.4 Deformation induced, CFTR mediated ATP release from RBCs in controlling vascular tone

Originally it was believed that oxygen transport from blood vessel, within the tissue occured by passive diffusion across the oxygen (O_2) gradient maintained by the uniform consumption of O_2 by the target tissue itself.[45,51] However this model failed to explain the observed sensitivity of the vasculature to changes in physiological O_2 levels inside the target tissues.[45,52] In 1993, studies conducted by Stein *et al.* reported that the amount of the O_2 present is more important in controlling oxygen supply than oxygen tension, suggesting the role of RBCs as a oxygen sensor in controlling vascular caliber.[45,52]

RBCs contain millimolar amounts of adenosine 5-triphosphate (ATP) in their cytosol. It has been shown using various experimental animal models and in human RBCs, that when traveling through microvasculature, RBCs release nanomolar to low micromolar amounts of ATP upon being subjected to mechanical deformation.[45,53,54] This ATP binds to the purinegic receptor (P2Y) on the vascular endothelium resulting in synthesis and release of the vasodilator nitric oxide (NO) resulting in vasodilation followed by an increase in blood flow to the target tissue. ATP is metabolized by enzymes present in the circulation, therefore this effect is likely localized.[45,54,55]

The mechanism by which ATP is released remains somewhat controversial, although several components of the signaling pathway have been identified over the fast few years. However, it has been proven that ATP release from RBCs occurs only in the presence of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is a cyclic adenosine monophosphate (cAMP) activated chloride channel expressed mainly in epithelial cells. However, CFTR also regulates the transport of other components.

The currently accepted mechanism suggests that ATP release occurs through a G-protein coupled signal transduction pathway (Figure1.4).[45,56] Receptor mediated activation of hetero-trimeric G protein will lead to the activate adinylyl cyclase enzyme, increasing cAMP within RBCs. This increased cAMP will lead to the activation of protein kinase-A, eventually activating CFTR, prompting the ATP release.

ATP release is mediated by pathway that involves various membrane bound proteins and enzymes, thus, oxidative damage to the RBC membrane can lead to decreased ATP release, which will eventually lead to impaired O_2 supply to the target tissues.

1.5 Proinsulin C-peptide as a potential treatment regime for diabetes

Until recently, microvascular complications arising from diabetes were attributed to elevated glucose levels and its consequences such as oxidative stress and activation of the polyol pathway. But recently with new findings in diabetic pathogenesis, it is believed that a deficiency of circulating C-peptide in the blood stream has a significant role, especially in type 1 diabetic complications.[57-59] Earlier, it was believed that microvascular complications are much more aggressive and rapidly developed in type 1 individuals in comparison to that of type 2.[60] Only in light of recent findings has it become clear that an essential lack of circulating C- peptide has contributed to these accelerated disease progressions and secondary damages in type 1 individuals.[57,60,61]

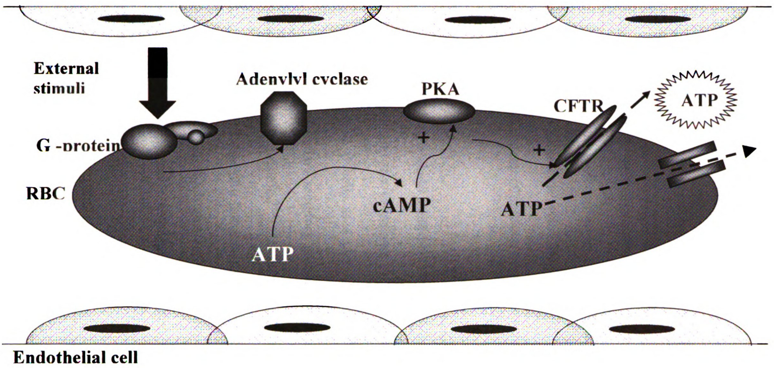

Figure 1.4: Proposed pathway for ATP release form RBCs. G-protein is activated as a respond to external stimuli (such as mechanical deformation, hypoxia). G protein will leads to the activate adinylyl cyclase, increasing cAMP within RBCs. This increased cAMP will lead to the activation of protein kinase-A, eventually activating CFTR, prompting the ATP release. Whether ATP is channeled through CFTR or other channel is not very clear.

In fact, currently, there are studies suggesting acute administration of C-peptide can improve vascular, kidney and neural dysfunction in type 1 diabetes.[59] It is now increasingly evident that C-peptide, in combination with insulin, is successful in controlling diabetic complications rather than insulin itself.

Although C-peptide was discovered in 1967, up until the 1990's, little or no biological significance was attributed to its function.[62] In fact, as indicated by the name "Connecting peptide", C-peptide was thought to be physiologically inert beside. providing the spatial arrangement necessary for the secondary and tertiary folding of the A and B chains of insulin.[62]

C-peptide is a single chain small peptide consisting of 31 amino acids connecting the N-terminal B-chain and the C-terminal A-chain.[63] It is synthesized during insulin biosynthesis in pancreatic β cells as a part of proinsulin.[63] Later, it is cleaved from proinsulin, stored in secondary secretory granules and released into the blood stream in equimolar amounts with insulin.[64,65] Even though the half life of insulin in the bloodstream falls in the range of a few minutes, C-peptide remains for hours. As a result of this, C-peptide is utilized as a biomarker for insulin.[66-69] Also, this longer half life of C-peptide in the bloodstream reinforces the claim that suggest at high hormone concentration, C-peptide suppresses the non-hypoglycemic effect of insulin and at lower concentration it enhances the insulin effect.[57,70-72]

Despite these new findings that suggest bioactivity of C-peptide, unequivocal lack of knowledge concerning the mechanism of C-peptide activity (as well as its inability to diminish complications in a certain percentage of the treated individuals during studies conducted), limits the use of C-peptide as a potential treatment option in diabetes. In fact, there are major drawbacks that stand against the notion of C-peptide as a bioactive peptide.

In a conventional way of thinking, a bioactive peptide exerts its effect by binding as a ligand to a specific molecule known as "receptor".[73] In the case of ligand receptor binding, there is a specific moiety in the ligand, as well as a receptor, that act as an active site for binding.[73] However, there is no known receptor for C-peptide in any of the target cells such as RBCs. Also, studies indicating not only the full-length native C-peptide, but also its C-terminal pentapeptide fragment is reported to exert physiological effects confirm the lack of the receptor based interaction for proinsulin C-peptide.[73]

Recently, non receptor mediated membrane interactions in C-peptide activity has been suggested. In fact, in 1999 Rigler *et al.* demonstrated that C-peptide binds specifically in nanomolar concentration to cell membranes, possibly to a G-protein-coupled receptor, resulting in internalization of the peptide.[59] Clinical studies conducted with type 1 diabetes patients who lack endogenous C-peptide production show that administration of C-peptide could increase regional blood flow in several tissues.[58,69,74] In addition to the binding data, they have shown that C-peptide involves the activation of Ca^{2+} dependent signaling pathway, stimulation of Na^+/K^+ ATP ase and endothelial nitric oxide synthase (eNOS) activation. In fact, all of these factors are related to the improvement of blood flow through the microvasculature, indicating the importance of C-peptide in glycemic control.[75,76]

Also, it has shown by our group that upon interacting with transition metal ions such as Cr^{3+}, Fe^{2+}, C-peptide increases both glucose uptake in to RBCs and ATP release from the RBCs, indicating a novel possible role of C-peptide in controlling blood glucose levels.[58]

1.6 Channeling glucose via different pathways and polyol pathway up-regulation in diabetes.

Upon entering the digestive track, food is broken down in to nutrition components such as proteins, carbohydrates and fats and then eventually to the buildings blocks of these macromolecules such as amino acids, triglycerols, monosaccharides and oligosaccharides, prior to absorption in to the bloodstream.[4] Glucose, a monosaccharide of vast importance, is the ubiquitous source of energy and important metabolic intermediate in living cells.[4,11] Also, glucose is the primary source of energy for the brain, and hence its availability influences psychological processes.[4]

Glucose is a simple six carbon aldohexose with two stereoisomers (L and D) with only the D form being bioactive, and hence available in the dietary intake.[4] In aqueous solutions, the glucose molecule exhibits an equilibrium between in an open-chain (acyclic) form and a ring (cyclic) form.[4] The cyclic form, a result of a covalent bond between the aldehyde C atom and the C-5 hydroxyl group, form a six-membered cyclic hemiacetal and it is the predominant species at physiological pH.[4]

Glucose is taken up into the circulation and distributed in the body in a manner dependant upon the energy demand of the cells.[4] In healthy subjects, a portion of absorbed glucose is stored as glycogen in the liver and the remaining fraction is then distributed among various cells based on their bio-energy needs.[4] Glucose, once entered into the target organ via facilitated diffusion, is immediately converted in to G6P to prevent reverse diffusion.[4] The majority of the G6P will undergo oxidation via glycolysis to enter anaerobic or aerobic respiration depending on the type of the cell, producing the much needed ATP energy.[4] Some fraction of the G6P will be channeled through the pentose phosphate pathway to generate a redox equivalent and five carbon sugars.[77]

Under hyperglycemic conditions, activation of polyol/sorbitol pathway occurs to open another route to channel the excess glucose entering the cell.[78] The polyol pathway is a non insulin dependent metabolic alternation for glucose.[4]

The polyol pathway consists of two enzymatic reactions catalyzed by aldose reductase (AR) and sorbitol dehydrogenase (Figure 1.5).[1,79,80] AR catalyzes the rate determining step converting glucose to sorbitol, utilizing the coenzyme NADPH.[81,82] Then sorbitol dehydrogenase (SDH) oxidizes generated sorbitol in to fructose reducing nicotinamide adenine dinucleotide (NADH) in the process.[83]

Although the polyol pathway is less functional to nonfunctional in normal cells, it is known that non insulin dependant diabetes (NIDDM) patients show increased polyol pathway activity. [80,84] It has been reported that the high glucose level increases activity of both aldose reductase and sorbitol dehydrogenase.[85]

In RBCs, where the PPP is the sole NADPH producer, activation of AR that consumes NADPH, will further escalate oxidative stress. (Fig 1.3) In fact, this is

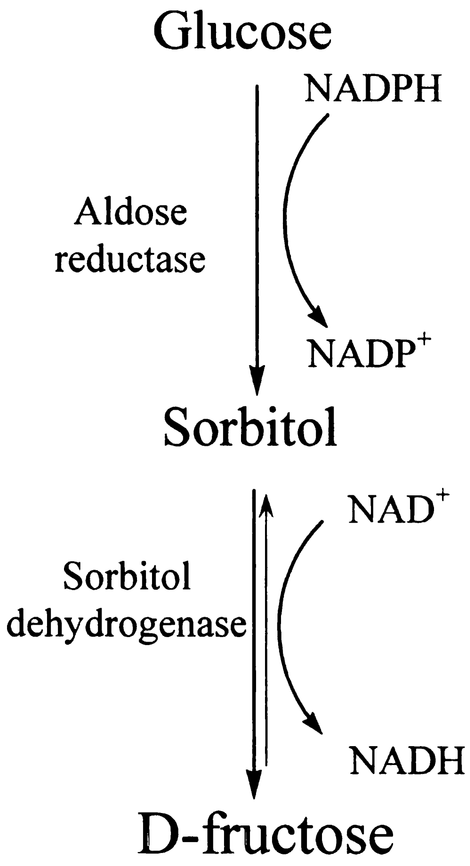

Figure 1.5: Schematic representation of the sorbitol pathway. Pathway consists of two enzymes aldose reductase and sorbitol dehydrogenase. AR reduces glucose in to sorbitol, while reducing NADPH. Then SDH oxidizes C_2 of sorbitol, forming fructose. Free NADH is generated in the process.

believed to be the main cause of such diabetic complications as retinopathy, neuropathy and nephropathy and cataracts in diabetic individuals.[85] Therefore, the sorbitol level in RBCs is an indication of the sorbitol accumulated in the retina of an eye and static nerve.[85-87]

Collectively, as a result, early detection and development of an effective treatment plan that includes life style changes such as diet and exercise, and new findings from conducted research, suggest that control of the pathogenesis of diabetes mellitus can be successful. Thus, despite the vast amount of money and research dedicated toward finding a cure for diabetes, diabetes still remains somewhat of an unsolved mystery with an ever-increasing occurrence world wide.

Therefore, understanding the molecular basis of RBC derived vascular regulation, contribution of various metabolic pathways to the oxidative status of RBCs and finally a factor that enables regulation of glucose in non insulin dependent targets will enable the improvement of the diagnosis and treatment options in diabetes associated vascular complications.

The work described here attempts to quantitatively determine the activity of the PPP enzyme G6PD by monitoring multiple parameters such as NADPH and ATP simultaneously in the presence of hyperglycemia. Also, the relation between proinsulin C-peptide, G6PD activity and the sorbitol pathway was investigated.

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Chapter Two

QUANTITATIVE ANALYSIS OF G6PD ACTIVITY AND SIMULTANEOUS DETECTION OF NADPH LEVEL AND RBC DERIVED ATP RELEASE

Since the late 19th century, cardiovascular diseases (CVD) were thought to be associated with diabetes.[1,2] These speculations were further strengthened with the discovery of insulin in 1922 [3-6] followed by improvements in complications such as renal failure, diabetes induced infections, and a marked increase in mortality and morbidity resulting from CVD in people with diabetes.[1] In fact, some scientists suggest that research and development during the past two decades has fueled a view of diabetes as an unusually severe form of cardiovascular disease (CVD) with a poor prognosis.[7,8]

According to the 2007 diabetes statistics released by Center for Disease Control and Prevention, diabetic patients shows up to a 3-fold increase in coronary heart disease events, independent of other traditional CVD risk factors, in comparison to their non-diabetic counterparts.[7] There are reports indicating that the physical characteristics of red blood cells (RBCs) such as deformability, plays a role in CVD pathogenesis in diabetic individuals.[9,10] Also there are reports indicating that damage to the oxidative defense system lead to the less deformable RBCs.[11] Therefore, understanding the effect of hyperglycemia on major contributors to the oxidative defense such as glutathione, NADPH levels and G6PD activity will provide beneficial effects for treatments and prevention of CVD in diabetes.

2.1 G6PD Structure and function

Human glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49, β-D-glucose-6-phosphate; NADP oxido-reductase) is an X – linked (Gene is located on X-chromosome) enzyme that is classified as a NADP$^+$-dependant dehydrogenase.[12,13] Also, G6PD is the enzyme that catalyses the first and rate determining step of the hexose monophosphate shunt or pentose phosphate pathway (PPP) and is located in the cytosol of animal, plant and microbacterial cells.[12,14-18] In addition, G6PD is found in the mitochondria of animal cells and the chloroplast of metaphytes or green plants.[9,15]

In solutions, G6PD exerts a rapid equilibrium between a dimeric and tetrameric form and this equilibrium is affected by pH and ionic strength.[15,19,20] Even though there is evidence suggesting that only the dimeric form is biologically active, this issue remains controversial to date because little is known about the biological significance of the interconversion.[12,15] However, it is widely accepted that the monomer of G6PD is biologically inactive, indicating the importance of the subunit contact for it's catalytic function and Michaelis-Menten kinetic properties.[12] Both the amino acid sequence and DNA sequence of the G6PD gene for almost all G6PD variants are known, and the crystal structure of the human Canton G6PD variant (most common G6PD variant) has been solved with up to 2.5 Å resolution. [12,15]

Being an X-linked enzyme, deficiencies in G6PD activity are more prominent in males compared to females.[17,21] G6PD deficiency is the most commonly occurring enzymopathy in humans, but remains somewhat benign unless triggered by the intake of such oxidative agents as premaquine or foods like fava beans.[15,17,21] Also, the occurrence of the G6PD deficiency disease remains somewhat geographical with high frequent outbreaks in malaria-endemic regions.[21] This, combined with the high frequency polymorphism (over 150 different human varieties) of the G6PD gene among different populations, has made G6PD very interesting to biochemists, geneticists and medical researchers.[17,21]

G6PD is recognized as an important antioxidant enzyme, especially in RBCs and in the microvasculature.[18,22] G6PD activity is the main source of NADPH in the body and in RBCs; in fact in RBCs, G6PD is the sole producer of NADPH.[19,23] NADPH is an important cofactor utilized by many antioxidant defense enzymes, therefore signifying the importance of G6PD activity in oxidant defense.[19] It has been shown in isolated adult cardiomyocytes that G6PD translocation to the cell membrane and G6PD activity increase with increasing oxidative stress.[22]

Disturbing the redox balance of the cell, caused by deficiency in G6PD activity, can lead to dysregulation of cell growth and signaling, abnormal embryonic development, altered susceptibility to viral infection as well as increased susceptibility to degenerative diseases.[24] In-fact there are reports suggesting that G6PD activity is inhibited in diabetic individuals.[20] Though the mechanism by which inhibition occurs remains unclear to date, there are two suggested pathways. One such possible mechanism was glycation induced inactivation of G6PD.[20] The other proposed mechanism suggests that high circulating glucose levels result in the activation of protein kinase A and subsequent phosphorylation leads to G6PD inhibition.[19,23]

It is well known G6PD activity is an indicator of cell age.[25] In 1986 Gross *et a.l* separated RBCs into four different age fractions and reported that G6PD activity decreased as the cell age.[25] Therefore understanding the changes in oxidant defense as cell age may lead to understanding the hyperglycemia induced decrease in G6PD activity and its effect on physiological functions of the cell.

Also, diabetes patients, and those individuals who are G6PD deficient, exhibit a high prevalence of hypertension and other cardiovascular complications.[22,26] Understanding the underlying effects of reduced G6PD activity, and hence lowered NADPH levels, in the RBCs of diabetic individuals may be beneficial in explaining its resultant effects on the microvasculature and complications in the cardiovascular system.[19]

2.2 NADPH as an indicator of G6PD activity

The common method for determining G6PD activity is by monitoring the increase in NADPH absorbance at 340 nm.[19,20] In this study, a fluorescence based detection scheme was used to monitor cellular NADPH levels.

Fluorescence based detection scheme

NADPH was determined using the Vybrant Cytotoxicity Assay Kit (Invitrogen Corp., Carlsbad, CA). In this assay, NADPH produced by G6PD is utilized by the enzyme diaphorase to reduce resazurin to the fluorescent product resorufin as shown in figure 2.1 (a). The resultant emission was measured at 587 nm.

2.3 Dehydroepiandrosterone, as a G6PD inhibitor

Commonly known as the "fountain of youth" or "the mother of all the hormones", dehydroepiandrosterone (DHEA) (Figure 2.1 (b)) is a naturally occurring steroid that is produced in the adrenal glands, gonads, adipose tissue, brain and the skin.[27,28] Even though it is produced in different parts of the body, more than 90 % of the circulating DHEA in human body is produced by the adrenal glands.[29] Though the exact function of DHEA is not well defined, it acts as a precursor for androgens and sex hormones such as testosterone and estrogen. [27,29]

DHEA seems to exert contrasting effects on individuals with diabetes. There are reports suggesting that administration of DHEA exerts beneficial effects on type 2 diabetes subjects.[29-31] Coleman and coworkers reported that dietary administration of DHEA to type 2 diabetic mice induces remission of hyperglycemia and insulin sensitivity.[29-32] Interestingly, DHEA acts as a non-competitive steroid inhibitor of G6PD and therefore will increase the oxidative stress on the cells by decreasing cellular NADPH, hence weakening antioxidant defense.[16,33-38]

There are also reports indicating that G6PD deficient/inhibited Chinese hamster ovary cells are more sensitive to effects of ionizing radiation and oxidizing agents such as diamide.[16] Diamide is a yellow, non-hygroscopic small diazene dicarbonyl oxidant that reacts rapidly with GSH, oxidizing it to the dimeric form GSSG.[39,40] Also decrease in deformation-induced RBC-derived ATP release upon treatment of RBCs with DHEA was reported by our group.[34]

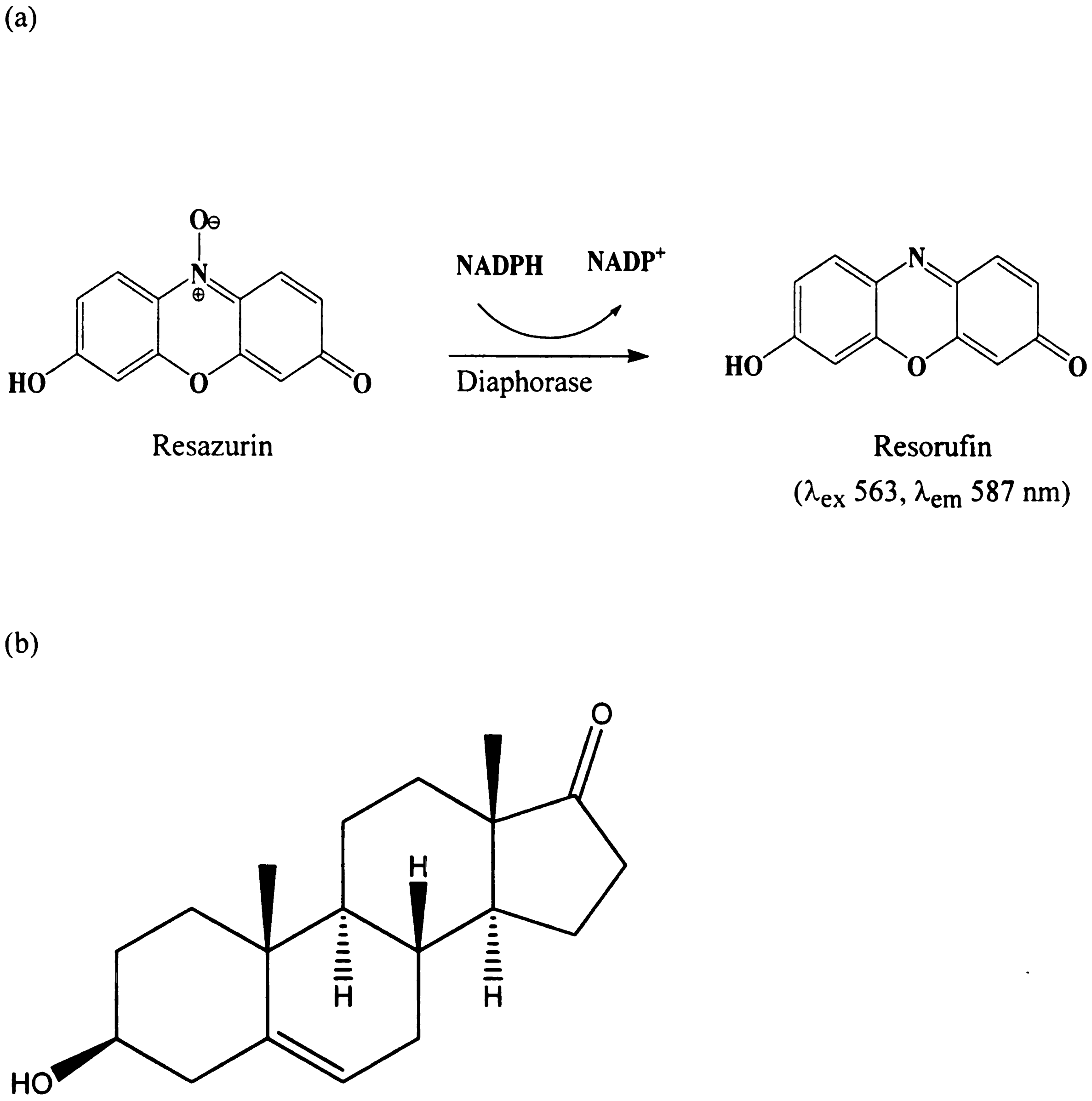

Figure 2.1: (a) Fluorescence-based NADPH detection scheme. Enzyme diaphorase converts non fluorescence resazurin to red fluorescence resorufin with the expense of NADPH. (b) Dehydroepiandrosterone, a non competitive steroid inhibitor of G6PD. DHEA is naturally produced from cholesterol in a human body but the circulating concentration readily decreases by the age of 20.

