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MADUSHI UPENDRIKA RATHTHAGALA

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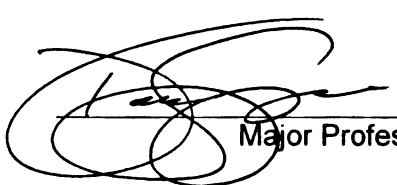

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DEFINING A NOVEL ROLE OF HYDROXYUREA ON ERYTHROCYTES

By

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ABSTRACT

DEFINING A NOVEL MECHANISM OF HYDROXYUREA ON ERYTHROCYTES

By

Madushi Upendrika Raththagala

Recent findings implicate the red blood cell (RBC) as a determinant in physiological events that regulate the blood flow in the circulatory system. It is currently known that circulating RBCs contain millimolar amounts of adenosine tri phosphate (ATP) and release a fraction of that ATP under certain physiological stimuli. It is hypothesized that oxidative stress and certain drug metabolites can affect the ability of the RBC to release ATP and thereby have an effect on pathophysiological conditions in disease status.

A novel fluorescence based assay using monochlorobimane (MCB) in conjunction with the standard addition method was developed to quantitatively determine glutathione (GSH) levels in RBCs without any separation of GSH from the complex biological matrix. The average GSH concentration of a 1% RBC solution was 0.042 ± 0.002 mM for 6 rabbits, which translates to a cellular concentration of 362 ± 20 amol/RBC. The ability to perform dynamic measurements provides a means to evaluate the effect of oxidant stressors on RBCs. GSH concentrations were determined in RBCs subjected to the specific oxidant, diamide, at 5 minute time intervals for up to 45 minutes. The results obtained from this study greatly contributed to the establishment of the relationship between oxidant stress, a weakened antioxidant system and RBC derived ATP release. The importance and necessity

of such a method is in developing microfluidic-based diagnostic tools, metabolomics studies, and systems biology applications.

Subsequently, a novel micro flow based chemiluminescence technique was employed to quantify and determine the effect of hydroxyurea, the currently approved therapy for sickle cell disease (SCD), on ATP release from RBCs. The release of ATP from RBCs increased from 0.350 ± 0.063 to 0.591 ± 0.105 μmol/l for n = 8 rabbits when these RBCs were incubated for 20 min with 50 μM hydroxyurea. This observation was further verified using varying concentrations of hydroxyurea (25-200 μM) and the highest ATP release was observed when a 7% hematoctrit of RBCs incubated with 100 μM hydroxyurea. Interestingly, the same trend of ATP release from RBCs was observed for a 7% hematoctrit of RBCs incubated in varying concentrations of spermine NONOate (125-1000 nM) for 10 minutes. Further experimentation involved in the identification of the NO source as well as defining the mechanism that exerts modulation of RBC derived ATP release in the presence of hydroxyurea. It was determined that hydroxyurea was resulting in an increased flux of calcium into the RBCs. These results suggested that the observed modulation of ATP release from RBCs in the presence of hydroxyurea is essentially a NO mediated effect. It is believed that NO produced within the RBC via NOS enzyme modulates the RBC deformability, thereby facilitating the modulation of ATP release from RBCs, accordingly. Finally, the development and utilization of novel analytical techniques enabled the investigation of two currently interesting biochemical pathways in RBCs; the effects of oxidant stressors and NO on RBC derived ATP release, which could ultimately be useful in developing therapies that benefit the vascular complications associated with diabetes mellitus and sickle cell disease.

~ To my parents, Ruchira, and Netuli, with love

ACKNOWLEDGEMENTS

Finishing up this dissertation, memories of experiences I have had the over the past four and half years flashes across my mind. I never imagined being on this path, but today I'm grateful to those who inspired and directed me towards higher education. Without them, I would not have achieved anything at all.

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CHAPTER 1

Red blood cell, structure, function and clinical implications

1.1 Red blood cell characteristics

Erythrocytes, commonly referred to as red blood cells (RBCs), are the most common type of blood cell in a vertebrate's vascular system.[1] RBCs are highly specialized cells specifically designed for the purpose of oxygen transport from the environment to the respiratory tissues in the body.

Human RBCs are small, biconcave disc shaped cells. The average mean volume of RBC is ~ 82-95 fl while the diameter ranges from 6-8 μm.[2, 4] Human RBCs occupy 45% of the total blood volume and carry 4.5-6.5 x 10^6 of RBCs in 1 μl of blood.[5] These cells are much smaller than the average cell in the body, although the biconcave shape results in the highest possible area for a given volume allowing for a shorter diffusion path for oxygen. Moreover, mature human RBCs lack nuclei and organelles such as mitochondria and golgi bodies and therefore are incapable of synthesizing their own proteins, lipids and nucleic acids.[6] All of these modifications are essentially deigned to provide enough space for the oxygen carrying protein, hemoglobin and an average RBC contains about 270,000 million hemoglobin molecules or 28-34 g of hemoglobin per dl of RBCs. In this respect, RBCs have often been considered (and oversimplified) as a "bag of hemoglobin" designated only for oxygen transport throughout the body.

In reality, RBCs contribute to many important steps in regulatory mechanisms of cellular functions other than their major role of oxygen transport in the body. They do carry important enzymes and substrates for several metabolic pathways and antioxidant defense mechanisms.[7] These processes are essential for RBC survival, as well as for the maintenance of the mechanical properties of the RBC. Indeed, the human RBC travels through the circulatory system about 500,000 times during its life time of ~120 days.[8] Therefore, the RBC membrane is also specially designed to withstand the mechanical stress placed on it within the circulation. Maintenance of RBC deformability is necessary for its proper functioning and is dependant on essentially three factors; internal viscosity, the geometry of the cell and the RBC membrane deformability.[9]

1.2 RBC membrane structure and deformability

The RBC membrane consists of a lipid bilayer and an underlying skeletal protein network (cytoskeleton). The skeletal network of the RBC membrane is a dense, filamentous hexagonal shaped protein network, directly laminating the inner leaflet of the lipid bilayer via the interaction of integral proteins present in the bilayer.[10, 11] The major component of the cytoskeleton is spectrin, which is a rod like protein composed of two non identical subunits of 240 kD (α sub unit) and 220 kD (β sub unit) intertwining side by side.[12, 13] The α and β subunits attach side to side in an anti parallel manner forming the heterodimer. The tetrameric structure formed by these two heterodimeric proteins is the predominant form in the cytoskeleton and attaches to several other proteins to form the skeletal protein network. Spectrin predominantly interacts with actin proteins

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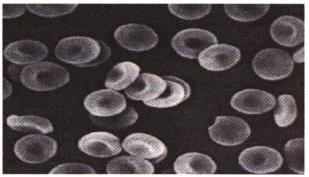

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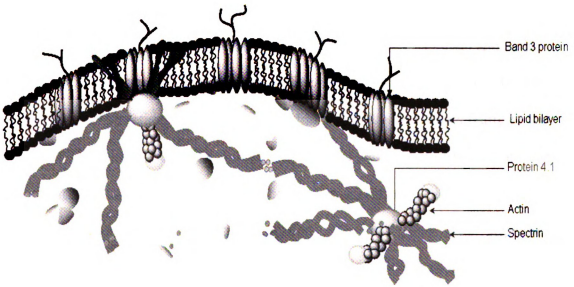

Figure 1.1 Figure illustrating the size and the shape of human erythrocyte and the membrane characteristics.[10] Scanning electron micrograph of human erythrocyte shows the biconcave disc shape cell (a).[14] Schematic representation of the RBC cytoskeleton shows the attachment of the cytoskeletal proteins (b).[15]

creating the spectrin-actin lattice like network underlying the lipid bilayer. Actin is present in oligomers of 12-20 actin filaments, and the ratio of actin to spectrin in the cytoskeleton consists of one actin oligomer to six spectrin tetramers. The attachment of the spectrin-actin lattice to the membrane bilayer is through the protein ankyrin.[16, 17] RBC ankyrin is an asymmetric globular protein of 215 kD that contains binding sites for both spectrin and the anion transporter protein (band 3) in the bilayer. Band 3 is a glycoprotein of ~93,000 bp and is the most abundant protein of the human RBC membrane.[15] It is important in the transport of chloride and bicarbonate across the membrane. In addition to interacting with ankyrin, band 3 has a high affinity for RBC membrane glycolytic enzymes, as well as to hemoglobin molecules in the cytoplasm.[18]

Protein 4.1 in the cytoskeleton helps the tight binding of spectrin and actin, which are otherwise very weak. It is composed of four major domains and has shown intriguing multifunctional structural properties by attaching vertically and horizontally to the lipid bilayer and the skeletal network. Protein 4.1 is also a component of the ternary complex that comprises the junctional complex of the cytoskeleton.[19, 20] In addition, there are several other accessory proteins associated with the cytoskeletal structure and, collectively, all of these molecules provide RBC shape, stability and deformability.

The ability of RBCs to change geometry under the influence of mechanical forces and revert back to their original shape is important for proper functioning of the cell. Therefore, the structure and deformability of the RBC membrane has been the subject of many investigations for many decades due to their importance in blood rheology. The proteins of the cytoskeleton has arranged in a definite way that preserves the geometry of the cell while allowing a temporarly shape change when traveling through the arterioles

and capillaries. Failure to maintain some level of deformability is often associated with rheological complications or in other words microcirculatory disturbances leading to several pathological conditions, such as hemolytic anemia, sickle cell disease, sperocytosis and diabetes mellitus.[9, 21,10, 22] Therefore, a systematic study of deformation and flow, provides insight into some remarkable properties of the RBC that are associated with its own unique structure and pathological consequences.

1.3 Metabolic pathways of RBC

Oxygen transport is a vital process in all living systems. In humans, hemoglobin has specialized functionality and structure to transport oxygen to skeletal muscles.[6] However, RBCs also contain hundreds of other organic and inorganic molecules in the cytoplasm in order to perform different functions for their survival. Some of these functions comprise generation of metabolic energy (ATP) and reducing agents (NADH and NADPH).[23] RBCs need energy to maintain osmotic balance and volume (via the many ATP dependant ion pumps), replenish the adenine nucleotide pool, and maintain membrane deformability and to protect cells through anti oxidant defense mechanisms.[2, 7, 8] All of these functions require the existence of several metabolic pathways including glycolysis, the pentose phosphate pathway (PPP), the 2,3, diphosphoglycerate shunt and nucleotide metabolic pathways.[23]

Among them, glycolysis is the predominant metabolic pathway present in the RBC for producing ATP. Glucose is the main energy source or the starting point of the pathway and it produces 2 ATP and 2 NADH molecules per glucose via anaerobic

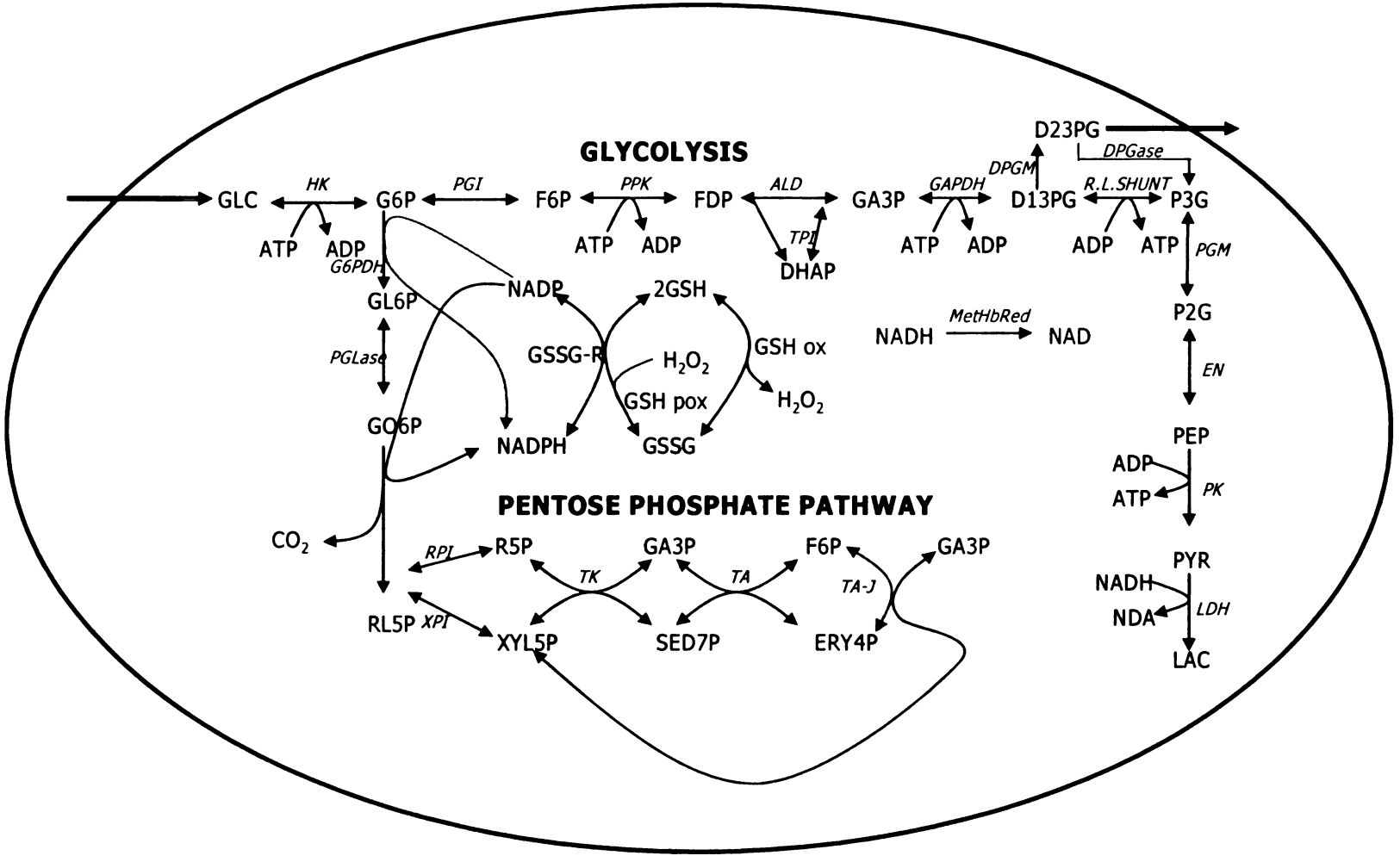

Figure 1.2 Schematic representations of the important metabolic pathways in RBCs. Glycolysis, pentose phosphate pathway, and the GSH regeneration pathways are interconnected and dependent upon NADPH and ATP production.[2]

glycolysis. The lack of mitochondria in the RBC prevents glucose from being oxidized aerobically through the citric acid cycle; hence the final products of glycolysis are lactate, ATP and NADH. The PPP contributes to the redox status of the cell by producing 2 NADPH molecules per glucose molecule.[24] Glucose is converted to glucose-6-phosphate by glucose-6-phosphate dehydrogenase (G6PD), a key regulatory enzyme in the RBC PPP. The major function of the PPP is to produce ribose sugar for nucleotides and to maintain the continuous supply of NADPH.[2] NADPH is used as an obligatory substrate for glutathione maintenance and the PPP is the only way of producing NADPH in RBCs. One molecule of NADPH is produced by reducing a $NADP^+$ and is used as a hydrogen donor molecule for the reducing reaction of glutathione reductase. The activity of the GSH redox cycle is dependant upon the activity of G6PD, and healthy cells maintain the reducing environment within the cells by properly maintaining the glutathione antioxidant status and the PPP. It is believed that under normal circumstances only 1/60 of the G6PD activity is utilized, but when the oxidant stress is high than the normal G6PD activity is increased according to the oxidant stress placed on the cells.

Any abnormality of the RBC metabolic pathways may lead to serious disease conditions, as these metabolic functions are interconnected via different steps and conditions in the RBC. However, our current understanding of the relationship between the metabolic deficiencies of RBCs and disease pathogenesis is lacking. Recent research is focused on generating a quantitative overview of metabolism and its regulation through a systems biology approach where all the factors involved for certain problems are evaluated.[25] Therefore, rapid detection methods, simultaneous analysis of different metabolites, and miniaturization are key steps to consider.

1.4 Antioxidant systems of RBCs

Oxidative stress, a pronounced pro-oxidant state, is the increased production of reactive oxygen species or the decrease in the reducing capacity of cellular redox couples (or weakening of the antioxidant systems).[26, 27] The destructive effects of the stress are determined by the extent of the imbalance resulting from the production of reactive species and detoxification ability of the cellular system. Reactive oxygen species, which include free radicals and peroxides, are formed in various cellular reactions. Increased lipid peroxidation, protein oxidation and DNA damage through reactive oxygen species or impairment of antioxidant defense systems in cells have lead to many pathological conditions including diabetes mellitus, atherosclerosis, Parkinson's disease, and Alzheimer's disease.[28-30]

Antioxidants are the cells' foremost supply of protection against various reactive oxygen species that include free radicals and peroxides. Presence of a working antioxidant mechanism is crucial for the protection of enzymes, membrane proteins and lipids from excessive oxidants and free radicals, as well as to maintain hemoglobin in its ferrous form without being oxidized. Erythrocytes are prone to higher oxidative damage due to the exposure of high oxygen content, thereby necessitating maintenance of their structural and functional proteins via an antioxidant defense mechanism.[31] Disturbance of the redox balance by the production of reactive species such as hydroxyl, superoxide, peroxinitrites, alkoxy or peroxyl radicals and peroxides will lead to RBC membrane damage, death or many pathophysiological consequences. Cellular components in RBCs such as lipids, proteins and enzymes can be exposed to reactive species, thus making

them susceptible to oxidative damage.[31] Among them, thiol containing proteins (eg., spectrin) are subjected to oxidation via its cystein thiols.[14] Disulfide bonds can be formed in the presence of oxidants and this could lead to the destruction of important membrane properties. Also, maintaining hemoglobin in the reduced state is essential for gas exchange and transport. Some hemoglobin molecules also attach to the cell membrane, hence, is important to maintain RBC mechanical properties. Therefore, it is a prerequisite to have an antioxidant system in cells, especially in RBCs.

The most abundant non-enzymatic antioxidant found in the RBC is the reduced form of glutathione (GSH).[32] This tri-peptide thiol (γ-L-glutamyl-L-cysteinyl glycine) acts as a major bio reducing agent via its high electron donating capacity linked to the sulfhydryl (-SH) group. GSH is present in millimolar concentrations in most mammalian cells, which makes it the most concentrated intracellular antioxidant in the RBC.[33, 34] GSH exists in two forms, reduced (GSH) and oxidized (GSSG). In healthy cells, the GSSG:GSH ratio is 1/10, however, modification of this physiological ratio is often an indicator of a disease state that may result in various pathological events. The proper GSSG:GSH ratio is maintained through tight regulation of GSH synthesis and the GSH redox cycle. Glutathione reductase converts oxidized glutathione back to its reduced form at the expense of NADPH produced via the PPP. Another GSH redox cycle enzyme, glutathione peroxidase, is also important in removing hydrogen peroxide from cells. It reduces H_2O_2 to H_2O while oxidizing GSH to GSSG. G6PD is an important redox cycle enzyme, as it is important for the activity of glutathione reductase by supplying the NADPH pool. The combined effects of GSH redox cycle enzymes maintain the reducing

environment within the cell, and thereby protect RBC characteristics within its activity of 120 days.

1.5 Novel roles of RBCs in the vascular system

1.5.1 ATP as a signaling molecule

It has become increasingly clear that, in addition to functioning as an oxygen transporter in the body, RBCs have other roles that can be considered important steps in the regulation of many cellular functions. One such role is its ability to release adenosine triphosphate (ATP), which has been identified as a potential regulator of vascular resistance or vessel diameter.[6, 21, 35, 36] ATP, which is released from the erythrocyte into the circulation, can bind to P_2Y purinergic receptors present in the vascular endothelial cells resulting in the synthesis and release of nitric oxide (NO).[37-39] NO synthesized in endothelial cells can be released both abluminally and luminally. When released abluminally, NO can interact with the vascular smooth muscle cells, stimulating vascular relaxation, and thereby an increase in vascular caliber.[38]

The relationship between NO synthesis in the vascular endothelium in response to RBC-derived ATP has not been established until 1995 when Sprague *et al.* reported on the role of the RBC as a determinant in vascular resistance.[40] It is generally accepted that increased shear stress is the major stimulus for endothelial NO production.[41] However, Sprague *et al.* showed that alterations in the shear stress did not stimulate NO synthesis in isolated perfused rabbit lungs in the absence of rabbit RBCs.[40] In other words, they concluded that the RBC is a determinant of endothelial derived NO synthesis.

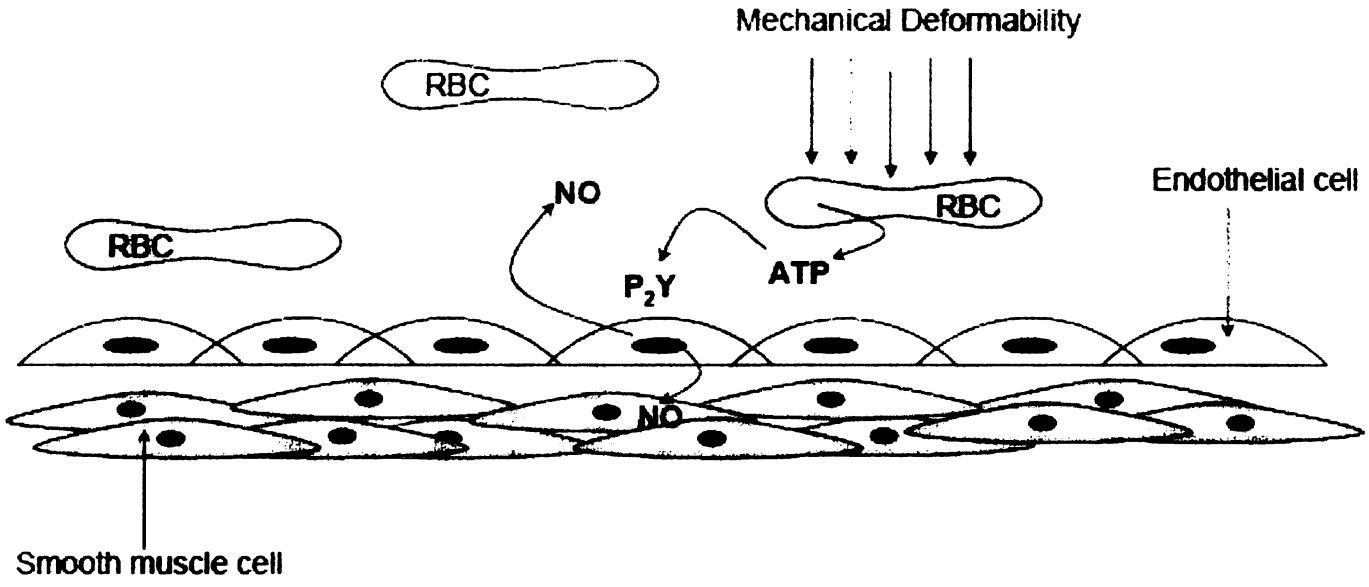

Figure 1.3 Schematic representation of the effect of ATP released from RBCs on the regulation of vascular caliber. The released ATP in response to mechanical deformation of RBCs can stimulate the NO synthesis in the endothelial cell. Next, the NO can diffuse into underlying smooth muscle layer, thereby triggering the smooth muscle relaxation.

In 1996, Sprague *et al.* further demonstrated the possible mechanism that links RBCs to NO synthesis.[36] They proposed that the ATP is released from RBCs in response to mechanical deformation and evokes the synthesis and release of NO from the endothelial cells lining the pulmonary circulation.[36, 42, 43] This hypothesis was supported by several previous findings. First, ATP is a signaling molecule and stimulates NO synthesis.[43] Second, ATP is the major adenine nucleotide present in the cell and is present in millimolar concentrations in RBCs. Third, it has a recognized role in signaling pathways of other cells.

Several analytical methods have been employed to determine the concentration of deformation induced ATP release from RBCs. In filtration methods, the RBCs are forced through small pores of filters, collected and assayed for ATP release. Although, this method of producing deformation induced ATP was successful in early applications, it has some limitations that prevent on-line measurement of ATP release from RBCs.[44] The new yet simple technique developed by Spence *et al.* enabled the detection of ATP release from RBCs upon passage through microbore tubing.[45] Moreover, this method resulted an improved understanding of the mechanisms that regulate vascular resistance under *in vivo* conditions as this method mimics closely the *in vivo* physiological conditions found in the circulation.

1.5.1.1 Mechanism of ATP release from RBCs

Red blood cells release ATP in response to certain physiological stimuli such as reduced oxygen tension or hypoxia, membrane deformation and a decrease in pH.[46,47] It

has also been recently reported that RBCs release increased amounts of ATP in the presence of pharmacological stimuli.[48] Low nanomolar levels of ATP are sufficient for the activation of purinergic receptors present in the endothelial cells, and thereby may regulate the vascular tone.[43] However, ATP is a large, charged molecule and is unable to cross the membrane through diffusion. Therefore, the presence of a specific mechanism that induces ATP release to the extracellular environment is necessary for the efflux of ATP from the cell.[49]

To date, the key components required for the release of ATP from RBCs have been identified. In initial studies, it was determined that ATP crosses the membrane via the ATP binding cassette (ABC), an ion channel protein. Next, the cystic fibrosis trans-membrane conductance regulator protein (CFTR), which is indeed an ABC family protein, was identified as the protein required for ATP release from erythrocytes.[50] The finding that CFTR protein activity was a requisite for the signaling mechanism of ATP release from cells led to further characterization of the pathway.

Specifically, it is now well established that heterotrimeric G protein (Gi,)[49, 51] adenylyl cyclase,[52] protein kinase A (PKA) and CFTR are important components of the signal transduction pathway of RBC-derived ATP release.[46,49,53] The first step of the pathway is the receptor mediated activation of G protein in response to either physiological stimuli such as mechanical deformation, pH or hypoxia or pharmacological stimuli such as iloprost or mastoparan.[49] The first step of the ATP release mechanism is the activation of G proteins. Catalytic activity of adenylyl cyclase can be stimulated by both Gαs and Gαi protein subtypes. For example, the heterotrimeric protein $G_{\alpha i1}$, $G_{\alpha i2}$ and $G_{\alpha i3}$ subunits have been identified in human and rabbit RBC membranes

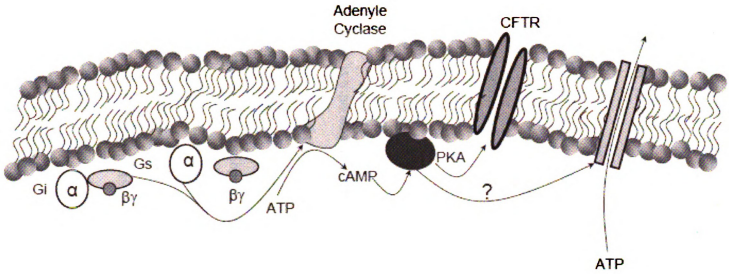

Figure 1.4 The proposed mechanism of ATP release from human erythrocytes. The first step of the mechanism is the activation of the G proteins in response to certain physiological stimuli such as shear stress or hypoxia. Then the cascades of events lead to the extrusion ATP molecules from RBCs. Abbreviations: Gs and Gi are hetertrimeric G proteins; PKA- protein kinase A; CFTR- cystic transmembrane conductance regulator protein.

related to ATP release from RBCs.[49] Activation of the specific sub component of the G protein stimulates adenylyl cyclase present in the membrane and thereby increases intracellular concentrations of 3'-5' cyclic adenosine mono phosphate (cAMP) within the cell. Adenylyl cyclase is important in PKA activity and it is indeed important in the phosphorylation of CFTR, and thereby initiating the translocation of ATP extracellularly.[54] However, to date, research to reveal the protein that is responsible for the ATP extrusion is still undergoing. It is either the direct involvement of the CFTR protein or another protein that is activated upon CFTR phosphorylation.

1.5.2 NO and RBCs

Nitric oxide is a paramagnetic, heterodiatomic free radical that exists as a gas at room temperature. It is one of the 10 smallest molecules in nature, making it one of the most rapidly diffusible molecules. The diffusion coefficient (D) of NO is approximately 3300-3800 $\mu m^2/s$ in aqueous solutions and also it can rapidly diffuse through membrane structures due its solubility in hydrophobic phases.[55]

The Nobel Prize for medicine or physiology was awarded to Robert Furchgott, Louis Ignarro, and Ferid Murad in 1998 for identifying NO as the endothelial-derived relaxing factor. Since then, this small free radical has been identified in a variety of beneficial and detrimental physiological roles through direct and indirect reactions due to its complex chemistry.[56]

Several important physiological roles of NO in biological systems including cardiovascular, immune, and nervous systems have been identified. It is well established

that NO is involved in vascular homeostasis by regulating the vascular resistance, smooth muscle relaxation and inhibiting RBC and platelet adhesion in the circulatory system.[57,58,59] NO synthesized in the endothelium affects 20-30% of basal human blood flow.[60]

However, there has been considerable debate over the interaction and physiological role between endothelium-derived NO and RBCs as well as the involvement of RBC-derived NO in the vascular function.[61] For example, a considerable amount of NO synthesized from the endothelium interacts with blood components known to have an effect on vascular homeostasis. However, it has long been known that NO reacts very fast with hemoglobin in RBCs; in fact hemoglobin is a scavenger of NO in biological systems. Initially, the high reaction rates of hemoglobin with NO have taken away the potential significance of NO on RBCs. However, the observation of varied reaction times for free hemoglobin as compared to hemoglobin encapsulated in RBCs suggested a possible role of NO in RBCs.[62] The erythrocyte membrane and the unstirred plasma layer around the RBC create a diffusional barrier to NO, thereby preserving the reactivity within circulation.[63] However, for free hemoglobin, the reaction rate is only dependant on the diffusion rate of NO to the heme pocket. NO reacts with oxyhemoglobin to form methemoglobin and nitrate, and with deoxyhemoglobin to form iron-nitrosyl-hemoglobin.[60] It is accepted that the rapid reaction of the heme group of hemoglobin and NO produce a heme-iron nitrosyl adduct (Hb(FeNO)), although no bioactivity is found upon this formation.[64]

Several researchers have shown that NO bioactivity is maintained through the formation of nitrosothiols.[65,66] Biochemical studies confirm that S-nitrosylation at 2 cysteine residues of the β subunit of hemoglobin is conserved in mammals and birds. S-

nitrosohemoglobin (SNOHb) is formed under oxygenated conditions of Hb(FeNO) where heme –to cys transfer occurs.[64] This finding was supported by the fact that NO may be transported as SNOHb in erythrocytes. SNOHb is known to be associated predominantly with the RBC membrane in humans, and more specifically, is bound to the cysteine residues in the hemoglobin-binding cytoplasmic domain of the anion exchanger AE1.[67]

It has been reported in the literature that RBCs can serve as oxygen responsive transducers of vasoconstrictor and vasodilator activity and this is partly due to NO bioactivity.[64] It has also been shown in the literature that SNOHb is formed upon oxygenation of (Hb(FeNO)) by heme to cysteine NO transfer, and transfer from low mass – nitrosothiols.[64,68,69] Continuous cycling of hemoglobin between oxygenated and deoxygenated states enables the rapid binding and release of NO. It is known that formed SNO-Hb is a stable in the oxygenated state of hemoglobin, but gains vasodilator potency upon deoxygenation of hemoglobin. However, the detection of SNOHb and other NO metabolites in biological systems is challenging due to its presence in low concentrations and the instability of the species. The inconsistent findings of NO derived species have also fueled the arguments over the physiological importance of these species.[57] Despite the debate over the "real" transport form, SNOHb formation is proposed way of maintaining and transporting the bioactivity of NO in the vascular system.

NO is produced by the nitric oxide synthase enzyme (NOS) in cells. NOS utilizes the amino acid L-arginine, converting it into citrulline and NO.[70] The enzyme requires

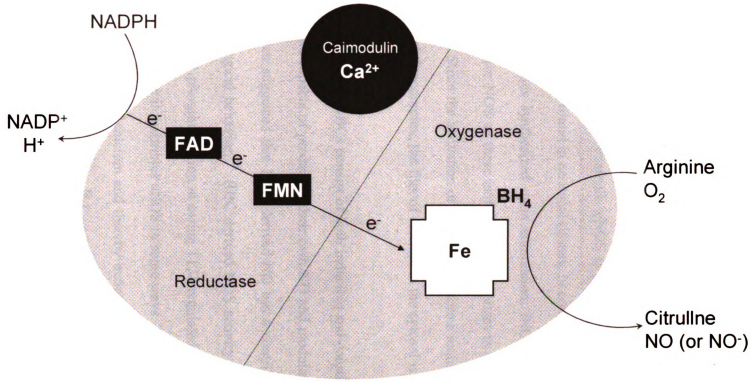

Figure 1.5 Figure illustrating the electron transport of NOS enzyme from reductase domain to oxygenase domain through the cofactors NADPH, FAD, FMN to heme and BH4 moiety via the activation of calcium-calmodulin complex.[70]

several cofactors such as FAD, FMN, tetrahydrobiopterin, and heme.[72] All of these cofactors are needed for the electron transport through the two domains. NOS exists in three different isomers commonly known as endothelial NOS (eNOS or NOS III), neuronal NOS (nNOS or NOS I) and inducible NOS (iNOS or NOS II) based on location and activity.[73-75] For example, eNOS is present predominantly in the endothelial cells, while nNOS is found in neuronal tissues. In contrast, iNOS can be seen in wide variety of tissues and is synthesized upon stimulation. iNOS and eNOS can be categorized as constitutive NOS as these isoforms are important in maintaining the basal NO levels in tissues. Since the activation of these two isoforms requires the formation of the calcium-calmodulin complex, the flux of calcium in the cytosol is the starting step of the synthesis of NO.

Human RBCs consist of both inducible and constitutive forms of NOS genes and thus are capable of producing their own NO and modulating several functions.[76] Also, it has been suggested that RBC derived NO has a contributory role in maintaining RBC physiological behavior.[77] RBC derived NOS activity depends on the intracellular Ca^{2+} levels and phosphorylation at serine 1177 regulated by the phoshatidylinositol-3-kinase (P13K) where it resembles eNOS in some ways. This enzyme is functionally active even at basal levels of calcium and thereby maintains RBC deformability and supports RBC passage through capillaries.[78] Considering the facts of NO synthesis, it is possible that both extracellular and intracellular sources of NO can affect cells in the circulatory system. Several researchers have suggested that NO is effect on RBC deformability is modulated by both endothelial and RBC derived NO, but the exact mechanism of the effect of NO on RBC deformability is still not clear.[79] It has been shown that NO

inhibitors have an effect on erythrocyte deformability in septic shock.[80, 81] Also, NO donors such as spermine NONOate increase the cell deformability. In addition, it has been found that erythrocyte deformability was influenced by polymorphonuclear leukocytes through a mechanism involving NO release.[82] It is also known that NO can modulate (either increase or decrease) erythrocyte deformability within a certain range of NO concentrations, as stated previously. In contrast, it was reported that NO inhibits hypoxia-induced ATP release from erythrocytes. Sprague *et al.* reported that NO released luminally from endothelial cells may function in a negative feedback manner to inhibit ATP release from erythrocytes.[38] They further believed that this inhibition occurs through a G protein activated pathway.[83,84] However, these findings have not taken the attention away from the potential positive effects of NO and its effect on RBCs in the microcirculation.

The clinical implications of RBC NOS activity is evolving and therapeutic interventions to enhance NO production via enhancing NOS activity has been considered in therapies for sickle cell disease, heart failure, and ischemia-repurfusion injury. Conversely, suppressing this activity is a better therapeutic approach for sepsis, where higher NO production stiffens the membrane and inhibits the RBC passage through the capillaries.[78]

1.6 Red blood cells and its involvement in disease status

Even though RBCs have been subjected to many investigations since its recognition as an oxygen carrier, little attention has been given to their involvement

indisease status. However, recent studies have demonstrated the effect of metabolic deficiencies or membrane abnormalities of RBCs on clinical complications of several diseases, where the major cause of the disease is not thought to be RBC related. Diabetes mellitus, pulmonary hypertension, cystic fibrosis and sickle cell disease are some of the diseases that fall into this category. Therefore, the study of RBC and its relevance in disease pathogenesis is vital for the advancement of medicine and drug discovery.

1.6.1 RBCs and diabetes mellitus

Diabetes mellitus, commonly known as diabetes, is a chronic disease of disordered metabolism, resulting in abnormally high glucose levels in the blood stream. Diabetes affects 23.6 million people or ~ 8% of the United States population.

The cause of hyperglycemia or high glucose levels in the blood is due to a combination of hereditary and environmental factors. The known basis of the disease is either the loss or diminished production of the insulin hormone by the pancreatic β cells or the decreased response or sensitivity of insulin to insulin receptors on the cell membrane. The net result of poor insulin usage is reduced intake of glucose by the cells, thereby increasing the concentration of glucose in the blood stream. Long term complications of diabetes include retinopathy, nephropathy, neuropathy, and vascular diseases. Hypertension, increased risk of stroke and heart failure are often associated with people suffering from diabetes.

Since 1921, diabetes has been treated and controlled by externally supplying the insulin hormone. To date, research has been centered on insulin alone for the treatment of

diabetes. Nevertheless, diabetes is a chronic disease with no cure that requires short term and long term management to prevent other complications associated with the disease. However, in recent years, research has involved attempts at better understanding the role of RBCs in diabetes and its complications.

Oxidative stress is a major component of molecular, cell and tissue damage in a number of diseases including diabetes. Numerous recent studies have shown that patients with diabetes mellitus have increased oxidative stress and an impaired antioxidant defense system.[28, 85] Free radicals can react with proteins, lipids, proteins, carbohydrates, etc, thereby affecting the overall physiology of the living system.[86]

Oxidative stress in diabetic RBCs is known to be higher in comparison to the healthy RBC. Moreover, diabetic-induced oxidative damage is higher in RBCs in comparison to other tissues in the body, largely because RBCs are carriers of oxygen. Also, the RBC is known to have a higher content of iron and polyunsaturated fatty acids, which are prone to oxidative damage.[87] Diabetic RBCs are subjected to a high glucose environment within the circulation and this hyperglycemic condition leads to non-enzymatic protein glycation in RBCs, especially of hemoglobin and other membrane proteins.[88] It has been shown that the interaction of glucose with proteins can form glycosylated proteins through the spontaneous chemical reaction of glucose with amino groups of the proteins.[89, 90] Moreover, glucose is prone to auto oxidation that can produce highly reactive oxygen species as well as other charged molecules. All of these molecules can damage the RBC itself. Schwartz *et al.* have shown that spectrin, ankyrin and protein 4.2 are the most glycosylated RBC proteins.[88] Interestingly, all of these proteins are

components of the RBC cytoskeleton, which provides evidence for the genesis of the decreased deformability of diabetic RBCs. It is well established that type 2 diabetic RBCs are less deformable in comparison to RBCs obtained from healthy humans.[91] Many studies have indicated that one aspect of diabetes is the abnormal rheological properties of RBCs.[88] These abnormalities may be due to the decreased deformability of the membrane, RBC cytoplasm viscosity and increased erythrocyte aggregation. Collectively, all of these properties suggest that protein glycosylation and increased oxidative damage results in decreased deformability of RBCs. However, there are other features of the RBC that may result in oxidative damage in diabetic RBCs that need to be considered. One of these features is a weakened antioxidant system.

A weakened antioxidant system has been observed in patients with diabetes. It has been reported that GSH metabolism is altered in both type 1 and type 2 diabetes patients. This is supported by findings that chronic hyperglycemia increases the activity of the polyol pathway and free radical generation, which leads to increased GSH oxidation. Moreover, activation of the aldose reductase pathway reduces NADPH concentrations thereby diminishing GSH regeneration.[86] It is well established that G6PD, a key regulatory enzyme in the PPP, is deficient in the RBCs of diabetic patients.[28] Because NADPH is produced exclusively via the PPP in RBCs, a deficiency of G6PD diminishes the availability of NADPH for glutathione regeneration, ultimately resulting in a decreased activity of the GSH antioxidant defense system (which can further affect the overall oxidative stress in diabetic RBCs).[32]

Erythrocytes, via their ability to release ATP in response to physiological stimuli, have been shown to be a determinant in maintaining vascular resistance. Carrol *et al.* has

shown that diabetic RBCs release lower amounts of ATP when compared to the RBCs obtained from healthy humans.[85]Sprague *et al.* reported that the RBCs obtained from type 2 diabetic patients have reduced expression of the Gi protein. Gi is an important component of ATP release from RBCs and reduced expression of this protein leads to deceased production of cAMP, which subsequently leads to less RBC-derived ATP release.[54] This suggests that defects in RBC physiology may affect the vascular reactivity, the major complication associated with diabetic patients.[92]

Taken collectively, increased oxidative stress, an impaired antioxidant system, and hereditary factors such as deficient enzyme/protein activities may lead to pathophysiological complications associated with diabetes and it is important to have a better understanding of these mechanisms for a better therapeutic approach to disease management. However, until recently, no attempts were taken to demonstrate the relationship among the antioxidant system, enzyme deficiencies and the ATP release from RBCs. Carrol *et al.* and Subasinghe *et al.* provided evidence for the first time showing the importance of antioxidant systems on ATP release from RBCs.[93] Importantly, this work enabled a quantitative determination of ATP release due to the disturbance of the antioxidant system.

1.6.2 RBCs and sickle cell disease

Sickle cell disease (SCD) is an autosomal recessive disease affecting more than 75,000 people in the US. It is the most common genetic disease affecting African-Americans (0.15% are homozygous and 8% have the sickle trait).[94] The characteristic

feature of this hereditary disease is a single amino acid mutation of the β subunit of the hemoglobin molecule. Normal hemoglobin (HbA) consists of 2 α globin and 2 β globin chains. The substitution of glutamate to valine results in a new type of hemoglobin called hemoglobin S (HbS). Formation and the presence of the HbS in the RBC results in the majority of pathophysiological conditions associated with the disease.[68, 95]

Patients with SCD suffer from a wide range of major clinical manifestations, including acute episodes of pain, stroke, acute chest syndrome, leg ulcers and organ damage.[96] These RBCs when traveling through the microvasculature, are subjected to large shape changes under deoxygenated conditions giving rise to the "sickle" shaped cells. The primary reason of sickling is the polymerization of the HbS molecules in the RBC under deoxygenated conditions. Formation of sickled cells is the critical point in vaso occlusive crisis, which is the main complication associated with the disease. Under deoxygenated conditions, these HbS molecules can polymerize into long fibers thereby increasing the RBC rigidity. High rigidity or decreased deformability leads to vascular obstruction and ischemia. Moreover, these cells have a shorter life span (16 days) compared to healthy RBCs (120 days) and are prone to hemolytic destruction (lysis) due to decreased deformability.[97, 98] Also, the damaged cell membrane results in increased adhesion to the endothelium enhancing the chances of vaso occlusion crisis, stroke and pulmonary hypertension.[99] Finally, activation of endothelial cells, platelets, and the coagulation system leads to clinical implications associated with the disease. These factors make the patients anemic and often in need of blood transfusions. As a result, SCD is considered a chronic rheological disease and a significant contributor to blood flow failure.

Even though the molecular basis of SCD has been known for decades, there was no promising therapy until 1995. The revolutionary publication of the critically important therapy was a breakthrough in the management of SCD. Hydroxyurea, a ribonucleotide reductase inhibitor, is a proven treatment for sickle cell disease.[100] The widely accepted mechanism of action of hydroxyurea is its ability to induce fetal hemoglobin (HbF) production in RBCs. However, the exact mechanism of increased HbF production by hydroxyurea is still not clear. Moreover, the beneficial effects of hydroxyurea do not always correlate with the increased HbF production, suggesting there may be an additional mechanism of hydroxyurea in SCD patients.

The improvement of rheological properties including increased whole cell deformability, reduced red cell density and improved erythrocyte hydration status have been observed with patients receiving hydroxyurea.[101] However, the mechanism of action of hydroxyurea in relation to improved rheological properties still remains to be elucidated, especially regarding the improvements of vascular properties, although various mechanisms have been proposed in the recent literature.

Even though SCD was originally considered a genetic disease, its major pathophysiologycal complications are associated with the alteration of vascular properties. SCD patients are known to have stiffened RBCs, low NO bioavailability, increased oxidative stress, increased RBC- endothelial interactions, all of which can be categorized under the impairment of one or more metabolic functions of the RBCs. Therefore, an understanding of the RBC properties, especially the factors that affect the regulation of blood flow, would be beneficial for patients having SCD. Moreover,

defining the mechanisms hydroxyurea in SCD would be helpful in developing novel therapies to overcome the clinical complications associated with the disease

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CHAPTER 2

Dynamic monitoring of glutathione in erythrocytes, without a separation step, in the presence of an oxidant insult

2.1 Glutathione

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine) is the most abundant, non enzymatic, low molecular mass, thiol-containing tripeptide found in virtually all the mammalian cells, plants and microorganisms.[1,2] Its facile electron donating capacity, linked to the sulfhydryl (SH) group of cysteine has made glutathione a potent antioxidant and a convenient cofactor in living systems.[3] Glutathione is present in millimolar concentrations (1-10 mM) inside the cell cytosol (85-90%) or other aqueous phases, although the actual concentrations are varying depending on the cell type.

Glutathione exists in two forms. The antioxidant, or reduced form, is conventionally known as glutathione (GSH) while the oxidized form is known as glutathione disulphide or GSSG. The GSH molecule is made up of three amino acids, glutamate, cystein and glycine, covalently linked end to end by the peptidic γ linkage. GSSG is formed by two GSH tri-peptides linked by a disulfide bond. The intracellular glutathione is in the reduced state, which represents ~90% of the total glutathione content in the cells. The oxidized concentrations are generally low but can be increased under various oxidative stresses or pathological conditions.

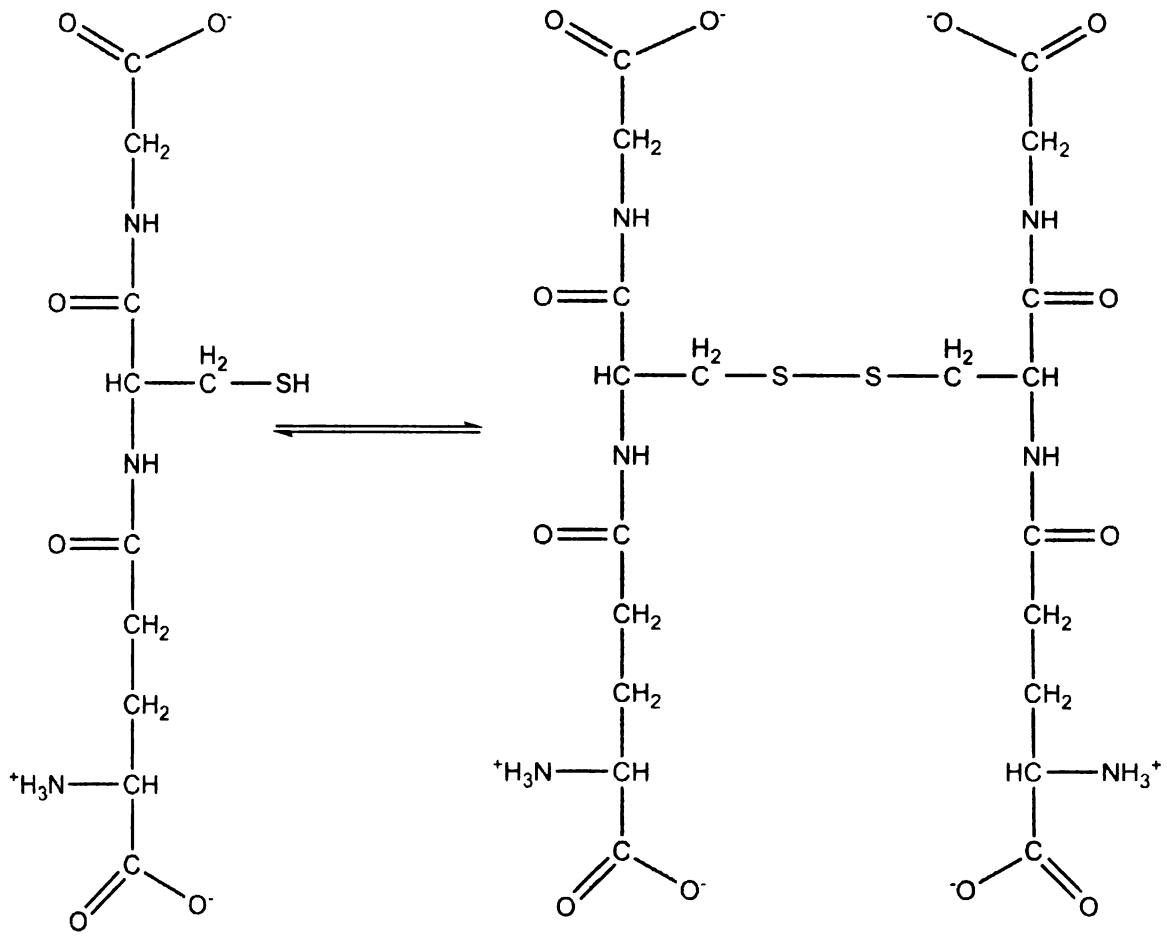


Figure 2.1 Structures of reduced glutathione (GSH) and oxidized glutathione (GSSG)

GSH/GSSG is the major redox couple that determines the antioxidant capacity of the cell. The ratio of GSH:GSSG has been shown to be an indicator of oxidative stress and therefore can be used as an effective measure of a diseased state.[5]

The most important function of glutathione in red blood cells (RBCs) is to detoxify the reactive oxygen species, free radicals and reactive elecrophiles.[5] Being the most abundant antioxidant in RBCs, GSH converts into an oxidized form (GSSG) while

reducing the various oxidants present in the cells. However, oxidized GSH or GSSG must revert back to its reduced form to maintain its activity. The conversion of GSSG to GSH is known as GSH regeneration.[1] Glutathione reductase is necessary for the regeneration process in the presence of NADPH, a cofactor produced in the pentose phosphate pathway. Importantly, this pathway is the only means of producing NADPH in the erythrocyte for glutathione regeneration.

GSH is synthesized from L-glutamate, L-cysteine, and glycine in two ATP-dependant pathways. The first step is the synthesis of γ-glutamylcysteine from L-glutamate and L-cysteine via the activity of γ-glutamylcystein synthase enzyme. Next, a glycine residue is added to the C-terminal of γ-glutamylcystein by the enzyme glutathione synthase. Even though GSH synthesis is the only source of glutathione for cells, the intracellular GSH concentrations are dependant on both *de novo* synthesis of GSH and the activity of the GSH redox cycle.

The GSH redox cycle is composed of several enzymes, namely, glutathione peroxidase (GPx), glutathione reductase (GR), and glucose-6-phosphate dehydrogenase (G6PD).[6] GPx is involved in neutralizing H_2O_2 to H_2O while oxidizing the GSH to GSSG. However, GSSG is then converted back to GSH by the enzyme GR. G6PD is important in the generation of the NADPH cofactor for the GSH regeneration process. The GSH redox cycle is a tightly regulated process and helps protect the cell from excessive oxidative damage. In addition to the GSH/GSSG redox couple, there are several other redox couples that maintain the reducing environment of the cell. However, the GSH redox status is important in maintaining the steady state value of the intracellular redox potential as GSH exists in 100-1000 folds higher than the reduced

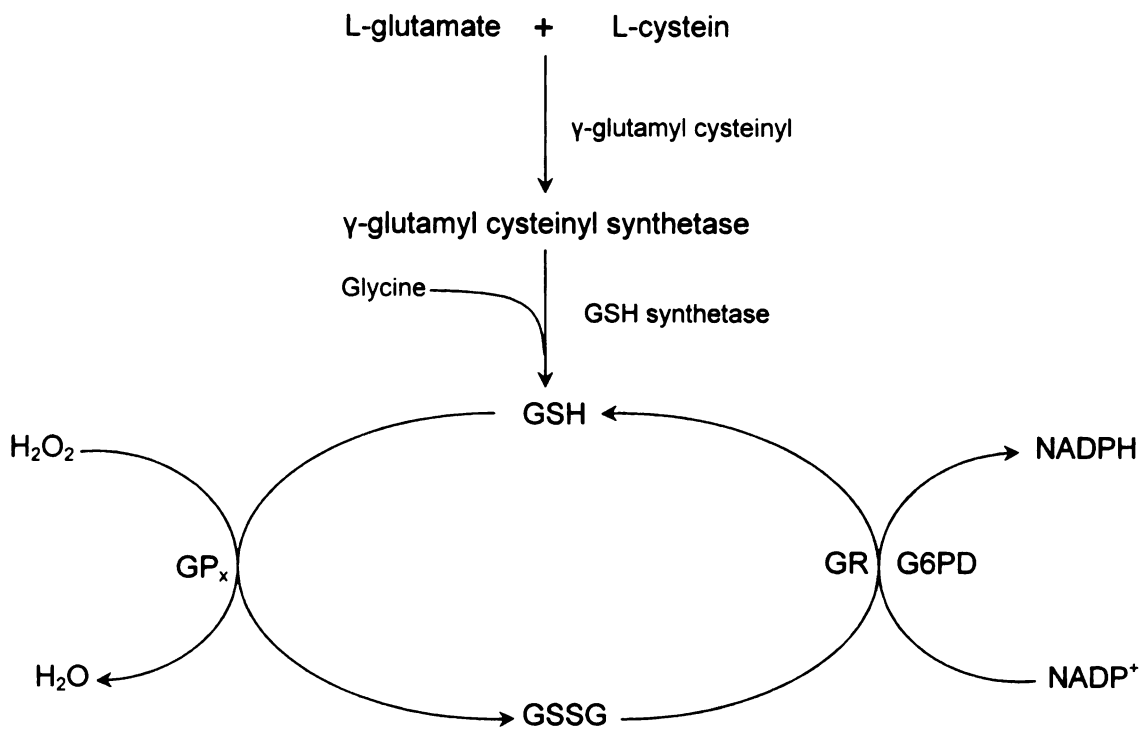


Figure 2.2 Schematic representation of the regulation of GSH levels in RBCs. GSH synthesis is a two step ATP dependant enzymatic mechanism that involves γ-glutamyl cysteinyl synthetase and glutathione synthetase. GSH redox cycle involves three enzymes; GPx- glutathione peroxidase, G6PD- glucose-6-phosphate dehydrogenase, and GR- glutathione reductase

form of other redox couples.Modifications of the GSH cycle or enzyme levels may result in an imbalance of the antioxidant:oxidant ratio leading to cellular structure fragility or various pathophysiological conditions. In addition to its function as an antioxidant or antitoxin, GSH is involved in diverse biological processes in living systems including protein synthesis, enzyme catalysis, transmembrane transport, cell metabolism.[5] Numerous studies have associated alterations in RBC GSH concentrations with various diseases including diabetes mellitus,[7-9] Alzheimer's disease,[10] HIV,[11] multiple sclerosis[12] and several types of cancers.[13,14] In all of these disease conditions, deleterious imbalances between the production and removal of reactive oxygen species and free radicals, as well as depleted GSH levels have been observed. Therefore, the redox balance between GSH and GSSG, or GSH regeneration, is important in maintaining cellular homeostasis.

2.2 Oxidative stress, glutathione, and diabetes mellitus

RBCs are subjected to higher oxidative stress compared to other cell types due to their continuously high levels of intracellular oxygen and high iron contents. GSH is known to be the most concentrated antioxidant found in RBCs and acts as the redox buffer maintaining iron in its reduced state for hemoglobin function and fighting against oxidative stress to maintain cellular deformability. In other words, RBC GSH levels are crucial in maintaining the RBC's ability to overcome oxidative stress-induced conditions during the RBC's lifetime of 120 days.[15]

As mentioned above, higher oxidative stress has been linked to many diseases including diabetes mellitus.[16,17] A weakened oxidant defense mechanism, higher

production of oxidant species, and the decreased deformability of diabetic RBCs have been known to exist for decades, but no study linked the oxidant stress of RBCs to the complications associated with diabetes mellitus. A weakened antioxidant system is often the result of decreased activity of glucose-6-phosphate dehydrogenase (G6PD), while the higher production oxidants are an effect of an increased rate in protein glycation by glucose. In addition, the decreased RBC deformability of diabetes may be due to the oxidation of membrane proteins such as spectrin in the presence of oxidants.

It has been previously reported that RBCs are a required component for maintaining vascular resistance via their ability to release adenine triphosphate (ATP), a known stimulus of NO. The amount of ATP released from RBCs is dependant on the deformability of RBCs, therefore the vascular complications associated with diabetes include a higher occurrence of strokes, heart attacks and the high probability of having hypertension. However, linking a physical characteristic of RBCs (e.g; RBC deformability) to a weakened oxidant system is difficult as it requires experimental methods to quantify the physiological consequence of oxidant insult placed on the RBCs. Quantifying the GSH levels upon oxidative stress would be the first step in such a determination as GSH levels represent the overall oxidant status of cells. However, currently available methods for GSH quantification require separation of the cellular constituents prior to measurement. These procedures are time consuming and therefore, measuring an immediate physiological response from the RBCs upon oxidant insult cannot be determined. Therefore, the objective of this study is to develop a new technique to quantitatively determine GSH levels in RBCs under oxidant stress in order to understand the effects of GSH depletion on disease diagnostics and disease pathogenesis.

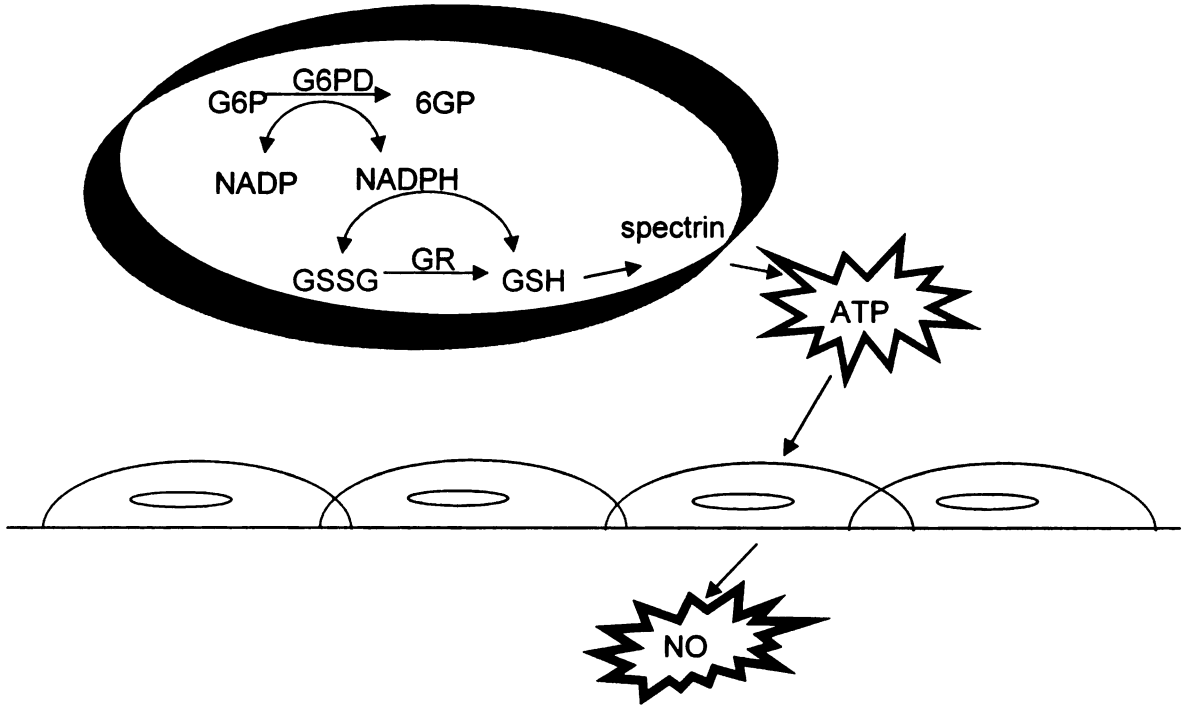


Figure 2.3 Proposed mechanism of the involvement of increased oxidative stress of RBCs on vascular complications of diabetes mellitus. The deficiency of G6PD activity decreases the intracellular NADPH levels thereby affect GSH regeneration process. Lower intracellular GSH levels may affect the RBC deformability, consequently the ATP release from RBCs. The decreased amounts of ATP could affect the NO synthesis from vascular endothelium leading to the vascular complications associated with the disease.[18]

2.3 Glutathione measurements: currently available methods and limitations

Numerous methods for glutathione measurements in different cell types have been described in the literature. Early attempts to measure GSH in various cell types included enzymatic,[19,20] colorimetric and fluorometric based assays. These methods employed potent glutathione reductase inhibitors, such as N-ethylmaleimide for enzymatic assays, and several probes such as 5-5'-dithiobis-(2-nitrobenzoic) acid (DTNB or Ellman's reagent), monochlorobimane (MCB) or monobromobimane (MBB) for fluorometric assays. However, many of these methods have insufficient detection limits and low precision that limit biological sample analysis.