2.4 RBC deformability and CFTR mediated ATP release from RBCs

Adenosine triphosphate (ATP), commonly known as a "universal energy carrier" or "molecular currency" is a multifunctional nucleotide generated and consumed within the body in a multitude of anabolic and catabolic reactions. ATP is mainly generated during respiration in non-photosynthetic organisms and during photosynthesis in photosynthetic organisms, and is readily consumed by many anabolic and catabolic enzymes.[41,42] It is involved in signal transduction pathways and acts as a substrate for kinases, a group of enzymes that catalyze the transfer of a phosphate group from high energy donors, to specific target molecules.[41,42]

It is well known that RBCs contain millimolar concentrations of ATP, and upon being subjected to a stimuli such as mechanical deformation, O_2 tension (hypoxia) or low pH, these RBCs release ATP into the lumen of the vessel.[43-46] It was reported that RBCs typically release nanomolar to low micromolar amounts of ATP.[34,45-47] This ATP can activate the purinergenic receptors on the vascular endothelial cells that will stimulate the synthesis of nitric oxide (NO), a known vasodialator.[34,43-47] This NO induces the production of cyclic guanosine monophosphate (cGMP) that will eventually lead to smooth muscle relaxation and an increase in blood flow to the target tissue. [46]

Previously, our group has shown that the amount of ATP released from the RBCs upon mechanical deformation is significantly decreased if the RBCs are subjected to oxidant stress prior to the deformation.[34] Based on these results it was hypothesized that in type 2 diabetic individuals, where RBCs are under constant oxidative stress and lowered G6PD activity and hence lowered NADPH levels, reduction of ATP release upon deformation is pronounced.[33,34] Our results suggested that when RBCs were subjected to an oxidative stress with 20 μM diamide (a known oxidant), ATP release from these RBCs was decreased by 63 %.[34] This decreased level of ATP release may impair blood distribution and increase the risk of developing cardiovascular complications in vivo.[33,34]

2.5 Simultaneous detection of G6PD activity and its effect on ATP release

The G6PD enzyme is a determinant in the antioxidant status of the RBC and is also used as an indicator of cell age.[48-50] Also, deformation-induced RBC-derived ATP release depends upon the deformability of the cell membrane and, hence, the antioxidant defense system. However, it is unknown if the relationship between the antioxidant status and RBC-derived ATP release occurs immediately or over a period of time. Therefore, it is important to be able to detect both G6PD activity (via the determination of NADPH) and deformation-induced ATP release from RBCs. In order to demonstrate a direct relationship between G6PD activity and deformation-induced ATP release from RBCs, a simultaneous microflow detection scheme was developed.

2.6 Experimental

2.6.1 Collection of RBCs

All surgical procedures involving animals used in this study were performed under protocols approved by the appropriate Animal Investigation Committee or Institutional Review Board. Rabbit RBCs were obtained from male New Zealand White rabbits (2.0-2.5 kg). Rabbits were anesthetized with ketamine (8.0 mg kg^{-1}, im) and xylazine (1.0 mg kg^{-1}, im) followed by pentobarbital sodium (15 mg kg^{-1} iv). After tracheotomy, the rabbits were mechanically ventilated (tidal volume 20 mL kg^{-1}, rate 20 breaths min^{-1}; Harvard ventilator). A catheter was placed into a carotid artery, heparin (500 units, iv) was given, and after 10 min, animals were exsanguinated. Human blood was obtained by venipuncture and collected in to a heparinized syringe. Human diabetes subjects are adults, mostly African Americans with hemoglobin A1c value of 7 % or greater.[33]

2.6.2 Preparation of RBCs

To prepare the RBCs from the collected blood sample, whole blood was centrifuged at × 500 g at 4°C for 10 min. The plasma and buffy coat were removed and stored for other experiments within the laboratory. RBCs were resuspended and washed 3 times in a physiological salt solution (PSS). The PSS was prepared by combining TRIS buffer (25 mL, prepared by mixing 50.9 g of TRIS in 1 L of distilled and deionized water (DDW)) and Ringer's buffer (25 mL, prepared by mixing 164.2 g NaCl, 7.0 g KCl, 5.9 g $CaCl_2.2H_2O$, and 2.5 g $MgSO_4$ in 1 L DDW). After the addition of dextrose (0.50 g) and albumin bovine fraction IV (fatty acid free, 2.5 g) to the above mixture, the solution was diluted to 500 mL with DDW and the pH was adjusted to 7.35- 7.45. The PSS was then triple filtered using a filter with 0.45 μm pores (Corning, Fischer Scientific). Cells were prepared on the day of use and studied within 8 h of removal from the animal or human subject.

2.6.3 Reagents

All reagents were prepared in DDW or PSS unless otherwise noted.

Resazurin stock was prepared by dissolving the content of the vial in 75 μL of analytical grade dimethyl sulfoxide (DMSO) to make a 4 mM resazurin stock solution. Next, the resazurin-reaction mixture was prepared by diluting 75 μL of the resazurin stock solution to 10.00 mL in the solution prepared by using the component C (lyophilized mixture of diaphorase, G6P and NADP+) and D (0.5 M tris, pH = 7.5) according to the instructions provided with the Vibrant Cytotoxicity assay kit.[34]

A 200 μM NADPH stock solution was prepared by dissolving NADPH (0.0043 g) in 25 mL of DDW. NADPH standards (0.0- 20.0 μM) were prepared by diluting the stock solution of NADPH in DDW, accordingly.[33]

20 μL of 1000 U mL^{-1} G6PD stock solution was diluted with DDW to 4.00 mL to make a 5 U mL^{-1} G6PD solution.

A 100 μM ATP stock solution was prepared by dissolving ATP (0.0619 g) in DDW and diluting to 1.00 L in DDW. ATP standards (0.0 - 1.5 μM) were prepared by diluting appropriate aliquots of the stock ATP solution in PSS.[34]

To prepare the luciferin/luciferase mixture required for chemiluminescence determination of ATP, luciferin (2 mg, Sigma) was dissolved in 20 mL of DDW. A 5 mL aliquot of the luciferin solution was added to a vial containing luciferin/luciferase (FLE-50, Sigma).[34]

A 2 mM stock solution of diamide was prepared by adding 0.0344 g of diamide to 100 mL of DDW. A 1 mL aliquot of the diamide stock solution was added to 9 mL of wash buffer to create a buffered 200 μM solution of diamide.[34]

2.6.4 Instrumentation

All non – flow fluorescence measurements were performed with a Shimadzu R5301 scanning spectrofluorometer. All chemiluminescence-based ATP measurements were conducted using a photomultiplier tube (PMT; Hamamatsu Corporation, Hamamatsu, Japan) placed in a light excluding box (Figure 2.2.). Flow based fluorescence measurements were carried out using a Jasco FP-2020 intelligent flow-through fluorescence detector with 16 μL flow cell.

2.7 Methods

2.7. 1 Optimization of NADPH detection parameters

Fluorescence detection of NADPH was optimized under non-flow based conditions prior to the application to flow-based system.

2.7.1.1 Determination of the incubation time for resazurin

50 μL of G6PD (5 U mL^{-1}) was diluted with PSS to 100 μL. The resazurin – reaction mixture (100 μL) was added and resulted fluorescence intensity was measured (ex. 563 nm, em. 587 nm) as a function of time for 65 minutes.

2.7.1.2 Qualitative detection of NADPH in rabbit, control and type 2 diabetes RBCs

A 0.04 % rabbit RBC sample was prepared by diluting the appropriate volume of RBCs in PSS. The resazurin reaction mixture (100 μL) was added to a solution of RBCs (0.04% HCT, 100 μL) and the content was mixed thoroughly and incubated at 37 °C for

30 min. The fluorescence intensity of the resorufin was measured (ex. 563 nm, em. 587 nm) using a scanning spectrofluorometer under static conditions.

To verify the change in fluorescence emission was the result of G6PD activity, RBCs were incubated with 100 μM DHEA, a known inhibitor of G6PD, for 30 min prior to addition to the reaction mixture. Finally, to identify the involvement of G6PD in ATP release from RBCs upon deformation, RBCs were incubated with 2 μM diamide (± 100 μM DHEA) for 20 min and monitored for G6PD activity as described above.

2.7.2 Chemiluminescence detection of RBC derived ATP release

To quantitatively determine ATP release, the luciferin/luciferase mixture was placed in a 500 μL syringe. ATP standards, or RBCs, were placed in the second syringe and both solutions were pumped through 30 cm sections of microbore tubing having an internal diameter of 50 μm at a rate of 6.7 μL min^{-1} using a dual syringe pump (Harvard Apparatus, Holliston, MA). The streams containing the luciferin/luciferase mixture and ATP standard/RBCs were combined (figure 2.2 (b)) at a mixing T-junction with an internal volume of 560 nL (Upchurch Scientific, Oak Harbor, WA). The combined stream flowed through a segment of microbore tubing having an internal diameter of 75 μm, allowing the detection of the resultant chemiluminescence from the reaction of the ATP (either in standard form or that released from RBCs) using a PMT (Hamamatsu Corporation, Hamamatsu, Japan) placed in a light excluding box. The polyimide coating was removed from the microbore tubing on the segment over the PMT to facilitate light transport through the tubing and to the PMT. (fig 2.2.)

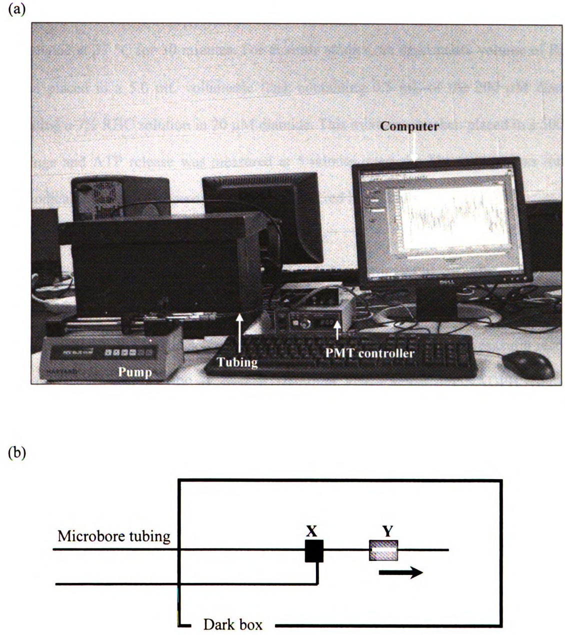

Figure 2.2 (a) Experimental setup used for ATP release measurements. (b) Schematic representation of the PMT and microbore tubing connection inside the light excluding box. X and Y represent the connecting "Tee" and window with the PMT underneath for chemiluminescence detection respectively. → indicates the direction of flow.

For studies involving DHEA, 7% RBCs (10.0 mL) were prepared by adding 1 mM DHEA (1.0 mL) to make 7% RBCs in 100 μM DHEA and this solution was incubated at 37 °C for 30 minutes. For diamide studies, an appropriate volume of RBCs were placed in a 5.0 mL volumetric flask containing 0.5 mL of the 200 μM diamide creating a 7% RBC solution in 20 μM diamide. This mixture was then placed in a 500 μL syringe and ATP release was measured at 5 minute intervals. The syringe was rotated periodically to ensure a homogeneous mixing of red blood cells.

2.7.3 Flow through detection of NADPH levels in rabbit red blood cells

A 0.02 % hematocrit of RBCs was used in all the experiments where NADPH was quantitatively measured. To determine NADPH, an aliquot of the resazurin reaction mixture was placed in a 500 μL syringe (Hamilton, Fisher Scientific). NADPH standards, or the RBCs, were placed in another syringe and both solutions were pumped through 30 cm sections of microbore tubing, having an internal diameter of 50 μm (Polymicro Technologies, Phoenix, AZ), at a rate of 1.0 μL min^{-1} using a dual syringe pump (Harvard Apparatus, Boston, MA). The two streams were combined at a mixing T-junction having an internal volume of 560 nL (Upchurch Scientific, Oak Harbor, WA). The now combined content was allowed to flow through a 90 cm long segment of microbore tubing having an internal diameter of 150 μm. The larger bore tubing resulted in a slower linear rate of the stream, thus enabling the required incubation period prior to detection of the resultant fluorescence using a flow-through fluorescence detector (Jasco, FP-2020 fluorescence detector). The total time taken for a single measurement was 30 min. The fluorescence intensity was measured at 587 nm (excitation at 563 nm). The same instrumental setup and experimental procedure described above was used to quantitate the cellular NADPH in diabetic and human control RBCs.

2.7.4 Separation of RBCs into age-based fractions and measurement of NADPH levels and ATP release

RBCs were separated into fractions based on cell density centrifugation.[51] In a 15 mL tube, 2 mL of a solution of Percoll (Sigma Chemical Co, St. Louis, MO) having a density of 1.115 g mL^{-1} were added to the bottom of the tube. Next, 2 mL of a similar solution of Percoll having a density of 1.105 g mL^{-1} were slowly added on to the top of the first 2 mL of the higher density solution. Finally, 1 mL of RBCs (70% hematocrit) was added to the top of the Percoll layers. The tube containing the Percoll and RBCs was then centrifuged at 3000 x g at 4 °C for 15 min. After centrifugation, two layers of RBCs are present; those having a density of less than the 1.105 g mL Percoll solution will appear at the top of the tube and represent those RBCs having the lowest density; these RBCs are considered the "younger" of the RBCs in the original RBC sample[37,51,52] (Figure 2.3). Those below the higher density Percoll solution are the denser, or aged, RBCs. These separated RBC fractions were removed by pipette and diluted to a hematocrit of 7% using the PSS described above. ATP release and NADPH levels measurements were made with 7% and 0.02% hematocrit RBCs, respectively, using the methods described above.

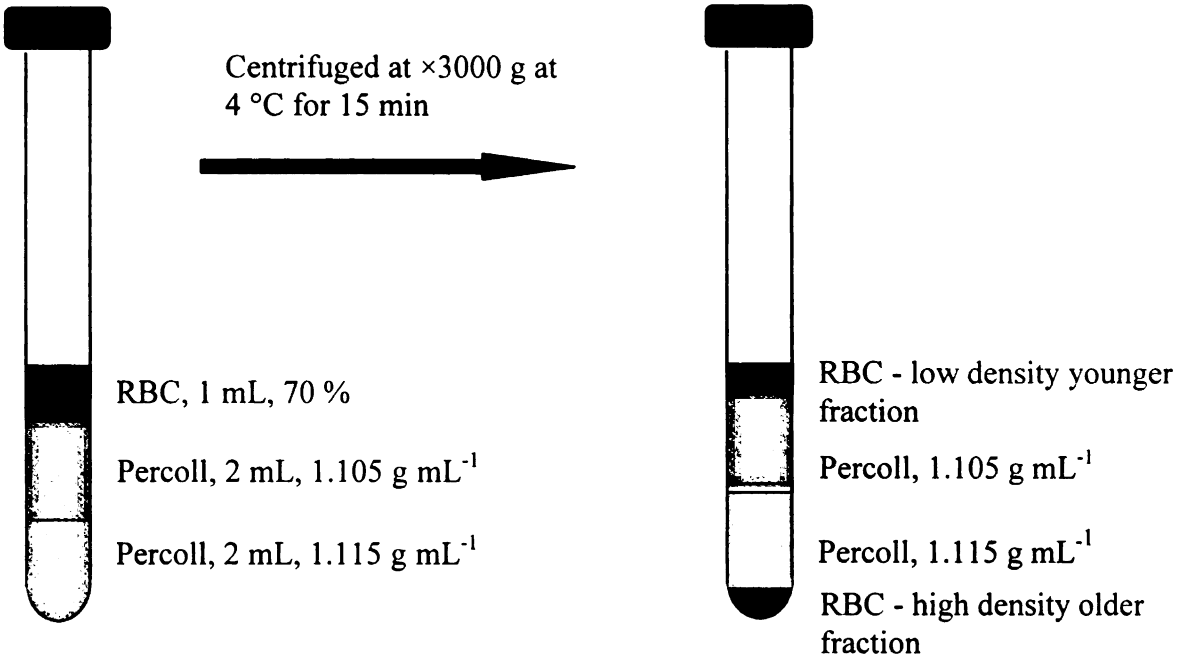

Figure 2.3: Pictorial representation of the separation of RBCs into fractions based on the age. Two Percoll solutions with slightly different densities were placed in a microcentrifuge tube and RBCs were introduced slowly to the same tube. The content was centrifuged at ×3000 g at 4°C for 15 min. RBCs were separated based on density (therefore age). The small RBC layer between two Percoll layers represents the RBCs that were not separated.

2.7.5 Simultaneous detection Scheme

In order to determine both cellular NADPH levels (hence, G6PD activity) and deformation-induced RBC-derived ATP release simultaneously, under continuous flow conditions, the experimental setup shown in Figure 2.4 was employed. Here, NADPH was measured using the previously described resazurin/resorufin fluorescence based assay while ATP was measured with the firefly luciferin/luciferase chemiluminescence. This setup mainly consists of dual syringe pumps, PMT, fluorescence detector and various lengths of fused silica microbore tubing (internal diameters of 50, 75 and 150 μm and an external diameter 363 μm, Polymicro Technologies, Phoenix, AZ) and connectors. (Upchurch Scientific Inc., Oak Harbor, WA)

Deformation was achieved by using 50 μM internal diameter microbore tubing whereas longer incubation times needed for fluorescence detection was achieved by application of microbore tubing with larger inner diameter (150 μm). Microbore tubing carrying different reacting agents were connected with a mixing tee (see figure 2.2 (b)) and the exciting stream from mixing tee was flowed in to a segment of 75 μm i.d. microbore tubing, for chemiluminescence measurements using the PMT setup explained above.

2.7.6 Simultaneous detection of NADPH levels and deformation induced RBC ATP release

A required volume of RBCs was placed in a 10 mL volumetric flask and was diluted with PSS to prepare a 7 % hematocrit of RBCs. Next, approximately 285 μL of this solution were diluted up to 100 mL in DDW to create a solution of RBCs with hematocrit of 0.02 %.

For the simultaneous determination of ATP and NADPH, two dual syringe pumps were employed as shown in figure 2.4. NADPH standards or the 0.02 % RBCs were placed in one syringe and the resazurin reaction mixture was placed in another and both were pumped at a rate of 1.0 μL min^{-1} through 50 μm internal diameter tubing. The content was combined at a mixing-T and incubated for 30 min by passing through the 90 cm long, 150 μm internal diameter microbore tubing. The resultant fluorescence was detected online with the flow-through fluorescence detector mentioned above.

Simultaneous to the NADPH measurements, the luciferin/luciferase mixture was placed in a 500 μL syringe. ATP standards, or RBCs, were placed in the second syringe and both solutions were pumped through 30 cm sections of microbore tubing having an internal diameter of 50 μm at a rate of 6.7 μL min^{-1} using the dual syringe pump mentioned above. The streams containing the luciferin/luciferase mixture and ATP standard/RBCs were combined at the mixing T-junction. The combined stream flowed through a segment of microbore tubing having an internal diameter of 75 μm, allowing the detection of the resultant chemiluminescence from the reaction of the ATP (either in standard form or that released from RBCs) using a PMT setup described in section 2.5.5. These measurements were repeated with the RBCs in the presence of the DHEA. The identical experimental setup and procedure were occupied to monitor control and human RBCs.

2.8 Results and discussion

Reactive oxygen species (ROS) such as superoxides, peroxides and hydroxyl radicals are generated during various cellular functions.[22] Under normal biological conditions these generated ROS are transformed into non-harmful species by the cellular antioxidant defense mechanism.[22] However, it is well known that the antioxidant defense system of the RBCs obtained from people with type 2 diabetes is deficient in comparison to those RBCs obtained from healthy individuals.[53-55] This weakened antioxidant status is believed to be the result of decreased levels of the cofactor NADPH due to the reduced activity of G6PD.[43,56,57] In fact, several studies have confirmed that RBCs having a down-regulated G6PD gene, show lowered basal NADPH concentrations.[15]

NADPH acts as a substrate for GSH_{red}, an enzyme that maintains GSH in its reduced, antioxidant form.[58] Upon oxidant insult, GSH is oxidized to its dimeric form GSSG, thereby protecting important cellular components and membrane proteins from being subjected to oxidant stress.[59] Upon decrease of NADPH, GSH_{red} activity decreases, thereby resulting in lower levels of reduced GSH and subsequent weakening of the cellular antioxidant defense. Also, it is well established that hyperglycemic conditions up-regulate metabolism and activate various biochemical pathways such as the sorbitol pathway that leads to overproduction of ROS.[22,60] This overproduction of ROS can diminish the already weakened antioxidant defense system and permanently damage important cellular components.[60]

When subjected to oxidation by ROS, membrane protein such as spectrin undergo structural modifications and change its specific three-dimensional structure, hence its function.[10,11,61] In fact, the cytoskeleton formed by spectrin is of extreme importance to

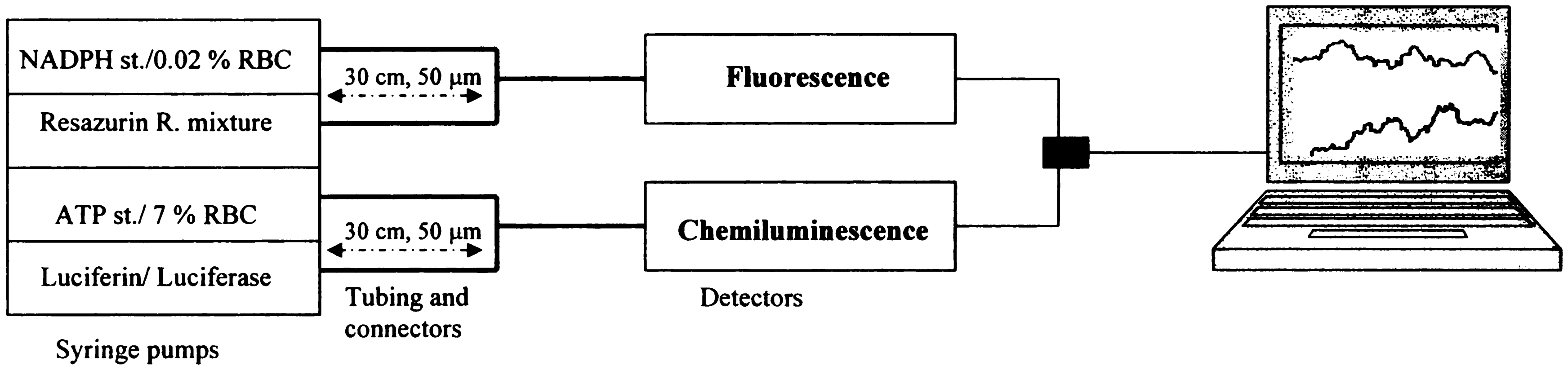

Figure 2.4: Schematic representation of the flow-based system employed to quantitatively and simultaneously determine the concentration of NADPH and deformation induced ATP release from the RBCs obtained from either rabbits or humans. The data acquisition program was written using labVIEW8 (National Instrumentation, Austin, TX).

RBCs, allowing them to undergo shape changes when traveling through the microvasculature.

Recently, our group and several other groups have shown that RBCs release nanomolar to low micromolar amounts of ATP when subjected to a mechanical deformation while traveling through the microvasculature.[34,62] This ATP can bind to the P2Y receptor on the endothelial cell of the vessel wall and stimulate endothelial NO synthase (e-NOS), stimulating synthesis of NO, a known vasodilator, thereby participating in the control of vascular caliber.[34,45,62]

We hypothesized that a decrease in the PPP, an increase in ROS and weakened antioxidant defense would lead to a more stiffened RBC membrane. These stiffened RBCs will undergo reduced deformation and an accompanying decrease in ATP release, facilitating complications in the microvasculature and the importance of understanding G6PD activity and its effect on RBC derived ATP release. But neither we, nor others, have studied whether this effect is a consequence of a long time event or an immediate result of the G6PD inhibition.

As the first step towards establishing the relationship between G6PD activity and RBC-derived ATP release, a scheme to monitor G6PD activity under continuous flow conditions, an essential element of the circulation was designed. Though UV absorption of NADPH is the most commonly utilized technique for measuring G6PD activity, this method carries certain drawbacks such as interference from NADH and reduced detection limits in comparison to fluorescence. Therefore, a fluorescence based resazurin/resorufin assay was used for the NADPH measurements.

In order to optimize the experimental conditions used to measure NADPH in RBCs, resazurin was incubated with G6PD in the presence of $NADP^+$, G6P and diaphorase. A linear increase in the fluorescence intensity as a function of a time was observed over a 70 min. period and then stabilized due to resazurin becoming the limiting reagent (Figure 2.5). A 30 min. incubation period was selected for further studies as it provides adequate intensity changes, as well as a shorter incubation time.

Upon optimization of the experimental conditions, the next step was to apply these optimized conditions to RBCs. To further clarify that the changes are due to G6PD activity, the RBCs were incubated with DHEA (a known G6PD inhibitor), and then treated with diamide (a known oxidant) prior to the NADPH measurements (Figure 2.6). These data confirm the reduction of cellular NADPH levels upon G6PD inhibition. However diamide did not have a significant effect ($p > 0.02$) on NADPH levels in comparison to control RBCs nor did it have any effect on G6PD inhibited RBCs. Also, the effect of DHEA after a 60 min. incubation was not significantly different from effect at 30 min. Therefore a 30 min. incubation time was used in further studies.