Recently, several new techniques couple the separation and detection techniques to quantify intracellular GSH in different cells and tissues. High performance liquid chromatography (HPLC)[2,21-23] and capillary electrophoresis (CE)[24] are widely used to quantify GSH or GSH:GSSG in biological samples. HPLC coupled to electrochemical,[25,26] fluorescence,[27-29] and ultra violet (UV) absorbance[30-33] detection methods are currently in wide use for the determination of GSH. Usually, UV measurements are taken after derivatization with DNTB or with 2,4-dinitropyenyl derivatives.[31] Fluorescent measurements are carried out after separating GSH using HPLC and then derivatized with several reagents such as monobromobimane (MBB),[27,29] ortho-phthalaldehyde (OPA), 1-dimethylaminonapthalene-5-sulphonyl chloride (dansyl chloride),[28,34] or ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F).[2] Moreover, HPLC can be coupled to mass spectrometric analysis.[35,36] Mass spectrometric detection is known to be much more sensitive compared to HPLC with UV, and HPLC

with fluorescence detection. More recently, CE with electrochemical detection, laser induced fluorescence detection, and UV detection were reported to be a highly sensitive method of quantifying GSH or GSH:GSSG in cellular systems.[15, 37, 38]

While these methods have been successful in measuring GSH and, in the case of those employing HPLC and CE, the GSH:GSSG ratio, each method has limitations. Generally, one of the more challenging areas of biological analysis is overcoming matrix effects. Large numbers of molecules in smaller volume, resembling structural features of different molecules and instability of the molecules have complicated biological sample analysis.

For example, methods that employ DNTB are often limited by its lack of specificity for GSH. Fluorescence based assays using bimane derivatives in erythrocytes have required the separation of cellular constituents. Those methods employing HPLC and CE have provided the separation necessary to quantify GSH in biological samples. GSH is a water soluble compound, thus extraction of GSH from biological matrices is a challenge without employing a derivatization step. Also, the chemical instability of the molecule, especially the susceptibility of the thiol group to oxidation, renders the quantitative determination even more difficult.

Several analytical techniques have been developed to quantify GSH levels in RBCs. However, wide differences in GSH concentrations in RBC samples have been reported in the literature and are probably due to the various sample preparation and analysis methods.[22] Improper sample preparation, collection, pre-treatment and storage of the sample for long periods of time can cause large variations of the GSH content in cells.[39,40] Moreover, the ability of GSH to undergo autooxidation prevents quantifying the

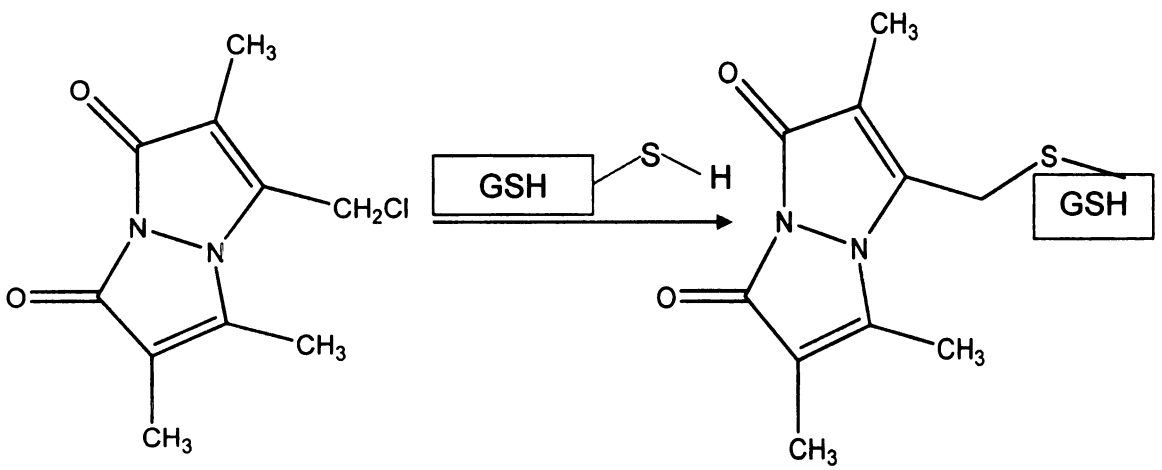
actual amounts of GSH inside the cells. Acid treatment is often carried out for RBC lysis, but this may be harmful for actual quantification as the GSH oxidation diminishes the GSH content in cells leading to possible underestimated values in the literature.[15] Sample preparation by acid treatment of proteins can lead to precipitation of oxyhemoglobin, oxygen release and free radical generation, thereby altering the GSH redox cycle. Recently, Zinellu *et al.* described a new method in which GSH oxidation was minimized by using cold water for RBC lysis followed by membrane filtration for the separation of cellular constituents.[15] However, this method is not successful in large sample analysis especially for diagnostic purposes because of the high expense in using membrane filters. In summary, all techniques described in the literature employ either a separation method or a derivatization step that results in a complicated analysis.

Quantitative determination of GSH is extremely difficult in RBCs compared to other cell types because of their complex matrix, particularly in the presence of hemoglobin in RBCs. A single RBC contains about 200 – 300 million hemoglobin molecules or 11-18 g/dl of hemoglobin content. Hemoglobin absorbs light ranging from the UV to the visible, preventing spectroscopic determination of different analytes in the RBC. Therefore, the separation of hemoglobin from the RBCs is necessary for most analytical techniques that employ UV and fluorescence detection. Taken collectively, improvements in proper sample preparation techniques and detection techniques are vital in quantifying GSH in biological samples.[15] Moreover, the determination of GSH in erythrocytes can potentially benefit clinical diagnosis at the early stages of disease. Therefore, a rapid method with appropriate detection limits and high reproducibility is a necessity for the analysis of biological sample.

2.4 Fluorescent determination of GSH using standard additions

Considering the drawbacks and limitations of the currently available methods of quantifying intracellular GSH, a new fluorescence based assay was developed to quantify GSH in erythrocytes. The primary objective of developing a new method is to have a rapid, reliable and more sensitive way of quantifying GSH in erythrocytes without separating GSH from other cellular constituents.[4] The necessity of having such a method is its utilization in disease diagnosis, metabolomics studies and microfluidic/lab on a chip based applications.

The new technique employs the fluorescent probe, MCB in combination wih the standard addition method. The use of MCB as a fluorescence probe for GSH determinations has been previously reported.[41,42]



MCB MCB-GSH conjugate

Figure 2.4 GSH reacts with non fluorescent MCB to form the fluorescent MCB-GSH conjugate

However, when used in conjunction with the method of standard additions to overcome the complex matrix of the erythrocyte, it is possible to perform a quantitative determination of the GSH levels in cells.[4] The method of standard addition is a powerful technique when the sample matrix is difficult or impossible to duplicate. This method is beneficial for RBC studies since it compensates for complex matrix interferences. The standard addition method employed in this assay was validated by the linear relationship obtained between the response and the analyte of interest.

Importantly, this new method can be performed without any sample preparation beyond isolation of the RBCs from the whole blood. Moreover, because no physical separation of GSH from the cell matrix is required, this method can be used to determine the GSH redox status in a dynamic manner when performed in a continuous flow stream.[4] Thus, this technique enables a constant monitoring of a small portion of the overall cellular metabolism in real time.

2.5 Experimental

2.5.1 Generation of Washed Red Blood Cells

RBCs were prepared on the day of use. For obtaining rabbit RBCs, male New Zealand White rabbits (2.0-2.5 kg) were anesthetized with ketamine (8.0 mg/kg) and xylazine (1.0 mg/kg) followed by pentobarbital sodium (15 mg/kg iv). After tracheotomy, the rabbits were mechanically ventilated (tidal volume 20 ml/kg, rate 20 breaths/min; Harvard ventilator). A catheter was placed into a carotid artery, heparin (500 units, iv) was administered, and after 10 min, animals were exsanguinated. Blood was collected

into vials and the RBCs separated from other formed elements and plasma by centrifugation at 500 x g at 4 °C for 10 min. The supernatant and buffy coat was removed by aspiration. Packed RBCs were resuspended and washed three times in PSS [PSS; in mM; 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21.0 tris(hydroxymethyl)aminomethane, and 11.1 dextrose with 5% bovine serum albumin, pH adjusted to 7.4].[4]

2.5.2 Methods and materials

Fluorescence Determination of GSH using Standard Additions

The standard addition method was employed to perform quantitative determinations of GSH. The GSH standards were prepared by combining 100 µl of a 1.0% hematocrit of RBCs and varying amounts (0, 4.0, 8.0, 12.0, 16.0, and 20.0 µl) of a 1.0 mM stock solution of standard GSH (Sigma) in separate vials. Also added to the mixture to aid in the labeling of the MCB probe was 50 µl of 100 units ml^{-1} of glutathione -S- transferase, (Sigma). The GSH stock was prepared by dissolving 0.0307 g of GSH (Sigma) in 18.0 MΩ distilled/deionized water (DDW). Next, varying volumes of buffer were added to each vial such that, upon addition of 100 µl of 250 µM MCB (Molecular Probes), the final volume would be equal to 1 ml. After the addition of the MCB to the RBC/GSH/GST//buffer mixture, a 10 minute incubation period was allotted to allow the MCB to react with GSH. Following the incubation period, the GSH-MCB fluorescence was measured (ex. 370 nm, em. 478 nm) for each of the prepared vials. The fluorescence intensity was plotted as a function of volume of GSH stock added to each vial.[4]

Monitoring GSH upon Oxidant Attack

GSH was monitored in both static (non-flow) and dynamic (continuous flow) systems. To demonstrate the ability of MCB to monitor the GSH redox status in erythrocytes following an oxidant attack in the absence of flow, a 20 μM diamide solution (Sigma) was added to 5000 μL of RBCs (1% hematocrit) to intentionally oxidize GSH to GSSG.[43] A stock solution of 2 mM diamide was prepared by dissolving 0.0344 g of diamide in 100 ml of DDW. 100 μl of RBCs were removed every 5 minutes and used to prepare samples containing a 0.1 % hematocrit of RBCs, GST, and MCB. The changes in fluorescence were monitored every 5 minutes for up to 90 minutes (except for the first reading; this reading was taken at 10 minutes because the MCB-GSH reaction was given a minimum of about 10 minutes before a signal was measured).

2.6 Results and Discussion

Following the 10 minute incubation period, the MCB fluorescence in the presence of RBCs was measured (ex. 390 nm, em. 520 nm) and found to be lower than the fluorescence of MCB without any RBCs (figure 2.5 a, b).[4] The RBCs contain GSH thus, it was expected that the fluorescence signal would be higher in the presence of RBCs and lower when MCB was measured in the absence of any GSH source. Samples (1000 μl) containing 250 μM MCB and 100 μM GSH were prepared with and without 100 μl of 1.0% hematocrit of RBCs (figure 2.5–c, d). Again, the samples that contained RBCs resulted in fluorescence emission that was lower than samples without any RBCs.[4]

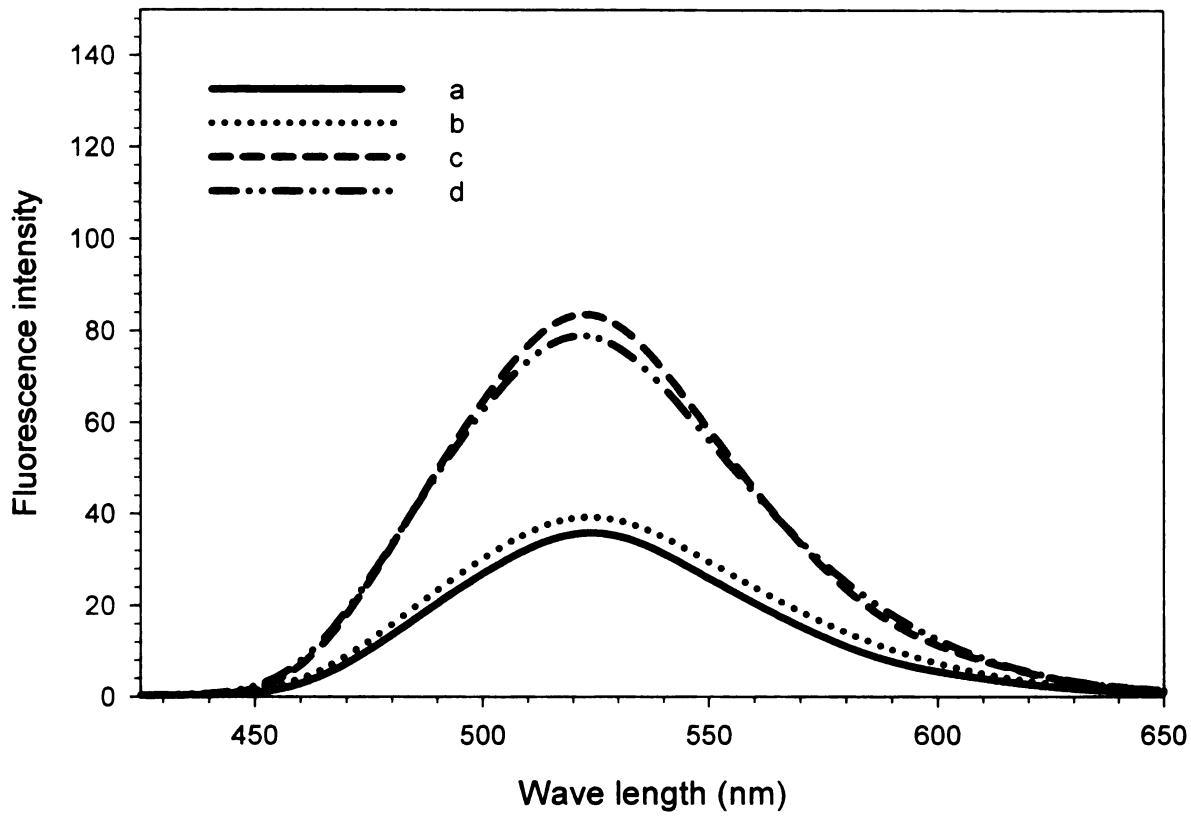


Figure 2.5 Emission spectra of the initial attempts of quantifying GSH and MCB. The fluorescent intensity of 0.1% RBCs (a) were lower than MCB without RBCs (b). The fluorescence intensity of MCB probe in 100 μM standard GSH (c) was higher than the MCB probe in the presence of 0.1% RBC that was prepared in a 100 μM standard GSH solution(d).[4]

It was suspected that the high molar extinction coefficient of hemoglobin at 390 nm was interfering with the excitation of the MCB. In fact, the molar absorptivity of oxyhemoglobin at 390 nm and 370 nm are 167,748 cm^{-1}/M and 88,176 cm^{-1}/M, respectively. This data, and the literature values, imply the interference of RBC hemoglobin in the fluorescent measurement of GSH with MCB. Therefore, by changing the excitation wavelength of MCB from 390 nm to 370 nm, the absorbance of hemoglobin could be significantly decreased while still providing adequate excitation of the probe.

Standards were prepared as described above and incubated for 10 minutes prior to acquiring the fluorescence spectra (ex. 370 nm, em. 478 nm). GSH was quantified from the calibration curve (figure 2.8) using the peak emission intensities shown in figure 2.7. The GSH concentration in the RBCs were then determined from the y-intercept (b) multiplied by the concentration of the GSH stock solution of 1 mM (C_{stock}) divided by the product of the slope (m) and volume of RBCs added to each standard that was measured (V_{RBC}) (1).[4]

$$GSH_{RBC} = bC_{stock}/mV_{RBC} \qquad (1)$$

Preparing the standards directly in the complex matrix of the sample, the hallmark feature of the standard addition method, allowed for the quantitative determination of GSH in erythrocytes without separation of cellular constituents or any sample pretreatment.[4] The GSH concentration per RBC from the data in figure 2.7 was determined to be 0.047 ± 0.003 mM.[4] The overall standard deviation was calculated using the standard deviations about the slope and intercept, respectively. This value is within

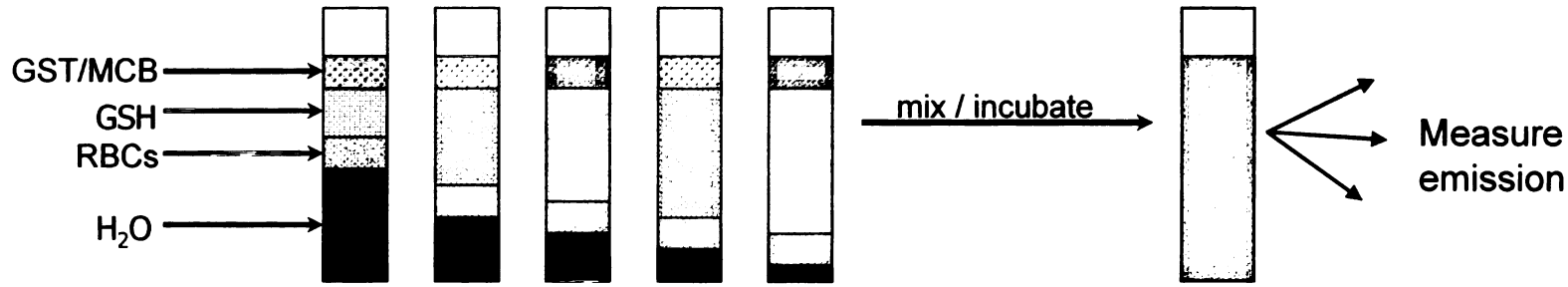


Figure 2.6 Order of reagent addition for GSH determination using the method of sanded additions. Varying volumes of water (730 – 750 µl) are added to a standard cuvette, followed by 100 µl of a 1% RBC sample. Next, 100 µl of MCB and varying amounts of GSH (resulting in a 1.0 ml mixture) were added simultaneously, and the reagents were allowed to react for 10 minutes prior to obtaining the fluorescence emission at 478 nm.[4]

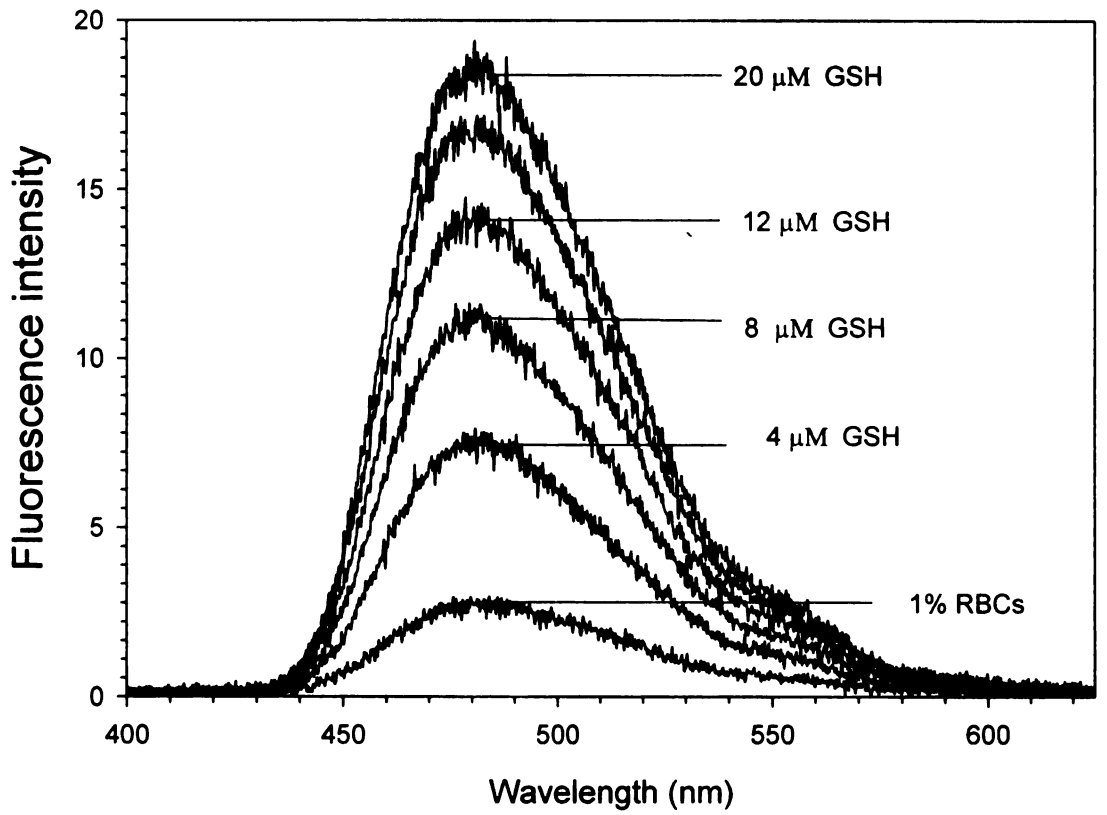


Figure 2.7 The standard addition method was used to quantify GSH in erythrocytes by adding increasing volumes of 1 mM standard GSH to mixture of 0.1% RBCs, 250 μM MCB and 2.5 U/μl GST to make 4-20 μM final GSH concentrations, respectively.[4]

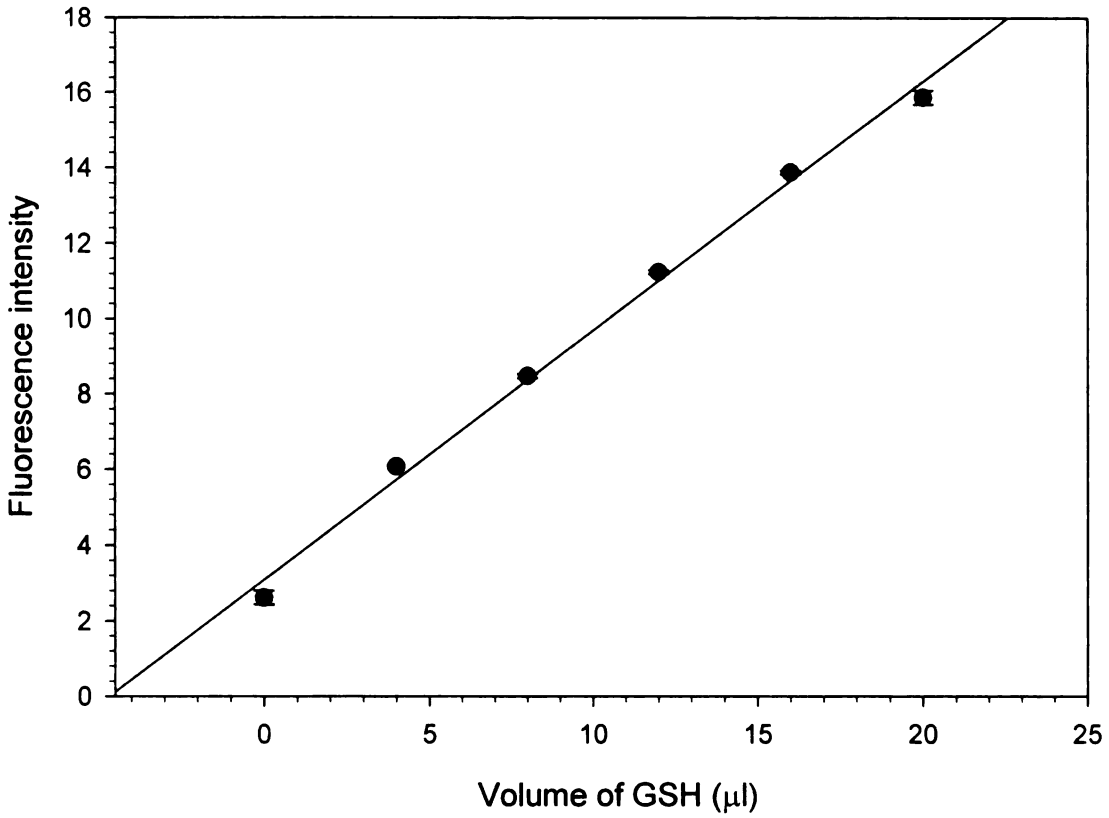


Figure 2.8 Calibration plot obtained for the standard addition method. The fluorescent intensities were plotted against the volume of standard GSH solutions added to each RBCs sample. The resulting equation of the line $y = 0.651x + 3.08$ ($R^2 = 0.9948$) was used to determine the GSH concentration in the RBCs.[4]

the range of concentrations published for GSH in mammalian erythrocytes but are somewhat higher than the previously reported for human RBCs.[44] By incorporating the volume of the RBC (87 fl), it was also determined that the amount of GSH per cell was ~400 amol. The average GSH concentration found in the bulk sample for the RBCs from n=6 different rabbits was 0.042 ± 0.002 mM. This number translates to a cellular concentration of 3.99 ± 0.25 mM/RBC in the sample and 362 ± 20 amol/cell.[4] The precision in these results are represented as a standard error of mean.

In order to provide evidence that the signal measured was actually due to the MCB reacting with GSH, and not other thiols found in the cellular matrix, a sample was spiked with a known amount of authentic GSH prior to preparing the standards used in the standard addition determination.[4] The amount of GSH found in the RBC sample was 0.046 mM. However, upon addition of 0.50 μmoles of authentic GSH to the sample it was determined that 99.8% percentage of the spiked GSH was recovered. These results suggest that standard addition method can be employed to successfully determine GSH levels in the RBCs without any sample preparation steps other than separating the RBCs from the whole blood. Also, these results suggest that the MCB probe is specific for GSH in these studies and that the fluorescence signal is due to the formation of the MCB/GSH fluorescence emission.

GSH functions as the most abundant non-enzymatic antioxidant in RBCs. As such, it is thought to protect other proteins from forming extensive oxidation products. Once subjected to an oxidant insult, the RBC attempts to maintain normal levels of GSH through GR-catalyzed reduction of the GSSG back to the protective GSH form. This dynamic reduction-oxidation process is continuous in cells. Therefore, it is imperative

that a system by designed that enabled quantitative monitoring of the redox status of the molecule of interest in the cell (GSH).

To demonstrate the ability of MCB to monitor the glutathione redox status in erythrocytes following an oxidant attack, 100 μl of 2 mM diamide were added to 5 ml of RBCs (1 % hematocrit) to intentionally oxidize GSH to GSSG. Diamide is useful in studying the GSH redox cycle because it is known to oxidize GSH specifically at a higher rate than the other thiols.[45] Aliquots of RBCs (100 μl) were removed every 5 minutes and used to prepare 1 ml samples containing 0.1% hematocrit of RBCs and 250 μM MCB. The changes in fluorescence were monitored every 5 minutes for up to 90 minutes. Figure 2.9 shows the initial decrease in fluorescence due to the GSH being oxidized to GSSG in the RBCs. However, after approximately 20 minutes, the GSH is slowly started regenerating due to the RBCs ability to maintain proper intracellular oxidant status.[18] The decrease in fluorescence emission exemplifies the ability of the diamide to oxidize the GSH to the dimer. Importantly, the emission profile also shows the ability of the RBC to return to levels of GSH that are statistically equivalent to the original values prior to oxidant insult.

However, when the oxidant insult is higher (40 μM), the regeneration ability was delayed by about 30 minutes compared to 20 μM diamide (figure 2.10). This observation was further validated by using a higher concentration of diamide (100 μM). In that instance, the ability of GSH to regeneration was lost due to the high oxidant stress. These studies clearly demonstrate that the GSH levels are dynamic and redox cycle changes in response to oxidants. Therefore, these results can be used in hypothesizing the exact mechanism of action of GSH under oxidative stress

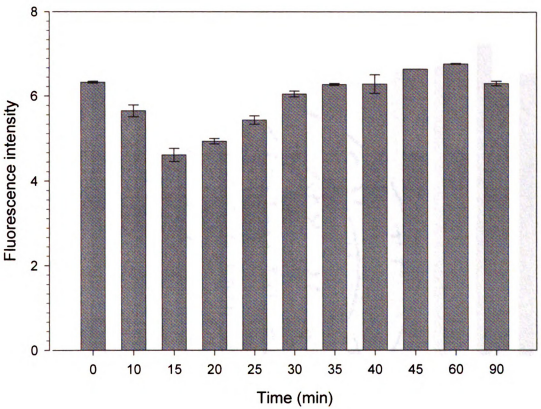


Figure 2.9 The ability of MCB to monitor changes in reduced glutathione concentration in erythrocytes was demonstrated upon the addition of 20 μM diamide to a 1% HCT sample of RBCs. The first bar in the graph is the reading for no diamide added and thus represents the amount of GSH originally present before the addition of the oxidant. As expected, the GSH levels deceases immediately after the addition of oxidant insult, but returned to the near initial after about 30 minutes.

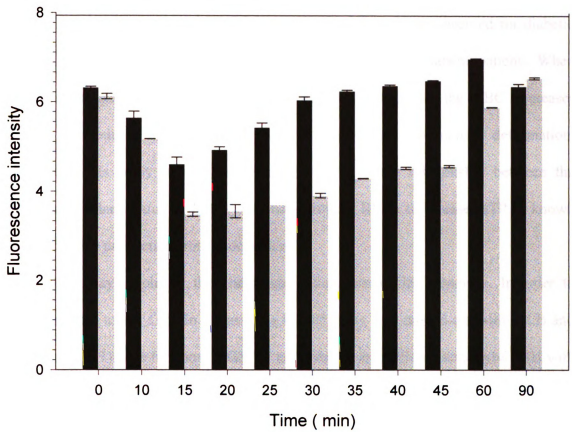


Figure 2.10 The effect of concentration of diamide on GSH regeneration ability was demonstrated in 20 and 40 μM diamide concentrations. The black bars represent the GSH levels of RBCs in 20 μM diamide, while gray bars represents the GSH levels of RBCs in 40 μM diamide. GSH regeneration ability was delayed in 30 minutes for 0.1% RBCs incubated with 40 μM diamide compared to 0.1% RBCs incubated with 20 μM diamide.

Moreover, the results shown in figure 2.9 and 2.10 greatly contributed to our understanding of the effect of oxidant stress on the RBC ATP release ability. The change of GSH levels and ATP release from RBC were observed in the presence of diamide and followed the same trend for over 45 minutes. Specifically, there was a rapid decrease of both GSH levels and the ATP release from RBCs after 15 minutes of incubation with diamide. These results also suggest that the reduced ATP release observed for diabetic RBCs may be due to the weakened antioxidant systems found in diabetic patients. When the RBCs are subjected to oxidant stress, the deformability of the RBC decreases resulting in reduced ATP release from RBCs under mechanical deformation. Collectively, this study was the first demonstration of the relationship between the antioxidant oxidant status in RBCs and the ability of RBCs to release ATP, a known stimulus for NO production from endothelium.[18]

This assay employed the glutathione-S-transferase (GST) enzyme. In order to demonstrate the use of GST in the assay, a kinetic study was carried out with MCB and GSH (figure 2.11). The function of GST is to catalyze conjugation reactions of GSH with different electrophiles present in the cells. This enzyme is important in detoxification reactions due to its ability to conjugate sulfydryl groups with other free radicals and reactive oxygen species. In this assay, the use of GST increases the reaction rate of MCB and GSH in RBCs allowing lower incubation time with the fluorophore. The average time of incubation of MCB with GSH is ~18 min, but in the presence of 2.5 U/μl of GST enzyme, the incubation time was decreased by 50%. This is important in evaluating the redox status of GSH upon oxidant attack as GSH regeneration can be observed within 20 minutes of incubation time with diamide. Furthermore, this assay can be employed in

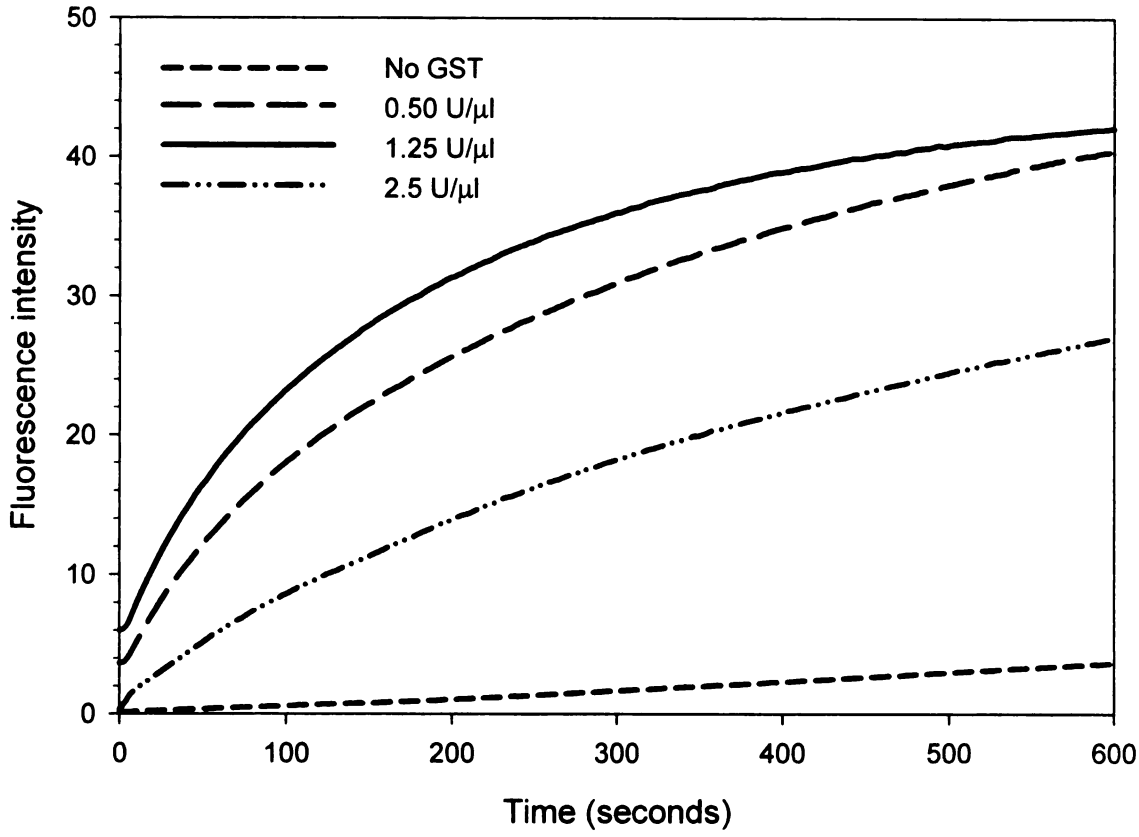


Figure 2.11 A kinetic study demonstrating the importance of GST in quantifying GSH in erythrocytes. The change of fluorescent intensity as a function of time is shown for three GST concentrations (0.5-2.5 U/µl).

continuous flow measurements. The continuous flow method will be able to monitor the redox status of glutathione at a rate that is limited by the data acquisition frequency. Also this assay is amenable to microchip studies and continuous flow studies that are important in evaluating the redox status of RBCs in disease status.

Such a dynamic measurement scheme is important in when attempting to measure the effects of oxidant stressors on RBCs, especially considering that patients with type 2 diabetes have been reported to have lower levels of GSH in their RBCs in comparison to healthy controls.[4] Moreover, recently, it has been shown that oxidative stress has a direct effect on the ability of erythrocytes to release NO stimulating ATP.[4] In fact, the trends in the ability of erythrocytes to release ATP upon mechanical deformation were similar to the ability of GSH to be remained in its antioxidant form or reduced form of GSH.[4]

2.7 Conclusions

GSH, the most abundant non-enzymatic antioxidant in RBCs, has been determined in both static and flow systems. Although GSH has been determined using numerous techniques, the work presented here represents the first quantitative determination of GSH using a bimane probe without any prior separation step.[4] However, cells are dynamic and homeostatic systems and will attempt to return to their pre-oxidative attack conditions. Therefore, it is imperative that the measurement of GSH oxidation to the GSSG dimer occur in near real time with the method that is fast and simple.[4] This type of monitoring would not be possible with any type of system that required a discrete sampling or injection like HPLC or CE.[4] Moreover, this assay system

was employed in establishing the importance of antioxidant status of erythrocytes in the ability of the erythrocytes to release ATP, a known nitric oxide stimulus.

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CHAPTER 3

Modulation of erythrocyte derived ATP release in response to hydroxyurea and nitric oxide

3.1 Hydroxyurea on vasculature

Sickle cell disease (SCD), an autosomal recessive disorder, is one of the most prevalent genetic disorders in the world.[1] It was the first genetic disease to be identified at the molecular level, with the single amino acid mutation of glulamate to valine in the β sub unit of hemoglobin resulting in the formation of a new hemoglobin molecule called hemoglobin S (HbS). This abnormal HbS alters the quaternary structure of hemoglobin in deoxygenated conditions resulting in the sickle shaped formation of the RBCs. Sickling of RBCs is responsible for the majority of the pathogenic events that result in the clinical aspects of the disease.

Even though it was the first disease to be identified at the molecular level in 1949, until the 1990s there was essentially no therapy for improving the complications associated with the disease. In 1995, Charache *et al* and Schechter *et al* showed the possibility of using hydroxyurea as a new therapeutic agent in the management of SCD.[2-4] Since then, hydroxyurea has been used as a clinically proven therapy for SCD in variety of situations.[5]

Hydroxyurea, a hydroxylated derivative of urea, is an inhibitor of the ribonucleotide reductase enzyme. It is water soluble and also easily absorbs into the

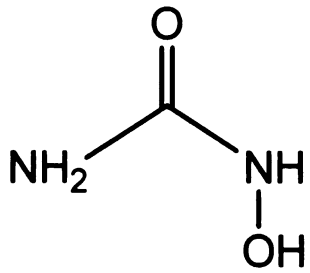

Figure 3.1 Structure of hydroxyurea

body and hence therapeutically easy to administer. The prime rationale of the beneficiary effects of hydroxyurea in SCD is its ability to induce fetal hemoglobin (HbF) levels in RBCs.[6]

The increase in HbF alters the kinetics and thermodynamics of HbS polymerization, thereby reducing the probability of sickling of the RBCs in deoxygenated conditions.[7] Several studies have suggested the possible mechanism of the augmentation of HbF upon hydroxyurea treatment. However, the exact mechanism of action still needs to be elucidated. Nevertheless, hydroxyurea appears to have many other beneficial effects in SCD patients through other possible mechanisms. The main evidence for this explanation comes from the clinical studies carried out in patients undergoing hydroxyurea treatment. Specifically, the main characteristics of SCD are painful vasoocclusive crisis, acute chest syndrome, and hemolytic anemia. Interestingly, many clinical improvements have been observed in patients undergoing hydroxyurea treatment even before any detectable rise in HbF in the RBC. Conversely, clinical improvements

have been observed upon hydroxyurea treatment have diminished upon discontinuing the treatment even though HbF levels are significant in the blood stream. These observations suggest other possible pathways of action for hydroxyurea in SCD.

Membrane damage is often associated with the sickling process and the damaged membrane is thought to increase adherence of RBCs to the endothelial cells and damage the endothelium.[3] Abnormal interactions between sickled RBCs and endothelial cells are the primary factors in instigating the microvascular occlusions in SCD, leading to painful vasoocclusive crisis.[8] One of the important benefits of hydroxyurea treatment in SCD, other than the HbF increase, is the reduction in red cell- endothelial cell interaction. Erythrocytes from hydroxyurea treated patients have a reduced adhesion to the endothelium and reduced expression of cytokines and other adhesion molecules.

Sickled RBCs are more susceptible to lysis; hence hemolytic anemia cell lysis is a widespread symptom of SCD. Apart from that, these cells are less deformable than those obtained from healthy controls and contribute to the abnormalities in blood flow. These factors indeed contribute to the major pathophysiological complications associated with the disease. Chronic hemolytic anemia, vasoocclusive crisis and acute chest syndrome are among the wide spectrum of clinical manifestations of the disease and all these symptoms share the rheological abnormalities of the vascular system. As a result, SCD is considered a disease of the circulatory system, even in the presence of its genetic basis. Interestingly, hydroxyurea is known to improve rheological characteristics such as increased red cell deformability, improved RBC hydration and reduced red cell density.[9] Zoumbos *et al* have demonstrated the improved deformability of red blood cell membrane upon treatment with hydroxyurea.[10] In contrast, an *in vitro* study performed by

Kim-Sharpio *et al* have demonstrated reduced RBC deformability at very high concentrations of hydroxyurea.[11]

Reduced NO bioavailability in SCD is an important component of the abnormalities associated with vascular function.[12] Intracellular HbS polymerization destabilizes the RBC membrane, resulting in RBC lysis and increased cell free hemoglobin content in the plasma. Decreased levels of NO in the vasculature result from the fast reaction of NO with cell free hemoglobin and reactive oxygen species.[1] Hydroxyurea has an effect on increasing the bioavailability of NO in SCD. It is known to exert some of its effects via the formation of NO.[13-15] Patients who undergo hydroxyurea treatment have higher levels of NO metabolites in the blood stream, suggesting the possibility of NO formation by hydroxyurea. However, the mechanism of NO formation by hydroxyurea is still not clear and requires further investigation

3.2 Nitric Oxide on vasculature

Nitric Oxide (NO) has been recognized as an ubiquitous intra and extra cellular signaling molecule in diverse pathophysiological processes such as cardiovascular homeostasis, neurotransmission and non specific immune responses. This small, diatomic free radical is considered as one of the most important signaling molecules in the cardiovascular system. It is well established that NO is involved in vascular homeostasis by regulating the vascular resistance, smooth muscle relaxation and inhibiting RBC and platelet activation, aggregation and adhesion in the circulatory system.[16.17]

NO is synthesized in the vascular endothelium in response to physiological stimuli and contributes to ~ 20-30% of the basal human blood flow.[17] NO synthesized in

endothelial cells can be released both abluminally and luminally.[18] When released abluminally, NO can interact with the vascular smooth muscle cells underlying the endothelium, stimulating vascular relaxation, and thereby resulting in an increase in vascular caliber. NO released luminally, which is released into the vessel, can interact directly with the components of the blood and thereby contribute to several physiological events.[18,19]

NO is produced enzymatically by the nitric oxide synthase (NOS) protein and requires five cofactors; flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (H_4B), nicotinamide adenine dinucleotide phosphate (NADPH) and a heme group for its full activity.[20] Among the three NOS isoforms present in mammalian cells, endothelial NOS (eNOS) and neuronal NOS (nNOS) require activation by the calcium – calmodulin complex. In the endothelium, NO is generated during the conversion of L- ariginine to L-citrulline by NOS upon activation by the calcium – calmodulin complex. Synthesis of NO by endothelial cells is a tightly regulated process and significantly contributes to regulation of the vascular caliber.

In addition to the production of NO by endothelial cells, human RBCs do contain both inducible and constitutive forms of NO synthase genes, and thus have the capability of producing their own NO and modulating several functions.[21,22] Therefore, it has been suggested that RBC derived NO has a contributory role in regulating the physiological behavior of the RBC as well as contributing to the intravascular NO pool. Therefore, NO can be considered as an intra- and extracellular signaling molecule that mediates several physiological functions in regulating the vasculature.

The bioavailability of NO in the vasculature depends on the NO diffusing from the endothelium and NO produced by other cells. However, the high reactivity between hemoglobin and NO was determined to be the reaction that resulted in a decreased bioactivity of NO in the vascular system. In addition, several studies have shown the encapsulation of hemoglobin in RBCs and the RBC free zone near the vessel wall have dramatically reduced the consumption of NO by hemoglobin compared to the reaction rate of cell free hemoglobin with NO and thereby provide evidence for the preservation of bioactivity of NO in the vasculature.[23,24] It has been experimentally shown that NO reaction with RBC encapsulated hemoglobin is ~1000 times slower than the reaction with cell free hemoglobin.

Apart from the diffusion barrier model of NO preservation in the cardiovascular system, there has been another mechanism that explains the preservation of NO in the vasculature. The formation of nitrosylhemoglobin [HbFe(II)NO] and S-Nitrosohemoglobin (SNOHb) in RBCs is of current interest, suggesting that NO is the third molecule transported by hemoglobin in the vasculature.[25,26] However, while this conservation of NO by reaction with hemoglobin is still attractive, it is a controversial concept. According to the SNOHb model, NO reacts first with the deoxygenated hemoglobin in oxygenated RBCs forming [HbFe(II)NO], which as has no vasodilator activity. NO is then transferred to cystein -98 residue of the β subunit of hemoglobin upon oxygenation to form SNOHb. NO can be released by SNOHb under deoxygenated conditions in an allosteric mechanism, thereby preserving the NO bioavailability in the vasculature.[17,27] SNOHb is stable in oxygenated RBCs, but easily releases NO in deoxygenated environments, thereby resulting in its RBCs vasodilator potency.[28] The

release of NO operates through the anion exchanger protein AE1 at the RBC membrane/cytosol interface.

Despite the controversies of NO reactivity preservation in the vasculature, it is well established that NO plays a central role in the cardiovascular system. Impaired release of NO by RBCs and endothelial dysfunction can limit the bioavailability of NO in the vascular system and is known to have direct implications in several disease conditions. Impaired release of NO by the RBC or dysfunction of RBCs NO synthesis by the RBC have been associated with the pathogenesis of hematologic, thrombotic, and ischemic disorders.[26] More specifically, RBC-NO processing impairment has been associated with diabetes,[29] SCD,[30] pulmonary hypertension,[31-33] and heart failure.[34] Detailed experimentation of disease conditions have suggested the possibility of impaired vasodilation by RBC derived NO or different amounts of SNOHb in RBCs. It is evident from studies that irregulation of blood flow may originate at different stages, and the concentrations of the NO, or the bioavailability of that NO, result in pathological complications.[26] Finally, it is now established that RBCs provide a novel vasodilator activity by producing NO via the NOS pathway and preserving NO via SNOHb formation.

3.3 Effect of hydroxyurea and NO on erythrocyte derived ATP release

It has been reported that ATP released from RBCs in response to several physiological stimuli such as mechanical deformation and hypoxia is a stimulus for endothelial derived NO synthesis.[35,36] The released ATP from RBCs can bind to the P2Y

receptors on the vascular endothelial cells, thereby stimulating the production of NO in the endothelial cells. The produced NO can diffuse in to the underlying smooth muscle layer where it can participate in the regulation of vascular caliber. Therefore, RBC derived ATP is considered as a determinant of regulating vascular resistance.[37]

RBC deformability plays a major role in proper functioning of the RBCs in relation to efficient oxygen and carbon dioxide distribution in the microvasculature. The ability of RBCs to release ATP in response to mechanical deformation and its importance in vascular resistance have led to the exploration of small molecules, metabolites or drugs that alter the deformability of RBCs. These molecules may open up a new field of current medical research for potential therapeutic applications in rheological abnormalities associated with disease conditions.

Hydroxyurea is the currently the only FDA approved treatment for patients with sickle cell anemia. The improvements of rheological properties such as increased cellular deformability and improved blood flow have been observed in patients undergoing hydroxyurea treatment. There are also several studies associated with RBC deformability and hydroxyurea. This relationship between hydroxyurea and RBC deformability has inspired us to investigate the effect hydroxyurea on RBC derived ATP release. However, there is no exact mechanism to demonstrate the ability of hydroxyurea in regulating blood flow or rheological properties in sickle cell patients. NO is another important molecule and is recognized as a potent endogenous vasodilator. Several researches have suggested that NO modulates RBCn deformability.[21, 38-40] Korbut *et al* have shown that NOS synthase inhibitors such as N-nitro-L-arginine methyl ester (LNAME) have an effect on erythrocyte deformability in septic shock.[38, 41]

In addition, it has been shown by the same authors that RBC deformability was influenced by polymorphonuclear leucocytes through a mechanism involved in NO release.[39] Furthermore, NO donors such as spermine NONOate have been shown to be involved in increased deformability of RBCs. In contrast, there has been a study showing reduced RBC deformability in response to NO.[42] These contradictory findings have taken attention away from the potential significance of NO in the microcirculation. Moreover, these inconsistencies led us to explore more closely the effect of NO in RBC mechanical properties. Also, the relationship of hydroxyurea and NO in RBC deformability provided new insights and inspired studies to examine the effect of these small molecules on RBC derived ATP release.As both hydroxyurea and NO have the ability to modulate RBC deformability, it is possible to hypothesize that hydroxyurea and NO can modulate the RBC derived ATP release and there by contribute to the rheological properties of erythrocytes.

3.4 Chemiluminescence measurement of deformation induced ATP release from RBC

Chemiluminescence is the emission of electromagnetic radiation as a result of a chemical reaction. When the light emitting reaction is arising from a living organism, it is commonly known as bioluminescence. Fire flies (*Photinus pyralis*) emit light by the reaction of luciferin and molecular oxygen. The rate of this reaction is very slow, thus luciferase, a bioluminescence producing enzyme, in fire flies catalyzes the reaction with the use of ATP. This enzymatic reaction is specific for ATP and has high quantum efficiency. Therefore, luciferin/luciferase reaction with ATP can be employed to

quantitatively determine reactions in biological samples. As shown in figure 3.1 luciferin and ATP combine at the active site of the luciferase forming luciferyl-AMP intermediate. Molecular oxygen reacts with the luciferyl-AMP forming an excited luciferyl -AMP* state. Upon relaxing, the excited intermediate emits a visible range light (green glow). The chemiluminescence intensity is proportional to the amount of luciferin molecules excited.

The attractiveness of chemiluminescence as a detection technique in bioanalytical applications is its simplicity. The reaction does not require any light source other than the light emitting reaction, making chemiluminescence a simple technique with no interference from an excitation source with high sensitivity and good limits of detection. The simple instrumentation used to detect the emitted light makes the assay robust and easy to use. A photomultiplier tube (PMT) is the most widely used detector in chemiluminescence. Good sensitivity, broad dynamic range and applicability to a wide range of reactions have made the PMT a traditional chemiluminescence detector. The use of chemiluminescence, in concert with a model that mimics the RBC circulation in the arterioles and capillaries, has been employed to determine the ATP release from RBCs in response to mechanical deformation.[43-45] Continuous pumping of RBCs through silica microbore tubing is close to the physiological conditions, thereby exposing RBCs to a mechanical stress that would be similar in the circulation. This novel microflow technique has advantages over the previously employed filtration method used by Sprague *et al* to quantify RBC derived ATP release. The filtration technique employs vacuum induced flow of RBCs through a membrane filter to place mechanical stress on RBCs. The release of ATP through filtration is detected using the same ATP assay, but in

an "off line" manner. This may lower the detection limits and lower the sample throughput.[44]

In contrast, the method employed in this study was able to monitor the chemiluminescence intensity in near real time over a longer period of time. This is advantageous in monitoring the real time changes in ATP release from RBCs in response to physiological stimuli. Also this method enabled a more clear understanding of the parameters that affect RBC derived ATP release *in vivo* as it mimics the internal circulation in a *in vitro* platform that allow understanding of the change of rheological properties on RBC derived ATP release.[46]

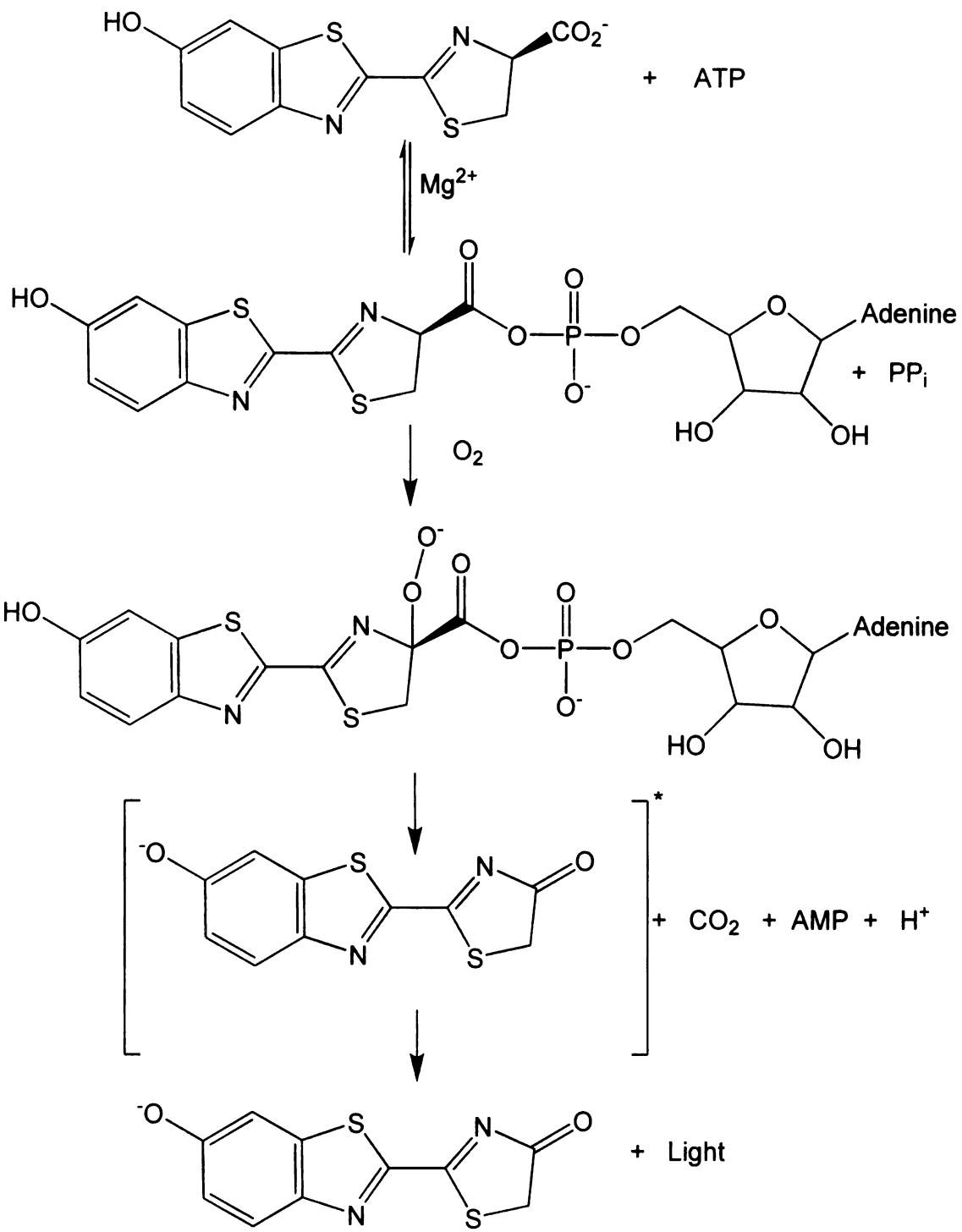

Figure 3.2 Schematic mechanism of the chemiluminescence generation from luciferin and luciferase in the presence of ATP. Note the importance of molecular oxygen in the generation of the intermediate of excited oxyluciferin.

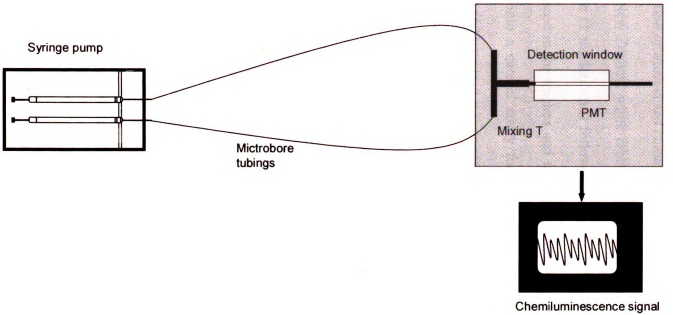

Figure 3.3 A Schematic representation of the instrumentation used to quantify the deformation induced ATP release from erythrocytes. RBCs are pumped through two different silica microbore tubing where the RBCs are deformed. The RBCs mix at the T junction emitting the light by the chemiluminescence.

3.5 Experimental

3.5.1 Methods and materials

Generation of washed red blood cells

Male, New Zealand white rabbits were anesthetized with ketamine (8 ml/kg, i.m.) and xylazine (1 mg/kg, i.m.) followed by pentobarbital sodium (15 mg/kg i.v.). A cannula was placed in the trachea and the animal was ventilated with room air. A catheter was placed into a carotid artery for administration of heparin and for phlebotomy. After heparin (500 units, i.v.), animals were exsanguinated. Human blood was obtained by venipuncture without the use of a tourniquet (antecubital fossa) and collected into a heparinized syringe. Blood was centrifuged at 500 x g at 4°C for 10 min. The plasma and buffy coat was discarded. RBCs were resuspended and washed x 3 in a physiological salt solution [PSS; in mM, 4.7 KCl, 2.0 $CaCl_2$, 140.5 NaCl 12 $MgSO_4$, 21.0 tris(hydroxymethyl)aminomethane, 11.1 dextrose with 5% bovine serum albumin (final pH 7.4)]. Cells were prepared on the day of use.

Preparation of ATP standards

All the reagents were purchased from Sigma chemical company, St. Louis, MO unless otherwise noted. A 100 μM stock solution of ATP was prepared by adding 0.0551 g of ATP to 1000 ml of distilled and deionized water (DDW). The standard solutions (0, 25, 50, 100, 150 nM) were made by adding 0, 67.5, 125.0, 187.5, 250.0, and 375.0 μl of 100 μM ATP stock solution to PSS, respectively. All the standards were prepared in 25

ml volumetric flasks on the day of use. PSS was prepared by first combining 25 ml of Tris buffer and 25 ml of Ringer's buffer and then adding 0.50 g of D-Glucose and 2.50 g of bovine albumin fraction V (fatty acid free). The entire solution was then diluted to 500 ml by adding DDW and adjusted the pH to 7.35-7.45. The PSS solution was filtered three times using a 0.45 μm filter (Corning, Fisher Scientific) before use.

Preparation of Luciferin-Luciferase reaction mixture

Firefly extract containing luciferin and luciferase was dissolved in 5.0 ml of DDW; an additional 0.002 g of synthetic luciferin was added to the firefly mixture to enhance the sensitivity. Chemiluminescence intensity generated by ATP and luciferin-luciferase reaction was measured with a PMT.

Other Reagents

Spermine NONOate: Spermine NONOate solution was prepared by dissolving 1.0 mg of spermine NONOate (Cayman Chemicals, Ann Arbor, MI) in 1 ml of 0.20 M NaOH. The concentration of the resulting spermine NONOate solution is 38 mM. The ranges of concentrations were prepared by adding different volumes of the stock solution in deoxygenated PBS 7.4 buffer.

Hydroxyurea: For studies involving hydroxyurea, a 4 mM hydroxyurea solution was prepared by dissolving 0.004 g of hydroxyurea (Sigma Aldrich, St. Louis, MO) in 25.0 ml of PSS. The range of concentrations (0 – 200 μM) were made by diluting appropriate volumes (0 – 500 μl) of the stock solution in a solution of 7% RBCs made in PSS. When

preparing the hydroxyurea treated RBCs, hydroxyurea was first added to PSS before adding RBCs to create a homogenously treated RBC solution. RBC samples were then incubated for 20 minutes at room temperature before the measurement portion of the analysis.

Diamide: For studies involving diamide, a 2 mM stock solution was prepared by dissolving 0.0344 g of diamide in 100 ml of DDW. The final concentration of 20 μM diamide was made by adding 1.0 ml of 2 mM diamide in 9.0 ml of PSS.

3.5.2 Instrumentation

Chemiluminescence measurement of deformation induced RBC derived ATP release

All RBC solutions were diluted to a hematocrit of 7% in PSS. To measure the RBC derived ATP release, the luciferin- luciferase reaction mixture was placed in a 500 μl syringe (Hamilton, Fischer Scientific). The RBC solution was placed in another 500 μl syringe and both syringes were loaded on to a syringe pump (Harvard Apparatus, Boston, MA) and connected to two 25 cm sections of silica microbore tubing having an internal diameter of 50 μm and an outer diameter of 362 μm (Polymicro Technologies, Phoenix, AZ). The microbore tubes were then connected to a T-junction (Upchurch Scientific, Oak Harbor, WA) having a volume of 256 nl. The combined contents were passed through a 6 cm section of microbore tubing having an internal diameter of 75 μm. The polyimide coating was removed from the tubing and placed on the PMT. The resultant

chemiluminescence produced by the RBC derived ATP and the luciferin-luciferase reaction was detected by a PMT housed in a light excluding box (figure 3.3).