Biaglow *et al.* has shown that cell growth and bio-reduction of thiol species decrease in Chinese hamster ovary cells on a time and concentration dependant manner upon incubation with DHEA due to decreased NADPH levels.[16] Also, there are reports indicating that overexpression of G6PD results in an increase in NADPH and a decrease of the damaging ROS.[22] Therefore, as expected, the effect of DHEA was profound in RBCs where G6PD is the sole NADPH producer.

NADPH levels were also measured in human control and type 2 diabetes RBCs under non-flow-based conditions. As shown in the data in Fig. 2.7, the NADPH level in

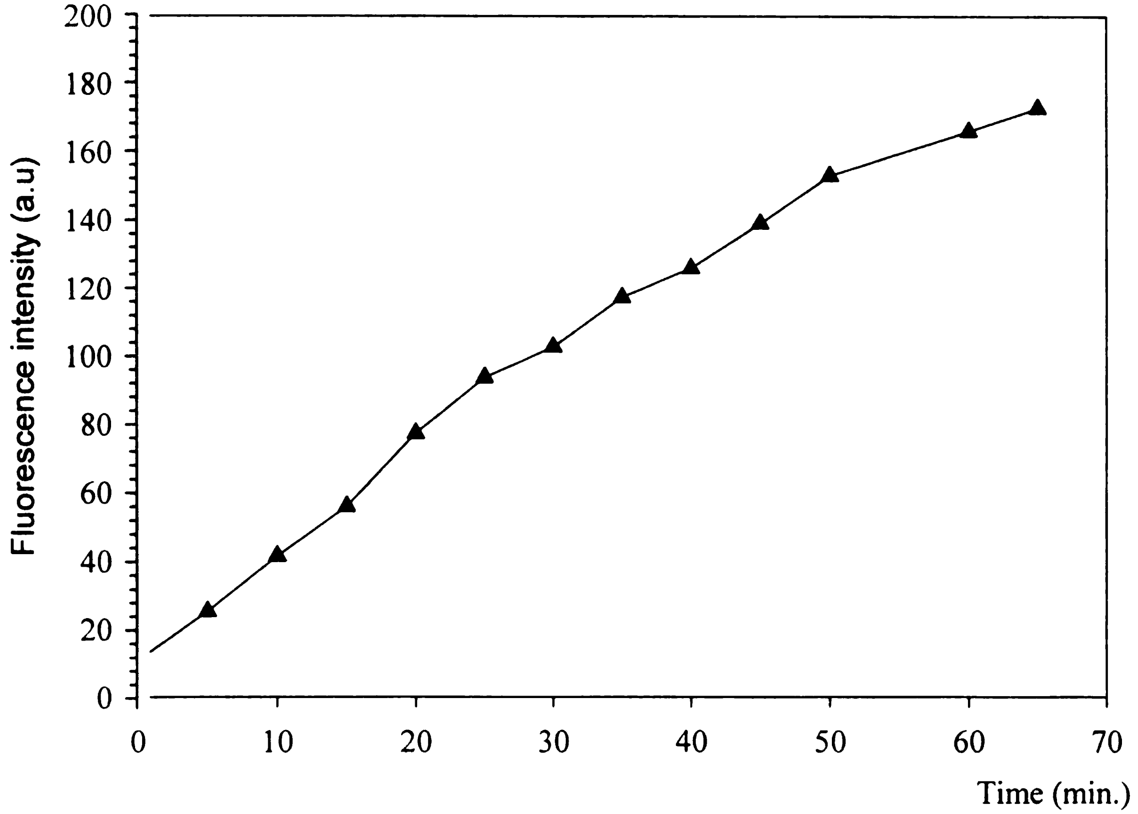

Figure 2.5: Kinetic study - optimization of the incubation time for resazurin. Resultant fluorescence intensity was measured over a period of 70 min. at 583 nm. Fluorescence intensity was increased linearly as a function of time up to 70 min. period.

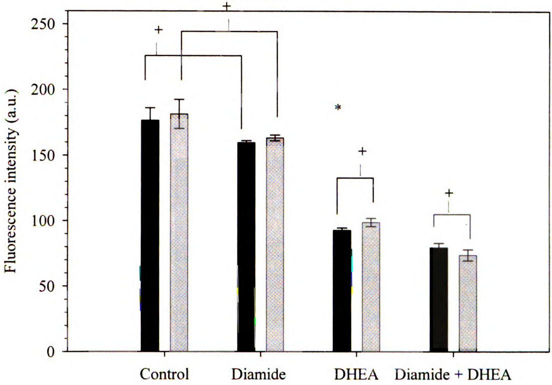

Figure 2.6: The effect of DHEA on G6PD activity (n = 6 rabbits). Gray bars represent 30 min. incubation with DHEA whereas black bars represent the 60 min. incubation. Diamide alone did not affect G6PD activity, nor did it have any further effect on G6PD activity in the presence of DHEA. The asterisks represent those values significantly different from the control ($p < 0.001$) while the crosses indicate NADPH levels that are statistically equivalent to the control ($p > 0.02$)

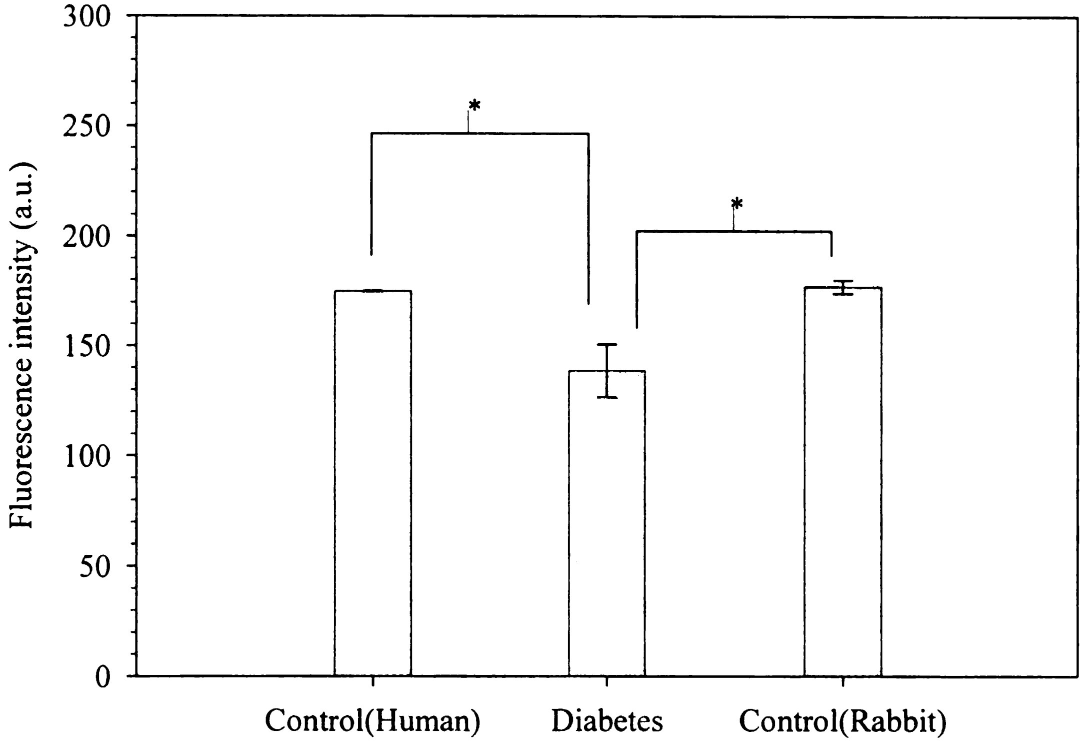

Figure 2.7: NADPH levels in rabbit, control human and diabetes RBCs measured under non flow conditions. The NADPH level in the RBCs obtained from the patients with diabetes was significantly different from the healthy human controls and rabbit RBCs ($p < 0.01$). $n = 5$ samples.

the RBCs obtained from patients with diabetes was about 30% less than healthy human controls and rabbit RBCs. These results follow the same trend as previously published ATP release data from RBCs of people with type 2 diabetes.[34] To provide further evidence that the G6PD activity may be a major determinant of RBC-derived ATP release, ATP release from the G6PD-inhibited and diamide-treated rabbit RBCs were measured. As shown in Fig 2.8, RBCs that were subjected to diamide alone were able to recover from the oxidant insult (diamide addition) as evident by the recovery of ATP release seen in measurements taken after 20 min.[34] The ability to recover from the diamide insult is similar to previous data generated by our group.[34] However RBCs incubated in DHEA did not recover in the same amount of time as RBCs incubated in diamide alone. In fact, the ATP release never recovered, even after a 40 min incubation period (Figure 2.8). These data, combined with the NADPH measurements in the presence of diamide incubated RBCs (Figure 2.6), suggest that inhibition of the G6PD enzyme, a major component of the pentose phosphate pathway, may be a determinant in the ability of RBCs to release ATP upon deformation.

Upon successful monitoring of the cellular NADPH levels and the resulting G6PD activity, it is essential to evaluate the relationship between the G6PD activity, deformation induced RBC-derived ATP release, in order to understand the relation between elevated cardiovascular complications and diabetes. The first step toward establishing such a correlation would be to assess the NADPH levels hence G6PD activity under continuous flow, an essential element of microcirculation.

We successfully extended our fluorescence detection scheme to a flow based measurement scheme. For calibration and quantification purposes, NADPH standards (0

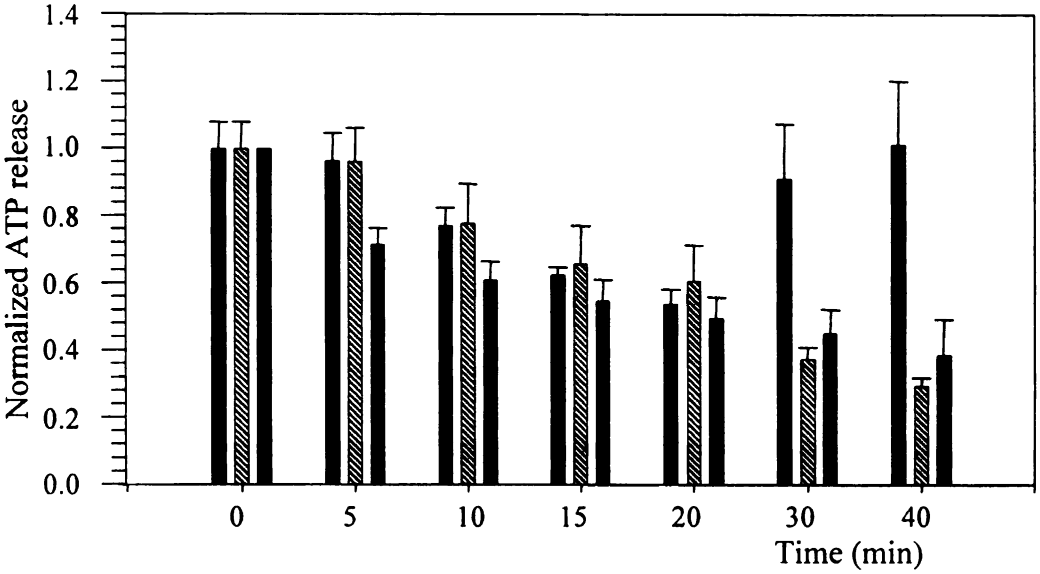

Figure 2.8: Deformation induced ATP release from RBCs in the absence and presence of a G6PD inhibitor is shown. The gray bars show the average ATP release ($n = 6$ rabbits) at various time intervals measured from RBCs that were subjected to 20 μM diamide alone. The black bars show the ATP release from aliquots of the RBCs after the addition of diamide; however, these RBCs were first incubated with 100 μM DHEA for 30 min in order to inhibit the G6PD activity. The bars with lines show ATP release from RBCs that were incubated with DHEA alone. The asterisks represent those values significantly different from the initial ($t = 0$ min, $p < 0.001$) while the crosses indicate ATP values that are statistically equivalent to the initial value at $t = 0$ min ($p > 0.02$).

– 20 mM) (Fig 2.9) were measured prior to measuring NADPH levels in RBCS. Once the method was established using NADPH standards, NADPH levels in normal rabbit RBCs and chemically aged RBCs (by inhibiting G6PD with DHEA) were measured. As summarized in Figure 2.10 (b), the DHEA-induced aging (via inhibition of the G6PD and the pentose phosphate pathway) resulted in a decrease in NADPH concentration from 13.2 ± 1.8 μM (normal RBCs) to 8.0 ±1.1 μM (RBCs incubated in DHEA) in a 0.02% solution of RBCs, a 39% decrease in NADPH concentrations.

It is well established that as cells age, the G6PD activity within the cell decreases resulting in a smaller RBC that is more dense and, importantly, less deformable. Therefore, to further verify the relationship between G6PD activity and ATP release, RBCs from healthy rabbits were separated based on cell density centrifugation as described above. The cellular NADPH levels and deformation induced ATP release were measured in these young and old fractions obtained from the whole sample of RBCs. As shown in figure 2.9, both NADPH concentration in, and deformation-induced ATP release from, the RBCs are significantly different in the younger and older fractions. As expected, both NADPH levels and ATP release from RBCs obtained from the less dense, "younger" fraction are greater than more dense RBCs with less G6PD activity. In the younger fraction, the NADPH level is 11 % greater compared to the older fraction, while the ATP release was 56 % greater in the younger RBC fraction compared to the older portion of the cells. See Figure 2.11.

Although there are reports suggesting a relation between decreased G6PD activity and increased RBC stiffness, this is the first attempt to monitor the consequences of these characteristics simultaneously and quantitatively while permitting the forces required

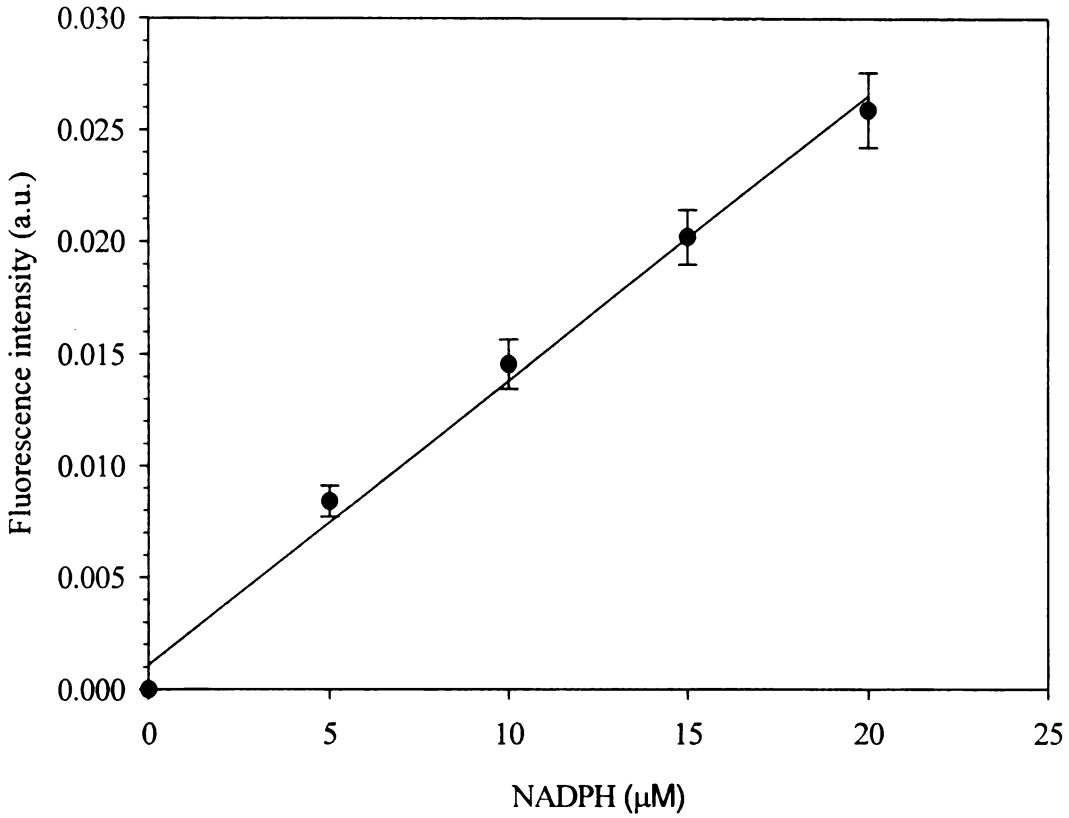

Figure 2.9: NADPH standards measured using flow based fluorescence detection scheme. Fluorescence intensity changes linearly within the NADPH concentration range of 0 – 20 μM ($r^2 = 0.998$). n = 7 sample sets.

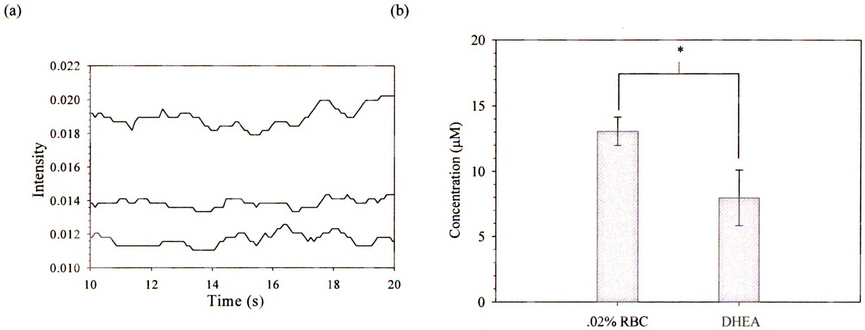

Figure 2.10: Quantitative measurement of cellular NADPH levels using flow-through fluorescence detection. (a) An NADPH standard (lower trace) in comparison with NADPH levels in 0.02% RBCs (top trace) and chemically aged RBCs (middle trace). (b) Quantitative representation of NADPH levels in 0.02% rabbit RBCs and DHEA inhibited rabbit RBCs ($n = 6$ rabbits). There is a 39% decrease in NADPH concentration upon incubation with the G6PD inhibitor DHEA for 30 min. The values in the absence and presence of DHEA are significantly different ($p < 0.01$).

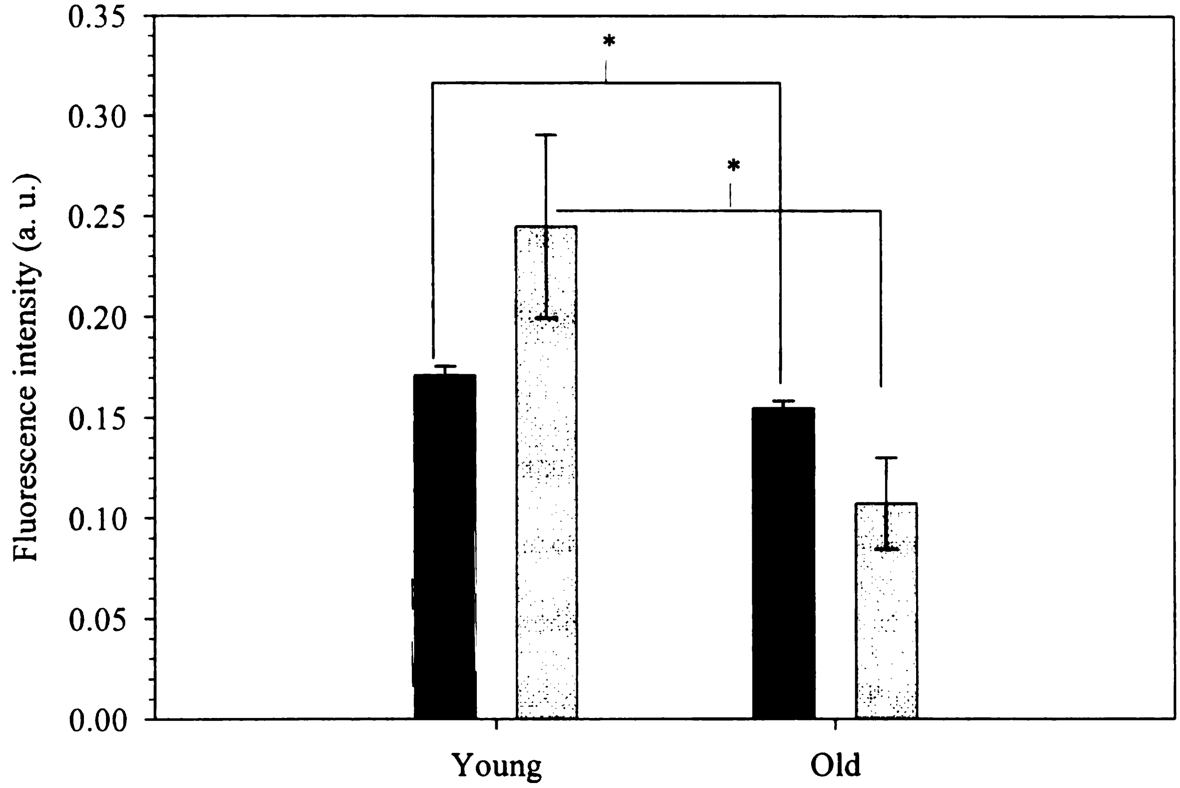

Figure 2.11: NADPH levels and deformation induced RBC derived ATP release from young and old fractions of rabbit RBCs. The black bars represent the fluorescence intensity corresponding to NADPH while the gray bars represent the chemiluminescence intensity corresponding to ATP release from the RBCs ($n = 3$ rabbits). The values between the two groups are significantly different ($p < 0.01$).

(flow) to induce the ATP release. Both NADPH and ATP were measured simultaneously, using the experimental setup shown in figure 2.4. As expected, a direct relationship between decreased NADPH levels and the decreased values of deformation-induced RBC-derived ATP is observed. Results showed in figures 2.12 and 2.13 indicate that the decrease in ATP release is proportional to the decrease in the cellular NADPH levels. Upon inhibition with DHEA, the ATP release decreased to 0.06 ± 0.02 μM, down from an initial value of 0.21 ± 0.07 μM in 7% RBCs. In a similar manner, the initial concentration of NADPH (12.73 ± 0.50 μM) decreased to 8.62 ± 0.29 μM upon addition of DHEA.

As the last step towards understanding the relationship between oxidative stress, G6PD activity and ATP release in diabetic subjects, we monitored these parameters in RBCs obtained from diabetic and healthy individuals. There is an approximate 50% decrease in NADPH concentrations and a 78% decrease in ATP release from the RBCs from diabetic patients as compared to healthy individuals (Figure 2.14)

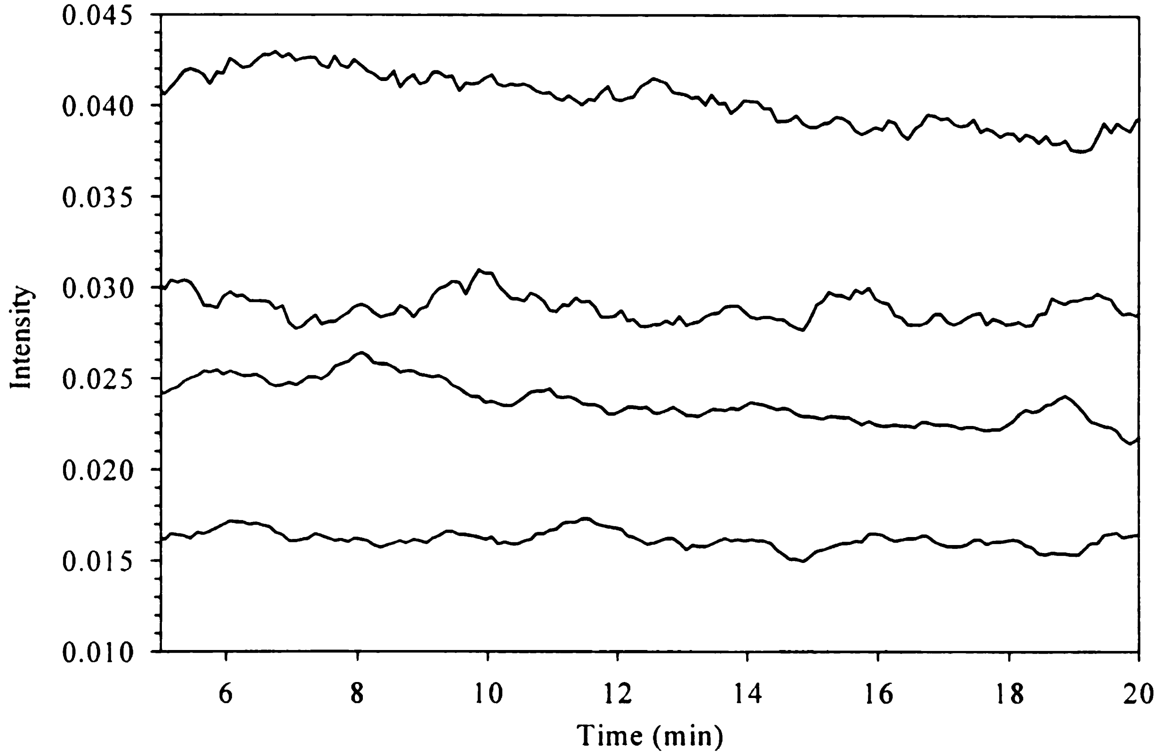

Figure 2. 12: Qualitative representation of simultaneous detection of NADPH levels and deformation-induced ATP release from rabbit RBCs. NADPH levels were measured using a fluorescence-based G6PD assay, while ATP release was measured using chemiluminescence. The bottom two traces represent ATP release from normal RBCs (higher of the pair) and G6PD inhibited (lower of the pair) RBCs. The top two traces represent NADPH levels in 0.02% RBCs in the absence (higher of the pair) and presence (lower trace of the pair) of DHEA.