3.6 Results and Discussion

It is well known that RBCs release ATP in response to mechanical deformation. It has also been reported that the amount of ATP released from RBCs is proportional to the overall RBC deformability. The previous works by other groups have also demonstrated the effect of hydroxyurea on RBC deformability. However, there was no study that determines the ability of hydroxyurea to release ATP from RBCs. In order to investigate the effects of hydroxyurea on RBC derived ATP release, as well as the cellular deformability, a 7% solution of RBCs was incubated in PSS containing 50 μM hydroxyurea for 20 minutes. The resultant ATP release from RBCs was measured using the microflow technique described. As expected, an increased release of ATP was observed for 7% RBCs incubated with 50 μM hydroxyurea compared to the control 7% RBCs (figure 3.4).

This primary data obtained from figure 3.3 demonstrates that deformation induced ATP release from RBCs is increased when subjected to hydroxyurea treatment. RBCs typically contain millimolar levels of ATP in the cytosol. However, the above data does not eliminate the possibility that the released ATP is not due to RBC lysis.

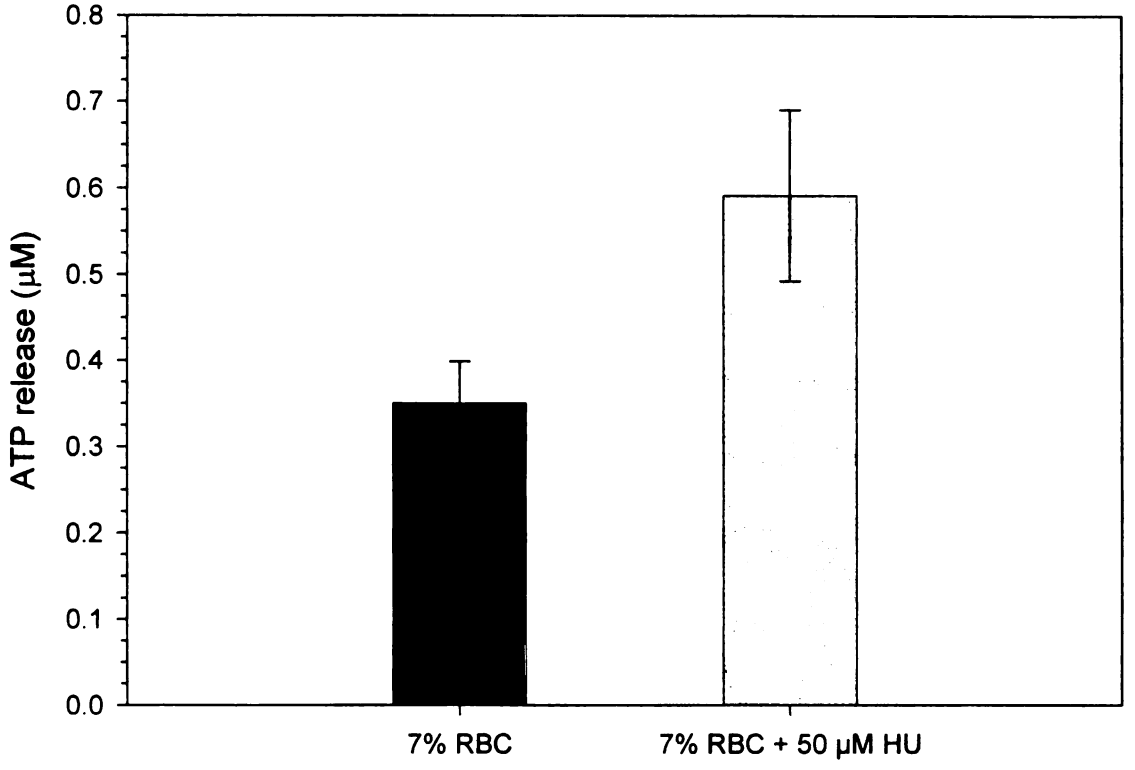

Figure 3.4 Quantitative determination of ATP release from RBCs in the presence of hydroxyurea using flow through chemiluminescence detection. The black bar represents the ATP release from a 7% hematoctrit of RBCs while the gray bar represents the ATP release from a 7% hematoctrit of RBCs incubated with 50 μM hydroxyurea for 20 minutes. The two bars are significantly different ($P < 0.03$) and error bars are reported as SEM for n = 8 rabbits.

Therefore, another experiment was designed to provide evidence that the increased ATP release was not due to RBC lysis. Glbenclamide, a known inhibitor of the CFTR protein, was employed to inhibit the signal transduction pathway of ATP release from RBCs. As CFTR is a required component of the pathway, the inhibition of the protein results in decreased ATP release. Figure 3.5 shows decreased ATP release from RBCs incubated with glybenclamide followed by hydroxyurea treatment compared to the RBCs incubated with hydroxyurea alone. A slight increase of ATP release from RBCs, compared to untreated RBCs, could be a possibility of partial inhibition of CFTR protein or the insufficient incubation time of RBCs and glybenclamide. If the cells have been lysed by the hydroxyurea treatment, one would still expected to see an increase in ATP release from RBCs incubated with 100 μM glybenclamide. The results from this study (shown in figure 3.5) confirm that the effect of hydroxyurea on RBC derived ATP release is not due to cell lysis.

The above experiments did not verify that the ATP release was deformation induced. Thus, another experiment was carried out to distinguish if the effect from hydroxyurea was deformation induced. Similar experimental conditions to those described above were followed for a non flow based measurement where the ATP release was determined after incubating the 7% RBC solution for 20 minutes in hydroxyurea without subjecting the RBCs to flow. Interestingly, hydroxyurea did not increase as much as RBC derived ATP release in the non flow system, compared to the flow based experiments suggesting the ability of hydroxyurea to modulate the RBC deformability (figure 3.7).

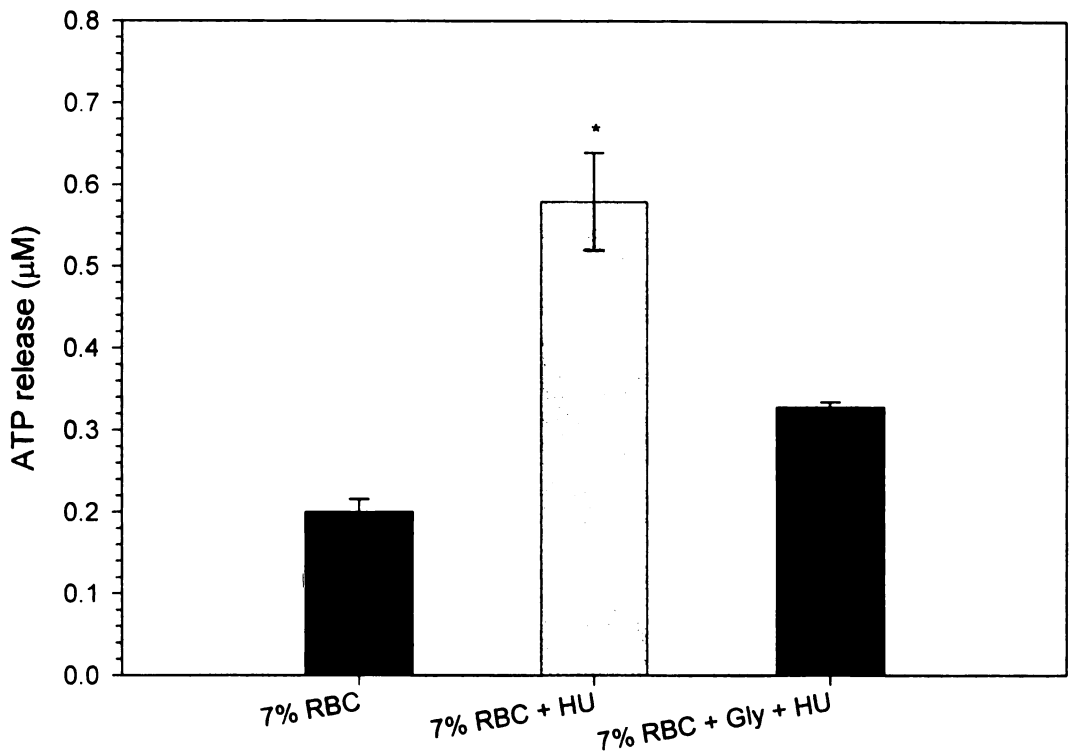

Figure 3.5 The effect of glybenclamide on ATP release from RBCs in the presence of hydroxyurea. The gray bar represents the ATP release from a 7% hematoctrit of RBCs incubated with 50 μM hydroxyurea while the dark gray bar represents the ATP release from a 7% hematoctrit of RBCs incubated with 100 μM glybenclamide followed by 50 μM hydroxyurea. The value of (*) is significantly different from others ($P<0.01$) and the error bars are reported as SEM for n = 5 rabbits.

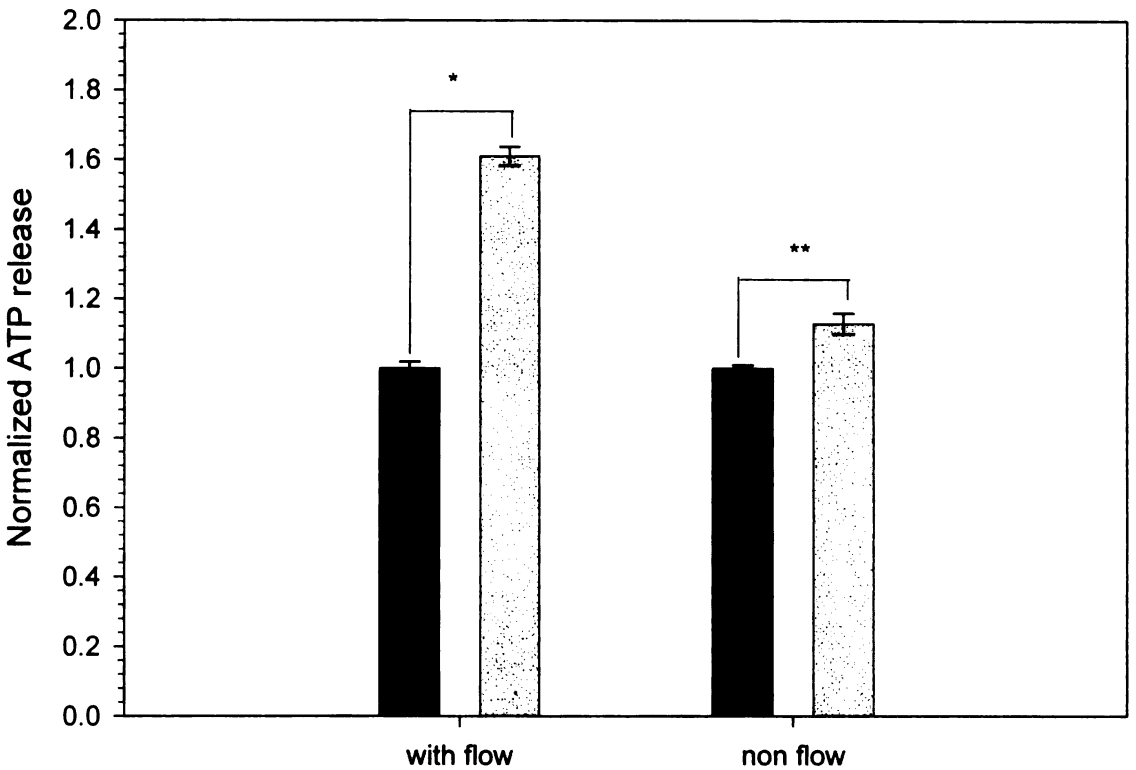

Figure 3.6 The increased ability of hydroxyurea to release ATP from RBCs under flow conditions. The black bars represent the ATP release from a 7% hematoctrit of RBCs while the gray bars represent the ATP release from a 7% hematoctrit of RBCs incubated with 50 μM hdroxyurea for 20 minutes. The bars corresponding to flow conditions (*) are significantly different ($P<0.001$). However, the bars denoted as (**) are different at $P<0.05$. Error bars were reported as SEM for $n = 4$ rabbits.

Encouraging results were obtained for hydroxyurea treated RBCs in vitro; therefore a series of experiments were carried out to determine the hydroxyurea concentration that resulted in the highest ATP release. The data in figure 3.7 and 3.8 show the effect of hydroxyurea concentration on RBC derived ATP release. RBC derived ATP release increased up to a concentration of 100 μM hydroxyurea in 7% RBCs. However, as shown, the release of ATP decreases, eventually reaching a value equivalent to the 7% RBC solution without hydroxyurea. This data was initially confusing, but repeated experiments indeed conferred the same observations. These data suggested the possibility of the NO forming ability of hydroxyurea, as NO has been shown to modulate the erythrocyte deformability. Many studies have indicated the ability of hydroxyurea to produce NO, but the mechanism of NO producing reactions by hydroxyurea is still not clear and somewhat controversial. However, the similarity between hydroxyurea and NO on cellular deformability led to a more detailed examination of the effect of NO on RBC derived release of ATP.

Initially, a 7% solution of rabbit RBCs were incubated with 100 nM spermine NONOate for 10 min followed by the quantitative determination of ATP. An increase in ATP release was observed for the 7% RBCs incubated with spermine NONOate compared to the control 7% rabbit RBCs incubated for 10 minutes in the absence of NONOate. (figure 3.9). The principle rationale behind this experiment was NO's ability to modulate the RBC deformability. Also, as stated above, the NO producing ability of hydroxyurea was taken into consideration as many proposed mechanisms were proposed for NO generation by hydroxyurea

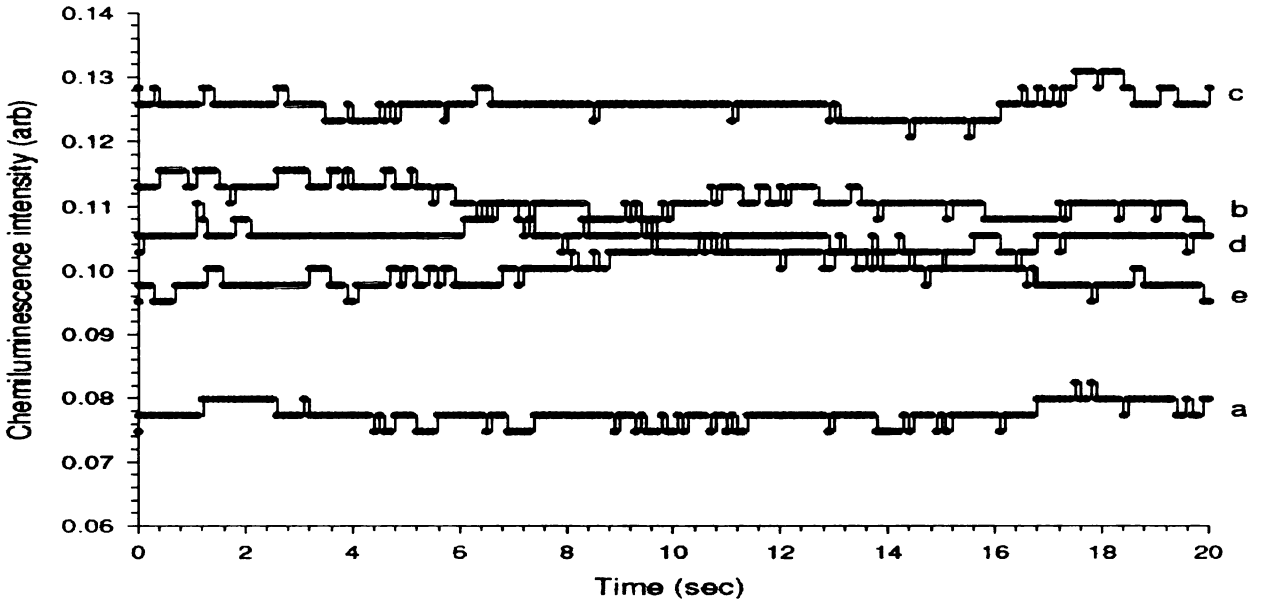

Figure 3.7 Chemiluminescence intensity change for RBC samples treated with varying concentrations of hydroxyurea. (a) is the chemiluminesence intensity recorded for 7% hematoctrit of RBCs reacted with luciferin-luciferase mix in the flow system while b, c, d, and e represent the 7% hematoctrit of RBCs incubated with 50,100,150,200 μM hydroxyurea, respectively. The signal intensities were taken at a rate of 10 points/sec during a 20 second time period.

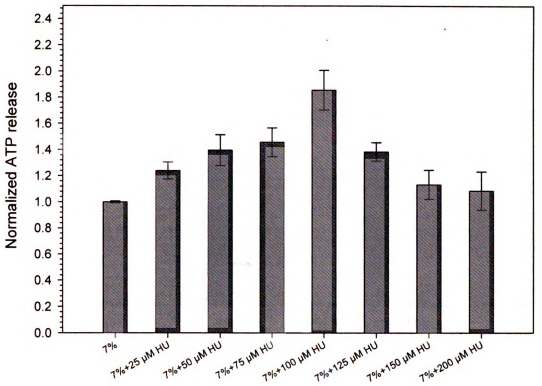

Figure 3.8 Effect of hydroxyurea concentration on deformation induced ATP release from RBCs. Highest ATP release was observed for 7% hematoctrit of RBCs incubated with 100 μM hydroxyurea for 20 minutes. After that, the ATP release started decreasing reaching to a value that was not significantly different from untreated RBCs at 200 μM hydroxyurea. The error bars are reported as SEM for n =7 rabbits.

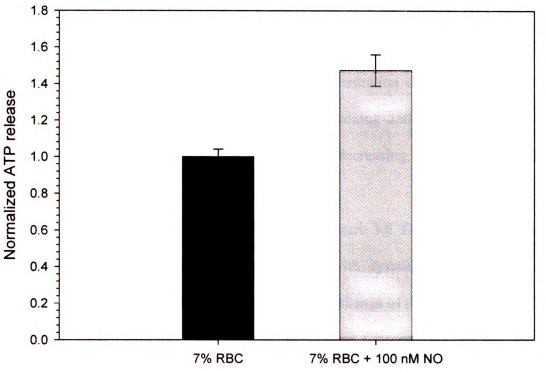

Figure 3.9 Quantitative measurement of deformation induced ATP release from 7% RBCs in the presence and absence of NO donor, spermine NONoate. The black bar represents the ATP release for untreated hematocrit of 7% RBC, while the gray bar represents the 100 nM NONOate treated hematoctrit of 7% RBCs. n=5 and the error or bars are reported as SEM.

The results obtained during the preliminary studies led to further experimentation involving NO and ATP release with different spermine NONOate concentrations. A series of 7% RBC samples were made using varying spermine NONOate concentrations (0 – 1000 nM) and incubated for 10 minutes. Deformation induced ATP released was measured using the luciferin- luciferase reaction. As shown in figure 3.10 and 3.11 the amount of ATP release from the RBCs was concentration dependant and the highest amount of ATP was released for the sample containing 250 nM spermine NONOate. Subsequently, the ATP release from RBCs started decreasing; reaching a value that was less than the normal release of ATP from 7% RBCs.

These data showed the same trend as in figure 3.8 The initial increase of ATP release may be due to the interaction of NO with cystein residues in hemoglobin (formation of SNOHb) leading to a conformational change of the hemoglobin. This could lead in deformational changes in the RBC membrane. Conversely, the decreased release of ATP from RBCs at higher spermine NONOate concentrations may be due to the higher oxidative stress caused by reactive nitrogen species in RBCs. Higher oxidant stress could damage the membrane properties; especially the oxidation of spectrin, which can reduce the RBC deformability.

The change in deformability was observed indirectly by measuring ATP release from RBCs. Therefore, diamide, a known oxidant, was used to oxidize cell membrane proteins such as spectrin. Upon oxidation, spectrin forms dimers making the cell membrane less deformable. The ATP release from RBCs is proportional to cell deformability; therefore, the same technique described previously can be

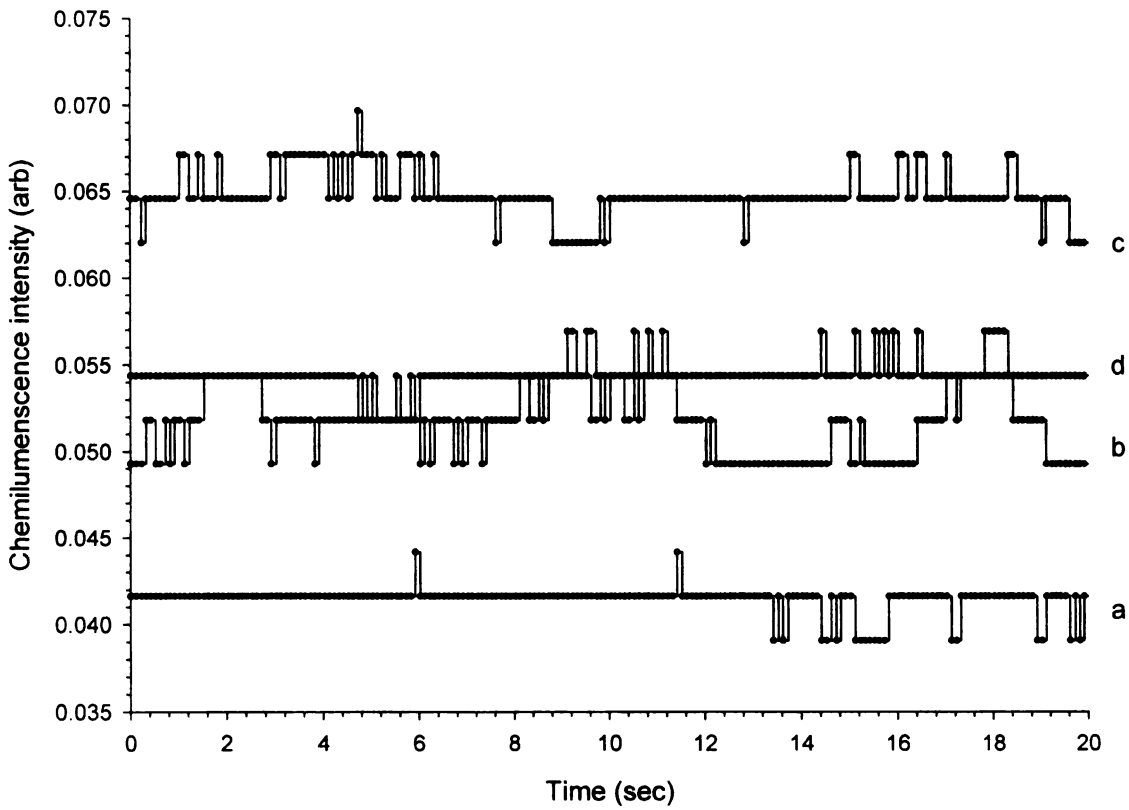

Figure 3.10 Chemiluminescence intensity change for RBC samples treated with varying concentrations of spermine NONOate. Spectrum (a) is the chemiluminesence intensity recorded for 7% hematoctrit of RBCs reacted with luciferin-luciferase mix in the flow system while spectra b, c, d, and e represent the 7% hematoctrit of RBCs incubated with 100, 250, 500 nM spermine NONOate, respectively. The signal intensities were taken at a rate of 10 points/sec for 20 seconds time period.

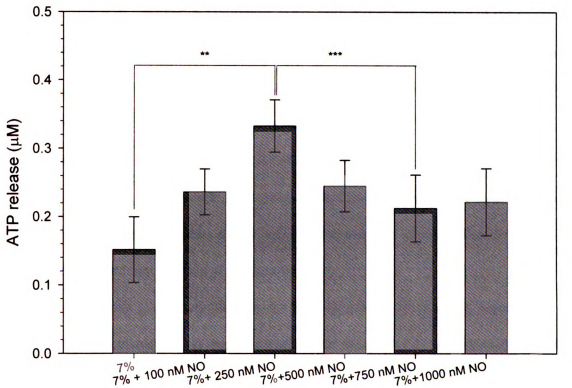

Figure 3.11 Effect of spermine NONOate on deformation induced ATP release from RBCs. The ATP release was initially increased gradually with increasing concentration of NO donor and the highest release was observed for 7% RBCs incubated with 250 nM Spermine NONOate. Then, the ATP release started decreasing gradually reaching to a point at 1000 nM where the ATP release was not significantly different from the initial untreated 7% RBCs. The two bars represented as (*) are significantly different in $P< 0.01$ and the two bars represented as (***) are significantly different in $P<0.05$. n = 4 rabbits and the error bars are reported as SEM

employed to investigate the effect of NO on diamide treated RBCs. The effect of NO on RBCs derived ATP release was not prominent when the cells were first incubated with 20 μM diamide followed by 100 nM spermine NONOate (figure 3.12).The chemiluminescence intensity was measured after a 10 minute incubation with the NO

In conclusion, both hydroxyurea and NO were shown to have an effect on modulating the RBC derived ATP release. Also, both molecules resulted in the release of different amounts of ATP from RBCs in response to mechanical deformation, and most interestingly, correlated perfectly. The increased ATP release was observed up to a certain concentration and then started decreasing. This study clearly demonstrates the relationship between hydroxyurea and NO on erythrocyte derived ATP release. However, the data do not provide evidence for a proper mechanism of action of hydroxyurea on ATP release. It suggests the ability of hydroxyurea to change the overall RBC deformability in a concentration dependant manner.

Hydroxyurea is a proven therapy for SCD because it helps in the reduction in the number of vasoocclusive crisis, hospitalization and the rate of blood transfusions. More over, it reduces the chances of having acute chest syndrome, the most dangerous complication of SCD. All these improved clinical complications could be related to improved blood flow. As ATP is a determinant in endothelial derived NO production and vasodilatation, the ability of hydroxyurea to release ATP from RBCs could be another mechanism of action of hydroxyurea in SCD patients. To date, it has been shown that hydroxyurea can improve the cellular deformability, hydration status and reduce the RBC- endothelial interactions. This study may be another attempt of

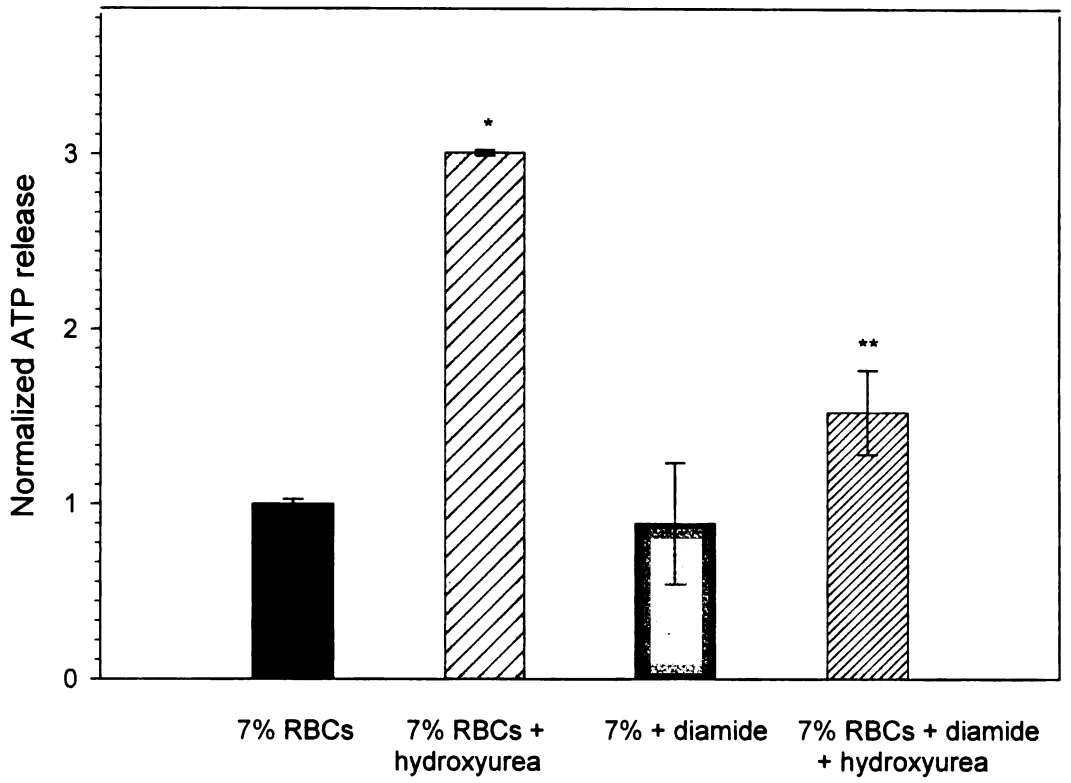

Figure 3.12 The effect of cellular oxidant, diamide on RBC ATP release in the presence of spermine NONOate. The black bar represents the ATP release for hematocrit of 7% RBCs while gray bar with medium lines represents the ATP release for 7% RBCs in 250 nM spermine NONoate. Obviously, the lowest ATP release was found to the 7% RBCs incubated with 20 μM diamide (dark gray bar). However, the ATP release is not increased significantly for 7% RBC incubated first with 20 μM diamide followed by 250 nM spermine NONoate. The (*) and (**) are significantly different in $P<0.001$ for n =3 rabbits and the error bars are reported as SEM.

demonstrating a possible relationship of hydroxyurea in relation to the improved blood flow in sickle cell patients undergoing hydroxyurea treatment. Further experimentation is necessary to conclude this novel mechanism of action described by the effect of hydroxyurea on erythrocyte ATP release.

3.7 Conclusions

It is well accepted that RBCs release ATP in response to mechanical deformation. There have also been reports demonstrating the effect of hydroxyurea on RBC deformability. However, this is the first study that demonstrates the effect of hydroxyurea on ATP release from RBCs. The effect is only seen in the flow system, where RBCs are subjected to continuous shear stress, suggesting a contributory role of hydroxyurea on RBC membrane properties. The modulation of ATP release from RBCs is dependant on varying hydroxyurea concentration. Moreover, the NO donor compound, spermine NONOate gave the same trend as hydroxyurea, suggesting a mechanism of NO formation by hydroxyurea within RBCs. The formed NO is thought to responsible of the observed ATP release from RBCs, as NO is known mediator of RBC deformability.