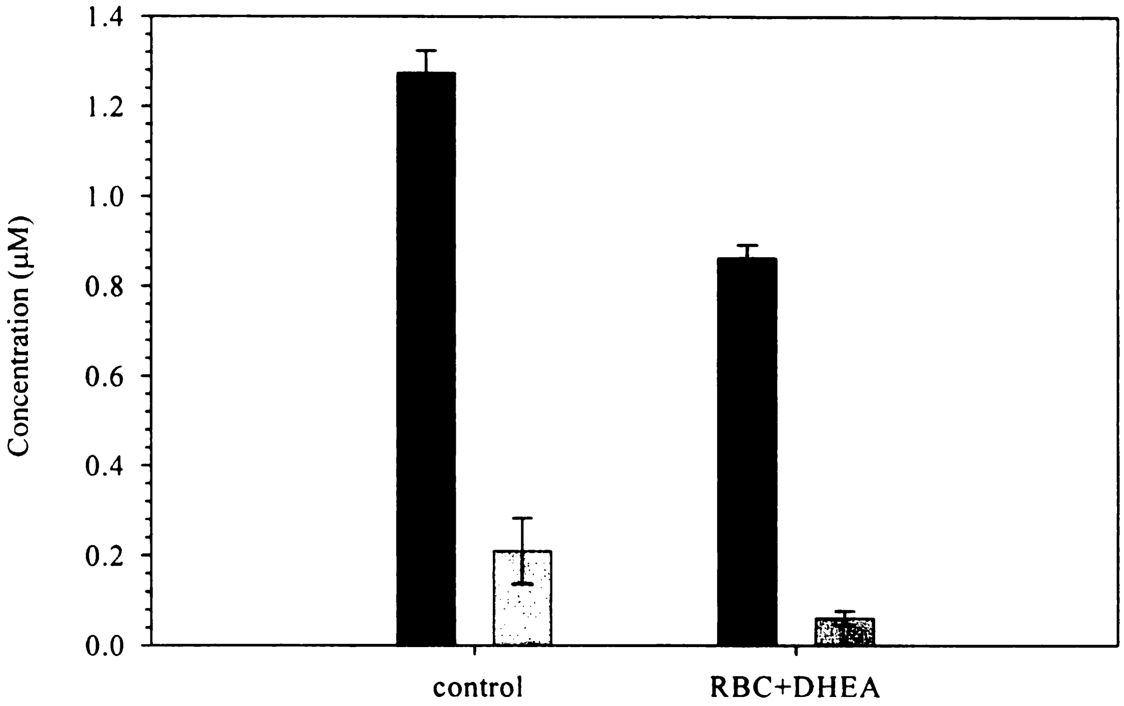

Figure 2.13: Quantitative representation of simultaneously detected NADPH levels and deformation-derived ATP release. The black bars represent NADPH $\times 10^{-1}$ concentration ($n = 5$ rabbits), while the gray bars represent ATP release ($n = 5$ rabbits). The values between the two groups are significantly different ($p < 0.01$).

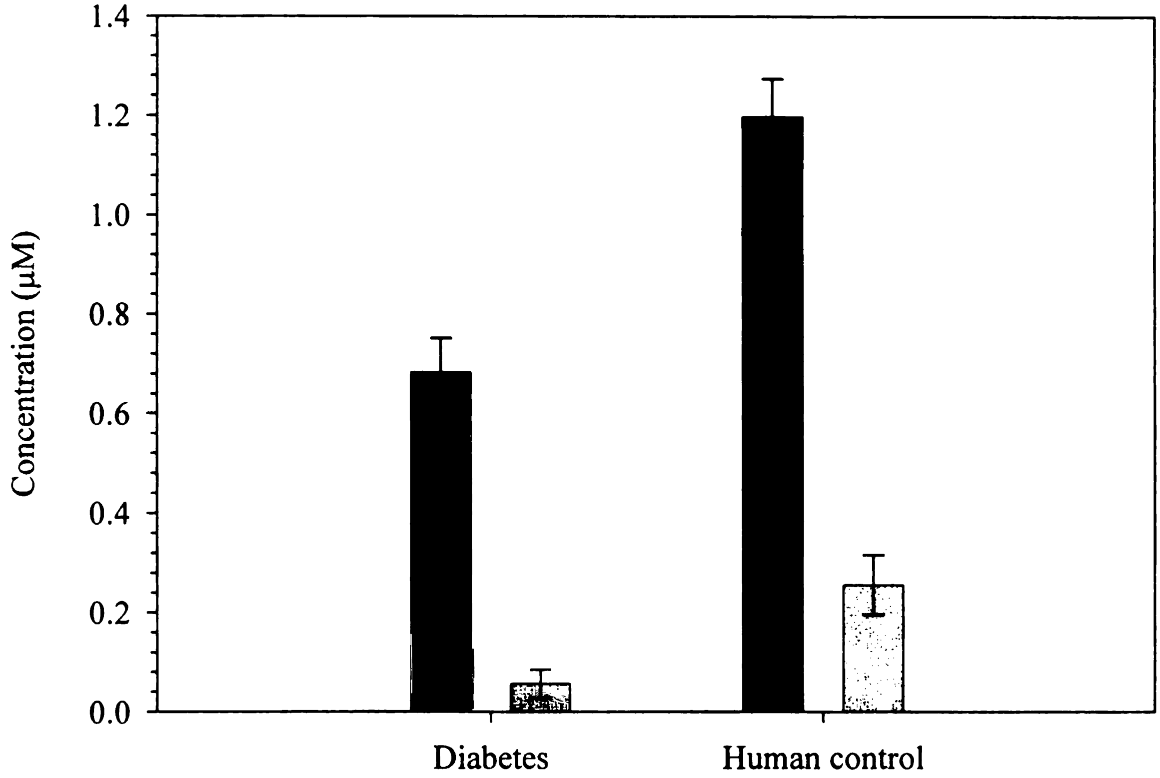

Figure 2.14: Measurement of cellular NADPH levels and ATP release from RBCs obtained from people with type 2 diabetes and healthy controls. The black bars represent NADPH $\times 10^{-1}$ ($n = 5$ individuals) and the gray bars represent ATP release ($n = 8$ individuals). There is an approximate 50% decrease in NADPH concentrations and a 78% decrease in ATP release from the RBCs from diabetic patients as compared to healthy individuals. The values between the two groups are significantly different ($p < 0.01$).

2.9 Conclusions

In type 2 diabetes, RBCs are under high oxidative stress and are believed to be less deformable, leading to lowered levels of deformation-induced ATP release. In an attempt to understand simultaneous changes in G6PD activity and deformation induced RBC derived ATP release, we successfully developed and extended an NADPH measurement scheme to continues flow based system, which is an essential condition in microcirculation, with the expectation of application of simultaneous detection. As expected cellular NADPH levels decrease by 39 % upon inhibition of enzyme G6PD signifying its importance in antioxidant defense.

When subjected to oxidative stress, if unprotected, oxidation of important membrane proteins will leads to a stiffening of the cell membrane. This effect is pronounced in RBCs where G6PD is the sole producer of NADPH, an essential cofactor in the antioxidant defense mechanism. In type 2 diabetes, RBCs are under high oxidative stress and are believed to be less deformable, leading to lowered levels of deformation-induced ATP release. Also G6PD activity is an indication of cell aging and upon aging cell membranes become more stiffened due to a weakened oxidative defense system.

Here we were able to measure cellular NADPH levels in the RBC (via a fluorescence-based assay) and deformation-derived ATP release from the RBC (via chemiluminescence assay) simultaneously, under continuous flow conditions. Results are consistent with the expected pattern of a direct relationship between NADPH concentrations in the RBC and deformation-derived ATP release from these cells. With a decrease in NADPH concentration, there is a decrease in deformation-derived ATP release from the RBC. A similar trend was observed with chemically aged (via inhibition of G6PD) RBCs, as well as young and old fractions of RBCs separated from whole blood. RBCs obtained from people with type 2 diabetes showed the same trend confirming that oxidative stress leads to a decrease in deformation-derived ATP release. Due to the ability of ATP to stimulate NO production in other cells (e.g., endothelial cells and platelets), the RBC thus becomes a potential determinant of blood flow in the diabetic circulation. Perhaps more evident from the work reported here, however, is the potential use of NADPH and ATP as biomarkers of oxidative stress in the RBC.[33]

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Chapter 3

ROLE OF C-PEPTIDE AND EFFECT OF DIABETES INDUCED OXIDATIVE STRESS ON C-PEPTIDE ACTIVITY

3.1 C-peptide as a bioactive peptide

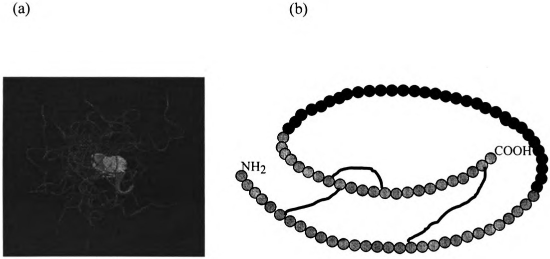
Proinsulin connecting-peptide, commonly known as a proinsulin C- peptide is a small 31 amino acid peptide synthesized and secreted together with the hormone insulin from the islets of Langerhans.[1] Its name was also inspired by the fact that insulin is composed of two peptide chains named as chain "A" and "B".[1]

The discovery of proinsulin by Steiner and his coworkers in 1965 is not only the milestone in the elucidation of the biosynthesis of proteins, but also leads to the complete understanding of insulin biosynthesis followed by the discovery of C-peptide.[1, 2] In 1968, Chance and coworkers isolated proinsulin from crystalline porcine insulin.[1, 3] They evaluated the amino acid sequence of a 33-amino acid peptide, linking the A and B chains of insulin and described it as a "connecting peptide".[1, 3, 4] During the same year Steiner and Clark successfully demonstrated that the primary role of proinsulin appeared to be to facilitate efficient formation of the disulfide bonds of insulin, leading to further investigations of C-peptide.[1, 4, 5] Subsequent research followed, leading to the isolation and analysis of human proinsulin and eventual preparation and sequence determination of human C-peptide.[1, 4, 6-10] Figure 3.1 depicts the structure and amino acid sequence of human C-peptide.

Upon investigating the role of C-peptide, studies conducted on rats indicated the kidney as the main organ responsible for the degradation of proinsulin and C-peptide.[11, 12] However extended studies on the possible physiological function and activity of C-peptide under various conditions were concluded to be negative by most researchers, despite some reports stating otherwise.[1, 13] Subsequently, it was concluded that C-peptide is devoid of any biological activity.[1 12, 13]

During the following years, it was discovered that equivalent amounts of insulin and C-peptide were secreted from the pancreas. Therefore measuring C-peptide in the circulation became an important diagnostic tool for various diseases associated with either pancreatic β cells or insulin.[1, 14] Between 1973 to 1976 there was a considerable increase in immunological studies involving C-peptide, exploring the possibility of using C-peptide as diagnostic tool.[1, 15-17] Currently, C-peptide assays are used in a large variety of studies, such as liver metabolism, ketoacidosis, insulin-induced hypoglycemia, oral glucose tolerance test, comparing portal and peripheral blood, diagnosing patients with islet cell tumors and examining children with juvenile diabetes.[1, 17, 18]

Despite its successful passive role as a diagnostic tool, C-peptide was thought to be biologically inert for most of its 40 years of existence since its discovery in 1967.[19, 20] In fact, except for the importance of using C-peptide as a unique biomarker for insulin biosynthesis, C-peptide was considered to have little or no importance beyond its role as a mere integral part of proinsulin.[21, 22]



10
Glu-Ala-Glu-Asp-Leu-Gln-Val-Gly-Gln-Val_Glu-Leu-Gly-

20
Gly-Gly-Pro-Gly-Ala-Gly-Ser-Leu-Gln-Pro-Leu-Ala-Leu-

30
Glu-Gly-Ser-Leu-Gln

Figure 3.1: (a) Structure of human proinsulin C-peptide (b) Schematic representation of the human proinsulin structure with chains A and B of insulin connected by C-peptide. (c) Amino acid sequence of human C-peptide

During the late 1990's, interest in C-peptide research was renewed.[1, 23-25] New studies reported by Wahren *et al* concluded that C-peptide has properties of an intracrine peptide hormone.[1, 26] In fact, a clinical study with 139 patients, conducted by Ekberg and coworkers has shown that C-peptide treatment for 6 months improves sensory nerve function in early-stage type 1 diabetic neuropathy.[1, 26] During the 1990s, reports surfaced indicating that C-peptide was able to ameliorate complications associated with diabetes in rat models, including vascular and neural dysfunction, glucose utilization and renal function.[27, 28] Other reports followed, demonstrating the ability of C-peptide to improve blood flow in the skin muscles and microvasculature.[29, 30] Also, it was reported that C-peptide was capable of improving the deformability of RBCs.[31] C-peptide has also been reported to increase endothelial nitric oxide synthase (eNOS) activity.[32, 33]

As increasing number of studies were directed towards the biological role of C-peptide, more beneficial effects were observed. Beneficial effects observed with both pancreas transplants and the combined replacement of insulin and C-peptide in treatment of type 1 diabetes are such examples.[24] The present stand on C-peptide has been summarized by Wahren *et al* as a "C-peptide is a bioactive peptide."[26]

Recently, our group has shown that RBCs treated with metal activated C-peptide release increased levels of ATP, even in the absence of flow (mechanical deformation) or hypoxia.[34, 35] RBCs were able to release increased concentrations of ATP for a period of about 24 h when incubated with C-peptide bound to Fe^{2+}, whereas binding of C-peptide to Cr^{3+} results in sustained ATP release over a period of 3 to 5 days.[34, 35] Further studies indicated that the ATP release is related to an increase in glucose transport across the RBC membrane.[34, 35]

C-peptide, when activated with metal ions such as Cr^{3+}, Zn^{2+} and Fe^{2+} stimulates glucose uptake in to rabbit RBCs.[34, 35] There are reports suggesting that there are milimolar levels of Zn^{2+} in the islets of Langerhans where proinsulin C-peptide is synthesized and secreted.[36-40] Therefore it is highly likely that C-peptide is associated with Zn^{2+} upon secretion from the pancreas. Thus, C-peptide, when complexed to a metal, has properties similar to insulin, facilitating glucose transport in to RBCs.[34] However, insulin acts upon the glucose transporter GLUT 4 and RBCs do not have GLUT 4 transporters. Therefore, metal-activated C-peptide may contribute to the proper blood glucose maintenance through activation of GLUT 1, the major glucose transporter on the RBC.[34]

Beyond the experimental level, no current studies were successfully extended to the therapeutic level of C-peptide replacement in preventing and ameliorating diabetic complications.[1] Also, despite recent findings there are debates about the biological activity of the C-peptide. These arguments appear to be based on three major facts that C-peptide lack relative to other bioactive peptides.

One such negative concern with C-peptide is that despite elevated concentrations of circulating C-peptide, people with type 2 diabetes develops complications associated with the disease. Recently, we reported that the ATP released from the RBCs of patients with type 2 diabetes was approximately 50% less in comparison to the ATP released from healthy controls.[41, 42] Further investigations in to this issue indicated a decrease in G6PD activity, a common trait in the diabetic RBC, leads to a less deformable cell eventually decreasing ATP release, and this effect is indeed acute or immediate rather than chronic.[42] Interestingly, there were reports suggesting that ATP release was decreased in the RBCs of patients with type 2 diabetes due to improper accumulation of cyclic AMP levels in the RBCs of these patients.[43] Therefore, under such circumstance C-peptide activation of glucose transport and subsequent ATP release from the RBCs may be impaired or less effective due to other complications within the diabetic red cells itself.[34]

Another negative concept of C-peptide is despite recent efforts, a receptor for C-peptide has not been reported.[44] C-peptide does not assume a stable, defined conformation in solutions. [19, 45, 46] This lack of a defined tertiary structure also facilitates the arguments of a non-receptor based interaction.[44] However, it is highly probable that a receptor for C-peptide binding has yet to be discovered.[46] A possible explanation to the absence of tertiary structure is that coordination of Zn^{2+} to negatively charged amino acid side chains (4 glutamic acid sites and 1 aspartic acid site)[4, 6, 10] and/or C-terminal of the C-peptide molecule may result in the formation of a stable secondary structure that facilitates intracellular signal transduction via conformation-dependent interactions between the C-peptide–metal complex and a cell membrane or unidentified cell-surface receptor.[34]

Due to lack of a receptor, C-peptide upon activation with Zn^{2+}, binds to RBC membrane to exert its biological insulin-like activity. Here it is hypothesized that RBCs are subjected to oxidative stress for long periods (e.g. type 2 diabetes individuals) changes occur at the cell membrane surface that affect C-peptide binding to the RBC membrane. In order to further evaluate this hypothesis, it is necessary to understand the fundamentals of the RBC membrane structure and effect of hyperglycemia on that structure.

3.1.1 RBC membrane and effect of hyperglycemia on membrane structure

Like any other mammalian cell membrane, the RBC membrane is a selectively permeable lipid bilayer, outlining the cell. The cell membrane is mainly composed of an asymmetric bilayer of phospholipids and different types of associated proteins such as integral proteins providing passage for various ions and hydrophilic compounds, membrane bound enzymes involved in catalytic processes, various receptors both extracellular and intracellular involving signal transduction, and finally they also provide attachment points to spectrin forming the cytoskeleton.

Amphipathic phospholipids, which are the main constituent of cell bilayer, spontaneously arrange so that the hydrophobic "tail" regions are shielded from the surrounding polar fluid, causing the more hydrophilic "head" regions to associate with the cytosolic and extracellular faces of the resulting bilayer. The arrangement of hydrophilic heads and hydrophobic tails of the lipid bilayer act as a barrier preventing diffusion of polar solutes such as amino acids, nucleic acids, carbohydrates, proteins, and ions) across the membrane, but generally allow for the passive diffusion of hydrophobic molecules.[47]

In bilayers, the lipids are organized non-randomly.[47] In general, the outer leaflet of the eukaryotic cell membrane and other internal organelles are enriched with phosphatidylcholines where as in inner leaflet, flippases and scramblases concentrate anionic lipids such as phosphatidyl serine (PS), phosphatidic acids and phosphatidylethanolamine creating an extra barrier to passage of charged species across.[47, 48]

Lipid asymmetry in the membrane originated from the vectoral biosynthesis in which most eukaryotic glycerophospholipids are synthesized on the cytoplasmic side of the endoplasmic reticulum (ER), whereas sphingolipids are either synthesized or modified on the lumenal side of the ER and Golgi apperatus.[47, 49] In oder to maintain bilayer balance, some newly synthesized lipids are translocated to the side of the membrane opposite to that of the site of synthesis.[47, 49] This translocation process is facilitated either by slow movement of lipids from one layer to the other or by activation of flippases, a family of transmembrane phospholipid transporter enzymes.[47, 49, 50] In RBCs, both binding of cytoskeleton to the anionic inner leaflet and PS flippase activity helps to maintain PS in the inner membrane.[50]

Under normal conditions cells are capable of withstanding changes in membrane polarity, but reports suggest that certain pathological conditions such as exposure to high glucose levels results in the translocation of PS from the inner leaflet of the membrane to the outer leaflet resulting in changes in cell membrane polarity.[50-52] Alterations in membrane polarity has profound effects on cell function.[50, 52] In fact, if C-peptide exerts its activity by interacting/internalization with the cell membrane, then increases in the negative charge on the outer layer of phospholipids will exert repulsive effects on C-peptide. Therefore it is important to understand the C-peptide's ability to bind to the RBC membrane under hyperglycemic conditions as well as under high oxidative stress.

3.1.2 Human C-peptide Enzyme Linked Immuno Sorbent Assay (ELISA)

By 1969, a differential immunoassay with proinsulin and insulin antibodies was developed allowing the detection of C-peptide in human serum and plasma.[53, 54] Enzyme

linked immuno sorbant assay (ELISA) is a commonly used test in biological experiments that requires the measurement of the concentration of a substance in a biological sample using the binding of an antibody, or antibodies, to its antigen.[54, 55] The immunoassay has the advantage of highly specific binding of an antibody to its antigen.[54, 55]
Antibodies, also known as immunoglobulins (Ig) are globulin proteins that are found in the bloodstream of vertebrates, and are used by the immune system to identify and neutralize foreign particles such as infectious bacteria and viruses.[55] The typical structure of an antibody consists of Y shaped structural unit made with two heavy chains and two light chains (Figure 3.2 a). Though all the antibodies posses structural similarity of the Y shape and four chains, there is a small region at the tip of the protein known as the "hypervariable region" that is extremely variable, allowing for millions of possible various antibodies.[56] Each of these variants binds to a different and specific target antigen allowing the immune system to recognize wide diversity of antigens.[56]

Antigens are substances that induced the generation of antibody and are usually proteins or polysaccharides. The unique part of the antigen recognized by an antibody is called an epitope and these epitopes bind with their antibody in a highly specific interaction allowing antibodies to identify and bind only their unique antigen in the midst of the different molecules that are present in the bloodstream.

In the sandwich ELISA used for the detection of C-peptide, a monoclonal C-peptide antibody is affixed to a surface of a plate or ELISA well, and then a specific antigen (e.g.: C-peptide) is added (Figure 3.2.b).[54, 55] Once the antigen is immobilized, a biotinylated detection antibody is added and forms complex with antigen. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins

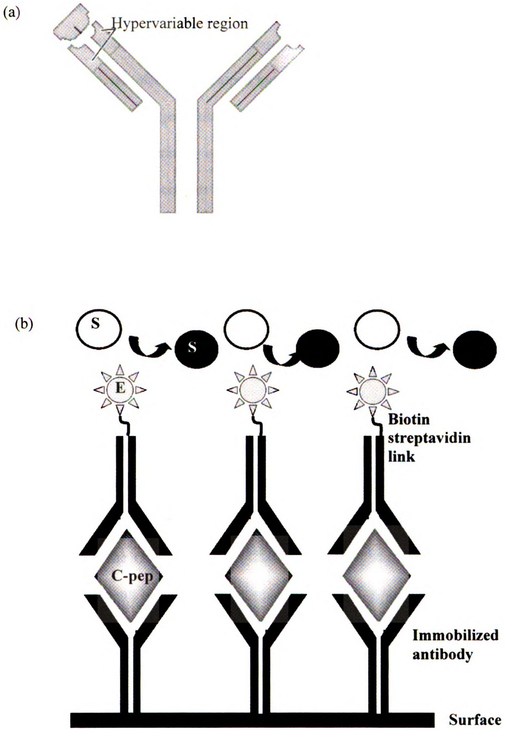


Figure 3.2: (a) Schematic representation of an antibody (b) Schematic representation of C-peptide ELISA where C-peptide is sandwiched between immobilized C-peptide-antibody and another biotinylated C-pep-antibody. Then Streptavidin linked enzyme was allowed to conjugate with detection antibody followed by addition of substrate.

or antibodies that are not specifically bound.[54] After the washings, bioconjugation of streptavidin linked horseradish peroxidase to the detection antibody is enabled. After the final wash step, the plate is developed by adding an enzymatic substrate that is converted in to some detectable signal (usually fluorescence or absorbance) by enzymatic reaction, which indicates the quantity of antigen present in the sample.[54] Use of a C-peptide ELISA provides the accounts of C-peptide interaction with RBC membrane under various oxidative stress conditions but provides no understanding of the it's effect on glucose transport in to cells.

3.1.3 Insulin like activity of C-peptide; ^{14}C-labeled glucose transport in to the RBC

Glucose, being the primary energy substrate for anaerobic glycolysis, is an essential component in metabolism. Being a polar molecule, free passage of glucose across cell membranes is restricted and requires the assistance of a specific transporter protein. In mammalian cells, glucose transport is facilitated by two major families of structurally related glucose transporters.[57] These are the GLUT and SGLT family of transporters.[57] The GLUT family, or the facilitative glucose transporter family, allows energy-independent, bidirectional, glucose transport (facilitated diffusion) in most tissues and cells.[57] The Na+/glucose cotransporter family, or the SGLT, mediates an active, Na+-linked transport process against an electrochemical gradient.[57] SGLT transporters are mostly seen in intestine and kidney epithelial cells. The GLUT family is the more important glucose transporter and consists of thirteen members divided into three major classes based on their protein structure.[57]

GLUT 4 is the major insulin-dependant glucose transporter and is commonly found in fat storage adipose tissues and skeletal and cardiac muscle cells.[58] Moreover, GLUT 4 is the most studied glucose transporter in diabetic pathogenesis.[58] Even though GLUT 4 is an insulin dependant glucose transporter, it is not the major glucose transporter in the RBCs or blood brain barrier, a pivotal component in glucose consumption in the human body.[58]

In fact, GLUT 1, the first and the most characterized glucose transporter, equilibrates the glucose across the RBC membrane.[58, 59] In addition, GLUT 1 is commonly found in fetal tissues and in the blood brain barrier.[59] The structure of GLUT 1 consists of 12 membranespanning α-helical proteins, each with 20 amino acids.[59] There are reports suggesting that glucose transport rates in to the RBCs of diabetic individuals are significantly less compared to that of healthy individuals. [60, 61] Also, there are reports indicating that the decrease in glucose entry in the RBC membranes of diabetic patients was due to a GLUT1 change in structure – mostly the outer domain of the glucose transporter.[61] These findings indicate that reduction in non-insulin-mediated glucose uptake plays an important pathogenic role in disorders of carbohydrate metabolism and associated complications in both the macro-and microvasculature in diabetic patients.[60, 61]

In fact, our group has previously shown using ^{14}C labeled glucose, that in the presence of metal activated C-peptide, glucose transport in to RBCs is up-regulated, indicating that metal activated C-peptide has an insulin like effect on the glucose uptake by RBCs, possibly by activating the glucose transporter GLUT 1.[34] As indicated previously, one problem with this finding is that the effect of C-peptide seems to diminish in the case of type 2 diabetic individuals where there is a elevated C-peptide levels during disease development.[34] In an effort to further investigate the possible method of C-peptide action and drawbacks in use of C-peptide as a diabetes treatment option, we further evaluated the ^{14}C labeled glucose transport under increased oxidative stress, a common environmental effect in diabetes.

Finally, to support our hypothesis, we further evaluated C-peptide binding to RBC membranes using C-peptide ELISA and changes in membrane PS content in the presence of oxidative stress and hyperglycemia and its effect on C-peptide activity and binding. In order to extent C-peptide therapy as a treatment option in diabetes, it is important to understand the possible ways of overcoming the factors hindering the insulin like C-peptide activity, allowing C-peptide to interact with the RBC membrane and exert its effect. Therefore, we further investigated the effect of certain pharmaceutical drugs used in treatment for diabetes, namely, metformin.

Metformin is the most popular oral anti-diabetic prescription drug in the United States.[62] Also, it is one of only two oral anti-diabetic drugs approved by World Health Organization as a treatment for diabetes. Metformin belongs to the biguanide class of anti-diabetic drugs and was discovered in 1957.[62, 63] Though it's potential for anti-diabetic effect was known for almost 30 years, it was not used in diabetic treatment in the United States until the late 1990s. Currently, it is the only biguanide currently in use, attributing to the reduced side effects on gastrointestinal complications compared to other drugs of the same class .[62]

There are reports suggesting metformin acts as an insulin sensitizer.[64-66] Also there are reports suggesting metformin, when used in type 2 diabetes individuals with cardiovascular complications, seems to alleviate their cardiovascular complications.[62, 67]

3.2 Experimental

Rabbit and Human RBCs were collected and prepared using the same techniques applied in chapter two. Rat blood was obtained from 5-month type 2 diabetic rats (BB/ZDB, blood glucose 24.0 ± 1.9 mM) and matching controls (blood glucose 5.0 ± 0.4 mM) according to the procedures approved by Animal Investigation Committee at Wayne State University (Detroit, MI) and prepared according to the same procedure described in chapter 2.

3.2.1 Reagents

Reagents were prepared in DDW or PSS unless otherwise noted. For ^{14}C labeled glucose transport studies, low glucose (0.55 mM glucose) PSS was prepared by adjusting the amount of glucose added appropriately.

C-peptide stock (8.3×10^{-6} M) solution was prepared by dissolving 99.9 % purified human C-peptide (0.25 mg) in 10.0 mL of DDW. This solution was stored in the 4 °C and used over a period of two weeks. From the C-peptide Stock, 250 μL was diluted up to 25.00 mL to prepare 8.3×10^{-8} M C-peptide solution.

Cr^{3+} stock (8.0×10^{-5} M) solution was prepared by dissolving $CrCl_3$ (0.0107 g) up to 500.00 mL in DDW. Then 31.9 mL of the Zn^{2+} stock was diluted up to 25.00 mL to make 1.02×10^{-7} M Zn^{2+} solution.

Zn^{2+} (8.0×10^{-5} M) stock solution was prepared by dissolving $ZnCl_2$ (0.0055 g) up to 500.00 mL in DDW. Then 31.9 mL of the Zn^{2+} stock was diluted up to 25.00 mL to make 1.02×10^{-7} M Zn^{2+} solution.

Metformin (0.0207 g) were dissolved in DDW (10.00 mL) to make 0.0125 M metformin solution.

3.2.2 Instrumentation

All the fluorescence measurements were performed with a Horiba-Jobinyvon Fluromax-4 spectrofluorometer. All chemiluminescence-based ATP measurements were conducted using a photomultiplier tube (PMT; Hamamatsu Corporation, Hamamatsu, Japan) placed in a light excluding box. Absorption measurements for the C-peptide immunoassay were conducted using a Molecular Devices Spectra Max M5^e platereader. ^{14}C labeled glucose transport measurements were taken using a Wallac 1450 microbeta plus liquid scintillation counter.

3.2.3 Effect of G6PD inhibition on ATP release from C-peptide treated red blood cells

5.00 mL of 14% hematocrit RBCs in 200 μM DHEA was prepared by diluting an appropriate volume of RBCs in PSS containing DHEA. This mixture was incubated for 30 min. Meanwhile in a second microcentrifuge tube, C-peptide (600 μl) was mixed with the Zn^{2+} (490 μL) or Cr^{3+} (490 μL) and the content was diluted up to 2.50 ml with PSS. At the end of a 30 min. incubation period, 2.50 mL of 14 % RBCs in 200 mM DHEA solution was transferred to the second centrifuge tube containing metal activated C-peptide to make 5.00 ml of 7% G6PD-inhibited RBCs in metal activated C-peptide. The remaining 2.50 mL of the 14% RBCs were diluted up to 5.00 ml with PSS to make a control of G6PD-inhibited RBCs. Control RBC samples and RBCs in metal activated C-peptide were prepared in a similar manner. Next, the samples were allowed to incubate for 6 hr. at room temperature. The resultant ATP release was measured using the chemiluminescence assay as described above.[41, 67]

At the end of the incubation period, 100 μL of RBCs were placed in a standard disposable fluorimetric cuvette. Then, 100 μL of a luciferin/ luciferase (L/L) solution was added. The content of the cuvette was mixed and the chemiluminescence intensity was measured 15 s after the L/L addition. The resultant space current from the PMT, which is proportional to the ATP-induced chemiluminescence, was recorded as a potential by a data acquisition board controlled by a program written with labVIEW8 (National Instruments, Austin, TX, USA).

3.2.4 Effect of G6PD inhibition on ^{14} C labeled glucose uptake in to RBCs

Experiments were performed using RBCs that had been washed in a low glucose concentration (0.55 mM) PSS. The solution preparation for C-peptide and all inhibitors remained the same as for previous studies. Additionally, 1.554 Bq ^{14}C-labeled glucose was added to the low glucose PSS. This created a ratio of ^{14}C labeled glucose:non-radio labeled glucose of 1:10. RBCs were added, creating a 7% hematocrit solution and allowed to incubate for a period of 4 hrs. The RBCs were then centrifuged at 500×g for 10 min. The resultant cells were resuspended in low glucose PSS. After another centrifugation (at 500×*g*), the RBCs were lysed with bleach and combined with scintillation cocktail and the radioactivity was measured using a scintillation counter over an integrations period of 1 min. for each sample. G6PD activity was inhibited by incubating RBCs with DHEA for 30 min. prior to the incubation with ^{14}C labeled glucose.

3.2.5 Effect of G6PD inhibition on C-peptide binding to red blood cell membrane

A 14 % solution of G6PD inhibited RBCs was prepared by incubating RBCs in appropriate volume of DHEA diluted in PSS. This solution incubated at room temperature for 30 min. 20 nM C-peptide and Zn^{2+} solutions were prepared by diluting 250.0 and 63.8 μL of C-peptide stock (8.3×10^{-6} M) and Zn^{2+} stock (8.0×10^{-5} M) solutions, respectively, in DDW. C-peptide (600 μL) and Zn^{2+} (490 μL) were placed in a 14 mL microcentrifuge tube and diluted up to 2.50 mL with PSS. Next, 2.50 mL of 14 % G6PD inhibited RBCs was added to this immediately to make a 7 % solution of G6PD inhibited RBCs in 20 nM metal activated C-peptide and the content of the tube was incubated for 4 hrs. A solution of 7 % RBCs in 20 nM metal activated C-peptide, 7 % RBCs and 7 % G6PD inhibited RBCs were prepared in the same manner.

At the end of the 4 hr incubation period, the content of the vials were centrifuged (× 500 g) for 10 min. and the cells were resuspended in PSS. After another centrifugation (at 500×*g*), the RBCs were lysed and the C-peptide ELISA was performed using a Human C-peptide ELISA kit (LINCO research, St. Charles, MO). The resulting absorbance was measured at 450 nm using the Molecular Devices Spectra Max $M5^e$ platereader.

3.2.6 Effect of G6PD inhibition and elevated glucose levels on PS translocation on RBC membrane

Experiments were performed using RBCs that had been incubated in a high glucose concentration (15 mM) PSS, in addition to the normal PSS. The 7 % hematocrit solutions of RBCs, G6PD inhibited (with DHEA) RBCs and RBCs in high glucose (15 mM) were prepared by diluting appropriate volumes of RBCs in appropriate PSS containing the required inhibitors. These solutions were then allowed to incubate at room temperature for 4 hrs. In order to evaluate the effect of metformin, each sample was divided in to two equal portions and an appropriate volume of metformin was added to one to make a RBCs suspension in 30 μM metformin while adding PSS to the set of controls. After 2 hrs of incubation time, 2 μL of the 7 % RBCs were diluted in 498 μL of 1X binding buffer (100 mM HEPES/ NaOH, pH 7.5 containing 1.4 M NaCl and 25 mM $CaCl_2$) provided with the Annexin V-FITC apoptosis detection kit (Sigma) to prepare a cell suspension of approximately 1×10^6 cells/ mL . To this, 5 μL of annexin labeled with the fluorescence dye fluorescein isothiocyanate (FITC) was added. After a 10 min incubation, the samples were centrifuged (× 500 g) for 5 min and the cells were rewashed and resuspended prior to measuring the fluorescence emission using the Horiba-Jobinyvon Fluromax-4 spectrofluorometer. (Excitation 487 nm and emission 515 nm)

3.3 Results and discussion

Despite the fact that it is one of the most studied pathological conditions, diabetes still remains dangerously epidemic with no cure and well over 171 million people suffering worldwide.[68-71] As scientists and researchers struggle to understand and explore the secrets behind the pathology and resultant associated complications, interest has focused on a wide variety of factors other than insulin. C-peptide, despite its original proclaim as a biologically inert compound, has been revived with a renewed interest almost 40 years after its discovery in 1967.[20] Despite the ever-increasing reports suggesting the possible insulin like activity of C-peptide in ameliorating the complications in type 1 diabetes, the issue still remains some what controversial.[34]

While groups of researchers strongly suggest that the C-peptide is bioactive other groups argue otherwise.[26] However during the last decade, an increasing number of reports have appeared describing the beneficial effects of C-peptide in diabetic complications especially, in type 1 diabetes.[72, 73] In fact, there are reports that when administrated in replacement doses, C-peptide seems restore the diabetes induced changes in both kidney and peripheral nerves system in type 1 animal models.[14, 20, 72] In addition, there are reports including clinical studies indicating C-peptides ability to improve blood flow in skin, skeletal muscle tissues, myocardium and nerves tissues.[20]

Recently our group reported that C-peptide, in the presence of transition metal ions such as Cr^{3+} and Fe^{2+}, could increase the amount of ATP released from RBCs and also increase the glucose uptake in to the RBCs.[34, 35] In fact, we reported a 70 % increase in ATP release and a 31 % increase of glucose transport in comparison to control RBCs and purified C-peptide (no metal added), after incubation of the RBCs for 6 h in a C-peptide activated with Fe^{2+} or Cr^{3+}. These findings clearly identify C-peptide is bioactive and its activity depends upon metal binding.[34]

Even though C-peptide exerts beneficial effects on ameliorating type 1 diabetic complications, similar effects were not observed with type 2 diabetes making annotations of biological activity as somewhat doubtful.[72] In this study, we attempt to address the particular issue by investigating the effect of hyperglycemia induced oxidative stress and resulted structural modifications and their effect on C-peptide activity.

Previously we have shown that in type 2 diabetes individuals, the glucose-6-phosphate dehydrogenase (G6PD) enzyme was deficient and resulted in the immediate decrease in RBC derived ATP release.[41,42] As the first step of understanding C-peptide's mode of action, as well as its inability to ameliorate complications in type 2 patients, we inhibited the G6PD enzyme activity with DHEA and then these G6PD inhibited RBCs were subjected to 6 hr incubation with Cr^{3+} activated C-peptide. As expected, upon incubation with C-peptide, the ATP release from control RBCs increased by 58 % (Figure 3.3). However, the ATP release from G6PD inhibited cells only increased by 27 % indicating that effect of C-peptide is diminished when the RBCs were subjected to oxidative stress. This results were further confirmed by an observed 32 % increase of ATP release from RBCs incubated with diamide a know oxidant [41] prior to the C-peptide treatment.

Though our original studies with C-peptide were conducted with Cr^{3+}, it is well known that there are milimolar levels of Zn^{2+} in pancreatic islets where C- peptide is synthesized and therefore it is logical to assume that Zn^{2+} has a higher chance to bind to C-peptide compared to Cr^{3+}. Therefore Zn^{2+} was selected for further studies and 10 nM C-peptide incubated in 10 nM Zn^{2+} was used unless otherwise noted. C-peptide concentrations used were in accordance with the physiological limits.

Similar trends of ATP release were observed upon treating RBCs with C-peptide activated using Zn^{2+}. As shown in figure 3.4. ATP release increased by 57 % in control

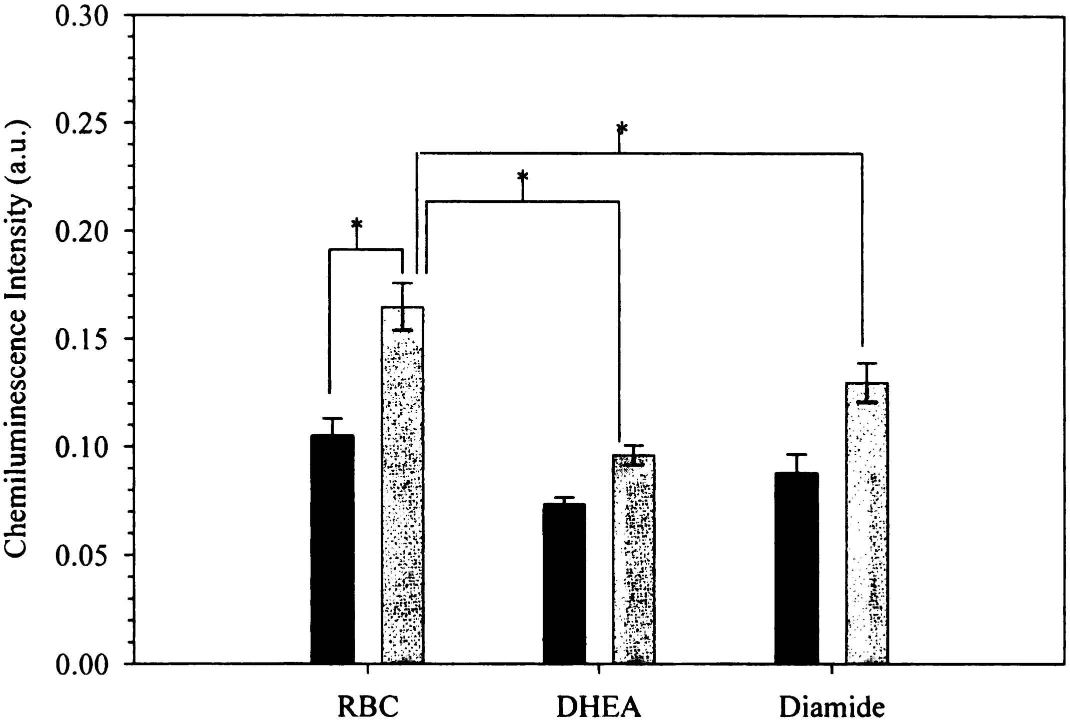


Figure 3.3: Effect on ATP release from rabbit RBCs upon C-peptide activated with Cr^{3+}. The gray bars represent the metal activated C-peptide treated RBCs and black bars represent the RBCs with out C-peptide. DHEA was used to inhibit the G6PD activity and diamide is a known oxidant. n = 6 rabbits. * represent the values that are significantly different from each other $p < 0.001$.

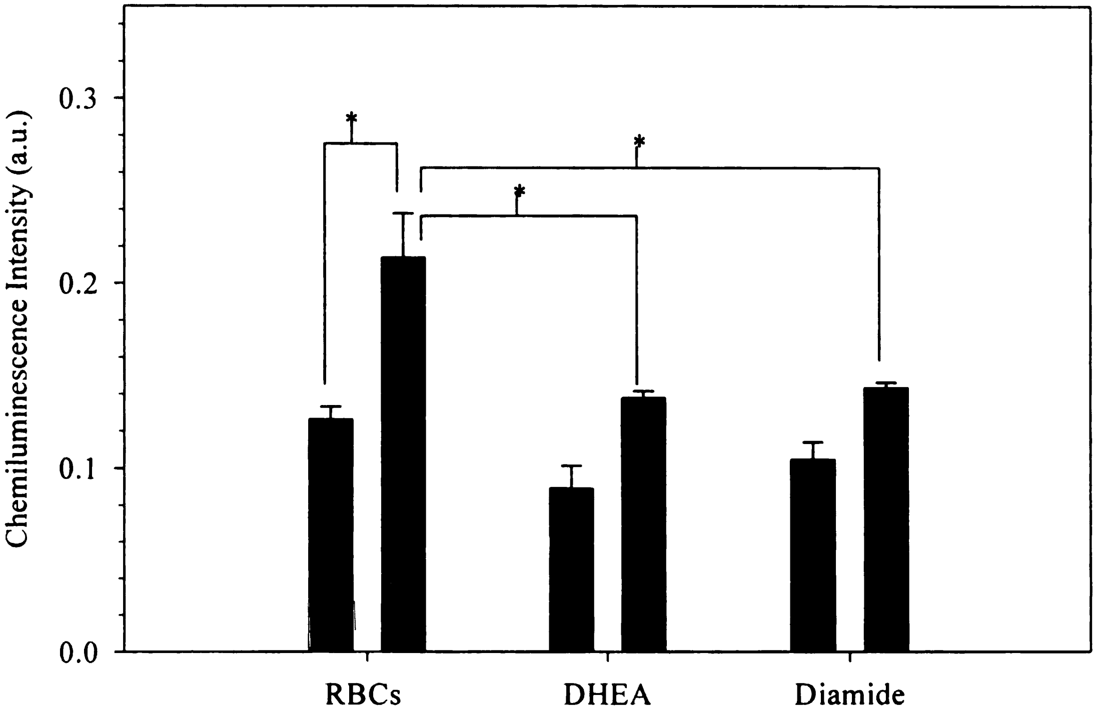


Figure 3.4: Effect on ATP release from rabbit RBCs upon C-peptide activated with Zn^{2+}. The gray bars represent the metal activated C-peptide treated RBCs and black bars represent the RBCs with out C-peptide. DHEA was used to inhibit the G6PD activity and diamide is a known oxidant. n = 9 rabbits. * represent the values that are significantly different at $p < 0.001$.

RBCs in comparison to 30 % and 32% increase in DHEA and diamide treated RBCs respectively further confirming oxidative stress indeed affects the C- peptide induced ATP release . However, it is inconclusive whether the decrease in ATP release is a result of a direct inhibition of C-peptide activity or false positive results occurred due to reduced ATP release from RBCs subjected to the oxidative stress.[41]

Previously, we reported C-peptide increased glucose uptake in to RBCS.[34] To provide further evidence that the exposure to high glucose concentration and resultant oxidative stress inhibits metal-activated C-peptide facilitated glucose transport into the RBC, ^{14}C-labelled glucose was included in the PSS at a 1:10 ratio with unlabelled glucose creating competition between the different glucose isotopes. As shown in Figure 3.5 the amount of glucose entering the RBC did not increase with comparison to the control C-peptide treated sample. In fact when G6PD activity was inhibited glucose transport increased only by less than 10 % compared to the 35 % increase in control RBC sample treated with C-peptide. In fact, there is no significance difference ($P < 0.001$) of ATP release in C-peptide treated G6PD inhibited RBCs from RBCs without C-peptide.

While this increase in glucose transport was not as high as the ATP released from the RBCs, it should be noted that the ratio of radio-labeled glucose to unlabelled glucose was not stoichiometrically equivalent. In addition, we have previously shown that RBCs incubated with the metal ions in the absence of C-peptide did not result in any increase in glucose transport across the RBCs membrane or RBC-derived ATP release.[34] This result indeed confirms C-peptide activity is hindered by increased oxidative stress. We propose that C-peptide excerts its bioactivity via membrane mediated interaction followed by activation of GLUT 1; therefore, it is important to understand the structural changes that

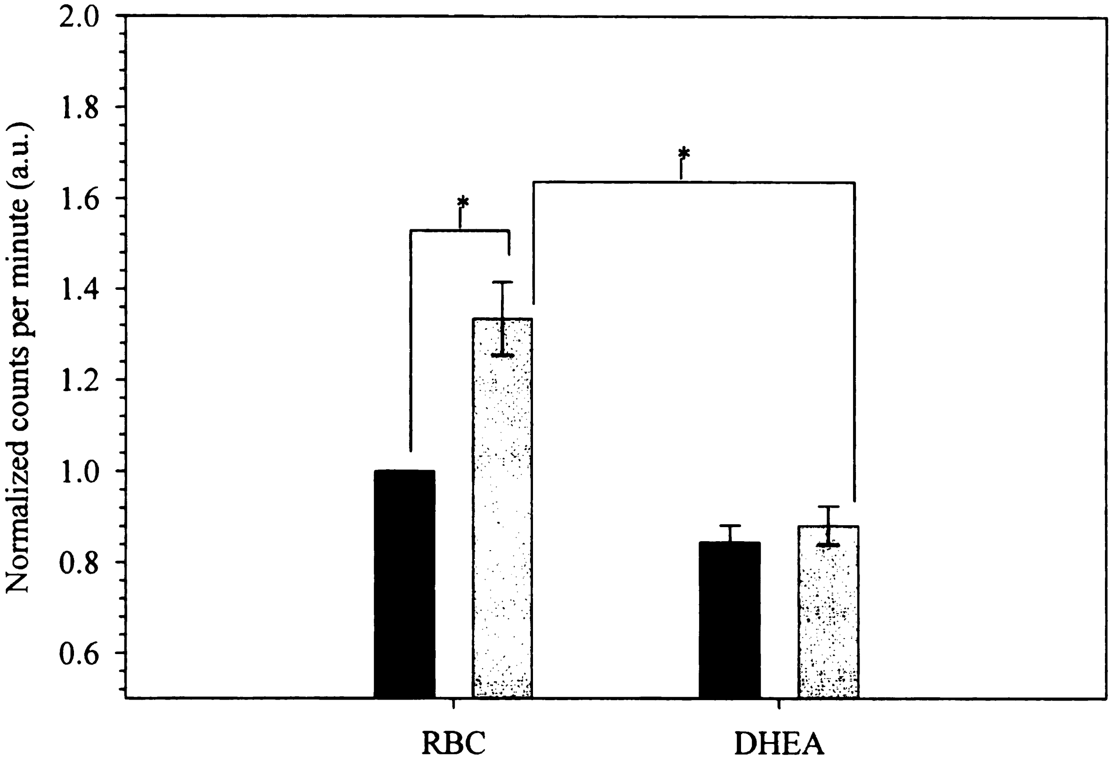


Figure 3.5: Effect of G6PD inhibition on C-peptide mediated ^{14}C labeled glucose uptake in to the RBCs. The gray bars represent the metal activated C-peptide treated RBCs and black bars represent the RBCs with out C-peptide. DHEA was used to inhibit the G6PD activity and diamide is a known oxidant. n = 8 rabbits. * represent the values that ate significantly different $p < 0.001$.

the RBC membrane undergoes under oxidative stress, and its effect on C-peptide's possible mode of action.