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CHAPTER 4

Effect of hydroxyurea on nitric oxide production in erythrocytes: A novel mechanism for hydroxyurea in sickle cell disease

4.1 Sickle cell disease and nitric oxide bioavailability

The pathophysiology of sickle cell disease has been traditionally characterized by the polymerization of hemoglobin S (HbS) in erythrocytes under deoxygenated (hypoxic) conditions resulting in the occlusion of blood vessels.[1] The occlusion of blood vessels, or commonly known as vasoocclusion can contribute to pain crisis, acute chest syndrome, organ dysfunction and in severe situations, death. In contrast, recent studies have shown that there are other pathological complications associated with the severity of the disease in addition to HbS polymerization.[2] One such condition is the deficiency of the endogenous vasodilator, nitric oxide (NO) in the vasculature.[3-5]

NO, a gas at room temperature, is a small diatomic free radical. The solubility of NO is 1.9 mM/atm at 25 °C in aqueous solutions, but increases 6-8 fold in non polar environments such as in cellular membranes.[6] The smaller size and the neutrality enable it to diffuse through cellular structures at a rate of 3300 $\mu m^2/s^{-1}$.[7] NO is also a highly reactive free radical and its half life depends on the initial concentration and the composition of the cellular system. Typically, the half life is 1-3 s in physiological solutions, although several studies have indicated a longer half life in physiological conditions. The high diffusibility and the short life time have made this molecule suitable

as a signaling molecule in various physiological processes. Therefore, NO dependant vasodilatation is one such important process in vascular homeostasis.

NO is synthesized continuously in the endothelium from L-arginine and is catalyzed by the eNOS. NO released from the endothelium can diffuse into the underlying smooth muscle cells and thereby activates smooth muscle relaxation process. In smooth muscle cells, NO first binds to the heme group of soluble guanylyl cyclase and triggers the production of cyclic guanylyl mono phosphate (cGMP) which in turn activates the cGMP dependant protein kinases (PKs). Activation of PKs results in the decreased levels of the intracellular calcium ion concentrations and this whole process results in smooth muscle relaxation.[8,9] Therefore, NO, produced by the endothelium, plays a central role in vascular homeostasis by contributing to the control of normal vascular tone. In addition, NO is involved in reduced platelet aggregation and reduced RBC adhesion in the vascular system contributing to smoother blood flow.

However, as mentioned above, patients with SCD suffer from reduced NO bioavailability. Therefore, NO related mechanisms in the vascular system, i.e., regulation of blood flow, reduced interactions of RBCs with the endothelium[10,11] and inhibition of platelet aggregation are also affected in patients having SCD. All of these conditions have been associated with an increased occurrence of several vascular complications such as vaso occlusive crisis, acute chest syndrome,[12] persistent pulmonary hypertension,[13]etc,. Moreover, all of these complications are known to be an effect of reduced bioavailability of NO in the vascular system and endothelium dysfunction and the increased consumption of NO by cell free hemoglobin are the known causes of reduced NO bioavailability.

Endothelium dysfunction in SCD is the inability of the endothelium to produce NO by the NOS pathway.[14] This may be due to low plasma arginine levels or increased arginase activity in the plasma of SCD patients.[15] Arginase is predominant in young RBCs. Higher production of young RBCs and the higher hemolysis rate of RBCs in SCD could increase the plasma arginase levels, thereby deplete the arginine reserves in the plasma of SCD patients. The depletion of arginine may affect the synthesis of NO by the endothelium as arginine is the substrate for the enzymatic synthesis of NO in the endothelium.[3]

Moreover, the consumption of NO by cell free hemoglobin is considered as the main reaction of decreased levels of NO in SCD patients.[4] The rate of hemolysis of RBCs is high in sickle RBCs due to their abnormal shape, rigidity of the membrane (decreased deformability) and the increased adhesiveness to the endothelium. In contrast, NO bioavailability is preserved in the normal vasculature due to the laminar flowing blood and the compartmentalization of hemoglobin inside RBCs. The cell free hemoglobin reacts 1000 times faster than the hemoglobin in RBCs. In fact, SCD patients have higher levels of cell free hemoglobin in the plasma, and thus consume NO rapidly, dramatically reducing the NO bioavailability. Therefore, the increased hemolytic rate provides the resistance to NO dependent blood flow in SCD patients.

Both factors described above, i.e. NO scavenging by hemoglobin and the endothelial dysfunction, have a synergistic effect on NO bioavailability and play an important role in vascular instability when the patients undergo vascular crisis or acute chest syndrome where the hemolysis rate is even higher compared to steady state SCD. In

addition to vascular instability, patients may suffer pulmonary hypertension, priapism, stroke, and leg ulcers depending on the location of impaired bioavailability occur.

Considering the factors that affect NO bioavailability in SCD patients, several therapeutic approaches have been under investigation to restore the NO levels in plasma. One approach is to directly deliver the NO and NO donor compounds to the circulation. Inhalation of NO gas is a FDA approved treatment but requires specialized handling and administration because of the noxious effects of that gas at higher concentrations. Also, some studies have shown the use of promising NO pharmaceutical, sodium nitrite as the NO donor, where the reaction takes place in RBC hemoglobin-derived nitrite reductase activity. Another possible therapy would be the arginine supplementation where the NO availability is enhanced by the increased NOS activity.[1,16]

In fact, it has been suggested that hydroxyurea, the clinically proven treatment for SCD, has an additional role as a NO donor in treatment of sickle cell disease.[17,18] However, the mechanism of NO production *in vivo* by hydroxyurea is not very well understood and is currently under thorough investigation. However, it is a possibility that clinical improvements, especially the increased rheological properties observed in patients that undergo hydroxyurea treatments, are mediated by NO induced vasodilatation or reduced platelet activation.

4.2 NO forming reactions of hydroxyurea

Primarily, the interests of investigating the possible mechanisms of hydroxyurea on SCD by means other than induction of HbF levels, arrived from the clinical

improvements observed from SCD patients, receiving hydroxyurea before any rise in HbF levels in the blood. Moreover, the NO forming ability of hydroxyurea has been proposed as a secondary, but a beneficial effect of hydroxyurea on SCD patients. However, the exact mechanism of NO formation from hydroxyurea is still not clear and under investigation.

4.2.1 *In vitro* NO formation from hydroxyurea

The molecular structure of hydroxyurea, which contains a N-O bond, has led investigators to focus the attention on researching the NO donor properties of the hydroxyurea molecule. It is known that the formation of NO from hydroxyurea requires oxidation of the molecule. Considering this fact, several mechanisms have been proposed for the formation of NO via oxidation of the hydroxyurea molecule and are shown in figure 4.1. Pathways 1 and 2 occur through the one electron oxidation of hydroxyurea forming the nitroxide radical intermediate. Pathway 3 occurs through the formation of hydroxylamine followed by three electron oxidation producing NO. Supporting the proposed mechanisms, hydroxyurea has been shown to react with variety of oxidants *in vitro* to form nitroxide or the NO free radical. Among them, the reaction of hydroxyurea with heme proteins, hydrogen peroxide and Cu-Zn-SOD have been thouroughly studied.[19]

Several research groups have shown the ability of heme groups to oxidize hydroxyurea to the NO free radical under *in vitro* conditions.[20-23] Stolze *et al* first reported the methemoglobin and nitrosylated hemoglobin (HbNO) formation from

A

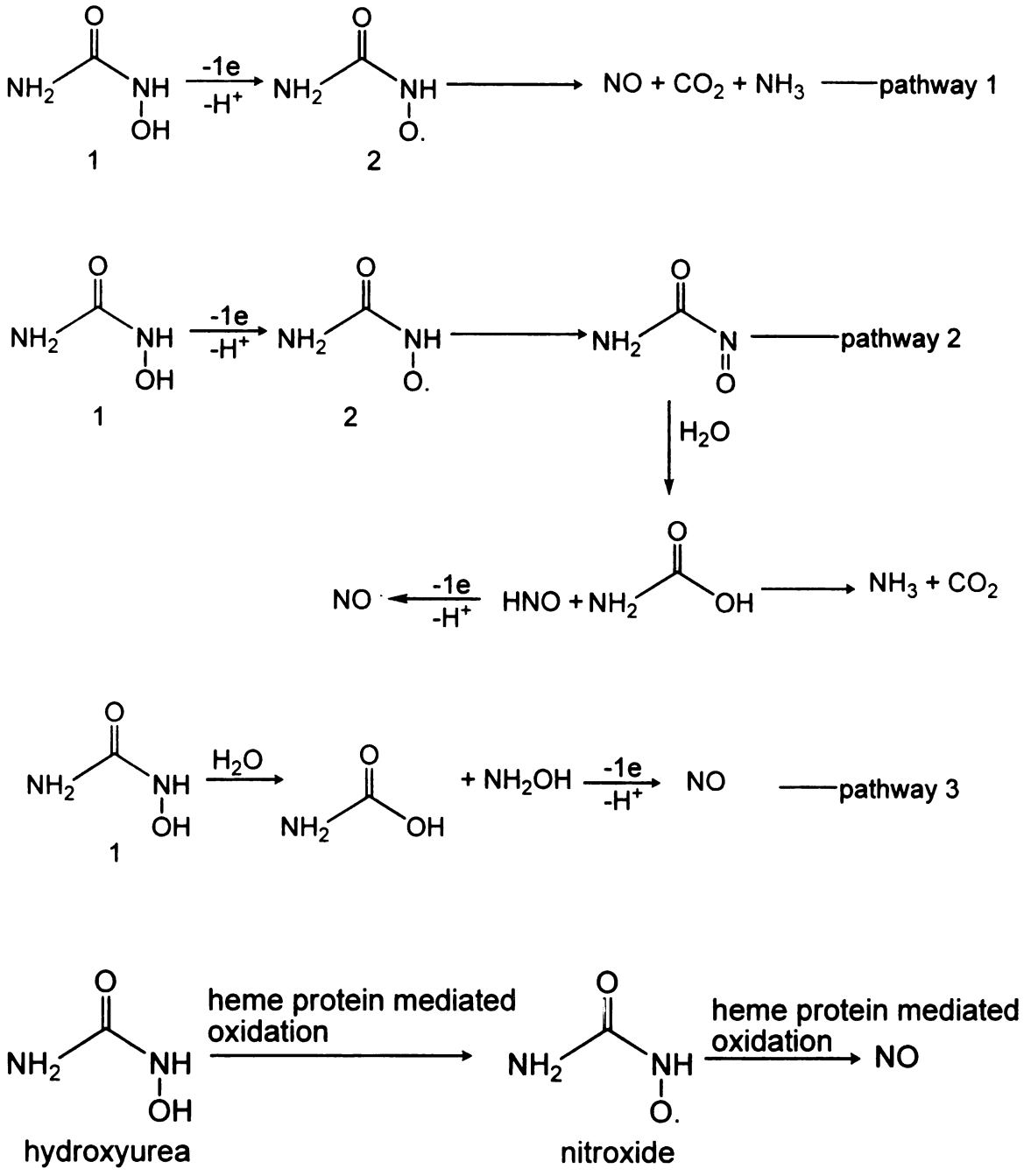

Figure 4.1 Three general pathways that depicts the formation of NO from hydroxyurea under *in vivo* or *in vitro* conditions **(A)**.[17] Proposed mechanism of the formation of NO by hydroxyurea in the presence of heme proteins **(B)**.[22]

hydroxyurea.[23] Furthermore it was observed that aminocarbonylaminooxyl (H_2N-CO-NH_2) radical and the low spin ferric methemoglobin with hydroxyurea (MetHb-NHOH-CO-NH_2). Another study by Pacelli *et al* showed the mechanism of NO formation by hydroxyurea through oxidation by hemoglobin to form the nitroxide radical intermediate. The nitroxide radical is further decomposed *in vitro* to give rise to the NO free radical.[22]

The several studies confirmed the same results and now it is well accepted that hydroxyurea reacts with oxyhemoglobin, deoxyhemoglobin and methemoglobin under *in vitro* conditions to form HbNO complex, proving the formation of the NO free radical from hydroxyurea (figure 4.2). Also these studies have confirmed the S-nitrohemoglobin formation by the reaction of hydroxyurea and methemoglobin.

4.2.2 *In vivo* NO formation from hydroxyurea

In vivo detection of NO is extremely challenging and difficult due to the rapid reaction of NO with oxygen and heme proteins. However, several studies have shown the formation of NO and NO derivatives in the blood stream upon hydroxyurea treatment using chemiluminescence, electrochemical and electron paramagnetic resonance spectroscopy methods.[24,25] Increased levels of plasma NO metabolites such as nitrite, nitrate, nitrosyl hemoglobin and S-nitrosohemoglobin have been observed with patients undergoing hydroxyurea treatment providing evidence of a novel mechanism of hydroxyurea. As NO is a known vasodilator in the vascular system, the clinical efficacy of hydroxyurea on SCD is known to mediate through regulating the blood pressure and flow.

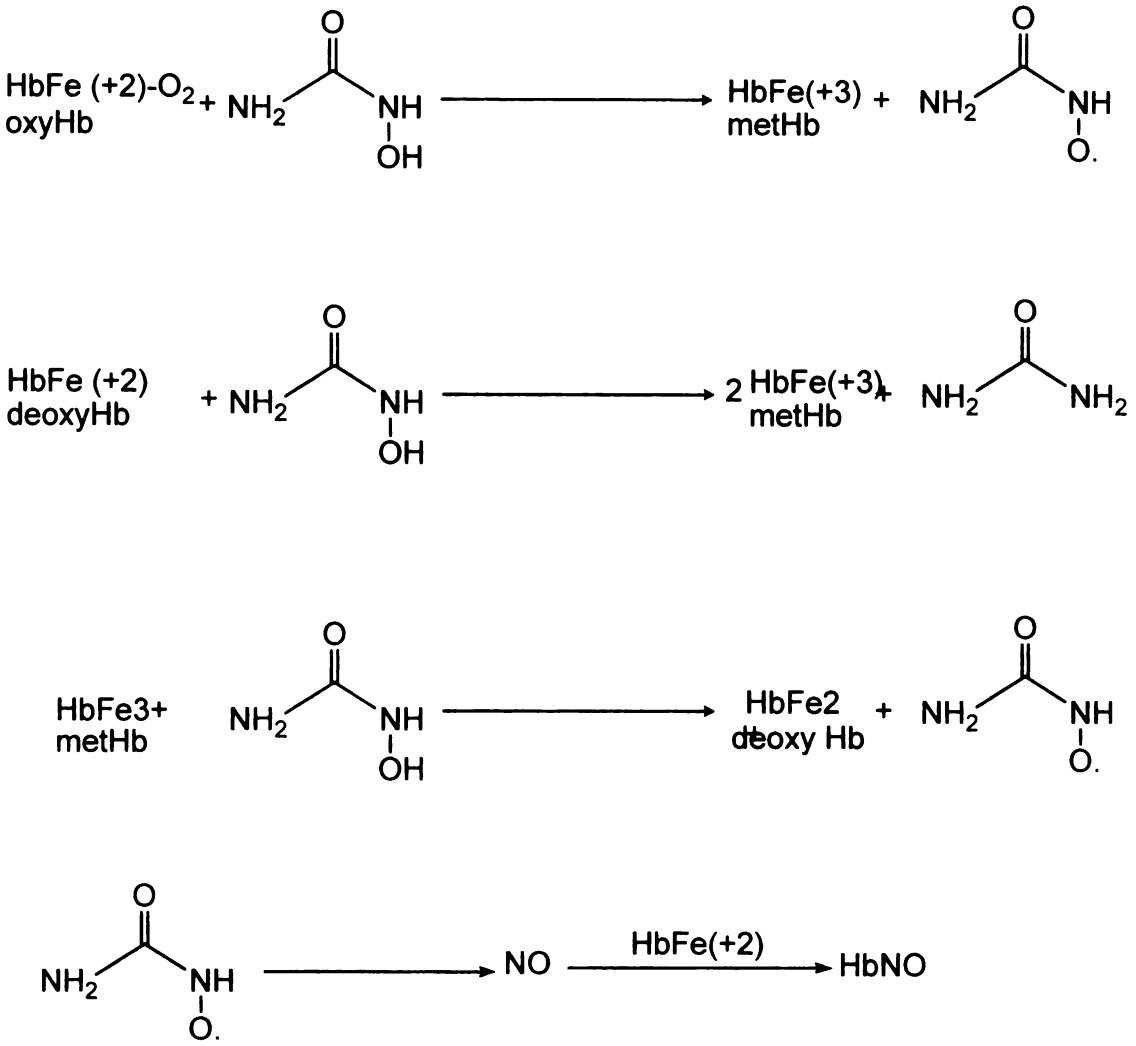

Figure 4.2 The reactions of HbNO formation by hydroxyurea from the reaction of oxy, deoxy, and methemoglobin.[17] The reactions of hydroxyurea with oxyhemoglobin and methemoglobin results in the formation of nitroxide radical which further oxidizes to form molecular NO.

Huang *et al* reported the high levels of HbNO levels in rats treated with hydroxyurea suggesting the formation of NO by hydroxyurea metabolism.[26] The same group further tested this observation on human volunteers. Oral administration of hydroxyurea on SCD patients produced detectable levels of HbNO in the blood stream, but the study did not confirm mechanism of NO formation via the metabolism of hydroxyurea *in vivo*.[25] However, there have been reports and a consideration that *in vivo* reactions forming NO from hydroxyurea with hemoglobin are not sufficient to account for the NO levels quantified *in vivo*. The incubation of 10 mM hydroxyurea with whole blood having oxygenated and deoxygenated hemoglobin did not give detectable levels of HbNO within a 2 h period.[27] These reactions occur at a slow rate, and that they do not demonstrate support for the increased production of NO metabolites formed upon hydroxyurea treatment *in vivo*.[20,28] Moreover, the sample calculations and kinetic stidies revealed that only a small fraction of hemoglobin has reacted with hydroxyurea under physiological conditions. Therefore, other different pathways that produce NO *in vivo* may account for the NO forming reactions by hydroxyurea, and should account for the *in vivo* production of NO metabolites from hydroxyurea. The catalase mediated pathway,[28] horseradish peroxidase catalysed pathway[29] and urease dependant pathways[27] are other sources of NO forming reactions are the potential *in vivo* formation of NO from hydroxyurea.

Interestingly, the effect of NO on HbF induction was proposed recently. The induction of HbF by hydroxyurea is known to mediate through the redox inactivation of the tyrosyl radical of ribonucleotide reductase enzyme, an effect that can be mediated by NO and vasodilators. Furthermore, higher levels of hydroxyurea activates cyclic guanylyl

mono phosphate (cGMP) production, which in turn activates the protein kinase which required for HbF production.[31] In addition to functioning as a vasodilator, the induction of cGMP levels have been observed in patients undergoing hydroxyurea treatment. It is well known that NO stimulates cGMP production, thereby mediating vasodilatation via smooth muscle relaxation. Recently, Nahavandi *et al*, have shown for the first time that NO derived donor compounds such as hydroxyurea open up a new therapeutic strategy in NO based therapy for SCD.[32]

Another important finding in relation to the NO forming ability of hydroxyurea was the decreased levels of NO metabolites during the vaso-occlusive crisis. Administration of single doses of hydroxyurea resulted in increase of NO in the plasma of patients on long term hydroxyurea treatment, and the levels remained above the physiological values for about a 12 h period. Also, the increased cGMP levels were observed for a 6 h period for the patients on single hydroxyurea treatment. In summery, these factors provide evidence for another possible mechanism of hydroxyurea; specifically, hydroxyurea behaving as a NO donor. Considering the half life of NO in physiological systems, there is a possibility that hydroxyurea may act as a NO donor as well as a stimulator for NO production.[31,33]

Moreover, it has been reported that L-arginine, the substrate for NOS, increased NO production only in the presence of hydroxyurea, implying the use of hydroxyurea as a NOS stimulus. Furthermore, Cokic *et al* have shown the induction of eNOS as well as the stimulation of cGMP levels in endothelial cells upon hydroxyurea treatment.[30] However, there is no evidence suggesting that hydroxyurea improves the rheological properties of erythrocytes via a NOS pathway in erythrocytes.

Collectively, the studies carried out *in vivo* clearly show a measurable increase in NO metabolites following the administration of hydroxyurea. However, to date, there is no strong evidence of the *in vivo* oxidant, or the site of the metabolism of hydroxyurea in respect to the formation of NO.[17]

4.3 A novel mechanism of action for hydroxyurea involving erythrocytes

It is now well established that hydroxyurea exerts its beneficial effects in SCD via a number of additional mechanisms other than the induction of HbF levels. Extensive research has been conducted over the last decade to identify these mechanisms, and many research groups have shown reduced red cell- endothelial interactions and improved rheologial properties of RBCs upon hydroxyurea treatment in SCD patients. Moreover, SCD is now being considered as a rheological disorder of RBCs, despite the fact that HbS polymerization due to the genetic mutation causes the major pathogenic events. Therefore, the investigation of the effects of hydroxyurea on RBC rheological properties would be beneficial in treating SCD patients in an effective way.

Decreased or loss of membrane deformability is a characteristic feature of sickle RBCs. This would impair blood flow in small arterioles and capillaries, contributing to vaso-occlusion and pain crisis. Improved RBC membrane deformability has been observed with SCD patients undergoing hydroxyurea treatment.[34] However, the mechanism of modulating the RBC deformability by hydroxyurea was not in a priority in investigations involving to hydroxyurea. The main reason for this may be due to the controversial findings of erythrocytes deformability in response to different hydroxyurea

concentrations. Several studies have shown increased RBC deformability with hydroxyurea, while other studies have shown decreased RBC deformability upon hydroxyurea treatment.[35,36]

The studies cited above provide a good base to study the effect of hydroxyurea on RBC derived ATP release. It is well established that ATP, a known stimulus of endothelial derived NO synthesis, is released in response to mechanical deformation of the RBC membrane. Therefore, if hydroxyurea has an effect on RBC deformability, there is a good possibility that it has an affect on RBC derived ATP release. None of the studies in the literature have shown the effect of ATP release from RBCs in response to hydroxyurea.

In line with expectations, as described in chapter 3, RBCs released higher amounts of ATP under mechanical deformation in response to hydroxyurea treatment. However, the amount of ATP release varied with hydroxyurea concentration, reaching a maximum at 100 μM hydroxyurea concentration in a 7% hematoctrit of RBCs. These studies suggested the possibility of changing deformability of RBCs in the presence of hydroxyurea, as ATP release is an indirect way of measuring RBC membrane deformability. However, the exact mechanism of hydroxyurea affect on RBC derived ATP was not clear from these studies. The same trend for ATP release from RBCs was observed for the NO treated RBCs suggesting the ability of hydroxyurea to form NO. Furthermore, there have been studies showing the effect of NO on RBC deformability. However, the controversial findings of the effect of NO on deformability have taken away the potential significance of this effect. Therefore, experiments were carried out to see the effect of NO on RBC derived ATP release. Interestingly, the same trend as that

seen with hydroxyurea was observed for NO treated RBCs as well. This study led the hypothesis that NO formed from hydroxyurea is responsible for the increased ATP release from RBC in response to mechanical deformation. Considering the reactions of NO formation by hydroxyurea, it is further hypothesized that hydroxyurea can induce NOS activity of RBCs thereby affect the NO production in RBCs. Moreover, the produced NO can improve the RBC membrane deformability thereby increase the release of ATP from RBCs.

4.4 Experimental

4.4.1 Methods and materials

Generation of washed red blood cells

RBCs were prepared on the day of use. For obtaining rabbit RBCs, male New Zealand White rabbits (2.0-2.5 kg) were anesthetized with ketamine (8.0 mg/kg) and xylazine (1.0 mg/kg) followed by pentobarbital sodium (15 mg/kg iv). After tracheotomy, the rabbits were mechanically ventilated (tidal volume 20 ml/kg, rate 20 breaths/min; Harvard ventilator). A catheter was placed into a carotid artery, heparin (500 units, iv) was administered, and after 10 min, animals were exsanguinated. Blood was collected into vials and the RBCs separated from other formed elements and plasma by centrifugation at 500 x g at 4 °C for 10 min. The supernatant and buffy coats were removed by aspiration. Packed RBCs were resuspended and washed three times in PSS [PSS; in mM; 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21.0

tris(hydroxymethyl)aminomethane, and 11.1 dextrose with 5% bovine serum albumin, pH adjusted to 7.4].

4.4.1.1 Chemiluminescence detection of ATP

Preparation of ATP standards

All the reagents were purchased from Sigma chemical company, unless otherwise noted. A 100 μM stock solution of ATP was prepared by adding 0.0551 g of ATP to 1000 ml of distilled and deionized water (DDW). The standard solutions (0, 25, 50, 100, 150 nM) were made by adding 0, 67.5, 125.0, 187.5, 250.0, and 375.0 μl of 100 μM ATP stock solution to PSS. All the standards were made in 25 ml volumetric flasks and made the day of use. PSS was made by first combining 25 ml of Tris buffer and 25 ml of Ringer's buffer and then adding 0.50 g of D-Glucose and 2.50 g of bovine albumin fraction V (fatty acid free). The entire solution was then diluted to 500 ml by adding DDW and adjusting the pH to 7.35-7.45. The PSS solution was filtered three times using 0.45 μm filter (Corning, Fisher Scientific) before use. All the standards were made on the day use to calibrate the chemiluminescence signal on the PMT.

Preparation of luciferin-luciferase reaction mixture

Firefly extract (contains luciferin and luciferase) was dissolved in 5.0 ml of DDW. Next, 0.002 g of synthetic luciferin was added to the firefly mixture to enhance the sensitivity. The chemiluminescence intensity generated by the ATP/luciferin-luciferase reaction was measured.

Chemiluminescence measurement of deformation induced RBC derived ATP release

All RBC solutions were diluted to a 7% hematoctrit in PSS. To measure the RBC derived ATP release, the luciferin- luciferase reaction mixture was placed in a 500 µl syringe (Hamilton, Fischer Scientific). The RBC solution was placed in another 500 µl syringe (Hamilton, Fischer Scientific) and both syringes were loaded on to a syringe pump (Harvard Apparatus, Boston, MA) and connected to two sections of silica microbore tubing having an internal diameter of 50 µm and an outer diameter of 362 µm (Polymicro Technologies, Phoenix, AZ). The microbore tubes were then connected to a T-junction (Upchurch Scientific, Oak Harbor, WA) having a volume of 256 nl. The combined contents were passed through a 6 cm microbore tubing having an internal diameter of 75 µm. The resultant chemiluminescence produced by the RBC derived ATP and the luciferin-luciferase reaction was detected by a PMT housed in a light excluding box (shown in chapter 3). The polyimide coating was removed from the tubing and placed over the PMT for measurement.

Non flow chemiluminescence measurements of RBC derived ATP release

The RBC solutions were diluted to 7% hematocrit and incubated in 50 µM hydroxyurea for 20 minutes in normal PSS and calcium free PSS. The control 7% RBCs were incubated in normal PSS and calcium free PSS. Next, 100 µl of incubated RBC samples were mixed with 100 µl of luciferin-luciferase, followed by measurement of the mix chemiluminescence intensity after 15 seconds by the PMT housed in the light excluding box.

Other reagents

L-NAME: A 1 mM stock solution of L-NAME was prepared by dissolving 0.003 g of L-NAME in 10 ml of phosphate buffered saline (pH 7.4)

Hydroxyurea: For studies involving hydroxyurea, a 4 mM hydroxyurea solution was prepared by dissolving 0.004 g of hydroxyurea (Sigma Aldrich, St. Louis, MO) in 25.0 ml of PSS. The range of concentrations (0 – 200 μM) were made by diluting appropriate volumes (0 – 500 μl) of the stock solution in a solution of 7% RBCs made in PSS. When preparing the hydroxyurea treated RBCs, hydroxyurea was first added to PSS before adding RBCs to create a homogenously treated RBC solution. RBC samples were incubated for 20 minutes at room temperature before the measurement portion of the analysis.

C-peptide: 0.25 mg of C-peptide was dissolved in 10 ml of DDW to make an 8.3 μM C-peptide stock solution. Next, 250 μl of the stock solution is dissolved in 25 ml of DDW and from that solution, 600 μl was taken to make the 10 nM working solution of C-peptide. The working solution is dissolved in a zinc containing medium where the concentration of both C- peptide and zinc are equimolar in solution.

4.4.1.2 Atomic absorbance spectroscopy (AAS) studies for calcium measurements in RBCs

Preparation of calcium standards for atomic absorption spectroscopy

The Ca^{2+} standards (2.50, 25.0, 250.0, 2500.0 μg/l or ppb) were prepared by a serial dilution of a 10,000 μg/l stock solution of standard $CaCO_3$ in 25 ml volumetric

flasks. The $CaCO_3$ stock solution was prepared by dissolving 0.004 g of $CaCO_3$ in 1000 ml of 18.0 MΩ DDW.

Preparation of RBC samples for atomic absorption spectroscopy

RBCs of a 7% hematocrit solution were incubated with 50 μM hydroxyurea for 20 minutes. Next, the RBC sample was centrifuged at 500 x g at 4 °C for 10 min to remove the calcium containing supernatant. The packed RBCs were resuspended and washed three times in calcium free PSS. Calcium free PSS was made by using calcium free Ringers buffer, but the ionic strength was balanced by substituting an equivalent amount of $MgSO_4$ [PSS; in mM; 4.7 KCl, , 3.2 $MgSO_4$, 140.5 NaCl, 21.0 tris(hydroxymethyl)aminomethane, and 11.1 dextrose with 5% bovine serum albumin, pH adjusted to 7.4]. Finally the washed RBCs were lysed in DDW, and the membrane ghosts were removed by centrifuging at 3000 x g at 4 °C for 10 min. The supernatant was then taken for the AAS studies.

4.4.1.3 Electron paramagnetic resonance spectroscopy (EPR) for HbNO detection in RBCs

Preparation of RBC samples for EPR

RBC solutions were diluted to a 7% hematocrit and incubated in 50 μM hydroxyurea for 20 minutes in normal PSS and calcium free PSS as needed. The incubated samples were transferred to 3 mm quartz tubes and were frozen immediately in liquid nitrogen.

Preparation of NO standards for EPR

The NO standards using Spermine NONOate were prepared as followed. The 38 μM spermine NONOate solution was prepared by diluting 10 μl of 38 mM stock spermine NONOate solution in 10 ml of deoxygenated PBS buffer. Next, a series of standard solutions (20, 40, 100, 200, 1000 μM) were prepared by using varying volumes of 38 μM spermine NONOate solution in a total of 5000 ml deoxygenated PBS buffer. The PBS buffer was deoxygenated in separate vials by the effervescence of nitrogen gas for 4 minutes.

For some experiments, NO standards were prepared by dissolving gaseous NO in deoxygenated PBS. A Schlink line vacuum system was employed in deoxygenating the PBS 7.4 buffer. NO gas was bubbled into the airtight flasks having deoxygenated PBS buffer to make the stock solution of NO (1.9 mM). The series of dilutions were made by diluting the appropriate volumes of the NO stock solution in deoxygenated PBS buffer in air tight flasks.