Conventional methods by which bioactive peptides exert their effect is via binding to its specific receptor at the target tissue or organ.[14] Proinsulin C-peptide has neither conserved amino acid sequences through the various species, nor any known receptor, supporting the claims that it is biologically inert.[14]

The first evidence suggesting a possible C-peptide interaction with cell membranes was reported in 1986 using radioactive ^{125}I labeled rat C-peptide.[74] Recently, Wharen and co-workers have shown that C-peptide in nanomolar concentrations binds to the cell surface, suggesting the possibility of a G-protein coupled membrane receptor.[14] Our studies also indicated the possible membrane mediated interaction followed by activation of the GLUT 1 transporter.[34]

The ability of exogenously added C-peptide (activated with Zn^{2+}) to bind to RBC membrane; under normal and oxidative stress C-peptide ELISA was performed. Our group has evidence suggesting that metal activation is required for C-peptide to bind to the RBC membrane. After incubation of normal and G6PD inhibited (by incubating with DHEA) RBCs with 20 nM peptide for 4 hrs, cells were washed, centrifuged and lysed and ELISA was performed. As shown in figure 3.6, the amount of C-peptide present in the centrifuged RBCs decreased from 73 % in regular RBCs to 25 % in RBCs incubated with DHEA, further suggesting the inability of proper C-peptide membrane interaction under type 2 diabetic conditions.

In summary, all the studies we have conducted so far suggest that not only does C-peptide bioactivity occur via activation of GLUT 1 by interacting with RBC

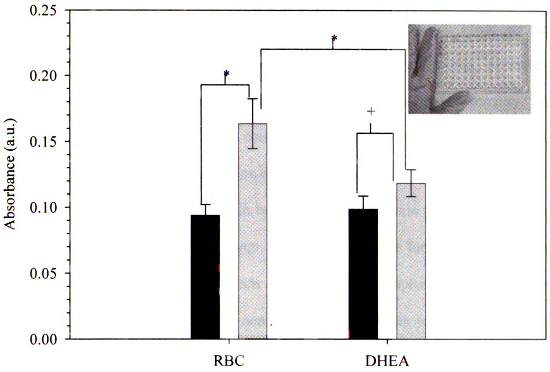


Figure 3.6: Effect of G6PD inhibition and resulted oxidative stress on C-peptide binding to the RBC. The gray bars represent the metal activated C-peptide treated RBCs and black bars represent the RBCs with out C-peptide. DHEA was used to inhibit the G6PD activity and diamide is a known oxidant. n = 6 rabbits. * represent the values that ate significantly different at $p < 0.001$. A picture of the ELISA assay (96-well plate) is shown in upper left hand corner.

membrane but also changes in the RBC itself under hyperglycemia and oxidative stress will cause a decrease in expected C-peptide bioactivity.

Under normal conditions, the RBC maintains asymmetry of membrane lipids by transporting anionic phospholipids such as PS into the inner leaflet. There are reports suggesting certain pathological conditions such as hyperglycemia promote the translocation of PS in to the outer membrane, disturbing regular lipid asymmetry.[75] Also, it is known that as a cell ages the PS content of the outer leaflet increases.[75] We believe this change in phospholipid composition exerts repulsive effects on C-peptide bioactivity.

Annexin V is a cellular protein belonging to the annexin protein group found in all the living organisms including fungi. Though the specific function of the protein is unknown, Annexin V selectively binds to the anionic phosphatidyleserine. Therefore Annexin V is used as a probe in the annexin A5 affinity assay to detect cells that have expressed PS on the cell surface, as commonly found in apoptosis as well as other forms of cell death. Here we investigated the PS content in the outer membrane of regular, G6PD inhibited and RBCs incubated in high glucose using FITC labeled annexin V. FITC is commonly used fluorescence dye with 494 nm excitation and 521 nm emission. (Figure 3.7)

As shown in figure 3.8 both G6PD inhibition as well as incubation with high glucose for 5 hrs resulted in a marked increase in annexin binding to the cell membrane of human RBCs. In fact incubation with 15 mM glucose for 5 hrs increased annexin binding to membrane by 41 % whereas G6PD inhibition increased that value by 28 % ($p < 0.001$).

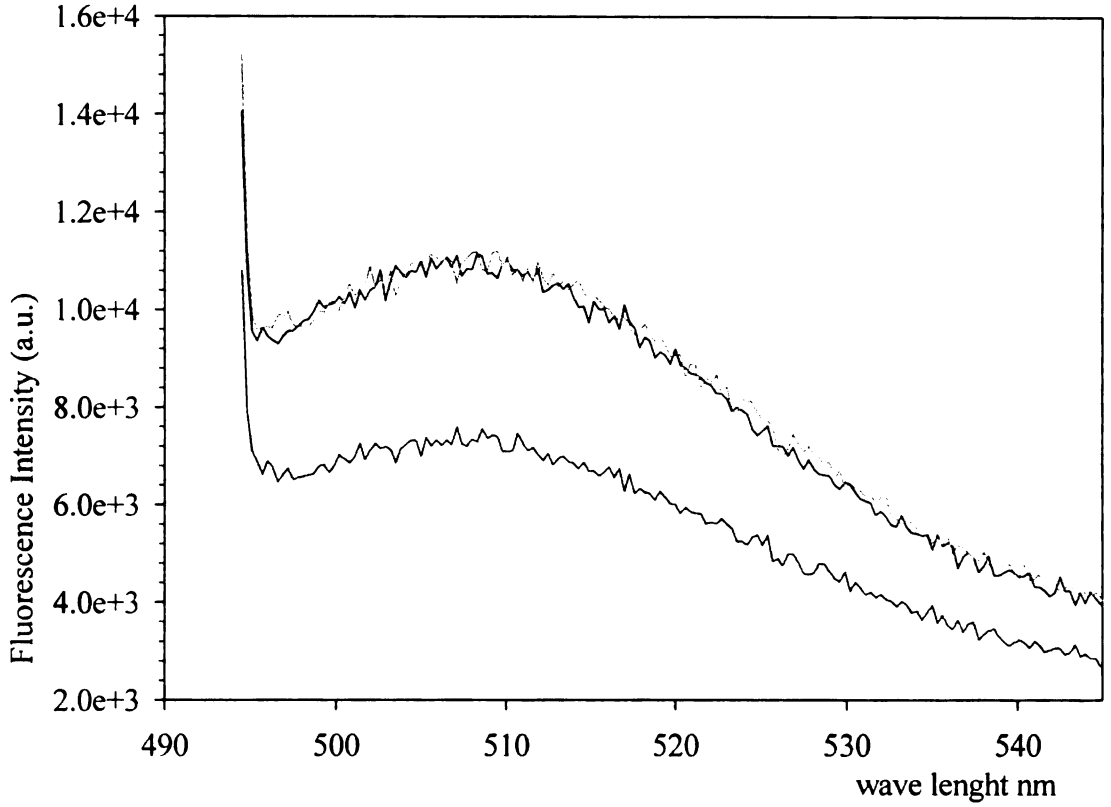


Figure 3.7: Annexin binding to the control, G6PD inhibited and RBCs incubated in 15 mM glucose. Row fluorescence intensity data of control RBCs and RBCs incubated in 15 mM glucose. Lower trace represents the control RBCs where as upper two traces represent RBCs incubated in DHEA and 15 mM glucose.

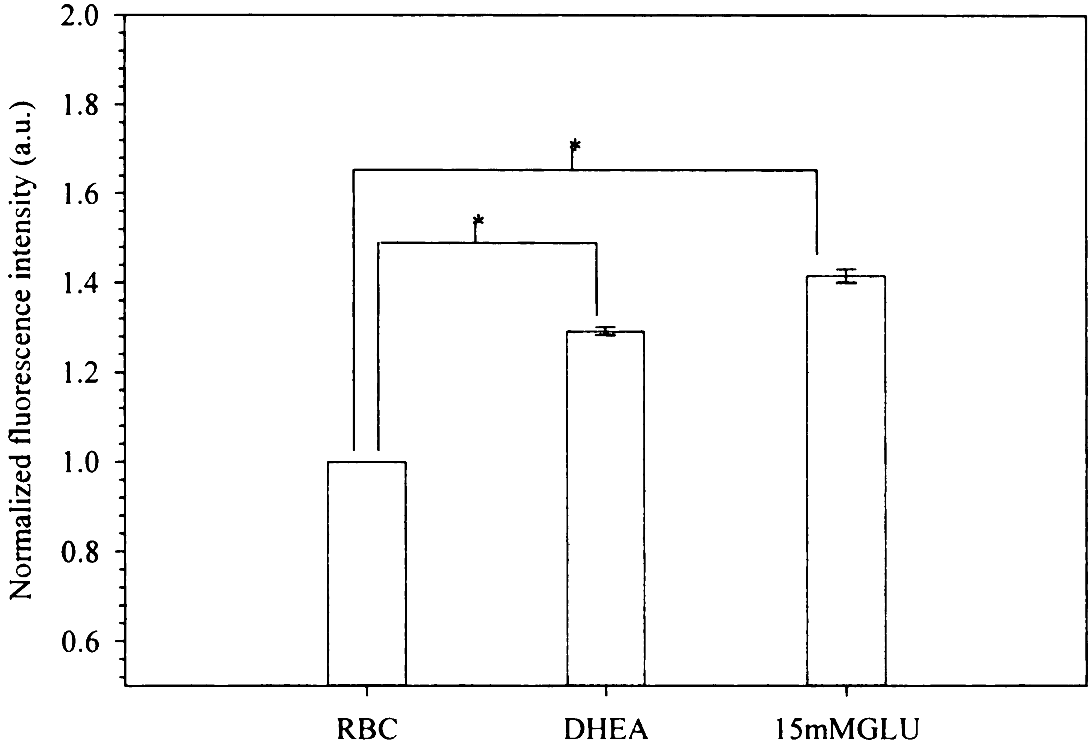


Figure 3.8: Quantitative representation of annexin binding to the control, G6PD inhibited and RBCs incubated in 15 mM glucose. Upon inhibition of G6PD (with DHEA) annexin binding increase by 28.2 % and incubation with glucose increased the annexin binding by 41 %. n = 3 individuals. * represent the values that are significantly different $p < 0.001$.

As mentioned above metformin is one of only two oral anti-diabetic drugs approved by the World Health Organization as a treatment for diabetes, and there are reports suggesting metformin acts as an insulin sensitizer.[64-66] Also, there are reports suggesting that metformin, when used in type 2 diabetes individuals with cardiovascular complications, seems to alleviate their cardiovascular complications.[62, 67]

Here we evaluated the ability of the prescription drug metformin to enhance the C-peptide effect on type 2 diabetic rat RBCs. As shown in figure 3.9 type 2 rats have 62 % higher annexin binding in comparison to the RBCs obtained from control rats. Upon incubation with 30 μM metformin for 2 hrs the amount of annexin binding to RBC membrane decreased by 28 %. In a separate study carried out in our laboratory, we measured the ATP lease from these RBCs after incubating them with C-peptide (activated with Zn^{2+}) for 4 hrs and then incubated with 30 μM metformin for 2 hrs. The increase in normalized ATP release from type 2 rat RBCs (31.2 %) was significantly less than that of the control rats (78.4 %) with $p < 0.01$ Upon treatment with 30 μM metformin ATP release from both control and type 2 rat RBCs increased by almost 100 % and the two values are not significantly different from each other. Similar trends were observed with C-peptide binding to the RBCs as well as C-peptide stimulated glucose transport in to the RBCs confirming the fact that metformin indeed enhances the effect of C-peptide activity in type 2 diabetes RBCS.

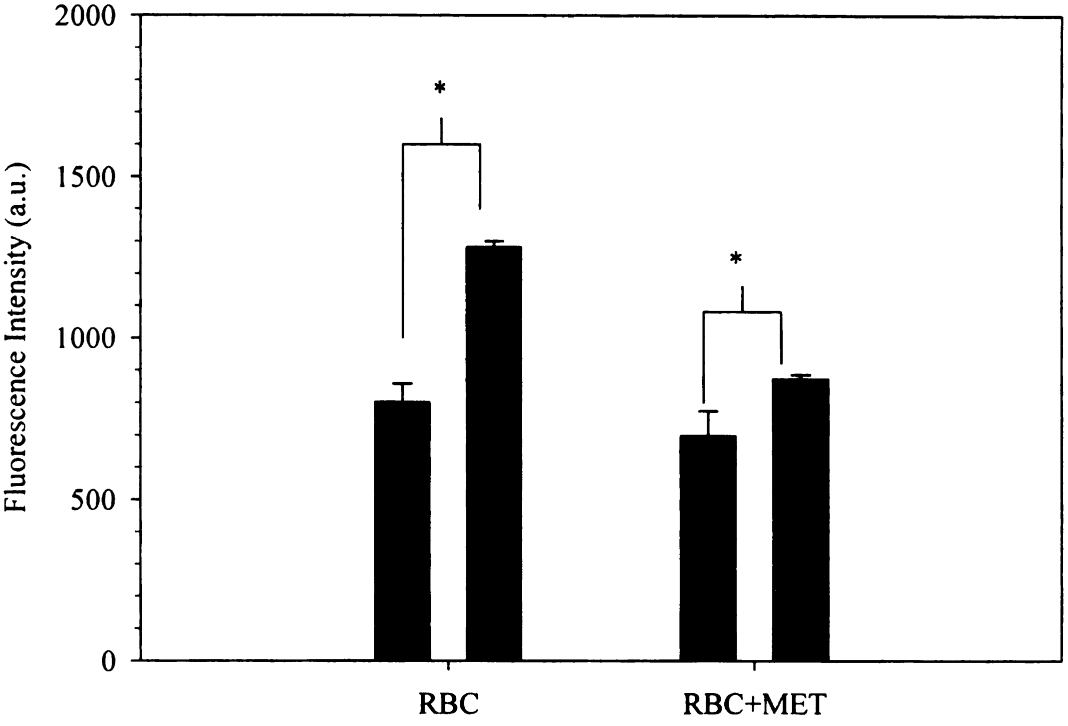


Figure 3.9: Annexin binding to the control and type 2 rat RBCs in the presence and absence of diabetic treatment drug metformon (30 μM). Black bars represent the control rats and the gray bars represent the type 2 diabetic rats. Incubation with metformin seems to mask the overall negative charge of PS facilitating C-peptide interaction with membrane. n = 5 ratts . * represent the values that are significantly diffaternt $p < 0.001$.

3.4 Conclusions

Proinsulin C-peptide, when activated with transition metals such as Zn^{2+} increases the RBC derived ATP release by 50 % above its original value. However, when RBCs were subjected to hyperglycemia or oxidative stress, the C-peptide effect was decreased. This increase in ATP release can lead to an increase in endothelial NO synthase activity resulting in increased NO levels leading to enhanced vasodilation and improved blood flow in to the target organs.

We propose that C-peptide upon activating with Zn^{2+} interacts with the RBC membrane, leading to insulin like affects by up-regulating the glucose uptake through GLUT 1. This effect was decreased when the RBCs were subjected to oxidative stress prior to C-peptide treatment. In fact, when the RBCs were subjected to a type 2 diabetic like environment, either by incubating with high glucose or by inhibiting G6PD activity, the effect of C-peptide on the RBC derived ATP release, ^{14}C labeled glucose transport and C-peptide binding to the RBCs was decreased.

It is known that hyperglycemia induces the PS translocation from the inner leaflet to the outer leaflet of cell membrane creating a net negative charge.[48, 76] Similar effect was observed in cells that are exposed to hyperglycemia or oxidative stress, decreasing the interaction between proinsulin C-peptide and RBC membrane. However, when these cells were incubated with prescription drug metformin, amount of the annexin V binding to the membrane decreased indicating the ability of metformin to masks the charge repulsion between C-peptide and RBC membrane.

Collectively we can state that C-peptide is indeed bioactive when interact with metal ions such as Zn^{2+}, and the method by which it exerts its bioactivity seems to be via some membrane based interaction and this effect seems to diminish when cells are subjected to an oxidative stress. Use of prescription drug in combination with metal activated C-peptide replacement may improve C-peptides ability to ameliorate complications in diabetes therefore acting as a “sensitizer” for C-peptide.

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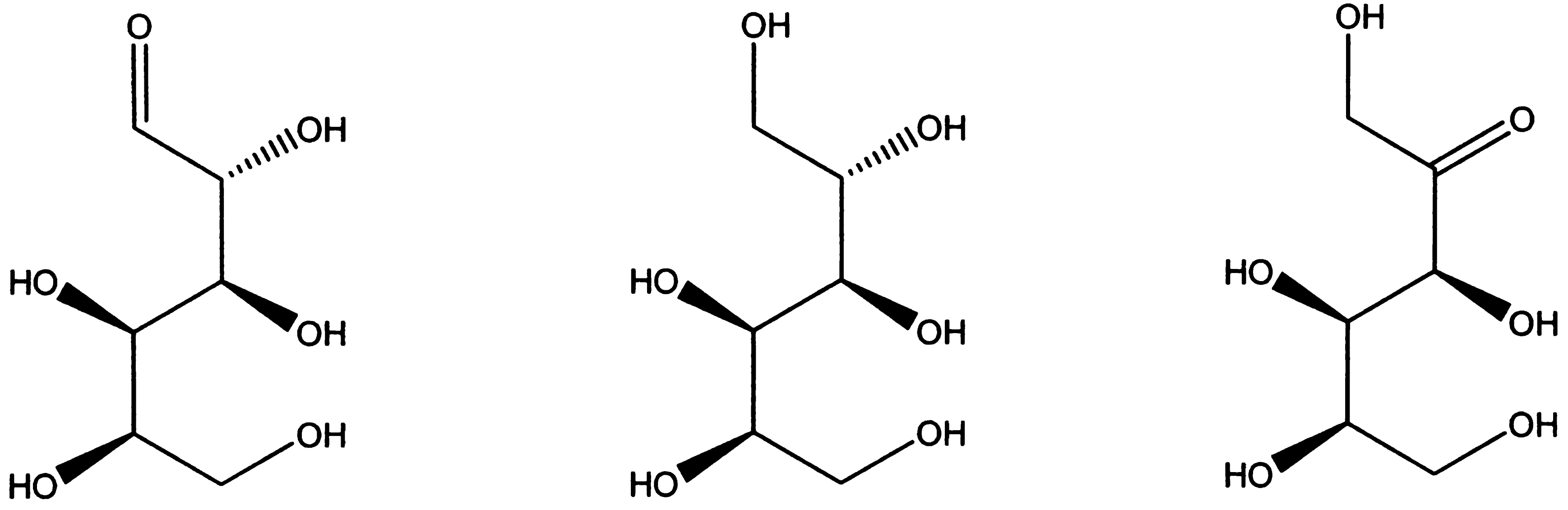
Chapter 4

EFFECT OF POLYOL PATHWAY AND G6PD ACTIVITY IN DIABETES RBCs

Glucose, derived from the Greek word *glykys*, meaning "sweet", is a simple monosaccharide that holds the central position of metabolism of living organisms including plants, animals and microorganisms.[1] This relatively energy rich monosaccharide is the main product of photosynthesis and the starting material for respiration in both eukaryotes and prokaryotes, thus serving as a life sustaining fuel source.[1, 2] Glucose contains the six carbon sugar (hexose) with an aldehyde group making it an aldohexose (figure 4.1).[1] Glucose has two stereoisomers L and D, but only D-glucose is biologically active.[1, 2] D-glucose is often referred to as dextrose monohydrate or simply as dextrose, especially in the food industry. The mirror-image of the D-glucose molecule, L-glucose, cannot be metabolized by living organisms/cells in the biochemical process known as glycolysis.[1, 2]

Under normal conditions, upon entering to the blood stream via absorption from the digestive system, the fate of glucose will vary on the energy status of the cells/organism.[1] There are three major possible pathways that the glucose can take.[1] A portion of the absorbed glucose will be stored as high molecular weight polymers such as glycogen in animals (or starch in plants) without altering the cytosolic osmolarity, for rapid utilization at a later time.[1, 2] Another portion of absorbed glucose will be transported



Glucose Sorbitol Fructose

Figure 4.1: Structures of glucose (a) Sorbitol (b) and fructose (C). Sorbitol is sugar alcohol of both glucose and fructose. Oxidation at C1 of glucose or C2 of fructose will result in sorbitol.

in to every tissue and cell within organism to be oxidized to pyruvate via glycolysis, which may undergo further oxidation in the citric acid cycle of aerobic respiration depending on the location and organism, producing ATP energy required for cellular functions.

Glucose is such an efficient fuel that it will generate more ATP per O_2 molecule in comparison to other fuel sources such as fat.[1] Glucose is not only a fuel molecule, but also is a remarkably versatile precursor that supplies array of metabolic intermediates for various biochemical reactions. Therefore, a third portion of absorbed glucose will enter the pentose phosphate pathway (PPP), to be converted in to other sugars and intermediates essential for other important biosynthetic and/or metabolic pathways (e.g., synthesis of DNA and RNA and phospholipid synthesis).[1]

In healthy individuals, all circulating sugars with the exception of glucose are controlled by the liver, whereas glucose concentrations are controlled mainly by insulin and several other factors.[3] In other words, glucose homeostasis in the human body is primarily maintained through peripheral glucose uptake and utilization with the activation and regulation of insulin, regulation of glycogen break down in the liver via glucagon, regulation of hepatic glucose production and the PPP via glucose-6-phosphate dehydrogenase.[1]

In diabetic individuals, this tight regulation of blood glucose levels is disrupted as a result of impaired insulin action. In the pathogenesis of diabetes, either the pancreas does not produce insulin or the cells are resistant to the insulin activity, thereby hindering the uptake and storage of glucose by tissues that depend upon insulin activity for glucose uptake such as muscle cells. This insulin resistance results in hyperglycemia or increased blood glucose levels.[4] The abnormally high blood glucose levels will lead to the activation and up-regulation of another metabolic fate of glucose named polyol pathway.[5]

4.1 Channeling glucose via polyol pathway

Glucose is transported in to the cells via a facilitated diffusion by two major families of structurally related glucose transporters known as the glucose transporter (GLUT) and sodium-dependent glucose cotransporters (SGLT).[6] GLUT4 is the insulin-dependant glucose transporter, found in adipose tissues and striated muscle such as skeletal and cardiac muscles.[7] In diabetes, due to insulin deficiency or resistance, glucose utilization by these is tissues diminished exerting severe glucose strain on non-insulin dependant glucose users such as RBCs and neurons.[4, 7-9] As a result, these cells will experience high glucose influx and disturb cellular homeostasis.