EPR measurements and data processing

All the EPR measurements were carried out in a Bruker ESP-300E spectrometer operating at 9.46 GHz using a Bruker ST4102 (TE102) microwave cavity. An Oxford Instruments ESR-900 liquid helium cryostat together with a model ITC-502 temperature controller was used to maintain the sample temperature at 4.2 K. The typical EPR settings were as follows; power 5 mW, field modulation frequency 100 kHz, field modulation amplitude 2.8 G, conversion time 327 ms, scan average 16. Each spectrum of either

spermine NONOate treated or hydroxyurea treated RBCs were obtained by subtracting the EPR spectrum of untreated RBCs.

4.5 Results and discussion

The similar trend of ATP release in the presence of varying concentrations of hydroxyurea and spermine NONOate led us to further investigate the NO producing ability of hydroxyurea in RBCs. According to the hypotheses, NO is produced from hydroxyurea, within RBCs, and the produced NO modulates the cellular deformability of RBCs, thereby increasing the release of RBC derived ATP upon mechanical deformation.

EPR spectroscopy experiments have been used by several groups to demonstrate *in vivo* and *in vitro* production of NO from hydroxyurea.[23,24] In fact, EPR spectroscopy is a reliable method of detecting and quantifying free radical species in biological systems. The interaction of NO with hemoglobin results in the formation of the HbNO molecule and is also paramagnetic. The detection of HbNO in RBCs has been used as an indicator molecule for previous hydroxyurea studies.

Even though, it had been previously performed, a series of experiments were carried out to investigate the ability of NO formation from hydroxyurea within the concentration range used for the RBC derived ATP release measurements (25- 200 μM). Figure 4.3 shows the chgaracteristic EPR spectrum for Fe2+- NO comeplex. The two peaks represent the g values at 6 and 2, respectively. In our experiments we were focused on the peak at g=2, where a large negative dip represents the heme nitric oxide complex.

First, an EPR experiment was carried out at 4 K to detect the HbNO signal in

RBC samples treated with hydroxyurea. NO reacts rapidly with deoxygenated hemoglobin to give HbNO. However, in this case, the RBCs were not subjected to deoxygenation in order to preserve the physiological conditions of the circulation. In the circulation, the majority of RBCs (98%) is in the oxygenated state while a small percentage (~1-2%) will be in the deoxygenated state. Considering the concentrations of hydroxyurea (25-200 μM) employed in the ATP release studies, a 100 μM hydroxyurea solution was first chosen to incubate with the RBCs and try to detect any HbNO formation from hydroxyurea derived NO.

Figure 4.4 shows the EPR signal obtained for RBCs incubated with 100 μM hydroxyurea for 20 min. The spectrum obtained for the hydroxyurea treated RBCs was subtracted from the spectrum obtained from untreated RBCs to obtain the difference spectrum, which is in fact showing the HbNO signal due to hydroxyurea. The subtraction method was applied to eliminate the EPR signal for anycopper containing protein, cerruloplasmin (g = 2.06 where g is the spectroscopic splitting factor) in RBCs as it overlaps almost the same field where HbNO occurs. The difference spectrum was compared with the difference spectrum obtained from RBCs incubated with a known concentration of spermine NONOate (100 μM) (figure 4.5). The comparison of the two subtracted spectra obtained from hydroxyurea and spermine NONOate is shown in figure 4.6 for comparison. The triplet hyperfine coupling and the g values at 2.01 are the characteristic features of HbNO formation.[37,38]

Subsequently, a series of NO treated RBCs were prepared (frozen immediately after addition of varying concentrations of NO standard solution) to quantify the HbNO signal in RBCs at 4.2 K. The standard calibration plot shown in figure 4.7 and 4.8

confirms the formation of HbNO in RBCs with varying NO concentrations. The EPR experiment to detect the HbNO signal in RBCs treated with gaseous NO was successful even though the triplet hyperfine coupling is not observed.

Unlike the NO standards, hydroxyurea did not produce a measurable HbNO signal in the EPR spectrum. This may be either due to the lack of formation of NO from hydroxyurea or the insufficient amount of NO formation in RBCs to be detected by EPR. Unfortunately, current EPR techniques are not sensitive enough to measure the basal levels of NO (< 200 nM) in biological tissues.[39] If the 100 μM hydroxyurea results in nanomolar levels of NO, this would not be the ideal technique to detect smaller NO concentrations in RBCs. Moreover, the studies that have been carried out by other groups have utilized millimolar concentrations of hydroxyurea to obtain detectable HbNO triplet hyperfine structure. Therefore, at this point, these experiments neither prove nor disprove our hypothesis as it is difficult to interpret the results with the noted experimental limitations. The experiment was performed with a concentration of hydroxyurea that resulted in the highest ATP release from the RBCs. Therefore, further experimentation with was not continuied for the other hydroxyurea concentrations.

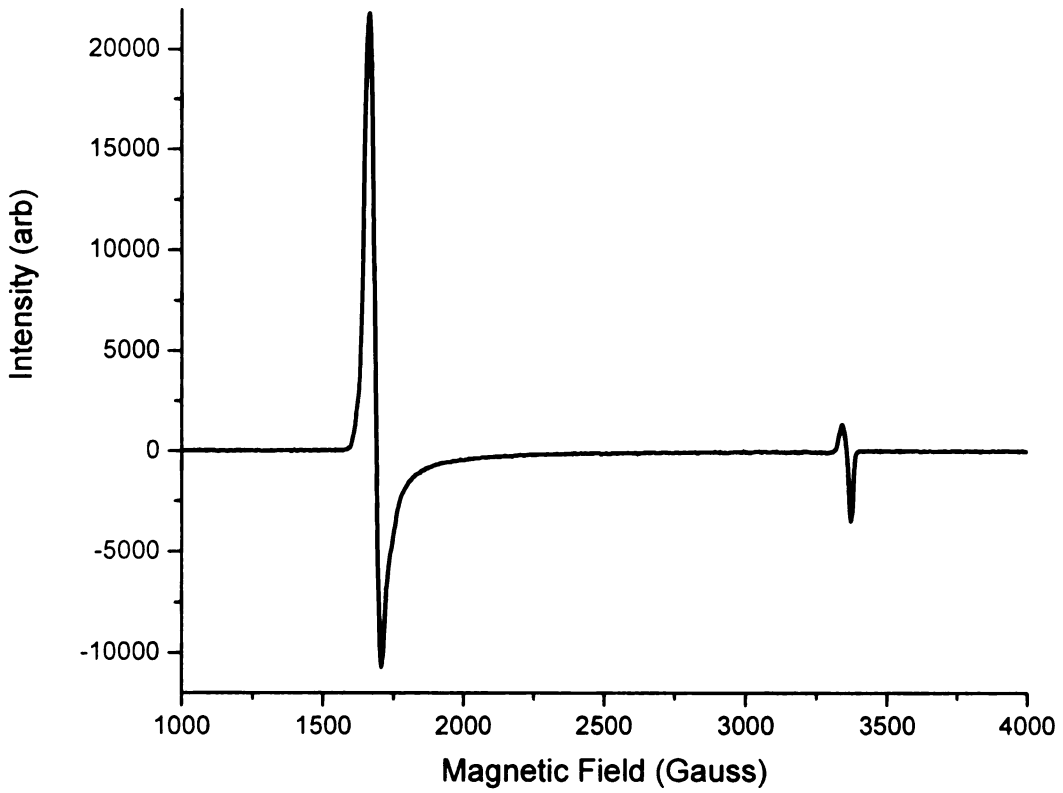

Figure 4.3 An EPR spectrum showing the characteristic peaks for Fe^{2+}-NO complex at g = 6 and g = 2.

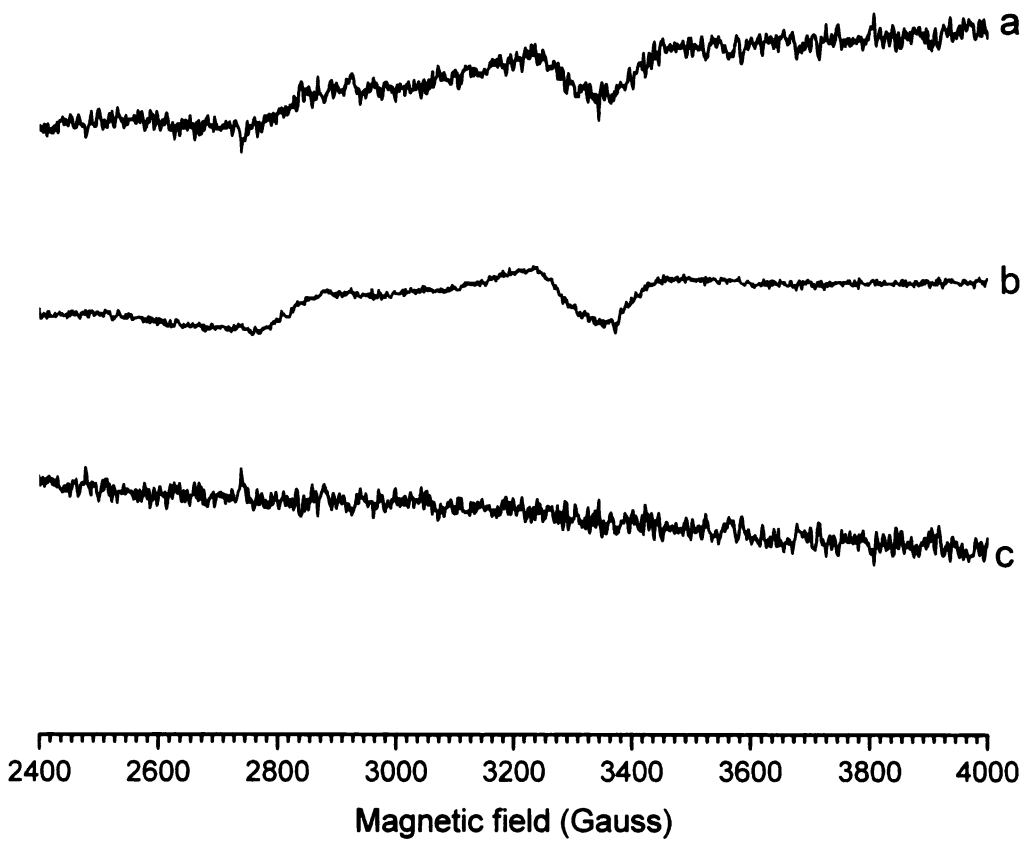

Figure 4.4 Typical EPR spectra obtained from control RBCs (a) and for 100 μM hydroxyurea treated RBCs (B). Spectrum C is the difference obtained by subtracting b from a. The EPR settings were as follows; power 5 mW, frequency 9.46 GHz, field modulation amplitude 2.8 G, conversion time 327 ms, temperature 4.2 K, scan average 4.

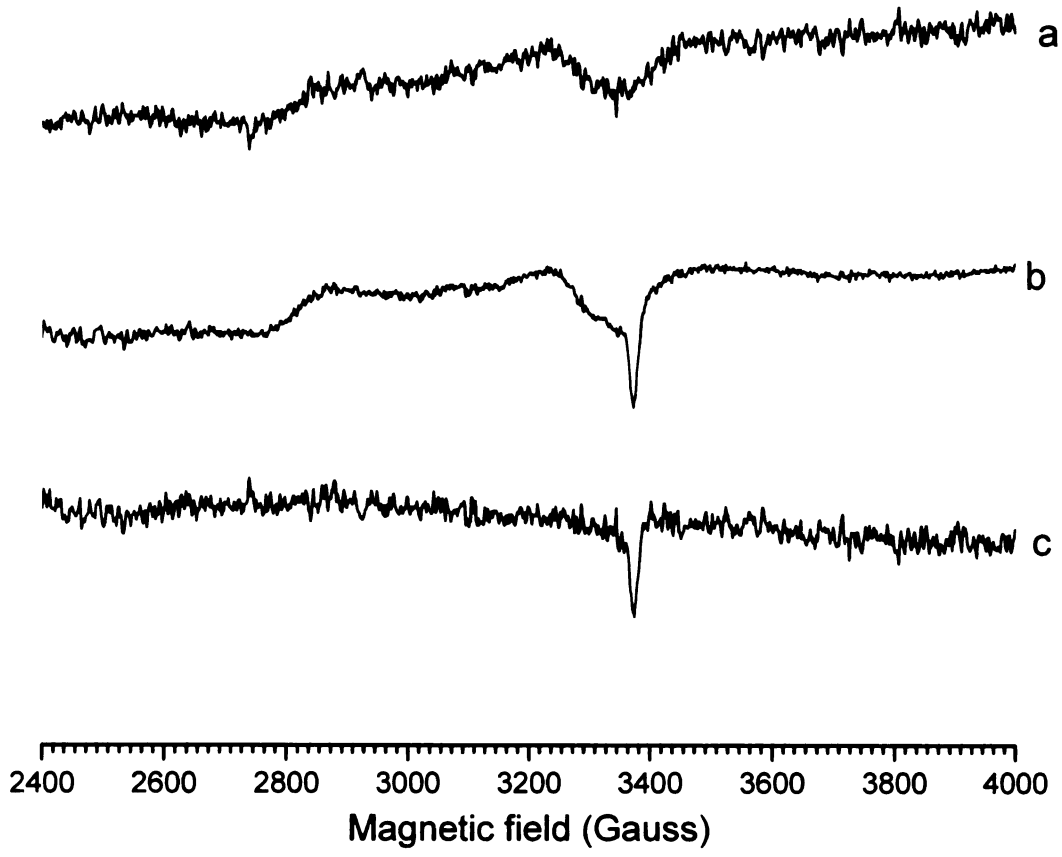

Figure 4.5 Typical EPR Spectra obtained from control RBCs (a) and 100 μM spermine NONOate treated RBCs (b). Spectrum (c) is the difference obtained by subtracting (b) from (a). A peak at g=2.01 represents the HbNO signal.The EPR settings were as follows; power 5 mW, modulation frequency 9.46 GHz, field modulation amplitude 2.8 G, conversion time 327 ms, temperature 4.2 K, scan average 4.

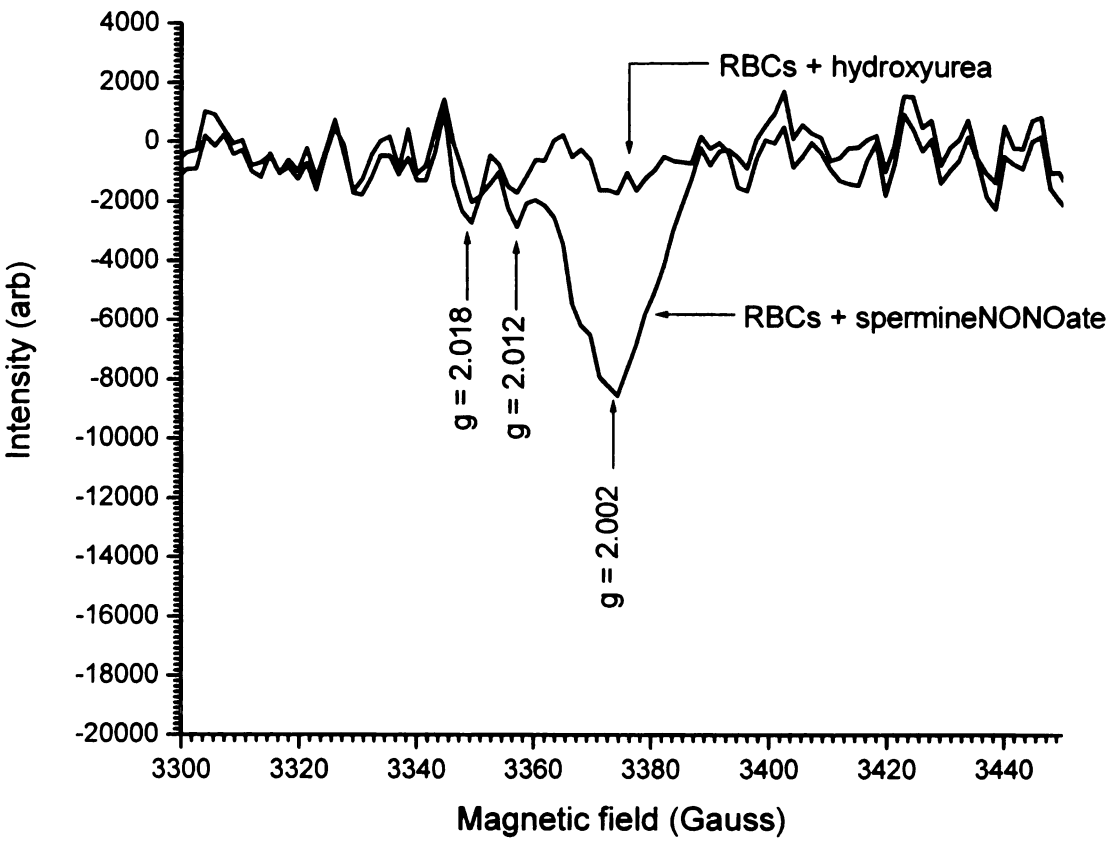

Figure 4.6 The difference spectra obtained for spermine NONOate treated RBCs and hydroxyurea treated RBCs. The triplet hyperfine structure at g =2.01 is the characteristic signal of HbNO in RBCs. The triplet hyperfine structure corresponding to the g values of HbNO in NO treated RBCs is not significant in hydroxyurea treated RBCs. The EPR settings were as follows; power 5 mW, modulation frequency 9.46 GHz, field modulation amplitude 2.8 G, conversion time 327 ms, temperature 4.2 K, scan average 4.

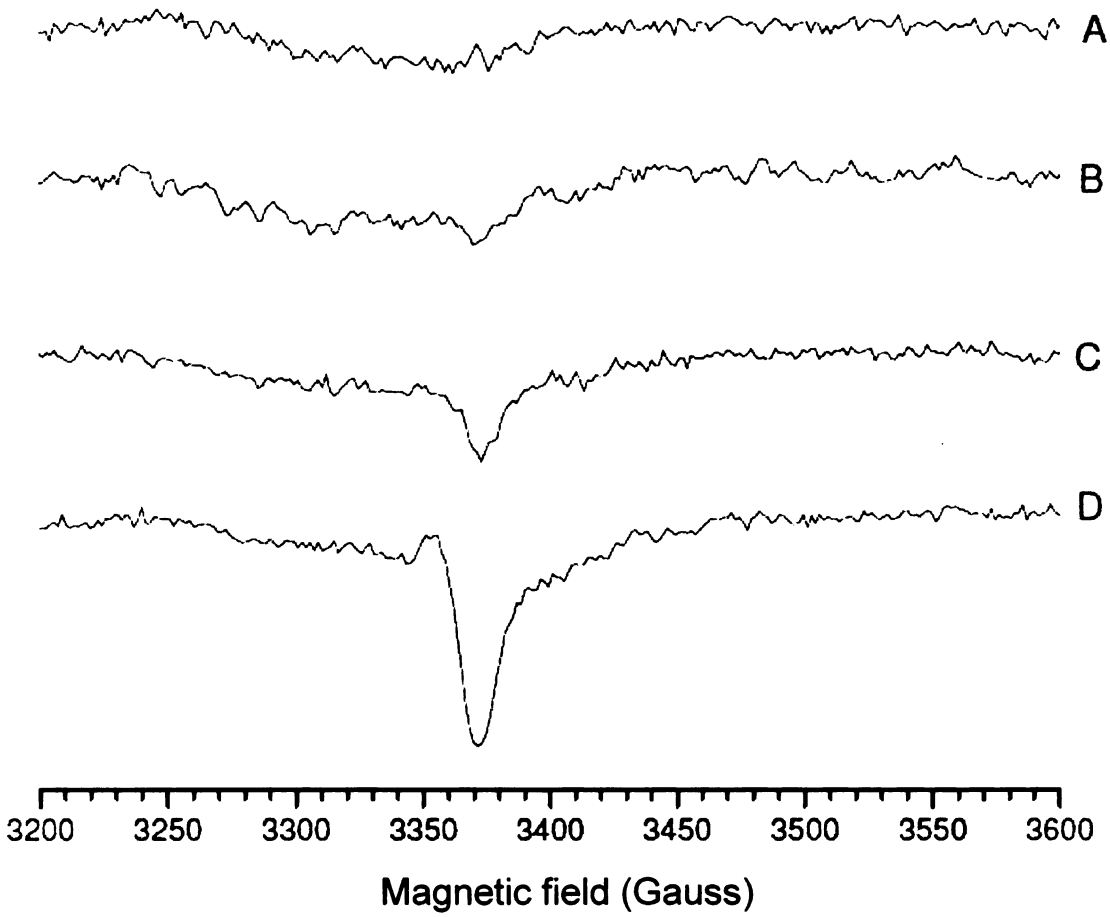

Figure 4.7 Typical EPR spectra obtained for the calibration curve of NO treated RBCs. A: untreated RBCs. Spectra B, C, and D were obtained from RBCs treated with 20, 40 and 100 μM spermine NONOate respectively. The EPR conditions were as follows: power 5mW; frequency 9.46 GHz, sweep time 15 minutes, temperature 4.2 K, scan average 4.

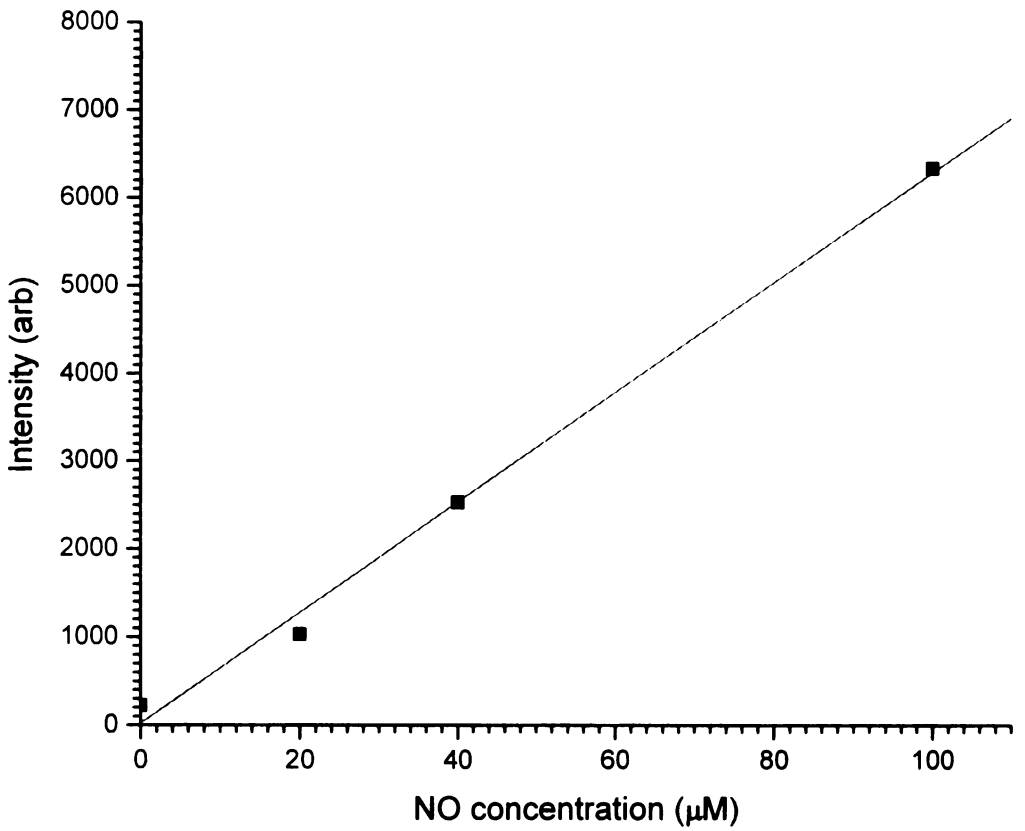

Figure 4.8 Calibration curve of HbNO signal obtained from the reaction of RBCs and gaseous NO. The peak heights of the HbNO signals are plotted against the varying NO concentrations of gaseous NO (20-100 μM).

The primary objective of this study was to investigate the mechanism of hydroxyurea on erythrocyte derived ATP release. Therefore, an attempt was made to investigate the effect of hydroxyurea on NOS activity in the RBCs. The RBCs are known to have the NOS enzyme in order to help preserve the RBC deformability within its life time of 120 days.[40,41] A 7% hematocrit of RBCs were first treated with 100 μM L-NAME, a known inhibitor for the NOS enzyme, to inhibit the NO production in RBCs. Next, another 7% hematoctrit of rabbit RBCs were incubated with 100 μM L-NAME for 30 minutes, followed by 50 μM hydroxyurea for 20 minutes. The incubated samples were pumped through silica microbore tubing having an internal diameter of 50 μm. The ATP release from the RBCs was measured using the flow based system described in chapter 3.

The reason for performing this experiment was to examine the effect of NO inhibition on RBC derived ATP release. As shown in figure 4.9, the the ATP release was lower in L-NAME treated RBCs, when compared to untreated RBCs, suggesting the involvement of NO on erythrocyte derived ATP release. Interestingly, a decreased ATP release from RBCs was observed for RBCs incubated with L-NAME, followed by hydroxyurea, compared to hydroxyurea treated RBCs. Therefore, this study suggested the involvement of hydroxyurea on NOS activity, and possibly RBC derived NO synthesis. In order to investigate these observations in depth, the mechanism of NO synthesis was taken into consideration. It is well known that RBCs carry eNOS or the constitutive form of NOS. The calcium-calmodulin (Ca-CAM) complex formation is the first step in the activation of eNOS pathway in cells.

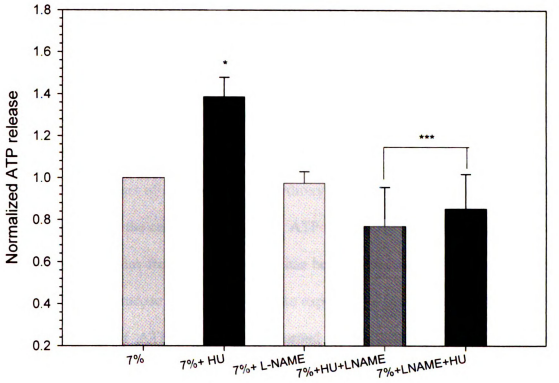

Figure 4.9 Measurement of ATP release from RBCs in the presence of L-NAME and hydroxyurea. RBCs incubated with L-NAME resulted in the lowest release of ATP from RBCs (third bar) showing the importance of intra erythrocyte NO production on ATP release. Hydroxyurea did not have any effect on ATP release in the presence of L-NAME indicating an important function of hydroxyurea on NOS in RBCs. The values between (*) and (**) are significantly different ($P < 0.04$). n = 4 rabbits and the error bars are represented as SEM.

In order to investigate the NO synthesis in RBCs in detail, an experiment was performed to examine whether there is an effect of the calcium content on RBC derived ATP release. Figure 4.10 shows the ATP release from RBCs in response to hydroxyurea in PSS and calcium free PSS. There is no net effect on RBC derived ATP release for hydroxyurea treated RBCs; compared to normal RBCs in calcium free PSS. This implicates the involvement of calcium in the mechanism for hydroxyurea activated RBC derived ATP release. However, this study did not confirm or eliminate the possibility of an overall impairment of biochemical mechanisms in the absence of calcium. In order to determine the impact of calcium ions on hydroxyurea activity, a calcium free buffer was used to investigate the cellular mechanisms. ATP release was measured in the presence of C-peptide in calcium free PSS. C-peptide has been shown to increase the ATP release from RBC via a glucose uptake pathway. As expected, C-peptide treated RBCs released higher amounts of ATP, compared to normal RBCs, confirming that the observed increase in ATP release from RBCs with hydroxyurea in calcium free buffer is not due to an impairment of all the physiological processes in the absence of calcium (data not shown).

Another experiment was designed to measure the intracellular calcium concentrations in RBCs. Iinitially, the calcium concentration was measured in using fluorescence spectroscopy with an intracellular calcium fluorophore (fluo-4 AM cell permeate, Molecular Probes). However, these experiments did not produce sufficient fluorescence and the differences of the fluorescence intensity between samples were not significant. This may be due to the matrix effects of the RBCs or due to the low

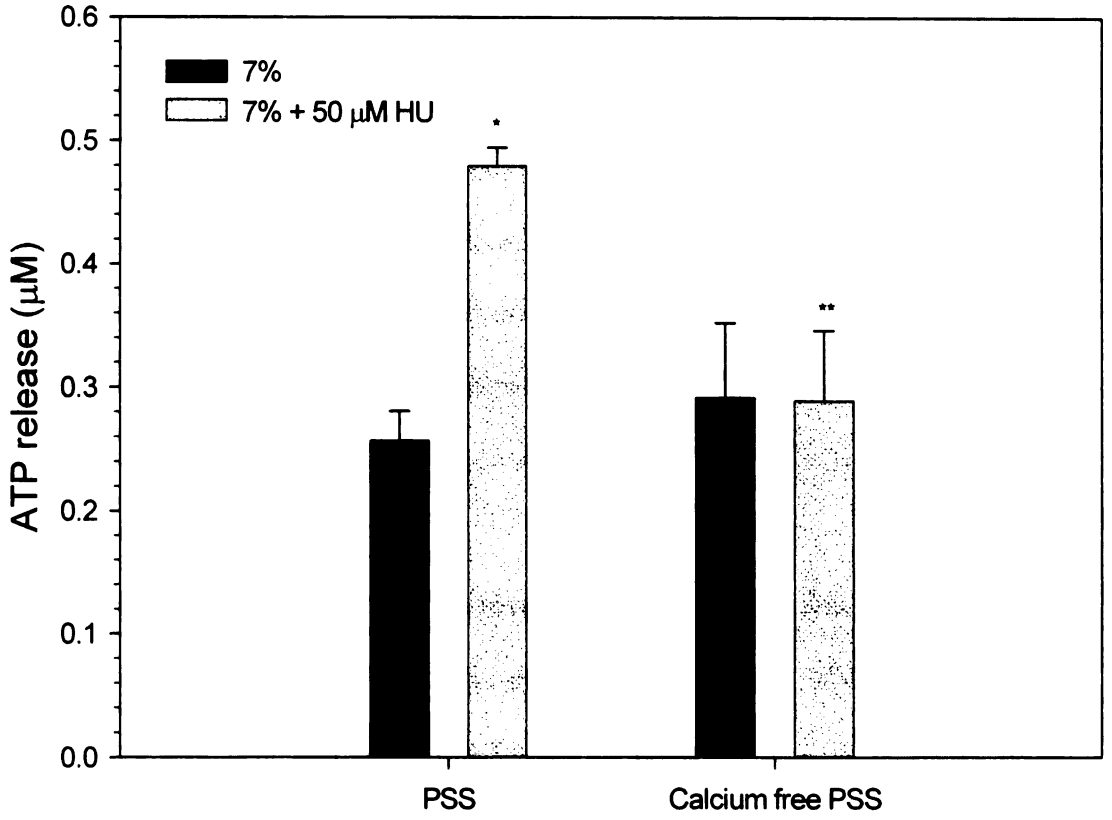

Figure 4.10 Measurement of the ATP release from RBCs in the presence and absence of calcium ions in the medium. Black bars represent the ATP release of untreated RBCs and gray bars represents the ATP release from RBCs treated with 50 μM hydroxyurea. Increased ATP release is observed for hydroxyurea treated RBCs in PSS while no significant increase of ATP release from RBCs is observed for RBCs treated with hydroxyurea in the calcium free PSS. The data represented as (*) and (**) are significantly different in $P < 0.01$. $n = 5$ rabbits and the error bars are reported as SEM.

sensitivity of the instrument. Therefore, atomic absorption spectroscopy (AAS) was employed as it is a standard method for calcium quantification with high sensitivity. The RBCs were first incubated with 50 μM hydroxyurea for 20 minutes. Lysed RBC samples were then prepared as described above and determined by AAS. The figure 4.11 shows an increase of calcium uptake in the presence of hydroxyurea in comparison to the untreated RBCs. Calcium concentrations for RBCs were obtained from the calibration curve using different calcium concentrations. The values shown in figure 4.11 represent the calcium content of a 10 % hematocrit of RBCs. Taking the hematoctrit and the volume of the RBC (80 fl) into consideration, the RBC calcium concentration was calculated. The concentrations were 67 and 105 nM for RBCs in calcium free PSS and normal PSS, respectively.