Upon entering the cells, glucose is phosphorylated by the enzyme hexokinase (with the expense of ATP) in to glucose-6-phosphate (G6P), and depending on the energy states of the cells, G6P will enter either glycolysis or PPP.[1, 10] Glycolysis utilization is saturated under hyperglycemic condition, thereby, exerting excessive glucose strain on cell and activating polyol pathway. Disruption of the osmotic regulation of cells, resulting from glucose accumulation, will also act as catalytic forces driving more and more glucose to be metabolized via the polyol pathway.[3, 11]

The polyol pathway, also known as the sorbitol pathway, consists of two reactions, one reduction step and the other an oxidation step (Figure 4.2).[4, 12] In the first step, glucose is reduced in to the alcohol sorbitol (Figure 4.1.b.) by the NADPH-dependant enzyme aldose reductase (AR).[4, 11] During the second step this sorbitol is oxidized to fructose (Figure 4.1.c.) by the NAD^+-dependant enzyme sorbitol dehydrogenase (SDH), reducing NAD^+ in to NADH. There are reports suggesting that hyperglycemic conditions in cells, leads to the activation of both aldose reductase and SDH.[4, 11]

The first enzyme of the polyol pathway is aldose reductase (EC: 1.1.1.21; alcohol:NADPH oxidoreductase) which belongs to the aldo-keto reductase enzyme family.[13] Most of the enzymes belonging to this family are involved in detoxification processes as they catalyze the reduction of a wide variety of substrates such as aliphatic and aromatic aldehydes, monosaccharides, steroids and polycyclic aromatic hydrocarbons.[14] Human AR is a monomeric protein of 315 amino acids with a molecular mass of about 36 kD.[14]

Human AR is a cytosolic enzyme widely distributed among the retina and the lens of the eye, kidney and various reproductive organs.[14] In the presence of NADPH, AR catalyses rate determining step of the polyol pathway reducing glucose to its corresponding alcohol sorbitol while oxidizing NADPH to $NADP^+$.[4, 11, 12, 14-16] Under normal physiological conditions, AR is capable of converting a minor fraction of glucose (< 3 %) in to sorbitol before glucose is phosphorylated, but does not occur to an extent that can be harmful to the tissue.[4, 14] This is due to the fact that glucose, at normal physiological conditions, is not the preferred substrate for AR. The specificity constant (k_{cat} / K_m), a measure of how efficiently an enzyme converts a substrate into product is only $2.8 \times 10^2\ M^{-1}min^{-1}$, which is considerably low value in comparison to the normal range of $10^{10}\ M^{-1}s^{-1}$.[14]

Sorbitol dehydrogenase (E.C. 1.1.1.14) is the second enzyme of the polyol pathway.[17] It oxidizes sorbitol back to fructose, reducing NAD^+ to NADH to complete the pathway. Human SDH (hSDH) is a tetramer of four identical catalytically active subunits with each subunit consisting of 356 amino acid residues (38 kD)[18, 19] and one catalytic zinc atom.[20] According to the X-ray crystal structure of hSDH (3.0 Å resolution), coenzyme NAD(H) binding reversibly to the each subunit and single zinc atom housed at the large catalytic domain is essential for the hSDH activity.[18]

Once sorbitol is formed, SDH readily and reversibly catalyses the oxidation of the C_2 of sorbitol producing fructose (Figure 4.2) with the reduction of NAD^+ to NADH.[18] In-fact SDH is capable catalyzing the reaction in the reverse direction too ($K_{\text{equilibrium}}$ sheep liver SDH = 3.7 nM).[5] It is reported that high pH facilitates the forward reaction whereas low pH facilitates the reverse reaction.[5]

As mentioned earlier, glucose is not the preferred substrate for AR, making the significance of the polyol pathway limited under normal glycemic conditions.[12] But there are reports suggesting some beneficial effects observed with the sorbitol pathway.[21] One such report is that sorbitol acts as a neutral organic osmolyte, regulating osmotic pressure of the cells hence maintaining the whole body homeostasis and correct cell volumes.[3]

Also there are reports suggesting AR plays an important role in cellular detoxification by removing reactive aldehyde species.[22-24] Though the antioxidant system is capable of handling reactive oxidant species (ROS) in the case of excessive ROS formation, it would cause increased lipid peroxidation followed by lipid aldehyde formation.[24] While glutathione (GSH) scavenges these aldehydes forming GSH-lipid aldehydes molecules, AR readily oxidizes these aldehydes forming GSH-lipid alcohol

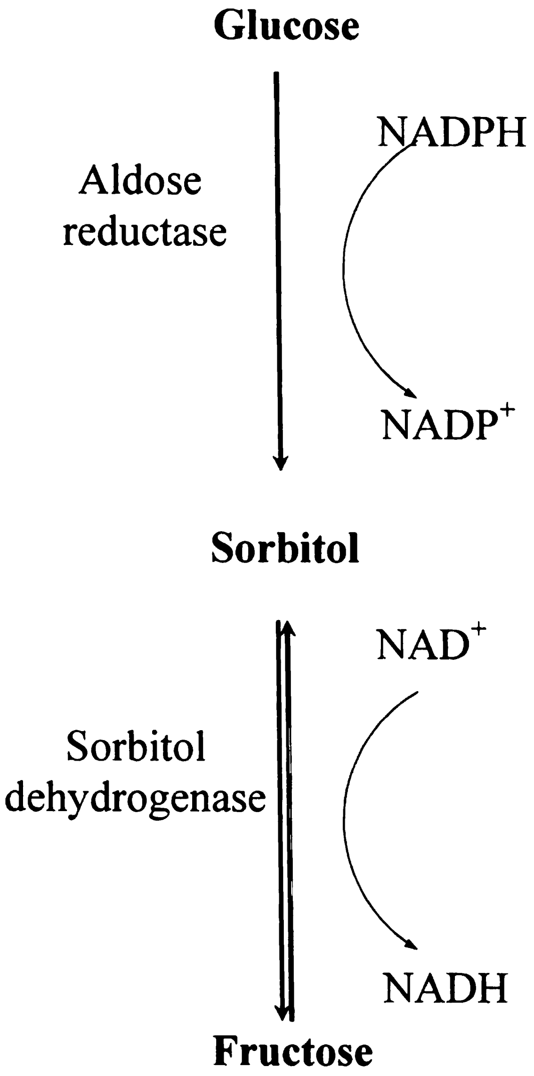


Figure 4.2: Schematic representation of the polyol pathway. Glucose is oxidized to its alcohol sorbitol by AR, while reducing NADPH. Then SDH oxidizes C_2 of sorbitol, forming a ketose, fructose. Free NADH is generated in the process.

and actively eliminates then from cells.[23, 24] In addition, fructose produced by SDH acts as energy source for sperm and sorbitol is involved in controlling absorption in renal medulla by regulating the osmosis.[14]

Under hyperglycemic conditions, the excess glucose is removed from the body through increased urine production. This process will lead to dehydration in vivo and cells are forced to maintain their volume by accumulating organic osmolytes, predominantly sorbitol. In addition to the overloaded glycolysis and impaired PPP, this process will also leads to the up-regulation of the polyol pathway under hyperglycemia.[25]

4.2. Up-regulation of sorbitol pathway leads to complications in diabetes

This up-regulation of polyol pathway has been linked to the onset of progressive microvascular complications leading to the retinopathy, nephropathy and neuropathy, observed in a portion of patients with diabetes especially type 1 diabetes.[3] Diabetic retinopathy is a vascular disorder affecting the microvasculature of retina and it was estimated that 75 % of more diabetes patients eventually develop retinopathy.[13] Though the mechanism by which up-regulation of polyol pathway increase hyperglycemia related injuries is not well understood, some of these complications are attributed to the physical accumulation of sorbitol and fructose within the cells, changes is osmosis and oxidative stress resulted from sorbitol and NADH accumulation and NADPH depletion.[3, 4, 11, 13]

Cells, that are living under normal physiological conditions maintains optimum (NAD^+/NADH):($NADP^+$/NADPH) ratio. One negative effect of this up-regulated activity of both AR and SDH enzymes is consumption of NADPH and production of NADH, impairing the regular redox balance of cell.[10, 11, 26] In fact both accumulation of NADP+

and NADH in the cells will leads to a state call "Pseudohypoxia", increasing the oxidative potential of the cells, and therefore increasing the oxidative damage to such important cellular components.[10, 11, 26]

Glutathione in its reduced state is a major form of antioxidant defense and cofactor NADPH is required for regeneration of GSH from its oxidized dimmer (GSSG).[27-29] This reduction of NADPH levels will cause both weakening of the antioxidant defense and increase in the oxidative stress,[26] and this situation is far worse in RBCs where glutathione is the most abundant nonenzymatic antioxidant and G6PD, the only NADPH producer is also impaired due to increased blood glucose levels.[25],[28-30]

These suggestion were further backed up by the extensive evidence provided in the literature suggesting that the inhibition of AR leads to amelioration of diabetic neuropathy ,retinopathy and nephropathy.[4] Bravi *et al.* reported that upon inhibition of AR with inhibitor tolrestat both sorbitol and GSH/GSSG were resoted to near normal value in RBCs obtained from type 2 diabetes individuals.[26] However these reports on reduced NADPH levels due to up-regulated sorbitol pathway seem contradictory.[10]

Unlike glucose, sorbitol does not bypass cell membrane therefore accumulating inside the cells.[11] In-fact there are repots suggesting elevated D-sorbitol levels in lens and peripheral nervous tissues in patients with diabetes suggesting its contribution to diabetic retinopathy and neuropathy.[16]

Nonenzymatic glycation is a post translational modification of protein structure by binding of a sugar such as glucose or fructose with out enzyme involvement and the extent of modification has been found increased in a variety of proteins such as the lens crystalline, the red cell membrane proteins, the peripheral nerve proteins, serum albumin in diabetic patients.[31] In most tissues where polyol pathway is active, the concentration of fructose is similar to that of glucose. In-fact in diabetes subjects, the level of fructose in the lens of an eye may rise 23-fold becoming twice as concentrated as glucose.[31]

Fructose is known to be ten fold higher glycation agent compare to the glucose.[11, 31] This is attributed to the fact that Schiff base formation rate by fructose is about 8-fold faster comparison to the that of glucose.[31] Therefore accumulation of fructose within the cell can leads to glycation of important proteins causing sever damage in comparison to elevated glucose levels hence disrupting regular functions of these proteins and is a major contributor to the diabetic retinopathy.[11, 31]

All these findings collectively suggest that it is important to be able to monitor sorbitol levels in cells to assets the diabetic related damages especially in the cells in which sorbitol pathway is active.

4.3. Monitoring the sorbitol level in RBCs

Since human RBCs are metabolically capable of glucose-sorbitol-fructose conversion (polyol pathway), and RBCs are more prone to oxidative stress compared to other cells RBC sorbitol levels can be use as an indicative of diabetic control.[16, 17] There are reports suggesting that RBC sorbitol level reflect the sorbitol level in lens and sciatic nerve, the longest and widest nerve that supplies nerves to most of lower half of the human body.[32] In addition to that sorbitol levels in RBCs may be useful too in assessing the damages caused to the tissues that are less accessible in comparison to RBCs, in clinical level especially in developing countries where numbers of patients are maximum and resourced and technology is scarce.[33, 34]

In-fact many different analytical techniques are being used to detect the sorbitol levels in RBCs. Among these methods are liquid chromatography and gas-liquid chromatography with refractive index detector, enzymatic techniques coupled to absorption or fluorescence detection schemes.[16, 32]

As shown in figure 4.2, sorbitol dehydrogenase converts sorbitol in to fructose, while reducing non fluorescence NAD^+ to fluorescence NADH. Nicotinamide adenine dinucleotide (NAD^+/NADH), discovered by British biochemists Arthur Harden and William Youndin in 1906 is a dinucleotide, coenzyme /cofactor found in most living cells. NADH is mainly involved in catabolic reaction undergoing oxidation, but also contributes as a substrate/co-factor for certain enzymes.[35]

Both NAD^+ and NADH strongly absorb in the UV region of the spectrum, due to presence of adenine base. (NAD^+ an NADH absorb respectively at 295/339 nm).[5, 36, 37] This difference in the ultraviolet absorption spectra makes them easily distinguishable in a simple absorption measurement couple to an enzyme assay.[36]

Also NADH in solution has an emission peak at 460 nm and a fluorescence lifetime of 0.4 ns, while the oxidized form of the coenzyme does not fluoresce.[37] These changes in fluorescence are also used to measure changes in the redox state of living cells, through fluorescence spectroscopy and microscopy.[36] During this study increase in NADH fluorescence was used to monitor RBC sorbitol levels.

Here we hypothesize that oxidative stress induced G6PD inhibition, coupled with elevation in polyol pathway activity will increase the oxidative stress on diabetes RBCs even further. There are reports suggesting that sorbitol activity increase with increasing blood glucose levels.[38] Also there are reports suggesting that G6PD activity is inhibited under hyperglycemic conditions.[25] But in RBCs G6PD is the only producer of NADPH. Therefore investigation of the effect of G6PD inhibition on sorbitol pathway activity was conducted and also the effect of metal activated C-peptide on sorbitol levels of regular RBCs and G6PD inhibited RBCs were also investigated.[39]

4.4 Experimental

Rabbit and Human RBCs were collected and prepared using the same techniques applied in chapter two.

4.4.1 Reagents

Reagents were prepared in DDW or PSS unless otherwise noted.

A Sorbitol stock solution 0.1 M Sorbitol was prepared by dissolving D-sorbitol (0.18217 g) in DDW up to 10.00 mL. Then 10.0 μL of this solution was diluted to make 10.00 mL of 100 μM sorbitol solution.

A 150 Units/ mL sorbitol dehydrogenase enzyme solution was prepared by diluting the content of the vial in an appropriate volume of cold DDW. Stock solution was divided in to aliquots of 0.133 mL and frozen till the day of use.

A 0.03 M $ZnSO_4$ solution was prepared by dissolving $ZnSO_4.7H_2O$ (0.2628 g) up to 5.00 mL with DDW.

Then a 10.00 mL solution of 0.475 M NaOH was prepared by dissolving NaOH pellets (0.19 g) in DDW.

A 100.00 mL solution of 0.15 M TRIS-HCl buffer (pH=8.6) containing 4.5 mM ethylenediaminetetraacetic acid (EDTA) was prepared by dissolving TRIS (1.882 g) and

EDTA (0.1675 g) in DDW and adjusting pH up to 8.6 with 2 M HCl and diluting up to mark with DDW. Then NAD^+ (0.0199 g) and 0.133 mL of 150 Units/ mL sorbitol dehydrogenase enzyme was diluted up to 10.00 mL using the above mentioned TRIS-HCl-EDTA buffer to prepare a sorbitol dehydrogenase reaction mixture ($SDH_{re\text{-}mix.}$).

Low glucose (55 μM) PSS was prepared by using the same procedure described in chapter 2, by replacing 0.5 g of glucose with 0.005 g of glucose. Then 10.00 mL of each 5.5, 7.5, 8.5 and 10.5 mM glucose PSS solutions were prepared by dissolving appropriated weight of glucose in PSS.

4.4.2 Instrumentation

All the fluorescence measurements were made using Horiba-jobinyvon Fluromax-4 spectrofluorometer.

4.4.3 Detection of sorbitol levels in red blood cells upon G6PD enzyme inhibition and C-peptide treatment

5.0 mL of 14% hematocrit RBCs in 200 μM DHEA was prepared by diluting appropriate volume of RBCs in a PSS containing DHEA. This mixture was incubated for 30 min. Meanwhile in a second microcentrifuge tube C-peptide (600 μL) was mixed with the Zn^{2+} (490 μL) and content was diluted up to 2.50 mL with PSS. At the end of 30 min. incubation period 2.50 mL of the 14 % RBCs in 200 μM DHEA solution was transferred to the second centrifuge tube containing metal activated C-peptide to make a 5.00 mL of 7% G6PD inhibited RBCs in metal activated C-peptide. Remaining 2.50 mL of the 14% RBCs were diluted up to 5.00 mL with PSS to make control G6PD inhibited

RBCs. Control RBC sample, RBCs in 1 nM Zn were prepared in a similar manner. Then the samples were allowed to incubate for 6hr. at room temperature.

At the end of incubation period, RBCs were lysed by diluting up to 7.00 mL with DDW then the proteins were precipitated by adding 1.00 mL of 0.475 M NaOH followed by 1.00 mL of 0.3 M $ZnSO_4$. Then the content of the tube was thoroughly mixed by vortexing and was centrifuged for 10 min. at ×2000 g. Then 1.00 mL of the resulted supernatant was placed in a vial and then 500 μL of SDH $_{\text{re-mix}}$ was added to it and mixed thoroughly and was incubated at 37 °C for 30 minutes. Then the resulted fluorescence for NADH was measured (Ex 366 nm, Em 474 nm).

4.4.4 Effect of elevated glucose levels on polyol pathway

7 % RBC sample in PSS containing X mM glucose was prepared by diluting appropriate volume of RBCs in corresponding PSS solution. (X= 5.5, 7.5, 8.5 and 10.5) Contents were incubated at room temperature for 5 hrs. Then the cellular sorbitol levels were measured using the same procedure described above.

4.5 Results and discussion

In order to monitor sorbitol levels in RBCs, method described by Shinohara *at el* was occupied due to the convenience of the method in comparison to the available other method such as chromatographic techniques.[33, 34] In this method sorbitol accumulated within the cell was allowed to react with sorbitol dehydrogenase enzyme in the presence of exogenously introduced NDA^+ ensuring the occurrence of the forward reaction. (Figure 4.2)

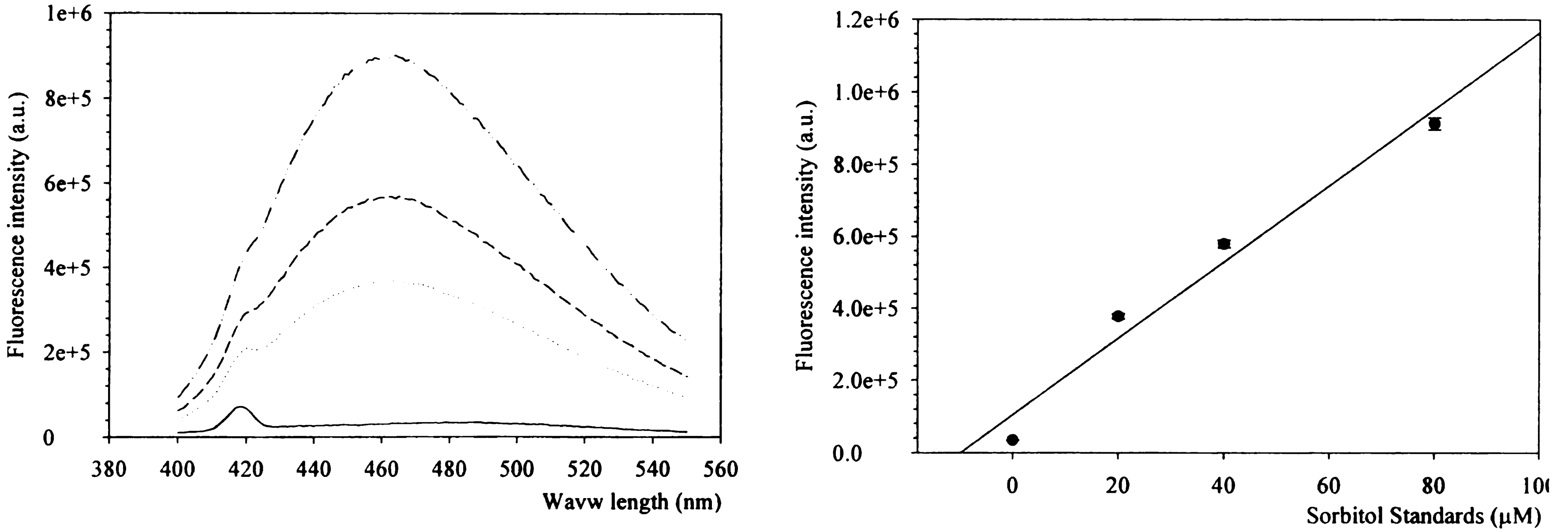


Figure 4.3: Fluorescent measurement for sorbitol standards. (a) Fluorescence intensity values for sorbitol standards 0, 20, 40 and 80 μM. (b) Quantitative data for the same set of standards. Y= 10605.6 x + 10457.6 and $r^2 = 0.97$. ($\lambda_{max} = 474$ nm)

Fluorescence intensity changes in sorbitol standards 0-80 μM were conducted daily prior to the measurements in for quantification and error elimination purposes. As expected linear increase in fluorescence intensity ((r^2 = 0.97) with increasing sorbitol concentration was observed (Figure 4.3). However, any conclusive data on sorbitol levels upon G6PD enzyme inhibition was not observed. In-fact these studies were conducted on RBC samples obtained from 14 different rabbits, and it appears that sorbitol level increased by 18.5 % upon G6PD inhibition in 6 samples and it decreased by 25.3 % in 8 samples (Figure 4.4). Despite the C-peptide's ability to increase glucose uptake to the RBCs[39], similar trend was seen with the RBCs treated with metal activated C-peptide treated RBCs. Here normalized sorbitol was increases by 47 % (0.58 ± 0.01) and was decreased by 17 % (1.22 ± 0.08).

In order to further clarify the method used here is indeed precise RBCs were spiked with sorbitol standards and resulted fluorescence intensity was measured. Values obtained from this method were slightly greater than the sorbitol standards alone, nonetheless not significantly different ($p<0.005$). Successful measurement of sorbitol levels in type 1 diabetes sample was performed in an attempt to further verify the method used here indeed accurate and executed with out any experimental errors (Figure 4.5).

As the next step change in sorbitol level in RBCs incubated in PSS containing varying levels of glucose was studied. Here RBCs were incubated with PSS containing 5.5, 7.5, 8.5 and 10.5 mM glucose (See figure 4.6) with the expectation of observing increase in sorbitol levels with increasing glucose concentration. However, very interesting observation was made upon analysis of the data acquired. As shown in figure 4.6 normalized fluorescence intensity corresponding to the NADH levels in the RBCs,

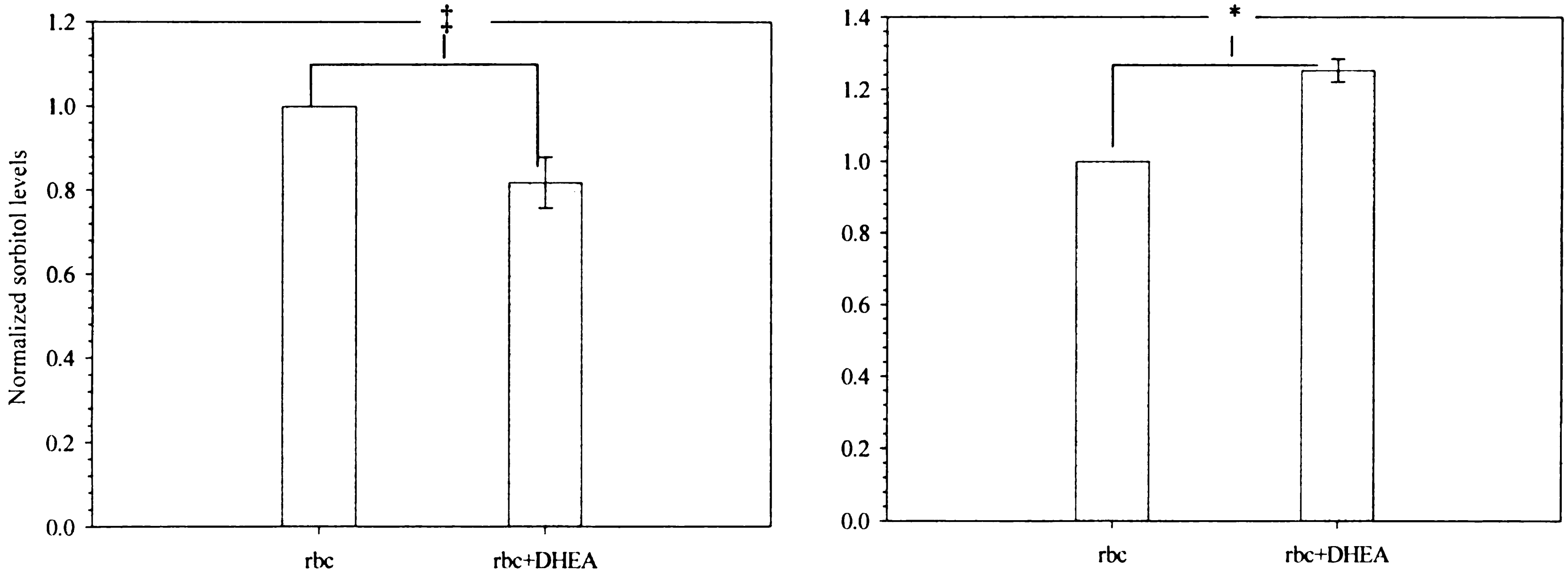


Figure 4.4: Normalized sorbitol level in G6PD inhibited (by DHEA) RBCs. (a) Normalized sorbitol decreased by 18.5 % when G6PD was inhibited. n= 8 (b) Normalized sorbitol level increased by 25.2 % upon G6PD inhibition. n= 6. Date represent mean ± SE. rabbits and * represent the values that are significantly different ($p<0.001$) whereas + represent ($p<0.005$).

hence normalizes RBC sorbitol level was decreased from 1.00 ± 0.0 to 0.87 ± 0.03, when glucose concentration was increased from 5.5 mM (regular blood glucose value) to 7.5 mM. Then when the concentration of glucose was further increased up to 10.5 mM normalized fluorescence intensity increased up to 1.08 ±0.05. These values were in fact significantly different ($p<0.005$) from each other indicating that with increase in blood glucose level, sorbitol levels show a slight decrease followed by increase larger than original value.