This observation was further investigated using EPR spectroscopy as the initial EPR studies were carried out in phosphate buffered saline (PBS pH 7.4) where the calcium concentration was zero. RBCs were incubated with 50 μM hydroxyurea for 20 minutes in normal PSS and calcium free PSS. Interestingly, a higher HbNO signal was observed for RBCs in the normal PSS, where calcium is present, compared to the RBCs that were in calcium free buffer. The signal subtraction method was employed here to eliminate the interference from the cerruloplasmin protein (figure 4.12, and 4.13). A comparison of the difference spectra obtained for hydroxyurea treated RBCs in calcium free PSS and normal PSS is shown in figure 4.14. The triplet structure obtained at $g = 2.0$ confirmed the presence of HbNO signal for calcium containing buffer, suggesting the involvement of Ca^{2+} in the NO formation of hydroxyurea. The absence of calcium in the medium could be the reason for not initially detecting the HbNO signal for RBCs treated

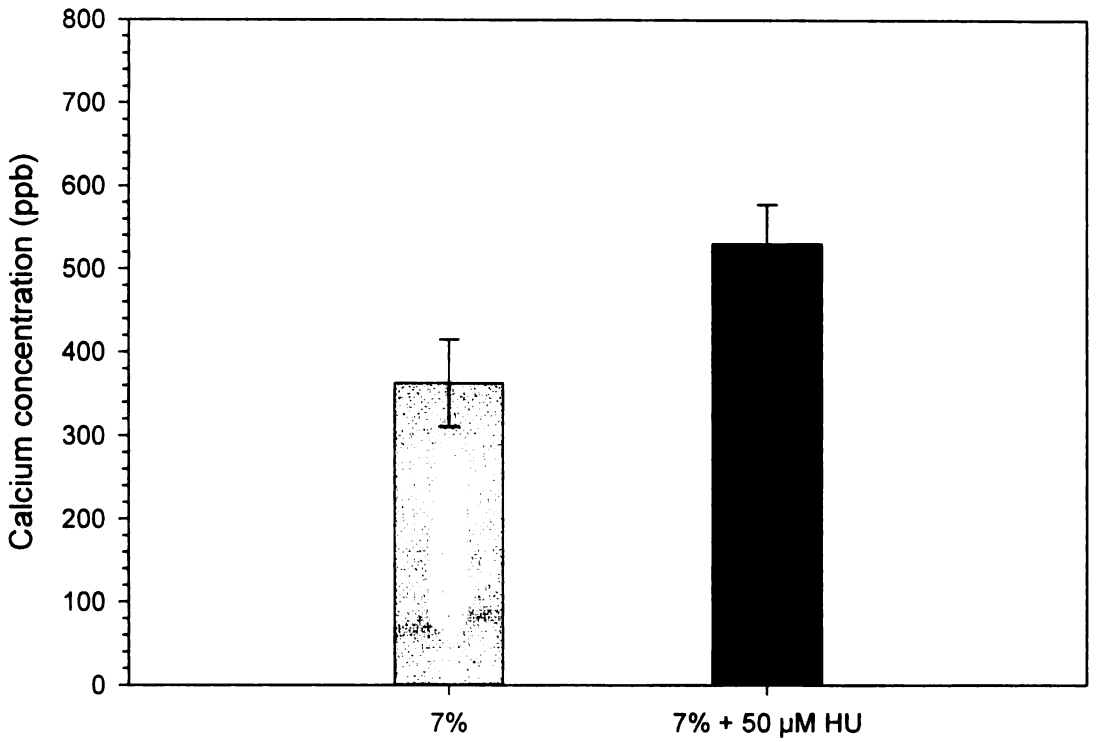

Figure 4.11 Atomic absorption spectroscopic study of calcium quantification in RBCs. The first bar (gray) represents the calcium concentration in the cytoplasm of hematocrit of 10% RBCs. The second bar (black) represents the calcium concentration in the cytoplasm of hematocrit of 10% RBCs incubated with 50 µM hydroxyurea solution for 20 minutes. The values are significantly different in $P < 0.025$. $n = 7$ rabbits and the error bars are represented as SEM.

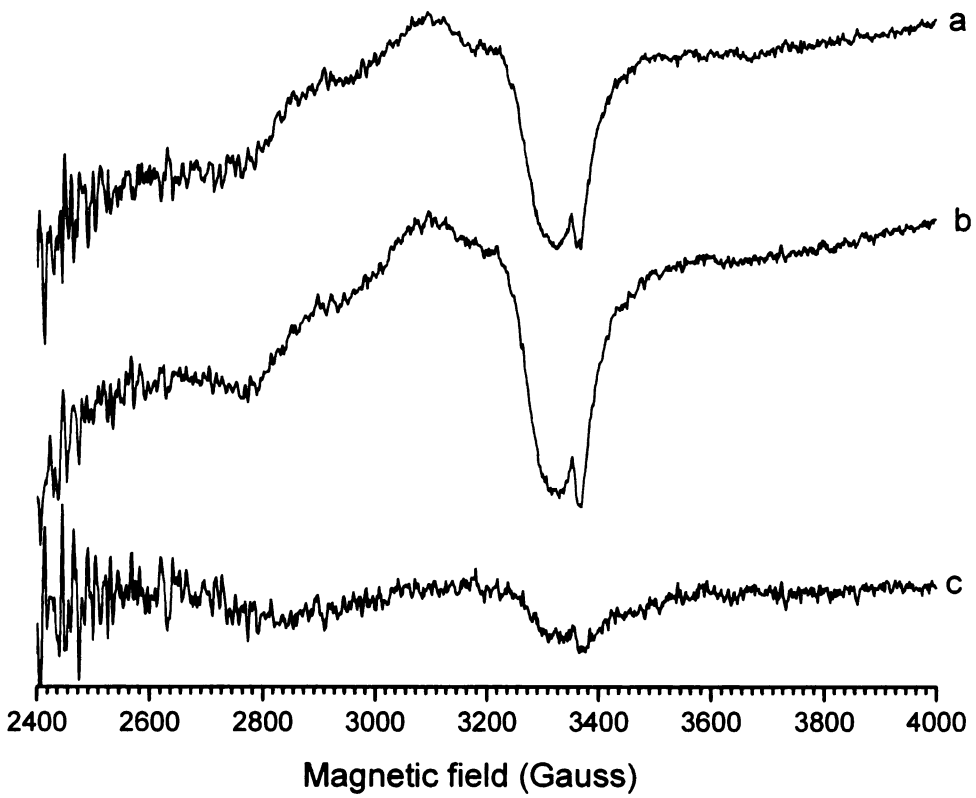

Figure 4.12 Typical EPR Spectra obtained from control RBCs (a) and for 50 μM hydroxyurea treated RBCs in normal PSS (b). Spectrum (c) is the difference spectra obtained by subtracting (b) from (a). The EPR settings were as follows; power 5 mW, modulation frequency 9.46 GHz; field modulation amplitude 2.8 G, conversion time 327 ms, scan average 16.

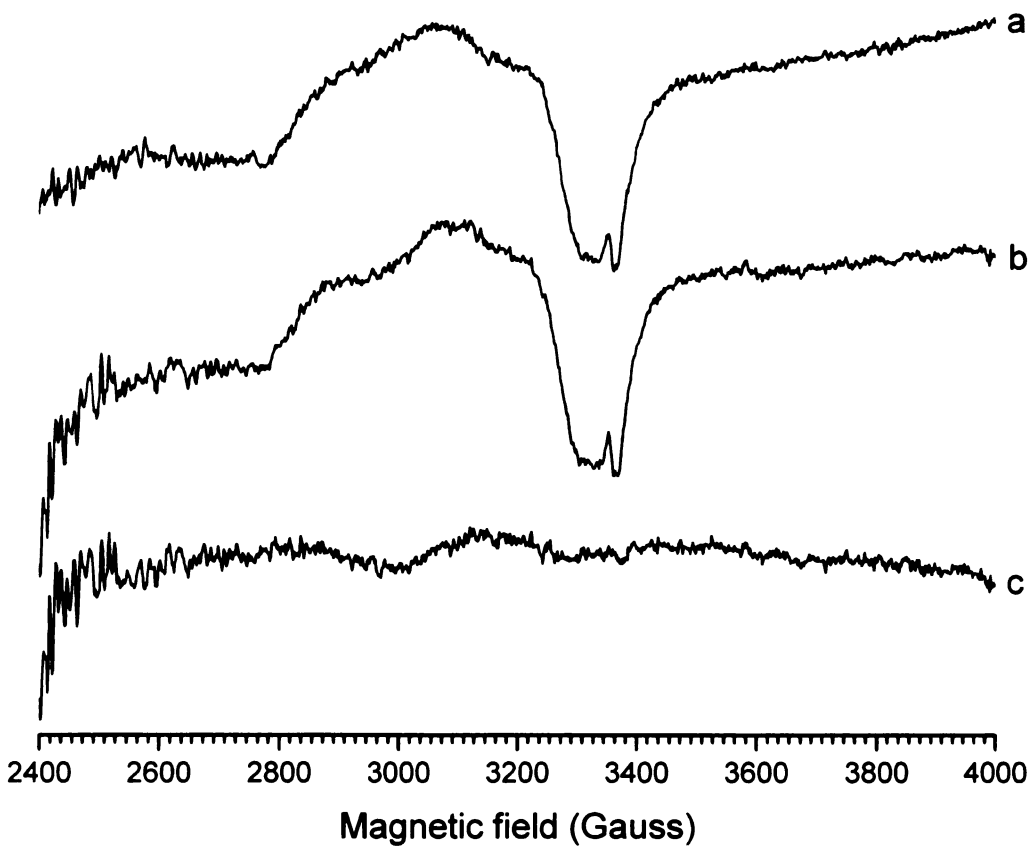

Figure 4.13 Typical EPR Spectra obtained from control RBCs (a) and for 50 μM hydroxyurea treated RBCs in calcium free PSS (b). Spectrum c is the difference spectra obtained by subtracting (b) from (a). The EPR settings were as follows; power 5 mW, modulation frequency 9.46 GHz, field modulation amplitude 2.8 G, conversion time 327 ms, temperature 4.2 K, scan average 16

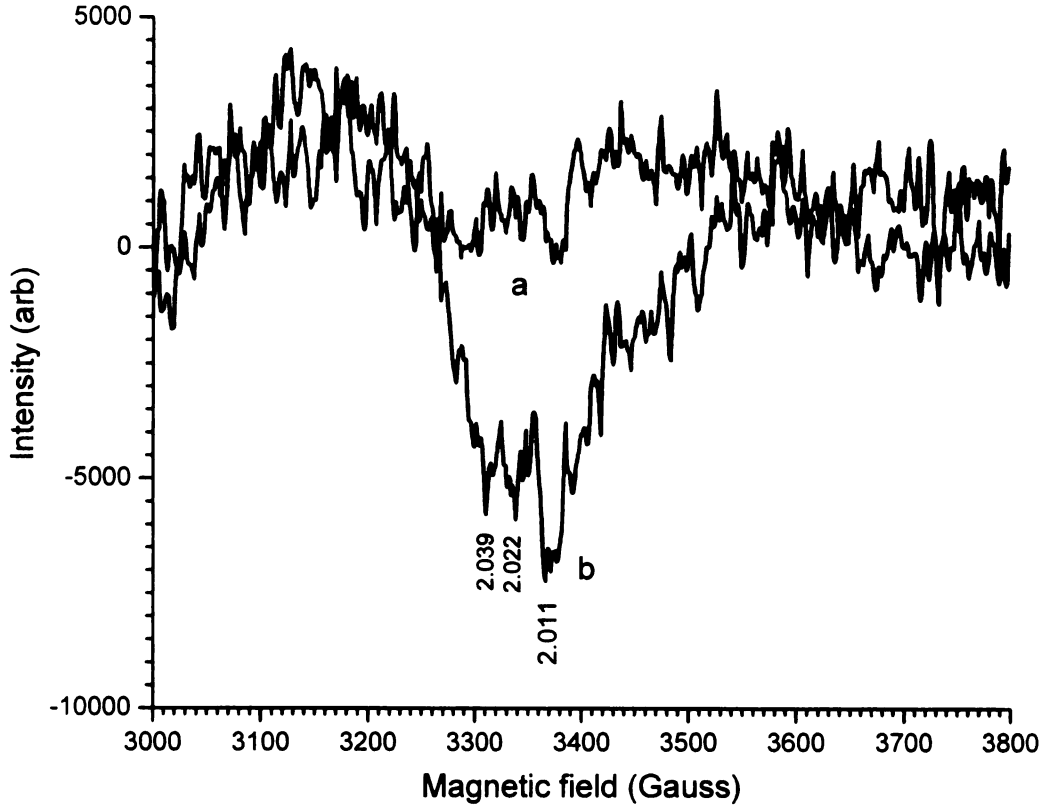

Figure 4.14 The difference EPR spectra obtained from RBCs incubated with 50 μM hydroxyurea in calcium free PSS (a) and in calcium containing PSS (b).The triplet hyperfine structure with g values at 2.02 indicates the presence HbNO in spectrum (b). However, the corresponding peaks of HbNO are not significant in spectrum (b). The EPR settings were as follows; power 5 mW, modulation frequency 9.46 GHz, field modulation amplitude 2.8 G, conversion time 327 ms, temperature 4.2 K, scan average 16

with hydroxyurea. However, the EPR data are in the preliminary level and require further investigation.

The results obtained using AAS and EPR for calcium studies were initially thought as a paradigm of the currently approved theory for the role of calcium in SCD. Higher intracellular calcium concentrations (five to ten fold increase compared to normal RBCs) are known to be a characteristic feature of sickle RBCs.[42] Moreover, a higher influx of calcium has been observed during the deoxygenation of sickle RBCs suggesting a possible effect of high calcium levels on sickled RBC water loss or dehydration. In that instance, a higher calcium uptake by hydroxyurea wouldn't benefit nor improve the rheological properties.

However, many studies have failed to demonstrate any measurable activity increase of the K+ channel or calcium pumps in sickled RBCs compared to healthy human RBCs. Therefore, it is difficult to find a correlation between calcium content and the dehydrogenation of RBCs, or any other related pathological event associated with the disease.[43] In line with these observations, Lew *et al* proposed a possible explanation for this behavior in 1985. According to their theory, sickle cell RBCs and to a lesser extent normal RBCs carry calcium in intracellular vesicles.[44,45] These endocytic vessels are formed from the endocytosis of the RBC membrane and contain a calcium ATPase pump.[46] The compartmentalization of sickle cell calcium limits the available ionized calcium levels in the cytoplasm, even though the calcium uptake was higher upon deoxygenation.[45] Most of the calcium in the intracellular vesicles is in the form of precipitates of inorganic or organic phosphates. Furthermore, Murphy *et al* measured the ionized calcium levels in oxygenated sickled RBCs, oxygenated normal RBCs, as well as

upon deoxygenation of sickled RBCs. This was an important study because the total calcium content in the cells exists in three locations. The majority of calcium is found in the cell membranes while lesser amounts are in the vesicle or in the ionized form in the cytosol of the RBCs. There was no difference of ionized calcium concentrations between oxygenated normal and sickle RBCs. Moreover, there was no significant increase in the ionized calcium content in the cytoplasm upon deoxygenating sickled RBCs.[47]

The calcium containing intracellular vesicles are a characteristic feature of sickle cells. Therefore, it is possible to suggest that these calcium vesicles are not involved in the signaling pathways or other biochemical pathways in sickled RBCs. Intracellular calcium is important in the NO synthesis in all the cell types containing the constitutive form of NOS and RBCs are not an exception. Therefore, an influx of calcium can increase the formation of calcium-CAM complex, thereby increasing the NO synthesis within the cell.

The results obtained from these studies clearly demonstrate the influence of hydroxyurea on NOS synthesis. Moreover, the AAS studies provide a novel mechanism of hydroxyurea on erythrocytes. The ability of increasing the calcium content in RBCs is intriguing and novel and provides a good correlation with NOS activity. Therefore, it is possible to conclude that hydroxyurea increases the intracellular calcium concentrations in RBCs, thereby increasing the synthesis of NO due to an increase in the calcium-CAM complex. An earlier study has shown the ability of hydroxyurea to increase the intracellular calcium concentration in endothelial cells by immobilizing the calcium pools in the endoplasmic reticulum. However, the work here shows for the first time that an increase in calcium influx occurs upon addition of hydroxyurea to RBCs. Furthermore,

this study suggests a new theory on the formation of NO by hydroxyurea. In addition, this work supports the findings of both increased and decreased deformability of RBCs upon hydroxyurea and NO addition. When the hydroxyurea concentration is optimal, it stimulates the NO synthesis by increasing the intracellular calcium concentration, which in turn increases the cellular deformability. Actually, the reason of modulating the deformability of RBCs with hydroxyurea is via NO production. NO has the ability to interact with the membrane bound hemoglobin molecules and this interaction can affect the membrane deformability. However, when NO production is high in response to high doses of hydroxyurea on RBCs, reactive nitric oxide species (RNS) can be formed, affecting the overall deformability of the cell membrane. RNS species, as well as NO, oxidize membrane proteins, especially the spectrin molecules, thereby affecting the cell membrane. Moreover, it is also known that NO itself acts to inhibit the activity of NOS via a negative feedback inhibition pathway.

Even though the deformability was not measured directly in this study, the whole explanation of deformability was supported by the results obtained from chapter 3. A change in ATP release from RBCs with different concentrations of hydroxyurea and NO suggested a change in deformability of RBCs. The work in this thesis explains the possible mechanism of action of hydroxyurea on erythrocytes in regulating blood flow in SCD. The NO, a known local vasodilator in the circulatory system, is being produced by hydroxyurea helping to maintain sickled RBC deformability, which is otherwise lost. This could lead in the uninterrupted blood flow in the capillaries and arterioles even in deoxygenated conditions.

4.6 Conclusions

SCD is a genetic disease with a significant rheological impairment of the vascular system. Hydroxyurea is a proven therapy for SCD and the mechanisms of the action of the clinical improvements of the treatment have yet to be elucidated.

EPR and AAS studies along with the chemiluminescence data have shown the effect of hydroxyurea on cytosolic calcium concentrations in RBCs. The higher calcium influx within the cytoplasm would increase the NO production by NOS in RBCs as calcium is an important component of the NO synthesis. Previous studies have shown the effect of NO on RBCs deformability. In fact, RBCs release ATP in response to mechanical deformation. Therefore, the observed ATP release from RBCs in response to hydroxyurea could be the interaction of NO with the RBC membrane, thus resulting the ATP release from RBCs. However, this study did not measure the RBC deformability directly, but the fact that ATP release in response to mechanical deformation suggest that the deformability is responsible for the modulation of the ATP release of RBCs. Overall, the studies in this chapter 3 and 4 provide evidence for a novel mechanism of hydroxyurea on erythrocytes in relation to the improvement of the blood flow.

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CHAPTER 5

Overall conclusions and future directions

5.1 Overall conclusions

The erythrocyte is a highly specialized but a very simplified cell. In fact, the lack of a nucleus and the intracellular organelles are especially designed for efficient oxygen and carbon dioxide transport through out the body by means of intracellular hemoglobin molecules. In that respect, the function of red blood cells (RBCs) have been oversimplified to oxygen transport, but these cells possess number of important metabolic pathways for important physiological processes and for their survival. Glycolysis, and the pentose phosphate pathway are two such pathways that exist in the RBCs for the production of ATP and NADPH. In addition, RBCs maintain cellular deformability via the strong antioxidant system present in the cells to allow passage through the small capillaries without damaging the cell.

It is very well accepted that RBCs release ATP, a known stimulus for NO synthesis, in response to mechanical deformation.[1,2,3]It has also been demonstrated that RBCs are a required component of the vasodialation via its ability to stimulate NO.[4,5] Both these findings have become important in identifying a novel role of RBCs in relation to regulation of the blood flow in small arteries and capillaries. Therefore, identification of the metabolic pathways or factors that regulate the RBC derived ATP release is necessary for better understanding this important and novel role of RBCs.

However, experimentation with RBC metabolic processes is challenging and difficult due to the complex matrix found in RBCs. We have developed a new fluorescent based method to quantify the major antioxidant in RBCs, glutathione (GSH), without a separation of the cellular constituents.[6] This method utilizes the previously known fluorescence probe, MCB, in conjunction with the standard addition method. The advantage of this method is its ability to quantify GSH in RBCs in the presence of hemoglobin, a major interfering molecule of spectroscopic analysis of biological samples. The value obtained for the bulk sample for the RBCs from n=6 different rabbits was 0.042 ± 0.002 mM. This number translates to a cellular concentration of 3.99 ± 0.25 mM/RBC in the sample and 362 ± 20 amol/cell. These values were within the acceptable concentration range of the previously published values.

Moreover, this technique was successfully employed in the investigation of the effect of antioxidant system in RBC derived ATP release. Our group was the first to report quantitatively that the diabetic RBCs release less amount of deformation induced ATP release in comparison to the ATP release of healthy non diabetic RBCs.[7] It is well established that diabetes RBCs are less deformable and also have a weakened antioxidant system. But there was no single study that linked these two factors to the diabetes complications. We believe that the decreased ATP release from diabetic RBCs directly involve in the diminished NO production from endothelium thereby affect the regulation of blood flow. This could be the reason for higher incidence of heart attacks, strokes, as well as, higher probability of having pulmonary hypertension in diabetic patients. In that respect, the development of a novel fluorescent based assay for glutathione measurements in RBCs without separating the cellular material greatly contributed to establish the

relationship between the weakened antioxidant system found in diabetic patients and the decreased ATP release in relation to the stiffening of RBCs membranes. The GSH concentrations and the ATP release from RBCs subjected to similar oxidant stress were monitored for over 45 minutes in 5 minute intervals. Interestingly, two graphs demonstrated the similar trends suggesting the relationship between the oxidant stress and the ATP release from RBCs. Moreover, this new techniques was a rapid and simple method to investigate the effect of oxidant stressors on cellular antioxidant system within the 45 minutes time period compared to the existing methods which could have taken more than 18 hours for a single assay.

Therefore, the necessity of such a system is not only limited to accurate glutathione measurement in RBCs, but also important in using in a large scale diagnostic tool for disease diagnosis. Furthermore, this method is amenable to quantitative determination of glutathione in a microfluidic chip which would be the ultimate useful in metabolomic studies of RBCs. Also, this method could be used in quantifying glutathione levels in a continuous flow system which could mimic physiological conditions of the circulatory system.

The second part of the thesis is focused on investigating the effect of hydroxyurea, a proven therapy for sickle cell disease, on erythrocytes. The hypothesis of the modulation of ATP release from RBCs in response to hydroxyurea was based on the literature of increased/decreased deformability of RBCs in the presence of hydroxyurea. The micro flow technique with a chemilumincence detection was employed to determine the effect of hydroxyurea on deformation induced ATP release from RBCs subjected to varying concentrations of hydroxyurea for 20 minutes. The results obtained for this study

led further determination of the exact mechanism of action of hydroxyurea on deformation induced ATP release. Investigating the NO forming ability of hydroxyurea was the first step towards the postulation of the mechanism, and ATP release was measured in the same way for RBCs incubated with varying concentrations of NO donor, spermine NONOate. Interestingly, a similar trend was observed for both hydroxyurea and NONOate treated RBCs, suggesting the involvement of NO on RBC derived ATP release from hydroxyurea. Further experimentation provided evidence for the involvement of hydroxyurea on the activity of NOS in RBCs. However, the mechanism of hydroxyurea on NO production was not understood until the atomic absorbance spectroscopic studies proved the increased uptake of calcium by RBCs in the presence of hydroxyurea. This observation was further confirmed by EPR spectroscopy studies as NO peak was only observed for hydroxyurea treated RBCs only in the presence of calcium

Therefore, we were successful in defining a novel role of hydroxyurea on erythrocytes in sickle cell patients. The mechanism of the increased release of ATP from RBCs is due to the increased uptake of calcium by RBCs in the presence of hydroxyurea thereby, increasing the production of NO via the activation of RBC NOS enzyme. This may be one of the mechanisms of the beneficiary effects observed in sickle cell patients receiving hydroxyurea before any rise in HbF in RBCs. The increased ATP release from RBCs could improve the local regulation of blood flow as ATP is a known stimulus for the vasodilator, NO in vascular system. This would be a very important mechanism explaining the reduced occurrence of vasoocclusive crisis, stroke, and heart attacks of patient's receiving hydroxyurea.[8]

In addition to defining a new mechanism of hydroxyurea on RBCs, this study provided evidence for a new pathway of NO formation from hydroxyurea. The NO formation via the activity of NOS in RBCs may be one of the possible reasons of increased levels of NO metabolites in RBCs of patients receiving hydroxyurea.

However, the ATP release started decreasing when the hydroxyurea concentration exceeded 100 μM. The reason of decreased amount of ATP release when the hydroxyurea concentration is higher may be due to the overproduction of NO in the RBCs. This could essentially develop increased oxidative stress in RBCs as NO can rapidly reacts with oxygen species creating reactive nitrogen and oxygen species. This could damage the RBC membrane and the deformability can be lost when the hydroxyurea concentration is higher.

Overall, this thesis contributes towards the understanding of the importance of the strong antioxidant system of RBCs in maintenance of the RBC membrane deformability and RBC derived ATP release.[6,7] Moreover, the work on this thesis defined a novel possible mechanism of hydroxyurea on erythrocytes which could be helpful in regulating the blood flow of sickle cell patients receiving hydroxyurea. Taken collectively, all these findings were based on the detection and quantification of important metabolites or elements such as ATP, GSH, HbNO, and calcium found in RBCs which were once known to be challenging and complex for analysis in the presence of hemoglobin. Therefore, in addition to the elucidation of two important pathways of RBCs in relation to disease pathogenesis and survival, this work greatly contributed towards the development and utilization of simple, and rapid analytical method in quantification and analyzing the biological samples in physiologically relevant conditions.

Taken collectively, this dissertation provides insight into the importance of exogenous and endogenous metabolites/molecules or therapeutic agents on red blood cell deformability and subsequent ATP release. Also, a development of a new technique of glutathione measurement facilitates the simultaneous detection of GSH in microfluidic devices which would be an ideal tool for disease diagnosis in future. Moreover, understanding of the exact mechanism of action of hydroxyurea will be beneficial in SCD treatment more efficiently and effectively and will guide to future aspects of SCD disease management.

5.2 Future directions

The future remaining work of GSH studies is the analysis of GSH levels in RBCs in the microfluidic chip. The multichannel microfluidic chip would be useful in multi sample analysis of GSH and can be eventually employed commercially as a GSH quantifying tool in disease diagnosis. There is not much to be done in that aspect other than the optimization of the fluorescence detection under the fluorescence microscope in the presence of RBCs.

However, for the studies involving hydroxyurea and RBCs, elucidating the calcium channels responsible for the calcium uptake from RBCs in the presence of hydroxyurea would be necessary as the calcium uptake pathway is still unknown. This could be done by specifically inhibiting the existing calcium channels in RBCs and then investigating the effect of ATP release from RBCs. These studies would give a better understaning of the newly hypothsiszed mechanism of hydroxyurea on RBCs.

Moreover, much of the work has been carried out in rabbit RBCs. It is necessary and important in repeating these studies in sickle cell RBCs to clearly demonstrate the beneficiary effects of hydroxyurea on sickle cell RBCs of humans. It is assumed to obtain the same results for rabbit RBCs and human RBCs due to the similarity of RBCs between the two species. The experiments with the sickle cell RBCs would determine quantitatively, the amounts of ATP release from RBCs in the presence of hydroxyurea.

Finally, an experiment that demonstrates the effect of hydroxyurea on NO synthesis by endothelial cells would be advantageous in proving the proposed mechanism of action of hydroxyurea on erythrocytes. This could be done in a microchip developed to grow endothelial cells as well as to passage red blood cells through the microchannels. An ideal chip design would be able to deform RBCs as they passage through the channels and the released ATP due to the mechanical deformation should diffuse in the endothelial cells grown on the chip, and stimulate the NO synthesis. The multi channels on the same chip would allow the NO production from endothelial cells in untreated RBCs as well as RBCs treated with different concentrations of hydroxyurea.

Also, a study that involves the demonstration of the interaction of RBCs and endothelial cells in the presence of hydroxyurea could be done on a microchip based model that mimics the circulation. This could be an important study as hydroxyurea is known to reduce the adhesion of sickle RBCs to endothelial cells. Also, this is considered as an additional mechanism of action of hydroxyurea on improved rheological properties of the vascular system observed in sickle cell patients.

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