This observed trend is similar to the change in glutathione levels in rabbit RBCs upon oxidant insult from diamide, a known oxidant.[27, 30] Also similar trend was observed with NO levels derived from vascular endothelial cells upon stimulation with ATP in a different study conducted in our laboratory.

Previously more than one group has reported that upon incubation of RBCs in high glucose, increase in sorbitol levels were observed.[33, 34] At a glance this results seems to contradict the results observed during this study. But upon through investigation it was clear most of these studies were conducted at glucose levels that are higher than 11.5 mM up to 27.8 mM which are two to tree folds increase in comparison to blood glucose in sever diabetise.[33, 34] But our study was conducted in range of 5.5 mM to 10.5 mM. In-fact during this study, sorbitol level in RBCs incubated in 17.5 mM glucose was measured by us and increase in normalizes sorbitol levels were observed. There for it is possible that these previous studies were conducted overlooking the possible decrease in sorbitol levels upon slight increase in blood glucose. However further through investigation upon this observation is required prior to apply these results in any attempts to evaluate changes in sorbitol pathway activity in diabetes.

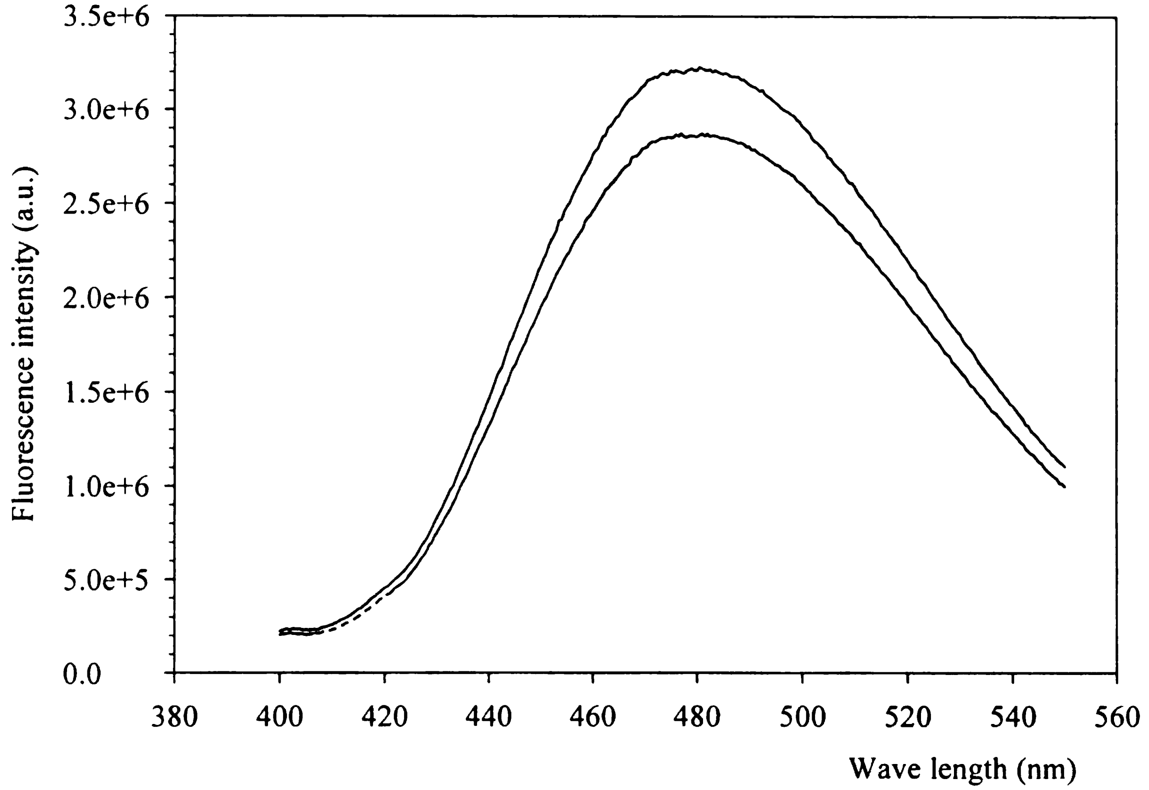


Figure 4. 5: Sorbitol levels in type 1 diabetes and control RBCs. Upper trace represent the Type 1 diabetes RBCs and lower trace represent the control RBCs. Two values are significantly different ($p < 0.005$).

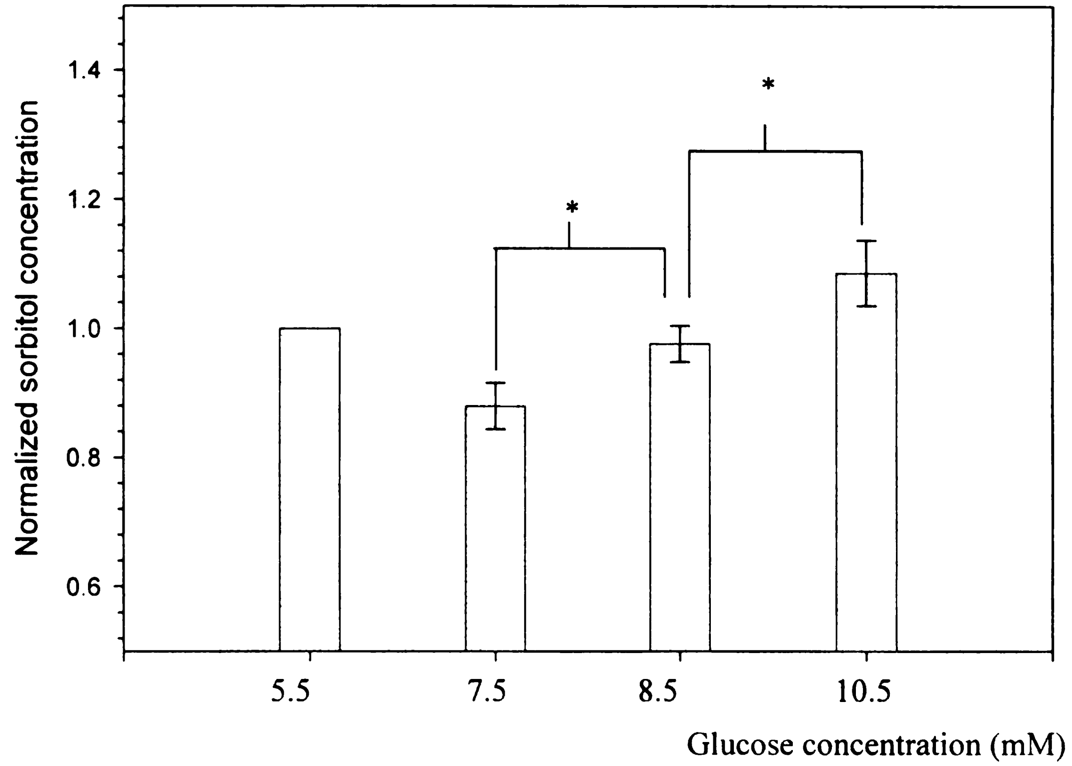


Figure 4.6: Normalized fluorescence intensity of RBCs incubated in PSS containing increasing amounts of glucose. Normalized fluorescence intensity decrease from 1.00 ± 0.0 to 0.87 ± 0.03 when glucose concentration increase from 5.5 mM to 7.5 mM. However fluorescence intensity increased up to 1.08 ±0.05 when glucose concentration was increased up to 10.5 mM. Date represent mean ± SE. n= 4 rabbits and * represent the values that are significantly different ($p<0.005$)

4.6 Conclusions

Effects of G6PD inhibition on cellular sorbitol levels in rabbit RBCs were some what inconclusive. In-fact upon G6PD inhibition sorbitol levels were increased in 43 % of the samples and it was deceased in other 57 %. During this study sorbitol dehydrogenase based NADH fluorescence method was occupied for the detection of cellular sorbitol.[33, 34] Though this method is widely used technique, sorbitol standards and sorbitol levels in type 1 human diabetes RBCs were measured in order to eliminate the experimental errors associated and further verification of the method. Very conclusive, expected increase in sorbitol levels were observed with both standards and human type 1 RBCs. Similar results were observed with RBCs incubated with metal activated C-peptide. Therefore it is necessary to further investigate on this aspect.

Despite the common beliefe that hyperglycemia increase the cellular sorbitol levels, interesting observation was made with the RBCs incubated with increasing glucose concentration PSS. When glucose levels were changed from 5.5 mM to 7.5 mM, decrease in sorbitol levels were observed. But when changing from 7.5 mM up to 17.5 mM increase in sorbitol level is increased. This observed trend coincide with the changes in cellular glutathione levels upon introduction of diamide[30] and also change in platelet derived nitric oxide levels upon addition of ATP treatment.

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Chapter Five

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

Although the etiology of the disease is not well defined, diabetic mellitus can be defined as a disordered metabolism followed by hyperglycemia resulting from either insufficient secretion of endogenous insulin or resistance of the target tissues to the insulin action, triggered by autoimmune response, viral infection or genetic cause.[1,2] Based on the pathogenesis of the disease, diabetes is subdivided in to various types, with type 1 and 2 being the most commonly occurring types. While exogenously administrated insulin and other currently approved medications address some symptoms and complications of the disease, numerous other complications affecting cardiovascular system, nerves or kidney and lens and retina of an eye are yet to be understood.[3,4]

In type 2 diabetes, RBCs are under high oxidative stress and are believed to be less deformable, leading to lowered levels of deformation-induced ATP release.[5-7] In an attempt to understand simultaneous changes in G6PD activity and deformation induced RBC derived ATP release, as NADPH measurement scheme was successfully developed and extended to a continuous flow based system, which is an essential element in the microcirculation.[7] As expected, cellular NADPH levels decreased by 39 % upon inhibition of G6PD signifying its importance in antioxidant defense.[7]

When subjected to oxidative stress, if unprotected, oxidation of important membrane proteins will lead to a stiffening of the cell membrane.[6,8,9] This effect is pronounced in RBCs where G6PD is the sole producer of NADPH, an essential cofactor in the antioxidant defense mechanism.[9-12] In type 2 diabetes, RBCs are under high oxidative stress and are believed to be less deformable, leading to the lowered levels of deformation-induced ATP release.[6,7] Also G6PD activity is an indication of the cell age and upon aging, cell membranes are believed to become more stiffened.[7,13]

Here cellular NADPH levels in the RBC (via a fluorescence-based assay) and deformation-derived ATP release from the RBC (via chemiluminescence assay) were measured simultaneously, under continuous flow conditions.[7] Results were consistent with the expected pattern indicating a direct relationship between NADPH concentrations in the RBC and deformation-derived ATP release from these cells.[7] With a decrease in NADPH concentration, there is a decrease in deformation-derived ATP release from the RBC and vice versa (Figure 5.1).[7]

A similar trend was observed with chemically aged (via inhibition of G6PD) RBCs, as well as young and old fractions of RBCs separated from whole blood.[7] RBCs obtained from people with type 2 diabetes showed the same trend confirming that oxidative stress leads to a decrease in deformation-derived ATP release.[6,7] Due to the ability of ATP to stimulate NO production in endothelial cells and even platelets, the RBC thus becomes a potential determinant of blood flow in the diabetic circulation. Perhaps more evident from the work reported here, however, is the potential use of NADPH and ATP as biomarkers of oxidative stress in the RBC.[7]

Since its discovery in 1967 proinsulin C-peptide was thought to be devoid of any biological activity up until late 90s'. [14] However, with the recent developments in

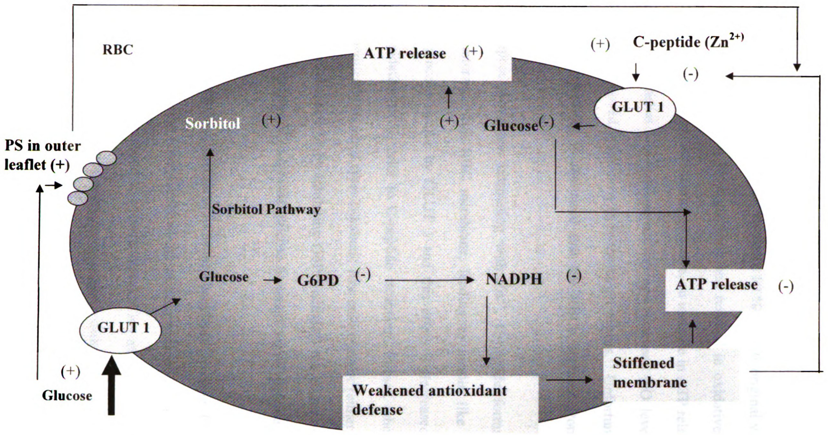

Figure 5. 1: Schematic representation of the result summary. (+) indicated the increase in concentration or pathway activity where as (-) indicates decrease

research involving C-peptide, new light has been shed on its possible biological role.[15] In-fact, proinsulin C-peptide, when activated with transition metals such as Zn^{2+}, increases the RBC derived ATP release by about 50 % over its original value. However, when RBCs were subjected to hyperglycemia and resulted in oxidative stress, the C-peptide effect was decreased. As mentioned above, an increase in ATP release can lead to an increase in endothelial NO synthase activity and increased NO levels resulting in vasodilation and improved blood flow in to the target organs.[16] Unfortunately, our data suggests C-peptide activity is decreased that the RBCs obtained from people with diabetes.

We propose that upon interacting with Zn^{2+}, C-peptide forms a secondary structure that interacts with RBC membrane, leading to insulin like effect by up-regulating the glucose uptake by GLUT 1 and this effect is decreased if RBCs are subjected to oxidative stress prior to C-peptide treatment. In-fact, when RBCs were subjected to chemically induced type 2 diabetic like environment (either by incubating with high glucose, diamide or by inhibiting G6PD activity) the effect of C-peptide on RBC derived ATP release, ^{14}C labeled glucose transport and C-peptide binding to the RBCs were decreased.

It is known that hyperglycemia induces the phosphatidyleserine (PS) translocation from the inner leaflet to the outer leaflet of the cell membrane creating a net negative charge in the cells that are exposed to hyperglycemia. This amount of PS present in the cell membrane was detected using annexin V (tagged with FITC) binding to the PS present in outer membrane.[17] It appears this overall charge alteration diffuses the interaction between proinsulin C-peptide and RBC membrane. Upon incubation of these cells with prescription metformin, the one of the two drugs approved by the world health organization (glybacamdie is the other one) for diabetes, binding of annexin V to membrane was reduced indicating its ability to masks the charge repulsion between C-peptide and RBC membrane.

Therefore, collectively it can be stated that C-peptide is indeed bioactive when interact with metal ions such as Zn^{2+}, and the method which it exert its bioactivity seems to be via some membrane based interaction and this effect seems to diminish when cells were under oxidative stress. Use of prescription drug metformin in combination with metal activated C-peptide replacement may improve C-peptides ability to ameliorate complications in diabetes therefore acting as a "snsitizer" for C-peptide.

However, results observed here with the metformin is rather indirect. Though annexin binding to PS is a specific interaction and increase in ATP release was observed with the combined C-peptide and annexin treatment, direct evidence for such interaction between C-peptide, metformin and annexin is needed.

It is well know that the polyol pathway is up-regulated in diabetes individuals leading to the accumulation of glucose-alcohol sorbitol in cells.[18] These elevated sorbitol levels were capable of triggering series of biological complications leading to retinopathy, neuropathy and nephropathy in diabetes.[18] However, for sorbitol pathway activation, a continuous supply of NADPH is required. In the RBCs, G6PD is the sole producer of NADPH and in the case of diabetes RBCs, G6PD activity is inhibited, resulting in lowered NADPH levels. Here, the effects of down-regulated G6PD activity on RBC sorbitol levels were evaluated.

Effects of the G6PD inhibition on cellular sorbitol levels in rabbit RBCs were some what inconclusive. In-fact, upon G6PD inhibition sorbitol levels were increased in 43 % of the samples analyzed and it was deceased in other 57 %. During this study sorbitol dehydrogenase based NADH fluorescence method was employed for the detection of cellular sorbitol.[19,20] Though this method is a widely used technique, sorbitol standards and sorbitol levels in human type 1 diabetes RBCs were measured in order to eliminate the experimental errors associated and further verification of the method and expected trend was observed with the both standards and the human type 1 RBCs. However, ambiguity was observed with both G6PD inhibited RBCs and RBCs incubated with metal activated C-peptide providing inconclusive results. However, since sorbitol is a food additive, dietary intake can affect the overall assay results. Therefore it is necessary to further investigate this aspect.[20]

Despite the common beliefe that hyperglycemia increases the cellular sorbitol levels, an interesting observation was made with the RBCs incubated with increasing glucose concentration PSS. When glucose levels were changed from 5.5 mM to 7.5 mM, a decrease in sorbitol levels were observed. But when changing from 7.5 mM up to 17.5 mM increase in sorbitol level is increased. This observed trend coincide with the changes in cellular glutathione levels upon introduction of diamide[6] and also change in platelet derived nitric oxide levels upon addition of ATP treatment.

Finally we can conclude this study by stating that hyperglycemia and oxidative stress induced by hyperglycemia caused both structural and physiological changes in RBCs. Thereby monitoring changes in various metabolites enables the predictions, early detection and treatment in diabetes mellitus.

5.2 Future directions

Ultimate goal of the all the above studies conducted are to use the knowledge of behavior of the various metabolites in the RBCs in prediction, diagnosis and treatment of diabetes and other cardiovascular complications. In-fact, detecting multiple components from a single RBC sample, with out the requirement of sample separation within a flow-based system will enable improved in vitro determinations of drug efficacy and cellular response to administered drugs.

The first step towards such measurement scheme was taken in above described work with development of dual detection scheme. Therefore, incorporation of measurement schemes of metabolites such as ATP, NADPH, NADH and also antioxidant GSH[21] in to a single measurement scheme on a PDMS (polydimethylsiloxane) microchip will be an interesting future work. This incorporation of microfluidic chip will proved the ability to better mimic the in vivo circulation, while monitoring in real time, changes in multiple metabolites present within the RBC.

Multiple sclerosis (MS) is an autoimmune disease in which the immune system attacks the central nervous system leading to the destruction of myelin sheaths (demyelination). Myelin sheaths are cells that wrap around the axon of the neuron, electrically insulating and protecting axon (Figure 5.2 and 5.3). There are reports indicating that demyelination in MS was caused by increased NO levels. Also, there are reports indicating that administration of steroids such as estrogens ameliorate the pain episodes in MS patients.

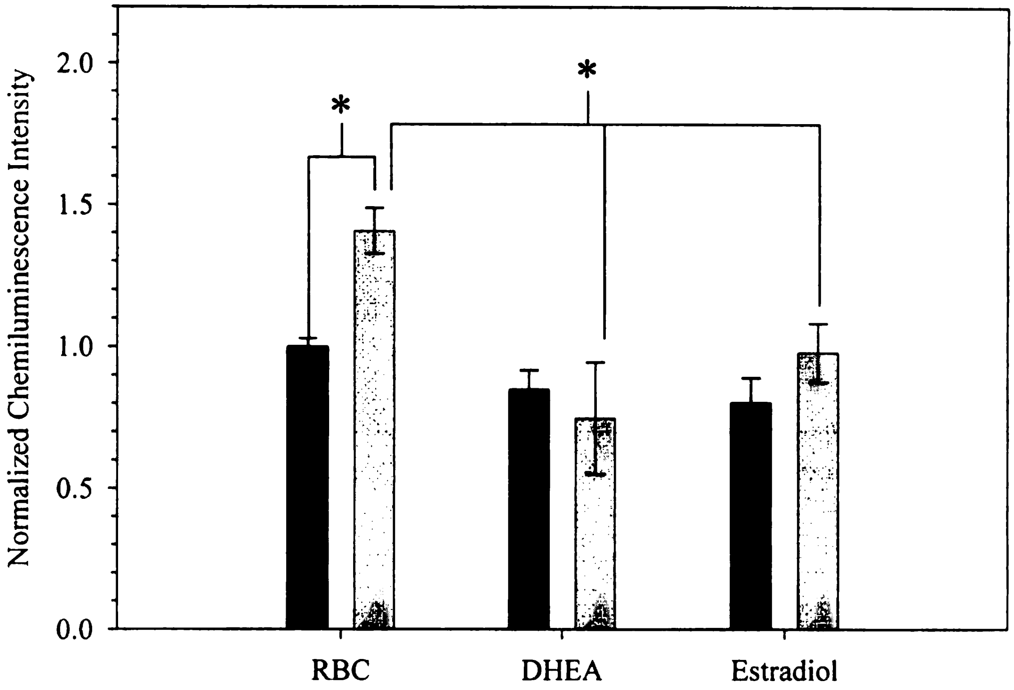

Figure 5. 2: Effect of estradiol on ATP release from RBCs. Upon incubation with estradiol ATP release from RBCs decrease even in the presence of ATP stimuli C-peptide (activated with Zn^{2+}). Black bars represent RBCs with out C-peptide and gray bars represent gray bars with C-peptide. n=5 rabbits and ‡ represent values that are significantly dirrerent ($p < 0.001$).

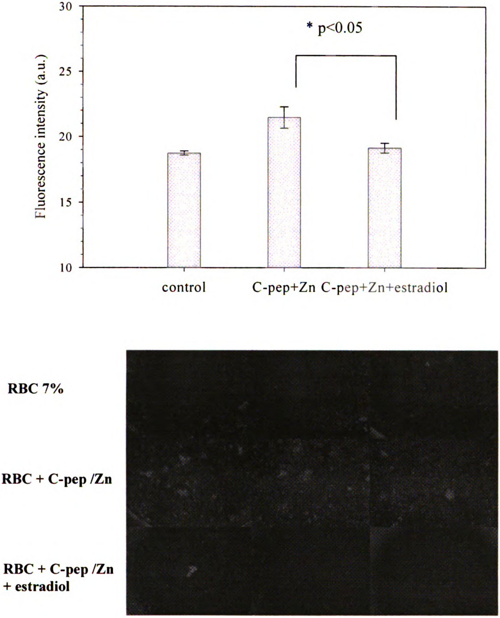

Figure 5. 3: Effect of estradiol on RBC derived ATP induced NO production from bPAEcells. (a) Qualitative representation and qualitative representation (b) of NO formed in bPAEC when stimulated with ATP released from RBCs incubated in estradiol in the presence and absence of C-peptide using method and the device described by Genes *et al.*[22] NO production decreased when cells are treated with estradiol even in the presence of C-peptide. n = 1 rabbit. Represent values significantly different ($p < 0.05$)

In a separate study conducted in our group it was discovered that RBCs of MS patients shows increased levels of ATP release upon mechanical deformation. In a closer infection, it was very clear that estrogens are structural analogs of DHEA. Therefore it is possible that these estrogens exerts DHEA like effect on RBCs hence lowering the amounts of ATP release which will leads to decrease levels on NO production ameliorating the autoimmune destruction and pain relapses in MS.

In fact, preliminary studies on effect of estradiol (a component of estrogen) on ATP release from RBCs were conducted. Here RBCs and RBCs treated with metal activated (Zn^{2+}) C-peptide was treated with estradiol. C-peptide was used as an ATP stimulator and RBCs incubated in DHEA was used as a control experiment. Both ATP release from RBCs[7] and NO[22] release from bovine pulmonary artery endothelial cells stimulated with RBC derived ATP was measured (Figure 5.1). Interestingly, decrease in both ATP release and NO was observed supporting our hypothesis. Therefore investigation of effects of DHEA, G6PD activity and effect of estrogen on G6PD activity and RBC derived ATP release will be an interesting future study that can be conducted using the duel detection system developed here.

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